QUORUM-SENSING RECEPTOR-LIGAND INTERACTIONS IN

PSEUDOMONAS AERUGINOSA

Amelia Renee McCready

A DISSERTATION
PRESENTED TO THE FACULTY
OF PRINCETON UNIVERSITY
IN CANDIDACY FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

RECOMMENDED FOR ACCEPTANCE
BY THE DEPARTMENT OF
MOLECULAR BIOLOGY

Advisor: Bonnie L. Bassler

April 2019
Abstract

Quorum sensing is a cell-to-cell communication mechanism that bacteria use to orchestrate collective behaviors. Quorum sensing relies on the production, release, accumulation, and detection of extracellular signal molecules called autoinducers. The opportunistic pathogen *Pseudomonas aeruginosa* possesses multiple quorum-sensing systems, each composed of a receptor-autoinducer pair. Two of the *P. aeruginosa* quorum-sensing receptors, LasR and RhlR, are canonical LuxR-type receptors, meaning that they closely resemble the founding quorum-sensing receptor, as well as quorum-sensing receptors present in other species of Gram-negative bacteria. The cognate autoinducers for LuxR-type receptors are homoserine lactones. When bound to their ligands, LuxR-type receptors function as transcription factors that activate hundreds of genes underpinning collective behaviors. This work explores LasR and RhlR ligand binding preferences to understand how each of these receptors interacts with cognate and non-cognate ligands and how receptor-ligand interactions drive regulation of downstream quorum-sensing products. First, the work shows how LasR distinguishes between similar homoserine lactones as well as how LasR preferentially selects its own ligand from a mixture of related molecules. Second, the work reports RhlR ligand-independent mutants that display constitutive activity *in vivo*, providing insight into the enigmatic RhlR structure. Furthermore, the research demonstrates how the activities of RhlR ligand-independent mutants are kept in check with respect to regulation of quorum-sensing products. Finally, this thesis uses a RhlR ligand-independent mutant to reveal how RhlR works in conjunction with the PqsE thioesterase to regulate the production of the critical virulence factor pyocyanin.
Acknowledgments

I want to thank many people who helped me to get to this point in my academic career. First and most importantly I want to thank my amazing parents: Mark and Patricia McCready. You have always supported my dreams and encouraged my career choices. You bought me microscopes and crystal growing kits and took me to many museums. You supported me both financially and emotionally, and I am forever grateful. I also appreciate the wonderful love and support of the rest of my family, particular my kind and loving grandparents: Elgy and Eva Joyce Quick and John and Geraldine McCready. I am also thankful to my cool “little” brother Sam…who I sincerely hope is prepared for me to call him every time my car makes a funny noise for the rest of my natural life.

I am so thankful for the amazing friends that I have made over the years most especially, my Notre Dame Piccolos: Bethany, Catherine, Janelle, Kate and Molly. It’s hard to imagine that we have been friends for almost ten years now because band camp really doesn’t seem that long ago. We have been through a lot since then and I have never had sisters, but you all are the next best thing. To my Friday night buddy: Sister-Amanda. I can’t believe you actually want to listen to me talk about bacteria, but even if you are just pretending, thanks! Thank you to all of the wonderful friends I have met through Princeton, particularly: Cathy, Jared, Krystal and Elizabeth. I have really enjoyed the sushi and the baking. But most of all, it has been really great to commiserate with you all. Knowing that I am not in this alone has been really reassuring. I can’t wait to see where you all go in your amazing scientific careers!

There are two phenomenal women scientists who also been instrumental in my career. First, I want to thank Dr. Patricia Champion. She taught me so much about genetics, bacteria and laboratory work. Most importantly, she pushed me to go to Princeton, which
was one of the best decisions I have ever made. I also want to thank my advisor Dr. Bonnie L. Bassler. Quorum sensing is the best, and you are a wonderful advisor. You have taught me so much! I truly do not understand how you manage everything you do. You have cultivated a wonderful lab, and I so incredibly grateful that I got to be a part of it. It has been an honor to learn from you. I know that they say that you should never meet your heroes, but they are wrong. I work for my hero, and I still think you are incredible.

I want to thank Princeton University and the whole Department of Molecular Biology. I am so glad that I was accepted here and even happier that I chose it. The people here are wonderful and helpful and I truly believe that this department is a special place. Thank you to my thesis committee Dr. Fred Hughson and Dr. Zemer Gitai. I appreciate your input on my research as well as you agreeing to read this thesis. Thank you to the rest of the Bassler Lab. You are all amazing scientists and I am humbled that I get work…and each lunch amongst you. Particularly thank you to Dr. Julie Valastyan and Dr. Jon Paczkowski. Julie, you keep this lab running, and both your science and life advice have been so invaluable to me. You were great bay-mate! To Jon, thank you for helping me with all of the biochemistry! I learned so much working with you, and I really hope that you crystallize RhlR someday. If anyone can do it, you can!

Finally, I would like to thank my partner Tony. Thank you for all of your love and support. Your passion for what you do inspires me to work harder, and your support makes me feel like I can do anything. I am so excited for our life together. Thank you for moving to Philadelphia with me. I know that you have a rough commute ahead of you, but the fact that you are willing to follow me, so that I can follow my dreams, means everything to me.
# TABLE OF CONTENTS

Abstract........................................................................................................................................... iii

**CHAPTER ONE: Introduction ........................................................................................................ 1**  
*Pseudomonas aeruginosa* LuxR-type quorum-sensing circuits.............................................. 2  
Quorum sensing controls virulence in *P. aeruginosa*................................................................. 5  
Quorum sensing regulates biofilm formation in *P. aeruginosa*.............................................. 7  
Inhibition of *P. aeruginosa* quorum sensing........................................................................... 8  
LasR ligand sensitivity and selectivity......................................................................................... 10

**CHAPTER TWO: Structural determinants driving homoserine lactone ligand selection in the Pseudomonas aeruginosa LasR quorum-sensing receptor .......................................................... 11**  
INTRODUCTION ......................................................................................................................... 11  
RESULTS ...................................................................................................................................... 13  
LasR responds to multiple HSL autoinducers........................................................................... 13  
LasR S129 mutations alter ligand sensitivity and ligand discrimination.............................. 20  
The LasR L130F substitution increases LasR ligand sensitivity............................................... 24  
LasR ligand selectivity and sensitivity influence the timing and strength of *in vivo* quorum-sensing-controlled behaviors................................................................. 28  
Structural basis underlying LasR ligand preferences.............................................................. 29  
DISCUSSION .............................................................................................................................. 37

**MATERIALS AND METHODS .............................................................................................. 40**

**ACKNOWLEDGEMENTS ........................................................................................................ 46**

**SUPPLEMENTAL METHODS ................................................................................................. 46**

**SUPPLEMENTAL FIGURES AND TABLES ........................................................................... 54**

**CHAPTER THREE: An autoinducer-independent RhlR quorum-sensing receptor enables analysis of RhlR regulation and distinguishes two roles for PqsE in pyocyanin production in Pseudomonas aeruginosa.......................................................... 67**

INTRODUCTION ......................................................................................................................... 67  
RESULTS ...................................................................................................................................... 69  
A screen for ligand-independent RhlR mutants....................................................................... 69  
Mutations in LasR that are homologous to those in RhlR* do not confer ligand independence to LasR ................................................................. 73  
RhlR and RhlR* protein purification and analyses................................................................. 73  
RhlR* drives biofilm formation in *P. aeruginosa*................................................................. 79  
RhlR* activates pyocyanin production...................................................................................... 81  
PqsE catalytic activity is not required for RhlR* to control pyocyanin production................. 82  
The thioesterase-independent PqsE function in pyocyanin production depends on active RhlR ......................................................................................... 83  
*P. aeruginosa* harboring RhlR* is pathogenic in a *Caenorhabditis elegans* infection assay .............................................................................................................. 85
RsaL prevents RhlR* from overstimulating production of quorum-sensing-controlled products

DISCUSSION

MATERIALS AND METHODS

ACKNOWLEDGEMENTS

SUPPLEMENTAL FIGURES AND TABLES

CHAPTER FOUR: Conclusions and Applications

CHAPTER FIVE: Literature Cited
CHAPTER ONE: Introduction

*Pseudomonas aeruginosa* is an opportunistic human pathogen that causes severe nosocomial infections in patients with compromised immune systems, infects patients with introduced medical devices, and causes mortality in cystic fibrosis sufferers (4). *P. aeruginosa* thrives in diverse environments and is capable of colonizing many different tissues. Furthermore, *P. aeruginosa* forms resilient biofilms and possesses multiple efflux pumps that make it naturally resistant to a variety of common antibiotics. These factors present challenges for the treatment of *P. aeruginosa* infections and make the development of novel treatment strategies an urgent priority. *P. aeruginosa* uses the inter-cellular communication process, called quorum sensing, to optimally deploy its virulence factors and to control biofilm formation. Therefore, studies of *P. aeruginosa* quorum sensing could provide opportunities to inhibit *P. aeruginosa* infections.

![Figure 1.1: Quorum sensing orchestrates bacterial collective behaviors.](image)

Bacteria produce and detect small molecule signals called autoinducers (purple triangles) to coordinately activate group behaviors. At low cell density they act as individuals, but at high cell density they act as a collective.
**Pseudomonas aeruginosa LuxR-type quorum-sensing circuits**

Quorum sensing is a mechanism of chemical communication that bacteria employ to monitor cell density and coordinate group behaviors (5). Quorum sensing relies on chemical signal molecules called autoinducers that bacteria produce, release, and detect (6, 7). At low cell density, autoinducer concentration is low and bacteria act as individuals. As cell density increases, autoinducer concentration likewise increases until a critical concentration is reached, at which point bacteria coordinately detect the presence of the autoinducers, and in response, activate expression of genes underpinning group behaviors (Fig 1.1). Gram-negative bacteria, including *P. aeruginosa*, often use cytoplasmic quorum-sensing receptors called LuxR-type receptors (8, 9). Typically, LuxR-type receptors consist of a ligand binding domain and a DNA binding domain, and they act as transcription factors that are only active when complexed with their cognate autoinducers (3, 10, 11). LuxR-type receptors use homoserine lactones as autoinducers, and these molecules possess identical lactone head groups and acyl tails that vary in length and decoration (12).

*P. aeruginosa* possesses two LuxR-type quorum-sensing receptors: LasR and RhlR, which detect the homoserine lactones called N-3-oxo-dodecanoyl-L-homoserine lactone (3OC12HSL) and N-butyryl-L-homoserine lactone (C4HSL), respectively (Fig 1.2). 3OC12HSL is synthesized by LasI, and C4HSL is synthesized by RhlI (6, 13). Like most other LuxR-type receptors, LasR and RhlR are inactive in the absence of a ligand (6, 14). However, when the cognate autoinducers are present, these proteins bind them, dimerize, are stabilized, and act as transcription factors. LasR sits at the top of the quorum-sensing cascade and, in addition to activating other genes, acts as a transcriptional activator of the gene encoding RhlR. RhlR, following detection of C4HSL, then activates its own set of target genes.
In addition to stimulating RhlR production, LasR also activates transcription of the gene encoding a quorum-sensing negative regulator called RsaL (15). RsaL, a transcription factor, accumulates in late stationary phase and prevents the over-production of quorum-sensing products, many of which are expensive public goods (16, 17). Indeed, previous studies demonstrate that a ΔrsaL strain of P. aeruginosa overproduces a virulence factor called pyocyanin (15). By preventing excessive production of virulence factors and other public goods, RsaL presumably enables P. aeruginosa to optimally manage its resources. Furthermore, because it allows P. aeruginosa to halt the production of many products, it has been suggested that RsaL is involved in allowing P. aeruginosa to adapt rapidly to

**Figure 1.2: The Pseudomonas aeruginosa LuxR-type quorum-sensing circuit.** The LasR receptor detects an autoinducer called 3OC_{12}HSL, made by the synthase LasI. The LasR:3OC_{12}HSL complex activates many genes including rhlR which encodes the second receptor RhlR. RhlR detects two autoinducers, C_{4}HSL synthesized by RhlI and an “alternative autoinducer” synthesized by PqsE. When complexed with either of these RhlR activates its own regulon. RsaL is a negative regulator activated by LasR. It binds directly to the downstream quorum-sensing genes and prevents their overproduction.
changing environments (18). By possessing opposing activities, together, LasR and RsaL set the quorum-sensing window of activity.

Compared to other receptors in the LuxR family, RhlR has some unique features. Most LuxR-type receptors bind tightly to and are solubilized by their cognate autoinducers. For example, LasR binds 3OC_{12}HSL with low nanomolar affinity and this interaction has enabled purification and crystallization of the LasR ligand binding domain (3, 19). In contrast, RhlR has micromolar affinity for C_{4}HSL (20). Furthermore, RhlR is not solubilized by C_{4}HSL, making biochemical analyses of RhlR difficult (14). Thus, little is known about the structure or behavior of RhlR because it has never been purified. Bioinformatic analyses suggest that RhlR shares some homology with other LuxR-type receptors especially with respect to its putative ligand binding pocket. Beyond its biochemical intractability, another apparently unique feature of RhlR is that in addition to C_{4}HSL, RhlR responds to a second, alternative autoinducer (1). The identity of the alternative autoinducer is unknown, but it is believed to enable RhlR to drive a distinct regulon of quorum-sensing genes compared to the genes regulated when C_{4}HSL is bound. There is evidence that the alternative autoinducer is synthesized by a thioesterase called PqsE (21). This alternative autoinducer appears to be important in the context of acute *P. aeruginosa* infection, and consistent with this notion, murine infection studies demonstrate that a Δ*rhlI* strain of *P. aeruginosa* is virulent, whereas both the Δ*rhlR* and the Δ*pqsE* strains are avirulent (1, 21). These data suggest that under some conditions RhlR relies on the alternative autoinducer rather than C_{4}HSL to stimulate virulence factor production and regulate infectivity.
**Quorum sensing controls virulence in *P. aeruginosa***.

Microarray and RNAseq analyses reveal that LasR and RhlR control the transcription of hundreds of genes in *P. aeruginosa*, including many virulence genes and genes required for biofilm formation (22). Some of these genes are regulated jointly, and others are regulated by either LasR or by RhlR (14). One of the products that LasR and RhlR jointly control is a secreted virulence factor called pyocyanin. Pyocyanin is a green-blue pigmented phenazine molecule that has been shown to cause profound cellular damage in the host (23). Amongst other effects, pyocyanin increases the free radical population in host cells and imposes oxidative stress on the cell. Pyocyanin production begins with either of the two *phz1* and *phz2* operons, which encode the enzymes required for production of a pyocyanin precursor molecule called phenazine-1-carboxylic acid (PCA) (Fig 1.3) (24). PCA is processed by the enzyme PhzM in 5-methylphenazine-1-carboxylic acid betaine and this product is processed by PhzS, to produce pyocyanin (24).

It has been suggested that two *phz* operons exist to allow differential regulation of pyocyanin production under diverse environmental conditions (25). For example, reports suggest that *phz1* is the primary active operon in liquid culture, while *phz2* is the primary active operon under surface growth conditions (25). The two *phz* operons have distinct promoters and appear to be regulated differently. The promoter of *phz1* contains a classic Lux-box, an element recognized by LuxR-type activators, and indeed, the *phz1* promoter can be bound by LasR and RhlR to stimulate its expression (26). In contrast, the sequence of the *phz2* promoter is distinct from *phz1* and it does not possess a Lux-box, suggesting that it may not be directly regulated by LasR and RhlR. Nonetheless, RhlR is required for the activation of both *phz1* and *phz2* and the production of pyocyanin (27). In addition to RhlR, PqsE is also required for the production of pyocyanin, possibly due to its role in
biosynthesis of the alternative RhlR autoinducer (21, 28). This thesis examines RhlR ligand binding and RhlR and PqsE regulation of pyocyanin in Chapter 3.

In addition to pyocyanin, quorum sensing also regulates many other virulence factors in *P. aeruginosa*. Elastase and rhamnolipid are two quorum-sensing-controlled virulence factors that are discussed here because they are used as reporters of *P. aeruginosa* quorum sensing throughout this thesis. Elastase is a protease that breaks down elastin, a crucial component of the host extra-cellular matrix, (29-31). Elastase is encoded by the *lasB* gene which is primarily activated by LasR (8). Rhamnolipid, a glycolipid surfactant that plays a role in *P. aeruginosa* swarming motility and biofilms is harmful because it disrupts epithelial cell tight-junctions in the host (32-34). The genes that encode rhamnolipid, *rhlA* and *rhlB*, are primarily activated by RhlR during stationary phase (35). Furthermore, *rhlA* appears to require both RhlR complexed with C₄HSL and the alternative autoinducer (1).
**Quorum sensing regulates biofilm formation in *P. aeruginosa*.**

*P. aeruginosa* biofilms present one of the major difficulties that clinicians face in treating infections (36). Bacterial populations living in biofilms are up to a 1000-fold more resistant to antibiotics than isogenic planktonic populations (37). Quorum sensing controls *P. aeruginosa* biofilm formation by activating production of important exoproducts like phenazines (including pyocyanin) as well as Pel polysaccharides and surfactants (34, 38). There is substantial evidence that quorum-sensing regulators are necessary for *P. aeruginosa* biofilm formation under diverse conditions (1, 39, 40). The PA14 strain of *P. aeruginosa* used in the present work forms biofilms with a characteristic rugose center and smooth periphery on Congo red plates, and Δ*rhlI* and Δ*rhlR* mutants are defective for biofilm formation (1). In addition to forming biofilms on surfaces, *P. aeruginosa* also forms streamer-like biofilms under flow conditions (39). Streamers prevent normal flow in models of medical devices such as stents and catheters (41). Furthermore, quorum sensing is required for streamer formation because a Δ*lasR* strain of *P. aeruginosa* does not form...
streamers under flow conditions (39). Collectively, these findings demonstrate that quorum sensing is crucial for *P. aeruginosa* to form resilient biofilms.

**Inhibition of *P. aeruginosa* quorum sensing**

In light of the crucial role that quorum sensing plays in *P. aeruginosa* virulence, as well as the shortage of effective treatments for *P. aeruginosa* infections, there is currently avid interest in developing quorum-sensing inhibitors as potential therapeutics. The logic is that interruption of quorum-sensing communication will make pathogens, such as *P. aeruginosa*, that rely on cell-cell communication for virulence unable to successfully mount infections. Additionally, because quorum sensing is not required for bacterial survival, it is suggested that quorum-sensing inhibitors would not be as prone to resistance development compared to traditional antibiotics. One of the major strategies undertaken to interfere with *P. aeruginosa* quorum sensing is to identify inhibitors of LasR and RhlR that bind to and prevent these receptors from activating transcription of virulence genes. Different research groups have attempted to identify LasR and RhlR inhibitors. Some have screened natural product libraries to identify molecules that inhibit quorum-sensing products. Such work revealed compounds including one isolated from Australian algae called Furanone-56 and another from garlic extract called ajoene (42, 43). Each of these compounds do reduce the production of quorum-sensing products, however, further study has revealed that both compounds have off target effects and do not bind directly to RhlR or LasR to act as inhibitors (43).

Another approach for identifying LasR and RhlR inhibitors has been to apply inhibitors discovered against other LuxR-type receptors to the *P. aeruginosa* LuxR-type receptors. One such compound is meta-bromo-thiolactone (mBTL). mBTL is structurally
similar to a compound called chlorolactone (CL), that was originally identified as an inhibitor of the Chromobacterium violaceum LuxR-type receptor CviR (44, 45). CL locks CviR into an inactive conformation (44). mBTL has been shown to bind LasR in the ligand binding pocket with similar affinity as 3OC$_{12}$HSL (46). mBTL also binds RhlR, and based on homology, presumably also interacts with the ligand binding pocket. At very high concentrations, mBTL does inhibit production of some $P$. aeruginosa quorum-sensing products including pyocyanin, however, it is primarily an agonist of both LasR and RhlR (14). Therefore, mBTL is not likely to be a viable therapeutic agent. Nevertheless, mBTL has proven useful as a research tool compound. As mentioned above, RhlR cannot be solubilized by C$_4$HSL, but it has been solubilized with mBTL (14). This work describes the first successful purification of RhlR using mBTL as the ligand (Chapter 3).

Most inhibitor screens have targeted LasR. Initially, LasR seemed like a promising target because of its position at the top of the $P$. aeruginosa quorum-sensing cascade. However, recent studies show that the LasR ligand binding pocket is large, enabling it to accommodate the 3OC$_{12}$HSL ligand. As a consequence, LasR also responds robustly to shorter chain homoserine lactones (47). Moreover, the LasR ligand binding pocket can adopt multiple conformations enabling binding of multiple synthetic ligands bearing different moieties, all of them as agonists (46). The remarkable flexibility of the LasR ligand binding pocket makes designing LasR inhibitors difficult. Finally, LasR appears to be important during acute infections, but during chronic infections and under starvation conditions, LasR is often found to be mutated and inactivated (48) possibly reducing its importance as a target for inhibition. Under such conditions, RhlR apparently bypasses LasR to restore the production of virulence factors (49). RhlR could therefore be a superior candidate for the development of quorum-sensing inhibitors. This work examines the
ligand binding pocket of RhlR and develops tools for studying both its structure and its regulation (Chapter 3).

**LasR ligand sensitivity and selectivity**

Several of the LuxR-type receptor ligand binding domains have been crystallized, including that of LasR. These structures have revealed that portions of the binding pockets are well conserved. Particularly well conserved are the residues in the binding pockets that interact with the lactone ring and the carbonyl on C3 of the acyl tail. In LasR, these residues are tyrosine 56 and tryptophan 60, respectively (3). These structures have also revealed that there are variations in each receptor that allow them to accommodate the appropriate homoserine lactone (3, 50). The finding of the flexibility of the LasR ligand binding pocket is interesting when considering how *P. aeruginosa* lives in nature, as it is known to encounter other species of bacteria (51) some of which produce homoserine lactones similar to 3OC12HSL, and which are indeed bound by LasR. This thesis addresses how LasR selects its cognate autoinducer amongst other similar molecules (Chapter 2). The work also examines which amino acid residues dictate the shape and flexibility of the LasR ligand binding pocket. Interestingly, some LuxR-type receptors, such as TraR from *Agrobacterium tumefaciens* show extreme specificity for a cognate homoserine lactone ligand, while others like SdiA from *Escherichia coli* are promiscuous like LasR and respond to multiple ligands. This thesis explores how the structures of LuxR-type receptors dictate ligand specificity or ligand promiscuity (Chapter 2).
CHAPTER TWO: STRUCTURAL DETERMINANTS DRIVING HOMOSERINE LACTONE LIGAND SELECTION IN THE *PSEUDOMONAS AERUGINOSA* LASR QUORUM-SENSING RECEPTOR

INTRODUCTION

Quorum sensing is a cell-cell communication process that enables bacteria to collectively control behavior (52). Quorum sensing relies on the production, release, and detection of extracellular signal molecules called autoinducers (53-55). At low cell density, autoinducer concentration is low, and bacteria act as individuals. As cell density increases, autoinducer concentration also rises. Under this condition, autoinducers bind to cognate receptors, initiating the population-wide regulation of genes underlying collective behaviors.

Many species of Gram-negative bacteria use acylated homoserine lactones (HSLs) as autoinducers (6, 7, 13, 56, 57). HSL autoinducers possess identical lactone head groups, but they vary in acyl chain length and decoration. The chain modifications promote specificity between particular HSL autoinducers and partner receptors (12). There are two kinds of HSL receptors. First, there are LuxR-type receptors, which are cytoplasmic HSL-binding transcription factors that possess variable ligand binding domains (LBD) and well-conserved helix-turn-helix DNA binding domains (DBD) (10, 58). There are also LuxN-type receptors, which are membrane-spanning two-component signaling proteins that bind HSLs in their periplasmic regions and transduce information regarding ligand occupancy internally by phosphorylation/dephosphorylation cascades (5, 52, 59).
Ligand specificity has been examined in the founding member of the LuxN receptor family from *Vibrio harveyi* (60). LuxN is exquisitely selective for its cognate autoinducer 3OHC_{4}HSL. Specific amino acids were identified in the predicted LuxN transmembrane spanning region that confer selectivity for chain length and for chain decoration. Longer HSLs competitively inhibit LuxN, suggesting that, in mixed-species consortia, *V. harveyi* monitors the vicinity for competing species, and in response to their presence, exploits LuxN antagonism to delay the launch of its quorum-sensing behaviors, thus avoiding loss of expensive public goods to non-kin.

Some analyses of HSL preference in LuxR-type receptors have also been performed (61). TraR from *Agrobacterium tumefaciens* excludes non-cognate HSLs (10, 62-64), LasR from *Pseudomonas aeruginosa* detects several long chain HSLs (65), and SdiA from *Escherichia coli* is highly promiscuous and avidly responds to HSLs with variable chain lengths (66-68). Comparison of structures of the LasR and TraR LBDs suggests that increased hydrogen bonding to the ligand in TraR, compared to LasR, accounts for the selectivity difference (65). Here, we systematically explore the LasR response to 3OC_{12}HSL and non-cognate HSLs. We use mutagenesis to establish the amino acid determinants that enable LasR to discriminate between HSLs. We identify LasR S129 as an amino acid residue that, when altered, decreases the LasR response to 3OC_{12}HSL and consequently, restricts the set of HSLs capable of activation. In contrast, we find that LasR L130F improves the LasR response to 3OC_{12}HSL, and as a result, allows LasR to respond to a broader set of HSLs than wildtype LasR. We also investigate how LasR ligand sensitivity and promiscuity affect the timing and strength of quorum-sensing control of *P. aeruginosa* behaviors. Finally, we solve crystal structures of the LasR LBD L130F bound to non-cognate autoinducers to establish the structural basis underlying ligand selectivity.
and sensitivity. We find that a flexible loop located near the ligand binding pocket promotes ligand promiscuity in the wildtype protein. This loop exists in SdiA, which is promiscuous, but not in TraR which is highly specific for its cognate ligand. We propose that evolution has established a balance between ligand discrimination and ligand sensitivity in LasR. Because LasR requires higher concentrations of non-cognate autoinducers than its cognate autoinducer for activation, this tradeoff could allow LasR to robustly respond to its own signal molecule, even in the presence of other bacteria that are producing HSLs. Nonetheless, because LasR can detect high concentrations of other HSLs, P. aeruginosa would be capable of reacting to the presence of these other bacteria when it is outnumbered.

RESULTS

LasR responds to multiple HSL autoinducers.

To investigate the preference LasR displays for different HSLs, we employed a plasmid reporter system in which transcription from a LasR-controlled promoter fused to luciferase (plasB-lux) was assessed in E. coli. Arabinose-inducible lasR was cloned on a second plasmid (35, 69). A set of HSLs differing in both carbon chain length and in functionality at the C-3 position was synthesized using a modification of a previously reported method (see Supplementary Methods and (70). Fig 2.1A shows reporter output following addition of 100 nM of the cognate autoinducer, 3OC_{12}HSL, and four other HSLs (3OC_{14}HSL, 3OC_{10}HSL, 3OC_{8}HSL, and 3OC_{6}HSL). All five HSLs activated LasR, but to differing levels. 3OC_{12}HSL, 3OC_{14}HSL, and 3OC_{10}HSL elicited maximal LasR activity, and 3OC_{8}HSL and 3OC_{6}HSL stimulated 7-fold and 18-fold less activity, respectively. Using dose response analyses, we examined these 5 compounds and 7 other HSLs harboring different functionalities on the C-3 carbon in combination with the various chain
lengths (Table 2.1 and Table 2.S1). 3OC\textsubscript{12}HSL was the most potent ligand, with an EC\textsubscript{50} of 2.8 nM. 3OC\textsubscript{14}HSL was half as potent with an EC\textsubscript{50} of 6.2 nM. From there, the EC\textsubscript{50} values followed the order 3OC\textsubscript{10}HSL < 3OC\textsubscript{8}HSL < 3OC\textsubscript{6}HSL. The Supplementary Table (Table 2.S1) shows data for the C-3 modifications, and that the ketone versions of the molecules are the most potent for every chain length. For this reason, we used the five HSLs shown in Table 2.1 for much of the remainder of this work. In all assays, appropriate concentrations of HSLs were chosen based on EC\textsubscript{50} data from the dose response assays (Table 2.1 and Table 2.S1).
Figure 2.1: LasR is activated by multiple homoserine lactone autoinducers. A) LasR-dependent bioluminescence was measured in *E. coli*. Arabinose-inducible LasR was produced from one plasmid and the *lasB-lux* reporter construct was carried on a second plasmid. 0.1% arabinose was used for LasR induction. B) Elastase activity was measured from Δ*lasI* *P. aeruginosa* using elastin-Congo red as the substrate. In A and B, 100 nM of the designated HSLs were tested. Two technical replicates were performed for each biological sample and 3 biological replicates were assessed. Error bars denote standard deviations of the mean. Paired 2-tailed t-tests were performed comparing each compound to the DMSO control. P-values: * <.05, ** <.01, *** <.0001. C) Comparison of LasR LBD protein levels in whole cell lysates (W) and in the soluble fractions (S) of *E. coli* cells harboring DNA encoding the LasR LBD on a plasmid. 1 mM IPTG was used for LasR LBD induction and either 1% DMSO or 10 µM of the indicated HSL was supplied. In all lanes, protein from .05 OD of cells was loaded. Results are representative of 3 trials. D) Thermal shift analyses of purified LasR LBD bound to 3OC₁₀HSL (left), 3OC₁₂HSL (middle), and 3OC₁₄HSL (right) without (designated DMSO) and following supplementation with an additional 10 µM of the indicated HSLs. Normalized fluorescence represents the first derivative of the raw fluorescence data (2) Each line represents the average of 3 replicates.
We measured \textit{in vivo} LasR activity in response to the test HSLs using an elastase assay (8). Elastase is encoded by \textit{lasB} and thus, is driven by the same promoter as we employed in the above recombinant \textit{E. coli lux} reporter assay. For the elastase analyses, we used a $\Delta$lasI \textit{P. aeruginosa} strain that makes no endogenous 3OC$_{12}$HSL. When supplied at 100 nM, all of the test compounds elicited some elastase activity, but 3OC$_{12}$HSL stimulated the highest elastase production (Fig 1B). To ensure that 100 nM was an appropriate ligand concentration for this assay, we measured elastase activity at 3OC$_{12}$HSL concentrations ranging from 1 nM to 1 $\mu$M (Fig 2S1). We do note that in \textit{P. aeruginosa}, 3OC$_{14}$HSL and 3OC$_{8}$HSL stimulated lower activity than expected based on their EC$_{50}$ values in \textit{E. coli}. Indeed, as shown below, these two molecules had reduced activity in all assays in all \textit{P. aeruginosa} strains used here. While we do not know the underlying molecular mechanism, we suspect that efflux pumps present in \textit{P. aeruginosa} but not in \textit{E. coli} could be responsible. For example, the \textit{P. aeruginosa} MexAB-OprM efflux pump can eliminate homoserine lactones as evidenced by reductions in EC$_{50}$ values in the $\Delta$mexAB-oprM mutant (71). Nonetheless, our results indicate that with respect to the HSLs we tested, LasR is most responsive to its cognate autoinducer 3OC$_{12}$HSL. However, non-cognate HSLs can induce production of the quorum-sensing product elastase, and presumably other quorum-sensing-regulated outputs.

LasR and most other LuxR-type receptors fold around their cognate HSL ligands. Thus, they are only soluble, capable of dimerizing, and capable of binding DNA when ligand is present (3, 63). The results in Fig 2.1A and 2.1B suggest that LasR can fold around HSLs in addition to 3OC$_{12}$HSL. To verify this notion, we tested whether LasR could be solubilized by non-cognate HSLs. To do this, we grew \textit{E. coli} producing the LasR LBD in the presence of each of the HSL compounds in our collection (Fig 2.1C shows the five test
compounds and Fig 2.S2A shows the eleven other compounds in the collection). Consistent with previous results, in the absence of any ligand (DMSO control), the LasR LBD is present in the whole cell lysate, but not in the soluble fraction, indicating that the protein is insoluble (14, 72). With respect to the main HSL test compounds, all except for 3OC₆HSL solubilized the LasR LBD (Fig 2.1C). Nonetheless, we could only purify to homogeneity the LasR LBD bound to the ligands containing the longer acyl chains: 3OC₁₂HSL, 3OC₁₄HSL, and 3OC₁₀HSL. Together, the results in Fig 2.1A-C suggest that although 3OC₈HSL and 3OC₆HSL can bind to and activate LasR, their interactions must be less stable than ligands with longer acyl chains. To test this hypothesis, we performed thermal shift analyses on LasR LBD-ligand complexes without and with the addition of either the same or a different HSL. The LasR LBD bound to 3OC₁₀HSL, 3OC₁₂HSL, and 3OC₁₄HSL had apparent melting temperatures of 42.3 °C, 49.1 °C, and 50.5 °C, respectively (Fig 2.1D, black lines) showing that LasR stability increases with increasing ligand chain length. Notably, the LasR LBD is slightly more stable when bound to the non-cognate ligand 3OC₁₄HSL, than when bound to the cognate ligand 3OC₁₂HSL. The discrepancy between the enhanced stability of purified LasR LBD:3OC₁₄HSL relative to LasR LBD:3OC₁₂HSL in the thermal shift assay and the higher EC₅₀ LasR displays for 3OC₁₄HSL compared to 3OC₁₂HSL could result from increased hydrophobic interactions with the C14 chain in the stably purified complex that do not drive LasR activation.

Exogenously supplied autoinducers can shift the apparent melting temperature of an existing receptor-ligand complex if the added ligand has the ability to stabilize the unfolding protein as it releases the pre-bound ligand. Importantly, exogenously supplied HSLs can only stabilize the LasR LBD if they have affinities that are equal to or higher than the originally bound ligand (69). For the LasR LBD:3OC₁₀HSL complex,
exogenously added 3OC\textsubscript{10}HSL, 3OC\textsubscript{12}HSL, and 3OC\textsubscript{14}HSL stabilize the LasR LBD, increasing the apparent melting temperature 3.4 °C, 6.4 °C, and 5.5 °C, respectively (Fig 2.1D). By contrast, the LasR LBD:3OC\textsubscript{12}HSL and the LasR LBD:3OC\textsubscript{14}HSL complexes could be further stabilized by exogenously supplied 3OC\textsubscript{12}HSL and 3OC\textsubscript{14}HSL, but not by 3OC\textsubscript{10}HSL (Fig 2.1D). While the short acyl chain HSLs could activate LasR with high micromolar EC\textsubscript{50} values in the \textit{plasB-lux} reporter assay, 3OC\textsubscript{6}HSL and 3OC\textsubscript{8}HSL did not stabilize the LasR LBD protein sufficiently in \textit{E. coli} for purification, presumably due to their low affinities (Fig 2.1C and Table 2.1). Thus, they could not be tested in the traditional thermal shift assay. They also did not enhance the stabilization of the LasR LBD pre-bound with other ligands, analogous to the inability of 3OC\textsubscript{10}HSL to stabilize the LasR LBD:3OC\textsubscript{12}HSL complex as it melted (Fig 2.1D). Together, our results indicate that in an environment containing a mixture of HSL autoinducers, LasR, while capable of detecting several HSLs, will preferentially detect long chain HSLs, with superior preference for its own autoinducer 3OC\textsubscript{12}HSL, followed closely by 3OC\textsubscript{14}HSL.
Table 2.1: EC$_{50}$ values (nM) for LasR HSL compounds in the *plasB-lux* assay

<table>
<thead>
<tr>
<th>Homoserine Lactone</th>
<th>WT</th>
<th>S129C</th>
<th>S129W</th>
<th>S129F</th>
<th>S129T</th>
<th>S129M</th>
<th>L130F</th>
</tr>
</thead>
<tbody>
<tr>
<td>3OC$_{12}$HSL</td>
<td>2.8</td>
<td>5.9</td>
<td>77</td>
<td>177</td>
<td>870</td>
<td>6600</td>
<td>2.0</td>
</tr>
<tr>
<td>3OC$_{14}$HSL</td>
<td>6.2</td>
<td>12</td>
<td>13</td>
<td>41</td>
<td>4100</td>
<td>NR</td>
<td>5.3</td>
</tr>
<tr>
<td>3OC$_{10}$HSL</td>
<td>8.0</td>
<td>32</td>
<td>2600</td>
<td>4980</td>
<td>4500</td>
<td>NR</td>
<td>5.0</td>
</tr>
<tr>
<td>3OC$_{8}$HSL</td>
<td>900</td>
<td>2900</td>
<td>2200</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>81</td>
</tr>
<tr>
<td>3OC$_{6}$HSL</td>
<td>2400</td>
<td>4400</td>
<td>NR</td>
<td>55000</td>
<td>NR</td>
<td>NR</td>
<td>1400</td>
</tr>
</tbody>
</table>

NR Denotes Non-responsive

95% Confidence intervals for EC$_{50}$ values are provided in Table 2.S2
LasR S129 mutations alter ligand sensitivity and ligand discrimination.

Our above results show that LasR detects multiple HSL ligands with different preferences. To alter those preferences, we mutagenized the LasR ligand binding pocket using the existing LasR LBD crystal structure as a guide (3). Among the set of mutants that we made, we identified one residue of interest: LasR S129. Previous work demonstrated that alteration of serine to alanine at this position transformed some LasR activators into inhibitors and vice-versa (73, 74). Moreover, the LasR LBD structure suggests that S129 is part of the network that interacts with the ligand acyl chain (3). Finally, S129 is well conserved across LuxR-type receptors, so we predicted that it could play a crucial role in ligand binding (75). We constructed LasR S129C, S129W, S129F, S129T, and S129M and examined their activities in the *E. coli* *lasB-lux* reporter assay, using 1 µM of each compound (Fig 2.2A). Wildtype LasR and LasR S129C showed some response to every test compound. Each of the other four mutants failed to respond to one or more of the HSL test compounds (Table 2.S1 Fig 2.2A). We used western blot analysis to confirm that all of the LasR S129 mutant proteins were produced at similar levels (Fig 2.S2B), demonstrating that the phenotypes are due to alterations in responses to ligands. Dose response analyses with the complete set of HSLs enabled us to obtain EC$_{50}$ values for each mutant. These values reveal that each LasR S129 mutant displays reduced activity with 3OC$_{12}$HSL compared to wildtype LasR (Table 2.1 and Table 2.S1). Moreover, there exists a consistent relationship between the activity LasR mutants display with 3OC$_{12}$HSL and the range of HSLs to which they respond. Mutants with the highest activity with 3OC$_{12}$HSL interact with a larger range of HSLs than mutants with reduced activity with 3OC$_{12}$HSL. For example, LasR S129M had the highest EC$_{50}$ for 3OC$_{12}$HSL and it showed no response to any other HSL in our set of test compounds (Table 2.1 and Table 2.S1). Conversely,
LasR S129C responded to all of the HSLs we tested except 3OHC₈HSL, C₈HSL, and 3OHC₆HSL, and it has the lowest EC₅₀/highest activity with 3OC₁₂HSL (Table 2.1 and Table 2.S1). With respect to EC₅₀ for 3OC₁₂HSL, our alleles followed the pattern wildtype < LasR S129C < LasR S129W < LasR S129F < LasR S129T < LasR S129M. They followed the exact reverse order in terms of breadth of response to different HSLs. These findings and the location of S129 in the LasR ligand binding pocket, support the proposal that substitutions disrupt a crucial hydrogen bond between S129 and the ketone in the autoinducer acyl chain, thus negatively affecting ligand sensitivity (73).
Figure 2.2: LasR S129 mutants alter LasR responses to HSL autoinducers. A) Wildtype LasR and LasR S129 mutant bioluminescence from the *E. coli* *p*las*B*-lux reporter. 1 µM of the indicated HSLs were provided (see Fig 2.1, panel A for detail). B) Wildtype LasR and LasR S129F-driven elastase activity in Δ*lasI* *P. aeruginosa*. 10 µM of the indicated HSLs were provided (see Fig 2.1, panel B for detail). In panels A and B, 2 technical replicates were performed for each biological sample and 3 biological replicates were assessed. Error bars denote standard deviations of the mean. Unpaired t-tests were performed to compare each mutant LasR-HSL combination to that of wildtype LasR with the same compound. Due to space constraints, statistics for panel A are provided in Table S3. In panel B, P-values: ** <.01, *** <.0001. C) Comparison of LasR S129F LBD protein levels in whole cell lysates (W) and in the soluble fractions (S) of *E. coli* cells harboring DNA encoding the LasR S129F LBD on a plasmid. 1 mM IPTG was used for LasR S129F LBD induction and either 1% DMSO or 10 µM of the indicated HSL was supplied. In all lanes, protein from .05 OD of cells was loaded. Results are representative of 3 trials. D) Thermal shift analyses of purified LasR S129F LBD bound to 3OC<sub>12</sub>HSL (left) and 3OC<sub>14</sub>HSL (right) without (designated DMSO) and following supplementation with an additional 10 µM of the indicated HSLs. Normalized fluorescence represents the first derivative of the raw fluorescence data (2). Each line represents the average of 3 replicates.
To explore the hydrogen bonding role of the amino acid at position 129 on ligand selection, we focused on LasR S129F. Our rationale was that a serine to phenylalanine substitution eliminates hydrogen bonding and, moreover, because the LasR S129F mutant has an intermediate phenotype in our assays (i.e., not the strongest and not the weakest), this allele would allow us to probe activity in vitro and in vivo. First, we examined the consequences of the LasR S129F alteration in vivo by incorporating the lasR S129F mutation onto the chromosome of the ΔlasI P. aeruginosa strain and assaying elastase activity (Fig 2.2B). We used 10 µM of each test HSL compound because increased ligand concentration is required to activate LasR S129F relative to wildtype LasR (see Table 2.1). LasR S129F was capable of promoting elastase production, but only in response to a subset of the HSLs that were capable of stimulating wildtype LasR, and as noted, in every case, at a higher ligand concentration than required for wildtype LasR.

To investigate the mechanism underlying the reduction in LasR S129F response to the HSL test compounds, we assessed whether it could be solubilized by them. Fig 2.2C shows that, similar to wildtype LasR, LasR S129F is not solubilized by our shortest test compound 3OC₆HSL, but unlike wildtype LasR, LasR S129F is also not solubilized by 3OC₈HSL. These results support our above finding regarding the inability of 3OC₈HSL to elicit a response from LasR S129F and they underpin the low sensitivity LasR S129F displays for 3OC₆HSL (See Fig 2.2A and Table 2.1). To evaluate the stability of LasR S129F, we used thermal shift measurements. The LasR S129F:3OC₁₂HSL complex had an apparent melting temperature of 35 °C showing that it is markedly less stable than the wildtype LasR:3OC₁₂HSL complex (Fig 2.1D and Fig 2.2D). Both 3OC₁₂HSL and 3OC₁₄HSL stabilized LasR S129F during melting and release of pre-bound 3OC₁₂HSL. Unlike wildtype LasR, (Fig 2.1D), LasR S129F preferred 3OC₁₄HSL over 3OC₁₂HSL (Fig 2.2D)
consistent with its 4-fold lower EC$_{50}$ for 3OC$_{14}$HSL than for 3OC$_{12}$HSL (Table 2.1). These findings indicate that the loss of hydrogen bonding between the C-1 ketone and the S129 residue can be compensated by enhanced hydrophobic interactions with the C$_{14}$ chain.

**The LasR L130F substitution increases LasR ligand sensitivity.**

Based on our results with the LasR S129 mutants, we suspected that other LasR autoinducer binding pocket residues located in the vicinity of the ligand acyl chain could participate in ligand selection. For this reason, we exchanged L130 and L128 for phenylalanine residues. As above, we tested their responses to the set of representative HSLs using the *E. coli* plasB-lux reporter assay (Fig 2.3A, Table 2.1, and Table 2.S1). Table 2.1 shows that, surprisingly, LasR L130F exhibited a lower EC$_{50}$ for every autoinducer tested. Shorter chain HSLs showed the most dramatic increases in activity. LasR L128F also conferred improved sensitivity to some short chain HSLs (Table 2.S1), but its phenotype was less pronounced than that of LasR L130F, so we further analyzed the LasR L130F mutant here. Fig 2.3A shows the wildtype LasR and the LasR L130F responses to a low concentration (50 nM) of the test HSLs. At this concentration, LasR L130F was roughly equivalent to wildtype LasR with respect to the response to 3OC$_{12}$HSL, 3OC$_{14}$HSL, and 3OC$_{10}$HSL. However, LasR L130F was approximately 5-fold more responsive to 3OC$_{8}$HSL than wildtype LasR and it was modestly more responsive to 3OC$_{6}$HSL. *In vivo* examination of LasR L130F ligand responses support these findings. When we assayed low concentrations (50 nM) of the test compounds, wildtype LasR stimulated elastase production only in response to 3OC$_{12}$HSL, whereas LasR L130F responded to 3OC$_{14}$HSL, 3OC$_{10}$HSL, and 3OC$_{8}$HSL in addition to 3OC$_{12}$HSL (Fig 2.3B).
We used thermal shift analyses to explore the mechanism underlying the increased sensitivity of the LasR L130F mutant for HSLs. We used 3OC_{12}HSL and 3OC_{14}HSL as our test ligands. Compared to the wildtype LasR LBD, the LasR LBD L130F is more stable when bound to each ligand (Fig 2.4A), suggesting that the L130F alteration increases the overall stability of the LasR protein. We exploited this feature to successfully purify the LasR LBD L130F bound to 3OC_{8}HSL. As mentioned above, we could not purify the wildtype LasR LBD bound to 3OC_{8}HSL. We performed thermal shift analyses on the LasR LBD L130F bound to 3OC_{8}HSL, 3OC_{10}HSL, 3OC_{12}HSL, and 3OC_{14}HSL to which we added different HSLs. Similar to the wildtype LasR LBD, exogenous addition of HSLs with long acyl chains further enhanced the stability of the LasR LBD L130F:HSL complexes compared to when HSLs with shorter acyl chains were added (Fig 2.1C and Fig 2.4B). For instance, the LasR LBD L130F:3OC_{8}HSL stability was enhanced by the

Figure 2.3: LasR L130F displays enhanced responses to HSL autoinducers. A) Wildtype LasR and LasR L130F-driven bioluminescence from the *E. coli* plasB-lux reporter. 50 nM of the indicated HSLs were provided (see Fig 2.1, panel A for detail). B) Wildtype LasR and LasR L130F-driven elastase activity in ΔlasI *P. aeruginosa*. 50 nM of the indicated HSLs were provided (see Fig 2.1, panel B for detail). In both panels, 2 technical replicates were performed for each biological sample and 3 biological replicates were assessed. Error bars denote standard deviations of the mean. Unpaired t-tests were performed to compare each LasR L130F-HSL combination to that of wildtype LasR with the same compound. P-values: * <.05, *** <.0001.
addition of 3OC₈HSL, 3OC₁₀HSL, 3OC₁₂HSL, and 3OC₁₄HSL but not 3OC₆HSL (Fig 2.4B). Protein solubility analyses track with these findings; long acyl chain ligands solubilize the LasR LBD L130F protein whereas short chain ligands do not (Fig 2.4C and Fig 2.S2C). Consistent with our EC₅₀ values, chain length appears to be the most important factor driving protein solubility and stabilization for both the LasR LBD and the LasR LBD L130F (Fig 2.S2C and Fig 2.4B).
Figure 2.4: The LasR LBD L130F is more stable than the wildtype LasR LBD. A) Thermal shift analyses of purified LasR LBD (solid lines) and LasR LBD L130F (dotted lines) bound to 3OC_{12}HSL (orange) or 3OC_{14}HSL (green). Each line represents the average of 3 replicates. B) Thermal shift analyses of purified LasR LBD L130F bound to 3OC_{8}HSL (top left), 3OC_{10}HSL (bottom left), 3OC_{12}HSL (top right), and 3OC_{14}HSL (bottom right) without (designated DMSO) and following supplementation with an additional 10 µM of the indicated HSLs. In panels A and B, normalized fluorescence represents the first derivative of the raw fluorescence data (2). C) Comparison of LasR L130F levels in the whole cell lysates (W) and the soluble fractions (S) of *E. coli* cells harboring DNA encoding LasR LBD L130F on a plasmid (see Fig 2.1, panel C for details). Either 1% DMSO or 10 µM of the indicated HSL molecule was added.
**LasR ligand selectivity and sensitivity influence the timing and strength of in vivo quorum-sensing-controlled behaviors.**

LasR L130F responds to HSLs at lower concentrations than does wildtype LasR, whereas LasR S129F requires higher concentrations (Table 2.1). Thus, we predicted that introducing these alleles into *P. aeruginosa* should influence its quorum-sensing responses in opposing manners. To test this prediction, we assayed pyocyanin production over time as the quorum-sensing readout in response to 3OC₁₂HSL, in a ΔlasI *P. aeruginosa* strain containing wildtype *lasR*, *lasR S129F*, or *lasR L130F*. For comparison, we also evaluated pyocyanin production over time in wildtype *P. aeruginosa* under the same conditions (Fig 2.5A). Guided by the EC₅₀ value of LasR with 3OC₁₂HSL, we tested an appropriate low (50 nM) and high concentration (1µM) of 3OC₁₂HSL to enable us to observe responses from the wildtype and each mutant. Consistent with their relative EC₅₀ values, when a low concentration of 3OC₁₂HSL (50 nM) was added, the strain with LasR L130F made significantly more pyocyanin than the strain with wildtype LasR at every time point (Fig 2.5A). At this HSL concentration, the strain carrying LasR S129F never activated pyocyanin production, presumably because the concentration of 3OC₁₂HSL was far below the EC₅₀ for LasR S129F (Fig 2.5A). When the same assay was performed with 1µM 3OC₁₂HSL, rapid and maximal pyocyanin output occurred for *P. aeruginosa* carrying both wildtype LasR and LasR L130F. Furthermore, this high ligand concentration reveals that LasR S129F can drive pyocyanin production in response to 3OC₁₂HSL, albeit weakly and only after 5 hours (Fig 2.5B). These results suggest that altering the sensitivity of LasR can change the production of quorum-sensing products such as pyocyanin.
Structural basis underlying LasR ligand preferences.

To define the molecular basis enabling LasR to accommodate non-cognate autoinducers, we determined the structures of the LasR LBD L130F bound to 3OC\textsubscript{10}HSL and 3OC\textsubscript{14}HSL. As mentioned, the structure of the wildtype LasR LBD bound to 3OC\textsubscript{12}HSL was reported previously (3). We used LasR LBD L130F for our studies, reasoning that its enhanced stability might allow crystallization with non-cognate ligands. Indeed, we were able to determine the structures of LasR LBD L130F:3OC\textsubscript{10}HSL and LasR LBD L130F:3OC\textsubscript{14}HSL to 2.1 Å and 1.9 Å, respectively (Table 2.S4). This resolution shows unambiguous ligand density enabling us to compare our structures with the LasR LBD:3OC\textsubscript{12}HSL structure (Fig 2.6A and Fig 2.S4A, S4B). The side chain of F130 is buried in a hydrophobic pocket distal to the ligand binding pocket (Fig 2.S4C and 2.S4D).

Figure 2.5: Wildtype LasR, LasR S129F, and LasR L130F display distinct pyocyanin production phenotypes in response to high and low concentrations of 3OC\textsubscript{12}HSL. Pyocyanin production was measured in \(\Delta\text{lasI}\) \(P.\ aeruginosa\) strains over the growth curve. Y-axis “Pyocyanin” is the amount of pyocyanin pigment (OD\textsubscript{695} nm) over cell density (OD\textsubscript{600} nm). Designations are: red, DMSO control; blue, wildtype LasR; green, LasR S129F; pink, LasR L130F. Concentrations used are: A) 50 nM 3OC\textsubscript{12}HSL, B) 1 \(\mu\text{M}\) 3OC\textsubscript{12}HSL. Data show the mean of 3 biological replicates. Error bars denote standard deviations of the mean. 2-way ANOVAs were performed to compare LasR S129F and LasR L130F to wildtype LasR under each condition. A 2-way ANOVA was also performed to compare wildtype LasR with 3OC\textsubscript{12}HSL and with DMSO alone. P-values: * <.05, *** <.0001.
Compared to leucine, phenylalanine provides increased hydrophobic interactions with amino acid residues L23, L30, F32, I35, L114, L118, L128, L151, and L154. These interactions may stabilize LasR, in turn allowing it to accommodate an expanded set of HSLs compared to wildtype LasR.

To understand how LasR can accommodate multiple long chain HSL ligands in its binding site, we used the crystal structures to examine the binding pocket in detail. In all the structures, the lactone head groups have the exact same placement, likely due to hydrogen bonding between the lactone ring ketone moiety and residue W60 (Fig 2.6B, 2.6C). The head group ketone and the two ketones on the acyl chain are further stabilized by an extensive hydrogen bonding network consisting of residues Y56, R61, D73, T75, W88, Y93, and S129 (Fig 2.6B, 2.6C). In the three structures, the C10, C12, and C14 chains take similar paths until carbon 6, where C10 and C14 diverge from the path taken by C12 chain. As a result, C14 and C12 fill the same volume (Fig 2.S4A). However, the absence of two carbons in the C10 chain compared to the C12 chain could account for the increase in EC50 and lower stability of the LasR LBD L130F:3OC10HSL complex compared to the LasR LBD:3OC12HSL and LasR LBD L130F:3OC14HSL complexes (Fig 2.S4B).
Figure 2.6: Crystal structures of LasR LBD L130F bound to 3OC$_{10}$HSL and 3OC$_{14}$HSL. A) Crystal structures of LasR LBD L130F:3OC$_{10}$HSL (gold) and LasR LBD L130F:3OC$_{14}$HSL (magenta) compared to the wildtype LasR LBD:3OC$_{12}$HSL structure (blue, modified from Bottomley et al. 2007, PDB: 2UVO). The bottom images show 90-degree rotations of the crystal structures relative to the images above. In the two top right-most structures, the asterisks highlight the LasR loop region that includes residues 40-51 and that undergoes a conformational shift when 3OC$_{14}$HSL is bound. B) Structural comparison of LasR LBD:3OC$_{12}$HSL (protein: blue, ligand: orange; modified from PDB: 2UVO) (3) and LasR LBD L130F:3OC$_{10}$HSL crystal structure (protein: gold, ligand: magenta). Amino acids drawn in stick format show important residues for lactone head binding (Y56, W60, R61, D73, T75, W88, Y93, and S129) and acyl chain binding (G38, L40, A50, I52, A70, V76, L125, and A127). C) Structural comparison of LasR LBD:3OC$_{12}$HSL (protein: blue, ligand: orange; modified from, PDB: 2UVO) (3) and LasR LBD L130F:3OC$_{14}$HSL crystal structure (protein: magenta, ligand: green). Amino acids drawn in stick format are the same as in panel B. Note the shift in the position of the loop region corresponding to residues 40-51.
We next investigated whether there were any structural rearrangements in the crystals that could account for the expanded HSL binding capabilities of LasR L130F. An 
~2 Å shift occurs in the loop corresponding to residues 40-51 (highlighted by asterisks in Fig 2.6A and displayed in Fig 2.S4D, 2.S4E) in the LasR LBD L130F:3OC₁₄HSL structure compared to the LasR LBD L130F:3OC₁₀HSL and the LasR LBD:3OC₁₂HSL structures (Fig 2.6B, 2.6C), and we propose the change in the placement of this loop underpins the altered binding capabilities of LasR L130F. Our structures with the different HSLs are consistent with recently reported data showing that the LasR loop conformation can be altered by synthetic agonists and, moreover, potency of the non-native ligands correlates with the thermal stability of the complexes (47).

In each of our newly described structures, there are two LasR monomers in the asymmetric unit. In the LasR LBD L130F:3OC₁₄HSL structure, the crystallographic contacts made by the loop regions in monomer A and monomer B differ slightly, while in the LasR LBD L130F:3OC₁₀HSL structure, the monomer A and monomer B loop regions are similar, and each makes crystal contacts similar to those in monomer B in the LasR LBD L130F:3OC₁₄HSL structure. It was possible that crystal contacts, rather than ligand binding, drove the different loop conformations in the LasR LBD L130F:3OC₁₄HSL structure. Because we know that the conformations of the loop regions in the previously published wild-type LasR LBD:3OC₁₂HSL structure are not influenced by crystallographic contacts, we can reliably compare the positions of the loop regions between LasR LBD L130F:3OC₁₄HSL monomer A and monomer A from the LasR LBD:3OC₁₂HSL structure. Both are free from crystallographic contacts allowing us to conclude that the loop shifts in our crystal structures are ligand-induced (Fig. 2.6A, 2.6C).
The crystal structures show that the majority of the LasR LBD exists as an interacting core that binds the lactone head and a portion of the acyl chain, whereas the loop consisting of residues 40-51 appears to act as a deformable region that enables LasR to accept ligands with different length acyl chains (Fig 2.6B, 2.6C). In the LasR LBD L130F:3OC₁₄HSL structure, Y47 lies parallel to the ligand (Fig 2.6C). We contrast this arrangement to the orientation of Y47 in the LasR LBD L130F:3OC₁₀HSL and LasR LBD:3OC₁₂HSL structures (Fig 2.6C and Fig 2.S4E, Fig 2.S4F). In those cases, Y47 lies perpendicular to the ligands. We suggest that the ability of Y47 to adopt multiple conformations helps accommodate the different ligands. To test this possibility, we mutated LasR residue Y47. LasR Y47R and LasR Y47S displayed decreased sensitivity to both 3OC₁₂HSL and 3OC₁₄HSL in the E. coli reporter assay (Fig 2.S5) suggesting that the loop provides interactions with the ligand chains that foster increased protein stability, allowing LasR to be activated. As previously noted, the packing of Y47 against the ligand acyl chain shields the ligand binding pocket from the bulk solvent (76). We infer that alterations at this site, and perhaps in the loop region generally, lead to protein instability.

To understand the structural basis underlying specificity and promiscuity in this family of proteins, we compared all reported structures of the LBDs of LuxR-type receptors to our structure of LasR LBD L130F:3OC₁₄HSL. The structures are SdiA LBD:3OC₈HSL, CviR LBD:C₆HSL, TraR LBD:3OC₈HSL, and QscR:3OC₁₂HSL (Fig 2.7A, and Fig 2.S6). Similar to what we show here for LasR, SdiA is promiscuous with respect to ligand selectivity (66-68). A loop similar to the one we pinpoint in Fig 2.6A as critical for LasR to accommodate different ligands, exists in the SdiA LBD (Fig 2.7A). Likewise, the orphan P. aeruginosa LuxR-type receptor QscR contains such a loop (Fig 2.S6), and QscR displays ligand promiscuity (11, 77). Conversely, CviR and TraR display strict specificity.
for their cognate autoinducers (10, 63, 78, 79). No such large loop exists in the CviR and TraR LBD structures (Fig 2.7A). Compared to the 12-residue loop in LasR and the 10-residue loop in SdiA, the corresponding CviR and TraR loops are 8 and 4 residues long, respectively. These findings are consistent with the idea that this flexible loop confers ligand promiscuity.

In addition to the differences in the overall structures of the receptors, we noted different conformations for the acyl chains in the LasR LBD structures compared to those in the other LuxR-type receptors. The autoinducer acyl chains in the CviR, TraR, and SdiA LBD structures have similar conformations within the ligand binding pockets terminating between residues Y88 and M89 for CviR, Y61 and F62 for TraR, and Y71 and Q72 for SdiA. By contrast, the acyl chains of the different ligands in the LasR LBD L130F structures orient their terminal carbons toward the opposing face of the ligand binding pocket (Fig 2.7B). These different paths appear to be driven by the hydrophobic interactions we described above for the different LasR ligands (Fig. 2.6B, 2.6C). We identified eight hydrophobic residues (G38, L40, A50, I52, A70, V76, L125, and A127) that dictate the shape of the ligand binding pocket in LasR. Indeed, these residues have generally hydrophobic characteristics in all LuxR-type proteins, but the size of each sidechain varies (Fig. 7B). For example, A127 in LasR corresponds to F132 in SdiA. The smaller residue in LasR accommodates the altered path taken by its autoinducer, allowing the terminal carbon of the acyl chain to bind proximal to residue A127. A bulkier residue at this position, as in SdiA, sterically hinders this path for the ligand, forcing the acyl chain to bend in the opposite direction. Thus, in SdiA, F132 forces the terminal carbon of 3OC8HSL to bind distally. CviR I153 and TraR M127 appear to perform analogous roles.
Figure 2.7: A conserved flexible loop region confers promiscuity to LuxR-type receptors. A) Top images: structural comparison of the LuxR-type receptors LasR LBD L130F:3OC_{14}HSL (magenta) and SdiA LBD:3OC_{8}HSL (green, modified from Nguyen et al., 2015, PDB: AY17) that exhibit promiscuity with respect to ligand binding. Bottom images: structural comparison of the LuxR-type receptors CviR LBD:C_{6}HSL (pink, modified from Chen et al., 2011, PDB: 3QP1) and TraR LBD:3OC_{8}HSL (silver, modified from Zhang et al., 2002, PDB: 1L3L) that display strict ligand specificity. B) Structural comparison of the protein:ligand interfaces for LasR LBD L130F:3OC_{14}HSL (top left, magenta), SdiA LBD:3OC_{8}HSL (top right, green), CviR LBD:C_{6}HSL (bottom left, pink), and TraR LBD:3OC_{8}HSL (bottom right, silver). Residues that make important hydrophobic sidechain interactions in each protein:ligand complex are shown in stick format and named.
To investigate whether a bulkier residue at position 127 would prevent LasR from binding to HSL ligands with long acyl chains, we constructed LasR A127W and assayed it in our *E. coli* plasB-lux reporter assay with 3OC\(_12\)HSL, 3OC\(_{10}\)HSL, 3OC\(_8\)HSL, and 3OC\(_6\)HSL. Fig 2.8 shows that LasR A127W did not respond well to 3OC\(_{12}\)HSL, therefore, we could not determine an EC\(_{50}\) value. However, the LasR A127W mutant did exhibit responses to shorter chain HSLs. The EC\(_{50}\) value of LasR A127W for 3OC\(_{10}\)HSL was 3530 nM, ~500 times higher than wildtype LasR for 3OC\(_{10}\)HSL (8 nM). LasR A127W had EC\(_{50}\) values similar to wildtype LasR for 3OC\(_8\)HSL and 3OC\(_6\)HSL. Indeed, in the case of 3OC\(_8\)HSL, the LasR A127W EC\(_{50}\) was somewhat lower than wildtype LasR (244 nM and 885 nM, respectively; Fig 2.8). We suggest that the large hydrophobic tryptophan side chain at position 127 cannot accommodate the long chain 3OC\(_{12}\)HSL ligand, but the protein is responsive to 3OC\(_8\)HSL and 3OC\(_6\)HSL because the enlarged amino acid side chain in the protein compensates for the missing carbons on the ligands. This finding suggests that larger amino acid residues at A127 improve the LasR response to shorter chain HSLs by enabling more stable protein-ligand complexes to form.
DISCUSSION

*P. aeruginosa* often occupies niches containing other bacterial species that produce HSL autoinducers (80). Our results show that LasR can, with reduced sensitivity, detect non-cognate HSLs and in response, activate transcription of quorum-sensing target genes. We suggest that determining the mechanisms that promote or restrict ligand access to LasR is important for understanding how the *P. aeruginosa* quorum-sensing response could be naturally or synthetically manipulated. Here, we identified mutations that alter how effectively LasR interacts with particular HSLs. Changing S129 increases LasR specificity for long chain ligands over short chain ligands but at a cost of reduced ligand potency, the consequence of which is delayed and dampened quorum-sensing output by *P. aeruginosa* (Fig 2.5A and 2.5B). In contrast, the LasR L130F alteration increases LasR response to 3OC12HSL, but, again at a cost, this time, the penalty is a diminished ability to discriminate against other HSL ligands. We presume, based on the pyocyanin data in Fig 2.5, such a
change would lead to premature and increased quorum-sensing activity. Such mis-timed and mis-regulated release of public goods may not benefit \textit{P. aeruginosa}.

We propose that LasR has evolved to finely balance ligand sensitivity with ligand promiscuity. We investigated the published sequences of hundreds of clinical isolates of \textit{P. aeruginosa} and S129 and L130 are strictly conserved (81) suggesting that they are crucial for \textit{P. aeruginosa} fitness. Striking the ideal balance between LasR ligand sensitivity and ligand specificity may mean that detection of some non-cognate HSLs must be tolerated, although importantly, only at higher concentrations relative to the cognate autoinducer.

We propose that LasR promiscuity could serve an important function in the environment (i.e., soil) and in eukaryotic hosts where \textit{P. aeruginosa} encounters other species of bacteria. For example, in the soil, \textit{Pseudomonas putida} produces 3OC\textsubscript{12}HSL, 3OC\textsubscript{10}HSL, 3OC\textsubscript{8}HSL, and 3OC\textsubscript{6}HSL (82). \textit{Klebsiella pneumoniae}, which resides in human airways, produces C\textsubscript{12}HSL (83). LasR promiscuity may allow \textit{P. aeruginosa} to “eavesdrop” on its competitors. Nonetheless, wildtype LasR detects 3OC\textsubscript{12}HSL more efficiently than other HSLs. Therefore, when \textit{P. aeruginosa} is at high cell density, it is likely that 3OC\textsubscript{12}HSL out-competes all other autoinducers. By contrast, when \textit{P. aeruginosa} is at low cell density, provision of non-cognate ligands made by other bacterial species that are at higher cell density could induce \textit{P. aeruginosa} to activate its quorum-sensing behaviors prematurely. It could be beneficial for \textit{P. aeruginosa} to engage in such behavior, for example, to enable the synthesis of toxic defensive products such as pyocyanin and rhamnolipids that endow \textit{P. aeruginosa} with an advantage over competing species (84, 85). Beyond defensive products, perhaps some of the quorum-sensing-controlled products produced under such conditions can be used exclusively by \textit{P. aeruginosa} and not by competing species. If so, LasR promiscuity could grant \textit{P. aeruginosa}
aeruginosa a “last ditch” opportunity to survive in environments in which it is vastly outnumbered by competing species.

Unlike LasR, SdiA, and QscR, other quorum-sensing receptors are exquisitely specific (e.g., TraR and CviR). Thus, evolution can build receptors to possess or to lack ligand specificity. Presumably, the particular niche in which a quorum-sensing bacterium resides derives the optimal receptor ligand-detection preference strategy. Promiscuous quorum-sensing receptors could be superior when quorum sensing stimulates the production of defensive products that aid in competition with other bacterial species. Conversely, a receptor possessing strict ligand specificity could be optimal in cases in which quorum sensing activates production of vital public goods that require protection from cheaters.

Our combined genetic, biochemical, and structural work revealed the molecular basis for non-cognate autoinducer recognition by LasR. A key flexible loop present in LasR, SdiA, and QscR appears to endow these receptors with the ability to bind to multiple HSL ligands. Conversely, the shorter and apparently less flexible loop present in TraR and CviR confers high specificity. Indeed, this structure-function analysis may help explain why a competitive inhibitor of CviR, chlorolactone (CL), behaves as an agonist for LasR (14). These findings are particularly enlightening when considering attempts to design inhibitors that specifically target different LuxR-type receptors. The flexible loop and hydrophobic residues in LasR near the acyl chain binding site will need to be taken into account when developing small molecule inhibitors that target LasR or other LuxR-type proteins that possess this feature. Designing molecules that target the flexibility of the loop region and/or that destabilize the protein could be explored for promiscuous receptors, such as LasR. By contrast, focused screening around molecules that resemble CL could yield
inhibitors for LuxR-type proteins that display strict specificity for their cognate autoinducers, such as CviR.

MATERIALS AND METHODS

Site directed mutagenesis

Mutations in *lasR* were constructed on the pBAD-A-*lasR* plasmid (69). Primers were designed using the Agilent Quikchange primer design tool and PCR with pFUltra polymerase (Agilent). PCR reactions were treated with DpnI to eliminate parental plasmid DNA and the plasmids with the mutant *lasR* genes were transformed into One Shot TOP10 chemically competent *E. coli* cells (Invitrogen). Reactions were plated on LB agar plates containing ampicillin (50 µg/mL) and individual mutants were verified via sequencing with primers for the *lasR* gene (ARM203 and ARM204). Primers and strains used in this work are listed in Tables 2.S5 and 2.S6, respectively.

*P. aeruginosa* strain construction

In-frame, marker-less *lasR* mutations were engineered onto the chromosome of *P. aeruginosa* PA14 using pEXG2-suicide constructs with gentamicin selection and *sacB* counter selection (86, 87). The *lasR* gene and 500 bp (base pairs) of flanking regions were cloned into pUCP18 (88). Site directed mutagenesis was performed as described above to construct point mutations in plasmid-borne *lasR*. The DNA carrying the mutant *lasR* genes was obtained from pUCP18 by restriction enzyme digestion with BamHI and EcoRI (NEB), and subsequently, the fragments were ligated into pEXG2. The recombinant pEXG2 plasmids were transformed into *E. coli SM10*λpir and, from there, the plasmids were mobilized into Δ*lasI* *P. aeruginosa* PA14 via biparental mating (1, 89). Exconjugants
were selected on LB plates containing 30 $\mu$g/mL gentamicin and 100 $\mu$g/mL irgasan after overnight growth at 37 $^\circ$C. After recovery, 5% sucrose was used to select for loss of the plasmid. Candidate mutants were patched onto LB plates and LB plates containing 30 $\mu$g/mL gentamicin to select against the resistance marker. Colony PCR was performed on gentamicin sensitive patches with primers that annealed 500 bp upstream and downstream of lasR (ARM455 and ARM456). These PCR products were sequenced with lasR forward and reverse primers (ARM203 and ARM204).

**E. coli plasB-lux reporter assay for LasR activity**

The development of an assay that reports on LasR activity in response to exogenous ligands using luciferase as the readout has been described previously (69). In brief, 2 $\mu$L of overnight cultures containing plasB-luxCDABE and pBAD-A with either wildtype lasR or mutant lasR alleles were back diluted into 200 $\mu$L LB medium and placed into clear-bottom 96-well plates (Corning). The plates were shaken at 30$^\circ$C for 4 h and 0.1% arabinose was added to each well along with a test HSL at the concentrations designated in the text and figures. To perform dose response analyses, 1 mM of each HSL was serially diluted 3-fold 10 times, and 2 $\mu$L of each dilution was added to the wells. Higher or lower HSL concentrations were assayed when EC$_{50}$ values did not fall into this range. Plates were shaken at 30$^\circ$C for 4 h. Bioluminescence and OD$_{600}$ were measured using a Perkin Elmer Envision Multimode plate reader. Relative light units were calculated by dividing the bioluminescence measurement by the OD$_{600}$ nm measurement. Non-linear regression was performed in Graphpad Prism6 to obtain EC$_{50}$ values.
Elastase assay

The *P. aeruginosa* PA14 Δ*lasI* strains carrying either wildtype or mutant *lasR* genes were grown overnight with shaking at 37° C in LB medium. Cultures were back diluted 1:50 in 3 mL of LB and grown for an additional 8 h with shaking at 37° C. Strains were back diluted 1:1000 into 3 mL of LB medium and test HSLs or an equivalent volume of DMSO were added to each culture. These cultures were grown overnight at 37° C with shaking. 1 mL of each culture was removed and the cells were pelleted by centrifugation at 16,100 x g. The supernatant was removed and filtered through a .22 µm filter (Millipore) and 100 µL of supernatant was added to 900 µL of 10 mM Na₂HPO₄ containing 10 mg of elastin-Congo red substrate (Sigma-Aldrich). These preparations were incubated at 37° C for 2 h. The mixtures were subjected to centrifugation at 16,100 x g for 10 min. The resulting supernatants were removed and OD₄₉₅ nm measured with a Beckman Coulter DU730 spectrophotometer against a blank of H₂O.

Thermal shift assay

Thermal shift analyses of 6xHis-LasR LBD and 6xHisLasR L130F LBD bound to HSLs were performed as previously described (69). In short, ligand-bound protein was diluted to 5 µM in reaction buffer (20 mM Tris-HCL pH 8, 200 mM NaCl, and 1 mM DTT (dithiothreitol)) containing DMSO or 10 µM HSL test compound in 18 µL total volume. The mixtures were incubated at room temperature for 15 min. 5000x SYPRO Orange (Thermo-Fisher) in DMSO was diluted to 200x in reaction buffer and used at 20x final concentration. 2 µL of 200x SYPRO Orange was added to the 18 µL sample immediately prior to analysis. Samples were subjected to a linear heat gradient of 0.05 °C/s, from 25 °C
to 99 °C in a Quant Studio 6 Flex System (Applied Biosystems) using the melting curve setting. Fluorescence was measured using the ROX reporter setting.

**Pyocyanin time course**

Overnight cultures of the *P. aeruginosa* wildtype, ΔlasI, ΔlasI lasR S129F, and ΔlasI lasR L130F strains were grown in LB medium with shaking at 37° C. 1.5 mL of each culture was diluted into 50 mL of fresh LB medium. 3OC_{12}HSL was added at the concentrations described in the text and figures and the cultures were shaken at 37° C for an additional 3 h. From there forward, 1 mL aliquots were removed every 30 min for 300 min and cell density (OD_{600} nm) was measured immediately using a Beckman Coulter DU730 Spectrophotometer. The aliquots were subjected to centrifugation at 16,100 x g for 2 min and the clarified supernatants were removed. The OD_{695} nm of the supernatants were measured. Pyocyanin activity was determined by plotting the OD_{695} nm/OD_{600} nm over time for each strain.

**Protein production, purification, and crystallography**

Recombinant 6xHis-LasR LBD and 6xHis-LasR LBD L130F proteins bound to 3OC_{8}HSL, 3OC_{10}HSL, 3OC_{12}HSL, or 3OC_{14}HSL were purified as previously described for LasR LBD:3OC_{12}HSL using Ni-NTA affinity columns followed by size exclusion chromatography (69). 6xHis-LasR LBD bound to 3OC_{10}HSL and 6xHis-LasR LBD bound to 3OC_{14}HSL were crystallized by the hanging drop diffusion method in a solution of 200 mM Mg(NO_{3})_{2} and 20% PEG 3350. Diffraction data were processed using the HKL-3000 software package (90). The structures were solved using Phaser in Phenix (91, 92) by molecular replacement, with the structure of LasR LBD:3OC_{12}HSL used as the search
model (3). The space group was P12_11. Model building was performed using Coot (93, 94) and further refinement was accomplished using Phenix (91). Images of the structures were generated with PyMOL (95). We note that the R_free is higher in the higher resolution structure of LasR LBD L130F:3OC_{14}HSL than it is for the lower resolution structure of LasR LBD L130F:3OC_{10}HSL. The data from the LasR LBD L130F:3OC_{14}HSL structure were of lower quality than that for the LasR LBD L130:3OC_{10}HSL structure resulting in a higher R_free value in the highest resolution bin for the LasR LBD L130F:3OC_{14}HSL structure, and therefore, an overall higher R_free value. Lowering the resolution cut-off for LasR LBD L130F:3OC_{14}HSL and re-refinement in Phenix did not lead to a demonstrable change in the R_free value.

**Protein solubility assay**

*E. coli* BL21 DE3 (Invitrogen) containing plasmid-borne 6xHis-LasR LBD or 6xHis-LasR LBD L130F were grown overnight and back diluted 1:500 in 20 mL of LB medium containing ampicillin (100 µg/mL). Cultures were grown to OD_{600} of 0.5 and protein production was induced with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). Upon addition of IPTG, the desired test HSL was also added at a final concentration of 10 µM, and the cultures were incubated at 25 °C with shaking for 4 h. Cells were harvested at 3,000 x g and resuspended in lysis buffer (500 mM NaCl, 20 mM Tris-HCl pH 8, 20 mM imidazole, 5% glycerol, 1 mM EDTA, and 1 mM DTT). The cells were lysed using sonication (1 s pulses for 15 s with a 50% duty cycle). The fraction we call the whole cell lysate was harvested after sonication. The soluble fraction was isolated by centrifugation at 32,000 x g. Samples were subjected to electrophoresis on SDS-PAGE gels (Biorad) and
imaged with an Image Quant LAS4000 gel dock using the chemiluminescent setting (GE Healthcare).

**Western blotting to assess LasR protein production levels**

Whole cell lysates and soluble fractions from *E. coli* carrying wildtype 6xHis-LasR and 6xHis-LasR S129C, S129F, S129M, S129T, S129W, and L130F were prepared as described in the preceding section, and then quantified using a Pierce BCA Protein Assay Kit (Thermo-Fisher). Samples were subjected to immunoblotting using a procedure adapted from (96). 10 µg of each sample was loaded into each well, and samples were probed with a monoclonal antibody to the 6xHis tag (Thermo-Fisher) followed by goat anti-mouse IgG2b cross adsorbed secondary antibody HRP (Thermo-Fisher). Both antibodies were used at 3 µg/mL. The blot was imaged with an Image Quant LAS4000 gel dock using the trans-illumination setting (GE Healthcare). Samples were tested in triplicate.

**Statistical methods**

In all experiments, values are the average of 3 biological replicates, each of which was assessed in 2 or 3 technical replicates as noted. For EC$_{50}$ analyses, wildtype LasR was used as the control. EC$_{50}$ values that appear in multiple experiments represent the mean from all experiments (Table 2.1, Fig 2.8, Table 2.S1 and Fig 2.S5). In these cases, 2 technical replicates of 3 biological replicates were assayed for every protein/molecule in each experiment. 95% confidence intervals were calculated for each EC$_{50}$ value in Table 2.1, and are provided in Table 2.S2. Error bars represent standard deviations of the means. 2-tailed t-tests were performed to compare experimental groups as noted in the figures. 2-
way ANOVAs were performed to compare LasR variants to wildtype LasR as noted in Fig 2.5. P values: *<.05, **<.01, ***<.0001

ACKNOWLEDGEMENTS

We thank Dr. Fred Hughson and Dr. Philip Jefferey for assistance with crystallography. We also thank Dr. Chari Smith and the entire Bassler group for insightful ideas about this research. This work was supported by the Howard Hughes Medical Institute, National Institutes of Health Grant 5R37GM065859, and National Science Foundation Grant MCB-1713731 (B.L.B.), NIGMS T32GM007388 (A.R.M.), and a Jane Coffin Childs Memorial Fund for Biomedical Research Postdoctoral Fellowship (J.E.P.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.

Accession Numbers

The coordinates and structure factors for the LasR LBD bound to 3OC10HSL and 3OC14HSL have been deposited in the Protein Data Bank with the accession codes 6MVN and 6MVM, respectively.

SUPPLEMENTAL METHODS

Homoserine lactone synthesis

Unless otherwise indicated, all temperatures are expressed in °C. All reactions were conducted at room temperature unless otherwise noted. 1H-NMR spectra were recorded on a Varian VXR-400, or a Varian Unity-400 at 400 MHz field strength. Chemical shifts are
expressed in parts per million (ppm, \( \delta \) units). Coupling constants \( (J) \) are in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), quintet (quin), or broad (br). Mass spectrometry analyses were performed on a Sciex API 100 using electrospray ionization (ESI). LCMS was carried out using a C-18 reverse phase column (2.1 ID, 3.5 micron, 50 mm). The column conditions were 98% water with 0.05% TFA and 2% methanol (MeOH) to 100% MeOH over 5.5 min. Analytical thin layer chromatography was used to verify the purity as well as to follow the progress of reaction(s). Unless otherwise indicated, all final products were at least 95% pure as judged by HPLC / MS.

**General procedure for the synthesis of homoserine lactones**

To a solution of (3S)-3-aminotetrahydrofuran-2-one (1.00 eq, HBr salt) and Et\(_3\)N (3.00 eq) in dichloromethane (DCM) was added a solution of the acid chloride (1.00 eq) in DCM. The resulting reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then diluted with H\(_2\)O (5 mL), and extracted with DCM (3 x 5 mL). The organic layers were combined, washed with brine (10 mL), dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure to give a residue. The residue was purified by silica gel column chromatography to give the desired HSL as a white solid.

\((S)-N-(2-\text{oxytetrahydrofuran}-3-\text{yl})\text{hexanamide} \text{(BB0189)}\)

Gradient elution with Petroleum ether/Ethyl acetate (EtOAc) = 3/1 to 1/1 afforded the title compound (425 mg, 96% yield, 98% purity by LC/MS) as a white solid. \(^1\)H NMR (CDCl\(_3\)) \(\delta = 6.26 \text{ (s, 1H)}, 4.63 \text{ (m, 1H)}, 4.50 \text{ (td, } J = 1.0, 5.6, 1\text{H)}, 4.33 \text{ (m, 1H)}, 2.87 \text{ (m, 1H)}, 2.27 \text{ ppm}\).
(S)-N-(2-oxotetrahydrofuran-3-yl)octanamide (BB0192)

Gradient elution with Petroleum ether/EtOAc = 3/1 to 1/1 afforded the title compound (270 mg, 96% yield, 99% purity by LC/MS) as a white solid. \(^1\)H NMR (CDCl\(_3\)) \(\delta = 5.94\) (s, br, 1H), 4.54 (m, 2H), 4.29 (ddd, \(J = 5.9, 9.5, 11.3, 1H\)), 2.88 (m, 1H), 2.26 (t, \(J = 7.7, 2H\)), 2.13 (m, 1H), 1.63 (m, 2H), 1.30 (m, 8H), 0.89 (t, \(J = 6.6, 3H\)); MS (ESI) calculated for C\(_{12}\)H\(_{21}\)NO\(_3\): m/z = 227; found: m/z = 228 (M+H).

(S)-N-(2-oxotetrahydrofuran-3-yl)decanamide (BB0195)

Gradient elution with Petroleum ether/EtOAc = 10/1 to 1/1 afforded the title compound (632 mg, 94% yield, 99% purity by LC/MS) as a white solid. \(^1\)H NMR (CDCl\(_3\)) \(\delta = 6.01\) (s, br, 1H), 4.55 (ddd, \(J = 5.7, 8.6, 11.6, 1H\)), 4.47 (t, \(J = 9.2, 1H\)), 4.29 (ddd, \(J = 6.1, 9.4, 11.2, 1H\)), 2.87 (m, 1H), 2.25 (t, \(J = 7.7, 2H\)), 2.12 (m, 1H), 1.66 (m, 2H), 1.30 (m, 12H), 0.88 (t, \(J = 6.8, 3H\)); MS (ESI) calculated for C\(_{14}\)H\(_{25}\)NO\(_3\): m/z = 255; found: m/z = 256 (M+H).

(S)-N-(2-oxotetrahydrofuran-3-yl)dodecanamide (BB0198)

Gradient elution with Petroleum ether/EtOAc = 10/1 to 1/1 afforded the title compound (632 mg, 94% yield, 99% purity by LC/MS) as a white solid. \(^1\)H NMR (CDCl\(_3\)) \(\delta = 5.94\) (s, br, 1H), 4.60-4.45 (m, 2H), 4.29 (m, 1H), 2.90 (m, 2H), 2.25 (t, \(J = 7.2, 2H\)), 2.14 (m, 1H), 1.64 (m, 2H), 1.35-1.22 (m, 16H), 0.88 (t, \(J = 6.7, 3H\)); MS (ESI) calculated for C\(_{16}\)H\(_{29}\)NO\(_3\): m/z = 283; found: m/z = 284 (M+H).
General Procedures for the synthesis of 3-oxo homoserine lactones

Procedure A: To a stirring solution of 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum’s acid) (1.00 eq) and dimethylaminopyridine (DMAP) (2.00 eq) in DCM at 0°C was added a solution of the acid chloride (1.00 eq) in DCM. The resulting reaction mixture was allowed to warm to room temperature and stirred for 12 h. The reaction mixture was diluted with DCM (30 mL) and washed with cold 2N HCl (3 x 30 mL). The organic layer was separated, dried over Na₂SO₄, filtered and concentrated under reduced pressure to give a crude product. The crude product was dissolved in anhydrous 1,4 dioxane (5 mL), and then (3S)-3-aminotetrahydrofuran-2-one (1.20 eq, HBr salt) and Et₃N (1.00 eq) were added. The resulting reaction mixture was degassed by purging with N₂ 3 times, then heated to 100°C for 12 h under an N₂ atmosphere. The reaction mixture was cooled to room temperature, diluted with H₂O (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (15 mL), dried over Na₂SO₄, filtered, and concentrated under reduced pressure to give a residue. The residue was purified by silica gel column chromatography to give the desired 3-oxo HSL as a white solid.

Procedure B: To a stirring solution of 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum’s acid) (1.00 eq) and DMAP (1.05 eq) in DCM at 0°C was added N,N’-dicyclohexylcarbodiimide (DCC) (1.10 eq) followed by the requisite carboxylic acid (1.00 eq). The resulting reaction mixture was allowed to warm to room temperature and stirred for 12 h. The reaction mixture was filtered through a pad of Celite to remove precipitated solids and concentrated under vacuum. The crude material was diluted with EtOAc (30 mL) and washed with cold 2N HCl (3 x 30 mL). The organic layer was separated, dried over Na₂SO₄, filtered and concentrated under reduced pressure to give a crude product. The
crude product was dissolved in anhydrous 1,4 dioxane, and then (3S)-3-aminotetrahydrofuran-2-one (1.00 eq, HBr salt) and Et₃N (1.00 eq) were added. The resulting reaction mixture was degassed by purging with N₂ 3 times, then heated to 100°C for 12 h under an N₂ atmosphere. The reaction mixture was cooled to room temperature, diluted with H₂O (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (15 mL), dried over Na₂SO₄, filtered, and concentrated under reduced pressure to give a residue. The residue was purified by silica gel column chromatography to give the desired 3-oxo HSL as a white solid.

(S)-3-oxo-N-(2-oxotetrahydrofuran-3-yl)hexanamide (BB0187)

Following Procedure A, elution with Petroleum ether/EtOAc = 1/1 afforded the title compound (285 mg, 29% yield, 97% purity by LC/MS) as a white solid. ¹H NMR (400MHz, CDCl₃) δ = 1H NMR (300 MHz, CDCl₃) δ=7.85 (s, 1H), 4.67 (ddd, J = 6.7, 1H), 4.57 (td, J = 1.3, 4.2, 1H), 4.34 (m, 1H), 3.52 (s, 2H), 2.82 (m, 1H), 2.56 (t, J = 7.2, 2H) 2.33 (m, 1H), 1.64 (m, 2H), 0.98 (t, J = 6.5, 3H); MS (ESI) calculated for C₁₀H₁₅NO₄: m/z = 213; found: m/z = 214 (M+H).

(S)-3-oxo-N-(2-oxotetrahydrofuran-3-yl)octanamide (BB0190)

Following Procedure A, elution with Petroleum ether/EtOAc = 1/1 afforded the title compound (220 mg, 37% yield, 99% purity by LC/MS) as a white solid. ¹H NMR (400MHz, CDCl₃) δ = 7.66 (s, br, 1H), 4.60 (m, 1H), 4.48 (t, J = 9.0 Hz, 1H), 4.29 (m, 1H), 3.47 (s, 2H), 2.77 (m, 1H), 2.53 (t, J = 7.3, 2H), 2.23, m, 1H), 1.61 (m, 2H), 1.31 (m, 4H), 0.90 (t, J = 6.6, 3H); MS (ESI) calculated for C₁₂H₁₉NO₄: m/z = 241; found: m/z = 242 (M+H).
(S)-3-oxo-N-(2-oxotetrahydrofuran-3-yl)decanamide (BB0193)

Following Procedure A, gradient elution with Petroleum ether/EtOAc = 10/1 to 2/1 afforded the title compound (75 mg, 15% yield, 99% purity by LC/MS) as a white solid. 
$^1$H NMR (400MHz, CDCl$_3$) δ = 7.68 (s, br, 1H), 4.60 (m, 1H), 4.48 (t, $J$ = 9.1, 1H), 4.28 (m, 1H), 3.47 (s, 2H), 2.77 (m, 1H), 2.52 (t, $J$ = 7.3, 2H), 2.22 (m, 1H), 1.59 (m, 2H), 1.27 (m, 8H), 0.88 (t, $J$ = 6.2, 3H); MS (ESI) calculated for C$_{14}$H$_{23}$NO$_4$: m/z = 269; found: m/z = 270 (M+H).

(S)-3-oxo-N-(2-oxotetrahydrofuran-3-yl)dodecanamide (BB0196)

Following Procedure A, gradient elution with Petroleum ether/EtOAc = 5/1 to 2/1 afforded the title compound (940 mg, 56% yield, 99% purity by LC/MS) as a white solid. 
$^1$H NMR (400MHz, CDCl$_3$) δ = 7.67 (s, br, 1H), 4.58 (m, 1H), 4.48 (m, 1H), 4.28 (m, 1H), 3.47 (s, 2H), 2.75 (m, 1H), 2.52 (t, $J$ = 7.3, 2H), 2.22 (m, 1H), 1.59 (m, 2H), 1.27 (m, 12H), 0.88 (t, $J$ = 6.2, 3H); MS (ESI) calculated for C$_{16}$H$_{27}$NO$_4$: m/z = 297; found: m/z = 298 (M+H).

(S)-3-oxo-N-(2-oxotetrahydrofuran-3-yl)tetradecanamide (BB0219)

Following Procedure B, gradient elution with Petroleum ether/EtOAc = 20/1 to 1/1 afforded the title compound (3.73 g, 46% yield, 98% purity by LC/MS) as a white solid. 
$^1$H NMR (400MHz, CDCl$_3$) δ = 7.67 (d, $J$ = 5.3, 1H), 4.60 (ddd, $J$ = 6.8, 8.6, 11.4, 1H), 4.48 (dd, $J$ = 9.2, 9.2, 1H), 4.28 (ddd, $J$ = 5.9, 9.4, 11.0, 1H), 3.47 (s, 2H), 2.74 (m, 1H), 2.53 (t, $J$ = 7.5, 2H), 2.23 (m, 1H), 1.59 (m, 2H), 1.26 (m, 16H), 0.91 (t, $J$ = 6.8, 3H); MS (ESI) calculated for C$_{18}$H$_{31}$NO$_4$: m/z = 325; found: m/z = 326 (M+H).
General procedure for the synthesis of 3-hydroxy homoserine lactones

To a stirring solution of 3-oxo HSL (1.00 eq) in DME (3 mL) at 0 °C was added NaBH$_4$ (0.35 eq). The resulting reaction mixture was stirred at 0 °C for 2 h. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by silica gel column chromatography to give the desired 3-hydroxy HSL as a white solid.

3-hydroxy-N-((S)-2-oxotetrahydrofuran-3-yl)hexanamide (BB0188)

Elution with EtOAc/CH$_3$CN = 50/1 afforded the title compound (96 mg, 45% yield, 96% purity by LC/MS) as a white solid. $^1$H NMR (400MHz, CDCl$_3$) $\delta$ = 6.64 (dd, br, $J$ = 5.2, 20.0, 1H), 4.61 (m, 1H), 4.49 (t, $J$ = 8.8, 2H), 4.30 (ddd, $J$ = 6.0, 9.5, 11.0, 1H), 4.04 (bs, 1H), 3.18 (dd, $J$ = 3.0, 17.1 Hz, 1H), 2.44 (m, 1H), 2.37 (m, 1H), 2.20(m, 1H), 1.53 - 1.39 (m, 4H), 0.94 (t, $J$ = 6.8, 3H); MS (ESI) calculated for C$_{10}$H$_{17}$NO$_4$: m/z = 215; found: m/z = 216 (M+H).

3-hydroxy-N-((S)-2-oxotetrahydrofuran-3-yl)octanamide (BB0191)

Gradient elution with EtOAc/CH$_3$CN = 80/1 to 70/1 afforded the title compound (96 mg, 32% yield, 99% purity by LC/MS) as a white solid. $^1$H NMR (400MHz, DMSO-d$_6$) $\delta$ = 8.31 (s, br, 1H), 4.57 (m, 1H), 4.48 (m, 1H), 4.33 (m, 1H), 4.20 (m, 1H), 3.79 (m, 1H), 2.37 (m, 1H), - 2.16 (m, 3H), 1.40-1.11 (m, 8H), 0.86 (t, $J$ = 6.6, 3H); MS (ESI) calculated for C$_{12}$H$_{21}$NO$_4$: m/z = 243; found: m/z = 244 (M+H).

3-hydroxy-N-((S)-2-oxotetrahydrofuran-3-yl)decanamide (BB0194)

Gradient elution with EtOAc/CH$_3$CN = 80/1 to 70/1 afforded the title compound (106 mg, 38% yield, 99% purity by LC/MS) as a white solid. $^1$H NMR (400MHz, CDCl$_3$) $\delta$ = 6.63
(d, br, \( J = 20.4, 1\)H), 4.59 (m, 1H), 4.49 (m, 1H), 4.30 (m, 1H), 4.03 (m, 1H), 3.15 (d, br, \( J = 14.0, 1\)H), 2.81 (m, 1H), 2.44 (m, 1H), 2.38 (m, 1H), 2.20 (m, 1H), 1.53 - 1.22 (m, 12H), 0.89 (t, \( J = 6.2, 3\)H); MS (ESI) calculated for C\(_{14}\)H\(_{25}\)NO\(_4\): m/z = 271; found: m/z = 272 (M+H).

3-hydroxy-N-((S)-2-oxotetrahydrofuran-3-yl)dodecanamide (BB0197)

Elution with EtOAc/CH\(_3\)CN = 100/1 afforded the title compound (137 mg, 47% yield, 99% purity by LC/MS) as a white solid. \(^1\)H NMR (400MHz, DMSO-\(d_6\)) \( \delta = 8.30 \) (dd, \( J = 8.1, 11.6, 1\)H), 4.54 (m, 2H), 4.33 (dt, \( J = 1.4, 8.8, 1\)H), 4.19 (m, 1H), 3.79 (m, 1H), 2.38 (m, 1H), 2.22-2.06 (m, 3H), 1.38-1.18 (m, 16H), 0.86 (t, \( J = 6.7, 3\)H); MS (ESI) calculated for C\(_{16}\)H\(_{29}\)NO\(_4\): m/z = 299; found: m/z = 300 (M+H).
Figure 2.S1: Elastase activity in response to different concentrations of 3OC\textsubscript{12}HSL. Elastase activity was measured from ΔlasI P. aeruginosa at the designated concentrations of 3OC\textsubscript{12}HSL. Elastin-Congo red was used as the substrate. Two technical replicates were performed for each biological sample and 3 biological replicates were performed. Error bars denote standard deviations of the mean.
Figure 2.52: Stability and expression of LasR and LasR mutants in the presence of HSLs. A) Comparison of wildtype LasR LBD protein levels in whole cell lysates (W) and in the soluble fractions (S) of E. coli cells harboring DNA encoding the LasR LBD on a plasmid. B) Western blot analysis of lysates of E. coli carrying arabinose-inducible 6xHis-tagged WT LasR, the designated LasR S129 mutant proteins, and the LasR L130F protein. Proteins were produced in E. coli grown in medium containing 100 µM 3OC12HSL and 10% L-arabinose. 10 µg of total protein was loaded in each lane. The blot was probed with antibody to the 6xHis tag followed by an HRP-containing secondary antibody. C) As in panel A with LasR LBD L130F. In panels A and C, 1 mM IPTG was used for LasR induction and either 1% DMSO or 10 µM of the indicated HSL was added. See Figure 2.1, panel C of the main text for details.
Figure 2.S3: Wildtype P. aeruginosa pyocyanin production over time. Pyocyanin production was measured in *P. aeruginosa* over the growth curve. Y-axis “Pyocyanin” is the amount of pyocyanin pigment (OD$_{695}$ nm) over cell density (OD$_{600}$ nm). 3 biological replicates were assessed. Error bars denote standard deviations of the mean.
Figure 2.S4: Electron density for the LasR LBD L130F protein near residue F130 and HSL ligands. A) A simulated annealing omit map, contoured at 1σ, shows the electron density (gray) around the HSLs in the LasR LBD L130F bound to 3OC_{14}HSL (green) and 3OC_{12}HSL (orange, from data in Bottomley et al., 2007). This comparison shows that 3OC_{14}HSL and 3OC_{12}HSL occupy nearly the same volume within the ligand binding pocket. W60 and T75 are labeled to provide perspective. B) A simulated annealing omit map, contoured at 1σ, shows the electron density around the HSL in the LasR LBD L130F bound to 3OC_{10}HSL (magenta). W60 and T75 are labeled to provide perspective. C) A simulated annealing omit map, contoured at 1σ, shows the electron density (gray) around LasR residue F130 and the surrounding hydrophobic residues (magenta). F130 interacts with L23, L30, F32, I35, L114, L118, L128, V147, L151, and L154 in the LasR LBD L130F:3OC_{14}HSL structure. The panel depicts the perspective highlighting the F130 interactions with F32, I35, L128, V147, and L151 and these residues are labeled. D) Cartoon representation of the LasR LBD L130F ligand binding pocket depicting the orientation of F130 relative to L23, L30, F32, I35, L114, L118, L128, V147, L151, L154, and 3OC_{14}HSL (green). Cartoon is shown at 80% transparency to enable all relevant residues to be observed. E) A simulated annealing omit map, contoured at 1σ, shows the electron density (gray) around the ligand in the LasR LBD (gold) bound to 3OC_{10}HSL (magenta) and highlights the nearby flexible loop region (orange). F) A simulated annealing omit map (gray), contoured at 1σ, shows the electron density around the ligand in the LasR LBD (magenta) bound to 3OC_{14}HSL (green) and highlights the nearby flexible loop region corresponding to residues 40-51 (orange).
Figure 2.S5: LasR Y47R and LasR Y47S have lower affinities for 3OC₁₂HSL and 3OC₁₄HSL than wildtype LasR. Bioluminescence from the plasB-lux reporter driven by wildtype LasR (blue), LasR Y47R (red), and LasR Y47S (green) (See Figure 2.1, panel A of the main text for details). 3OC₁₂HSL (A) or 3OC₁₄HSL (B) were added at the designated concentrations. Two technical replicates were performed for each biological sample and 3 biological replicates were assessed. Error bars depict standard deviations of the mean. The EC₅₀ values were obtained by performing dose response assays for each HSL and analyzing the resulting data using non-linear regression analysis.
Figure 2.S6: QscR contains a flexible loop region. Structural comparison of the LuxR-type receptor LasR LBD L130F:3OC\textsubscript{14}HSL (magenta) and QscR LBD:3OC\textsubscript{12}HSL (orange, modified from PDB: 3SZT) (11), both of which exhibit promiscuity with respect to ligand binding.
Table 2.S1: E $C_{50}$ values (nM) for LasR and HSL compounds in the *plasB-lux* assay

<table>
<thead>
<tr>
<th>Ligand</th>
<th>WT</th>
<th>S129C</th>
<th>S129W</th>
<th>S129F</th>
<th>S129T</th>
<th>S129M</th>
<th>L128F</th>
<th>L130F</th>
</tr>
</thead>
<tbody>
<tr>
<td>3OC12HSL</td>
<td>2.8</td>
<td>5.9</td>
<td>77</td>
<td>177</td>
<td>870</td>
<td>6600</td>
<td>3.1</td>
<td>2.0</td>
</tr>
<tr>
<td>3OH3C12HSL</td>
<td>39</td>
<td>170</td>
<td>320</td>
<td>2900</td>
<td>NR</td>
<td>NR</td>
<td>37</td>
<td>23</td>
</tr>
<tr>
<td>C12HSL</td>
<td>10</td>
<td>71</td>
<td>630</td>
<td>941</td>
<td>970</td>
<td>NR</td>
<td>5.1</td>
<td>6.3</td>
</tr>
<tr>
<td>3OC13HSL</td>
<td>6.2</td>
<td>12</td>
<td>13</td>
<td>41</td>
<td>4100</td>
<td>NR</td>
<td>2.9</td>
<td>5.3</td>
</tr>
<tr>
<td>3OC14HSL</td>
<td>8.0</td>
<td>32</td>
<td>2600</td>
<td>4980</td>
<td>4500</td>
<td>NR</td>
<td>5.3</td>
<td>5.0</td>
</tr>
<tr>
<td>3OH3C14HSL</td>
<td>270</td>
<td>1470</td>
<td>38700</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>560</td>
<td>110</td>
</tr>
<tr>
<td>C14HSL</td>
<td>400</td>
<td>4200</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>1200</td>
<td>170</td>
</tr>
<tr>
<td>3OC15HSL</td>
<td>900</td>
<td>2900</td>
<td>2200</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>1100</td>
<td>81</td>
</tr>
<tr>
<td>3OH3C15HSL</td>
<td>38000</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>67000</td>
<td>17100</td>
</tr>
<tr>
<td>C15HSL</td>
<td>27000</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>4000</td>
<td>5900</td>
</tr>
<tr>
<td>3OC16HSL</td>
<td>2400</td>
<td>4400</td>
<td>NR</td>
<td>55000</td>
<td>NR</td>
<td>NR</td>
<td>1200</td>
<td>1400</td>
</tr>
<tr>
<td>3OH3C16HSL</td>
<td>20000</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>20100</td>
<td>2200</td>
</tr>
</tbody>
</table>

NR denotes non-responsive
Table 2.S2: 95% Confidence intervals for EC$_{50}$ values shown in Table 1

<table>
<thead>
<tr>
<th>Homoserine Lactone</th>
<th>LasR Allele</th>
<th>WT</th>
<th>S129C</th>
<th>S129W</th>
<th>S129F</th>
<th>S129T</th>
<th>S129M</th>
<th>L130F</th>
</tr>
</thead>
<tbody>
<tr>
<td>3OC$_{12}$HSL</td>
<td></td>
<td>2-4</td>
<td>4-10</td>
<td>55-120</td>
<td>140-230</td>
<td>760-1000</td>
<td>2000-8800</td>
<td>1.5-2.5</td>
</tr>
<tr>
<td>3OC$_{14}$HSL</td>
<td></td>
<td>5-10</td>
<td>9-14</td>
<td>10-14</td>
<td>35-48</td>
<td>3200-5100</td>
<td>NR</td>
<td>3-6</td>
</tr>
<tr>
<td>3OC$_{10}$HSL</td>
<td></td>
<td>3-17</td>
<td>23-49</td>
<td>1000-8100</td>
<td>3500-7600</td>
<td>3500-5700</td>
<td>NR</td>
<td>3-7</td>
</tr>
<tr>
<td>3OC$_{3}$HSL</td>
<td></td>
<td>400-1700</td>
<td>1800-5300</td>
<td>1200-6500</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>60-100</td>
</tr>
<tr>
<td>3OC$_{3}$HSL</td>
<td></td>
<td>1600-4200</td>
<td>2300-5300</td>
<td>NR</td>
<td>24000-70000</td>
<td>NR</td>
<td>NR</td>
<td>580-1600</td>
</tr>
</tbody>
</table>

NR denotes Non-responsive

Table 2.S3: P values for LasR S129 mutants compared to wildtype LasR with HSL compounds in the plasB-lux assay.

<table>
<thead>
<tr>
<th>Homoserine Lactone</th>
<th>LasR Allele (compared to wildtype)</th>
<th>S129C</th>
<th>S129F</th>
<th>S129M</th>
<th>S129T</th>
<th>S129W</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td></td>
<td>.522</td>
<td>.849</td>
<td>.845</td>
<td>.001</td>
<td>.415</td>
</tr>
<tr>
<td>3OC$_{12}$HSL</td>
<td></td>
<td>.116</td>
<td>.407</td>
<td>4.35e$^{-8}$</td>
<td>6.15e$^{-5}$</td>
<td>.141</td>
</tr>
<tr>
<td>3OC$_{14}$HSL</td>
<td></td>
<td>.264</td>
<td>.007</td>
<td>1.23e$^{-7}$</td>
<td>1.39e$^{-7}$</td>
<td>.318</td>
</tr>
<tr>
<td>3OC$_{10}$HSL</td>
<td></td>
<td>.101</td>
<td>1.51e$^{-7}$</td>
<td>1.38e$^{-7}$</td>
<td>1.38e$^{-7}$</td>
<td>1.53e$^{-6}$</td>
</tr>
<tr>
<td>3OC$_{3}$HSL</td>
<td></td>
<td>3.67e$^{-5}$</td>
<td>5.32e$^{-6}$</td>
<td>5.15e$^{-6}$</td>
<td>5.12e$^{-6}$</td>
<td>5.44e$^{-6}$</td>
</tr>
<tr>
<td>3OC$_{3}$HSL</td>
<td></td>
<td>.008</td>
<td>1.53e$^{-9}$</td>
<td>1.40e$^{-9}$</td>
<td>1.39e$^{-9}$</td>
<td>1.75e$^{-9}$</td>
</tr>
</tbody>
</table>
### Table 2.S4: Crystallographic Statistics

<table>
<thead>
<tr>
<th>Dataset</th>
<th>LasR LBD L130F:3OC&lt;sub&gt;14&lt;/sub&gt;HSL</th>
<th>LasR LBD L130F:3OC&lt;sub&gt;10&lt;/sub&gt;HSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space Group</td>
<td>P 1 2 1 1</td>
<td>P 1 2 1 1</td>
</tr>
<tr>
<td>Unit Cell Dimensions (Å)</td>
<td>(a = 44.88, b = 70.77, c = 53.82, \alpha = \beta = \gamma = 90^\circ)</td>
<td>(a = 42.66, b = 62.12, c = 51.32, \alpha = \beta = \gamma = 90^\circ)</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30.0-1.90 (1.98-1.90)</td>
<td>30.0-2.13 (2.20-2.13)</td>
</tr>
<tr>
<td>Unique Reflections</td>
<td>25887</td>
<td>13464</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.6 (86.0)</td>
<td>96.4 (76.0)</td>
</tr>
<tr>
<td>&lt;Redundancy&gt;</td>
<td>2.5 (1.7)</td>
<td>2.0 (1.6)</td>
</tr>
<tr>
<td>(R_{merge})</td>
<td>0.102 (0.379)</td>
<td>0.076 (0.664)</td>
</tr>
<tr>
<td>(&lt;I&gt;/&lt;\sigma&gt;)</td>
<td>14.4 (1.1)</td>
<td>17.4 (1.4)</td>
</tr>
<tr>
<td>Total atoms</td>
<td>2742</td>
<td>2669</td>
</tr>
<tr>
<td>(R_{work}) (%)</td>
<td>22.3 (39.5)</td>
<td>17.94 (22.3)</td>
</tr>
<tr>
<td>(R_{free}) (%)</td>
<td>26.5 (48.7)</td>
<td>23.44 (33.6)</td>
</tr>
<tr>
<td>Average B-factor</td>
<td>29.0</td>
<td>31.5</td>
</tr>
<tr>
<td>R.m.s. deviation from ideality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.007</td>
<td>0.007</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.21</td>
<td>1.02</td>
</tr>
<tr>
<td>Dihedral angles (°)</td>
<td>17.88</td>
<td>17.49</td>
</tr>
<tr>
<td>Phi-Psi values (Ramachandran)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most favored</td>
<td>97.19</td>
<td>99.07</td>
</tr>
<tr>
<td>Additionally allowed</td>
<td>2.81</td>
<td>0.93</td>
</tr>
<tr>
<td>Outliers</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.S5: Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ to 3’</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARM203</td>
<td>cggttttcttgagctggaacgc</td>
<td>LasR forward primer</td>
</tr>
<tr>
<td>ARM204</td>
<td>aacggeccataatggcggcgtac</td>
<td>LasR reverse primer</td>
</tr>
<tr>
<td>ARM357</td>
<td>acgctgaggtcagcgcgccgag</td>
<td>lasRS129T forward</td>
</tr>
<tr>
<td>ARM358</td>
<td>ctcggcgcctgctactgtcaggt</td>
<td>lasRS129T reverse</td>
</tr>
<tr>
<td>ARM371</td>
<td>cagctgagccagcgcgcgcaga</td>
<td>lasRS129C forward</td>
</tr>
<tr>
<td>ARM372</td>
<td>tgcggecctgtgctactgtcaggt</td>
<td>lasRS129C reverse</td>
</tr>
<tr>
<td>ARM375</td>
<td>cttcagctggagaaagcgcgccagtt</td>
<td>lasRS129F forward</td>
</tr>
<tr>
<td>ARM376</td>
<td>aactcgccgcggctgttctcagcttgggaag</td>
<td>lasRS129F reverse</td>
</tr>
<tr>
<td>ARM425</td>
<td>cttcagctggagcagcgcgcgcaga</td>
<td>lasRS129W forward</td>
</tr>
<tr>
<td>ARM426</td>
<td>actcgccgcgtgctgctactgtcaggt</td>
<td>lasRS129W reverse</td>
</tr>
<tr>
<td>ARM437</td>
<td>cttcagctggagctcgccgcgcag</td>
<td>lasRL130F forward</td>
</tr>
<tr>
<td>ARM438</td>
<td>ggccgcgtgcggctcagctgagga</td>
<td>lasRL130F reverse</td>
</tr>
<tr>
<td>ARM441</td>
<td>ctcggcgcgtgctgctactgtcaggt</td>
<td>lasRS129M forward</td>
</tr>
<tr>
<td>ARM442</td>
<td>cttcagctggagctcgccgcgcag</td>
<td>lasRS129M reverse</td>
</tr>
<tr>
<td>oJP819</td>
<td>gctagagctcatgcagcgcgcagtt</td>
<td>lasRA127W forward</td>
</tr>
<tr>
<td>oJP820</td>
<td>ggcggcctgttcgctgctgcaggt</td>
<td>lasRA127W reverse</td>
</tr>
<tr>
<td>ARM455</td>
<td>taatagtccagcgcacctgagaaagtggctgtgtgcgcc</td>
<td>lasR pEXG2 upstream forward</td>
</tr>
<tr>
<td>ARM456</td>
<td>taatagtccagcgcacctgagaaagtggctgtgtgcgcc</td>
<td>lasR pEXG2 downstream reverse</td>
</tr>
<tr>
<td>ARM470</td>
<td>taatcctgagctgagctgagaggtggctgtgtgcgcc</td>
<td>pUCP18 lasR upstream forward</td>
</tr>
<tr>
<td>ARM471</td>
<td>tattaggatccttccgagacgctgagaggtggctgtgtgcgcc</td>
<td>pUCP18 lasR downstream reverse</td>
</tr>
<tr>
<td>ARM472</td>
<td>ctcgactacacgccgacgctgagaggtggctgtgtgcgcc</td>
<td>pUCP18 lasR overlapping forward</td>
</tr>
<tr>
<td>ARM473</td>
<td>tgggagcagacgccgtgtgtgtgtgcgcc</td>
<td>pUCP18 lasR overlapping reverse</td>
</tr>
</tbody>
</table>
Table 2.S6: Strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCBPP-PA14</td>
<td>Wild type, generous gift from Dr. George O'Toole</td>
<td>(Kukavica-Ibruli et al., 2008)</td>
</tr>
<tr>
<td>SM51</td>
<td><em>P. aeruginosa ΔlasI</em></td>
<td>(Mukherjee et al., 2017)</td>
</tr>
<tr>
<td>JP113</td>
<td><em>E. coli</em> pBad-A-lasR PA14 pCS26-lasB-lux</td>
<td>(Paczkowski et al., 2017)</td>
</tr>
<tr>
<td>AM05</td>
<td><em>E. coli</em> pBad-A-lasR-S129F pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM06</td>
<td><em>E. coli</em> pBad-A-lasR-S129C pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM31</td>
<td><em>E. coli</em> pBad-A-lasR-S129M pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM32</td>
<td><em>E. coli</em> pBad-A-lasR-S129T pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM28</td>
<td><em>E. coli</em> pBad-A-lasR-S129W pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM29</td>
<td><em>E. coli</em> pBad-A-lasR-L130F pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM30</td>
<td><em>E. coli</em> pBad-A-lasR-L128F pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM51</td>
<td><em>E. coli</em> pBad-A-lasR-Y47R pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM53</td>
<td><em>E. coli</em> pBad-A-lasR-Y47S pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM59</td>
<td><em>E. coli</em> pBad-A-lasR-G38A pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM60</td>
<td><em>E. coli</em> pBad-A-lasR-G38I pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM61</td>
<td><em>E. coli</em> pBad-A-lasR-G38L pCS26-lasB-lux</td>
<td>This study</td>
</tr>
<tr>
<td>AM41</td>
<td><em>P. aeruginosa ΔlasI lasR-S129F</em></td>
<td>This study</td>
</tr>
<tr>
<td>AM43</td>
<td><em>P. aeruginosa ΔlasI lasR-L130F</em></td>
<td>This study</td>
</tr>
<tr>
<td>pJP14</td>
<td><em>E. coli</em> DH-IBP-8xHis-lasR:LBD (1-170)</td>
<td>(Paczkowski et al., 2017)</td>
</tr>
<tr>
<td>AM58</td>
<td><em>E. coli</em> DH-IBP-8xHis-lasR:LBD-L130F (1-170)</td>
<td>This study</td>
</tr>
<tr>
<td>SM10lpir</td>
<td><em>E. coli thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu</em></td>
<td>(Simon et al., 1983)</td>
</tr>
<tr>
<td>pEXG2</td>
<td>Allelic exchange vector with pBR origin, gentamicin</td>
<td>(Borlee et al., 2010)</td>
</tr>
</tbody>
</table>
resistance, \textit{sacB}, generous gift from Dr. Joseph Mougous

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pUCP18</td>
<td>\textit{E. coli-Pseudomonas Amp}^r shuttle vector</td>
</tr>
<tr>
<td>(Schweizer., 1991)</td>
<td></td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>\textit{E. coli B F– dcm ompT hsdS}(r– m –) \textit{gal} $\lambda$(DE3)</td>
</tr>
<tr>
<td>Agilent</td>
<td></td>
</tr>
<tr>
<td>One Shot Top10</td>
<td>\textit{E. coli F- mcra} $\Delta$(mrr-hsdRMS-mcraBC) $\varphi$80lacZ$\Delta$M15 $\Delta$lacX74 recA1 araD139 $\Delta$(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG</td>
</tr>
<tr>
<td>Thermo-Fisher</td>
<td></td>
</tr>
</tbody>
</table>
Chapter Two was adapted from the publication:

CHAPTER THREE: AN AUTOINDUCER-INDEPENDENT RHLR QUORUM-SENSING RECEPTOR ENABLES ANALYSIS OF RHLR REGULATION AND DISTINGUISHES TWO ROLES FOR PQSE IN PYOCYANIN PRODUCTION IN PSEUDOMONAS AERUGINOSA

INTRODUCTION

Quorum sensing is a process of intercellular communication that bacteria use to coordinate group behaviors (5, 53, 55, 97). Quorum sensing relies on the production, release, and group-wide detection of signaling molecules called autoinducers (6, 7, 98). At low concentrations of autoinducer, bacteria act as individuals. At high concentrations of autoinducer, bacteria act as collectives, initiating behaviors that are beneficial when undertaken in unison by the group. Many species of Gram-negative bacteria use LuxR-type quorum-sensing receptors to orchestrate group behaviors (8, 13, 99). LuxR-type receptors are transcription factors that, as they fold, typically bind to and are stabilized and activated by cognate homoserine lactone (HSL) autoinducers (6, 100). The opportunistic pathogen Pseudomonas aeruginosa employs two LuxR-type quorum-sensing receptors, LasR and RhlR, that interact with the cognate autoinducers N-3-oxo-dodecanoyl-L-homoserine lactone (3OC\textsubscript{12}HSL) and N-butyryl-L-homoserine lactone (C\textsubscript{4}HSL), respectively (8, 13). 3OC\textsubscript{12}HSL and C\textsubscript{4}HSL are produced by the LasI and RhlI synthases, respectively. LasR activates the genes encoding RhlR and RhlI, in addition to its own regulon, so the two quorum-sensing systems function in tandem (35, 54). RhlR also responds to a second autoinducer, designated the “alternative autoinducer”, whose identity remains unknown (1). The alternative autoinducer is produced by the PqsE thioesterase. When bound to either
C₄HSL or the alternative autoinducer, RhlR activates transcription of many genes, including those required for virulence factor production and biofilm formation (22, 40).

LasR has been studied extensively; multiple LasR structures have been solved and the activities of wildtype and mutant LasR variants have been characterized (3, 8, 73, 101). RhlR, by contrast, is understudied, primarily as a consequence of its biochemical intractability. The C₄HSL autoinducer does not stabilize recombinant RhlR, which has precluded purification of the protein (14). One possible explanation underlying these difficulties is that RhlR does not bind C₄HSL particularly tightly, as evidenced by a micromolar EC₅₀ (20). We contrast that value to the nanomolar EC₅₀ that LasR, which can be purified, exhibits for 3OC¹₂HSL (101). A synthetic ligand called meta-bromo-thiolactone (mBTL) has been used to successfully solubilize RhlR, enabling some preliminary analyses of activity in recombinant *E. coli* (14). We use mBTL in some of the studies in the present work.

To accelerate studies of RhlR, here, we identify a RhlR mutant, RhlR Y64F W68F V133F, which we call RhlR*, that is stable and displays constitutive activity in the absence of any ligand. Threading analyses predict that all three of the RhlR* mutations reside in the ligand binding site, presumably allowing RhlR* to adopt a conformation mimicking the ligand-bound state (102). RhlR* properly regulates quorum-sensing-controlled traits, including virulence factor production and biofilm formation. Furthermore, we exploit RhlR* to demonstrate that, together, RhlR and PqsE promote distinct regulation of the virulence factor called pyocyanin under particular environmental conditions. In this regard, we demonstrate that PqsE plays two roles, one that depends on thioesterase catalytic activity and one that does not. RhlR* is capable of bypassing the thioesterase-dependent PqsE activity but not the thioesterase-independent activity. Finally, we show that the
negative regulator, RsaL, prevents RhlR* from hyper-stimulating quorum-sensing-controlled genes. RhlR* represents a valuable tool for exploring RhlR structure and function.

RESULTS

A screen for ligand-independent RhlR mutants.

To overcome issues with RhlR biochemical intractability, we took a genetic approach to identify RhlR mutants amenable to purification that might, moreover, provide insight into the RhlR structure, ligand binding mechanism, and regulation of transcriptional activation. Toward this goal, we performed a screen for mutants of RhlR that could function in the absence of a ligand. We used our previously reported E. coli reporter assay that harbors rhlR on one plasmid, and a RhlR-activated prhlA-lux transcriptional fusion on a second plasmid (19). rhlA encodes a rhamnolipid biosynthetic enzyme required for virulence (103, 104). On the first plasmid, we replaced wildtype rhlR with a library of rhlR point mutants. The logic underlying the screen is as follows: Wildtype RhlR requires its cognate autoinducer C₄HSL for activation. Therefore, E. coli carrying wildtype rhlR and prhlA-lux produces no light unless exogenous C₄HSL autoinducer is provided. We provided no autoinducer to the E. coli cells. We introduced the rhlR mutant library and screened the recombinants for those that produced light, reasoning that such E. coli must harbor RhlR alleles that function independently of a ligand. We identified two RhlR mutants, RhlR Y64F and RhlR W68F, that produced 37-fold and 2.5-fold more light, respectively, than the background level produced by wildtype RhlR (Fig 3.1A). In neither case did the mutants elicit maximal luciferase activity comparable to that stimulated by wildtype RhlR in the presence of saturating (10 µM) C₄HSL (Fig 3.1A, dotted line).
Furthermore, increased light production occurred when C₄HSL or mBTL was provided to the *E. coli* reporter strain harboring RhlR Y64F or RhlR W68F (Fig 3.S1). Therefore, RhlR Y64F and RhlR W68F, while harboring intrinsic autoinducer-independent activity, remain capable of ligand-driven activation.
Figure 3.1: RhlR mutants that do not require a ligand to activate gene expression. RhlR- and LasR-controlled bioluminescence was measured in *E. coli*. Arabinose-inducible RhlR or LasR was produced from one plasmid and a *prhlA-lux* (for RhlR) or *plasB-lux* (for LasR) reporter construct was carried on a second plasmid. 0.1% arabinose was used to induce RhlR and LasR. A) Light production driven by wildtype RhlR and the designated RhlR mutants in the absence of the C₄HSL ligand. B) RhlR-dependent bioluminescence was measured as in A for wildtype RhlR (blue) and RhlR* (purple) in response to the specified concentrations (nM) of C₄HSL (top) and mBTL (bottom). C) Light production driven by wildtype LasR and the designated LasR mutants in the absence of the 3OC₁₂HSL ligand. The color coding in panels A and C depicts the corresponding LasR and RhlR mutants. They are as follows: LasR (RhlR): Y56F (Y64F), W60F (W68F), A127F (V133F), Y56F W60F (Y64F W68F), Y56F A127F (Y64F V133F), W60F A127F (W68F V133F) and Y56F W60F A127F (Y64F W68F V133W, from here forward called RhlR*). In A and C, the dotted lines represent the maximum light produced by wildtype RhlR and LasR in response to saturating (10µM) C₄HSL or saturating (10 nM) 3OC₁₂HSL, respectively. Data show the mean of 3 biological replicates and 2 technical replicates for each biological replicate. Error bars represent standard deviations. For A and C, independent *t*-tests were performed comparing each mutant to wildtype. P-values: ns >.05, ** <.01 *** <.001.
We wondered whether a RhlR mutant could be obtained that harbored maximum transcriptional activation capability and was impervious to stimulation by a ligand. One possibility was the double RhlR Y64F W68F mutant, which we constructed, but its phenotype was similar to the single RhlR Y64F mutant (Fig 3.1A). Thus, we sought additional mutations in RhlR that could be tested for enhancement of the ligand-independent phenotypes of the RhlR Y64F, RhlR W68F, or RhlR Y64F W68F mutants. We have previously reported that mutations at LasR A127 alter LasR responses to HSLs, and our companion structural analyses revealed that the A127 residue lies in the LasR ligand binding pocket and interacts with the autoinducer acyl tail (3, 101). V133 is the residue in RhlR equivalent to A127 in LasR, so we explored its role in ligand-driven activation of RhlR. We constructed RhlR V133F and found that the mutant displayed a partially ligand-independent phenotype, but one that was stronger than that of either the RhlR Y64F or RhlR W68F mutant (Fig 3.1A). Combining the RhlR V133F mutation with the Y64F or W68F mutation further enhanced RhlR ligand-independent activity to approximately that made by wildtype RhlR in the presence of saturating autoinducer (Fig 3.1A). The RhlR Y64F W68F V133F triple mutant exhibited maximum constitutive activity, exceeding that produced by wildtype RhlR in the presence of saturating autoinducer. In the absence of autoinducer, the triple mutant produced approximately 10,000 times more light than did wildtype RhlR in the absence of autoinducer (Fig 3.1A). Provision of C4HSL or mBTL did not further increase the activity of RhlR Y64F W68F V133F (Fig 3.1B and Fig 3.S1). Thus, RhlR Y64F W68F V133F appears to be fully active with no ligand bound. In the remainder of this work, we refer to RhlR Y64F W68F V133F as RhlR*.
Mutations in LasR that are homologous to those in RhlR* do not confer ligand independence to LasR.

To investigate whether the residues we pinpointed as conferring ligand-independent activity on RhlR also do so on LasR, we constructed the analogous set of single, double, and triple LasR mutants. These variants are: LasR Y56F, LasR W60F, LasR A127F, LasR Y56F W60F, LasR Y56F A127F, LasR W60F A127F, and LasR Y56F W60F A127F. We examined the activities of these LasR mutants along with wildtype LasR in our previously reported *E. coli* plasR plasB-lux reporter system (105). All of these mutants responded, at least partially, to exogenous 3OC₁₂HSL, mBTL, or both agonists. (Fig 3.S2), and none of the LasR variants stimulated luciferase activity in the absence of added ligand (Fig 3.1C). Thus, the effects of mutations in RhlR do not transfer to LasR even though RhlR Y64 (LasR Y56) and RhlR W68 (LasR W60) are well conserved residues amongst the LuxR family of receptors (106). The residue at RhlR V133 (LasR A127) is not as well conserved among LuxR family members. However, the residue at this position is typically hydrophobic (A, I, L, F, M, or V) (101).

RhlR and RhlR* protein purification and analyses.

We characterized different RhlR* biochemical activities to learn how it functions without a ligand. First, as a proxy for folding, we compared the solubility of RhlR* to that of wildtype RhlR bound to mBTL, the only ligand known to be capable of solubilizing RhlR (Fig 3.2A) (14). We expressed wildtype rhlR and rhlR* from the T7 promoter in *E. coli*. In the case of wildtype RhlR, we grew the recombinant strain in the absence and presence of 100 µM of mBTL. In the case of RhlR*, we did not add any ligand. As reported previously, RhlR was insoluble in the absence of the mBTL ligand (14) and in the presence
of mBTL, a substantial portion of the RhlR protein was present in the soluble fraction (Fig 3.2A). RhlR* was soluble at levels comparable to that of the RhlR:mBTL complex (Fig 3.2A) showing that unlike wildtype RhlR, RhlR* can fold when it is not bound to a ligand. Addition of mBTL to *E. coli* expressing RhlR* did not enhance its solubility (Fig 3.2A), further indicating that RhlR* folds without a ligand.
Figure 3.2: Purification of RhlR:mBTL and RhlR*. A) Comparison of whole cell lysate (WCL) and soluble (S) fractions from E. coli cells overexpressing RhlR or RhlR* in the absence of molecule (DMSO) or in the presence of 100 µM mBTL. “L” denotes ladder and the 25 kDa band is designated. B) The final step in the purification of the full-length RhlR:mBTL and RhlR* proteins. Top: Coomassie stained gels with lanes loaded with 1% total volume from peak fractions 7-12 from the Superdex-200 size exclusion column. Bottom: Immunoblots of the peak fractions from the gels above using an anti-RhlR antibody (1). “L” and “I” denote ladder and input, respectively. Molecular weight markers are designated in between the gels. C) Activity of the E. coli prhlA-lux reporter strain (see Fig 3.1) in response to released mBTL from purified RhlR protein. The assay was carried out as described for Fig 3.1. As controls, 1% DMSO (no molecule; left-most bar) was added and pure mBTL (right-most bar) was assayed at 10 µM.
We purified the RhlR:mBTL complex (Fig 3.2B shows the final fractions; Fig 3.S3A shows the preceding purification step) using a protocol similar to one we developed for purification of LasR bound to homoserine lactones and analogs (see Materials and Methods and (101)). We confirmed the presence of RhlR protein by immunoblotting with a RhlR-specific antibody (Fig 3.2B). We confirmed that mBTL was present by heating the purified RhlR:mBTL complex to 95 °C to denature the protein and allow release of the ligand. We assayed the released ligand using the E. coli rhlR and prhlA-lux reporter strain (Fig 3.2C). Released mBTL was also verified by mass spectrometry (Fig 3.S3B). We used the identical protocol to purify RhlR* with no ligand added (Fig 3.2B). To our knowledge, this is the first report of the purification of a functional RhlR:ligand complex, and also, of course, the first time a RhlR variant has been purified without a ligand. The yield of RhlR* was low, and its purity in the peak fraction was approximately 70% compared to >95% purity for the wildtype RhlR:mBTL complex (Fig 3.2B). These issues hindered our ability to perform multiple biochemical analyses for comparison of RhlR* to RhlR:mBTL. To overcome this issue, we next purified maltose binding protein-tagged RhlR: mBTL and RhlR* (called MBP-RhlR:mBTL and MBP-RhlR*, Fig 3.S4A). MBP-RhlR required the presence of mBTL to become soluble and enable purification, while MBP-RhlR* did not. MBP-RhlR solubility was enhanced ~2-fold by mBTL (Fig 3.S4A) and we return to this point below. Thus, MBP-RhlR and MBP-RhlR* are governed by principles similar to those of the native proteins, but, in the case of MBP-RhlR*, with markedly increased yield.

To test whether the MBP tag interfered with RhlR function, we compared the DNA binding capabilities of RhlR:mBTL and MBP-RhlR:mBTL using a gel-shift assay to assess binding to rhlA promoter DNA. rhlA expression is RhlR-dependent and the promoter contains a rhlL-box sequence that is required for RhlR promoter binding in vivo (Fig 3.S4B)
Both RhlR:mBTL and MBP-RhlR:mBTL bound to the *rhlA* promoter in a concentration dependent manner and, moreover, there were no differences between MBP-RhlR:mBTL and RhlR:mBTL DNA binding (Fig 3.S4C). These results gave us confidence that the MBP tag does not interfere with RhlR activity, and thus MBP-RhlR* would be suitable for assessment of its function. Indeed, exactly like MBP-RhlR:mBTL, MBP-RhlR* bound to the *rhlA* promoter as well as two other RhlR-dependent *rhl*-box containing test promoters, *rhII* and *hcnA* (Fig 3.3A and Fig 3.S4B). Neither protein bound to control DNA amplified from an intergenic region of the *P. aeruginosa* chromosome (Fig 3.S4D). Using isothermal titration calorimetry and the *rhl*-box consensus sequence (108), we determined the $K_d$ for MBP-RhlR:mBTL and MBP-RhlR* for DNA to be 23 nM and 34 nM, respectively (Fig 3.3B). Thus, RhlR* functions essentially identically to RhlR bound to ligand, at least with respect to DNA binding.

RhlR* functions in our *E. coli* reporter assay and it can bind DNA *in vitro* with no ligand present. We thus wondered whether RhlR* can, in fact, bind a ligand. To test this possibility, we purified MBP-RhlR* in the presence of mBTL. MBP-RhlR* solubility was enhanced ~2-fold when mBTL was present (Fig 3.S4A, right side). For comparison, wildtype MBP-RhlR solubility was enhanced at least 10-fold by mBTL (Fig 3.S4A, left side). Mass spectrometry showed that mBTL was present in MBP-RhlR* (Fig 3.S4E). Thus, while the RhlR* variant activates gene expression in a ligand-independent manner that is not enhanced by mBTL (Fig 3.1 and Fig 3.S1B), it can bind mBTL (Fig 3.S4A, 3.S4E).
Figure 3.3: MBP-RhlR:mBTL and MBP-RhlR* bind equally well to DNA. A) Electrophoretic mobility gel shifts showing 300 bp biotin-labeled DNA fragments containing the rhlA (top), rhlI (center), and hcnA (bottom) promoters incubated with different concentrations of or MBP-RhlR:mBTL or MBP-RhlR*. “Ub” and “B” denote unbound DNA and DNA bound to protein, respectively. The probe DNA was used at 30 ng with 500, 200, 100, 50, 30, 20, and 10 ng of the specified protein going from left to right on the gels. The right-most lane shows the no protein control (designated by the dash). B) Isothermal titration calorimetry analyses of and MBP-RhlR:mBTL (top) and MBP-RhlR* (bottom) binding to the rhl-box consensus DNA sequence (ACCTGCCAGATTTGCAGGT). 1 µM protein and 20 µM DNA were used in the reaction. The calculated K_d values for MBP-RhlR:mBTL and for MBP-RhlR* for the rhl-box consensus sequence are 23 nM and 34 nM, respectively. DP and ΔH denote differential power and enthalpy, respectively.
RhlR* drives biofilm formation in *P. aeruginosa*.

Our *in vitro* analyses show that unliganded-RhlR* functions identically to wildtype RhlR bound to an autoinducer or an autoinducer mimic. To examine the consequences of RhlR* on quorum sensing *in vivo*, we engineered *P. aeruginosa* with *rhlR* on the chromosome at its native location in an otherwise wildtype strain and in a Δ*rhlII* Δ*pqsE* strain. Our rationale for the second strain is that by deleting *rhlII* and *pqsE*, we eliminate endogenous production of both of the RhlR autoinducers; C₄HSL (synthesized by RhlII) and the alternative autoinducer whose identity is not known (synthesized by PqsE). We measured the effect of RhlR* on biofilm formation in both strains. Wildtype *P. aeruginosa* produces biofilms with a rugose center and a smooth periphery on Congo red plates (Fig 3.4) (1, 21). By contrast, the *P. aeruginosa* Δ*rhlII* Δ*pqsE* strain forms hyper-rugose biofilms because it is defective for phenazine production (Fig 3.4) (1, 21). Both the wildtype and the Δ*rhlII* Δ*pqsE* *P. aeruginosa* strains harboring RhlR* produced biofilms with morphologies similar to the wildtype (Fig 3.4). These results suggest that, *in vivo*, RhlR* is active, ligand independent, and capable of promoting normal biofilm formation. Furthermore, the presence of autoinducers has no effect on the RhlR*-driven phenotype as shown by the similar biofilms made by the *rhlR* and the *rhlR* Δ*rhlII* Δ*pqsE* strains.
Figure 3.4: RhlR* stimulates biofilm formation in *P. aeruginosa* in the absence of an autoinducer. Colony biofilm phenotypes of the designated *P. aeruginosa* strains following growth for 120 h on tryptone Congo-red medium. Scale bar is 2 mm. Images are representative of three biological replicates and two technical replicates for each biological replicate.
RhlR* activates pyocyanin production.

RhlR is a crucial regulator of *P. aeruginosa* virulence. Unlike wildtype RhlR, RhlR* activity is not subject to the cell-density-dependent accumulation of autoinducer. For this reason, we wondered if RhlR* was capable of proper regulation of virulence factor production. To investigate this issue, we measured pyocyanin production, which depends on RhlR (13). Wildtype and *rhlR*^*^ P. *aeruginosa* strains produced, respectively, 26-and 21-fold more pyocyanin than the Δ*rhlR* strain (Fig 3.5A). Thus, RhlR* can substitute for RhlR to control pyocyanin production. Interestingly, the Δ*rhlI* Δ*pqsE* and *rhlR*^*^ Δ*rhlI* Δ*pqsE* strains produced almost no pyocyanin (Fig 3.5A). This result suggested that, unlike for biofilm formation and rhlA transcription, RhlR* could require an autoinducer to promote pyocyanin production. To investigate this possibility, we investigated the reliance of RhlR* on RhlI and on PqsE for pyocyanin production. The Δ*rhlI* strain produced almost no pyocyanin while the *rhlR*^*^ Δ*rhlI* strain produced pyocyanin at a level equivalent to the *rhlR*^*^ strain (Fig 3.5A). These results show that C_4_HSL is not required for RhlR* to activate pyocyanin production. The Δ*pqsE* strain produced almost no pyocyanin, a phenotype that has been reported previously (28, 109). Furthermore, Fig 3.5A shows that the *rhlR*^*^ Δ*pqsE* strain also did not make pyocyanin. Thus, RhlR* cannot bypass the need for PqsE in pyocyanin production. There are two interpretations for these results. First, the PqsE-dependent alternative autoinducer is absolutely required for RhlR* to function in pyocyanin production. Alternatively, PqsE has an additional role in pyocyanin production that is distinct from its role in alternative autoinducer biosynthesis. We favor the latter interpretation based on our above results showing that PqsE is dispensable for the RhlR* protein to fold and bind DNA, to function in biofilm formation, and to properly control rhlA transcription. We provide additional evidence for this interpretation below.
PqsE catalytic activity is not required for RhlR* to control pyocyanin production.

We wanted to discover whether, in the context of RhlR*, PqsE is required for synthesis of the alternative autoinducer or whether PqsE has some other, unknown, role in pyocyanin production. A PqsE crystal structure has revealed the PqsE thioesterase catalytic site (110), and previous studies have demonstrated that the catalytic residues are essential for production of both pyocyanin and the alternative autoinducer (21, 111). We reasoned that if PqsE catalytic activity was not necessary for RhlR* to activate pyocyanin production, it would demonstrate that the alternative autoinducer is not required for RhlR* to function in pyocyanin production. Moreover, if RhlR* was capable of bypassing PqsE catalysis, then PqsE must play some other non-catalytic role in pyocyanin biosynthesis. For this analysis, we exploited two previously reported PqsE mutants PqsE S273A and PqsE F276A that are stably produced in P. aeruginosa but have no thioesterase catalytic activity (21, 110, 111). We transformed plasmids carrying pqsE S273A or pqsE F276A into the rhlR* ΔrhlI ΔpqsE and ΔrhlI ΔpqsE P. aeruginosa strains and measured pyocyanin production. Introduction of pqsE S273A and pqsE F276A into the ΔrhlI ΔpqsE strain harboring wildtype rhlR, did not enable pyocyanin production (Fig 3.5B). By contrast, partial pyocyanin production was restored in the rhlR* ΔrhlI ΔpqsE pqsE S273A and rhlR* ΔrhlI ΔpqsE pqsE F276A strains. Specifically, they made, respectively, 7-fold and 3.6-fold more pyocyanin than the rhlR* ΔrhlI ΔpqsE strain (Fig 3.5B). These results suggest that PqsE has both thioesterase-dependent and thioesterase-independent roles in pyocyanin production, and, crucially, the RhlR* allele provides us a means to distinguish between them. Wildtype RhlR requires both PqsE activities to promote pyocyanin production, whereas RhlR* is able to bypass the thioesterase-dependent activity of PqsE but requires
the thioesterase-independent function for pyocyanin production in this assay. We can also conclude that, with respect to pyocyanin production, the thioesterase-dependent PqsE function involves the alternative autoinducer whereas the thioesterase-independent function does not.

The thioesterase-independent PqsE function in pyocyanin production depends on active RhlR.

Given that RhlR* cannot stimulate pyocyanin production without PqsE, but it can stimulate pyocyanin production if catalytically inactive PqsE is present (Fig 3.5B), PqsE must perform a thioesterase-independent function in pyocyanin production. Furthermore, we hypothesized that active RhlR is required for PqsE to perform this role because catalytically-inactive PqsE cannot stimulate pyocyanin production in the ΔrhlI ΔpqsE strain (Fig 3.5B). The ΔrhlI ΔpqsE strain does not produce C₄HSL or the alternative autoinducer. Therefore RhlR, while present in the ΔrhlI ΔpqsE strain, is inactive. To test this hypothesis, we supplemented the ΔrhlI ΔpqsE pqsE S273A and ΔrhlI ΔpqsE pqsE F276A strains with 10 μM C₄HSL and compared their pyocyanin production with that of wildtype P. aeruginosa that activates RhlR via endogenously-produced autoinducers. Indeed, C₄HSL activation of RhlR restored partial pyocyanin production to the ΔrhlI ΔpqsE pqsE S273A and ΔrhlI ΔpqsE pqsE F276A strains (Fig 3.5C). We cannot do the analogous experiment in which we add the alternative autoinducer to activate RhlR because its identity is unknown. Nonetheless, the experiment with C₄HSL allows us to conclude that the thioesterase-independent PqsE activity requires active RhlR to stimulate pyocyanin production.
Figure 3.5: RhlR* stimulates pyocyanin production in \textit{P. aeruginosa} in the absence of an autoinducer. A) Pyocyanin production was measured in the designated \textit{P. aeruginosa} strains. The percent pyocyanin production is quantified as pyocyanin production over cell density and compared to that produced by wildtype \textit{P. aeruginosa} which was set to 100%. Bars are representative of three biological replicates and two technical replicates for each biological replicate. Error bars depict standard deviations. Unpaired \(t\)-tests were performed to compare pyocyanin production from each strain to that produced by the \(\Delta rhlR\) strain. P-values: ns >.05, *** <.001. B) Pyocyanin production was measured as in panel A for the designated \textit{P. aeruginosa} strains. Unpaired \(t\)-tests were performed to compare pyocyanin production from each strain to that produced by the \(rhlR^* \Delta rhlI \Delta pqsE\) strain. P-values: ns >.05, * <.05, ** <.01, *** <.001. C) Pyocyanin production was measured as in panel A from the \(\Delta rhlI \Delta pqsE\ pqsE-S273A\) and \(\Delta rhlI \Delta pqsE\ pqsE-F276A\ \textit{P. aeruginosa}\) strains with and without addition of 10 \(\mu\)M \(C_4\)HSL. The values were compared to that made by wildtype \textit{P. aeruginosa} that produces endogenous \(C_4\)HSL and to which no \(C_4\)HSL was added, which was set to 100%. Bars are representative of four biological replicates and two technical replicates for each biological replicate. Paired \(t\)-tests were performed to compare pyocyanin production with and without added \(C_4\)HSL for each of the two mutant strains. P-values: *** <.001.
**P. aeruginosa harboring RhlR** is pathogenic in a *Caenorhabditis elegans* infection assay.

To examine the function of RhlR* in the context of an animal infection, we employed the *C. elegans* fast kill assay (112). In this assay, *P. aeruginosa* rapidly kills *C. elegans* in a pyocyanin-dependent manner. The assay steps are as follows: *P. aeruginosa* cells are incubated for 48 h on petri plates, enabling them time to produce pyocyanin, after that, nematodes are added, and killing is subsequently assessed (112, 113). Wildtype and *rhlR*+ *P. aeruginosa* killed over 95% of the *C. elegans* within 24 h (Fig 3.6). By contrast, the Δ*rhlI ΔpqsE* strain was highly attenuated, killing only 2% of the nematodes. (Figure 3.6). These results parallel our findings in the pyocyanin production assay and show, again,
that RhlR* functions \textit{in vivo}. Surprisingly, however, the \textit{rhlR* ΔrhlI ΔpqsE} \textit{P. aeruginosa} strain killed 74\% of the \textit{C. elegans}. These results differ from the above pyocyanin assay, in which RhlR* could not override the absence of PqsE to make detectable pyocyanin. We suspect that the differences in the results with the \textit{rhlR* ΔrhlI ΔpqsE} strain could be due to the markedly different growth conditions for \textit{P. aeruginosa} between the two assays. It has previously been suggested that PqsE-driven pyocyanin production depends on growth conditions -- liquid versus surfaces (21). We hypothesized that the thioesterase-dependent and thioesterase-independent PqsE activities could vary according to the growth condition. Specifically, we predicted that, on surfaces, the thioesterase-dependent PqsE activity dominates, and because RhlR* can bypass this one of the two PqsE activities (Fig 3.5B), the \textit{rhlR* ΔrhlI ΔpqsE} strain is capable of killing a substantial percentage of the nematodes. In contrast, in liquid culture, the thioesterase-independent PqsE activity dominates (Fig 3.5A) and because RhlR* cannot bypass the thioesterase-independent PqsE activity, the \textit{rhlR* ΔrhlI ΔpqsE} fails to produce pyocyanin. With this idea in mind, we reasoned that full pyocyanin production, and thus the wildtype level of \textit{C. elegans} killing, could be achieved by the \textit{rhlR* ΔrhlI ΔpqsE} strain if we supplied it with the non-bypassable thioesterase-independent PqsE activity. To do this, we introduced the catalytically-defective PqsE alleles \textit{pqsE S273A} and \textit{pqsE F276A} into the \textit{rhlR* ΔrhlI ΔpqsE} strain. The \textit{rhlR* ΔrhlI ΔpqsE pqsE S273A} and the \textit{rhlR* ΔrhlI ΔpqsE pqsE F276A} strains both killed \textasciitilde 95\% the nematodes, so these two strains are fully virulent (Fig 3.6). This result indicates that the difference in virulence between the wildtype strain and the \textit{rhlR* ΔrhlI ΔpqsE} strain is due to the thioesterase-independent activity of PqsE. Finally, we note that the \textit{ΔrhlI ΔpqsE pqsE S273A} and \textit{ΔrhlI ΔpqsE pqsE F276A} strains that possessed wild type RhlR rather than RhlR*, killed fewer than 5\% of the \textit{C. elegans} (Fig 3.6). These data support our
above findings that the thioesterase-independent PqsE activity relies on an active RhlR to drive pyocyanin production (Fig 3.5C). Collectively, these data show that RhlR* does not need an autoinducer to kill nematodes and that the thioesterase-dependent PqsE activity plays the primary role in pyocyanin production on surfaces, at least under the conditions we use.

Figure 3.7: RsaL prevents RhlR* from overstimulating RhlR-controlled gene expression. A) Pyocyanin production was measured in the designated P. aeruginosa strains as in Figure 5. Production from the wildtype was set to 100%. Bars represent the mean of 3 biological replicates and 2 technical replicates for each biological replicate. Error bars represent standard deviations. Independent t-tests were performed to compare pyocyanin production from each strain to that produced by the wildtype strain. P-values are ns >.05, *** <.001. B) Colony biofilm phenotypes of the designated P. aeruginosa strains as in Figure 4. Scale bar is 2 mm. Images are representative of three biological replicates and two technical replicates for each biological replicate.
**RsaL prevents RhlR* from overstimulating production of quorum-sensing-controlled products.**

Many RhlR-controlled products are public goods that are energetically expensive to produce. Thus, one could imagine that it would be detrimental for *P. aeruginosa* to harbor a constitutive RhlR allele that hyper-stimulates quorum-sensing-controlled gene expression. However, curiously, all of our above results show that *P. aeruginosa* containing RhlR* is essentially wildtype for biofilm formation (Fig 3.4), pyocyanin production (Fig 3.5A), and virulence in nematodes (Fig 3.6). While our experiments do not address survival of *P. aeruginosa* carrying RhlR* in the wild, they nonetheless suggest that some component exists that puts a brake on RhlR* activity to normalize RhlR-controlled traits. The obvious candidate is RsaL, a transcriptional regulator that represses quorum-sensing-activated genes (15, 18). To test this hypothesis, we measured pyocyanin production in late stationary phase (RsaL accumulates during stationary phase (16)) in wildtype, rhlR*, ∆rsaL, and rhlR* ∆rsaL strains. Both strains containing the ∆rsaL mutation produced significantly more pyocyanin than did the corresponding wildtype and rhlR* *P. aeruginosa* strains (Fig 3.7A). We also tested the role of RsaL in curbing RhlR* activity in biofilms. Unlike wildtype and rhlR* *P. aeruginosa*, both the ∆rsaL and rhlR* ∆rsaL strains produced biofilms that were smooth (Fig 3.7B). These biofilms resemble those made by the ∆rhlI mutant suggesting that the ∆rsaL and rhlR* ∆rsaL strains over-produce phenazines (1). However, the biofilms made by the ∆rsaL and rhlR* ∆rsaL contained voids at the centers, which we do not understand (Fig 3.7B). Together, these results show that RsaL negatively regulates pyocyanin production, and in the context of our work, functions to keep unregulated RhlR* in check.
DISCUSSION

RhlR, a central component of the *P. aeruginosa* quorum-sensing system, controls many genes, including those required for biofilm formation and virulence factor production. Here, we report RhlR*, a constitutive RhlR allele that is stably produced and that functions without an agonist bound. There are dozens of studied LuxR-type receptors, of which RhlR is one. Almost all are unstable and inactive absent a ligand. We note that EsaR and a few other LuxR-type receptors are exceptions in that they operate by a mechanism distinct from the vast majority of LuxR-type receptors, RhlR included. Specifically, EsaR binds DNA and activates transcription when no ligand is bound and EsaR is inactive when bound to an autoinducer (114, 115). Amongst the ligand dependent LuxR-receptors, RhlR* represents a new type of mutant and its lack of ligand-dependence enables previously inaccessible possibilities for its study.

RhlR* contains three mutations: Y64F, W68F, and V133F. Currently, there is no RhlR crystal structure, but the locations of the RhlR* mutations provide potential insight into the RhlR structure. Based on threading, each of the RhlR* mutations reside in the putative RhlR ligand binding site and we suspect that the three large hydrophobic phenylalanine residues fill and stabilize the hydrophobic ligand binding pocket, essentially mimicking the bound ligand, and in so doing, stabilize the protein (102). This idea is supported by our previous work with LasR demonstrating that a phenylalanine substitution at LasR L130 (RhlR L136) in the LasR ligand binding pocket made the LasR L130F protein overall more stable than wildtype LasR, but not ligand independent (101). Furthermore, mutation of the autoinducer acyl-tail binding LasR residue A127 to tryptophan prevented binding of 3OC_{12}HSL and enhanced binding to homoserine lactones possessing shorter tails suggesting that the large residue partially occupied the ligand binding site (101).
However, neither of these LasR substitutions conferred constitutive activity to LasR, nor did the LasR Y56F W60F A127F mutant made here (Fig 3.1C). We suggest that one reason RhlR may be more amenable to stabilization by bulky substitutions than LasR is because RhlR, to accommodate C₄HSL, possess an intrinsically smaller binding pocket than LasR (which naturally accommodates 3OC₁₂HSL), and a smaller binding pocket is more easily filled and stabilized by bulky groups.

Our analyses of in vivo RhlR* phenotypes show that the protein is functional with no autoinducer present, and that in this state, it can drive biofilm formation and nematode killing (Figs 3.4 and 3.6). The role of RhlR* in activating pyocyanin production was less straightforward concerning its reliance on PqsE (Fig 3.5A). Our analysis revealed that PqsE has two distinct roles in pyocyanin production, one that is thioesterase dependent and one that is thioesterase independent. The thioesterase-dependent role appears to involve production of an alternative autoinducer, and this function is bypassed by RhlR* (Fig 3.5B). The thioesterase-independent PqsE role remains undefined but is required for RhlR* to activate pyocyanin production in liquid but not on surfaces (Figs 3.5A, 3.5B and 3.6). This result, combined with earlier ones (21), provide increasing evidence that *P. aeruginosa* virulence products are regulated differently under particular environmental conditions. Our present work suggests that the thioesterase-dependent PqsE activity is crucial on surfaces. Furthermore, recent evidence demonstrates that the alternative autoinducer, and thus, the thioesterase-dependent PqsE activity are required for acute infection in mice (21). By contrast, here we, along with an earlier report (116), find that *P. aeruginosa* requires the thioesterase-independent PqsE activity to produce pyocyanin in liquid culture. We do not know what role the thioesterase-independent PqsE activity plays in the *P. aeruginosa* lifecycle outside of the laboratory. Previous reports have demonstrated
that PqsE 273A and PqsE S276A do not possess thioesterase activity (21, 111). However, some thioesterases can perform more than one catalytic activity (117). Thus, at present, we cannot eliminate the possibility that PqsE catalyzes a reaction that produces a molecule responsible for the thioesterase-independent role of PqsE. Nonetheless, because the ΔrhlI ΔpqsE pqsE S273A and ΔrhlI ΔpqsE pqsE F276A strains carrying wildtype rhlR were incapable of pyocyanin production unless we exogenously activated RhlR (Fig 3.5C), we suggest that the thioesterase-independent PqsE activity requires functional autoinducer-bound RhlR to stimulate pyocyanin production.

RhlR* behaved similarly to wildtype RhlR in all of our assays. Most surprising to us was that high-level constitutive activation of RhlR did not cause increased gene expression or hyper-production of quorum-sensing-controlled products. These findings suggested to us that evolution has built checks into the *P. aeruginosa* quorum-sensing system that protects it against excessive stimulation. We suggest that system brakes exist both upstream and downstream of RhlR. First, the upstream brake is LasR, which is required for activation of rhlR expression. Thus, constitutive RhlR* is incapable of prematurely activating target gene expression because its own expression depends on LasR, and LasR only functions at high cell density when its cognate autoinducer, 3OC₁₂HSL, has accumulated. This idea is supported by previous work demonstrating that provision of excess C₄HSL to *P. aeruginosa* cultures does not prematurely activate expression of RhlR target genes (118). Although, LasR directs the proper timing of RhlR (and RhlR*) activity, RhlR*, once made, could drive excessive activation of its target genes, but our results show this does not happen. We propose that this cap on activity is due to RsaL, that acts as a second, downstream brake on RhlR (and RhlR*) activity (Fig 3.7). RsaL represses transcription of RhlR-activated target genes preventing their
overproduction (17). Collectively, our results demonstrate that while RhlR is responsible for activating a large regulon of genes in *P. aeruginosa*, two buffering mechanisms are present and ensure that RhlR production is constrained to a proper window and its activity does not exceed the tolerable range.

Our discovery of RhlR* and its characterization provides initial insight into its biochemical activities, how it functions in conjunction with PqsE, and possibly the shape of the ligand binding pocket. The RhlR* protein offers an unparalleled opportunity for crystallization, given that wildtype RhlR and RhlR mutants reported to date have been intractable to structural analysis. Furthermore, because there is a connection between RhlR activity and PqsE activity, RhlR* could be a useful tool to discover the mechanism underlying the thioesterase-independent role of PqsE. Finally, because RhlR* is constitutively active, it could be used to identify RhlR inhibitors, possibly fostering development of anti-quorum-sensing therapeutics for *P. aeruginosa*, fulfilling an urgent medical need.

**MATERIALS AND METHODS**

**Mutant rhlR library construction:**
Random mutations in *rhlR* were generated using the Diversify PCR Random Mutagenesis Kit (Takara; mutagenesis level 5 protocol) with the primers ARM289 and ARM290 (Table 3.S1). Mutagenized DNA was digested with XhoI and SacI (NEB) and ligated into pBAD-A using T4 ligase (NEB). The resulting plasmids were transformed into One-shot TOP10 *E. coli* chemically competent cells (Invitrogen) along with *prhlA-luxCDABE*. Reactions were plated on LB agar rectangular plates containing ampicillin and kanamycin. Colonies were arrayed into black clear-well 96-well plates (Corning) using a BM3-BC colony
handling robot (S&P Robotics Inc) and screened for luciferase production. Hits were sequenced using primers ARM209 and ARM210. Candidate rhlR mutants were re-engineered in pBAD-A-rhlR using a previously reported site directed mutagenesis protocol (101). Primers and strains used in this work are listed in Tables 3.S1 and 3.S2, respectively.

Construction of lasR mutants:
LasR alleles containing mutations homologous to those studied here in RhlR were constructed using pBAD-A carrying lasR and a previously reported site directed mutagenesis protocol (101). Mutations were sequenced using primers ARM203 and ARM204 (101, 105).

P. aeruginosa strain construction:
In-frame, marker-less rhlR mutations were engineered onto the chromosome of P. aeruginosa PA14 as previously described (1). Briefly, the rhlR gene and 500 base pairs of upstream and downstream flanking regions were cloned into pUCP18 (88). rhlR mutants were constructed using pEXG2-suicide constructs with gentamicin selection and sacB counter selection (86, 87). Candidate rhlR constructs were sequenced with rhlR forward and reverse primers (ARM209 and ARM210). Primers and strains generated using this method are listed in Tables S1 and S2, respectively.

E. coli prhlA-lux assay for RhlR activity and plasB-lux assay for LasR activity:
We have previously described a method to assess RhlR activity in response to exogenous ligands, which relies on luciferase as a reporter (19). In brief, 2 µL of overnight cultures of Top 10 E. coli carrying a plasmid harboring prhlA-luxCDABE and the pBAD-A plasmid
carrying either wildtype or mutant rhlR alleles were back diluted into 200 µL LB medium and aliquoted into clear-bottom 96-well plates (Corning). The plates were shaken at 30 °C for 4 h, at which time 0.1% arabinose was added to each well. To measure responses to a single concentration of autoinducer, either 2 µL of DMSO or 10 µM of C4HSL in DMSO was added to each well. To measure responses to different concentrations of ligands, ten 3-fold serial dilutions of 10 mM C4HSL or 10 mM mBTL were made into DMSO, and 2 µL of each dilution was added to appropriate wells. In all assays, the plates were shaken at 30 °C for 4 h. Bioluminescence and OD₆₀₀ were measured using a Perkin Elmer Envision Multimode plate reader. Relative light units were calculated by dividing the bioluminescence measurement by the OD₆₀₀ nm measurement. The assay to measure LasR and mutant LasR activity is identical to that for RhlR except the reporter plasmids contain lasR and plasB-luxCDABE and 3OC₁₂HSL (serially diluted from 100 µM) was used as the autoinducer instead of C₄HSL.

**RhlR expression and purification:**

Full-length RhlR/RhlR* (cloned into pET23b) and MBP-RhlR/MBP-RhlR* (cloned into pMALC2x) were overexpressed in BL21 *E. coli* cells using 1 mM IPTG at 25 °C for 4 h in the presence (for wildtype RhlR) or absence (for RhlR*) of 100 µM mBTL. Cells were pelleted at 16,100 x g and resuspended in lysis buffer (500 mM NaCl, 20 mM Tris-HCl pH 8, 20 mM imidazole, 1 mM EDTA, 1 mM DTT, 5% glycerol). Resuspended cells were lysed using sonication (1 s pulses for 15 s). The soluble fraction from each preparation was isolated using centrifugation 32,000 x g. For RhlR:mBTL and RhlR*, protein was prepared for heparin column binding by diluting the samples 5-fold in buffer A (20 mM Tris-HCL pH 8, 1 mM DTT). Protein was loaded on a heparin column (GE Healthcare) and eluted
using a linear gradient from buffer A to buffer B (1 M NaCl, 20 mM Tris-HCl pH 8, 1 mM DTT). Peak fractions were assessed by SDS PAGE analysis and pooled. Pooled fractions were concentrated for size exclusion chromatography using a Superdex-200 (GE Healthcare) column in 200 mM NaCl, 20 mM Tris-HCl pH 8, and 1 mM DTT. Pooled fractions were concentrated to 2 mg/mL, flash frozen, and stored at -80 °C. To confirm the presence of mBTL, 100 µL of fractions 7-12 from Superdex-200 columns were heated to 95 °C for 15 min. Denatured protein was removed by centrifugation at 20,000 x g and 100 µL of the clarified supernatants were added to 900 µL of the reporter strain. For MBP-RhlR:mBTL and MBP-RhlR*, soluble fractions were applied to amylose resin (NEB) and incubated at 4 °C for 1 h. Bound protein was eluted from the resin using 10 mM maltose in lysis buffer and collected via gravity flow. Elution was repeated 10 times using 1 mL elution volumes. Fractions were pooled and concentrated. Concentrated protein was applied to a Superdex-200 column as described above.

**LC-MS/MS analysis of mBTL:**

600 µL of acetonitrile was added to 20 µg of RhlR:mBTL, MBP-RhlR:mBTL, or MBP-RhlR* and heated at 40 °C for 1 h to extract ligand from the protein. The sample was concentrated, and an equivalent of 2 µg was injected on an LTQ Orbitrap XL coupled to a Shimadzu HPLC (Thermo Scientific) for analysis as reported previously (119).

**Electrophoretic mobility shift assay:**

The rhlA, rhlI, and hcnA promoter sequences were amplified using PCR. The 5’-end of the forward primer for each pair was labeled with biotin (IDT). The labeled probe was incubated with 0, 10, 20, 30, 50, 100, 200, 500 ng of purified RhlR:mBTL, MBP-
RhlR:mBTL, or MBP-RhlR* in binding buffer (20 mM Tris-HCl pH 8, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 1.5 mg/mL poly-IC, 50 µg/mL BSA, and 10% glycerol) at room temperature for 15 min. DNA-protein complexes were subjected to electrophoresis on 6% DNA retardation gels (Invitrogen). DNA was visualized using the chemiluminescent nucleic acid detection module (Thermo Scientific). Briefly, DNA was transferred to a membrane, crosslinked, and incubated with blocking buffer for 15 min with shaking. The membranes were next incubated in stabilized streptavidin-horseradish peroxidase conjugate in blocking buffer for 15 min with shaking. The membranes were then washed 4 times for 5 min with wash buffer followed by incubation in substrate equilibration buffer for 5 min with shaking. Chemiluminescent substrate working solution was prepared by mixing equal parts luminol solution and stable peroxide solution. The membrane was removed from substrate equilibration buffer and incubated with the prepared chemiluminescent solution for 5 min without shaking. After incubation, the excess liquid was decanted and the blot was imaged on an Image Quant LAS4000 gel dock using the luminescence setting (GE Healthcare).

**Isothermal titration calorimetry:**

Isothermal titration calorimetry (ITC) was performed using a MicroCal PEAQ-ITC instrument (Malvern). 20 µM of *rhl*-box consensus sequence DNA (ACCTGCCAGATTTGCAGGT) was titrated into a cell containing 1 µM of MBP-RhlR:mBTL or MBP-RhlR* at 25 °C with a stirring speed of 1,000 rpm. Initial injection volume for the DNA was 0.4 µL and every subsequent injection was 2 µL. Consensus sequence DNA was resuspended in buffer to match the MBP-RhlR* and MBP-
RhlR:mBTL S200 buffer. Data fitting was performed with the PEAQ-ITC Analysis software (Malvern).

**Colony biofilm assay:**

The protocol for this assay was adapted from (1). In brief, 1.5 µL of overnight cultures of *P. aeruginosa* strains grown in 1% tryptone broth were spotted onto 60 x 15 mm plates containing 10 mL of 1% tryptone medium, 1% agar, 40 mg/L Congo red dye, and 20 mg/L Coomassie brilliant blue dye. Biofilms were grown for 120 h at 25 °C. Images were acquired using a Leica stereomicroscope M125 mounted with a Leica MC170 HD camera at 7.78x zoom.

**Pyocyanin production assay:**

Overnight cultures of *P. aeruginosa* grown in LB medium were diluted 1:50 in 25 mL of LB medium and agitated for 8 h at 37 °C. 1 mL aliquots were removed from the culture and cell density (OD_{600} nm) was measured immediately using a Beckman Coulter DU730 Spectrophotometer. The aliquots were next subjected to centrifugation at 16,100 x g for 2 min and the clarified supernatants were removed and filtered through 2 µm filters. The OD_{695} nm of each supernatant was measured. Pyocyanin activity was determined by plotting the OD_{695} nm/OD_{600} nm over time for each strain. Pyocyanin assays on stationary phase cultures were performed as above, except the cultures were incubated for 24 h prior to harvesting.
**C. elegans fast kill assay:**

This procedure was adapted from (112). Briefly, 10 µL of the *P. aeruginosa* strains under study were spread onto 3.5 cm peptone-glucose-sorbitol agar medium plates (PGS) and grown for 24 h at 37 °C followed by 24 h at 25 °C. Thirty age-matched L4 *C. elegans* nematodes were placed onto each plate containing the bacteria. *C. elegans* were scored as live or dead at 4, 6, 8 and 24 h by stroking each animal with a pick and assessing signs of movement. The percentage of live nematodes was calculated by dividing the number of live worms by the total number of worms on each plate and multiplying by 100.

**ACKNOWLEDGMENTS**

We thank Dr. Sampriti Mukherjee for the generous gift of *P. aeruginosa* strains. We thank Dr. Tharan Srikumar and Dr. Saw Kyin of the Princeton University Proteomics and Mass Spectrometry Core Facility as well as Dr. Venu Vandavasi of the Princeton University Biophysics Core Facility. We also thank the entire Bassler group for their insightful ideas about this research. This work was supported by the Howard Hughes Medical Institute, National Institutes of Health Grant 5R37GM065859, National Science Foundation Grant MCB-1713731 (B.L.B.), NIGMS T32GM007388 (A.R.M.), and a Jane Coffin Childs Memorial Fund for Biomedical Research Postdoctoral Fellowship (J.E.P.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.
**SUPPLEMENTARY FIGURES AND TABLES**

**Figure 3.S1: Wildtype and mutant RhlR responses to different ligands.** A) RhlR-controlled bioluminescence was measured in *E. coli*. Arabinose-inducible RhlR was produced from one plasmid and the prhlA-lux reporter construct was carried on a second plasmid. 0.1% arabinose was used for RhlR induction. Light production driven by wildtype RhlR and the designated RhlR mutants is shown in response to the specified concentrations (nM) of C₄HSL (left) and mBTL (right). Data show the mean of 3 biological replicates and 2 technical replicates for each biological replicate. Error bars represent standard deviations.
Figure 3.S2: Wildtype and mutant LasR responses to different ligands. LasR-controlled bioluminescence was measured in *E. coli*. Arabinose-inducible LasR was produced from one plasmid and the *plasB-lux* reporter construct was carried on a second plasmid. 0.1% arabinose was used for LasR induction. Light production driven by wildtype LasR and the designated LasR mutants is shown in response to the specified concentrations (nM) of 3OC_{12}HSL (left) and mBTL (right). The key describing the correspondence of LasR alleles to RhlR alleles is provided in the legend to Figure 1 of the main text. Data show the mean of 3 biological replicates and 2 technical replicates for each biological replicate. Error bars represent standard deviations.
Figure 3.S3: Purification of RhlR:mBTL. A) Shown are the results from the initial step of purification of RhlR:mBTL by heparin chromatography. Top: UV$_{280}$ chromatogram of the peak fractions from the heparin column. AU denotes arbitrary units. Center: Coomassie-stained gel analysis of peak fractions 24-33 (shown by the dotted lines). 1% total volume from peak fractions was loaded in each lane. Bottom: Immunoblot of peak fractions 24-33 using an anti-RhlR antibody. Molecular weight markers are designated to the right of the gel. “L” and “I” denote ladder and input, respectively. B) Extracted ion chromatogram of 1 µM mBTL control sample (top) and the mBTL released from 2 µg of purified RhlR protein (bottom, see pooled fractions from Fig 3.2B).
Figure 3.S4: Purification and characterization of MBP-RhlR:mBTL and MBP-RhlR*.

A) The soluble fractions from lysed E. coli cells expressing MBP-RhlR or MBP-RhlR* that had been grown in the presence or absence of mBTL were incubated, in bulk, with amylose resin and eluted with 10 mM maltose in lysis buffer (see Materials and Methods). Seven 1 mL fractions were collected and 1% of the total volume of each fraction was subjected to SDS PAGE analysis. “L” denotes ladder and the 70 kDa band is designated.

B) DNA sequences from -300 to -1 bp of the rhlA, rhlI, and hcnA promoters. Red sequences show the rhl-boxes.

C) Electrophoretic mobility gel shift showing the 300 bp biotin-labeled rhlA promoter sequence incubated with increasing concentrations of RhlR:mBTL and MBP-RhlR:mBTL. “Ub” and “B” denote unbound DNA and DNA bound to protein, respectively. The probe DNA was used at 30 ng with 500, 200, 100, 50, 30, 20, and 10 ng of the specified protein going from left to right on the gels. The right-most lane shows the no protein control (designated by the dash).

D) Electrophoretic mobility gel shift showing a biotin-labeled 300 bp fragment of intergenic control DNA with different concentrations of RhlR:mBTL and MBP-RhlR:mBTL. “Ub” denotes unbound DNA. The probe DNA was used at 30 ng with 500, 200, 100, 50, 30, 20, and 10 ng of the specified protein going from left to right on the gels. The right-most lane shows the no protein control (designated by the dash).

E) Extracted ion chromatogram of ligand released from purified MBP-RhlR* that was expressed in E. coli grown in the presence (top) or absence (bottom) of mBTL.
Table 3.S1: Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARM2 03</td>
<td>CGGTTTTCTTGGAGCTGGAAACGC</td>
<td>LasR sequencing primer</td>
</tr>
<tr>
<td>ARM2 04</td>
<td>AACCGGCCATAATGGCGCAGCTAC</td>
<td>LasR sequencing primer</td>
</tr>
<tr>
<td>ARM2 09</td>
<td>GGTGGGACGGTGGTTGCGTAGCG</td>
<td>RhlR sequencing primer</td>
</tr>
<tr>
<td>ARM2 10</td>
<td>CAGATGAGGGCCACGGCGCG</td>
<td>RhlR sequencing primer</td>
</tr>
<tr>
<td>ARM2 89</td>
<td>TTAATCTCGAGATGGAGGAATGACGAG</td>
<td>RhlR mutagenesis primer XhoI</td>
</tr>
<tr>
<td>ARM2 90</td>
<td>AATTGAGCTCTCAGATGGACACG</td>
<td>RhlR mutagenesis primer SacI</td>
</tr>
<tr>
<td>ARM3 33</td>
<td>AGGCCTTGGGAAAGGTGCATGACGAC</td>
<td>RhlR Y64F forward</td>
</tr>
<tr>
<td>ARM3 34</td>
<td>GAGGTCCATGGCACCTTCTCCGACGAC</td>
<td>RhlR Y64F reverse</td>
</tr>
<tr>
<td>ARM3 35</td>
<td>CTGGTATCGCTCTCAGAAGGGGCCACGAC</td>
<td>RhlR W68F forward</td>
</tr>
<tr>
<td>ARM3 36</td>
<td>CCTATCCCAAGGGCTTCTCTGGAGCCATGAC</td>
<td>RhlR W68F reverse</td>
</tr>
<tr>
<td>JP890</td>
<td>CGCGCCGCACGGGAAAAGGAAAGCTGACGAC</td>
<td>RhlR V133F forward</td>
</tr>
<tr>
<td>JP891</td>
<td>GACCGGGACCAGGGAGGGAGGGAGGGACGAC</td>
<td>RhlR V133F reverse</td>
</tr>
<tr>
<td>ARM3 51</td>
<td>GCGGCCGGGAAAGGTGGCCGACGAC</td>
<td>LasR Y56F forward</td>
</tr>
<tr>
<td>ARM3 52</td>
<td>CTTCATCGTGCGCAACCTTCCCGGACGAC</td>
<td>LasR Y56F reverse</td>
</tr>
<tr>
<td>ARM3 45</td>
<td>GTAATGCTCGCGGAAGGGCGGCGACGAC</td>
<td>LasR W60F forward</td>
</tr>
<tr>
<td>ARM3 46</td>
<td>AACTACCCGGCAGCCAGTCCGACGAC</td>
<td>LasR W60F reverse</td>
</tr>
<tr>
<td>ARM5 45</td>
<td>CACGCTGAGCTAGGAAAGCCGACGAC</td>
<td>LasR A127F forward</td>
</tr>
<tr>
<td>ARM5 46</td>
<td>CGCGACCAGACTCGGGCTCCTCCGTAGCGAC</td>
<td>LasR A127F reverse</td>
</tr>
<tr>
<td>ARM5 28</td>
<td>TTAAGGCAAGCTTGCAGCGGAGCCCGAC</td>
<td>RhlR upstream HindIII</td>
</tr>
<tr>
<td>ARM5 29</td>
<td>AGCGTTCTGAGGCGCGAGCTAGCGAC</td>
<td>RhlR downstream PstI</td>
</tr>
<tr>
<td>SP103</td>
<td>AGGAGGAAGCTTTACCGGCTAGCGTTCCAGATGTCG</td>
<td>RsaL deletion upstream HindIII</td>
</tr>
</tbody>
</table>
Table 3.S2: Strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM381</td>
<td>PrhlA-mNeonGreen</td>
<td>(1)</td>
</tr>
<tr>
<td>JP113</td>
<td><em>E. coli</em> pBad-A-lasR PA14 pCS26-lasB-lux</td>
<td>(19)</td>
</tr>
<tr>
<td>JP114</td>
<td><em>E. coli</em> pBad-A-rhlR PA14 pCS26-rhlA-lux</td>
<td>(19)</td>
</tr>
<tr>
<td>SM10pir</td>
<td><em>E. coli</em> thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu</td>
<td>(89)</td>
</tr>
<tr>
<td>AM10</td>
<td><em>E. coli</em> pBad-A-rhlR-Y64F PA14 pCS26-rhlA-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM11</td>
<td><em>E. coli</em> pBad-A-rhlR-W68F PA14 pCS26-rhlA-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM26</td>
<td><em>E. coli</em> pBad-A-rhlR-Y64F W68F PA14 pCS26-rhlA-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM75</td>
<td><em>E. coli</em> pBad-A-rhlR-V133F PA14 pCS26-rhlA-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM76</td>
<td><em>E. coli</em> pBad-A-rhlR-Y64F V133F PA14 pCS26-rhlA-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM77</td>
<td><em>E. coli</em> pBad-A-rhlR-W68F V133F PA14 pCS26-rhlA-lux</td>
<td>This Study</td>
</tr>
</tbody>
</table>

- **SP104**: TTCAGCATGCTTGCGGCTCGAGTTCCGTGTTGACACATCGCGCAGCTCGAGT

- **SP105**: AGGAGGAACTTAAACGGCTGAGTTCCAGATGT

- **SP106**: CTCCTTCTAGAAGACCTGCATCGAGCT

- **JP484**: AGGCTCTGCAAGTGCTCTAT

- **JP485**: TTCACCCTCCAAAATTT

- **JP492**: GAATGAGGTGATTTAAATGA

- **JP493**: GTCGGGGAAGGCAGGTAGTG

- **JP516**: TATTACATATGAGGAATGACGGAGGCTTTTT

- **JP517**: TATTAGAGCTCTAGATAGTGCAGCGCCG
<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM78</td>
<td><em>E. coli</em> pBad-A-rhlR-Y64F W68F V133F PA14 pCS26-rhlA-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM82</td>
<td><em>E. coli</em> pBad-A-lasR-A127F PA14 pCS26-lasB-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM01</td>
<td><em>E. coli</em> pBad-A-lasR-Y56F PA14 pCS26-lasB-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM04</td>
<td><em>E. coli</em> pBad-A-lasR-W60F PA14 pCS26-lasB-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM83</td>
<td><em>E. coli</em> pBad-A-lasR-Y56F A127F PA14 pCS26-lasB-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM84</td>
<td><em>E. coli</em> pBad-A-lasR-W60F A127F PA14 pCS26-lasB-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>AM85</td>
<td><em>E. coli</em> pBad-A-lasR-Y56F W60F A127F PA14 pCS26-lasB-lux</td>
<td>This Study</td>
</tr>
<tr>
<td>SM384</td>
<td>ΔrhlI PrhlA-mNeonGreen</td>
<td>(1)</td>
</tr>
<tr>
<td>SM568</td>
<td>ΔrhlIΔpqsE PrhlA-mNeonGreen</td>
<td>(21)</td>
</tr>
<tr>
<td>AM79</td>
<td>rhlIR* PrhlA-mNeonGreen</td>
<td>This Study</td>
</tr>
<tr>
<td>AM80</td>
<td>ΔrhlI rhlIR* PrhlA-mNeonGreen</td>
<td>This Study</td>
</tr>
<tr>
<td>AM81</td>
<td>ΔrhlI ΔpqsE rhlIR* PrhlA-mNeonGreen</td>
<td>This Study</td>
</tr>
<tr>
<td>SM48</td>
<td>ΔrsaL</td>
<td>This Study</td>
</tr>
<tr>
<td>AM86</td>
<td>ΔrsaL rhlIR*</td>
<td>This Study</td>
</tr>
<tr>
<td>AM87</td>
<td>ΔrhlIΔpqsE PrhlA-mNeonGreen pUCP18-Plac-pqsE-S273A</td>
<td>This Study</td>
</tr>
<tr>
<td>AM88</td>
<td>ΔrhlIΔpqsE PrhlA-mNeonGreen pUCP18-Plac-pqsE-F276A</td>
<td>This Study</td>
</tr>
<tr>
<td>AM89</td>
<td>ΔrhlIΔpqsE rhlIR* PrhlA-mNeonGreen pUCP18-Plac-pqsE-S273A</td>
<td>This Study</td>
</tr>
<tr>
<td>AM90</td>
<td>ΔrhlIΔpqsE rhlIR* PrhlA-mNeonGreen pUCP18-Plac-pqsE-F276A</td>
<td>This Study</td>
</tr>
<tr>
<td>One Shot Top10</td>
<td><em>E. coli</em> F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara/leu) 7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Thermo-Fisher</td>
</tr>
<tr>
<td>pEXG2</td>
<td>Allelic exchange vector with pBR origin, gentamicin resistance, sacB</td>
<td>(86)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>generous gift from Dr. Joseph Mougous</td>
</tr>
<tr>
<td>JP157</td>
<td><em>E. coli</em> pET23b-rhlR</td>
<td>This Study</td>
</tr>
<tr>
<td>JP158</td>
<td><em>E. coli</em> pET23b-rhlR*</td>
<td>This Study</td>
</tr>
<tr>
<td>JP159</td>
<td><em>E. coli</em> pMAL-rhlR</td>
<td>This Study</td>
</tr>
<tr>
<td>JP160</td>
<td><em>E. coli</em> pMAL-rhlR*</td>
<td>This Study</td>
</tr>
</tbody>
</table>
Chapter Three was adapted from the publication:

CHAPTER FOUR: CONCLUSIONS AND APPLICATIONS

The work in this thesis provides new insight into the structure and regulation of the central *P. aeruginosa* quorum-sensing receptors, LasR and RhlR. Specifically, the work demonstrates that LasR can, with high affinity, detect non-cognate homoserine lactones, some of which are known to be produced by other bacterial species. These findings imply that in mixed species environments, *P. aeruginosa* could employ LasR to “eavesdrop” on its competitors and alter its behavior based on their presence. This hypothesis remains to be tested. The work here also shows that, by altering LasR sensitivity and selectivity, quorum-sensing timing and strength of response can be likewise altered. The consequent effects, if any, on the fitness of the *P. aeruginosa* population needs to be investigated in the context of mixed species consortia and during infection.

This work also examines RhlR function and regulation. Little was previously known about RhlR but, because RhlR controls production of many of the most critical virulence factors and components required for biofilm formation, and because RhlR activity apparently cannot be bypassed during chronic infections, RhlR may prove a valuable target for the development of anti-quorum-sensing therapeutics. Chapter 3 of this thesis presents a constitutively active autoinducer-independent RhlR mutant, called RhlR*. RhlR* represents a novel type of LuxR-receptor mutant. Because RhlR* is constitutively active, it may be a useful tool for conducting inhibitor screens. Specifically, RhlR* could be screened against a library of compounds to identify those that decrease its inherent activity. Such a screen for RhlR inhibitors would likely be performed using recombinant *E. coli* for ease, to avoid off-target effects, and to circumvent issues with efflux from *P. aeruginosa*. In such a screen, targeting RhlR* would be preferable over wildtype RhlR because RhlR* does not require an agonist for stability and activation. Because the identity
of the alternative autoinducer is currently unknown it cannot be added as an agonist, thus any screen using wildtype RhlR would need to employ C4HSL. If inhibitors could be identified with this strategy, there is no reason to suspect that, subsequently, in *P. aeruginosa*, they would prevent RhlR from activating genes in response to the alternative autoinducer. This issue is particularly problematic considering the crucial role the alternative autoinducer plays in virulence factor production. The work here shows that RhlR* can activate genes that rely on both C4HSL and the alternative autoinducer. Therefore, screening against RhlR* may aid in identifying inhibitors with broad functionality.

RhlR* is stable and soluble in the absence of an autoinducer. These features make it a valuable tool for further biochemical analyses, and potentially, crystallography to identify the RhlR structure. RhlR* may also prove useful for studies of RhlR regulation. The work here used RhlR* to discover a thioesterase-independent activity of PqsE, but RhlR* could likewise accelerate studies of other components of the *P. aeruginosa* quorum-sensing system. In the future it would be interesting to examine the effects of RhlR* of non-RsaL-controlled products. Furthermore, RNAseq comparisons between wildtype and *rhlR* *P. aeruginosa* strains should be undertaken to examine whether differential regulation of targets occurs. Finally, *P. aeruginosa* carrying *rhlR* should be examined in a murine infection model because it is known that RhlR and PqsE are critical for acute infection. Such an analysis could validate and extend the *C. elegans* infection studies presented here.

One of the most important mysteries surrounding RhlR is the identity of the alternative autoinducer. Once that compound is known, how it is bound by RhlR, and how the RhlR-alternative autoinducer interactions are connected to PqsE activity could be
investigated. It will also be worthwhile to examine if and how RhlR interacts with each of its two autoinducers under different environmental conditions. Work performed here combined with previous studies (1, 27) show that RhlR regulation of target genes is not identical in liquid culture compared to on surfaces. Thus, further investigation, with a focus on the functions of the two autoinducers is required. Preliminarily, it appears that the alternative autoinducer plays its most important roles on surfaces such as in the context of a biofilm and during infection of the mammalian (mouse) lung.

Findings in this thesis and in other studies (1, 21, 120) provide evidence that PqsE is central to *P. aeruginosa* virulence. However, while it is known that PqsE is involved in the production of the RhlR alternative autoinducer and plays some thioesterase-independent activity in pyocyanin production, its precise roles in virulence remain undefined. An important question to answer is: what is the thioesterase-independent activity of PqsE? This activity appears to be critical in liquid culture, but dispensable on surfaces. Therefore, mutagenesis of PqsE followed by a screen for variants that produce pyocyanin on surfaces but not in liquid culture could reveal residues involved in the PqsE thioesterase-independent activity, possibly revealing insight into the thioesterase-independent PqsE mechanism/activity. Also, because the thioesterase-independent activity of PqsE requires active RhlR, it would also be interesting to explore whether the thioesterase-independent activity of PqsE drives production of RhlR-controlled products in addition to pyocyanin. RNAseq analyses comparing transcriptomes of wildtype, Δ*pqsE*, and Δ*pqsE pqsE-F273A* (i.e., thioesterase inactive) *P. aeruginosa* strains could provide the needed information. Such an analysis, by identifying distinctly regulated genes, could reveal clues as to the thioesterase-independent PqsE function.
Exploring PqsE further could be critical for successfully designing *P. aeruginosa* quorum-sensing inhibitors for several reasons. First, because PqsE is central to *P. aeruginosa* virulence, PqsE itself could be a viable therapeutic target. Second, because the alternative autoinducer is essential for infection, it is possible that RhlR could be successfully controlled by designing competitive inhibitors that resemble the alternative autoinducer. Third, if the thioesterase-independent function of PqsE is broadly involved in virulence, then inhibiting the RhlR-alternative autoinducer complex may not be sufficient to arrest *P. aeruginosa* virulence. In that case, direct inhibition of PqsE may prove a superior strategy. In either case, more information on PqsE and its interactions with RhlR are required to successfully undertake such an investigation.

In summary, this work provides tools for further study of the central quorum-sensing receptors LasR and RhlR. In the case of LasR, the identification of highly sensitive and selective mutants could aid enable discovery of such mutants in LuxR-type receptors from other quorum-sensing bacteria. Likewise, the identification of RhlR* suggests that constitutive alleles could be discovered for other LuxR-type proteins, and moreover, used to pinpoint interacting partner components and their precise roles. Beyond the development of new reagents, the work in this thesis provides insight into the features of LuxR-type receptor proteins that drive ligand promiscuity and specificity. Finally, this work provides insight into how the enigmatic RhlR receptor is regulated and how its partner PqsE controls virulence factor production. Together, the findings presented here can act as a foundation for the further study of quorum sensing in *P. aeruginosa* specifically and, more generally, other quorum-sensing bacteria that rely on LuxI-LuxR types of quorum-sensing systems.
CHAPTER FIVE: LITERATURE CITED


