QUORUM SENSING INHIBITION IN *PSEUDOMONAS AERUGINOSA*: INVESTIGATIONS INTO MECHANISM AND RESISTANCE DEVELOPMENT

by

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Under the supervision of Professor Helen E. Blackwell

At the University of Wisconsin-Madison

ABSTRACT

The opportunistic pathogen *Pseudomonas aeruginosa* uses quorum sensing (QS) to regulate many of its virulence traits. It has a QS system based on *N*-acyl L-homoserine lactone (AHL) signal molecules that are produced by LuxI-type synthase enzymes and sensed by intracellular LuxR-type receptor proteins. We have developed several QS inhibitors (QSIs) that strongly inhibit LuxR-type proteins and can be used as mechanistic probes and potential antivirulence treatments. The research described in this thesis explores the mechanisms by which these QSIs function and the potential for resistance to develop to such antivirulence agents. Using competitive growth studies of *P. aeruginosa* QS mutants under infection-relevant conditions, we first demonstrated that two discrete obstacles impede the spread of QSI resistance: (1) a small number of QSI-resistant mutants cannot produce enough signal to induce QS, and (2) group-beneficial QS-regulated traits render QSI-resistant bacteria susceptible to cheating by QSI-sensitive neighbors. Having shown that resistance will likely not spread quickly to QSIs, we aimed to better understand how QSIs function in order to improve their potency. We tested the activity of several non-native QS modulators on site-directed mutants of the LasR QS receptor. Our data strongly suggested that the synthetic ligands bind LasR in an orientation analogous to its

native signal molecule, *N*-(3-oxo)-dodecanoyl L-homoserine lactone (OdDHL), and that the interactions of their lactone head groups with Trp60 are important in governing whether they activate or inhibit LasR. This information can be leveraged to design more potent QSIs. Also, we hypothesized that active efflux decreases the potency of LasR inhibitors. Indeed, we found that the MexAB-OprM multidrug efflux pump was responsible for substantial losses in potency for every QSI tested except 5,6-dimethyl-2-aminobenzimidazole (DMABI), which may serve as a scaffold for the design of efflux-resistant QSIs. Lastly, since *P. aeruginosa* and many other microbes frequently live in polymicrobial communities, their QS circuits may be influenced by signals produced by neighboring bacteria. To that end, we characterized the ligand-activation specificity of LasR and a related protein, AbaR from *Acinetobacter baumannii*, and developed a new model to predict the promiscuity with which bacteria in polymicrobial environments respond to other QS signals.

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Helen E. Blackwell

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The CBI program was instrumental in my development as a scientist, and I am grateful to Laura Kiessling for coordinating this program and encouraging me to be a part of it for the past four years. The literature seminars and research talks exposed me to wide variety of important research topics and challenged me to think critically about results, conclusions, and future research directions. The professors and students in this program were excited to contribute their unique backgrounds and skills, and together they made it a very enjoyable an informative experience. Additionally, students in the CBI program directly helped my projects proceed by offering experimental help or insightful questions that guided research directions. A few key CBI trainees who helped in these ways are Dr. Rebecca Lennen, Dr. Brad Ryland, Julia Schwartzman, Darryl Wesener, and Bennett Fox.

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Anthony, his wife Audrey, and my two friends Dan and Monica have also been great sources of support and healthy distraction over my graduate school career.

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LIST OF ABBREVIATIONS

AHL	*N*-acyl-L-homoserine lactone
AbaI	*A. baumannii* synthase enzyme
AbaR	*A. baumannii* receptor protein
Ap	ampicillin
BHL	*N*-butanoyl-L-homoserine lactone
BLAST	basic local alignment search tool
Boc	*tert*-butoxycarbanoyl
bp	base pair
BSA	bovine serum albumin
Cb	carbenicillin
Cm	chloramphenicol
CAA	casamino acids
CFU	colony forming unit
CI	confidence interval
dba	dibenzylideneacetone
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	*N*,*N*'-dicyclohexylcarbodiimide
DD	decylamine decanoyl chloride
dH_2O	distilled water
DIC	*N*,*N"*-diisopropylcarbodiimide
DIEA	diisopropylethylamine
DIPEA	*N,N*-diisopropylethylene
DMABI	5,6-dimethyl-2-aminobenzimidazole

DMAP	4-(dimethylamino)pyridine
DMAPA	dimethylaminopropylamine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
EC_{50}	half-maximal effective concentration
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetracetic acid
EI	electron impact
ESI	electrospray ionization
ExpR2	*P. carotovora* receptor protein
FL500/540	GFP fluorescence (excitation 500 nm; emission 540 nm)
Fmoc	fluorenylmethyloxycarbonyl
GC	gas chromatography
GFP or gfp	green fluorescent protein
Gm	gentamicin
h	hours
HL	homoserine lactone
HPLC	high performance liquid chromatography
IC_{50}	half-maximal inhibitory concentration
IPTG	isopropyl β-D-1-thiogalactopyranoside
Km	kanamycin
LacZ	β-galactosidase
LasB	elastase B enzyme
LasI	autoinducer synthase protein in *Pseudomonas aeruginosa*

LasR	autoinducer receptor protein in *Pseudomonas aeruginosa*
LB	Luria Bertani
LuxI	autoinducer synthase protein in *Vibrio fischeri*
LuxR	autoinducer receptor protein in *Vibrio fischeri*
LVAgfp	unstable variant of green fluorescent protein
m/z	mass-to-charge ratio
MS	mass spectrometry
MW	weight average molar mass
NA	not applicable or not available
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
O/N	overnight
OD_{600}	optical density at 600 nm
OdDHL	*N*-3-oxo-dodecanoyl-L-homoserine lactone
OHHL	*N*-3-oxo-hexanoyl-L-homoserine lactone
OH-dDHL	*N*-3-hydroxyl-hexanoyl-L-homoserine lactone
OOHL	*N*-3-oxo-octanoyl-L-homoserine lactone
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	polymerase chain reaction
QS	quorum sensing
QSI	quorum sensing inhibitor
QscR	quorum sensing control repressor
QSM	quorum sensing media
R	QSI-resistant mimic strain of *P. aeruginosa*

RhlI	autoinducer synthase protein in *P. aeruginosa*
RhlR	autoinducer receptor protein in *P. aeruginosa*
rt	room temperature
S	QSI-sensitive mimic strain of *P. aeruginosa*
SAR	structure-activity relationship
s.e.m.	standard error of the mean
Sm	streptomycin
Tc	tetracycline
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
Tn	transposon
Tp	trimethoprim
TraR	*A. tumefaciens* receptor protein (Transfer)
Tris	trisaminomethane
Ts	tosyl
VB	Vogel-Bonner

CHAPTER 1:

BACTERIAL COMMUNICATION: WHAT IS IT? HOW DO THEY DO IT? AND WHAT HAVE WE LEARNED ABOUT IT?

1.1 Introduction

Bacteria are fascinating tiny organisms that have a tremendous impact on human lives and livelihood. These small organisms (or "microorganisms") are typically about 2–3 μm long. This length is almost a million times shorter than a human (on average 1.7 meters). Bacteria are often rod-shaped or spherical, but can also look like spirals and other shapes (see **Figure 1.1**). Just like humans, bacteria contain long chains (approximately 5 million base pairs) of deoxyribonucleic acid (DNA) that comprehensively encodes how a given bacterial cell should be constructed. This DNA instruction manual tells the bacterial cell how to make each of its thousands of proteins (large molecules that perform most tasks in a cell).

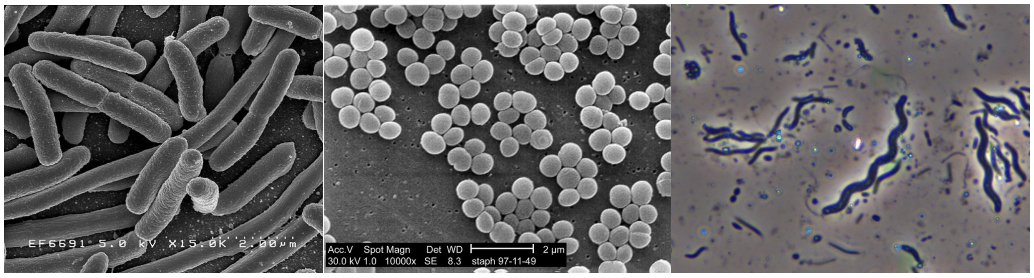

Figure 1.1. Microscopy images of bacteria.
(Left) *Escherichia coli* (i.e., *E. coli*) cells that are about 2.5 μm long. (Middle) *Staphylococcus aureus* cells that are about 0.5 μm in diameter. (Right) *Spirillum winogradskyi* cells that are about 3–4 μm long. All three images are public domain, accessed from Wikipedia.

Perhaps one of the most fascinating things about bacteria is how they can adapt to environmental changes. Imagine a bacterial cell as a self-sustaining factory of robots. It has a set of instructions that govern every aspect of how the factory should run (the DNA). And, it has thousands of different robots all with important functions (the proteins). Some robots maintain the physical structure of the factory. Some robots work to make sure all the other robots have fuel. Some robots make new robots as old ones break or as the factory determines that its output needs require a different collection of robots. Some robots get rid of waste or fix broken robots, and so

much more. Bacterial cells are similar—we need only replace the word "robot" with "protein" and the word "factory" with "cell" in the previous sentences to see that. Good factories must adapt to their situation. An ideal automobile factory would be able to make any vehicle possible. Supply demands change, and perhaps the automobile factory should no longer make trucks, but instead make hybrid cars. In that case, an ideal factory would recognize this need, go to its instructions, and make robots that will break down the robots that are useful only for producing large trucks and use that material instead to make the robots needed to produce smaller hybrid cars. Many bacteria are incredibly adaptable, just like this ideal factory. As a few examples, they can adjust their set of proteins in order to feed on certain food sources when those are abundant, to secrete poisons (toxins) into the environment when they are around enemies, and to produce useful products to maintain a symbiotic relationship with a host organism.[1, 2]

Not only are bacteria fascinating in how they can respond to stimuli, but they are also incredibly important for human life (see **Table 1.1**). Most people are familiar with disease-causing bacterial pathogens, which are certainly significant to humans,[3] but bacteria intersect with our lives in many other ways. Unless we regularly brush our teeth, bacteria growing in our mouths produce acid that causes cavities.[4] Commensal bacteria on us and inside us help to digest food, train our immune system, and protect us from pathogens.[5, 6] Bacteria also play harmful and beneficial roles in agriculture and environmental maintenance. Plant pathogens cause disease that can wipe out crops,[7, 8] but other bacteria are essential players in collecting (or "fixing") nitrogen from the environment to help feed legumes and naturally fertilize soil.[2] Furthermore, bacteria break down dead organic material to return nutrients to the soil that eventually find their way through the food chain back to us.[9, 10] Not only do bacteria break down material to provide nutrients, but they also degrade toxins for bioremediation.[11, 12] Lastly, bacteria play significant roles in industrial processes. For the pharmaceutical industry, many drugs are molecules that

bacteria make for their own purposes and we have co-opted for our uses.[13] The food industry relies heavily on bacteria for the production of yogurt, cheese, and many other products.[14] However, industries also have to overcome challenges of bacterial contamination that spoils products and the formation of sessile bacterial colonies (or "biofilms") that clog and corrode pipes.[15] As we can see, bacteria are small but amazingly complex organisms that play key roles in practically every aspect of human life. Therefore, bacteria are certainly worthy of study to uncover their myriad functions and behaviors and leverage these discoveries for human well-being.

Table 1.1. Selected intersections of bacteria with human life.

Importance of bacteria	Reference
Cause pathogenic infections	3
Oral bacteria cause cavities	4
Improve digestive health and train immune system	5, 6
Destroy crops	7, 8
Fix nitrogen for legumes	2
Break down dead organic material	9, 10
Break down toxins in environment	11, 12
Synthesize life-saving drugs	13
Produce foods (e.g., yogurt and cheese)	14
Impede industrial processes	15

In the earlier days of microbiology, it was believed that each bacterial cell functioned on its own as an individual organism (i.e., each "factory" from the illustration above works on its own in isolation from other factories). Increasingly, we are instead finding that they often function as communities that are more productive when they cooperate with one another.[16, 17] In the case of troublesome bacteria, this "increased productivity" of communities could be a bad thing (for example, communities of bacteria form biofilms that are difficult to treat in human infections and on industrial equipment[15]). In the case of beneficial bacteria, however, the increased productivity makes them more helpful (for example, communal nodules of bacteria fix nitrogen in the roots of

legumes[2]). Therefore, within the study of bacteria, the sub-study of how bacteria function within communities has grown increasingly important.

This introductory chapter will address a few fundamental conceptual questions about bacterial communication. We will then shift to briefly explain three central questions in the study of bacterial communication that are addressed in the subsequent chapters of this thesis: (1) do bacteria of different species "speak the same language?"; (2) how can we artificially enter into the dialog?; and (3) can bacteria become resistant to our interference with their communication (as bacteria frequently become resistant to antibiotics)? We close with a brief and more technical summary of the five chapters of this thesis.

1.2 Fundamental Questions Regarding Bacterial Life in Communities

1.2.1 Why would bacteria live in communities?

Across many levels of living organisms, we frequently observe life in communities. At least two main reasons are useful to explain why living in communities can be more beneficial than living alone. First, division of labor can make a community of workers more efficient than each worker performing all the tasks without specialization. Division of labor is often connected with the industrial revolution, but the concept is much older than that. Communities throughout history comprised people who specialize in farming, cooking, battling, raising children, or making useful tools.[18, 19] The comforts of our society today are, in part, due to the fact that we specialize at given tasks. An accountant doing excellent accounting, a farmer engaged in excellent farming, a manufacturer building excellent tools, a chef cooking delicious food, an entertainer providing exceptional enjoyment, a child care-provider providing nuturing care and education for children, and a physician providing excellent healthcare combine together for a richer and more secure existence than individuals building their own shelter, finding their own food, and healing their

own illnesses (**Figure 1.2A**). What may come as a surprise, however, is that humans are not alone in specialization of labor. When many animals mate, one parent gathers food while the other stays home to protect offspring. Even insects display clear division of labor: ants have queens and males for reproduction, and minor and major workers to perform the various tasks of maintaining the colony.[20]

A second clear advantage of communal living is that certain behaviors are only possible when performed in larger groups because a threshold strength is needed. The example of carrying a piano is illustrated in **Figure 1.2B**, but one could also consider protection from attackers or even workers unions gaining strength in numbers. Even if every member of a community is performing the same task, often a single member is not capable of completing the task on his/her own, and therefore a community is needed.

These two advantages of communal living are also displayed in bacteria. Some bacteria have been observed to benefit from division of labor. For example, many bacteria form miniature dandelion-like "fruiting body" structures in which some bacteria turn into spores (like the fluffy white seeds of a dandelion) and other bacteria form a sporulation stalk (like a dandelion stem) to hold the spores in the air for greater dispersal to distant fertile environments.[16] This division of labor of the bacteria, where some become spore cells and others become stalk cells, enables the bacteria to colonize distant environments that they could not otherwise reach as individuals (**Figure 1.2C**). Secondly many bacteria are known to require a threshold amount of strength to successfully complete a task. Often this manifests itself in the secretion of molecules that modify the environment around the bacteria (**Figure 1.2D**). For example, bacteria need the element iron in order to grow well, so if they are in an environment that has low levels of iron, they actually produce and secrete molecules called siderophores that will diffuse away, bind tightly to iron atoms, and if they passively diffuse back to the cell, the cell will import the siderophore-bound

iron. Since the siderophores can freely diffuse, this behavior is only beneficial if the bacteria are fairly dense in their environment. Otherwise, the siderophore bound to iron may never return to the cell. As an illustration, imagine we go fishing by shooting fish with darts, and after the fish has been shot, it floats to the surface. The problem is that the fish can float away from you quickly, so it is practically useless to shoot a fish when you are alone in a wavy ocean (like it is useless to try to lift a piano by yourself in **Figure 1.2B**). However, if you were in a tiny pond packed tightly with boats of other people, it is very productive for all of you to shoot fish. Even if the fish you shot floats away from you, a fish that someone else shot will float to you, so everyone should end up with fish. Therefore, the dense community of fishers enables the feeding of each person. Likewise, a dense community of bacteria enables the accession of iron via siderophores.

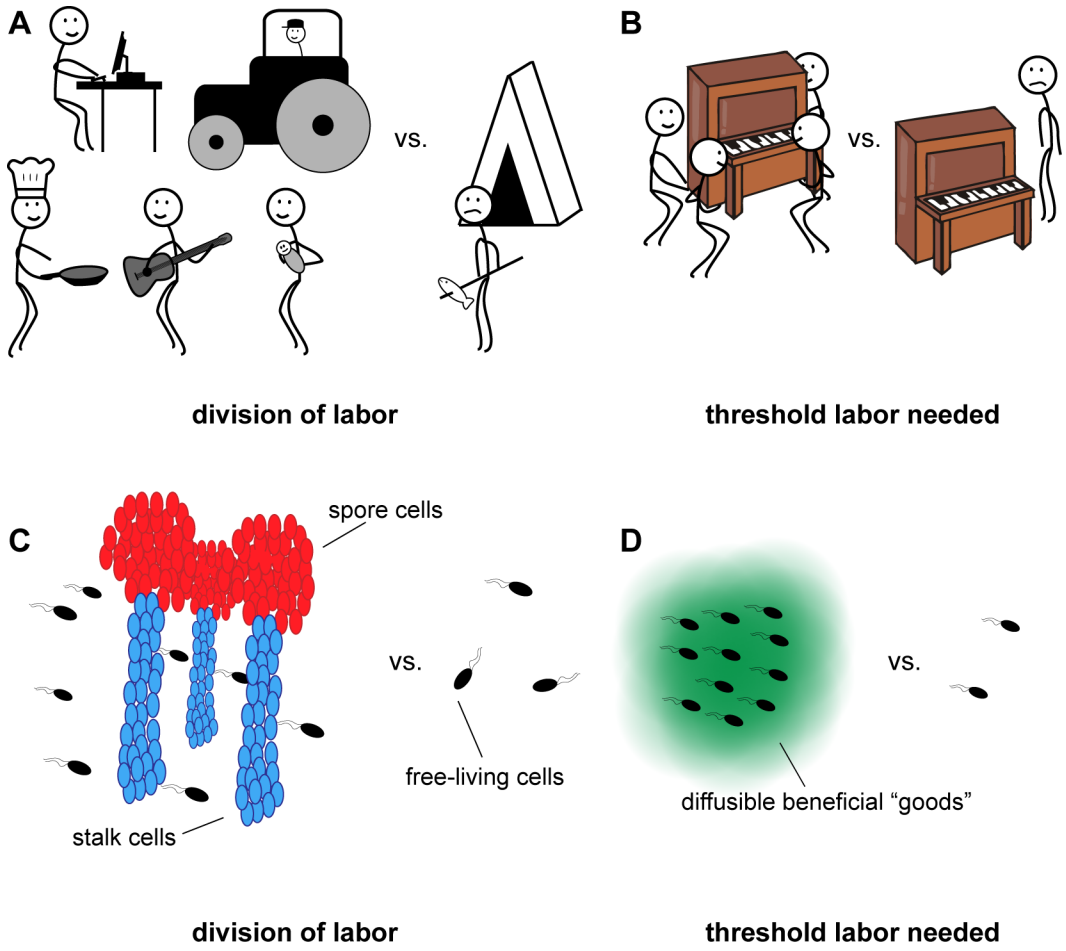

Figure 1.2. Benefits to living in communities.
(A) Humans benefit from communal living by adopting division of labor, which is more efficient than living as individuals. (B) Living in community also allows humans to perform tasks that require greater strength than an individual can muster (like lifting a heavy piano). (C) Bacteria likewise benefit from division of labor. Illustrated here are bacteria that differentiate into reproductive spore cells and non-reproductive stalk cells that hold the spore cells at a higher elevation to promote greater dissemination. (D) Bacteria also benefit from communal living by densely producing shared diffusible resources, like siderophores, which would be present at sub-functional concentrations if the bacterial density was low.

1.2.2 Why would communication be important?

We have established that communal living can have at least two major benefits for humans, bacteria, and other organisms. However, organisms are often capable of surviving on their own when necessary. They simply need to change the way that they behave. In every example in **Figure 1.2**, the organisms must discern whether to function as a community or not, and they use

communication to make this decision. For the case of division of labor, an accountant who is the lone survivor of a plane crash in the wilderness should not start crunching numbers—that would be a waste. Instead the accountant must alter his/her behavior to meet the necessities of life until he/she reaches a community again. Similarly, a few bacteria should not try to form a stalk when no dispersal spores are around. For the case of a necessary threshold strength, a single person should not try to lift a piano—he/she would waste energy and likely get hurt. Likewise, a single bacterium should not exert the metabolic cost to produce and secrete siderophores or other diffusible factors if the bacterium is not in a dense environment with other organisms that are also secreting these products. In all of these situations, the actors need to know if there are enough others around who are willing to cooperate in order to make their effort worthwhile. Communication is thus critical. Communication is the means by which the organisms can discern if sufficient cooperators are present to warrant community behavior. To borrow a word from governance proceedings, organisms use communication to determine if they have reached a "quorum" before acting. Hence this process in bacteria is called "quorum sensing."[21]

1.2.3 How do bacteria communicate?

Communication methods that enable humans to decide whether community behavior is prudent involve our five senses (**Figure 1.3**). We hear sounds and we see images to determine if other people are present, if they seem willing to work together with us, and if they have a good plan. Simpler organisms appear to rely more on other senses, such as smell and taste. For example, insects leave chemical trails that attract one another.[20] Smell and taste are analogous to the mechanism by which bacteria sense their quorum. Our noses and mouths have special nerve cells that contain receptor proteins, which each selectively bind specific odor and flavor molecules (like the malodor and perfume molecules shown in the right panel of **Figure 1.3A**).

Upon binding its specific molecule, a receptor protein changes shape and causes the nerve cell to send a signal to the brain indicating the presence of that specific odor or taste molecule (like a lock is selectively activated by a specific key, **Figure 1.3B**). Bacteria also have proteins that are selectively bound and activated by molecules that enable them to "smell" or "taste" the presence of other bacteria. If many bacteria are present, a high concentration of "scent" molecules is present, and thus the bacteria "know" they should cooperate (**Figure 1.3C**). A memorable analogy of how bacteria communicate their density is to consider blindfolding and gagging sweaty people and asking them to pick up a piano. A group of lifters will smell each other and know they should be able to lift the piano, but a lonely lifter will realize from the lack of odor that he/she should wait for more help to arrive. Likewise, if bacteria "smell" a quorum density of sibling bacteria, they "know" it's worth secreting costly, but beneficial, diffusible nutrient-acquiring factors.

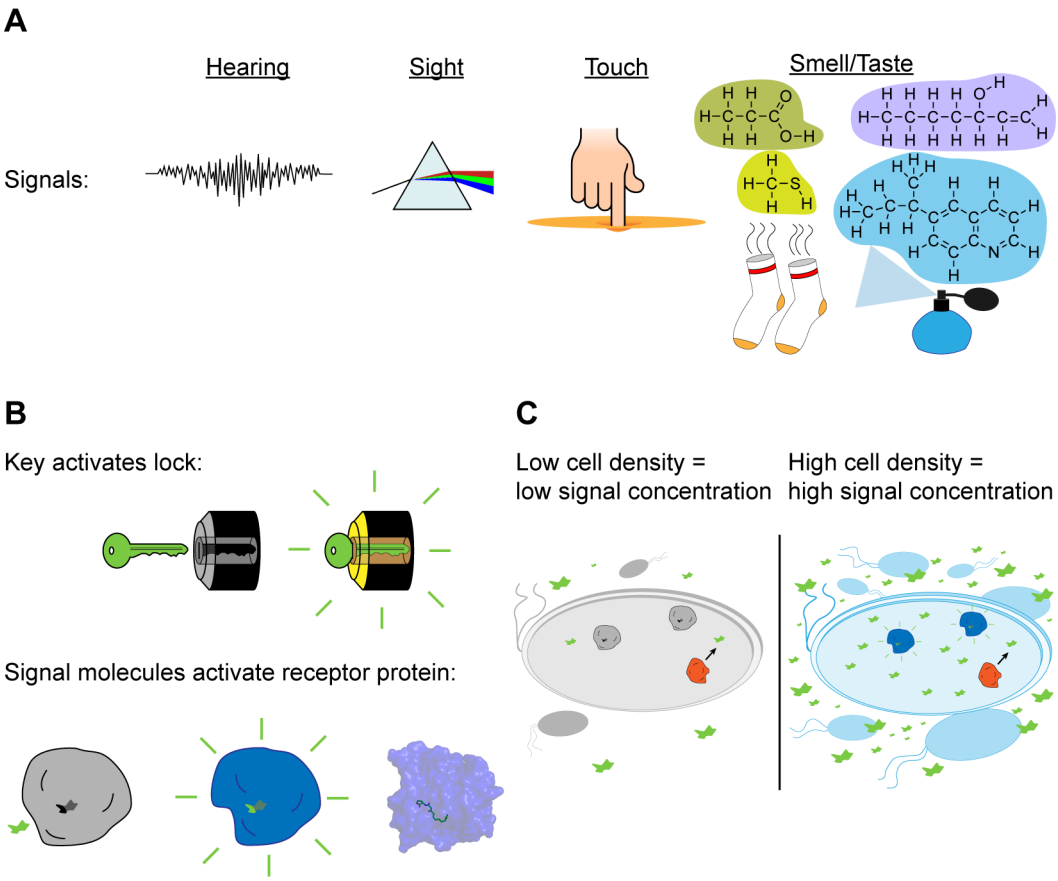

Figure 1.3. Mechanisms of perceiving communication signals.
(A) Humans perceive communication signals by hearing sound waves, seeing electromagnetic waves, feeling physical forces, and smelling and tasting specific molecules. The two molecules above the socks are malodors that resemble body odor and decaying matter, whereas the molecules above the perfume bottle are scents that resemble lavender and wood. (B) Molecules are smelled and tasted by humans, bacteria, and other organisms via specific receptor proteins that are activated when they bind their cognate signal molecule (like a lock is activated when it binds the correct key). To the right is a real molecular-resolution picture of a receptor protein (blue, LasR from *Pseudomonas aeruginosa*) bound to its signal molecule (green, *N*-(3-oxo)-dodecanoyl L-homoserine lactone). (C) Illustration of bacterial quorum sensing. A protein in bacteria (orange) synthesizes signal molecules (green), whose concentration correlates to the bacterial density. When the bacterial density reaches a threshold, the signal (or "scent") concentration is high enough to bind to and activate their receptor proteins (grey to blue), which causes the bacteria to change behavior.

1.3 Specific Research Questions

1.3.1 Do bacteria of different species "speak the same language?"

Many different kinds of bacteria exist, so how do they sense if they are surrounded by their siblings or by others (possibly even ones that could hurt them)? The answer is relatively simple:

different species make different molecules and respond primarily to their unique molecule (like humans not understanding a dog's barks, or more subtly, humans not understanding different languages). Interestingly, some bacteria respond to their own signals *and* also those produced by others, but other bacteria only sense and respond to their own.[22-27] An analogy is my brother only understands English, my sister-in-law is fluent in English and Spanish, and my friend regularly communicates in English, Malay, Korean, and three very different dialects of Chinese. Different bacterial species likewise display varying degrees of multilingual fluency (**Figure 1.4**). We were able to develop a model to explain how some bacteria distinguish between the different signals and others do not. Furthermore, we proposed a method for predicting if less studied species will be "multilingual" or not. This work is presented in **Chapter 6**.

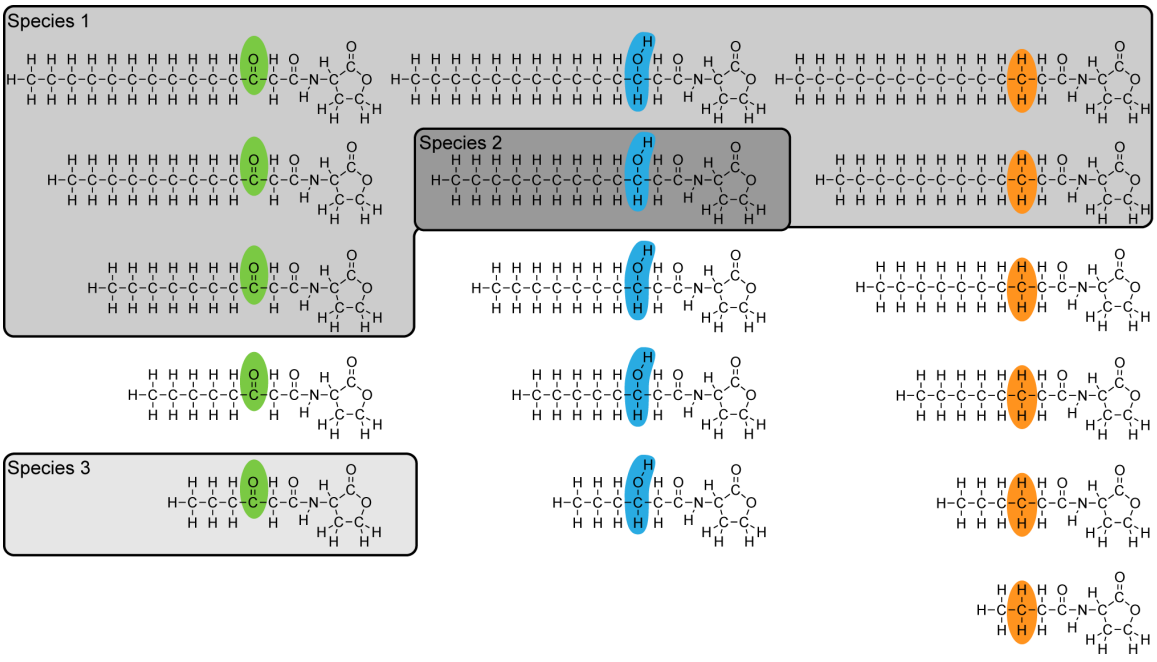

Figure 1.4. Examples of chemical "languages" used by bacteria.
Several signaling molecules are displayed that vary in length and in composition at the circled position. Species 1 is "multilingual" in that it can perceive many different signal molecules, but the two other species can only recognize one signal (i.e., they are "monolingual").

Interestingly, there are important benefits and harms to responding to signals from other organisms (**Figure 1.5**). Bacteria can have three different responses to signals from another organism: (1) respond to the alternative signal as if it were their own signal and perform cooperative activity, (2) ignore the signals made by others and only perform the cooperative activity if enough identical members are around, and (3) be inhibited by the signal made by another organism and stop the cooperative activity—even if a quorum of identical members are around. Each of these cases can be beneficial in certain scenarios (**Figure 1.5**). If the other bacterium is the same species or a closely related one that will cooperate, then it is good to respond to its signal positively and work together—like multilingual people who work together to lift a piano. If the other bacterium is a different species that has a neutral impact, then it is best for the initial bacterium to simply ignore the neutral bacterium's signal and only perform the cooperative behavior if there is a quorum of cooperative partners. In the piano-lifting example, this would be like having some calm, friendly dogs around—they will not help lift the piano, but they also should not be too much of an impediment to piano lifting, so its best to just ignore them. Lastly, if the neighbors will actually have a detrimental impact on the initial bacteria, the bacteria should respond in a negative manner to this competing neighbor. Even if they have sufficient numbers to perform their task, they should not do it. This case is akin to having sufficient help to lift a piano but then noticing a ferocious bear nearby. Even though you can lift the piano, it is better to leave the piano and run away. Since we see bacteria that respond positively, negatively, and neutrally toward signals from other bacteria, we suspect that the bacteria have evolved to respond properly to "good," "neutral," and "bad" neighbors. There is likely much more to discover about communication between different bacterial species, as we are just beginning to identify the complex mixtures of bacterial species that live together in varied environments (e.g., in our digestive systems, in the soil, and even deep in the sea[28]). Our work will help lay a

foundation for characterizing how these complex mixtures of organisms communicate with each other.

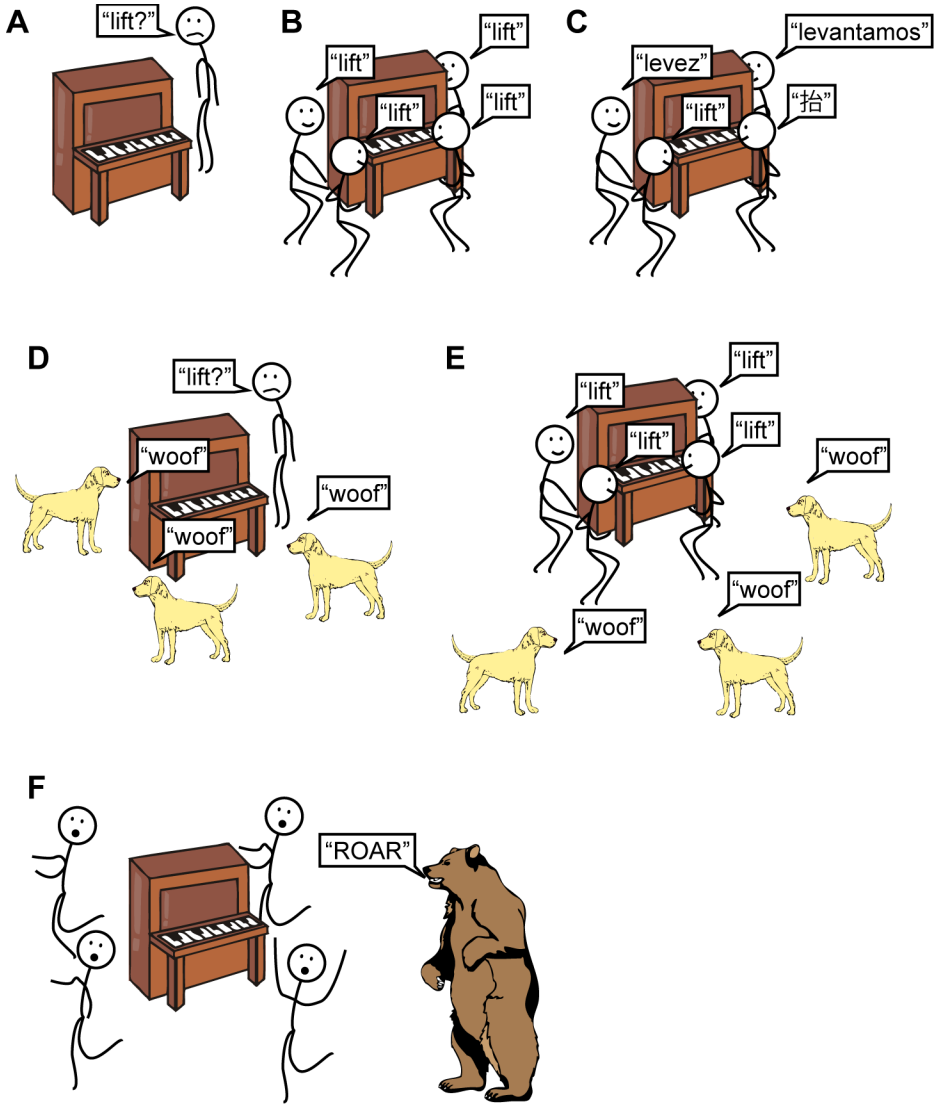

Figure 1.5. Examples of healthy responses to different types of neighbors.
(A) When alone, it is best to not waste energy lifting a piano. (B,C) When with a quorum of cooperative partners (whether they speak identical languages or not), it is possible to successfully lift a piano. (D) When surrounded by only unhelpful partners, it is best to not waste energy lifting a piano. (E) When a quorum of cooperative partners and several unhelpful (but harmless) partners are present, it is best to ignore the unhelpful partners and lift the piano. (F) When a harmful partner is present, it is best to not lift the piano (but instead focus on protection), even if a quorum of cooperative lifters is present.

1.3.2 How can we artificially enter into the dialog?

As stated above, bacteria communicate via quorum sensing in order to determine whether to perform communal behavior that often has harmful or beneficial impacts on humans (e.g., harmful biofilms that are difficult to clear from infections and industrial equipment or beneficial nitrogen-fixing nodules on legume roots). If bacteria used speech to communicate, we would try to find the right frequencies of sound to prevent them from hearing each other. If bacteria used vision to see each other, we might try to blind them with light. However, since bacteria communicate using chemical signals that bind selectively to receptor proteins, we can design specific molecules to bind to the receptor proteins to disrupt their communication. To illustrate, we return briefly to the analogy of sweaty, blindfolded, and gagged people who are asked to lift a piano. They are willing to lift the piano when they smell many others because they trust that together they can succeed. However, if we spray strong air freshener in the room, the people will no longer realize that they have a quorum that is sufficient to lift the piano, so they will leave it resting on the ground. This illustration is bizarre, but on a molecular level, it is not too different from our efforts to inhibit bacterial quorum sensing. Smell occurs when an odor molecule binds to a scent receptor protein in the nose (like a key fits into a lock) and activates the protein to send a signal to the brain. Air fresheners can function by binding a receptor protein slightly differently in a way that blocks it from binding the scent molecule, but does not activate the receptor protein (like an almost correct key getting stuck in a lock). We seek the same goal of blocking receptor proteins in bacteria without activating them (**Figure 1.6A**). Instead of preventing sweaty people from lifting a piano, however, our laboratory's main focus has been on preventing pathogenic bacteria from forming biofilms, migrating to infect new tissues, and producing toxins that destroy host cells.

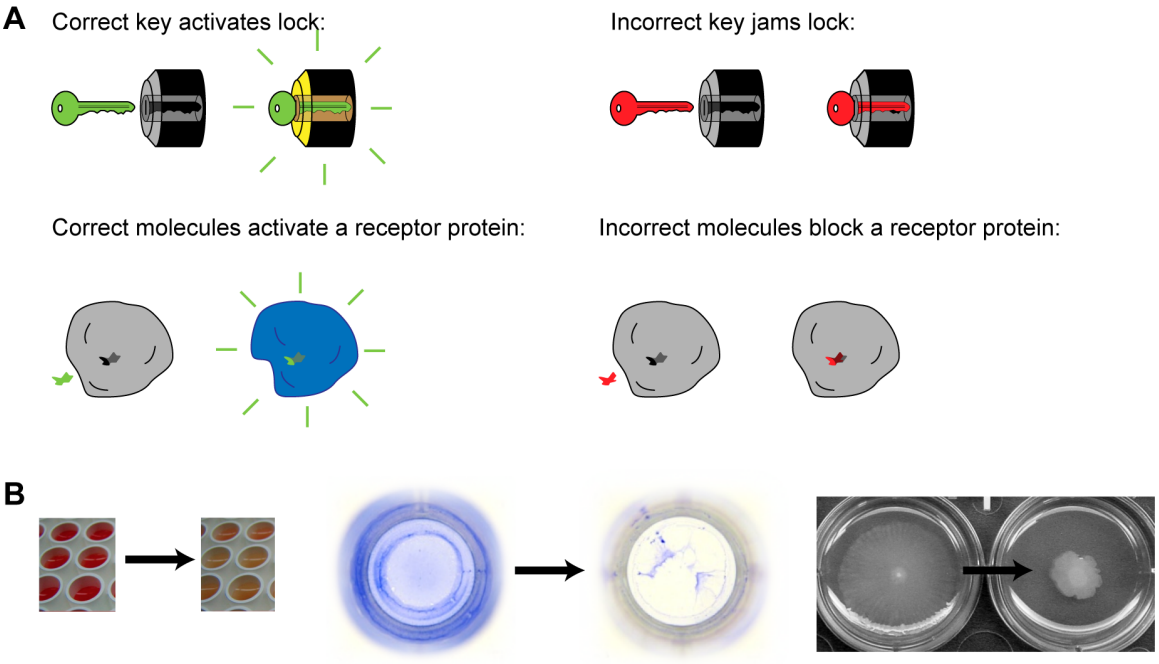

Figure 1.6. Blocking quorum sensing receptor proteins inhibits virulence of pathogens.
(A) Bacteria sense that they have a quorum density when signal molecules (green) bind to and activate receptor proteins (grey to blue) like a key binds to and activates a lock. Just like an incorrect key can fit in a lock and jam it in an inactive state, alternative molecules (red) can be synthesized that block a receptor protein from binding its signal and therefore prevent activation. (B) Images of bacterial virulence prevented by addition of quorum sensing inhibitors (left to right): lysis of red blood cells by *S. aureus* (image from Tal-gan, et al.[29]), biofilm formation by *P. aeruginosa* (image from Frei, et al.[30]), and swarming motility by *A. baumannii* (image from Stacy, et al.[31]).

As **Figure 1.6B** shows, our laboratory has successfully inhibited quorum sensing in multiple bacteria to prevent virulent behaviors (toxin production by *S. aureus* in the left image, biofilm formation by *P. aeruginosa* in the middle image, and swarming motility by *Acinetobacter baumannii* in the right image). However, many of our molecules that inhibit quorum sensing have drawbacks. Most are not very stable and therefore block communication for only a short period of time.[32-34] Other molecules are not very potent,[35] and therefore would require unreasonably large amounts of material to be used in real-life applications outside of controlled laboratory conditions. Also, for any treatment of disease in animals or plants, molecules must be found that cause no appreciable harm to these host organisms. All of these challenges provide the

opportunity to develop improved inhibitors of quorum sensing. To develop improved inhibitors, a better understanding of how the current ones function and their shortcomings is crucial.

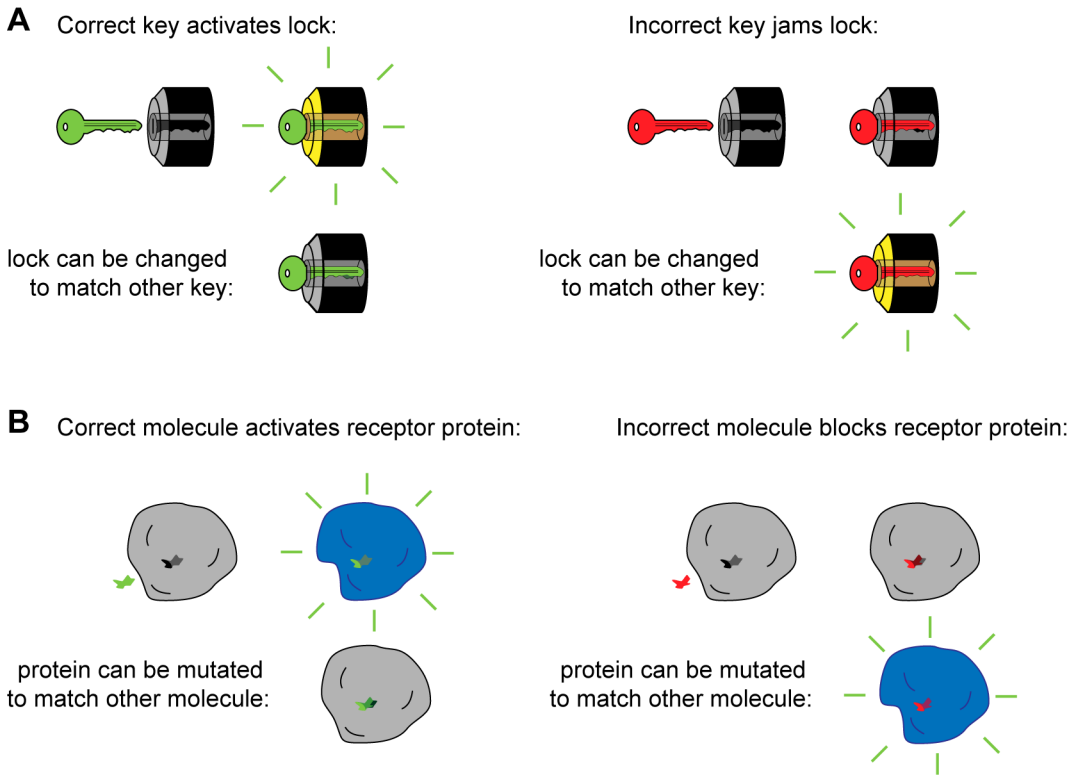

Figure 1.7. Mutation of quorum sensing receptors can allow activation by an inhibitor. (A) If an incorrect key jams a lock, the lock could theoretically be altered to properly fit this "incorrect" key and instead be activated by it. (B) Similarly, receptor proteins can be mutated to accommodate for different signal molecules and actually be activated by molecules that used to be inhibitors and be inhibited by molecules that used to be activators.

In **Chapters 3** and **4** of this thesis, we discovered why certain molecules are good at inhibiting quorum sensing and why other molecules are good at actually mimicking the natural communication signals to activate quorum sensing (i.e., function as proper keys that activate the lock). Three major techniques allowed us to make these discoveries. First, we studied 3-dimensional pictures that show how the receptor protein binds its signal molecule (like seeing a blueprint of a lock with the proper key inside of it). These images are the result of X-ray

crystallography, which is a challenging process of forming a crystal of the protein of interest (not unlike crystals of salt or sugar) and then shooting X-rays at the crystal and analyzing the pattern formed by the X-rays when they exit the crystal. However, on its own, this method neither tells us which aspects of the signal molecule are most important for activating the receptor protein (like certain peaks and valleys on a key could be more important than others), nor does it tell us what an ideal inhibitor would look like. To address those questions, we synthesized a set of different molecules that are similar to the signal but are subtly different in ways that could prevent activation (like making several keys that miss a certain peak or valley present in the true key). We then tested the activity of the molecules, and saw some that completely lost activity. These molecules likely cannot bind the protein, like a key that will not even fit in the lock. We also observed molecules that still activate the protein well, which indicate that the altered portion is not very important for binding and activating the protein. Lastly, we observed molecules that block the activity of the protein. These molecules likely bind the protein, but do not activate it, like a wrong key stuck in a lock. These are the communication blockers (or "quorum sensing inhibitors") that can prevent the harmful behavior of pathogens. The third technique used to confirm that certain aspects of a molecule-protein interaction are important for activating or blocking the protein was as follows: we modified (or "mutated") the protein and observe how those mutations changed the ability of the molecule to activate or inhibit the protein. We discovered mutations to the protein that cause it to be *inhibited* by a molecule that *activates* the original protein, and we found mutations that cause the protein to be *activated* by a molecule that *inhibits* the original protein (**Figure 1.7**). Therefore, we confirmed that those molecule-protein interactions (like interactions between a lock and specific ridges on a key) are important for activation and inhibition. By combining all three of these techniques (investigating structural images, testing different molecules for protein activation and inhibition, and testing the activation

and inhibition of mutated proteins), we discovered many crucial interactions that determine activation and inhibition of a quorum-sensing receptor protein in the pathogenic bacterium *P. aeruginosa*.

In **Chapter 5** of this thesis, we investigated another factor that determines whether a quorum sensing inhibitor will be active. Many of these communication-blocking molecules need to enter inside the bacterial cell in order to carry out their function, and unfortunately, we found that many of these molecules are excreted or "effluxed" (i.e., spit out) of the cell before they can perform their function. This situation is akin to giving a baby medicine. It could be an amazing medicine that will completely heal the baby, but if the baby keeps spitting it out, the medicine will not stay in the baby long enough to work. Although most quorum sensing inhibitors were strongly effluxed from the pathogenic bacterium *P. aeruginosa*, we found that some molecules were effluxed far less than others and therefore should be more active. The clear next step is to find molecules that are *both* very good at blocking the receptor *and* stay inside the cell. Other researchers in our laboratory are continuing this work to develop optimized quorum sensing inhibitors that exhibit both of these critical traits.

1.3.3 Can bacteria become resistant to our interference with their communication?

In the future, the interference of bacterial communication might be used to control infections in humans and plants, and could also be used to prevent biofilms in industry. If they see widespread use, the question arises of how bacterial populations will respond to these treatments over time. The pervasive use of antibiotics has caused antibiotic resistance to arise and spread through bacterial populations rapidly (See **Figure 1.8A**). Would resistance likely arise and quickly spread in the case of quorum sensing inhibition? This is an important research question for the field.

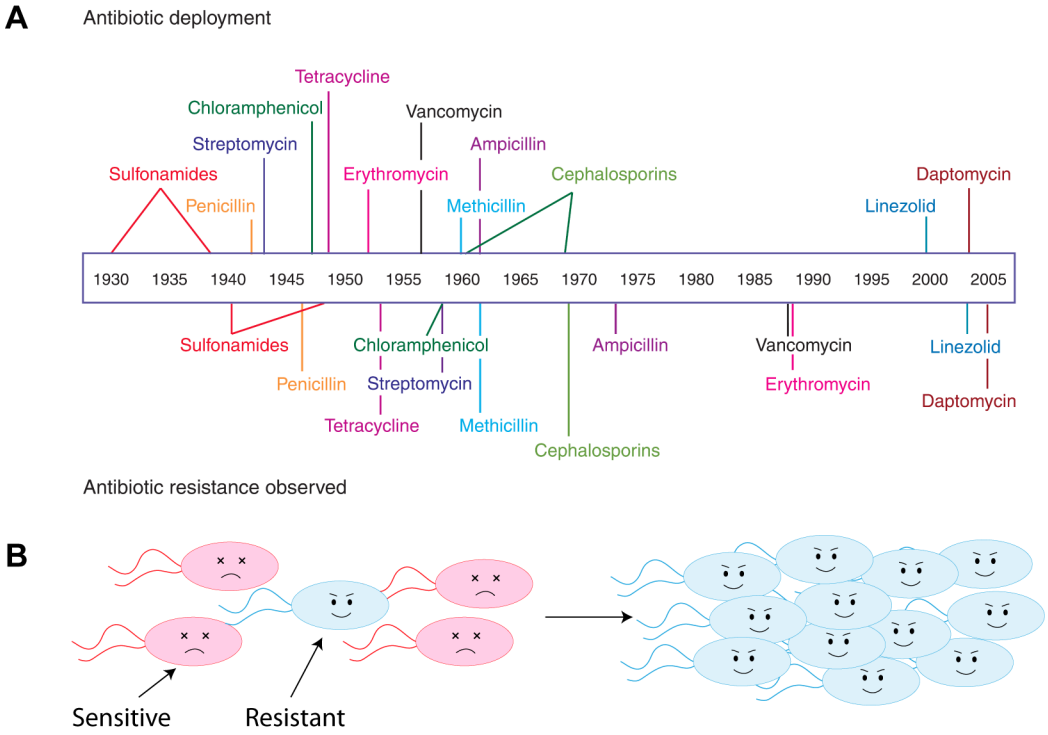

Figure 1.8. Spread of resistance to antibiotics.
(A) Diagram demonstrating the quickness with which resistance to new antibiotics has been observed (image from Clatworthy and Hung[36]). (B) Antibiotic resistance spreads quickly because only the resistant bacteria (blue) survive and reproduce.

When we consider antibiotics, the cause of their rapid spread is very clear. Antibiotics prevent the growth of all the bacteria except the resistant ones, so only the resistant bacteria multiply and eventually take over their local population and then disseminate widely (**Figure 1.8B**). Many different mechanisms of resistance exist: bacteria can start strongly effluxing antibiotics, the proteins that are blocked by the antibiotics can mutate to no longer be blocked, or the bacteria can even develop the ability to degrade the antibiotics. Acquiring a resistance mechanism is a very rare occurrence, so resistant bacteria are initially present at extremely low levels. However, because only the resistant ones can grow, that tiny fraction of resistant bacteria quickly overtakes the entire population.

We expect bacteria to be able to develop resistance to molecules that inhibit quorum sensing. All three previously mentioned mechanisms of antibiotic resistance could also apply to quorum sensing inhibitors (increased efflux, mutation of receptor protein, or degradation of inhibitor). Furthermore, the behaviors that are prevented by quorum sensing inhibitors are generally beneficial to the bacterial population, so resistance would be advantageous for growth. However, in the case of quorum sensing inhibition resistance, the competition that arises between the resistant bacterium and those around it that still have blocked communication is very different than the competition that arises in the case of traditional antibiotics. The experiments presented in **Chapter 2** demonstrate that two distinct obstacles are present that serve as barriers to the spread of resistance to communication inhibitors (**Figure 1.9**). The first obstacle is due to the fact that resistance should initially arise at low levels. If only a single bacterium is resistant, all the other bacteria are still blocked from communicating. This communication blocking not only prevents the other bacteria from sensing each other's signals, but it also prevents them from making signal themselves. So, although the single resistant bacterium is capable of sensing other bacteria, insufficient signal is present to activate its receptor proteins. As an illustration, this scenario is akin to blindfolded and gagged people being asked to lift a piano, and all but one of the people are incapable of smelling *and producing* body odor. Even though there are sufficient people present to lift the piano, and even though one person can smell, he/she does not know there are enough others to lift because they are not producing odor.

The second obstacle to the spread of resistance is that the behaviors regulated by communication are often communal behaviors that are susceptible to "cheating." If one child brings a lunch to a room of hungry children and then leaves to use the restroom, it is quite possible that he/she will not have much of a lunch remaining upon returning to the room. Similarly, if a single bacterium is resistant and can now secrete siderophores into the

environment, these siderophores bind iron ions, but then freely diffuse and can be taken up by other bacteria. Therefore, the resistant bacterium incurs a cost to make the siderophores, but only gets a fraction of the beneficial iron; whereas the neighboring communication-blocked bacteria get iron without any cost. Thus, *the resistant bacteria can actually be in worse shape* than the bacteria that are still sensitive to the quorum sensing inhibition—the opposite of the case of traditional antibiotics. Experiments reported in **Chapter 2**, in which we grew mutants of the pathogen *P. aeruginosa* together and observed which one outcompetes the other, provided strong empirical support for the existence of these two barriers.

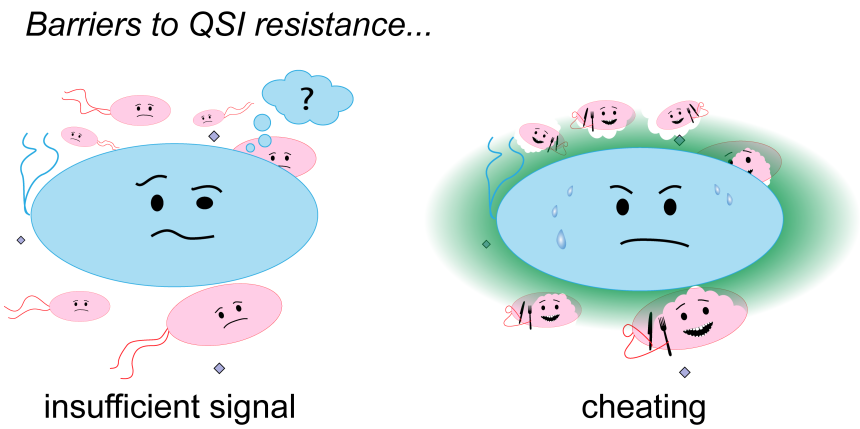

Figure 1.9. Obstacles that prevent the spread of resistance to quorum sensing inhibition (QSI). (Left) When only a few resistant bacteria (blue) are present, they cannot produce enough signal (green diamonds) to induce a quorum sensing response. (Right) When the quorum sensing response of a resistant bacterium does turn on, it secretes shared resources (green halo) that benefit non-resistant bacteria around it. Since the non-resistant bacteria benefit but have no cost to produce the resources, they are termed "cheaters." The cheaters can be more fit than the resistant bacterium, and they prevent the spread of the resistant one.

1.4 Technical Summary of the Dissertation Scope and Organization

This thesis is divided into five chapters that fit into four categories at the frontier of quorum sensing (QS) research. First, **Chapter 2** discusses work to investigate the obstacles that should prevent the spread of resistance to QS inhibition. Next, **Chapters 3** and **4** report the results and conclusions of studies aimed at gaining a better molecular-level understanding of the activating

and inhibiting behavior of QS modulators. Then **Chapter 5** reports the discovery that *P. aeruginosa* readily effluxes most QS modulators, thereby decreasing their activity. Lastly, **Chapter 6** reports the analysis of the signal-response promiscuity (or multilingualism) of two QS receptors and provides a model to explain their different degrees of promiscuity and predict the promiscuity of hundreds of unstudied homologous receptor proteins. Each chapter was written to stand alone from the other chapters and can thus be read independently. Finally, **Chapters 2** and **6** contain appendices that describe ongoing work.

1.4.1 Chapter 2: Tests of the likelihood of resistance spreading to QS inhibitors

Since the advent of inhibiting virulence in model systems by QS inhibitors (QSIs), researchers have suggested that "antivirulence" QS inhibition therapies should be less susceptible to the spread of resistance compared to traditional antibiotics. The rationale for this hypothesis was rarely well articulated, and until recently, had no true empirical support.[37, 38] Using competitive growth experiments with QS mutants of the opportunistic pathogen *P. aeruginosa*, the study reported in **Chapter 2** empirically confirmed the existence of two discrete obstacles that decrease the likelihood of resistance spreading to QS inhibitors: (1) a few resistant mutants spontaneously arising will produce insufficient native signal to induce QS, and (2) the expression of QS-regulated group-beneficial phenotypes can make the resistant bacteria less fit than the QSI-sensitive bacteria (see **Figure 1.9**). This finding supports the hypothesis that resistance should spread slower to QSIs than to traditional antibiotics and therefore encourages the further development of QSIs.

1.4.2 Chapters 3 & 4: Uncovering the ligand-receptor interactions that lead to activation and inhibition of LasR and other QS receptor proteins

Our laboratory and others have developed several non-native ligands that activate and inhibit LasR and other LuxR-type QS receptors.[39-47] It was still unclear how these ligands bound to their receptors and how subtle differences between ligands determined whether they would activate or inhibit the receptor. By performing site-directed mutagenesis on the *P. aeruginosa* LasR protein, and then testing the ability of these mutants to be activated and inhibited by several ligands, we found that the ligands likely bind in very similar manners to the native signal molecule, *N*-(3-oxo)-dodecanoyl L-homoserine lactone (OdDHL) (**Figure 1.10A**), and that interactions between the ligands and Trp60 can govern whether LasR is active or inactive (**Figure 1.10B**).

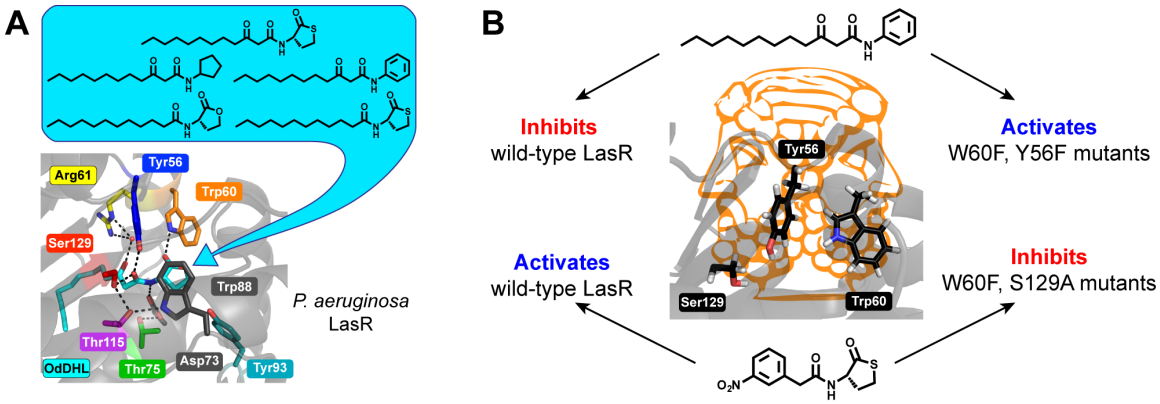

Figure 1.10. Site-directed mutagenesis of the LasR QS receptor uncovers important molecular interactions for activation and inhibition by native signal and non-native QS modulators.
(A) Mutational analysis of the LasR ligand-binding pocket suggest that OdDHL analogs bind the ligand-binding pocket in nearly identical manners to OdDHL. (B) W60F, Y56F, and S129A mutations to the LasR protein markedly flip the activity of the aniline inhibitor (top) to an activator and the thiolactone activator (bottom) into an inhibitor.

1.4.3 Chapter 5: Discovery of the influence of active efflux on the activity of synthetic QS modulators

QS in *P. aeruginosa* has consistently been difficult to strongly inhibit.[35, 48] Due to the prevalence of multidrug efflux pumps in this opportunistic pathogen,[49] we hypothesized that its efflux pumps were tempering the activity of QS modulators. Through genetic and chemical knockouts of efflux, we found that the MexAB-OprM drug efflux pump efficiently excreted nearly every QS modulator, which substantially decreased their activities. One recently discovered QS modulator (5,6-dimethyl-2-aminobenzimidazole, DMABI,[30] see **Figure 1.11**) was found to be immune to efflux, and is therefore a promising lead scaffold for the design of QS modulators with improved potencies in *P. aeruginosa*.

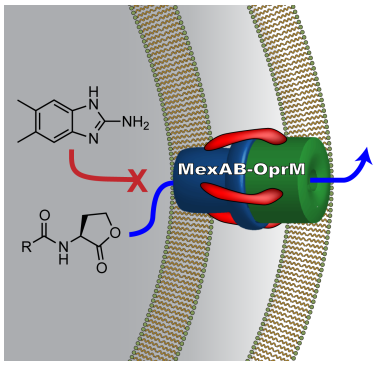

Figure 1.11. MexAB-OprM multidrug efflux pump decreases the potency of most QS modulators in *P. aeruginosa*.
The QS-inhibitory effects of DMABI (top) are not impacted by efflux; therefore DMABI is a promising scaffold for efflux-resistant QS modulators (image from Moore, et al.[50]).

1.4.4 Chapter 6: Delineating the causes of varying signal selectivities of QS receptor proteins

With improved DNA-sequencing technology, we are increasingly finding environments that house many bacterial species living in community.[28] Many infections are made up of multiple bacterial species,[51] but also commensal polymicrobial communities on and inside humans are relevant for health.[5, 6] These communities can contain several different proteobacteria all capable

of producing and sensing similar AHL QS signals. Cross-talk between their QS systems could alter the behavior of each species and the community as a whole, so delineating how species will respond to the QS signals of other organisms is crucial. Using a comprehensive set of naturally produced signal molecules and synthetic analogs, we evaluated the signal-response promiscuity of *P. aeruginosa* and *A. baumannii*—bacteria that are similarly robust environmental bacteria that act as opportunistic pathogens of humans and are frequently found in polymicrobial infections.[51, 52] We not only found that the LasR QS receptor of *P. aeruginosa* is more promiscuous than AbaR of *A. baumannii*, but we also developed a model to explain this relative promiscuity based on analysis of sequence alignments and structural data for many LuxR-type proteins (see **Figure 1.12**). This new model may allow researchers to predict the signal promiscuity of unstudied organisms, and our work should also help in the development of organism-specific QS modulators as chemical probes to investigate how entire communities respond to changes in the QS activity of a single member.

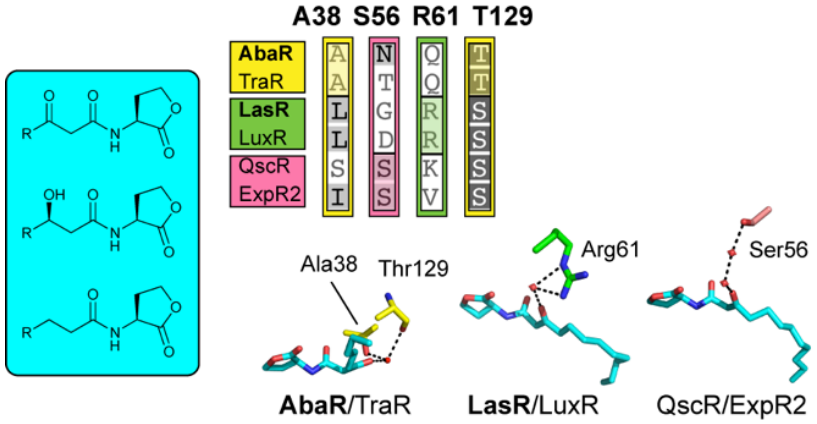

Figure 1.12. Hypothesized specificity determinants for LuxR-type proteins.
Residues Ala38 and Thr 129 in *A. baumannii* AbaR and *Agrobacterium tumefaciens* TraR, residue Ser56 in *P. aeruginosa* QscR and *P. carotovorum* ExpR2, and Arg61 in *P. aeruginosa* LasR and *Vibrio fischeri* LuxR correspond with the signal specificity of these receptor proteins for AHL ligands of different lengths and oxidation states (left, with cyan background). The residues are involved in hydrogen bonds to the 3-keto position of the native signal in crystal structures of TraR,[53] LasR,[54] and QscR[22] (bottom right).

1.4.5 Future directions (appendices to Chapter 2 and Chapter 6)

Several avenues for new research were opened by the work reported in this thesis. In particular, two research projects are currently being pursued by other members of our laboratory. The appendix to **Chapter 2** discusses the implications that population expansion can have on the spread of QSI resistance. Rationale and preliminary results are presented that suggest that QSI resistance will spread in populations that are growing on solid structured surfaces if the population can expand and colonize new space. Data show that spread of QSI resistance under these conditions is still substantially less likely than the spread of resistance to traditional antibiotics. This work is being continued by a current graduate student in our lab, Kimberly Tyler. The appendix to **Chapter 6** reports the initial stages of work to test the importance of the hypothesized ligand-response selectivity determinants in LuxR-type proteins. Site-directed mutagenesis is being performed on the *P. aeruginosa* LasR receptor protein. Our model predicts that one set of mutations will cause LasR to become more promiscuously activated by several ligands, and another set should make it more selective to only a few ligands. This work is being continued by a current undergraduate student in our lab, Trevor Schell.

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CHAPTER 2:

COMPETITION STUDIES CONFIRM TWO MAJOR BARRIERS THAT CAN PRECLUDE THE SPREAD OF RESISTANCE TO QUORUM-SENSING INHIBITORS IN BACTERIA

Contributions: J. P. G. designed and performed all experiments and analyzed all data. J. P. G. and H. E. B. wrote the manuscript. Kimberly A. Tyler aided in performing experiments described in the appendix.

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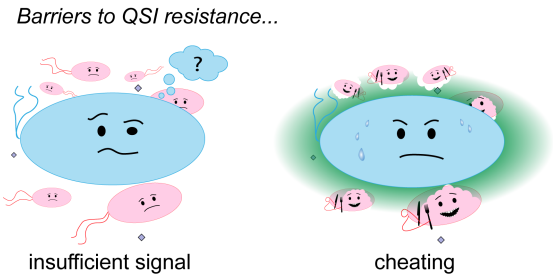
Abstract.



The growing threat of antibiotic resistance necessitates the development of novel antimicrobial therapies. Antivirulence agents that target group-beneficial traits in microorganisms (i.e., phenotypes that help the cells surrounding the producer cell instead of selfishly benefiting only the producer cell) represent a new antimicrobial approach that may be robust against the spread of resistant mutants. One prominent group-beneficial antivirulence target in bacteria is quorum sensing (QS). While scientists are producing new QS inhibitors (QSIs) at an increasing pace for use as research tools and potential therapeutic leads, substantial work remains in empirically demonstrating a robustness against resistance. Herein we report the results of *in vitro* competition studies in *Pseudomonas aeruginosa* that explicitly confirm that *two separate barriers* can impede the spread of resistance to QSIs: (1) insufficient native QS signal levels prevent rare QSI-resistant bacteria from expressing their QS regulon, and (2) group-beneficial QS-regulated phenotypes produced by resistant bacteria are susceptible to cheating by QSI-sensitive neighbors—even when grown on a solid substrate with limited mixing to mimic infected tissue. These results underscore the promise of QSIs and other antivirulence molecules that target group beneficial traits as resistance-robust antimicrobial treatments, and provide support for their further development.

2.1 Introduction

Microbes play essential roles in our world. However, these organisms can also have devastating effects on human health and productivity via pathogenic infection. The spread of antibiotic resistance is gradually disarming our society and causing increased morbidity, mortality, and costs associated with infection. In the U.S. alone, antibiotic-resistant hospital-acquired infections kill an estimated 50,000–100,000 annually[1] and cost the U.S. society approximately $35 billion per year.[2] This current toll, coupled with the increasing trajectory of resistance development, makes the spread of antibiotic resistance one of the world's greatest health concerns.[3]

In view of these challenges, both chemists and biologists have become interested in developing "evolution-proof" drugs that are robust against the development of resistance by microbes.[4, 5] This represents a substantial undertaking, however. Traditional antibiotics are inherently highly susceptible to resistance, evidenced by the fact that resistance often appears in clinics only a few years after the first therapeutic use of a new antibiotic.[5] Antibiotics prevent the growth of all drug-sensitive bacteria, but allow resistant bacteria to grow. This scenario presents an extraordinarily strong selective pressure for a single resistant bacterium to propagate through the entire population. In the past decade, "antivirulence" drugs that do not directly kill bacteria, but instead prevent pathogens from expressing their detrimental phenotypes, have garnered increased attention.[4, 5] In particular, antivirulence agents that specifically target *group-beneficial* virulence traits (i.e., phenotypes that, when expressed, help not only the bacterium expressing them, but all of its neighbors as well) may allow for a fundamentally decreased rate of resistance spread within infections. This "resistance robustness" is possible because a resistant bacterium that arises would not selfishly benefit from its resistance, but instead would help its antivirulence-

drug-sensitive peers at a cost to itself.[6-8] Examining resistance spread to such antivirulence approaches was the broad goal of the current study.

Our research group and others have focused on bacterial quorum sensing (QS), a widespread cooperative trait, as one such group-beneficial antivirulence target.[9-11] QS is an intercellular chemical signaling mechanism that bacteria use to monitor their local cell densities.[12, 13] In QS, bacteria constitutively produce low levels of diffusible signal molecules. As the bacteria multiply in a confined space, more signal accumulates until it reaches a threshold concentration that both activates an auto-induction loop for increased signal production and also induces the expression of a set of genes that are beneficial for growth in a cell-dense environment. Viable QS inhibition strategies include competitive inhibition of signal binding to receptor proteins,[11, 14-19] inhibition of signal synthesis,[20] and sequestration and degradation of signals.[10, 21] Several small molecule- and macromolecule-based approaches are shown in **Figure 2.1**. Small molecule QSIs targeting QS receptors have arguably seen the most intense study so far.

QS is a compelling resistance-robust antivirulence target because it involves two levels of group behavior that could lead to *two barriers to the spread of resistance*. First, each individual cell depends on the other cells in the population to produce signal molecules in order for sufficient signal to accumulate to induce QS gene expression.[22] Second, many of the genes that are activated by QS are themselves group-beneficial (e.g., biofilm formation and secreted diffusible factors like proteases, siderophores, and toxins).[23-25] These group-beneficial genes could render a bacterium that expresses its QS regulon susceptible to "cheating" by neighboring bacteria that lack functioning QS systems. The cheaters benefit from the production of these common goods by QS-active bacteria, yet they do not reciprocate by producing the goods themselves. The existence of QS cheaters in bacterial populations has been extensively demonstrated both *in vitro*[24, 26-28] and *in vivo*,[29-31] but studies on the implications for resistance to

QSIs are few and yield conflicting results.[31-33] For example, one study by Wood and co-workers found that resistance to the QSI furanone C-30 (shown in **Figure 2.1**) via increased drug efflux can spontaneously arise and spread when the opportunistic pathogen *Pseudomonas aeruginosa* is grown in a minimal medium with adenosine as the sole carbon source.[32] However, another study by Mellbye and Schuster suggested that QSI resistance does not spread when *P. aeruginosa* is grown in a minimal medium with bovine serum albumin (BSA) as the only carbon source[33]—a competitive growth condition that has shown reasonable correlation with competition studies in animal infections and clinical isolates.[26, 27, 29, 30] The authors did not use a small molecule QSI in this latter study, but rather used a pair of *P. aeruginosa* mutants that mimic QSI resistance.[33]

We sought to reconcile these previous results in order to guide future QSI and antivirulence research in general. In the current study, we explicitly evaluated two unique obstacles that could preclude the spread of resistance to inhibitors of QS receptors. First, we hypothesized that a few resistant bacteria that spontaneously arise in a population of QSI-sensitive bacteria would not produce sufficient signal molecules to turn on QS and therefore would have no fitness advantage over sensitive bacteria. Second, in a situation where a few resistant bacteria might overcome the first obstacle and express their QS-regulated genes, we hypothesized that those resistant bacteria would be outcompeted by QSI-sensitive bacteria cheating off of the common goods produced by the resistant bacteria[22]—even in a physically structured environment that models tissue infections. We explicitly examined these two barriers to resistance *in isolation from each other* using QS mutants of *P. aeruginosa*. The results of the competition studies described herein confirmed our hypotheses and provide strong support for QS inhibition as a potential resistance-robust approach to antimicrobial therapy.

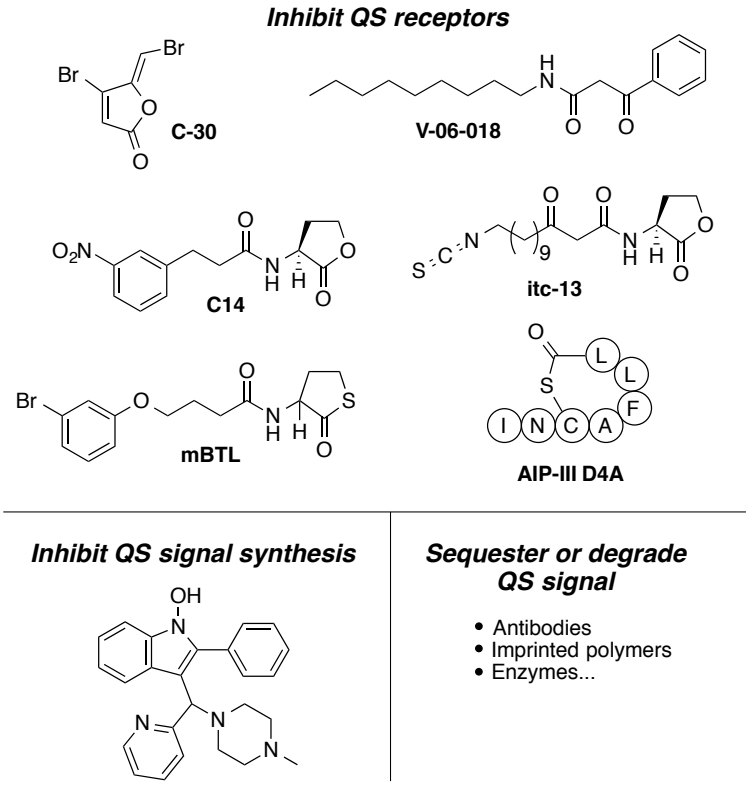


Figure 2.1. Common QS inhibition strategies.
(Top) Representative competitive inhibitors of signal binding to QS receptor proteins are C-30,[19] V-06-018,[18] C14,[17] itc-13,[16] mBTL,[15] and AIP-III D4A[14]). (Bottom) Inhibitors of signal synthesis[20] and availability[10, 21] have also been explored.

2.2 Results and Discussion

2.2.1 Development of experimental conditions

We chose the Gram-negative pathogen *P. aeruginosa* for study because of its prevalence in antibiotic-resistant infections[3] and its well-characterized QS system. This pathogen uses primarily LuxR/LuxI-type circuits for QS (i.e., the *las* and *rhl* systems),[13] with which it controls the production of an arsenal of virulence factors and growth into impermeable biofilms in infections.[23, 34] *P. aeruginosa* infection models in mice have demonstrated that QS is important for abundant growth in infections, presumably due to the QS-regulated production of secreted proteases and siderophores and defenses against the immune system.[35-37] As such, common

nutrient-rich growth media that do not require QS for growth are poor models of *in vivo* infection growth. We therefore utilized QS-selective growth media for the current study, in which the supplied carbon sources can only be utilized by bacteria after digestion by QS-regulated enzymes.[26-28, 32, 33, 38] Since *P. aeruginosa* regulates many phenotypes by QS, some of which are "selfish" (i.e., primarily aid growth of the individual expressing the phenotype) and many of which are "group-beneficial" (i.e., significantly aid growth of neighboring bacteria as well as the individual expressing the phenotype),[23] both selfish and group-beneficial QS-selective media were used.[26, 27]

The selfish QS-selective medium contained adenosine as the only carbon source, because adenosine metabolism in *P. aeruginosa* requires the production of nucleoside hydrolase (Nuh), a periplasmic protein that is under QS control. In turn, the group-beneficial QS-selective medium contained the protein BSA as the main carbon source. Metabolism of BSA requires the presence of an extracellular protease to liberate simple peptide nutrients, which can be used not only by the bacterium that secreted the protease, but also by cheating neighbors that do not secrete proteases. A predominant secreted protease in *P. aeruginosa* is elastase B (LasB), which is produced under QS control. We found that both media were QS selective, since wild-type *P. aeruginosa* PAO1 grew substantially better (i.e., reached maximal cell density over five days faster) than a QS mutant strain (Δ*lasR*, Δ*rhlR*) that lacked functional LuxR-type receptors (**Figures 2.2A** and **2.2B**; see *Section 2.4.1* for details of strains).

At the outset, we considered treating *P. aeruginosa* with small molecule QSIs in these QS-selective media and monitoring whether resistance arose and spread over time.[32] However, the QSIs in *P. aeruginosa* that are free of off-target growth affects do not sufficiently inhibit QS. For example, two of the most potent LasR receptor inhibitors, V-06-018 reported by Greenberg and co-workers[18] and *N*-(3-nitrophenylacetanoyl)-L-homoserine lactone (C14) reported by our

laboratory[17] (**Figure 2.1**), still allow for at least 40% of the native level of LasB protease production in *P. aeruginosa*. This partial inhibition would lead to a weak QS-based selective pressure that would preclude a definitive selection experiment in our media. In addition, while furanone C-30 (**Figure 2.1**) is a widely studied QSI and has been shown to inhibit some QS-regulated behaviors in *P. aeruginosa* by more than 90%,[19] we observed significant non-QS-based growth inhibition by this compound at the concentrations necessary for QS inhibition (see *Section 2.5.1* and **Figure 2.11**). As highlighted above, Wood and co-workers used this QSI in their recent study to demonstrate that resistance can quickly arise to QSIs.[32] However, we believe that C-30's off-target growth effects impose non-QS-based selective pressures for the spread of resistance. In fact, such off-target growth effects are a feature we and other research groups explicitly avoid in the ongoing design of improved QSIs.[11]

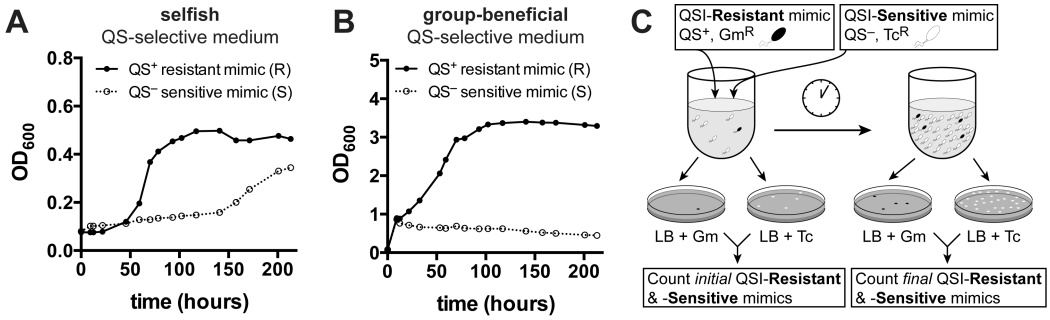


Figure 2.2. Demonstration of selective media and experimental setup.
(A) Growth curves for monoculture QS^+ (R) and QS^- (S) strains grown in QSM + 0.1% adenosine (selfish QS-selective medium). (B) Growth curves for R and S in QSM + 0.1% CAA + 1% BSA (group-beneficial QS-selective medium). In both QS-selective media, monoculture R grew substantially better than monoculture S. (C) Schematic of the competition studies performed herein. A QSI-resistant mimic (PAO1::mini-Tn7-GFP-Gm^R) and a QSI-sensitive mimic (*ΔlasR*, *ΔrhlR,* Tc^R) were mixed and grown in QS-selective media. Initial and final ratios of resistant:sensitive bacteria were calculated by counting colony forming units (CFUs) of resistant mimics on gentamicin-containing plates and CFUs of sensitive mimics on tetracycline-containing plates. Gm^R = gentamicin resistant, Tc^R = tetracycline resistant.

Thus to test resistance to a future ideal QSI in the current study, we instead utilized a pair of *P. aeruginosa* strains to mimic a QSI-sensitive bacterium and a QSI-resistant bacterium

(**Figure 2.3**).[33] To mimic a QSI-sensitive bacterium (termed S hereafter) having its QS system chemically knocked down nearly 100% by a potent and selective QS receptor inhibitor, we used the *P. aeruginosa* QS mutant strain (Δ*lasR*, Δ*rhlR*). To mimic a QSI-resistant bacterium (termed R hereafter) having a functioning QS system in the presence of a QSI, we used wild-type *P. aeruginosa* PAO1. The QS mutant had no observable growth defects (see **Figure 2.6B**), except in QS-selective media (**Figures 2.2A** and **2.2B**). These two strains therefore model sensitivity and resistance to an ideal QSI that completely inhibits QS with no off-target effects (**Figure 2.3**).

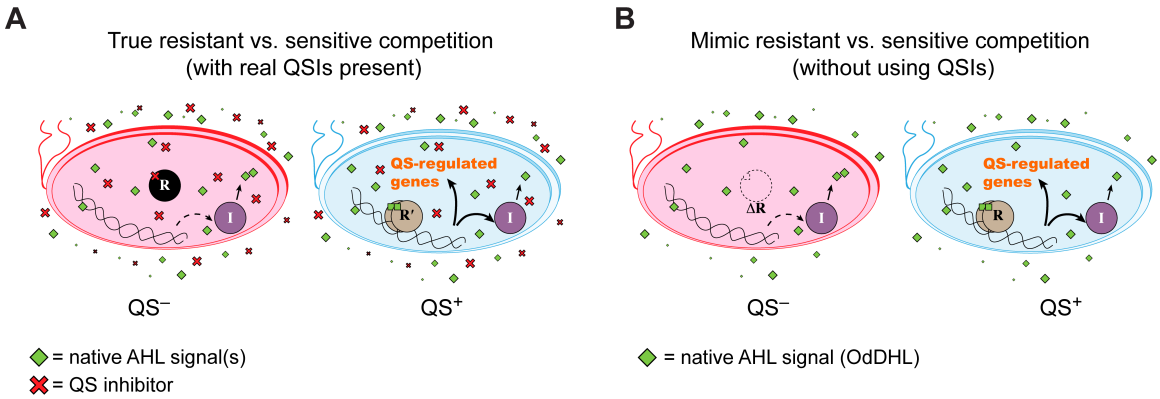


Figure 2.3. Comparison of a "true" QSI resistance competition to the experimental mimic competition in this study.
"R" circles represent QS receptor proteins, and "I" circles represent QS signal synthase proteins. In the true case (A), the wild-type bacteria have chemically knocked down QS (red), and the resistant mutants are still capable of QS even in the presence of the QSI (blue). In the mimic case (B), a *P. aeruginosa* Δ*lasR* Δ*rhlR* mutant has a genetically knocked down QS system (red) to mimic the QSI-sensitive strain, and the resistant mimic is wild-type PAO1 (blue), which is fully capable of QS under the experimental conditions. In both panels, substantial native AHL signals are shown, but if the resistant bacteria are rare, much less signal will actually be present (due to poor signal production by the QS-inhibited strains).

To model a few resistant bacteria arising spontaneously under QSI treatment, we grew populations of the QSI-sensitive mimics (S) seeded with a small number of QSI-resistant mimics (R). We labeled strains R and S with different antibiotic resistance markers, which allowed us to readily count the R/S ratio before and after growth to determine if the few resistant bacteria were more fit and spread through the population (**Figure 2.2C**). In all subsequent experiments,

resistance spread was quantified by calculating the relative fitness of R vs. S, which is the final ratio of R/S divided by the initial R/S ratio (see *Section 2.4.5*).[39] Relative fitness values > 1 indicated a spread of resistance had occurred.

2.2.2 First barrier to resistance: non-quorate signal levels

We first tested the hypothesis that a population of QSI-sensitive bacteria treated with a QSI would not produce sufficient signal to activate the QS system of a small number of QSI-resistant "infiltrators." Therefore, if such QSI-resistant mutants were to arise, they would not be more fit than neighboring QSI-sensitive bacteria. An example case is the development of a mutant that effluxes a QSI efficiently. The effective concentration of the QSI would be lower for that cell, so its QS system would no longer be inhibited. However, that cell would still require a quorum level of native QS signal in order to express its QS-regulated genes. Since the other cells in the population are still inhibited, they would not express sufficient QS signal to induce QS in the resistant bacterium (**Figure 2.4A**).

We examined this barrier by competing the resistant mimic (R) with the sensitive mimic (S) in co-culture in the "selfish" QS-selective media. By using the selfish media, we excluded any potential fitness effects due to cheating (i.e., the second hypothesized barrier, see below), and therefore explicitly tested the existence of only the first barrier. We mixed the bacteria at three different initial proportions of R (50%, 1%, and 0.01%). In the extreme case that 50% of the population was initially resistant, sufficient native QS signal should be produced for the resistant bacteria to be quorate, as shown by quantifying the amount of native QS signal (*N*-(3-oxo)-dodecanoyl-L-homoserine lactone, OdDHL) extracted from grown culture (see **Figure 2.7**). However, at initial proportions at or below 1% R, the population should remain non-quorate, even

at high total bacterial densities (**Figure 2.7**). The most relevant case in an infection would be very low levels of resistant bacteria spontaneously arising (<< 1%), thus we tested 0.01% R.

In each competitive co-culture trial at quorate levels of R (i.e., 1:1 R/S ratio), the fitness of R was greater than S (i.e., relative fitness R/S > 1, **Figure 2.4B**). However, when R was seeded as ≤ 1% of the population (relevant to resistance arising as << 1% of an infection), the resistant mimics *did not* consistently outcompete their QSI-sensitive neighbors. These data support the hypothesis that low levels of QSI-resistant bacteria are incapable of expressing their QS genes, and therefore have no fitness advantage over QSI-sensitive neighbors. We note that this finding expands on the recent work of Mellbye and Schuster (as introduced above),[33] who demonstrated that QSI resistance failed to spread in a *group-beneficial* selective medium. The authors concluded that resistance failed to spread in their experiment because the phenotypes under QS control (i.e., production and secretion of proteases) were group-beneficial. Our results now demonstrate that *even using a selective pressure based on a selfish QS-regulated phenotype*, QSI resistance should not spread due to a dependence on signal production. This result is significant, as it argues that QSI resistance could be even less likely to spread than previous work has suggested.

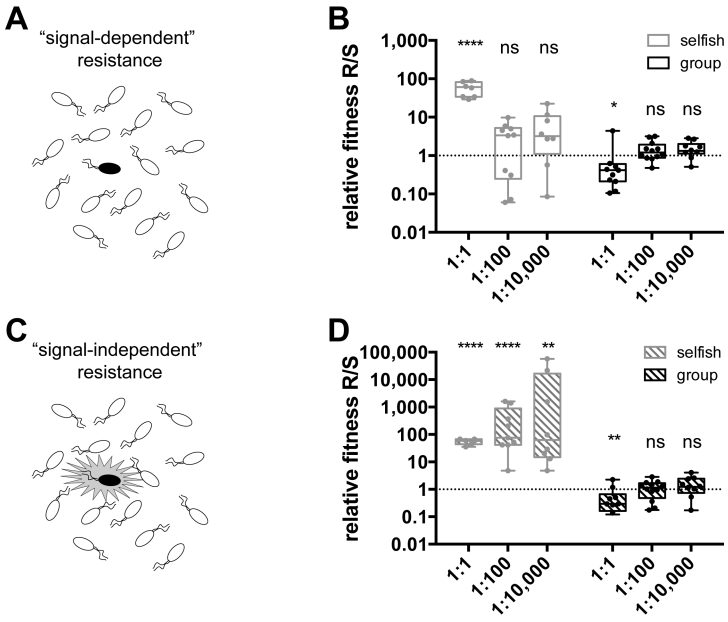


Figure 2.4. Relative fitness of resistant (R) versus sensitive (S) mimic strains.
R and S were grown in co-culture with selfish phenotype selection (adenosine carbon source, grey) and group-beneficial phenotype selection (BSA carbon source, black). (A) Schematic demonstrating a non-quorate rare signal-dependent QSI-resistant mutant. (B) Relative fitness of signal-dependent R vs. S. (C) Schematic demonstrating a signal-independent QSI-resistant mutant that can express its QS regulon, even when rare. (D) Relative fitness of signal-independent R vs. S. Relative fitness values > 1 indicate that the resistant mimic is more fit and will spread. Data are represented as box and whisker plots. Each dot is an individual data point. Boxes encompass the inner quartiles, and horizontal lines are median values. Whiskers extend to the furthest data points. The statistical significance of relative fitness deviations from 1 were tested via paired t-tests comparing the logarithm of the final R/S ratio to the logarithm of the initial R/S ratio for each sample (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, not significant (ns) $p > 0.05$).

Our data were initially difficult to reconcile with the work of Wood and co-workers using furanone C-30 (*vide supra*).[32] While our results suggest that improved drug efflux mutants should not have a fitness advantage when they are at low levels in a QS-inhibited population, Wood and co-workers showed that improved-efflux mutants (via overexpression of the MexAB-OprM drug efflux pump) were able to readily spread under C-30 treatment. As mentioned above, we believe that the selective pressure present in this previous study is due to furanone C-30 imposing general, non-QS-related growth-inhibitory affects on *P. aeruginosa* in minimal media, and is not due to the QSI activity of C-30. If C-30 were an ideal QSI without off-target effects, we contend

that low levels of improved efflux mutants would not spread through a population. Therefore, the Wood study and our study work together to underscore the need to design better QSIs that do not suffer from off-target affects that can select for resistance.

An insightful conclusion from the work of Wood and co-workers is that many *P. aeruginosa* strains in chronic infections are already resistant to QSIs via overexpression of the MexAB-OprM pump.[32] This mode of small molecule resistance is common and presumably arises due to the selective pressure imposed by previous treatment with antibiotics. Consequently, we believe that ideal resistance-robust QSIs should (1) not have non-selective growth inhibitory effects and (2) not be susceptible to the same resistance mutations as traditional antibiotics that are used before (or in conjunction with) the QSI. Our findings suggest that if such QSIs are developed, they will be robust against the spread of signal-dependent resistance mechanisms, regardless of the selfishness of the QS-regulated phenotypes needed for growth. In this context, recent studies in our laboratory have shown that certain QSI scaffolds can evade active efflux in *P. aeruginosa* via the MexAB-OprM pump,[40] which serves to leverage these compounds for future development.

2.2.3 Second barrier to resistance: group-beneficial QS-regulated genes

Although signal-dependent mechanisms of resistance would be thwarted by the first barrier described above (e.g., efflux pump overexpression, QSI degradation, or target protein modification to become immune), additional mechanisms of resistance are conceivable that would not require quorate levels of native signal in order to express the QS regulon. Examples of "signal-independent" resistance mechanisms include mutations that lead to constitutive expression of the QS regulon or cause the QS receptor protein to respond to the QSI as an agonist instead of an antagonist. The latter mechanism has been observed with mutations of the QS receptors CviR[41] and LuxR[17, 42] in *Chromobacterium violaceum* and *Vibrio fischeri*, respectively.

These resistance mechanisms would enable rare resistant bacteria to express different genes than their QSI-sensitive neighbors, which could give them a fitness advantage (see **Figure 2.4C**). In these cases, we reasoned that a second barrier would arise to inhibit the spread of resistance: cheating by QSI-sensitive bacteria off the group-beneficial phenotypes expressed by the QSI-resistant bacteria. QS-based cheating has been demonstrated previously *in vitro*[24, 26, 27] and in infections *in vivo*.[29, 30] These past studies have explored whether low levels of cheaters could invade a population of cooperative bacteria. In contrast, we sought to address the opposite question in the current study, by investigating whether low levels of QS-cooperators can outcompete QS-cheaters. This question is directly relevant to the situation of QSI-resistant cooperators arising in a population of QSI-sensitive cheaters. To our knowledge, the only other study that investigated this situation is that of Mellbye and Schuster (*vide supra*);[33] however, the authors did not test very low initial levels of resistance (<<1% of the cells), which should be the most relevant condition to initial stages of resistance spread. Furthermore, this past work mimicked signal-dependent resistance mechanisms and therefore could have underestimated the ability of signal-independent mechanisms to spread. We therefore explicitly tested the existence of this second barrier by performing new competition experiments with low initial frequencies of signal-independent QSI-resistant cooperators to test whether QSI-resistant bacteria could be more fit and spread.

To imitate signal-independent resistance mechanisms, we developed experimental protocols where the resistant mimics were artificially coerced to express their QS-regulated genes even when they were rare in the population. For selection based on a selfish phenotype, the native *P. aeruginosa* QS signal OdDHL was added to the selection medium to induce expression of *nuh*. To induce production of LasB in the group-beneficial selection, the resistant mimic was engineered to constitutively express *lasB* (see *Sections 2.4.3* and *2.4.4* and **Figures 2.8** and **2.9**).

Using these procedures, we found that the signal-independent resistant mimics were substantially more fit than the sensitive mimics (i.e., relative fitness R/S > 1) under selfish selection, even when the signal-independent resistant mimics initiate at low levels in the population (see **Figure 2.4D**). In line with our initial hypothesis, however, *signal-independent resistant mimics were not more fit than sensitive mimics when growth was dependent on the group-beneficial QS-regulated production of LasB* (relative fitness R/S ≤ 1; **Figure 2.4D**). In total, these results demonstrate, for the first time, that even signal-independent resistant bacteria are incapable of spreading when the QS-regulated selective pressures at work are group-beneficial, which provides a second barrier to the spread of QSI resistance. These results are especially compelling in light of recent experiments with *in vivo* mouse infection models[29] and cystic fibrosis clinical isolates,[30] which suggest that group-beneficial selective pressures are significant in *P. aeruginosa* infections.

2.2.4 Effect of local population structure on resistance spread

The competition studies above, as well as related experiments,[32, 33] were all performed in well-mixed liquid culture. In reality, many infections have a more spatially structured, biofilm appearance.[43, 44] As **Figure 2.5A** illustrates, structured populations on solid matrices can keep secreted goods closer to the bacteria that produce them.[39, 45] As such, the impact of population structure on QS resistance spread could be significant. To examine this phenomenon, we converted the group-beneficial selective medium described above to a solid growth medium[45] and repeated the competition studies with initial ratios of 1:100 R/S. Multiple degrees of population structure were tested by altering the plating technique to obtain interspersed monoclonal patches of R and S that were each 0.1 mm, 1 mm, or 1 cm diameter (**Figures 2.5B–D**). Larger monoclonal patches should provide greater sharing of goods among the resistant mimics because

the resistant cells are on average closer to other resistant cells than sensitive cells. We reasoned that this cooperativity should provide the resistant mimics with a greater advantage over the sensitive mimics. Our results showed only when the patches were very large (1 cm diameter) did the signal-independent resistant mimics have an advantage (**Figure 2.5E**).

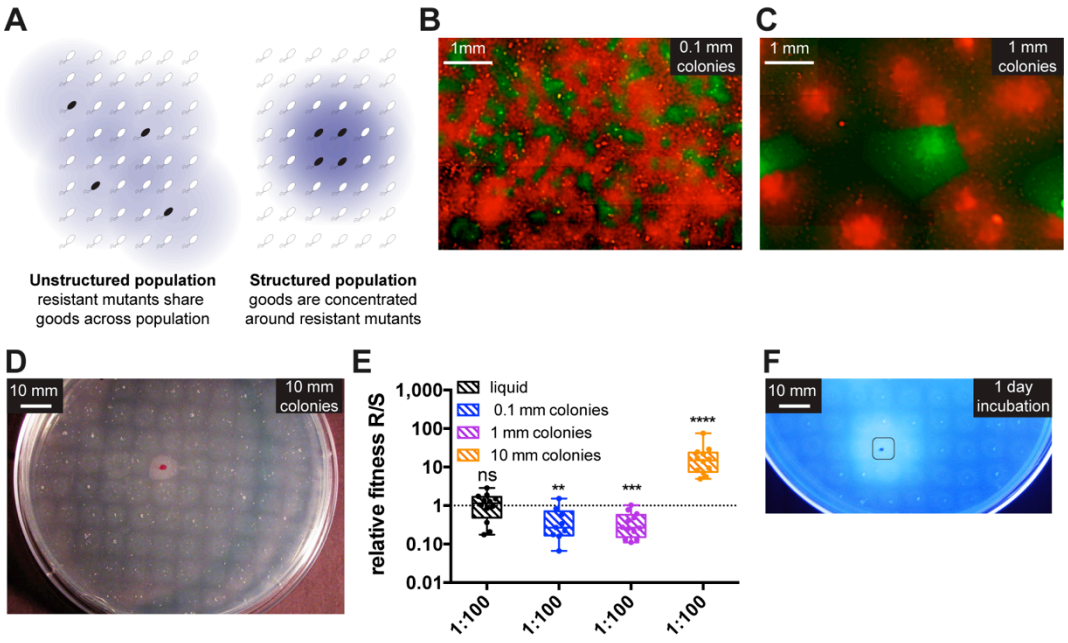


Figure 2.5. Structured environments retain susceptibility to cheating.
(A) Schematic showing that a well-mixed culture would quickly distribute goods away from the producers and also prevent producers from making monoclonal patches of high goods concentrations. (B–D) Images demonstrating the increasing degrees of population structure that were tested. In B–C, fluorescence microscopy was used to visualize 1:10 ratios of R producing GFP (green) and S producing mCherry (red). In D, a 1:100 ratio of R vs. S was visualized without magnification: the macrocolony marked with a red dot is R, and the 99 other spots on the plate are S. (E) Relative fitness of signal-independent R vs. S in liquid group-beneficial medium and in solid group-beneficial medium with different degrees of population structure. Data analysis was analogous to that described in **Figure 2.4**. (F) Image visualizing the diffusion of protease-digested goods after one day of growth of a 1:100 ratio of R vs. S. The bright halo around the single R colony (circled) is diffused protease-digested fluorogenic substrate. The substrate diffusion indicates the distance over which QSI-sensitive bacteria can cheat off neighboring QSI-resistant bacteria.

We hypothesized that the necessary patch size for a resistance advantage was related to the distance that goods diffuse from the producer. To test the diffusion distance of the protease-digested goods, we added a LasB substrate to the plate that fluoresces once cleaved by LasB and

then repeated the competition experiment above with 1 cm colony spacing. After only one day of incubation (before substantial selective growth has occurred), the digested product had diffused approximately 1 cm past the edges of the resistant colony (see **Figure 2.5F**). A similar degree of diffusion has previously been reported for siderophores through agar.[45] Since the 1 cm population structure was on the same size scale as the goods diffusion (as in the right image of **Figure 2.5A**), the goods stayed mostly near the large protease-producing resistant colony, making the resistant bacteria more fit. However, when the patches were smaller, the diffused goods benefitted hundreds to tens of thousands of surrounding QSI-sensitive patches, which enabled substantial cheating. Together, these results indicate that resistance should not spread within a microbial population under group-beneficial selection, even if grown in a viscous or spatially structured environment, as long as the colony-to-colony distance is smaller than the diffusion distance of secreted goods. Although diffusion rates through 1.5% agar are unlikely to directly correlate to diffusion in infections *in vivo,* we note that the 1 cm separation distance needed for the spread of resistance is more than 100× larger than the monoclonal colony separation observed within reported images of biofilm infection biopsies (≤ 0.1 mm).[43]

2.3 Conclusions and Outlook

We expect QSI-resistant mutants to arise in nature.[32, 41] However, at the outset of this study, we hypothesized that the QSI-resistant mutants would struggle to overtake their population relative to traditional antibiotic resistant mutants. The competition studies reported herein provide the first empirical evidence that (1) QS-signal-dependence is sufficient to impede the spread of many mechanisms of QSI resistance, (2) cheating is sufficient to impede the spread of even *signal-independent* mechanisms of QSI resistance under *in vivo*-relevant group-beneficial selection, and (3) reasonable degrees of population structure on a solid matrix still do not enable

signal-independent QSI-resistant bacteria to spread. While our experiments were designed to directly mimic resistance to QSIs that block QS receptor function, we believe that the results apply broadly to the other QS inhibition strategies (i.e., inhibition of signal synthesis, and sequestration and degradation of signal molecules; **Figure 2.1**). As previous research has shown good correlation between protease-based *in vitro* selective pressures and *in vivo* selective pressures in mouse infections and human cystic fibrosis lungs,[26, 27, 29, 30] we are optimistic that these barriers to the spread of resistance will be relevant in infections. Ongoing research in our laboratory is focused on studying the impact of multispecies cultures[46, 47] and population expansion[48] on the spread of QSI resistance, as these have recently been shown to affect microbial competition.

We close by highlighting an additional potential advantage of QS inhibition (or other antivirulence approaches) compared to traditional antibiotics; namely, these approaches should not affect the growth of nonpathogenic bacteria in natural environments. A major cause of the prevalence of resistance in pathogens is that antibiotics select for resistance in the harmless bacteria in human guts and in the environment.[49, 50] As resistance genes become more abundant in environmental bacteria, the genes have an increased likelihood of transferring to neighboring pathogens.[51] Since QSIs and other antivirulence approaches are likely narrow spectrum and only affect fitness in specific settings, they should not broadly increase the prevalence of resistance genes in the environment.[5] When this feature is coupled with the results described herein, which indicate that two unique barriers impede QSI-resistant pathogens from outcompeting their QSI-sensitive neighbors, we conclude that QS inhibition and other antivirulence approaches have substantial promise as resistance-robust therapeutics.

2.4 Experimental

2.4.1 Strains and routine growth conditions

All strains and plasmids used are listed in **Table 2.1**. The QSI-resistant mimic strain (R) (GFP^+, Gm^R) and the QSI-sensitive mimic strain (S) (*ΔlasR*, *ΔrhlR*, Tc^R) were constructed from the same parent *P. aeruginosa* PAO1 strain to avoid any fitness differences due to microevolution.[52] To construct the gentamicin-resistant "QSI-resistant mimic" strain, a mini-Tn7 transposon harboring GFP and *aaaC*1 (i.e., gentamicin resistance, Gm^R) was inserted into the neutral *att*Tn7 site of the PAO1 chromosome by 4-parental mating between *P. aeruginosa* PAO1 and *E. coli* strains harboring pUXBF-13,[53] pRK600,[54] and pMiniTn7-gfp2,[55] followed by citric acid selection on Vogel-Bonner (VB) minimal medium (57.4 mM K_2HPO_4, 8.37 mM $NaNH_4HPO_4$, 0.811 mM $MgSO_4$, 9.52 mM citric acid[56]) + gentamicin.[57] Single colonies were restreaked on VB + gentamicin, and an individual colony from the second plate was picked (named strain PAO1-Tn7-gfp/Gm^R, also called "R") and verified for Tn7 insertion both by observing GFP fluorescence and by colony PCR with primers Tn7-GlmS (5'–AATCTGGCCAAGTCGGTGAC–3') and Tn7R109 (5'–CAGCATAACTGGACTGATTTCAG–3').[55]

Table 2.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description*	Reference
E. coli		
DH5a	F⁻, j80d*lacZ*DM15D(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(rk⁻, mk⁺) *phoA supE*44 λ⁻ *thi*-1 *gyrA*96 *relA*1	Invitrogen
S17-1 λ*pir*	*recA pro thi hsdR⁻ hsdM⁺* RP4-2-Tc::Mu-Km::Tn7 λpir; Sm^R Tp^R	[58]
JLD-271	K-12 Δ*lacX74 sdiA271::*Cam; Cm^R	[59]
P. aeruginosa		
PAO1	Wild-type, isolated by B. Holloway from human wound	[60]
PA14	Wild-type, isolated by M. Schroth from human wound	[61]
PAO-JP3	PAO1 Δ*lasR::*Tc^R *rhlR::*Tn*501*; Tc^R Hg^R	[62]
PAO1-Tn7-gfp/Gm^R	PAO1 with mini-Tn7-based insertion of constitutive GFP Mut3b expression behind a $P_{A1/O4/03}$ promoter; QSI-resistant mimic R; Gm^R	This study
PAO-JG33	PAO1 Δ*lasR::*Tc^R; Tc^R	This study
PAO-JG35	PAO1 Δ*lasR::*Tc^R Δ*rhlR*; QSI-sensitive mimic S; Tc^R	This study
PAO1-Tn7-*lasB*	PAO1 with mini-Tn7-based insertion of constitutive *lasB* expression behind a *tac* promoter; R-*lasB*; Sm^R	This study
PAO-JG35-Tn7-*lasB*	PAO-JG35 with mini-Tn7-based insertion of constitutive *lasB* expression behind a *tac* promoter; S-*lasB*; Sm^R	This study
Plasmids		
pUX-BF13	Mini-Tn7 helper plasmid; Ap^R	[53]
pRK600	Conjugation helper plasmid; Cm^R	[54]
pBK-miniTn7-gfp2	Mini-Tn7 plasmid expressing GFP Mut3b & Gm^R; Ap^R	[55]
pEX18-Gm	Gene-replacement vector; *sacB oriT* Gm^R	[63]
pJG038	pEX18-Gm with Δ*lasR*::Tc^R cassette	This study
pJG055	pEX18-Gm with markerless Δ*rhlR* cassette	This study
pMP7605	pBBR1 MCS-5 derivative expressing the mCherry gene under a *tac* promoter, Gm^R	[64]
pMP7607	pBK-miniTn7-KmΩSm1 derivative expressing the mCherry gene under a *tac* promoter, Ap^R	[64]
pML27	P_{tac}-*lasB* expression plasmid; *lacIq* Ap^R	[65]
pJG068	pBK-miniTn7-KmΩSm1 derivative expressing *lasB* under a *tac* promoter, Ap^R	This study
pJN105L	Arabinose-inducible expression plasmid for *lasR*; Gm^R	[66]
pPROBE-KL	*lasI'-gfp[LVA]* transcriptional fusion derivative of pPROBE-KT;[67] Km^R	[40]

*Abbreviations: Sm^R, streptomycin resistance; Tp^R, trimethoprim resistance; Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance; Gm^R, gentamicin resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance.

The same parental PAO1 strain was mutated by homologous recombination to construct the tetracycline-resistant "QSI-sensitive mimic" strain. To replace the *lasR* gene with a tetracycline

resistance (Tc^R) cassette, an approximately 5 kb Δ*lasR*::Tc^R cassette was amplified from PAO-JP3[62] genomic DNA with primers D060 (5'–GCTGCTCGGCTTCTGGGTG–3') and D061 (5'–ACGTTTGCCCCGCTACTGG–3'). The amplified region contained a *KpnI* site and an *EcoRI* site that were used to clone into *KpnI/EcoRI*-cut pEX18Gm.[63] The resulting plasmid pJG038 was transformed into *E. coli* S17-1 λpir by electroporation and then transferred into PAO1 by conjugation. Individual merodiploid colonies that were isolated by selection on VB + gentamicin plates were picked and selected for the second recombination event by growth on LB + 15% sucrose at 30 ºC. Strain PAO-JG33 was isolated, and sequencing of DNA amplified from its *lasR* region confirmed the gene deletion. PAO-JG33 produced significantly less green pigment than wild-type PAO1 and displayed virtually no ability to degrade an elastin congo red substrate compared to wild-type PAO1 (using a previously reported method;[17] **Figure 2.6A**).

We next deleted the *rhlR* gene from PAO-JG33 because others have shown that *rhlR* can compensate for *lasR* mutants.[27, 68] To delete the *rhlR* gene, an approximately 1 kb upstream portion was amplified from PAO1 genomic DNA with primers D062 (5'–ACTGGATTCCAACGTGCCCGAGCAG–3') and D063 (5'–ACTTCTAGATGCAGTAAGCCCTGATCGATAAAATG–3'), and an approximately 900 bp downstream portion was amplified from PAO1 genomic DNA with primers D083 (5'–ACTTCTAGAAACTTCCACCACAAGAACATCCAGA–3') and D066 (5'–ACTAAGCTTCCAAATCCCGGAATGCAGG–3'). The two pieces were cut with *XbaI*, ligated together, reamplified with primers D062 and D066, digested with *BamHI* and *HindIII*, and ligated into *BamHI/HindIII*-cut pEX18Gm. The resulting plasmid pJG055 was introduced into PAO-JG33 via the same mating method as above for pJG038. After selection on VB + gentamicin and counter-selection on LB + sucrose, strain PAO-JG35 was isolated. Sequencing of DNA amplified from its *rhlR* region confirmed the gene deletion. PAO-JG35 cleaved even less elastin congo red

substrate than PAO-JG33 (**Figure 2.6A**). PAO-JG35 (also called "S") did not grow differently from PAO1 or PAO1-Tn7-gfp/Gm^R (or "R") in non-QS-selective media (**Figure 2.6B**), but did grow significantly slower than the R strain in QS-selective media (see **Figures 2.1A** and **2.1B** in main text). For microscopy, pMP7605[64] was conjugated into strain S by mating with *E. coli* S17-1 λpir containing pMP7605, followed by selection on VB + gentamicin.

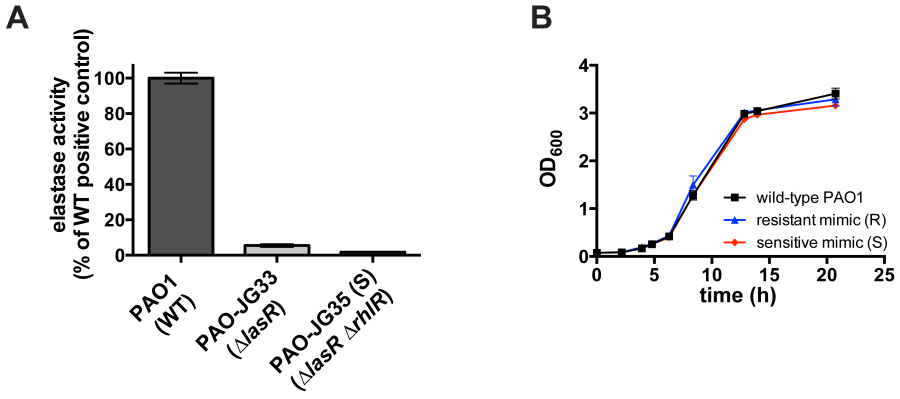


Figure 2.6. Elastase activity and growth curves of wild-type *P. aeruginosa* PAO1 compared to mutants.
(A) Elastase activity quantified by digestion of an elastin congo red substrate. Values were normalized to the activity of wild-type PAO1. (B) Growth curves for R and S strains and wild-type PAO1 in QSM + 1% CAA (non-QS-selective minimal medium). No fitness difference was observed over the 20 h growth period. Error bars in both plots represent s.e.m. (n = 3).

To construct the "signal-independent" constitutive *lasB*-producing QSI-resistant (R-*lasB*) mimic strain, the P$_{tac}$-*lasB* cassette from pML27[65] was first amplified with primers D108 (5'–CAGGCTGAAAATCTTCTCTCATCC–3') and D110 (5'–AAAACTTAAGCCTGAACTTTAGACCGGGTTC–3'). The amplified DNA was cut with *KpnI* and *AflII*. Likewise, pMP7607[64] was cut with *KpnI* and *AflII*, which removed the mCherry gene. The cut P$_{tac}$-*lasB* insert was ligated into the cut pMP7607 to yield pJG068. The mini-Tn7 cassette harboring P$_{tac}$-*lasB* was inserted into the PAO1 genome via the same procedure as for adding the GFP-Gm^R cassette to construct strain R above, except that Pseudomonas isolation agar (PIA, 2%

peptone, 0.69 mM $MgCl_2$, 57 mM K_2SO_4, 1.36% agar, 2% glycerol, 25 mg/mL irgasan) + streptomycin was used for selection instead of VB + gentamicin. The procedure was carried out on both wild-type PAO1 and PAO-JG35 to obtain PAO1-Tn7-*lasB* (termed R-*lasB*) and PAO-JG35-Tn7-*lasB* (termed S-*lasB*). R-*lasB* had greater protease activity than strain R, and S-*lasB* had much greater protease activity than strain S (**Figure 2.9A**), confirming insertion. Insertion was also confirmed by colony PCR with primers Tn7-GlmS (5'–AATCTGGCCAAGTCGGTGAC–3') and Tn7R109 (5'–CAGCATAACTGGACTGATTTCAG–3').[55]

All plasmids were conjugated into *P. aeruginosa* strains by mating with *E. coli* S17-1::λpir. Luria-Bertani (LB) broth (1% peptone, 0.5% yeast extract, 0.5% NaCl, EMD Millipore) was used for all cloning, *E. coli* growth, and *P. aeruginosa* overnight cultures. The QS-selective medium[26, 38] (QSM; 49.3 mM Na_2HPO_4, 50.0 mM KH_2PO_4, 4.8 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, 0.60 mM $CaCl_2$, 25 μM $FeSO_4$, 0.162 μM $(NH_4)_6Mo_7O_{24}$, 38 μM $ZnSO_4$, 14 μM $MnCl_2$, 1.6 μM $CuSO_4$, 0.86 μM $CoCl_2$, 1.9 μM BH_3O_3, 5.5 μM $NiCl_2$, 6.7 μM EDTA, pH 7.0) was prepared and stored as 7 QSM components (QSMCs) that were mixed and sterilized prior to use: 10× QSMC-1 (493 mM Na_2HPO_4, 500 mM KH_2PO_4, autoclaved), 100× QSMC-2 (0.483 M $MgSO_4$, autoclaved), 100× QSMC-3 (0.757 M $(NH_4)_2SO_4$, autoclaved), 500× QSMC-4 (0.299 M $CaCl_2$, autoclaved), 500× QSMC-5 (3.60 mM $FeSO_4$, sterile filtered), 5,000× QSMC-6 (0.809 mM $(NH_4)_6Mo_7O_{24}$, sterile filtered), 1,000× QSMC-7 (38.1 mM $ZnSO_4$, 18.0 mM $FeSO_4$, 14.2 mM $MnCl_2$, 1.56 mM $CuSO_4$, 0.861 mM $CoCl_2$, 1.89 mM BH_3O_3, 5.47 mM $NiCl_2$, 6.72 mM EDTA, pH 2.1, sterile filtered). The components were added to H_2O to dilute each up to 1×, the carbon source(s) were added (1% BSA and 0.1% CAA for group-beneficial or 0.1% adenosine for selfish), and the mixture was sterile filtered. To make solid QSM, the H_2O and an amount of agar that would give a final concentration of 1.5% was autoclaved, and the sterile components were added to the

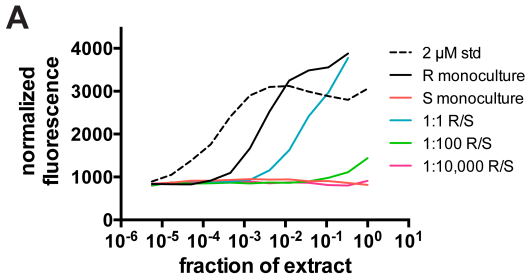
agar/water mixture after cooling to 58 ºC. Aliquots of 1% BSA and 0.03% CAA were added from a 10× sterile-filtered stock immediately before pouring the plates. Bacteria were grown at 37 ºC with 200 rpm shaking unless noted otherwise. When needed, antibiotics were used at the following concentrations: 15 mg/mL gentamicin, 20 mg/mL tetracycline, 500 mg/mL streptomycin, 100 mg/mL ampicillin, and 50 mg/mL kanamycin.

2.4.2 Quantification of OdDHL produced by mixtures of R and S strains

To verify that non-quorate levels of the *P. aeruginosa* OdDHL signal molecule were present with R/S ratios ≤ 1:100, OdDHL was extracted from QSM + 0.1% CAA cultures upon reaching stationary phase and quantified using a bioreporter assay (**Figure 2.7**). The bioreporter assay quantified OdDHL by measuring LasR-activated GFP production by the heterologous reporter *E. coli* JLD21/pJN105L + pPROBE-KL.[40] Inoculation and growth were performed as described in the Methods section of the main text, except 5 μL aliquots of undiluted rinsed cultures were used to inoculate 5 mL portions of media in 14 mL culture tubes. After 10 h of growth, the cultures reached stationary phase, and they were pelleted. An unincubated 2 μM control was also prepared, where 4 mL of medium (without bacteria) was treated with 2 μM OdDHL from a 4 mM DMSO stock.

A 4-mL aliquot of culture supernatant or control was extracted with 3 × 2 mL ethyl acetate, and the ethyl acetate was evaporated. Each isolated residue was redissolved in 300 μL LB + gentamicin + kanamycin and serially diluted by factors of 3 into LB + gentamicin + kanamycin. A 180 μL portion of these dilutions was added to 20 μL portions of the *E. coli* JLD21/pJN105L + pPROBE-KL bioreporter that had been grown overnight in LB + gentamicin + kanamycin and added to 96-well black-walled, clear-bottom microtiter plates. Arabinose was added to the mixtures at a final concentration of 0.4% to induce LasR production. These bioreporter cultures

were incubated at 37 ºC with 200 rpm shaking for 7 h. Fluorescence (λ_{ex}: 500/27 nm, λ_{em}: 540/25 nm) was read using a plate reader and normalized by OD_{600}. Dilution-response curves were obtained (**Figure 2.7A**), and the concentrations of OdDHL present in the QSM + 0.1% CAA cultures were approximated by observing the curve shifts compared to the 2 µM control (**Figure 2.7B**). The 1:100 R/S mixture produced approximately 300 pM OdDHL, which was nearly 1000× less than wild-type PAO1 and well below the quorate concentration (**Figure 2.7B**). These results strongly suggest that rare R (≤ 1%) should not be capable of expressing its QS regulon under the conditions tested in this study.



B

Approximate concentration of OdDHL produced in QSM + 0.1% CAA, 10 h	
R monoculture	200 nM (100%)
S monoculture	<30 pM (<0.02%)
1:1 R/S	25 nM (12.5%)
1:100 R/S	**300 pM (0.15%)**
1:10,000 R/S	**<30 pM (<0.02%)**

Figure 2.7. Quantification of the native *P. aeruginosa* QS signal, OdDHL, in mixed R/S cultures. OdDHL was extracted from QSM + 0.1% CAA cultures grown for 10 h and serially diluted for quantification by using a LasR bioreporter assay. (A) Dilution-response curves for extracts of various bacterial cultures showing fluorescence of the LasR bioreporter strain relative to its OD_{600}. The standard (std) curve was 2 µM OdDHL that was not incubated with bacteria; the other curves were for 2 µM OdDHL incubated with bacterial cultures. (B) The amount of OdDHL produced by each culture (approximated based on the amount of dilution required for the 2 µM standard to give comparable activity to the extracts). Ratios ≤ 1:100 R/S produced << 1% of as much OdDHL as wild-type *P. aeruginosa*, which should not be sufficient for QS-regulated gene expression over the conditions tested in the current study.

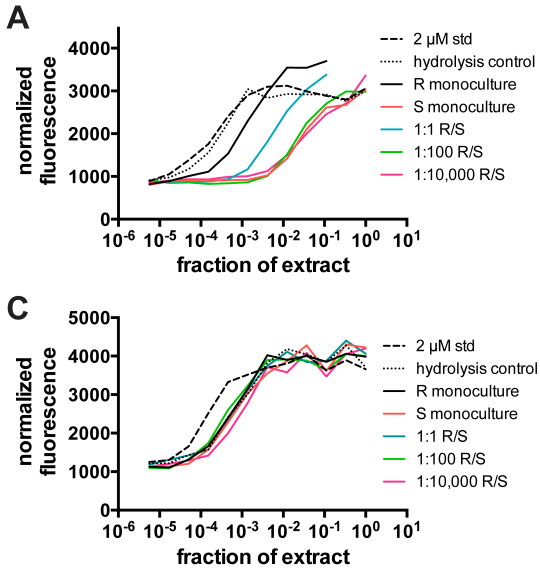
2.4.3 Degradation of OdDHL in P. aeruginosa cultures

To mimic "signal-independent" QSI resistance, we first tried to induce QS in the R strain when it was rare by the exogenous addition of 2 µM OdDHL. We were concerned, however, that OdDHL might degrade in the media during the course of the experiment. Although previous reports indicate that OdDHL is fairly stable to non-enzymatic lactonolysis in buffered media,[69, 70] this signal remains susceptible to enzymatic degradation by the many acylases produced by

P. aeruginosa.[71-73] If these enzymes degrade OdDHL substantially within hours, then the exogenous addition of OdDHL potentially would not be sufficient to activate QS-regulon expression when R is rare. We tested the extent to which exogenously added OdDHL was degraded by the bacteria under our growth conditions (in 0.1% CAA to mimic the initial stages of growth in both 1% BSA + 0.1% CAA, and in 0.1% adenosine). The experimental protocol was the same as that for quantifying production of OdDHL outlined above, except that 2 μM OdDHL was exogenously added to each sample from a 4 mM stock solution (final DMSO concentration 0.1%) prior to incubation. An additional control of OdDHL incubated at 37 ºC in the growth medium without bacteria was included to measure non-enzymatic lactonolysis (i.e., background hydrolysis). As above, the final dilution-response curves were compared to the dilution-response curve of a 2 μM standard (**Figures 2.8A** and **2.8C**), and concentrations of OdDHL remaining after incubation were approximated by the shift observed relative to the 2 μM standard (**Figures 2.8B** and **2.8D**).

After 10 h of incubation in the CAA medium, nearly no background hydrolysis of OdDHL had occurred (by analysis of the "no bacteria" control), but growth with *P. aeruginosa* caused substantial degradation (**Figure 2.8B**). The 1:100 R/S culture had only 1% of the initial OdDHL remaining (20 nM). This level of degradation was not observed in the adenosine medium with *P. aeruginosa*; 30% of the OdDHL remained after an entire day, which was very similar to the level of background hydrolysis after one day (**Figure 2.8D**). At this rate, multiple days would be required for OdDHL to degrade below quorate levels in the adenosine medium. Therefore, these results indicate that exogenous addition of OdDHL is a viable strategy for mimicking "signal-independent" resistance in the selfish adenosine selective medium. However, since OdDHL is 99% degraded within hours in the group-beneficial BSA selective medium, QS cannot be induced reliably via exogenous OdDHL addition in this medium. Instead, the R-*lasB* strain was used,

which constitutively expresses *lasB* under a *tac* promoter without the need of OdDHL (see below).



B

Approximate concentration of OdDHL remaining in QSM + 0.1% CAA, 10 h	
hydrolysis control	2 µM (100%)
R monoculture	650 nM (30%)
1:1 R/S	125 nM (5%)
≤1:100 R/S	**20 nM (1%)**

D

Approximate concentration of OdDHL remaining in QSM + 0.1% adenosine, 26 h	
hydrolysis control	650 nM (30%)
all cultures	650 nM (30%)

Figure 2.8. Degradation of OdDHL by *P. aeruginosa* cultures.
OdDHL was extracted from cultures that initially had 2 µM OdDHL added prior to incubation with bacteria. The OdDHL extracts were diluted and quantified by calculating the degree of dilution required of the 2 µM OdDHL standard to reach the same activity as the extract. (A–B) OdDHL extracted from QSM + 0.1% CAA cultures grown for 10 h. Cultures of 1:100 and 1:10,000 R/S readily degraded OdDHL to 1% of its initial concentration after only 10 h. (C–D) OdDHL extracted from QSM + 0.1% adenosine cultures grown for 26 h. All adenosine cultures lost OdDHL at a much slower rate than the CAA cultures. The OdDHL degradation in adenosine medium was presumed to be mostly due to non-enzymatic hydrolysis because the remaining levels of OdDHL in this medium were indistinguishable from the level of OdDHL in the no bacteria control.

2.4.4 Confirmation of active protease secretion by non-quorate R-lasB

Others have reported that the *P. aeruginosa xcp* protein secretion machinery is required to secrete active LasB, and that *xcp* expression is also regulated by QS.[74, 75] Therefore, we reasoned at the outset that constitutive expression of *lasB* under a *tac* promoter could possibly not translate to secretion of active LasB under non-quorate conditions due to lack of *xcp* expression. However, we found that that active LasB was indeed secreted from P_{tac}-*lasB* cells under non-quorate

conditions by testing supernatant for ability to digest a LasB substrate (azocasein),[76, 77] as described below.

To compare protease production by strains R and S to strains R-*lasB* and S-*lasB*, cultures were grown in QSM + 0.1% CAA in 96-well microtiter plates (200 µL in each well) for 20 h at 37 ºC with shaking at 200 rpm. The cells were pelleted, and 25 µL of the supernatant was added to 75 µL of 1% azocasein (Sigma-Aldrich) in buffer (50 mM Tris, pH 7.8). The digestion was incubated for 5 h at 37 ºC, and remaining azocasein was precipitated with 100 µL of 15% trichloroacetic acid (TCA) in H_2O. After centrifuging at 16,000 × *g* for 10 min, 109 µL of supernatant was added to 91 µL of 1 M NaOH, and absorbance (λ = 440 nm) was read using a plate reader. Absorbance of a negative control (digestion by 1× M9 salts) was subtracted from each sample. The P_{tac}-*lasB* cassette induced production of active LasB even in the QS^- (*ΔlasR*, *ΔrhlR*) S-*lasB* strain for both replicates tested (**Figure 2.9A**), demonstrating that active QS is not needed to secrete active LasB from our "signal-independent" system.

We also tested the ability of rare R-*lasB* to produce near quorate levels of active LasB by repeating the protease assay, but with 1:100 R/S (non-quorate negative control), 1:100 R-*lasB*/S (non-quorate, constitutive *lasB*), and 1:1 R/S (quorate positive control). Since less overall protease should be produced by the 1:100 mixtures (due to only 1% of the cells being capable of producing protease when quorate compared to 50% in the 1:1 mixture), 5 × 200 µL of each 1:100 culture were grown. The supernatants were pooled, and 500 µL was concentrated 20-fold to approximately 25 µL with a 10 kDa MW cut-off spin filter (Millipore Amicon Ultra – 0.5 mL) before addition to azocasein and incubation as described above. CFUs were also determined from the final cultures. The final protease activity was normalized by both the degree of supernatant concentration used (20-fold concentration vs. no concentration) and the ratio of R/S (or R-*lasB*/S) in the culture to obtain the protease activity produced per R (or R-*lasB*) cell. Data are represented

as percentage of digestion by the 1:1 R/S positive control (**Figure 2.9B**). The 1:100 R-*lasB*/S culture produced significantly more protease than the 1:100 R/S culture, and nearly as much protease (per R cell) as the 1:1 R/S positive control. These data confirm that P_{tac}-*lasB* indeed enables the "signal-independent" QSI-resistant mimic to secrete active LasB, even in the absence of quorate signal levels.

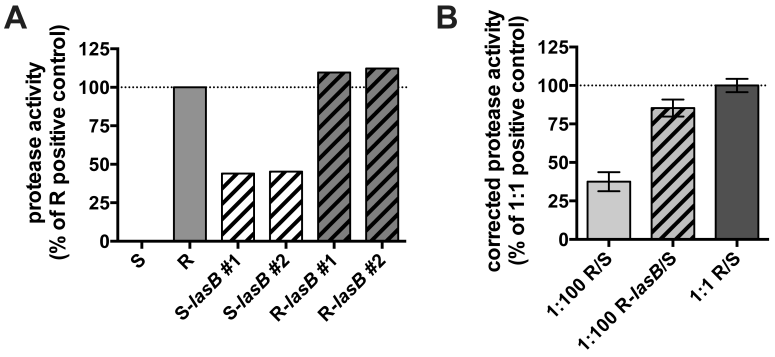


Figure 2.9. Confirmation of LasB production by the "signal-independent" R-*lasB* mutant.
(A) Plot comparing protease activity (azocasein digestion) of supernatants from bacterial monocultures grown in QSM + 0.1% CAA. Each bar is a single trial with one replicate (#1 and #2 represent two trials with the same strain). Protease activities were normalized to the positive control (strain R). S-*lasB* produced nearly half the protease activity as the R positive control in monoculture, indicating that QS is not essential for the secretion of moderate levels of active LasB when it is expressed heterologously under P_{tac}. (B) Plot comparing azocasein digestion of supernatants of mixed bacterial cultures grown in QSM + 0.1% CAA. Protease activities were corrected for the percentage of R (or R-*lasB*) cells present and normalized to the "quorate" positive control (1:1 R/S). Non-quorate levels of R-*lasB* (1:100 R-*lasB*/S) produced nearly as much protease per R cell as the quorate 1:1 R/S positive control, confirming that P_{tac}-*lasB* induces expression of active LasB under non-quorate, rare R conditions. Error bars represent s.e.m. (n = 3).

2.4.5 Competitive growth experiments

Overnight cultures of strains R and S were mixed in different ratios to final volumes of 500 mL. The mixtures were rinsed 2x with M9 salts (47.9 mM Na_2HPO_4, 22.0 mM KH_2PO_4, and 8.56 mM NaCl) to remove products from overnight growth. The mixtures were serially diluted and plated on LB + antibiotic plates (one gentamicin or streptomycin and one tetracycline). R and S colony-forming units (CFUs) were counted on their respective antibiotic plates and used to determine the initial R/S ratios. For liquid competition experiments, 2 mL of a 1:10 dilution of the

rinsed mixtures was inoculated into wells of 96-well microtiter plates containing 198 mL of the QS-selective growth media[38] supplemented with either 1% BSA (Sigma-Aldrich) and 0.1% casamino acids (CAA; Acros) for group-beneficial selection or 0.1% adenosine (Sigma-Aldrich) for selfish selection. To mimic signal-independent resistance in the adenosine conditions, 1 mL of the native QS signal OdDHL (Sigma-Aldrich) was added from a 400 mM DMSO stock solution (final concentration of 2 mM, with 0.5% DMSO). Growth was monitored by OD_{600} measurements using a microplate reader (Synergy™ 2, BioTek® Instruments, Inc., see **Figure 2.10** for representative growth curves). When the cultures reached stationary phase or grew for 150 h (whichever occurred first), they were serially diluted in M9 salts, and plated on LB + antibiotic plates. CFUs were counted for calculation of final R/S ratios. In case biofilms formed during the course of extended growth, cultures were thoroughly resuspended and mixed by pipetting up and down and scraping the sides and bottoms of the wells before serial dilution and CFU determination. To avoid complications due to evaporation, only the inner wells of the 96-well plates were inoculated, and the outer wells were filled with sterile water or media. At the end of growth, the inoculated wells still contained > 170 mL liquid.

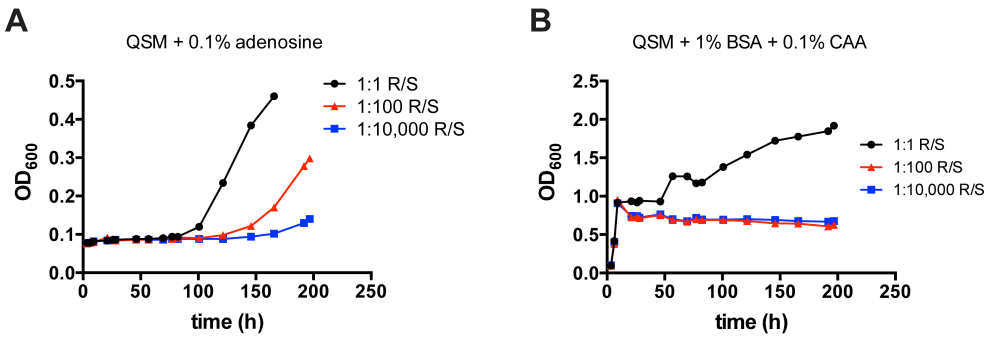


Figure 2.10. Representative bacterial growth curves from competition experiments.
(A) Growth curves of R/S co-cultures in the selfish QSM + 0.1% adenosine QS-selective medium. (B) Growth curves of R/S co-cultures in the group-beneficial QSM + 1% BSA + 0.1% CAA QS-selective medium. As expected, slower growth is observed in both media when R is present at a lower frequency. Theoretically, selective growth could potentially still occur even when no overall growth is apparent, as an increase in the growth of rare R bacteria could be "masked" by a lack of growth by the larger S population.

For solid competition experiments, the same media recipe was used except 1.5% agar was added, and 0.03% CAA was used instead of 0.1%. Plates were inoculated by three different methods to afford different degrees of population structure. To make a finely mixed population, sterile glass beads were used to spread 100 μL of a $10^2\times$ dilution of the rinsed mixed R/S culture. The $10^2\times$ dilution deposited individual bacteria that were approximately 0.1 mm apart from each other, which grew into 0.1 mm diameter microcolonies by metabolizing the 0.03% CAA carbon source. To make an intermediately mixed population, a $10^4\times$ dilution of the rinsed mixed R/S culture was used; this led to more sparsely deposited bacteria that grew to form 1 mm diameter microcolonies after CAA digestion. Finally, to examine the least degree of mixing, 1 μL aliquots of rinsed R and S monocultures were individually spotted approximately 1 cm apart on a grid that filled the 10 cm diameter plate with 100 spots (see **Figures 2.5B**, **2.5C**, and **2.5D** in main text for images of different degrees of population structure). After inoculation, the plates were incubated at 30 ºC. When the plates had thick growth and pigment production or had been grown for 12 days (whichever occurred first), cells were resuspended from the plate using 3 x 2 mL rinses with M9 salts and scraping with a bent glass pipet. The resuspensions were serially diluted and plated on LB + antibiotic plates for CFU counting.

For all competition studies, "relative fitness R/S" (v) was calculated by the method of Ross-Gillespie et al.:[78] $v = (x_1(1-x_0))/(x_0(1-x_1))$, where x_0 and x_1 are the initial and final resistant mimic frequencies, respectively. Values of $v > 1$ indicate that the resistant mimic outcompeted the sensitive mimic (i.e., resistance is spreading). Values of $v \leq 1$ indicate a lack of resistance spread.

2.4.6 Microscopy

Bacterial population structure was imaged on a Zeiss AX10 Imager.M2 epifluorescent microscope with a HXP 120 C Lamp using the 2.5×/0.12 FLUAR objective in conjunction with an AxioCam MR monochrome camera controlled by AxioVision (Rel 4.8.2) software (Carl Zeiss MicroImaging). GFP and mCherry filters were used. Samples were prepared identically to those for solid competition experiments except that the S strain harbored pMP7605.[64] Microscopy was performed directly through the agar plate after incubation at 30 ºC for 6 days. The microscope's field of view was not sufficiently large to display several microcolonies, so nine partially overlapping images were taken with the same exposure settings and overlaid without modification using Adobe® Photoshop. The composite images were cropped to have flush edges and to be of the same size.

2.4.7 Measurement of the diffusion distance of LasB-digested common goods

The LasB substrate (2-aminobenzoylalanyl-glycyl-leucyl-alanyl-4-nitrobenzylamide; Peptides International) was added to the QSM + 1% BSA + 0.03% CAA agar mixture from a DMSO stock. The final concentration was 80 mM substrate with 0.03% DMSO. After incubation with bacteria at 30 ºC for 24 h, cleaved substrate was imaged with a UV transilluminator (312 nm; TFP-M/WL, Vilber Lourmat) in conjunction with the FOTO/Analyst Apprentice system (Fotodyne, Inc.).

2.5 Supplementary Notes, Figures, and Tables

2.5.1 Determination of non-QS-dependent, off-target growth effects of brominated furanone C–30.

We initially considered performing rounds of growth in QS-selective media in the presence of small molecule QSIs to test for the development and spread of QSI-resistant mutants. However, the known QSIs of *Pseudomonas aeruginosa* either appear to have off-target effects or do not fully inhibit QS-regulated phenotypes in wild-type *P. aeruginosa*. We were concerned that one of the best reported QSIs, furanone C-30,[19] had off-target growth-inhibitory effects due to preliminary studies in our laboratory and because a very similar molecule, (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (brominated furanone BF8, **Figure 2.11C**), was shown to have alternative non-QS-related targets in *P. aeruginosa*.[79] To examine this issue, we grew *P. aeruginosa* in our QS-selective and non-QS-selective media in the presence of C-30 to test if there were concentrations of C-30 that slowed growth only under QS-selective pressure. Our experimental protocol is outlined below.

P. aeruginosa strain PA14 overnight culture was rinsed twice with 1× M9 salts by centrifugation and resuspension, and was resuspended in an equal volume of 1× M9 salts. The rinsed culture was diluted 10-fold with 1× M9 salts, and 2 μL of the dilution was added to 198 μL of various media containing furanone C-30 (Sigma-Aldrich) in a 96-well microtiter plate. The media tested were Luria-Bertani (LB, nutrient-rich), QSM + 1% CAA (minimal, non-QS-selective), and QSM + 0.1% adenosine (QS-selective). Three different concentrations of furanone C-30 were tested (15 μM, 50 μM, and 150 μM) in each medium; C-30 was added from 200× DMSO stocks, so that each well contained 0.5% DMSO, including the negative control. Three separate wells of each condition were prepared. The cultures were incubated at 37 ºC with 200

rpm shaking, and OD_{600} was read periodically over 30–120 h, depending on the growth medium (**Figure 2.11A**).

Since the magnitude of shift in the growth curve depends on the general rate of growth in the medium, the faster growing media (LB and QSM + 1% CAA) appeared to have less of a shift than the slow-growing medium (QSM + 0.1% adenosine). To account for this artifact while quantifying the effects of C-30, specific growth rates (μ) were calculated for each condition and normalized to the DMSO control in each medium. To calculate specific growth rate, first the growth time required to reach an OD_{600} of half the maximum OD_{600} ($OD_{600,mid}$; indicated with a line on the plots, **Figure 2.11A**) was calculated from the growth curve. Thereafter, the number of generations required for growth from inoculation to that OD_{600} was approximated by dividing the midpoint OD_{600} by the initial OD_{600}, taking the logarithm of that quotient, and dividing it by log(2). The average generation time was then calculated by dividing the growth time to $OD_{600,mid}$ by the number of generations to reach $OD_{600,mid}$. Finally, specific growth rate (μ, in h^{-1}) was calculated by dividing ln(2) by this average generation time. To convert the specific growth rates to relative growth rates, each value was divided by the mean specific growth rate for the DMSO control in that medium. Each sample was treated independently for the calculations; thus, the final error bars represent a triplicate of individual relative growth rates (**Figure 2.11B**).

Although C-30 only minimally slowed growth of *P. aeruginosa* in LB medium (corroborating a recent report by Wood and co-workers[32]), this compound inhibited the growth rate in the *non-QS-selective* minimal medium (CAA) by approximately 25% at concentrations as low as 15 μM. Further, the degree of growth inhibition in the QS-selective medium (adenosine) was *no greater* than the degree of growth inhibition in the non-QS-selective medium (CAA; **Figure 2.11B**)—indicating that the growth inhibitory effect of C-30 is not primarily due to its QS-inhibitory activity. The QSM + 1% CAA conditions were repeated on a separate day with both

P. aeruginosa strains PA14 and PAO1, and similar growth effects were caused by C-30. Since these off-target growth effects would certainly cause a non-QS-based selective pressure for resistance under our experimental conditions, we chose not to use C-30 as a QSI in the current study. As stated in the main text, other reported QSIs do not inhibit elastase B production substantially. For example, two of the most potent reported *P. aeruginosa* QSIs that act *via* the LasR receptor (V-06-018[18] and *N*-(4-bromophenylacetanoyl)-L-homoserine lactone[17]) cannot inhibit elastase B production in wild-type *P. aeruginosa* PAO1 by greater than 60%, which would provide too weak of a QS-dependent selective pressure to obtain unambiguous results. We therefore developed a model system using mutant *P. aeruginosa* strains that mimics resistance development to an ideal QSI that inhibits QS at nearly 100% with no off-target effects (see main text and **Figure 2.3** for a detailed schematic of our model).

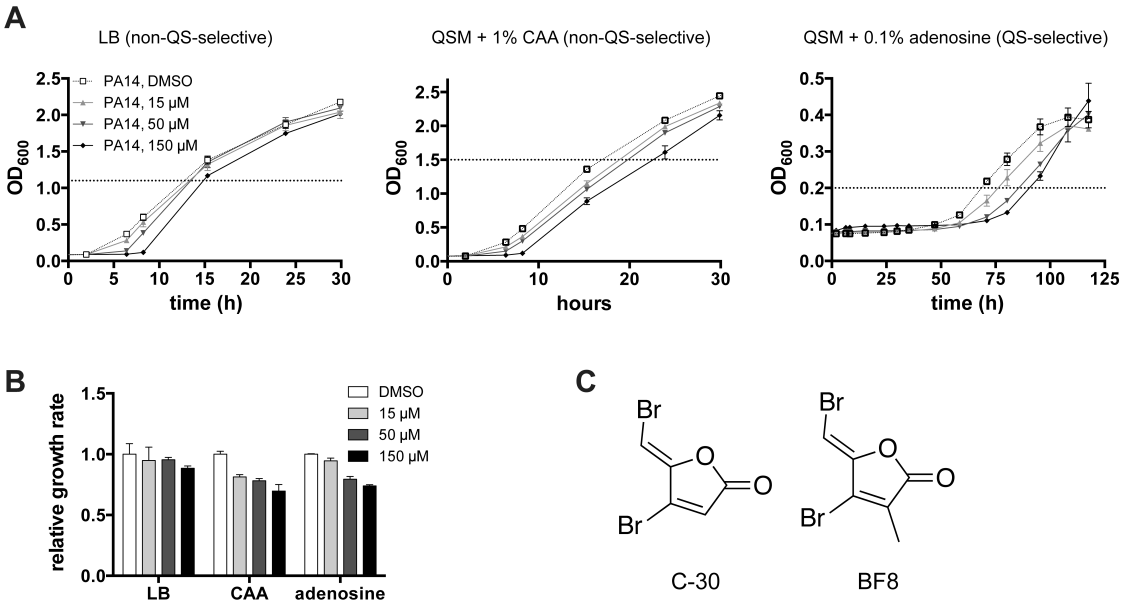


Figure 2.11. Growth inhibitory effects of brominated furanone C-30 on *P. aeruginosa* PA14.
(A) Growth curves of PA14 in LB, QSM + 1% CAA (non-QS-selective minimal medium), and QSM + 0.1% adenosine (QS-selective minimal medium) with increasing concentrations of C-30. Error bars represent s.e.m. (n = 3). Dashed lines indicate the half-maximal OD_{600} ($OD_{600,\ mid}$) used for calculating average growth rates. (B) Average growth rates of PA14 with increasing concentrations of C-30. Values are normalized to the average PA14 growth rate in that medium with no C-30 added. Error bars represent s.e.m. (n = 3). C-30 clearly slowed the growth rate of *P. aeruginosa* by ~25% not only in the QS-selective medium (adenosine), but also in a comparable *non-QS-selective* medium (CAA)—indicating that the growth inhibitory effect of C-30 is not primarily due to its QS-inhibitory activity. (**C**) The chemical structures of brominated furanones C-30 and BF8 (another known QS modulator in *P. aeruginosa*) are shown for comparison. BF8 is known to have non-QS targets in *P. aeruginosa*.[79]

2.5.2 Basis for adenosine-based selfish QS-dependent selective pressure.

To complement the group-beneficial QS-selective pressure, we also employed a selfish QS-selective pressure. Heurlier et al. discovered that *lasR* mutants of *P. aeruginosa* do not grow with adenosine as the sole source of carbon.[80] Adenosine metabolism is dependent on QS because nucleoside hydrolase (Nuh)—the enzyme responsible for cleavage of the *N*-glycosidic bond of inosine in the degradation of adenosine (see **Figure 2.12**)—is produced under control of the Las QS system.[23, 80]

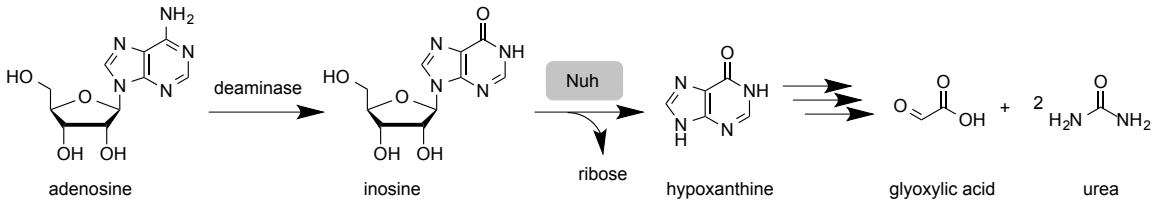


Figure 2.12. Degradation of adenosine by *P. aeruginosa*.
After deamination of adenosine, nucleoside hydrolase (Nuh, shaded grey) cleaves the resulting inosine into ribose and hypoxanthine, which are further degraded into simpler molecules.[80, 81] Nuh production is regulated by QS.[23]

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2.7 Appendix: Impact of Population Expansion on the Spread of QSI Resistance (Future Direction)

2.7.1 Population expansion can promote the fitness benefit of group-beneficial traits

In the studies reported in this Chapter, the QSI-resistant mimics only had a fitness advantage when given a structured surface with relatively large monoclonal patches (approximately 1 cm), which provided for a higher concentration of the common goods around the resistant bacteria (see **Figure 2.5**). We initially reasoned that such a large monoclonal patch of resistant bacteria spontaneously arising would be a relatively low probability event: for example, several neighboring bacteria would need to all spontaneously develop resistance-conferring mutations, or a single resistant cell would need to leave the colony, evade the immune system, find a good niche, and reproduce to form a quorate monoclonal colony. However, we discovered that another mechanism to develop large monoclonal patches is more likely: the stochastic coarsening of an expanding mixed population (see **Figure 2.12**). As illustrated well by a diagram from Hallatschek et al.[1] (shown in **Figure 2.12A**), when a finely mixed population of unique organisms expands outward, random fluctuations in growth rates can cause several protruding rays of bacteria to be cut off by encroaching neighboring rays (e.g., as the red patch is cut off by the surrounding green ones). Hallatschek et al. showed that this process can rapidly form a few large monoclonal patches from a finely mixed population of *E. coli* cells that differ only by the fluorescent protein that they express (**Figure 2.12B**). These monoclonal patches (that readily arise from stochastic processes in the absence of a selective pressure) are on the size scale necessary for asymmetric sharing of common goods that could cause cooperative common goods producers (like QSI-resistant cells) to be more fit than cheating non-producers (like QSI-sensitive cells). Indeed, van Dyken et al. showed that solid structure with expansion enabled a cooperative *Saccharomyces cerevisiae* strain to outcompete a cheating *S. cerevisiae* strain (**Figure 2.12C**), whereas when the

mixed population was not allowed to expand, the cooperative strain was less fit. Another study has recently drawn similar conclusions using the same species of yeast.[2] We therefore sought to test if population expansion would enable larger monoclonal patches of QSI-resistant *P. aeruginosa* mimics to form, have a fitness advantage, and spread.

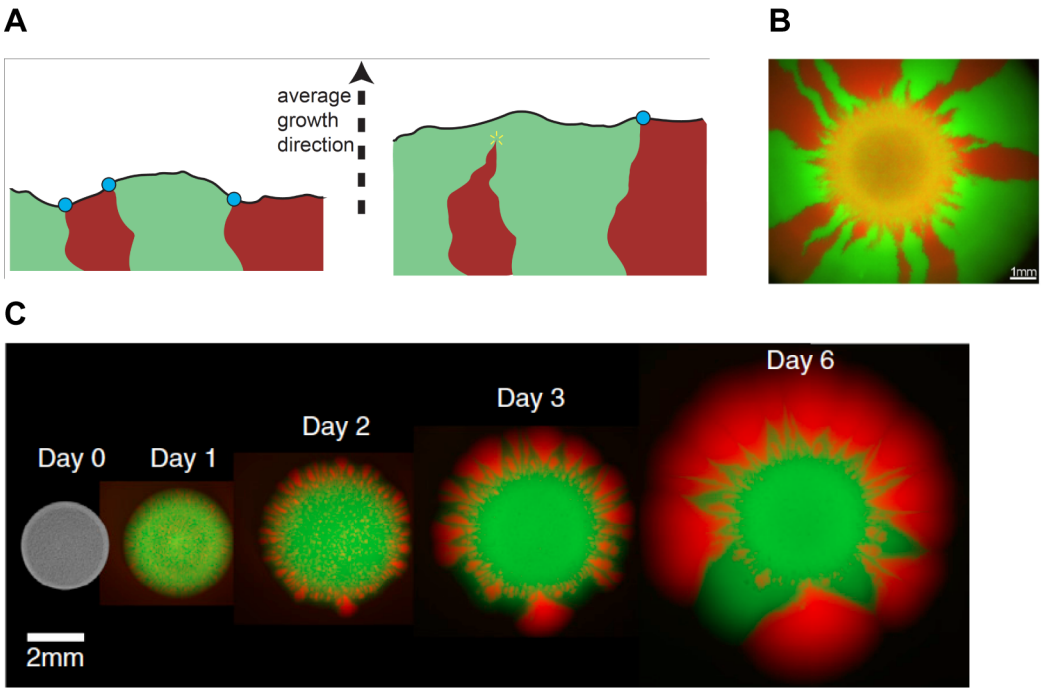

Figure 2.13. Population expansion promotes the fitness benefit of group-beneficial traits.
(A,B) Images from Hallatschek et al.[1] demonstrating a finely mixed colony experiencing coarsening as it grows outward, both as a cartoon and as a photograph with fluorescently labeled *E. coli* cells. (C) Image from van Dyken et al.[3] demonstrating that expansion allows cooperative *S. cerevisiae* (red) to outcompete "cheating" *S. cerevisiae* mutants (green).

2.7.2 QSI resistance spreads under expansion conditions

To test the hypothesis that population expansion on a solid surface would enable the spread of QSI resistance, we repeated the same competition experiments from **Figure 2.5**, but instead of spreading the bacteria across the entire surface of the plate, a 1 µL spot of the 1:10:000 R/S bacterial mixture was placed on the center of the plate at a dilution that should contain

approximately 30 resistant cells and 300,000 sensitive cells. As the left image of **Figure 2.13A** illustrates, the GFP-labeled QSI-resistant mimics (green) spread substantially and nearly overtook the population, corresponding to a fitness advantage of greater than 10,000:1 for the QSI-resistant mimics relative to the mCherry-labeled QSI-sensitive mimics. These results indicate that population expansion certainly does enable the spread of QSI resistance for *P. aeruginosa* in our model system.

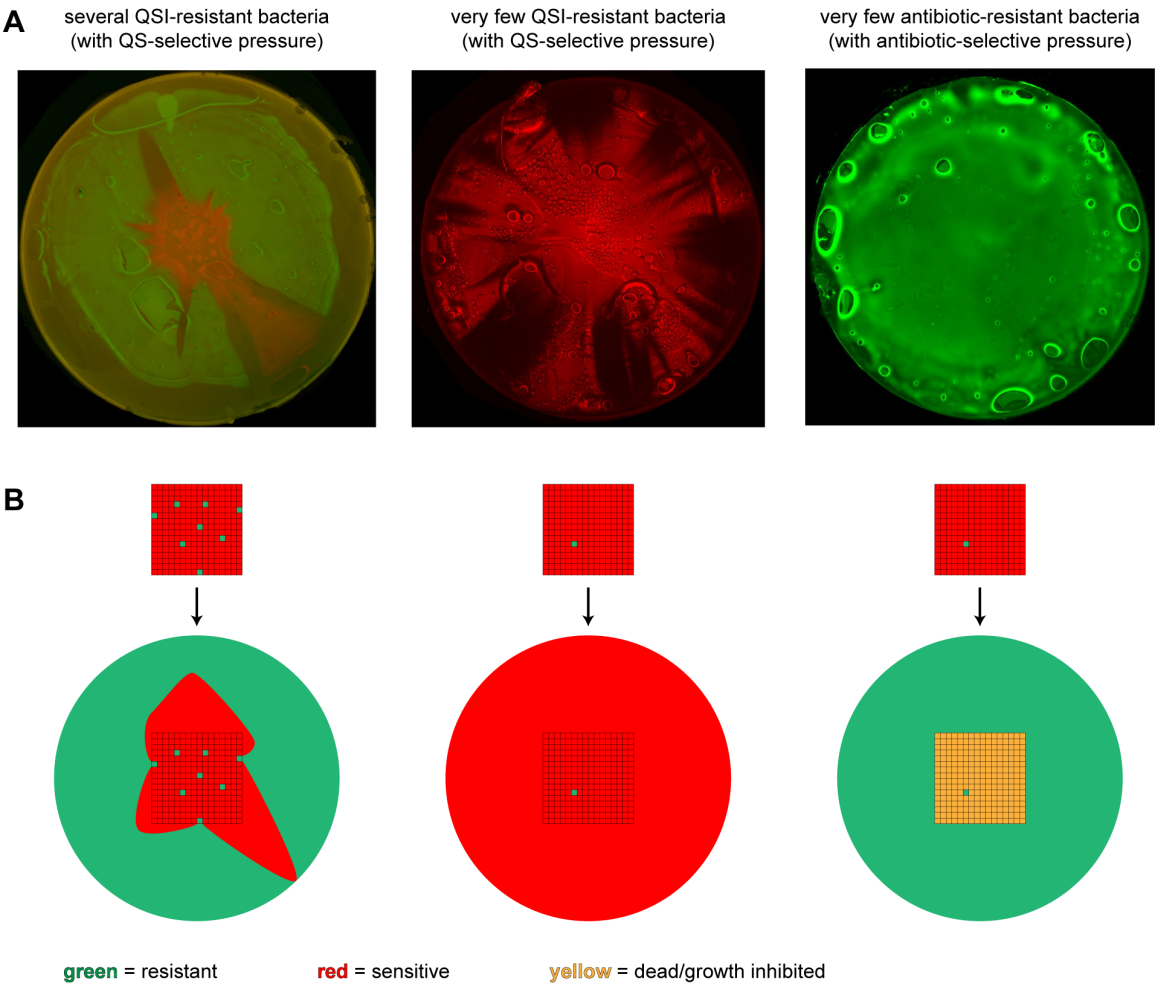

Figure 2.14. ***P. aeruginosa*** **QSI-resistant mimics spread on solid surface with population expansion.** (A) Competitive growth on QS-selective media with expansion allowed. Images were obtained by scanning on a Typhoon FLA 9500 scanner (GE Healthcare) excited at 473 nm and 532 nm wavelengths. Plates were prepared as described in Chapter 2, except 30 µg/ml gentamicin was added to the antibiotic-selective plate (right). Cells were inoculated onto the center of 10 cm-diameter plates from diluted mixtures of rinsed overnight cultures in 1 µL aliquots and grown at 30 ºC for 12 days. The left plate was inoculated with approximately 30,000 total cells of a 1:10,000 ratio R/S (containing approximately 30 resistant cells), and the right two plates were inoculated with approximately 3 million total cells of a $1{:}10^6$ ratio R/S (containing approximately 3 total resistant cells). For the QSI resistance plates, the same strains were used as in **Figure 2.5**. For the antibiotic resistance plate, the same R strain was used, but the S strain was wild-type PAO1 with an mCherry- and streptomycin-conferring miniTn7 transposon genomically incorporated.[4] The middle image shows patches lacking fluorescence. These patches are likely due to poor growth of the QSI-sensitive mimics. (B) Cartoon illustrating competitive growth of the expanding populations. Cartoons match the condition of the photograph directly above them.

2.7.3 An obstacle to the spread of QSI resistance with expansion: resistance cells must be on the edge of the colony

Although we demonstrated that population expansion allows for the spread of QSI-resistance, we reasoned that for the resistant bacteria to form a monoclonal patch via population coarsening, they would need to be on the edge of the initial finely mixed population. As the left cartoon in **Figure 2.13B** illustrates, resistant (green) cells on the edge can expand to form monoclonal patches; however, if the only resistant cell(s) are trapped in the middle of the mixed population, they would presumably not benefit from the expanding population (middle cartoon, **Figure 2.13B**). We tested this possibility by making the QSI-resistant mimics more rare in the population (1:1,000,000 R/S ratio with greater than 3 million cells spotted), and indeed, under these conditions no resistant bacteria (green) were observed (**Figure 2.13A**, middle).

We were concerned that since, on average, approximately three viable cells on the plate were resistant, then perhaps this one plate lacked any viable QSI-resistant mimics. We therefore tested 25 other plates and did not observe resistance spreading on any of them. Furthermore, we directly compared this condition to the spread of resistance to a conventional antibiotic (gentamicin). We mixed a GFP-labeled gentamicin-resistant wild-type strain of *P. aeruginosa* (Gent-R) with an mCherry-labeled gentamicin-sensitive wild-type strain of *P. aeruginosa* (Gent-S) at the same 1:1,000,000 R/S ratio and spotted the same number of cells onto the same media but now containing 30 μg/mL gentamicin. In this experiment, the only fitness difference between the two strains was their resistance to the gentamicin that is present. Under these conditions, all 10 plates tested showed only resistant cells (green) after growth—indicating a complete spread of antibiotic resistance (**Figure 2.13A**, right). Therefore, we believe that the lack of resistance spread under the QSI-resistant case is not due to a complete absence of resistant cells, but rather, when QSI-resistant cells are rare, they have a low likelihood of residing on the edge of the population and

therefore will not spread. Furthermore, these data present a stark contrast with antibiotic selective pressure that does not appear to require resistant bacteria to reside on the edge. This outcome is likely because the gentamicin-sensitive cells in the mixed population either die or cannot divide at all, and thus are incapable of sealing off the edges and preventing the escape of internal resistant cells (**Figure 2.13B**, right). In essence, under an antibiotic selective pressure, every resistant cell in a population is "on the edge" and capable of escaping and forming high-fitness cooperative monoclonal patches. Accordingly, the spread of QSI resistance has an additional obstacle compared to resistance to traditional antibiotics—the resistant bacteria must be on the edge to spread.

In view of these results, we developed a simple mathematical model to describe the likelihood of resistant bacteria residing on the edge and spreading, and we have initiated studies to empirically test and refine this model. The model (presented in **Figure 2.14**) is based on a binomial distribution that describes the probability of a certain number of resistant bacteria to randomly reside on the edge of a square population, given a certain number of total bacteria and a certain R/S ratio. We note that this preliminary model contains several oversimplifications: (i) it assumes two-dimensional growth and no thickness to the colony; (ii) it assumes the "edge" is only the width of one cell; and (iii) it assumes that resistance has an equal likelihood of arising at every position within the colony. Nonetheless, we believe this model is a good starting point, and it can be adjusted as necessary if it does not match empirical data.

Binomial expression f(x) describes the probability of **x** resistant cells (R) being on the edge:

(i.e., probability that given **n** opportunities, the event will happen ("success") **x** times, given that the probability of the event happening each opportunity is **p**)

$$f(x) = \binom{n}{x} p^x (1-p)^{n-x} \qquad \binom{n}{x} = \frac{n!}{x!\,(n-x)!}$$

n = number of R cells present in the population (i.e., number of opportunities for an R cell to be on the edge. population size * fraction R in population)
x = number of R cells on an edge (variable of this probability mass function)
p = probability of an R cell being on an edge, given one trial (see below)

"**n** choose **x**" is the number of permutations for **x** "successes" from a set of **n** trials

$$p = \frac{\text{edge cells}}{\text{total cells}} = \frac{\text{tot cells} - \text{inner cells}}{\text{tot cells}} = \frac{w^2 - (w-2)^2}{w^2}$$

w = width of population in # of monoclonal patches

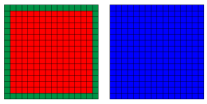

Figure 2.15. Binomial expression to describe the likelihood of resistant bacteria randomly being on the edge of a two-dimensional colony.

To test our model, we have started to repeat the assays displayed in **Figure 2.13** with different R/S ratios and numbers of cells. We are now comparing the number of resistant bacteria that actually escape from the finely mixed population (i.e., the number of green streaks projecting from the center) to the number of resistant bacteria that the model predicts to reside on the edge of the mixed population. If these two values do not match, we will refine the model. Obvious initial modifications would be to increase the size of the edge to include more than a single cell width or to add a third dimension of thickness to the colony, but certainly other permutations to the model are possible and can be readily explored.

In conclusion, the results described in this Appendix indicate that expansion allows for the spread of QSI resistance on solid surfaces from well mixed bacterial populations. However, the resistant bacteria need to be on the edge of the population—a requirement that we observed not to be present for the spread of antibiotic resistance, and therefore should make the spread of QSI resistance less probable than the spread of antibiotic resistance. We are currently testing and

refining a model to quantify the reduced likelihood of the spread of resistance to QSIs compared to conventional antibiotics.

2.8 References for Appendix

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CHAPTER 3:

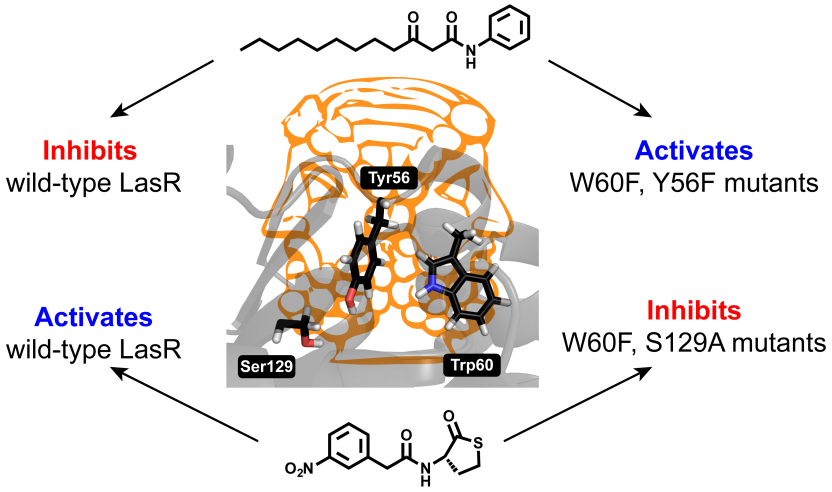
MUTATIONAL ANALYSIS OF THE QUORUM-SENSING RECEPTOR LASR REVEALS INTERACTIONS THAT GOVERN ACTIVATION AND INHIBITION BY NON-LACTONE LIGANDS

Contribution: J. P. G., C. E. M., and H. E. B. designed the research and wrote the paper; J. P. G. and C. E. M. analyzed data; J. P. G. prepared mutant LasR strains, performed fluorescence assays, and docking and NBO analysis; C. E. M. synthesized ligands OdDHL, **1**, **2**, and **3** and performed β-galactosidase assays; T. L. S. performed β-galactosidase assays; F. M. R. synthesized and characterized ligand TP-5.

This chapter has been submitted for publication, in part, under the same title – Reference: Gerdt, J. P., McInnis, C. E.,* Schell, T. L., Rossi, F. M., and Blackwell, H. E. (2014) Mutational analysis of the quorum-sensing receptor LasR reveals interactions that govern activation and inhibition by non-lactone ligands, *Submitted*.

*these authors contributed equally

Abstract.



Gram-negative bacteria use *N*-acyl L-homoserine lactone (AHL) quorum sensing (QS) signals to regulate the expression of myriad pathogenic and mutualistic phenotypes. Non-native AHL analogs can strongly attenuate QS receptor activity and thereby QS signaling; however, we currently lack a molecular understanding of the mechanisms by which most of these chemical probes elicit their agonistic or antagonistic profiles. In the current study, we investigated the origins of striking activity profile switches (i.e., receptor activator to inhibitor, and vice versa) observed upon alteration of the lactone head group in certain AHL analogs. Reporter gene assays of mutant versions of the *Pseudomonas aeruginosa* QS receptor LasR revealed that interactions between the ligands and Trp60, Tyr56, and Ser129 govern whether these ligands behave as LasR activators or inhibitors. Using this knowledge, we propose a model for the activation and inhibition of LasR by non-native AHL analogs—encompassing a subtly different interaction with the binding pocket to a global change in LasR conformation.

3.1 Introduction

While bacteria were once considered simple organisms that functioned as single cells, we now know that they often live as multicellular societies cooperating and competing with each other to exploit the resources in their surroundings.[1] Any society needs communication between its members, and bacteria use quorum sensing (QS) as a mechanism to sense their local population densities. When a "quorum" of bacteria is reached, the microbes alter their behavior to a phenotype that is more appropriate for a dense, cooperative environment. Such phenotypic changes often involve secretion of substances that can aid siblings and harm competitors (e.g., digestive enzymes, siderophores, and toxins),[1, 2] Notably, many bacterial pathogens use QS to initiate attack on a host only when they have amassed in a sufficient population number to overwhelm the host response. The link between pathogenesis and QS has attracted considerable recent interest to this communication network as a potential anti-infective target.[3-5] In turn, many symbionts use QS to initiate mutually beneficial relationships with their hosts, perhaps most conspicuously that between legumes and nitrogen-fixing rhizobia.[6]

Among the proteobacteria, QS is mostly achieved through the biosynthesis and subsequent concentration-sensing of *N*-acyl L-homoserine lactone (AHL) signals. Within a given species, each bacterium synthesizes the same AHL constitutively at a low level (via LuxI-type synthases). Most AHLs can freely diffuse into and out of the cell. If the bacteria accumulate in an enclosed environment, the AHL concentration increases until it reaches a threshold intracellular level sufficient for productive binding and activation of its target receptor protein (termed a LuxR-type protein), which then alters the transcription of QS-regulated genes.[2, 7] A typical LuxR-type protein is LasR from the opportunistic pathogen *Pseudomonas aeruginosa*. When its cognate signal AHL (*N*-(3-oxo)-dodecanoyl L-homoserine lactone, OdDHL, **Figure 3.1**) is present at a quorate concentration, LasR binds this signal and is stabilized by it in an active, dimerized form

that recognizes certain promoters and recruits transcriptional machinery to induce QS gene expression.[8]

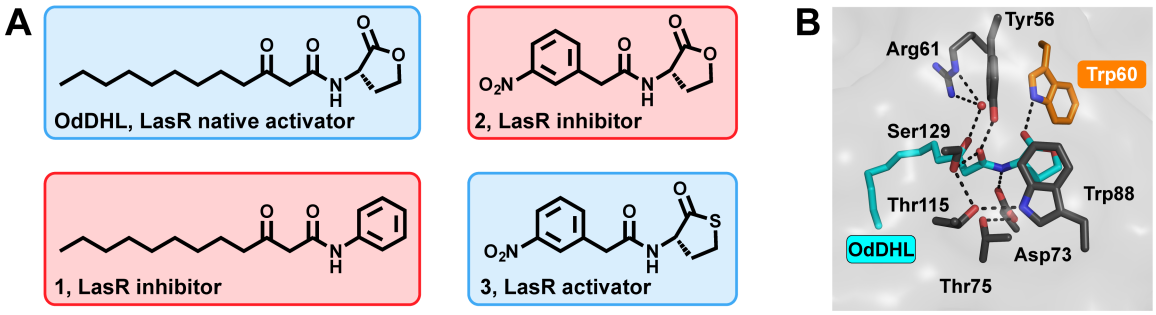


Figure 3.1. Impact of AHL head group on ligand activity in LasR.
(A) Two pairs of ligands that share common acyl tails but have differing heads groups that govern LasR activation or inhibition. (B) View of the OdDHL binding site in the $[LasR:OdDHL]_2$ X-ray crystal structure.[9] Trp60 (highlighted in orange) hydrogen bonds to the lactone head group of OdDHL (cyan). Other residues that hydrogen bond with OdDHL or are part of a hydrogen-bonding network to OdDHL are displayed in grey. Hydrogen bonds are displayed as black dashed lines.

As QS is dependent on the exchange of chemical signals, there is significant interest in the development of chemical probes that can prevent QS signal-receptor binding and alter QS outcomes. Indeed, the ability to modulate QS with non-native molecules has tremendous implications for artificially disrupting or promoting both pathogenic and mutualistic behavior.[10-12] The spatial and temporal control afforded by chemical probes can enable a deeper understanding of important microbial phenotypes and possibly have direct therapeutic potential.[3, 4, 13] As therapeutics, QS inhibitors have a prospective advantage over traditional antibiotic therapies, since recent sociomicrobiology studies suggest that resistance is likely to spread more slowly to QS inhibitors (that target virulence phenotypes) than to traditional antibiotics (that target growth).[14, 15] As such, QS inhibition is emerging as an important "anti-virulence" approach.[4, 5]

Some of the most well studied chemical modulators of LasR are AHL analogs that have altered acyl tails, altered lactone heads, or both.[10, 16, 17] Generally, more attention has been given

to variation in the acyl tail, but recently we and others have synthesized non-lactone versions of AHLs with the objective of both obtaining LasR ligands with enhanced hydrolytic stability and expanding our understanding of the structural features of the lactone head group that control ligand activity.[18-24] These ligands certainly exhibit enhanced stabilities relative to lactone analogs—but more interestingly, some of our non-lactone ligands also have the *opposite activity* on LasR compared to their lactone analog. For example, aniline ligand **1** (**Figure 3.1A**) is an analog of the native activating ligand OdDHL, but it is a good *inhibitor* of LasR in reporter assays and in QS phenotypic assays.[19, 20] Other analogs of OdDHL with aniline head groups have also shown LasR inhibitory activity.[17, 19, 22] In addition, we observed that thiolactone **3** (**Figure 3.1A**) is a moderate *activator* of LasR, in contrast to its direct lactone analog **2**, which is instead a good inhibitor of LasR.[18, 25] These dramatic activity switches are seemingly caused by relatively subtle changes in ligand structure; however, the molecular bases for these flips in activity are unclear. In fact, there is virtually no information about the molecular mechanisms by which *any non-native AHL analog* modulates LuxR-type receptors.[26, 27] Elucidating the causes of receptor activation versus inhibition by AHL analogs would not only improve our understanding of the molecular foundations of AHL-based QS, but would also augment our ability to design more potent molecular probes to modulate this signaling pathway. Toward this broad goal, we examined the origins of the activity flipping observed for the non-lactone LasR modulators **1** and **3** in the current study.

Herein, we report our investigations of interactions of LasR with non-lactone AHL analogs through the systematic mutagenesis of specific residues in the LasR native ligand binding site. We selected the residues for modification through study of the reported X-ray crystal structures of the LasR *N*-terminal ligand binding domain (residues 1–173, out of 239) bound to OdDHL,[9, 28] which permitted us to hypothesize determinants for LasR activation or inhibition by non-lactone

AHLs. We subsequently tested these hypotheses via reporter gene assays using mutant LasRs. A similar mutagenesis approach was recently shown to be successful for studying the interactions of AHLs bearing non-native acyl groups with CviR, a LuxR-type receptor from *Chromobacterium violaceum*.[26] As the structures of analogs **1**–**3** closely approximate native AHLs, and analogs **1** and **2** act via competitive inhibition, we reasoned that they would also target the LasR ligand binding site; we therefore mutated the residues therein (**Figure 3.1B**). We found that mutation of Trp60, Tyr56, and Ser129 in LasR (**Figure 3.1B**) drastically flipped the activity of alternate head group ligands **1** and **3**. These observations led to the development of a new model by which AHL analogs with different head groups exert opposite effects on LasR activity. As this model is further refined, we believe it will inform the design of next-generation QS modulators with heightened activities. The flipped-activity mutations identified in this work also have further implications—for the development of resistance to QS inhibitors and for use in synthetic biology. We end with a discussion of these two prospects.

3.2 Results and Discussion

3.2.1 Importance of Trp60 in governing LasR activation and inhibition by non-lactone ligands

Structural data for LuxR-type proteins bound to AHL ligands remains very limited.[29] However, each X-ray crystal structure of LasR and its homologs bound to an AHL reveals a hydrogen bond between the Trp60 (or homologous) side chain NH and the AHL lactone carbonyl,[9, 26, 28, 30-32] and the Trp60 residue is highly conserved in LuxR-type proteins.[29] We hypothesized that the differential activity of alternative head-group ligands **1** and **3** toward LasR could be derived from different interactions of their non-lactone head groups with Trp60. To test this hypothesis, we mutated Trp60 in LasR to a phenylalanine residue, which has a smaller side chain that lacks a hydrogen-bond donor, but retains significant p character. We tested the activity

of the mutant LasR using a β-galactosidase reporter in an *Escherichia coli* background (see *Section 3.4.4*). This W60F mutant was only moderately impaired at responding to OdDHL (**Tables 3.1** and **3.2**), but the activities of ligands **1** and **3** in the W60F mutant almost completely reverted back to the activities exhibited by their lactone counterparts (OdDHL and **2**, respectively) in both wild-type and W60F LasR (**Table 3.2**). Ligand **1**, which is an inhibitor of wild-type LasR, reverts to being an *activator* in the W60F mutant like OdDHL. Conversely ligand **3**, which activates wild-type LasR, reverts to being an *inhibitor* in W60F, analogous to ligand **2**. We termed this interesting observation to be "Janus" behavior (after the two-faced Roman god of transitions), because the ligands transition between two vastly different activities depending on the identity of residue 60 in LasR. Although prior mutations to LasR homologs have demonstrated altered responses to AHLs,[26, 33] this observation is unique in that it involves only a single residue replacement, and this replacement flips both an inhibitor into an activator *and* an activator into an inhibitor. To verify that this Janus activity was not an artifact of the β-galactosidase reporter, we also tested ligands **1** and **3** in the W60F LasR mutant using a fluorescence reporter,[34] and observed the same Janus profile. The striking reciprocal activities of these two ligands in wild-type LasR versus mutant LasR are apparent in **Figure 3.2A,B**.

Table 3.1. EC_{50} values for OdDHL in LasR mutants.

	WT	**Y56F**	**W60F**	**S129A**	**R61M**	**T75V**	**T115V**	**D73L**	**W88F**
EC_{50}[a]	10 nM	10 nM	75 nM	50 nM	600 nM	0.5 nM	3500 nM	inactive	inactive

[a]Data are geometric means of biological triplicates. Error did not exceed ×/÷ 1.4 nM.

Table 3.2. Activity of OdDHL and ligands 1–3 in wild-type and W60F LasR.
Shading highlights a lack of significant activity.

	wild-type				W60F			
ligands	**activation**[a]	**EC_{50}**[b]	**Inhibition**[a,c]	**IC_{50}**[bc]	**Activation**[a]	**EC_{50}**[b]	**Inhibition**[a,c]	**IC_{50}**[b,c]
OdDHL	100%	10 nM	–	–	73%	75 nM	–	–
1	3%	–	54%[d]	4800 nM	58%	1100 nM	-17%	–
2	0%	–	80%	510 nM	1%	–	81%	3700 nM
3	82%[d]	5100 nM	-13%[c]	–	-13%	–	98%	550 nM

[a] ligands screened at 10 μM, arithmetic mean of biological triplicate is shown. No s.e.m. exceeded 20%. Negative values for activation or inhibition mean the ligand is an inhibitor or activator, respectively, instead.
[b] geometric mean of biological triplicate is shown. No arithmetic s.e.m. of log-transformed data exceeded 0.14 (corresponding to an EC_{50} geometric standard error of ×/÷ 1.4[35]).
[c] for inhibition, ligands were tested against EC_{50} of OdDHL (see **Table 3.1**).
[d] data previously reported.[18, 19]

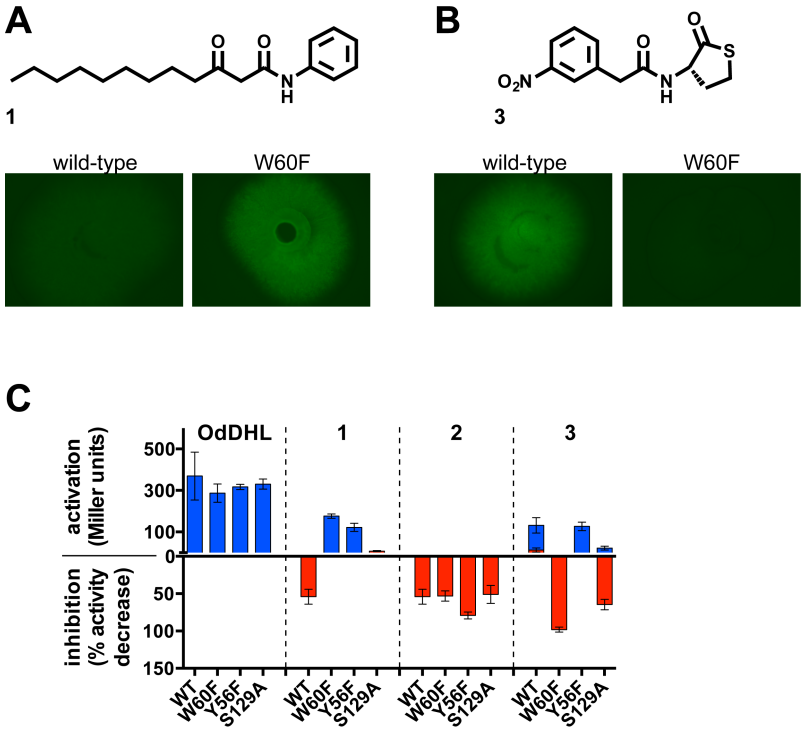


Figure 3.2. "Janus" ligand-protein behavior.
(A,B) Differential activation of green-fluorescent protein (GFP) expression by wild-type and W60F LasR in the presence of 10 μM ligands 1 and 3. (C) β-galactosidase reporter assays of wild-type LasR and three mutants with "Janus" behavior. Activation assays were performed by adding ligand at 10 mM, and activity is reporter as Miller units on the positive y-axis. Inhibition assays were performed by adding ligand at 10 mM and OdDHL at its EC_{50} value for that mutant (see **Table 3.1**), and activity is reported on the negative y-axis as the % decrease in activity relative to only OdDHL being present. Error bars represent s.e.m. of a biological triplicate.Impact of AHL head group on ligand activity in LasR.

3.2.2 Importance of other LasR residues in governing activation or inhibition by non-lactone ligands

We were interested in ascertaining whether this Janus activity was unique to the Trp60 residue or if other hydrogen-bonding residues were similarly important at governing non-lactone ligand activity in LasR. We therefore mutated every residue in the LasR ligand-binding pocket that hydrogen bonds to OdDHL or is part of a hydrogen-bonding network with other residues that hydrogen bond with OdDHL (as shown in the $[LasR:OdDHL]_2$ structure, **Figure 3.1B**). The residues were mutated to amino acids that were of comparable size but lacked hydrogen-bonding ability (Y56F, R61M, D73L, T75V, W88F, T115V, and S129A). We constructed β-galactosidase reporters for each LasR mutant in *E. coli*, analogous to the W60F LasR mutant reporter above, and tested OdDHL and ligands **1**–**3** for mutant LasR activation and inhibition in each reporter. The Y56F and S129A mutants also showed Janus behavior (**Figure 3.2C**), but none of the other LasR mutants displayed such flipped activity relative to wild-type (**Figure 3.3A**). These non-Janus mutants were generally less strongly modulated by the ligands, presumably due to a missing polar interaction that leads to weaker ligand binding. Interestingly, unlike the W60F mutation that caused both ligand **1** and ligand **3** to flip activity from inhibitor to activator (and vice versa), the Y56F mutation only flipped the activity of ligand **1** from an inhibitor to an activator, and the S129A mutation only flipped the activity of ligand **3** from an activator to an inhibitor (**Figure 3.2C**). Ligand **3** remained an activator in the Y56F mutant, while ligand **1** displayed minimal activity in the S129A mutant. Both of the Tyr56 and Ser129 side chains are shown to engage in hydrogen bonds with the amide carbonyl of OdDHL in the $[LasR:OdDHL]_2$ structure (**Figure 3.1B**). Taken together, these LasR mutant data suggest that interactions of QS modulators **1**–**3** with these two amide-binding residues (Tyr56 and Ser129), along with Trp60, are important determinants for LasR activation and inhibition.

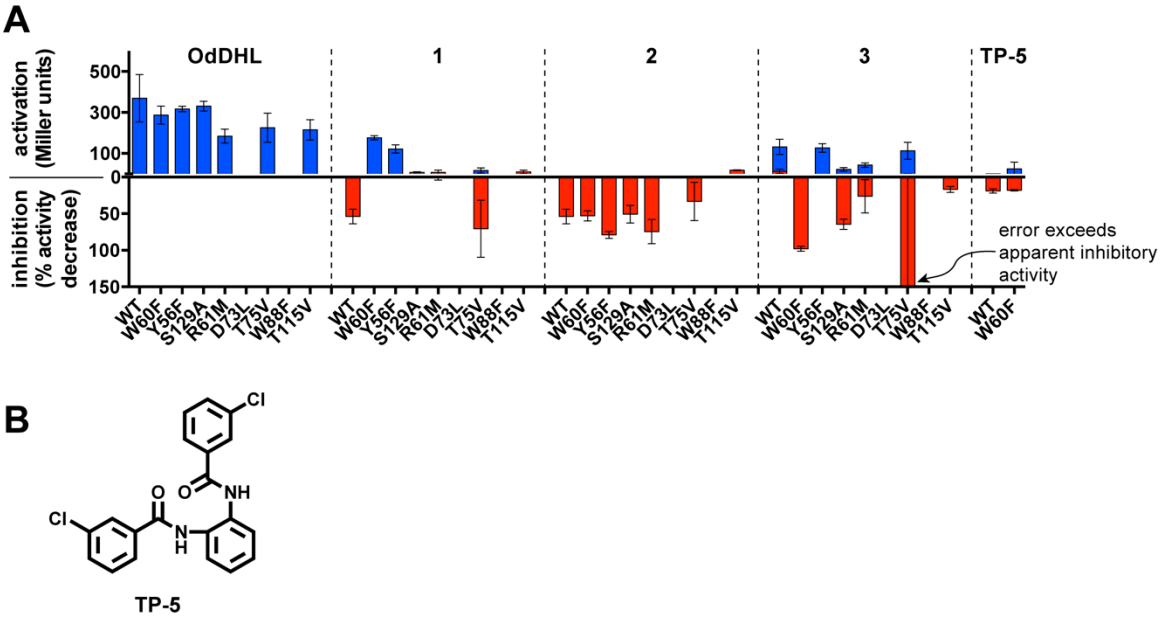


Figure 3.3. Activity profiles of ligands in LasR mutants.
(A) Activation and inhibition data for ligands **1**–**3** in selected LasR mutants. Data for the ligand TP-5 (structure shown in panel (B)) in wild-type LasR and the W60F mutant are also included. Activation assays were performed by adding ligand at 10 μM, and activity is reported on the positive y-axis in Miller units. Inhibition assays were performed by adding ligand at 10 μM and OdDHL at its EC_{50} value for that mutant (see **Table S3**), and activity is reported on the negative y-axis as the % decrease in activity relative to only OdDHL being present. Error bars represent s.e.m. of a biological triplicate. Inhibition data for ligand **3** in the T75V mutant demonstrated poor reproducibility, but since the activation data for **3** in the T75V mutant was comparable to that for **3** in wild-type LasR, we ruled out Janus behavior for ligand **3** in this mutant.

Trp60 has previously been hypothesized to be important for LasR:non-lactone ligand interactions. In 2006, Jog et al. proposed that altered interactions with Trp60 could explain why different stereoisomers of OdDHL analogs with cyclohexanol or cyclopentanol head groups activate LasR to different degrees;[24] this study was performed prior to the report of the $[LasR:OdDHL]_2$ structure and was instead based upon analysis of the structure of TraR, a LuxR-type protein from *Agrobacterium tumefaciens*. In 2009, Zou and Nair modeled the synthetic tri-phenyl LasR inhibitor, TP-5[36] (shown in **Figure 3.3B**) into their X-ray crystal structure of LasR bound to an activating ligand (a related tri-phenyl derivative, TP-3), and suggested that a possible cause of the observed inhibitory activity for TP-5 (assuming this ligand targets the same ligand

binding site) was the poor alignment of TP-5's chlorine atom with the Trp60 side chain NH for a halogen bond.[28] We tested this hypothesis by examining the activity of TP-5 in the W60F LasR mutant. No Janus behavior was observed for TP-5 in this mutant—its LasR inhibitory activity was unchanged, and no LasR activation was observed (**Figure 3.3A**). These data reveal that mutation of Trp60 to Phe does not perturb interactions of TP-5 with LasR. Other mutations to Trp60 are necessary to gain further insights into the nature of Trp60 interactions with TP-5, if any. Nevertheless, these data for TP-5 indicate that this non-lactone inhibitor may make alternate contacts with LasR relative to ligands **1**–**3**.

3.2.3 Model for LasR inhibition by non-lactone QS modulators

In view of the reporter strain data above, we developed a model to explain the activation and inhibition activity of these alternate head group ligands (**1** and **3**) in wild-type and mutant LasRs. Since wild-type LasR is inhibited by ligand **1** and the W60F mutation reverts **1** to an activator, we suspected that Trp60 interacts unfavorably with the aniline head of ligand **1**, which leads to an inactive LasR conformation. The replacement of tryptophan with the smaller phenylalanine residue relieves this unfavorable interaction, making the binding of ligand **1** compatible with the active conformation of LasR. To test this model at a more molecular level, Autodock[37] was used to computationally dock ligand **1** into the OdDHL-binding site of wild-type LasR and the (presumed) OdDHL-binding site of the W60F LasR mutant. We used the $[LasR:OdDHL]_2$ structure reported by Bottomley et al. for these computational studies[9] (see *Section 3.4.6*). In agreement with our model, the lowest energy poses showed that the hydrogen *ortho* to the amide in the aniline head group of ligand **1** was consistently clashing with the NH of the Trp60 side chain (**Figure 3.4A**, orange arrow), whereas the W60F mutation enabled this *ortho* hydrogen to fit between two phenylalanine hydrogens with significantly longer atom-to-atom distances

(**Figure 3.4A**). We of course are cautious to avoid firm structural conclusions based on docking results; nonetheless, the computational study suggests that Trp60 can indeed have unfavorable interactions with the aniline head of ligand **1** that are relieved by the W60F Janus mutation. This finding can be viewed as a "bump-hole" phenomenon,[38, 39] where the aniline head provides a subtle "bump" that is sterically incompatible with Trp60 but is accommodated for by a "hole" formed by a tryptophan-to-phenylalanine mutation. To our knowledge, bump-hole approaches to modulating LuxR-type proteins with small molecules are yet to be reported, and this finding with LasR and ligand **1** provides impetus for exploration of this powerful chemical biology technique for the study of AHL-type QS.

Regarding ligands **2** and **3**, the wild-type activity data suggest that the thiolactone head group of ligand **3** enables it to bind wild-type LasR in a manner compatible with receptor activation, whereas the lactone ligand **2** binds LasR in a manner incompatible with receptor activation (**Table 3.2**). Since the W60F mutation abrogates this activation by ligand **3**, we suggest that the thiolactone is able to form a favorable hydrogen-bonding interaction that stabilizes Trp60 in LasR's active conformation, but the lactone of ligand **2** does not engage in this stabilizing interaction. Although natural bond order (NBO) analysis suggests that thiolactone carbonyls are not intrinsically better hydrogen bond acceptors than lactone carbonyls (i.e., they do not have a greater negative charge indicating higher basicity, see **Table 3.3**), docking of **3** into the LasR ligand binding site with Autodock[37] showed that the larger size of the thiolactone ring in **3** can position its carbonyl closer to the Trp60 side chain NH and also slightly further into the pocket (**Figure 3.4B**). Therefore, a model that matches our data and is molecularly reasonable posits that the thiolactone ligand **3** is better positioned to hydrogen bond with Trp60 and hold it in an orientation that stabilizes the active LasR conformation, while the smaller lactone of ligand **2** is improperly positioned for this active state stabilization. The W60F mutation causes both ligands

to lose this hydrogen bond and therefore neither can position Phe60 in a conformation necessary for LasR activation. This model corroborates and serves to refine the earlier proposal by Jog et al. (*vide supra*), which invoked a prominent role for Trp60 in positioning the head groups of certain OdDHL analogs for differential LasR activation.[24]

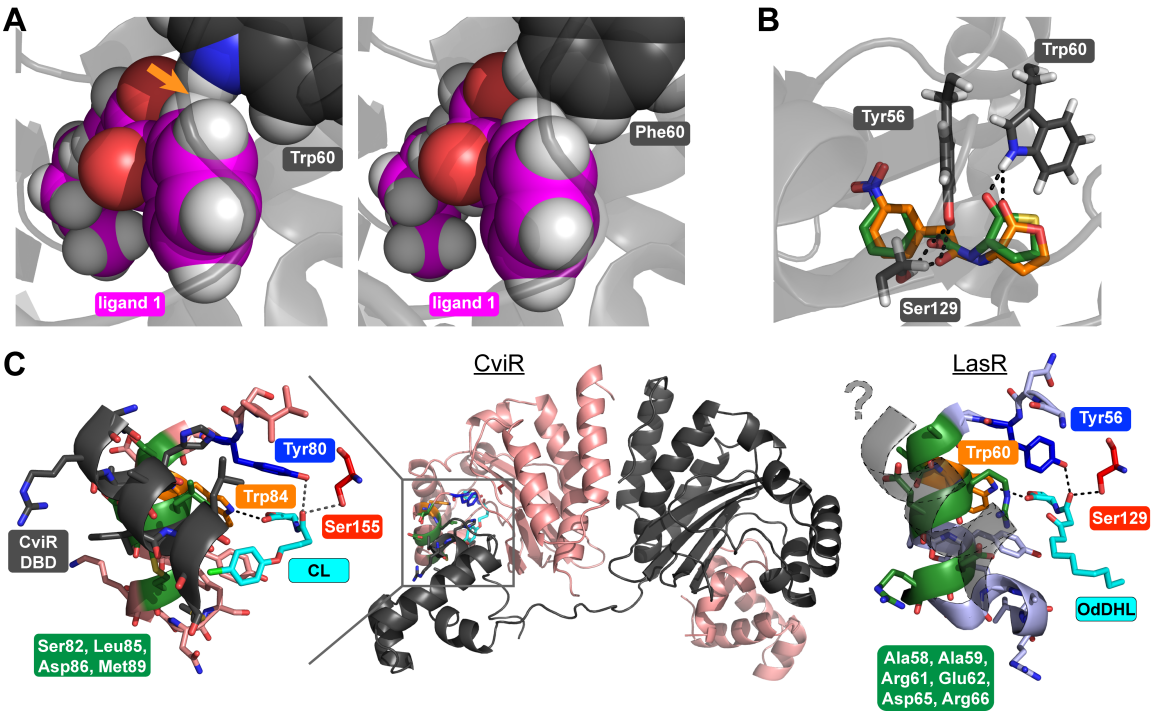


Figure 3.4. Model for Trp60 governing LasR activation vs. inhibition.
(A) Images from automated docking of ligand 1 (magenta) into wild-type and W60F LasR, displaying steric clash between ligand 1 and Trp60 (see orange arrow) that is relieved in the W60F mutant. (B) Image from automated docking of ligands 2 and 3 (orange and green, respectively) into wild-type LasR. Residues Tyr56 and Ser129 are shown hydrogen-binding to the ligand amide carbonyls, and Trp60 is shown hydrogen-binding to the lactone and thiolactone carbonyls with O···H distances of 2.2 Å and 1.8 Å, respectively. (C) Images of X-ray crystal structures of $[CviR\text{-}CL]_2$[26] and $[LasR\text{-}OdDHL]_2$.[9] Ligands (cyan) are shown interacting with Trp84 (CviR) and Trp60 (LasR) residues (orange), which are adjacent to residues (green) in an alpha helix that interacts with a binding partner (grey) in the inactive crossed-domain $[CviR\text{-}CL]_2$ structure (left inset). A hypothesized binding partner for LasR is displayed in transparent grey (right). Tyr56 and/or Ser129 homologous residues (blue and red, respectively) interact with the amide carbonyl to position the ligand lactone head near Trp60. Hydrogen bonds are displayed as dashed lines. "Janus" ligand-protein behavior.

Table 3.3. Charge on the C=O oxygen for γ-butyrolactone vs. γ-butyrothiolactone (i.e., the head groups of ligands 2 and 3) as determined by NBO analysis.

Compound	Charge
lactone	-0.57922
thiolactone	-0.56123

Finally, the observed importance of residues Tyr56 and Ser129—the side chains of which are known to hydrogen bond to the amide carbonyl of OdDHL (**Figure 3.1B**)—can be connected to this same model by considering how they position the ligands near Trp60. In the case of ligand **1**, Tyr56 may be essential in holding the ligand such that the aniline head group clashes with Trp60, allowing it to behave as a LasR inhibitor. In the case of ligand **3**, Ser129 may be essential in holding the ligand such that the thiolactone hydrogen bonds with Trp60, thereby engendering LasR activation.

It was still unclear how different orientations of Trp60 within the ligand binding site, presumably caused by different ligand interactions, could regulate whether LasR was in an active or inactive conformation. However, scrutiny of the reported X-ray structure of CviR bound to a non-native AHL ligand (CL) provided some insights.[26] CL is a CviR antagonist, and the $[CviR:CL]_2$ homodimer structure revealed a crossed-domain conformation (**Figure 3.4C**), where the ligand-binding domain of each CviR monomer was bound to the DNA-binding domain of the other monomer. This conformation splayed the DNA-binding domains far apart from each other (relative to that observed in $[LuxR\text{-type protein:native AHL}]_2$ complexes), and the authors proposed that this structure is unable to bind DNA, belaying the mode of action of the antagonist CL. We observed that in this presumably inactive conformation, CviR engages in a protein-protein interaction adjacent to its Trp60 homologous residue (between residues Ser82, Leu85, Asp86, and Met89 adjacent to Trp84 and residues Thr246, His247, Ile249, and Val250 in the DNA-binding domain). We speculate that LasR could also form a homologous inactivating

protein-protein interaction between residues near Trp60 (Ala58, Ala59, Arg61, Glu62, Asp65, Arg66) and the LasR DNA-binding domain or an alternative binding partner (as shown in **Figure 3.4C**). In support of this hypothesis, previous work on CviR showed that subtly different interactions between its ligands and Met89—a residue in the same inactivating protein-protein interface that interacts with the *acyl tail* of CviR-binding ligands—could alone drastically change whether the CviR dimer preferred the active or inactive conformation.[26] We propose here that subtle positioning changes to Trp60 in LasR can similarly translate to the formation of the hypothesized inactivating protein-protein interaction. In total, our model posits that ligands with *alternative head groups* interact differently with Trp60 to alter Trp60's conformation enough to govern whether or not an adjacent inactivating protein-protein interaction is favorable. This model is consistent with the Janus behavior of the W60F, Y56F, and S129A mutants with ligands **1** and **3**, is logical on a molecular level, and relates well to the recently reported model for CviR inhibition by alternative tail ligands.[26] Further studies are certainly necessary to test this model and are ongoing in our laboratory. Mutagenesis of the residues hypothesized to form inactivating protein-protein interactions near Trp60 is an important next step, as are structural studies of both wild-type LasR and W60F LasR bound to ligand **1**. Structures of full length LasR, to augment the reported *N*-terminal ligand binding domain structures,[9, 28] would be particularly revealing with regard to probing interactions in the DNA binding domain.

3.3 Implications and Conclusions

The results reported herein yield a new model to explain LasR activation and inhibition by AHL analogs with non-native head groups. However, they also afford two other important implications. First, the observation that a single amino acid mutation (W60F or Y56F) can make LasR immune to QS inhibitor **1**, as well as similar findings with other LuxR-type proteins,[26, 33]

demonstrates that a likely mechanism of resistance to QS inhibitors is mutation of LuxR-type proteins. We find it particularly interesting that the W60F and Y56F LasR mutants became *activated* by an inhibitor, which would lead to a "signal-independent" mechanism of QS-inhibitor resistance (i.e., native OdDHL signal would not be required for QS activation of the mutant as long as the inhibitor-turned-activator is present). We have recently discussed the implications of such signal-independent QS-inhibitor resistance pathways,[15] However, as we and others have shown, QS inhibitors should hold a decreased spread of resistance compared to traditional antibiotics due to the competition that arises after a resistant bacterium appears.[1, 14, 15] Therefore, even though resistance can readily develop (via the LuxR-type receptor mutations examined here or other potential paths), it should not spread easily, and the application of QS inhibitors as resistance-robust microbial control agents still holds significant promise.

The second interesting implication of the three LasR mutants (W60F, Y56F, and S129A) is their potential use in bioengineering applications. The emerging field of synthetic biology requires the precise control of gene expression.[40] For complex applications, this requires several orthogonal gene regulatory elements. Some of the most common inducible gene-expression systems suffer from cross-talk and are based on metabolic regulation, which can influence metabolism as an undesired side-effect.[41] Thus AHL-based QS systems have found significant recent utility in expanding the toolbox of orthogonal inducible gene-expression elements.[33, 42-44] A limitation of natural AHLs is their relative hydrolytic instability,[45] which is alleviated substantially by replacing their lactone head groups with stable non-lactone variants (such as **1** and **3**).[17-24] The W60F, Y56F, and S129A LasR mutants reported herein, which respond in opposite ways to the same non-lactone ligands, enable methods of complex regulated gene expression that could provide for interesting experiments with mixed bacterial populations. For example, a gene in one organism could be induced by addition of ligand **1**, and then addition of

ligand **3** would *both* afford induction of a gene in a second organism *and* shut off the first. This mixed-population "pulse-chase" technique would have the benefit of not requiring the removal of the initial signal molecule from the medium, and concomitantly uses ligands that should not influence metabolism and are more hydrolytically stable than native AHLs. Many other experimental scenarios are conceivable, as well.

In conclusion, we have delineated new insights into the mechanisms by which non-lactone ligands influence activation and inhibition of LasR—a QS receptor that regulates the virulence of the prevalent opportunistic pathogen *P. aeruginosa*. Our site-directed mutagenesis and structural analyses provide the first empirical evidence demonstrating that aniline and thiolactone ligand head groups interact differently with Trp60 relative to lactone head groups, and that this differential interaction is sufficient to govern complete flips between marked activation and inhibition of LasR. Based on the $[CviR:CL]_2$ co-crystal structure,[26] we proposed a model by which different interactions with Trp60 could translate to an inactive conformation of LasR. The reported data and model are significant because the field has practically no information about the molecular mechanisms by which synthetic activators and inhibitors interact with LasR. As this new model is further explored, we believe that it should provide insights for the design of new synthetic LasR modulators with improved efficacies. For example, we could intentionally design ligands that will displace Trp60 more drastically. Alternatively, if we discover that the hypothesized inactivating protein-protein interaction is in fact operative in LasR, we can attempt to rationally stabilize or destabilize it to control LasR activity. Lastly, looking beyond the mechanistic outcomes of this study, the exciting discovery of Janus behavior between different LasR mutants both demonstrates a mechanism by which signal-independent resistance can develop to QS inhibition and holds implications for engineered gene regulation.

3.4 Experimental

3.4.1 Compound handling and reagents

Stock solutions of synthetic compounds (10 mM) were prepared in DMSO and stored at room temperature in sealed vials. OdDHL, **1**, **2**, and **3** were synthesized as previously reported.[18, 19, 46] The synthesis and characterization of the TP-5 ligand is reported in *Section 3.4.9*. Solvent resistant polypropylene (Corning Costar cat. no. 3790) and clear polystyrene (Corning Costar cat. no. 3997) 96-well microtiter plates were used as appropriate.

3.4.2 Bacterial strains, media, and growth conditions

The bacterial strains used in this study were *E. coli* DH5α [F^- φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*] and *E. coli* K-12 derivative JLD271.[47] See **Table 3.4** for a list of strains and plasmids used in this study. *E. coli* was cultured at 37 ºC in Luria-Bertani (LB) medium and on LB plates with 1.5% agar. For selection and maintenance of plasmids, gentamicin, ampicillin, and kanamycin were used at 15 μg/ml, 100 μg/ml, and 50 μg/ml, respectively.

Table 3.4. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description*	Reference
E. coli		
DH5a	F$^-$, j80d*lacZ*DM15D(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(rk$^-$, mk$^+$) *phoA supE*44 λ$^-$ *thi*-1 *gyrA*96 *relA*1	Invitrogen
JLD-271	K-12 Δ*lacX74 sdiA271::*Cam; Cm^R	[47]
Plasmids		
pJN105L	Arabinose-inducible expression plasmid for *lasR*; Gm^R	[48]
pSC11	*lasI-lacZ* transcriptional fusion reporter plasmid; Ap^R	[49]
pPROBE-KL	*lasI'-gfp[LVA]* transcriptional fusion derivative of pPROBE-KT;[50] Km^R	[34]
pJG002	Y47F mutant analog of pJN105L	This study
pJG007	Y56F mutant analog of pJN105L	This study
pJG008	W60F mutant analog of pJN105L	This study
pJG009	R61M mutant analog of pJN105L	This study
pJG010	D73L mutant analog of pJN105L	This study
pJG011	T75V mutant analog of pJN105L	This study
pJG012	Y93F mutant analog of pJN105L	This study
pJG0013	T115V mutant analog of pJN105L	This study
pJG014	S129A mutant analog of pJN105L	This study

*Abbreviations: Cm^R, chloramphenicol resistance; Gm^R, gentamicin resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance.

3.4.3 Construction of mutant LasR reporter strains

Site-directed mutagenesis was carried out on the LasR-expressing plasmid pJN105L[48] by overlap extension polymerase chain reaction (PCR)[51] (see **Table 3.5** for PCR primers). The mutagenized *lasR* genes were digested with *EcoRI* and *XbaI* and cloned into *EcoRI/XbaI*-cut pJN105L.[48] The mutant pJN105L variants were sequenced to verify mutagenesis and transformed via electroporation into the *E. coli* DH5α/pSC11[49] reporter strain and selected on LB + gentamicin + ampicillin plates.

Table 3.5. PCR primers for site-directed mutagenesis of LasR

Mutation	Forward primer sequence[a]	Reverse primer sequence[a]
flanking primers	CGATTAGAATTCTTAAGAAGAACGTAGCGCTATG	CCACGCTCTAGAGGCAAGA
Y56F (TAC→TTC)	TCGGCAAC**TTC**CCGGCCGC	GCGGCCGG**GAA**GTTGCCGA
W60F (TGG→TTC)	GGCCGCC**TTC**CGCGAGCATT	AATGCTCGCGG**AAG**GCGGCC
R61M (CGC→ATG)	GGCCGCCTGG**ATG**GAGCATTACG	CGTAATGCTC**CAT**CCAGGCGGCC
D73L (GAC→CTC)	GCGGGTC**CTC**CCGACGGTCA	TGACCGTCGG**GAG**GACCCGC
T75V (ACG→GTG)	GGTCGACCCG**GTG**GTCAGTCACT	AGTGACTGAC**CAC**CGGGTCGACC
W88F (TGG→TTC)	GCCGATTTTC**TTC**GAACCGTCCA	TGGACGGTTC**GAA**GAAAATCGGC
T115V (ACC→GTC)	GTATGGGCTG**GTC**ATGCCGCTGC	GCAGCGGCAT**GAC**CAGCCCATAC
S129A (AGC→GCC)	GCGCGCTG**GCC**CTCAGCGT	ACGCTGAG**GGC**CAGCGCGC

[a]**Bold** = mutated codon

3.4.4 β-galactosidase reporter gene assays

All assays were conducted as previously reported[18, 19] for the *E. coli* wild-type LasR strain DH5α/pJN105L + pSC11.[48] Absorbance measurements were obtained using a Biotek Synergy monochromator plate reader running Gen5 v1.05 software. A 600 nm filter was used for reading bacterial cell density. Filters of 420 nm and 550 nm were used for Miller-type absorbance assays. OdDHL EC_{50} values were calculated for all LasR mutant strains (**Table 3.1**) by reported dose-response methods.[18] Synthetic ligands were tested for LasR activation at 10 μM in each strain. Similarly, ligands were tested for LasR inhibition at 10 μM against OdDHL at its EC_{50} value (**Table 3.1**) for the mutant LasR strain.

3.4.5 Fluorescence microscopy

To prepare the fluorescent reporter strains, the same pJN105L-derived mutant plasmids were transformed into *E. coli* JLD271/pPROBE-KL [34] via electroporation and selection on LB + gentamicin + kanamycin. Microscopy was performed with a Zeiss AX10 Imager.M2 epifluorescent microscope with a HXP 120 C Lamp using the 2.5×/0.12 FLUAR objective in conjunction with an AxioCam MR monochrome camera controlled by AxioVision (Rel 4.8.2)

software (Carl Zeiss MicroImaging). GFP filters was used. Microscopy was performed directly through an LB agar plate containing 10 μM **1** or **3** and 0.4% arabinose after inoculation with approximately 10^5 CFU of the *E. coli* fluorescent reporter strains and incubation at 37 ºC for 9 h.

3.4.6 Ligand docking

All four ligands discussed in this work were computationally docked into wild-type LasR and W60F LasR structures using AutoDock v.4.2.[37] The LasR structure was generated from the $[LasR:OdDHL]_2$ structure (pdb 2UV0).[9] Using SYBYL-X 2.1.1 (Certara, L.P.), the native ligand and all water molecules were removed. To generate the structure of the W60F mutant, Trp60 was replaced with Phe. For both structures, all hydrogen atoms were added. The four ligands (OdDHL and **1**–**3**) were built and geometry optimized in Sybyl using the Powell method with 0.05 kcal/(mol*Å) gradients and 100 maximum iterations, using Simplex initial optimization, Tripos force field, and Gasteiger-Huckel charges. Docking dimensions were set in ADT Autodock Tools and were centered on the binding pocket with a size that included the entire pocket. Autodock was then used to dock all four ligands with 30 trials, population size of 100, random starting position and conformation, translation step range of 2.0 Å, rotation step ranges of 50°, elitism of 1, mutation rate of 0.02, cross-over rate of 0.8, local search rate of 0.06, and 1,000,000 maximum energy evaluations. As a test of the docking quality, the docking of OdDHL was compared to its position in the original X-ray crystal structure (pdb 2UV0), and it was observed to overlap extremely well (**Figure 3.5A**). The lowest energy poses that bound in the pocket similarly to OdDHL are displayed in **Figure 3.4A,B** and are representative of other low-energy poses. Ensembles of all the docking poses are shown in **Figure 3.5**. All structural images were generated in PyMol 1.3 (Schrödinger, LLC).

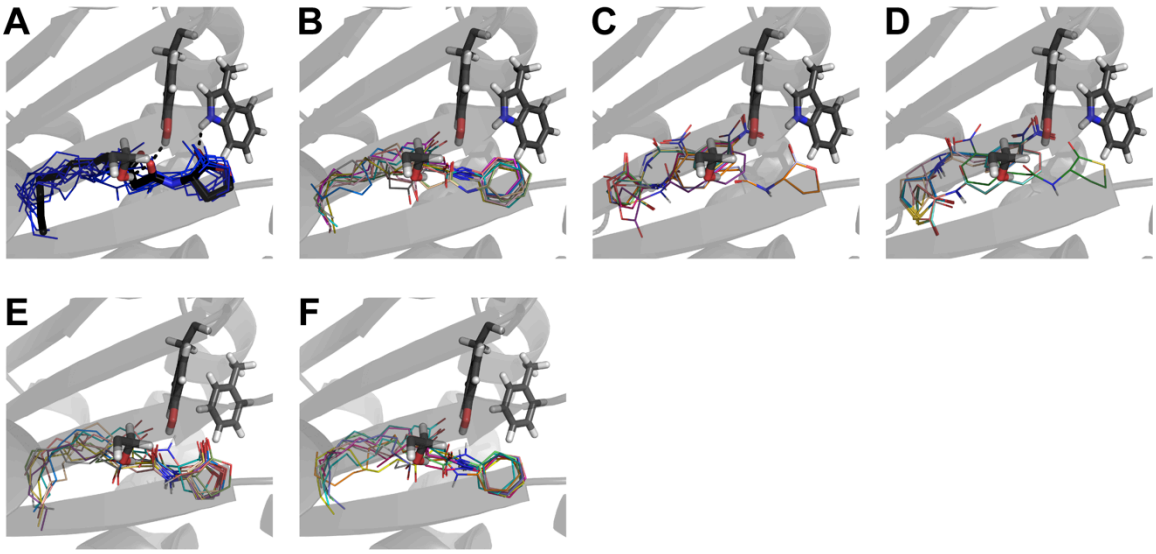


Figure 3.5. LasR docking images
Images of the 10 lowest energy poses from the LasR:ligand docking experiment. Residues Tyr56, Trp60/Phe60, and Ser129 are displayed in grey. (A) OdDHL docked into wild-type LasR ligand binding site. Docked structures are shown in blue; X-ray crystal structure placement of OdDHL shown in black; hydrogen bonds to OdDHL from X-ray structure shown with black dashed lines. (B) Ligand **1** docked into wild-type LasR ligand binding site. (C) Ligand **2** docked into wild-type LasR ligand binding site. Only 2 poses are similar to OdDHL. (D) Ligand **3** docked into wild-type LasR ligand binding site. Only 1 pose is similar to OdDHL. (E) OdDHL docked into W60F LasR mutant ligand binding site. (F) Ligand **1** docked into W60F LasR mutant ligand binding site.

3.4.7 Computational evaluation of the relative basicity of the C=O oxygen of γ-butyrolactone vs. γ-butyrothiolactone

Simplified analogs of the homoserine lactone and homocysteine thiolactone head groups were prepared in Pymol 1.3 (Schrödinger, LLC) from reported X-ray crystal structures. An *N*-trimethylacetyl homoserine lactone X-ray crystal structure[52] was imported into Pymol 1.3, and the acetylamide group was replaced with a hydrogen to yield γ-butyrolactone (see **Figure 3.6** for image and **Table 3.6** for coordinates). The simplified thiolactone (i.e., γ-butyrothiolactone, **Figure 3.6** for image and **Table 3.7** for coordinates) was similarly prepared in Pymol 1.3 from an *N*-acetyl homocysteine thiolactone X-ray crystal structure.[53] These two molecules were then subjected to NBO analysis at the B3LYP/6-3111G(2d,p) level of theory as implemented in

WebMO 14.[54] The resulting energies for the lactone and thiolactone model systems were −306.589720195 Hartree and −629.48733841 Hartree, respectively. Cartesian coordinates for each molecule are listed below. The NBO analysis also revealed that the C=O oxygen of the lactone has a slightly (but significantly) greater negative charge than that of the thiolactone, indicating that the lactone is inherently a slightly better hydrogen bond acceptor (see **Table 3.3**).

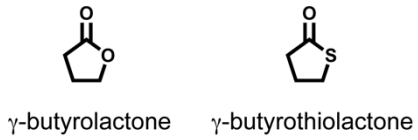


Figure 3.6. Lactone and thiolactone structures used for NBO analysis.

Table 3.6. Cartesian coordinates for the γ-butyrolactone submitted to NBO analysis.

	X	Y	Z
C	1.257324	-0.855309	0.105198
C	1.441899	0.660992	-0.068692
C	0.017446	1.232222	0.034984
C	-0.892441	-0.014707	0.005382
O	-0.147772	-1.128772	-0.110255
O	-2.089863	-0.015256	0.027782
H	-0.233309	1.814651	-0.851398
H	-0.087253	1.864079	0.917279
H	1.864418	0.888918	-1.047909
H	2.101541	1.072959	0.691925
H	1.501081	-1.182551	1.113320
H	1.809236	-1.445014	-0.624665

Table 3.7. Cartesian coordinates for the γ-butyrothiolactone submitted to NBO analysis.

	X	Y	Z
C	0.090003	1.418714	0.182346
C	0.985459	0.189769	0.018436
O	2.181192	0.211153	-0.046270
S	0.004047	-1.281037	-0.056390
C	-1.556543	-0.403376	0.183566
C	-1.311468	1.020128	-0.270310
H	-2.017490	1.606222	0.241667
H	-1.294496	1.320448	-1.286677
H	-2.349219	-0.850958	-0.269423
H	-1.586247	-0.437414	1.041134
H	0.130840	1.490961	1.219840
H	0.357612	2.326708	-0.358366

3.4.8 General experimental for chemical synthesis

All chemical reagents were purchased from commercial sources (Sigma-Aldrich and Fisher) and used without further purification. ^{1}H NMR spectra were recorded in deuterated NMR solvents at 400 MHz on a Brucker Avance-400 spectrometer. Chemical shifts are reported in parts per million (ppm, δ) using tetramethylsilane (TMS) as a reference, and couplings are reported in hertz (Hz). Electrospray ionization (ESI) MS data were obtained using a Waters (Micromass) LCT^{TM} system. This instrument uses a time-of-flight analyzer. Samples were dissolved in methanol and sprayed with a sample cone voltage of 20. Melting point (mp) was recorded on a DigiMelt MPA 160 melting point apparatus and is uncorrected.

*3.4.9 Synthesis of TP-5 (structure shown in **Figure 3.3B**).*

1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (0.287 g, 1.50 mmol), *o*-phenylenediamine (0.081 g, 0.75 mmol) and dimethylaminopyridine (0.018 g, 0.15 mmol) were sequentially added to a suspension of *m*-chlorobenzoic acid (0.234 g, 1.50 mmol) in dichloromethane (5 mL). The reaction mixture was stirred for 24 h, diluted with dichloromethane (25 mL), and extracted with 1 M HCl (3 x 25 mL) followed by saturated aqueous sodium

bicarbonate (3 x 25 mL). The organic layer was dried with anhydrous magnesium sulfate. Evaporation of solvent gave TP-5 as a white solid (0.136 g, 47% yield). mp = 190–192 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.41 (s, 1H), 8.02 (s, 1H), 7.87 (d, *J* = 7.8 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 1H), 7.47 (t, *J* = 7.9 Hz, 1H), 7.37–7.28 (m, 1H), 6.91–6.78 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 165.2, 135.1, 135.0, 132.3, 130.4, 130.0, 128.1, 126.5, 125.9, 125.6. HRMS calculated for $C_{20}H_{15}Cl_2N_2O_2$ $[M+H]^+$ 385.0506; found 385.0512.

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CHAPTER 4:

UNRAVELING THE CONTRIBUTIONS OF HYDROGEN-BONDING INTERACTIONS TO THE ACTIVITY OF NATIVE AND NON-NATIVE LIGANDS IN THE QUORUM-SENSING RECEPTOR LasR

Contribution: J. P. G., C. E. M., and H. E. B. designed the research and wrote the paper; J. P. G. and C. E. M. analyzed data; J. P. G. prepared mutant LasR strains and sequence alignments; C. E. M. synthesized ligands and performed β-galactosidase assays; T. L. S. performed β-galactosidase assays.

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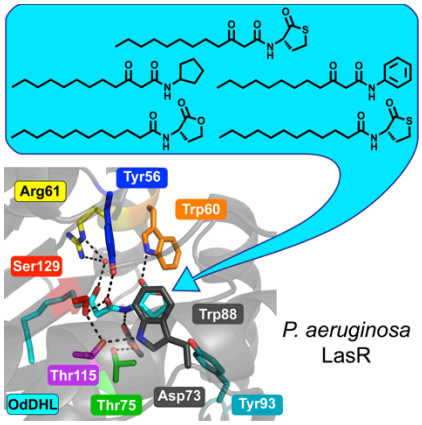
*these authors contributed equally

Abstract.



Quorum sensing (QS) via the synthesis and detection of *N*-acyl L-homoserine lactone (AHL) signals regulates important pathogenic and mutualistic phenotypes in many bacteria. Over the past two decades, the development of non-native molecules that modulate this cell-cell signaling process has become an active area of research. The majority of these compounds were designed to block binding of the native AHL signal to its cognate LuxR-type receptor, and much effort has focused on LasR in the opportunistic pathogen *Pseudomonas aeruginosa*. Despite a small set of reported LasR structural data, it remains unclear which polar interactions are most important for either (i) activation of the LasR receptor by its native AHL signal, *N*-(3-oxo)-dodecanoyl L-homoserine lactone (OdDHL), or (ii) activation or inhibition of LasR by related AHL analogs. Herein, we report our investigations into the activity of OdDHL and five synthetic analogs in wild-type LasR and in nine LasR mutants with modifications to key polar residues in their ligand binding sites. Our results allowed us to rank, for the first time, the relative importance of each LasR:OdDHL hydrogen bond for LasR activation and provide strong evidence for the five synthetic ligands binding LasR in a very similar orientation as OdDHL. By delineating the specific molecular interactions that are important for LasR modulation by AHLs, these findings

should aid in the future design of synthetic modulators of LasR (and homologous LuxR-type receptors) with improved potencies and selectivities.

4.1 Introduction

Bacteria, much like higher organisms, must alter their behavior to have optimal fitness in changing environments. For example, if nutrients are limited, they swim to a new environment,[1] change their metabolic flux,[2] or even enter into spore states.[3] In addition to sensing the presence of nutrients, bacteria perceive which organisms are around them and the density in which they are packed in order to regulate whether or not to secrete toxins (e.g., antibiotics, hemolysins, and reactive small molecules) and to produce shared resources (e.g., siderophores, enzymes, and even light).[4, 5] Many bacteria sense their own population densities in a process called quorum sensing (QS). In QS, bacteria biosynthesize a small molecule or short peptide signal that is either secreted or diffuses out of the bacterial cell and then can disperse throughout the environment.[6-8] As the bacteria replicate and their density increases within a confined space, the concentration of signal in this environment likewise increases.[9] Once signal levels reach a threshold concentration, the signals engage in productive interactions with bacterial receptor proteins that ultimately result in changes to gene expression. In the case of Gram-negative bacteria, the signal molecules are primarily *N*-acyl L-homoserine lactones (AHLs) that are biosynthesized by LuxI-type enzymes. These AHL signals are sensed by intracellular LuxR-type receptors, which upon binding signals, typically form active dimers and function as transcription factors to induce expression of QS-regulated genes.[6, 7]

Numerous bacteria that are relevant to human health use QS to regulate pathogenic or mutualistic behaviors. For example, the opportunistic pathogen *Pseudomonas aeruginosa* waits until it amasses a quorum before it expresses many virulence genes that harm the host organism.[4] The notorious human pathogen *Staphylococcus aureus* also uses QS to control a broad range of virulence phenotypes.[5] Alternatively, nitrogen-fixing rhizobia use QS to regulate their conversion into mature root nodules that help feed legumes.[10] Because QS is dependent on signal-receptor

binding, blocking this binding event with a synthetic ligand represents a logical approach to artificially control virulent and beneficial phenotypes in bacteria. To this end, our laboratory and others have developed small molecules that competitively bind QS receptors and modulate the myriad phenotypes that they regulate.[11-17] In particular, we have heavily focused on developing inhibitors and activators of the LuxR-type receptor LasR, which is a primary regulator of virulence in *P. aeruginosa*.[18] Such modulators of LuxR-type receptors have significant utility as chemical probes to study QS with both temporal and spatial control. Notably, because of the selfless communal nature of QS, QS inhibitors are likely to provide a weaker selective pressure for resistance relative to traditional antibiotics that directly inhibit bacterial growth.[19-21] For these reasons and others, the development of small molecule and macromolecular QS inhibitors as anti-virulence agents has attracted considerable attention.[22-26]

Despite much past research, we still know relatively little about which ligand-receptor interactions are critical for activation or inhibition of LasR and other LuxR-type QS receptors. The first structures of a LuxR-type protein (TraR from *Agrobacterium tumefaciens*) bound to a native AHL were reported over a decade ago,[27, 28] but additional structural data of LuxR-type proteins with either native or non-native ligands remains scarce.[29] The paucity of both structural and biochemical data is primarily due to LuxR-type proteins being difficult to manipulate *in vitro*. These proteins are unstable without a ligand bound,[27, 30-34] the ligands can be difficult to exchange once bound,[31, 32] and often the proteins aggregate and become insoluble when bound to inhibitors.[35] Even when bound to its native AHL signal (*N*-(3-oxo)-dodecanoyl L-homoserine lactone, OdDHL; **Figure 4.1**), only structures of the truncated N-terminal ligand-binding domain LasR have been solved to date.[35-37] Further, there have been only two reported studies of LuxR-type proteins bound to non-native ligands: first, the LasR N-terminal domain bound to triphenyl (TP)-type synthetic activators,[35] and second, full-length CviR (from *Chromobacterium*

violaceum) bound to three AHLs exhibiting differing degrees of partial agonism (octanoyl L-homoserine lactone, decanoyl L-homoserine lactone, and the "chlorolactone" inhibitor CL).[38]

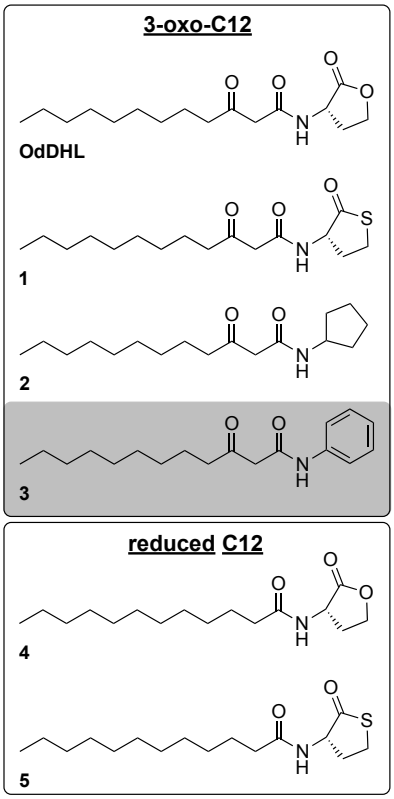


Figure 4.1. Native AHL ligand OdDHL and the OdDHL analogs examined in this study.
The compounds are divided into groups containing the same tails (3-oxo-C12 or reduced C12) but varying heads (lactone, thiolactone, cyclopentyl, or phenyl). The single LasR inhibitor (**3**) is shaded grey; the others (OdDHL, **1**, **2**, **4**, and **5**) are all LasR activators.

While these previous structural studies afford some insights into the binding of AHL and non-AHL ligands to LasR and its homologs, they fall short of delineating both the specific ligand-receptor interactions that are most important for activation of LasR and the modes by which non-native OdDHL-like analogs bind to LasR and affect its activity. This information is crucial for our efforts to design improved synthetic LasR modulators, and provided the motivation for the current study. Our investigations reported herein centered on two broad goals: (i) to uncover the

relative importance of each hydrogen-bonding residue in the LasR ligand binding pocket for activation by its native ligand OdDHL, and (ii) to develop a better molecular-level understanding of how non-native ligands bind and activate LasR. The latter goal focused on a set of five analogs that closely mimic OdDHL with non-native head groups (**1**–**3**), an altered acyl tail (**4**), or both (**5**) (shown in **Figure 4.1**). In light of the challenges outlined above in manipulating LuxR-type proteins *in vitro*, we used bacterial cell-based β-galactosidase reporter-gene assays on site-directed mutants of LasR in order to investigate specific ligand-receptor interactions. Our results allowed us to rank, for the first time, the significance of each hydrogen-bonding residue in the LasR ligand-binding pocket for receptor activation. Interestingly, we also discovered one mutation that made LasR *more sensitive* to OdDHL. In turn, the results for the non-native ligands strongly supported our hypothesis that straight-chain OdDHL analogs that lack certain hydrogen-bonding moieties, whether they are activators or inhibitors, can still bind the LasR ligand-binding pocket in nearly an identical manner as OdDHL. This finding provides the first empirical evidence of the binding mode of the non-native activators (**1**, **2**, **4**, and **5**), and affords further support for the predicted binding mode of aniline inhibitor **3**.[39]

4.2 Results and Discussion

4.2.1 Importance of hydrogen-bonding residues for LasR activation by OdDHL

We first sought to understand the relative importance of each hydrogen-bonding residue in the LasR ligand-binding pocket for receptor activation. Although X-ray crystal structures reveal the likely interactions present between LasR and a bound ligand (OdDHL or TP-type ligand),[35, 36] they fail to tell us the relative importance of these interactions for LasR activation. To our knowledge, the field still lacks a comprehensive mutational analysis of the hydrogen bonding residues in the ligand-binding pocket of LasR. A catalog of previously reported mutations to

polar residues in the ligand-binding pocket of LasR and its related homologs LuxR (*Vibrio fischeri*), TraR (*A. tumefaciens*), and RhlR (*P. aeruginosa*) is presented in **Table 4.5**. We selected nine residues in the LasR ligand-binding pocket for site-directed mutagenesis (**Figure 4.2**). The side chains of eight of these residues appear to either hydrogen bond directly with OdDHL or hydrogen bond with another LasR residue that hydrogen bonds with OdDHL (i.e., Tyr56, Trp60, Arg61, Asp73, Thr75, Trp88, Thr115, and Ser129), as revealed in the structure reported by Bottomley et al.[36] Closer scrutiny of this LasR structure indicated that the side chain of the ninth residue, Tyr93, could conceivably hydrogen bond with OdDHL if this ligand was slightly reoriented in the pocket, so we also included the Tyr93 residue in our studies. Each residue was mutated to an approximately isosteric residue that lacked a hydrogen-bonding side chain (Asp→Leu, Thr→Val, Trp→Phe, Tyr→Phe, Ser→Ala, Arg→Met, **Table 4.1**). We tested the activity of OdDHL in each mutant LasR using a β-galactosidase reporter-gene assay in an *Escherichia coli* background (see *Section 4.4.5*). The EC_{50} values for OdDHL and the maximal activity levels (at OdDHL concentrations well above the EC_{50} values) were determined for each mutant (listed in **Table 4.1**). These values and activity levels were then compared to those for OdDHL in wild-type LasR. In addition, we also gauged each residue's importance for LasR activation from an evolutionary perspective by calculating the percent conservation of each residue among 100 of LasR's most closely related homologs (see *Section 4.5.3* and **Table 4.6** for additional details).

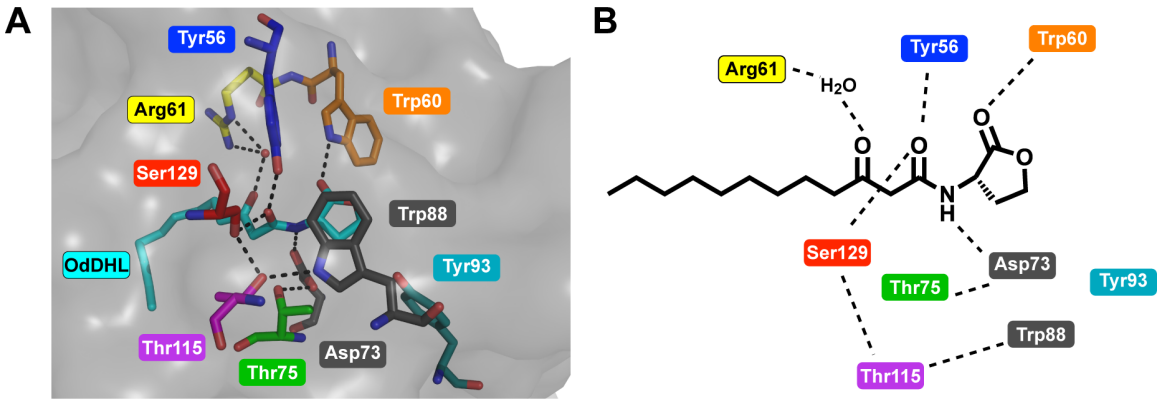


Figure 4.2. LasR residues mutated in this study.
(A) Image from the LasR X-ray crystal structure (OdDHL shown in cyan)[36] and (B) a corresponding cartoon illustrating suspected hydrogen-bonding interactions (dashed black lines) between eight different LasR residues and OdDHL. Tyr93 is also shown, as it could hydrogen bond with OdDHL if the ligand reorients slightly in the pocket.

Surprisingly, one of the LasR mutants (T75V) actually had a greater than 10× *lower* EC_{50} value for OdDHL relative to wild-type LasR (**Table 4.1**). Such a hypersensitive LasR mutant, to our knowledge, has no literature precedence, although other work has revealed hypersensitive mutants of the *Pantoea stewartii* EsaR receptor.[40] We propose that the suspected polar interaction between Thr75 and Asp73 in wild-type LasR (see **Figure 4.2**) decreases the sensitivity of LasR to OdDHL. When this polar interaction was removed by the T75V mutation, the pocket was presumably able to reorient in a manner that promoted improved binding of OdDHL. Interestingly, an alignment with 100 of LasR's most closely related homologs showed that hydrophilic residues at this position are relatively rare (see **Table 4.1** and **Table 4.6**). Most homologs possess an isoleucine or valine at this position instead of a threonine, which like our T75V mutant, would not allow interactions with Asp73. It is intriguing that LasR has a lower-than-maximal sensitivity for its native ligand, given that over evolutionary history one would assume it has had the opportunity to sample valine at the T75 position. These data suggest that *P. aeruginosa* might have a fitness benefit by being *less sensitive* to OdDHL. If true, this is a surprising discovery, as it would require the synthesis of more OdDHL signal and would

therefore be less efficient. Perhaps a counteracting advantage of lessened sensitivity would be the decreased risk of accidental activation by stochastic fluctuations in signal concentration or by similar signals produced by neighboring bacterial species. Additional experiments are currently underway with this mutant, as well as with less sensitive mutants, to characterize the fitness implications of higher and lower sensitivities to the OdDHL signal.

Table 4.1. EC_{50} values and maximal activation by OdDHL for wild-type and mutant LasRs, and the evolutionary conservation of each mutated residue.

mutation	EC_{50} (nM)[a]	maximal activation (% vs. wild-type)[b]	identity of residue in 100 LasR homologs[c]
wild-type	10	–	–
T75V	0.5	106%	26% (*53% Val, 15% Ile*)
D73L	*inactive*	0%	100%
W88F	*inactive*	0%	100%
Y93F	10	93%	4%
Y56F	10	102%	73%
S129A	50	103%	66% (20% Thr, 5% Cys)
W60F	75	73%	98%
R61M	600	56%	14%
T115V	3500	109%	46% (28% Ser, 7% Cys)

[a] EC_{50} value is the concentration of OdDHL at which the activity is half the maximum activity for that mutant. Values are geometric means of biological triplicates (s.e.m. of log-transformed data $\leq \pm 0.14$, which corresponds to antilog errors of $\times/\div$ 1.4, n = 3). Representative dose curves are shown in **Figure 4.6**.
[b] Maximal activation levels of the mutants relative to wild-type LasR were determined using OdDHL concentrations much higher than the EC_{50} value in that strain. 10 μM was sufficiently high to reach maximal activation for all strains except T115V, which had a substantially higher EC_{50} value. 100 μM was used for T115V instead. Values are means of biological triplicates (s.e.m. $\leq \pm$ 23%, n = 3).
[c] LasR was aligned with 100 closely related homologs, and the conservation of each residue of interest is reported (see *Section 4.5.3* and **Table 4.6** for entire alignment data).

The other LasR mutants behaved as expected, showing varying degrees of reduced sensitivity to OdDHL. Two mutants were completely inactive (D73L and W88F), suggesting that these residues play a critical role in LasR activation by OdDHL. This finding corroborates published data for other mutations to these residues in LasR and to their homologous residues in LuxR, TraR, and RhlR.[41-46] We note that these two residues are universally conserved among 100 of LasR's closely related homologs (**Table 4.1**). This finding provides a satisfying correlation

between the evolutionary conservation of the residues and their importance for activity. We cannot say with certainty that the D73L and W88F LasR mutants are inactive due to their inability to bind OdDHL—the residues could be necessary for protein folding regardless of the ligand. Nevertheless, since Asp73 and Trp88 are in the ligand-binding pocket, it is reasonable to suspect their importance is related to ligand binding. Furthermore, Asp73 appears to hydrogen bond with a ligand amide in every crystal structure of a ligand-bound LasR homolog—even with the TP-1, TP-3, and TP-4 ligands that are structurally distinct from AHLs[35]—underscoring the likely importance of interactions between Asp73 and LasR activating ligands.

Among the other LasR mutants evaluated, two displayed essentially no change (Y93F and Y56F) in activation by OdDHL relative to wild-type LasR, and two showed moderate decreases in activity (S129A and W60F) (**Table 4.1**). The results for the Y56F and W60F mutants were comparable to a previous report;[47] however, the Y56F, S129A, and W60F mutations were less detrimental than similar mutations reported in TraR, LuxR, and RhlR, which almost or completely obliterated the activities of these related receptors (**Table 4.5**).[42, 45, 46, 48] The Y93F mutation has not been previously examined in any LuxR-type protein. Gratifyingly, the relative activity trend for these four LasR mutants is largely consistent with their relative degree of conservation: Tyr93 is unconserved (4%) and Tyr56 is only moderately conserved (73%), while Ser129 is either Ser, Thr, or Cys in 91% of the homologs and Trp60 is nearly universally conserved (98%). Therefore, mutant activity and residue conservation match well for the T75V, D73L, W88F, Y93F, Y56F, S129A, and W60F mutations in LasR.

Mutation of Arg61 in LasR, which binds the OdDHL 3-keto carbonyl, was found to be detrimental for LasR activation (60-fold increase in EC_{50} relative to wild-type, **Table 4.1**), consistent with previous studies on LasR and LuxR.[49] However, despite its importance, Arg61 is not conserved among LasR homologs. This apparent inconsistency can be explained in two

reasonable ways: first, many of the 100 LasR homologs examined herein have a native AHL ligand that lacks a 3-keto group and they therefore do not have an evolutionary benefit to engage in such a hydrogen bond (e.g., *C. violaceum* CviR,[50] *Ralstonia solanacearum* SolR,[51] and *Rhodopseudomonas palustris* RpaR[52]), and second, some homologs are able to bind the 3-keto via alternative residues (e.g., Ala38 and Thr129 in *A. tumefaciens* TraR[27, 28] and Ser56 in *P. aeruginosa* QscR[53]). Turning to the final LasR mutant (T115V), we found that Thr115 is very important for LasR activation by OdDHL (T115V exhibits a greater than 100-fold increase in EC_{50} relative to wild-type, **Table 4.1**), corroborating prior reports in both LasR and TraR.[45, 54, 55] Although it does not directly hydrogen bond to OdDHL in the reported X-ray crystal structures of LasR,[35, 36] it appears to serve as a "linchpin" of a hydrogen-bonding network between Trp88 and Ser129 (**Figure 4.2**). Surprisingly, Thr115 is not universally conserved—19% of the nearest LasR homologs do not contain a side-chain capable of hydrogen bonding in that position. Therefore, similar to Arg61, hydrogen bonds supplied by Thr115 to properly orient the OdDHL hydrogen-bonding network are likely supplied by other residues in LasR homologs.

Systematically screening these nine LasR mutants in the same reporter system allowed us to rank the relative importance of the five LasR hydrogen bonds to OdDHL as follows: amide NH > 3-keto C=O > lactone C=O > amide C=O. Of note, this trend does not match the predicted hydrogen-bonding strengths of the residues (e.g., amide carbonyls are substantially more basic than ester and ketone carbonyls); therefore, the importance of the hydrogen bonding residues is likely dependent on factors other than bond strength. Nonetheless, this order largely matches the degree of conservation of the residues that bind these moieties in LasR homologs. We believe that this information could be leveraged for the design of new non-native agonists with potentially increased potencies; for example, designing ligands to interact with Asp73 and Arg61 will likely be more important than designing ligands to interact with Tyr56. In addition to determining this

ranking, we also gained insights into the relative importance of four residues that do not directly hydrogen bond with OdDHL. One abolished activity (W88F), one dramatically decreased activity (T115V), one had no effect (Y93F), and remarkably, one was over 10× *more sensitive* than wild type to OdDHL (T75V). As discussed above, LasR may have evolved to have a lower sensitivity to its native ligand, and we are currently using this mutant to explore the fitness implications of QS signal sensitivity.

*4.2.2 Importance of hydrogen-bonding residues for LasR activation by thiolactone ligand **1***

After examining the impact of the mutations in LasR on OdDHL activation, we next tested the ability of ligands with varying head groups and acyl tails (**1**–**5**, see **Figure 4.1**) to activate and inhibit the seven active LasR mutants identified above. We hypothesized that these ligands, which resemble OdDHL, bind the orthosteric ligand-binding pocket in the same orientation as OdDHL. Our results supported this hypothesis. The thiolactone analog of OdDHL (ligand **1**) exhibits comparable agonistic activity as OdDHL in wild-type LasR,[56, 57] and it behaved in an equivalent manner to OdDHL in activating the LasR mutants (**Figure 4.3**), except it had a significantly lower EC_{50} value for the R61M mutant (i.e., an ~8× decrease relative to OdDHL; **Table 4.2**). These data suggest that ligand **1** binds in the pocket nearly identically to OdDHL. We propose that the differences in activity between OdDHL and **1** (**Table 4.2**) in wild-type (slight), Y56F (slight), S129A (slight), and R61M (moderate) are due to slightly different interactions with Trp60. A previous docking study of ours on a related thiolactone AHL analog and LasR supports this hypothesis.[39] This computational study demonstrated that due to its larger ring size, the thiolactone head group could interact with Trp60 with a subtly different positioning, and we reason that such an effect could also be operative for **1** with LasR. Overall, the data for agonist **1**

suggest that it binds very similarly to OdDHL, and provide impetus for the further study of AHL analogs with thiolactone head groups as improved LasR modulators.[56]

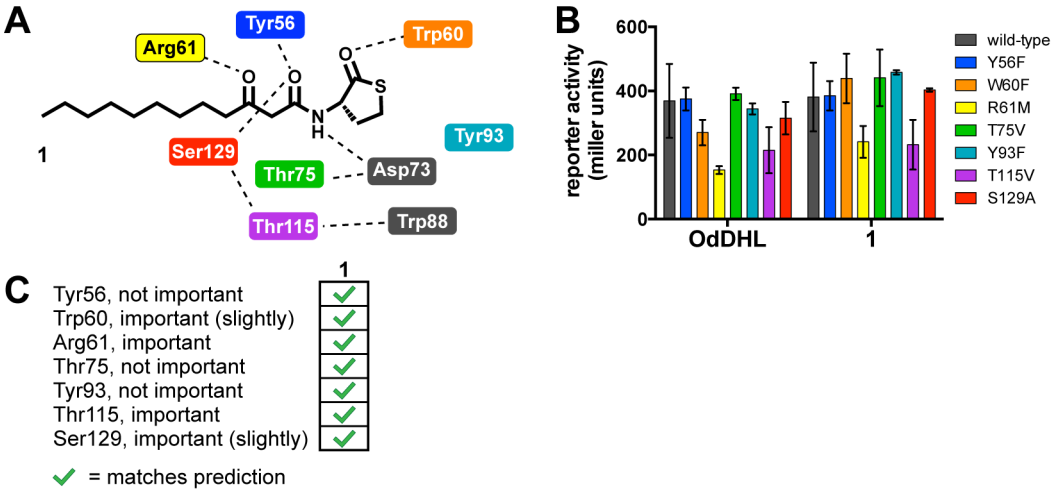


Figure 4.3. Activation of mutant LasRs by thiolactone ligand 1.
(A) Predicted interactions made by ligand **1** in the binding pocket of LasR (identical to OdDHL). (B) Activation of each mutant by OdDHL and its thiolactone analog **1**, each at 10 μM. Error bars indicate s.e.m. from a biological triplicate (n = 3) (C) Predicted and actual importance of interactions for activation by **1**. A green check indicates the prediction was correct. See also **Table 4.2**.

Table 4.2. EC_{50} values for wild-type and mutant LasR activation by OdDHL and ligand 1.

mutation	OdDHL EC_{50} (nM)[a]	Ligand **1** EC_{50} (nM)
wild-type	10	5
T75V	0.5	0.5
Y93F	10	10
Y56F	10	5
S129A	50	30
W60F	75	70
R61M	600	80
T115V	3500	3500

[a] EC_{50} value is the concentration of OdDHL at which the activity is half the maximum activity for that mutant. Values are geometric means of biological triplicates (s.e.m. of log-transformed data ≤ ± 0.14, which corresponds to antilog errors of ×/÷ 1.4, n = 3). Representative dose curves are shown in **Figure 4.7**.

*4.2.3 Importance of hydrogen-bonding residues for LasR activation and inhibition by non-lactone ligands **2** and **3***

We predicted that LasR *activation* by cyclopentyl ligand **2** and LasR *inhibition* by aniline ligand **3** would depend on Arg61, Ser129, and Thr115 because (i) these residues were important for OdDHL activity (**Table 4.1**), and (ii) no interactions between these residues and the ligands should be affected by changing the ligand head group from a lactone (as in OdDHL) to a cyclopentyl or a phenyl (as in **2** and **3**, respectively) (**Figure 4.1 and Figure 4.4A,B**). In addition, ligand activity was initially expected to not depend on Trp60 (because ligands **2** and **3** lack the ability to hydrogen bond with Trp60, as seen in **Figure 4.4A,B**) or Tyr56, Thr75, and Tyr93 (as they were not significantly important for OdDHL activity, as shown in **Table 4.1**). Activation data for ligand **2** strongly agreed with these hypotheses, as only the R61M, S129A, and T115V mutations were significantly detrimental for LasR activation by **2** (**Figure 4.4C,E**). This finding suggests that **2** binds LasR similarly to OdDHL and simply lacks a polar interaction with Trp60.

As we previously reported,[39] aniline ligand **3** was unexpectedly observed to exhibit two-faced "Janus" behavior—i.e., it transitions from a good inhibitor into a moderate agonist—in certain LasR mutants (Y56F and W60F, **Figure 4.4C,D)**. Apart from this Janus behavior (which itself is consistent with binding LasR similarly to the native OdDHL[39]), every other residue that hydrogen bonds with OdDHL was important for the LasR inhibitory activity of **3** (**Figure 4.4D,E**). The only mutation that did not have a consistent negative impact on LasR inhibition was T75V, which was expected because this mutation only improved the sensitivity of LasR to OdDHL (**Table 4.1**). Therefore, inhibitor **3** likely binds LasR in a very similar orientation to OdDHL, but as we suggested in our earlier study,[39] the phenyl head group probably makes different interactions with the Trp60 residue to lead to an inactive conformation. Interestingly, we also uncovered a third Janus mutation for ligand **3** in this study. The Y93F mutation, which appeared to have no impact

on OdDHL activity in LasR (**Table 4.1**), flipped the activity of **3** into an agonist in this mutant (**Figure 4.4C,E**). In retrospect, this observation makes sense because Tyr93 would be near the phenyl head group of **3** if it binds similarly to OdDHL, and this Tyr→Phe mutation could provide slightly more space for ligand **3** to bind without displacing Trp60 or other residues from their active conformations. Essentially, this can be considered a "bump-hole" type phenomenon,[58, 59] with the mutation Y93F providing a "hole" in which to accommodate the non-native head group "bump" of ligand **3**. We previously posited that a related bump-hole interaction could belay the Janus behavior for **3** in the W60F mutant.[39] Collectively, these data strongly support the hypothesis that ligands **2** and **3** bind the LasR ligand binding pocket in orientations analogous to that of OdDHL; ligand **2** binding leads to an active LasR conformation, but ligand **3** binding leads to a subtly different, yet inactive LasR conformation.

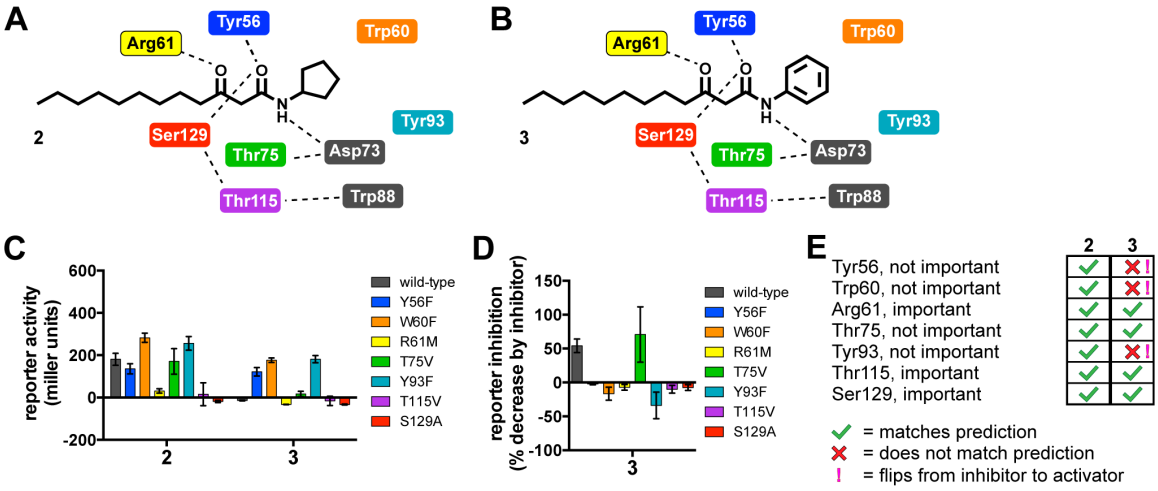


Figure 4.4. Activation and inhibition of mutant LasRs by non-lactone ligands 2 and 3. (A,B) Predicted interactions made by ligands **2** and **3** in the binding pocket of LasR. These ligands lack polar interactions with Trp60. (C) Activation of each mutant by **2** and **3**, each at 10 μM for all mutants. (D) Percent inhibition of each mutant by **3** (100% inhibition is complete shutdown of β-galactosidase production). Ligand **3** was added at 10 μM, and OdDHL was present at the EC_{50} value for the given mutant. Negative inhibition values indicate *activation* greater than that afforded by only OdDHL at its EC_{50} concentration. Error bars for all plots indicate s.e.m. from a biological triplicate (n = 3). Data for ligand **3** has been partially reported previously.[39] (E) Predicted and actual importance of interactions for activation by **2** and inhibition by **3**. A green check indicates the prediction was correct. A red X indicates a substantial deviation from the expectation, and a magenta exclamation point indicates a flip from inhibition to activation activity.

*4.2.4 Importance of hydrogen-bonding residues for LasR activation by ligands **4** and **5***

In view of our results thus far, LasR activation by ligands **4** and **5** (the lactone and thiolactone analogs of OdDHL lacking the 3-keto moiety, respectively; **Figure 4.1**) were expected to depend primarily on interactions with Trp60, Thr115, and Ser129, if the ligands bind similarly to OdDHL. This hypothesis follows from a similar rationale as above: (i) these three residues were important for OdDHL activity (**Table 4.1**), and (ii) no interactions between these residues and the ligands should be affected by loss of the 3-keto group on the ligand tail (**Figure 4.1** and **Figure 4.5A,B**). In turn, the R61M mutation was expected to have a minimal impact on LasR activation because ligands **4** and **5** should already be incapable of forming a hydrogen bond with Arg61. The Y56F, T75V, and Y93F mutations were also predicted to have minimal effects on the activities of **4** and **5**, as these mutations were not detrimental to OdDHL activity (**Table 4.1**).

Since ligand **4** was known to have an EC_{50} value in wild-type LasR significantly less than 10 μM (40 nM[60]), we screened ligands **4** and **5** at 100 nM instead of at 10 μM in the LasR mutants to increase the chances of seeing differential activity. As expected, the T115V mutation destroyed LasR activation by **4** and **5**, and the W60F and S129A mutations significantly decreased LasR activation by these two analogs (**Figure 4.5C**). None of the other mutations had a substantial impact on activity, except Y56F, which had a small but significant impact on LasR activation by ligand **4**. Most telling was the minimal impact of the R61M mutation on the activities of **4** and **5**. Even though this mutation dramatically affects the activities of **1**–**3**, it had perhaps only a slight effect on ligand **4** and no significant effect on **5**. This result strongly supports these two ligands binding similarly to OdDHL, because in that orientation, they would be incapable of hydrogen bonding with Arg61 and thus would be unaffected by an R61M mutation. We note that structural data for LasR with ligands **1**–**5** would help to conclusively answer this question and many of the other interesting questions arising from this study. Nevertheless, the close correlation of the results with our expectations for all five synthetic ligands in the LasR mutants provides the first empirical evidence for these compounds binding to LasR in the same orientation as OdDHL.

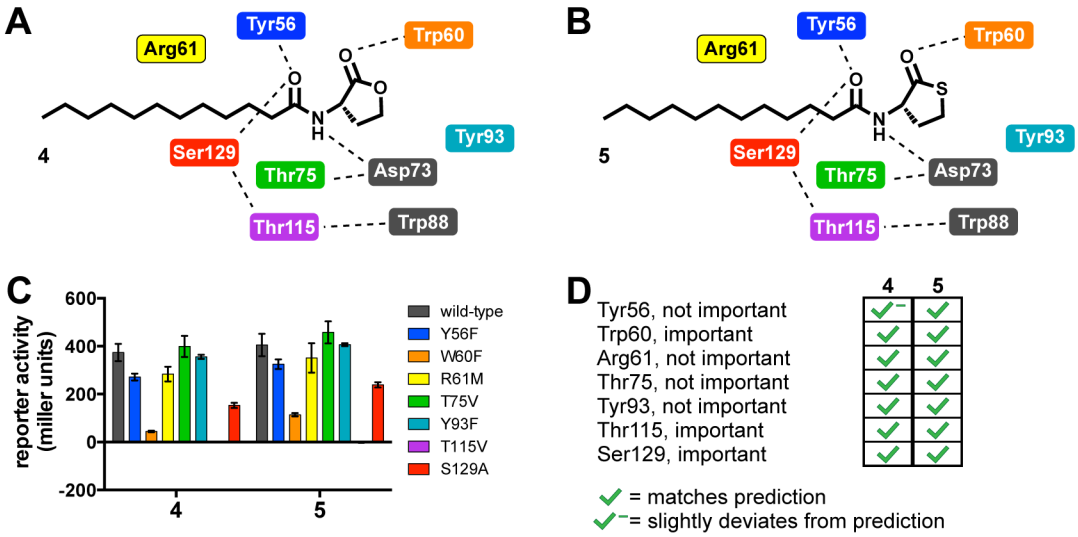


Figure 4.5. Activation of mutant LasRs by ligands 4 and 5.
(A,B) Predicted interactions made by ligands **4** and **5** in the binding pocket of LasR. These ligands lack polar interactions with Arg61. (C) Activation of each mutant by **4** and **5**, each at 100 nM for all mutants. Error bars for all plots indicate s.e.m. from a biological triplicate (n = 3). (D) Predicted and actual importance of interactions for activation by **4** and **5**. A green check indicates the prediction was correct, and a green check-minus indicates the prediction was slightly incorrect.

4.3 Summary and Conclusions

We have performed detailed investigations into the activity of OdDHL and five synthetic analogs on both wild-type LasR and on nine LasR mutants with modifications to their ligand-binding sites. While structural data have been reported for LasR complexed to its native ligand (OdDHL) and to selected non-native TP agonists,[35, 36] prior to the work reported herein, the relative importance of each receptor-ligand polar interaction and the binding modes of AHL-derived LasR modulators were largely unknown. Analysis of the activity profiles for each LasR mutant with OdDHL revealed the following hierarchy of importance for its hydrogen bonds to LasR: amide NH > 3-keto C=O > lactone C=O > amide C=O. This ranking serves to clarify the interactions that should be maintained in the design of next-generation synthetic LasR modulators. One LasR mutation, T75V, actually afforded a mutant that is *more sensitive* to OdDHL than wild-type LasR. Interestingly, Thr75 is often a valine or isoleucine in LasR

homologs, leading us to propose that *P. aeruginosa* actually gains some fitness advantage by having a less-than-maximal sensitivity to OdDHL. Analysis of LasR mutant activity with ligands **1**–**5** strongly supported the hypothesis that all five of these ligands bind the orthosteric ligand-binding pocket in the same orientation as OdDHL, but simply lack certain hydrogen-bonding interactions. Ligand **3** was the unique LasR inhibitor investigated in this study, and as reported previously, the W60F and Y56F mutations flip it to an activator.[39] We also identified a third, new "Janus" mutation for **3** (Y93F), the location of which is hypothesized to be near the binding location of ligand **3**'s phenyl head group. This discovery further supports our model that aniline ligand **3** binds similarly to OdDHL, but makes subtly different interactions that lead to an inactive LasR conformation.[39] In total, this study serves to deepen our understanding of LasR:OdDHL interactions and augments current LasR:OdDHL structural data by providing an expansive set of new mutant activity data with a series of closely related ligands. Moreover, it provides very strong evidence (in the absence of high-resolution structures) that several non-native ligands bind the LasR ligand-binding pocket in a mode similar to OdDHL—findings that will be helpful in guiding efforts to design new non-native LasR modulators.

4.4 Experimental

4.4.1 Compound handling and reagents

OdDHL and ligands **1**–**5** were synthesized as previously reported.[56, 61, 62] Stock solutions of compounds (10 mM) were prepared in DMSO and stored at room temperature in sealed vials. Solvent-resistant polypropylene (Corning Costar cat. no. 3790) and clear polystyrene (Corning Costar cat. no. 3997) 96-well microtiter plates were used as appropriate. All biological reagents were purchased from Fisher and used according to enclosed instructions.

4.4.2 Instrumentation

Absorbance and fluorescence measurements were obtained using a Biotek Synergy monochromator plate reader running Gen5 v1.05 software. A 600 nm filter was used for reading bacterial cell density. Filters of 420 nm and 550 nm were used for Miller-type absorbance assays.

4.4.3 Bacterial strains, media, and growth conditions

The bacterial strain used in this study was *E. coli* DH5α [F^- φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*]. *E. coli* was cultured at 37 ºC in Luria-Bertani (LB) medium and on LB plates with 1.5% agar. For selection and maintenance of plasmids, gentamicin and ampicillin were used at 15 μg/ml and 100 μg/ml respectively.

4.4.4 Construction of mutant LasR reporter strains

Mutant LasR strains were based on the *E. coli* LasR β-galactosidase reporter strain reported by Lee et al.[34] Site-directed mutagenesis was carried out on the LasR-expressing plasmid pJN105L[34] by overlap extension polymerase chain reaction (PCR) (see **Table 4.3** for PCR primers).[63] The mutagenized *lasR* genes were digested with *EcoRI* and *XbaI* and cloned into *EcoRI/XbaI*-cut pJN105L. The mutant pJN105L variants were sequenced to verify mutagenesis and transformed via electroporation into the *E. coli* DH5 α/pSC11[64] reporter strain and selected on LB + gentamicin + ampicillin plates. Mutants Y56F, W60F, R61M, D73L, T75V, W88F, T115V, and S129A were previously reported.[39] Mutant Y93F is new to this study (see **Table 4.4** for complete list of strains and plasmids).

Table 4.3. Primers for site-directed mutagenesis.

Mutation	Forward primer sequence[a]	Reverse primer sequence[a]
flanking primers	CGATTAGAATTCTTAAGAAGAACGTAGCGCTATG	CCACGCTCTAGAGGCAAGA
Y56F(TAC→TTC)	TCGGCAAC**TTC**CCGGCCGC	GCGGCCGG**GAA**GTTGCCGA
W60F(TGG→TTC)	GGCCGCC**TTC**CGCGAGCATT	AATGCTCGCGG**AAG**GCGGCC
R61M(CGC→ATG)	GGCCGCCTGG**ATG**GAGCATTACG	CGTAATGCTC**CAT**CCAGGCGGCC
D73L(GAC→CTC)	GCGGGTC**CTC**CCGACGGTCA	TGACCGTCGG**GAG**GACCCGC
T75V(ACG→GTG)	GGTCGACCCG**GTG**GTCAGTCACT	AGTGACTGAC**CAC**CGGGTCGACC
W88F(TGG→TTC)	GCCGATTTTC**TTC**GAACCGTCCA	TGGACGGTTC**GAA**GAAAATCGGC
Y93F(TAC→TTC)	ACCGTCCATC**TTC**CAGACGCGAA	TTCGCGTCTG**GAA**GATGGACGGT
T115V(ACC→GTC)	GTATGGGCTG**GTC**ATGCCGCTGC	GCAGCGGCAT**GAC**CAGCCCATAC
S129A(AGC→GCC)	GCGCGCTG**GCC**CTCAGCGT	ACGCTGAG**GGC**CAGCGCGC

[a]**Bold** = mutated codon

Table 4.4. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description*	Reference
E. coli		
DH5a	F$^-$, j80d*lacZ*DM15D(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(rk$^-$, mk$^+$) *phoA supE*44 λ$^-$ *thi*-1 *gyrA*96 *relA*1	Invitrogen
Plasmids		
pJN105L	Arabinose-inducible expression plasmid for *lasR*; Gm^R	[34]
pSC11	*lasI-lacZ* transcriptional fusion reporter plasmid; Ap^R	[64]
pJG002	Y56F mutant analog of pJN105L	[39]
pJG005	W88F mutant analog of pJN105L	[39]
pJG008	W60F mutant analog of pJN105L	[39]
pJG009	R61M mutant analog of pJN105L	[39]
pJG010	D73L mutant analog of pJN105L	[39]
pJG011	T75V mutant analog of pJN105L	[39]
pJG012	Y93F mutant analog of pJN105L	This study
pJG0013	T115V mutant analog of pJN105L	[39]
pJG014	S129A mutant analog of pJN105L	[39]

*Abbreviations: Gm^R, gentamicin resistance; Ap^R, ampicillin resistance

4.4.5 Reporter gene assays

β-galactosidase reporter assays were conducted as previously reported for the wild-type LasR strain.[56, 61] All LasR mutant strains were evaluated for activation by OdDHL at both 10 μM and through dose-response analysis. Non-native ligands were tested for LasR activation at 10 μM in each strain. Similarly, ligands were tested for LasR inhibition at 10 μM against OdDHL at its EC_{50} value for the mutant LasR strain.

4.4.6 Determining conservation of homologous residues between LasR homologs

A Concise Microbial Protein BLAST search was performed using blastp on all proteins from complete genomes in the protein cluster database (ProtClustDB) using the LasR sequence aag04819.1 (https://www.ncbi.nlm.nih.gov/genomes/prokhits.cgi). The top 100 hits were imported into Geneious Pro 5.5.8 and aligned with a ClustalW alignment using the BLOSUM cost matrix with gap open cost of 10 and gap extend cost of 0.1. See full alignments in *Section 4.5.3*. The frequency of each amino acid at the mutated residues was cataloged within Geneious (comprehensive data in **Table 4.6**, summary presented in **Table 4.1**).

4.5 Notes

4.5.1 Catalog of previous mutations to active-site polar residues in LuxR-type proteins

Table 4.5 presents a selection of relevant previously reported mutations to LasR and homologous LuxR-type proteins.

Table 4.5. Catalog of LasR mutants generated in this study and selected previously reported data for LuxR-type proteins with related mutations.

This study	*Previous studies*			
LasR mutation	LuxR-type protein	Mutation	Effect on activity with native ligand	Reference
Y56F	LasR	Y56C	not determined	[65]
	LasR	Y56F	no change	[47]
	LuxR	Y62F	significant decrease in sensitivity	[42]
	TraR	Y53F	moderate decrease in sensitivity	[45]
	TraR	Y53S	inactive or major decrease in sensitivity	[45]
W60F	LasR	W60F	moderate decrease in sensitivity	[47]
	LuxR	W66H	inactive	[42]
	TraR	W57F	inactive or major decrease in sensitivity	[45]
	TraR	W57Y	inactive or major decrease in sensitivity	[45]
R61M	LasR	R61M	moderate decrease in sensitivity	[49]
	LuxR	R67M	inactive or major decrease in sensitivity	[49]
D73L	LasR	D73E	loss in activity (sensitivity not reported)	[41]
	LasR	D73N	not determined	[66]
	LuxR	D79N	inactive	[42, 43]
	TraR	D70E	inactive	[44]
	TraR	D70N	inactive	[44]
	TraR	D70S	inactive or major decrease in sensitivity	[45]
	TraR	D70V	inactive or major decrease in sensitivity	[45]
	RhlR	D81E	did not bind native ligand *in vitro*	[46]
T75V	LasR	T75K	not determined	[65]
W88F	LasR	W88Y	loss in activity (sensitivity not reported)	[41]
	LasR	W88C	not determined	[67]
	LuxR	W94D	inactive	[42]
	LuxR	W94Q	could not be stably expressed	[48]
Y93F	none			
T115V	LasR	T115I	isolated PAO1 mutant had major deficiencies in producing OdDHL, BHL, elastase, and nucleoside hydrolase	[54, 55]
	TraR	T115I	significant decrease in sensitivity	[45]
S129A	TraR	T129S	no change	[45]
	TraR	T129A	inactive or major decrease in sensitivity	[45]
	TraR	T129V	significant decrease in sensitivity	[45]
	TraR	T129L	inactive or major decrease in sensitivity	[45]
	TraR	T129I	inactive or major decrease in sensitivity	[45]
	TraR	T129F	inactive or major decrease in sensitivity	[45]
N/A*	CviR	M89A	moderate decrease in sensitivity	[38]
	CviR	M89S	moderate decrease in sensitivity	[38]
	CviR	M89L	no change	[38]
	CviR	M89F	no change	[38]
	CviR’	S89M/ N77Y	maximum activity substantially decreased	[38]
N/A*	QscR	S56G	moderate decrease in sensitivity	[53]
	QscR	S56T	slight decrease in sensitivity	[53]

* Nearby residues in CviR and QscR not examined in this study; data included here to be comprehensive

4.5.2 Dose-response curves

Dose-response curves from which the EC_{50} values in **Tables 4.1** and **4.2** were calculated are presented in **Figures 4.6** and **4.7**.

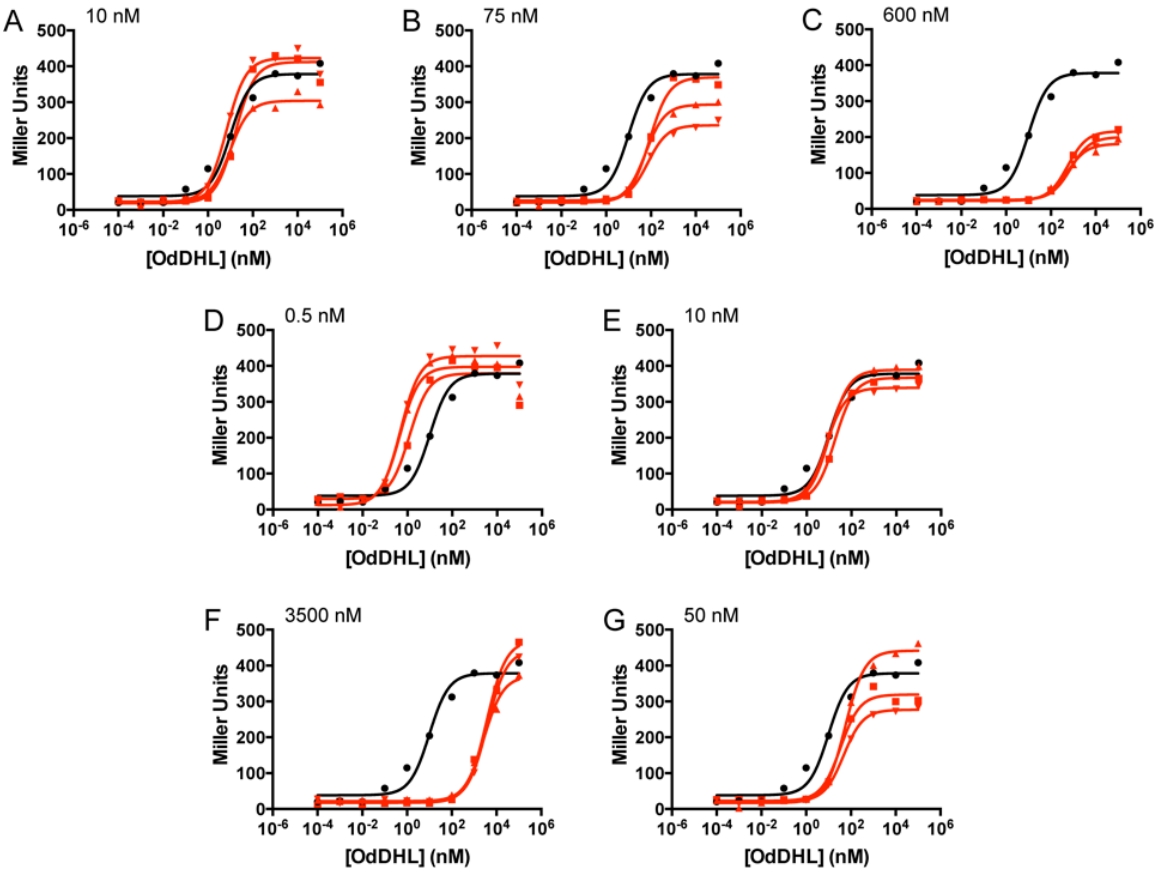


Figure 4.6. Dose-response curves for mutant LasRs with OdDHL.
OdDHL dose-response curves used to calculate EC_{50} values for OdDHL in each LasR mutant: (A) Y56F, (B) W60F, (C) R61M, (D) T75V, (E) Y93F, (F) T115V, and (G) S129A. For each plot, the three red curves compose a biological triplicate of data for OdDHL in the mutant, and the black curve is a representative replicate for OdDHL in wild-type LasR for comparison. The geometric mean of each EC_{50} value is displayed on each plot and in **Table 4.1**.

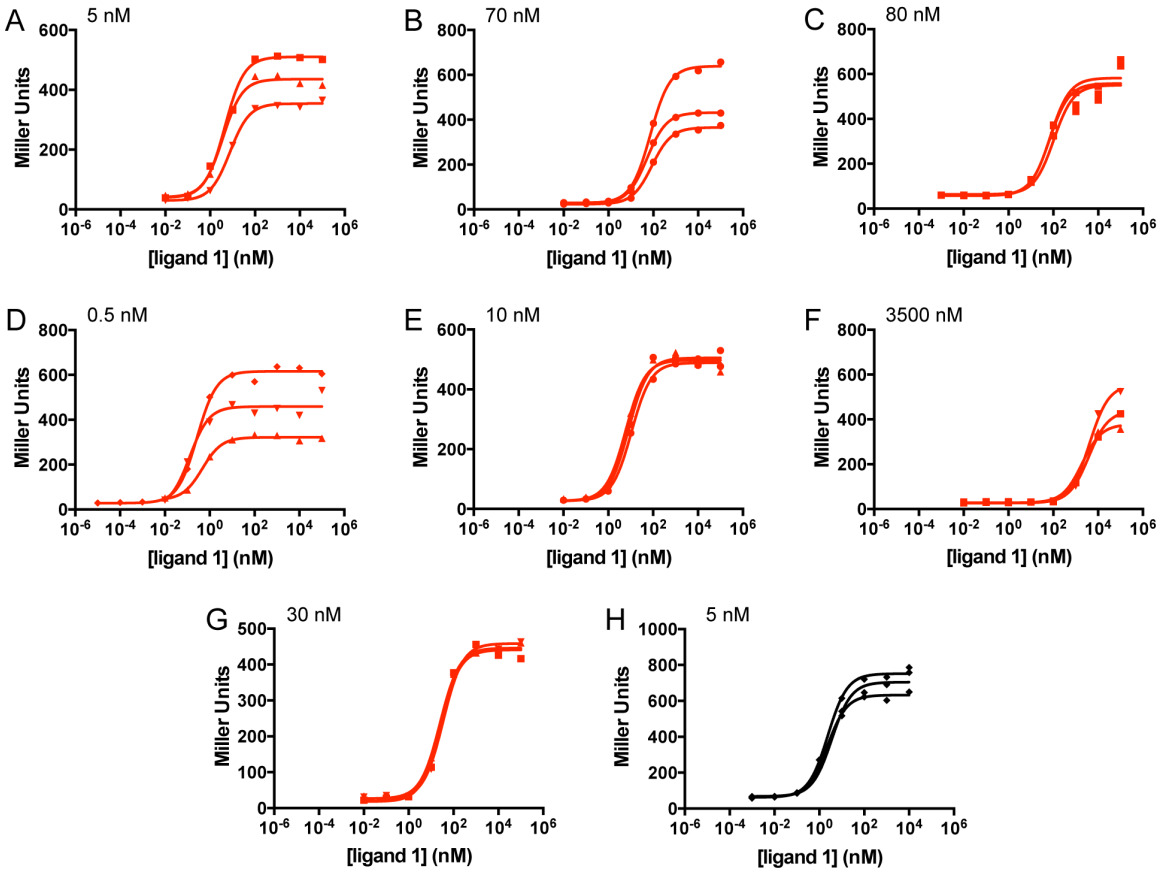


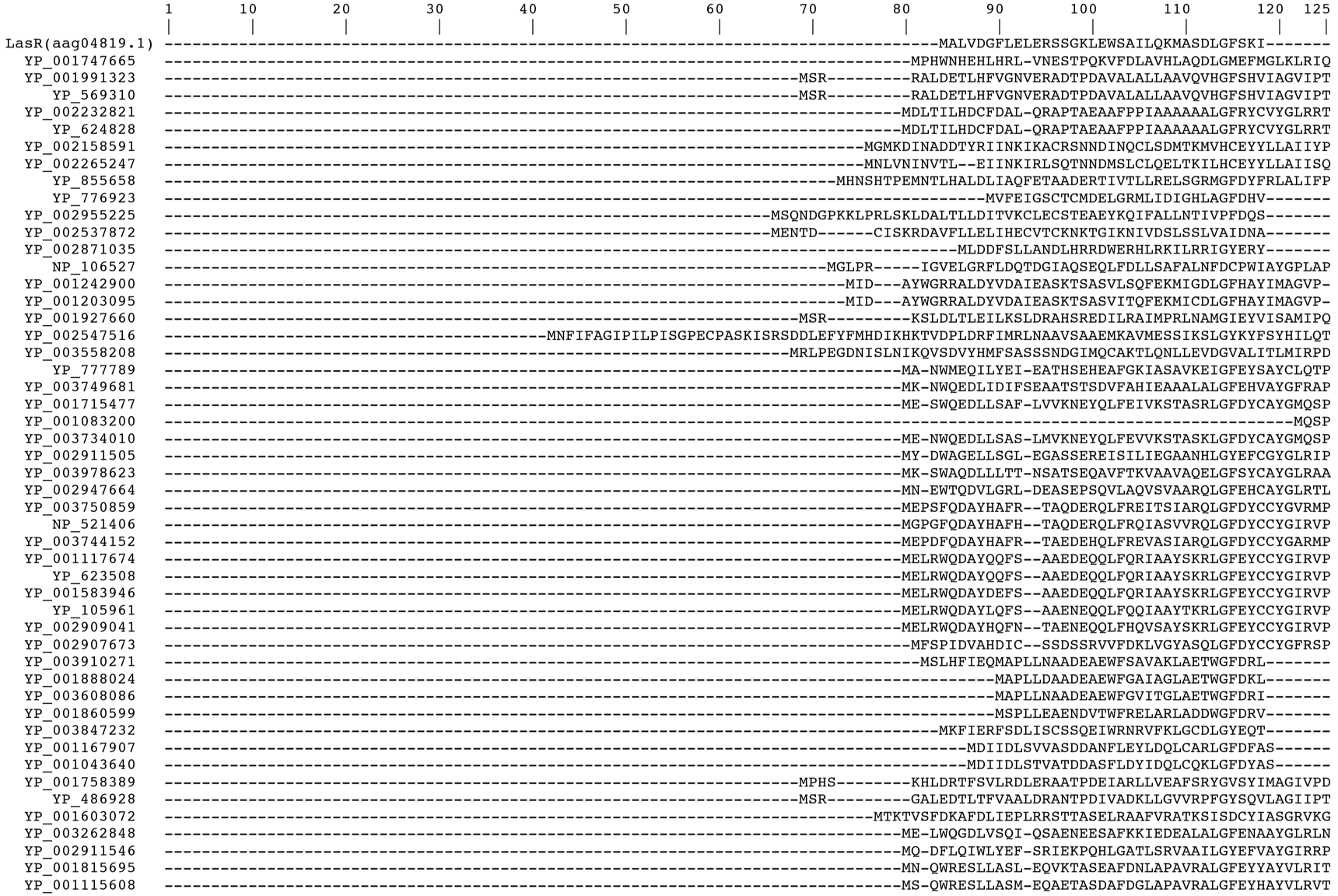
Figure 4.7. Dose-response curves for mutant LasRs with ligand 1.
Ligand **1** dose-response curves used to calculate EC_{50} values for **1** in each LasR mutant: (A) Y56F, (B) W60F, (C) R61M, (D) T75V, (E) Y93F, (F) T115V, and (G) S129A. Panel (H) shows the dose-response curve for **1** in wild-type LasR. For each plot, the three curves compose a biological triplicate of data for **1**. The geometric mean of each EC_{50} value is displayed on each plot and in **Table 4.2**.

4.5.3 Entire residue conservation data and sequence alignments

Table 4.6 on the following page lists the abundance of each amino acid at the mutated positions in all 100 aligned LasR homologs (a summary is reported in **Table 4.1)**. The next six pages contain the full sequence alignments from which the percent identities were derived. LasR is listed first, followed by the other 100 proteins. The mutated residues are boxed for clarity.

Table 4.6. Conservation among 100 LasR homologs at mutated sites in this study.
See following page for table. The next six pages show the full sequence alignments from which this table was derived. The residues marked with boxes are those that were mutated in this study.

	Y56		W60		R61		D73		T75		W88		Y93F		T115		S129	
residue	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%
A	1	1.0	0	0.0	4	4.0	0	0.0	3	3.0	0	0.0	5	6.8	16	16.2	0	0.0
C	3	3.0	0	0.0	1	1.0	0	0.0	0	0.0	0	0.0	1	1.4	7	7.1	5	5.1
D	0	0.0	0	0.0	0	0.0	99	100.0	0	0.0	0	0.0	3	4.1	0	0.0	0	0.0
E	0	0.0	0	0.0	2	2.0	0	0.0	0	0.0	0	0.0	4	5.5	0	0.0	0	0.0
F	4	4.0	0	0.0	10	10.1	0	0.0	0	0.0	0	0.0	15	20.5	0	0.0	1	1.0
G	0	0.0	0	0.0	2	2.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.0	2	2.0
H	3	3.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
I	0	0.0	0	0.0	5	5.1	0	0.0	15	15.2	0	0.0	3	4.0	1	1.0	0	0.0
K	1	1.0	0	0.0	6	6.1	0	0.0	0	0.0	0	0.0	6	8.0	0	0.0	0	0.0
L	1	1.0	1	1.0	12	12.1	0	0.0	1	1.0	0	0.0	8	11.0	0	0.0	2	2.0
M	1	1.0	0	0.0	10	10.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
N	1	1.0	0	0.0	2	2.0	0	0.0	0	0.0	0	0.0	4	5.5	0	0.0	4	4.0
P	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Q	0	0.0	0	0.0	7	7.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
R	0	0.0	0	0.0	14	14.1	0	0.0	0	0.0	0	0.0	9	12.3	0	0.0	0	0.0
S	0	0.0	0	0.0	4	4.0	0	0.0	1	1.0	0	0.0	1	1.4	28	28.3	65	65.7
T	2	2.0	0	0.0	2	2.0	0	0.0	26	26.3	0	0.0	3	4.1	45	45.5	20	20.2
V	0	0.0	0	0.0	18	18.2	0	0.0	53	53.5	0	0.0	8	11.0	1	1.0	0	0.0
W	10	10.1	97	98.0	0	0.0	0	0.0	0	0.0	99	100.0	0	0.0	0	0.0	0	0.0
Y	72	72.7	1	1.0	0	0.0	0	0.0	0	0.0	0	0.0	3	4.1	0	0.0	0	0.0
Gap	0	–	0	–	0	–	0	–	0	–	0	–	26	–	0	–	0	–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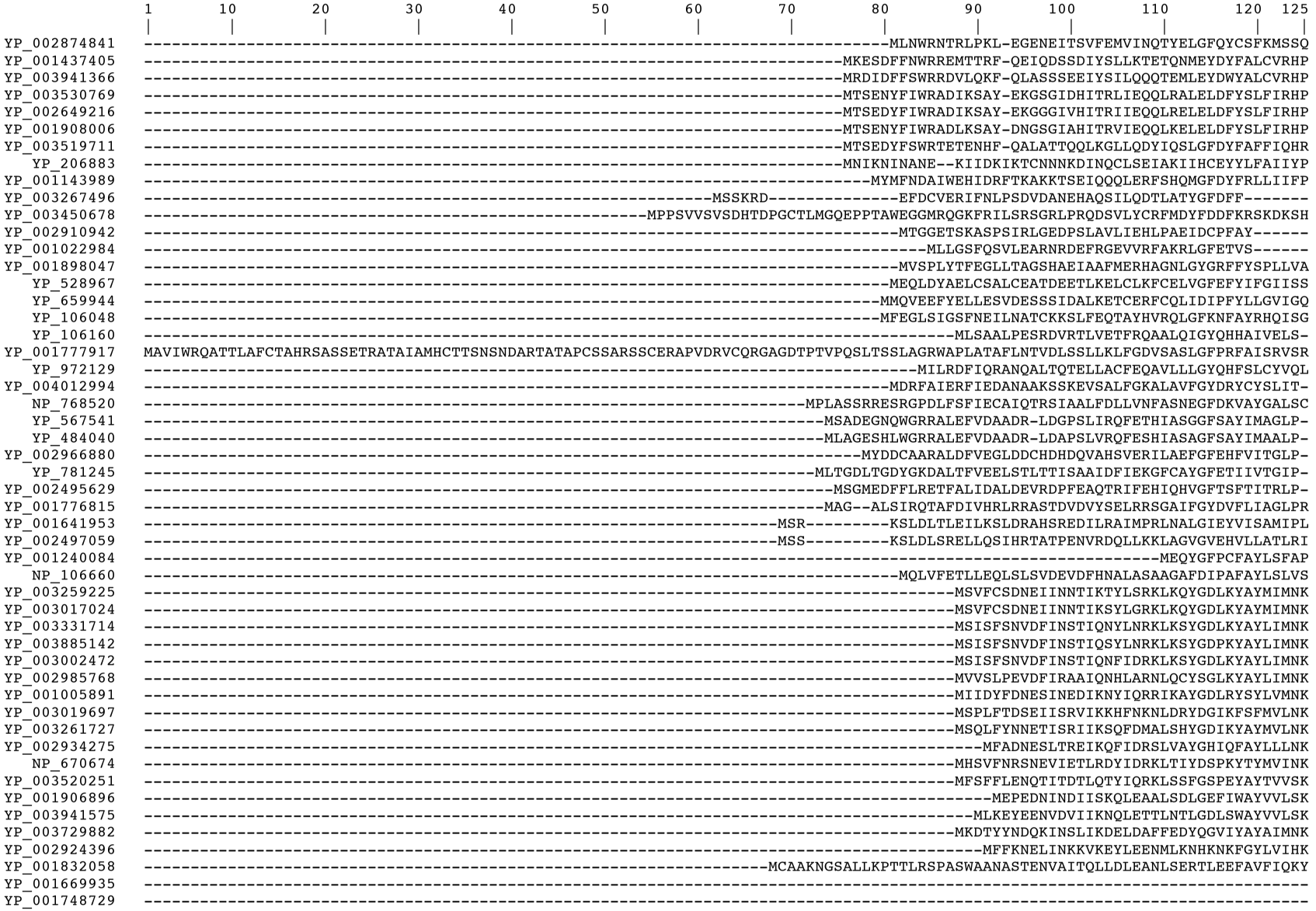
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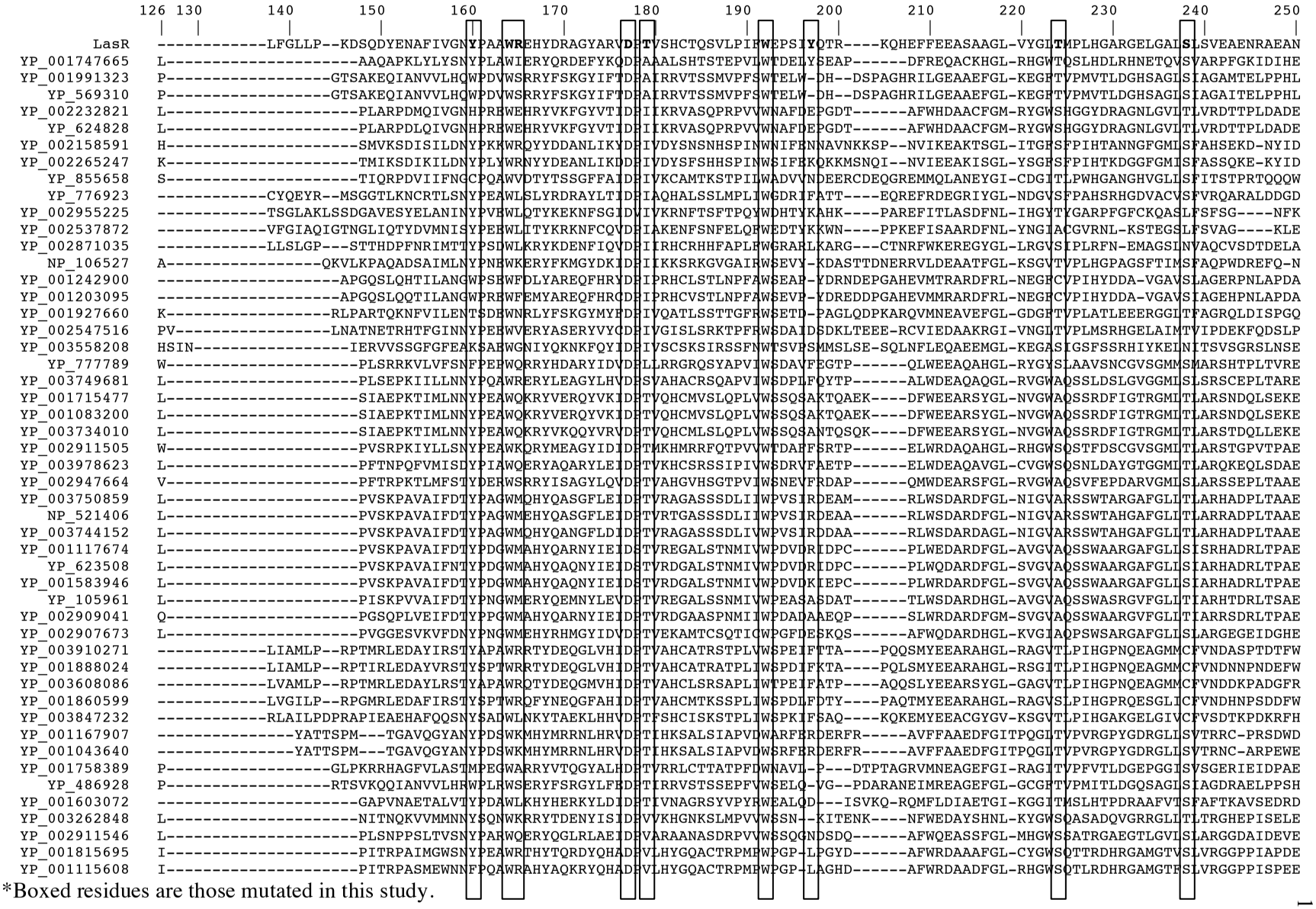
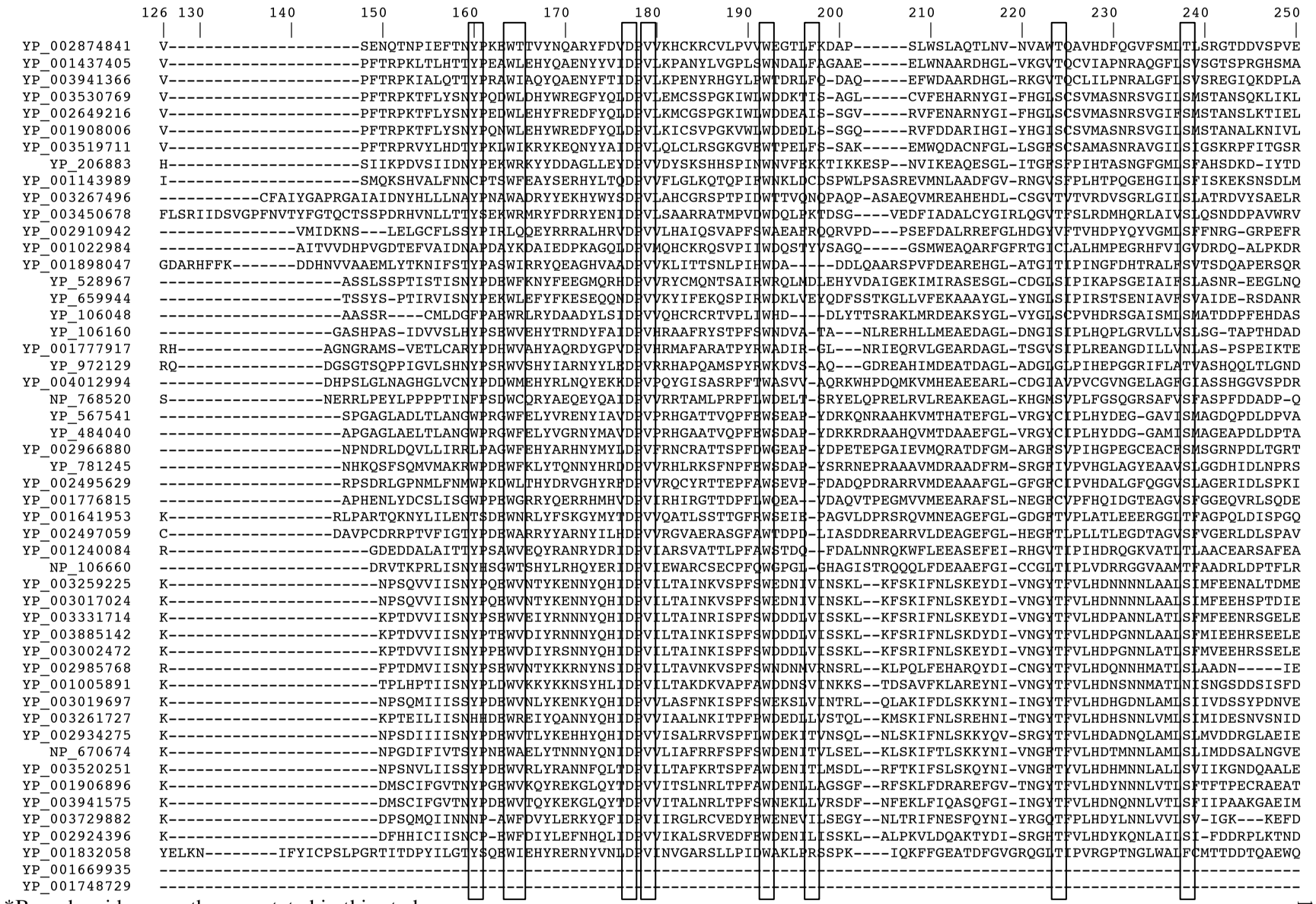
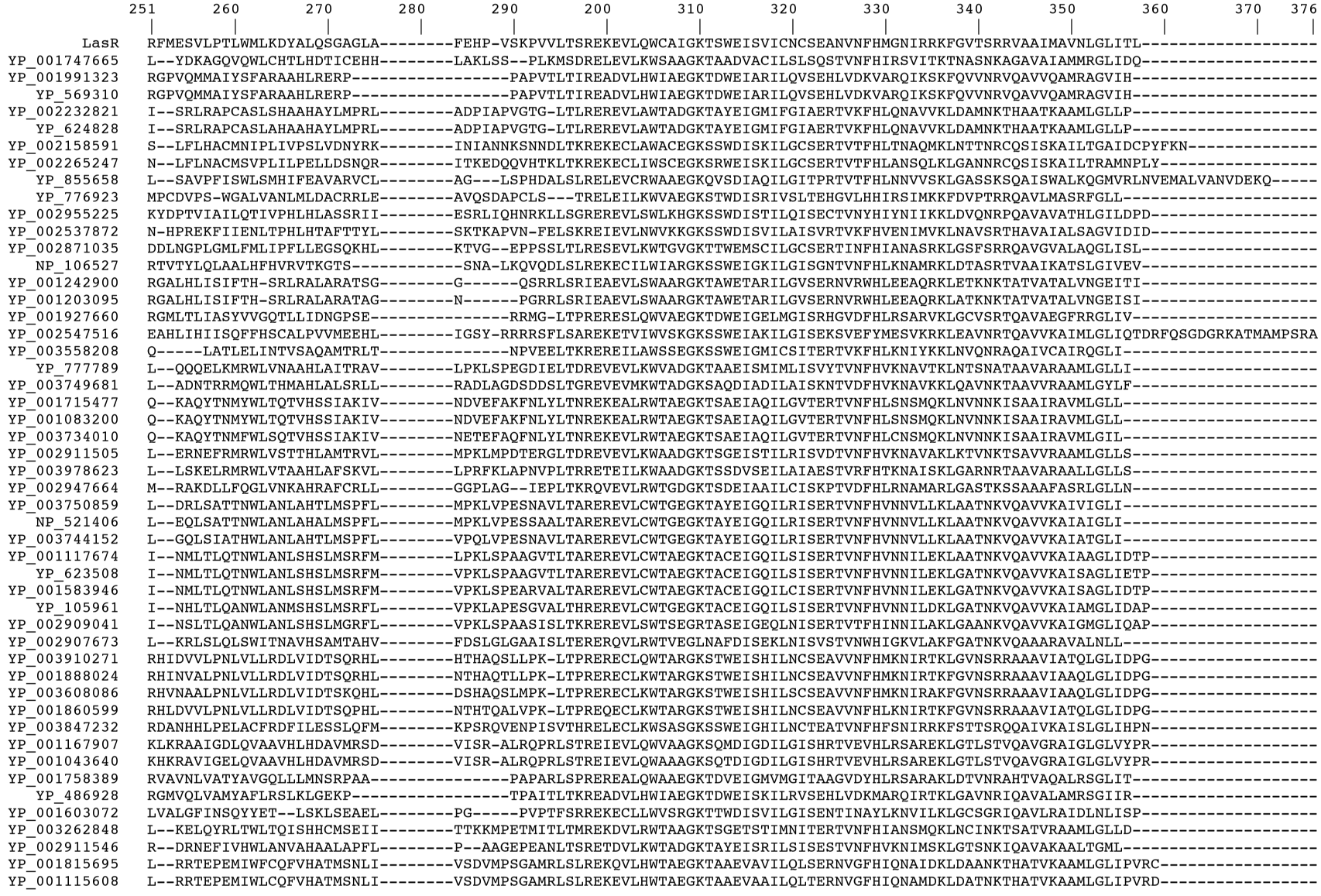


*Boxed residues are those mutated in this study.



*Boxed residues are those mutated in this study.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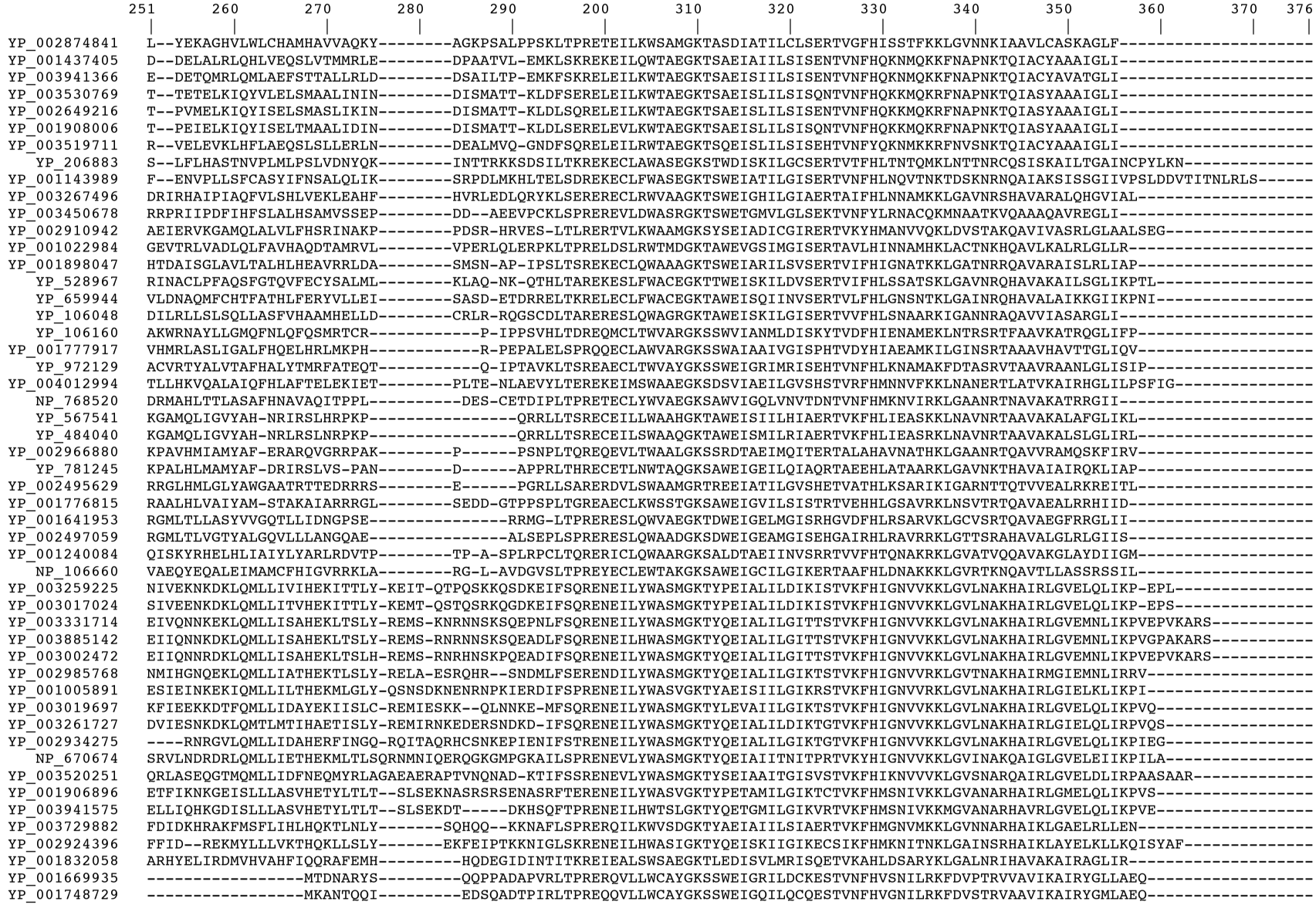
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CHAPTER 5:

ACTIVE EFFLUX INFLUENCES THE POTENCY OF QUORUM SENSING INHIBITORS IN *PSEUDOMONAS AERUGINOSA*

Contribution: J. P. G. initiated the project and prepared the efflux-pump mutant strain of *P. aeruginosa*. J. D. M. performed all bacterial screening assays. N. R. Eibergen prepared the pPROBE-KL reporter plasmid. J. D. M. and H. E. B. wrote the manuscript.

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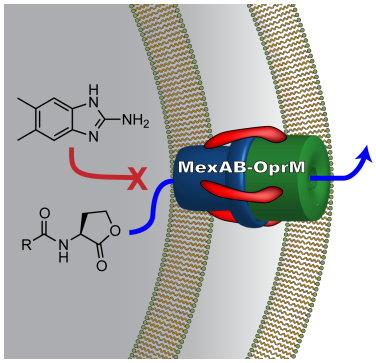
Abstract.



Many bacteria regulate gene expression through a cell-cell signaling process called quorum sensing (QS). In proteobacteria, QS is largely mediated by signaling molecules known as *N*-acylated L-homoserine lactones (AHLs) and their associated intracellular LuxR-type receptors. The design of non-native small molecules capable of inhibiting LuxR-type receptors, and thereby QS, in proteobacteria is an active area of research, and numerous lead compounds are AHL derivatives that mimic native AHL signals. Much of this past work has focused on the pathogen *Pseudomonas aeruginosa*, which controls an arsenal of virulence factors and biofilm formation through QS. The MexAB-OprM drug efflux pump has been shown to play a role in the secretion of the major AHL signal in *P. aeruginosa*, *N*-(3-oxododecanoyl) L-homoserine lactone. In the current study, we show that a variety of non-native AHLs and related derivatives capable of inhibiting LuxR-type receptors in *P. aeruginosa* display significantly higher potency in a *P. aeruginosa* Δ(*mexAB-oprM*) mutant, suggesting that MexAB-OprM also recognizes these compounds as substrates. We also demonstrate that the potency of 5,6-dimethyl-2-aminobenzimidazole, recently shown to be a QS and biofilm inhibitor in *P. aeruginosa*, is not affected by the presence or absence of the MexAB-OprM pump. These results have implications for the use of non-native AHLs and related derivatives as QS modulators in *P. aeruginosa* and

other bacteria, and provide a potential design strategy for the development of new QS modulators that are resistant to active efflux.

5.1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen responsible for life-threatening infections in immunocompromised patients, such as those suffering from AIDS, burn wounds, or cystic fibrosis.[1-3] These infections are often refractory to treatment with common antibiotics due to the emergence of multidrug-resistant (MDR) strains of *P. aeruginosa*, which has prompted an urgent need for the development of orthogonal treatment strategies. Targeting virulence phenotypes rather than general cell growth represents one potential strategy. Significant advances toward such "anti-virulence" therapies include the inhibition of biofilm formation,[4] toxin production,[5] bacterial adhesion factors,[6] and quorum sensing (QS).[7-10] Small molecules that target these pathways could find broad use as both research tools and new therapeutic agents. In particular, the design of QS inhibitors for *P. aeruginosa* and other bacterial pathogens has attracted significant attention over the past ~20 years.[11, 12] The efficacy of such compounds as QS inhibitors in *P. aeruginosa* is the focus of the current study.

QS is widespread in bacteria and allows the coordination of gene expression with bacterial population density.[13, 14] This intercellular communication pathway is mediated by small molecules or peptides (i.e., autoinducers) that vary in concentration as a function of cell number. At high cell densities, the signals reach a sufficient concentration to bind and activate QS receptors, which subsequently regulate transcription of primarily group-beneficial genes. Proteobacteria use *N*-acylated L-homoserine lactones (AHLs) as their main autoinducers. The AHL signals are generated by LuxI-type synthases and sensed by cytoplasmic LuxR-type receptors. *P. aeruginosa* has two LuxI/LuxR pairs, LasI/LasR and RhlI/RhlR, which produce and sense *N*-(3-oxododecanoyl) L-homoserine lactone (OdDHL) and *N*-butanoyl L-homoserine lactone (BHL), respectively. QscR, an additional "orphan" or "solo" LuxR-type receptor in *P. aeruginosa*, lacks an associated LuxI-type synthase; instead, it also recognizes the OdDHL

signal produced by LasI. LasR is believed to play an important role in controlling virulence in *P. aeruginosa* and regulates the production of elastase B, exotoxin A, and the biosynthesis machinery for a number of metabolites related to host tissue breakdown.[15, 16] Furthermore, clinical isolates of *P. aeruginosa* strains lacking a functional *las* system are less virulent in animal infection models, suggesting that successful LasR inhibition could significantly attenuate *P. aeruginosa* virulence.[17-20]

Our laboratory and others have synthesized and examined a range of non-native AHLs as LasR and QscR modulators in *P. aeruginosa*.[21-24] Of the >150 compounds in our in-house libraries of AHLs, we have identified a number of LasR inhibitors using an *E. coli* reporter strain to measure LasR-mediated transcriptional activation.[25-29] However, the potencies of these compounds in *P. aeruginosa* LasR reporter strains are generally muted in comparison.[30] Meijler and co-workers observed similar effects in their studies of both covalent and non-covalent inhibitors of LasR in related *E. coli* and *P. aeruginosa* reporter strains.[31] In general, the efficacy of small molecule drugs is often lower in *P. aeruginosa* relative to many other Gram-negative bacteria due to decreased membrane permeability, enhanced active efflux, or a combination of both factors,[32-35] which prompted us to consider the possibility that these features could also influence the potency of our synthetic LasR modulators.

In 1999, Iglewski and co-workers showed that OdDHL passively diffuses across the *P. aeruginosa* cell membrane (albeit at a ~10-fold slower rate than the shorter-chain autoinducer BHL) and that the presence of the efflux pump MexAB-OprM significantly reduces the intracellular concentration of OdDHL, suggesting that MexAB-OprM recognizes OdDHL as a substrate.[36] In concurrent work, Poole and co-workers demonstrated that a *P. aeruginosa* mutant capable of MexAB-OprM overexpression produced reduced levels of QS-regulated virulence factors, presumably due to low levels of intracellular OdDHL.[37] MexAB-OprM is a member of

the resistance-nodulation-division (RND) family of efflux pumps, which are a main class of pumps in Gram-negative bacteria known to contribute to intrinsic and acquired resistance to exogenous compounds.[38] Given that RND pumps often possess broad substrate profiles, we reasoned that active efflux could play a role in reducing the potency of our AHL-derived LasR inhibitors in *P. aeruginosa*, and that, in particular, MexAB-OprM recognizes these non-native AHLs in a similar manner as it does OdDHL. Recent studies by Gotoh and co-workers—showing that MexAB-OprM can transport a small set of naturally occurring 3-oxo-acyl HLs (with 8–14 carbon acyl tails)—provide support for this hypothesis.[39, 40,41] To our knowledge, systematic investigations into the effect of active efflux on AHL-derived and non-AHL QS inhibitors are yet to be reported.

Herein, we report our analysis of the potency of synthetic LasR antagonists in the presence and absence of the MexAB-OprM drug efflux pump. We generated a *P. aeruginosa* Δ(*mexAB-oprM*) mutant and used a GFP reporter of LasR activity to examine compound antagonism trends relative to a strain with an operative pump. We demonstrate that both OdDHL and non-native AHL analogues suffer significant decreases in potency when subjected to active efflux by MexAB-OprM, but a previously reported biofilm and LasR inhibitor in *P. aeruginosa*, 5,6-dimethyl-2-aminobenzimidazole (DMABI)[42] does not display this efflux-induced reduction in potency

5.2 Results

5.2.1 P. aeruginosa MexAB-OprM reduces the potency of OdDHL

We began our study by comparing the potency of OdDHL in a *P. aeruginosa* mutant lacking both the AHL synthases LasI and RhlI (PAO-JP2; i.e., pump-active) and a mutant lacking both AHL synthases and the MexAB-OprM pump (PAO-JG21; i.e., pump-mutant). Both strains

contained a functional LasR receptor and reported LasR activity via a p*lasI*-LVAgfp reporter plasmid (see **section 5.5**). Dose responses for LasR activation by OdDHL in the pump-active and pump-mutant *P. aeruginosa* strains were obtained. As we hypothesized, OdDHL was a more potent activator of LasR in the pump-mutant strain relative to the pump-active strain (**Figure 5.1**). The EC_{50} value shifted from 95 nM in the pump-active PAO-JP2 to 6.6 nM in the pump-mutant PAO-JG21. This > 10-fold increase in potency for OdDHL in the pump-mutant strain largely correlates with prior experiments by Iglewski and co-workers that showed at least a 3-fold increased cellular concentration of [^{3}H]-OdDHL in a *P. aeruginosa* Δ(*mexAB-oprM*) mutant.[43]

We next evaluated the possibility that other RND efflux pumps could be involved in active efflux of OdDHL in *P. aeruginosa*. The *P. aeruginosa* genome contains up to 12 RND efflux systems, seven of which have been directly shown to recognize a wide range of drugs.[33, 44] If other RND pumps recognize OdDHL as a substrate, we reasoned that the addition of a non-specific inhibitor of RND-type pumps would result in a further decreased EC_{50} value for OdDHL. Dose responses for LasR activation by OdDHL in the pump-active PAO-JP2 were performed in the presence of the non-specific RND-type pump inhibitor, Phe-Arg-β-naphthylamide (PAβN; **Figure 5.1**).[45] The EC_{50} value for OdDHL in PAβN-treated pump-active PAO-JP2 was 9.0 nM, which was statistically indistinguishable from the EC_{50} value of 6.6 nM for OdDHL in the untreated pump-mutant PAO-JG21 ($p > 0.60$ as measured by a t-test). To ensure that the effects of PAβN inhibition and Δ(*mexAB-oprM*) mutation were not additive, an OdDHL dose response analysis was also performed in pump-mutant PAO-JG21 in the presence of PAβN. The EC_{50} value for OdDHL in this experiment was 7.2 nM, again indistinguishable from the values for the untreated pump-mutant and the PAβN-treated pump-active strain (**Figure 5.1**). These data support the hypothesis that OdDHL is not a substrate of other *P. aeruginosa* RND pumps.

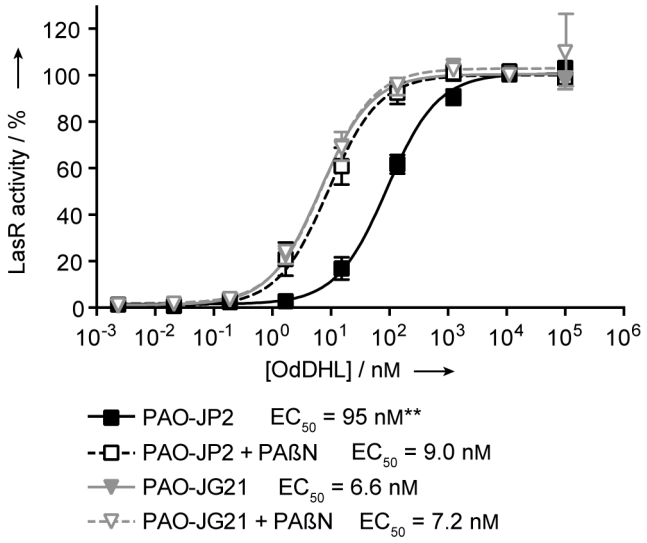


Figure 5.1. MexAB-OprM decreases the potency of OdDHL in *P. aeruginosa*.
Dose responses are shown for OdDHL in pump-active PAO-JP2 and pump-mutant PAO-JG21 in the presence and absence of the non-specific efflux pump inhibitor PAßN. LasR activity was measured by expression of the *lasI*::*gfp*[LVA] transcriptional fusion. Both pump-active PAO-JP2 and pump-mutant PAO-JG21 displayed a similar dynamic range of response to OdDHL, but PAO-JG21 showed stronger LasR activity at lower OdDHL concentrations. The potency of OdDHL in the pump-active PAO-JP2 in the presence of PAßN was comparable to the potency of OdDHL in the pump-mutant PAO-JG21 in the absence of PAßN. No additive effect on the increased potency of OdDHL was observed in the pump-mutant PAO-JG21 in the presence of PAßN. Error bars represent SEM of four trials for each strain. ** – $p < 0.01$ as measured by a t-test performed on the log(EC_{50}) of each trial.

5.2.2 P. aeruginosa Δ(mexAB-oprM) and E. coli LasR reporters respond similarly to non-native AHLs

As highlighted above, our prior biological evaluation of non-native AHL libraries revealed a number of potent LasR inhibitors using an *E. coli* reporter strain to measure LasR-mediated transcriptional activation.[25-27] However, the activities of these compounds in *P. aeruginosa* LasR reporter strains were generally muted.[30] The increased potency of OdDHL in pump-mutant PAO-JG21 (shown above) suggested that efflux by MexAB-OprM might contribute to the discrepancies observed for AHL-induced modulation of LasR activity in *P. aeruginosa* as compared to that in *E. coli*. We thus performed side-by-side screens of our ~150-member non-native AHL library (shown in **Figures 5.4**–**5.7**) for LasR antagonistic activity in the *E. coli*

JLD271, pump-active PAO-JP2, and pump-mutant PAO-JG21 reporter strains in an attempt to resolve the source of these discrepancies. In these competitive antagonism assays, non-native compound was screened against OdDHL at its EC_{50} value in each of the reporter strains. To facilitate more rapid-throughput screening in *E. coli*, a new fluorescence-based reporter, pPROBE-KL, was designed for use in JLD271 (see *section 5.5.4*). The plasmid pPROBE-KL harbors a *lasI* promoter that has been transcriptionally fused to *gfp*, allowing for the production of GFP upon activation of LasR.

The reporter gene assays yielded several interesting data trends (see **Figures 5.8–5.13** for full primary data). The LasR inhibitory activities of our non-native AHLs were modest in PAO-JP2, with only 11 AHLs showing ≥25% LasR inhibition (structures shown in **Figure 5.3A**; percent inhibition values listed in **Table 5.3**). In contrast, the AHLs were significantly more active in PAO-JG21, with 84 compounds showing ≥25% LasR inhibition, and 11 compounds showing ≥80% LasR inhibition (**Table 5.4**). The number of strong LasR inhibitors in *E. coli* was similar to that in PAO-JG21: 77 compounds showed ≥25% LasR inhibition, and 13 compounds showed ≥80% LasR inhibition (**Table 5.5**).[46]

Analysis of these three sets of assay data revealed that the LasR antagonism trends observed for non-native ligands in *E. coli* and in the *P. aeruginosa* pump-mutant were strikingly conserved. Several of the strongest LasR inhibitors were shared amongst these two strains (e.g., AHLs **E3, E5**, **E10**, and **E27**; **Tables S2–S3**). To more quantitatively compare the three sets of assay data, we calculated Pearson product-moment correlation coefficients, which are commonly used to measure the linear dependence of two measured variables (**Figure 5.2**).[47] The LasR inhibitory activities of compounds in *E. coli* correlated much more strongly to pump-mutant PAO-JG21 (r = 0.7848) than to pump-active PAO-JP2 (r = 0.2834) (**Figure 5.2B** vs. **Figure 5.2A**). Additionally, a least-squares linear regression of the data in **Figure 5.2B** returned a trend line highly suggestive

of one-to-one correspondence of LasR inhibitory activities. Notably, the strong correlation of antagonistic activities occurs despite the fact that membrane permeability and expression levels of LasR may not be similar in the *E. coli* and *P. aeruginosa* strains. These results suggest that—despite the differences between *E. coli* and *P. aeruginosa*—active efflux by MexAB-OprM is a primary cause of the discrepancies between the LasR inhibitory activities of our non-native AHLs in pump-active *P. aeruginosa* and the LasR inhibitory activities of the same compounds in the *E. coli* reporter.

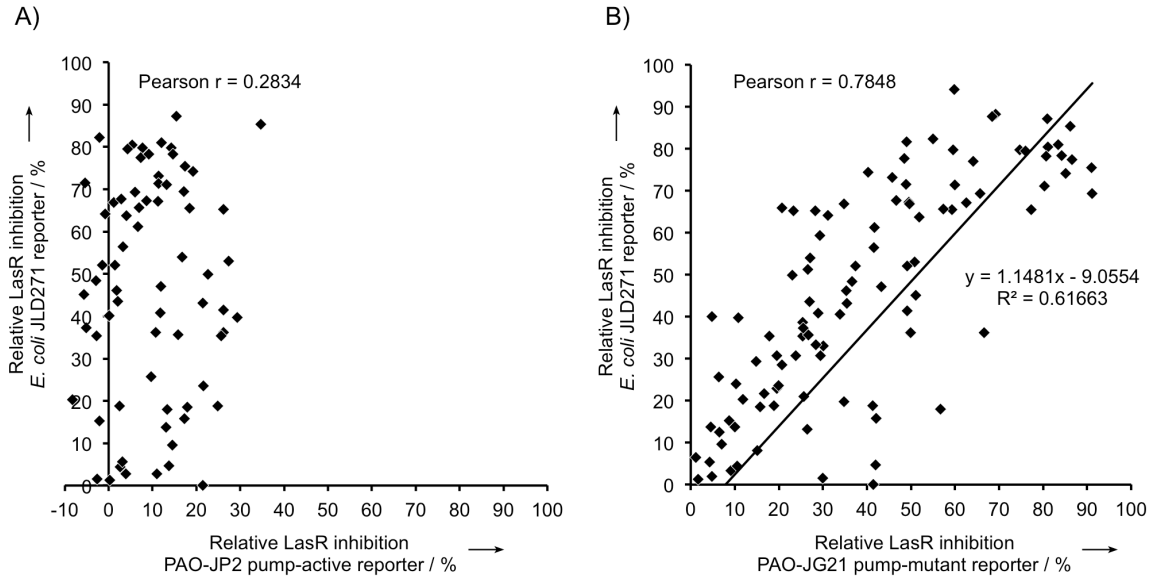


Figure 5.2. Comparative analyses of LasR inhibition data for non-native AHLs in *P. aeruginosa* and *E. coli* LasR reporter strains.
Non-native AHLs show decreased LasR inhibition in A) pump-active *P. aeruginosa* PAO-JP2 as compared with pump-mutant *P aeruginosa* PAO-JG21, but inhibitory activities strongly correlate in B) *E. coli* JLD271 as compared with pump-mutant PAO-JG21. Linear correlations of data sets in A) and B) were compared by calculating the Pearson product-moment correlation coefficient (Pearson r). Each point represents a different non-native AHL. Data were obtained from three separate trials. Error bars are not included for clarity, but SEM of all primary screening data did not exceed ±10%.

5.2.3 MexAB-OprM reduces potency of non-native AHLs and related inhibitors

The results from the LasR antagonism screens above suggest that effective QS inhibitors in wild-type *P. aeruginosa* will require attributes that allow them to bypass the mechanism of

MexAB-OprM efflux. As previously mentioned, evading efflux is an important issue in the development of effective antibiotics,[32, 35, 48] and in fact any small molecule agent acting via an intracellular target, in *P. aeruginosa*. While the primary data above indicated that non-native AHL activities were influenced by MexAB-OprM, we sought to determine if certain types of acyl chain structures rendered an AHL more or less susceptible to efflux by MexAB-OprM.

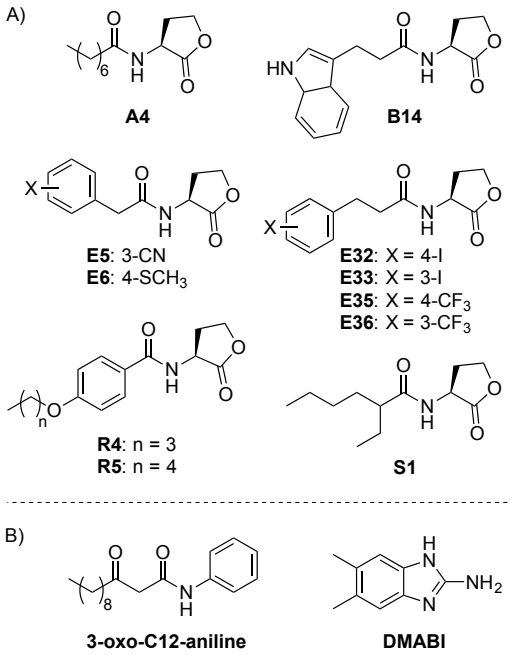


Figure 5.3. Structures of compounds subjected to LasR antagonism dose-response analyses in this study.
A) AHLs exhibiting ≥25% inhibition of LasR in the primary LasR antagonism screen in pump-active *P. aeruginosa* PAO-JP2. B) Non-AHL-based compounds chosen due to their activities in QS phenotypic screens in wild-type *P. aeruginosa*.

We reasoned that compounds acting as poor substrates of MexAB-OprM would likely show similar potencies in both pump-active and pump-mutant *P. aeruginosa* strains. To determine LasR inhibitory potencies in both strains, the compounds had to exhibit a full antagonism dose response curve in both the PAO-JP2 and PAO-JG21 reporter strains. Accordingly, we were limited to the compounds that inhibited LasR activity in the pump-active PAO-JP2 primary

screen, which were the 11 AHLs capable of ≥ 25% LasR inhibition (shown in **Figure 5.3A**). Dose–response analyses for LasR inhibition were performed in the presence of OdDHL at its EC_{50} in each strain. We also included in this dose-response analysis an OdDHL analogue, 3-oxo-C12-aniline, which lacks the hydrolysable lactone head group (**Figure 5.3B**). Prior studies by our laboratory have shown that 3-oxo-C12-aniline is capable of inhibiting LasR activity by 30% in *P. aeruginosa* and by 55% in *E. coli* (using related reporter constructs).[49] Spring and co-workers have also reported that 3-oxo-C12-aniline is capable of strongly inhibiting pyocyanin production in wild-type *P. aeruginosa*, a well-known QS-controlled phenotype.[50] Because 3-oxo-C12-aniline inhibits *P. aeruginosa* pyocyanin production significantly better than non-native AHLs, while only showing modest activity in LasR reporter assays, we were interested to learn if 3-oxo-C12-aniline was able to evade efflux by MexAB-OprM.

Table 5.1. IC_{50} values for LasR inhibition by selected compounds in *P. aeruginosa* PAO-JP2 and PAO-JG21.[a]

	PAO-JP2		PAO-JG21		
Compound	IC_{50} (μM)	95% CI (μM)	IC_{50} (μM)	95% CI (μM)	Fold change of IC_{50}
A4	1.0[b]	0.21-4.9	0.12[b]	0.021-0.70	8.3
B14	15[b]	2.9-34	0.95[b]	0.43-2.1	15
E5	12	6.5-21	3.1	2.6-3.8	3.9
E6	≥ 480	-	19	12-33	≥ 25
E32	6.0	3.2-11	0.26[b]	0.043-1.5	23
E33	15[b]	9.1-24	0.20[b]	0.062-0.65	75
E35	5.7[b]	1.7-19	0.16[b]	0.061-0.40	35
E36	14[b]	2.5-81	0.34[b]	0.11-1.1	41
R4	≥ 570	-	41	30-57	≥ 13
R5	≥ 230	-	55	38-78	≥ 4.2
S1	≥ 710	-	24	16-34	≥ 29
3-oxo-C12-aniline	11	4.2-23	1.7	1.3-4.2	6.5
DMABI	**2.3**	1.3-4.1	**1.4**	0.81-2.5	**1.6**

[a] Antagonism assays were performed in the presence of 100 nM OdDHL in PAO-JP2 and 10 nM OdDHL in PAO-JG21. [b] Antagonism dose response exhibited non-monotonic behavior, showing an increase in activity at high concentrations. We have previously identified this phenomenon,[28] and studies are currently underway to identify the mechanistic basis for the trend.

The IC_{50} values for the 11 AHLs and 3-oxo-C12-aniline in the pump-active PAO-JP2 and pump-mutant PAO-JG21 LasR reporter strains are listed in **Table 5.1**. Compounds **E6**, **R4**, **R5** and **S1** showed incomplete inhibition of LasR in pump-active PAO-JP2 at the solubility limit of the compound; thus, only a lower bound on the IC_{50} value could be determined. Overall, the potencies of the tested compounds were strongly affected by the presence of MexAB-OprM, with

each of the IC_{50} values measured in pump-active PAO-JP2 being significantly different ($p < 0.05$) than the IC_{50} values measured in pump-mutant PAO-JG21. In fact, seven of the 12 compounds experienced a loss in potency of an even greater magnitude (up to 75-fold for AHL **E33**) than that observed for OdDHL (~10-fold; **Figure 5.1**). For certain AHLs, the fold-change in potency upon removal of MexAB-OprM was affected by subtle alterations in compound structure. For example, phenylacetanoyl HLs **E5** and **E6**, differing in only their substituents at the 3- or 4- position on the aromatic ring, show clear differences in their potency shifts between the two strains (3.9 vs. ≥ 25 fold-changes, respectively). While the phenylpropionoyl HLs (**E32**, **E33**, **E35,** and **E36**) exhibited consistently high fold-changes in IC_{50} value upon deletion of MexAB-OprM (23–75), the position of the iodine substituent on the aromatic ring (i.e., in **E32** and **E33**) appeared to cause a greater shift in potency relative to the trifluoromethyl substituent in **E35** and **E36**. The more modest shifts in potency for **E5** and 3-oxo-C12-aniline (3.9 and 6.5-fold changes, respectively) relative to other compounds in **Table 5.1** suggest that they may represent scaffolds that partially evade efflux. This modest shift for 3-oxo-C12-aniline also suggests that factors apart from active efflux are contributing to its significantly heightened phenotypic inhibitory activity in *P. aeruginosa* relative to non-native AHLs. It may be the case that the absence of a hydrolysable lactone head group in 3-oxo-C12-aniline contributes to a longer half-life of the active inhibitor, increasing its observed efficacy in the long-term (> 12 h) pyocyanin assays.[50]

5.2.4 DMABI is equally potent in pump-active and pump-mutant P. aeruginosa

The results above indicated that MexAB-OprM can recognize a wide variety of AHL derivatives with non-native acyl tails and 3-oxo-C12-aniline, with a non-native head group. In an attempt to uncover compound classes that resist efflux-induced reductions of potency and further explore the promiscuity of MexAB-OprM, we sought to test the potency of other QS modulators

in *P. aeruginosa* with structures distinct from AHLs. Recently, our laboratory identified 2-aminobenzimidazoles (2-ABIs) as a potent class of biofilm inhibitors and dispersers in *P. aeruginosa*.[42] In particular, the compound 5,6-dimethyl-2-aminobenzimidazole (DMABI) is a potent inhibitor of biofilm growth in wild-type *P. aeruginosa* (IC_{50} at 24 h = 4 μM) and a modest (~40%) inhibitor of LasR activity in *P. aeruginosa* PAO-JP2 (harboring a p*lasI*-LVAgfp reporter plasmid; **Figure 5.17**). We tested the LasR inhibitory activity of DMABI in both the MexAB-OprM pump-active and pump-mutant LasR reporter strains of *P. aeruginosa* to determine the susceptibility of the 2-ABI scaffold to active efflux. While the maximum inhibition of LasR activity by DMABI was modest (40%), the IC_{50} values calculated from the dose–response inhibition curves in PAO-JP2 and PAO-JG21 were statistically indistinguishable (2.3 vs. 1.4, respectively; **Table 5.1**). This result indicates that the potency of DMABI is not affected by MexAB-OprM in *P. aeruginosa*. DMABI was also subjected to dose-response analyses in the presence of 25 μg/mL PAβN using both PAO-JP2 and PAO-JG21 LasR reporter strains. In each case, the presence of the non-specific pump inhibitor had no effect on DMABI potency (**Figure 5.17**), suggesting that DMABI is not recognized as a substrate by other RND-type pumps in *P. aeruginosa*.

5.3 Discussion

The results reported herein demonstrate that the RND efflux pump MexAB-OprM in *P. aeruginosa* has a significant effect on the potency of both native and non-native AHLs as LasR modulators. We first showed that OdDHL is approximately 10-fold more potent in a *P. aeruginosa* Δ(*mexAB-oprM)* mutant strain relative to a pump-active strain, which corroborates a previous report showing that deletion of MexAB-OprM increases the intracellular concentration of OdDHL.[36] Because MexAB-OprM has a clear influence on OdDHL potency, we suspected that

similar perturbations could also occur with our non-natural AHLs and related analogs. Furthermore, we reasoned that the presence of the MexAB-OprM efflux pump in *P. aeruginosa* could account for the discrepancies that we have previously observed between the activities of our synthetic AHLs in *P. aeruginosa* versus those in *E. coli* LasR reporter strains.[30] We tested this hypothesis by performing side-by-side assays of our non-native AHL libraries in pump-active and pump-mutant *P. aeruginosa* LasR reporter strains and in an *E. coli* LasR reporter strain. We found that the LasR inhibitory activities of our compounds in *E. coli* much more strongly matched those in the pump-mutant *P. aeruginosa* compared to pump-active *P. aeruginosa*. These data suggest that the presence of the MexAB-OprM pump strongly affects the activities of non-native AHLs in *P. aeruginosa*. Although *E. coli* does express a homologous RND pump, AcrAB-TolC, this pump has a significantly different substrate profile and has not been shown to export any AHLs in *E. coli*.[32, 51] Our experiments provide a clear illustration that LasR activity screens performed using *E. coli* reporters allow elucidation of SARs describing a compound's ability to potentially bind to and modulate LasR, but they do not necessarily provide an accurate representation of the molecule's intracellular availability in *P. aeruginosa*.

We next focused on identifying LasR inhibitors that might evade active efflux and therefore retain potency as LasR inhibitors in *P. aeruginosa* by performing dose-response analyses on 11 potent non-native AHL inhibitors in the pump-active and pump-mutant *P. aeruginosa* LasR reporter strains. We also examined the activity of an AHL analog that lacked the native lactone head group, 3-oxo-C12-aniline. The majority of these LasR inhibitors showed a significant loss of potency in the pump-active reporter (> 10-fold), despite possessing a wide variety of acyl tail functionalities (straight-chain alkyl, branched alkyl, aryl, and heterocyclic; see **Figure 5.3A**), which suggests that MexAB-OprM is likely promiscuous across a broad range of AHL derivatives. Although none of the tested AHLs fully resisted potency effects due to active efflux,

we noted that subtle changes in acyl chain structure for certain AHLs elicited varied responses to MexAB-OprM mutation (e.g., **E5** vs. **E6** and **E32** vs. **E33**). RND-type pumps homologous to MexAB-OprM are present in a number of Gram-negative bacteria that use LuxR/LuxI-type QS circuits (e.g., *Acinetobacter baumannii*, *Agrobacterium tumefaciens*, *Burkholderia* spp., etc.).[33] Though mutations of efflux pumps in some species have had varied effects on QS-controlled phenotypes,[52, 53] our results have implications for future SAR studies of AHL-derived modulators of LuxR-type receptors performed in Gram-negative bacteria. It may be the case that variations in QS modulatory activity are a result of not only a compound's differential ability to interact with a LuxR-type receptor, but also a compound's differential susceptibility to active efflux.

Our observation that MexAB-OprM can recognize a range of AHLs as substrates prompted us to consider alternative chemical scaffolds that might inhibit QS while also bypassing MexAB-OprM efflux in *P. aeruginosa*. We recently identified a class of *P. aeruginosa* biofilm inhibitors known as 2-ABIs,[42] which were shown to inhibit LasR through an, as of yet, undetermined mechanism. Given their potent biofilm inhibitory activities in wild-type *P. aeruginosa*, we surmised that the compounds could be inherently resistant to active efflux. We tested the most potent lead compound, DMABI, and found that the presence of MexAB-OprM did not decrease the compound's potency. We also measured the potency of DMABI in the presence of PAßN to determine if DMABI is recognized by any other RND pumps present in PAO-JG21. Co-incubation with PAßN showed no significant change in DMABI potency in either PAO-JP2 or PAO-JG21, confirming the QS-inhibitory efficacy of DMABI is not significantly impacted by any RND pump in *P. aeruginosa*.

We currently have three hypotheses to explain the mechanism by which DMABI evades a loss in potency due to efflux by *P. aeruginosa*: 1) MexAB-OprM and other RND-type pumps do not recognize DMABI as a substrate, 2) the diffusion rate of DMABI into the cell is so rapid that

active efflux is negligible, or 3) DMABI acts upon an extracellular target. Regardless of the mechanism, the unique ability of DMABI to evade reduction of potency as a QS inhibitor will be further studied and leveraged to design more potent probes capable of QS inhibition in wild-type *P. aeruginosa* strains and even possibly for prevalent multidrug-resistant *P. aeruginosa* strains that overexpress RND-type pumps.[33] Current efforts in our laboratory are focused on identifying the mechanism of biofilm and LasR inhibition by DMABI and designing new compounds that more strongly antagonize LasR, while retaining DMABI's resistance to efflux by RND pumps.

5.4 Summary and Conclusions

The design of small molecules capable of blocking QS pathways, and thereby virulence, in the opportunistic pathogen *P. aeruginosa* is of significant interest. Numerous non-native AHLs and related analogs have been reported that are capable of modulating the activities of LuxR-type receptors in *P. aeruginosa* (LasR, RhlR, and QscR). In this study, we determined that a variety of non-native AHLs capable of inhibiting the LasR receptor in *P. aeruginosa* display significantly higher potency in a *P. aeruginosa* Δ(*mexAB-oprM*) mutant. These data suggest that the presence of the MexAB-OprM pump, and thus active efflux, strongly affects the activities of non-native AHLs in *P. aeruginosa*. We also demonstrated that while a variety of AHL analogues appear to be recognized as substrates by MexAB-OprM, a compound structurally distinct from the AHLs—DMABI—does not display this efflux-induced reduction in potency. To our knowledge, this study represents the first systematic investigation of the role of active efflux in the activity profiles of small molecule QS inhibitors in Gram-negative bacteria. Our results have implications for the use of QS modulators in *P. aeruginosa* and potentially other proteobacteria, and provide a prospective design strategy (based on 2-ABI derivatives) for the development of new QS modulators that may be resistant to active efflux.

5.5 Experimental

5.5.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in **Table 5.2**. Bacteria were grown at 37 ºC in Luria-Bertani (LB) medium unless otherwise noted. *P. aeruginosa* strain PAO-JP2 harboring p*lasI*-LVAgfp was grown in the presence of carbenicillin (300 µg/mL). As expected, the Δ(*mexAB-oprM)* strain PAO-JG21 was more sensitive to antibiotic selection and thus was grown in the presence of 100 µg/mL carbenicillin. *E. coli* strain JLD271 harboring plasmids pPROBE-KL and pJN105L was grown in the presence of kanamycin (50 µg/mL) and gentamicin (10 µg/mL). All overnight cultures were shaken at 200 rpm.

Table 5.2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant properties	Source or reference
Strains		
P. aeruginosa		
PAO-JP2	PAO1 *lasI*::Tet *rhlI*::Tn501-2; Hg^R Tc^R	[54]
PAO-JG21	PAO-JP2 Δ(*mexA-mexB-oprM*)	This study
E. coli		
DH5α	*E. coli* strain for transformation	[55]
S17-1	Mobilizer strain	[56]
JLD271	K-12 Δ*lacX74 sdiA271*::Cam; Cl^R	[57]
Plasmids		
pEX18Gm	Gm^R; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18; pEX100T backbone Gm^R	[58]
pJG034	pEX18Gm with markerless Δ(*mexAB-oprM*)	This study
p*lasI*-LVAgfp	*lasI*'-gfp[LVA] transcriptional fusion; Cb^R	[59]

5.5.2 Chemicals

Non-native AHLs, 3-oxo-C12-aniline, and 5,6-dimethyl-aminobenzimidazole (DMABI) were synthesized as described previously.[28, 42, 49, 60, 61] Structures of the entire in-house AHL library evaluated in this study are shown in **Figures 5.4**–**5.7**. *N*-(3-oxo)-dodecanoyl L-homoserine lactone (OdDHL) was purchased from Sigma-Aldrich. The pump inhibitor Phe-Arg-β-naphthylamide (PAßN) was purchased from Chem-Impex, International.

5.5.3 Construction of P. aeruginosa mexAB-oprM mutant (PAO-JG21)

To construct PAO-JG21, an 898-base pair (bp) upstream portion of *mexA* and a 521-bp downstream portion of *oprM* were amplified from *P. aeruginosa* PAO1 genomic DNA by PCR

with the following primers: 5′–ACTAAGCTTCAATACATGGACGTCGGG–3′ (native HindIII site underlined) and 5′–ACTGAATTCGGAGATCGGCGACAGCACC–3′ (added EcoRI site underlined) for *mexA*; and 5′–ACTGAATTCGACCTGTCGACCACCGGCA–3′ (added EcoRI site underlined) and 5′–AGTTCTAGAGATGTCCGGGCGCCGT–3′ (added XbaI site underlined) for *oprM*. The EcoRI sites incorporated by the internal primers were digested and used to ligate the two portions together, yielding an in-frame deletion with an EcoRI scar. This construct was PCR amplified and ligated into the pEX18Gm vector using HindIII and XbaI sites to form pJG034. The pJG034 plasmid was introduced into *E. coli* S17-1::λpir by electroporation and transferred to *P. aeruginosa* PAO-JP2 by conjugation and citric acid + antibiotic selection on Vogel-Bonner minimal medium[62] supplemented with gentamicin (15 µg/ml). The resulting gentamicin-resistant merodiploid colonies were counterselected on LB agar plates supplemented with 5% sucrose, which yielded colonies that were gentamicin sensitive and sucrose resistant. The *mexAB-oprM* region of one mutant colony (PAO-JG21) was PCR amplified and sequenced to verify the markerless in-frame deletion.

5.5.4 Construction of pPROBE-KL

To construct plasmid pPROBE-KL, a 317-bp fragment spanning the upstream region of *lasI* (-282 to +35 relative to the *lasI* translational start codon) was amplified by PCR. The primers were 5′–GAATTAGGATCCGCAGGTTCTCGCCATTC–3′ (native BamHI site underlined) and 5′–GAATAGGAATTCTCGAACTCTTCGCGCCG–3′ (added EcoRI site underlined). The PCR-generated fragment was digested with EcoRI and BamHI and subsequently ligated with BamHI/EcoRI-digested pPROBE-KT to generate pPROBE-KL.

5.5.5 LasR reporter assay protocol

To evaluate the LasR inhibitory activities of our synthetic compounds in *P. aeruginosa*, PAO-JP2 or PAO-JG21 harboring the plasmid p*lasI*-LVAgfp were grown overnight as described above. An appropriate amount of synthetic compound stock solution (or OdDHL stock solution, as a control) in DMSO was added to wells in 96-well microtiter plates, with final DMSO concentrations (after addition of cells) not exceeding 1%. The overnight culture was diluted 1:100 in fresh LB medium and was grown to an OD_{600} of 0.3. Subculture was treated with OdDHL (100 nM in PAO-JP2 or 10 nM in PAO-JG21) by adding the appropriate amount of an OdDHL stock solution in DMSO (10 mM or 1 mM). The subculture was then dispensed in 200 µL portions into each synthetic compound-treated well of the microtiter plate.

Plates were incubated at 37 ºC for 6 h, and GFP production was monitored using a Biotek Synergy 2 plate reader (Excitation: 500 nm, Emission: 540 nm) and quantified with Gen5 1.05 software. The final OD_{600} of each well was measured to normalize GFP production to cell density. In LasR antagonism assays, the normalized fluorescence of each compound competing against OdDHL was reported relative to the normalized fluorescence of the OdDHL-only positive control. All synthetic compounds were tested in triplicate, and three separate trials were performed using unique cultures. Antagonism dose–response analyses were performed by testing compounds over a range of concentrations in this LasR reporter assay. IC_{50} values and 95% confidence intervals were calculated using GraphPad Prism 4 software. Dose response analyses of OdDHL were performed in a similar manner, except that the bulk subculture was not treated with OdDHL; instead, varying concentrations of OdDHL were added to the 96-well microtiter plates before addition of subculture.

To evaluate the LasR inhibitory activities of our synthetic compounds in *E. coli*, *E. coli* JLD271 harboring plasmids pPROBE-KL and pJN105L was grown overnight as described above.

Antagonism assays were performed as those in *P. aeruginosa* with the following modifications: Overnight cultures were diluted 1:10 with fresh LB medium containing kanamycin (50 µg/mL) and gentamicin (10 µg/mL). Solid arabinose was added to the subculture to a final concentration of 0.5%, and a DMSO stock of OdDHL was added to the subculture to a final concentration of 2 nM. The subculture was immediately dispensed into compound-treated 96-well microtiter plates. The plates were shaken at 200 rpm for 4 h at 37 ºC.

LasR agonism and antagonism assays in the presence of PAßN were performed by adding PAßN from a 50 mg/mL H_2O stock to cultures immediately prior to plating to yield a final PAßN concentration of 25 µg/mL.

5.6 Supplementary Figures, and Tables

Supplementary figures and tables are presented on the following pages.

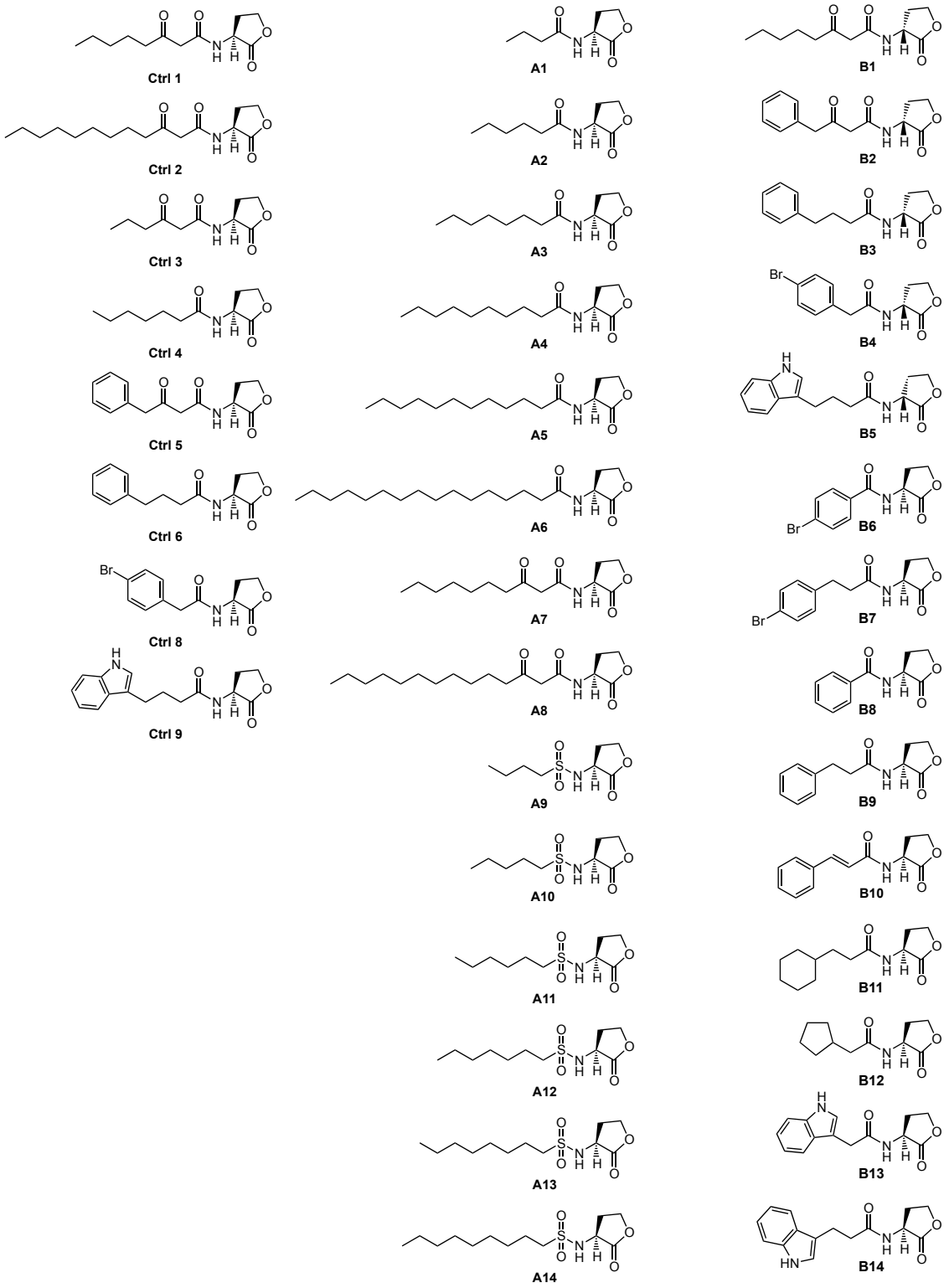


Figure 5.4. AHLs from the control, A, and B libraries examined in this study.
Compound numbering is analogous to our earlier publications.[28]

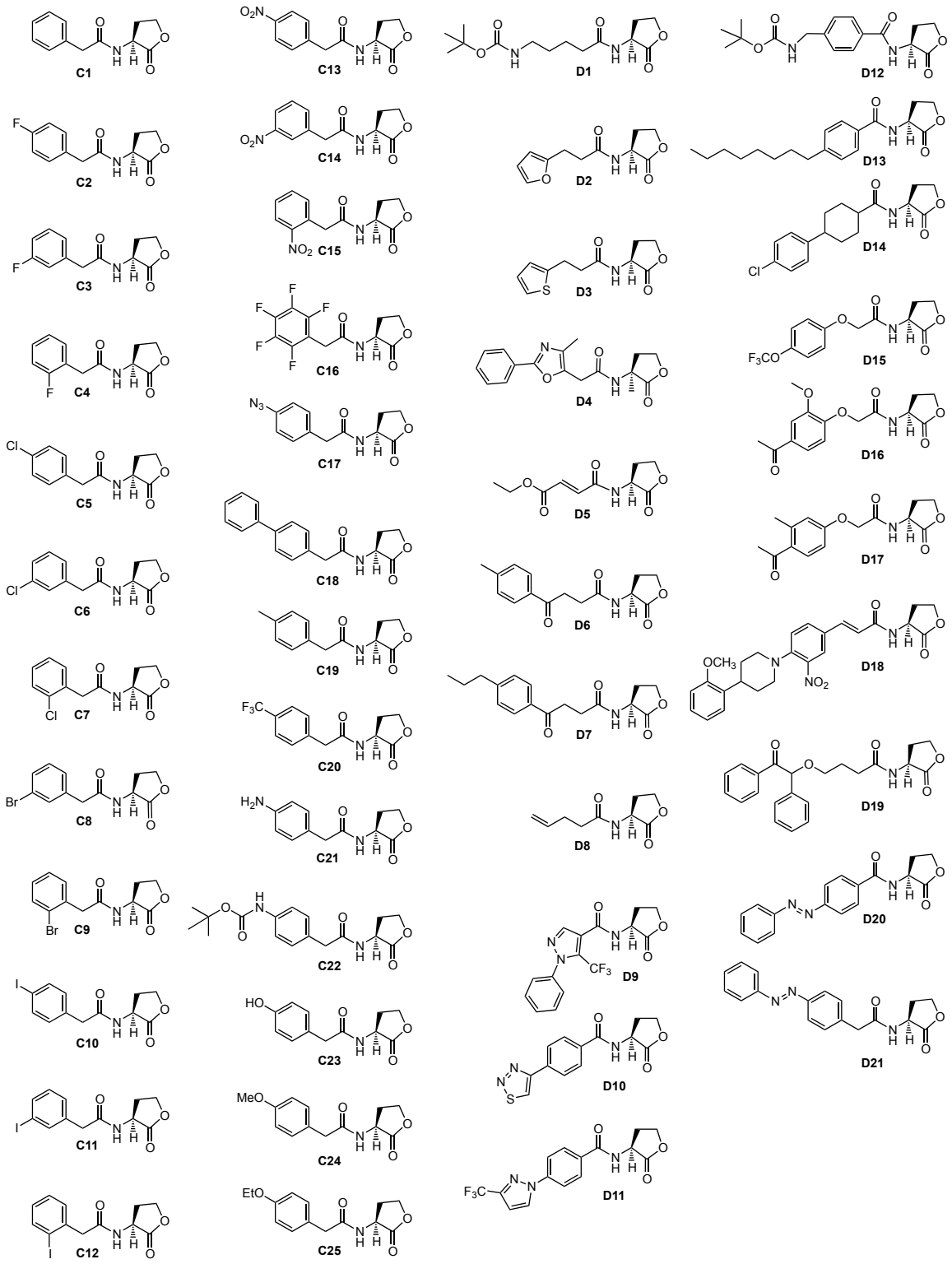


Figure 5.5. AHLs from the C and D libraries examined in this study. Compound numbering is analogous to our earlier publications.[28]

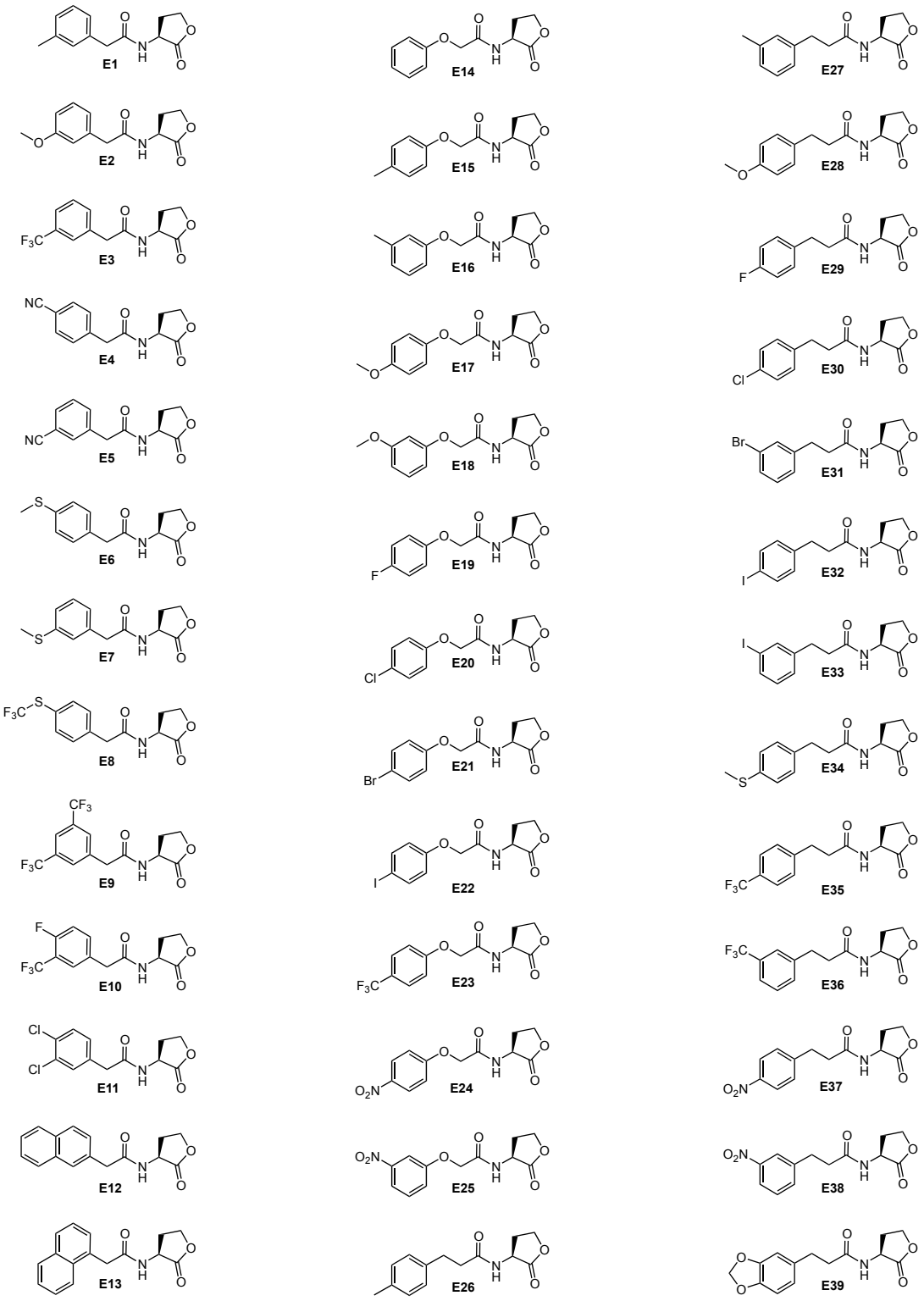


Figure 5.6. AHLs from the E library examined in this study.
Compound numbering is analogous to our earlier publications.[27]

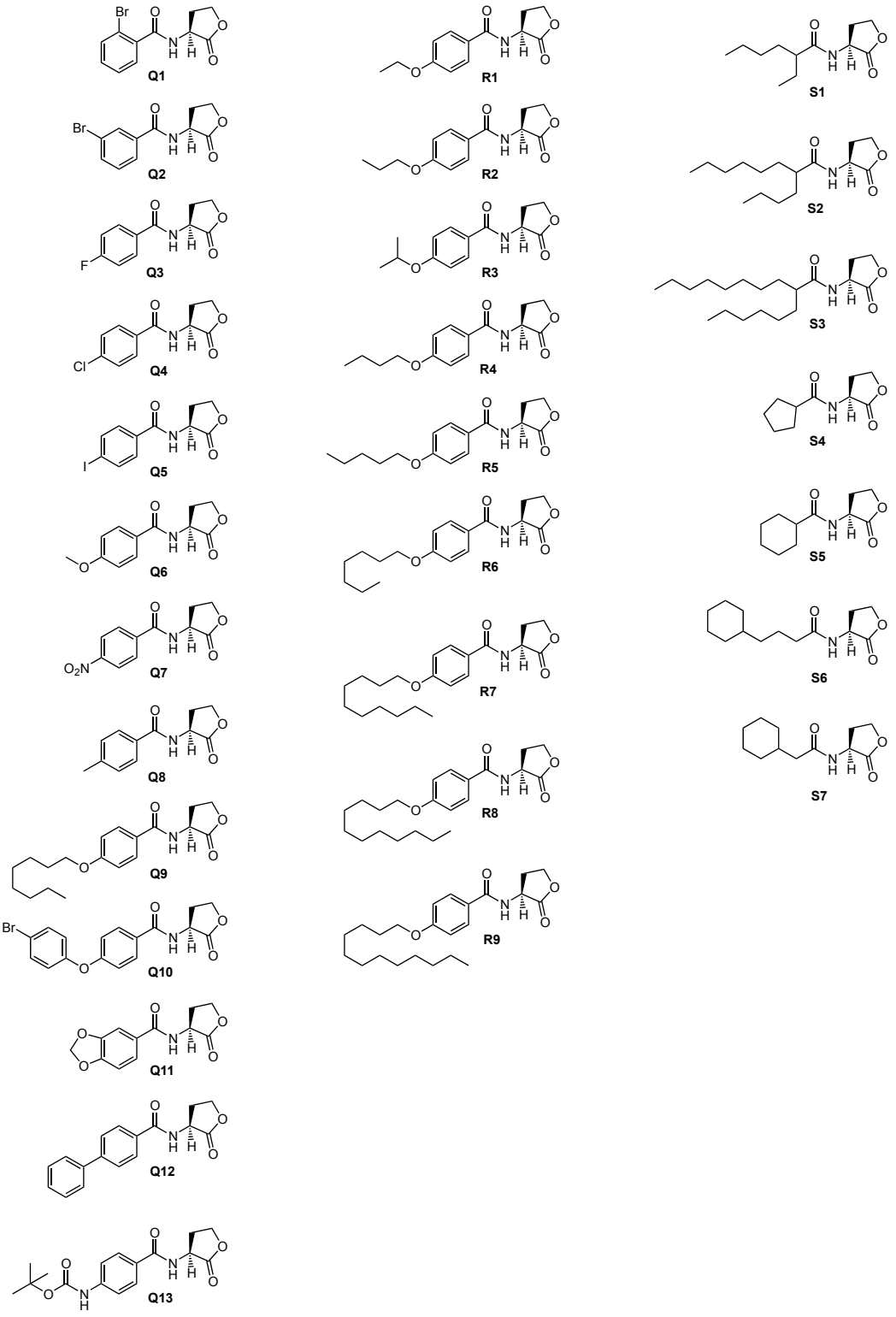


Figure 5.7. AHLs from the Q, R, and S libraries examined in this study. Compound numbering is analogous to our earlier publications.[61]

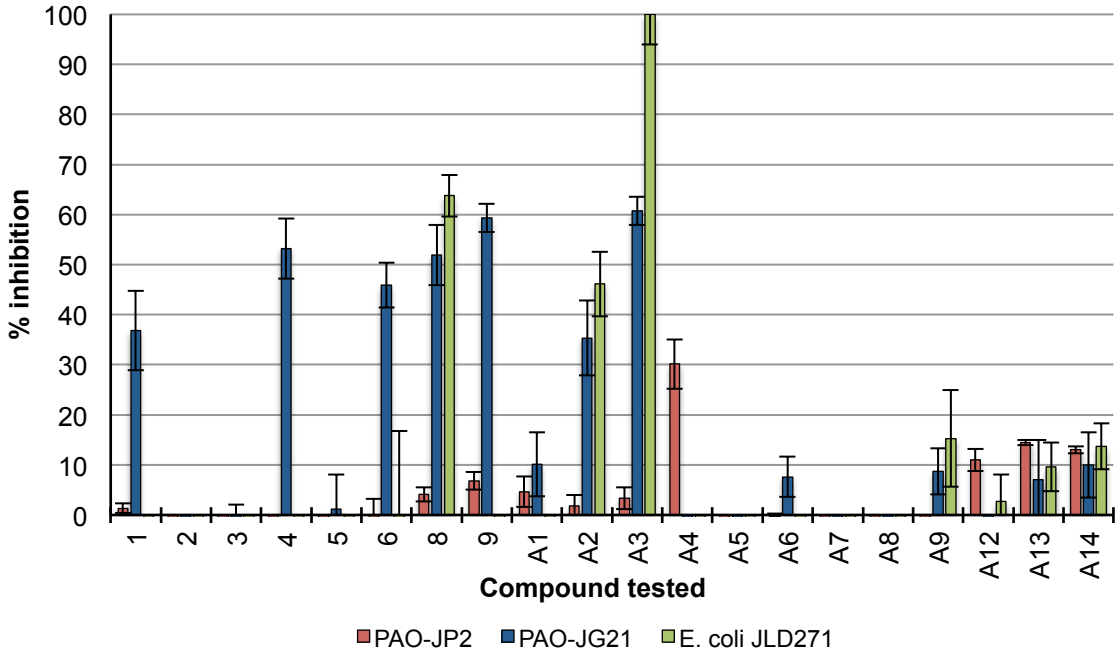


Figure 5.8. Primary LasR antagonism screening data for the control and A libraries in *P. aeruginosa* PAO-JP2 + p*lasI*LVAGFP, *P. aeruginosa* PAO-JG21 + p*lasI*LVAGFP, and *E. coli* JLD271 + pPROBE-KL.

Antagonism assays were performed using the following compound concentrations and controls:

PAO-JP2: 10 µM of synthetic ligand against 100 nM OdDHL
Positive control (100 % inhibition) = 2 µL DMSO (no AHL added)
Negative control (0 % inhibition) = 100 nM OdDHL

PAO-JG21: 10 µM of synthetic ligand against 10 nM OdDHL
Positive control (100 % inhibition) = 2 µL DMSO (no AHL added)
Negative control (0 % inhibition) = 10 nM OdDHL

JLD271: 10 µM of synthetic ligand against 2 nM OdDHL
Positive control (100 % inhibition) = 2 µL DMSO (no AHL added)
Negative control (0 % inhibition) = 2 nM OdDHL

All fluorescence data were background-corrected by subtracting the negative control fluorescence value (wells containing reporter strain + 2 µL DMSO only) from the experimental value. Percent (%) LasR activity was measured by normalizing background-corrected value to fluorescence value obtained in wells containing reporter strain + OdDHL. Percent (%) LasR inhibition = 100% – % LasR activity.

All compounds were screened in triplicate over 3 separate trials. Error bars represent the SEM of 3 trials.

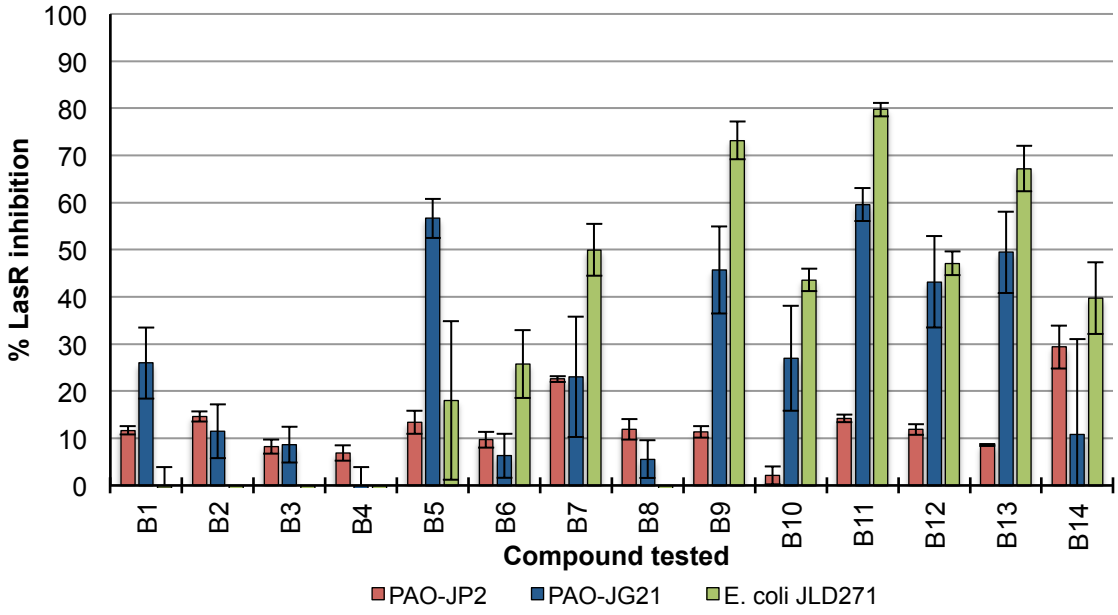


Figure 5.9. Primary LasR antagonism screening data for B library in *P. aeruginosa* PAO-JP2 + p*lasI*LVAGFP, *P. aeruginosa* PAO-JG21 + p*lasI*LVAGFP, and *E. coli* JLD271 + pPROBE-KL. Antagonism assays were performed as described in **Figure 5.8**.

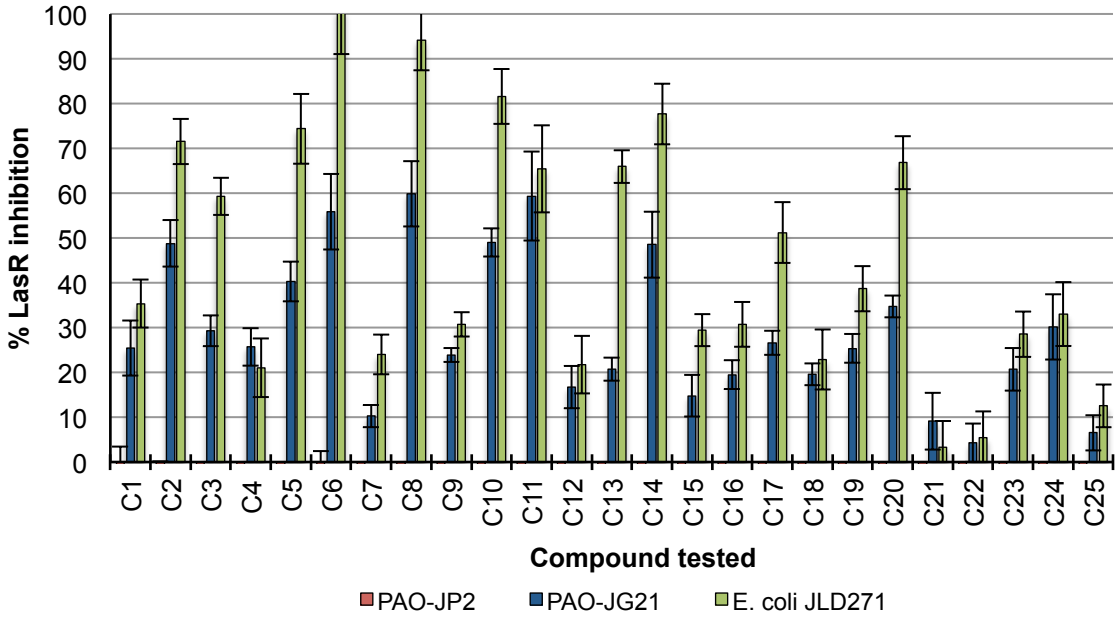


Figure 5.10. Primary LasR antagonism screening data for C library in *P. aeruginosa* PAO-JP2 + p*lasI*LVAGFP, *P. aeruginosa* PAO-JG21 + p*lasI*LVAGFP, and *E. coli* JLD271 + pPROBE-KL. Antagonism assays were performed as described in **Figure 5.8**.

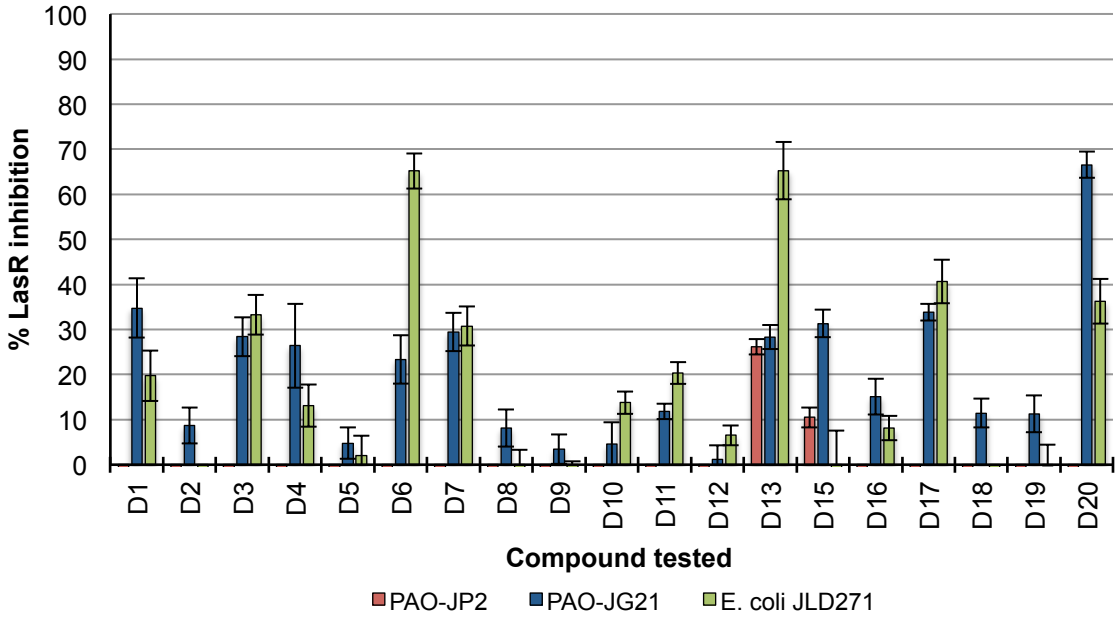


Figure 5.11. Primary LasR antagonism screening data for D library in *P. aeruginosa* PAO-JP2 + p*lasI*LVAGFP, *P. aeruginosa* PAO-JG21 + p*lasI*LVAGFP, and *E. coli* JLD271 + pPROBE-KL. Antagonism assays were performed as described in **Figure 5.8**.

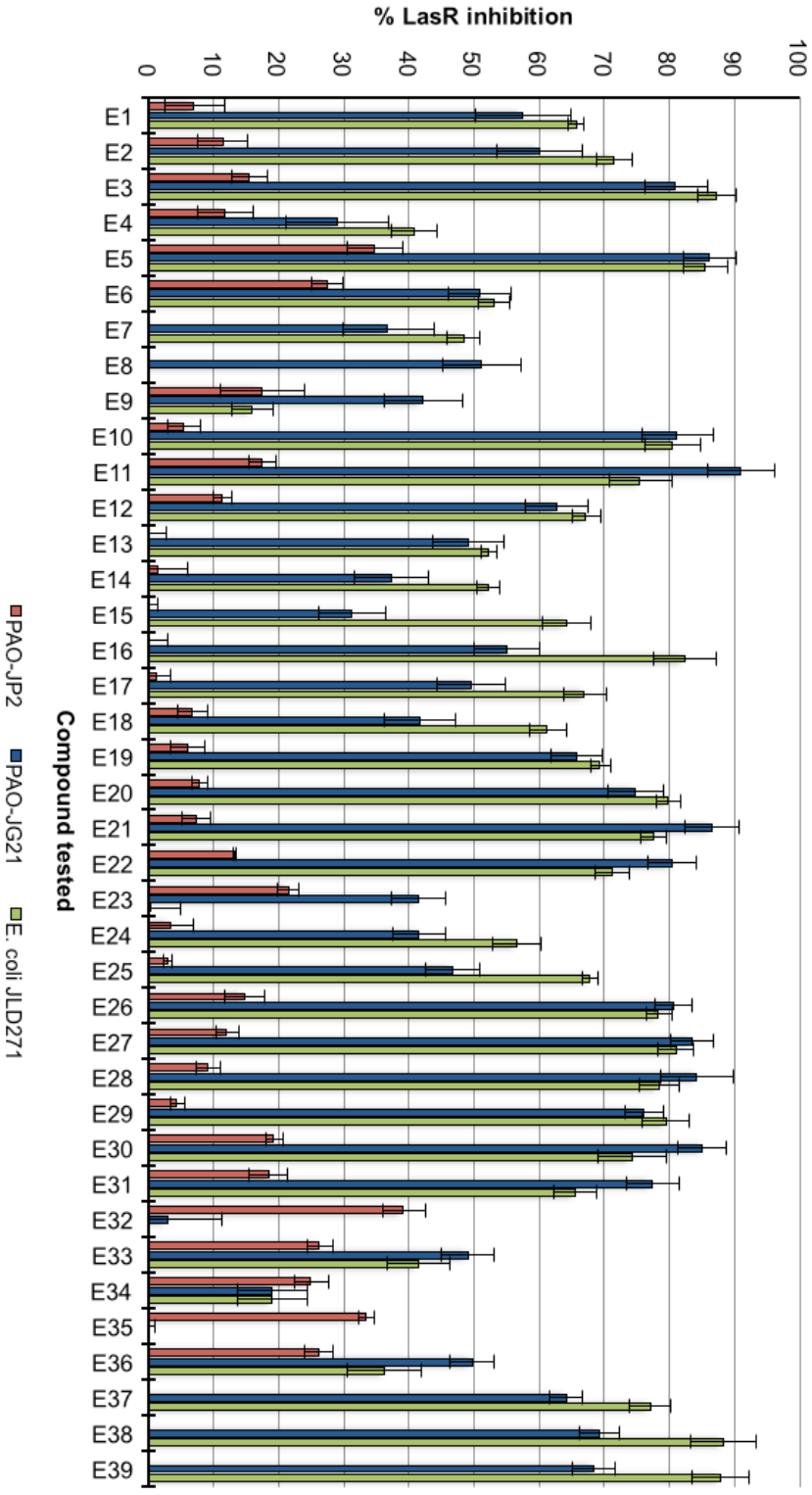


Figure 5.12. Primary LasR antagonism screening data for E library in *P. aeruginosa* PAO-JP2 + p*lasI*LVAGFP, *P. aeruginosa* PAO-JG21 + p*lasI*LVAGFP, and *E. coli* JLD271 + pPROBE-KL. Antagonism assays were performed as described in **Figure 5.8**.

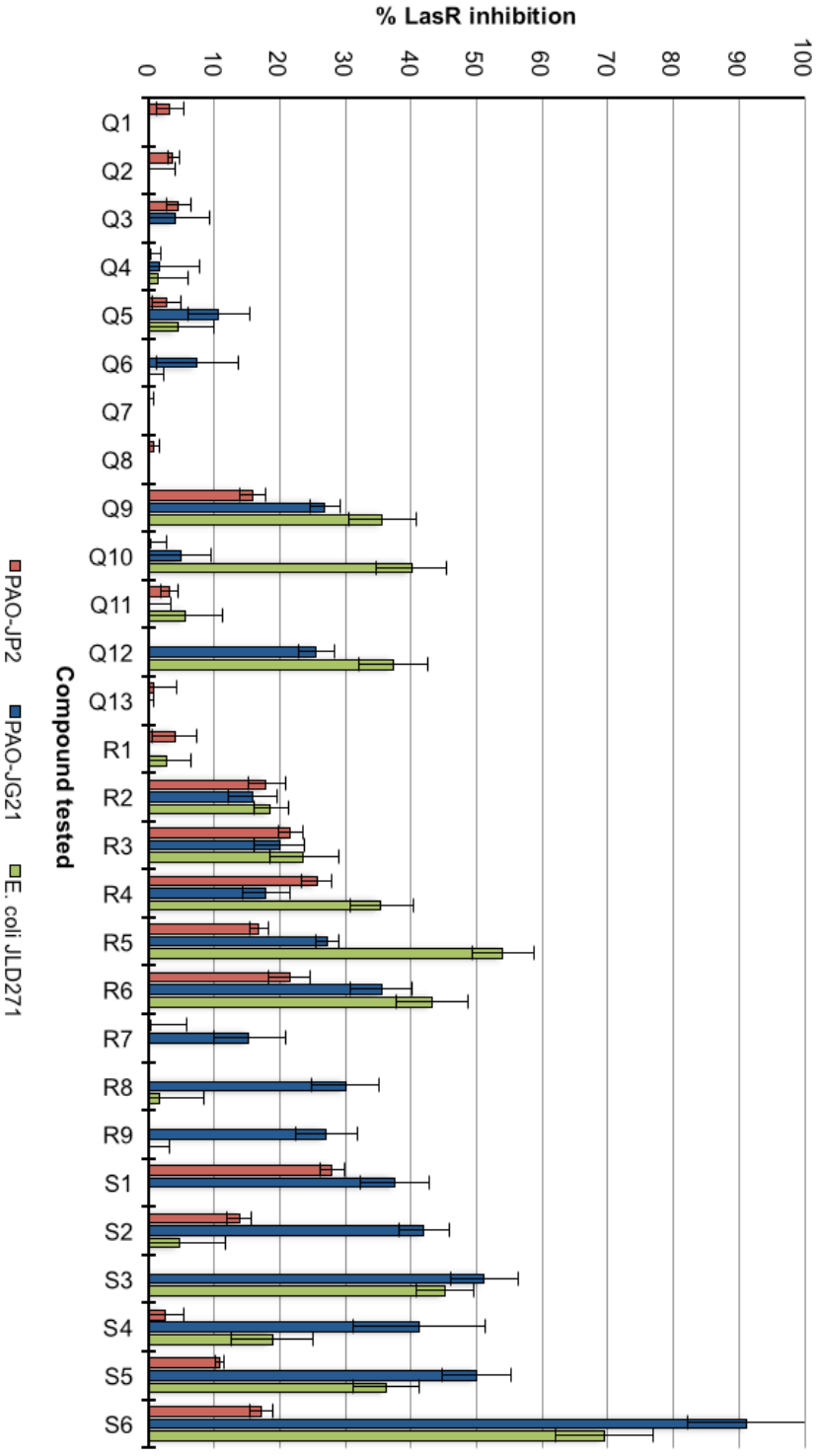


Figure 5.13. Primary LasR antagonism screening data for Q, R, and S libraries in *P. aeruginosa* PAO-JP2 + p*lasI*LVAGFP, *P. aeruginosa* PAO-JG21 + p*lasI*LVAGFP, and *E. coli* JLD271 + pPROBE-KL.
Antagonism assays were performed as described in **Figure 5.8**.

Table 5.3. List of the most active AHL-derived LasR antagonists identified in the *P. aeruginosa* PAO-JP2 (pump-active) GFP reporter screen and accompanying inhibition data.
Primary data from plots in **Figures 5.8–5.13**.

	Compound	LasR inhibition (%)
≥ 25% inhibition	**A4**	30
	B14	29
	D13	26[a]
	E5	35
	E6	27
	E32	39
	E33	26
	E35	33
	E36	26
	R4	25
	S1	28

[a] Compound displayed limited solubility at concentrations greater than 1 μM.

Table 5.4. List of the most active AHL-derived LasR antagonists identified in the *P. aeruginosa* PAO-JG21 (pump-mutant) GFP reporter screen and accompanying inhibition data.
Primary data from plots in **Figures 5.8–5.13.**

	Compound	LasR inhibition (%)
≥ 80% inhibition	**E3**	81
	E5	86
	E10	81
	E11	91
	E21	87
	E22	80
	E26	81
	E27	83
	E28	84
	E30	85
	S6	91

	Compound	LasR inhibition (%)	Compound	LasR inhibition (%)	Compound	LasR inhibition (%)
≥ 25% inhibition	**Ctrl 1**	37	**D1**	35	**E21**	87
	Ctrl 4	53	**D3**	28	**E22**	80
	Ctrl 6	46	**D4**	26	**E23**	41
	Ctrl 8	52	**D7**	29	**E24**	42
	Ctrl 9	59	**D13**	28[a]	**E25**	47
	A2	35	**D15**	31	**E26**	81
	A3	61	**D17**	34	**E27**	83
	B1	26	**D20**	67	**E28**	84
	B5	57	**E1**	57	**E29**	76
	B9	46	**E2**	60	**E30**	85
	B10	27	**E3**	81	**E31**	77
	B11	60	**E4**	29	**E33**	49
	B12	43	**E5**	86	**E36**	50
	B13	49	**E6**	51	**E37**	64
	C1	25	**E7**	37	**E38**	69
	C2	49	**E8**	51	**E39**	68
	C3	29	**E9**	42	**Q9**	27
	C4	26	**E10**	81	**Q12**	26
	C5	40	**E11**	91	**R5**	27
	C6	56	**E12**	63	**R6**	35
	C8	60	**E13**	49	**R8**	30
	C10	49	**E14**	37	**R9**	27
	C11	59	**E15**	31	**S1**	38
	C14	30	**E16**	55	**S2**	42
	C17	35	**E17**	50	**S3**	51
	C19	25	**E18**	42	**S4**	41
	C20	35	**E19**	66	**S5**	50
	C24	30	**E20**	75	**S6**	91

[a] Compound displayed limited solubility at concentrations greater than 1 μM.

Table 5.5. List of the most active AHL-derived LasR antagonists identified in the *E. coli* JLD271 GFP reporter screen and accompanying inhibition data.
Primary data from plots in **Figures 5.8–5.13.**

	Compound	LasR inhibition (%)
≥ 80% inhibition	**A3**	99
	B11	80
	C6	100
	C8	94
	C10	82
	E3	87
	E5	85
	E10	80
	E16	82
	E20	80
	E27	81
	E38	88
	E39	88

	Compound	LasR inhibition (%)	Compound	LasR inhibition (%)	Compound	LasR inhibition (%)
≥ 25% inhibition	**Ctrl 8**	64	**C20**	67	**E20**	80
	A2	46	**C23**	29	**E21**	77
	A3	99	**C24**	33	**E22**	71
	B6	26	**D3**	33	**E24**	56
	B7	50	**D6**	65	**E25**	68
	B9	73	**D7**	31	**E26**	78
	B10	44	**D13**	65[a]	**E27**	81
	B11	80	**D17**	41	**E28**	78
	B12	47	**D20**	36	**E29**	79
	B13	67	**E1**	66	**E30**	74
	B14	40	**E2**	71	**E31**	66
	C1	35	**E3**	87	**E33**	41
	C2	72	**E4**	41	**E36**	36
	C3	59	**E5**	85	**E37**	77
	C5	74	**E6**	53	**E38**	88
	C6	100	**E7**	48	**E39**	88
	C8	94	**E10**	80	**Q9**	36
	C9	31	**E11**	75	**Q10**	40
	C10	82	**E12**	67	**Q12**	37
	C11	65	**E13**	52	**R4**	35
	C13	66	**E14**	52	**R5**	54
	C14	78	**E15**	64	**R6**	43
	C15	29	**E16**	82	**S3**	45
	C16	31	**E17**	67	**S5**	36
	C17	51	**E18**	61	**S6**	69
	C19	39	**E19**	69		

[a] Compound displayed limited solubility at concentrations greater than 1 μM.

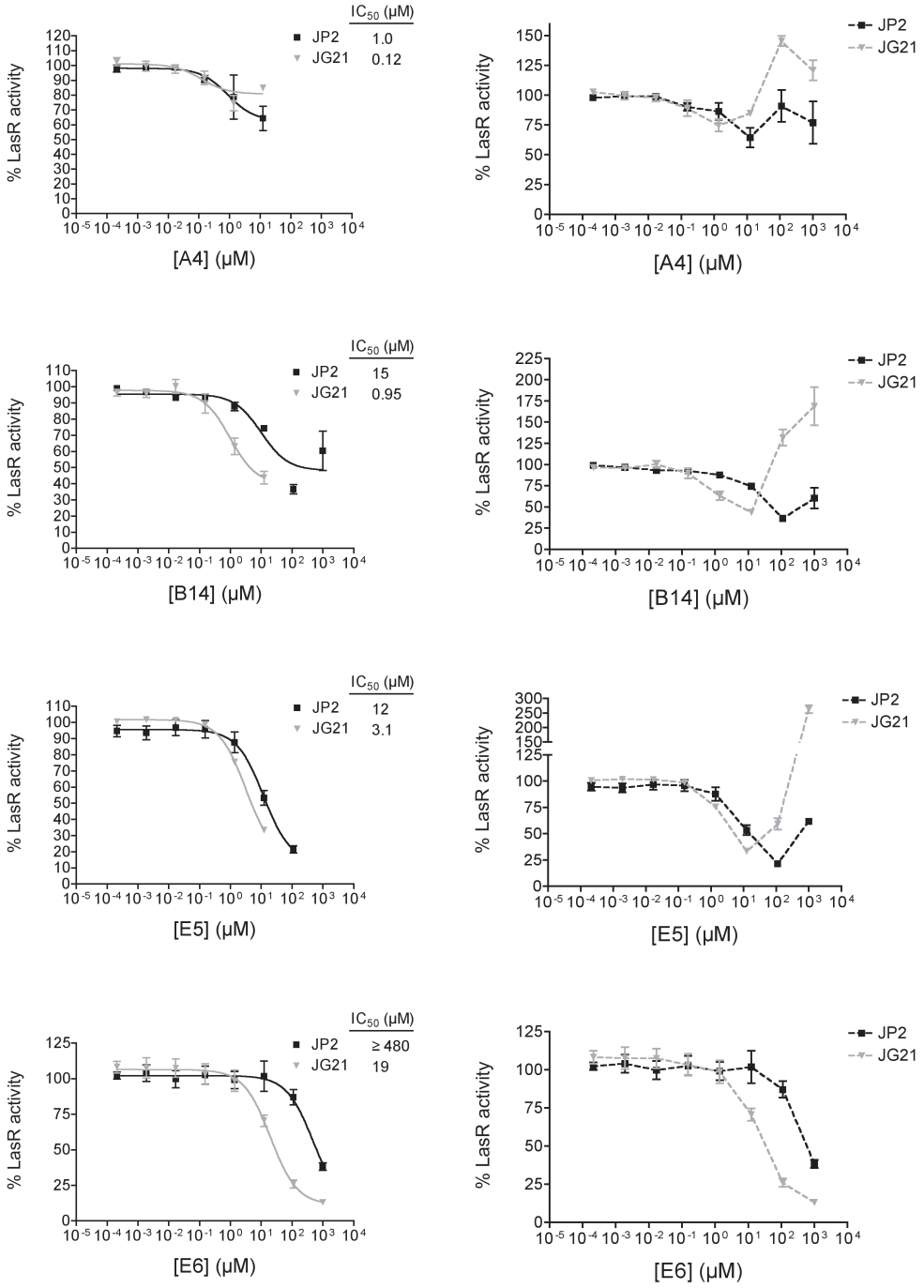


Figure 5.14. LasR antagonism dose responses and IC_{50} values for AHLs A4, B14, E5, and E6 in *P. aeruginosa* PAO-JP2 and PAO-JG21.

Plots on the left are truncated to show the dose response curves of the compounds in the inhibitory concentration range for each strain. Plots on the right show the full dose response including non-monotonic behavior, if applicable. Synthetic compounds were screened against 100 nM and 10 nM OdDHL in PAO-JP2 and PAO-JG21, respectively, over varying concentrations. IC_{50} values were calculated from the truncated plots using GraphPad Prism. Error bars, SEM of n = 3 trials.

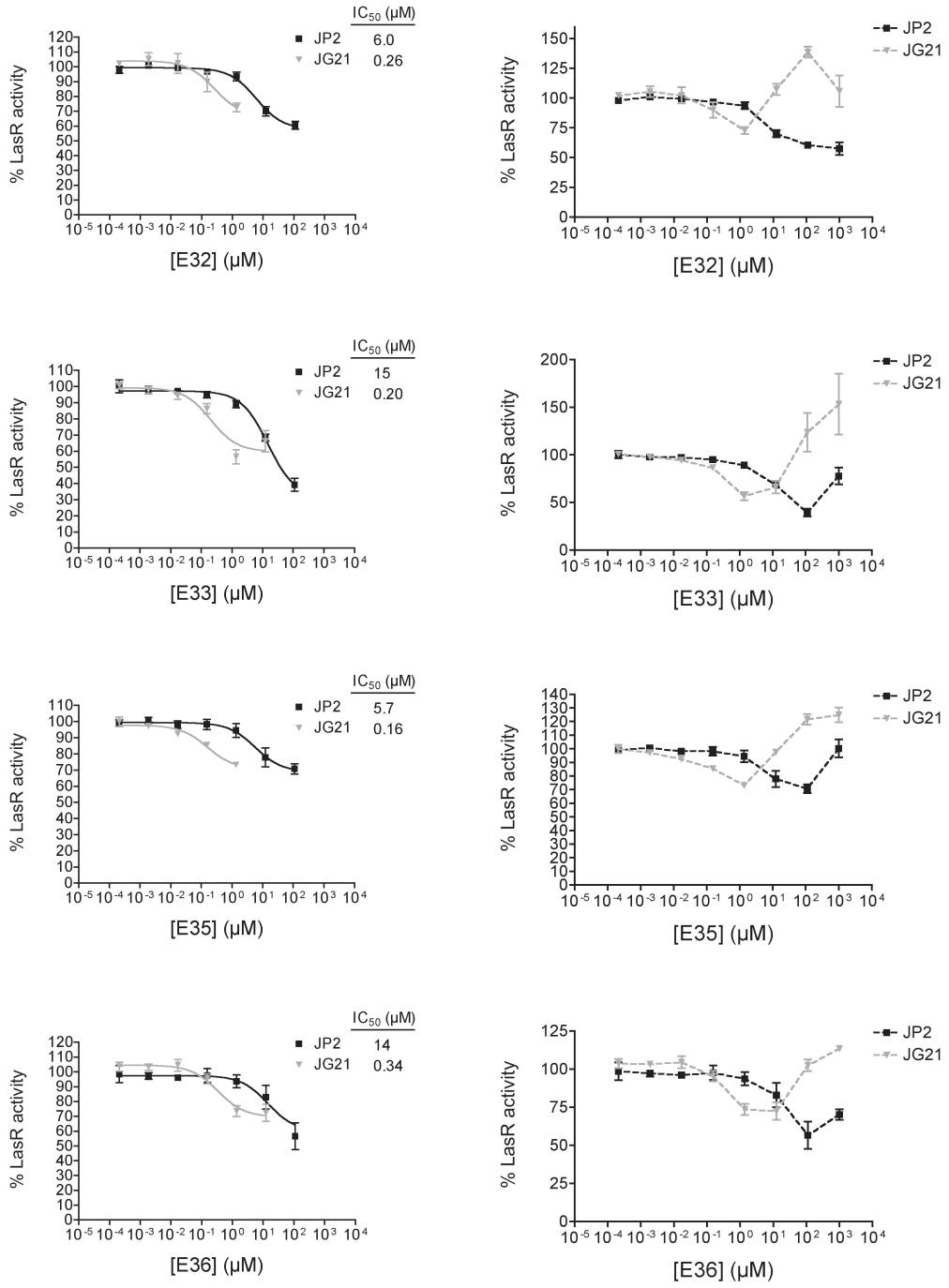


Figure 5.15. LasR antagonism dose responses and IC_{50} values for AHLs E32, E33, E35, and E36 in *P. aeruginosa* PAO-JP2 and PAO-JG21.

Plots on the left are truncated to show the dose response curves of the compounds in the inhibitory concentration range for each strain. Plots on the right show the full dose response including non-monotonic behavior, if applicable. Synthetic compounds were screened against 100 nM and 10 nM OdDHL in PAO-JP2 and PAO-JG21, respectively, over varying concentrations. IC_{50} values were calculated from the truncated plots using GraphPad Prism. Error bars, SEM of n = 3 trials.

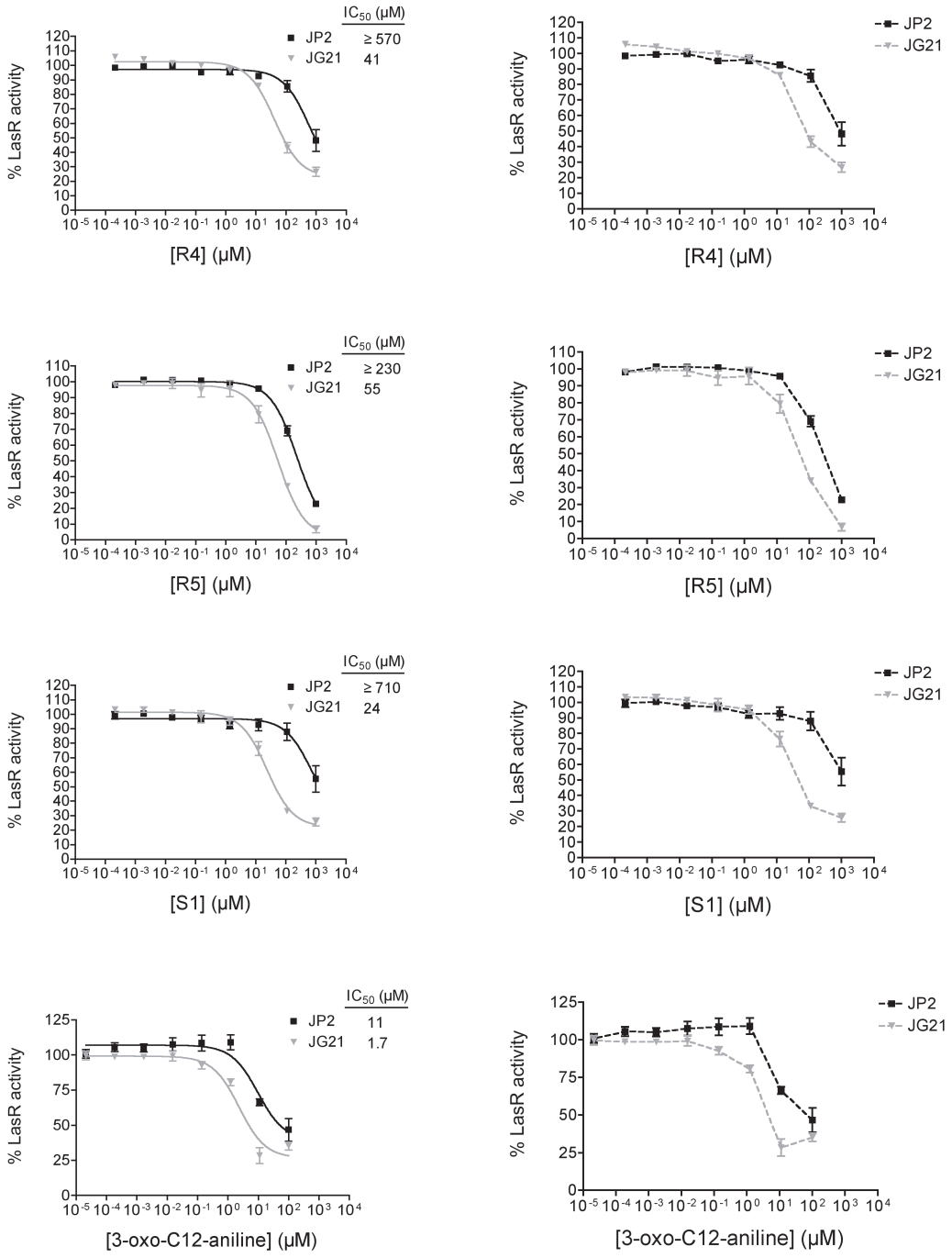


Figure 5.16. LasR antagonism dose responses and IC_{50} values for AHLs R4, R5, S1, and 3-oxo-C12-aniline in *P. aeruginosa* PAO-JP2 and PAO-JG21.

Plots on the left are truncated to show the dose response curves of the compounds in the inhibitory concentration range for each strain. Plots on the right show the full dose response including non-monotonic behavior, if applicable. Synthetic ligands screened against 100 nM and 10 nM OdDHL in PAO-JP2 and PAO-JG21, respectively, over varying concentrations of inhibitor. IC_{50} values were calculated from the truncated plots using GraphPad Prism. Error bars, SEM of n = 3 trials.

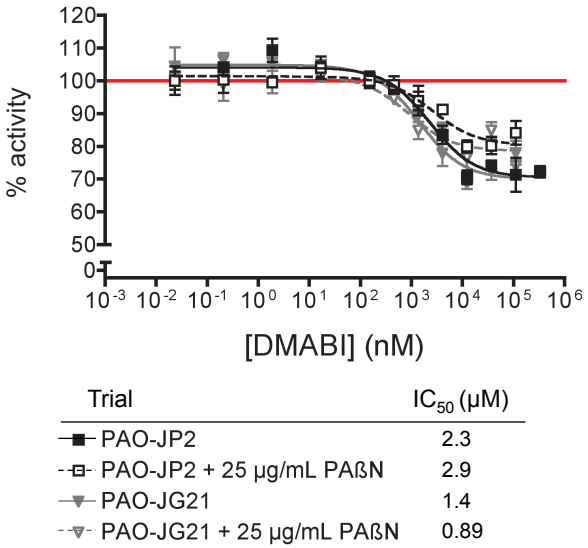


Figure 5.17. LasR antagonism dose response curves for DMABI in *P. aeruginosa* PAO-JP2 and PAO-JG21 in the absence and presence of PAßN.
DMABI potency was not affected by removal of MexAB-OprM or addition of the RND pump inhibitor PAßN. Antagonism assays were performed using the following compound concentrations and controls:
PAO-JP2:
Variable concentrations of DMABI against 100 nM OdDHL
Positive control (100 % inhibition) = 2 µL DMSO (no AHL added)
Negative control (0 % inhibition; red line) = 100 nM OdDHL

PAO-JG21; PAO-JP2 + PAßN; PAO-JG21 + PAßN:
Variable concentrations of DMABI against 10 nM OdDHL
Positive control (100 % inhibition) = 2 µL DMSO (no AHL added)
Negative control (0 % inhibition; red line) = 10 nM OdDHL
All fluorescence data were background-corrected by subtracting the negative control fluorescence value (wells containing reporter strain + 2 µL DMSO only) from the experimental value. Percent (%) LasR activity was measured by normalizing the background-corrected value to the fluorescence value obtained in wells containing reporter strain + OdDHL. Percent (%) LasR inhibition = 100% – % LasR activity.
IC_{50} values were calculated using GraphPad Prism. Error bars, SEM of n = 3 trials.
The % activity scale on the y-axis has been zoomed to show that LasR inhibition by DMABI, while modest, is significant relative to the negative control.

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CHAPTER 6:

LIGAND-RESPONSE SELECTIVITIES OF THE QUORUM SENSING RECEPTORS AbaR AND LasR FROM *ACINETOBACTER BAUMANNII* AND *PSEUDOMONAS AERUGINOSA* REVEAL NEW INSIGHTS INTO LIGAND RECOGNITION BY LUXR-TYPE PROTEINS AND THE POTENTIAL FOR INTERSPECIES SIGNALING IN GRAM-NEGATIVE BACTERIA

Contribution: J. P. G. performed sequence and structure analysis. D. M. S. and J. P. G. analyzed data. D. M. S., J. P. G., and H. E. B. wrote the manuscript. D. M. S., J. R. B., and H. X. synthesized AHLs and performed bacteriological assays.

Trevor L. Schell is currently carrying on the work described in the appendix.

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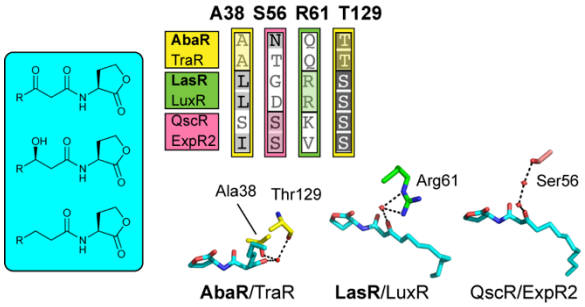
Abstract.



The influence that bacteria living in polymicrobial communities have on human life is tremendous. The complex interactions between the bacteria within these communities are only beginning to be understood, but one form of cross-species interaction is interplay between their quorum-sensing (QS) communication systems. In the current study, we tested the likelihood that the QS systems of two related opportunistic pathogens (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) that frequently arise in polymicrobial infections would be affected by the QS signals of neighboring species. Through the synthesis and screening of a focused library of native and non-native *N*-acyl L-homoserine lactone (AHLs), we found that the AbaR LuxR-type receptor protein of *A. baumannii* is highly selective for its its native *N*-(3-hydroxydodecanoyl) L-homoserine lactone signal. However, the homologous LasR receptor of *P. aeruginosa* is more promiscuously activated by AHLs, suggesting that LasR QS is more susceptible to activation by neighboring species. To explain the observed difference in signal selectivity, we developed a model based on (i) the activity profiles of these proteins and the previously reported profiles of the related LuxR-type receptors *Agrobacterium tumefaciens* TraR, *Vibrio fischeri* LuxR, *P. aeruginosa* QscR, and *Pectobacterium carotovora* ExpR2, (ii) sequence alignments, and (iii) structural data for TraR, LasR, and QscR. We propose that LuxR-type receptor selectivity depends predominantly on the identity of residues Ala38, Ser56, Arg61, and Thr129. This model may help to predict the signal selectivities of hundreds of less-studied LuxR-

type QS receptors from bacteria that grow in polymicrobial communities and possibly respond in interesting ways to one another's signals. In addition, we discovered AHLs that could be used to selectively activate LasR and selectively inhibit AbaR in polymicrobial infection models.

6.1 Introduction

Our world is home to an estimated 4–6 × 10^{30} living prokaryotes at any given moment.[1] These microbes predominantly grow in communities containing many species that cooperate and compete for optimal fitness.[2-4] Such polymicrobial communities have tremendous implications on human life and livelihood. For example, bacterial infections are often caused by a mixture of pathogenic species.[3, 5, 6] Beyond infections, however, we are increasingly appreciating the importance of the human microbiome (i.e., the mixture of hundreds of species of microbes that live on us and inside us) for human health.[7, 8] Communities of microbes are also essential for the production of food. Microbiomes in livestock,[9] on plants,[10] and in the soil[11] are crucial for the production of meat and produce and for the general health of the environment. Other foods, like cheese, are directly made by communities of microbes.[12, 13] Polymicrobial biofilms also can have an impact on important industrial processes that shape our daily lives.[14, 15]

In these communities, different species are all secreting different factors (e.g., small molecules, peptides, proteins) into the environment that can affect one another, and these complicated interactions can result in significant benefit or harm to the local environment or to host organisms. One way that the microbes can interact is through cross-talk of quorum sensing (QS) systems. Bacterial QS is generally viewed as a mechanism by which bacteria sense their population density and alter their behavior for optimal fitness under that density (although other explanations for the phenomenon exist[16, 17]). In QS, the bacteria synthesize and secrete a small molecule or peptide signal that can diffuse into the environment. Only when the density of bacteria is sufficiently high does the signal accumulate to a concentration at which it productively binds receptor proteins in the bacterial cells that then induce changes in gene expression. In the case of the Gram-negative proteobacteria phylum, bacteria typically use *N*-acyl L-homoserine lactone (AHL) signals that are biosynthesized by LuxI-type enzymes and sensed by receptor

proteins of the LuxR-type transcriptional regulator class.[18, 19] QS is typically viewed as a method by which bacteria monitor the density of their own species (allowing them to "count themselves"), but since many microbes that QS live in close proximity, it is likely that their signals function as cues that activate or disrupt the QS systems of other microbes,[4, 20, 21] and likewise microbes could eavesdrop on their neighbors and alter their behavior appropriately.[22, 23] Indeed, examining the modes by which and the extent to which bacteria use QS for interspecies—and potentially even interkingdom—sensing is an area of significant and increasing research interest.[24, 25]

For over 10 years the AI-2 quorum sensing system has been shown to be widespread among bacterial species and allows neighboring species to substantially impact the behavior of each other, but AHL-based QS is generally considered to function more for intraspecies communication.[19] However, initial examples have arisen demonstrating the impact of AHL-based QS cross talk on the behavior of polymicrobial populations. In the knot disease of the olive tree, the presence of non-pathogenic *Pantoea agglomerans* and *Erwinia toletana* aggravate the disease caused by *Pseudomonas savastanoi* pv. *savastanoi* in a QS-dependent manner.[26] It has also been shown that *Pseudomonas aeruginosa* can turn on the QS system of *Burkholderia cepacia* in mixed biofilms in lung infection models,[27] which mimic the mixed biofilms frequently observed in CF lungs.[28] Computational studies suggest that such cross-species signal interactions could have tremendous implications on the stability of microbiomes and their behavior.[29]

Genome sequencing technology is now allowing for the rapid identification of the organisms present in a given environment and the likely communication genes that they possess. For example, we know that in the human microbiome, 43 species of bacteria are present that have LuxR/SdiA-type receptor proteins, and at least 11 species have genes that should produce AHL signal molecules.[30] However, tools to discover the actual degree of chemical cross-talk between

species within a population and how that cross-talk influences the population's behavior are limiting. Two tools that would help the community substantially are (i) a method to use protein sequence of LuxR-type proteins to predict the promiscuity with which different LuxR-type proteins respond to signal molecules produced by other species, and (ii) small molecule probes that selectively activate and inhibit the QS of single species. The ability to predict promiscuity based on sequence would allow researchers to leverage the vast and growing metagenomic sequence data of polymicrobial environments to develop informed hypotheses of what QS cross-talk could be present. Then to compliment such insights, the addition of species-selective QS modulators to turn off or on the QS of individual species would allow for the interrogation of the impact of QS cross-talk on the *behavior* of the communities with the exquisite spatial and temporal control allowed by chemical probes.[31] The development of such a method and chemical tools was the motivation for the current study.

Herein, we report our investigations into the selective activation and inhibition of the similar LuxR-type receptor proteins AbaR of *Acinetobacter baumannii* and LasR of *P. aeruginosa* using AHL ligands. There were three main objectives for this study. First, we aimed to determine the limits of previously observed[32] AbaR ligand selectivity and LasR ligand promiscuity. Second, we sought to discover AHLs that can serve as chemical probes to selectively activate or inhibit the QS systems of only LasR or only AbaR to use to modulate their QS orthogonally in polymicrobial communities. Lastly, we aimed to use the ligand-promiscuity data reported herein, along with screening data of other LuxR homologs to develop a model that explains why some LuxR-type proteins are more selective than others, which could possibly help to predict the promiscuity of hundreds of LuxR-type proteins based on their amino acid sequences. We indeed confirmed a striking signal-response selectivity for AbaR and greater promiscuity for LasR. We also discovered a suite of synthetic ligands that can serve as tools to selectively activate LasR and

selectively inhibit AbaR, but no ligands were uncovered that do the reverse. Lastly, we developed a new model based on the known activities of *A. baumannii* AbaR,[32] *P. aeruginosa* LasR,[33, 34] *Agrobacterium tumefaciens* TraR,[33, 34] *Vibrio fischeri* LuxR,[33, 34] *P. aeruginosa* QscR,[35, 36] and *Pectobacterium carotovora* ExpR2,[37] as well as their amino acid sequences and the X-ray crystal structures of LasR, [38] TraR,[39] and QscR,[40] that explains the relative signal selectivity of these ligands and provides initial predictions about the selectivity of less-studied LuxR-type proteins.

6.2 Results and Discussion

6.2.1 Rationale for receptor selection

Several reasons prompted the choice of AbaR and LasR for focused study. First, an initial study suggested that these two receptors have different degrees of selectivity for their native signal molecules,[32] even though their native signals are very similar: AbaR naturally responds to *N*-(3-hydroxydodecanoyl) L-homoserine lactone (OH-dDHL) to regulate virulence in *A. baumannii*,[32, 41, 42] and LasR naturally responds to *N*-(3-oxo-dodecanoyl) L-homoserine lactone (OdDHL) to regulate several virulence phenotypes in *P. aeruginosa*.[43-46] Therefore studying these proteins could yield explanations to what makes a protein more selective for a given signal. Furthermore, these two species reside in polymicrobial contexts where cross-talk is possible: they are both widespread in the environment, where they live in proximity to hosts of other microbes, and they are both opportunistic pathogens of immunocompromised humans, where they often form polymicrobial infections,[6, 47-49] sometimes with each other.[50, 51] Lastly, because of widespread antibiotic resistance in these opportunistic pathogens, the study of their virulence is important for human health.[48, 52]

6.2.2 Library design and synthesis

The native AHLs of AbaR and LasR (OH-dDHL and OdDHL) only differ in oxidation state at the 3-posititon on their acyl chain. To systematically test the impact of acyl chain length and 3-position oxidation state on activity in AbaR and LasR, we selected aliphatic AHL ligands **5**–**20** for study, of which **9**, **11**–**15**, and **20** are new ligands to our growing AHL libraries (see **Figure 6.1**). Ligands **1**, **5**–**8**, **10**, and **16**–**19** have been previously synthesized and evaluated in LasR and AbaR but were re-evaluated here under the same conditions as the new ligands (see **Table 6.3** for original AHL identities and references) to facilitate legitimate data comparison.[32-34] The hydroxyl-containing ligands **11**–**15** were prepared as diastereomeric mixtures, whereas ligand **2** is the purely ®-OH-dDHL diastereomer. Many of these aliphatic-tail AHLs are, in fact, naturally occurring signal molecules for various proteobacteria. Therefore, testing their ability to modulate the AbaR and LasR QS receptors will likely not only provide chemical probes but should also elucidate the likelihood of *A. baumannii* and *P. aeruginosa* to be affected by cross-talk with neighboring proteobacteria in real polymicrobial communities.

Since some of the most active non-native AHLs contain substituted phenyl groups in the acyl tail (e.g., ligands **3** and **4**),[32, 34, 36, 53-55] we were interested in examining the effect that altering the 3-position oxidation state for ligands with substituted phenyl groups can have on their activity profiles in AbaR and LasR. Therefore, we examined the *N*-(phenylbutanoyl) L-homoserine lactone (PBHL) ligands **21**–**26**, which have unsubstituted or chlorine- or iodine-substituted phenyl groups and varying 3-position oxidation states (see **Figure 6.1**). Within this set of compounds, ligands **22**, **23**, and **26** constitute new AHLs for our in-house libraries, and ligands **3**, **4**, **21**, **24**, and **25** have been previously evaluated in LasR and AbaR (see **Table 6.3**).

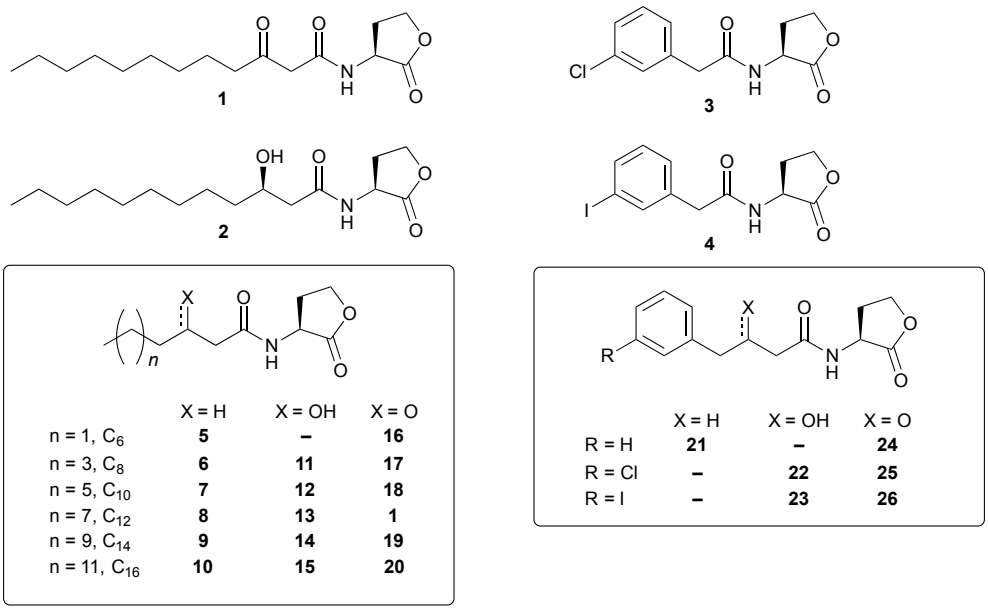


Figure 6.1. AHLs evaluated in this study.
The natural AHLs of LasR (**1**) and AbaR (**2**), and the non-native AHLs evaluated in this study (**3**–**26**) are displayed. Although non-native to AbaR and LasR, many of the aliphatic-tail ligands **5**–**20** are naturally produced by other species, and therefore are likely present when *P. aeruginosa* and *A. baumannii* reside in polymicrobial environments.

6.2.3 AbaR is highly selective for aliphatic-tail AHLs with 12-carbon chains and 3-position hydroxyls

Since our earlier study suggested that AbaR was particularly sensitive to acyl chain length and oxidation state,[32] we sought to more rigorously characterize the exact limits of which chain lengths and oxidation states would activate or inhibit this receptor's activity and directly compare these limits to those for LasR activation and inhibition. We examined aliphatic AHLs **5**–**20** for AbaR and LasR activation and inhibition using published bacterial reporter strains (see **section 6.4** for full details). In these assays, activated AbaR and LasR induce the production of a β-galactosidase enzyme, the amount of which can be quantified by its ability to cleave a colorimetric substrate. Activation (or agonism) is tested by adding AHLs and quantifying β-galactosidase activity. Competitive inhibition (or antagonism) is tested by adding native ligand

(OH-dDHL for AbaR, OdDHL for LasR) along with the AHL and quantifying the decrease in β-galactosidase activity caused by the AHL. The primary assay data resulting from these screens is listed in **Table 1**. These data are also plotted in an alternate format in **Figure 6.2**, which highlights the dependence of AbaR and LasR activity on ligand length and oxidation state.

Table 6.1. AbaR and LasR primary agonism and antagonism assay data and EC_{50} and IC_{50} values with confidence intervals for the aliphatic-tail AHLs[a]

Acyl Chain	AHL	Activation (%)[b]	EC_{50} value (μM)[c]	Inhibition (%)[d]	IC_{50} value (μM)[e]
AbaR					
®-3-OH-dDHL	**2**	100	0.699		
Methylene					
C_6	**5**	0		67	
C_8	**6**	0		79	9.87 (8.28–11.8)
C_{10}	**7**	0		71	9.64 (8.94–10.4)
C_{12}	**8**	0		40	
C_{14}	**9**	0		32	
C_{16}	**10**	1		14	
Hydroxyl					
C_8	**11**	0		79	3.06 (1.91–4.90)
C_{10}	**12**	29		6	
C_{12}	**13**	75	10.3 (7.18–14.7)[f]	-93	
C_{14}	**14**	31		-7	
C_{16}	**15**	0		23	
Oxo					
C_6	**16**	2		48	
C_8	**17**	7		86	2.65 (0.892–7.90)
C_{10}	**18**	6		69	1.60 (0.826–3.09)
C_{12}	**1**	43	7.53 (5.92–9.59)[f]	-53	
C_{14}	**19**	11		5	
C_{16}	**20**	0		6	
LasR					
OdDHL	**1**	100	5.79 (2.04–16.4)		
Methylene					
C_6	**5**	13		-14	
C_8	**6**	0		63	1.29 (0.961–1.74)[g]
C_{10}	**7**	34		57	
C_{12}	**8**	92	59.6 (51.4–69.0)	-59	
C_{14}	**9**	77	72.2 (36.7–142)[f]	-44	
C_{16}	**10**	26		-29	
Hydroxyl					
C_8	**11**	0		75	1.60 (0.686–3.73)[g]
C_{10}	**12**	38		31	0.112 (0.0748–0.168)[g]
C_{12}	**13**	85	248 (124–495)[f]	-46	
C_{14}	**14**	103	270 (149–487)	-43	
C_{16}	**15**	35	511 (294–889)[f]	-6	
Oxo					
C_6	**16**	17		1	
C_8	**17**	16		58	0.260 (0.199–0.339)
C_{10}	**18**	94	154 (73.4–323)	-53	
C_{12}	**1**	100	5.79 (2.04–16.4)	-81	
C_{14}	**19**	103	17.5 (15.4–19.8)	-56	
C_{16}	**20**	88	105 (40.1–275)[f]	-44	

[a] See **Figures 6.4**, **6.5**, **6.9**, and **6.10** for plots of primary data and **Figures 6.6**, **6.7**, **6.8**, **6.11**, **6.12**, **6.13**, and **6.15** for plots of dose response curves. All assays performed in biological triplicate.
[b] AHLs evaluated at 100 μM and normalized to **2** at 100 μM for AbaR and evaluated at 10 μm and normalized to **1** at 10 μM for LasR (set to 100%). Errors are displayed in **Figure 6.2**.
[c] EC_{50} values determined by testing AHLs over a range of concentrations (200 pM – 200 μM). 95% confidence intervals for the EC_{50} values are displayed.
[d] AHLs evaluated at 100 μM against **2** at 700 nM for AbaR and evaluated at 10 μM against **1** at 10 nM for LasR. Errors are displayed in **Figure 6.2**.
[e] IC_{50} values determined by testing AHLs over a range of concentrations (10 nM – 100 μM) against **2** at 700 nM for AbaR and against **1** at 10 nM for LasR. 95% confidence intervals for the IC_{50} values are displayed.
[f] Dose response curve reached a plateau over concentrations tested, yet the level of the maximal induction was lower than that for **2** for AbaR or **1** for LasR; EC_{50} value calculated from this dose response curve.
[g] Dose response curve upturned at higher concentrations and did not reach 100% inhibition over concentrations tested (prior to upturn), IC_{50} value calculated from partial antagonism dose response curve reported in **Figures 6.15** and **6.16**.

In the side-by-side test for receptor activation, AbaR was indeed far more selectively activated than LasR (**Figure 6.2**). All lengths of the fully reduced aliphatic AHLs (**5**–**10**) were incapable of activating AbaR, and most were instead strong competitive inhibitors of AbaR activation by its native ligand OH-dDHL (**2**). In particular, aliphatic AHLs with chain lengths below 12 carbons (**5**–**7**) were the strongest inhibitors. Fully oxidized ligands (ketone at the 3-position) were slightly better at activating AbaR than the reduced aliphatic AHLs; however, only the 12-carbon AHL, OdDHL (**1**), was capable of appreciable AbaR agonism (~50%). All other chain lengths of the 3-oxo AHLs were incapable of significant activation, and in fact the shorter chain (C_6, C_8, C_{10}) ligands **16**–**18** were good competitive inhibitors of AbaR. The hydroxyl AHL class was the best at activating AbaR, which is not surprising given that the native ligand (OH-dDHL (**2**)) bears a 3-OH moiety. Since diastereomeric mixtures of these ligands (**11**–**15**) were tested, activities were less than would be expected for the diastereopure ligands (e.g., the activity of the diastereomeric mixture of C_{12} ligands, **13**, yields 75% activation compared to 100% by the purely *(R)*-OH-dDHL, **2**). Even in these diastereomeric mixtures, the C_{10} and C_{14} ligands activated AbaR by more than 25%. However, at longer and shorter chain lengths, the AHLs did not activate AbaR, but instead were competitive inhibitors—especially the shorter C_8 ligand **11**.

In total, AbaR can only be activated by ligands that vary by the slightest margin from its native ligand (OH-dDHL) in either oxidation state or chain length, but not both. Most of the aliphatic AHLs tested, especially those with shorter chains, were instead good competitive inhibitors of AbaR.

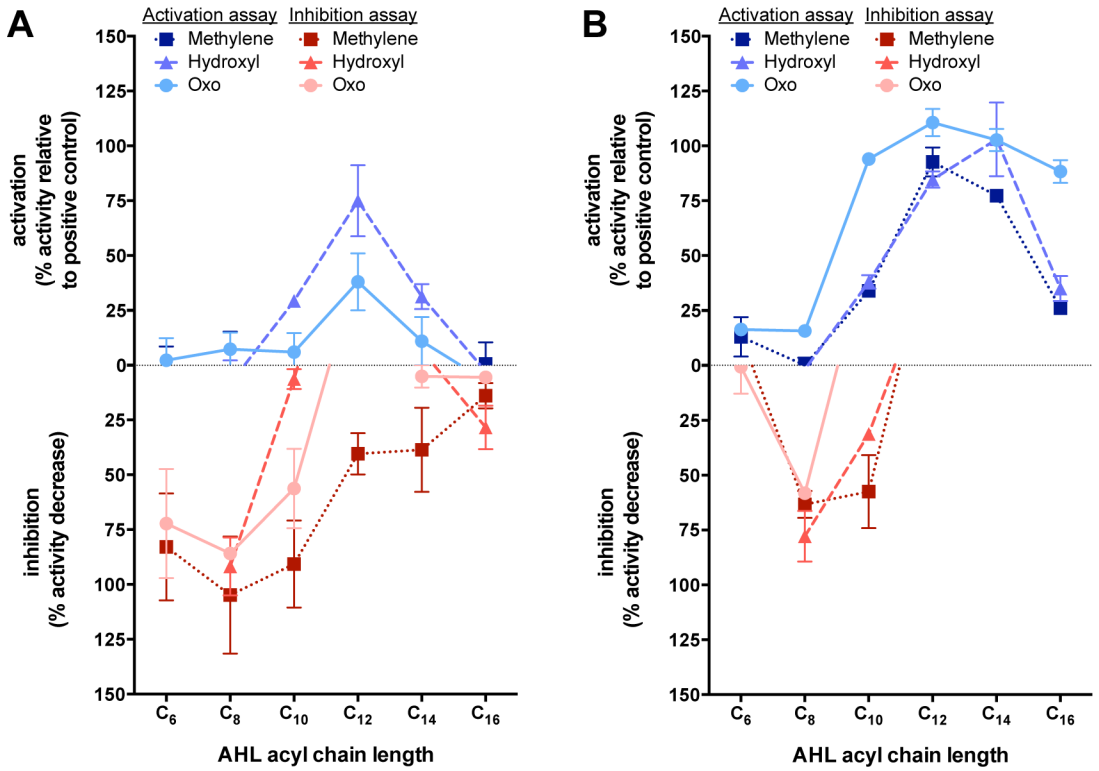


Figure 6.2. Activation and inhibition of AbaR (A) and LasR (B) by aliphatic AHLs.
Data is also presented in tabular form in **Table 1**. Description of assays is described in **Table 1** and in the **section 6.4**. Activation activity is reported with blue shades as percent of the activity induced by the receptor's native ligand (**2** for AbaR and **1** for LasR) on the positive y-axis. Inhibition activity is reported in red shades on the negative y-axis as the % decrease in activity relative to only negative ligand being present. Activities of fully reduced "methylene" AHLs **5**–**10** are displayed as dark-shaded squares with dotted lines, activities of 3-hydroxyl AHLs **11**–**15** are displayed as medium-shaded triangles with dashed lines, and activities of fully oxidized 3-oxo AHLs **16**–**18**, **1**, **19**, and **20** are displayed as light-shaded circles with solid lines. Error bars represent s.e.m. of a biological triplicate. "Negative" activation values (indicating inhibition) and inhibition values (indicating activation) were excluded for clarity.

In contrast, LasR was much more promiscuously activated by AHL ligands than AbaR (**Figure 6.2B**). AHLs with any of the three oxidation states were able to substantially activate

LasR (>75%) at the native-like C_{12} length (**8**, **13**, and OdDHL (**1)**). The reduced and hydroxyl ligands were still good agonists (>75%) at the C_{14} length (**9**, **14**), but were much weaker agonists at the C_{16} length (**10**, **15**) and at chain lengths less than C_{12}. Reduced and hydroxyl ligands of both lengths C_8 (**6**, **11**) and C_{10} (**7**, **12**) were competitive inhibitors of LasR, instead. LasR was activated by an even wider range of ligands with the native 3-oxo functionality: chain lengths of C_{10} through C_{16} (OdDHL (**1**), **18**, **19**, and **20**) all activated LasR >75%. Only the C_8 3-oxo ligand **17** was a competitive LasR inhibitor. In total, LasR can be activated by ligands of substantial variation in chain length or oxidation state, and can even tolerate ligands with variations in both traits.

Clearly both AHL acyl chain oxidation state and length play key roles for both AbaR and LasR, but the tolerance thresholds differ substantially. With regard to the biological relevance of these activity profiles, it is interesting that AbaR is so selective for activation by its native ligand, whereas LasR is more promiscuous. Both *A. baumannii* and *P. aeruginosa* are increasingly found in mixed microbial environments, where other species can be producing AHLs with various lengths and oxidation states.[6, 47-51] However, these two organisms appear to have opposite approaches to respond to the AHLs of other species: *A. baumannii* QS is not activated—but in fact inhibited—by these foreign signals, whereas *P. aeruginosa* QS is activated. The fitness implications of these two strategies is an exciting avenue for future research,[21, 56] and a potential biochemical rationale for this relative selectivity and promiscuity is discussed later on in this work (**Figure 6.3**). In addition, these assays revealed ligands that selectively activate LasR and have essentially no effect on AbaR (the C_{14} and C_{16} 3-oxo ligands **19** and **20**), ligands that have no effect on LasR and selectively inhibit AbaR (the C_6 methylene and 3-oxo ligands **5** and **16**), and finally, ligands that both activate LasR and inhibit AbaR (the C_{10} 3-oxo ligand **18,** and to a lesser extent, the C_{12} and C_{14} methylene ligands **8** and **9**). The identification and development of

ligands with LuxR-type receptor selectivity remains largely unchartered;[33, 34] thus, the discovery of these activity profiles for ligands **5**–**20** in AbaR and LasR is significant. These ligands can be applied for testing the impact of AbaR inhibition and LasR activation in mixed cultures of *A. baumannii* and *P. aeruginosa*—a condition that is relevant to infection populations.[50, 51]

6.2.4 AbaR and LasR are inhibited to similar degrees by aromatic AHLs of different 3-position oxidation states

We next examined the effects that altering the 3-position oxidation state in the non-native PBHL ligands (**21**-**26)** has on their agonistic and antagonistic activities in AbaR and LasR. As **Table 2** shows, all six PBHLs are inhibitors of AbaR, and all but ligand **21** are inhibitors of LasR. None of the ligands demonstrated appreciable agonist activity on either receptor (see **Figures 6.4** and **6.9**). Similarly, *N*-(phenylacetanoyl) L-homoserine lactones **3** and **4** are inhibitors of LasR (40–60%)[34] and AbaR (70–90%)[32] (see **Figures 6.4** and **6.9**). The 3-position oxidation state appeared to have no impact on the ability of each AHL to modulate AbaR (i.e., **21** was similar to **24**, **22** was similar to **25**, and **23** was similar to **26**). The oxidation state slightly impacted LasR inhibition, however, as the percent inhibition values for ligands **24** and **22** were somewhat higher than for ligands **21** and **25**, respectively, and the IC_{50} for ligand **26** was 4× lower than that for ligand **23**. There was no obvious trend of which oxidation state yielded the strongest inhibitory activity for this ligand class, however. With the chlorine-substituted PBHLs (**22** and **25)**, 3-hydroxyl provided better inhibition than 3-oxo, but the opposite was observed for the iodine-substituted ligands (**23** and **26**). With the unsubstituted phenyl ligands (**21** and **24**), 3-oxo was better than 3-methylene, so possibly some oxidation at the 3 position is better than none due to polarity or sterics, although more ligands would need to be tested to confirm this.

Table 6.2. AbaR and LasR primary antagonism assay data and IC_{50} values with confidence intervals for the aromatic-tail AHLs[a, b]

	AbaR		LasR	
AHL	Inhibition (%)[c]	IC_{50} value (nM)[d]	Inhibition (%)[c]	IC_{50} value (μM)[d]
21	56		0	
22	74	7.54 (6.81–8.34)	89	1.50 (0.778–2.89)
23	73	7.36 (5.74–9.44)	48	1.98 (1.26–3.10)
24	51		20	1.91 (0.885–4.10)
25	51	10.5 (7.16–15.4)	53	1.53 (0.734–3.17)
26	69	20.0 (9.28–43.0)	54	0.458 (0.191–1.10)

[a] See **Figures 6.5** and **6.10** for primary data and **Figures 6.8**, **6.13**, **6.14**, and **6.16** for plots of dose response curves. All assays performed in biological triplicate.
[b] All ligands were screened for ability to activate LasR and AbaR, too, and none showed appreciable agonist activity. See **Figures 6.4** and **6.9**.
[c] AHLs evaluated at 100 μM against **2** at 700 nM for AbaR or at 10 μM against **1** at 10 nM for LasR. Error ≤ ±10%.
[d] IC_{50} values determined by testing AHLs over a range of concentrations (10 nM – 100 μM) against **2** at 700 nM for AbaR or **1** at 10 nM for LasR**.**

Collectively, the aliphatic- and aromatic-tail AHLs tested herein provide ligands with a remarkable range of activity and selectivity profiles. These compounds can activate both AbaR and LasR (**13**), inhibit both AbaR and LasR (**6**, **7**, **11**, **17**), activate LasR only (**19**, **20**), inhibit AbaR only (**5**, **16**), or *both* activate LasR *and* inhibit AbaR at the same time (**18**). We still lack ligands that selectively activate AbaR and/or selectively inhibit LasR. Since the latter additional ligands would be particularly useful tools to study the impact of QS on polymicrobial cultures containing *P. aeruginosa* and *A. baumannii*, research is ongoing in our laboratory to discover modulators with these desired profiles. An additional approach to provide such activity profiles could be to apply bump-hole design. This strategy, which has been fruitful to selectively regulate signal receptors in human cells, involves mutating one receptor to have a unique "hole" that allows it to accommodate a small molecule modulator that has a matching unique "bump."[57] Therefore, only the mutated receptor is modulated by the ligand. We have recently reported subtle single-residue mutations in LasR that provide such a bump-hole scenario, causing mutant LasR to respond to ligands in opposite ways relative to wild-type LasR.[58] Generating *P. aeruginosa*

strains with these mutant LasR genes might allow for the temporally controlled selective inhibition of *P. aeruginosa* LasR QS and/or selective activation of *A. baumannii* AbaR QS in polymicrobial environments.

6.2.5 A model for LuxR-type receptor selectivity for native AHL signal

From these combined studies, the primary difference in AbaR:AHL and LasR:AHL SARs was that AbaR was only strongly activated by its native ligand; conversely, ligands of multiple oxidation states and chain lengths were good agonists of LasR. Thus, the question arose as to what protein features make a given LuxR-type receptor protein selective for its native ligand or more promiscuous. Understanding these features would provide a better molecular understanding of the LuxR-type QS mechanism, and importantly in the context of the broad goals of this study, allow us to gain insights into the possibility of mixed species QS signaling in polymicrobial environments. To address these questions, we compared the sequences of AbaR and LasR with other LuxR-type homologs to see if specific residues could be responsible for the differing structure activity relationships (SARs, see **Figure 6.3**). The amino acid sequences of AbaR and LasR were aligned with 13 other LuxR-type receptors that are present in bacterial species that produce AHLs with keto or hydroxyl groups at their 3 position (as these receptors most likely should respond to oxidized AHLs). At least four of these 13 additional receptors have well-characterized AHL SARs (TraR from *A. tumefaciens* with native ligand *N*-(3-oxo-octanoyl) L-homoserine lactone (**17**), LuxR from *V. fischeri* with native ligand *N*-(3-oxo-hexanoyl) L-homoserine lactone (OHHL, **16)**, QscR from *P. aeruginosa* with native ligand OdDHL (**1**), and ExpR2 from *P. carotovora* with native ligand OHHL (**16**); highlighted in **Figure 6.3A**).[33-37] Similarly to AbaR, TraR has been shown to be selectively activated by its native ligand **17**, and not by AHLs with other 3-position oxidation or acyl chain lengths.[33, 34] In contrast, LuxR, which

naturally responds to **16**, is moderately activated by a somewhat broader range of AHLs, similarly to LasR.[33, 34] Finally, QscR, an orphan receptor thought to naturally bind OdDHL (**1**), and ExpR2, a receptor that is the primary regulator of *P. carotovorum* virulence through detection of **16**, are activated by a broad range of AHLs with varied 3-position oxidation state and acyl chain length, as well as many other AHLs with aromatic groups.[35, 37]

In view of these SARs, we suspected to find sequence similarities between AbaR and TraR. Indeed, as can be seen in **Figure 6.3A**, these two receptors share identity at residues A38 (left yellow box), Q63/58 (green box), and T131/129 (right yellow box), among other positions (see full sequence alignment in **Figure 6.17**). Likewise, LasR and LuxR shared L36/42 (left yellow box), R61/67 (green box), and S129/137 (right yellow box)—all different from the homologous residues in TraR and AbaR. Finally, QscR and ExpR2 (the most promiscuous proteins) showed identity at the S56/48 position (pink box) and at S129/125 (right yellow box). We considered the possibility that the identities at these positions could be due to the proteins being generally closer related and less separated by genetic drift. To test that possibility, we performed a phylogenetic analysis of the 15 proteins and found that the proteins with similar selectivities and identity at these specific positions *do not* cluster together—indicating that they are not overall more similar (**Figure 6.3B**). Therefore, it is unlikely that the observed identities at these positions is due to genetic drift, which gives weight to the observed sequence identities at the positions that correlated with the selectivity of ligand responses (A38, S56, R61, and T129).

We next mapped these highlighted residues to the reported X-ray crystal structural data for TraR,[59] LasR,[38] and QscR[40] bound to their native AHL ligands, and we were delighted to discover that they play significant roles in ligand binding in the solid-state. The boxed residues appear to form hydrogen bonds to the 3-oxo groups of their ligands (Ala38 and Thr129 for TraR, Arg61 for LasR, and Ser56 for QscR, see **Figure 6.3C,D**). Furthermore, after careful scrutiny of these

structures, we clearly see three different modes of binding to the 3-oxo group, and these three modes correspond with the different observed ligand-response selectivities. In the TraR structure, the T129 side-chain and A38 amide carbonyl combine to form a water-mediated hydrogen bond with the 3-oxo of the AHL.[39, 60] Site-directed mutagenesis has demonstrated the importance of this interaction for TraR discriminating between different AHLs.[61] Since TraR is more discriminately activated than LasR,[34, 61] we would suspect a site less accommodating to alternative AHLs. Indeed, because the Thr129/Ala38 bond involves two protein residues, a ligand change would likely require a greater restructuring of the TraR protein compared to LasR, in which only one side chain (Arg61)[38] would be immediately affected. AbaR is the only protein in the alignment to share identity with TraR at both residues; we hypothesize that AbaR binds its native ligand in a similar mode, and that mode is the origin of its higher selectivity compared to LasR. Furthermore, the Arg67 of LuxR suggests that it binds the 3-oxo moiety of its native ligand similarly to LasR, explaining its mild-to-moderate promiscuity in our previous studies.[33, 34] In the QscR crystal structure, the ligand 3-oxo group hydrogen bonds with the protein's Ser56 through a chain of *two* water molecules.[40] It therefore makes sense that QscR has the most promiscuous activity,[33, 34] since the two waters could be excluded or shifted to accommodate other oxidation states at the 3-oxo positions (as previously noted by Churchill and coworkers[58]). ExpR2, the other promiscuous protein,[37] shares this serine residue, as well.

Therefore, for AbaR, we suspect that its native ligand selectivity originates in a TraR-like binding mode versus the more promiscuous LasR binding mode. More generally, we hypothesize that the residues homologous to Ala38/Thr129 in TraR, Arg61 in LasR, and Ser56 in QscR are important determinants of promiscuity of ligand binding for all LuxR-type proteins. Certainly, other determinants of ligand selectivity can exist in addition to these residues—for example, Arnold and co-workers showed a combination of mutations outside the binding pocket can confer

drastic changes in LuxR substrate selectivity,[62, 63] and the research teams of Churchill and co-workers and Chai and Winans have each shown that residues in the hydrophobic tail-binding regions of QscR and TraR, respectively, can help determine chain-length selectivity.[40, 61] Nonetheless, we believe the hypothesized selectivity determinants are simple, logical, and promising. Ongoing work will test this hypothesis by mutation of those four residues in multiple proteins and monitoring for the expected alterations in selectivity.

Finally, we return to the other nine LuxR-type receptors in our alignment analysis that have less characterized SARs (CarR from *P. carotovorum*, EsaR from *Pantoea stewartii*, ExpR1 form *P. carotovorum*, PpuR from *Pseudomonas putida*, PssR from *Pseudomonas savastanoi*, RhiR from *Rhizobium leguminosarum*, SpnR from *Serratia marcescens*, VanR from *Vibrio anguillarum*, and YpsR from *Yersinia pseudotuberculosis*) to probe if their sequences contained residues similar to Ala38/Thr129 in TraR, Arg61 in LasR, or Ser56 in QscR. Generally, these receptors matched either LasR/LuxR or QscR/ExpR2 better than TraR/AbaR. We therefore hypothesize that these LuxR homologs are activated by a broad range of non-native AHLs. In support of this hypothesis, we recently reported that ExpR1 displays LuxR-like activity with a focused AHL library.[37] Furthermore, YpsR is present in a bacterium (*Y. pseudotuberculosis*) that produces at least 24 different AHLs,[64] and PpuR and SpnR are present in bacteria (*P. putida* and *S. marcescens*) that each produce four separate signal molecules,[65, 66] suggesting that these receptor proteins naturally respond to a range of ligands. Future work remains to test the actual ligand selectivity of these receptors and therefore support or refute our model. However, the prospect of being able to predict ligand selectivity from sequence is an exciting possibility given the ease with which genomic sequence information can be obtained from mixed bacterial populations in nature. Its conceivable that this work could lead to future developments that enable researchers to discover which bacterial species are present in a native environment *and* how their

QS systems respond to one another's signals by simply investigating the metagenomic DNA sequence data from that environment.

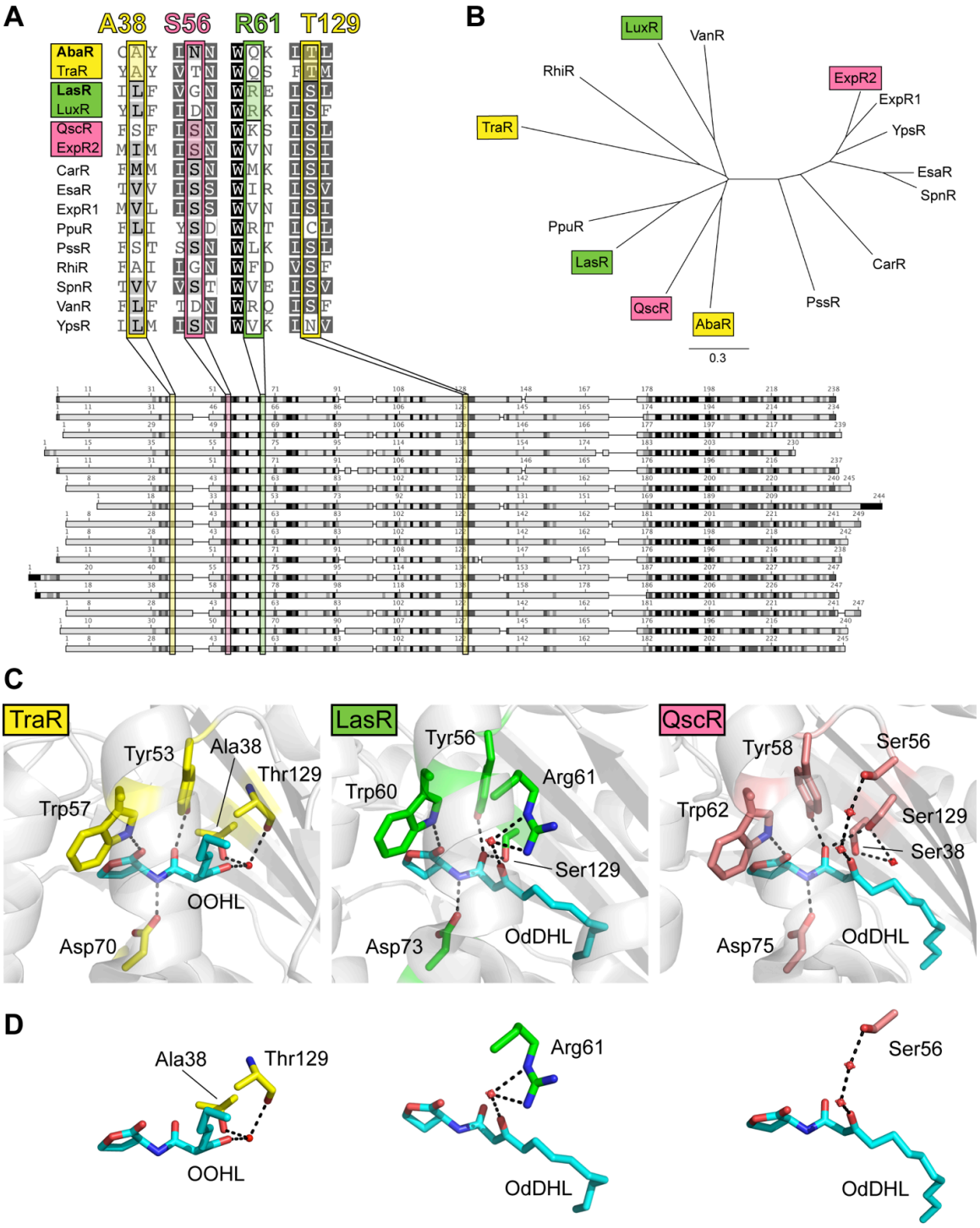

A
A38
S56
R61
T129
AbaR
TraR
LasR
LuxR
QscR
ExpR2
CarR
EsaR
ExpR1
PpuR
PssR
RhiR
SpnR
VanR
YpsR
B
LuxR
VanR
RhiR
ExpR2
ExpR1
YpsR
TraR
EsaR
SpnR
PpuR
LasR
CarR
QscR
PssR
AbaR
0.3
C
TraR
Tyr53
Ala38
Thr129
Trp57
OOHL
Asp70
LasR
Tyr56
Arg61
Trp60
Ser129
Asp73
OdDHL
QscR
Tyr58
Ser56
Trp62
Ser129
Ser38
Asp75
OdDHL
D
Ala38
Thr129
OOHL
Arg61
OdDHL
Ser56
OdDHL

Figure 6.3. Model to explain AHL-selectivity in AbaR, LasR, and other LuxR-type QS receptors. (A) Alignment of fifteen LuxR homologs present in bacteria known to produce oxidized (3-oxo or 3-hydroxyl) AHLs. See **Figure 6.17** for full alignment. Darker shading indicates higher degree of conservation. The boxed proteins are ones with well-characterized AHL SARs, separated by characterization as selective receptors (yellow: AbaR, TraR), moderately selective receptors (green: LasR, LuxR), and promiscuous receptors (pink: QscR, ExpR2). Four residues are highlighted that are consistent among proteins of the same selectivity category and are also involved in the proteins' interactions with the 3-oxo moiety of the ligand in published X-ray crystal structures (A38 and T129 for TraR, S56 for QscR, and R61 for LasR). (B) Phylogenic tree showing overall relatedness of the fifteen aligned proteins—the proteins with similar selectivities and alignments at residues 38, 56, 61, and 129 do not cluster together. (C) Images of the ligand-binding pocket of X-ray crystal structures of $[TraR\text{-}OOHL]_2$ (pdb 1L3L, left),[59] $[LasR\text{-}OdDHL]_2$ (pdb 2UV0, middle),[38] and $[QscR\text{-}OdDHL]_2$ (pdb 3SZT).[40] (D) Simplified versions of binding pocket images displaying only the residues involved in hydrogen-bonding with the 3-oxo ligand moiety. Black dashed lines indicate hydrogen bonds, and red spheres indicate water molecules. Sequence alignment (ClustalW with BLOSUM cost matrix) and phylogenic tree production (Jukes-Cantor genetic distance model with the neighbor-joining method and no outgrouping) were performed in Geneious software (Biomatters Ltd, New Zealand). Structures were viewed in PyMol™ software (Schrödinger, LLC). Species names and GenBank Accession numbers for the aligned sequences are as follows: AbaR, *A. baumannii*, EGJ67179.1; TraR, *A. tumefaciens*, AAC28121.1; LasR, *P. aeruginosa*, AAG04819.1; LuxR, *V. fischeri*, AAQ90196.1; QscR, *P. aeruginosa*, NP_250589.1; ExpR2, *P. carotovorum*, from Cui et al.;[66] CarR, *P. carotovorum*, AAC45995.1; EsaR, *P. stewartii*, AAA82097.1; ExpR1, *P. carotovorum*, AAX77679.1; PpuR, *P. putida*, AAM75413.1; PssR, *P. savastanoi*, CBM41481.1; RhiR, *R. leguminosarum*, AAA26360.2; SpnR, *S. marcescens*, AAN52499.1; VanR, *V. anguillarum*, AAC45213.1; YpsR, *Y. pseudotuberculosis*, AAD40485.1.

6.3 Summary and Outlook

This study has rigorously characterized the impact of AHLs with different chain lengths (C_6 through C_{16}), 3-position oxidation states (ketone, alcohol, and methylene), and gross structures (aliphatic versus aromatic) on activation and inhibition of the AbaR and LasR QS receptor proteins from the opportunistic pathogens *A. baumannii* and *P. aeruginosa*. We found that AbaR is exquisitely selective for its native ligand. Out of this focused library of 26 AHLs, it was only activated >50% by its native ligand, and only minimal changes (i.e., keto to hydroxyl oxidation or C_{12} to C_{10} or C_{14} length) could be tolerated to maintain any activation at all. These data demonstrate how *A. baumannii* QS should not be activated by the AHL signals of neighboring bacteria (in contrast to *P. aeruginosa* and most other proteobacteria that appear to have more promiscuous LuxR-type receptors). We speculate that over the course of evolutionary history, *A. baumannii* has undergone a selective pressure to avoid QS in the presence of other bacteria

that use AHL-based QS, but we are unaware of other evidence that support or refute this proposal. We also discovered AHL ligands that could be useful chemical probes to activate both receptors, inhibit both receptors, selectively activate LasR, selectively inhibit AbaR, and *both* activate LasR *and* inhibit AbaR as they grow in polymicrobial communities. By investigating sequence alignments and X-ray crystal structures, we were able to hypothesize ligand selectivity determinants for LuxR-type proteins and offer predictions of selectivity for receptors in less studied organisms. The deeper understanding of the *A. baumannii* and *P. aeruginosa* QS systems afforded by this study is significant because virulence phenotypes of both of these pathogens are regulated by QS,[32, 41-46] and both pathogens are of significant clinical concern due to their propensity to antibiotic resistance.[48, 52] Furthermore, they have both been observed extensively in polymicrobial infections where QS cross-talk is likely to occur. More broadly, we expect that the groundwork that this study helps to lay for the molecular mechanisms of cross-species communication will show its significance as we increasingly observe polymicrobial populations with complex behaviors in infections, agriculture, and the environment.

6.4 Experimental

6.4.1 General Experimental

All chemical reagents were purchased from commercial sources (Alfa-Aesar, Sigma-Aldrich, and Acros) and used without further purification. Solvents were purchased from commercial sources (Sigma-Aldrich and J.T. Baker) and used as obtained, with the exception of dichloromethane (CH_2Cl_2), which was distilled over calcium hydride immediately prior to use. Water was purified using a Millipore Analyzer Feed System.

^{1}H NMR spectra were recorded in deuterated NMR solvents at 300 MHz on a Varian Mercury-300 spectrometer. Chemical shifts are reported in parts per million (ppm, δ) using

corresponding solvents or tetramethylsilane (TMS) as a reference. Couplings are reported in hertz (Hz). Electrospray ionization (ESI) MS data were obtained using a Waters (Micromass) LCT^{TM} system. This instrument uses a time-of-flight analyzer. Samples were dissolved in methanol and sprayed with a sample cone voltage of 20. Electron impact (EI) MS data were obtained using a Waters (Micromass) AutoSpec® system.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Shimadzu system equipped with an SCL-10Avp controller, an LC-10AT pump, an FCV-10ALvp solvent mixer, and an SPD-10MAvp UV/vis diode array detector. A Restek Premier C18 column (5 μm, 4.6 mm x 250 mm) was used for all analytical RP-HPLC work. An Agilent Zorbax prepHT 300SB-C18 column (7 μm, 21.2 mm x 250 mm) was used for all preparative RP-HPLC work. Standard RP-HPLC conditions were as follows: flow rates were 1 mL min-1 for analytical separations and 9 mL min-1 for preparative separations; mobile phase A = water; mobile phase B = acetonitrile.

Compounds were synthesized according to previously reported protocols and purified by RP-HPLC (purities >95%).[32-34, 67, 68] Compounds that have been reported previously are listed in **Table 6.3** with original compound names and original publication. Characterization data are below:

Tetradecanoyl-L-homoserine lactone (9). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.00 ppm (dd, 1H, NH), 4.55 (m, 1H, C(O)OC**H**$_2$), 4.48 (t, 1H, C(O)NHC**H**-lactone), 4.30 (m, 1H, C(O)OC**H**$_2$), 2.88 (m, 1H, C**H**$_2$-lactone), 2.24 (m, 2H, CH_2C**H**$_2$C(O)), 2.13 (m, 1H, C**H**$_2$-lactone), 1.64 (m, 2H, C**H**$_2$$CH_2$C(O)), 1.2-1.4 (m, 20H, C**H**$_2$), 0.88 (t, 3H, C**H**$_3$).

3-hydroxy-octanoyl-L-homoserine lactone (11). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.11 ppm (m, 1H, NH), 4.58 (m, 1H, C(O)OC**H**$_2$), 4.45 (t, 1H, C(O)NHC**H**-lactone), 4.31 (m, 1H, C(O)OC**H**$_2$), 4.03 (m, 1H, C**H**(OH)), 2.82 (m, 1H, C**H**$_2$-lactone), 2.47 (ABX, 2H, CH(OH)C**H**$_2$C(O)), 2.19 (m,

1H, C**H**$_2$-lactone), 1.2-1.5 (m, 12H, C**H**$_2$), 0.88 (t, 3H, C**H**$_3$); matches previously reported NMR data.[69]

3-hydroxy-decanoyl-L-homoserine lactone (12). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.50 ppm (m, 1H, NH), 4.68 (m, 1H, C(O)OC**H**$_2$), 4.48 (t, 1H, C(O)NHC**H**-lactone), 4.32 (m, 1H, C(O)OC**H**$_2$), 4.01 (m, 1H, C**H**(OH)), 2.76 (m, 1H, C**H**$_2$-lactone), 2.42 (ABX, 2H, CH(OH)C**H**$_2$C(O)), 2.38 (m, 1H, C**H**$_2$-lactone), 1.2-1.6 (m, 8H, C**H**$_2$), 0.88 (t, 3H, C**H**$_3$).

3-hydroxy-dodecanoyl-L-homoserine lactone (13). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.61 ppm (m, 1H, NH), 4.58 (m, 1H, C(O)OC**H**$_2$), 4.47 (dt, 1H, C(O)NHC**H**-lactone), 4.30 (m, 1H, C(O)OC**H**$_2$), 4.01 (m, 1H, C**H**(OH)), 2.80 (m, 1H, C**H**$_2$-lactone), 2.41 (ABX, 2H, CH(OH)C**H**$_2$C(O)), 2.19 (m, 1H, C**H**$_2$-lactone), 1.2-1.5 (m, 8H, C**H**$_2$), 0.88 (t, 3H, C**H**$_3$); matches previously reported NMR data.[32, 70]

3-hydroxy-tetradecanoyl-L-homoserine lactone (14). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.55 ppm (dd, 1H, NH), 4.57 (m, 1H, C(O)OC**H**$_2$), 4.48 (t, 1H, C(O)NHC**H**-lactone), 4.29 (m, 1H, C(O)OC**H**$_2$), 4.01 (m, 1H, C(OH)**H**), 2.83 (m, 1H, C**H**$_2$-lactone), 2.37 (ABX, 2H, C(OH)C**H**$_2$C(O)), 2.18 (m, 1H, C**H**$_2$-lactone), 1.2-1.6 (m, 20H, C**H**$_2$), 0.88 (t, 3H, C**H**$_3$); matches previously reported NMR data.[70]

3-hydroxy-hexadecanoyl-L-homoserine lactone (15). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.55 ppm (m, 1H, NH), 4.57 (m, 1H, C(O)OC**H**$_2$), 4.45 (t, 1H, C(O)NHC**H**-lactone), 4.26 (m, 1H, C(O)OC**H**$_2$), 4.09 (m, 1H, C**H**(OH)), 2.81 (m, 1H, C**H**$_2$-lactone), 2.54 (ABX, 2H, CH(OH)C**H**$_2$C(O)), 2.39 (m, 1H, C**H**$_2$-lactone), 1.2-1.6 (m, 24H, C**H**$_2$), 0.88 (t, 3H, C**H**$_3$).

3-oxo-hexadecanoyl-L-homoserine lactone (20). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.72 (d, 1H, NH), 4.60 (m, 1H, C(O)OC**H**$_2$), 4.48 (dt, 1H, C(O)NHC**H**-lactone), 4.29 (m, 1H, C(O)OC**H**$_2$), 3.47 (s, 2H, C(O)C**H**$_2$C(O)), 2.75 (m, 1H, C**H**$_2$-lactone), 2.52 (t, 2H, CH_2C**H**$_2$C(O)), 2.24 (m, 1H, C**H**$_2$-lactone), 1.60 (m, 2H, C**H**$_2$$CH_2$C(O)), 1.26 (m, 20H, C**H**$_2$), 0.88 (t, 3H, C**H**$_3$).

3-hydroxy-4-(3-iodo-phenyl)butanoyl-L-homoserine lactone (23). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58 ppm (m, 2H, *o*- and *p*-ArC**H**), 7.18 (d, 1H, *o*-ArC**H**), 7.05 (t, 1H, *m*-ArC**H**), 6.82 (m, 1H, NH), 4.58 (m, 1H, C(O)OC**H**$_2$), 4.48 (t, 1H, C(O)NHC**H**-lactone), 4.29 (m, 2H, C(O)OC**H**$_2$ and C(OH)**H**), 2.71 (m, 3H, C**H**$_2$-lactone and ArC**H**$_2$C(OH)), 2.42 (m, 2H, C(OH)C**H**$_2$C(O)), 2.20 (m, 1H, C**H**$_2$-lactone); ESI-MS: expected m/z = 389.01, observed 411.7 $[M+Na]^+$.

3-oxo-4-(3-iodo-phenyl)butanoyl-L-homoserine lactone (26). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.65 ppm (d, 1H, *p*-ArC**H**), 7.58 (s, 1H, *o*-ArC**H**), 7.49 (d, 1H, NH), 7.18 (d, 1H, *o*-Ar**H**), 7.10 (t, 1H, *m*-Ar**H**), 4.58 (m, 1H, C(O)OC**H**$_2$), 4.52 (dt, 1H, C(O)NHC**H**-lactone), 4.30 (m, 1H, C(O)OC**H**$_2$), 3.78 (s, 2H, ArC**H**$_2$C(O)), 3.53 (s, 2H, C(O)**H**$_2$C(O)), 2.76 (m, 1H, C**H**$_2$-lactone), 2.19 (m, 1H, C**H**$_2$-lactone); ESI-MS: expected m/z = 387.00, observed 409.7 $[M+Na]^+$.

Table 6.3. AbaR and LasR primary antagonism assay data and IC_{50} values with confidence intervals for the aromatic-tail AHLs[a, b]

AHL name (as described here)	AHL name (as previously published)	Original Publication
1	**2** (OdDHL)	[33, 34]
2	®-OH-dDHL	[32]
3	**C6**	[33, 34]
4	**C11**	[33, 34]
5	**A2**	[33, 34]
6	**A3**	[33, 34]
7	**A4**	[33, 34]
8	**A5**	[33, 34]
9	**-**[a]	This study
10	**A6**	[33, 34]
11	**-**[a]	This study
12	**-**[a]	This study
13	**-**[a]	This study
14	**-**[a]	This study
15	**-**[a]	This study
16	**3**	[33, 34]
17	**1**	[33, 34]
18	**A7**	[33, 34]
19	**A8**	[33, 34]
20	**-**[a]	This study
21	**6**	[33, 34]
22	**-**	This study
23	**-**	This study
24	**5**	[33, 34]
25	**15**	[71]
26	-	This study

[a] Commercially available AHLs.

6.4.2 Biological reagents and strain information

All biological reagents were purchased from Fisher Scientific and used according to enclosed instructions. Luria–Bertani (LB) medium was prepared as instructed with pH = 7.0. Buffers and solutions (Z buffer, 0.1% (m/v) aqueous SDS, and phosphate buffer) for Miller absorbance assays in *A. baumannii* were prepared as described.[72] The *A. baumannii* M2 *abaI::lacZ* (Δ*abaI* reporter) strain was used for the AbaR bacteriological assay in this study.[73] The initial LasR reporter strain was *E. coli* DH5α [F^- φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+)

phoA supE44 λ^- *thi-1 gyrA96 relA1*] harboring the LasR expression vector pJN105L and a plasmid-born *lasI–lacZ* fusion (pSC11).[74] Bacterial cultures were grown in a standard laboratory incubator at 37 °C with shaking (200 rpm) unless noted otherwise. Absorbance measurements were obtained using a Biotek Synergy 2 microplate reader using Gen5 data analysis software. All bacteriological reporter assays were performed in triplicate. No AHL was found to inhibit growth over the time course of the assays in this study.

6.4.3 Compound handling

Stock solutions of synthetic compounds (10 mM and 1 mM) were prepared in DMSO and stored at 4 °C in sealed vials. The amount of DMSO used in small molecule screens did not exceed 2% (v/v). Solvent resistant polypropylene or polystyrene 96-well multititer plates were used when appropriate for small molecule screening. The concentrations of synthetic AHL ligand used in the primary antagonism and agonism assays and the relative ratios of synthetic ligand to **1** (10 μM : 10 nM) and **2** (100 μM: 0.70 μM) in the LasR and AbaR antagonism assays, respectively, were chosen to provide the greatest dynamic range between inhibitors and activators for each bacterial reporter strain. The concentration of **2** used in the antagonism assays was equal to its EC_{50} value in the *A. baumannii* (Δ*abaI*) reporter strain.[32] The concentration of **1** was twice its EC_{50} value in the *E. coli* reporter strain.

6.4.4 AbaR β-galactosidase protocol

For primary agonism assays, 2 μL of concentrated control or AHL stock solution (to give a final concentration of 100 μM) was added to wells in a 96-well multititer plate. An overnight culture of the *A. baumannii* (Δ*abaI*) reporter strain (OD_{600} = 1.2) was diluted 1:100 with fresh LB. A 198 μL portion of the diluted culture was added to each well of the multititer plate containing

AHLs. Plates were incubated statically at 37 °C for 18–24 h. The cultures were then assayed for β-galactosidase activity following the standard Miller assay method as we previously reported for this strain.[32, 72] Briefly, the OD_{600} of each well of the 96-well multititer plate was recorded. Next, 50 μL aliquots from each well were transferred to a solvent resistant 96-well multititer plate containing 200 μL Z buffer, 8 μL $CHCl_3$, and 4 μL 0.1%(w/v) aqueous SDS. This suspension was mixed via repetitive pipetting (30x), after which the $CHCl_3$ was allowed to settle. A 150-μL aliquot from each well was transferred to a fresh 96-well multititer plate, 20 μL of ONPG (4 μg mL^{-1} in phosphate buffer) was added to each well at time zero, and the plate was incubated at 55 °C for 20 min. Thereafter, the enzymatic reaction was terminated by the addition of 50 μL of 1 M Na_2CO_3. Absorbance at 420 and 550 nm were measured for each well using a plate reader, and Miller units were calculated according to standard methods.[72] Primary AbaR antagonism assays were performed in a similar manner except that the AHL was screened at 100 μM against 0.70 μM **2**.

6.4.5 LasR reporter gene assay (β-galactosidase)

An overnight culture of *E. coli* was diluted 1:10 with fresh LB containing 100 μg mL^{-1} ampicillin and 15 μg mL^{-1} gentamicin. The subculture was incubated with shaking at 37 °C until the optical density of 200 μL reached 0.27 (approximately 90 min). Arabinose (4 mg mL^{-1}) was then added to the culture to induce production of LasR. A 198-μL portion of the diluted culture was then added to each well of a multititer containing AHLs prepared in the same way outlined above. Plates were incubated statically at 37 °C until the optical density of the wells reach 0.45 (approximately 2 h). The cultures were then assayed for β-galactosidase activity. Briefly, the OD_{600} of each well of the 96-well multititer plate was recorded. Next, 50-μL aliquots from each

well were transferred to a solvent resistant 96-well multititer plate contain 200 μL Z buffer, 8 μL $CHCl_3$, and 4 μL 0.1% (w/v) aqueous SDS. This suspension was mixed *via* repetitive pipetting (~30 x), after which the $CHCl_3$ was allowed to settle. A 100-μL aliquot from each well was transferred to a fresh 96-well multititer plate, and 20 μL of substrate (ONPG, 4 μg mL^{-1} in phosphate buffer) was added at time zero. After 20 min incubation at 25 °C, the reaction was terminated by the addition of 50 μL of 1 M Na_2CO_3. Absorbance at 420 and 550 nm were measured for each well using a plate reader, and Miller units were calculated according to standard methods.[72]

6.5 Supplementary Figures

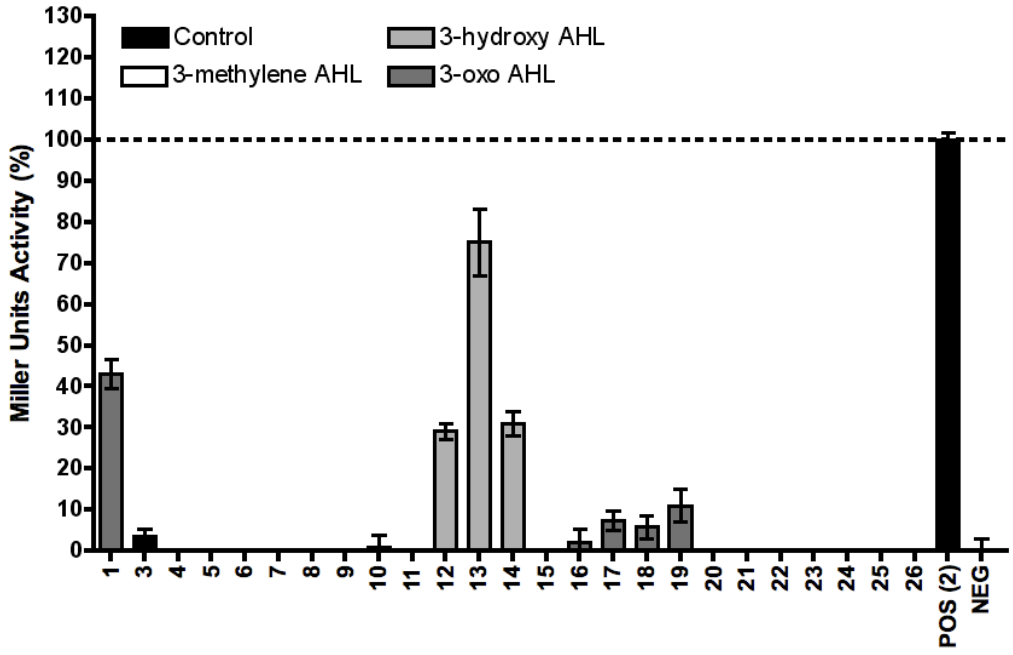


Figure 6.4. Primary agonism screening data for control compounds and non-native AHLs in *A. baumannii* (*ΔabaI*) reporter strain.
Agonism data for 100 μM synthetic ligand. Positive control (POS) = 100 μM **2**. Negative control (NEG) = DMSO without compound. Error bars in each plot indicate standard error of the mean of nine values.

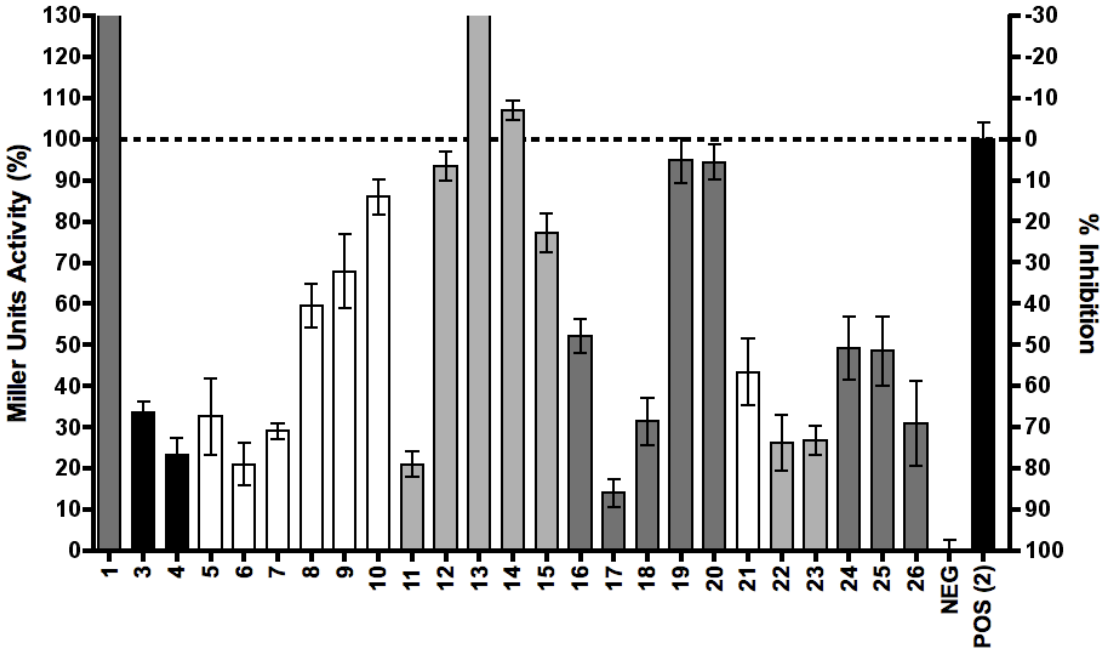


Figure 6.5. Primary antagonism screening data for the control compounds and non-native AHLs in *A. baumannii* (*ΔabaI*) reporter strain.
Antagonism data for 100 μM synthetic ligand tested against 700 nM **2**. Positive control (POS) = 700 nM **2.** Negative control (NEG) = DMSO without compound. Miller units report relative absorbance.

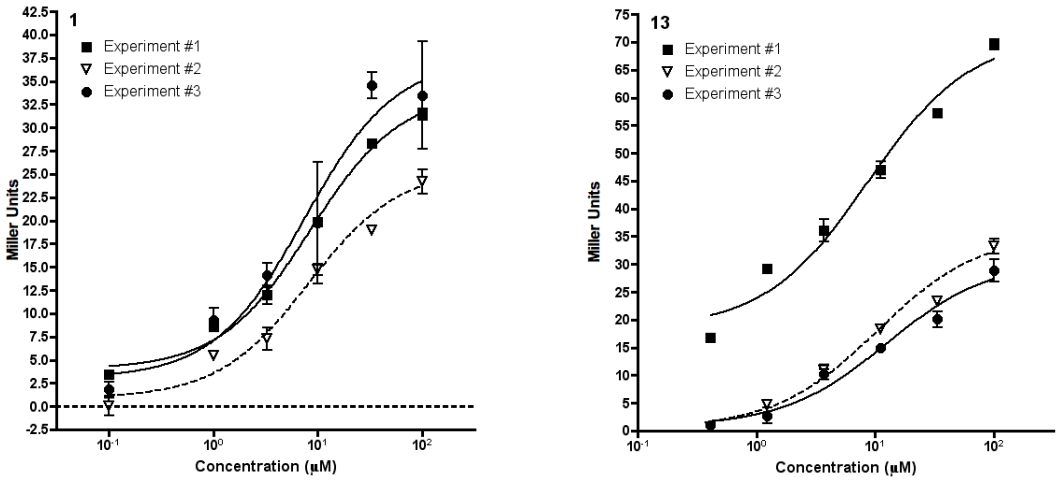


Figure 6.6. Agonism dose response curves for AHLs 1 and 13 in *A. baumannii* (*ΔabaI*) reporter strain.
Synthetic ligand screened over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

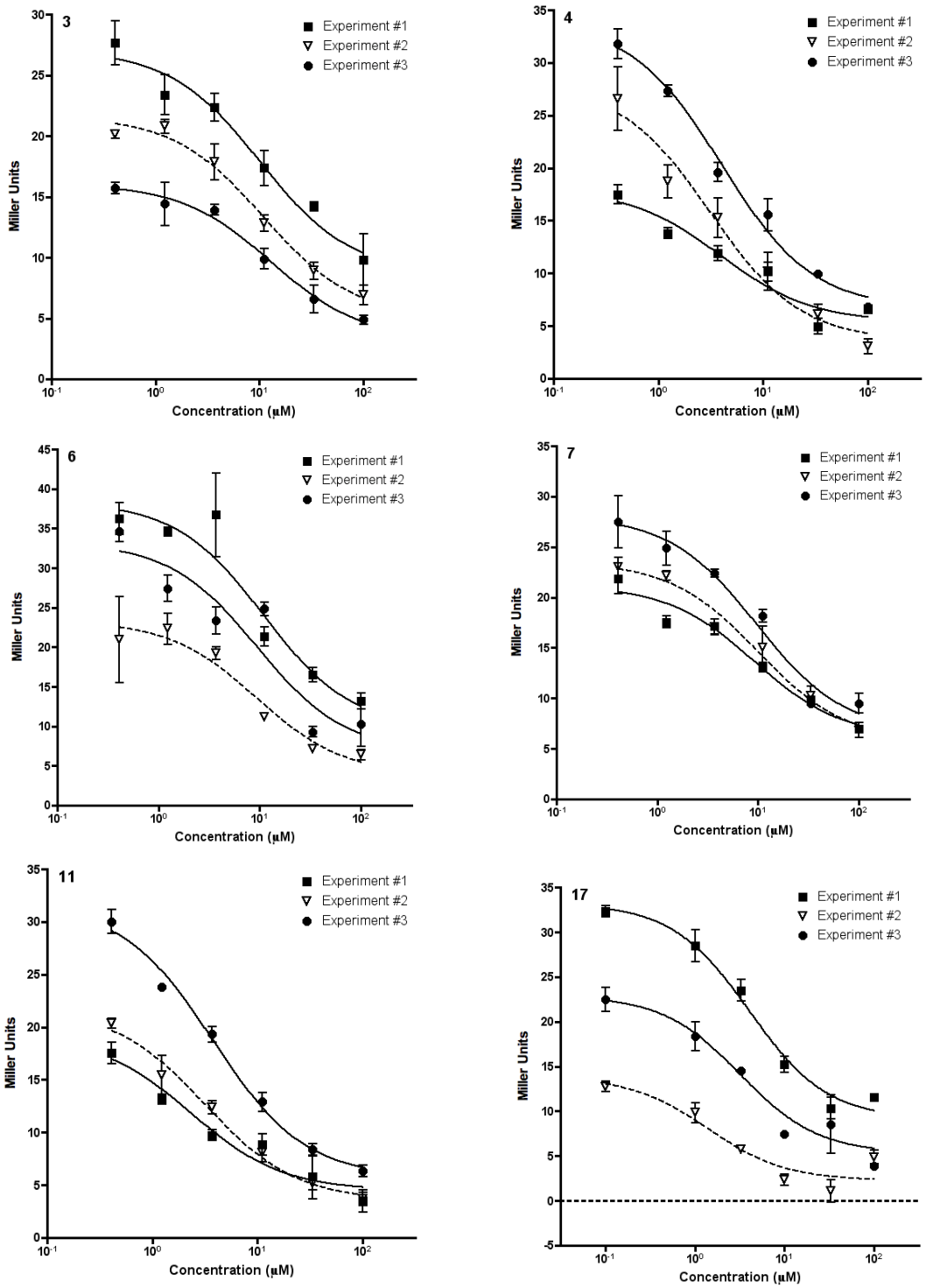


Figure 6.7. Antagonism dose responses for AHLs 3, 4, 6, 7, 11, and 17 in *A. baumannii (ΔabaI)* reporter strain.
Synthetic ligand screened against 700 nM **2** over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

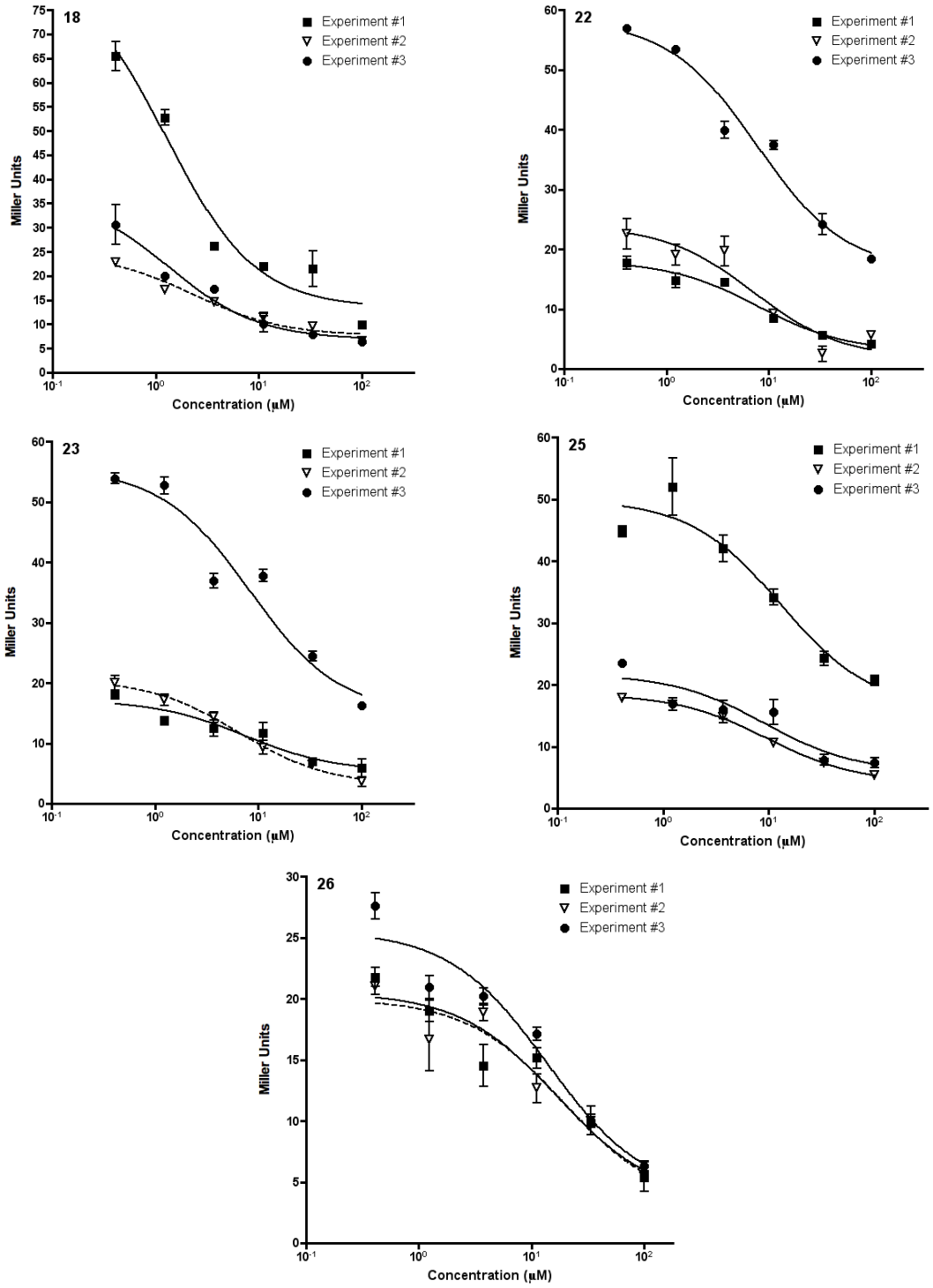


Figure 6.8. Antagonism dose responses for AHLs 18, 22, 23, 25, and 26 in *A. baumannii (ΔabaI)* reporter strain.
Synthetic ligand screened against 700 nM **2** over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

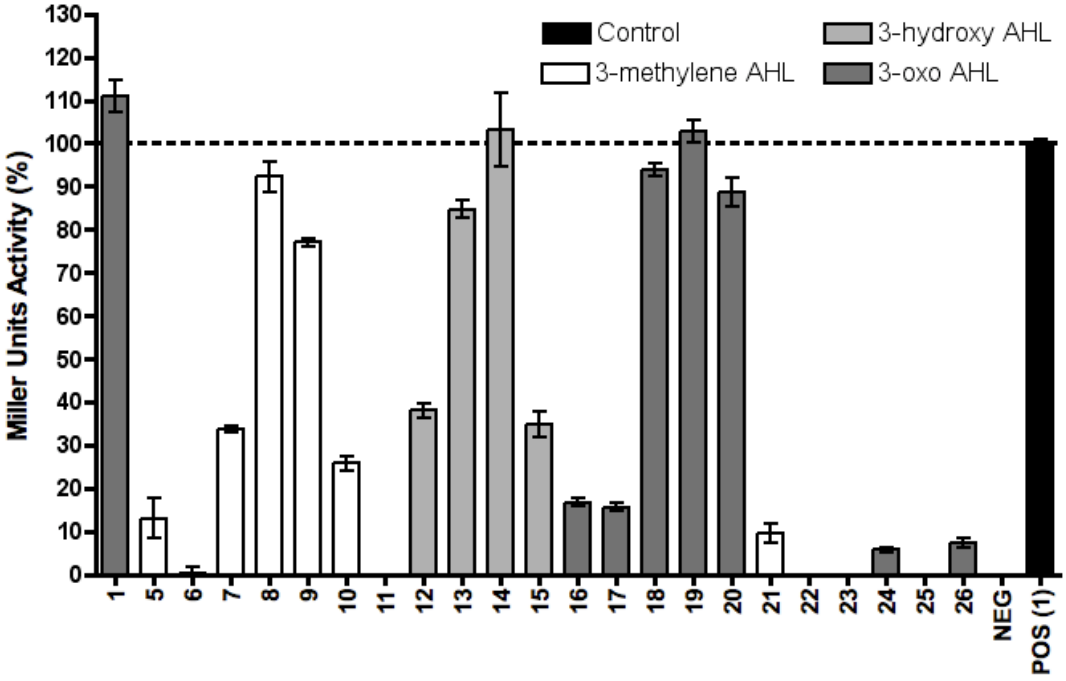


Figure 6.9. Primary agonism screening data for control compounds and non-native AHLs in *E. coli* pJN105L reporter strain.

Agonism data for 10 μM synthetic ligand. Positive control (POS) = 10 μM **1**. Negative control (NEG) = DMSO without compound. Error bars in each plot indicate standard error of the mean of nine values.

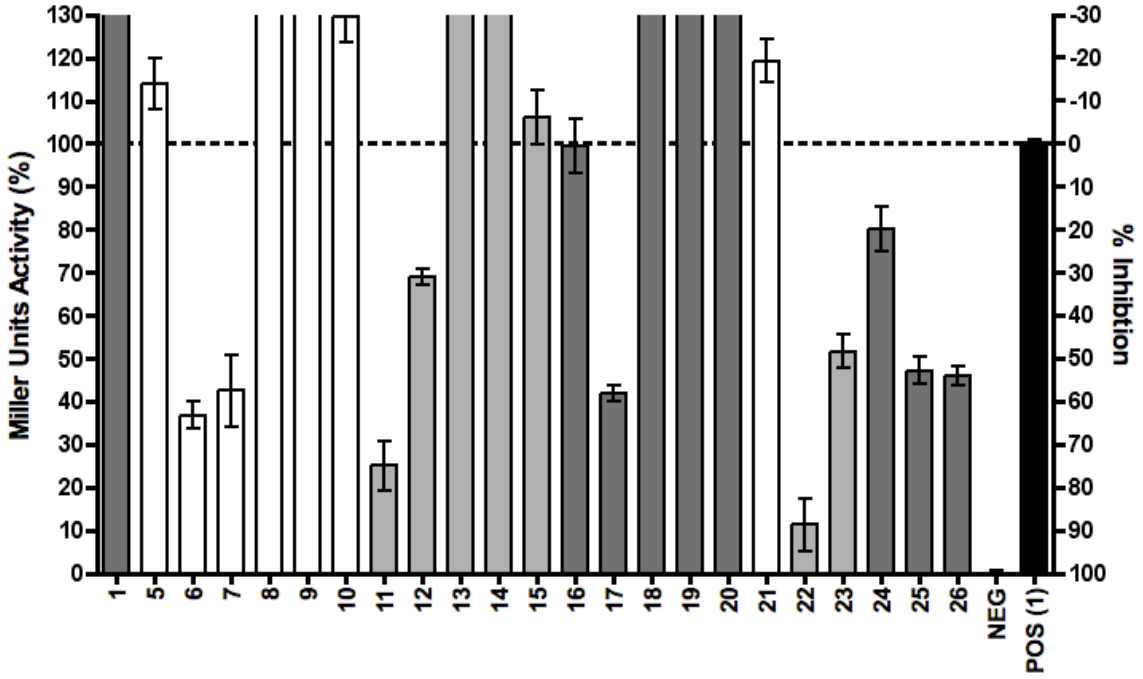


Figure 6.10. Primary antagonism screening data for the control compounds and non-native AHLs in *E. coli* pJN105L reporter strain.

Antagonism data for 10 μM synthetic ligand tested against 10 nM **1**. Positive control (POS) = 10 nM **1**. Negative control (NEG) = DMSO without compound. Miller units report relative absorbance.

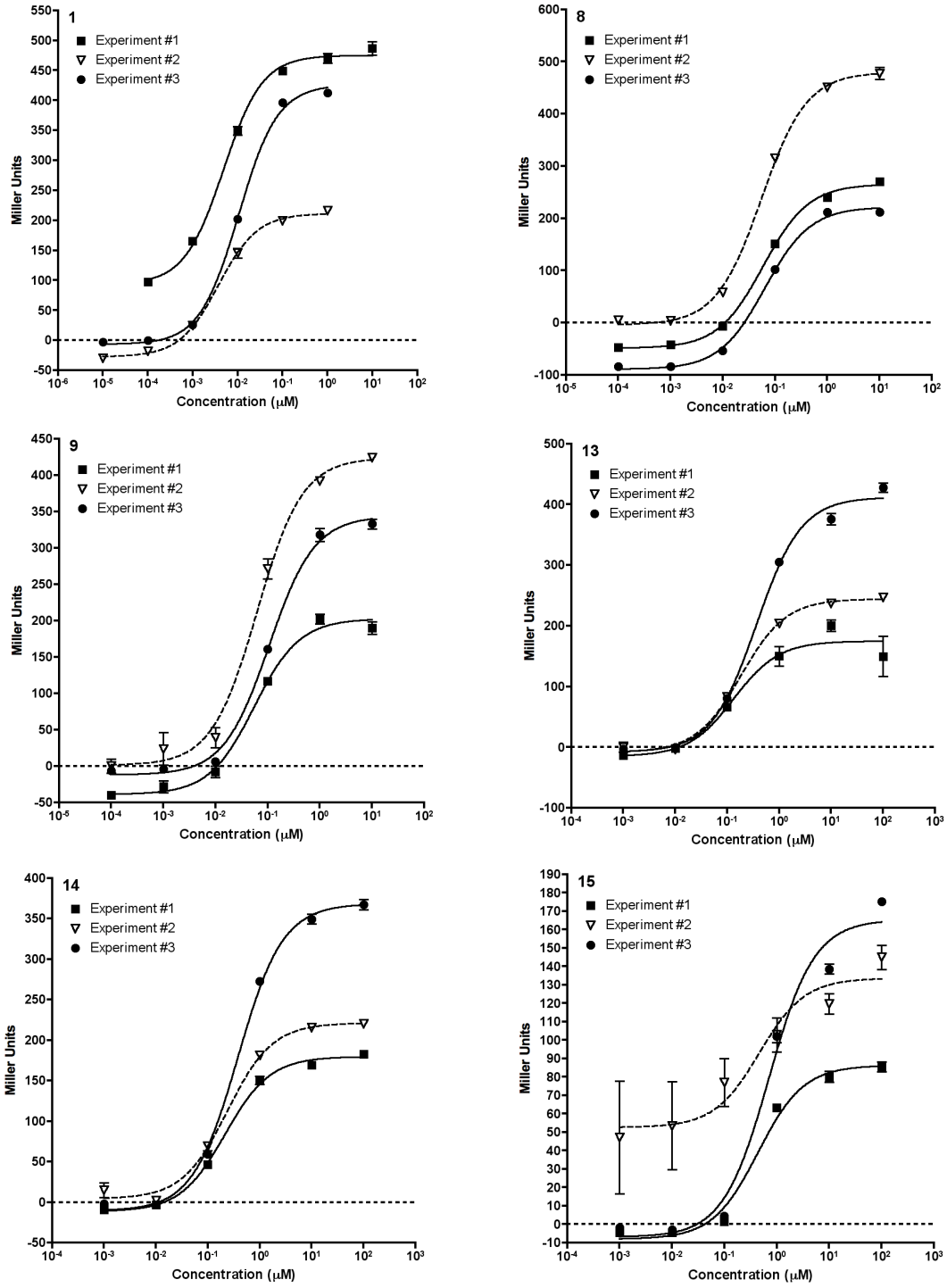


Figure 6.11. Agonism dose response curves for AHLs 1, 8, 9, 13, 14, and 15 in *E. coli* pJN105L reporter strain.

Synthetic ligands screened over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

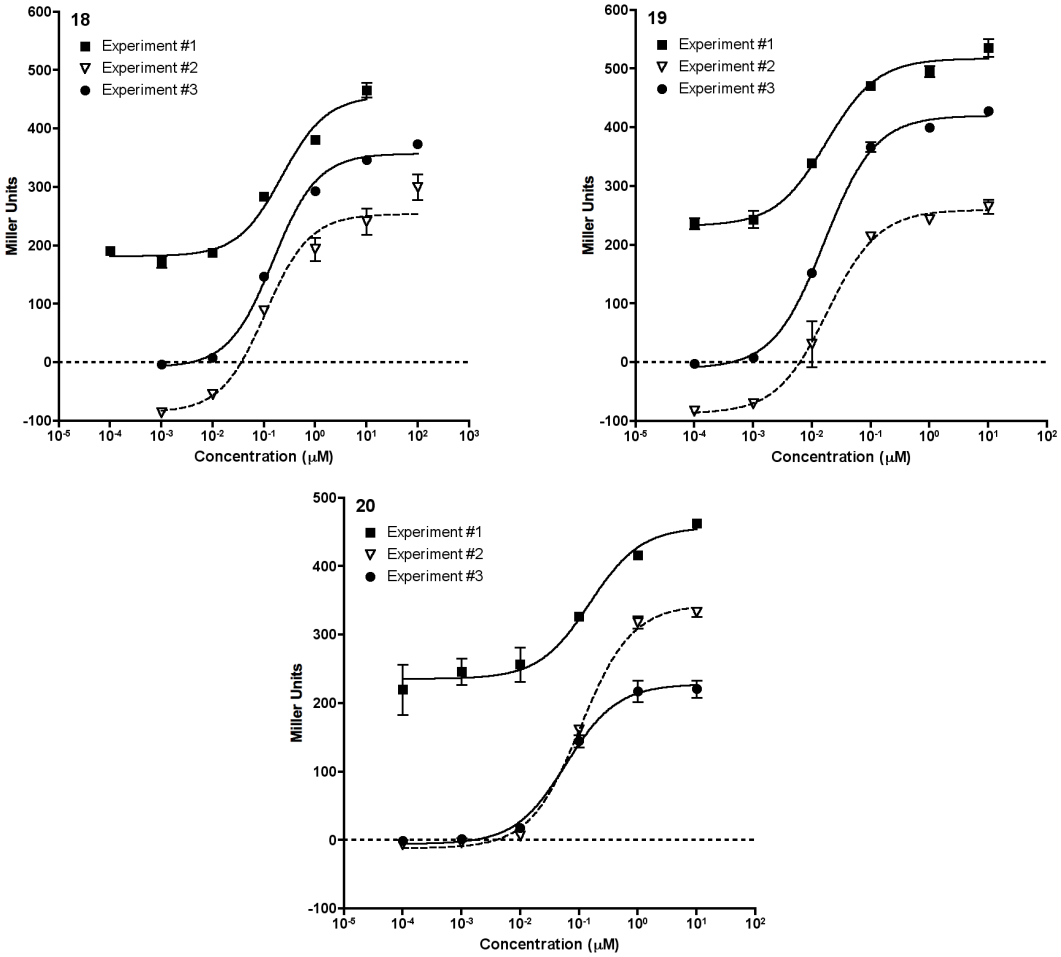


Figure 6.12. Agonism dose response curves for AHLs 18, 19, and 20 in *E. coli* pJN105L reporter strain.

Synthetic ligands screened over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

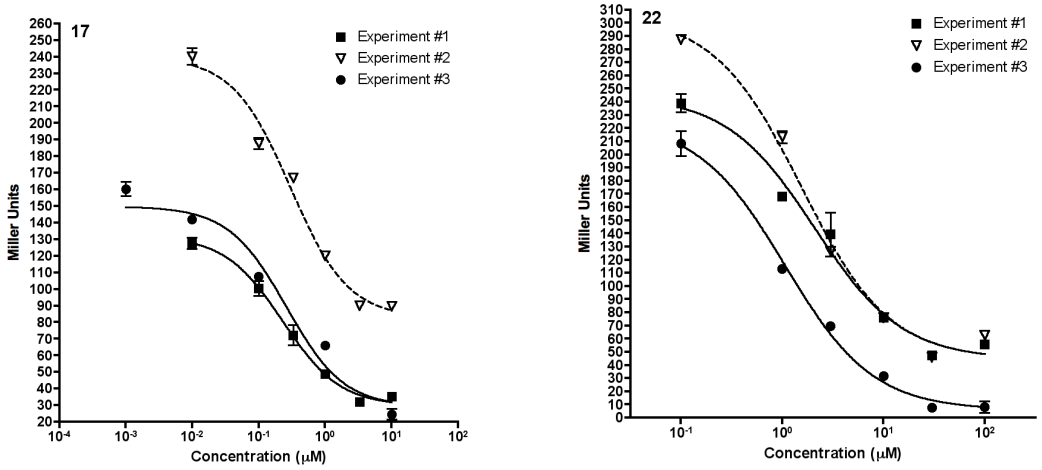


Figure 6.13. Antagonism dose responses for AHLs 17 and 22 in *E. coli* pJN105L reporter strain. Synthetic ligands screened against 10 nM **1** over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

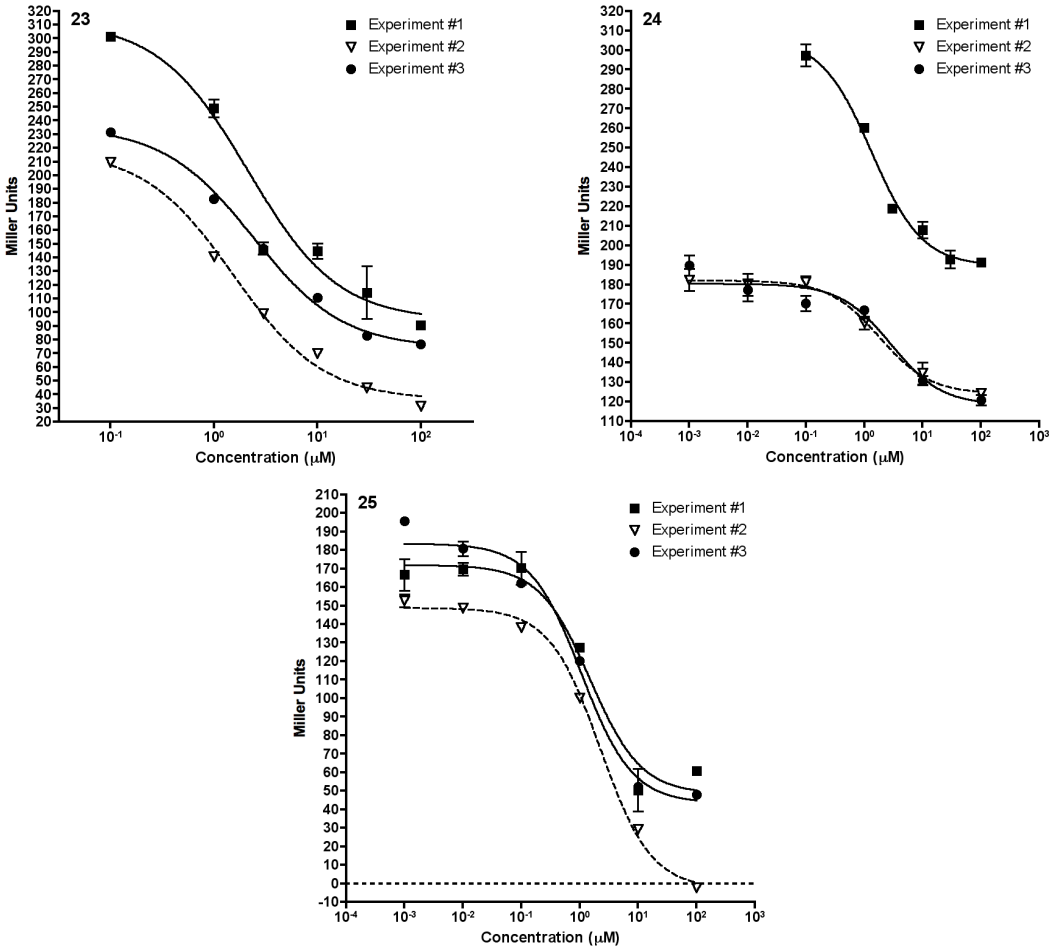


Figure 6.14. Antagonism dose responses for AHLs 23, 24, and 25 in *E. coli* pJN105L reporter strain. Synthetic ligands screened against 10 nM **1** over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

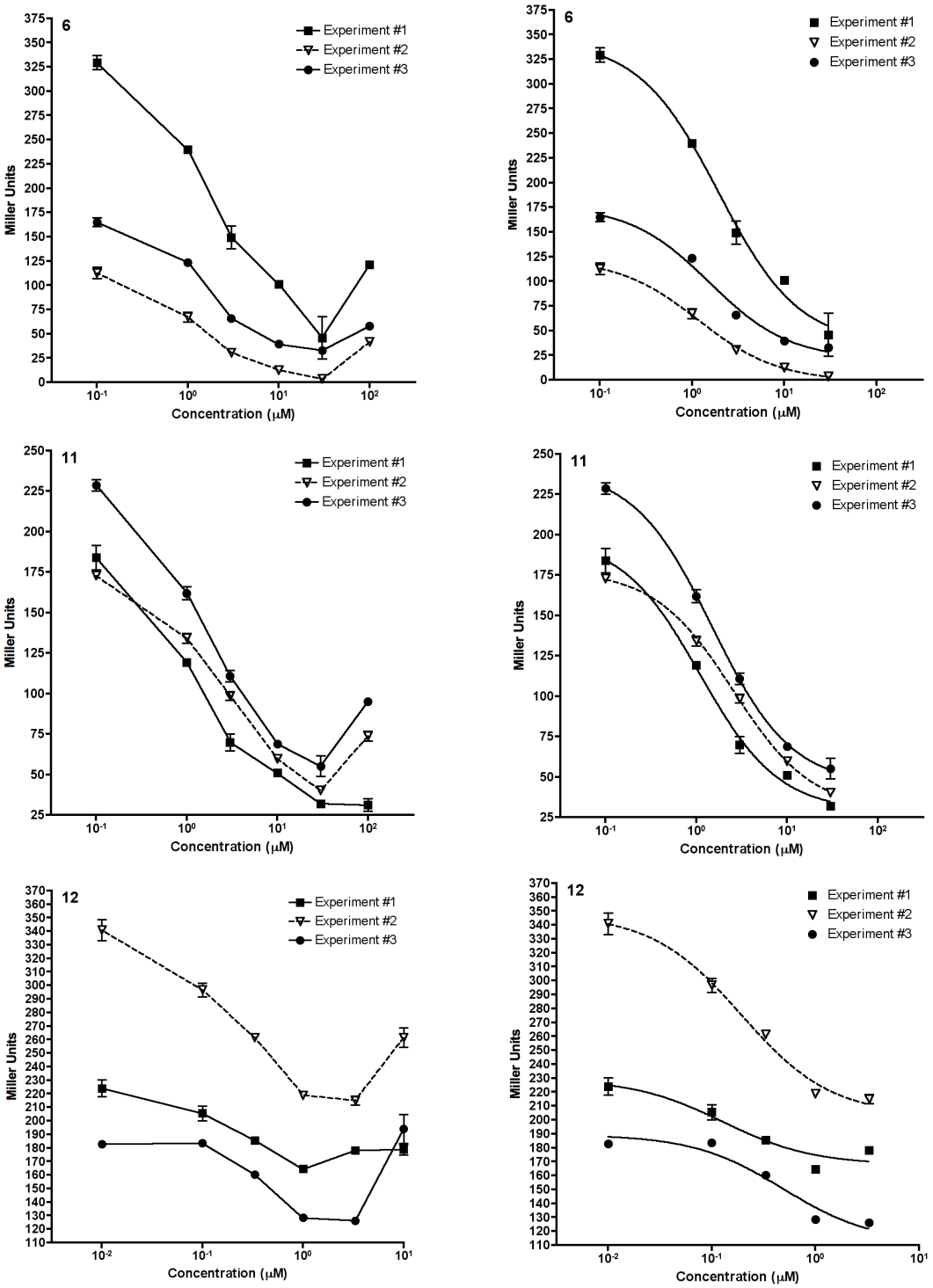


Figure 6.15. Antagonism dose responses for AHLs 6, 11, and 12 in *E. coli* pJN105L reporter strain. (left) Full antagonism dose responses, and (right) concentrations used to calculate IC_{50} values. Synthetic ligands screened against 10 nM **1** over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

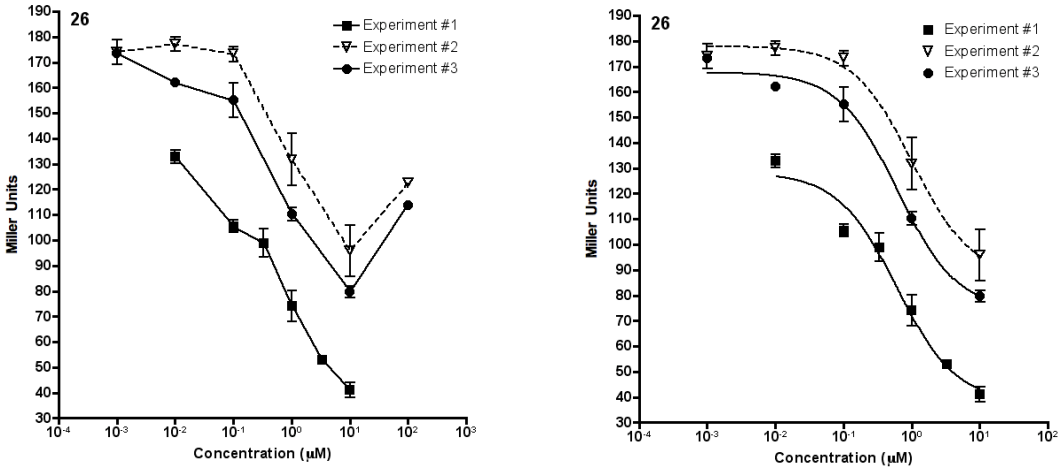
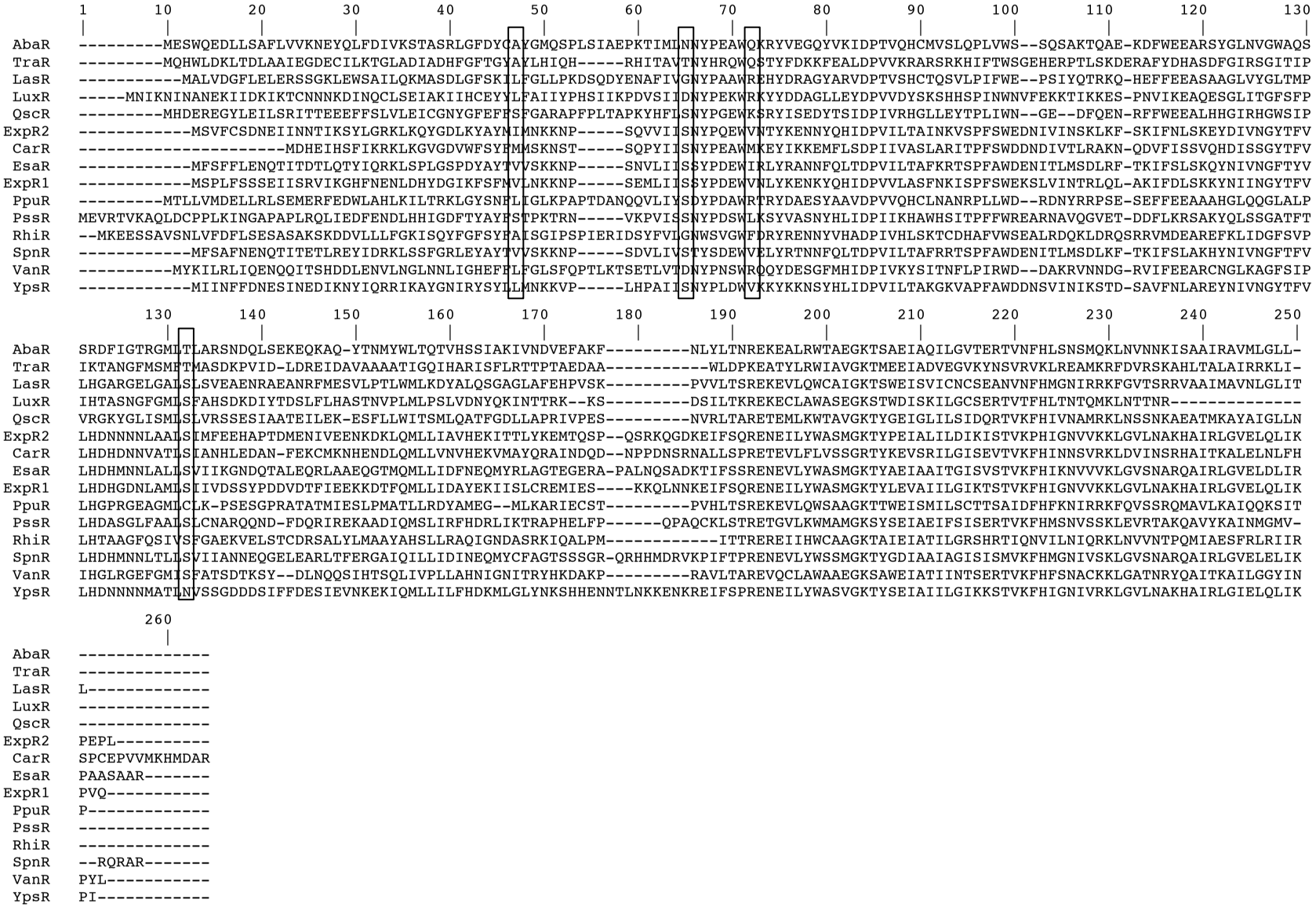


Figure 6.16. Antagonism dose response for AHL 26 in *E. coli* pJN105L reporter strain.
(left) Full antagonism dose responses, and (right) concentrations used to calculate IC_{50} values. Synthetic ligand screened against 10 nM **1** over varying concentrations. Error bars indicate standard error of the mean of triplicate values.

Figure 6.17. Sequence alignment of 15 LuxR-type proteins from bacterial species that produce 3-oxo and 3-OH AHLs.
(Following page) Full version of the sequence alignment that is displayed in **Figure 6.3**. See **Figure 6.3** for details. Key residues are boxed.


1 10 20 30 40 50 60 70 80 90 100 110 120 130
AbaR ---------MESWQEDLLSAFLVVKNEYQLFDIVKSTASRLGFDYCAYGMQSPLSIAEPKTIMLNNYPEAWQKRYVEGQYVKIDPTVQHCMVSLQPLVWS--SQSAKTQAE-KDFWEEARSYGLNVGWAQS
TraR ---------MQHWLDKLTDLAAIEGDECILKTGLADIADHFGFTGYAYLHIQH-----RHITAVTNYHRQWQSTYFDKKFEALDPVVKRARSRKHIFTWSGEHERPTLSKDERAFYDHASDFGIRSGITIP
LasR ----------MALVDGFLELERSSGKLEWSAILQKMASDLGFSKILFGLLPKDSQDYENAFIVGNYPAAWREHYDRAGYARVDPTVSHCTQSVLPIFWE--PSIYQTRKQ-HEFFEEASAAGLVYGLTMP
LuxR -----MNIKNINANEKIIDKIKTCNNNKDINQCLSEIAKIIHCEYYLFAIIYPHSIIKPDVSIIDNYPEKWRKYYDDAGLLEYDPVVDYSKSHHSPINWNVFEKKTIKKES-PNVIKEAQESGLITGFSFP
QscR ---------MHDEREGYLEILSRITTEEEFFSLVLEICGNYGFEFFSFGARAPFPLTAPKYHFLSNYPGEWKSRYISEDYTSIDPIVRHGLLEYTPLIWN--GE--DFQEN-RFFWEEALHHGIRHGWSIP
ExpR2 ----------MSVFCSDNEIINNTIKSYLGRKLKQYGDLKYAYMIMNKKNP-----SQVVIISNYPQEWVNTYKENNYQHIDPVILTAINKVSPFSWEDNIVINSKLKF-SKIFNLSKEYDIVNGYTFV
CarR ----------MDHEIHSFIKRKLKGVGDVWFSYFMMSKNST-----SQPYIISNYPEAWMKEYIKKEMFLSDPIIVASLARITPFSWDDNDIVTLRAKN-QDVFISSVQHDISSGYTFV
EsaR ----------MFSFFLENQTITDTLQTYIQRKLSPLGSPDYAYTVVSKKNP-----SNVLIISSYPDEWIRLYRANNFQLTDPVILTAFKRTSPFAWDENITLMSDLRF-TKIFSLSKQYNIVNGFTYV
ExpR1 ----------MSPLFSSSEIISRVIKGHFNENLDHYDGIKFSFMVLNKKNP-----SEMLIISSYPDEWVNLYKENKYQHIDPVVLASFNKISPFSWEKSLVINTRLQL-AKIFDLSKKYNIINGYTFV
PpuR ---------MTLLVMDELLRLSEMERFEDWLAHLKILTRKLGYSNFLIGLKPAPTDANQQVLIYSDYPDAWRTRYDAESYAAVDPVVQHCLNANRPLLWD--RDNYRRPSE-SEFFEEAAAHGLQQGLALP
PssR MEVRTVKAQLDCPPLKINGAPAPLRQLIEDFENDLHHIGDFTYAYFSTPKTRN-----VKPVISSNYPDSWLKSYVASNYHLIDPIIKHAWHSITPFFWREARNAVQGVET-DDFLKRSAKYQLSSGATFT
RhiR --MKEESSAVSNLVFDFLSESASAKSKDDVLLLFGKISQYFGFSYFAISGIPSPIERIDSYFVLGNWSVGWFDRYRENNYVHADPIVHLSKTCDHAFVWSEALRDQKLDRQSRRVMDEAREFKLIDGFSVP
SpnR ----------MFSAFNENQTITETLREYIDRKLSSFGRLEYAYTVVSKKNP-----SDVLIVSTYSDEWVELYRTNNFQLTDPVILTAFRRTSPFAWDENITLMSDLKF-TKIFSLAKHYNIVNGFTFV
VanR ----------MYKILRLIQENQQITSHDDLENVLNGLNNLIGHEFFLFGLSFQPTLKTSETLVTDNYPNSWRQQYDESGFMHIDPIVKYSITNFLPIRWD--DAKRVNNDG-RVIFEEARCNGLKAGFSIP
YpsR ----------MIINFFDNESINEDIKNYIQRRIKAYGNIRYSYLLMNKKVP-----LHPAIISNYPLDWVKKYKKNSYHLIDPVILTAKGKVAPFAWDDNSVINIKSTD-SAVFNLAREYNIVNGYTFV
130 140 150 160 170 180 190 200 210 220 230 240 250
AbaR SRDFIGTRGMLTLARSNDQLSEKEQKAQ-YTNMYWLTQTVHSSIAKIVNDVEFAKF---------NLYLTNREKEALRWTAEGKTSAEIAQILGVTERTVNFHLSNSMQKLNVNNKISAAIRAVMLGLL-
TraR IKTANGFMSMFTMASDKPVID-LDREIDAVAAAATIGQIHARISFLRTTPTAEDAA-----------WLDPKEATYLRWIAVGKTMEEIADVEGVKYNSVRVKLREAMKRFDVRSKAHLTALAIRRKLI-
LasR LHGARGELGALSLSVEAENRAEANRFMESVLPTLWMLKDYALQSGAGLAFEHPVSK---------PVVLTSREKEVLQWCAIGKTSWEISVICNCSEANVNFHMGNIRRKFGVTSRRVAAIMAVNLGLIT
LuxR IHTASNGFGMLSFAHSDKDIYTDSLFLHASTNVPLMLPSLVDNYQKINTTRK--KS---------DSILTKREKECLAWASEGKSTWDISKILGCSERTVTFHLTNTQMKLNTTNR----------
QscR VRGKYGLISMLSLVRSSESIAATEILEK-ESFLLWITSMLQATFGDLLAPRIVPES---------NVRLTARETEMLKWTAVGKTYGEIGLILSIDQRTVKFHIVNAMRKLNSSNKAEATMKAYAIGLLN
ExpR2 LHDNNNNLAALSIMFEEHAPTDMENIVEENKDKLQMLLIAVHEKITTLYKEMTQSP--QSRKQGDKEIFSQRENEILYWASMGKTYPEIALILDIKISTVKPHIGNVVKKLGVLNAKHAIRLGVELQLIK
CarR LHDHDNNVATLSIANHLEDAN-FEKCMKNHENDLQMLLVNVHEKVMAYQRAINDQD--NPPDNSRNALLSPRETEVLFLVSSGRTYKEVSRILGISEVTVKFHINNSVRKLDVINSRHAITKALELNLFH
EsaR LHDHMNNLALLSVIIKGNDQTALEQRLAAEQGTMQMLLIDFNEQMYRLAGTEGERA-PALNQSADKTIFSSRENEVLYWASMGKTYAEIAAITGISVSTVKFHIKNVVVKLGVSNARQAIRLGVELDLIR
ExpR1 LHDHGDNLAMLSIIVDSSYPDDVDTFIEEKKDTFQMLLIDAYEKIISLCREMIES----KKQLNNKEIFSQRENEILYWASMGKTYLEVAIILGIKTSTVKFHIGNVVKKLGVLNAKHAIRLGVELQLIK
PpuR LHGPRGEAGMLCLK-PSESGPRATATMIESLPMATLLRDYAMEG--MLKARIECST---------PVHLTSREKEVLQWSAAGKTTWEISMILSCTTSAIDFHFKNIRRKFQVSSRQMAVLKAIQQKSIT
PssR LHDASGLFAALSLCNARQQND-FDQRIREKAADIQMSLIRFHDRLIKTRAPHELFP------QPAQCKLSTRETGVLKWMAMGKSYSEIAEIFSISERTVKFHMSNVSSKLEVRTAKQAVYKAINMGMV-
RhiR LHTAAGFQSIVSFGAEKVELSTCDRSALYLMAAYAHSLLRAQIGNDASRKIQALPM----------ITTRREREIIHWCAAGKTAIEIATILGRSHRTIQNVILNIQRKLNVVNTPQMIAESFRLRIIR
SpnR LHDHMNNLTLLSVIIANNEQGELEARLTFERGAIQILLIDINEQMYCFAGTSSSGR-QRHHMDRVKPIFTPRENEVLYWSSMGKTYGDIAAIAGISISMVKFHMGNIVSKLGVSNARQAIRLGVELELIK
VanR IHGLRGEFGMISFATSDTKSY--DLNQQSIHTSQLIVPLLAHNIGNITRYHKDAKP---------RAVLTAREVQCLAWAAEGKSAWEIATIINTSERTVKFHFSNACKKLGATNRYQAITKAILGGYIN
YpsR LHDNNNNMATLNVSSGDDDSIFFDESIEVNKEKIQMLLILFHDKMLGLYNKSHHENNTLNKKENKREIFSPRENEILYWASVGKTYSEIAIILGIKKSTVKFHIGNIVRKLGVLNAKHAIRLGIELQLIK
260
AbaR ----------
TraR ----------
LasR L---------
LuxR ----------
QscR ----------
ExpR2 PEPL------
CarR SPCEPVVMKHMDAR
EsaR PAASAAR-------
ExpR1 PVQ-------
PpuR P---------
PssR ----------
RhiR ----------
SpnR --RQRAR-------
VanR PYL-------
YpsR PI---------

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6.7 Appendix: Site-Directed Mutagenesis To Enhance or Decrease the Specificity of LasR for its Native Ligand (Future Direction)

An interesting outcome of this study was our discovery of four particular residues in LasR that corresponded with the ligand-response specificity of multiple LuxR-type QS receptor proteins. To test the importance of these residues as native ligand-specificity determinants in LuxR-type receptors, we mutated these residues in *P. aeruginosa* LasR (positions 36, 54, 61, and 129) to the corresponding amino acids that are present in more selective LuxR type proteins (*A. baumannii* AbaR and *A. tumefaciens* TraR) and to the corresponding amino acids that are present in a more promiscuous LuxR type protein (*P. aeruginosa* QscR). Our goal is to then test the ligand-response specificity of these LasR mutants (see **Table 6.4** for mutated residues) to both OdDHL and other AHLs.

Table 6.4. Mutations suspected to make LasR more specific (like AbaR or TraR) or less specific (like QscR).

More specific		Less specific	
Receptor mimicked	**Mutations**	**Receptor mimicked**	**Mutations**
AbaR/TraR	L36A	QscR-type	L36S
TraR	G54T	QscR-type	G54S
AbaR	G54N	QscR-type	R61K
AbaR/TraR	R61Q	QscR-type	L36S, G54S, R61K
AbaR/TraR	S129T		
TraR	L36A, G54T, R61Q, S129T		
AbaR	L36A, G54N, R61Q, S129T		

Site-directed mutagenesis by overlap extension PCR[1] was carried out on the pJN105L LasR expression plasmid[2] in the same manner as described in **Chapters 3** and **4** of this thesis. The mutagenic primers for the single-residue mutants are listed in **Table 6.5**. For the three multi-mutation preparations, gBlocks® that incorporated all the mutations were obtained from Integrated DNA Technologies (see **Table 6.6** for sequences). Using the gBlocks® and an

overlapping PCR product encoding the C-terminal region of LasR, overlap extension PCR was carried out with the same flanking primers as above. Mutant lasR genes were digested, re-ligated into the pJN105L backbone, confirmed by Sanger sequencing, and incorporated into the DH5α/pSC11 β-galactosidase reporter.[2] Currently, these mutants are being screened for activity with 3-oxo, 3-hydroxyl, and 3-methylene AHLs of C_8, C_{10}, C_{12}, and C_{14} lengths (ligands **6**–**9**, **11**–**14**, **17**, **18**, **1**, and **19** from **Figure 1**). We expect the AbaR- and TraR-like mutations to render LasR more specific to a smaller set of AHLs, whereas the QscR-like mutations should confer greater promiscuity to LasR. Further studies may also be carried out performing similar mutations on other LuxR-type proteins and testing their activity.

Table 6.5. PCR primers for site-directed mutagenesis of LasR

Mutation	Forward primer sequence[a]	Reverse primer sequence[a]
flanking primers	CGATTAGAATTCTTAAGAAGAACGTAGCGCTATG	CCACGCTCTAGAGGCAAGA
L36A (CTG→GCG)	TCGAAGATC**GCG**TTCGGCCTG	CAGGCCGAA**CGC**GATCTTCGA
G54T (GGC→ACC)	TTCATCGTC**ACC**AACTACCCGGCC	GGCCGGGTAGTT**GGT**GACGATGAA
G54N (GGC→AAC)	TTCATCGTC**AAC**AACTACCCGGCC	GGCCGGGTAGTT**GTT**GACGATGAA
R61Q (CGC→CAA)	GCCGCCTGG**CAA**GAGCATTACG	CGTAATGCTC**TTG**CCAGGCGGC
S129T (AGC→AAC)	CGCGCTG**ACC**CTCAGCGTG	CACGCTGAG**GGT**CAGCGCG
L36S (CTG→AGC)	TCTCGAAGATC**AGC**TTCGGCCTG	CAGGCCGAA**GCT**GATCTTCGAGA
G54S (GGC→AGC)	TTCATCGTC**AGC**AACTACCCGGCC	GGCCGGGTAGTT**GCT**GACGATGAA
R61K (CGC→AAA)	GCCGCCTGG**AAA**GAGCATTACG	CGTAATGCTC**TTT**CCAGGCGGC

[a]**Bold** = mutated codon

Table 6.6. gBlocks® and PCR primer for production of LasR with multiple-mutations

Mutations	G-block sequence[a]
L36A(CTG→GCG), **G54T**(GGC→ACC), **R61Q**(CGC→CAA), **S129T**(AGC→AAC)	CGATTAGAATTCTTAAGAAGAACGTAGCGCTATGGCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTC AAGTGGAAAATTGGAGTGGAGCGCCATCCTGCAGAAGATGGCGAGCGACCTTGGATTCTCGAAGATC**GC G**TTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTC**ACC**AACTACCCGGCCGC CTGG**CAA**GAGCATTACGACCGGGCTGGCTACGCGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAG CGTACTGCCGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAAGC CTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCGCT G**ACC**CTCAGCGTGGAAGCGGAAAACCGGGCCGAGGCCAACCGTTTCATGGAGTCGGTCCTGCCGACCCT GTGGATGCTCAA
L36A(CTG→GCG), **G54N**(GGC→AAC), **R61Q**(CGC→CAA), **S129T**(AGC→AAC)	CGATTAGAATTCTTAAGAAGAACGTAGCGCTATGGCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTC AAGTGGAAAATTGGAGTGGAGCGCCATCCTGCAGAAGATGGCGAGCGACCTTGGATTCTCGAAGATC**GC G**TTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTC**AAC**AACTACCCGGCCGC CTGG**CAA**GAGCATTACGACCGGGCTGGCTACGCGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAG CGTACTGCCGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAAGC CTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCGCT G**ACC**CTCAGCGTGGAAGCGGAAAACCGGGCCGAGGCCAACCGTTTCATGGAGTCGGTCCTGCCGACCCT GTGGATGCTCAA
L36S(CTG→AGC), **G54S**(GGC→AGC), **R61K**(CGC→AAA), **S129** remains	CGATTAGAATTCTTAAGAAGAACGTAGCGCTATGGCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTC AAGTGGAAAATTGGAGTGGAGCGCCATCCTGCAGAAGATGGCGAGCGACCTTGGATTCTCGAAGATC**AG C**TTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTC**AGC**AACTACCCGGCCGC CTGG**AAA**GAGCATTACGACCGGGCTGGCTACGCGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAG CGTACTGCCGATTTTCTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAAGC CTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTCGCGGCGAACTCGGCGCGCT G**AGC**CTCAGCGTGGAAGCGGAAAACCGGGCCGAGGCCAACCGTTTCATGGAGTCGGTCCTGCCGACCCT GTGGATGCTCAA
forward primer paired with reverse flanking primer above to prepare C-terminal PCR product	GTCGGTCCTGCCGACCCTGTG

[a]**Bold** = mutated codon

6.8 References for Appendix

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