RhlR Quorum Sensing and Social Dynamics in Cystic Fibrosis-Adapted Isolates of *Pseudomonas aeruginosa*

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Abstract

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The opportunistic pathogen *Pseudomonas aeruginosa* is a major cause of airway infection in cystic fibrosis (CF) patients. *P. aeruginosa* employs several hierarchically arranged and interconnected quorum sensing (QS) regulatory circuits to produce a battery of virulence factors such as elastase, phenazines, and rhamnolipids. The QS transcription factor LasR sits atop this hierarchy, and activates the transcription of dozens of genes, including that encoding the QS regulator RhlR. Paradoxically, deleterious *lasR* mutations are frequently observed in isolates from CF patients with chronic *P. aeruginosa* infections. In contrast, mutations in *rhlR* are rare. We have recently shown that in CF isolates, the QS circuitry is often rewired such that RhlR acts in a LasR-independent manner. This dissertation explores how QS activity and social dynamics may differ in a rewired background through comprehensive analysis of *P. aeruginosa* E90, a LasR-null, RhlR-active chronic infection isolate.

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PREFACE

Until the late twentieth century, it was long believed that bacteria functioned as lone survivalists; early notions of bacteria as social creatures were largely unappreciated and dismissed with skepticism. The advent of PCR and DNA sequencing empowered scientists with the means necessary to characterize the genetic basis underlying the phenomenon of “autoinduction” i.e. response to or regulation by self-produced signals. Later, it was appreciated that many bacterial species are in fact capable of gene regulation via autoinduction and some can even sense the signals produced by other species. It is now well regarded that these signals serve as a means of chemical communication between bacterial cells and can drive inter- and intraspecies interactions as well as virulence. Since its conception, the study of bacterial communication or “quorum sensing” has paved the way for new areas of investigation such as novel therapeutic design and system biology applications.

CHAPTER I. A Brief History of Quorum Sensing in Gram-negative Bacteria

Quorum sensing (QS) refers to the mechanism through which bacterial cells differentially regulate gene expression in response to self-produced chemical signals, the local concentration of which correlates with population density (1–3). Some of the earliest and most notable pieces of evidence regarding QS in gram-negative bacteria originates from the study of light production by the marine species *Vibrio fischeri*, formerly called *Photobacterium fischeri.* In 1970, Nealson and colleagues had noted that synthesis of the enzyme luciferase coincides with the logarithmic phase of growth, at which point the culture medium is sufficiently “conditioned” by the growing *V. fischeri* cells. Furthermore,

when cells are inoculated in a “preconditioned” medium, i.e. a medium in which logarithmic-phase cells had been growing previously, luciferase synthesis occurs readily without the characteristic delay associated with lag phase (4). They hypothesized that light production is driven by secreted self-produced factors without the need for an external agent and referred to this phenomenon as “autoinduction” (4). It was proposed that these self-produced factors might play a role in chemical signaling, but this idea was largely unexplored for the next decade or so.

Further research regarding luminescence in *V. fischeri* brought forth two pivotal breakthroughs: 1) the identification of *luxI* and *luxR* as the genes required for regulation of the *lux* operon, which confers light production (5, 6) and 2) characterization of the extracellular signal that controls light production, *N*-3-oxohexanoyl-L-homoserine lactone, the first acyl-homoserine lactone (AHL) structure determined (7). However, it wasn’t until DNA sequencing became adopted as a common laboratory technique in the 1990s that quorum sensing research dramatically transformed. Scientists began uncovering a plethora of LuxI and LuxR-type systems in several bacterial species, all of which appeared to share a common theme: the *luxI* homolog encodes an autoinducer or signal synthase and the *luxR* homolog encodes a cognate signal receptor. Moreover, these LuxI and LuxR-type systems were found to regulate a diverse array of functions ranging from virulence to cell septation (1). Altogether, these early discoveries defined the concept of “quorum sensing”; that bacteria sense and secrete signals as a proxy for cell density so that metabolically costly functions are withheld until a sufficiently dense population can accrue the benefits of such functions.

Researchers also began to unveil the rich diversity of QS signals. Signals are not just reserved to AHL structures, but also include various fatty acids, quinolones, and derivatives of metabolic intermediates, to name a few. The different QS systems and signals of gram-negative bacterial species have been reviewed at length (3). Interestingly, a single organism is capable of producing multiple kinds of signals. For example, *Pseudomonas aeruginosa*, the model organism for this project, produces both AHLs and quinolones.

CHAPTER II. Quorum Sensing in *Pseudomonas aeruginosa*

QS circuitry

Pseudomonas aeruginosa is a gram-negative bacterium and opportunistic pathogen that is capable of causing severe infections in immunocompromised individuals. Many *P. aeruginosa* virulence factors, including phenazines, rhamnolipids, and hydrogen cyanide, are regulated by QS (8). QS in *P. aeruginosa* is complex and consists of two complete LuxI/LuxR QS systems: LasI/LasR and RhlI/RhlR (9, 10) (Fig. 1). The signal synthase LasI produces the signal N-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL). Above a certain concentration, 3OC12-HSL binds to and facilitates the dimerization of LasR (11). The LasR homodimer functions as a transcriptional activator promoting the expression of hundreds of genes including *rhlR* and *rhlI*, thereby linking the two acyl-homoserine lactone (AHL) QS regulatory circuits (9, 10). Similarly, RhlI produces the signal *N*-butanoyl-homoserine lactone (C4-HSL), which binds to RhlR, initiating transcription of an additional set of target genes that overlap somewhat with the LasR regulon(12, 13). There is an additional LuxR homolog in *P. aeruginosa* called QscR, which does not have a cognate signal. Instead, QscR binds to 3OC12-HSL and appears to raise the threshold for quorum sensing

through activation of a single linked operon(14, 15). Lastly, there is a third, non-AHL QS circuit in *P. aeruginosa* that involves a 2-heptyl-3-hydroxy-4-quinolone signal (*Pseudomonas* quinolone signal; PQS), which activates the transcription factor PqsR(16). PqsR and RhlR co-regulate the production of some extracellular products(17). It is important to note that the hierarchical arrangement with which the QS regulatory systems are described is primarily based on studies performed under a specific set of laboratory conditions (typically a culture grown in lysogeny broth or Luria-Bertani broth with shaking). As described later in this chapter, there are various circumstances under which this hierarchical cascade of gene regulation no longer applies.

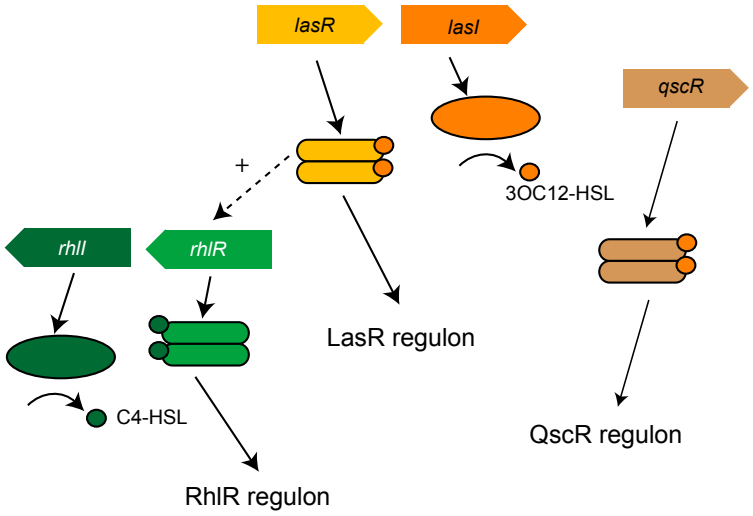


Figure 1. Overview of AHL quorum sensing in *P. aeruginosa*. The QS signal 3OC12-HSL is produced by LasI. Signal binding to LasR activates transcription of dozens of genes. A similar phenomenon occurs with RhlR and its cognate signal, C4-HSL. The *lasI* and *lasR* genes are genetically linked, as are *rhlR* and *rhlI.* QscR responds to 3OC12-HSL and activates a single operon, PA1895-1897. The dashed line signifies a regulatory relationship, as defined in the laboratory strains PAO1 and PA14. Note that the LasR and RhlR regulons partially overlap.

Nutritional and Environmental Factors Influence P. aeruginosa QS

Although the term “quorum sensing” might imply that gene expression is solely influenced by population density, in actuality an increase in cell density is necessary but insufficient to induce expression of most QS-regulated genes. In fact, induction of most QS-regulated genes does not occur until the population approaches stationary phase (8, 18). Aside from responding to fluctuations in cell density, the QS regulatory systems in *P. aeruginosa* also integrate several nutritional and environmental cues as discussed below.

Activation of the stringent response

RelA is a ribosome-associated protein that synthesizes nucleotide guanosine 3′,5′-bisdiphosphate (ppGpp) (19), an effector of the stringent response, a broadly conserved stress response elicited by bacterial cells during nutrient starvation, heat-shock, and various other stress conditions (20). In *Escherichia coli,* the stringent response also leads to activation of the alternative sigma factor RpoS, the regulon of which enhances long-term survival as the population transitions to stationary phase (21, 22).

In *P. aeruginosa*, homologs of both RelA and RpoS have been identified (23, 24). Further, RpoS has been shown to not only play a role in stress tolerance (25), but in QS gene regulation as well (26). Overexpression of *relA* induces expression of *rpoS* leading to premature production of quorum-regulated exoproducts irrespective of cell density (26). In the same study, the researchers found that expression of *lasR* and *rhlR* as well as their cognate signals are also prematurely activated in the *relA* overexpression background. A later study by Schafhauser *et al.* revealed that the stringent response also impacts the

PQS system. Using a stringent-response deficient mutant, they showed that PQS precursor levels were upregulated (27) likely due to reduced expression of LasR and RhlR, which repress the PQS system (16). Altogether, these findings demonstrate how the stringent response can upend the canonical quorum sensing hierarchy as LasR no longer regulates PQS when the stringent response is inactivated.

Iron starvation

In *P. aeruginosa*, the ferric uptake regulator (Fur) downregulates the expression of two small RNAs (sRNAs) encoded by *prrF1* and *prrF2* under iron-replete conditions. These two sRNAs otherwise prevent the synthesis of iron-containing proteins as a means of reserving this essential nutrient when iron levels are scarce (28). In addition to their role in iron homeostasis, the *prrF* sRNAs also impact PQS levels. Specifically, PrrF negatively regulates the *antABC* operon, which encodes enzymes necessary for the degradation of anthranilate, a precursor to PQS (29). Therefore, under iron-replete conditions, PqsR activity is low. The expression of the *antABC* operon is also known to be affected by QS regulators. RhlR and its cognate signal C4-HSL upregulate *antA* thereby repressing PQS (8). Inversely, PqsR represses *antA* expression (29). The ability of *P. aeruginosa* to integrate iron levels as a regulator of QS has important implications in the design of QS inhibitors as possible therapeutics. For instance, under conditions where iron is abundant, LasR in wild-type *P. aeruginosa* is shown to be the primary driver of virulence functions, whereas in low-iron environments, RhlR and PqsR play more prominent roles (30). These findings highlight the importance of testing a variety of growth conditions to assess the efficacy of QS inhibitors in the context of infection.

Phosphate limitation

Earlier reports noted that under nutrient rich conditions RhlR activity is dependent on LasR activation, but that in low-phosphate medium, *rhlR* expression is influenced by additional transcriptional regulators including Vfr and sigma factor σ^{54} (31). A later study by Jensen *et al.* sought to characterize the molecular mechanism linking phosphate levels to *rhlR* expression. They hypothesized that the phosphate-responsive two-component regulatory system PhoR-PhoB (32, 33) plays a role in driving *rhlR* expression. Using phosphate limiting conditions, they demonstrated that transcription of *rhlR* is significantly lower in a *phoB* mutant strain (34). Further, they showed that biosynthesis of hydroxy-alkyl-quinolones is also enhanced in low phosphate medium in a *phoB*-dependent manner demonstrating that phosphate levels can influence both AHL and PQS signaling. (34).

CHAPTER III. *Pseudomonas aeruginosa* in the context of cystic fibrosis

Cystic fibrosis (CF) is a genetic disease that affects over 70,000 people worldwide and is caused by mutations in both copies of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (35, 36). Mutations in the *CFTR* gene result in defective mucociliary clearance, which leads to an accumulation of abnormally thick mucus in the airways(37). Consequently, CF patients are at increased risk of lung infection by various pathogens including *P. aeruginosa*(38).

P. aeruginosa infects nearly 50% of all CF patients, with an 80% prevalence in adults(39). Once it is established in CF airways, *P. aeruginosa* is incredibly difficult to eradicate(38). Furthermore, about 20% of strains are multi-drug resistant(36). In response to the alarming increase in antibiotic resistance, recent research efforts have centered upon inhibiting *P. aeruginosa* virulence as an alternative therapeutic approach(40–43).

The expression of many *P. aeruginosa* virulence factors is regulated via QS. As discussed earlier (Fig. 1), in laboratory strains such as PAO1 and under laboratory conditions, LasR regulates RhlR and a non-AHL QS transcription factor, PqsR. LasR therefore has been considered a master QS regulator and mutations in *lasR* confer attenuated virulence in animal models(44, 45). Despite its importance in establishing infection, *lasR* is commonly mutated in chronic *P. aeruginosa* CF isolates(46–48). In some patients, the frequency of *lasR* mutant isolates has been reported to be greater than 50% (48, 49). Although a handful of single-nucleotide substitutions in *lasR* still yield functional protein, most mutations in either the signal- or DNA-binding domains yield a nonfunctional polypeptide (46). These early findings led to the notion that QS is not essential during chronic stages of infection, dampening enthusiasm for QS inhibitors as potential therapeutics. Contrary to this idea, we and others have shown that many LasR-null *P. aeruginosa* chronic infection isolates remain capable of engaging in QS activity through the RhlI/RhlR circuit(46, 50, 51).

Given the plasticity of the QS hierarchy, there are likely several mechanisms through which LasR-null clinical isolates can maintain RhlR activity. Recently, Kostylev et al. have

proposed that RhlR-active clinical isolates may emerge from a LasR-null background via mutation of the gene encoding the transcription factor MexT (52). Although it is apparent that CF strains “rewire” their QS circuitry so that RhlR is the key transcription factor, the RhlR regulon in a rewired background had not yet been described.

CHAPTER IV. RhlR quorum sensing in a *Pseudomonas aeruginosa* clinical isolate

We are interested in the regulatory remodeling of QS that occurs in isolates of P. aeruginosa from chronic infections, including those in CF. To begin to understand how RhlR-mediated QS in clinical isolates might be different from that of laboratory strains(46, 49, 51, 53), we studied a CF isolate called E90(54), which contains an inactivating, single base-pair deletion in *lasR*, and uses RhlR to mediate QS. E90 produces QS-regulated virulence factors at levels comparable to that of PAO1. We used RNA-seq to characterize the RhlR regulon of this isolate by comparing its transcriptome with that of an isogenic *rhlR* deletion mutant. We determined that the E90 RhlR regulon consists of over 83 genes including those that encode virulence factors. Using a three-dimensional tissue culture model, we also observed that E90 induces cell death in a RhlR-dependent manner. Together our data provide a much broader picture of the “rewiring” of QS that can take place in CF-adapted *P. aeruginosa*, while also providing a basis for elucidating RhlR-specific gene regulation without the confounding effects of LasR-dependent activation.

RESULTS

RhlR and C4-HSL-dependent QS activity is conserved in LasR-null isolate E90

We identified isolate E90 from a phenotypic survey of chronic infection isolates collected in the Early *Pseudomonas* Infection Control (EPIC) Observational Study (46). This isolate, an apparent *lasR* mutant, still engaged in activities that are putatively QS-regulated such as rhamnolipid, exoprotease, and phenazine production. The *lasR* gene of E90 features a 1 bp deletion at nucleotide position 170 (of 720), a frameshift mutation which results in a premature stop codon (at residue 114), in the signal-binding domain of LasR (55). To confirm that this single nucleotide polymorphism encodes a nonfunctional LasR polypeptide, we transformed the strain with a LasR-specific reporter plasmid consisting of *gfp* fused to the promoter region of *lasI,* which encodes the signal synthase and is strongly activated by LasR (56). GFP fluorescence in E90 transformed with this reporter plasmid was unmeasurable and mirrored that of a PAO1Δ*lasR* mutant (Fig. 2A). As a complementary approach, we measured the concentration of 3OC12-HSL produced by E90 using a bioassay. We found that after overnight growth, E90 produced 40 nM 3OC12-HSL, a very low amount compared to PAO1 (1.5 μM), and in contrast to PAO1Δ*lasR* for which no 3OC12-HSL was detected (Fig. 2B). Together these data suggested that E90 produces a small amount of 3OC12-HSL in a LasR-independent manner, but that the 3OC12-HSL was not important for activation of QS-regulated genes. In contrast, E90 produced approximately 8.3 μM C4-HSL after overnight growth, comparable to PAO1 (9.8 μM). Altogether, these data confirmed that E90 encodes a non-functional LasR, and suggested that if QS was active in this isolate, it was regulated by either RhlR, PqsR, or both transcription factors.

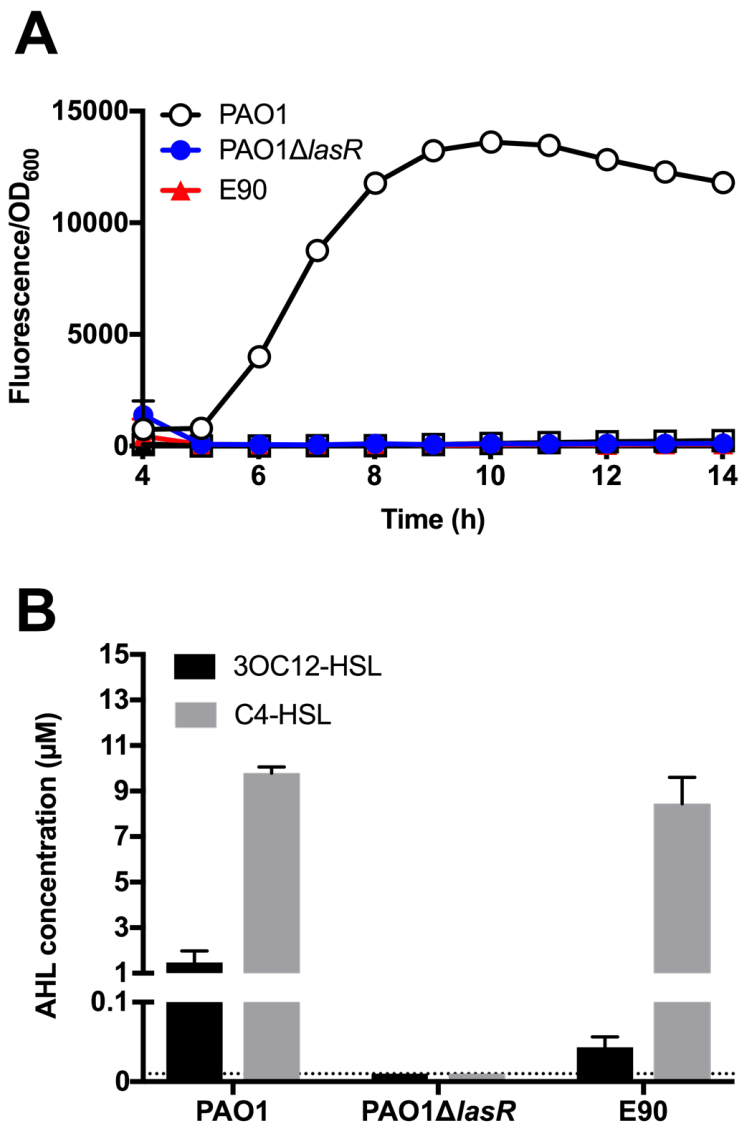
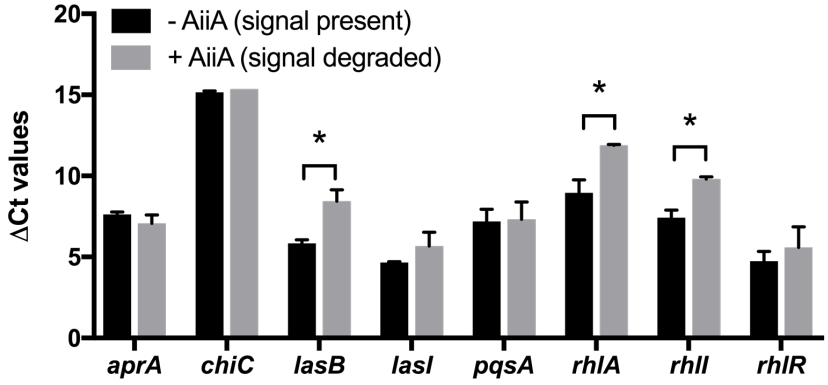


Figure 2. LasR activity is absent in E90, a cystic fibrosis-adapted chronic infection isolate. A) P_{lasI}-*gfp* reporter activity over time (Fluorescence/OD_{600}). PAO1, open circles; PAO1Δ*lasR*, blue circles; E90, red triangles. Data from the first three hours are excluded from analysis because cell density measurements were below the limit of detection. B) AHL signal concentrations. Black bars, 3OC12-HSL; grey bars, C4-HSL. The dashed line indicates the limit of detection for the 3OC12 and C4-HSL bioassay (10 nM in each case). Both the PAO1Δ*lasR* mutant and E90 produce concentrations of 3OC12-HSL and C4-HSL that are significantly different from PAO1 (p-value < 0.05 by t-test). For (A) and (B), means and standard deviation of biological replicates are shown (*n*=3). In some cases, error bars are too small to be seen.

To determine if E90 retained AHL-dependent QS, we examined the expression of several well-studied quorum-regulated genes in the presence or absence of the AiiA lactonase, an enzyme that degrades AHL signals(57). Using qRT-PCR, we observed that expression of *lasB* and *rhlA* were increased in the presence of AHLs (Fig. 3). These genes, which encode the exoprotease elastase and a rhamnosyltransferase involved in rhamnolipid production, were identified as QS-regulated in PAO1 (8, 58). The gene *rhlI*, which encodes the C4-HSL synthase, was also AHL-regulated (Fig. 3). Next we asked if the small amount of 3OC12-HSL produced by E90 (Fig. 2B) has a role in QS. Using a transcriptional reporter assay, we observed that expression from the *lasB* and *rhlA* promoters in the E90Δ*lasI* background was similar to that of wild-type, suggesting that 3OC12-HSL does not contribute to the QS activity observed in E90 (Fig. S1).



Gene name	Fold change
aprA	0.7
chiC	1.2
lasB	6.1
lasI	1.2
pqsA	1.1
rhlA	7.6
rhlI	5.3
rhlR	1.8

Figure 3. In isolate E90, expression of several canonical QS-regulated genes is AHL-dependent. The following target genes were measured in the presence or absence of AiiA lactonase using qRT-PCR: *lasI,* 3OC12-HSL signal synthase; *lasB,* elastase; *rhlI,* C4-HSL signal synthase; *rhlR,* RhlR, *pqsA,* coenzyme A ligase involved in *Pseudomonas* Quinolone Signal synthesis; *chiC,* chitinase; *aprA;* alkaline metalloprotease. The differences in threshold cycle (ΔCt) are measured relative to the housekeeping gene *rplU.* Fold changes in gene expression (table on right) are reported relative to cultures incubated with AiiA lactonase. Asterisks (*) indicate statistical significance ($p < 0.05$ by *t* test). Error bars represent the standard deviation for results of three independent experiments.

Previous work has shown that RhlR activity can be uncoupled from LasR regulation in LasR-null backgrounds (52, 59, 60). The *rhlR* gene in E90 encodes a protein that is identical to that of PAO1, although it contains three synonymous mutations, and there is no difference in the *rhlR* promoter at the Vfr binding sites or the putative Las box. Given that C4-HSL production is robust in E90 (Fig. 2B), we queried if this strain similarly engaged in RhlR-dependent QS activity. To address this question, we engineered a *rhlR* deletion in the E90 background to observe its effect on quorum-regulated phenotypes. We found that the E90Δ*rhlR* deletion mutant, like a *rhlI* mutant, exhibited undetectable *rhlA* promoter activity and produced little to no exoprotease and pyocyanin, consistent with the idea that RhlR regulates QS activity in E90 (Fig. 4). The phenotype could be complemented in the *rhlI* mutant by addition of C4-HSL. We also verified that differences in exoprotease and pyocyanin production observed between PAO1, E90, and PAO1 or E90-derived strains are not due to differences in growth under the conditions used for this assay (Fig. S2). As a whole, our results showed that the LasR-null isolate E90 retains QS activity in a RhlR- and C4-HSL-dependent manner, and suggested that regulation by RhlR in this strain parallels that of LasR in PAO1. Because RhlR-dependent QS regulation appears to be common in CF isolates (46, 50, 51), we reasoned that a study of the genes regulated by RhlR in this background would give insight into which QS-regulated gene products might be important in chronic CF infections. Furthermore, because *rhlR* is not regulated by LasR in E90 (and other clinical isolates), a study of the E90 QS transcriptome has the potential to disentangle genes that are regulated solely by RhlR from those that require both LasR and RhlR.

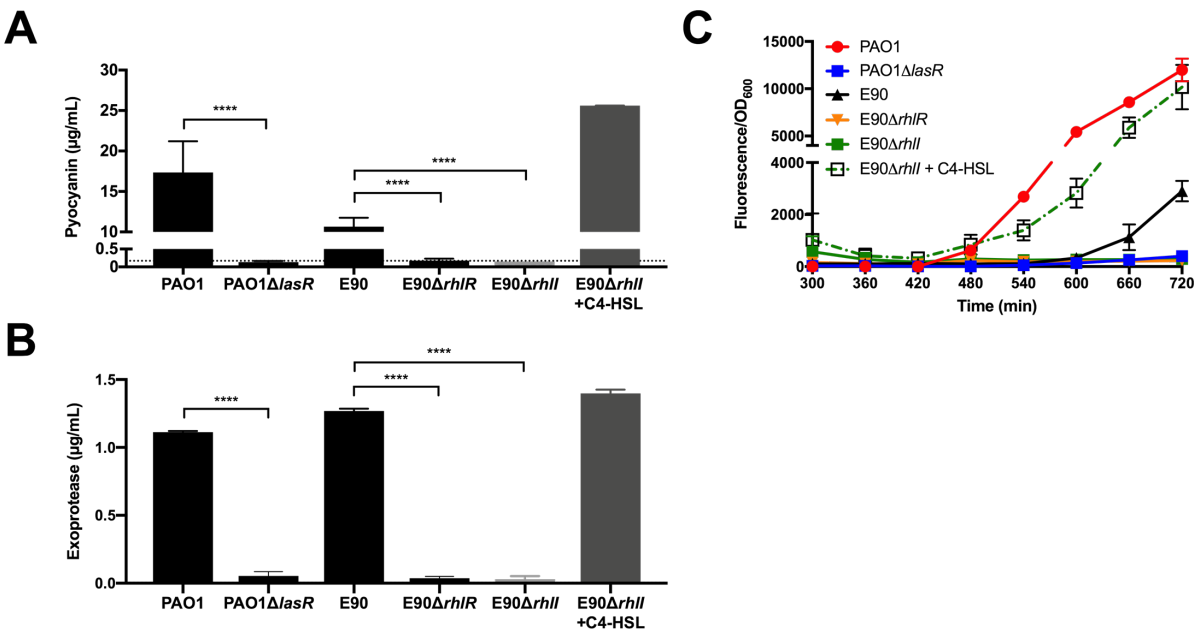


Figure 4. RhlR regulates QS in E90. Production of (A) pyocyanin or (B) protease in either PAO1, E90, PAO1Δ*lasR*, E90Δ*rhlR*, E90Δ*rhlI* or E90Δ*rhlI* with 10 µM C_4-HSL. The dashed line indicates the detection limit for the pyocyanin assay, which is 0.2 µg/mL. The detection limit (not shown) for the Pierce™ fluorescent protease assay is approximately 0.004 µg/mL. C) *rhlA-gfp* reporter activity over time. Data from the first five hours are excluded because cell density measurements were below the limit of detection of the plate reader. Error bars represent the standard deviation for results of three independent experiments. In some cases, error bars are too small to be seen. Both the PAO1Δ*lasR* mutant and E90 produce concentrations of pyocyanin or exoprotease that are significantly different from PAO1 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by t-test).

Identification of the RhlR regulon of E90

To determine which genes are regulated by RhlR, we performed an RNA-seq-based differential gene (DE) expression analysis comparing RNA collected from cultures of the parent strain E90 to the isogenic RhlR deletion mutant. First, we sought to generate a *de novo*-assembled genome for E90 to use as an RNA-seq mapping reference which would account for the potential genomic differences between E90 and reference strains of *P. aeruginosa*. Using a hybrid approach combining both short- and long-read high-throughput sequencing, we were able to assemble the genome of E90 into a single

circular contig of approximately 6.8 Mb that harbors 6650 annotated features (Fig. 5A; 6650 features total, 6503 protein coding sequences). In addition to being roughly 550 kb larger than the published sequence of laboratory strain PAO1 (61), the genome of E90 includes 862 features with no homology to PAO1. Also present is a 4.4 Mb inversion relative to PAO1, which includes an internal reorder of roughly 250 kb. The inversion appears to be the result of a recombination event between two roughly 5 kb repeat regions that do not have homology to PAO1, but flank the *rrnA/rrnB* region previously implicated in restructuring of the *P. aeruginosa* genome (61). A brief search of the E90 genome for *P. aeruginosa* genes previously reported to be under purifying selection in CF isolates revealed a nonsynonymous mutation in the gene coding for the probable oxidoreductase MexS (locus PAE90_2949/PA2491; nonsynonymous SNP), as well as the resistance-nodulation-division multidrug efflux membrane fusion protein precursor MexA (locus PAE90_0464/PA0425; 33bp deletion) (48).

Next, to facilitate a comparison to previously published QS regulons that also implemented an RNA-seq based approach (62), we grew strains in LB-MOPS to an OD_{600} of 2.0. The growth of E90 and E90Δ*rhlR* are indistinguishable in this medium under the conditions used for this experiment (Fig. S3). Our DE analysis identified 53 genes that were upregulated in the E90 vs. E90Δ*rhlR* comparison (Tables 1 and S1). Forty-four (83%) of these genes were identified as QS-regulated in a previous microarray study of PAO1 (8) (Fig. 5B) and 21 belong to the core quorum-controlled genome characterized in (62).

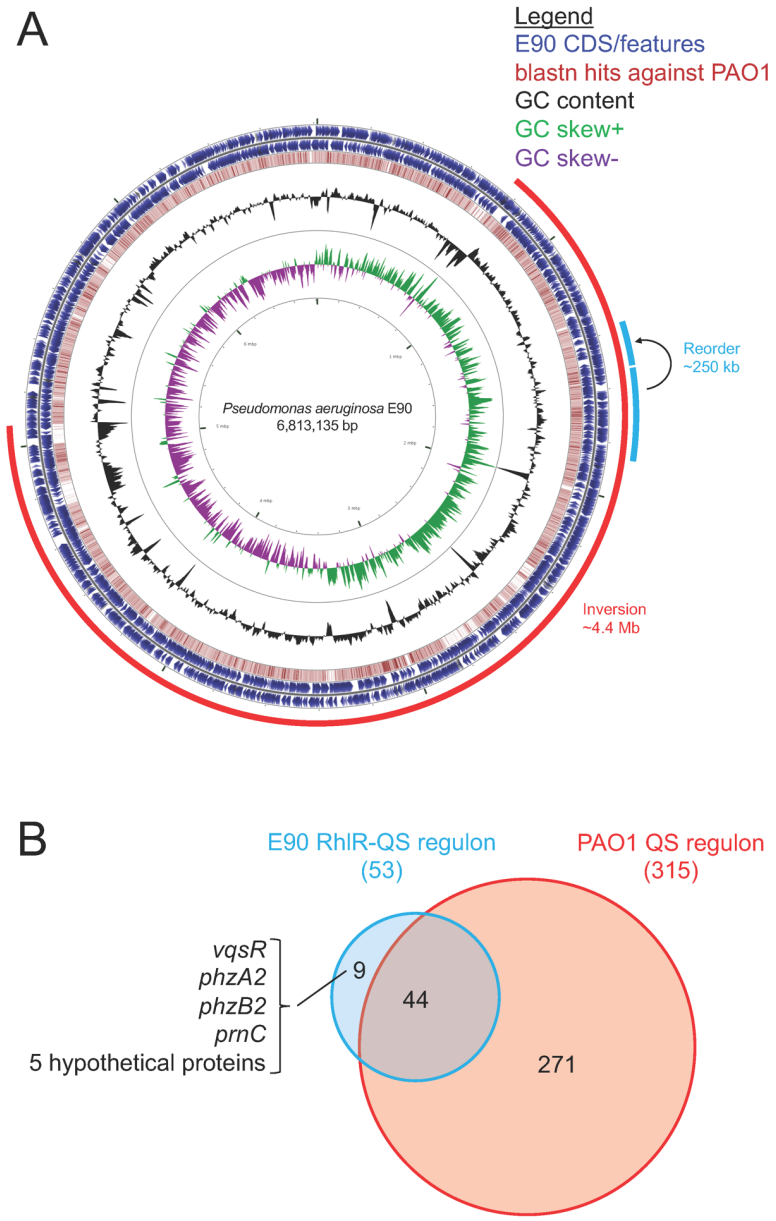


Figure 5. General features of the complete E90 genome and RhlR-QS regulon. (A) This circular representation of the E90 genome includes rings indicating the following features described from the outer-most to inner-most rings: annotated features of coding DNA sequences (blue) or ribosomal DNA (grey) on the forward (outer) or reverse (inner) strands; all-by-all blastn hits (red) in a comparison against PAO1_107 (nucleotide identity >40%); GC content deviation (black); GC skew (+, green; -, purple). Additional outer, partial rings indicate the 4.4 Mb inversion (bright red) and the 250 kb reorder (light blue). (B) Venn diagram highlighting genes shared between the E90 RhlR-induced regulon and the PAO1 QS-induced regulon as determined via microarray in (8). Lobes are scaled to approximate the relative size of each regulon.

Table 1. The 20 most highly RhlR-activated genes in isolate E90.

De novo ID[a]	Gene name[b]	Product description[c]	Fold change[d]	Fold change by RT-PCR[e]
PAE90_1621	*rhlB*	rhamnosyltransferase chain B	5688.8	
PAE90_1620	*rhlA*	rhamnosyltransferase chain A	1956.4	
PAE90_0833	*phzC1*	phenazine biosynthesis protein PhzC	129.4	
PAE90_1777		probable FAD-dependent monooxygenase	64.5	
PAE90_1773		conserved hypothetical protein	60.9	
PAE90_1775		probable short chain dehydrogenase	52.0	
PAE90_3307	*hcnA*	hydrogen cyanide synthase HcnA	35.0	
PAE90_1771		probable acyl carrier protein	32.6	
PAE90_3647		probable acyl carrier protein	32.1	
PAE90_1364	*lasB*	elastase LasB	32.1	
PAE90_0834	*phzB1*	probable phenazine biosynthesis protein	30.9	
PAE90_0835	*phzA1*	probable phenazine biosynthesis protein	29.0	
PAE90_1778		probable non-ribosomal peptide synthetase	28.3	
PAE90_1772	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III	23.5	
PAE90_1776		hypothetical protein	20.4	
PAE90_2705		**hypothetical protein**	**15.5**	**12.1**
PAE90_0837		**hypothetical protein**	**14.7**	**26.0**
PAE90_2723	***vqsR***	**VqsR**	**14.4**	**48.5**
PAE90_1770		hypothetical protein	13.2	
PAE90_0133		**hypothetical protein**	**12.2**	**10.6**

a. “PAE90” identification numbers correspond to locus tags in the E90 *de novo* genome.

b. Gene names from PAO1-UW reference annotation (PAO1_107; see Materials and Methods) available on the Pseudomonas Genome Database (https://www.pseudomonas.com).

c. Product descriptions from PAO1-UW reference annotation, with the exception of those genes not present in the PAO1 genome (see Materials and Methods) which are described as annotated in the *de novo* genome.

d. Fold change values were determined by DEseq as described in Materials and Methods

e. Bold: genes not previously identified as QS-regulated (Schuster et al., 2003). Fold-change values for these genes were confirmed by RT-PCR.

We also identified several well-known virulence genes including those that encode biosynthetic machinery required for rhamnolipid (*rhlAB*), hydrogen cyanide (HCN; *hcnABC*), elastase (*lasB*), and pyocyanin synthesis (*phzABC1*). Elastase is an exoprotease known to degrade various components of the innate and adaptive immune system including surfactant proteins A and D (63, 64). Rhamnolipid and pyocyanin have also been previously appreciated for their roles in airway epithelium infiltration and damage (65, 66). In addition, our RNA-seq analysis revealed *hsiA2,* the first gene in the cluster encoding the Second Type VI Secretion System, which facilitates the uptake of *P. aeruginosa* by lung epithelial cells (67).

While QS control of the phenazine biosynthesis pathway has been reported previously, only one of the two "redundant" operons ("*phz1"*; *phzA1-G1*) was identified in our study (3). Interestingly, our transcriptome analysis found that RhlR also regulates the first two genes of the second phenazine operon ("*phz2"*; *phzA2-G2*) in E90, albeit at a slightly lower level than *phz1*. Both operons encode nearly identical sets of proteins, each with the capacity to synthesize the precursor (phenazine-1-carboxylic acid) of many downstream phenazine derivatives, including the virulence factor pyocyanin (68). Despite their seemingly redundant function, *phz1* and *phz2* do not appear to be regulated in concert. In strain PA14, although *phz1* is more highly expressed than *phz2* in liquid culture, similar to what we observed in the E90 RhlR regulon, *phz2* actually contributes more to overall phenazine production in liquid culture (69). Furthermore, *phz2* is the only active *phz* operon in colony biofilms, and was the only *phz* operon implicated in lung colonization in a murine model of infection (69).

Moreover, we observed that the RhlR regulon included genes that likely confer a growth advantage in the CF lung. For example, *cbpD* encodes a chitin-binding protein shown to contribute to the thickness of biofilms, the development of which is important for nutrient acquisition and stress resistance (70). The *prnC* gene, which encodes a monodechloroaminopyrrolnitrin 3-halogenase, was also present in the E90 RhlR regulon, and has not been reported in previous *P. aeruginosa* transcriptomes nor is present in the PAO1 reference genome. PrnC has only previously been described in *P. protegens* (formerly *P. fluorescens*), where it is involved in the synthesis of pyrrolnitrin, an antifungal antibiotic (71).

Among the most highly regulated genes (8, 62) were those belonging to a conserved nonribosomal peptide synthetase (NRPS) pathway (PaE90_1770-1779; PA3327-3336). The products of this NRPS pathway have been identified as azetidine-containing alkaloids referred to as azetidomonamides (72). The biological significance of this widely conserved NRPS pathway in *Pseudomonas* species or what roles azetidomonamides may play in virulence or interspecies interaction is not well understood, but regulation by QS appears to be a common feature.

Our interrogation of the E90 RhlR regulon also revealed 30 genes that were RhlR-repressed; none of these genes were reported in previous reports of QS-repressed genes [3] and 19 are not present in the PAO1 genome. We found two genes of the *alpBCDE* lysis cassette, *alpB* and *alpC*, were repressed by RhlR in E90 under the conditions of our experiments. While induction of *alpBCDE*, via de-repression of the *alpA* gene, has been

shown to be lethal to individual cells, it may benefit infecting cells at the population level (73). We also observed down-regulation of the gene encoding the posttranscriptional regulatory protein RsmA by RhlR in E90. RsmA is nested in a host of regulatory machinery important in infection, and an *rsmA* mutant strain has been observed to favor chronic persistence and increased inflammation in a murine model of lung infection (74). Lastly, we identified RhlR regulation of phage loci not found in the PAO1 genome. The RhlR-repressed phage loci correspond to E90 genes PaE90_2433 through PaE90_2442.

RhlR is the primary driver of cytotoxicity in a lung epithelium model

Laboratory *lasR* mutant strains are less virulent than the WT in acute infection settings (75–77). However, as the RhlR-dependent QS regulon of E90 includes several factors implicated in virulence (Table 1), we hypothesized that E90 might be capable of inducing host cell death. To address this question, we incubated incubated either PAO1, E90, or engineered QS transcription-factor mutants in an *in vivo*-like three-dimensional (3D) lung epithelial cell culture model (A549 cell line) (78). The 3D lung cell model possesses several advantages over the standard A549 monolayer as an infection model, including increased production of mucins, formation of tight junctions and polarity, decreased expression of carcinoma markers, and physiologically relevant cytokine expression and association of *P. aeruginosa* with the epithelial cells (78, 79). Following an incubation period of 24 hours, we measured eukaryotic cell death of the 3-D cell cultures via cytosolic lactate dehydrogenase (LDH) release. Consistent with prior studies, WT PAO1 cytotoxicity is abrogated in a *lasR* deletion mutant strain; however, cytotoxicity of a PAO1 *rhlR* mutant strain is similar to that of the wild-type, because in this assay the secreted

products responsible for cytotoxicity are LasR-regulated in PAO1, with little or no contribution from RhlR. Strikingly, the opposite was true for E90: deletion of RhlR significantly reduces cytotoxicity. Addition of exogenous C4-HSL to an E90Δ*rhlI* deletion mutant restored cytotoxicity to WT levels (Fig. 6). This RhlR-dependent cytotoxicity might be related to the different timing of RhlR activation in E90, the specific set of genes regulated by RhlR in this strain, or both. Together, these results highlight the restructuring of QS gene regulation in this clinical isolate and underscore implications for virulence during chronic infection.

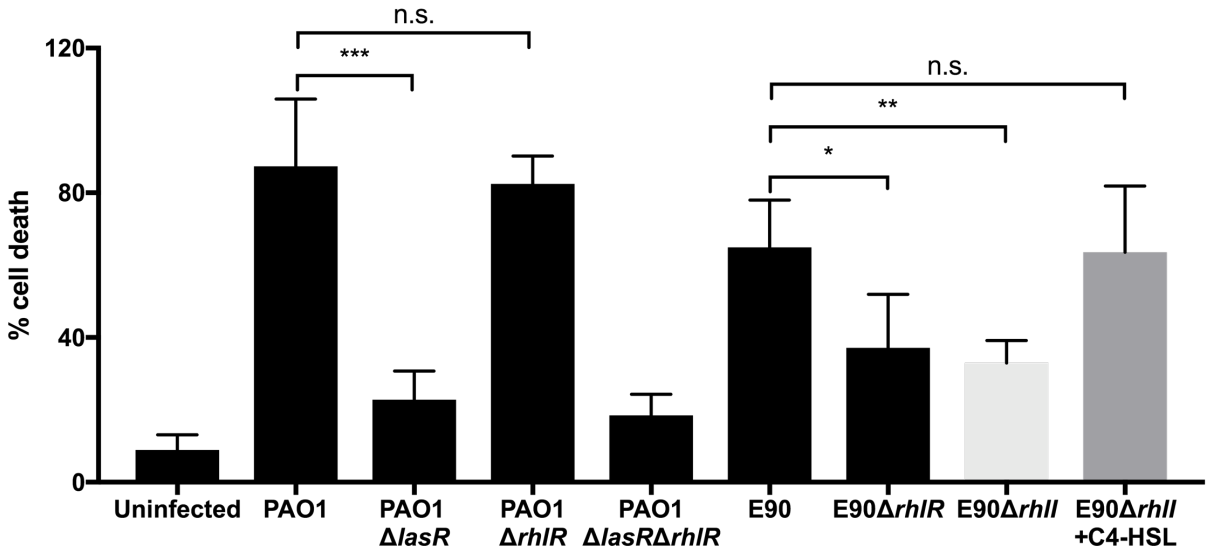


Figure 6. RhlR regulates cytotoxicity in E90, but not PAO1. We measured cell lysis (as a percentage of the total lactate dehydrogenase release caused by incubation with a lysis agent) of A549 cells incubated with PAO1, E90, or QS mutants of PAO1 or E90, or E90Δ*rhlI* with 10 μM C4-HSL. Asterisks (*) indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by OneWay ANOVA with a Bonferroni correction applied). Error bars represent the standard deviation for results of at least three independent experiments.

DISCUSSION

A substantial body of literature now suggests that the QS hierarchy of *P. aeruginosa* is adaptable and that *lasR* mutants can be rewired to be AHL QS proficient (46, 59, 60, 80). These rewired LasR-null clinical isolates retain the QS regulation of several exoproducts through the RhlI/RhlR circuit (46, 50). Prior studies examining a RhlR-dependent variant of PAO1 (52), and another LasR-null and RhlR-active clinical isolate(50), show that the parent strain outcompetes RhlR-null derivatives when grown in co-culture (50, 52). These findings support the notion that there is something inherently disadvantageous about mutation of RhlR and point to RhlR as a key QS transcription factor in chronic infections like CF.

We do not know the mechanism or genetic modifications that resulted in Las- independent RhlR activity in isolate E90. In strain PAO1, in which the hierarchy of QS was initially described, LasR mutants can readily evolve an independent RhlR QS system through inactivating mutations of *mexT*, which encodes a non-QS transcriptional regulator (52, 60). However, this is not the case in isolate E90, which possesses a functional *mexT* allele. We did observe that *rhlI* expression is upregulated by RhlR in E90 unlike in PAO1, where *rhlI* expression is predominately LasR-regulated (81). These data suggest that in E90 RhlR and RhlI may constitute a positive autoregulatory loop that may facilitate Las-independent RhlR activity. We are interested in investigating alternate mechanisms, other than inactivation of *mexT,* through which RhlR escapes LasR regulation in these rewired backgrounds.

In the present study, we aimed to identify which genes comprise the RhlR regulon in a clinical isolate, which may shed light on factors important for establishment or continuation of a chronic infection. Our RNA-seq analysis revealed that the E90 RhlR regulon bears a substantial amount of overlap with the suite of AHL-regulated genes previously identified in PAO1(8) and consists of virulence factors that are likely advantageous in the context of the CF lung.

A portion of the genes found to be RhlR-regulated in our transcriptomic analysis of E90 were not previously reported to be QS-regulated. These genes include *vqsR* (PAE90_2723/PA2591) and two genes of the *phz2* operon (PAE90_3614-3615/PA1899-1900). VqsR is itself a LuxR-homolog that serves to augment QS gene regulation, possibly through activation of the orphan QS receptor QscR, although the precise mechanism and biological outcomes of this interaction are still mysterious (82, 83). Our finding that the *phz2* operon, in addition to *phz1*, is activated by RhlR may reflect ongoing QS adaptation in our selected CF isolate. While E90 appears to produce slightly less pyocyanin in broth culture than PAO1, pyocyanin production by E90 may be comparatively greater in the biofilm lifestyle of the CF lung. The *phz2* locus, while showing roughly 98% nucleotide identity with *phz1*, has been shown to be responsible for nearly all the pyocyanin produced in biofilms by PAO1 and is the dominant contributor to murine lung colonization between the two loci (69). It is possible that some of these previously unreported QS-regulated genes were excluded from earlier transcriptome analyses(8, 62) due to different analysis approaches or methodology. Of particular note, we compared a *rhlR*-deletion mutant to the parent strain to derive our transcriptome, while some of these

previous studies used signal-synthase mutants with and without signal, which has been demonstrated, in the case of RhlR QS, to yield a different phenotype (84).

We also discovered RhlR-QS-regulation of many genes that are not present in the PAO1 genome. This list includes several hypothetical proteins activated as much as 15-fold in E90 compared to the RhlR mutant. The list also includes the gene encoding the halogenase PrnC, a protein involved in production of the antifungal antibiotic pyrrolnitrin which may be important in interspecific interactions in the CF lung (85). Our finding that RhlR-QS in E90 also appears to repress genes in the programmed cell death cassette *alpBCDE*, points to additional potential for QS regulation of population level interactions in CF-adapted strain E90.

FUTURE DIRECTIONS

The first study described herein provides a basis for characterizing the quorum sensing capacity of LasR-null clinical isolates. Although earlier studies had observed that RhlR-regulated activity may be retained in some LasR-null isolates, few attempts have been made to identify the totality of genes regulated by RhlR in this background. Our work expands upon previous findings by leveraging *de novo* genome sequencing and RNA-seq to characterize the RhlR quorum regulon in isolate E90. The scope of our analysis is limited by our examination of a single clinical isolate and laboratory growth conditions used for RNA-seq analysis. However, our data nevertheless provide a basis for understanding regulatory remodeling of QS activity and point to avenues for future investigation. Several important questions remain about QS in clinical isolates, including

whether or not there is a “core” regulon that is common to isolates that use either LasR or RhlR as the primary QS transcription factor.

Although we do not yet fully understand the biological significance of the RhlR-mediated suppression of the phage identified in this study, we are interested in exploring its role, if any, in fitness and inter- and intra-species competition in the near future. We note that had we used the PAO1 genome for read alignment we would have failed to identify the phage loci and a handful of other genes. These findings therefore argue in favor of using de novo genomes to improve transcriptome analyses of clinical and environmental isolates moving forward.

Strikingly, we found that in E90, RhlR but not LasR is the critical determinant of cytotoxicity in a three-dimensional lung epithelium cell aggregate model. Though our study did not reveal exactly which virulence factors are important for cell death in this model, our results nevertheless challenge the idea that LasR-null isolates are avirulent. Instead, our data argue that some virulence activity is conserved in rewired isolates, but that RhlR and not LasR is the primary regulator of several such functions. Additional experiments using animal models of infection are necessary to test this idea. In summary, our work sheds light on RhlR-specific gene regulation and reveals the potential breadth of QS activity and virulence functions retained in LasR-null, CF-adapted isolates.

CHAPTER V. RhlR-mediated Conflict and Cooperation in *P. aeruginosa*

INTRODUCTION

Cooperation, a social behavior that benefits other members of the population, is ubiquitous in the natural world and organisms have developed various strategies to stabilize cooperative behaviors(86–88). In bacterial populations, an example of cooperation is the production of public goods such as extracellular proteases and toxins, which are metabolically costly for individuals to produce, but can be utilized by all members of the group. Populations of cooperators are susceptible to invasion by defectors or “cheaters”, cells that forgo the metabolic burden associated with cooperation but still benefit from the public goods produced by cooperators (87). For this reason, cooperation is potentially unstable in the population and its ubiquity has puzzled evolutionary biologists.

Pseudomonas aeruginosa, a ubiquitous environmental gram-negative bacterium, has been used as a model organism for studying cooperation and conflict in bacterial populations (86, 89–93). Several of its potentially cooperative behaviors are regulated by quorum sensing (QS), a cell-cell communication system mediated by small signaling molecules. One example of a QS-regulated cooperative behavior is the production of the exoprotease elastase, which is required for growth using either bovine serum albumin or casein as a sole carbon and energy source (89, 94). Elastase is secreted into the extracellular milieu and proteolyzes casein into constituent amino acids or peptides, a readily usable carbon source. In a well-mixed environment, all cells in the population can benefit from elastase production regardless of whether or not they produce it themselves

(94); therefore elastase constitutes a “public good”. Previously, we and others have demonstrated that cheaters can eventually invade populations of QS-proficient cooperators (50, 89, 90) when *P. aeruginosa* PAO1 passaged in minimal media supplemented with 1% casein. These cheaters are QS-deficient and commonly harbor deleterious mutations in either *lasR* or *pqsR* (89)*;* however, no experimental evolution studies to date have reported mutations occurring in *rhlR* (50, 89). Similarly, in surveys of *P. aeruginosa* isolates from the CF lung, *rhlR* mutations are either rare or only occur decades following mutations in *lasR* (51).

The rarity with which *rhlR* mutations occur may reflect that loss of RhlR activity imparts a competitive disadvantage in co-culture with RhlR-proficient strains (50, 90, 91). Because *rhlR* mutations are uncommon in both experimental evolution studies as well as the CF lung, we hypothesize that a mechanism intrinsic to *P. aeruginosa* likely maintains RhlR QS within the population. One possible mechanism through which RhlR-null mutants are suppressed or outcompeted by RhlR-proficient strains is the RhlR-regulated production of, and resistance to, hydrogen cyanide (HCN) (50, 90). Other potential mechanisms, including production of phenazines by cooperators (95), have also been proposed. In light of recent studies including our own work that show LasR-null strains can evolve an active RhlR QS system independent of LasR regulation (52, 59, 60), we are interested in investigating how social dynamics may differ in a population that relies on RhlR instead of LasR to regulate cooperative behavior.

To answer questions pertaining to RhlR-mediated cooperation we studied the same LasR-null, RhlR active isolate described in the previous chapter, E90 (46). Because E90 uses RhlR to coordinate group behaviors such as elastase production (96), we refer to it as a "RhlR cooperator". In this study, we asked if RhlR cooperators are subject to cheating, as are LasR cooperators, and began to explore how RhlR stabilizes cooperation in the absence of a functional Las system. Understanding the role of RhlR in maintaining cooperation may offer insight on the possible advantages of hierarchical as opposed to parallel arrangement of QS circuits and shed light on the evolution of clinical isolates.

RESULTS

RhlR-null cheaters do not invade a population of RhlR cooperators

To investigate how social dynamics may differ in a population of RhlR cooperators, we serially passaged E90 in a minimal medium supplemented with casein ("casein broth") as the sole carbon and energy source for >100 generations, as described under Materials and Methods. Prior studies have shown that when strain PAO1 is serially passaged in casein broth, LasR-null mutants invariably invade the population and reach a frequency of about 20-30% (86, 89). LasR-null mutants are considered cheaters in this context because they do not produce elastase but still benefit from the production of elastase by QS-proficient or cooperating cells (94). Under some circumstances, the high frequency of social cheaters results in a population collapse by causing loss of a quorum.

We predicted that cheaters with *rhlR* mutations would invade the E90 population since inactivating RhlR would relieve the metabolic cost associated with producing public goods

including elastase in this background (Fig. 7). Unexpectedly, we did not detect the emergence of elastase-deficient cheats after 30 days of passage across 5 independent trials. To exclude the possibility that RhlR-null, constitutive elastase producers invaded the population instead, we performed Sanger sequencing on 5 elastase-positive isolates from each trial. We did not identify mutations in either the *rhlR* gene or its promoter sequence. The findings from our experimental evolution studies suggest that *rhlR* mutations are disfavored in the RhlR cooperator background.

Figure 7. In the LasR-null isolate E90, the ability to grow in casein broth is dependent on the RhlR-RhlI QS circuit. (Left to right): E90, E90 *rhlR,* and E90 *rhlI* monocultures grown for 24 h in minimal medium supplemented with 1% casein (wt/vol). Robust growth is evidenced by the production of the blue-green compound pyocyanin, which indicates that the population is quorate.

Mutations in *rhlR* confer a competitive disadvantage in co-culture

We initially hypothesized that RhlR mutants fail to invade the population because they exhibit growth defects compared to the parent strain. To test this idea, we engineered a clean deletion of *rhlR* in the E90 background and compared its growth kinetics and sensitivity to stationary phase stress in buffer Luria Bertani (LB) broth to the parent strain. We used LB for the remainder of our study because the RhlR-null mutant does not grow as a monoculture in casein broth. We found that the doubling time and growth yield of the RhlR-null mutant was similar to the parent strain after 24h of growth (~4-6x10^9 CFU/mL) (Fig. 8), ruling out the possibility that *rhlR* mutants are growth impaired.

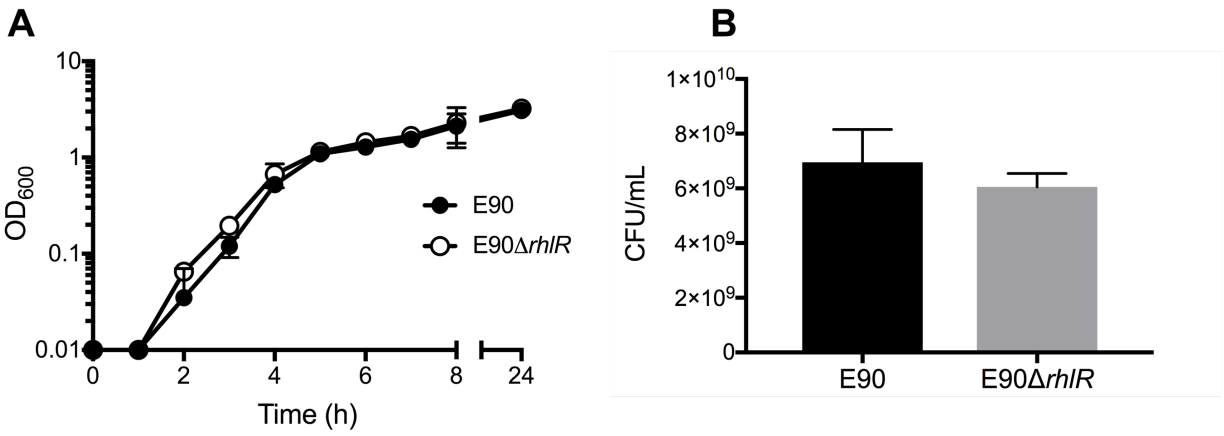


Figure 8. The RhlR-null mutant grows comparably to the RhlR cooperator in buffered Luria Bertani broth. A) Growth curve in MOPS-buffered LB in 18 mm culture tubes at 37°C. B) Cell yield following 24 h incubation. Data shown are the average and standard deviation of three biological replicates. In some cases, error bars are too small to be seen.

Next, we hypothesized that mutations in *rhlR* impart a disadvantage in competition. To assess the relative fitness of the *rhlR* deletion mutant depending on its frequency in the population, we co-cultured it with the parent strain at initial frequencies of 1%, 10%, or

50%. Using flow cytometry to count individual cells, we calculated the competitive index of the mCherry-tagged *rhlR* deletion mutant following three days of co-culture with serial passage every 24 h. We found that regardless of the initial frequency, the proportion of RhlR-null mutants in the population substantially decreased corresponding to a competitive index far below 1 (Fig. 9).

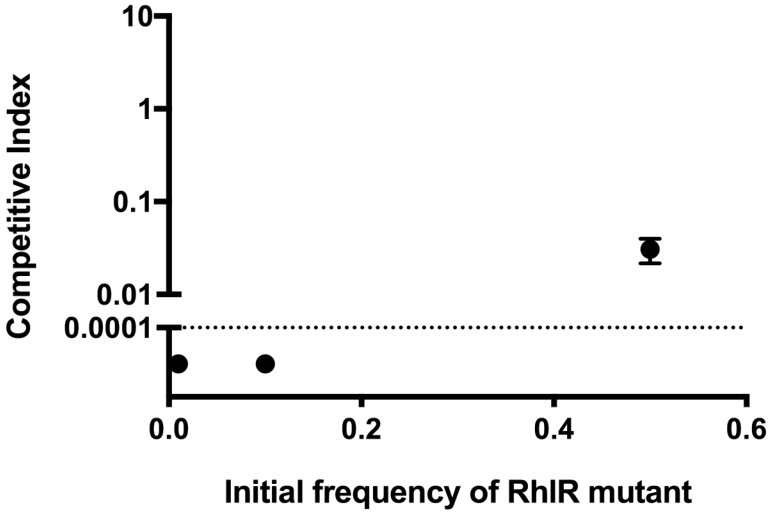


Figure 9. Mutations in *rhlR* confer a competitive disadvantage. The competitive index of the *rhlR* mutant is displayed on the y-axis. In relation to the parent strain, a competitive index of >1 indicates that the strain is more fit, =1 indicates that it is equally fit, and <1 indicates that it is less fit. The dashed line indicates the limit of detection for the flow cytometer. Data shown are the average and standard deviation of three biological replicates.

Because flow cytometry can enumerate both live and dead cells, we also patched ~100 individual colonies at the end of each co-culture experiment onto skim milk agar plates, as had been done previously (86, 90, 97), to survey the population for *rhlR* mutants. When plated onto skim milk agar, *rhlR* mutant colonies are distinguished by the absence of a "halo" or zone of clearance due to the inability to produce elastase. We found that our results from patching onto skim milk agar were similar to what we found by flow cytometry,

with a significant reduction in the fraction of cells that were RhlR-null. Our results suggest that *rhlR* mutants rarely emerge among RhlR cooperators because loss of RhlR activity confers a competitive defect.

The emergence of *rhlR* mutants is suppressed by an AHL-regulated mechanism

We tested whether the competitive advantage of RhlR cooperators is due to an intact RhlR-RhlI regulatory QS circuit. For this experiment, we co-cultured the parent RhlR cooperator with an isogenic *rhlI* (C4-HSL synthase) mutant or the *rhlR* mutant. Both the *rhlI* and *rhlR* mutants have an incomplete QS circuit and are incapable of engaging in RhlR QS in monoculture; however, we found that unlike the *rhlR* mutant, the *rhlI* mutant was equally fit as the parent strain in co-culture (Fig. 10).

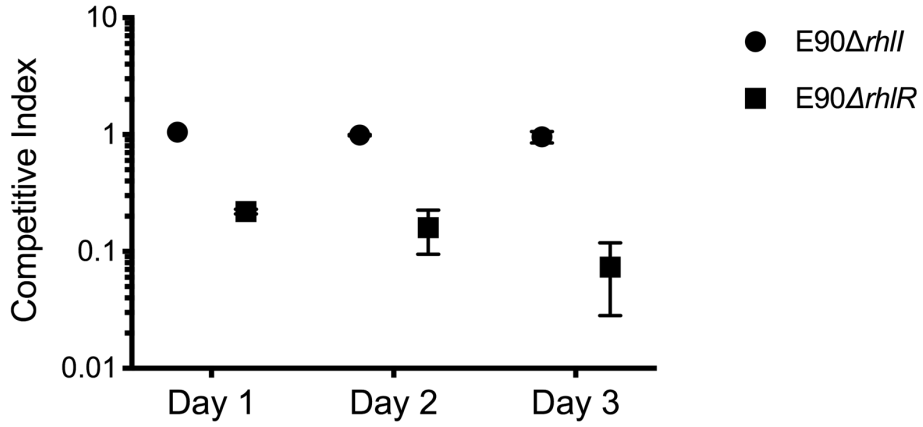


Figure 10. The relative fitness of a *rhlR* or *rhlI* mutant in co-culture with the RhlR cooperator in buffered LB. The competitive index of either the RhlR-null or RhlI-null mutant is displayed on the y-axis. In relation to the parent strain, a competitive index of >1 indicates that the strain is more fit, =1 indicates that it is equally fit, and <1 indicates that it is less fit. Data shown are the average and standard deviation of three biological replicates.

We reasoned that in contrast to the *rhlR* mutant, the RhlR activity of *rhlI* mutant is complemented *in trans* during co-culture with the parent strain since C4-HSL is freely diffusible across the membrane. As a complementary approach, we added AiiA lactonase, an enzyme that degrades AHLs (57), to the RhlR cooperator and *rhlR* mutant co-culture. The addition of lactonase was able to partially rescue the relative fitness of the *rhlR* mutant (Fig. 11). Additionally, we found that growth of a *rhlR* mutant monoculture is unperturbed by the addition of 10 μM C4-HSL, the concentration produced by the parent strain, demonstrating that the signal itself is not toxic (Fig. S4). We concluded that a C4-HSL-dependent mechanism confers a competitive advantage to RhlR cooperators.

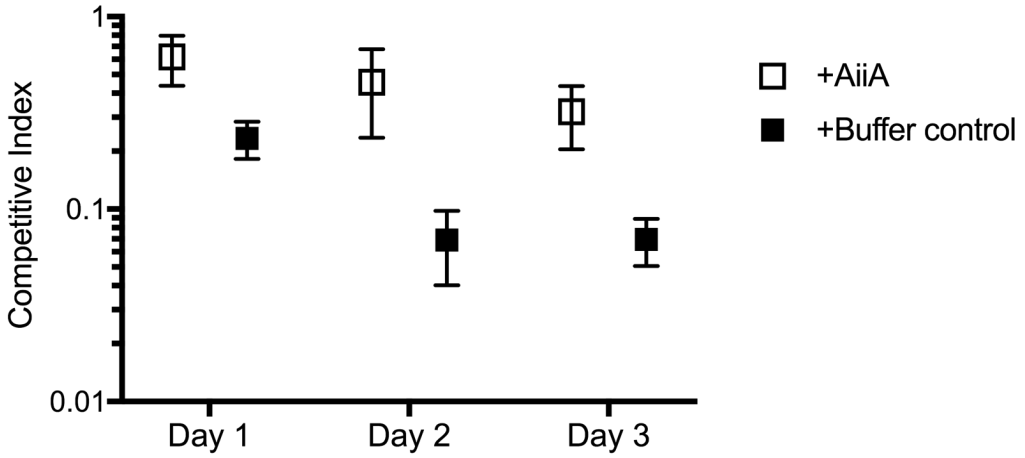


Figure 11. The addition of AiiA lactonase rescues the relative fitness of the *rhlR* mutant in co-culture with the parent RhlR cooperator. The competitive index of the *rhlR* mutant following addition of 100 μg/mL AiiA lactonase or a buffer control is displayed on the y-axis. In relation to the parent strain, a competitive index of >1 indicates that the strain is more fit, =1 indicates that it is equally fit, and <1 indicates that it is less fit. Data shown are the average and standard deviation of three biological replicates.

HCN production alone does not suppress *rhlR* mutants in the E90 background

Previous reports have highlighted the importance of hydrogen cyanide production in "policing" cheaters in QS populations of *P. aeruginosa* (50, 90). In PAO1, the RhlR-regulated production of HCN plays a prominent role in stabilizing cooperation; both *rhlR* mutants and HCN synthase mutants are unable to control LasR-deficient cheaters in the population (90). Recently, Chen *et al.* reported similar findings in E80, a LasR-null, RhlR-active *P. aeruginosa* isolate. In the latter study, *rhlR* mutants likely did not emerge in their experimental evolution studies because they have increased sensitivity to HCN than the parent strain (50). To test whether HCN production was responsible for the suppression of *rhlR* mutants in the E90 background, we competed a E90Δ*hcnC* mutant with the E90Δ*rhlR* mutant and found that the latter was still outcompeted in co-culture (Fig. 12). Our findings suggest that HCN production alone is not responsible for the maintenance of cooperation in E90. This observation is consistent with other strains of *P. aeruginosa*, which do not appear to produce HCN in concentrations sufficient to police cheaters (52). Next, we asked whether E90 secretes one or more other products that could impair RhlR-null mutants by assaying E90-spent supernatant for antimicrobial activity. To answer this question, we monitored the growth of E90Δ*rhlR* monocultures incubated with the cell-free supernatant of an E90 monoculture. However, we found that the E90-spent supernatant had no effect on the growth of the E90Δ*rhlR* mutant (Fig. S5).

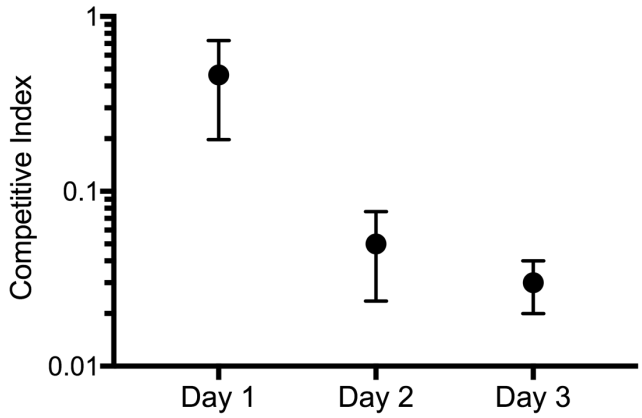


Figure 12. HCN production by cooperators does not control *rhlR* mutants in the E90 background. The competitive index of the RhlR-null mutant following co-culture with the HCN synthase-deficient RhlR cooperator is displayed on the y-axis. In relation to the parent strain, a competitive index of >1 indicates that the strain is more fit, =1 indicates that it is equally fit, and <1 indicates that it is less fit. Data shown are the average and standard deviation of three biological replicates.

Using RNA-seq to identify factors that enable E90 to outcompete *rhlR* mutants

A potential drawback to the experiment described in the previous section is that our preparation of cell-free supernatant may not capture products that are volatile, highly reactive, or transiently produced. Therefore, as a complementary approach we analyzed genes that are differentially expressed by the E90 parent strain using RNA-seq. To optimize the conditions used for RNA-seq, we performed a time course experiment to identify when the RhlR-null mutant gets outcompeted during co-culture with E90. Having learned that the RhlR-null mutants are outcompeted by t = 8 hours (Fig. 13), we reasoned that studying gene expression at this time point may offer insight regarding which RhlR-regulated factors confer a competitive advantage.

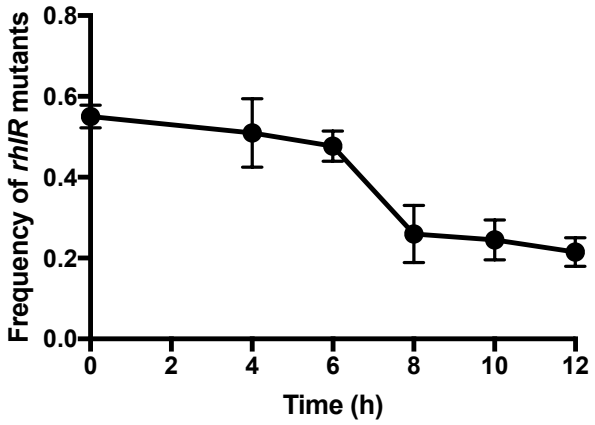


Figure 13. RhlR cooperators outcompete *rhlR* mutants during stationary phase. The medium is inoculated with a 1:1 ratio of E90 and E90Δ*rhlR* cultures, or an intial frequency of 0.5 for each strain. The frequency of the RhlR-null mutant during is displayed on the y-axis as a function of time in hours. Data shown are the average and standard deviation of three biological replicates.

At t = 8 hours, we found a total of 33 upregulated genes in the E90 background compared to the E90Δ*rhlR* mutant. The top 10 hits from our transcriptomic analysis are summarized below in Table 2 and include genes necessary for the production of rhamnolipids, azetidomonamides, as well as fatty acid and AHL signal synthesis.

Table 2. The 10 most highly regulated genes in E90 at t = 8 hours

Gene name[a]	Product description[b]	Fold change[c]
rhlA	rhamnosyltransferase chain A	11.6
rhlB	rhamnosyltransferase chain B	11.0
hcnA	hydrogen cyanide synthase	4.8
PA3330	probable short chain dehydrogenase	4.7
PA3328	probable FAD-dependent monooxygenase	4.2
PA3332	conserved hypothetical protein	4.2
PA3327	probable non-ribosomal peptide synthetase	4.2
lasB	elastase	4.1
acp1	acyl carrier protein; Acp1	4.1
acp3	acyl carrier protein; Acp3	4.1

a. Gene names from PAO1-UW reference annotation (PAO1_107; see Materials and Methods) available on the Pseudomonas Genome Database
b. Product descriptions from PAO1-UW reference annotation
c. Fold change values were determined by DEseq as described in Materials and Methods

DISCUSSION

P. aeruginosa is an opportunistic pathogen that infects nearly 50% of all CF patients with an 80% prevalence in adults (36). Despite the importance of the QS regulator LasR in establishing infection, our group and others have demonstrated that LasR is commonly mutated in chronic *P. aeruginosa* CF isolates (46–48). In contrast to *lasR*, the coding sequence of *rhlR* is largely conserved and we have shown that RhlR QS is maintained in many LasR-null isolates (46). In addition, prior experimental evolution studies performed under conditions requiring QS for growth have also failed to observe mutations in *rhlR*

(50, 89). While others have demonstrated that RhlR mutants are rare because they are likely outcompeted by RhlR-active strains (49, 50, 90), relatively little is known about the myriad strategies used to maintain RhlR-mediated QS or cooperative behavior.

To understand how social dynamics may differ in a LasR-null, RhlR-active, or "RhlR cooperator" background, we serially passaged E90 in minimal medium with casein as the sole carbon source, which requires QS-regulated production of elastase for growth. In E90, elastase production is dependent on RhlR. Interestingly, we found that RhlR-null cheats were consistently unable to emerge among the RhlR cooperators, a finding that is consistent with that of Chen *et al*. To understand why RhlR-null mutants are heavily disfavored, we studied the viability and relative fitness of an engineered, RhlR-null mutant. We found that RhlR-null mutants begin to be outcompeted by the parent strain during stationary phase due to an AHL QS-dependent mechanism. Currently, two strategies for maintaining QS in laboratory strains have been described: the RhlR-regulated production of and resistance to hydrogen cyanide (HCN) and regulation of rhamnolipid production (90, 92). However, we have shown that these strategies do not account for the phenotype we observe in the RhlR cooperator E90: a mutant deficient in HCN production is still able to outcompete the RhlR-null mutant and rhamnolipids are not required for planktonic growth.

In an attempt to discern whether RhlR cooperators secrete a factor that intoxicates or otherwise impairs *rhlR* mutants, we assayed the growth of the RhlR-null mutant in the presence of E90-spent supernatant. Although the E90-spent supernatant had no

bactericidal or bacteriostatic effect on the RhlR-null mutant, it is possible that the factor(s) may be highly volatile and would lose activity during our preparation of the cell-free supernatant. Therefore, we employed an RNA-seq approach to identify RhlR-regulated factors that might confer a competitive advantage over RhlR-null mutants. Our transcriptomic analysis revealed that the most upregulated genes include those necessary for the production of HCN, azetidomonamides, AHL signals, and fatty acids. Although we had previously determined that HCN production alone does not confer a competitive advantage, HCN may work in conjunction with other factors to outcompete RhlR-null mutants. Previously, our group showed that a combination of QS-regulated antimicrobials protected PAO1 from cheating by *Burkholderia multivorans* in a laboratory co-culture model (93). It is possible that E90 employs a similar strategy to prevent cheating by RhlR-null mutants. To test this hypothesis, the next logical step would be to engineer a combinatorial deletion mutant lacking two or more antimicrobials such as hydrogen cyanide or rhamnolipids, both of which were identified in our RNA-seq analysis (Table 2). An alternate hypothesis is that the products of the genes we identified confer a private benefit to E90, the effects of which are only apparent in co-culture with RhlR-null mutants.

FUTURE DIRECTIONS

Our findings highlight the diversity of strategies used to maintain RhlR quorum sensing and cooperation. While we have yet to identify exactly which combination of factors prevent RhlR-null mutants from emerging in the E90 background, our results nevertheless support the hypothesis that inhibiting the RhlR-RhlI QS circuit may be beneficial in the

context of a chronic infection. Even in a rewired background, the loss of RhlR activity appears to be disadvantageous. In theory, inhibiting RhlR activity could destabilize populations of QS-proficient *P. aeruginosa* as it would result in reduced expression of QS-regulated antimicrobials. Even if a small proportion of cells developed resistance to a RhlR inhibitor, a proof-of-concept study predicts that these resistant cells would still be dominated by sensitive cells (98). Recent research efforts have looked towards the design or discovery of small molecules that inhibit RhlR function (41, 43, 99); however, the long-term effects of RhlR inhibition on the evolutionary trajectory of clinical isolates remains unclear. Investigating how the use of a RhlR inhibitor may affect population dynamics or the emergence of resistance to QS-based therapies would help clarify the viability of such an approach.

SUPPLEMENTAL DATA

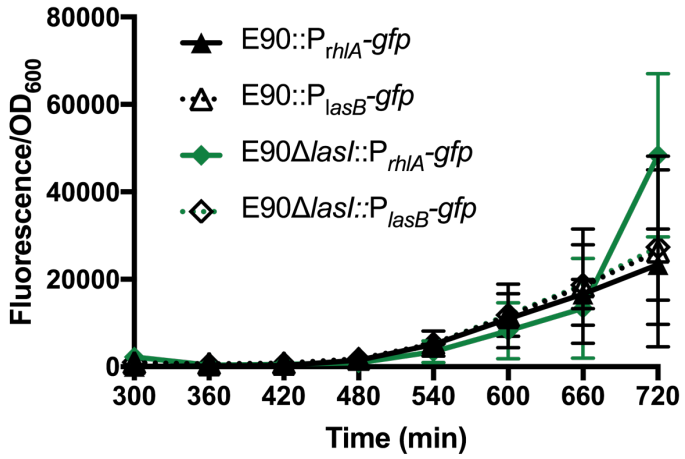


Figure S1. QS activity in E90 is not dependent on *lasI*. Expression of *lasB* or *rhlA* in either E90 or E90Δ*lasI*. Data from the first five hours are excluded because cell density measurements were below the limit of detection of the plate reader. Error bars represent the standard deviation for results of three independent experiments. Please note that in some cases, error bars are too small to be seen.

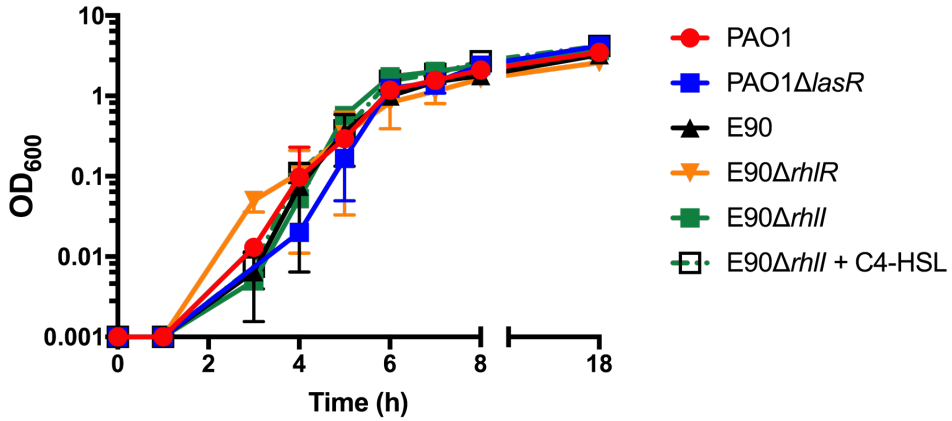


Figure S2. Growth curves of PAO1, E90, and PAO1 or E90-derived mutants in 3 mL of MOPS-buffered Luria Bertani broth. Error bars represent the standard deviation for results of three independent experiments. In some cases, error bars are too small to be seen.

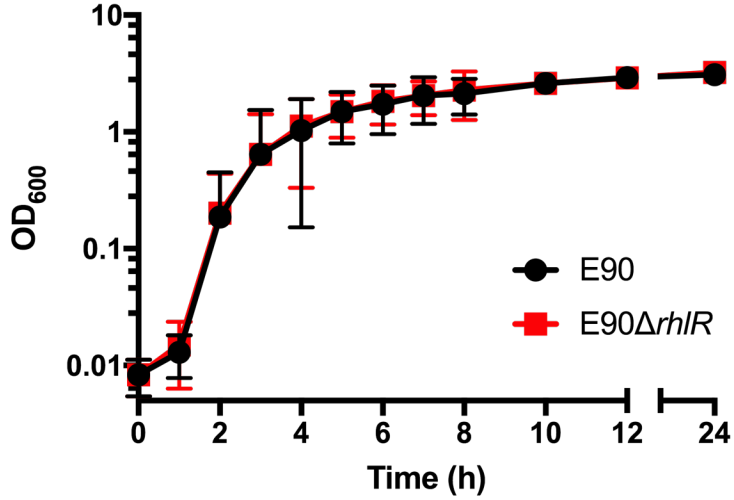


Figure S3. Growth curves of E90 and E90Δ*rhlR* in buffered Luria-Bertani Broth in 125-mL baffled flasks. Means and standard deviation of biological replicates are shown (n=3). In some cases, error bars are too small to be seen.

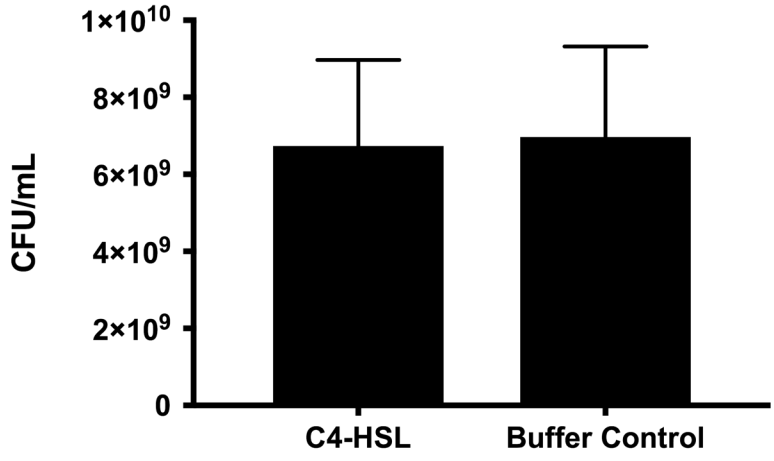


Figure S4. C4-HSL alone is not toxic to the RhlR mutant. Growth yield of the E90 *rhlR* mutant incubated with 10 μM exogenous C4-HSL or a buffer control (acidified ethyl acetate) after 18 h in buffered LB. Data are the average of three independent biological replicates.

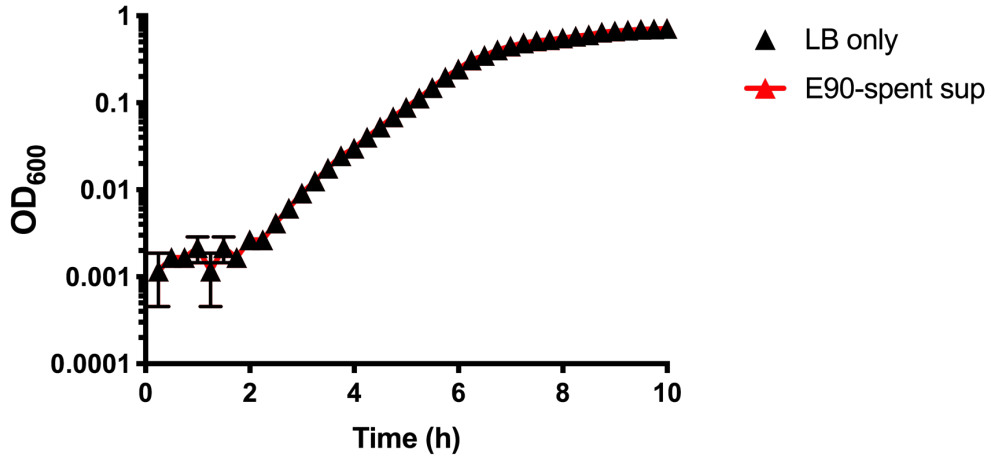


Figure S5. E90-spent supernatant does not perturb the growth of a RhlR-null mutant. Data are the average of three independent biological replicates. In some cases, error bars are too small to be seen.

Table S1. Complete list of RhlR-activated genes in strain E90.

De novo ID[a]	Gene name[b]	Product description[c]	Fold change	PAO1 ID[d]	A.A. % Identity[e]
PAE90_1621	*rhlB*	rhamnosyltransferase chain B	5688.8	PA3478	100.0
PAE90_1620	*rhlA*	rhamnosyltransferase chain A	1956.4	PA3479	99.7
PAE90_0833	*phzC1*	phenazine biosynthesis protein PhzC	129.4	PA4212	99.8
PAE90_1777		probable FAD-dependent monooxygenase	64.5	PA3328	100.0
PAE90_1773		conserved hypothetical protein	60.9	PA3332	100.0
PAE90_1775		probable short chain dehydrogenase	52.0	PA3330	100.0
PAE90_3307	*hcnA*	hydrogen cyanide synthase HcnA	35.0	PA2193	99.0
PAE90_1771		probable acyl carrier protein	32.6	PA3334	100.0
PAE90_3647		probable acyl carrier protein	32.1	PA1869	98.7
PAE90_1364	*lasB*	elastase LasB	32.1	PA3724	100.0
PAE90_0834	*phzB1*	probable phenazine biosynthesis protein	30.9	PA4211	99.4
PAE90_0835	*phzA1*	probable phenazine biosynthesis protein	29.0	PA4210	100.0
PAE90_1778		probable non-ribosomal peptide synthetase	28.3	PA3327	99.5
PAE90_1772	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III	23.5	PA3333	100.0
PAE90_1776		hypothetical protein	20.4	PA3329	99.8
PAE90_2705		**hypothetical protein**	**15.5**	*	*
PAE90_0837		**hypothetical protein**	**14.7**	*	*
PAE90_2723	***vqsR***	**VqsR**	**14.4**	**PA2591**	**100.0**
PAE90_1770		hypothetical protein	13.2	PA3335	99.6
PAE90_0133		**hypothetical protein**	**12.2**	*	*
PAE90_1774		cytochrome P450	10.9	PA3331	100.0
PAE90_2704		probable transcriptional regulator	10.6	PA2848	37.9
PAE90_1743	*lecB*	fucose-binding lectin PA-IIL	10.6	PA3361	100.0
PAE90_1779	*clpP2*	ClpP2	9.6	PA3326	100.0
PAE90_3305	*hcnC*	hydrogen cyanide synthase HcnC	8.2	PA2195	100.0
PAE90_3615	***phzA2***	**probable phenazine biosynthesis protein**	**7.3**	**PA1899**	**99.4**
PAE90_3306	*hcnB*	hydrogen cyanide synthase HcnB	7.2	PA2194	99.6
PAE90_1623	*rhlI*	autoinducer synthesis protein RhlI	7.0	PA3476	99.5
PAE90_2703		**Halogenase PrnC**	**6.5**	*	*
PAE90_4443		probable major facilitator superfamily (MFS) transporter	6.4	PA1131	99.1
PAE90_1769		probable major facilitator superfamily (MFS) transporter	6.2	PA3336	99.7

PAE90_2721		probable periplasmic spermidine/putrescine-binding protein	5.7	PA2592	100.0
PAE90_0911		hypothetical protein	5.2	PA4141	100.0
PAE90_1619		probable deoxycytidine triphosphate deaminase	4.8	PA3480	99.5
PAE90_3645	*lasA*	LasA protease precursor	4.0	PA1871	99.0
PAE90_6618		**hypothetical protein**	**3.8**	*	*
PAE90_3167	*ambD*	AmbD	3.8	PA2303	99.7
PAE90_4348		hypothetical protein	3.6	PA1216	100.0
PAE90_3035		**hypothetical protein**	**3.5**	*	*
PAE90_6270		hypothetical protein	3.5	PA5220	99.6
PAE90_4216	*lasI*	autoinducer synthesis protein LasI	3.4	PA1432	100.0
PAE90_3614	***phzB2***	**probable phenazine biosynthesis protein**	**3.4**	**PA1900**	**100.0**
PAE90_4343		hypothetical protein	3.1	PA1221	99.3
PAE90_3168	*ambE*	AmbE	3.0	PA2302	99.1
PAE90_2714		conserved hypothetical protein	3.0	PA5232	28.4
PAE90_0838	*phzM*	probable phenazine-specific methyltransferase	2.9	PA4209	99.7
PAE90_4868	*cbpD*	chitin-binding protein CbpD precursor	2.7	PA0852	100.0
PAE90_3166	*ambC*	AmbC	2.7	PA2304	99.4
PAE90_1624	*pheC*	cyclohexadienyl dehydratase precursor	2.6	PA3475	98.9
PAE90_4444	*rhlC*	rhamnosyltransferase 2	2.6	PA1130	98.8
PAE90_2866	*lecA*	LecA	2.4	PA2570	100.0
PAE90_3882	*hsiA2*	HsiA2	2.2	PA1656	100.0
PAE90_2720	*qteE*	quorum threshold expression element, QteE	2.2	PA2593	100.0

a. “PAE90” identification numbers correspond to locus tags in the E90 *de novo* genome.

b. Gene names from PAO1-UW reference annotation (PAO1_107) available on the Pseudomonas Genome Database (https://www.pseudomonas.com).
c. Product descriptions from PAO1-UW reference annotation (PAO1_107), with the exception of those genes not present in the PAO1 genome (see *) which are described as annotated in the *de novo* genome.
d. PAO1 identification numbers from PAO1-UW reference annotation (PAO1_107).
e. A.A., amino acid. Amino acid identity relative to PAO1-UW (PAO1_107) reference sequences listed under PAO1 ID.
* Denotes genes not present in the PAO1-UW genome (blastp bit score less than 50).
Bold denotes genes not previously identified as QS-regulated (Schuster et al., 2003).

Table S2. Complete list of RhlR-repressed genes in strain E90.

De novo ID[a]	Gene name[b]	Product description[c]	Fold change	PAO1 ID[d]	A.A. % Identity[e]
PAE90_2418		hypothetical protein	-2.0	*	*
PAE90_2416		hypothetical protein	-2.0	*	*
PAE90_2453		probable bacteriophage protein	-2.1	PA0640	49.5
PAE90_2415		Phage protein	-2.1	*	*
PAE90_2457		hypothetical protein	-2.1	*	*
PAE90_2420		hypothetical protein	-2.2	*	*
PAE90_2419	*rsmA*	RsmA	-2.2	PA0905	61.0
PAE90_2455		probable bacteriophage protein	-2.2	PA0641	63.4
PAE90_2459		hypothetical protein	-2.5	PA0647	50.0
PAE90_2444		hypothetical protein	-2.6	PA0636	40.9
PAE90_2460		hypothetical protein	-2.7	PA0648	70.3
PAE90_2446		probable bacteriophage protein	-2.7	PA0638	48.4
PAE90_2445		conserved hypothetical protein	-2.8	PA0637	40.0
PAE90_2428	*alpB*	AlpB	-2.8	PA0908	53.8
PAE90_2456		hypothetical protein	-2.9	*	*
PAE90_2433		Phage protein	-2.9	*	*
PAE90_2442		JK_16P	-3.0	*	*
PAE90_2443		hypothetical protein	-3.0	*	*
PAE90_2431		hypothetical protein	-3.0	*	*
PAE90_2432		62kDa structural protein	-3.0	*	*
PAE90_2438		hypothetical protein	-3.1	*	*
PAE90_2441		probable phage protein YPO2116	-3.1	*	*
PAE90_2439		hypothetical protein	-3.2	*	*
PAE90_2436		hypothetical protein	-3.3	*	*
PAE90_2429	*alpC*	AlpC	-3.3	PA0909	50.5
PAE90_2461		conserved hypothetical protein	-3.4	PA0629	77.5
PAE90_2440		hypothetical protein	-3.5	*	*
PAE90_2430		hypothetical protein	-3.8	*	*
PAE90_2435		hypothetical protein	-3.8	*	*
PAE90_2434		hypothetical protein	-3.8	*	*

a. “PAE90” identification numbers correspond to locus tags in the E90 *de novo* genome.

b. Gene names from PAO1-UW reference annotation (PAO1_107) available on the Pseudomonas Genome Database (https://www.pseudomonas.com).

c. Product descriptions from PAO1-UW reference annotation (PAO1_107), with the exception of those genes not present in the PAO1 genome (see *) which are described as annotated in the *de novo* genome.

d. PAO1 identification numbers from PAO1-UW reference annotation (PAO1_107).

e. A.A., amino acid. Amino acid identity relative to PAO1-UW (PAO1_107) reference sequences listed under PAO1 ID.

* Denotes genes not present in the PAO1-UW genome (blastp bit score less than 50).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are described below in Table S3. E90 is part of a collection of clinical isolates obtained via the Early *Pseudomonas* Infection Control Observational (EPIC Obs) Study(54). The isolates are from oropharyngeal and sputum samples from 5-12 year-old patients. Further details regarding the EPIC Obs study design and results have been described previously (54, 100).

For the transcriptional reporter assays as well as pyocyanin and AHL measurements, overnight cultures were started from single colonies grown in 3 mL of Luria-Bertani (LB) broth buffered with 50 mM morpholinopropanesulfonic acid (MOPS) in an 18 mm culture tube. For the cytotoxicity experiments, overnight cultures were started from single colonies in 5 mL of unbuffered LB broth. When appropriate, antibiotics were added at the following concentrations: 10 µg/mL gentamicin or 100 µg/mL ampicillin for *Escherichia coli*, and 100 µg/mL gentamicin for *P. aeruginosa*. Cells were grown at 37˚C with shaking at 250 RPM unless stated otherwise. For complementation experiments, synthetic C4-HSL (Cayman Chemical) was added to E90Δ*rhlI* deletion mutant cultures to a final concentration of 10 µM.

For directed evolution experiments in casein, overnight cultures were started from single colonies as described above then diluted 1:20 in 4 mL of casein broth, a minimal medium (101) supplemented with 1% casein (wt/vol) (Sigma Aldrich) as the carbon source. Cultures were then serially passaged 1:20 in casein broth for up to 30 days.

Table S3. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Description	Reference / source
P. aeruginosa strains		
PAO1	Wild-type strain	60
PAO1Δ*lasR*	PAO1 containing an unmarked, in-frame *lasR* deletion	
PAO1Δ*rhlR*	PAO1 containing an unmarked, in-frame *rhlR* deletion	88
E90	Chronic infection isolate from pediatric CF patient, 1 bp del (-C) at nt 170 in *lasR*	45
E90Δ*rhlR*	E90 containing an unmarked, in-frame *rhlR* deletion	This study
Other strains		
E. coli DH5α	F- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rk-, mk+) *phoA supE44* λ-*thi*-1 *gyrA96 relA1*	Invitrogen
S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	101
Plasmids		
pECP61.5	Contains an *rhlA-lacZ* translation fusion and IPTG-inducible *rhlR* , ApR	
pPROBE-GT	Broad-host-range pVS1/p15a GFP reporter, GmR	102
pJF01	pPROBE-GT with -502 through +31 of 5' region of *rhlA* inserted with HindIII and BamHI, GmR	45
pBS351	pPROBE-GT with -282 through +223 of 5' region of *lasI* inserted with HindIII and BamHI, GmR	45
pBS413	pPROBE-GT with -290 through +217 of 5' region of *lasB* inserted with SalI and BamHI, GmR	This study
pEXG2-Δ*rhlR*	pEXG2 with Δ*rhlR* deletion construct extending from -300 to +300 with respect to translation translation start site; GmR	88

LasR and RhlR activity

LasR and RhlR-specific promoter fusions constructed in pPROBE-GT have been described previously (46). Electrocompetent *P. aeruginosa* cells were prepared through repeated washing and resuspension of cell pellets in 300 mM sucrose(104).

Transformants were obtained by plating on LB agar supplemented with gentamicin and verified by PCR.

Experimental cultures were prepared as follows: first, overnight cultures were grown with the addition of 100 µg/mL gentamicin and 100 µg/mL AiiA lactonase, the latter inhibiting AHL-mediated QS(57). The addition of AiiA lactonase eliminates residual GFP fluorescence that would otherwise arise from previously induced reporter gene expression during overnight growth. Overnight cultures were then diluted to an optical density (OD_{600}, 1 cm pathlength) of 0.001 (approximately 1-5 $x10^6$ CFU/mL) in 3 mL MOPS-buffered LB supplemented with AiiA lactonase in 18 mm culture tubes. After these cultures grew to an approximate OD_{600} of 0.2, they were diluted to OD_{600} 0.001 in 400 µL of MOPS-buffered LB alone in a 48-well plate with a clear bottom (Greiner Bio-One). To prevent evaporation, strains were only grown in wells that did not line the edges of the plate and all empty wells were filled with 400 µL water. We monitored GFP fluorescence and OD_{600} at 30-minute intervals for 15 hours using a BioTek Synergy HI microplate reader (excitation: 489 nm, emission: 520 nm, gain: 80). All strains were grown at 37˚C with shaking for the duration of the assay. To account for differences in growth, results were normalized to OD_{600}. As a negative control, each strain was electroporated with an empty vector, which was used to establish a baseline level of background fluorescence. The fluorescence intensity was calculated by subtracting the background fluorescence from the total fluorescence measured at every time point. All experiments were performed in biological triplicate.

Construction of various E90-derived mutants

A homologous recombination approach was used to generate an in-frame deletion mutant (105, 106). Fragments flanking the gene of interest (GOI) was PCR-amplified from E90 genomic DNA and cloned into pEXG2 to yield pEXG2.GOI, which was then transformed into *E. coli* S17-1 in order to facilitate conjugal transfer of pEXG2.GOI into E90. Transconjugants were selected by plating on *Pseudomonas* isolation agar supplemented with gentamicin, and deletion mutants were counter-selected by plating onto LB agar with 10% (wt/vol) sucrose. Deletion of GOI was confirmed by PCR and targeted sequencing.

Co-culture experiments

Overnight cultures of the indicate strains were started from single colonies as described under “Bacterial strains and growth conditions”. On the day of the experiment, cultures were back-diluted 1:100 in 3 mL of buffered LB and grown until mid-log phase (OD_{600} 0.3-0.6). Inocula for the co-culture experiments were prepared from mid-log cultures. The initial frequencies of each strain were verified by performing flow cytometry as described previously (107) with the following modifications: ten thousand events were counted at the end of each co-culture experiment, and we detected the RhlR mutant using an mCherry tag (excitation: 580 nm, emission: 610 nm) and its competitors using a GFP tag (excitation: 488 nm, emission: 510 nm). Competitive indices were calculated by determining the final RhlR mutant-to-competitor ratio, then dividing it by the initial RhlR mutant-to-competitor ratio (108). A competitive index greater than 1 indicates that the RhlR mutant is more fit than its competitor, equal to 1; equally fit, and less than 1; less fit.

Assaying RhlR mutant growth in E90-spent supernatant

Overnight cultures were started from single colonies as described under "Bacterial strains and growth conditions". E90 cultures were then back-diluted 1:100 in 3 mL of buffered LB and grown until stationary phase: t = 8 h, OD_{600} 2.1 – 2.3. Cell-free supernatant was prepared by pelleting the E90 cells via centrifuge and filtering the supernatant using a 0.2 µm filter. On the day of the growth experiment, E90 *rhlR* mutant overnight cultures were back-diluted 1:1000 in 400 µL of a 1:1 mixture of buffered LB and E90-spent supernatant; the spent supernatant alone does not support growth. OD_{600} was measured in a 48-well plate (Greiner Bio-One) using a BioTek Synergy HI microplate reader as described under "LasR and RhlR activity".

AHL signal extraction and measurement

Experimental cultures were prepared from overnight cultures diluted to OD_{600} 0.001 in 3 mL of MOPS-buffered LB in an 18 mm culture tube. Experimental cultures were grown with shaking until they reached OD_{600} 2.0. Then, AHL signals were extracted from experimental cultures using acidified ethyl acetate as described elsewhere(109). We used an *E. coli* DH5α strain containing either pJN105L and pSC11 in conjunction with the Tropix® Galacto-Light™ chemiluminescent assay (Applied Biosystems) to measure 3OC12-HSL, or containing pECP65.1 to measure C4-HSL (14, 58, 110).

Protease and pyocyanin measurements

Experimental cultures were prepared from overnight cultures by diluting to OD_{600} 0.001 in 3 mL MOPS-buffered LB in 18 mm culture tubes. For secreted protease measurements, experimental cultures were grown with shaking for 18 h. Then, cells were

pelleted and 100 µL of filtered supernatant were collected to measure protease production using the FITC-Casein for Pierce Fluorescent Protease Assay Kit (ThermoFisher Scientific). For pyocyanin measurements, experimental cultures were grown with shaking for 18 h and pyocyanin was extracted from cultures as described previously(46). We grew strains in MOPS-buffered LB to remain consistent with the growth conditions used for RNA-seq analysis.

Cytotoxicity of three-dimensional A549 cell cultures

A three-dimensional lung epithelial cell culture model was generated by culturing A549 cells (ATCC CCL-185) on porous microcarrier beads in a rotating well vessel (RWV) bioreactor system, as described previously (78). A549 cells were grown in GTSF-2 medium (GE Healthcare) supplemented with 2.5 mg/L insulin transferrin selenite (ITS) (Sigma-Aldrich), 1.5 g/L sodium bicarbonate, and 10% heat-inactivated FBS (Invitrogen), and incubated at 37˚C under 5% CO_2, >80% humidity conditions. Infection studies were performed on cultures grown for 11 to 14 days in the RWV. Thereafter, 3-D cell cultures were equally distributed in a 48-well plate at a concentration of 2.5×10^5 cells/well (250 µL volume), and infected with the different strains at a targeted multiplicity of infection of 30:1 as described previously(79). All infection studies were performed in the above-described cell culture medium, with the exception that no FBS was added given the interference of serum compounds with QS signaling (111). After 24 h infection, the release of cytosolic lactate dehydrogenase (LDH) from 3-D lung epithelial cell cultures was determined using a LDH activity assay kit (Sigma-Aldrich) according to the manufacturer's instructions. A standard curve using NADH was included. The positive

control (theoretical 100% LDH release) was obtained by lysing 2.5 × 10^5 cells with 1% Triton-X100. All LDH release values were expressed as a percentage of the positive control.

RNA isolation and qRT-PCR

Overnight cultures were started from single colonies grown in 3 mL of MOPS-buffered LB in 18 mm culture tubes. Experimental cultures were prepared by diluting overnight cultures to OD_{600} 0.01 in 25 mL of MOPS-buffered LB in 125-mL baffled flasks. Experimental cultures were grown at 37˚C with shaking at 250 RPM. Approximately 1 x 10^9 cells were pelleted at OD_{600} 2.0 and mixed with RNA Protect Bacteria reagent (Qiagen) before being stored at -80˚C. Thawed cell pellets were resuspended in QIAzol reagent and mechanically lysed by bead beating. To extract RNA, we used the RNeasy kit (Qiagen) according to manufacturer's instructions. Isolated RNA was then treated with Turbo DNAse (Ambion) and purified using the MinElute cleanup kit (Qiagen). Three biological replicates were processed for each strain (E90 and E90Δ*rhlR*). Next, cDNA was prepared using the iScript™ cDNA Synthesis Kit (BioRad). Then, expression of target genes was analyzed by following the protocol for the iQ™ SYBR® Green SuperMix (BioRad) on a CFX96 Real-Time PCR cycler for a total of 40 cycles. We analyzed expression of the following genes: *lasI, lasB, rhlI, rhlR, rhlA, pqsA, chiC, aprA, vqsR* (PAE90_2723), PAE90_0133, PAE90_0837, and PAE90_2705. We used *rplU* as a reference gene.

Whole-genome sequencing, RNA-seq, and differential gene expression analysis

We generated the complete circular sequence of E90 using a *de novo* whole-genome sequencing approach. High-molecular weight (HMW) genomic DNA was isolated from overnight E90 liquid culture using the Genomic-tip 20/G kit (Qiagen). Genomic DNA was sequenced separately using the following two approaches. For short reads, genomic DNA was subjected to 300 bp PE sequencing on the Illumina MiSeq platform using TruSeq v3 reagents to yield approximately 20 M raw reads which were then groomed using Trimmomatic (v0.36; adapter trimming, paired reads only, Phred score cutoff=15) (112). For long reads, genomic DNA was prepared into two ligation-mediated (SQK-LSK109, Oxford Nanopore) libraries: one barcoded via PCR (EXP-PBC001) and the other via native barcoding (EXP-NBD114). Libraries were then subjected to sequencing on the Nanopore MinION platform using R9.4.1 pores. Nanopore reads were base-called and de-multiplexed using Guppy (v3.1.5, Oxford Nanopore), further groomed to remove adapters and for quality using Porechop (v0.2.4) (113), and final statistics determined in NanoPack (NanoPlot v1.27.0; NanoQC v0.9.1) (114) (read length N50=12kb; median read quality=Q12.6). All reads were then combined in a hybrid *de novo* assembly approach using the Unicycler pipeline (115), including short-read assembly via SPAdes(v3.13.0)(116), long-read assembly via Racon (v1.4.3), and polishing via Pilon (117), to yield the complete E90 genome. The E90 genome was then annotated using the RAST pipeline (118). The E90 genome is available via the National Center for Biotechnological Information (NCBI) under BioProject accession PRJNA559863.

For RNA-seq experiments, cultures were prepared, and RNA was extracted and purified as described above for qRT-PCR with 2 biological replicates per treatment. Genewiz, LLC performed rRNA depletion, library generation, and sequencing for all samples. RNA reads were obtained using the Illumina HiSeq platform with an average of 15.3M 150-bp paired-end raw reads per sample which were then groomed using Trim Galore (v0.4.3; https://github.com/FelixKrueger/TrimGalore). Reads were then aligned against the E90 genome and counted using the Subread/featureCounts suite of command line tools to produce a final count matrix of 4 by 6478 which was then loaded into the R statistical environment (119, 120). Differential expression (DE) analysis was performed using DESeq2 using a fold-change cut-off of 2 and an adjusted p=0.05 (121). The raw reads and count matrix associated with this transcriptome analysis have been deposited in the of the NCBI Sequence Read Archive under BioProject accession PRJNA559863.

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