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Small Molecule Disruption of Quorum Sensing Cross-Regulation in *Pseudomonas aeruginosa* Causes Major and Unexpected Alterations to Virulence Phenotypes

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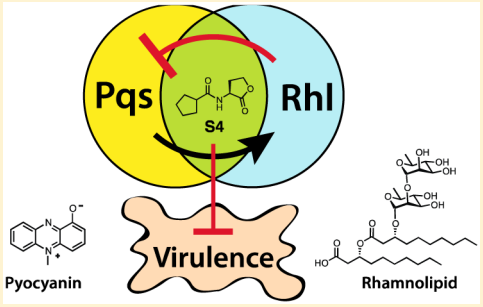
Supporting Information

ABSTRACT: The opportunistic pathogen *Pseudomonas aeruginosa* uses three interwoven quorum-sensing (QS) circuits—Las, Rhl, and Pqs—to regulate the global expression of myriad virulence-associated genes. Interception of these signaling networks with small molecules represents an emerging strategy for the development of anti-infective agents against this bacterium. In the current study, we applied a chemical approach to investigate how the Las-Rhl-Pqs QS hierarchy coordinates key virulence phenotypes in wild-type *P. aeruginosa*. We screened a focused library of synthetic, non-native *N*-acyl L-homoserine lactones and identified compounds that can drastically alter production of two important virulence factors: pyocyanin and rhamnolipid. We demonstrate that these molecules act by targeting RhlR in *P. aeruginosa*, a QS receptor that has seen far less scrutiny to date relative to other circuitry. Unexpectedly, modulation of RhlR activity by a single compound induces *inverse* regulation of pyocyanin and rhamnolipid, a result that was not predicted using genetic approaches to interrogate QS in *P. aeruginosa*. Further, we show that certain RhlR agonists strongly repress Pqs signaling, revealing disruption of Rhl-Pqs cross-regulation as a novel mechanism for QS inhibition. These compounds significantly expand the known repertoire of chemical probes available to study RhlR in *P. aeruginosa*. Moreover, our results suggest that designing chemical agents to disrupt Rhl-Pqs crosstalk could be an effective antivirulence strategy to fight this common pathogen.



INTRODUCTION

Quorum sensing (QS) is a method of intercellular communication used by microorganisms to assess their local population densities and coordinate the expression of group-beneficial phenotypes.[1−3] This process involves the release of a chemical signal, typically a small molecule or peptide termed an "autoinducer," into the environment where it accumulates at a concentration proportional to cell density. At a threshold signal concentration, and thus a threshold population, the autoinducer signal binds a cognate receptor protein that initiates discrete changes in gene expression. The Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa* uses QS to regulate biofilm formation, group motility, and an arsenal of excreted virulence factors in order to overwhelm host defenses and establish chronic infections, most often in immunocompromised individuals (e.g., suffering from cystic fibrosis, chronic wounds, or HIV).[4,5] Because of the emerging threat of multidrug resistance in *P. aeruginosa*, and other clinically relevant pathogens, researchers have sought to develop chemical agents that can attenuate virulence phenotypes without generating a strong selective pressure to evolve resistance (so-called "anti-virulence" therapeutics).[6,7] Due to their critical role in regulating virulence, QS circuits are particularly attractive targets for such compounds.[8,9] Indeed, several recent reports suggest that anti-QS agents will hold significant promise as resistance-robust drugs.[10−12]

Like many other Gram-negative bacteria, *P. aeruginosa* utilizes *N*-acyl L-homoserine lactones (AHLs) as autoinducers for QS (Figure 1).[1,3] The AHL signal is synthesized by a LuxI-type synthase and recognized by an intracellular LuxR-type receptor. *P. aeruginosa* possesses two such QS systems: Las and Rhl. LasI and RhlI produce the autoinducers *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL) and *N*-butyryl-L-homoserine lactone (BHL), respectively, that are bound by LasR and RhlR. Once activated by their native signals, LasR and RhlR homodimerize and act as transcription factors to regulate a specific set of genes. In turn, LasR and RhlR are repressed by the "orphan" LuxR-type receptor QscR, which also binds OdDHL.[13] There also exists a third QS system in *P. aeruginosa*, Pqs, which does not respond to AHLs (Figure 1).[14] The enzymes PqsA−D and PqsH produce the autoinducer 2-heptyl-3-hydroxy-4(1*H*)-quinolone, known as *Pseudomonas* quinolone signal (PQS). The LysR-type transcriptional regulator PqsR binds PQS and controls a separate regulon.[14−17]

Under standard laboratory growth conditions, there is a regulatory hierarchy between the three QS circuits in *P. aeruginosa* (shown schematically in Figure 1).[18] Namely, Las induces expression of both the Rhl and Pqs systems. Once active, the Pqs system positively regulates Rhl (through an

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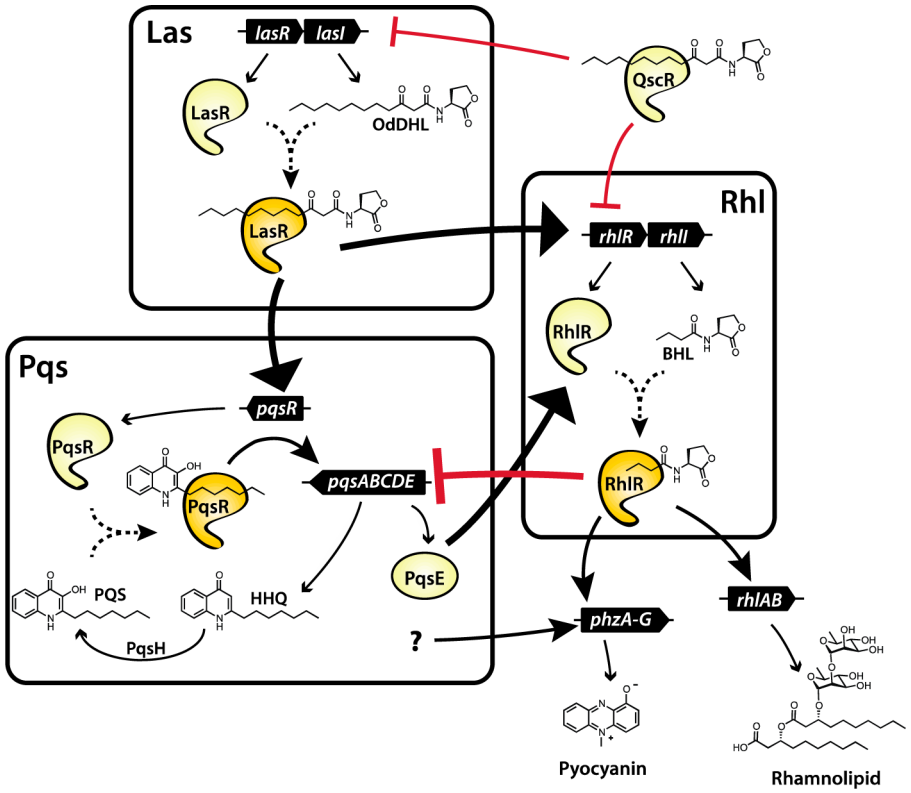

Figure 1. Quorum sensing in *P. aeruginosa*. LasR induces expression of both *rhlR* and *pqsR*. RhlR negatively regulates Pqs by suppressing *pqsA-E* expression. Pqs augments the Rhl system through an unknown mechanism involving PqsE. RhlR directly regulates pyocyanin and rhamnolipid synthesis. Pqs signaling is a primary regulator of pyocyanin, but the molecular mechanism of this regulation is currently unknown. Large arrows indicate major regulatory pathways between circuits. Solid arrowheads indicate positive regulation, while flat, red arrowheads indicate negative regulation.

unknown mechanism involving PqsE), while Rhl represses Pqs.[19−23] Las has typically been viewed as the master regulator of the QS systems; however, several studies have indicated that this regulatory hierarchy is nutritionally and environmentally dependent.[24−27] For example, Zhang and co-workers recently demonstrated that, under low phosphate growth conditions, Pqs and Rhl are able to activate through a Las-independent mechanism.[28] In addition, clinical isolates of *P. aeruginosa* often possess nonfunctional mutations in *lasR* yet maintain full virulence by expressing Rhl- and Pqs-dependent factors.[29,30]

Much remains to be learned about how environmental conditions, the relative timing of QS circuit activation, and crosstalk between systems combine to influence global QS-gene regulation in *P. aeruginosa*. In particular, the Rhl and Pqs regulons are closely associated, but the importance of the inverse regulation between these two circuits in coordinating virulence is unclear. Two virulence factors of significant interest controlled by Rhl and Pqs are the redox-active phenazine pyocyanin and the biosurfactant rhamnolipid. Pyocyanin induces oxidative stress and pro-inflammatory response in the airway epithelia during lung infection and its presence positively correlates with disease severity.[31,32] In turn, rhamnolipids play an important role in *P. aeruginosa* chronic infection by facilitating biofilm maturation and immune evasion.[33,34] Accordingly, elucidation of how cross-regulation between Rhl and Pqs influences these virulence phenotypes could allow for the design of novel, targeted antivirulence agents. A chemical approach is particularly poised to address these knowledge gaps.[9] Specifically, small molecule probes that are capable of altering the activities of individual QS receptors in wild-type cells would allow for the target receptor's role in both controlling specific phenotypes and regulating other QS circuits to be discerned with the native QS machinery intact.

Toward this goal, many research groups have targeted the individual QS systems in *P. aeruginosa* for inhibition with non-native small molecules and macromolecules, and have shown that these agents can attenuate certain virulence phenotypes in the wild-type bacterium.[8] In view of the regulatory hierarchy introduced above (Figure 1), the vast majority of these studies have focused on LasR. Notably, Greenberg,[35] Meijler,[36] Spring,[37] and our own laboratory,[38−42] among others, have reported compounds that inhibit LasR at low micromolar concentrations and reduce the production of various virulence factors (**1**−**3**, Figure 2B). Further, Janda and co-workers have generated monoclonal antibodies capable of sequestering OdDHL, effectively quenching the Las circuit.[43] More recent efforts have been aimed at developing inhibitors of the Pqs system.[44−47] For example, Hartmann and co-workers have prepared several small molecule antagonists of PqsR and PqsD that attenuate Pqs-associated virulence factors and biofilm development (**4**, Figure 2B).[45−47] Compounds that specifically target Rhl, however, are scarce.[48,49] Recently, Bassler and co-workers reported the first non-native AHL that modulates RhlR in vivo (**5**, or mBTL; Figure 2B).[50] This compound, proposed to act (at least in part) as a RhlR partial antagonist, strongly inhibits pyocyanin production and extends nematode survival in a *P. aeruginosa* infection model, suggesting that RhlR could be a promising target for antivirulence therapeutics. Many questions regarding the effects of such small molecule QS modulators persist, however. For example, how does chemical

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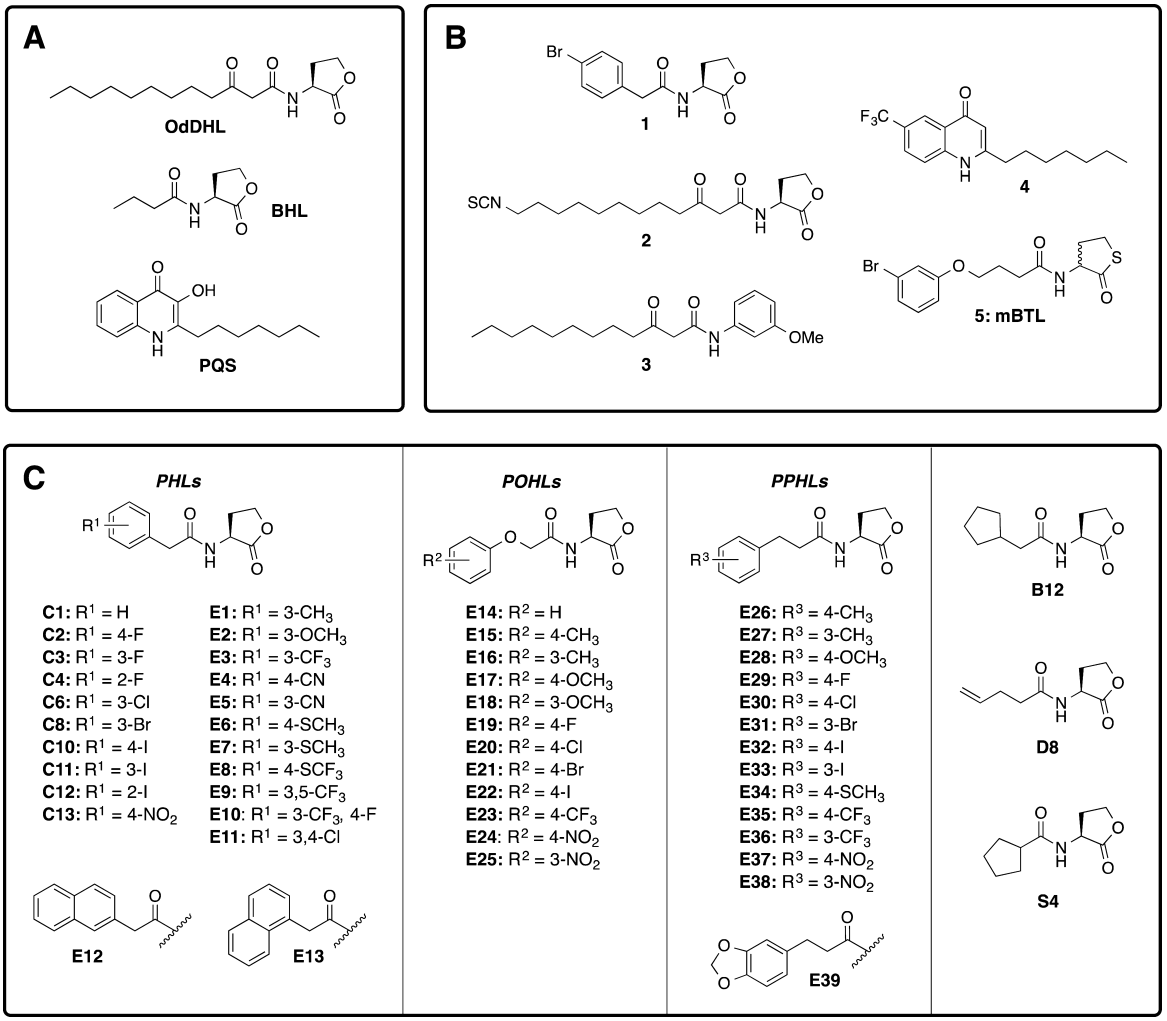

Figure 2. Structures of QS modulators. (A) The native autoinducers used by *P. aeruginosa* for QS. (B) Non-native AHLs reported to inhibit QS pathways in wild-type *P. aeruginosa*. AHLs **1**−**3** target LasR,[36−38] PQS analogue **4** targets PqsR,[45] and thiolactone **5** (mBTL) targets RhlR.[50] (C) Non-native AHLs used in this study. PHLs = phenylacetyl L-homoserine lactones; POHLs = phenoxyacetyl L-homoserine lactones; PPHLs = phenylpropionyl L-homoserine lactones. L-homoserine lactone head groups for **E12**, **E13**, and **E39** omitted for brevity.

modulation of one QS system in *P. aeruginosa* affect the activities of the others? Further, the above studies have focused solely on *inhibition* of specific receptors. How does overstimulation of QS affect virulence phenotypes in *P. aeruginosa*? A better understanding of these basic questions is important before effective antivirulence strategies can be realized.

In the present study, we applied a chemical approach to investigate how the Las-Rhl-Pqs QS hierarchy coordinates key virulence phenotypes in wild-type *P. aeruginosa*. We screened a focused library of synthetic, non-native AHLs for their ability to modulate production of the virulence factor pyocyanin and identified both inhibitors and inducers of this phenotype. We demonstrate that the tested compounds alter pyocyanin production primarily by modulating RhlR activity. Unexpectedly, RhlR agonists were revealed to be the strongest inhibitors of pyocyanin production, while RhlR antagonists induce pyocyanin. Consistent with this trend, several AHLs were found to potently inhibit or induce synthesis of rhamnolipid, a second virulence factor controlled directly by RhlR, albeit with *inverse* effects relative to those on pyocyanin production. Finally, we show that stimulation of the Rhl system by RhlR agonists can strongly suppress Pqs signaling in the wild-type organism. The compounds described herein greatly expand the existing portfolio of chemical tools available to study RhlR in *P. aeruginosa*.[48−50] Moreover, these results suggest that disruption of crosstalk between the Rhl and Pqs systems by small molecules can lead to dysregulation of virulence in wild-type *P. aeruginosa*, a finding with important implications for the design of QS-targeted antivirulence agents against this notorious pathogen.

■ EXPERIMENTAL SECTION

General Experimental Information. All absorbance measurements were made in 200 μL of solution in a clear 96-well microtiter plate (Costar 3370) using a Biotek Synergy 2 plate reader running Gen 5 software (version 1.05). Bacterial growth was assessed by measuring the culture cell density according to absorbance at 600 nm (OD_{600}). The concentration of DNA and RNA samples was determined using a NanoDrop spectrophotometer. All common materials and reagents used for RNA isolation, reverse transcription, and RT-qPCR were purchased from commercial sources and certified nuclease-free. Standard PCR reactions were performed using an Eppendorf MasterCycler Personal thermal cycler. Assay data were analyzed using Microsoft Excel for Mac 2011 and GraphPad Prism 6 for Mac OS X (version 6.0c).

AHLs from our compound libraries,[39−42] compound **4**,[45] and mBTL (**5**),[50] were synthesized as previously reported. OdDHL and

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PQS were purchased from Sigma-Aldrich. BHL was purchased from Cayman Chemical. Chlorophenol red-β-D-galactopyranoside (CPRG) was purchased from Roche. Stock solutions of synthetic compounds (40 or 100 mM) were prepared in DMSO and stored at −20 °C in sealed vials. The amount of DMSO used in small molecule screens did not exceed 1% (v/v). No compound had an effect on bacterial growth over the concentrations tested (Figure S2, Figure S3).

Bacterial Strains and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table S1. Full details regarding plasmid and strain construction are found in the Supporting Information. Plasmids pJN105R2 and pSC11-rhlI* were transformed iteratively into *Escherichia coli* JLD271 by electroporation to prepare the *E. coli* RhlR reporter strain. All media and reagents for bacterial culture were purchased from commercial sources. Bacteria were cultured in Luria−Bertani broth (LB) at 37 °C with shaking at 200 rpm unless otherwise noted. The *E. coli* RhlR reporter was grown in LB containing 100 μg/mL ampicillin and 10 μg/mL gentamicin. The *E. coli* PqsR reporter was grown in LB containing 100 μg/mL ampicillin. The *P. aeruginosa* PAO-JP2 LasR and RhlR reporters were grown in LB with 300 μg/mL carbenicillin. Freezer stocks of bacterial strains were maintained at −80 °C in 1:1 LB:glycerol.

Pyocyanin Assay Protocol. The amount of pyocyanin in *P. aeruginosa* culture supernatants was measured following the protocol of O'Loughlin et al. with modifications.[50] A 10 mL overnight culture of *P. aeruginosa* PAO1 was grown for 16 h. DMSO stock solutions of test compounds (40 or 60 mM) were prepared, serially diluted (if necessary), and 12.5 μL aliquots were added to sterile 15 mL borosilicate glass test tubes. An inoculating culture was prepared by diluting the overnight culture 1:100 into fresh LB medium, and 2.5 mL aliquots of this subculture were added to each test tube (final AHL concentration were 200 μM for primary screens and 300 μM to 0.05 μM for dose experiments, 0.5% DMSO). The cultures were grown for 17 h, and the final cell density measured by reading OD_{600}. Relative pyocyanin levels were measured by first pelleting 1 mL of well-mixed culture at 1,500 × *g* for 10 min, transferring 200 μL of the resulting supernatant to a clear, plastic 96-well microtiter plate, and reading absorbance at 695 nm. Data were normalized by dividing this absorbance value by the final OD_{600} and plotted relative to the DMSO positive control. We also evaluated pyocyanin levels following an extraction protocol[51] and found that this protocol gave identical results to the above method.

***P. aeruginosa* PAO-JP2 Reporter Gene Assay Protocol.** Assays for LasR and RhlR activity were performed in *P. aeruginosa* strains PAO-JP2/p*lasI*-LVA*gfp* and PAO-JP2/p*rhlI*-LVA*gfp*, respectively, according to a previously reported protocol,[52] with the following adaptations. For all assays using the RhlR reporter, OdDHL was added to the subculture at a final concentration of 100 nM in order to induce the production of RhlR (via LasR) immediately before the culture was dispensed into plates. For all antagonism assays, the concentration of native ligand utilized was approximately equal to its EC_{50} value in each bacterial reporter strain (238 nM OdDHL, 30 μM BHL; Figure S1). For LasR antagonism assays, non-native AHLs (100 μM) were screened against 200 nM OdDHL. For RhlR antagonism assays, non-native AHLs (100 μM) were screened against 100 nM OdDHL and 30 μM BHL. For LasR and RhlR agonism assays, non-native AHLs were tested at 100 μM, and activity was measured relative to 100 μM OdDHL or 900 μM BHL, respectively.

***E. coli* RhlR Reporter Gene Assay Protocol.** Assays for RhlR activity were performed in *E. coli* JLD271/pJN105R2/pSC11-rhlI* as previously reported,[39] with the following adaptations. A subculture was prepared by diluting an overnight culture 1:10 with fresh media containing appropriate antibiotics and incubated until it had grown to OD_{600} = 0.5. For RhlR antagonism assays, the concentration of native ligand utilized was approximately equal to its EC_{50} value (11 μM, Figure S1). For RhlR antagonism assays, non-native AHLs (100 μM) were screened against 10 μM BHL. For RhlR agonism assays, non-native AHLs were tested at 100 μM, and activity was measured relative to 900 μM BHL. Modifications were made to the standard Miller assay protocol to accommodate the use of the CPRG β-galactosidase substrate. After cell lysis, 150 μL of the aqueous layer was transferred from each well of the solvent-resistant plate to the wells of a new clear, flat-bottom 96-well microtiter plate. CPRG (4 mg/mL in phosphate-buffered saline) was added to each well in 25 μL volumes. Plates were incubated at 30 °C until the positive control wells developed a red color (approximately 30 min). The amount of processed CPRG substrate was assessed by measuring the absorbance of each well at 570 nm. Enzymatic activity was calculated using the following equation: Miller units $=1000 \cdot Abs_{570}/(OD_{600} \cdot t \cdot V)$ where t is the incubation time of substrate with lysate and V is the volume of culture lysed.

Rhamnolipid Assay Protocol. Rhamnolipid was quantified according to the method of Koch et al. with modifications.[53] A 10 mL overnight culture of *P. aeruginosa* PAO1 was grown for 16 h. A subculture was prepared by directly diluting the overnight culture 1:100 into fresh Minimal Medium (49.3 mM Na_2HPO_4, 50 mM KH_2PO_4, 4.8 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, 0.6 mM $CaCl_2$, 25 μM $FeSO_4$, 0.162 μM $(NH_4)_6Mo_7O_{24}$, 38 μM $ZnSO_4$, 14 μM $MnCl_2$, 1.6 μM $CuSO_4$, 0.86 μM $CoCl_2$, 1.9 μM boric acid, 5.5 μM $NiCl_2$, 6.72 μM EDTA, 0.6% glycerol in 18 MΩ deionized water; see Supporting Information for details regarding this medium). DMSO stock solutions of test AHLs were prepared (2 or 40 mM) and 10 μL aliquots were added to 15 mL sterile borosilicate glass test tubes, followed by 2 mL of bacterial subculture (final AHL concentration 10 or 200 μM, 0.5% DMSO). The resulting cultures were grown for 20 h. The final OD_{600} of the bacteria was measured, and the cells were pelleted at 1,500 × *g* for 10 min. The supernatant (1 mL) was transferred to a 1 dram glass vial and extracted twice with 1 mL of diethyl ether. The pooled organic fractions were evaporated to dryness and the resulting residue reconstituted in 200 μL deionized water. In a 1.7 mL plastic centrifuge tube, 50 μL of this extract was diluted into 450 μL of a solution of 0.19% (w/v) orcinol in 50% (v/v) concentrated H_2SO_4. The tubes were vortexed thoroughly to mix and incubated in an 80 °C heating block for 30 min. After briefly cooling to room temperature, 200 μL of the resulting yellow to yellow-orange solution was transferred to a clear 96-well microtiter plate and the absorbance at 421 nm measured. Data were normalized by dividing this absorbance value by the final OD_{600} and plotted relative to a DMSO positive control.

RNA Isolation and Reverse Transcription Protocols. A 10 mL overnight culture of *P. aeruginosa* PA01 was grown for 16 h. A subculture was prepared by directly diluting the overnight culture 1:100 into fresh LB. DMSO stock solutions (2, 60, or 100 mM) of test AHLs were prepared, and 12.5 μL aliquots were added to 15 mL borosilicate glass test tubes, followed by 2.5 mL bacterial subculture (final AHL concentration 10, 300, or 500 μM, 0.5% DMSO). The cultures were then grown for 8 h. Total RNA was harvested using Ambion PureLink RNA Mini Kits and subjected to both on-column DNase treatment with Ambion PureLink DNase and exogenous DNase treatment with Ambion DNA-free Kits. RNA integrity was confirmed by native gel electrophoresis of 1 mg of each total RNA sample in a 1% agarose Tris-Borate-EDTA gel and inspection of the rRNA bands for degradation. If needed, RNA samples were stored at −80 °C. Elimination of genomic DNA was confirmed by PCR (see Supporting Information). Reverse transcription was performed on 1 μg of intact, DNA-free total RNA using Applied Biosystems High Capacity cDNA Reverse Transcription Kits according to manufacturer's instructions. The resulting cDNA was diluted with nuclease-free water as necessary and stored at −20 °C.

qPCR Protocol. PCR primers for each gene of interest were designed using Primer3 software (Table S4). Primers were validated for their ability to amplify single products from *P. aeruginosa* PAO1 genomic DNA prior to use in qPCR experiments (see Supporting Information for full details regarding primer design, validation, and calibration curves). All qPCR experiments were performed on a Roche LightCycler 480 in white 96-well PCR plates (Roche) using iTaq Universal SYBR Green Supermix (Roche) according to manufacturer's protocol. cDNA stocks were diluted as necessary, and 5 μL aliquots were added to each well of a PCR plate followed by 15 μL primer master mix. Melt curves were obtained for every run, and any wells with multiple peaks were excluded from analysis. Data was normalized according to the method of Vandesompele[54] with *ampC*, *mreB*, *recA*, and *rpoD* used as reference genes.[55] Statistical analyses were

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performed on three independent biological replicates using a ratio-paired *t* test assuming a Gaussian distribution (using GraphPad Prism 6 software).

***E. coli* PqsR Reporter Gene Assay Protocol.** Assays for PqsR activity were performed in *E. coli* DH5α/pEAL08−2 as previously reported,[39] with the following modifications.[45,56] A subculture was prepared by diluting an overnight culture 1:100 with fresh medium containing appropriate antibiotics and incubated until it had grown to OD_{600} = 0.15. For PqsR antagonism assays, the concentration of native ligand (PQS) utilized was approximately equal to its EC_{50} value (7.5 nM, Figure S1). For PqsR antagonism assays, non-native AHLs (100 μM) were screened against 10 nM PQS. For PqsR agonism assays, non-native AHLs were tested at 100 μM, and activity was measured relative to 100 μM PQS. Plates were incubated for 2 h. To assess β-galactosidase activity, 20 μL of final culture was lysed, and 100 μL of the aqueous layer was transferred from each well of the solvent-resistant plate to the wells of a new clear, flat-bottom 96-well microtiter plate. CPRG (4 mg/mL in phosphate-buffered saline) was added to each well in 16.7 μL volumes. Plates were incubated at room temperature until the positive control wells developed a red color (approximately 7 min). The amount of processed CPRG substrate was assessed as described above.

RESULTS AND DISCUSSION

Selection of Test AHLs for Screening. Previous studies in our laboratory have resulted in the discovery of many non-native AHLs that are modulators of LasR activity in heterologous *E. coli* reporter strains.[38−41] However, compounds must be able to modulate LuxR-type receptor activity in the native *P. aeruginosa* background, a nontrivial barrier, in order to be most useful as chemical probes of QS. Accordingly, such ligands must overcome (i) the relative impermeability of the *P. aeruginosa* cell wall,[57] (ii) active efflux mechanisms,[58] and (iii) competition with the native signal molecules for receptor binding. To characterize the abilities of our non-native AHLs to affect LasR and/or RhlR activities in the native organism, we utilized two strains of *P. aeruginosa* PAO-JP2, a mutant that lacks functional AHL synthases (Δ*lasIrhlI*), harboring the GFP reporter plasmids p*lasI*-LVA*gfp* or p*rhlI*-LVA*gfp*.[59] We screened our entire in-house AHL collection[39−42] in these two reporter strains for both agonism and antagonism of LasR and RhlR (see Experimental Section). From these data (Table S2), we selected for further evaluation in wild-type *P. aeruginosa* a focused library of 52 non-native AHLs (Figure 2C) that were revealed to either agonize or antagonize LasR and/or RhlR (by ≥30%). Many of these were of the phenylacetyl L-homoserine lactone (PHL), phenoxyacetyl L-homoserine lactone (POHL), or phenylpropionyl L-homoserine lactone (PPHL) subclasses, which we have previously observed to be highly active scaffolds for LuxR-type receptor modulation.[38−41] In view of the aforementioned challenges, we reasoned that these compounds would have the greatest likelihood of modulating QS-controlled gene expression in wild-type *P. aeruginosa*.

AHLs Are Modulators of Pyocyanin Production. We next sought a robust assay with which we could correlate AHL-induced effects on a specific virulence phenotype with discrete changes to QS pathways in wild-type *P. aeruginosa*. We chose to monitor pyocyanin production, as this virulence factor is regulated by all three of this bacterium's canonical QS circuits. Specifically, pyocyanin biosynthesis is believed to be directly regulated by RhlR and Pqs signaling, while LasR is an indirect regulator, inducing each of the other two circuits (Figure 1).[60,61] As a result, genomic deletion of *lasR*, *rhlR*, or *pqsR* results in delayed or abolished pyocyanin production.[22,26,50,62] Thus, we reasoned that monitoring pyocyanin levels upon AHL treatment could yield useful insights into how QS controls the expression of this virulence phenotype in *P. aeruginosa* and provide clues as to which receptor (or receptors) should be targeted to most effectively limit pyocyanin production in the native organism.

We grew wild-type *P. aeruginosa* (PAO1) in the presence of 200 μM of compound and quantified the amount of pyocyanin in the culture supernatants. Gratifyingly, we found nine AHLs that inhibited pyocyanin levels by over 50% relative to a DMSO control (Figure 3; see Table S3 for data for all 52 AHLs). Of

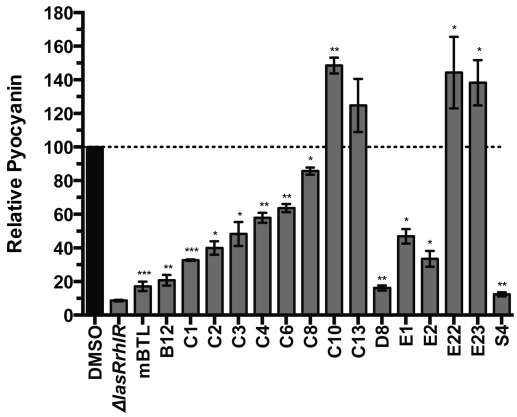

Figure 3. Pyocyanin assay data for selected non-native AHLs. All compounds tested at 200 μM in wild-type *P. aeruginosa* (PAO1). Relative pyocyanin levels were quantified after 17 h shaking incubation and normalized to the final cell density (OD_{600}). All values are plotted relative to a DMSO-treated positive control. PAO-JG35 (Δ*lasRrhlR*) was used as a negative control. Error bars represent the standard error of three independent experiments (n = 3). * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$.

these, short-chain AHLs **D8** and **S4** (Figure 2C) were the most active, inhibiting pyocyanin production by 84 and 88%, respectively. Encouraged by these results, we performed dose experiments and found that the IC_{50} values for **D8** and **S4** were approximately 9.9 and 6.8 μM, respectively, placing them among the most potent QS-based inhibitors of pyocyanin reported in wild-type *P. aeruginosa* (Figure S4).[45,50,63] Consistent with literature precedent,[50] we observed that mBTL inhibited pyocyanin by 83% (Figure 3) with an IC_{50} of 12 μM (Figure S4). Notably, we also found three AHLs in our primary screen that *induced* pyocyanin production in the wild-type organism. In particular, 4-iodo PHL **C10** and 4-iodo POHL **E22** effected a nearly 50% increase in pyocyanin levels compared to the DMSO control (Figure 3). The discovery of both inhibitors and inducers of pyocyanin production from our subset of AHLs was encouraging, as this then allowed us to correlate LasR and/or RhlR transcriptional activity to a spectrum of phenotypic outcomes (vide infra).

Pyocyanin Effects Are Caused by RhlR Modulation. To determine which QS receptor proteins were being targeted by our AHLs to elicit the observed effects on pyocyanin production, we circled back and correlated these phenotypic assay data to our reporter gene assay data. The LasR and RhlR reporter agonism and antagonism data for our lead pyocyanin inducers and inhibitors (including mBTL as a control) are listed in Table 1. Because LasR sits at the top of the canonical QS hierarchy in *P. aeruginosa*, we hypothesized that many of the identified pyocyanin inhibitors would be strong LasR antagonists and vice versa. However, we found that, excluding

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Table 1. LasR/RhlR Antagonism and Agonism Assay Data in the *P. aeruginosa* PAO-JP2 and *E. coli* Reporter Strains for the Lead AHLs

		PAO-JP2				*E. coli*	
	PAO1	LasR[b]		RhlR[b]		RhlR[b]	
compound[a]	pyocyanin inhibition (%)	antagonism (%)[c]	agonism (%)[d]	antagonism (%)[e]	agonism (%)[f]	antagonism (%)[g]	agonism (%)[h]
S4	88	31	8	12	105	−110	91
D8	84	15	10	−9	86	−95	81
mBTL	83	−61	99	−40	102	−122	83
B12	79	33	7	15	46	−52	79
C1	67	43	7	41	48	−64	84
E2	66	37	2	40	42	−60	86
C2	60	60	7	72	32	−47	71
C13	−25	31	9	56	2	60	11
E23	−38	47	5	85	−1	64	5
E22	−44	60	2	92	−1	74	7
C10	−49	33	7	54	2	84	1

[a]Compounds are sorted by pyocyanin percent inhibition. The horizontal line in the table separates pyocyanin inhibitors from inducers for clarity. Compounds tested at 100 μM in all assays. [b]See Experimental Section and Table S1 for full assay and strain information. For antagonism assays, values are given relative to bacteria treated with only the native AHL ligands. For agonism assays, values are given relative to bacteria treated with a concentration of native ligand found to give maximum reporter response. All values represent the mean of three biological replicates (n = 3), unless otherwise noted. Error did not exceed ±10%. [c]Assays performed in strain PAO-JP2/p*lasI*-LVA*gfp* in the presence of 200 nM OdDHL. [d]Assays performed in strain PAO-JP2/p*lasI*-LVA*gfp*. Percent agonism is given relative to 100 μM OdDHL. [e]Assays performed in strain PAO-JP2/p*rhlI*-LVA*gfp* in the presence of 100 nM OdDHL and 30 μM BHL. [f]Assays performed in strain PAO-JP2/p*rhlI*-LVA*gfp*. Percent agonism is given relative to 900 μM BHL. [g]Assays performed in strain JLD271/pJN105R2/pSC11-rhlI* in the presence of 10 μM BHL. Values represent the mean of two biological replicates (each run in triplicate). [h]Assays performed in strain JLD271/pJN105R2/pSC11-rhlI*. Percent agonism is given relative to 900 μM BHL.

mBTL, all of the lead compounds, no matter if they were pyocyanin inhibitors or inducers, exhibited approximately the same activity profile against LasR; namely, they were weak to moderate LasR inhibitors. For example, compounds **C2** and **E22** each antagonized LasR by 60%, yet the former is a pyocyanin inhibitor while the latter is a pyocyanin inducer. Neither **C2** nor **E22** displayed LasR agonism. The control compound mBTL was unique among pyocyanin inhibitors in that it behaved as a strong LasR agonist instead. From these data, we conclude that non-native AHLs do not induce pyocyanin through agonism of LasR. These data do not exclude LasR antagonism as a contributor to pyocyanin inhibition, but interestingly, our most potent pyocyanin inhibitors, **D8** and **S4**, appear to be two of the weaker LasR antagonists tested. Furthermore, **C2** was a significantly weaker pyocyanin inhibitor than **D8**, yet the reporter assay data revealed **C2** to antagonize LasR far more than **D8**. These trends indicate that LasR modulation by the AHLs is ultimately not responsible for the observed differences in pyocyanin production.

We next considered the effect of non-native AHLs on RhlR activity as observed in our PAO-JP2 reporter. Because deletion of *rhlR* abolishes pyocyanin production,[22] we hypothesized that RhlR agonists would be pyocyanin inducers while RhlR antagonists would be pyocyanin inhibitors. In contrast, we found that all of the pyocyanin inhibitors identified were strong RhlR *agonists* instead (Table 1). Furthermore, the magnitude of RhlR agonism correlated positively with pyocyanin inhibition, suggesting an inverse relationship between RhlR activity and this phenotype. The lead pyocyanin inhibitors **S4**, **D8**, and mBTL were the strongest RhlR agonists, activating the receptor by 105, 86, and 102%, respectively. Conversely, AHLs that induced pyocyanin production had no RhlR agonistic activity; these compounds tended to be strong RhlR antagonists. For example, AHLs **E22** and **E23** both induced pyocyanin production, yet each inhibited RhlR transcriptional activity by over 80% in the PAO-JP2 RhlR reporter strain.

Three compounds deviate slightly from the above trends. Namely, the pyocyanin inhibitors **C1**, **C2**, and **E2** each exhibited both significant RhlR agonism *and* antagonism. Because active LasR is needed to induce RhlR production in the PAO-JP2 reporter strain,[59] and because each of these compounds also displayed LasR antagonism (Table 1), we reasoned that the observed RhlR antagonism could be a "knock on" effect of LasR inhibition. To test this hypothesis, we examined each compound in a heterologous (*E. coli*) RhlR reporter (see Experimental Section). In this strain, we found that the pyocyanin inhibitors behave solely as RhlR agonists (Table 1). All five of the lead pyocyanin inhibitors showed over 70% RhlR agonism. Interestingly, all RhlR antagonism by the same compounds was abolished, presumably due to removal of interference by LasR. Similar to the PAO-JP2 RhlR reporter strain, the pyocyanin *inducers* exhibited strong RhlR antagonism (each by at least 60%; Table 1). From these PAO-JP2 and *E. coli* reporter data, we conclude that the tested AHLs modulate pyocyanin primarily by affecting RhlR activity in *P. aeruginosa*.

Intrigued by these unexpected trends, we next evaluated pyocyanin levels after exogenous treatment of wild-type *P. aeruginosa* with the native RhlR ligand, BHL (Figure 2A). We observed dose-dependent inhibition of pyocyanin levels by BHL with an IC_{50} of approximately 17 μM (Figure S4). This result indicates that agonism of RhlR is indeed responsible for the observed pyocyanin inhibition. When one examines the structures of the lead pyocyanin inhibitors, it is not entirely surprising that they behave as RhlR agonists (Figure 2C). Compound **D8** is identical to BHL with the exception of a terminal vinyl group, and **B12** and **S4** can be considered to be BHL analogues where the acyl tail is conformationally locked by a cyclopentyl moiety. Because of these structural similarities,

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as well as recent reports that LuxR homologues can accommodate non-native AHLs in their ligand binding sites,[64,65] we believe that the most likely mechanism by which our RhlR agonists (and antagonists) interact with RhlR is via competitive displacement of BHL in the ligand binding pocket.

In total, these assay data suggest that the pyocyanin induction by our AHLs is due to RhlR antagonism, whereas pyocyanin inhibition is a result of RhlR agonism. For each compound, RhlR modulation dictates the pyocyanin phenotypic outcome irrespective of its effect on LasR. Although the mild LasR antagonism observed for certain AHLs could contribute to pyocyanin inhibition, it is the RhlR activity that dominates. We note that in contrast to Bassler and co-workers, we found that the pyocyanin inhibitor mBTL only agonizes RhlR.[50] However, differences between mBTL and our lead compounds, such as molecular structure and activity against LasR, could indicate that mBTL attenuates pyocyanin production by a different mechanism. Possibly the most interesting result of our pyocyanin screen is the phenotypic discrepancy between our chemical genetic experiment (i.e., small molecule modulation of RhlR) and a traditional genetic approach (i.e., knockout of *rhlIR*[22,62]). The latter predicts that RhlR agonism should promote pyocyanin production; however, we observe the opposite. We believe these results are not in conflict. Rather, each of these experiments yields unique insight into how RhlR regulates pyocyanin production. The genetic experiments indicate that RhlR regulates a factor essential for pyocyanin synthesis. Our results with small molecules imply that RhlR also *suppresses* an additional factor required for pyocyanin production and that this effect dominates over any positive pyocyanin regulation by RhlR. We return to these implications and others below.

Potent RhlR Modulators Affect Rhamnolipid Production. To further evaluate the lead AHLs identified in the above assays, we examined their abilities to modulate an additional virulence phenotype directly controlled by RhlR in *P. aeruginosa*—rhamnolipid production. RhlR directly regulates expression of the enzymes responsible for rhamnolipid biosynthesis (*rhlAB*); thus, rhamnolipid production is greatly reduced in a *rhlR* mutant.[34,66] We expected that any non-native AHLs that were RhlR agonists would promote rhamnolipid synthesis, while RhlR antagonists would inhibit this phenotype. We cultured wild-type *P. aeruginosa* in the presence of eight of our lead AHLs and quantified the amount of rhamnolipid in the supernatant. Consistent with our hypothesis, the tested RhlR agonists strongly induced rhamnolipid production (Figure 4, see Table S3 for data for all 52 AHLs). Notably, compounds **D8** and **S4** induced rhamnolipids by over 200% relative to a negative control (at 200 μM; Figure 4). Subsequent studies revealed that these compounds are capable of stimulating rhamnolipid production at concentrations as low as 10 μM (Figure S5). Consistent with our data that mBTL is a RhlR agonist, this compound also induced rhamnolipid production to a large extent (Figure 4). In addition, the RhlR antagonists proved to be mild to moderate rhamnolipid inhibitors. Of these compounds, **E22** was the most active, inhibiting rhamnolipid production by approximately 60%. These additional phenotypic data strongly suggest that the non-native AHLs tested herein are directly modulating RhlR activity in *P. aeruginosa*.

Interestingly, for both the RhlR agonists and antagonists, the compound activity trends in the rhamnolipid assays were opposite of those observed in pyocyanin assays (see Figure 5). This observation may have consequences for the design of

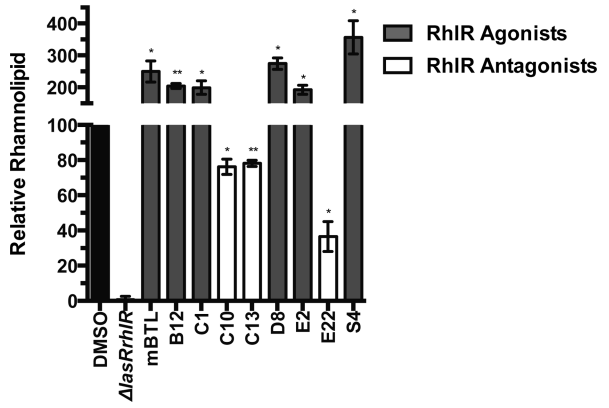

Figure 4. Rhamnolipid assay data for select non-native AHLs. Wild-type *P. aeruginosa* (PAO1) was grown in an optimized minimal media in the presence of 200 μM AHL for 20 h, after which the relative amounts of rhamnolipids produced were quantified via the orcinol test (see Experimental Section). The resultant values were normalized to the final cell density (OD_{600}). Data is plotted relative to a DMSO-treated positive control. PAO-JG35 (Δ*lasRrhlR*) was used as a negative control. Error bars represent the standard error of three independent experiments (n = 3). * = $p < 0.05$, ** = $p < 0.005$.

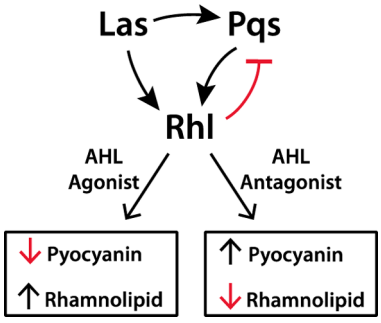

Figure 5. Summary of the modes by which modulation of RhlR activity by non-native AHLs affects pyocyanin and rhamnolipid production in *P. aeruginosa*. Agonism of RhlR results in inhibition of pyocyanin production and induction of rhamnolipid production. Antagonism of RhlR results in induction of pyocyanin production and inhibition of rhamnolipid production.

antivirulence therapeutics targeting QS pathways in *P. aeruginosa*. If modulation of RhlR with a small molecule results in the inhibition of one QS-controlled phenotype but the simultaneous promotion of another, care will need to be taken in targeting this receptor for infection control. However, we note that induction of rhamnolipid production may not be entirely adverse. Rhamnolipids are overproduced naturally during the dispersal of mature *P. aeruginosa* biofilms, so it could be possible for a RhlR agonist to inhibit pyocyanin while simultaneously suppressing biofilm development.[67] Indeed, we speculate that the biofilm inhibition by mBTL observed by Bassler and co-workers could be a result of rhamnolipid induction by this compound.[50]

RhlR Agonists Suppress PQS Signaling. The above data indicate that non-native AHLs can strongly affect RhlR transcriptional activity; however, the mechanisms by which RhlR modulation causes the observed effects on pyocyanin production remained unclear. Specifically, if RhlR positively regulates pyocyanin production, how does RhlR agonism result in reduced pyocyanin levels? Several studies have demonstrated that RhlR has a negative regulatory influence on genes

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associated with the Pqs system, namely, *pqsA-E* and *pqsR*.[19−23] Furthermore, Pqs signaling is known to be a major regulator of pyocyanin production.[16] Thus, it is plausible that agonism of RhlR with a small molecule could suppress the Pqs system and thereby inhibit a downstream phenotype under its control (i.e., pyocyanin).

To test this hypothesis, we treated wild-type *P. aeruginosa* PAO1 with our lead RhlR agonists and examined the mRNA transcript levels of 10 genes of interest by RT-qPCR. These genes included the Las and Rhl AHL synthases and receptors (*lasI*, *lasR*, *rhlI*, *rhlR*), two genes responsible for PQS synthesis and response (*pqsA*, *pqsR*), two genes involved in pyocyanin biosynthesis (*phzA*, *phzB*), and two genes necessary for rhamnolipid biosynthesis (*rhlA*, *rhlB*). Treatment with 500 μM of the RhlR agonist **S4** resulted in discrete expression changes in certain genes of interest relative to a DMSO-treated control (Figure 6A). Consistent with our phenotypic observations, **S4** strongly down-regulates *phzA* and *phzB* and up-regulates *rhlB*. In agreement with our analysis of the reporter gene data, **S4** also up-regulates *rhlI* and *rhlR* by 2-fold while having minimal effect on *lasI* and *lasR*. Finally, **S4** induces an approximate 5-fold down-regulation of *pqsA*, an enzyme essential for PQS synthesis, but has little effect on *pqsR*.[68] This gene expression signature suggests that RhlR agonism by **S4** results in suppression of PQS biosynthesis, which, in turn, leads to inhibition of pyocyanin production. Importantly, these trends hold true when **S4** is present at as little as 10 μM. Interestingly, we observed that *lasR* was induced 2-fold at this lower concentration (Figure 6A). Given that **S4** did not agonize LasR in the PAO-JP2 reporter assay (Table 1), this up-regulation is likely not due to direct effects on LasR. Additional experiments are required to determine the origin of this intriguing finding and are ongoing. Treatment with 500 μM of the RhlR agonist, **D8**, resulted in similar transcriptional suppression of *pqsA*, although the observed up-regulation in *rhlIR* and *rhlAB* with this compound was more muted (Figure 6B). We note that none of the lead AHLs displayed either agonism or antagonism of PqsR in an *E. coli* reporter strain (Table S5); thus, the down-regulation of *pqsA* observed here for **S4** and **D8** cannot be attributed to inhibition of PqsR activity. Identical RT-qPCR experiments were performed with the RhlR antagonists **C10** and **E22**; however, treatment with these AHLs did not result in observable changes in gene expression (Figure S7). We hypothesize that this is because RhlR antagonists must outcompete the native ligand, BHL, for receptor binding in the wild-type organism, while RhlR agonists complement existing BHL-mediated activation of RhlR.

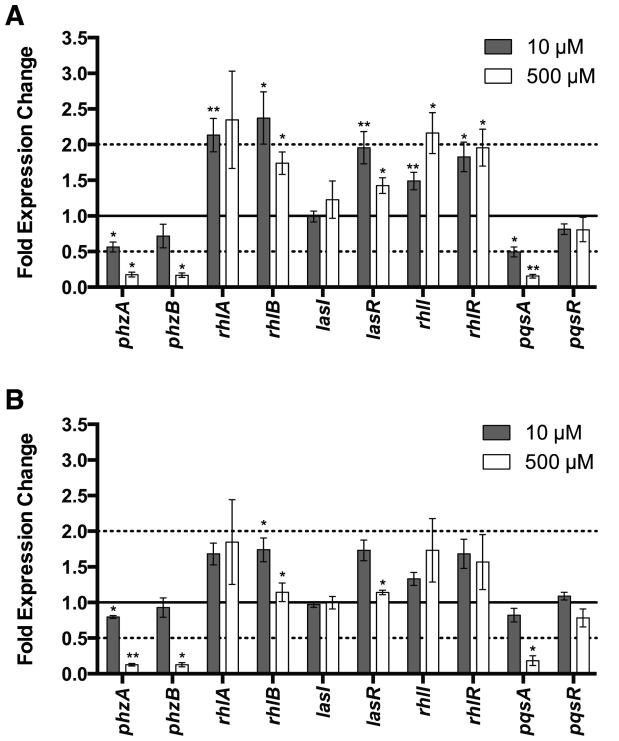

Figure 6. AHL-induced changes in QS gene expression observed by RT-qPCR. Wild-type *P. aeruginosa* (PAO1) was treated with 10 μM or 500 μM (A) **S4** or (B) **D8** for 8 h. Data indicate fold change relative to a DMSO-treated control. A value of 1 (solid black lines) indicates no change in gene expression upon compound treatment. Dotted lines indicate a 2-fold up- or down-regulation. Error bars represent the standard error of three independent experiments ($n = 3$). * = $p < 0.05$, ** = $p < 0.005$.

The results of our RT-qPCR experiments indicate that small molecule modulation of RhlR can induce significant changes in QS gene expression. Our observation that agonism of RhlR results in *suppression* of Pqs signaling is the most important, for the following reasons. First, it suggests a plausible mechanism by which small molecule agonism of RhlR results in inhibition of pyocyanin production. The Pqs system is believed to be a primary regulator of pyocyanin;[16] thus, RhlR-mediated down-regulation of genes responsible for PQS biosynthesis would significantly inhibit Pqs signaling and reduce the bacterium's capacity to synthesize pyocyanin. Our data suggest that even small increases in RhlR activity, induced by 10 μM compound, can inhibit Pqs signaling and lead to dysregulation of the normal pyocyanin phenotype. We note that timing of compound dosing also appears to be critical for disruption of this cross regulation. In fact, we observed no change in pyocyanin production when **S4** was added after mid-log phase of growth (Figure S8), suggesting that RhlR agonism can suppress Pqs *prior* to natural activation of Pqs signaling (typically at the outset of stationary phase[15]).

Second, these findings serve to reconcile the apparent discrepancy between pyocyanin assay data observed in a *rhlR* knockout and that observed upon small molecule RhlR agonism, as discussed above. Our data indicate that suppression of Pqs signaling by RhlR agonism can dominate over any positive regulation of pyocyanin by RhlR. Knockout of *rhlR*, however, abolishes this suppressive effect. Thus, one would not expect phenotypes observed in a *rhlR* mutant to necessarily predict those resulting from small molecule agonism of RhlR in the wild-type organism, where Rhl-Pqs cross-regulation remains intact. Third, because of the importance of this Rhl-Pqs cross talk, our findings indicate that targeting multiple QS circuits, either simultaneously or sequentially, may be a promising antivirulence strategy in *P. aeruginosa*. For instance, a cocktail of RhlR and PqsR/PqsD inhibitors may be more effective at inhibiting global virulence phenotypes than targeting one receptor alone.

■ SUMMARY AND CONCLUSIONS

In this study, we aimed to assess the effects of small molecule QS modulators, both agonists and antagonists, on global QS in *P. aeruginosa* with the goal of further defining the role of cross-regulation between its QS circuits in virulence. We screened a focused library of non-native AHLs for their ability to modulate

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production of two important virulence factors, pyocyanin and rhamnolipid, in wild-type *P. aeruginosa*. We identified both inhibitors and inducers of these phenotypes and show that the observed AHL-induced effects are primarily due to modulation of RhlR activity. Notably, compounds found to inhibit pyocyanin also *induced* rhamnolipid production, and vice versa. In addition, we show that RhlR agonists strongly inhibit Pqs signaling in wild-type *P. aeruginosa*.

The results presented herein have several important implications for both the design and discovery of new QS-based antivirulence agents. First, our findings provide additional and compelling evidence that RhlR is a tractable target for small molecule modulation. Our results indicate that both agonism and antagonism of RhlR can have large effects on important QS-controlled virulence phenotypes. We posit that this is due to RhlR's unique role in the QS hierarchy of *P. aeruginosa*. Because RhlR controls both its own regulon of virulence factors and plays an important role in regulating the Pqs system, RhlR perturbation by small molecules can drastically alter the expression of virulence factors primarily controlled by each circuit. Second, our study is the first to demonstrate that small molecule-mediated disruption of crosstalk between the Rhl and Pqs systems can inhibit a virulence phenotype in the wild-type organism. Inverse regulation between Rhl and Pqs is likely an important contributor to the maintenance of normal virulence phenotypes; thus, the results presented herein indicate that interference of cross-regulation between Rhl and Pqs is a plausible strategy to attenuate virulence. Lastly, this study further demonstrates the utility of a chemical approach for studying QS in wild-type *P. aeruginosa*, and in bacteria in general. These findings provide an important starting point for the design of effective antivirulence strategies and for future studies of the complex QS circuitry in *P. aeruginosa*. Ongoing work in our laboratory is aimed at delineating the environmental contexts under which chemical modulation of specific *P. aeruginosa* QS circuits is most effective, as well as designing new chemical scaffolds to selectively target individual receptors. These results will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Full details of bacterial strains and plasmids, reporter and phenotypic assay data, and RT-qPCR protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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