Chemical- and materials-based approaches to further the understanding of quorum sensing in bacteria

by

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A dissertation submitted in partial fulfillment of

The requirements for the degree of

Doctor of Philosophy

(Microbiology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2020

Date of final oral examination: April 7, 2020

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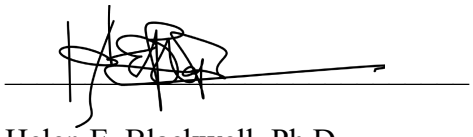
Abstract

Many common bacteria can communicate via a process called quorum sensing (QS), wherein small molecule or peptide signals are produced and detected in order for the bacterial population to monitor its cell density. Signal concentration increases with cell number, and at a sufficiently high concentration of signals, a variety of bacterial group behaviors can occur, including the production of virulence factors that play an important role in pathogenesis. As QS is fundamentally a chemical signaling process, where the structure and concentration of signal is critical to inter-cellular communication, there is significant and growing interest in the development of non-native molecules capable of intercepting QS in bacteria. Such compounds enable chemical biology approaches to further understand bacterial QS and the myriad processes controlled thereby, along with paving a route toward potential anti-infectives.

In this thesis, I outline a set of parallel yet integrated studies aimed at the development of (i) new molecules that modulate QS in bacterial pathogens and (ii) new materials-based approaches for the detection of quorate populations of bacteria and their group-associated products. I first describe a study characterizing the activity of a set of small molecules, originally designed to target one QS receptor in *Pseudomonas aeruginosa*, for their activity in two related and important QS receptors in the same bacterium. I identified multiple groups of compounds with novel activity profiles, including compounds that target (i) one receptor specifically, (ii) all three receptors in similar ways, and (iii) receptors in ways that could lead to synergistic QS outcomes. Next, I developed two materials-based approaches to screen for QS activity in *Staphylococcus aureus* and *P. aeruginosa*. The first of these approaches was based on the vesicle lysis test (VLT) to identify and screen for QS modulators in a high-throughput manner. The second method uses liquid crystal-infused slippery liquid infused porous surfaces (LC-SLIPS) to

easily detect the presence of QS-controlled surfactants with the unaided eye. Taken together, the experiments described herein further the understanding of QS in bacteria and provide new chemical tools and robust assay methods to study QS that will be useful to the growing field of sociomicrobiology.



Helen E. Blackwell, Ph.D.

Acknowledgements

"If you can meet with Triumph and Disaster and treat those two imposters just the same."
If, Rudyard Kipling

To Helen, Clint, Barb, and Joe – you saw something in me. Something that scared, dreaming high schooler wanted, something the girl who got on a plane for the first time wished for. Something that led to many questions, many answers, and many frustrating times, but eventually – a PhD. Thank you for taking me to sushi, for telling me you told me so, and for letting me cry in your offices.

To Elwood, Kelly, Jesse, Nick, Yi, Michael, Joe, and Michelle - You helped me, step by step, become a scientist and a better person. From not catching on fire to standing up for myself, you believed in me and you taught me so much.

To Jake, Stephen, Sara, Wenqi, Mike, Rebecca, and Emily, you helped me find my passion in coming up with ridiculous metaphors and the joy in passing them on to others.

To Harshit, Curran, and Benjamin - you asked me so many ridiculous questions that sparked so many amazing experiments. We never tried the chalk.

To my MDTP cohort, you were there when I was stressed, you were there when I was happy. You were my constant friends and my constant helpers - especially for a "microbiologist" in a chemistry lab!

To Kay, Mary, Andrea, and Jan. You helped me find peace. Noam, you helped me keep it.

To Savannah, my best friend. My girl-talker. My constant harbor in the storm. We both did the thing! Took me long enough.

To Ali and Jenny, high school is a strange place. Marching band is even stranger. I'm glad to have met and kept some weirdos like me and to find a mantra and a place where I could always remember to believe in and trust my teammates.

To my labmates, especially Korbin, Betty, Daniel, Matt, Maggie, Cassie, Tom, Kate+the Emmas for the laughs, the friends, the science-talk, for all sticking it out together in this strange ride.

To my friends and anyone who I've forgotten, thanks. I have a terrible memory, but there are also so many to thank 'cause I've had a lot of help.

To my family, who gave me a place to always fall back to, a comfort I always needed.

And to Michael, my almost-husband. For everything.

(and thank you to Rudyard Kipling for this wonderful poem)

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List of Abbreviations

%	percent
(-)	negative
(+)	positive
<	less than
>	greater than
°	degree
°C	degrees Celsius
5CB	4-Cyano-4'-pentylbiphenyl
Å	angstrom
Act.	activity
Ag	agonism
Agr	accessory gene regulator (QS system in *Staphylococcus aureus*)
AgrABCD	a protein of the accessory gene regulator system
AHL	*N*-acyl-L-homoserine lactone
AIP	autoinducing peptide
Ala, A	alanine
AmbBCDE	genes that produce IQS
Amp	ampicillin
Ant	antagonism
AprA	alkaline protease in *Pseudomonas aeruginosa*
AprXDEF	alkaline protease synthesis machinery

Asp, D	aspartic acid
ATCC	American type culture collection
B. subtilis	*Bacillus subtilis* subsp. *spizizenii*
BamHI	restriction endonuclease
BHI	brain heart infusion
BHL	*N*-butanoyl-L-homoserine lactone
BlaZ	beta-lactamase
bp	base pair
CF	cystic fibrosis
CI	confidence interval
cm	centimeter
coop	cooperative
COPD	chronic obstructive pulmonary disease
CPRG	chlorophenol red-beta-D-galactopyranoside
cSt	centistrokes
DBTAB	dodecyl-1,12-bis(trimethylammonium bromide
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMSO	dimethyl sulfoxide
DOPC	1,2-dioleoyl-sn-glycerol-3-phosphocholine
DPPC	1,2-dipalmitoyl-sn-glycerol-3-phosphocholine
DPPE	1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine

DTAB	dodecyltrimethylammonium bromide
E. coli	*Escherichia coli*
e.g.	*exempli grati*, meaning “for example”
E7	mixture of liquid crystals
EC_{50}	half-maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
ESI-Q-TOF	electrospray-ionization quadrupole time-of-flight mass spectrometry
ET	exofoliative toxin
etc.	*et cetera*, meaning “and the rest”
F	forward
FLIPS	ferro fluid liquid infused porous surfaces
fMet	*N*-formylmethionine
g	gram
Gent	gentamicin
GFP	green fluorescent protein
h, hr	hour
HAA	3-(3-hydroxyalkanoyloxy)alkanoate
HCl	hydrochloric acid
HF	high fidelity
HL	homoserine lactone
hla	gene encoding alpha hemolysin
hld	gene encoding delta hemolysin (aka delta toxin)
HPLC	high performance liquid chromatography

HTAB	hexadecyltrimethylammonium bromide
i.e.	id est, meaning "that is" as used for clarification
IC_{50}	half-maximal inhibitory concentration
iPOH	isopropyl alcohol
IQS	2-(2-hydroxylphenyl)-thiazole-4-carbaldehyde
L	liter
L. monocytogenes	*Listeria monocytogenes*
LacZ	beta-galactosidase
LasB	elastase
LasI	autoinducer synthase protein in *Pseudomonas aeruginosa*
LasR	autoinducer receptor protein in *Pseudomonas aeruginosa*
LB	Luria Bertani (*aka* lysogeny broth)
LC	liquid crystal
LPS	lipopolysaccharide
LUV	large unilamellar vesicle
LuxI	autoinducer synthase protein in *Vibrio fischeri* family of autoinducer synthase proteins
LuxR	autoinducer receptor protein in *Vibrio fischeri* family of autoinducer receptor proteins
LysR	family of transcriptional regulators that include PqsR
M	molar
Met, M	methionine
mg	miligram

min	minute
mL	milliliter
mM	millimolar
mol%	mole percent
MRSA	methicillin-resistant *Staphylococcus aureus*
MRSEC	Materials Research Science and Engineering Centers
mTorr	millitorr
MΩ	megaohm
N_2	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
NCPA	non-classical partial agonist
NIH	National Institutes of Health
nL	nanoliter
nM	nanomolar
nm	nanometer
NMR	nuclear magnetic resonance
non-coop	noncooperative
NSF	National Science Foundation
OD	optical density
OdDHL	*N*-(3-oxo)-dodecanoyl-L-homoserine lactone
ORF	open reading frame
p	plasmid

P	phosphorus
P. aeruginosa	*Pseudomonas aeruginosa*
PA1897	gene of unknown function, target of QscR
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	poly(ethyleneimine)
pH	negative log of hydrogen ion concentration
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PqsR	autoinducer receptor protein in *Pseudomonas aeruginosa*
PSM	phenol soluble modulin
PTFE	polytetrafluoroethylene
PUF	protein of unknown function
PVDMA	poly(2-vinyl-4,4-dimethylazlactone)
QS	quorum sensing
QS -	quorum sensing minus
QS +	quorum sensing plus
QscR	autoinducer receptor protein in *Pseudomonas aeruginosa*
QSI	quorum sensing inhibitor
QSM	quorum sensing modulator
R	reverse
R. leguminosarum	*Rhizobium leguminosarum*
RhlA	HAA synthase gene in *Pseudomonas aeruginosa*
RhlB	rhamnosyltransferase gene in *Pseudomonas aeruginosa*

RhlI	autoinducer synthase protein in *Pseudomonas aeruginosa*
RhlR	autoinducer receptor protein in *Pseudomonas aeruginosa*
RNA	ribonucleic acid
RP-HPLC	reversed-phase high performance liquid chromatography
rpm	revolutions per minute
RsaL	LasR inhibitory protein in *Pseudomonas aeruginosa*
s (or sec)	second
S. aureus	*Staphylococcus aureus*
S. enterica	*Salmonella enterica* subsp. e*nterica*
S. meliloti	*Sinorhizobium meliloti*
SalI	restriction endonuclease
SAR	structure-activity relationship
SD	standard deviation
SdiA	autoinducer receptor protein in *Escherichia coli* and *Salmonella enterica*
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SEM	scanning electron microscopy
SLIPS	slippery liquid infused porous surfaces
SrgE	effector in *Salmonella enterica*
subsp.	subspecies
tAIP	truncated autoinducing peptide
TCDA	10,12-Tricosadiynoic acid
TEM	transmission electron microscopy

TetM	tetracycline resistance protein
TFA	trifluoroacetic acid
TP	triphenyl
Tris	tris(hydroxymethyl)aminomethane
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin
UV	ultraviolet
V. fischeri	*Vibrio fischeri*
WT	wild type
YFP	yellow fluorescent protein
α	alpha
β	beta
β-gal	beta-galactosidase
β-ME	β-mercaptoethanol
δ	delta
μL	microliter
μM	micromolar

CHAPTER ONE: Introduction

Contributions: K. E. Nyffeler wrote the Chapter, K.E. Nyffeler and H. E. Blackwell edited the chapter together.

An Introduction to Quorum Sensing

General Quorum Sensing Background

In 1964, researchers discovered that competence in *Streptococcus pneumoniae* changed over the growth cycle and was dependent on the number of cells present.[1-2] A decade later, other scientists described a similar observation in which bacteria in the light organs of marine hosts only produced a bioluminescent compound when the bacteria were of a sufficiently large and dense population—a phenomenon they termed autoinduction (i.e., the regulation of behaviors without an exogenous signal).[3] A protein LuxR was identified in the marine bacteria (*Vibrio fischeri*) that appeared to control this cell density sensing process, and over the ensuing decades, considerable research into the function of this sensing mechanism expanded rapidly. The phenomenon involves the production of a signal, often a small molecule or peptide, that binds to a receptor only once a sufficient ligand concentration is reached (i.e., a large population of bacteria are present in a given environment). Thereafter, the activated receptor-ligand complex binds a promoter sequence directly or triggers binding of a separate protein to alter gene expression at high cell density. Myriad genes and phenotypes can be directly or indirectly controlled by this process. Oftentimes, one of the genes regulated by the receptor encodes a protein product responsible for production of the signal-producing molecule itself (e.g., a LuxI-type signal synthase). In some systems, production of the receptor has been observed to be similarly controlled. In either case, this interconnectivity leads to an auto-induction loop once the signal molecule—the "auto-inducer"—accumulates to sufficient levels.[4] (Figure 1)

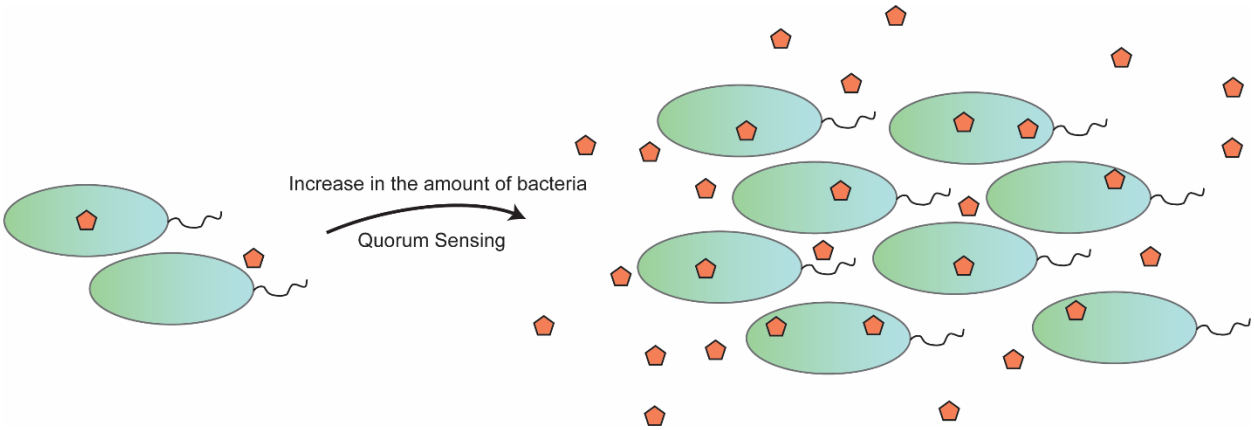

Figure 1. Generic autoinduction in bacteria, with increasing amount of autoinducer (orange) as bacterial population increases

In 1994, this process was named "quorum sensing" (QS) in a report by Fuqua, Winans, and Greenberg.[5] This choice of nomenclature is instructive since the auto-induction only occurs when a specific number of bacteria is reached (i.e., a "quorum"). Below this cell number, a lower basal amount of signal is produced and does not productively bind its receptor. Above the quorate cell number, productive receptor binding occurs and downstream processes are activated. Other types of QS beyond those controlled via LuxR-type receptors have been discovered with divergent proteins and chemical signals, from the widespread AI-2 system discovered in *Vibrio cholerae*[6] to the *agr* system in *Staphylococcus aureus*,[4] among others. Over time, the scope of QS has become clear, with the autoinduction phenomenon appearing in many prevalent pathogens (*S. aureus* in medical device biofilms, *Pseudomonas aeruginosa* in pneumonias) and symbionts (*e.g.*, *Rhizobia* in root nodulation, and *V. fischeri* in light production and nutrient acquisition).[7-10]

Quorum sensing in Pseudomonas aeruginosa

Medical relevance of P. aeruginosa

A 2019 report by the CDC[11] identified over 32,000 cases of antibiotic resistant *P. aeruginosa* in hospitalized Americans. *P. aeruginosa* is primarily an opportunistic pathogen; it presents the most danger to patients under respiratory distress. In fact, a study of over 3,000 children with cystic fibrosis (CF) found that *P. aeruginosa* infection was a strong predictor of mortality.[12] *P. aeruginosa* can also infect patients with other respiratory comorbidities, including chronic obstructive pulmonary disease (COPD)[13] and those on ventilators.[10] In addition to respiratory infections, *P. aeruginosa* can cause chronic wound infections.[14] In *P. aeruginosa*, QS tightly controls virulence. It is widely hypothesized that this observation is explained by the necessity for a pathogen to initiate expression of its virulence factors neither too early (before sufficient numbers exist) nor too late (after surveillance by innate immunity has already detected the infection).

Quorum sensing network in P. aeruginosa

Given this necessity for temporal regulation, *P. aeruginosa* is among the bacteria that use LuxR-type receptors as part of its QS system. The species has three interconnected LuxR-type receptors, with additional and disparate QS systems integrated into the broader circuit. The first of the three LuxR-type receptors to be discovered in *P. aeruginosa* was LasR. Like other LuxR-type receptors, LasR is a cytoplasmic transcription factor. LasR is responsive to the 12-carbon acyl homoserine lactone (AHL) *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (OdDHL). OdDHL is produced by its cognate synthase (LasI), which is upregulated by the LasR-OdDHL complex.[15] However, unlike other LuxR-type receptors, LasR does not bind and upregulate its own

promoter—LasR is primarily controlled by other factors and is highly responsive to the growth phases of the cell.[15-20] For many years, LasR was thought to be the master regulator of the three LuxR-type receptors. However, more recent work suggests that the second receptor to be discovered, RhlR, may figure more prominently in control, particularly in long-term infections in CF patients.[21] RhlR is responsive to *N*-butanoyl-L-homoserine lactone (BHL) produced by the AHL synthase RhlI. RhlR directly controls RhlI and itself, but again, is often under tight LasR control.[15] The third LuxR-type receptor, QscR was not discovered until relatively recently, partially because it lacks a paired synthase (this type of LuxR-receptor is termed an "orphan") and does not control a large number of target genes.[22] Until very recently, all that was known about QscR's role in *P. aeruginosa* QS was that it strongly bound OdDHL (LasI's product) and led to later induction of LasR and RhlR.[22-23] One report provided evidence that QscR inhibited RhlR and LasR binding by forming heterodimers with the other two proteins.[24] However, in 2018 the Dandekar laboratory discovered that QscR's only binding target is *PA1897*, an operon of unknown function beyond repressing LasR and RhlR activation. Indeed, QscR was dispensable for this repression when activation of the operon was controlled constitutively.[25]

Beyond LuxR-type receptors, PqsR is a LysR-type receptor in *P. aeruginosa* that interacts with RhlR and LasR and strongly controls the production of phenazine and quinolone virulence factors, including the blue-green redox active factor, pyocyanin.[26] PqsR is activated by LasR and can also activate RhlR and LasR.[26-28] However, RhlR represses *pqsA*, a protein involved in synthesis of PQS, the ligand of PqsR, so the regulation is not straightforward.[28] In the early 2010s, an additional QS system was discovered in *P. aeruginosa* that is thought to have a role in activation of RhlR and PqsR when LasR is absent or downregulated (*i.e.,* in latent CF infections or in low-phosphate conditions).[29-30] Collectively, these four QS proteins—LasR,

RhlR, QscR, and PqsR—are all interregulated and tightly control *P. aeruginosa* virulence in differing environmental conditions. (Figure 2)

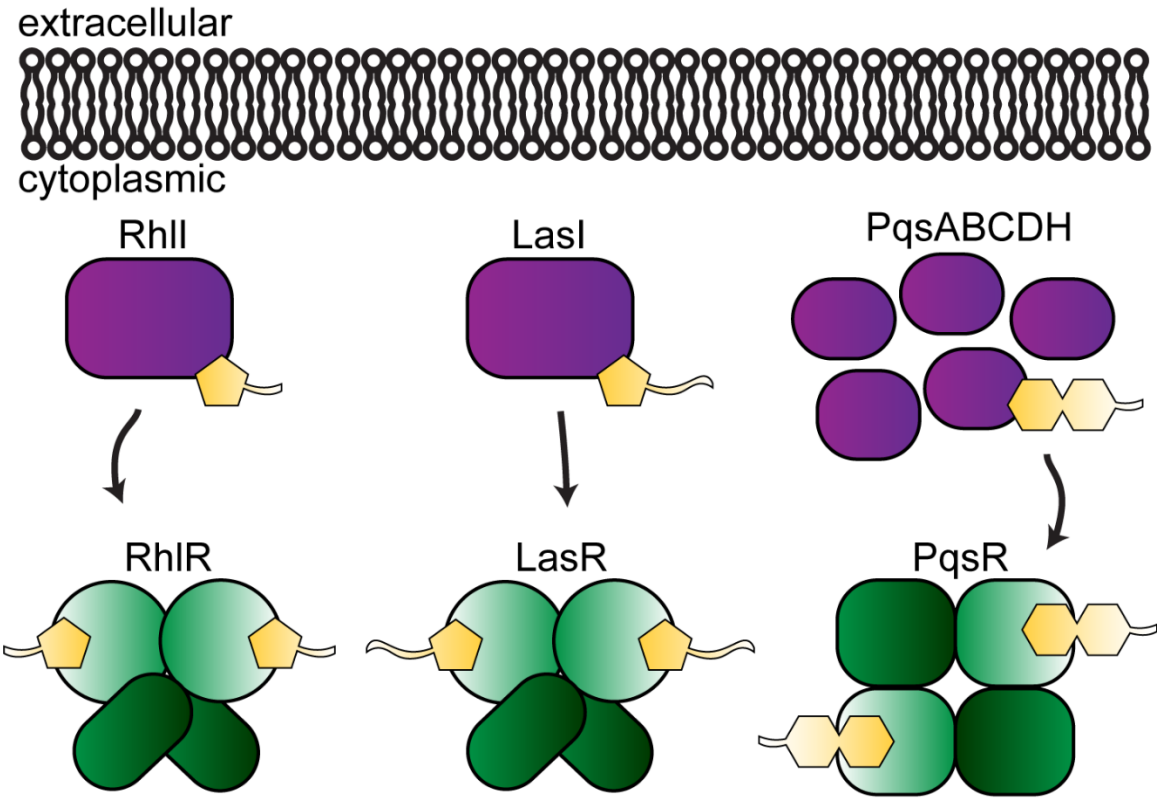

Figure 2. Rhl, Las, and Pqs systems in *P. aeruginosa*. Ligands are represented in yellow, ligand producing proteins in purple, and ligand binding and transcription in green.

QS-controlled virulence

Many *P. aeruginosa* toxins and virulence factors are QS controlled. Rhamnolipid is controlled primarily by RhlR, but also to a lesser degree directly via LasR.[31] This surfactant is important for invasion of epithelial cells[32] and uptake of hydrophobic compounds.[33-35] Rhamnolipid may even be used for encapsulation and delivery of other virulence factors.[36-37] Elastase is a protease primarily under LasR control, but also by RhlR to a lesser degree.[38] It

increases the permeability of the barrier between epithelial cells (cell-cell junctions),[39-40] an action that is exacerbated by QS-controlled exotoxin A (which prevents the restoration of the cell-cell junctions).[39, 41] Pyocyanin is yet another *P. aeruginosa* toxin; it gives the bacterium its characteristic blue-green color. It is primarily regulated by PqsR, but again, to a lesser degree LasR and RhlR.[42] Pyocyanin is part of a larger class of molecules known as phenazines that have a large host of toxic effects. Pyocyanin itself is a redox-active molecule and is thought to induce cytotoxicity via oxidative damage.[43]

Chemical control of P. aeruginosa *quorum sensing*

Because the ligands that ultimately control LuxR-type QS are small molecules, many laboratories have become interested in developing alternate ligands to control QS.[44-46] The long term goal of such ligand development is to find an anti-virulence compound, as it is hypothesized that such a pharmacological intervention could lead to decreased resistance development, relative to current antibiotics.[47-48] Beyond their development as therapeutics, however, QS modulators (QSMs) are also extremely useful as research tools. QSMs provide improved temporal and spatial control over classic genetic tools—they can be added and removed at will. Of the three LuxR-type receptors in *P. aeruginosa*, LasR's native ligand OdDHL has been most thoroughly studied from a structure-activity relationship (SAR) point of view, by testing larger and smaller acyl tails, making alterations to the lactone head group, adding non-native functionality to the acyl tail.[49-59] While many non-native QSMs are based on the native ligand, chemical scaffolds that target LasR have also been discovered via high-throughput screens of sizable small molecule libraries.[60-62] In addition, RhlR has become a very popular target as of late due to its aforementioned role in latent infection, while QscR has been explored to a lesser degree.[49, 63-65]

Given the interregulation of the *P. aeruginosa* receptors and QscR's inhibitory effects on the other two receptors, it is becoming increasingly important to understand how promiscuous a given QSM is, especially within an organism like *P. aeruginosa* that has multiple LuxR-type receptors. For example, because QscR and LasR share a native ligand, modulators which only target one of the two could be used to tease apart the two receptors' contributions to regulation of virulence and other factors. Few studies exist that examine the separate effects of QSMs on different LuxR-type receptor, particularly those within one organism. [65]

Transcriptional control of QS

While the structures of many of QSMs are based off that of the native ligand and thus presumed to be competitive binders, we currently lack much biochemical and structural data to support this hypothesis. X-ray crystal structures of LuxR-type receptors have been challenging to obtain due to their inherent low solubilities, particularly in the absence of ligand. Thus, it is difficult to determine if a QSM binds at the same location on a receptor, or to the same conformation of the receptor, as the native ligand. Increasing this uncertainty in *P. aeruginosa*, the activated LasR:OdDHL complex does not interact with every one of its promoters in the same manner; for example, LasR has been found to bind promoters both cooperatively (as a multimer of dimers) and non-cooperatively (as a single dimer).[38, 66] The *las* box is well documented for the better understood, non-cooperative binding site, but no consensus sequence has been determined for the secondary site further upstream.[38] The Iglewski laboratory studied two promoters via transcriptional fusion (promoter driving *lacZ* transcription).[67] The authors found that the concentration of OdDHL required to reach half-maximal activity was 10x higher for the cooperative promoter (*lasB*) than the noncooperative (*lasI*). While these promoters have

other differences beyond the *las* box, this finding should be expounded upon. To our knowledge, direct comparison of the effects of non-native QSMs on LuxR-type protein:DNA binding has never been reported between disparate promoters.

Quorum sensing in Staphylococcus aureus

Medical relevance of S. aureus

Staphylococcus aureus is a pervasive nosocomial and community-associated pathogen and uses QS to control many aspects of virulence. In one study of over 3 million bacterial isolates across the United States, *S. aureus* was identified in ~20% of all isolates from inpatients (the most frequent bacterial species detected) and ~15% of isolates from outpatients (the second most frequent bacterial species detected).[68] In addition, in 2005, ~50% of these isolates were MRSA (methicillin-resistant *S. aureus*) and up to 60% of all MRSA isolates were multi-drug resistant.[68] Toxic shock syndrome (TSS) is caused by *S. aureus* and often attributed to tampon colonization but may develop from surgical procedures as well. The condition is primarily associated with production of toxic shock syndrome toxin (TSST-1),[69] which is under the direct control of QS. [70-71] *S. aureus* can also form biofilm on implantable medical devices under the control of QS, leading to expensive removal of the implants and, sometimes, dangerous sepsis in the joints.[72-73] In addition to the bloodborne and deep tissue infections and associated intoxications, *S. aureus* also causes a host of skin infections, many of which are toxin driven. For example, staphylococcal scalded-skin syndrome is often mediated by exfoliative toxin (ET), which can cause painful blisters throughout the body.[74] ET is another of the large arsenal of toxins regulated by *S. aureus* QS.[75-77]

QS network in S. aureus

S. aureus utilizes a peptide-based QS system known as the accessory gene regulator (*agr*) system. The system consists of four proteins regulated by an auto-induction loop. AgrD, the precursor to the QS signal, is produced as a propeptide in the cytoplasm, processed by AgrB to cyclize the peptide, and finally post-translationally modified and exported to the extracellular milieu as the mature autoinducing peptide signal (AIP). The AIP signal then binds to AgrC, a transmembrane histidine kinase (HK). This binding event activates a phosphorelay leading to phosphorylation of the response regulator AgrA; thereafter, the activated AgrA dimerizes and binds to DNA. (Figure 3) AgrA's primary regulatory targets are RNAII (encoding *agrBDCA*) and RNAIII (encoding *hld*—i.e., delta toxin—and a regulatory RNA). *S. aureus* has evolved into four different *agr* specificity groups (I-IV), each defined by a unique AIP signals. Between *agr* groups, the source of variability is primarily located in AgrD, but also extends into portions of AgrB and AgrC.[4] Interestingly, different specificity groups are have been shown to interfere with each other, with each AIP inhibiting the other non-self AgrC.[78] This interference has been hypothesized to play a role in the groups' colonization of different niches on a host, but additional research is needed to understand the origins of this phenomenon.

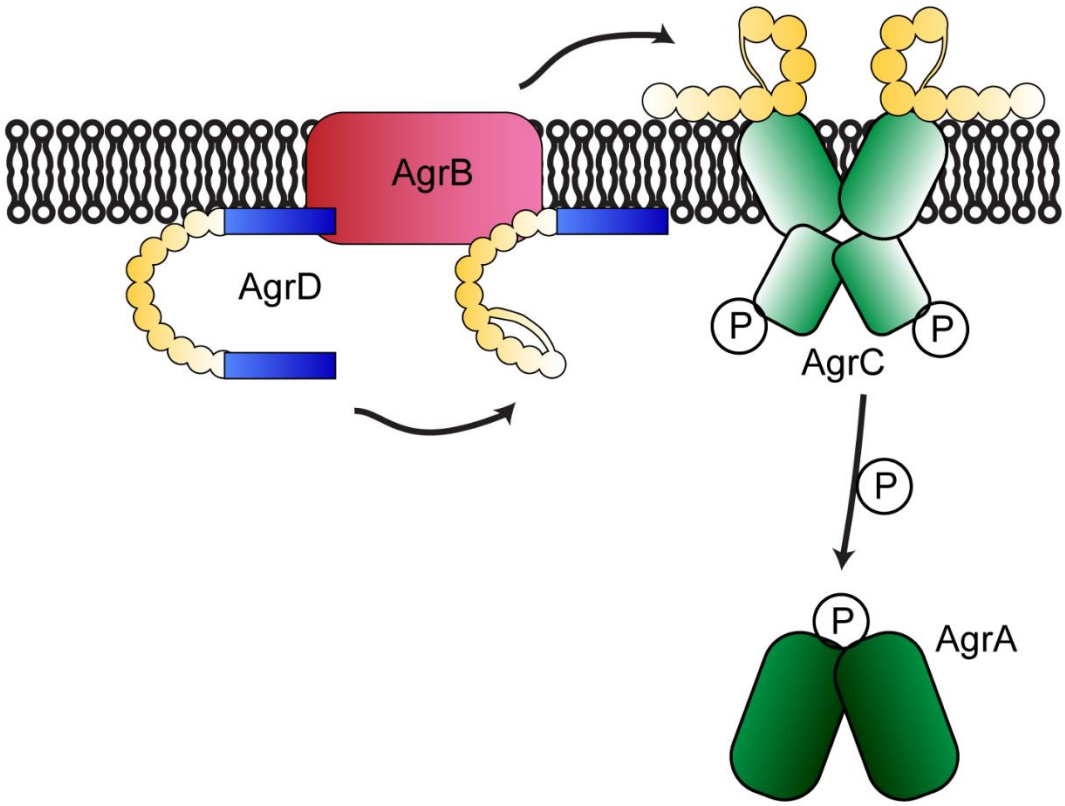

Figure 3. Quorum sensing schematic in *S. aureus*. AgrB (red) assists in the cyclization, export, and maturation of the propeptide AgrD (blue and yellow) to the final auto inducing peptide (AIP, yellow). The AIP then binds to the histidine kinase AgrC (light green), activating a phosphorelay that phosphorylates AgrA, the response regulator (dark green).

QS-controlled virulence in S. aureus

RNAIII is a main effector of virulence in *S. aureus* and, as highlighted above, is directly activated by AgrA. It contains open reading frames (ORFs) encoding various toxins, but also has a large number of regulatory functions. The exact mechanism of repression varies but is RNA-mediated and always prevents ribosome binding. Among the regulatory targets of RNAIII are *hla* (α-hemolysin, a toxin),[79] *spa* (surface protein A, binds immunoglobulins),[80-81] and *rot* (a transcriptional regulatory protein).[82-83] A large host of other toxins are also regulated by

downstream effects of RNAIII in *S. aureus*, including the exfoliative toxins mentioned earlier.[76-77] In *S. aureus*, a major group of these *agr*-controlled toxins are the phenol soluble modulins (PSMs). These are amphiphilic peptides and are divided into two major classes, the shorter α and the longer β PSMs.[84] Almost all of the PSM genes are regulated by the *agr* system, either by RNAIII or directly via AgrA binding.[85] PSMs have a number of virulence-related functions, although they of course vary between different types of PSMs (i.e., PSM α3 is cytolytic and β-type PSMs are not).[85] PSMs have been implicated in biofilm formation and dissemination[86] and in eukaryotic cell lysis.[87] Underscoring their importance in infection, PSM α deletion strains showed decreased wound size (in mice) and abscess volume (in rabbits).[88-90]

Chemical control of QS in S. aureus

The mature AIP signal is a macrocyclic peptide, with a thioester bridging the cysteine and the C-terminal residue. In view of its relatively simple, small molecule-like nature, numerous groups have pursued the design of peptidic ligands to interfere with QS in *S. aureus* and related species. Studies from our laboratory and others have examined systematic changes to the amino acids present in the AIPs (*e.g.*, alanine or D-amino acid scans), shortened or lengthened the AIP primary structure, and generated peptidomimetics of AIPs to achieve better stability and activity.[91-100] Other studies have uncovered synthetic AgrC modulators from natural sources.[101-104] Importantly, AgrC modulators do not have to penetrate the *Staphylococcal* cell, allowing for more diverse and higher molecular weight ligands to be probed. QSM development in *S. aureus* has not focused only on AgrC, however: AgrA has also been a target of high-throughput screens and a substantial number of effective inhibitors have been recently discovered.[105-107]

Materials-based solutions to identify QS-controlled surfactant production

QS-controlled surfactant production by bacteria

Given the degree to which QS controls virulence, factors produced under QS control can serve as highly useful read-outs for the presence of a quorate community of bacteria. Biosurfactants are easily identifiable QS-controlled factors. While the aforementioned *S. aureus* and *P. aeruginosa* produce QS-controlled biosurfactants (PSMs and rhamnolipid, respectively), there are numerous examples of other species of bacteria that also control biosurfactant production via QS. [108-113] There are several applications in which monitoring biosurfactant production would be informative: (1) as a straightforward readout for the performance of new QSMs, and (2) to identify the presence of a quorate population of bacteria.

Current methods to screen for new QSMs

In order to assess the utility of biosurfactant production as a readout for QS, the current methods available to screen for new QSMs should be considered. For *S. aureus*, a thorough review detailing the assays available for screen QS modulators of the *agr* system was reported by Quave and Horswill in 2014.[114] A majority of the assays are low-throughput: (i) low-throughput agar plate tests (*e.g.*, blood agar diffusion assays,[115] evaluation of colored products,[116] blue-white screening,[117] etc.) to visually detect the presence of secondary metabolites, (ii) low-throughput cell culture techniques (*e.g.*, invasion/adhesion assays,[118-119] cell lysis,[120] etc.) to detect the presence of virulence factors, and (iii) low-to-medium throughput chromatographic assays (*e.g.*, via HPLC[121] or MS[122] methods) to detect the presence of secondary metabolites. High-throughput assays are generally molecular in nature, for example requiring qPCR[123] or transcriptional fusions,[124] and thus more experimental design at the outset and interpretation

thereafter. Notably, the vesicle lysis test— developed by the Jenkins laboratory in 2010[125] — is one of the few non-chromatographic or molecular biology assays highlighted by Quave and Horswill as having potential utility for the straightforward and rapid-throughput detection of QSMs.[114] To our knowledge, this assay has never been used to screen for QSMs, especially in a high-throughput manner.[125]

Vesicle lysis test

In 2010, the Jenkins laboratory described an experimental test that readily distinguished *P. aeruginosa* and *S. aureus* from *E. coli*.[125] Briefly, the authors immobilized phospholipid giant unilamellar vesicles (GUVs) on a maleic anhydride substrate, and when cultures of certain bacteria were added to the loaded substrate, the vesicles released their sodium azide cargo and killed the *P. aeruginosa* and *S. aureus* but failed to release their cargo in the presence of *E. coli*.[125] The authors went on to demonstrate that the lysis was QS-linked.[126-127] They and others determined that the causative agents of vesicle lysis in these two pathogens were PSMs and rhamnolipids.[84, 126] In light of these results, and the robust nature of the assay, the vesicle lysis test could be an extremely powerful tool to screen for QSMs in *S. aureus*, *P. aeruginosa*, and potentially additional bacteria that produce surfactants. (Figure 4) More broadly, it could be deployed as a sensor for quorate, and potentially pathogenic, populations of bacteria in various environments.

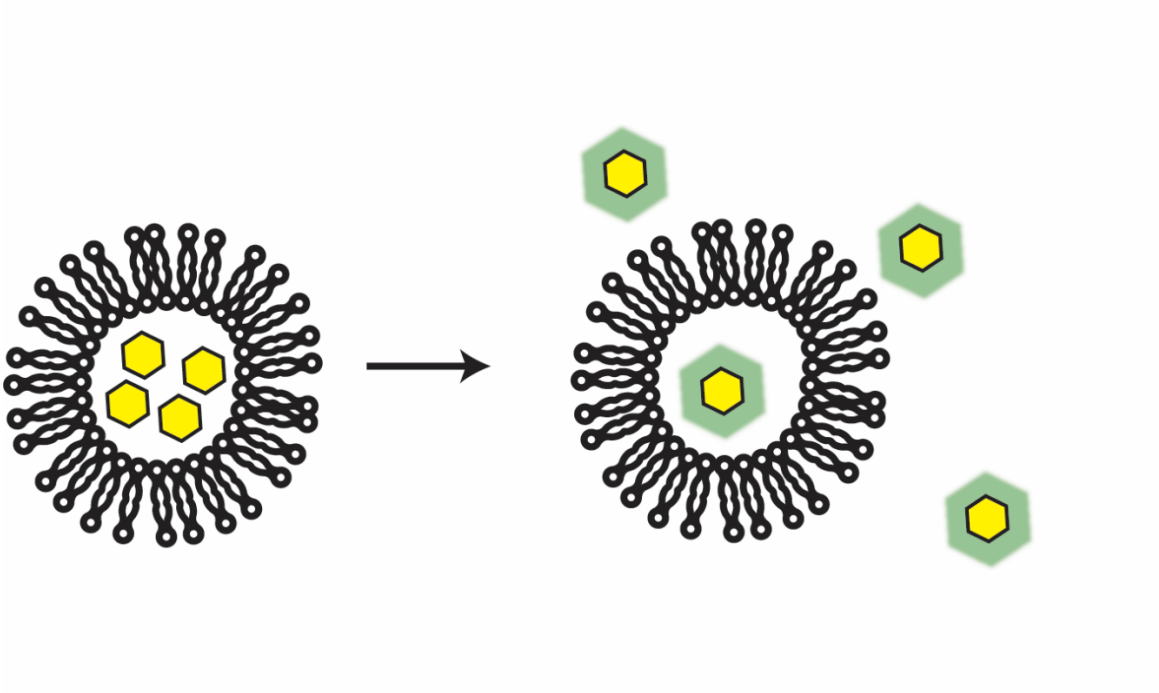

Figure 4. Schematic of the vesicle lysis test. Lipid bilayers encapsulating calcein (yellow hexagons) will release their cargo in the presence of a surfactant. Concentrated calcein is quenched and does not fluoresce but fluoresces upon release from the vesicles and thus dilution. (right)

Responsive materials to identify quorate bacterial populations

The Blackwell and Lynn laboratories at UW–Madison have been working together for a number of years to design novel QS-responsive soft materials and coatings.[128-132] One approach to such materials could involve non-covalent interactions between a QS-controlled metabolite and a responsive material. Current work from our two laboratories[133] has shown that a type of liquid crystal (LC) sensor could be used to identify a quorate population of bacteria. Thermotropic LCs exhibit a state of matter between liquids and solids: at the appropriate temperature, they have orientational properties as in a solid (each molecule orients in the same direction relative to every other molecule); however, they have no positional order (each molecule can move around other molecule, as long as they maintain that orientation). The LCs

will orient themselves in a specific way and changes in this orientation caused by the presence of certain bacterial secondary metabolites can be used for the detection of bacterial products. Out most recent study incorporated LCs in droplet form and showed that these droplets respond to the presence of quorate populations of *P. aeruginosa* through the sensing of both long-chain AHLs and rhamnolipids.[133] Another recent report demonstrated that the infusion of LCs into a slippery liquid-infused porous surface (SLIPS) allowed detection of non-biological surfactants.[134] Given these two reports, it is reasonable to imagine future studies exploring LC-infused SLIPS for the detection of QS-controlled products and thus sensing a quorate population of bacteria.

Dissertation Scope

Chapter 2: Selective and promiscuous chemical modulators of LuxR-type quorum sensing in Pseudomonas aeruginosa

As highlighted above, non-native molecules capable of intercepting QS represent useful tools to explore the role of this pathway in bacterial virulence. As individual bacterial species can use multiple QS systems to regulate virulence, and also commonly reside in mixed microbial communities with other bacteria capable of QS, chemical tools that are either selective for one particular QS system, or are "pan active" and target all QS pathways, are both of significant value. In this Chapter, we outline the analysis of a set of compounds reported to target one QS receptor in *Pseudomonas aeruginosa* for their activity in two other QS circuits in this pathogen, and the discovery of a set of molecules with novel activity profiles, including ligands that agonize all three QS systems, agonize one but antagonize the other two, or strongly antagonize just one.

Chapter 3: The vesicle lysis test for the facile identification of synthetic modulators of quorum sensing and biosurfactant production in bacteria

Chemical mimics of the native QS signals are valuable as research tools to explore the many unanswered questions about the mechanism of QS and could provide a pathway to potential anti-virulence approaches. However, robust methods for the rapid identification of non-native QS modulators (QSMs) are scarce. We reasoned that an assay for the presence of QS-controlled surfactants could be repurposed for the straightforward identification of QSMs. Herein, we applied the vesicle lysis test (VLT)—an assay that monitors the disruption of large unilamellar vesicles (LUVs) via fluorescence – to screen for QSMs. We generated robust calcein-loaded LUVs and showed that calcein release was QS-dependent and coincided with QS onset in both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We then demonstrated that the VLT can be used to identify known QSMs in both pathogens and demonstrate in *S. aureus* that this assay allows for quantitative measures of QSM activity. Lastly, we use the VLT to perform a high-throughput screen of a commercial small molecule library and discovered new small molecule QSMs. This study demonstrates a high-throughput and straightforward method of screening for QSMs via the detection of bacterial virulence factors.

Chapter 4: Anisotropic liquid infused surfaces: a platform for naked-eye detection of biosurfactants

As highlighted above, LCs alone have been used to sense the presence of quorate populations of bacteria and LC-infused slippery liquid infused porous surfaces (LC-SLIPS) have been used to sense the presence of surfactants. In this Chapter, we infused Teflon with LCs to create novel LC-SLIPS. After the simple addition of a droplet to a LC-SLIP surface at an angle, the droplet

with surfactant will slide slower than the "bare" droplet, leading to easy unaided-eye detection. We demonstrate that the speed at which the droplet rolls down these LC-SLIPS is surfactant- and concentration-dependent. We applied these LC-SLIPS to the detection of quorate populations of bacteria, and we discovered that they can readily sense PSMs produced by *S. aureus* and can differentiate QS + and QS- *S. aureus*. Overall, this study demonstrates a new use for SLIPS and demonstrates an easy, naked-eye approach to surfactant identification that could find application in a range of fundamental and applied contexts.

Appendix 1: Exploring the use of synthetic ligands to differentially modulate transcriptional activation of QS genes by LasR in Pseudomonas aeruginosa

In *Pseudomonas aeruginosa*, the QS receptor LasR (as the $[LasR:OdDHL]_2$ homodimer) has two alternate modes of promoter binding: (1) as a dimer of dimers (cooperative binding), and (2) as a dimer (noncooperative). These two binding modes differ by promoter. We hypothesize that, given these two binding modes, QSMs could differentially modulate the LasR regulon. In this Appendix, we outline the beginnings of our work to explore this hypothesis, including an explanation of the Gibson cloning we are using to create plasmids to test this hypothesis.

Appendix 2: A comparative analysis of synthetic QSMs in Pseudomonas aeruginosa: *New insights into mechanism, active efflux susceptibility, phenotypic response, and next-generation ligand design*

As highlighted above, considerable recent research has been devoted to the design of small molecules capable of modulating the LasR QS receptor in the opportunistic pathogen *Pseudomonas aeruginosa*. These molecules hold significant promise in a range of contexts;

however, as most compounds have been developed independently, comparative activity data for these compounds are scarce. Moreover, the mechanisms by which the bulk of these compounds act are largely unknown. This paucity of data has stalled the choice of an optimal chemical scaffold for further advancement. In this Appendix, we submit the best-characterized LasR modulators to standardized cell-based reporter and QS phenotypic assays in P. aeruginosa, and we report the first comprehensive set of comparative LasR activity data for these compounds. Our experiments uncovered multiple interesting mechanistic phenomena (including a potential alternative QS-modulatory ligand binding site/partner) that provide new, and unexpected, insights into the modes by which many of these LasR ligands act. The lead compounds, data trends, and mechanistic insights reported here will significantly aid the design of new small molecule QS inhibitors and activators in *P. aeruginosa*, and in other bacteria, with enhanced potencies and defined modes of action.

Appendix 3: Design, synthesis, and biochemical characterization of non-native antagonists of the Pseudomonas aeruginosa *quorum sensing receptor LasR with nanomolar IC_{50} values*

Small molecule tools that inhibit LasR activity would serve to illuminate its role in *P. aeruginosa* virulence, but we currently lack highly potent and selective LasR antagonists, despite considerable research in this area. V-06-018, an abiotic small molecule discovered in a high-throughput screen, represents one of the most potent known LasR antagonists but has seen little study since its initial report. In this Appendix, we report a systematic study of the structure−activity relationships (SARs) that govern LasR antagonism by V-06-018. We synthesized a focused library of V-06-018 derivatives and evaluated the library for bioactivity using a variety of cell-based LasR reporter systems. The SAR trends revealed by these

experiments allowed us to design probes with 10-fold greater potency than that of V-06-018 and 100-fold greater potency than other commonly used N-acyl-L-homoserine lactone (AHL)-based LasR antagonists, along with high selectivities for LasR. Biochemical experiments to probe the mechanism of antagonism by V-06-018 and its analogues support these compounds interacting with the native ligand-binding site in LasR and, at least in part, stabilizing an inactive form of the protein. The compounds described herein are the most potent and efficacious antagonists of LasR known and represent robust probes both for characterizing the mechanisms of LuxR-type QS and for chemical biology research in general in the growing QS field.

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CHAPTER TWO: Selective and Promiscuous Chemical Modulators of LuxR-type Quorum Sensing Receptors in *Pseudomonas aeruginosa*

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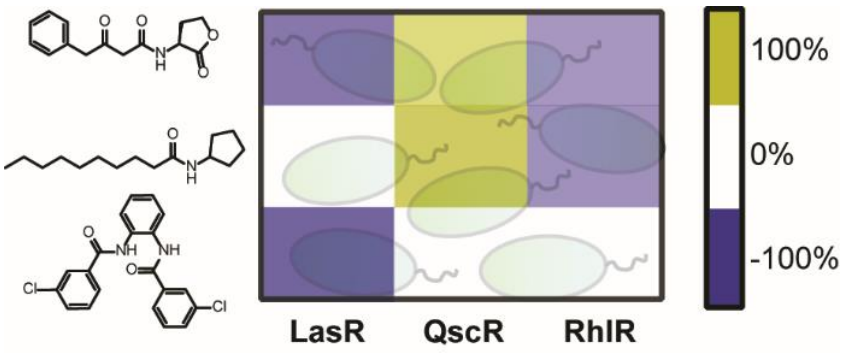
To be submitted to *ACS Chemical Biology*

Contributions: K. E. Nyffeler designed and performed experiments, K. E. Nyffeler and H. E. Blackwell wrote the text.

Abstract

Quorum sensing (QS) allows bacteria to assess their local cell density using chemical signals and plays a prominent role in the ability of common pathogens to infect a host. Non-native molecules capable of intercepting QS represent useful tools to explore the role of this pathway in bacterial virulence. As individual bacterial species can use multiple QS systems to regulate virulence and also commonly reside in mixed microbial communities with other bacteria capable of QS, chemical tools that are either selective for one particular QS system, or are "pan active" and target all QS pathways, are both of significant value. Herein, we outline the analysis of a set of compounds reported to target one QS receptor in *Pseudomonas aeruginosa* for their activity in two other QS circuits in this pathogen, and the discovery of a set of molecules with novel activity profiles, including ligands that agonize all three QS systems, agonize one but antagonize the other two, or strongly antagonize just one.



Introduction

Quorum sensing (QS) is a type of bacterial cell-cell communication that involves the production and detection of small molecule signals, or autoinducers.[1-3] The opportunistic pathogen *Pseudomonas aeruginosa*, along with many other common Gram-negative bacteria, use *N*-acyl L-homoserine lactones (AHLs) as QS signals. AHLs are produced at a low basal levels by LuxI-type synthases, and the concentration of AHL in the local environment increases with bacterial cell number. Once the AHL concentration reaches a threshold level within the cell (i.e., after a "quorate" population has amassed), productive binding of the AHL to its cognate intracellular LuxR-type receptor occurs. This binding event leads to a cascade of downstream transcriptional changes, including increased production of the LuxI-type synthase that generates more AHLs (i.e., autoinduction). In pathogens that use such LuxI/LuxR circuits for QS, such as *P. aeruginosa*, a large number of virulence genes are controlled via QS.[4-5] Indeed, in a number of infection models, including mice and *Caenorhabditis elegans*, QS has been shown to significantly contribute to infection.[6-7] This connection has attracted considerable attention to the inhibition of QS pathways as a potential pathway to block virulence in *P. aeruginosa*, along with in other related pathogens, and chemical strategies to intercept QS have become a major focus of research.[8-13]

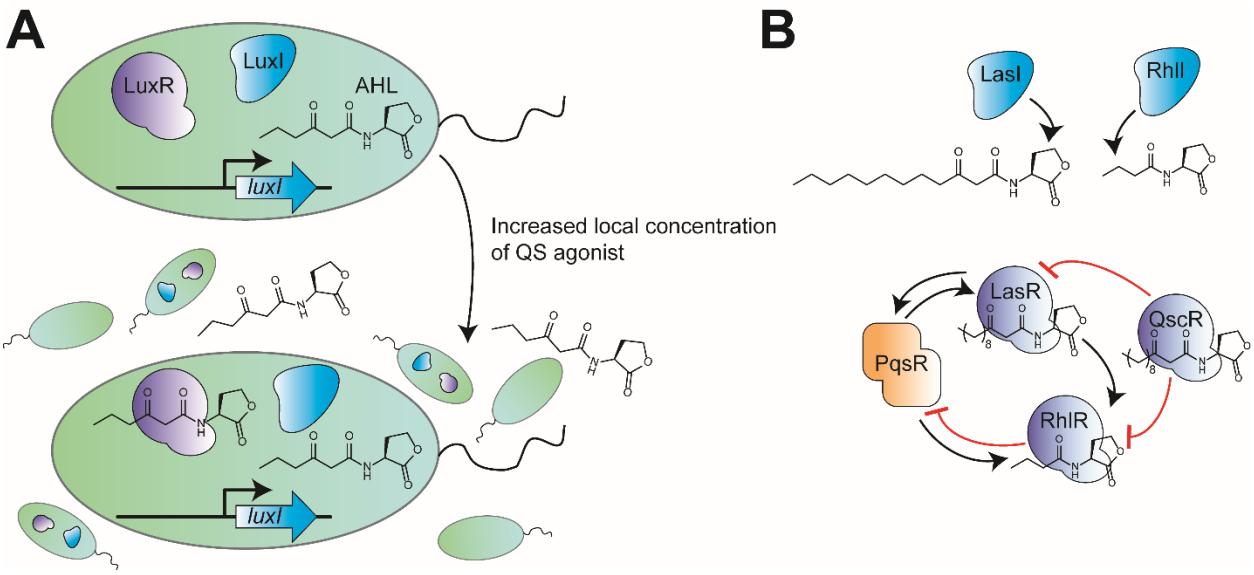


Figure 1: Schematics of quorum sensing (QS) processes. (A) Model of LuxI/LuxR-type QS in bacteria. (*Top*) The LuxI-type synthase (light blue) and LuxR-type receptor (dark blue) are produced at low levels at low cell density, and the local concentration of AHL signal (represented by *N*-(3-oxo)-hexanoyl-L-homoserine lactone here) is low. (*Bottom*) As cell number increases, AHL concentration likewise increases. Productive binding of the AHL to LuxR-type receptor typically promotes [AHL:receptor] homodimerization and subsequent transcriptional activation of QS-regulated genes. Among other downstream effects, the production of the LuxI-type synthase will be upregulated, yielding more AHL. (B) A simplified schematic of the QS system in *P. aeruginosa.* Red (inhibitory) or black (activation/production) arrows do not necessarily represent direct binding. The LysR-type receptor, PqsR, is in orange.

P. aeruginosa uses three LuxR-type receptors (LasR, RhlR, and QscR) and two LuxI-type synthases (LasI and RhlI) as part of its QS system. LasR and QscR are activated by the 12 -carbon AHL produced by LasI, *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (OdDHL), while RhlR is activated by the shorter, 4-carbon AHL, *N*-butanoyl-L-homoserine lactone (BHL), produced by RhlI (Figure 1A). LasR has been considered to function largely upstream of the other two receptors, serving to activate RhlR and be repressed by QscR. Most research targeting LuxR-type receptors in *P. aeruginosa* with chemical tools has focused on LasR.[14-16] However, recent studies of *P. aeruginosa* isolates from chronic lung infections indicate that LasR is actually nonfunctional and RhlR instead plays a leading role in virulence.[17] Even in acute

infections when LasR is active, a prominent role for the other receptors in the interregulation of each other and the production of virulence factors exists (Figure 1B).[17] Adding further complexity to the situation is the LysR-type receptor, PqsR, which is unrelated to the LuxR-type systems, regulated by a quinolone signal (PQS), and shown to modulate both LasR and RhlR.[18] Many questions about the roles of each receptor in infection remain. For example, QscR is only known to target a single operon,[19] the function of which is poorly understood but includes LasR and RhlR repression. Chemical tools are now beginning to be developed to delineate the function of all three of the LuxR-type receptors in *P. aeruginosa* QS, with potent ligands that modulate RhlR featuring prominently in the past ~6–7 years.[20-23]

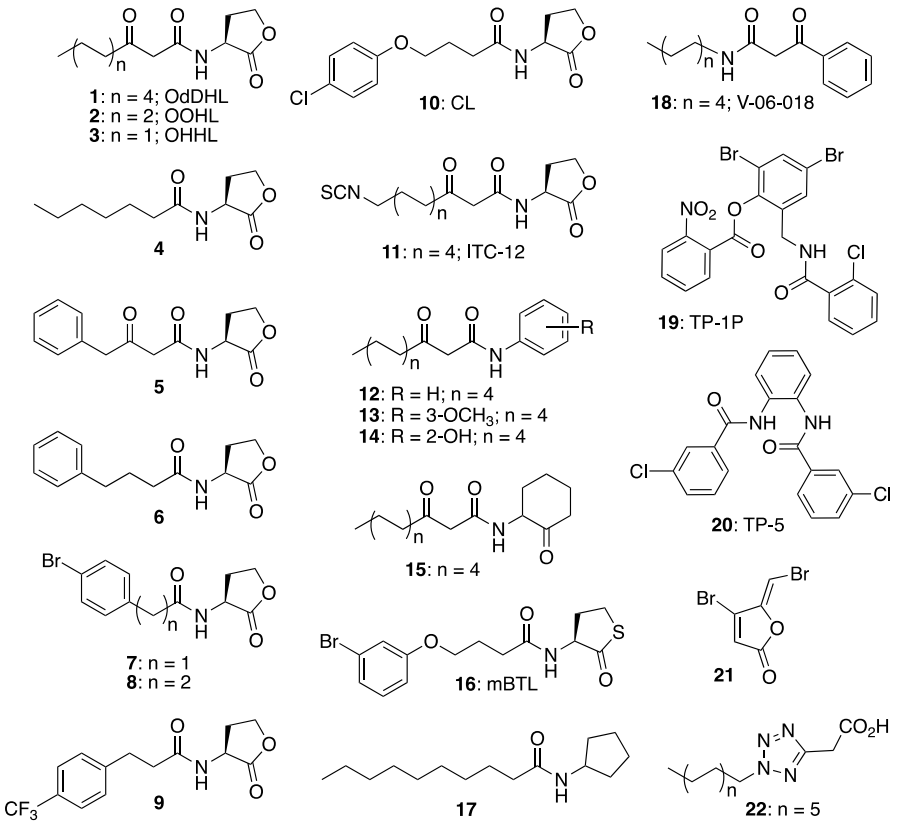


Figure 2: Structures of the compounds evaluated in the current study. Compound numbering reflects that used by Moore *et al*.[24] Common abbreviated names for certain compounds are included. Compound **1** (OdDHL) is the native AHL ligand for LasR.

In 2015, our laboratory performed a comparative activity study of a set of 21 small molecules reported to strongly modulate LasR (Figure 2), with a goal of identifying the most promising chemical scaffolds for further LasR probe development.[24] This study was motivated by the presumed prominence of LasR in *P. aeruginosa* QS at that time (*vide supra*); it revealed several non-AHL type compounds that strongly activated and inhibited LasR activity in cell-based reporter gene assays, along with a series of compounds that displayed differing levels of activity or even toxicity. The selectivity profiles of the majority of these compounds in *P. aeruginosa* remain unknown, however, as their effects on RhlR and QscR have not been investigated. In view of the growing prominence of RhlR in *P. aeruginosa* virulence and the questions about receptor interregulation in general in this pathogen, we sought to explore the activity of these compounds in RhlR and QscR further. Small molecules that could selectively modulate only one receptor, target all three receptors (i.e., "pan-active" or "promiscuous" ligands), or synergistically modulate multiple receptors (e.g., activating QscR and also inhibiting LasR) all are of significant interest and remain largely unexplored. Understanding the structural features that engender receptor selectivity, or lack thereof, could also guide the design of new chemical tools with either improved or novel activity profiles. Scrutiny of the LasR reporter assay data of Moore *et al.* revealed certain inconsistencies when compounds were tested in a heterologous reporter strain (i.e., *E. coli*) rather than the native organism,[24] suggestive that they could target different receptors/pathways in *P. aeruginosa* and providing additional motivation for this study. Herein, we report our investigations of this compound library for RhlR and QscR activity, a systematic analysis of their overall receptor activity profiles, and our discovery of both promiscuous and selective LuxR-type receptor modulators in *P. aeruginosa*, along with new insights into mechanisms of LasR inhibition in *P. aeruginosa*.

Results and Discussion

Despite the value of experiments in the wild-type background, examining the activity of non-native ligands in *P. aeruginosa* strains harboring a reporter construct for any one specific LuxR-type receptors can be challenging due to the interregulation of LasR, RhlR, and QscR. For example, LasR needs to be activated to allow for RhlR expression. This connection means that any compound that antagonizes LasR will also appear as a RhlR antagonist in *P. aeruginosa*, regardless of its effects on RhlR. Heterologous *E. coli* reporter systems have the advantage of isolating the receptor of interest from the others, as well as eliminating effects due to *P. aeruginosa*'s intrinsic mechanisms for drug resistance[25] and AHL efflux mechanism.[26] We thus used a heterologous *E. coli* strain (JLD271; a mutant lacking its native LuxR-type receptor *SdiA*)[27] to screen each compound from the Moore et al. study[24] for agonistic and antagonistic activity in either RhlR or QscR (see Methods for full details). Two *E. coli* reporter systems were generated, each with two plasmids: one plasmid to produce either RhlR or QscR under the control of arabinose, and the second plasmid containing a transcriptional fusion of a promoter region known to be regulated by RhlR or QscR (*rhlI* or *PA1897*, respectively) with *lacZ*. Receptor activity was then examined by measuring β-galactosidase activity in the presence of non-native ligand (to measure receptor agonism) or in competition against the native/preferred ligand for either receptor (BHL or OdDHL, respectively; to measure receptor antagonism).

Of the 21 compounds from the original set tested (**2**–**22**; Figure 2), we eliminated **21** due to its observed toxicity, even at moderate concentrations. For each of the remaining 21 compounds, we examined their activity over a range of concentrations in each receptor and obtained dose response agonism and antagonism curves (Figure 5). To fully gauge their receptor selectivity, we compared these activity profiles in RhlR and QscR to those reported by Moore et

al. in LasR using an analogous *E. coli* reporter system.[24] A side-by-side listing of the calculated EC_{50} and IC_{50} values for each compound in each receptor is shown in Table 1.

Table 1: Agonism and antagonism screening data in LasR, QscR, and RhlR for compounds 1–20[a]

	LasR Agonism[b]		**QscR agonism**		**RhlR agonism**		**LasR Antagonism**[b]		**QscR antagonism**		**RhlR antagonism**	
Comp.	EC_{50} (μM)[c]	Act. (%)[d]	EC_{50} (μM)	Act. (%)	EC_{50} (μM)	Act. (%)	IC_{50} (μM)[c]	Inb. (%)[d]	IC_{50} (μM)	Inb. (%)	IC_{50} (μM)	Inb. (%)
1: OdDHL	0.0018 (0.0016 – 0.0021)	100	0.0073 (0.0038 – 0.014)	100	Antag.	–	Ag.[j]	–	Ag.	–	>40	71
2	4.5 (3.0 – 6.7)	95[e]	0.0041 (0.0016 – 0.011)	95	>200	36[i]	0.078 (0.032 – 0.19)	35[e]	Ag	–	Ag.	–
3	>100[f]	30[e]	>10	56[i]	>40	57[i]	10.4 (5.3 – 21)	70[e]	Ag.	–	Ag.	–
4	Antag[g]	–[h]	0.30 (0.13 – 0.70)	70[e]	>40	59[i]	2.8 (1.1 – 6.8)	65	NCPA	27[e]	Ag.	–
5	Antag.	–	1.0 (0.62 – 1.6)	66[i]	Antag.	–	2.8 (1.3 – 6)	65	Ag.	–	32 (3.6 – 290)	49
6	NCPA	–	0.95 (0.61 – 1.5)	72[e]	Antag.	–	1.0 (0.34 – 3.2)	70[e]	NCPA	25[e]	23 (6.0 – 91)	62
7	NCPA	–	>10	55[e]	Antag.	–	3.5 (2.6 – 4.8)	75[e]	NCPA	50[e]	9.3 (3.9 – 22)	87

8	8.4 (4.5 – 16)	90[e]	0.017 (0.0068 – 0.042)	84[i]	>200	49[i]		0.16 (0.043 – 0.57)	45[e]	Ag.	–	Ag.	–
9	0.65 (0.29 – 1.4)	105	0.016 (0.0088 – 0.028)	99	>200	–		Ag.	–	Ag.	–	>200	44
10	33 (23 – 48)	60[e]	0.030 (0.017 – 0.053)	83[h]	Antag.	–		0.49 (0.1 – 23)	40[e]	Ag.	–	–	70
11	0.017 (0.014 – 0.02)	95	94 (14 – 620)	98	Antag.	–		Ag.	–	Ag.	–	–	85
12	0.92 (0.53 – 1.6)	40[i]	>100	31[i]	–	–		Ag.	–	Ag.	–	–	–
13	>100	–[i]	Antag.	–	Antag.	–		4.7 (1.9 – 12)	40[i]	>100	25	>200	25
14	0.096 (0.06 – 0.15)	85[i]	–	–	–	–		Ag.	–	–	–	–	–
15	0.24 (0.16 – 0.35)	90[i]	–	–	–	–		Ag.	–	–	–	–	–
16	0.013 (0.0067 – 0.025)	90[i]	0.066 (0.040 – 0.11)	79[h]	1.4 (1.2 – 1.7)	107		Ag.	–	Ag.	–	Ag.	–

17	–	–	0.62 (0.35 – 1.1)	91	Antag.	–	–	–	Ag.	–	13 (0.97 – 160)	51
18	Antag.	–	–	–	–	–	2.3 (0.89 – 6.1)	50	–	–	–	26
19	0.0078 (0.0047 – 0.013)	100	–	–	–	–	Ag.?	–	–	–	–	–
20	Antag.	–	–	–	–	–	70 (56 – 88)	85	–	–	–	–

[a] See Methods for details of reporter strains and assay protocols; see SI for full dose response curves. [b] LasR data reproduced from Moore et al.[24] [c] Values in parentheses indicate 95% Confidence Intervals (CIs) for the EC_{50} or IC_{50} values. [d] Denotes the largest level of activation or inhibition at any concentration tested, if greater than 25%. [e] Non-classical partial antagonist (NCPA). Denotes largest level of antagonism or agonism; see text and Figure 5. [f] A ">" indicates an estimate of the lower bound of potency, as a full dose-response curve could not be generated over the concentrations tested. [g] "Antag." indicates the compound showed < 25% agonism and was classified an antagonist. [h] Activity is less than 25% or an EC_{50} or IC_{50} could not be calculated. [i] Indicates partial agonist; i.e., a compound that does not have antagonism activity and does not reach 100% agonism.[24] [j] "Ag." indicates the compound showed < 25% antagonism and was classified an agonist.

Our screening of the compound collection in the other LuxR-type receptors in *P. aeruginosa* revealed that certain compounds do not target LasR directly. A notable example was the cyclopentyl AHL analog **17** (C10-CPA). In 2007, Ishida et al. reported that **17** displayed antagonistic activity against RhlR and LasR-driven promoters in a wild-type *P. aeruginosa* strain (PAO1).[28] Moore et al. went on to show that **17** acted as a LasR antagonist in a *P. aeruginosa*

strain (PAO-JP2, *ΔlasI rhlI*) harboring a LasR reporter, yet was inactive in a heterologous *E. coli* LasR reporter system.[24] We hypothesized that this activity trend could be due to **17**'s activity in RhlR, QscR, or potentially another target. Our reporter screening data in the *E. coli* RhlR and QscR reporter systems demonstrated that cyclopentyl analog **17** is a potent and relatively selective QscR agonist, exhibiting only very moderate RhlR antagonism. QscR's known inhibitory effects on LasR and RhlR could explain the mode by which **17** acts as a LasR antagonist in *P. aeruginosa*; that is, **17** agonizes QscR, and QscR, in turn, antagonizes LasR. Thus, **17** acts as an indirect LasR antagonist. Additional studies are required to fully confirm such a pathway, but this result highlights the potential value of exploiting the interegulated nature of the LasR, QscR, and RhlR circuit to obtain a specific outcome in *P. aeruginosa*. Similar to compound **17**, Moore et al. also found that tetrazole **22** could antagonize LasR in *P. aeruginosa* yet was inactive in an *E. coli* reporter for LasR. However, in contrast to **17**, we observed **22** was also inactive in the *E. coli* RhlR and QscR reporters, suggestive that **22** antagonizes LasR via a mechanism that does not include direct interaction with RhlR or QscR.

Consistent with this collection of compounds being originally shown or designed to target LasR, the largest set of receptor selective compounds identified was for LasR. The reporter assays revealed compound **18** (V-06-018), the most potent LasR antagonist in this collection, was highly selective for LasR over both RhlR and QscR. The weaker LasR antagonist, triphenyl (TP) derivative **20** (TP-5), was also found to be a highly selective for LasR over the other two receptors. The lack of activity for compounds **18** and **20** in QscR is perhaps the most notable, as QscR and LasR are maximally *activated* by the same natural ligand (OdDHL), strongly suggesting that the modes by which **18** and **20** inhibit LasR are not operative in QscR. We also found that the strongest LasR agonist in this collection, the other TP derivative (**19**; TP-1P)

displayed no appreciable activity in either RhlR or QscR in the *E. coli* reporter assays, which correlates with earlier reports of Greenberg and co-workers.[29] While **19** and **20** are to our knowledge the only TP-type analogs to be examined in QscR or RhlR , the apparent LasR selectivity, efficacy, and potency of this class of compounds further underscores their potential value as probe compounds. Indeed, this scaffold has been further examined recently by others,[29-30] and it will be interesting to learn if LasR selectivity is maintained in other TP-type analogs.

Apart from the phenyl OdDHL analog **12** and thiolactone **16** (mBTL), all of the non-LasR selective compounds were found to agonize QscR to some degree (**2**, **8**, **9**, and **10** being the most potent; Table 1). No strong QscR antagonists were identified in this study, underscoring again that, despite sharing OdDHL as their preferred/native ligand, the mechanisms of small molecule-mediated agonism and antagonism are different for LasR and QscR,[31-32] and that QscR is considerably more promiscuous in terms of agonist-type ligands relative to LasR. In turn, as OdDHL is known to be a RhlR antagonist,[33] results that repeat in this study, it makes sense that we find a relatively large number of RhlR antagonists in the collection (**5**, **6**, **7**, and **17** being the most potent, Table 1).

Interestingly, we discovered two compounds (3-oxo-aryl HL **5** and aryl HL **6**) that target all three of the LuxR-type receptors but do so differently. These compounds are antagonists of both RhlR and LasR, while simultaneously acting as QscR agonists. We term such complex behavior as "combinatorial antagonism." Compounds **5** and **6** have similar structures,[34] varying only in the oxidation state at carbon 3 of the acyl tail, and with the exception of slight non-monotonic behavior of **6** in LasR and QscR at high concentrations (i.e., displaying antagonism at low concentrations and agonism at high concentrations), they have nearly identical efficacies and potencies overall. We reason that a compound with this activity profile could exhibit a higher

level of global QS antagonism in *P. aeruginosa* than a compound that targets a single receptor alone, due to its combined effects (i.e., agonizing QscR antagonizes LasR, antagonizing LasR antagonizes RhlR, etc.). Testing this hypothesis in wild-type *P. aeruginosa* and in infection models is ongoing and could further illuminate the interconnectedness of the three receptors and its role at different timepoints.

Our screening also revealed that one compound, bromo thiolactone **16** (mBTL), was actually an agonist of all three receptors, with potencies and efficacies in RhlR and LasR comparable to that of each receptor's native ligand. In QscR, **16** has lower efficacy and is an order of magnitude less potent than OdDHL yet is still among the strongest QscR agonists identified in this study. Small molecules with broad LuxR-type receptor *agonism* are rare; it will be interesting to explore the activity of **16** in a wider swath of LuxR-type receptors to further examine its level of promiscuity. The activity profile of **16** in *P. aeruginosa* is distinct from that of **5** and **6**, setting up a potential conflict between the receptors in their control of each other (i.e., agonizing QscR should antagonize LasR (and thus also RhlR), but then this ligand also agonizes LasR and RhlR). Compound **16** thus provides a novel chemical strategy to examine the hierarchy of the LuxR-type receptor triumvirate in *P. aeruginosa*.

To examine the larger trends in activity of these compounds across the three receptors, we plotted their activity profiles in the reporter assays using a heat map (**Figure 3**). This heat map makes it quite easy to identify receptor-selective and efficacious compounds. Again, given that this compound set was designed to target LasR, it is unsurprising that nearly all of the compounds target LasR in some way, except for **17** and **22**. The heat map representation also nicely reveals "combinatorial antagonists"; aryl HLs **5**, as previously discussed. The non-classical partial agonism profile of **6** and **7** in LasR and QscR (antagonizing at low concentration

yet agonizing at high, the former trend obvious in the heat map) limits its utility as a probe molecule relative to **5** (see Table 1 and Figures 4 and 5).

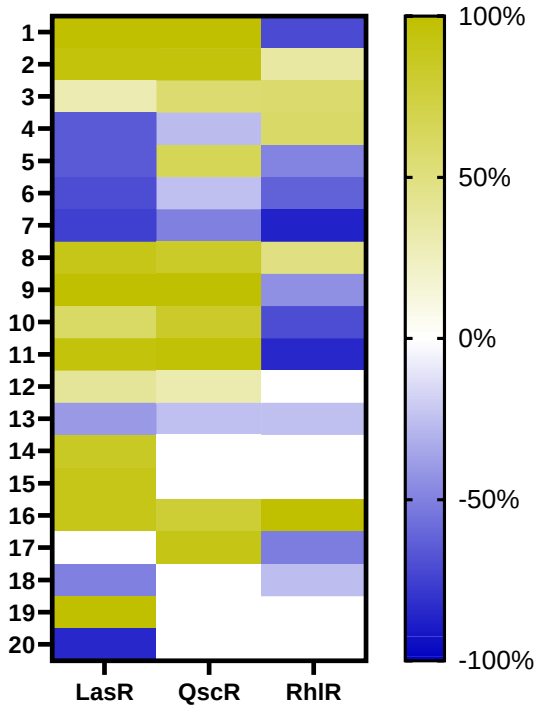


Figure 3: Heatmap of agonism and antagonism in LasR, RhlR, and QscR. Yellow (positive numbers) represents percent agonism, while blue (negative numbers) represents percent antagonism. A white cell indicates neither antagonism nor agonism. Compound **22** omitted as inactive in all three receptors. Compounds with non-monotonic activity profiles are shown as antagonists only, compounds with partial agonism activity are shown as agonists only.

In summary, we report investigations into the activity of a set of compounds for modulation of two LuxR-type receptors *in P. aeruginosa*, RhlR and QscR, and juxtapose these activity profiles to that observed in LasR. Receptor selective compounds were identified for LasR, along with a set of compounds that displayed novel combinatorial activity profiles by agonizing or antagonizing each of the receptors. Both sets of compounds can be applied to ask different and important questions into the role of each LuxR-type receptor in controlling QS and virulence in *P. aeruginosa*. Compound **5** could be used to block virulence pathways more

extensively than using a receptor selective specific antagonist. This strategy would have advantages over having to use multiple compounds or make multiple genetic knockouts, or if it is unknown which circuit controls a specific virulence factor. We are interested to learn if such compound activity profiles can be identified in other LuxR/LuxI-type systems, and if the potency and selectivity of the compounds reported herein in can be increased. In addition, selectivity is of interest to us and the broader field, especially when it comes to understanding the origin of that selectivity. QscR and LasR's differences could help us in determining these factors, given their similarities. In this vein, compounds **18**, **19**, and **20** could give us new insights into compound design; indeed, our laboratory just completed a systematic structure-activity relationship study of **18** and steps have been made by us and others to improve the profiles of **19** and **20**.[29, 35-36] We have also identified the cause of LasR inhibition by **17,** a discovery that highlights the importance of screening compounds in *E. coli* in addition to the native organism. Together, this study highlights a suite of compounds with unique activity profiles and underscores the importance of fully understanding the spectrum of compound activity when seeking to use them as tools in bacteria or mixed systems harboring multiple LuxR-type receptors. [37]

Acknowledgments

Financial support for this work was provided by the NIH (R35 GM131817). K. Nyffeler was supported in part by the UW−Madison NIH Chemistry−Biology Interface Training Program (T32 GM008505) and as part of a seed award from the UW–Madison NSF MRSEC (DMR-1720415).

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Supplemental Information

Table 2. Bacterial strains and plasmids used in this study.

Strains	**Properties**	**Ref**
Escherichia coli JLD271	K-12 *ΔlacX74 sdiA271::*Cam	[1]
Plasmids		
pJN105R2	arabinose-inducible RhlR expression vector	[2]
pJN105Q	arabinose-inducible QscR expression vector	[3]
pSC11-*rhlI*	*rhlI'-lacZ* transcriptional fusion	[2]
pSC11-*PA1897*	*PA1897'-lacZ* transcriptional fusion	[4]

METHODS

General. Compounds were purchased (Sigma-Aldrich) or synthesized via previously reported methods.[5-14] Stock solutions of compounds were prepared in DMSO and stored at -78 °C. Biological reagents and media were purchased from Goldbio, RPI, or Sigma-Aldrich and used according to enclosed instructions.

β-galactosidase reporter assay protocol. Full strain information (with references) in Table 2. Assays were performed as in previous studies.[2, 4, 15] A single colony of *E. coli* JLD271 was grown overnight in Luria-Bertani (LB) medium with 100 µg/mL Ampicillin and 10 µg/mL Gentamicin. For QscR reporter assays, the bacteria harbored pJN105Q and pSC11-*PA1897*,

while RhlR reporter assays harbored pJN105R2 and pSC11-*rhlI**. From that culture, a 1:10 dilution was made into LB supplemented with 100 µg/mL Ampicillin and 10 µg/mL Gentamicin and grown to an OD_{600} = 0.25. Arabinose (4 mg/mL) was added to induce protein production, and in an antagonism assay, native ligand (either OdDHL or BHL) at its EC_{50} was added. For QscR, the EC50 used was 10 nM, and RhlR EC50 was 10 µM. Aliquots (198 µL) of this culture was added to the well of a 96-well plate followed by 2 µL compound stock solution. For QscR, dose response curves were performed at a final compound concentration of 100 µM, and 10-fold compound dilutions. For RhlR assays, compound dose response curves were performed at a final concentration of 200 µM, with 5-fold compound dilutions (except for BHL, which was used at a concentration of 1 mM). DMSO final concentration kept below 2%. Positive agonism controls: 100 µM OdDHL for QscR, 1mM BHL for RhlR. Positive antagonism controls: DMSO, with EC50 of native ligand added to culture. Negative agonism and antagonism controls: DMSO. Plates were incubated at 37°C for 4 hours, and OD_{600} read. To a chem-resistant plate, 204 µL of Z-buffer and 8 µL of chloroform were mixed with 50 µL of culture. 150 µL of the aqueous layer was transferred to a clear plate and added to 25 µL of 4 mg/mL CPRG. Plates were then incubated for 45-60 min (for QscR) or 20 min (for RhlR), at 30°C and then absorbance (A_560 or A_570) was read. A Synergy 2 plate reader using Gen5 (v1.05) analysis software (Biotek) or a Envision 2105 Multimode plate reader using EnVision Manager (v1.14) analysis software (EnVision) was used for absorbance measurements. Prism 6 or 7 (GraphPad) was used for making graphs and calculating EC50 and IC50s using a three-parameter (QscR) or four-parameter fit (RhlR). Technical replicates were performed and the average graphed with error bars representing SEM.

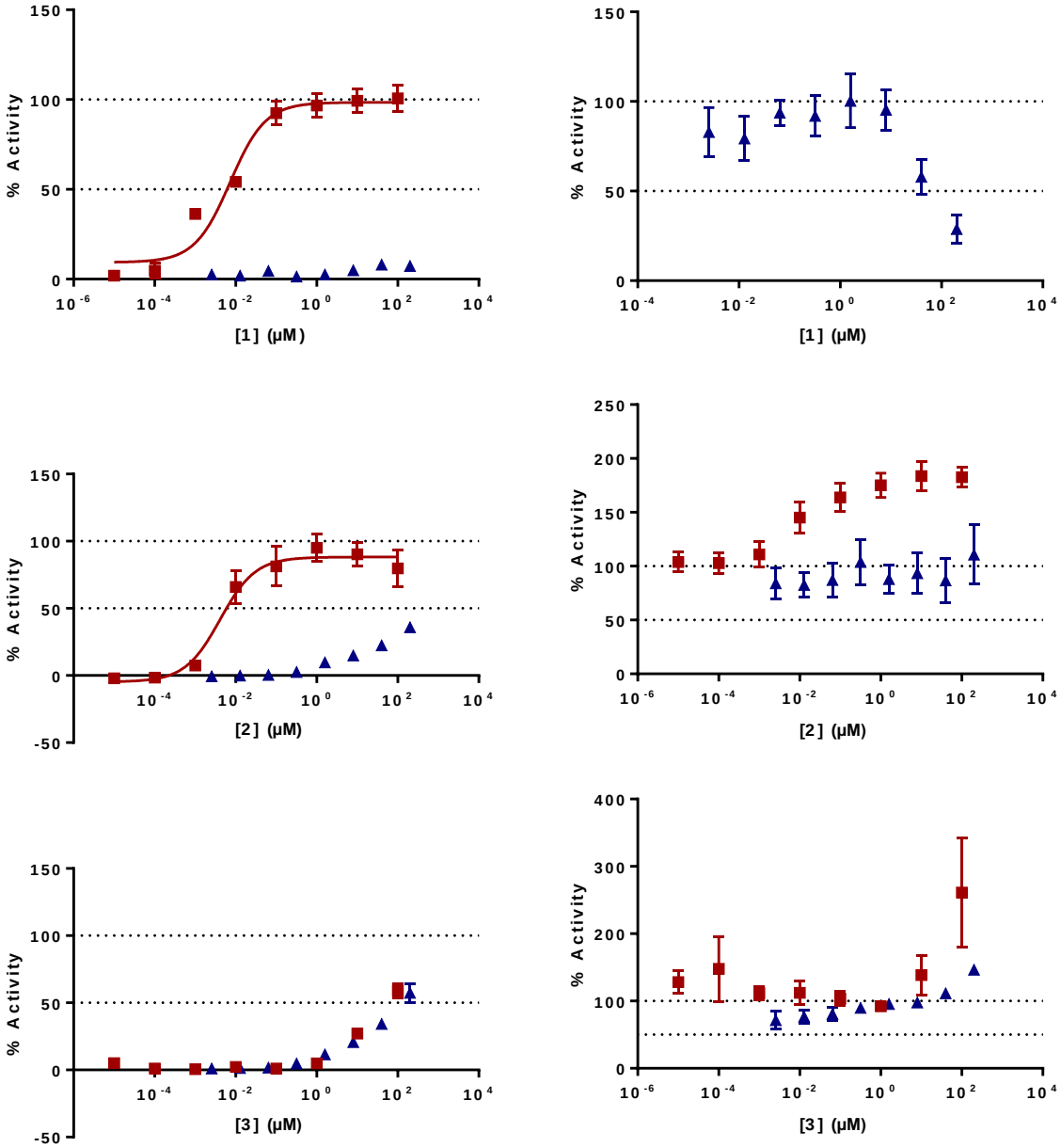
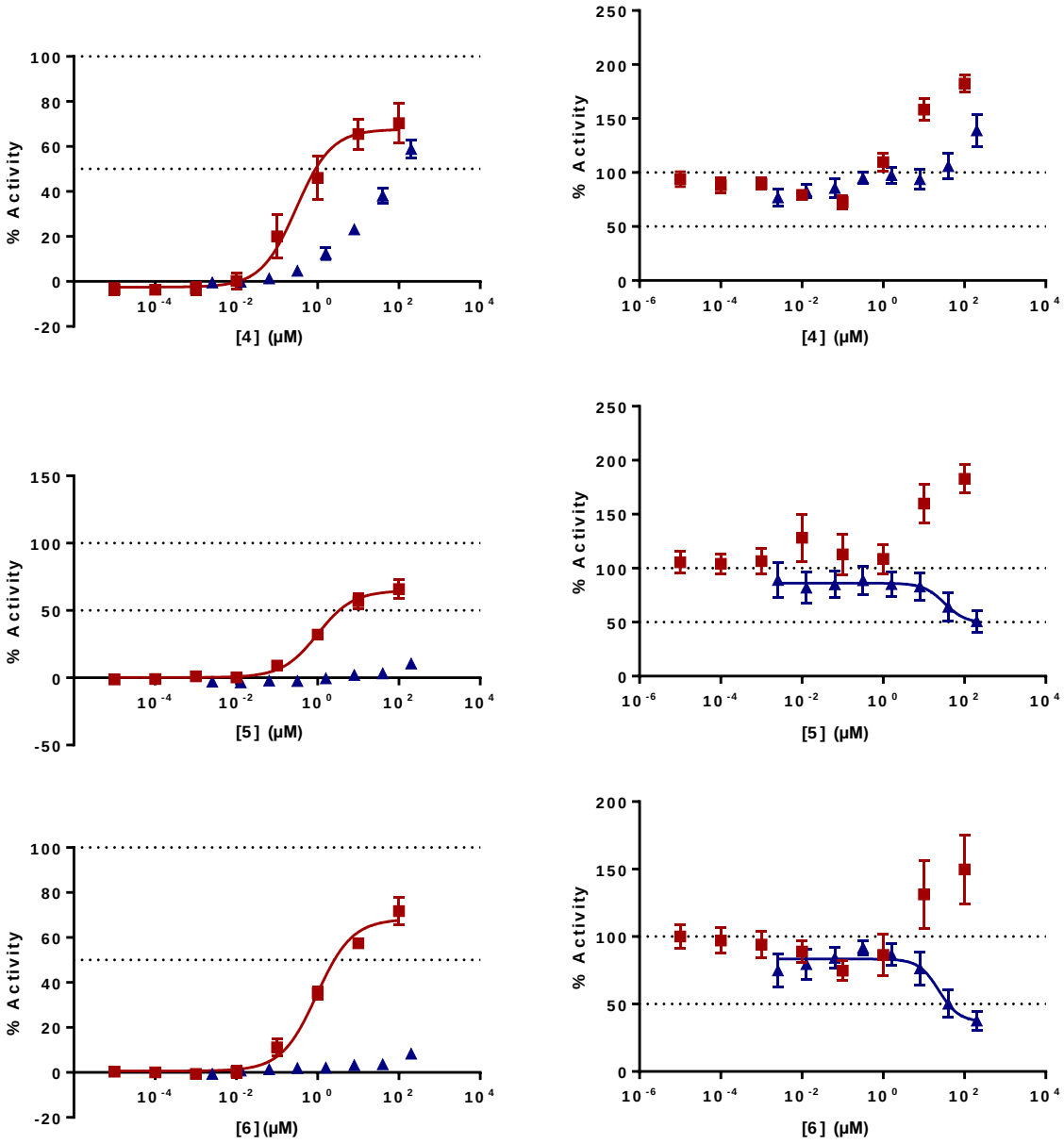


Figure 4. Dose-response agonism and antagonism curves in the *E. coli* QscR and RhlR reporters for all compounds in this study. QscR (red squares) and RhlR (blue triangles) agonism curves are shown on the left; antagonism curves are shown on the right. Compounds were screened for antagonism against 10 nM OdDHL (QscR) or 10 μM BHL (RhlR). See biological assay protocols for details of methods. Compound **1** is OdDHL. Error bars represent the SEM of ≥3 trials. Curves were fit only if an EC50 could be generated and Prism was able to generate a curve.



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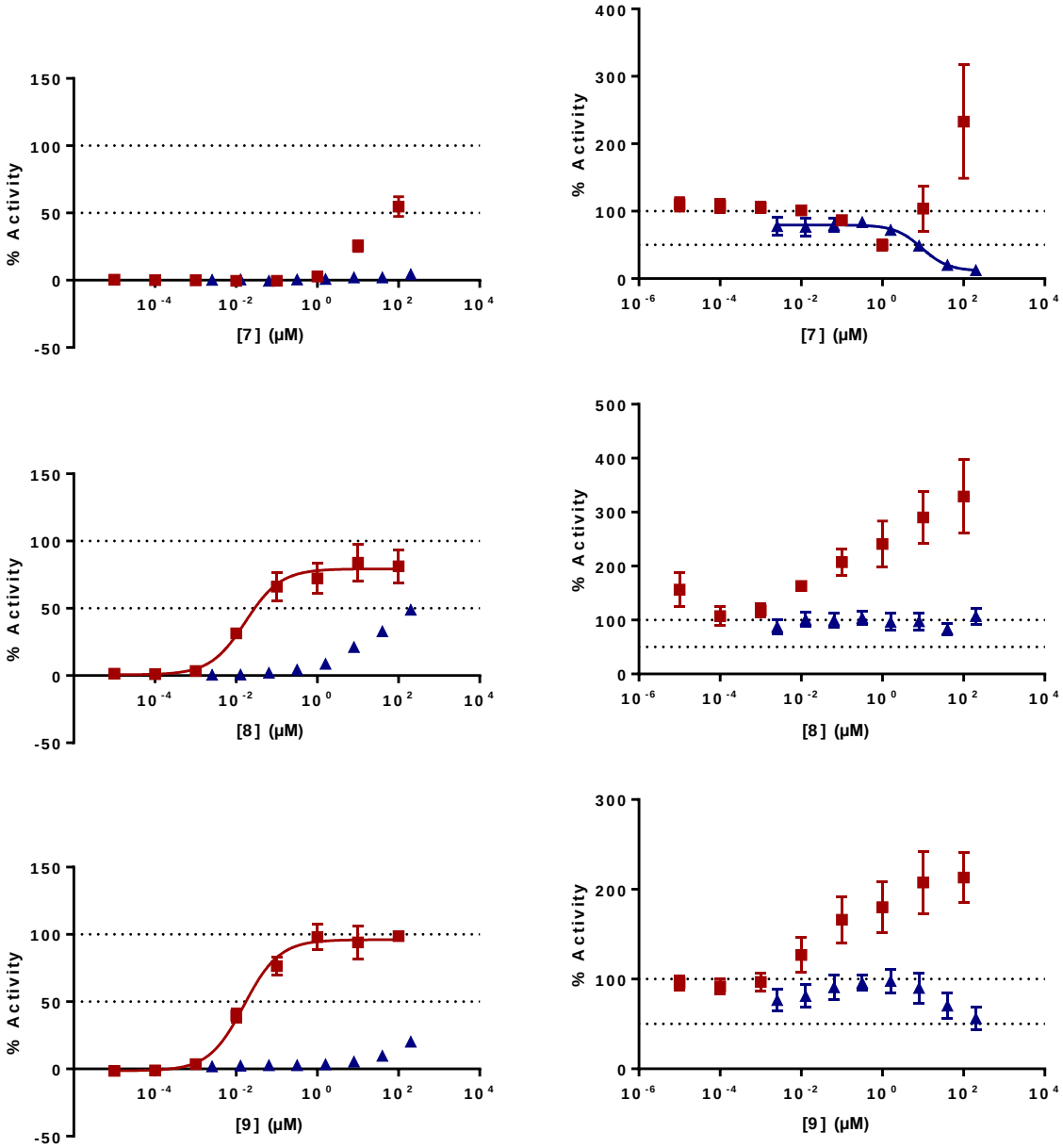

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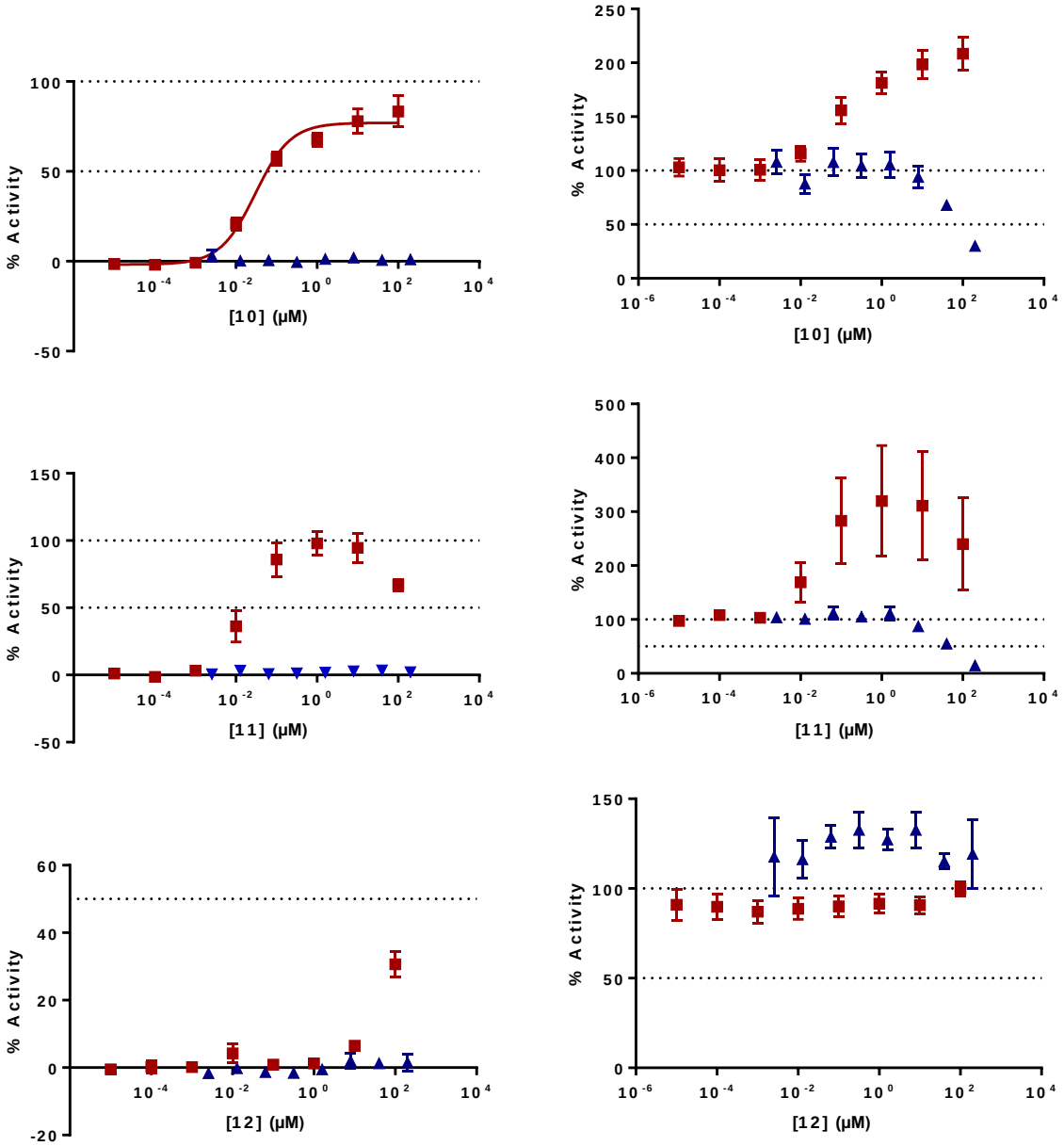

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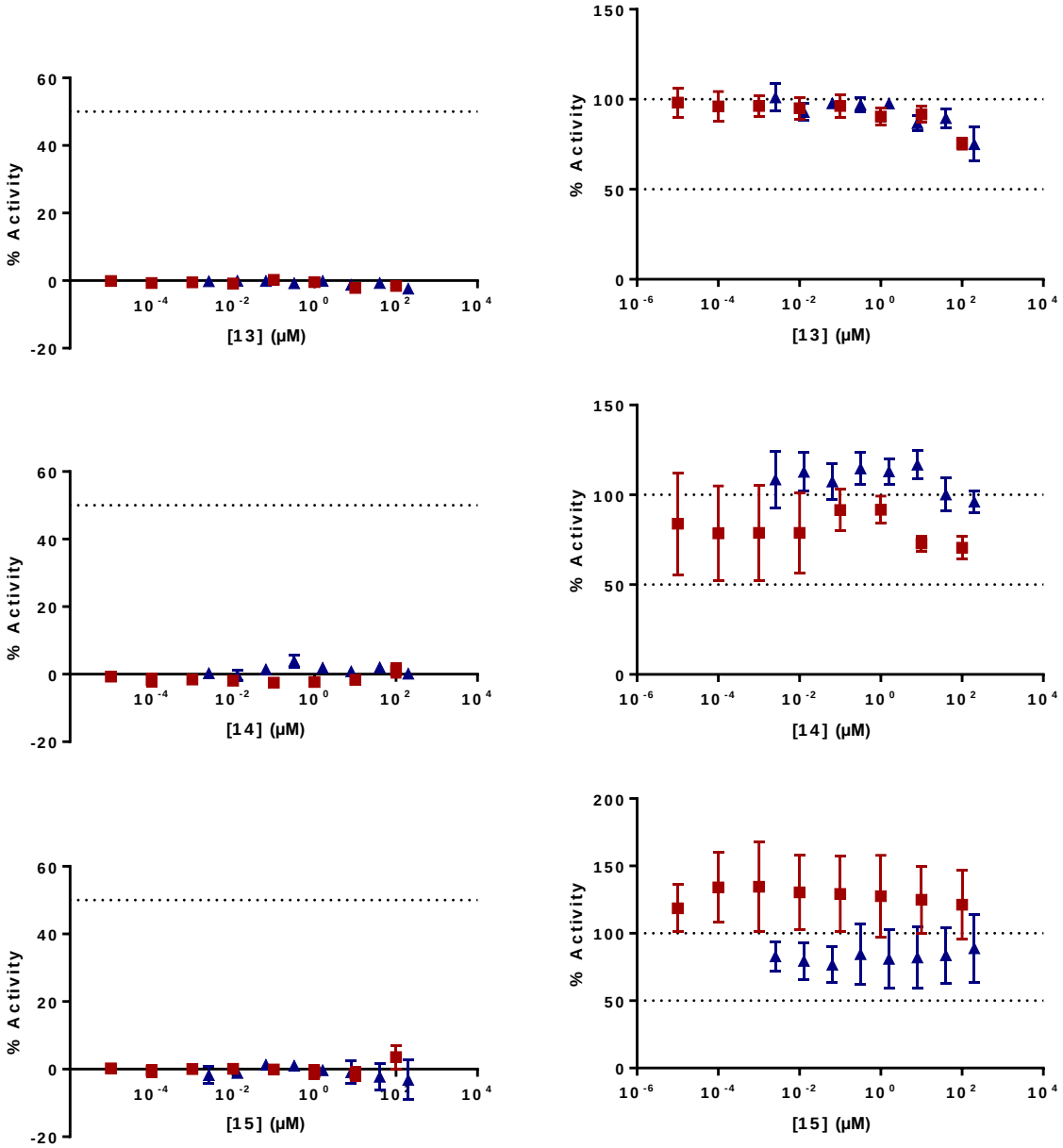

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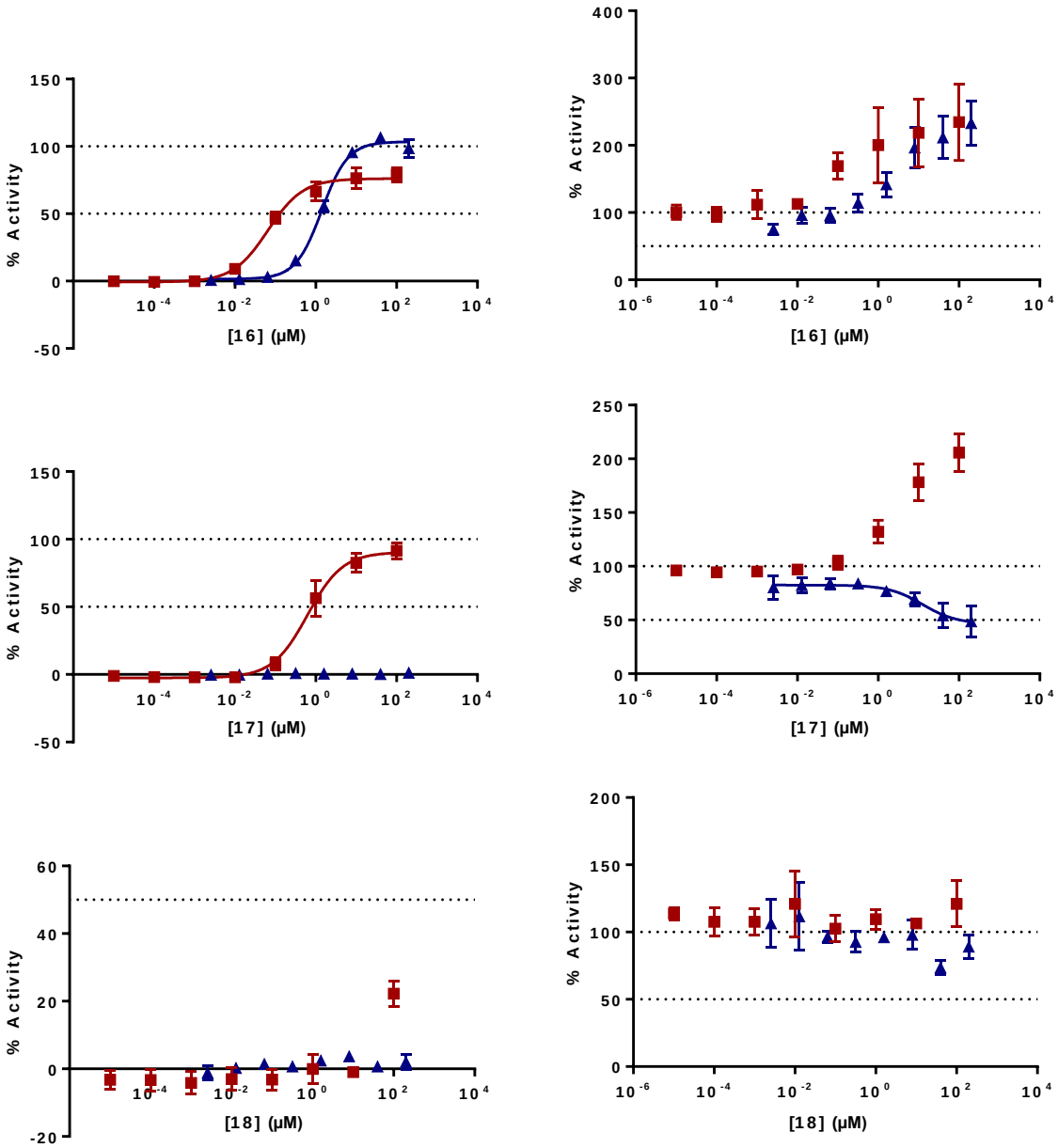

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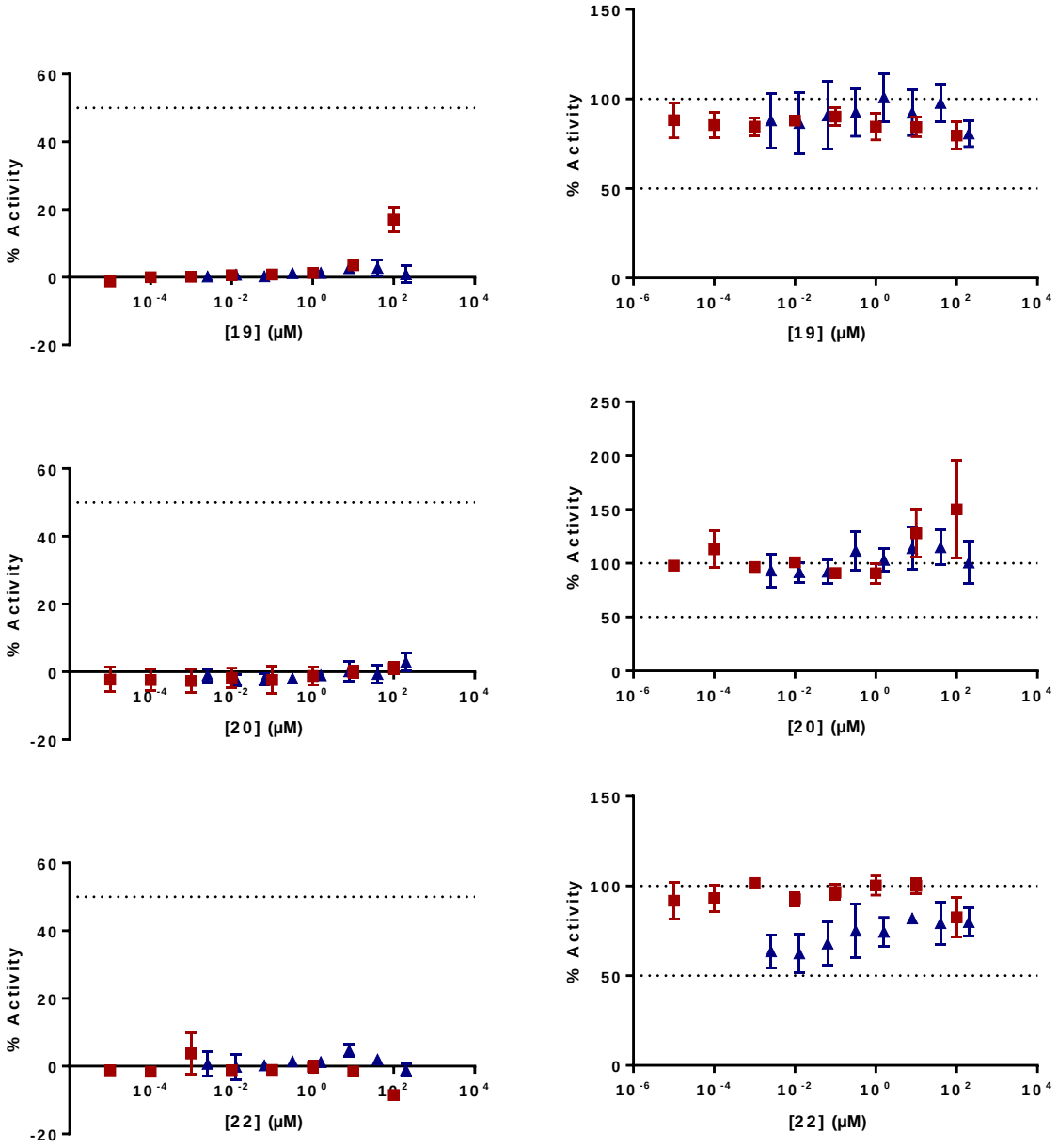

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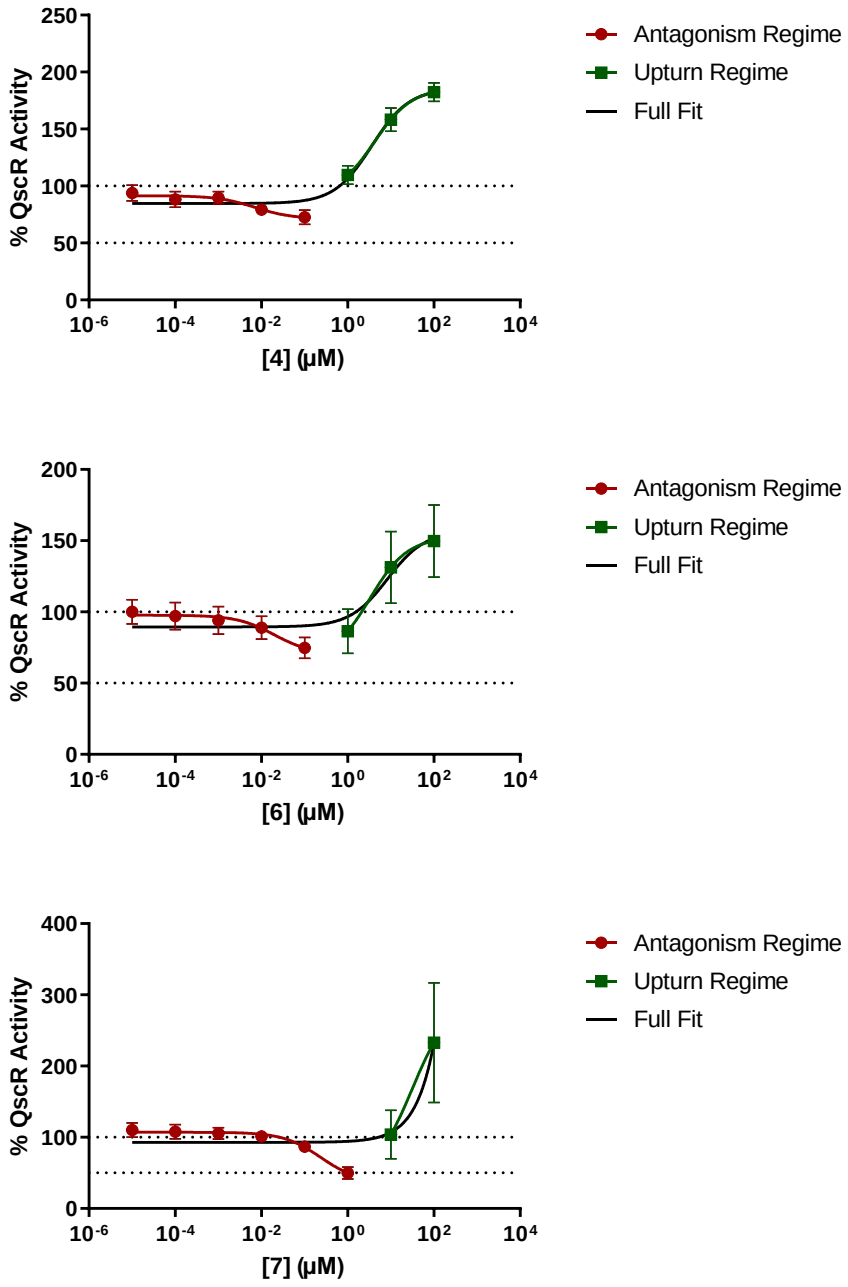

Figure 5. Dose-response curves for non-classical partial agonists **4**, **6**, and **7** in QscR. These data are from antagonism assays in the *E. coli* QscR reporter against 10 nM OdDHL. "Full fit" (black) represents a 3-parameter fit of the full antagonism dose-response curve with GraphPad Prism's 3-parameter fit; "Antagonism regime" (red circles) is the concentrations range over which the compound either has no effect or antagonizes QscR; "Upturn regime" (green squares) is the concentrations range over which the compound agonizes QscR. See biological assay protocols for details of methods. Error bars represent the SEM of ≥3 trials.

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CHAPTER THREE: A Vesicle Lysis Test for the Facile Identification of Synthetic Modulators of Quorum Sensing and Biosurfactant Production in Bacteria

Kayleigh E. Nyffeler, Tom J. Polaske, Curran G. Gahan, Korbin H.J. West, David M. Lynn, and Helen E. Blackwell

To be submitted to *ACS Infectious Diseases*

Contributions: K. E. Nyffeler and T.J. Polaske designed and performed experiments, C.G. Gahan assisted with experiments and formulated and prepared vesicles, and K.H.J. West assisted with HPLC and identification of δ-toxin. K. E. Nyffeler, T.J. Polaske, D.M. Lynn, and H. E. Blackwell wrote the text.

Abstract

Quorum sensing (QS) allows bacteria to assess their local cell density using chemical signals and activate group behaviors once they reach a threshold cell number. Many common pathogens use QS to control the production of a plethora of virulence factors, including surfactants. Chemical mimics of the native QS signals are valuable as research tools to explore the many unanswered questions about the mechanism of QS and could provide a pathway to potential anti-virulence approaches. However, robust methods for the rapid identification of non-native QS modulators (QSMs) are scarce. We reasoned that an assay for the presence of QS-controlled surfactants could be repurposed for the straightforward identification of QSMs. Herein, we applied the vesicle lysis test (VLT)—an assay that monitors the disruption of large unilamellar vesicles (LUVs) via fluorescence – to screen for QSMs. We generated robust calcein-loaded LUVs and showed that calcein release was QS-dependent and coincided with QS onset in both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We then demonstrated that the VLT can be used to identify known QSMs in both pathogens and demonstrate in *S. aureus* that this assay allows for quantitative measures of QSM activity. Lastly, we use the VLT to perform a high-throughput screen of a commercial small molecule library and discovered new small molecule QSMs. This study demonstrates a high-throughput and straightforward method of screening for QSMs via the detection of bacterial virulence factors.

Introduction

Many common bacteria utilize an intercellular signaling network to assess their population density and coordinate gene expression at high cell number to regulate group behaviors. This phenomenon is called quorum sensing (QS). QS is mediated by small molecule or peptide signals and allows bacteria to coordinate many activities that are deleterious to their hosts or the environment, including the production of sessile, drug-impervious biofilms and virulence factors that play roles in deadly infections. For example, *Staphylococcus aureus* is one of many pathogens that uses a cyclic peptide-based QS system to produce toxins and cause disease.[1] In turn, QS can also regulate a collection of symbiotic behaviors that benefit their host, such as the plant-associated bacteria *Rhizobium leguminosarum* and *Sinorhizobium meliloti* that utilize an acyl homoserine lactone (AHL)-based QS system to assist in root nodulation and nitrogen fixation.[2] In either symbiosis or pathogenesis, QS may control up to 10% of a bacterium's genome and have wide-ranging effects on its lifestyle and community-based behavior. There is significant interest in developing strategies to intercept this cell-cell signaling pathway as an approach to either mitigate or amplify certain bacterial behaviors, and also more generally, to ask basic mechanistic questions about QS and its role in a range of health, industrial, and environmental contexts.

The general QS mechanism involves bacteria producing their cognate QS signal, or autoinducer, at a basal level at low cell density (Figure 1).[3-5] This signal can be either passively or actively transported out of the cell into the bacterium's local environment and can diffuse into other cells. The concentration of signal will increase with cell number, and once a threshold concentration is achieved (inside or outside of the cell, dependent on pathway), the autoinducer will bind to its cognate intracellular or extracellular receptor. This ligand:receptor binding event

will signal the bacteria to alter gene expression levels to support community-based bacterial behaviors, including toxin production, biofilm growth, swarming, etc. As QS is dependent on the production and sensing of relatively simple chemical signals, considerable research has focused on the development of non-native molecules and other chemical strategies to either block or activate QS pathways.[6-8] Our group [9-15] and many others [16-20] have designed and synthesized a range of quorum sensing modulators (QSMs) that mimic the structures of the native QS signals. These compounds represent valuable tools to study QS, but many have limitations in terms of their potency, selectivity, and solubility and stability in biological media. For these reasons and others, the identification of alternate chemical scaffolds that are active as QSMs is of significant interest. New, high-throughput assay methods could find use in the discovery of such compounds, and the development of such assays was a motivation for the current study.

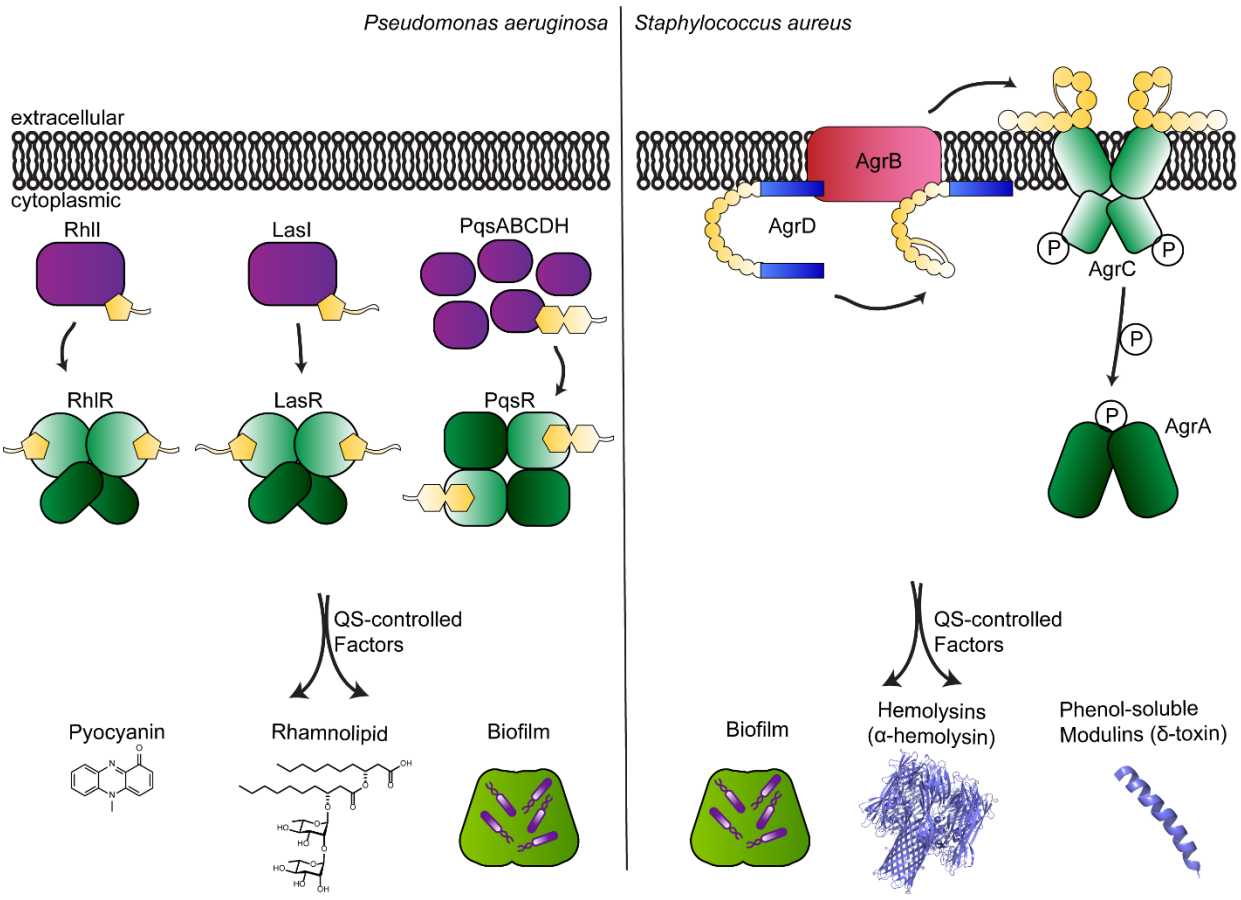

Figure 1. Schematic of the QS process in bacteria. *P. aeruginosa* is a Gram-negative bacterium (left) using LuxR-type and LysR-type receptors (green), while *S. aureus* uses the *agr* system (right). In *P. aeruginosa*, the signals (yellow) are produced by LuxI-type synthases (LasI and RhlI) and a suite of proteins for PQS (Pseudomonas quinolone signal, PqsABCDH, purple). They bind to the signal binding domains of the receptors (RhlR, LasR, and PqsR, light green), which then allow for productive binding of the DNA binding domains to DNA (dark green) and the production of a variety of factors, including pyocyanin, rhamnolipid, and biofilm. *S. aureus* AgrD is a precursor to the QS signal, made up of the final signal (yellow) and portions that get removed (blue). It is processed by AgrB (red), which assists in the cyclization, post-translational modification, and export of the peptide. It then binds to the histidine kinase AgrC (light green), which transduces the signal to the response regulator AgrA (dark green). QS controls factors including biofilm, hemolysins, and phenol-soluble modulins. Hemolysin PDBID 3ANZ, δ-toxin 2KAM.

One of the most common methods utilized to test for QS agonism or antagonism is the use of a cell-based reporter gene assay. In this assay, a transcriptional binding site for a known QS transcription factor is fused to a reporter gene. When the activated QS transcription factor

binds its cognate promoter, a detectable signal will be produced via transcription of the reporter gene (most commonly encoding green fluorescent protein (GFP) or β-galactosidase (β-gal)) that can be either quantitated directly or indirectly), allowing for QS activity to be measured. Depending on the signal output, reporter gene assays can be performed readily in a high-throughput format. However, this method involves genetic modification, which may not always be possible in certain bacteria. These assays are also commonly performed with the assumption that expression of the QS gene is directly correlated with a specific phenotype, but this is not always the case, with many bacteria controlling virulence (or other phenotypes) by additional and often indirect pathways.

A second common method for measuring QS activity active is to perform an assay that directly quantifies the production of QS-controlled factors, often called a "phenotypic" QS assay. For example, the soil bacterium *Chromobacterium violaceum* produces the bis-indole pigment violacein, which can be easily detected colometrically via its intense purple color.[21] Indeed, *C. violaceum* is often used as a "biosensor" strain for native QS signals as this color change is visible to the eye. Similarly, *Pseudomonas aeruginosa* uses QS to control the production of the blue secondary metabolite pyocyanin, which can be detected directly by HPLC, spectrophotometry, and electrochemical methods.[22-25] Biofilm production is often examined as a QS phenotype and can be quantitated using a range of spectrometric techniques,[26] but these methods suffer significantly from high error rates due to the extreme sensitivity of biofilm growth on environmental factors and are challenging to miniaturize. Semi-direct detection of QS-controlled virulence factors can also be performed. For example, *Staphylococcus aureus* produces hemolysin under the control of QS, and lysis of erythrocytes can be measured readily on a blood agar plate or in cultures as a gauge of hemolysin activity.[27] Assays of other QS-

controlled exoenzymes have also been developed for specific systems (e.g., elastase B in *P. aeruginosa* or cellulase in *Listeria monocytogenes*).[22, 28] Lastly, QS-controlled factors can be detected using molecular biology techniques, including qPCR and Western blots, although these experiments require an understanding of QS in the organism of interest at the genetic level. Overall, except for the organisms that generate known products that are straightforward to detect spectrophotometrically,[21, 29] these phenotypic assays are not used in a high-throughput, or even medium-throughput, manner.

A robust assay to test for QS activity that combines the high-throughput nature of the reporter gene assays with the benefits of directly measuring a known QS-controlled factor would be of broad utility for the identification of new QSMs. This paucity of rapid-throughout assays was underscored by Quave and Horswill in their 2014 review article on QS in *S. aureus* (but broadly applicable to the QS field in general),[5] and has not been directly addressed to date. One assay that attracted our attention as a potential assay for QSMs for further development, and also noted by Quave and Horswill, was the "vesicle lysis test".

The vesicle lysis test (VLT) was reported for bacteria in a study by the Jenkins laboratory in 2010,[30] in which they demonstrated that cargo encapsulated within a phospholipid vesicle could be selectively released only upon the growth of a bacterial strain that produces surfactants capable of vesicle cleavage. The ~100 nm vesicles were shown to be straightforward to prepare on scale with commercial phospholipids; different combinations of lipids could be used to heighten sensitivity to certain bacteria.[31] The encapsulated cargo in the vesicles was typically an antimicrobial agent or a self-quenching fluorescent dye, such as carboxyfluorescein or calcein.[32-33] Zhou et al. originally demonstrated that the vesicle lysis test was able to differentiate between the two pathogens (*S. aureus* and *P. aeruginosa*) that produce surfactants and a non-pathogenic

E. coli strain that does not produce surfactant, only releasing toxic sodium azide from the vesicles when the surfactant was present.[30] In a subsequent study from the Otto laboratory, Duong et al. mixed carboxyfluorescein-loaded phospholipid vesicles with a variety of cell-free *S. aureus* supernatants.[34] Upon release and dilution of the dye, an easily detectable fluorescence signal was observed. Duong et al. went on to show that the causative agent of vesicle lysis was a class of amphipathic peptides known as phenol-soluble modulins (PSMs) produced by *S. aureus* (and other Staphylococci) under the control of QS.[34] This finding was further corroborated by the Jenkins lab in 2014 with Laabei et al. demonstrating that a *S. aureus* QS mutant strain (QS-) failed to lyse their vesicles.[35] In a similar study, the Jenkins lab also identified the cause of vesicle lysis by *P. aeruginosa* cultures was rhamnolipid, a surfactant produced under QS control in this pathogen.[32] In view of the relative ease of both preparing the vesicles and performing the VLT assay, and the relatively large number of bacteria that are either known or suspected to produce surfactants under the control of QS, we reasoned that the VLT could provide a robust and potentially high-throughput assay for the identification of new QSMs.

Herein, we describe the use of the VLT for the discovery of bacterial QSMs. We began by optimizing the assay for the detection of quorate populations of both *S. aureus* and *P. aeruginosa*. We demonstrate that the timing of vesicle lysis by the bacteria correlates with the onset of gene transcription in a corresponding QS reporter strain. Thereafter, we show that vesicle lysis can be modulated by known chemical activators and inhibitors of QS. We also outline biochemical and genetic studies to determine the cause or pathway responsible for vesicle lysis in each pathogen. Lastly, we transition the assay to a high-throughput format in a 384-microwell plate and screen a commercial small molecule library to identify several novel inhibitors of *S. aureus* QS. Overall, this study demonstrates a robust, high-throughput assay to

screen for biosurfactant production in bacteria—a phenotype commonly under the control of QS—and thereby to identify QSMs and could find use in a broad range of applications.

Results and Discussion

Generation of calcein-loaded vesicles. As highlighted above, Jenkins and coworkers demonstrated that vesicle formulations could be produced that responded to the presence of biosurfactants in bacterial cultures.[36-41] This was accomplished using combinations of saturated lipids with cholesterol and a photopolymerizable alkyne-containing fatty acid (10, 12-tricosadiynoic acid) that can be crosslinked under UV light.[36-39] We sought to develop simpler vesicle-based systems that were still able to differentiate between wild-type (WT) and QS mutants of *S. aureus* and *P. aeruginosa*, yet were less technically challenging to produce than those described by Jenkins. Our goal was to make the VLT more accessible to groups unfamiliar with the fabrication of liposomes. For this reason, we decided to forgo the use of photopolymerizable lipids as they require an additional processing step. Our optimized vesicle formulation for *S. aureus* was composed of 70% DOPC and 30% cholesterol (mol%), whereas our optimized formulation for *P. aeruginosa* was composed of 68% DPPC, 2% DPPE, and 30% cholesterol (mol%) (see Methods).

For both formulations, calcein-loaded large unilamellar vesicles (LUVs) were produced using a modification of the thin film hydration method.[42-43] Dried phospholipid films were rehydrated in 70 mM calcein solution. The resulting vesicle suspension was frozen and thawed 5 times and then passed through a 100 nm polycarbonate filter. The vesicles were then separated from the unencapsulated calcein by size exclusion chromatography, and the concentration of phospholipids in solution was determined by ^{31}P NMR. This procedure resulted in vesicles that

were approximately 150 nm in diameter as determined by dynamic light scattering, which was similar to vesicle sizes generated using this method in previous reports (**Figure 13**).[44-46] Using these methods, we prepared ~4 mL aliquots of vesicles that were used over multiple experiments and stored for no longer than a week.

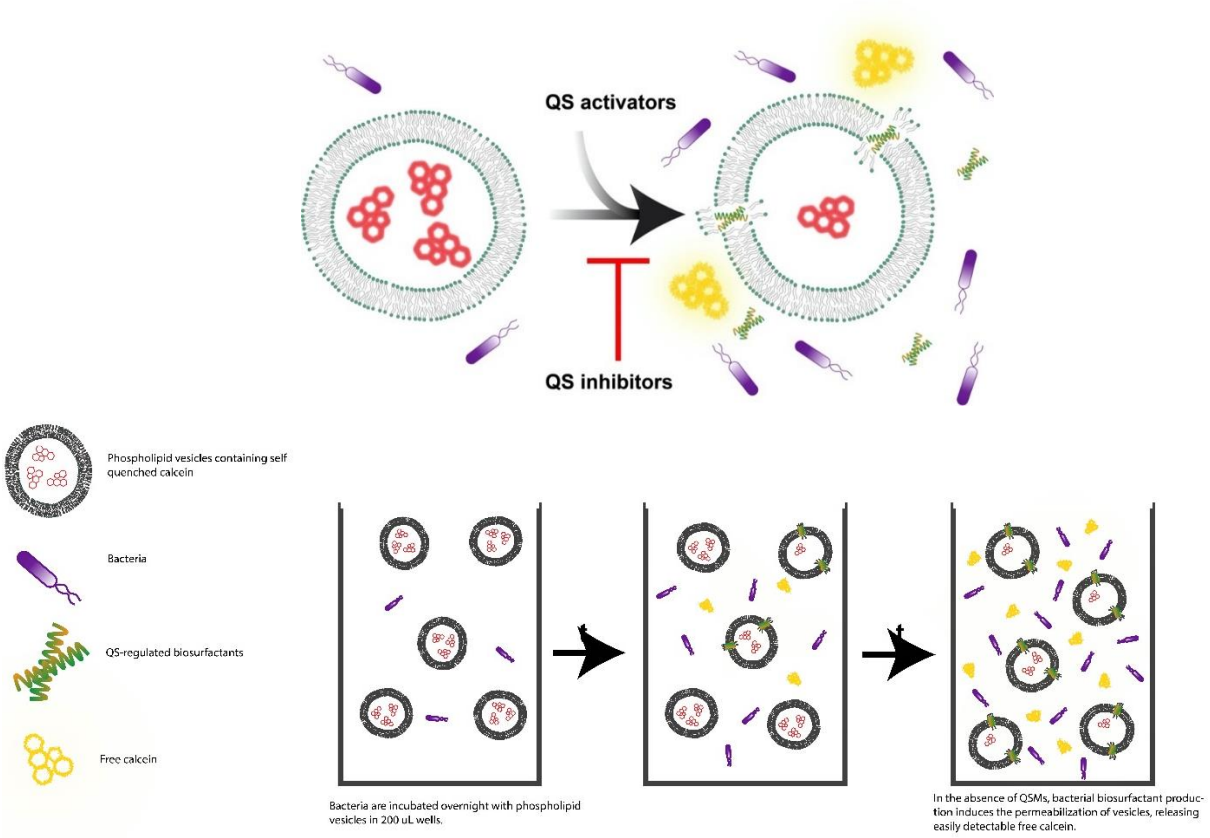

Figure 2. Schematic of the vesicle lysis test.

Detection of quorate bacterial populations with vesicle lysis test. With the two classes of vesicles in hand, we first sought to reproduce the results of the Jenkins group and others[34] and demonstrate that vesicle lysis occurs due to the production of biosurfactants by bacteria. We incubated the two classes of calcein-loaded vesicles with either WT or QS- strains of *S. aureus*

or *P. aeruginosa* (see Methods and Table 1 for details of strains). The *S. aureus* QS mutant lacks the AgrD and AgrB components of its agr-type QS system (Figure 1) and is unable to produce its native autoinducer peptide (AIP) signal. Similarly, the *P. aeruginosa* QS mutant lacks its enzymatic machinery for AHL QS signal production (i.e., LasI and RhlI; Figure 1). As seen in Figures 3A and 3B, both of the WT strains are able to lyse the vesicles and there is a distinct difference in the fluorescence signals produced by both the QS- strains, indicating that the presence of the QS system was a cause for vesicle lysis in both strains. No effect on cell growth was observed in either strain over the time course of the assay (12 h, Figure 9).

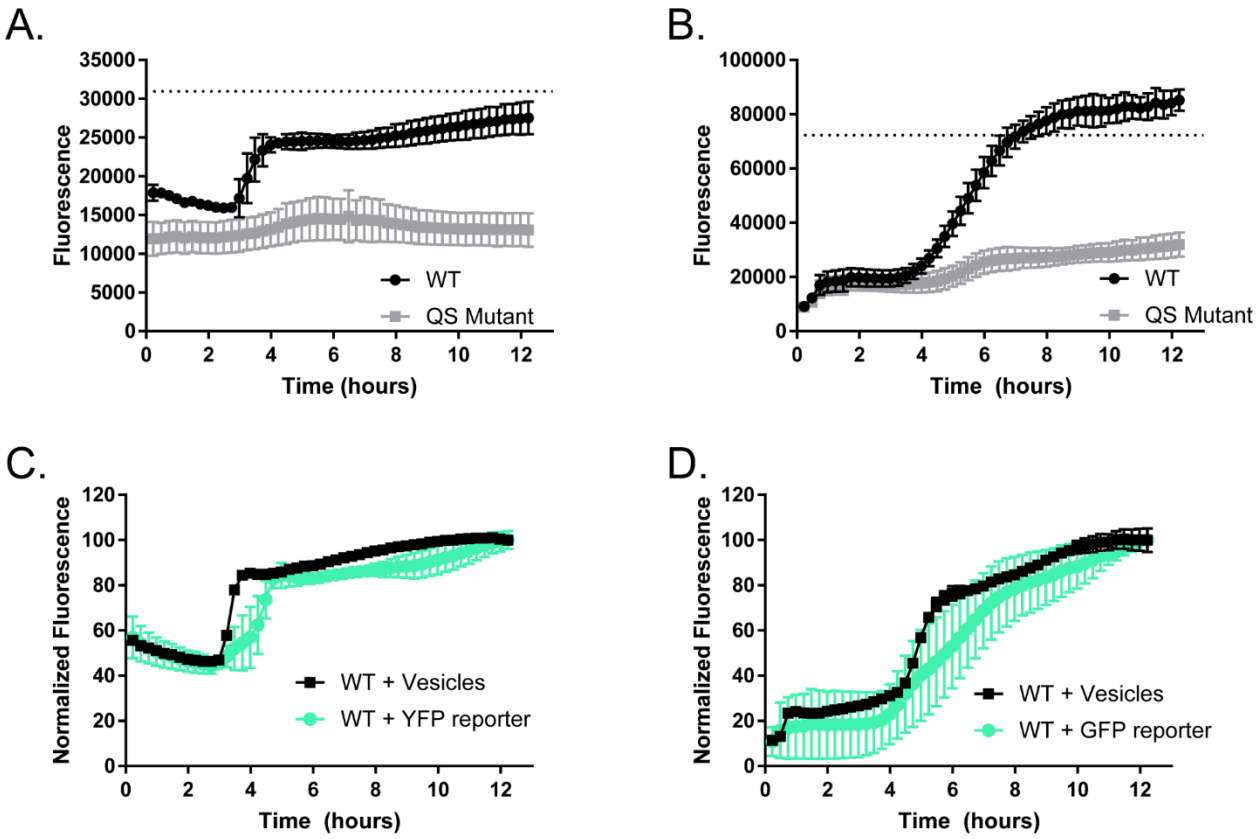

Figure 3. Vesicle lysis by *S. aureus* and *P. aeruginosa* (A) *S. aureus* WT (black circles) and QS mutant (gray squares) strains incubated with calcein-loaded vesicles. (B) *P. aeruginosa* WT (black circles) and QS mutant (gray squares) incubated with calcein-loaded vesicles. (C) *S. aureus* WT with calcein-loaded vesicles (black squares) and WT + YFP reporter (green circles). (D) *P. aeruginosa* WT with calcein-loaded vesicles (black squares) and WT + GFP reporter (green circles).

As QS onset is typically reported to occur at middle to late log phase of bacterial growth,[47-48] we were interested in whether the timing of vesicle lysis in the assay correlated with the onset of QS. We performed the VLT in WT bacteria containing QS reporter plasmids (encoding yellow fluorescent protein (YFP) for *S. aureus* and LVA-GFP for *P. aeruginosa*; see Table 1), and compared the timing of fluorescent protein production to vesicle lysis in the WT strains that lacked the plasmid. The intensities of the fluorescence signals in the VLT and the reporter systems varied considerably; therefore, to compare the VLT and reporter data, each of the timepoints was normalized to fluorescence intensity at the 12-hour timepoint. We observed a remarkable correlation between the time at which the fluorescent proteins were produced by the reporter strain and that of vesicle lysis (Figures 3C and 3D). Again, growth of the reporter strains was not substantially affected by the presence of the vesicles (Figure 9C and 9D). These results with the reporter strains support that the timing of QS onset and vesicle lysis is closely correlated for *S. aureus* and *P. aeruginosa*. We reasoned that a product generated under QS control was the cause for vesicle lysis, and looking to Jenkins past reports,[32, 49] it was most likely the known biosurfactants produced by these bacteria at high densities. We return to the nature of these QS products below.

Application of vesicle lysis test to the detection of QSMs. We next asked whether vesicle lysis could be either promoted or inhibited by the addition of exogenous QS activators (agonists) or inhibitors (antagonists), respectively, to the vesicles in the presence of bacteria. We chose a set of QSMs active in either *S. aureus* or *P. aeruginosa* for testing, including the native QS signals in each strain (Figure 4). For *S. aureus*, we chose the native peptide ligand (**1**), as well as four QS inhibitors of varying potency developed by our laboratory (**2**, **3**, **5**).[9, 50] Each likely acts via

binding to AgrC, the transmembrane receptor for the native ligand **1**, and blocking its histidine kinase function.[20] Compound **6**, or savirin, has been shown to inhibit QS in *S. aureus* via binding and inactivating AgrA, the downstream target of AgrC.[51] For *P. aeruginosa*, the native ligands of three of the QS transcriptional regulators (i.e., LasR, RhlR, and PqsR) were chosen (3-oxo-dodecanoyl HL (**7**), butanoyl HL (**8**), and the Pseudomonas quinolone signal (**12**), respectively). Rounding out the set were non-native AHL **9** that is a mimic of **8** and strongly agonizes RhlR, and two non-lactone AHL analogs that strongly antagonize LasR (**10**, reported by Greenberg and co-workers[18] and its furan analog **11**, reported by our lab[13]). For all the compounds tested, VLTs were performed with a QS- strain of the appropriate species to examine if the compounds alone lysed vesicles or had an effect on bacterial growth; none of the compounds cleaved vesicles or impacted growth at the concentrations tested (Figures 10 and 11).

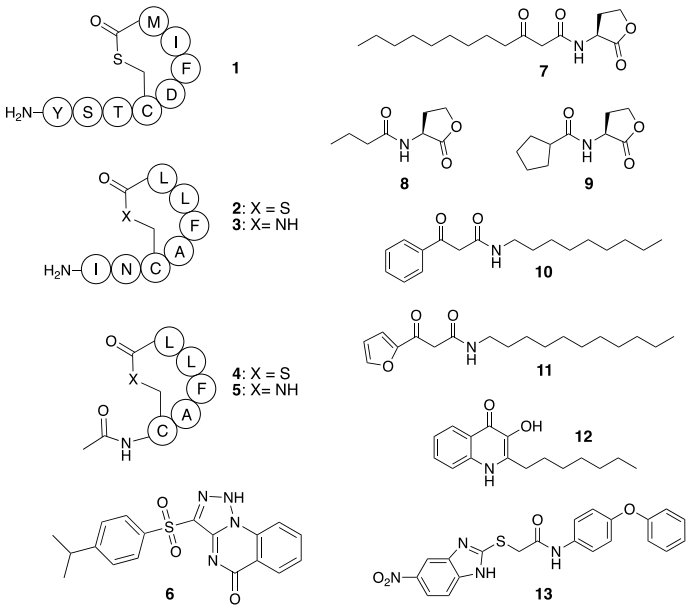

Figure 4. Compounds evaluated in this study. *S. aureus* native QS signal (**1**: AIP-I), *S. aureus* synthetic QS antagonists (**2**: AIP-III D4A; **3**: AIP-III D4A amide; **4**: tAIP-III D2A; **5**: tAIP-III D2A amide; and **6**: savirin), *P. aeruginosa* native QS signals (**7**: OdDHL; **8**: BHL; and **12**: PQS), *P. aeruginosa* synthetic QS agonist (**9**: S4), and *P. aeruginosa* synthetic QS antagonists (**10**: V-06-018; **11:** furan V-06-018 analog; and **13**: M64). Alternate compound names from original reports indicated.

We incubated the appropriate calcein-loaded vesicles with wild-type bacteria and each compound of interest at a concentration necessary for near-maximum inhibition or activation (or at the solubility limit) and monitored the fluorescence of the culture for 12 hours. In *S. aureus*, the QS inhibitors and activators had considerable effects on vesicle lysis. Four of the QS inhibitors (**2**, **3**, **5**, and **6**) completely abrogated vesicle lysis by wild-type *S. aureus* (Figure 5A), while the native QS agonist (**1**) strongly promoted vesicle lysis in the QS+ strain, shifting the start of lysis by approximately two hours compared to the vehicle (Figure 5B). In *P. aeruginosa*, the trends were similar for the LasR and RhlR modulators, but the magnitude of change between the vehicle and compounds was considerably smaller than that observed in *S. aureus*. The two LasR antagonists **10** and **11** delayed vesicle lysis by approximately two hours, although lysis eventually occurred to the same degree (Figure 5C). The results with the *P. aeruginosa* QS agonists varied based on their target receptors. The results for **7**, the native LasR agonist, were difficult to interpret. Rather than a drastic shift to earlier lysis we expected, we observed only a slightly earlier lysis and a much slower lysis over time. These differences could be due to effects of the surfactant-like nature of **7** and/or potentially other phenomena. (see Figure 10A). However, compounds **8** and **9**—the RhlR agonists—did show a decrease in the time to vesicle lysis (Figure 5D). While the behavior of the native QS signal **7** warrants further exploration, these collective data support the use of the vesicle lysis test to identify QSMs, especially in *S. aureus* where their effects in the assay were dramatic.

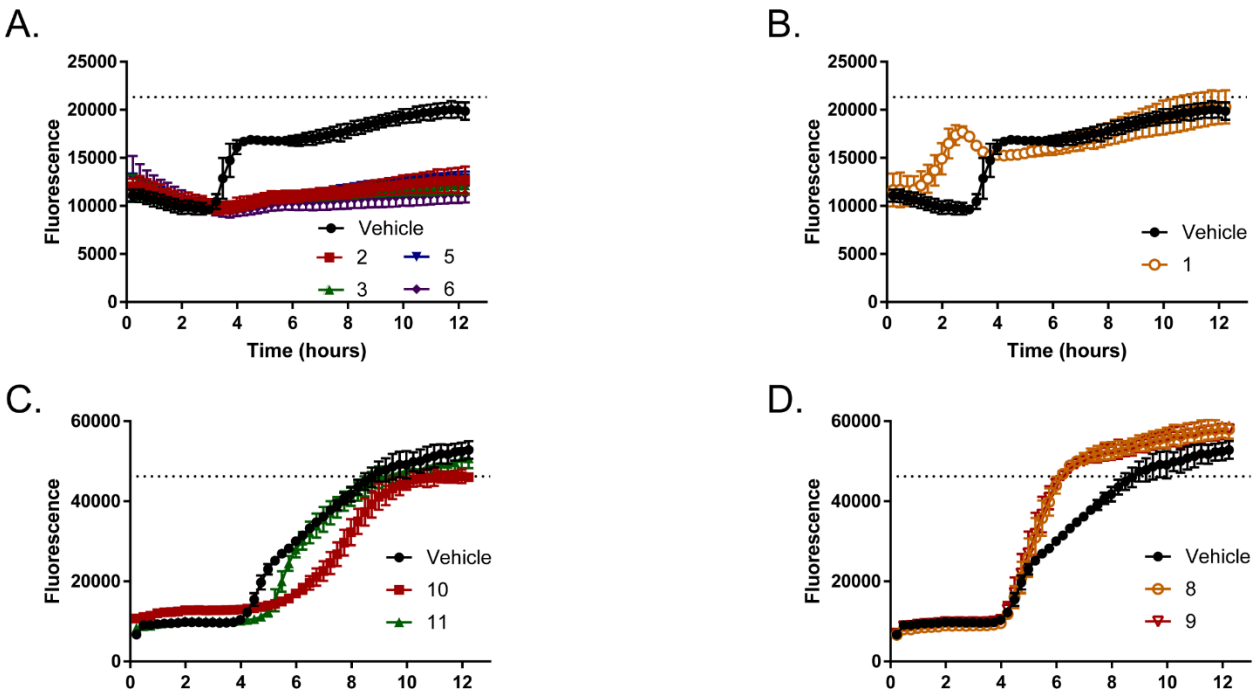

Figure 5. Application of vesicle lysis test to screen for QSMs. Each strain was incubated with the appropriate calcein-loaded vesicles**.** (A) *S. aureus* WT (QS+) and QS antagonists **2**, **3**, **5**, and **6**. (B) *S. aureus* mutant (QS-) and its native ligand (**1**)**.** (C) *P. aeruginosa* WT (QS+) and QS antagonists **10** and **11**. (D) *P. aeruginosa* WT (QS+) with QS agonists **8** and **9.**

We next examined the sensitivity of the VLT for QSMs by examining selected compounds over varied concentrations. For these experiments, we chose to use *S. aureus*, as the QS inhibitors tested had the most dramatic effect in this bacterium. We incubated *S. aureus*, calcein-loaded vesicles, and 3-fold dilutions of QS inhibitor **2** (starting at 100 nM). We observed that increasing concentrations of **2** directly correlated to the timing of delays in vesicle lysis (Figure 6A). We examined whether the potencies of different compounds (at the same concentration) could be discriminated using the VLT. Four QS inhibitors of varying potencies (**2–5**) were examined at 1 nM, and variable activities were observed (no lysis, lysis partway in between, and full lysis) that correlated with the relative potencies of the QSIs in cell-based reporter assays (**Figure 6B**).[9, 50] These results support the use of the VLT to determine the relative potencies of QS inhibitors, and that the timing of vesicle lysis can be correlated to compound concentration.

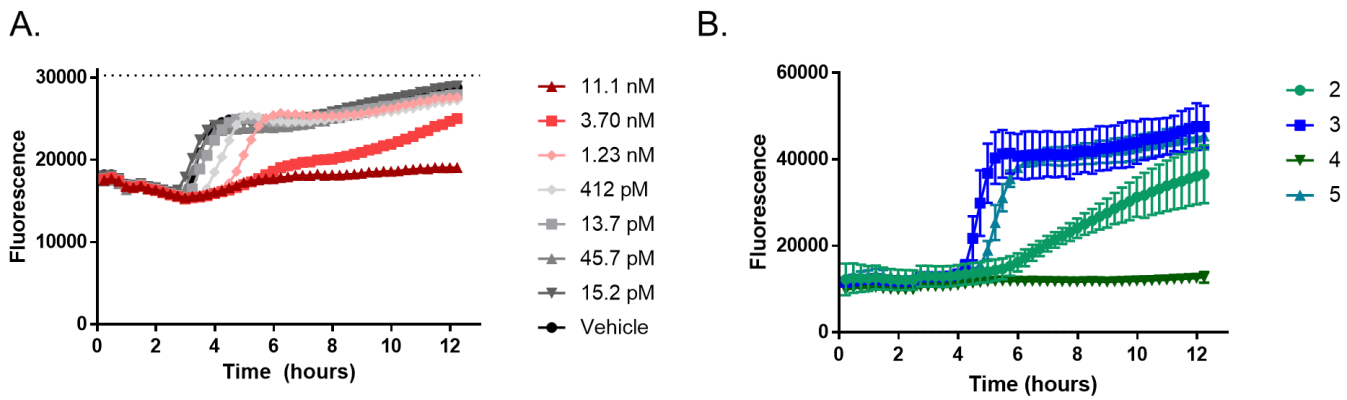

Figure 6. Application of the vesicle lysis test to screen QSMs at varying compound concentrations or with varying potencies. Bacteria were incubated with calcein-loaded vesicles. (A) *S. aureus* WT (QS+) with antagonist **2** at varied concentrations. (B) *S. aureus* WT (QS+) with antagonists **2**–**5** at 2 nM.

Investigations into the QS product or pathway responsible for vesicle lysis. As noted above, previous studies have linked the lysis of vesicles by *S. aureus* to the production of PSMs, a class of amphiphilic peptides produced under the control of QS.[52] We were interested to determine if a PSM in our *S. aureus* strain was the cause of lysis, and sought to identify the PSM(s) being produced. We examined a set of cell-free *S. aureus* supernatants using a reported HPLC protocol for PSM separations:[53] (i) WT with vehicle and (ii) QS mutant with vehicle (Figure 12). These HPLC analyses revealed two peaks that were present only in the WT + vehicle supernatants; inhibition of QS (via genetic knock-down) caused the disappearance of these peaks. The two peaks were determined by mass spectrometry to correspond to δ-toxin PSM and its formylated version, fMet-δ-toxin. As δ-toxin (and its formylated version) have been shown to lyse POPC vesicles previously,[34] and only our WT *S. aureus* strain was capable of lysing the vesicles developed herein, these data are supportive of these two PSMs being the causative agents of the vesicle lysis by our strain.

We were also interested to explore the cause for vesicle lysis by our *P. aeruginosa* strain. To this end, we screened the PqsR antagonist **13** (or M64) reported by Rahme and co-workers.[54]

We noticed that **13** had a higher inhibition of lysis than any other *P. aeruginosa* QS modulator. We note that RhlR, not PqsR, is the transcription factor that primarily controls rhamnolipid production in *P. aeruginosa*, and rhamnolipid was previously reported as the primary cause of vesicle lysis.[32] This unexpected result led us to ask if PqsR was actually controlling rhamnolipid production or if another, previously unidentified factor was leading to vesicle lysis by *P. aeruginosa*. To test this question, we first examined that activity of a *rhlA* mutant in the VLT, which cannot produce rhamnolipid or its amphiphilic precursor, HAA [3-(hydroxyalkanoyloxy)alkanoic acid]. We expected to see a decrease in vesicle lysis if rhamnolipid was not present, but instead we saw an increase in vesicle lysis in the *rhlA* mutant relative to WT (Figure 7). Next, we added the PqsR inhibitor **13** to the *rhlA* mutant and observed a drastic reduction in vesicle lysis relative to WT *P. aeruginosa* (Figure 7). These results suggest that PqsR plays a significant role in the mechanism of vesicle lysis, and that, in contrast to prior reports, rhamnolipid is not the major cause of lysis. Ongoing work is directed at determining the mechanism of lysis by *P. aeruginosa* and will be reported in due course.

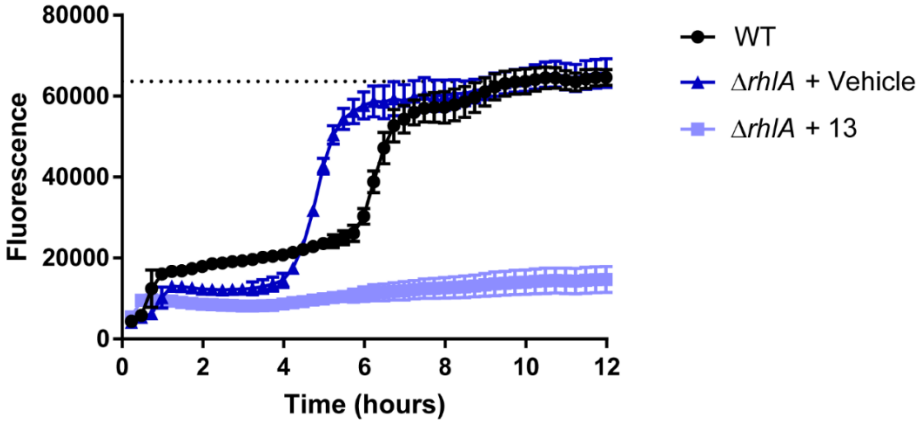

Figure 7. An alternate agent in *P. aeruginosa* appears to be causing vesicle lysis. Each strain is incubated with calcein vesicles. *P. aeruginosa* WT (black circles), *P. aeruginosa ΔrhlA* + vehicle (blue triangles), *P. aeruginosa ΔrhlA* + PqsR antagonist **13** (light blue squares).

Application of the VLT in a high-throughput screening format. Satisfied with the ability of our assay to discriminate between QSMs in *S. aureus*, we wanted to explore the suitability of the VLT to identify novel biosurfactant modulators (and thus likely QSMs) via high-throughput screening. The WT *S. aureus* strain again was examined, and the assay was reoptimized to accommodate a 384-microwell format (see Methods). We screened a commercial diversity library of 25,280 compounds (Life Chemicals, Inc.) at 10 μM for compounds capable of vesicle lysis in the presence of *S. aureus*. Approximately ~100 compounds resulted in <80% fluorescence emission relative to the vehicle control. Subsequent secondary screening was conducted to confirm the activity of potential hits and identify compounds with the strongest potency. Lead compounds that hit again in the secondary screen were subjected to reporter gene assays in *S. aureus*, eventually revealing a set of 6 compounds that could inhibit *agr* function. (Figure 14) Notably, two of these hit compounds were capable of inhibiting *agr* to virtually 100% at sub-micromolar concentrations. These compounds have approximately ~7-10x greater potencies than the aforementioned small molecule AgrA inhibitor, **6** (Figure 8), and further characterizing their structures and delineating their mode of *agr* inhibition will be a focus of a subsequent study. The identification of these compounds clearly supports the viability of the VLT as an assay conducive to high-throughput screening for QSMs.

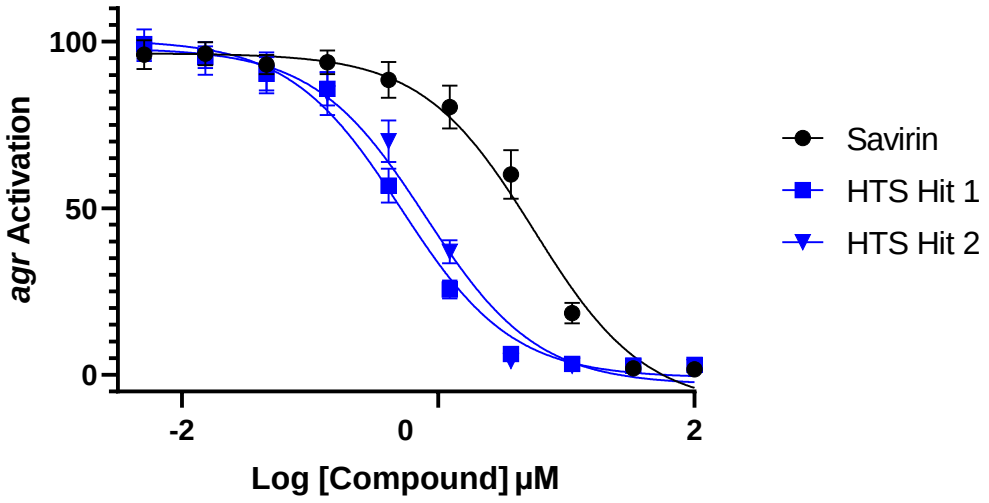

Figure 8. Dose-response analysis for high-throughput screen hit compounds 1 and 2 using YFP transcriptional reporter. Both HTS Hit 1 (IC_{50} = 0.5 µM) and HTS Hit 2 (IC_{50} = 0.7 µM) displayed greater potency than control compound **6** (IC_{50} = 5.5 µM) with near maximal *agr* inhibition. Error bars indicate standard error of the mean.

Summary and conclusions. In this work, we have demonstrated the validity of the VLT to screen for QSMs in bacteria. The VLT was found to be highly robust, straightforward to implement, and capable of discriminating between QSMs at different concentrations and of different relative potencies. We identified an agent responsible for lysis of our vesicles in *S. aureus*—two PSMs—and an alternate pathway responsible for lysis in *P. aeruginosa*, controlled via PqsR. The VLT was readily adapted to perform a high-throughput screen of small molecules for their ability to inhibit *S. aureus* surfactant production, presumably via QS. From this screen we identified 6 novel compounds that inhibit vesicle lysis. Follow on assays on the lead compounds in *agr* reporter assays revealed ~10 compounds capable of *agr* inhibition. These results support the use of the VLT assay for the straightforward identification of new QSMs in *S. aureus*.

The successful implementation of this assay in *P. aeruginosa* and *S. aureus* paves a route toward its application in other organisms. Bacterial-produced biosurfactants have been linked to immune cell toxicity and swarming, among numerous other downstream effects.[55-56] The ability to disrupt membranes is likely present in many bacteria, even as a non-QS controlled factor. Of the notorious ESKAPE group of pathogens,[57] surfactants have been identified from four of the six (including *P. aeruginosa* and *S. aureus*).[58-59] Accordingly, the VLT could be used to study surfactant production in these pathogens and possibly many other, even if the bacteria are not readily amenable to genetics or if the promoter responsible for biosurfactant production has not been identified. Monitoring biosurfactant production by different strains or clinical isolates could be useful also; for example, rhamnolipid production is correlated with heightened virulence in *P. aeruginosa* clinical isolate and is biosynthesized for commercial applications on scale. Ongoing work in our laboratories is focused on the application of the VLT in range of organisms and experimental contexts, and this work will be reported in due course.

Materials and Methods

Materials. 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC), cholesterol, filter supports and Mini extruder were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine (DPPE), dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl), Sephadex-G50 column size exclusion chromatography beads, triethylphosphine oxide, deuterium oxide (D2O) and calcein were purchased from Sigma (St. Louis, MO). Isopropanol (iPOH) and tris-base were purchased from Fisher Scientific (Waltham, MA). 100 nm polycarbonate extruder filters were purchased from Millipore (Billerica, MA).

Ethylenediaminetetraacetic acid (EDTA) was purchased from Acros Organics (Geel, Belgium). 10x concentrated phosphate buffered saline (PBS) solution was purchased from Dot Scientific (Burton, MI). Triton X-100 was obtained from Sigma (St. Louis, MO). LB Broth was obtained from Research Products International (Mount Prospect, IL). Brain Heart Infusion (BHI) Broth was obtained from Teknova (Hollister, CA). Deionization of distilled water was performed using a Milli-Q system (Millipore, Bedford, MA) yielding water with a resistivity of 18.2 MΩ. All materials were used as received without further purification unless otherwise specified.

Cell culture. All bacteria were grown at 37 °C and 200 rpm shaking unless otherwise specified. *P. aeruginosa* was grown in LB, and *S. aureus* was grown in BHI. (Table 1) AH1677 and PAO-JG35 with p*lasI*-LVAgfp were used for reporting QS-controlled transcription and were grown with 10 μg/mL chloramphenicol and 300 μg/mL carbenicillin, respectively. All other strains were grown without supplemented antibiotic. RN6390b and PAO1-PGSC were used as wild-type (QS +) strains for most experiments, while RN9222 and PAO-JG35 were used as QS- strains. As we had no interest in maintaining β-galactosidase activity in RN9222 (it served only as QS-), we did not maintain this strain with antibiotic. PAO1-T and PAO1-T *ΔrhlA* were used for testing **13**'s effect on rhamnolipid production – the different wild type was necessary given large variation among PAO1 isolates. [60]

Compound handling and preparation. Compounds were stored at -20 °C dry until resuspension in DMSO. Concentration of compound in DMSO varied with potency and apparent solubility. Final compound concentrations were: 10 μM for **1-5**, 20 μM for **6**, 1 mM for **8**, 200 μM for **9**, 100 μM for **7** and **10**, 10 μM for **11**, and 10 μM for **13**.

Production of calcein-containing phospholipid vesicles. Phospholipid vesicles were produced using the thin film hydration method.[61] Stock solutions of phospholipids in chloroform were mixed to the desired mole fraction and chloroform was removed using an N_2 stream or rotary evaporation followed by at least 1 hour under vacuum. Self-quenching solutions of calcein were prepared by combining calcein and PBS or a specially prepared calcein buffer (1 mM EDTA, 10 mM Tris-base, and 100 mM NaCl; pH 7.4) to a calcein concentration of 70 μM. The resulting solutions were titrated with aqueous 10 M NaOH until all calcein was dissolved and the solution reached pH 7.4 or pH 8 for PBS and the calcein buffer respectively. The resulting calcein solutions were added to the dried phospholipid films (PBS for *P. aeruginosa* formulations and the calcein buffer for the *S. aureus* formulations) for a final lipid concentration of 5 mg/mL. The resulting lipid solutions were vortexed vigorously and briefly sonicated in a bath sonicator. *S. aureus* formulations were then freeze thawed 5 times by alternating the vial between baths containing iPOH and dry ice and a 60 °C water bath. Vesicles prepared for the assay validation experiments were then passed at least 7 times through a 100 nm polycarbonate filter using an Avanti polar lipids Mini-prep extruder. Vesicles prepared for the high throughput screen were passed through the filter 3 times. The resulting vesicle solutions were then washed using a hand packed Sephadex-G50 column to remove calcein from the vesicle solution. The concentration of phospholipid in the solution was quantified using ^{31}P NMR, and the phospholipid concentration was adjusted to 2 mM for unsaturated lipid solutions and 1 mM for saturated lipid solutions.

96 well plate vesicle assay. 2mL or 10 mL cultures were inoculated with a colony of the strain of interest and allowed to shake overnight. From these, culture was directly diluted 1:50 in BHI for *S. aureus* or 1:100 in LB for *P. aeruginosa*.

178 µL of the diluted cell culture was mixed with 20 µL vesicle solution and 2 µL of compound solution (dissolved in DMSO at 100x working concentration) in a clear-bottom, black 96-well microtiter plate (Corning 3904). For controls, DMSO (vehicle) or Triton (1% final concentration) was substituted for compound. Plates were incubated in a Biotek Synergy 2 (Gen5 1.05 software) microplate reader at 37°C with shaking at the 'High speed' setting. Fluorescence (excitation 500 nm, emission 540 nm) and OD (absorbance, 600 nm) reads were performed every 15 minutes for 12 hours.

Compounds and serial dilutions were tested in technical triplicate in a single microtiter plate. GraphPad Prism 7 was used to generate curves and SD error from the replicates.

High-throughput screening and follow up analysis. Experimental compounds and positive control were delivered to 384 microwell plates in 25 nL DMSO. 50 µL of diluted cells (1:50 overnight *S. aureus* in BHI) containing 0.1 M phospholipid vesicles for a final concentration of 10 µM was added to plates. 250 nM of **2** and DMSO (vehicle) were used as the positive and negative controls respectively. Plates were statically incubated at 37°C for 24 hours at which fluorescence (483 excitation, 530 emission) and OD600 reads were taken. Of the 25,280 compounds, 92 (0.36%) resulted in fluorescence emission less than 80% compared to vehicle control. A secondary screen of these compounds was performed to confirm fluorescence inhibition, and 16 compounds were selected for follow up dose-response analysis in the 384-well vesicle lysis assay format (Figure 12). Of these, 5 compounds were deemed probable inhibitors of biosurfactant production and selected for further dose-response analysis using the *S. aureus agr* QS YFP reporter. The YFP reporter protocol was performed similarly to the method

previously by our group[62] and dose-response curves and IC_{50} values were generated using GraphPad Prism 8.3.1.

Acknowledgments

Financial support for this work was provided by the NIH (R21 AI135745). K. Nyffeler and T. Polaske were supported in part by the UW−Madison NIH Chemistry−Biology Interface Training Program (T32 GM008505); K. Nyffeler was also supported as part of a seed award from the UW–Madison NSF MRSEC *(*DMR-1720415*)*. C. Gahan was supported in part by an NSF Graduate Research Fellowship. We acknowledge the Manoil lab (U Washington) for the transposon strains used in this work (NIH P30 DK089507). NMR facilities at in the Department of Chemistry at UW-Madison are supported by the NSF (CHE-0342998). MS facilities in the UW-Madison Department of Chemistry were supported by the NSF (CHE-9974839).

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Supplemental Information

Table 1. Bacterial strains and plasmids used in this study.

	Referred to herein	Genotype/Phenotype	Reference or Source
Staphylococcus aureus			
RN6390b	WT	Wild type, *agr* group I (NTCC8325 cured of prophages[1])	Novick [2]
RN9222	QS -	RN6911 with pRN7035	Lyon et al. [3]
RN6911	N/A	*tetM*::*agr*, from RN6390	Novick et al. [1]
AH1677	QS reporter	USA300 LAC with pDB59	Kirchdoerfer et al. [4]
***Pseudomonas* aeruginosa**			
PAO1-PGSC	WT	Wild type	Obtained from Pseudomonas Genetic Stock Center
PAO-JG35	QS -	*lasR rhlR* mutant, from PAO1-PGSC	Gerdt and Blackwell [5]
PAO1-T	QS +	Wild type	WT from PA two-allele library [6]
PAO1-T Δ*rhlA* (PW6886)	Δ*rhlA*	*rhlA*-E08::IsphoA/hah	PA two-allele library [6]
Plasmids			
pDB59	QS reporter	*P3'-yfp*$_{10B}$ transcriptional fusion, Chl^R	Yarwood et al. [7]
pRN7035	QS -	*agr-P3::blaZ* fusion	Lyon et al. [3]
p*lasI*-LVAgfp	QS reporter	*lasI'-LVAgfp* transcriptional fusion, Car^R	De Kievit et al. [8]

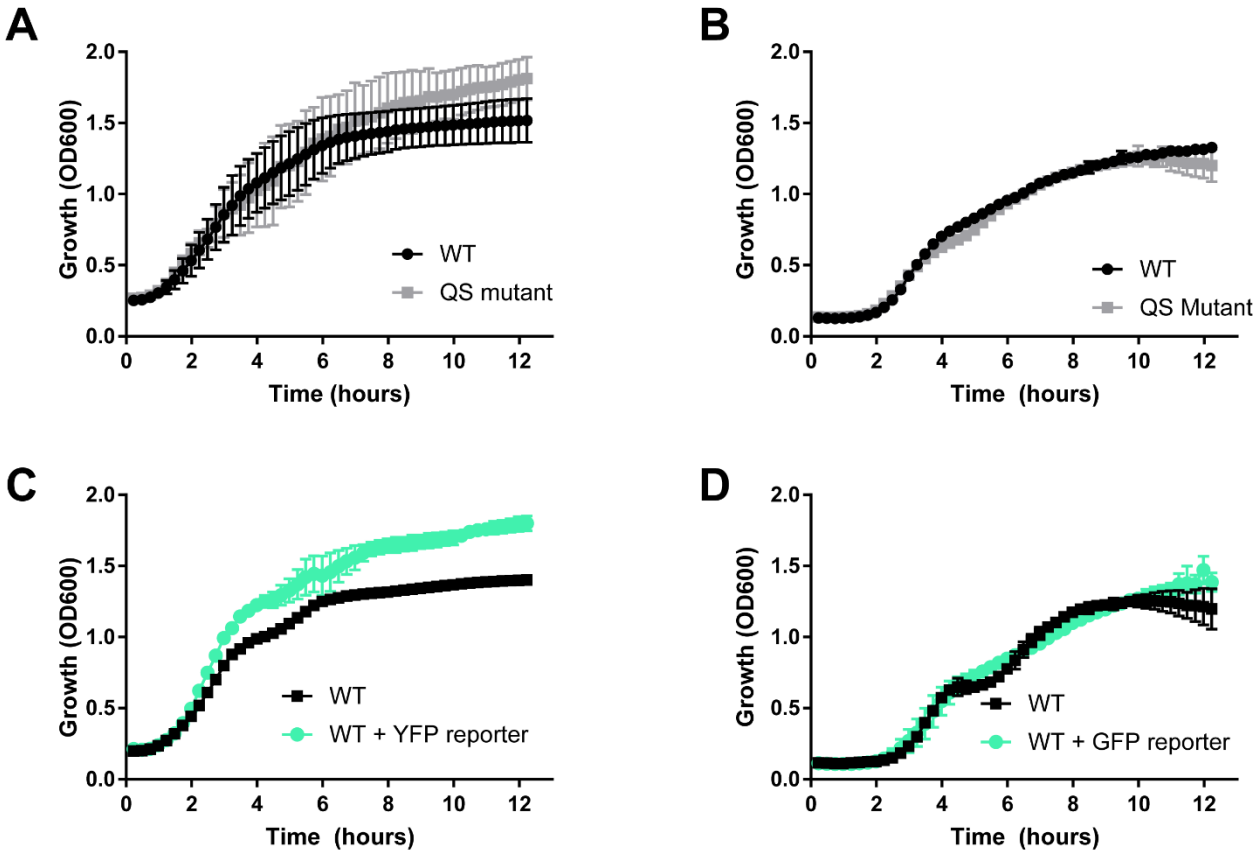

Figure 9. Growth curves for bacteria incubated in the presence of calcein-loaded vesicles. (A) Growth curves for *S. aureus* wild-type (black circles) and *S. aureus* QS mutant (gray squares) strains. (B) Growth curves for *P. aeruginosa* wild-type (black circles) and QS mutant (gray squares) strains. (C) Growth curves for *S. aureus* wild-type (black squares) and *S. aureus* wild-type + YFP *agr* reporter plasmid (green circles). (D) Growth curves for *P. aeruginosa* wild-type (black squares) and *P. aeruginosa* wild-type + GFP *las* reporter (green circles). See Methods section for full details of assays.

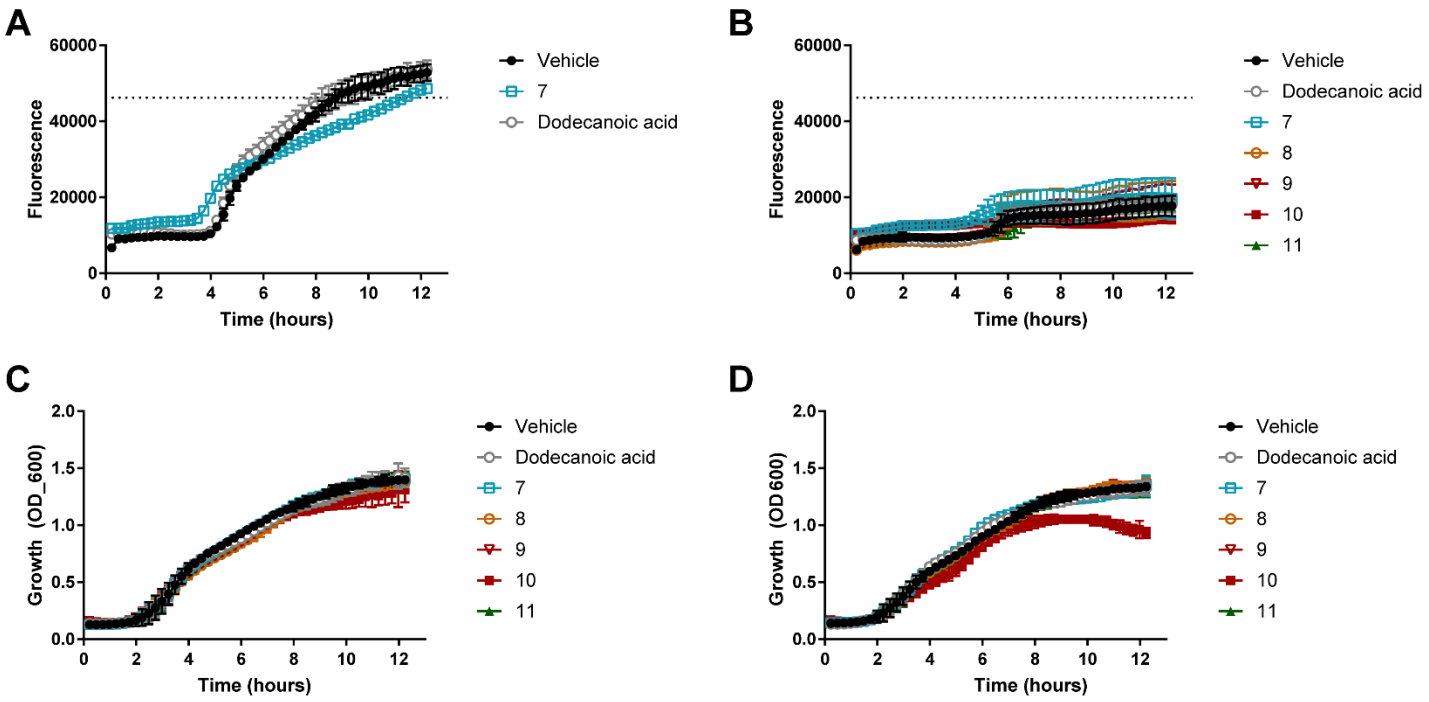

Figure 10. Fluorescence and growth curves for *P. aeruginosa* strains incubated in the presence of calcein-loaded vesicles and various compounds. (A) Fluorescence curves for *P. aeruginosa* wild-type with vehicle (DMSO), **7**, or dodecanoic acid. (B) Fluorescence curves for *P. aeruginosa* QS mutant with vehicle, dodecanoic acid, and compounds **7**–**11**. (C) Growth curves for *P. aeruginosa* wild-type with vehicle, dodecanoic acid, and compounds **7**–**11**. (D) Growth curves for *P. aeruginosa* QS mutant with vehicle, dodecanoic acid, and compounds **7**–**11**. See Methods section for full details of assays.

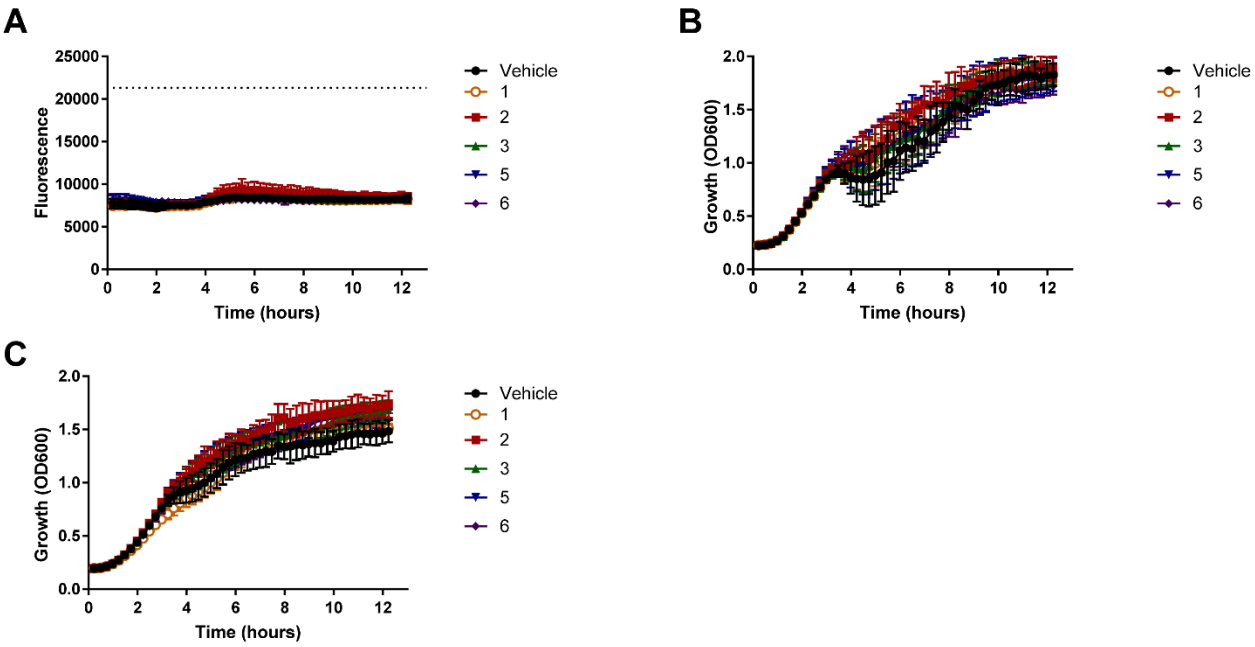

Figure 11. Fluorescence and growth curves for *S. aureus* strains incubated in the presence of calcein-loaded vesicles and various compounds. (A) Fluorescence curve for *S. aureus* QS mutant with vehicle or compounds **1**–**3**, **5**, or **6**. (B) Growth curves for *S. aureus* QS mutant with vehicle or compounds **1**–**3**, **5**, or **6**. (C) Growth curves for *S. aureus* wild-type with vehicle or compounds **1**–**3**, **5**, or **6**. See Methods section for full details of assays.

Identification of δ-toxin. 10 mL subcultures of *S. aureus* QS+ and QS- were grown overnight by diluting a previous overnight culture 1:50 in fresh BHI. This subculture was centrifuged at 16,100 g for 5 minutes. The supernatant was then passed through a PerkinElmer polytetrafluoroethylene (PTFE) syringe filter with 0.45 μm pore size. Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on the cell-free supernatant. A Shimadzu system equipped with a SCL-10Avp system controller, a DGU-14A degasser, LC-20AT solvent delivery unit, a SIL-10AF autosampler, a CTO-10AS column oven equipped with a manual injector, a SPD-M10A UV-Vis diode array detector, and a FRC-10A fraction collector was used. Solvent A was 18 MΩ water with 0.1% TFA, and solvent B was HPLC-grade

acetonitrile with 0.1% TFA. The column was a Kromasil Eternity C18 column (10 mm x 250 mm, 5 mm particle size with 100 Å pore size). A linear gradient of 0% solvent B -> 100% solvent B at 5 mL/min flow rate for 41 min. 4 mL of the filtered supernatant was injected. These peaks were lyophilized and analyzed with a Bruker II ESI-Q-TOF mass spectrometer.

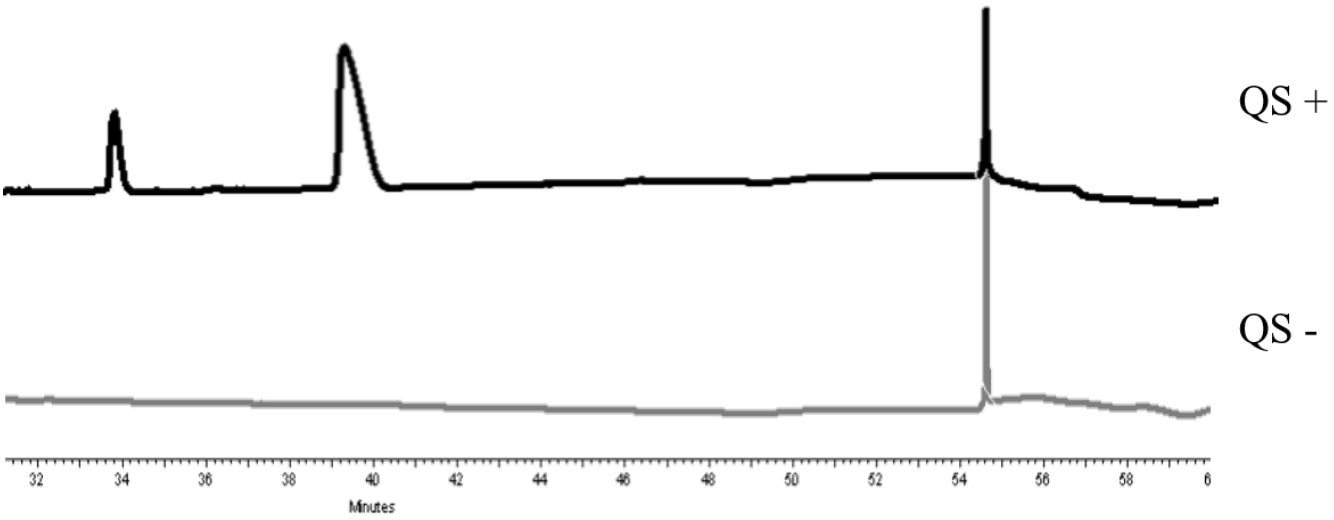

Figure 12. Except of HPLC trace from QS+ (black) and QS- (gray) strains of *S. aureus*.

NMR quantification of phospholipids. To determine the concentration of phospholipids in stock solutions, quantitative $^{31}P[^{1}H]$ NMR spectroscopy was performed in 90% H_2O and 10% D_2O using a Bruker Avance-400 spectrometer with a BBFO probe. A relaxation delay (D1) of 11s was determined by inversion-recovery pulse sequence experiments. 890 µL of a phospholipid vesicle sample in water was mixed with 10 µL of Triton-X or rhamnolipid solution and 100 µL of a 100 mM triethylphosphine oxide internal standard solution in D_2O. All spectra were referenced relative to the phosphorus peak of the internal standard (~δ65ppm). Acquisition parameters are as follows: PULPROG=zgig30, D1=11s, SW=405ppm, O2P=3.75ppm, NS=64, DS=4, LB=1.

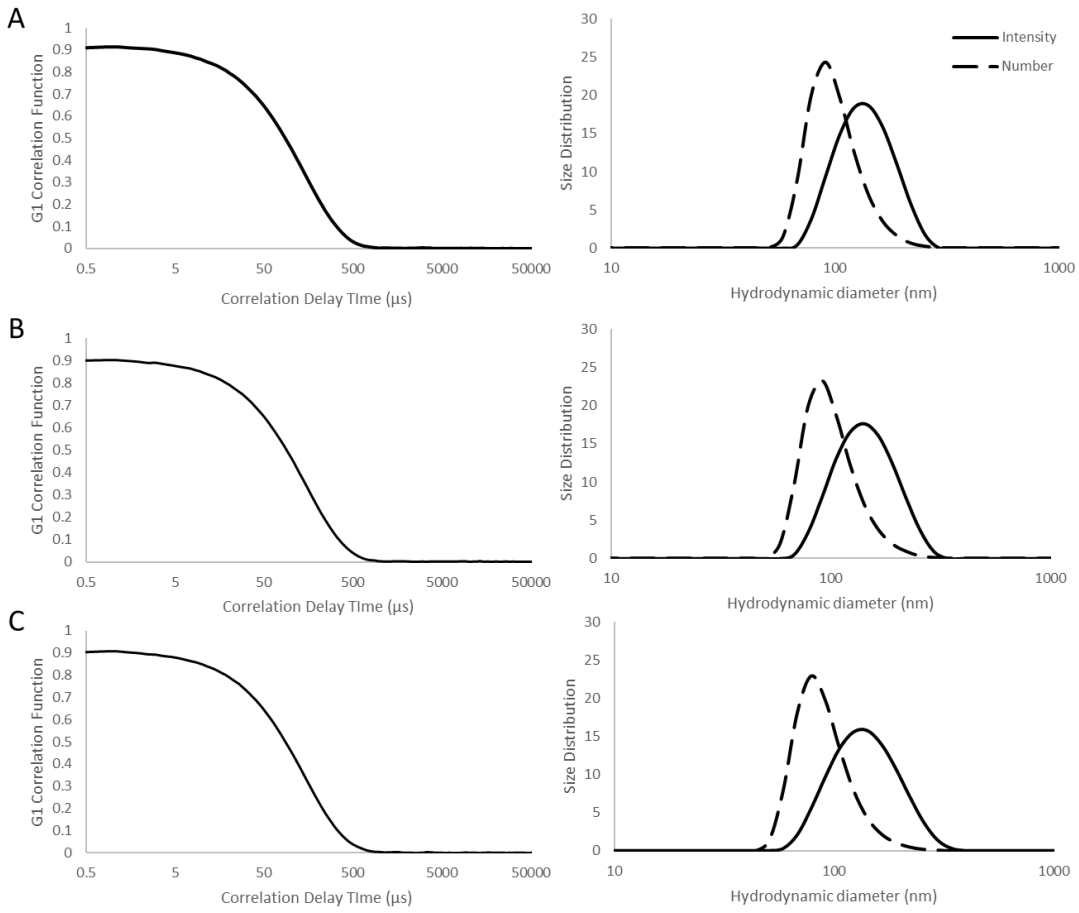

Figure **13**. Results of DLS analysis of the various vesicle formulations produced for use in the vesicle lysis test. (Left) Autocorrelation functions (ACFs) and (Right) corresponding intensity and number average size distributions for vesicles composed of (A) 68% DPPC, 30% Cholesterol and 2% DPPE, (B and C) 70% DOPC and 30% Cholesterol passed through a 100 nm PC membrane (B) 3 times and (A,C) 7 times. The ACFs and size distributions sown are the average of three independent measurements of a single vesicle sample.

High-throughput screening and follow-up protocol.
Experimental compounds and positive control **2** were delivered in 25 μL DMSO with an Echo 550 liquid handler from LabCyte Inc. into black 384-well microtiter plates from Corning**.** All experimental compounds were tested at 10 μM and 250 nM of **2** was used as positive control. 2 mL overnight cultures of WT *S. aureus* agr group-I were grown at 37°C with shaking at 200 rpm. Overnight cultures were diluted 1:500 in fresh BHI medium at which point vesicles were added for a final concentration of 1 mM ^{31}P. This vesicle/cell mixture was then added to the 384-well plates with a Multidrop 384 liquid-dispensing robot from Thermo Scientific to a total volume of 50 μL per well. Plates were statically incubated for ~24 hours at 37°C at which point fluorescence (excitation 483, emission 530) and OD (absorbance 600) reads were taken using a CLARIOstar® *Plus* microtiter plate reader from BMG Labtech running MARS data analysis software. Primary and secondary screen data was collected, and scatter plots generated using the Collaborative Drug Discovery Vault informatics platform. The dose-response experiment using the vesicle lysis assay was performed in a 384-well plate in technical duplicate with compound concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, and 20 μM. Dose-response curves for were generated using variable slope (4 parameter) non-linear regression analysis for compound inhibition with GraphPad Prism 8.3.1.

The follow up dose-response analysis of **HTS Hit 1** and **HTS Hit 2** was performed using the YFP transcriptional reporter for *S. aureus agr* group I. Compounds were purchased directly from Life Chemicals Inc. (**HTS Hit 1** and **2**) or AK Scientific (**6**) and DMSO stock solutions were prepared and stored at -20°C. Serial dilutions (3x) spanning 5 nM to 100 μL were delivered using 2 μL DMSO into black 96-well microtiter plates (Corning 3904). 2 mL overnight cultures were grown at 37°C with shaking at 200 rpm. Cultures were diluted 1:50 in fresh BHI media and 198 μL of this mixture was added to each well containing compound for a total volume of 200

μL per well. All plates contained a vehicle control consisting of 2 μL DMSO and 198 μL culture and a media control consisting 200 μL BHI. Plates were incubated for ~24 hours at 37°C with shaking at 200 rpm. Fluorescence (excitation 510, emission 544) and OD (absorbance 600) were taken with a PerkinElmer EnVision microtiter plate reader running Envision Manager software. Background fluorescence from BHI control was subtracted from raw fluorescence reads, OD_{600} corrected, and finally normalized to vehicle control. Dose-response curves and IC_{50}s were generated using 3 parameter non-linear regression analysis for compound inhibition with GraphPad Prism 8.3.1. All compounds were tested in technical triplicate with at least 3 biological replicates.

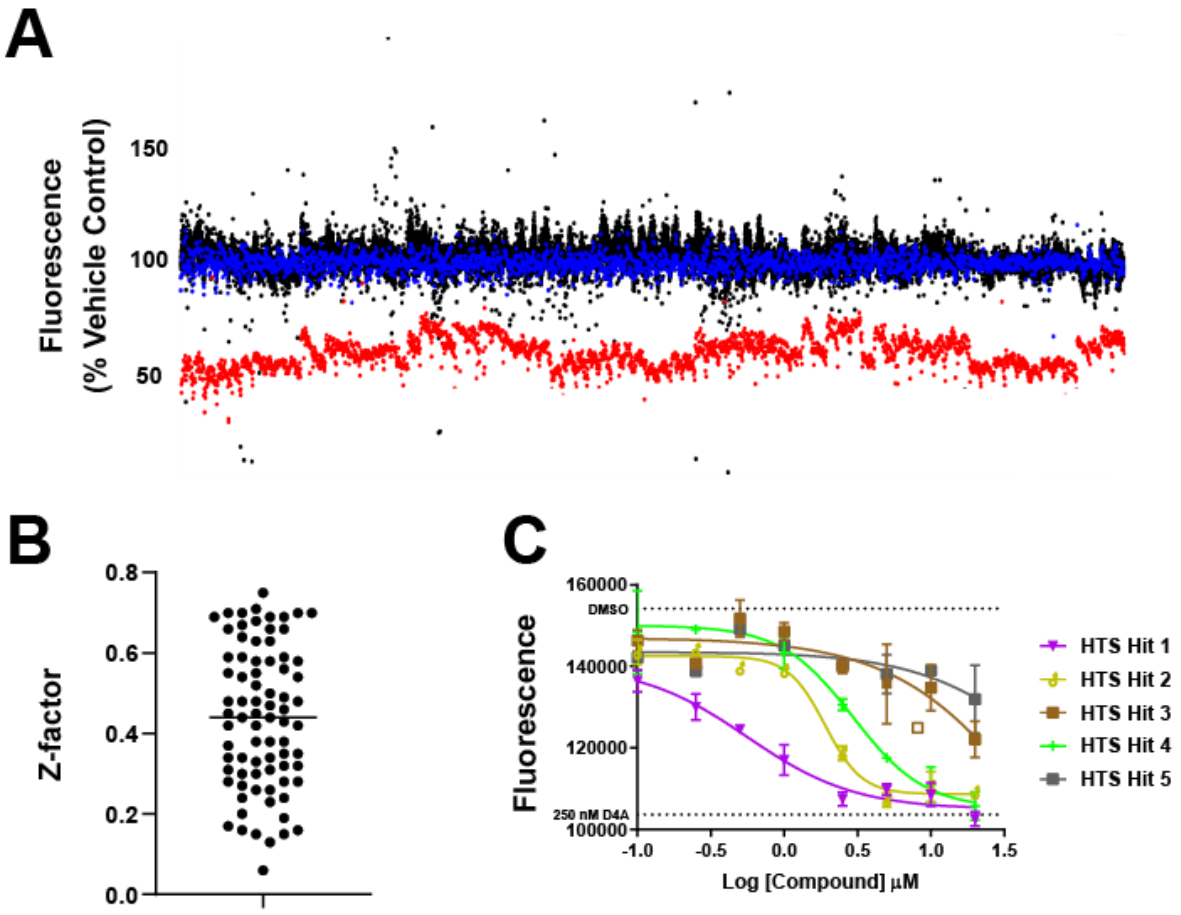

Figure 14. Summation of high-throughput screening results. (**A**) Scatter plot generated for primary screen of experimental compounds. Experimental compounds (black) which resulted in less than 80% fluorescence of vehicle control (blue) were selected for secondary screening to confirm activity. Positive control (red) typically yielded fluorescence values between 50% and 70% of vehicle control. (**B**) Z-factors of all 79 plates used in the primary high-throughput screen. All plates had positive z-factors with a median z-value of 0.42 (black bar). (**C**) Dose-response analysis of select compounds using the vesicle lysis test. Of the 14 compounds selected for dose-response analysis, 5 indicated probable inhibition of biosurfactant production and were subjected to further analysis using an *agr* YFP transcriptional reporter. Error bars represent SD. Vehicle and positive control (dashed lines) are also given.

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CHAPTER FOUR: Anisotropic Liquid Infused Surfaces: A Potential Platform for Naked-Eye Detection of Biosurfactants

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*authors contributed equally

To be submitted

H. Agarwal designed the material, H. Agarwal and K.E. Nyffeler performed experiments, H. Agarwal, K.E. Nyffeler, H.E. Blackwell, and D.M. Lynn wrote the text.

Abstract

Slippery liquid infused porous surfaces (SLIPS) are a new class of bioinspired materials that have gained immense interest as antifouling, anti-icing, anti-frosting, and smart surfaces. Over the years, researchers have expanded on the range of lubricating liquids and underlying matrices that can be used to fabricate SLIPS, broadening the range of functionality of these surfaces. In this Chapter, we expand upon our previously reported results about new opportunities to tune droplet mobility on SLIPS by using a thermotropic liquid crystal (E7) as the lubricating liquid for fabricating SLIPS. Aqueous droplets containing surfactants display a significantly higher sliding time compared to "bare" droplets on E7-infused SLIPS. Also, the sliding time of a surfactant-containing droplet depends on the surfactant concentration, electrolyte concentration, and surfactant structure. Our results are consistent with a physical picture that involves transient and reversible changes in the interfacial orientation of the LCs in these materials from "planar" to "homeotropic" when placed in contact with aqueous fluids containing surfactants. We demonstrate the potential utility of these new surface through the detection of several biologically important amphiphilic toxins produced by bacterial pathogens. Droplets of complex bacterial cultures containing biosurfactants (such as rhamnolipids and phenol soluble modulins (PSMs)) slide-off LC-SLIPS at significantly slower rates than cultures without surfactant and in a concentration dependents manner , enabling LC-infused SLIPS to be used as a fast, simple, and unaided-eye detection platform to report on surfactant. More broadly, as amphiphile production is dependent on cell density in many common bacteria, LC-infused SLIPS could thus also be used for the sensing of bacterial community formation, or quorum sensing.

Introduction

Slippery liquid-infused porous surfaces (SLIPS) and liquid-infused surfaces (LIS) comprise a relatively new classes of synthetic soft materials fabricated by the infusion of lubricating oily liquids into chemically compatible nanoporous, microporous, or topographically patterned surfaces.[1-3] Provided that the chemical properties of the lubricant and the underlying surfaces are suitably matched, these materials present a 'slippery' layer of mobile fluid at the surface that can repel other immiscible fluids or substances with which they come in contact. For example, SLIPS and LIS can shed droplets of aqueous solutions at very low sliding angles (e.g., $< 5°$), endowing these materials, and surfaces coated with them, with robust anti-icing,[1, 4-5] anti-frosting,[5] and anti-fouling[6-9] properties.

Aizenberg and co-workers reported the first examples of SLIPS in 2011 by infusing perfluorinated liquids into nanoporous polytetrafluoroethylene (PTFE) membranes.[1] Since that initial report, many groups have expanded on the range of lubricating liquids and underlying porous matrices that can be used to fabricate SLIPS, improve their chemical and physical stabilities in complex environments, and design multifunctional coatings with improved anti-fouling behaviors.[10] It is now broadly recognized that the properties of the infused oil can have substantial impacts on both the stability of the mobile liquid layer (e.g., the degree to which the infused oil can be displaced by a contacting fluid) and the mobility of droplets of aqueous fluid (e.g., droplets of water slide more slowly on SLIPS fabricated using higher viscosity oils, and more rapidly on coatings infused with lower viscosity liquids).

These latter observations have motivated recent work to explore the infusion of 'functional' oils with physicochemical properties that can be manipulated actively and dynamically. We reported recently that hydrophobic nanoporous polymer coatings fabricated by

covalent layer-by-layer (LbL) assembly could be infused with a thermotropic liquid crystal (LC), an anisotropic fluid, to design SLIPS that respond to chemical changes in their environments.[11] For example, SLIPS fabricated using the nematic LC E7 exhibited slippery properties that changed substantially and reversibly when a contacting liquid contained (or did not contain) an amphiphilic molecule, such as a surfactant. That past work demonstrated that LC-infused SLIPS can discriminate actively based on the chemical composition of a contacting fluid, suggesting new approaches for the manipulation of droplet mobility and a basis for the design of surfaces that permit 'naked-eye' detection of environmental analytes. More recently, Wang et al. reported the design of SLIPS fabricated using ferrofluids to yield so-called 'FLIPS' that can be transformed using magnetic fields to present either smooth or multiscale hierarchical surface features, providing surfaces that permit active control over the self-assembly of colloidal particles at the micrometer scale or the dislodging of bacterial biofilms at centimeter length scales.[12] This past work, combined with a steadily growing body of research on the functionalization and/or patterning of new rough or porous surfaces that can be used to host stable films of infused oils, has significantly expanded the range of potential applications of 'slippery' surfaces.[13-17]

The work reported here was motivated by our past observations of responsive behaviors in slippery LC-infused LbL coatings, and the potential of these materials to enable dynamic control over the mobility of immiscible fluid droplets. This current study sought to (i) explore the generality of this approach to the design of environmentally-responsive SLIPS, (ii) provide insight into key chemical and physical factors that govern the dynamic behaviors of these LC-infused materials and their responses to fluids of varying composition, and (iii) explore the

potential of LC-infused SLIPS to enable the development of new soft material platforms for the detection of environmental agents or the discovery of new chemical and biological agents.

In this Chapter, we demonstrate that thermotropic LCs can be infused into microporous PTFE thin films to yield LC-infused membranes that exhibit slippery behaviors and remain physically and functionally stable when contacted with a broad range of synthetic and biological aqueous fluids. We also show that droplets of aqueous fluids slide over the surfaces of these LC-infused materials at speeds that depend upon the composition of the fluid (e.g., the ionic strength of the fluid or the presence, absence, concentration, and structure of natural and synthetic amphiphiles). In general, sliding times on these LC-infused SLIPS increased significantly with increasing amphiphile concentration in the droplet, permitting the measurement of differences in sliding time to be used to estimate the concentration of amphiphiles in a solution. Our results are consistent with a physical picture that involves transient and reversible changes in the interfacial orientation of the LCs in these materials from ‘planar’ to ‘homeotropic’ when placed in contact with aqueous fluids containing surfactants.

In the last part of this chapter, we demonstrate that these materials can be used to report the presence of amphiphilic toxins in aqueous samples containing Gram-negative or Gram-positive bacteria. Large and readily observable differences in the sliding speeds of droplets obtained from cultures of bacterial human pathogens correlate to the presence or absence of amphiphiles produced by these pathogens, demonstrating an approach to the naked-eye detection of bacterial toxins. In a broader and more general context, the ability of these materials to translate molecular interactions at interfaces created between amphiphiles and planar thin films of LCs into large and readily-observed changes in the sliding times of small aqueous droplets could open the door to a broad range of other new applications for these liquid-infused materials.

Results and Discussion

Our past work demonstrated that the infusion of the thermotropic liquid crystal E7 into hydrophobic and nanoporous polymer coatings fabricated by reactive/covalent LbL assembly can be used to design SLIPS that respond actively to changes in the chemical composition of the contacting liquid (e.g., the presence or absence of surfactants).[11] In that study, we reported that aqueous droplets containing model amphiphiles such as sodium dodecyl sulfate (SDS) slide significantly slower over LC-infused SLIPS compared to droplets that did not contain surfactant, and that the sliding time depended on the structure of the surfactant (e.g., surfactants with longer aliphatic tails were observed to slide more slowly). These differences in sliding times as a function of amphiphile concentration and structure were not observed in otherwise identical SLIPS prepared using isotropic silicon oil, suggesting that the anisotropic nature and molecular orientation of the infused LC played a role in determining sliding speed. These novel behaviors could be technologically useful and could expand the range of potential applications for slippery liquid-infused materials; however, the nanoporous LbL coatings used in that past work have complex architectures that are poorly defined and cannot be readily or predictably tuned. LbL fabrication also requires multistep fabrication procedures that are not readily scalable. To address these challenges, and investigate the extent to which our the observations in our past studies might be generalized more broadly across broader classes of SLIPS, we sought to characterize the single-step infusion of LCs into commercially available porous PTFE membranes used as a matrix for the infusion of isotropic oils to design SLIPS in several past studies.[1, 7]

We first performed a series of experiments to determine whether PTFE membranes could be infused with nematic thermotropic LCs, and whether the resulting LC-infused membranes

were ‘slippery’ and chemically or physically stable upon contact with broad range of liquids. The infusion of thermotropic liquid crystal (E7 unless otherwise noted; the structure is shown in Figure 5) into porous PTFE membranes with pore sizes of 200 nm and thicknesses of 25-51 μm (SEM images showing top-down views of these membranes are shown in Figure 6) resulted in LC-infused SLIPS that allowed aqueous droplets to slide readily on the surface (see Materials & Methods for additional details of these experiments). Figure 1A shows top-down views of a 50 μL droplet of DI water (colored green using food coloring to enhance visual contrast) placed on an LC-infused SLIPS tilted at 20°; the droplet was observed to slide over a length of 4 cm in ~4 seconds. LC-infused SLIPS were also stable when contacted with a broad range of chemically complex liquids, including unfiltered lake water, acidic (1M HCl) and alkaline media (1M NaOH), skim milk, and human urine. As shown in Figure 7, 50 μL droplets of these different liquids placed on LC-infused SLIPS tilted at 20° were also observed to slide over a length of 4 cm in ~4 seconds, similar to the behaviors of droplets of MiliQ water.

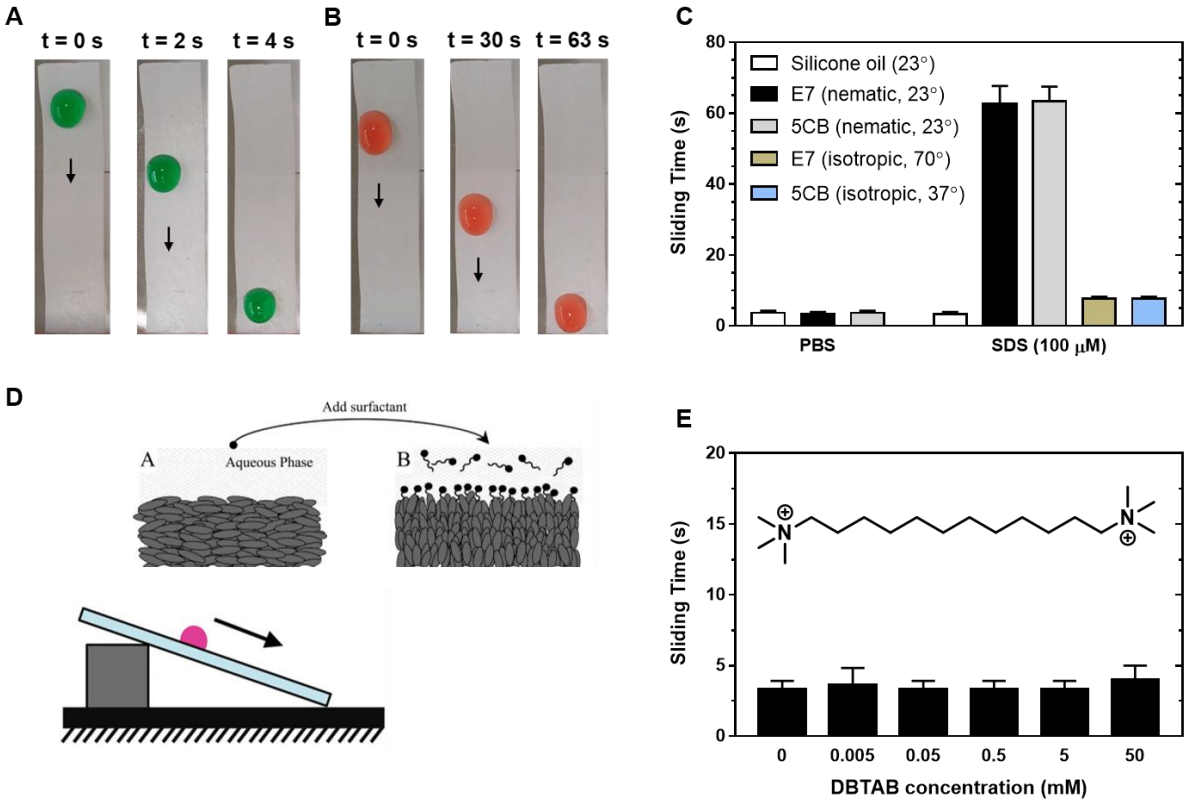

Figure 1. A,B) Images showing 'top-down' view at different time points of (A) a 'bare' PBS droplet (colored green) and B) a PBS droplet containing 100 μM SDS (colored orange) sliding down a E7-infused SLIPS tilted at 20°. C) Plot showing sliding behaviors of droplets of PBS or PBS droplets containing SDS (100 mM) on PTFE membranes infused with silicone oil (white), thermotropic liquid crystals E7 (black) and 5CB (gray) at 23° C, the gold bar and light blue bar shows the sliding behavior of an PBS droplet containing SDS on E7-infused SLIPS equilibrated at 70 °C and 5CB-infused SLIPS equilibrated at 37 °C respectively; results are expressed as the time required for a 50 μL droplet to slide 4 cm on a 'slippery' surface tilted at 20 °C. (D) Schematic showing the test format (pink colored droplet sliding down an inclined LC-SLIPS (colored light blue) and the consequent changes in the anchoring of LCs from planar to homeotropic upon adsorption of surfactant from the droplet to the droplet-SLIPS interface. (E) Plot showing the sliding time of 50 μL DBTAB (0.005–50 mM) -containing PBS droplets on E7-infused SLIPS tilted at 20°.

Previously, we reported that surfactant-containing droplets slide on LC-infused SLIPS fabricated by infusion of E7 into nanoporous PEI-PVDMA multilayer films more slowly compared to droplets of DI water, For example, 10 μL droplets of DI water containing 0.4×10^{-3} M SDS slid 10 times (~49 s) slower than DI water droplets that did not contain surfactant (~5

s).[11] We observed similar differences in the sliding behaviors of water droplets on LC-infused SLIPS fabricated by infusion of E7 into commercially available PTFE membranes. SDS-containing droplets were observed to slide very slowly compared to DI water droplets. As shown in Figure 1B, a water droplet containing 100 μM SDS (colored orange) slid over a length of 4 cm in ~63 s, compared to the ~4 s time required for a MiliQ droplet that did not contain surfactant (~4 s; Figure 1A). We also measured the sliding times of SDS-containing droplets on E7-infused SLIPS maintained at 70 °C, a temperature well above the nematic/isotropic transition temperature (~60 °C) of E7. As shown in the Figure 1C (yellow bar) the SDS-containing droplets slid over a length of 4 cm in ~8 s, a time that is significantly faster than that observed on surfaces infused with E7 in the nematic phase (~63 s). Additional experiments using SLIPS fabricated by the infusion of the nematic liquid crystal 5CB instead of E7 into PTFE membranes revealed similar results (the structure of 5CB is shown in Figure 5). As shown in Figure 1C, a 100 μM SDS-containing droplet slid ~15 times slower (~63 s) on 5CB-infused surfaces compared to droplets of DI water (~4 s). However, for 5CB-infused SLIPS maintained at 37 °C, a temperature above the nematic/isotropic transition temperature (~35 °C) of this LC, SDS-containing droplets slid appreciably faster (blue bar; ~8 s) (Figure 1C). Lastly, as also shown in Figure 1C, we note that no differences in sliding speed were observed between SDS-containing droplets and droplets of DI water on PTFE membranes infused with silicone oil, a model isotropic oil (both types of droplets slide over a length of 4 cm in ~4 s).

Overall, the results of these experiments demonstrate that the novel responsive sliding behaviors observed in our past studies using LbL coatings are preserved when using more well characterized commercial porous PTFE membranes. When combined, our results support the hypothesis that this behavior is the result of the properties of the infused LC, and not a result of

other unappreciated physical or chemical synergistic interactions between the LC and those more complex coatings. These results also suggest the possibility that the infusion of LCs could be used more generally to impart responsive behavior in SLIPS fabricated using a variety of other well-known hydrophobic matrices used to fabricate slippery surfaces in past studies.[10] We note that the results discussed above also suggested that the large differences in the sliding behaviors of droplets observed in the presence or absence of surfactant are the result of the anisotropic nature of the LC.

It is well understood that thermotropic LCs such as E7 and 5CB adopt so-called homeotropic anchoring when hosted at LC-air interfaces (i.e., the mesogens are generally aligned perpendicular to the interface), and that they adopt so-called planar anchoring when hosted at interfaces created between LCs and aqueous solutions (i.e., the mesogens are generally aligned parallel to the interface).[18-19] In addition, previous studies have reported that adsorption of surfactants such as SDS at aqueous/LC interfaces can result in an orientational transition in the anchoring of LCs from planar to homeotropic orientations at the interface.[18-19] In the experiments reported above, a droplet of DI water placed on an LC-infused SLIPS results in the formation of an aqueous/LC interface, at which the LCs near the interface would be expected to exhibit planar anchoring. A droplet of water containing a surfactant also results in the formation of an aqueous/LC interface; however, the surfactant molecules in the droplet should also adsorb at the aqueous/LC interface and promote homeotropic anchoring. We speculate that the slower sliding speeds of surfactant-containing droplets shown in Figure 1B result from dynamic changes in the anchoring of the LCs (see schematic in Figure 1D) as aqueous/LC interfaces are formed and surfactant adsorbs there. We note that we were unable to characterize the orientation of the LCs in the experiments described above using polarized light microscopy,[20] methods commonly used

to characterize the orientation of LCs at LC/aqueous interfaces, because of the complexities of the system used here, including the opacity and thickness of the PTFE membranes. We note further, however, that the results reported above, when combined with those of our past studies in LC-infused LbL coatings and the results reported below involving surfactants with different tail length and head groups, are consistent with this hypothesis.

We anticipate that any potential changes from planar to homeotropic anchoring that occur at aqueous/LC interfaces created by contact with aqueous droplets containing surfactant would occur and form continuously at that interface as the droplet slides along the surface. We did not observe changes in the velocities of droplets as they slid along LC-infused surfaces, providing general support for this hypothesis. It is, of course, possible that the concentration of surfactant in an aqueous droplet could become depleted if some of it remains bound at air/LC interfaces created in areas behind a sliding droplet (that is, sliding droplets could leave behind 'trails' of adsorbed surfactants as they move across a surface, which would result in a concomitant reduction in surfactant concentration in the droplet). We did not measure changes in surfactant concentration in the droplets in the studies performed here, and if surfactant depletion does occur, it did not occur to extents that resulted in significant changes in droplet sliding velocities at the surfactant concentrations and path lengths evaluated in the experiments above. We do note, however, that droplets of DI water placed on surfaces previously exposed to SDS-containing droplets were observed to slide over a distance of 4 cm over ~7 s, a time that is slower than the sliding times of DI water droplets on fresh LC-infused PTFE membranes that were never exposed to surfactant-containing droplets (~4 s, as described above). This difference in sliding speeds is generally consistent with the view that surfactant from sliding droplets could remain at LC/air interfaces after surfactant-laden droplets have moved along the surface. We note further

in this context that the sliding times of DI water droplets on 'used' LC-infused SLIPS returned to values of ~4 s that were indistinguishable from freshly-prepared surfaces after 'rinsing' with 3-5 additional droplets of DI water. This result suggests that the adsorption of surfactant, to whatever extent it may occur, is reversible. In general, we found it possible to use, rinse, and reuse these LC-infused SLIPS multiple times with no observable changes in subsequent droplet sliding behaviors.

To investigate further the role that homeotropic anchoring of LC may play in influencing droplet sliding speeds, we also evaluated the sliding speed of a aqueous droplets containing the cationic bolaform surfactant dodecyl-1,12-bis(trimethylammonium bromide) (DBTAB; structure shown in Figure 1E). DBTAB adopts a looped configuration at oil/water interfaces and has a much higher limiting surface area (~107 $Å^2$ at an air-water interface) compared to the limiting surface area of analogous classical surfactants (e.g., ~63 $Å^2$ for DTAB) that adopt tilted configurations at air/water interfaces.[18] Previously, it was reported that DBTAB promotes planar, rather than homeotropic, anchoring of 5CB at aqueous/LC interfaces at concentrations ranging from 0.01 mM to 100 mM. We found aqueous droplets containing between 5 μM to 50 mM DBTAB to slide over a distance of 4 cm in ~3 seconds (Figure 1E), a sliding time comparable to those of droplets of DI water alone, and a time that is substantially faster than those of droplets containing SDS. The concentrations evaluated here span the CMC of DBTAB (20-50 mM) and are above the observed onset of surface activity of DBTAB at the air-water interface (< 1 mM).[18] These results provide further support for the view that the large differences in sliding speeds observed for droplets containing single-tailed surfactants such as SDS and DTAB result from dynamic and surfactant-induced changes in the orientation of the LC from planar to homeotropic in regions of the SLIPS interface that are in contact with the droplets.

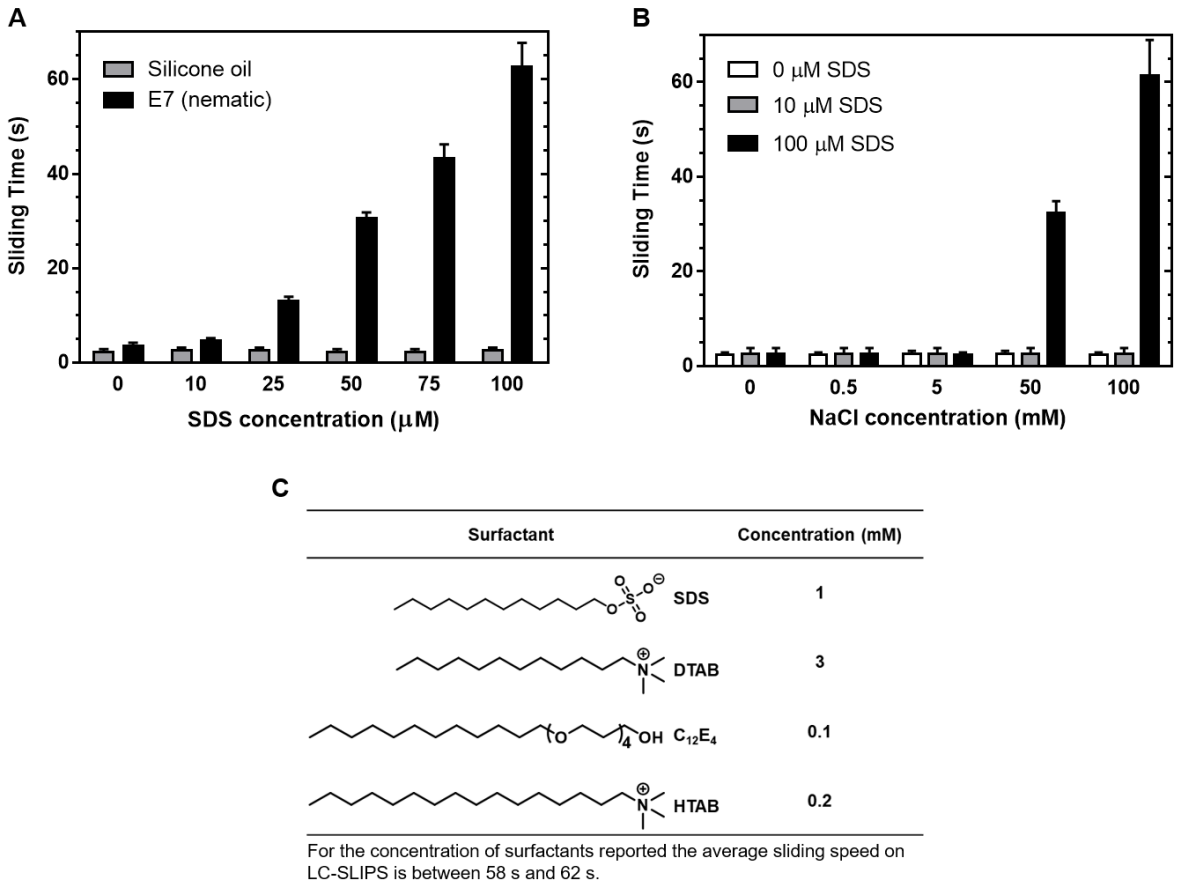

Figure 2. (A) Plot showing the sliding time of 50 μL droplets of PBS containing SDS (0–100 μM) on silicone oil-infused SLIPS (gray) and E7-infused SLIPS (black) tilted at 20°. (B) Plot showing the Influence of NaCl concentration (0 to 100 mM) on the sliding time of SDS-containing droplets; concentration of SDS was fixed at 0 μM (white), 10 μM (gray), and 100 μM (black). (C) Concentration of various surfactant in MiliQ for which the average sliding speed of 50 μL droplets is between 58 to 62 s on E7-infused SLIPS tilted at an angle of 20°.

The results of additional experiments characterizing the influence of surfactant concentration, salt concentration, and surfactant structure on the sliding speeds of surfactant-containing droplets on LC-infused SLIPS (Figure 2) were also consistent with the proposition that orientational changes in the anchoring of LCs can influence droplet sliding behaviors. Past studies have established that the anchoring of LCs at aqueous/LC interfaces is influenced strongly by the areal density of the surfactant molecules adsorbed at the interface and interactions between the surfactant tails and LCs. It has been demonstrated using 5CB-water

interface that, with increasing surfactant concentration, the limiting areal density of surfactant tails at the interface increases and results in homeotropic alignment of the LC. Similar results were obtained by increasing the electrolyte concentration, which can screen electrostatic repulsion between charged surfactant head groups. Finally, it has also been reported that the nature of the hydrophilic head group of the surfactant and the aliphatic chain length of the surfactant tail group can impact the limiting areal density of a surfactant at the air-water or oil-water interfaces.

As shown in Figure 2A (black bars), we observed that droplet sliding speeds increase substantially as the concentration of SDS in droplets of phosphate-buffered saline (PBS) was increased from 0 μM to 100 μM. Droplets of PBS containing 10 μM SDS slid rapidly (over ~5 s) over a distance of 4 cm on LC-infused SLIPS, whereas droplets containing 100 μM SDS exhibited sliding times of ~62 s. The sliding times of SDS-containing droplets exhibited uniform sliding times of ~3 s on control membranes infused with silicone oil regardless of SDS concentration (Figure 2A; gray bars).

Experiments using droplets containing a fixed concentration of SDS with different concentrations of electrolyte (NaCl) revealed manipulation of electrolyte concentration to also impact the sliding speed of surfactant-containing droplets on LC-infused SLIPS. As shown in Figure 2B, for 100 μM SDS solutions in MiliQ water (black bars), the addition of 100 mM NaCl increases the sliding time (~62 s) by 15 times compared to the addition of 0.5 mM NaCl. Varying the NaCl concentration of water droplets free of SDS over this same concentration range did not impact droplet sliding speeds (Figure 2; white bars). The sliding speeds of SDS-containing droplets were also not sensitive to NaCl concentration at lower concentrations of SDS (e.g., 10 μM; Figure 2; gray bars).

Finally, we investigated the influence of surfactant head and tail group structure on droplet sliding speeds using SDS, DTAB, HTAB, and the non-ionic surfactant $C_{12}E_4$ (structures shown in Figure 2C). For these experiments, we prepared surfactant solutions in DI water to decouple the impact of surfactant structure from that of salt concentration. We measured the sliding speeds of droplets containing different concentrations of these surfactants and identified the concentrations of each surfactant that resulted in average sliding speeds between 58 s – 62 s (Figure 2C). For surfactants with different head groups [SDS (anionic), DTAB (cationic), and $C_{12}E_4$ (non-ionic)] but identical aliphatic tail lengths (12 carbons), the measured concentrations were 1 mM, 3 mM, and 0.1 mM respectively. These values correlate with the limiting areal densities of these surfactants at air-water interfaces.[18] Finally, we also tested the impact of change in the aliphatic tail length keeping the similar headgroup on sliding speed of a surfactant-containing droplet on LC-SLIPS. Figure 2C also shows a comparison of results for DTAB and HTAB, which possess identical cationic head groups but different aliphatic tail lengths. The concentration of HTAB required to achieve an average sliding speed of ~60 s (200 μM) was found to be 15 times lower than that for DTAB (3 mM). This result is consistent with those of our past study using LC-infused LbL coatings and, more broadly, with the fact that as the alkyl chain length of a surfactant increases, the limiting areal density decreases and the longer alkyl chain lengths can penetrate deeper in the E7, which should lead to anchoring of E7 closer to the normal at aqueous/LC interfaces. Finally, we note that the addition of 100 mM NaCl to solutions of $C_{12}E_4$ did not result in changes to droplet sliding speeds, consistent with the fact that $C_{12}E_4$ is a non-ionic surfactant and, thus, the limiting areal density should not be affected by the addition of electrolyte.

Past studies have demonstrated the potential of different LC-based materials platforms, including both planar LC/air and aqueous/LC interfaces and aqueous LC emulsions comprised of free-floating micrometer-scale LC droplets suspended in water, to sense and report on the presence of different environmental amphiphiles (such as lipids, proteins, and surfactants) with remarkable sensitivity[21-23]. In past studies, changes in LC orientation promoted by the adsorption of amphiphiles was characterized using polarized light microscopy or by changes in the forward- and side-scattering of light using flow cytometry. Both of these analytical methods are effective, but also require specialized and expensive instrumentation and, in general, some degree of technical knowledge to interpret the sometimes complex results that arise from them. The LC-infused SLIPS reported here offer a new platform that translates factors that promote changes in the anchoring of LCs at aqueous interfaces (e.g., the presence of an amphiphile) to other readily observable macroscale phenomena (e.g., the rate at which a droplet of water slides across a surface).

The large and substantial differences in the sliding speeds of surfactant-containing and non-surfactant-containing droplets provide a straightforward and visual, ‘naked-eye’ approach for the detection of surfactants or other amphiphilic contaminants in aqueous environments. In the limit of high amphiphile concentration, this approach would require no special equipment or expertise to interpret (e.g., a droplet sliding over a short distance within 4 seconds can be readily distinguished from a droplet that requires 1 min to traverse the same distance). However, because sliding speeds are also observed to vary as a function of surfactant concentration (Figure 2A), it is possible that this approach could also be used to provide estimates of the concentration of an analyte in an aqueous solution using equipment as basic as a stopwatch, or by using computer image analysis. We note further in this context that, in cases where differences in droplet sliding

speeds may be difficult to distinguish, differences can be further magnified or enhanced by varying several simple extrinsic parameters, such as sliding angles or droplet volumes, that also have impacts on sliding speed. For example, the difference in sliding time (Δt) between a 50 μL droplet of DI water and a 50 μL droplet of water containing 100 μM SDS increases from 5 s to 70 s when the sliding angle of the SLIPS surface is reduced incrementally from 23° to 17°. Similarly, decreasing the volume of a droplet of water containing 100 μM SDS from 60 μL to 40 μL magnifies the difference in sliding time (Δt) from 8 s to 40 s. (Figure 8) We note that for charged surfactants, the results discussed above (Figure 2B) suggest that the detection limits of LC-infused SLIPS can also be manipulated or enhanced by modifying electrolyte concentration. When combined, modifications to both extrinsic and intrinsic parameters can be varied to increase or decrease droplet mobility and, in turn, influence the sensitivity of the response of an LC-infused SLIPS surface. Finally, as discussed above, because changes in LC anchoring on LC-infused SLIPS are transient and reversible, these materials also have the potential to be used and reused multiple times without affecting droplet sliding behaviors.

To demonstrate proof of concept and explore the potential of this approach to naked-eye detection, we performed a series of additional experiments to determine whether measurements of droplet sliding times on LC-infused PTFE membranes could be used to identify the presence of amphiphilic compounds produced by *Pseudomonas aeruginosa,* a bacterial pathogen that uses the amphiphilic small-molecule *N*-oxo-dodecanoyl-L-homoserine lactone (OdDHL) to regulate its quorum sensing (QS) system, and thus important group behaviors such as biofouling and the production of other amphiphilic toxins, such as rhamnolipids, that are detrimental in environmental and healthcare settings. We recently reported that free-floating microscale droplets of 5CB suspended in aqueous media can be used to detect and report the presence of

biologically-relevant concentrations of OdDHL and rhamnolipids, as well as an amphiphilic precursor to the biosynthesis of rhamnolipids [3-(3-hydroxyalkanoyloxy)alkanoic acid, (HAA)], using polarized light microscopy and flow cytometry (see Figure 9A for the structures of these amphiphiles).[24] The experiments described here sought to determine whether the amphiphilicity of these compounds could provide a basis for unaided, or naked-eye, detection of these bacterial products by simple measurement of the sliding speeds of droplets obtained from cultures of *P. aeruginosa* on the surfaces of LC-infused SLIPS.

We performed a series of initial experiments to measure the sliding times of 50 μL droplets containing known concentrations of OdDHL (over the range of 25–150 μM), rhamnolipids (over the range of 12.5–50 μg/mL), and HAA (over the range of 6.25-25 μg/mL) on E7-infused SLIPS (Figure 9). The concentration ranges used in these experiments were selected to encompass the range of biologically relevant concentrations of these amphiphiles. We also performed experiments using C4-AHL (over the range of 1-1000 μM), a short-tailed and non-amphiphilic analog of OdDHL that is also used by *P. aeruginosa* to regulate the production of rhamnolipids. Solutions of C4-AHL, OdDHL, and HAA were prepared in PBS containing 1% (v/v) DMSO to enhance solubility. Inspection of the results in Figure 9A-B shows that differences in the sliding times of C4-AHL and OdDHL-containing droplets compared to those of control droplets (containing only PBS and 1% DMSO) were not significant over the range of concentrations used here. Further inspection of these results, however, reveals substantial differences in the sliding times of droplets containing HAA (at concentrations ≥12.5 μg/mL, Figure 9C) or rhamnolipids (at concentration ≥ 25 μg/mL; Figure 9D) compared to control droplets. For example, droplets containing 12.5 μg/mL HAA or 25 μg/mL rhamnolipids slid over a distance of 4 cm in ~18 s or ~39 s, respectively, compared to control droplets, which slid much

more rapidly over the same distance (in ~3 s; SLIPS were maintained at a common sliding angle of 20° for these experiments). These results demonstrate that measurements of droplet sliding times on LC-infused SLIPS can be used to report on the presence (or absence) of QS-controlled amphiphiles such as HAA and rhamnolipids in aqueous solutions.

Past reports demonstrate that LC interfaces decorated by surfactants at concentrations incrementally below the threshold of what is required to promote a change in the anchoring of the LCs can be used to report on the presence of other, secondary amphiphilic species at lower concentrations than would be required using 'clean' or 'bare' LC interfaces. We reasoned that the sensitivity of droplet sliding times to rhamnolipid concentrations could be increased by preparing rhamnolipid solutions using water or buffer also containing low concentrations of SDS. To explore the potential of this approach, we prepared solutions of at different concentrations of rhamnolipid also containing 4 μM SDS (a concentration that, by itself, does not change the sliding time of an aqueous droplet significantly relative to DI water; see Figure 2A and the discussion above) and measured the sliding times on E7-infused SLIPS. As shown in Figure 10, the addition of low concentrations of SDS resulted in a two-fold reduction of the limit of detection for rhamnolipids, from 20 μg/mL to 10 μg/mL. We did not further optimize the conditions used here or explore the lower limit of detection that is possible using this approach. These results do suggest, however, other straightforward practical means, in addition to those described above, by which differences in droplet sliding times can be manipulated or magnified to enhance the potential utility of LC-infused SLIPS in the context of sensing.

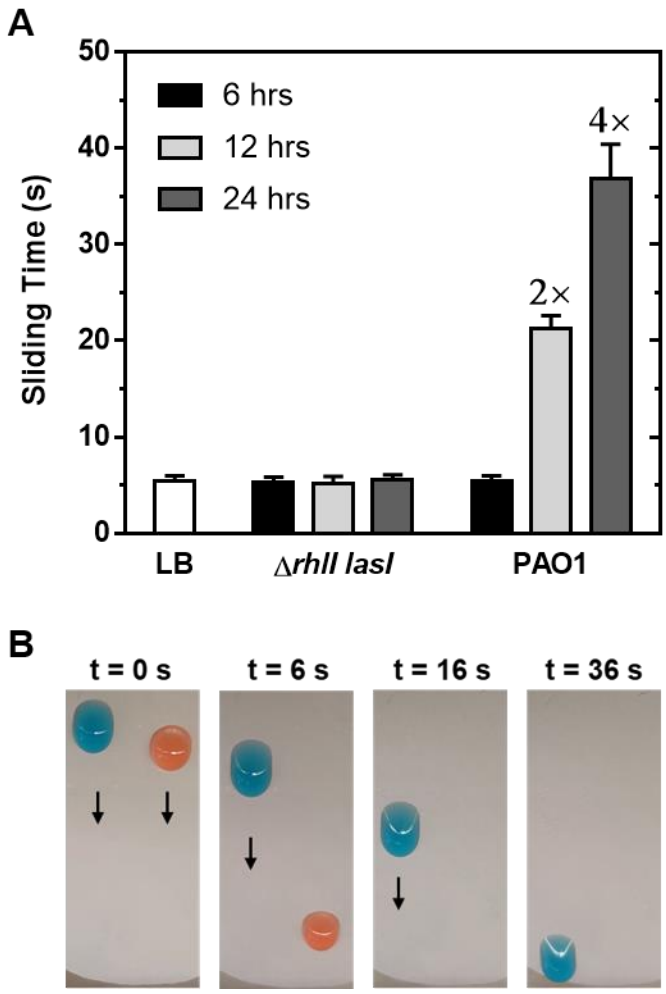

Figure 3. Surfactant in *P. aeruginosa* cell culture can be detected with LC-SLIPS. (A) Sliding time of LB media, *P. aeruginosa* WT (PAO1), and QS-mutant (*ΔrhlI lasI*) cell culture at 6 h (black), 12 h (light gray), and 24 h (dark gray). (B) Top-down view of WT *P. aeruginosa* culture (blue) and QS- (*ΔrhlI lasI*) (orange) at 0 s, 6 s, 16 s, and 36 s. Colors added with food coloring.

We next performed a series of biological experiments to determine whether LC-infused SLIPS could be used to monitor the production of these amphiphiles in live cultures of bacteria. For these experiments, we cultured two different strains of *P. aeruginosa*: WT and Δ*lasI rhlI* (the latter is a genetic mutant strain of *P. aeruginosa* lacking genes critical to QS and that is thus unable to produce QS-associated virulence factors, including rhamnolipids and HAA). These experiments were performed in LB culture medium with shaking at 37 °C (see Materials &

Methods for additional details). Aliquots of these bacterial cultures were removed at pre-determined time points (6, 12, and 24 hours) and the times required for 35 μL droplets of these samples to slide a distance of 4 cm at 20° incline were measured. Inspection of Figure 3A reveals that droplets of cultures of the Δ*lasI rhlI* mutant had short sliding times (~5 s) at incubation times of 6, 12, and 24 hours that were indistinguishable from the sliding time of LB medium alone. This result is consistent with the fact the Δ*lasI rhlI* mutant lacks genes critical to QS and, thus, is unable to produce either HAA or rhamnolipids. Further inspection of Figure 3A also reveals droplets taken from cultures of the WT mutant after 6 hours of incubation to slide rapidly (over ~5 s). This result is consistent with the observation of low, sub-quorate populations of bacteria at this early time point that are unable to produce HAA or rhamnolipids.

In contrast, droplets taken from cultures of the WT mutant after 12- and 24-hours of incubation did not slide on LC-infused SLIPS and, instead, spread on the surfaces of these materials, likely due to the presence of a significantly higher concentration of QS-controlled surfactants. Further dilution of these samples with LB medium reduced droplet spreading and enabled meaningful measurements of sliding times. Two-fold dilution of samples taken at 12 hours of incubation of the WT strain resulted in droplet sliding times of ~21 s (Figure 3A). Samples taken after 24 hours of incubation, which would be expected to contain higher concentrations of HAA and rhamnolipid, required additional dilution; four-fold dilution with LB medium yielded sliding times of ~36 s.

Overall, these results are consistent with an increase in the concentration of these QS-controlled amphiphiles in WT *P. aeruginosa* cultures over time. Additional experiments were performed using two other mutant strains (Δ*rhlA* and Δ*rhlB*) that lack functional proteins in the rhamnolipid biosynthetic pathway (Figure 11). RhlA is upstream of RhlB, so the Δ*rhlB* mutant

accumulates the intermediate HAA and does not convert it into rhamnolipids, while both HAA and rhamnolipid production is abrogated in Δ*rhlA*. As expected, droplets of Δ*rhlA* collected after different incubation periods of 6, 12 and 24 hours exhibited sliding times on LC-infused SLIPS that were fast and indistinguishable from those of LB medium alone (~5 s), whereas an increase in sliding times was observed in samples collected from Δ*rhlB* cultures at 12 and 24 hours, likely due to the presence of HAA (Figure 11). When combined, the results of these experiments demonstrate that measurements of the sliding times of droplets of bacterial cultures on LC-infused SLIPS can be used to identify the presence of two amphiphilic virulence factors (rhamnolipids and HAA) in cultures of *P. aeruginosa* and, in particular, distinguish between and monitor changes in the growth of sub-quorate and quorate populations of this human pathogen (see Figure 3B). Collectively, the results generated using the mutants described above also demonstrate that changes in droplet sliding times reported here are the result of the production of HAA and rhamnolipid, and not the result of other compounds produced by bacteria under these growth conditions.

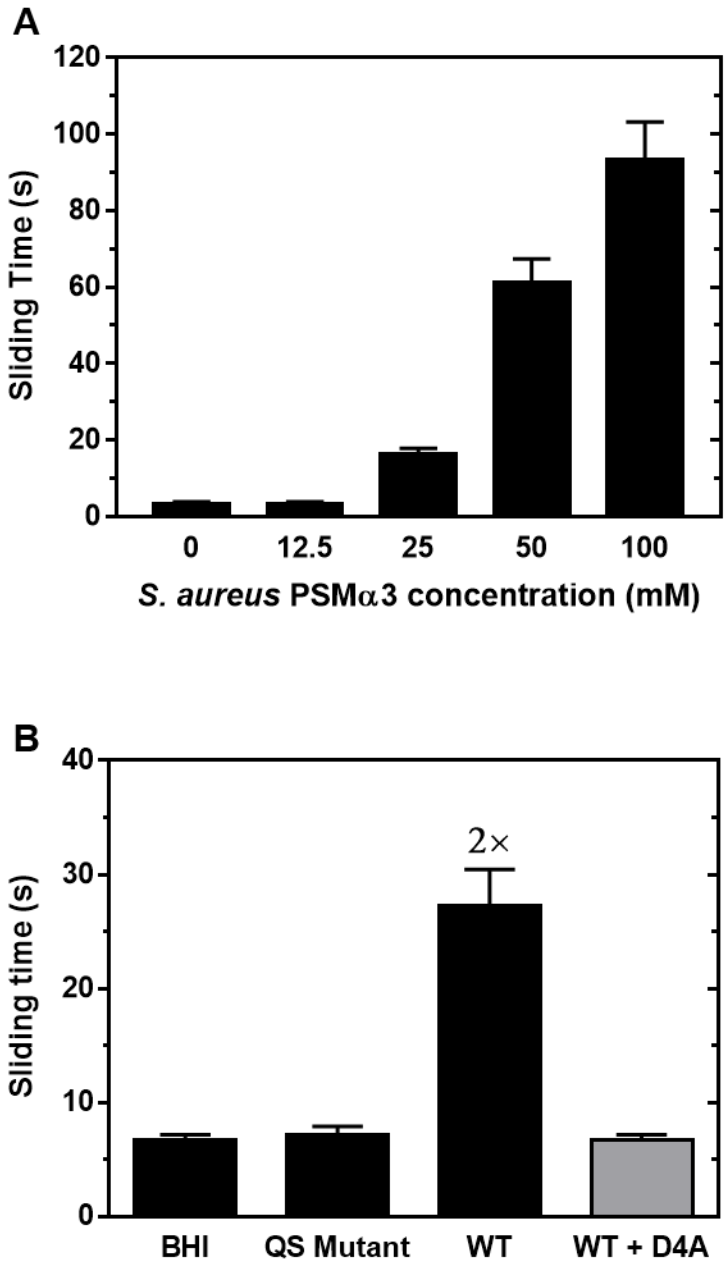

Figure 4. *S. aureus* products increase sliding time on LC-SLIPS. (A) Sliding time of purified *S. aureus* PSMα3 at 0 mM, 12.5 mM, 25 mM, 50 mM, and 100 mM. (B) Sliding time of BHI media 24-hour cultures of *S. aureus* QS mutant, WT (2x diluted), and WT + D4A (QS inhibitor).

We performed a final series of experiments to determine whether the results reported above could be used to identify the presence of amphiphilic toxins produced in another pathogen—namely, *Staphylococcus aureus*, a notorious Gram-positive human pathogen. It is

well known that *S. aureus* produces a family of amphiphilic peptides known as phenol soluble modulins (PSMs), also under the control of QS.[25] Amphiphilic peptides differ substantially in structure from single-tailed surfactants, and it was not clear at the outset of these studies whether PSMs could adsorb at aqueous/LC interfaces and change the anchoring of LCs. We therefore prepared solutions of PSMα3, one of several PSMs produced by *S. aureus*, at concentrations ranging from 12.5 mM to 100 mM in PBS and measured the sliding times droplets of these solutions on LC-infused SLIPS. As shown in Figure 4A, the sliding times of PSM-containing droplets increased substantially with an increase in the concentration of PSM in the droplet. For example, the sliding time increased from ~16 s to ~93 s with an increase in the concentration of PSMα3 from 25 mM to 100 mM (Figure 4A), suggesting that PSMs can interact with LCs and induce changes in the anchoring of LCs in ways that, at the least, lead to large changes in sliding behaviors that are similar to those observed above using conventional surfactants.

We performed additional experiments to determine whether LC-infused SLIPS could report detect the presence of PSMs in live cultures of *S. aureus* and, thereby, provide methods to monitor QS in communities of this pathogen. For these experiments, we cultured an *S. aureus* WT strain and a QS mutant strain (lacking AgrBD, proteins critical for QS) for 24 hours and measured the sliding times of the droplets of the culture on LC-infused SLIPS (additional details of these experiments can be found in the Materials & Methods). Droplets obtained from cultures of the WT strain slid significantly more slowly (over ~24 s) compared to droplets obtained from cultures of the QS mutant strain (~7s), consistent with the expected presence of PSMs in the WT culture (see Figure 4B). To provide support for this conclusion, we also performed experiments in which we added a known inhibitor of QS (AIP-III D4A, at a concentration of 1 μM) to cultures of the WT *S. aureus* strain and measured the sliding time of the culture after 24 hours.

AIP-III D4A has been demonstrated to fully inhibit *S. aureus* QS at concentrations ≥ 1 nM.[26] We observed the sliding speeds of droplets of cultures incubated in the presence of this inhibitor to be comparable (~7 s) to those of the QS mutant (see Figure 4B). Taken together, these results demonstrate that readily observed changes in the sliding times of droplets of *S. aureus* cultures can be used to identify the presence (or absence) of inhibitors of QS. These results thus also suggest a potential basis for the development of straightforward droplet-based bio-analytical screening assays that could be used as a tool to identify new synthetic inhibitors of bacterial QS. Experiments to this end are currently underway and will be reported in due course.

Materials and Methods

Materials. Sodium dodecyl sulfate (SDS, ACS grade, ≥99.0%), dodecyltrimethylammonium bromide (DTAB, ≥98.0%), hexadecyltrimethylammonium bromide (HTAB, ≥98.0%), silicone oil (η = 50 cSt), Brij 30 ($C_{12}E_4$), sodium chloride (NaCl, ACS grade, ≥99.0%) and N-oxo-dodecanoyl-L-homoserine lactone (OdDHL) were obtained from Millipore Sigma (Milwaukee, WI). The thermotropic liquid crystals 5CB and E7 were purchased from Jiangsu Hecheng Display Technology Co. (Jiangsu, China). Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate; pH 7.4) was prepared from OmniPur® 10× concentrate (Millipore Sigma, Milwaukee, WI). Unlaminated Teflon membrane filters (pore size = 0.2 μm, thickness = 25-51 μm) were purchased from Sterlitech Corporation (Kent, WA). Eutrophic lake water was locally sourced from Lake Mendota, Madison, WI. Nature's Touch skim milk was purchased from Kwik Trip (Madison, WI). Pooled human urine was purchased from Innovative Research Inc. (Novi, MI). Luria-Bertani medium (LB), Lennox formulation, was purchased from Research Products International (Mt. Prospect, IL). Brain heart infusion (BHI) medium was

purchased from Teknova (Hollister, CA). N-Butanoyl-L-homoserine lactone (BHL) was purchased from Cayman Chemical (Ann Arbor, MI). Dodecyl-1,12-bis(trimethylammonium bromide) (DBTAB) was a kind gift from Prof. Nicholas L. Abbott (Cornell University, Ithaca, NY). The phenol-soluble modulin PSM-alpha-3 was a kind gift from Prof. Samuel H. Gellman (UW-Madison, Madison, WI). Rhamnolipids (90% pure) were obtained from AGAE technologies (Corvallis, OR). 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA), [24] and AIP-III D4A[26] were synthesized according to previously reported methods. Water with a resistivity of 18.2 MΩ was obtained from a Millipore filtration system. All materials were used as received without further purification unless otherwise noted.

General Considerations. Scanning electron micrographs were acquired using a LEO 1550 scanning electron microscope at an accelerating voltage of 3 kV. Samples were coated with a thin layer of gold using a gold sputterer operating at 45 mA under a vacuum pressure of 50 mTorr for 1 min prior to imaging. Digital photographs and videos were acquired using a Samsung Galaxy S7 smartphone. Sliding time data were analyzed using Microsoft Excel and plotted using GraphPad Prism 7 (version 7.0h). For measurements of OD600 used to monitor cell growth, 200 μL of cell suspensions were added to a clear-bottomed 96-well plate (Corning 3370) and absorbance was measured at a wavelength at 600 nm using a Synergy 2 plate reader (Biotek) with Gen5 1.05 software. When OD600 was found to be above 1.0, the cell suspensions were diluted accordingly using fresh media until OD600 was in a readable range (< 1.0).

Preparation of SLIPS. SLIPS were prepared by depositing a lubricating liquid (5CB, E7, or silicone oil) on the top surface of a porous polymer membrane (supported on a glass slide) using

a pipette. The lubricating liquid was then spread using tweezers to form a uniform over-coated layer. Samples were allowed to stand for several minutes to allow the liquid to infuse into the porous membrane (evident by a visual change in the opacity of the membrane) through capillary wicking. The excess liquid was then removed from the surface by dabbing with weighing paper.

Characterization of Droplet Sliding Speeds. Characterization of the sliding speeds of droplets placed on the surfaces of LC-infused SLIPS was performed in the following general manner. LC-infused SLIPS were placed on a custom-made stage and the stage was attached to the moving arm of a digital protractor using binding clips. The digital protractor was set at a specified sample angle, and a pre-determined volume of aqueous solution was placed as a droplet on the surface of the liquid-infused surface. Sliding droplets were recorded on digital video, and the time required for droplets to slide a distance of 4.0 cm along the surface was measured using a digital timer. In some cases, aqueous solutions were prepared using food coloring to enhance visual contrast of the sliding droplets. For characterization of the sliding speeds of different bacterial strains, three biological replicates were performed. After each measurement, the surface was washed by depositing multiple droplets of MiliQ water and allowing them to slide down the surface until the sliding time of the water droplets returned to a value of ~3 s. For each surfactant solution, the sliding times of at least 3-5 droplets were measured and used to calculate an average sliding time with standard deviation. Each experimental series was performed on a common LC-infused slippery surface, with appropriate experimental controls to prevent variability in sliding time measurements between different LC-infused surfaces.

Bacterial Strains and Growth Conditions. All bacteria were grown at 37 °C, with shaking at 200 rpm. *S. aureus* was cultured in BHI medium; all other species were grown in LB medium. For all strains, an overnight culture was grown in a 15 mL glass tube (no more than 2 mL of culture) or a 25 mL Erlenmeyer flask (no more than 5-10 mL) to allow for sufficient aeration. For experiments using *S. aureus*: In a 25 mL Erlenmeyer flask, 50 µL of overnight culture (6390 or 9222) was added to 5 mL of BHI (1:100 dilution), and AIP-III D4A (if applicable) was added to achieve a final concentration of 1 µM. DMSO was added as a vehicle control (no greater than 2% final concentration) to cultures not containing this peptide. For experiments using *P. aeruginosa:* Bacteria were grown as reported previously.[24] Briefly, a culture of overnight bacteria was diluted 1:100 in 75mL of fresh LB medium and shaken for 24 h, unless otherwise specified. To induce RhlR phenotypes in PAO-SC4 (Δ*lasI rhlI*), a final concentration of 200 µM BHL was used. DMSO (no greater than 2%) was added to cultures as a vehicle control for experiments not involving AHL.

*note: WT (mPAO1) was used for all tests described in the text as "PAO1." PAO1-T was tested and showed no appreciable difference to mPAO1 (data not shown)

Table 1. Bacterial strains and plasmids.

	Referred to herein	**Genotype**	**Reference or Source**
Staphylococcus aureus			
RN6390b	*S. aureus* WT	Wild type, *agr* group I (NTCC8325 cured of prophages[27])	Novick[28]
RN9222	QS mutant	RN6911 with pRN7035	Lyon et al.[29]
RN6911	N/A	*tetM::agr*, from RN6390	Novick et al.[27]
Plasmid			
pRN7035	QS mutant	*agrCA* and *agr-P3::blaZ* fusion	Lyon et al.[29]
Pseudomonas aeruginosa			
PAO1	N/A	Wild type, isolated from wound	Holloway[30]
mPAO1	PAO1, WT	Wild type, derivative of Holloway's isolate	Gift from E.P. Greenberg[24]
PAO1-T	N/A	Wild type, derivative of Holloway's isolate	WT from PA two-allele library[31-32]
PAO-SC4	Δ*lasI rhlI*	*lasI rhlI* in-frame deletions	Gift from E.P. Greenberg[24]
PAO1 Δ*rhlB*	Δ*rhlB*	Unmarked, in-frame *rhlB* deletion	Smalley et al.[33]
PAO1-T Δ*rhlA* (PW6886)	Δ*rhlA*	*rhlA*-E08::IsphoA/hah	PA two-allele library[31-32]

Acknowledgments

Financial support for this work was provided by the NIH (R21 AI135745). K. Nyffeler was supported in part by the UW−Madison NIH Chemistry−Biology Interface Training Program (T32 GM008505); K. Nyffeler was also supported as part of a seed award from the UW–Madison NSF MRSEC *(*DMR-1720415*).* We acknowledge the Manoil lab (U Washington) for the transposon strains used in this work (NIH P30 DK089507).

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Supplemental Information

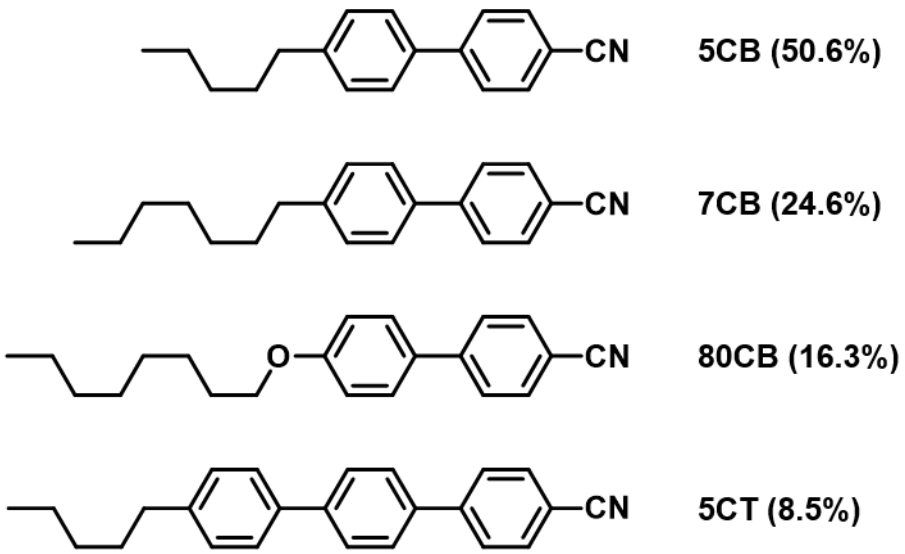

Figure 5. Thermotropic liquid crystal E7 is a proprietary combination of four different liquid crystals - 5CB, 7CB, 80CB, and 5CT.

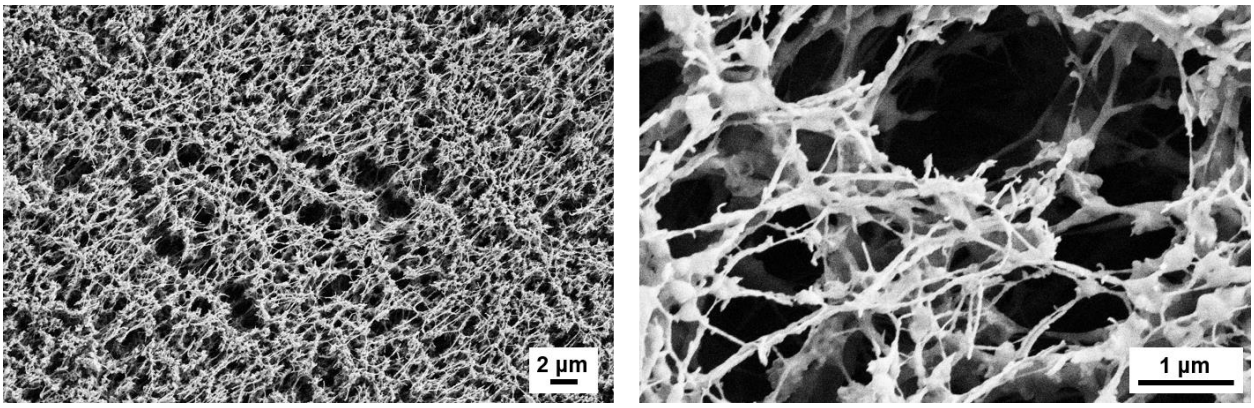

Figure 6. Low and high magnification 'top-down' SEM images of PTFE membrane showing nanoporosity

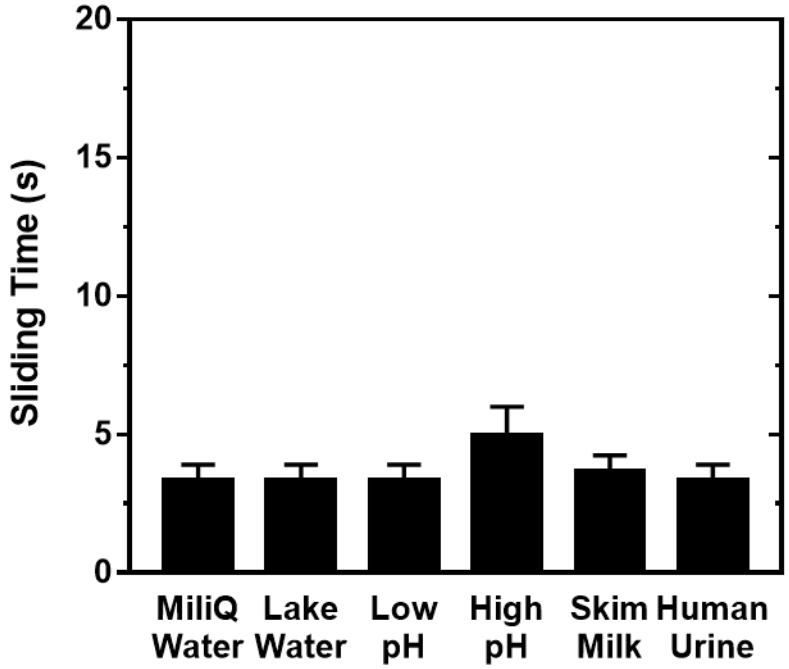

Figure 7. Plot showing sliding time of 50 μL droplets of chemically complex liquids (MiliQ water, unfiltered eutrophic lake water, low pH (1M HCl), high pH (1m NaOH), skim milk and pooled human urine) sliding on E7-infused SLIPS tilted at 20°.

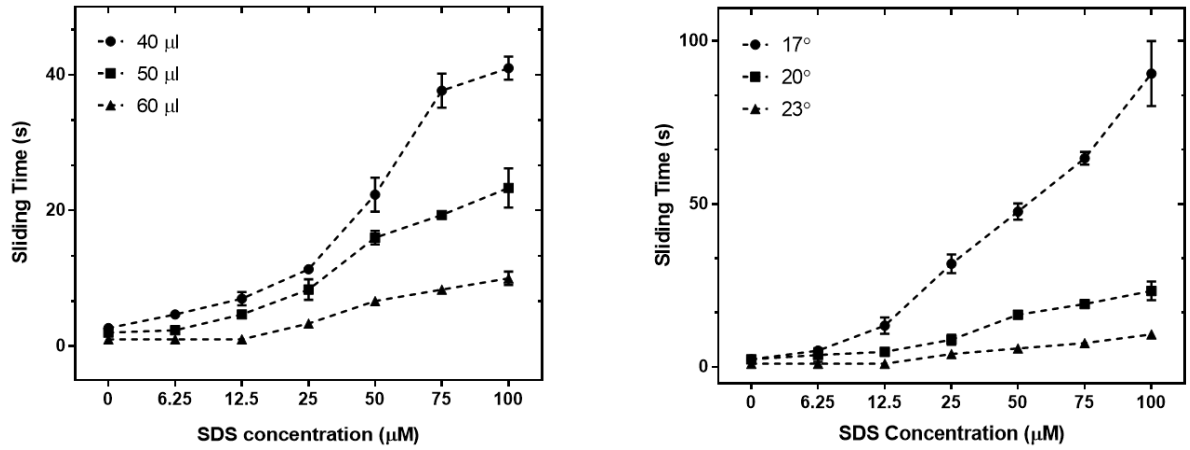

Figure 8. Dependence of sliding time on SDS concentration, (A) droplet volume, and (B) degree of inclination

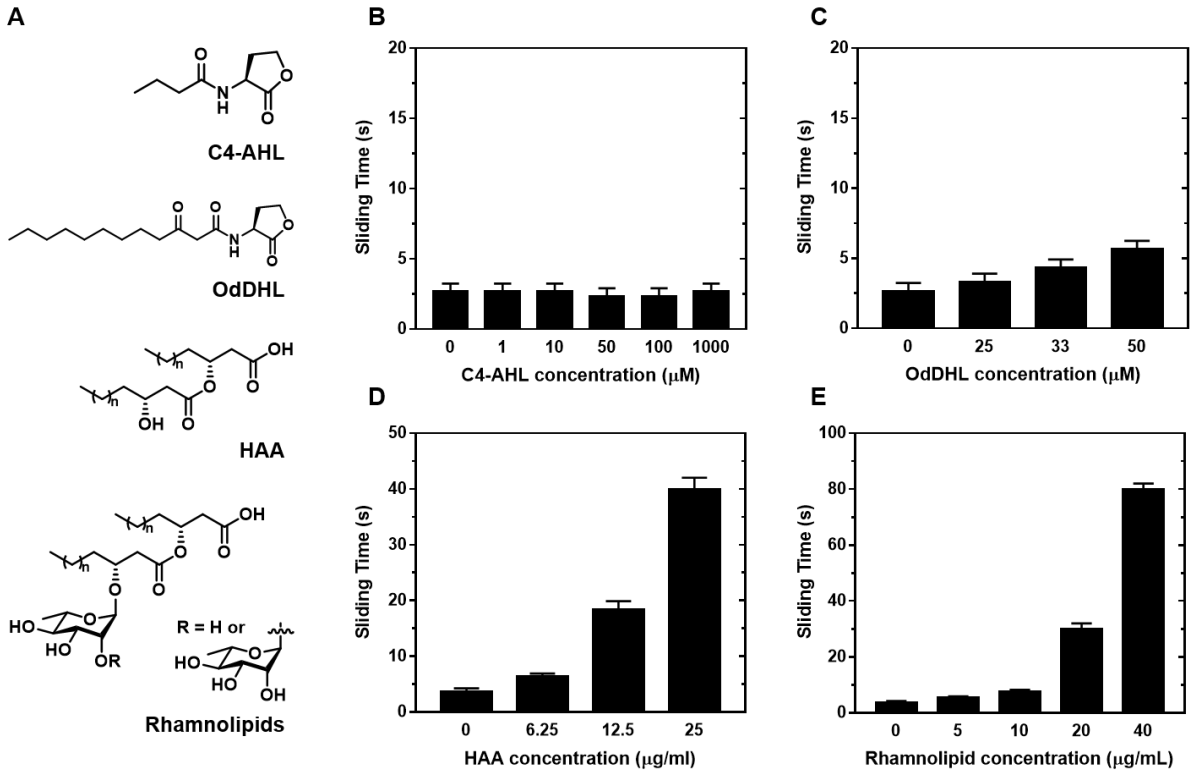

Figure 9. (A) Structures of various *P. aeruginosa* QS-controlled amphiphiles tested in this study. (B-E) Plots showing sliding time of 50 µL droplets of (B) C4-AHL, (C) OdDHL, (D) HAA, and (E) rhamnolipids on E7-infused SLIPS tilted at 20°; C4-AHL, OdDHL and HAA solutions were prepared in PBS with 1% (v/v) DMSO to enable higher solubility.

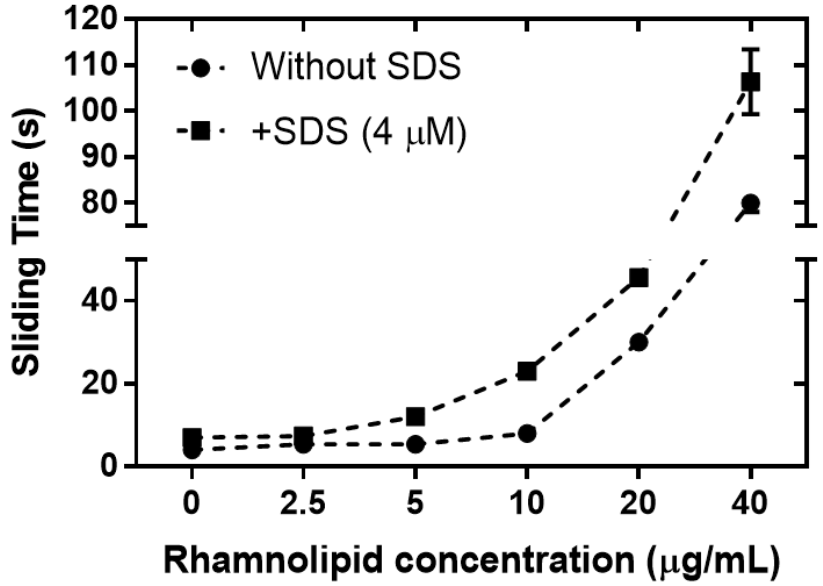

Figure 10. Plot showing the sliding time of rhamnolipid (0- 40 µg/ml) containing droplets on LC-SLIPS with SDS (4 µM; black squares) and without SDS (black circles).

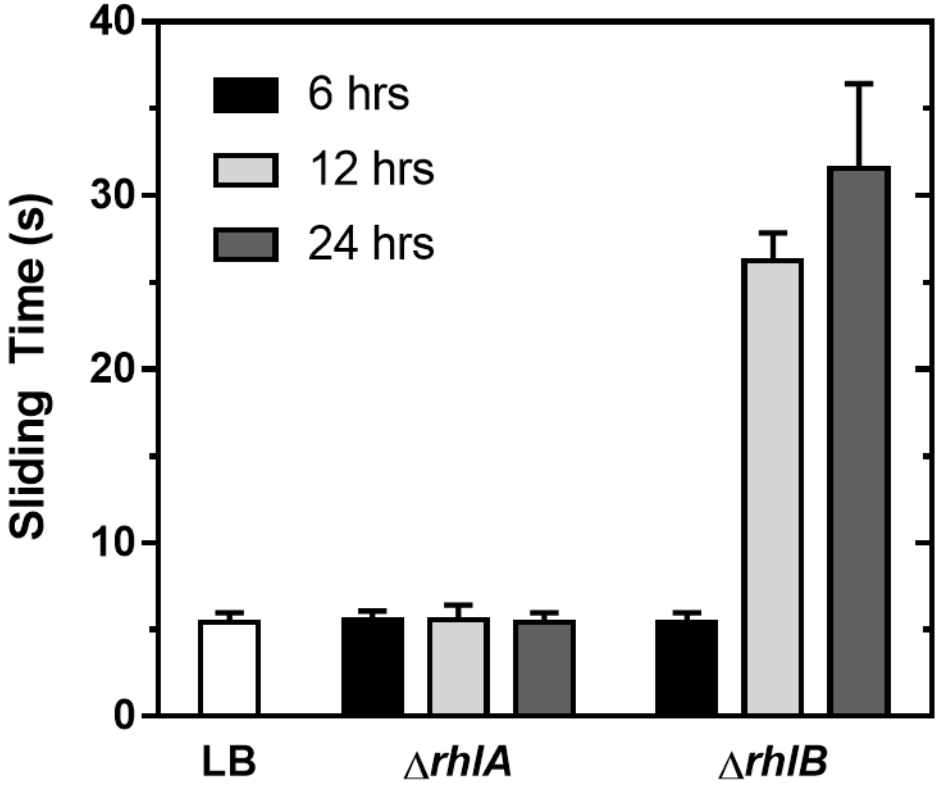

Figure 11. Effect of *P. aeruginosa* mutants on sliding time. Sliding time of LB media, Δ*rhlA*, and Δ*rhlB* at 6 hrs (black), 12 hrs (light gray), and 24 hrs (dark gray).

CHAPTER FIVE: Future Directions

Contributions: K. E. Nyffeler wrote the Chapter, K.E. Nyffeler and H. E. Blackwell edited the chapter together.

In this thesis, I have demonstrated the utility of screening of compounds in multiple receptors, particularly homologous ones within one organism. As described in Chapter 2, I identified specific and synergistic compounds in *P. aeruginosa*, as well as ones with new activities. In Chapters 3 and 4, I utilize this knowledge and information from other studies to develop and optimize new methods of sensing the presence of bacterial secondary metabolites. The first of these, in Chapter 3, demonstrates the utility of the previously-reported vesicle lysis test (VLT) in high-throughput screening to identify novel QSMs, where we have identified novel modulators of *S. aureus* QS, which represent brand new scaffolds for chemical synthesis. Lastly, I have discovered a new use for a novel material, LC-infused SLIPS, in sensing surfactants in bacterial culture.

Applications for compounds identified in Chapter 2

The information uncovered about the compounds in Chapter 2 points the way to ideal chemical scaffolds for future exploration as QS modulators. For example, the V-06-018 (**18**) and TP compounds (**19** and **20**) have been already explored as new ligand scaffolds in our laboratory, and they are—as revealed by work described herein—LasR-specific.[1-2] Additionally, compounds identified as synergistic (e.g., **5**) could be applied to *Caenorhabditis elegans* infection assays and other virulence assays that our laboratory is developing, to probe the simultaneous targeting of three LuxR-type receptors for optimal virulence control. These compounds may lead to increased virulence inhibition when compared to a receptor selective compound of similar activity but lacking the same synergistic effects.

Looking more broadly, interest in compounds that modulate multiple LuxR-type receptors comes from the fields of systems biology and microbial ecology. Studies like the one in

Chapter 2 are critical for studies involving large numbers of bacteria and understanding the role that QS plays in complex, mixed microbial communities. While this study was specific to *P. aeruginosa*, the screening of these compounds in other LuxR-type receptors in other strains will expand their scope and potential utility as research tools. Indeed, our laboratory already has begun efforts in this direction.[3-4]

Further applications of the VLT and exploration of our findings from the VLT so far

The successful use of the VLT in Chapter 3 to screen a large compound library paves the way for further implementation of this assay in other organisms, to screen for new QSMs and for the production of surfactants. Our laboratory and others have contributed to the development of QSMs in *Burkholderia* spp., *L. monocytogenes*, and *S. epidermidis*, all of which produce biosurfactants or vesicle lysis agents at least partially under QS control.[3, 5-6] Preliminary work (not included in this thesis) shows that the current vesicles are also sufficient for use in detection of PSMs in *S. epidermidis* and other groups of *S. aureus*, supporting the use of the VLT in other bacteria.

One of the strengths of VLT is for the potential identification of surfactants in less well-studied species, especially ones that are not amenable to genetic modification (e.g., incorporation of transcriptional reporters). In a species such as this, the VLT could be ideal for identification of a novel surfactant or of factors controlling the production of surfactant. If a novel surfactant appears to be present (i.e., vesicle lysis occurs), then steps can be taken to identify the surfactant. Chromatography could first be used to isolate fractions that may contain the product of interest. This could be followed by extraction or fractionation with an organic solvent (e.g., diethyl ether or methanol). Fractions could then be screened in the VLT to identify which contain the

surfactant of interest. If the sample is sufficiently pure, MS or GC techniques could then be used to identify the unknown surfactant. If the genome of the organism is fully or partially available, combing the genome for homologs of known surfactant-producing genes could assist in identification of the surfactant.

Our discovery of new QSMs in *S. aureus* via the VLT motivate many new experiments. First, we need to further explore their structures and activity profiles to determine mechanism of action. Such work could include chemical synthesis and SAR (structure-activity relationship) studies to explore the novel scaffolds uncovered.

In addition, experiments with compound **13** in this Chapter (M64, PqsR inhibitor) and *ΔrhlA* indicate that rhamnolipid may not be the sole agent of vesicle lysis in *P. aeruginosa*, a finding that needs to be further explored. More studies exploring mutants in the PqsR regulon are needed to determine the exact mechanism(s) of vesicle lysis and their relative contributions. One critical experiment to perform in the short term is to treat vesicles with exogenous rhamnolipid, to either confirm or refute the results of the Jenkins lab,[7] and thereby resolve if this surfactant is lysing our vesicles at all.

Further applications for the LC-SLIPs assay

In Chapter 4, we demonstrate the use of the LC-SLIPS to sense the presence of surfactant in bacterial cultures. The quantitative nature of this study provides an approach for the screening of rhamnolipid or PSMs from isolated bacterial cultures – both molecules that correlate with virulence in many cases. For example, one can imagine using LC-SLIPS to screen for rhamnolipid in clinical isolates of *P. aeruginosa*. This assay could be used to survey natural populations of bacteria (in clinical samples or in soil) to identify the diversity degrees of

rhamnolipid production. Finally, and in analogy to the VLT, the LC-SLIPs approach could be implemented for the rapid and visual screening of QSMs.

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APPENDICES

APPENDIX ONE: Exploring the use of synthetic ligands to differentially modulate transcriptional activation of QS genes by LasR in *Pseudomonas aeruginosa*

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K.E. Nyffeler and R.M. Gillis designed and performed experiments, K.E. Nyffeler, R.M. Gillis, and H.E. Blackwell wrote and edited the text.

Abstract

Quorum sensing is a method of bacterial cell-cell communication. In *P. aeruginosa*, QS controls up to 10% of the genome, including a large swath of virulence genes. In *Pseudomonas aeruginosa*, the QS receptor LasR (as the $[LasR:OdDHL]_2$ homodimer) has two alternate modes of promoter binding: (1) as a dimer of dimers (cooperative binding), and (2) as a dimer (noncooperative). These two binding modes differ by promoter. We hypothesize that, given these two binding modes, QSMs could differentially modulate the LasR regulon. Herein, we outline the beginnings of our work to explore this hypothesis, including an explanation of the Gibson cloning we are using to create plasmids to test this hypothesis.

Introduction

Quorum sensing (QS) involves bacterial cell-cell communication. QS bacteria produce and detect small molecule signals based on their population density. In QS in *Pseudomonas aeruginosa* and other Gram-negative species, *N*-acyl L-homoserine lactones (AHLs) function as the QS signal. When bacterial populations are sufficiently dense, a large portion of the genome is differentially expressed *via* QS, including a variety of virulence genes.[1-2] With these phenomena in mind, QS has been studied as a potential target for chemical modulation and therefore mitigation of virulence. Generally, QS modulators are of interest for their broad inhibition of virulence. However, targeted methods may be useful – i.e., specifically targeting the production of a specific factor to lessen disease severity, progression, or immune response. One method of specific targeting is to modulate specific QS proteins – small molecule modulators to target only one receptor have been developed by our group and others.

In *P. aeruginosa*, an opportunistic Gram-negative pathogen, QS controls up to 10% of the genome, actions primarily governed by the LuxR-type receptor, LasR.[1-2] LasR is activated by its native AHL ligand, *N*-(3-oxo)-dodecanoyl L-homoserine lactone (OdDHL). Traditionally, LasR has been considered to reside at the top of the QS hierarchy and exert control over a large number of other QS proteins, impinges on many virulence-critical systems. Therefore, although many molecules have been developed that are specific for LasR, these modulators would have an effect on a large number of operons. With that in mind, we were interested in discovering modulators that only affect a subset of LasR's regulatory roles. To accomplish this goal, we looked to reported differences in LasR's regulation, specifically the mechanism of LasR-promoter binding. We reasoned that it was unlikely that modulators would have an effect on close-to-consensus binding sites; indeed, work from the Greenberg laboratory shows

LasR:promoter binding happens via two broadly differing mechanisms: noncooperative (one LasR dimer) and cooperative (more than one LasR dimer).[3-4]

There may be a number of consequences of these two very distinct binding modes that one may consider. First, this cooperativity may have an effect on timing of LasR-dependent regulation. This was not borne out fully in studies, but it may still be a contributing factor.[3] Second, the apparent affinity of compound:LasR binding may be affected. Indeed, earlier work by the Iglewski lab demonstrated that a cooperative (*lasB*) promoter transcriptionally fused to beta-galactosidase displayed 10x lower affinity for OdDHL than the noncooperative (*lasI*) (i.e., it required ~10x less OdDHL to reach half-maximum receptor activation).[5] While we do not know that this observation is strictly due to the differences in cooperativity between promoters, it lends credence to the argument.

These differences in apparent OdDHL affinity in *lasB* and *lasI* becomes more important when one considers that, among laboratories developing and testing compounds, either promoter has been used to study compound activity, potentially leading to disparate activity profiles. To our knowledge, non-native modulator activity in multiple promoters has never been directly compared. We sought to undercover those differences and potentially learn more about LasR's multiple binding modes.

Results

We began by choosing promoters identified as cooperative vs. noncooperative (Table 1) that had virulence-relevant phenotypes. We chose two with cooperative promoters: (1) LasB produces elastase, a protein that contributes to host tissue damage, cytotoxicity, and immunological interference[6] (2) AmbBCDE, a set of non-ribosomal peptide synthase genes that

produce an alternate QS signal critical for virulence, particularly under low-phosphate conditions.[7-8] We also chose two genes with non-cooperative promoters: (1) AprXDEF, an unknown protein (AprX) and a set of three membrane proteins (AprDEF) required for secretion of an alkaline protease (AprA) [9-10] (2) RsaL, a small protein that represses LasR by competing for the *lasI* promoter.[11-12] We already possessed a *lasI* transcriptional fusion with which to compare results.

Table 1. Four promoter regions from *P. aeruginosa* chosen for cloning with their corresponding attributes. Information on cooperativity from[3-4]

Gene Number	**Gene or Operon**	**EMSA Result**	**Function**
PA1245-PA1248	*aprXDEF*	Non-coop	Alkaline protease
PA1431-PA1432	*rsaL lasI*	Non-coop	Regulatory + LasI Synthase
PA2305-PA2302	*ambBCDE*	Coop	IQS Production
PA3724	*lasB* OP1	Non-coop	Elastase
PA3724	*lasB* OP2	Coop	Elastase

To test activity in these different promoters, we chose 8 compounds with varying LasR activities. These activities were determined based on assays conducted with a reporter gene constructed with the *lasI* promoter.[13] Using traditional cloning methods, we constructed pSC11-*aprX*, a reporter plasmid identical to that used in the previous study (pSC11-*lasI*), but with the

aprX (non-cooperative) promoter. In all cases, we observed no significant difference between dose-response curves for a given compound for each promoter pair (Figure 1 and Table 2). For a given compound, the maximum percent LasR activation of each promoter pair consistently fell within the 95% confidence intervals. Similarly, for compounds in which an EC_{50} could be generated, the EC_{50} was not significantly different between pSC11-*lasI* and pSC11-*aprX*. However, the major difference between the assays of pSC11-*lasI* and pSC11-*aprX* was the relative strength of the two promoters. The pSC11-*lasI* extracts reached their peak absorbance of 1.0 in approximately 6 minutes, whereas the pSC11-*aprX* extracts took 45 minutes on average. This pattern was consistent among all of the tested compounds.

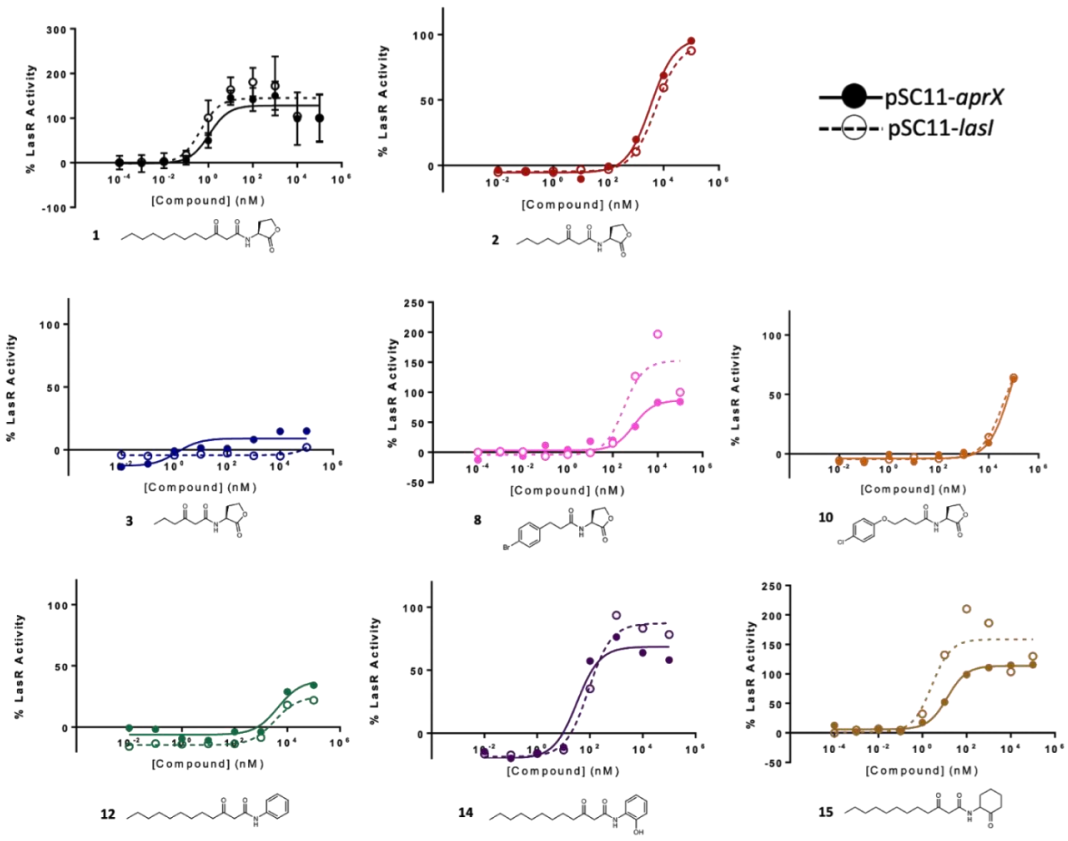

Figure 1. Dose-response curves for pSC11-*lasI* and pSC11-*aprX* reporter assays with their corresponding compounds. The numbers correspond to Library Compound labels given to the molecules in the study Moore *et al.* [13] Each pSC11-*aprX* assay was performed in technical triplicate, errors bars not shown.

Table 2. Percent LasR activity and EC_{50} values with 95% confidence intervals for pSC11-*lasI* and pSC11-*aprX* reporter assays and their corresponding compounds.

	EC_{50} (nM)		*Maximum % LasR Activity*	
Compound	***pSC11-lasI***	***pSC11-aprX***	***pSC11-lasI***	***pSC11-aprX***
1 (OdDHL)	1.7 (0.6-4.7)	2.2 (1.2-2.4)	110 (96-130)	91 (84-97)
2	5300 (4800-5700)	3400 (1200-9900)	93 (91-95)	97 (77-120)
3	-	-	11 (N/A)	9.0 (0-40)
8	320 (62-1400)	870 (73-5500)	150 (110-200)	87 (61-120)
10	-	-	92 (82-108)	110 (70+)
12	-	-	25 (19-31)	38 (24-56)
14	88 (38-198)	32 (15-69)	88 (74-100)	69 (58-79)
15	2.6 (0.3-20.9)	13 (1-120)	160 (120-200)	110 (87-140)

Discussion and Future Work

The results from this experiment suggest that there is no difference in the compounds' LasR activity as measured by either the *lasI* or *aprX* promoters. This supports our initial hypothesis that noncooperative promoters will have similar interactions with compound. However, the results are obviously limited and a more complete test of the hypothesis will use *lasB* and *ambB* promoters.

In order to confirm our findings and appropriately draw conclusions about the differences between the other promoter regions, along with accelerate our rate of progress, we plan to utilize Gibson Assembly[14] for the cloning of the plasmids and perform identical β-gal assays. From there, identifying differences in percent LasR activation or EC_{50} between the cooperative and non-cooperative promoter regions would be possible.

LasR was originally chosen as the target for this study because its regulon has been the most well studied. However, recent work on RhlR, another *P. aeruginosa* QS receptor, suggests

that this protein could be an even more desirable target for a future study of this nature.[15-17] The Bassler laboratory reported that RhlR bound not only to *N*-butanoyl-L-homoserine lactone (BHL), but also to a second, as of yet discovered, ligand.[16-17] Their data was consistent with a model where RhlR has two regulons, with one controlled by each ligand. This system could provide an interesting platform to explore compound-controlled differential gene expression.

Materials and Methods

General. Compounds (Sigma-Aldrich) were purchased or synthesized *via* previously reported methods, as outlined in Moore (2015).[13] Stock solutions of compounds were prepared in DMSO and stored at -80 °C. Biological reagents and media were purchased from Goldbio, RPI, or Sigma-Aldrich and used according to enclosed instructions. Cloning reagents were purchased from Zymo Research, Promega, or New England Biolabs, and used according to enclosed instructions.

β-galactosidase assay. β-galactosidase assay was undergone as in Chapter 2 or Moore.[13] The only modifications were the timing of CPRG incubation – 10 minutes for pSC11-*lasI* and ~45 minutes pSC11-*aprX*.

Traditional cloning. Four promoter regions of varying lengths were amplified from the *P. aeruginosa* genome using the following forward and reverse primers (Table 3)

Table 3. Amplified region length for each gene and corresponding primer sequences used for traditional cloning. SalI-HF cut sites are marked in red. BamHI-HF cut sites are marked in green. The amplified regions were designed to encapsulate a portion of the upstream region of the genes/gene operons and the beginning of the open reading frame. Relative to the translational start codon of each gene, the amplified region spanned from -320 to +27 for *ambB*, -477 to +27 for *aprX*, -326 to +27 for *lasB*, and -165 to +27 for *rsaL*.

ambB	347 bp	5'-CATGTCGACATAAGCAC CTATACGCCGTTGCAGGG-3'	5'-CATGGATCCTAACGGTA AGCCATGTCGCTCCTGCAT-3'
aprX	504 bp	5'-CATGTCGACCGGCACTTT GGTGCATAAGGATATAAC-3'	5'-CATGGATCCGTTCGCGGC TGCATTGAATAATCCCAT-3'
lasB	353 bp	5'-CATGTCGACAAGCGTGC AACTGATGATCGTCCACAT-3'	5'-CATGGATCCCAGGTCAA GCGTAGAAACCTTCTTCAT-3'
rsaL	192 bp	5'-CATGTCGACCGAACTCTT CGCGCCGACCAATTTG- 3'	5'-CATGGATCCGGGCTGTGT TCTCTCGTGTGAAGCCAT-3'

The fragments were digested with SalI-HF and BamHI-HF (New England Biolabs) and ligated to complementary SalI-HF/BamHI-HF-digested pSC11 plasmids, resulting in pSC11-*ambB*, pSC11-*aprX*, pSC11-*lasB*, and pSC11-*rsaL*. The plasmids were then transformed into Zymo Research Mix & Go! *E. coli* cells.

Gibson cloning. To improve procedural efficiency, Gibson Assembly cloning was subsequently performed in lieu of traditional cloning. Four regions identical to those produced by the traditional method were amplified in addition to the pSC11 plasmid.

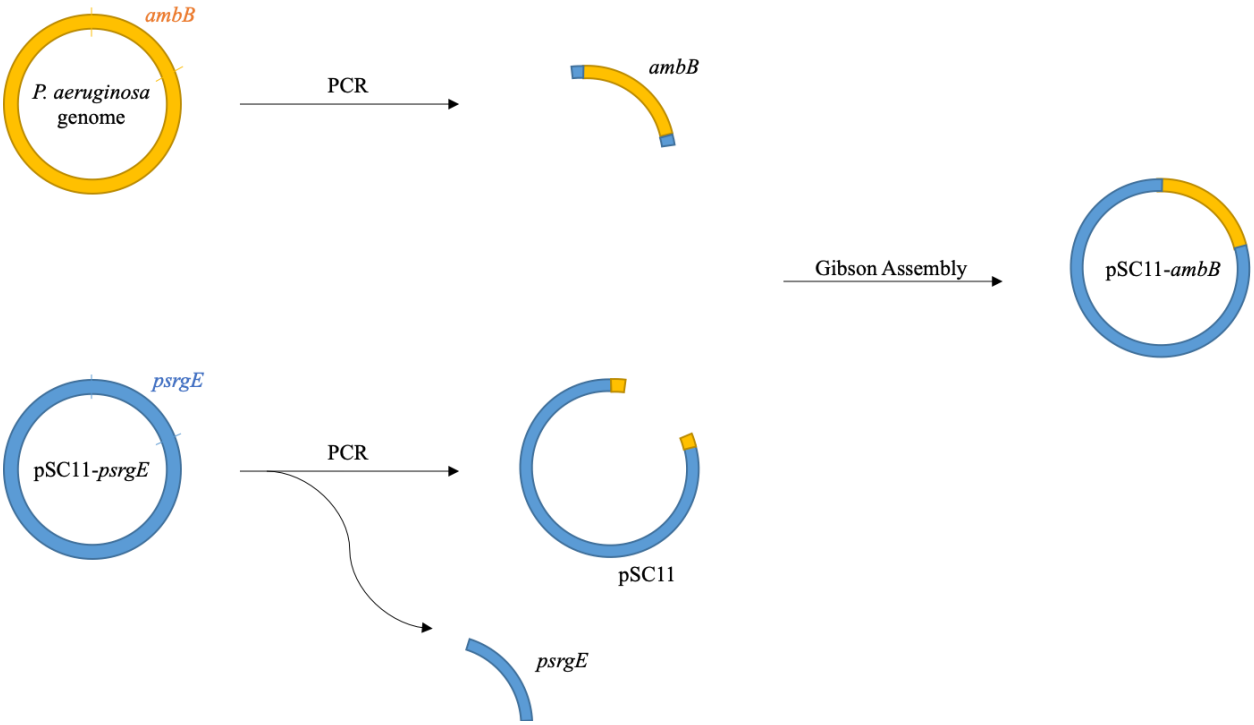

Figure 2. Schematic of Gibson cloning. Yellow fragments respond to the *P. aeruginosa* genome, and blue fragments respond to the pSC11 vector.

Table 4. Primer sequences used in Gibson Assembly cloning for each recombinant plasmid. Top row sequences were used for amplification of the pSC11 vector, and bottom row sequences were used for amplification of the *P. aeruginosa* genome regions described in traditional cloning.

pSC11-*ambB*-Gib	pSC11	5'-AACGGCGTATAGGTGCTTATGTCGACGTCACGCGTCCATG-3'	5'-CTAGAAGCTTCTAGCTAGAGGGTA-3'
	ambB	5'-TACCCTCTAGCTAGAAGCTTCTAGTAACGGTAAGCCATGTCGCT -3'	5'-CATGGACGCGTGACGTCGACATAAGCACCTATACGCCGTT-3'
pSC11-*aprX*-Gib	pSC11	5'-CCTTATGCACCAAAGTGCCGGTCGACGTCACGCGTCCATG-3'	5'-CTAGAAGCTTCTAGCTAGAGGGTA-3'
	aprX	5'-CCCTCTAGCTAGAAGCTTCTAGAGAGTTCGCGGCTGCATTGAATA-3'	5'-CATGGACGCGTGACGTCGACCGGCACTTTGGTGCATAAGG-3'
pSC11-*lasB*-Gib	pSC11	5'-CGATCATCAGTTGCACGCTTGTCGACGTCACGCGTCCATG-3'	5'-CTAGAAGCTTCTAGCTAGAGGGTA-3'
	lasB	5'-CCCTCTAGCTAGAAGCTTCTAGAGACAGGTCAAGCGTAGAAACCTT-3'	5'-CATGGACGCGTGACGTCGACAAGCGTGCAACTGATGATCG-3'
pSC11-*rsaL*-Gib	pSC11	5'-TGGTCGGCGCGAAGAGTTCGGTCGACGTCACGCGTCCATG-3'	5'-CTAGAAGCTTCTAGCTAGAGGGTA-3'
	rsaL	5'-CCCTCTAGCTAGAAGCTTCTAGAGAGGGCTGTGTTCTCTCGTGTG-3'	5'-CATGGACGCGTGACGTCGACCGAACTCTTCGCGCCGACCA-3'

Four primers were used in the construction of each recombinant plasmid: two for the amplification of the insert and two for the amplification of the vector. (Table 4) Each primer was designed with an overlapping, non-annealing end necessary for Gibson Assembly. Because of an issue with palindromic sequences near the area of ligation closer downstream of the insert, a small fragment was removed in the amplification of pSC11 to avoid double primer binding. This

deleted region was 24 bp in length for what would become pSC11-*ambB* and 21 bp in length for pSC11-*aprX*, pSC11-*lasB*, and pSC11-*rsaL*. The PCR amplified regions will be added to Gibson Assembly Master Mix and incubated, yielding the desired recombinant plasmids.

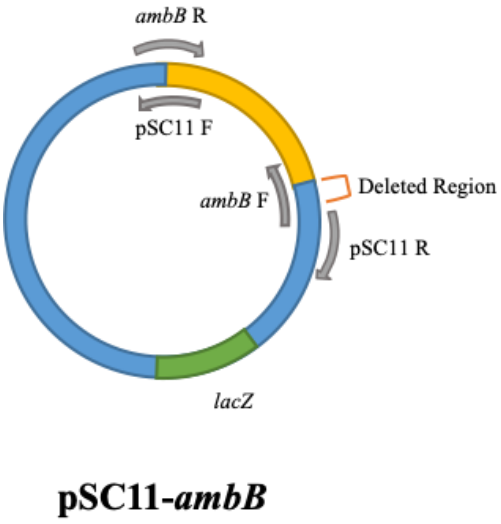

Figure 3. Schematic of Gibson primers on pSC11-*ambB*. The blue region corresponds to pSC11, the yellow region corresponds to the *P. aeruginosa* genome *ambB* insert, and the green region illustrates the presence of *lacZ* on the pSC11 plasmid. The orange bracket illustrates the region deleted from pSC11 after amplification.

Acknowledgments

Financial support for this work was provided by the NIH (R35 GM131817). K. Nyffeler was supported in part by the UW−Madison NIH Chemistry−Biology Interface Training Program (T32 GM008505) and as part of a seed award from the UW–Madison NSF MRSEC (*DMR-1720415*).

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APPENDIX TWO: A Comparative Analysis of Synthetic Quorum Sensing Modulators in *Pseudomonas aeruginosa*: New Insights into Mechanism, Active Efflux, Susceptibility, Phenotypic Response, and Next-Generation Ligand Design

J.D. Moore designed and performed experiments and synthesized QS modulators, F.M. Rossi synthesized QS modulators, K.E. Nyffeler performed LasR overexpression assays, J.D. Moore and H.E. Blackwell wrote the text.

Moore, J.D.; Rossi, F.M.; Welsh, M.A.; Nyffeler, K.E.; Blackwell, H.E.; *J. Am. Chem. Soc.* **2015**, *137*(46), 14626-14639

https://doi.org/10.1021/jacs.5b06728

JACS
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A Comparative Analysis of Synthetic Quorum Sensing Modulators in *Pseudomonas aeruginosa*: New Insights into Mechanism, Active Efflux Susceptibility, Phenotypic Response, and Next-Generation Ligand Design

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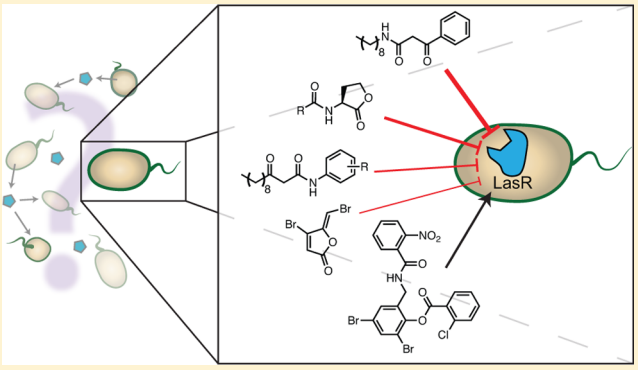
Supporting Information

ABSTRACT: Quorum sensing (QS) is a chemical signaling mechanism that allows bacterial populations to coordinate gene expression in response to social and environmental cues. Many bacterial pathogens use QS to initiate infection at high cell densities. Over the past two decades, chemical antagonists of QS in pathogenic bacteria have attracted substantial interest for use both as tools to further elucidate QS mechanisms and, with further development, potential anti-infective agents. Considerable recent research has been devoted to the design of small molecules capable of modulating the LasR QS receptor in the opportunistic pathogen *Pseudomonas aeruginosa*. These molecules hold significant promise in a range of contexts; however, as most compounds have been developed independently, comparative activity data for these compounds are scarce. Moreover, the mechanisms by which the bulk of these compounds act are largely unknown. This paucity of data has stalled the choice of an optimal chemical scaffold for further advancement. Herein, we submit the best-characterized LasR modulators to standardized cell-based reporter and QS phenotypic assays in *P. aeruginosa*, and we report the first comprehensive set of comparative LasR activity data for these compounds. Our experiments uncovered multiple interesting mechanistic phenomena (including a potential alternative QS-modulatory ligand binding site/partner) that provide new, and unexpected, insights into the modes by which many of these LasR ligands act. The lead compounds, data trends, and mechanistic insights reported here will significantly aid the design of new small molecule QS inhibitors and activators in *P. aeruginosa*, and in other bacteria, with enhanced potencies and defined modes of action.



INTRODUCTION

Many common bacteria use an intercellular chemical signaling process termed quorum sensing (QS) to coordinate local population density with group-beneficial behaviors.[1] In Gram-negative bacteria, QS is largely mediated by *N*-acylated L-homoserine lactone (AHL) signals, which are produced by LuxI-type enzymes and sensed by intracellular LuxR-type receptors (Figure 1).[2] The AHL ligands passively diffuse out of the cell and into neighboring cells; some bacteria also use active efflux to facilitate AHL dissemination.[3,4] As the bacterial population grows within the confines of a particular environment, the local concentration of AHL signal likewise increases. Once the AHL concentration reaches a threshold intracellular level (corresponding to a "quorate" bacterial population), productive binding of the AHL to its target LuxR-type receptor occurs. This binding event typically induces receptor dimerization, DNA binding, and subsequent transcriptional activation of QS target genes.

Numerous bacterial pathogens use QS to regulate the timing and extent of virulence factor production, thereby allowing them to amass until a sufficient population has been achieved to overwhelm a host immune response.[2] As QS is dependent on small molecule signals and the relative concentration thereof, there is substantial interest in the development of chemical strategies that disable QS signaling networks and thus stem or even prevent virulence. Such "anti-virulence" approaches could provide novel pathways to mitigate bacterial infection in humans, animals, and plants.[5−8] More fundamentally, chemical interventions could provide new insights into the mechanisms

Received: June 29, 2015
Published: October 22, 2015

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639

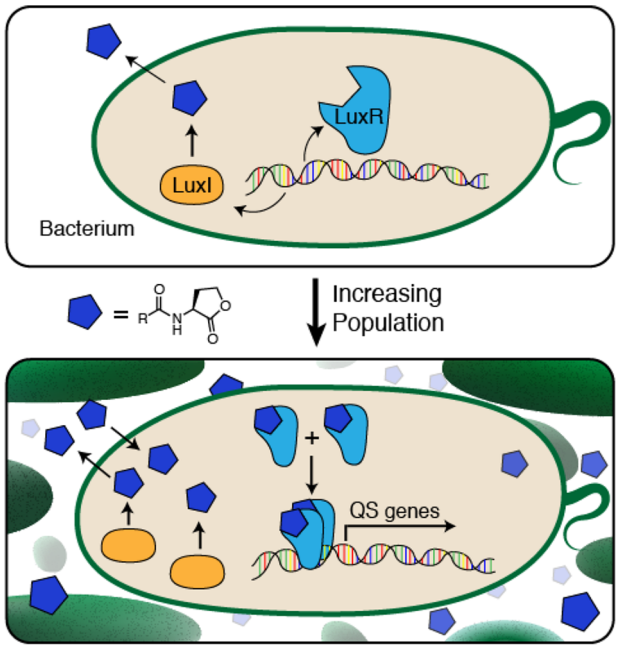


Figure 1. Simplified QS circuit in Gram-negative bacteria. LuxI-type synthases produce AHLs (blue pentagons) that can bind to cognate LuxR-type receptors. At high cell densities, activated receptors induce transcription of QS genes.

by which bacteria use QS to their advantage, insights that might not be readily elucidated using traditional genetic methods.[9−12]

The most common Gram-negative bacterium found in hospital-acquired infections, *Pseudomonas aeruginosa*, uses QS to regulate the production of numerous extracellular proteases, biofilm maturation factors, and toxins.[5] This opportunistic pathogen has become increasingly resistant to most current antibiotic therapies, so the need for the development of new approaches to treat *P. aeruginosa* infections is urgent.[13] Accordingly, chemical strategies to inhibit QS in *P. aeruginosa* have received significant recent attention. Such nonbactericidal, antivirulence approaches could also be particularly robust to resistance development, further enhancing their potential utility.[14,15]

P. aeruginosa has a relatively complex QS network that includes (at least) two LuxI/LuxR pairs: LasI/LasR and RhlI/RhlR. The *las* subnetwork utilizes *N*-(3-oxododecanoyl) L-homoserine lactone (OdDHL, **1**; Figure 2) as its signal, while the *rhl* subnetwork uses *N*-butyryl L-homoserine lactone (BHL). LasR and RhlR each activate discrete regulons involved in virulence; however, as LasR activates the *rhl* system, LasR has been a principle target of study for the development of small molecule QS modulators in *P. aeruginosa*.[16] The selection of LasR for investigation is further supported by the observation that *P. aeruginosa* LasR mutants have dramatically attenuated virulence and invasiveness in certain in vivo infection models.[17] Over the past ∼20 years, campaigns of rational design,[18−25] high-throughput screening,[26−28] and computational modeling[29,30] have revealed a large number of compounds reported to modulate LasR transcriptional activity; the bulk of these ligands are anticipated to directly compete with OdDHL for binding to LasR (albeit definitive mechanistic data is scarce; see below). Several of these compounds, both AHL-derived and otherwise, have been shown to modulate important QS-dependent virulence phenotypes in *P. aeruginosa* and certainly constitute chemical tools to study QS pathways in this pathogen.

That said, there remain significant challenges for the further design and application of non-native LasR ligands. The following three issues are perhaps most urgent: First and foremost, the majority of these compounds have been tested for activity in LasR using *widely variable* biological assays (see below). Furthermore, any systematic side-by-side comparisons of known LasR modulators have been extremely limited, typically comparing, at maximum, 2−4 control compounds to new ligands of interest.[11,21,24,25,31] Second, for the compounds for which LasR IC_{50} values have been calculated in *P. aeruginosa*, these values are typically only low-micromolar (in cell-based assays). Molecules with heightened potencies would undoubtedly be of value for both fundamental and applied QS research. Third, the scientific community has virtually no mechanistic information about how the known synthetic LasR ligands interact with the receptor (if they do so directly) and modulate its function. Slowing such studies is the fact that LasR, similar to many other LuxR-type receptors, is relatively unstable in the absence of native ligand (OdDHL), which has prevented the use of in vitro assays to directly assess small molecule antagonism. Collectively, these challenges preclude (i) the selection of a lead LasR ligand scaffold for advancement as a robust chemical probe, and (ii) the cultivation of new and informed ligand design strategies.

To date, the activities of reported LasR modulators typically have been measured using cell-based assays reliant on a genetically engineered reporter. Reporter gene assays have been performed in a wide array of *P. aeruginosa* and heterologous (*E. coli*) LasR-producing strains using many different reporter constructs and conditions, resulting in a broad range of reported ligand activities for LasR activation or inhibition.[32] Numerous research groups have also advanced lead compounds into *P. aeruginosa* bioassays that measure attenuation of QS-controlled virulence phenotypes,[16] but these studies are equally disparate in the phenotypes studied and in the experimental conditions used (for a listing, see Table S2). Confounding such assays is the fact that attenuating wild-type *P. aeruginosa* virulence phenotypes is often more difficult than simply disrupting LasR in an *E. coli* "biosensor" strain. Small molecule modulators must contend with a number of obstacles presented by *P. aeruginosa*, including but not limited to enzymatic degradation,[33] low membrane permeability,[34] active efflux,[35] and constitutive production of the native autoinducers.[36] Thus, compounds that fail in these assays may do so for reasons other than low intrinsic activity on LasR. Determining the most promising small molecule scaffolds for further development as LasR modulators—ideally, ones that subvert the aforementioned obstacles present in wild-type *P. aeruginosa*—is of paramount importance to researchers working at the growing interface of chemistry and biology in the QS field. Identifying such compounds was the motivation for the current study.

Herein, we report the first comparative analysis of the most promising synthetic LasR modulators reported to date. This set of compounds comprises natural and non-natural AHLs, AHL analogues, natural products, and structurally unique molecules (Figure 2). We began by comparing compound potency in a single *P. aeruginosa* LasR reporter strain, and thereafter examined these compounds for *direct* LasR modulation in a single *E. coli* LasR reporter. The activity trends uncovered in these standardized reporter studies were also recapitulated in our QS phenotypic assays in wild-type *P. aeruginosa*, most

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639

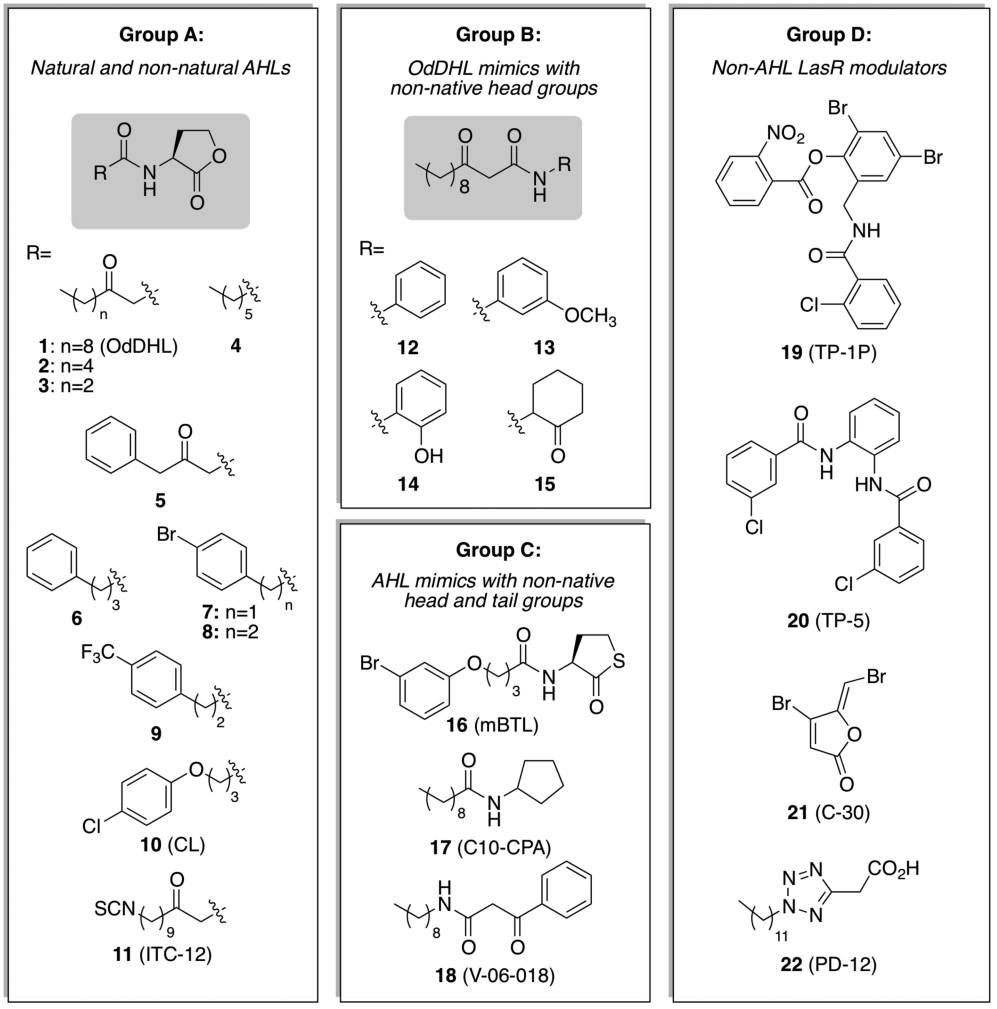


Figure 2. Structures of natural (**1**–**3**) and non-natural (**4**–**11**) AHLs, OdDHL mimics retaining the native 3-oxo-C12 tail (**12**–**15**), AHL mimics with non-native head and tail groups (**16**–**18**), and structurally unique compounds (**19**–**22**) chosen for evaluation of LasR modulatory activity and *P. aeruginosa* phenotypic response. Compounds were developed by the following laboratories: **4**, Winans and co-workers; **5**, **6**, Doutheau and co-workers; **7**–**9**, **12**, Blackwell and co-workers; **10**, **16**, Bassler and co-workers; **11**, Meijler and co-workers; **13**, Spring and co-workers; **14**, **15**, Suga and co-workers; **17**, Kato and co-workers; **18**–**20**, **22**, Greenberg and co-workers; **21**, Givskov and co-workers. See Supplementary Note S1 and Table S2 for key citations for each library member.

notably revealing two compounds capable of completely inhibiting the QS-dependent production of a key virulence factor. In the course of our investigations, we also discovered a series of interesting, and unexpected, dose–response phenomena for certain LasR modulators. These observations are significant, as they provide mechanistic insights—with respect to active efflux, receptor overexpression in heterologous strains, and the competitive or noncompetitive interactions of antagonists—that most likely apply not only to LasR, but also to the many other LuxR-type homologues found in bacteria.

EXPERIMENTAL SECTION

Chemical Reagents and Instrumentation. All chemical reagents and solvents were purchased from commercial sources (Acros, Alfa-Aesar, Fisher, Sigma-Aldrich) and used without further purification. See Supporting Information for details of NMR, HPLC, and MS instrumentation.

LasR Modulator Library Compounds. Compounds **1**–**4**, **15**, and **21** were purchased from Sigma-Aldrich. Compounds **5** and **6**,[18] **7**–**9**,[21] **10**,[11] **12**,[37] **13**,[25,38] **14**,[31] **16**,[11] **17**,[22] **18**,[11] **20**,[14] **21**,[39] and **22**[25] were synthesized as reported previously and yielded spectra that matched those reported. Compounds **11** and **19** (TP-1P) were generously supplied by the laboratories of Prof. Michael Meijler and Prof. Peter Greenberg, respectively. ^{1}H and ^{13}C NMR spectra of **19** (TP-1P) matched those reported by Janda and co-workers (see Supporting Information).[40] As the reported potencies of **19** (TP-1P) and its related isomer TP-1R are similar (an activity trend that we also observe; data not shown),[40] we examined only **19** herein. The two AHL analogues reported by Bassler and co-workers, **10** and **16** (evaluated previously as racemates),[11] were synthesized for this study in enantiopure form, using L-homoserine lactone and L-homocysteine thiolactone, respectively. Characterization data (HPLC, MS, and/or NMR) for compounds **1**–**4**, **10**, **11**, **13**, and **15**–**22** (i.e., those not characterized in our prior studies) are provided in the Supporting Information.

Compound Handling. Stock solutions of library compounds (100 mM, unless limited by solubility of the compound) were prepared in DMSO and stored at −20 °C in sealed vials. Solvent-resistant polypropylene or polystyrene 96-well microtiter plates (Costar) were used when appropriate for LasR reporter gene assays.

Biological Reagents and Strain Information. All standard biological reagents were purchased from Sigma-Aldrich or Gold Biotechnology and used according to enclosed instructions. Buffers and solutions for Miller absorbance assays in *E. coli* (Z buffer, 0.1% aqueous SDS, and phosphate buffer) were prepared as described

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626–14639

previously.[41] Water (18 MΩ) was purified using a Millipore Analyzer Feed System.

The bacterial strains and plasmids used in this study are listed in Table S1. Bacteria were grown in a standard laboratory incubator at 37 °C with shaking (200 rpm) in Luria−Bertani (LB) medium unless otherwise noted. Absorbance and fluorescence measurements were obtained using a Biotek Synergy 2 microplate reader using Gen5 1.05 data analysis software. All biological assays were performed in triplicate. EC_{50} and IC_{50} values, as well as respective 95% confidence intervals, were calculated using GraphPad Prism software (v. 4.0) using a sigmoidal curve fit (see Supplementary Note S6 for more information regarding curve fitting).

***P. aeruginosa* LasR Reporter Assay Protocol.** Compound activities in the *P. aeruginosa* LasR reporter strains were measured according to our previously reported method,[35] with the following modifications: Overnight cultures were grown for exactly 20 h; for antagonism assays in *P. aeruginosa* PAO-JP2, the 1:100 subculture was pretreated with 150 nM OdDHL; for antagonism assays in *P. aeruginosa* PAO-JG21, the 1:100 subculture was pretreated with 20 nM OdDHL. For full assay protocol, see Supplementary Note S3.

***E. coli* LasR Reporter Assay Protocol.** Compound activities in the *E. coli* JLD271 LasR reporter strain were measured according to previously reported methods (Blackwell and co-workers[21] for LasR reporter strain growth; Wolf and co-workers[42] for β-galactosidase activity measurement), with the following modifications: The *E. coli* Δ*sdiA* strain JLD271[43] was used to harbor the LasR expression and reporter plasmids pSC11 and pJN105L, respectively; the 1:10 subculture was grown to an OD_{600} of 0.450 before inducing LasR expression with 4 mg/mL L-arabinose and pretreating with 2 nM OdDHL; the cell permeabilization mixture was optimized to contain 200 μL Z-buffer, 8 μL $CHCl_3$, and 4 μL 0.1% aqueous SDS; the β-galactosidase substrate chlorophenol red-β-D-galactopyranoside (CPRG) was used, and thus no termination/quenching step was necessary. For full assay protocol, see Supplementary Note S4

***P. aeruginosa* LasR Overexpression/Reporter Strain Construction and Protocol.** The LasR overexpression plasmid pJN105L was introduced into *E. coli* S17−1::λpir by electroporation and then transferred to *P. aeruginosa* PAO-JP2 by conjugation and selection on LB supplemented with gentamicin (10 μg/mL) and tetracycline (12 μg/mL). Reporter assays measuring compound activities on LasR were performed as in the above *P. aeruginosa* assays, but L-arabinose (4 mg/mL) was added to subcultures immediately prior to dispensing subculture into compound-treated plates.

Elastase B Production Assay in Wild-Type *P. aeruginosa*. The activity of elastase B in *P. aeruginosa* culture supernatants was measured colorimetrically using an elastin-Congo red substrate.[44] A 10 mL overnight culture of *P. aeruginosa* PAO1 (wild-type) was grown for 16 h as described above. DMSO stock solutions of test compounds (10 mM) were prepared, and 2 μL aliquots were added to the wells of a clear plastic 96-well microtiter plate (Costar 3370). An inoculating subculture was prepared by pelleting an aliquot of the overnight culture at 1500*g* for 10 min, followed by resuspension of the cell pellet into a 100× volume of fresh LB medium (effecting a 1:100 dilution of the overnight). To each well, a 198-μL aliquot of subculture was added (final compound concentrations were 100 μM, with 1% DMSO), and the plates were incubated for 20 h. The final cell density was measured by reading OD_{600}. The cultures were pelleted by centrifugation of the assay plate at 2000*g* for 30 min, and 50 μL of supernatant from each well was transferred to a new 96-well plate. A 150-μL aliquot of 0.5% (w/v) elastin-Congo red conjugate (Elastin Products Co.) in Tris buffer (10 mM Tris, 1 mM $CaCl_2$, pH 7.2) was added to each well, and the plate sealed with a polypropylene storage mat (Costar 3080). The plate was incubated at 37 °C with shaking (200 rpm) while attached to a Labquake rotator (8 rpm) to ensure complete mixing. After 12 h, undigested elastin was pelleted by centrifugation at 1500*g* for 2 min, 100 μL of the supernatant was transferred to a new 96-well plate, and the absorbance at 490 nM was measured. Elastase B activity values for all cultures were background-corrected to that of wells containing no bacteria, then growth-normalized by dividing the resulting absorbance value by the final OD_{600} and plotted relative to a DMSO-treated *P. aeruginosa* PAO1 control. Elastase activity of *P. aeruginosa* PAO-JP2 was included in each experiment as a fully QS-inhibited positive control.

RESULTS AND DISCUSSION

LasR Modulator Library Curation. We selected 22 compounds for our comparative analyses based on a combination of the following factors: (i) noteworthy reported potency and/or efficacy as a LasR antagonist or agonist, (ii) ready synthetic tractability, (iii) commercial availability as a reported LasR modulator, and/or (iv) unique structural or QS-modulatory characteristics. The compound library was then divided into four distinct structural classes (Groups A−D; Figure 2), which roughly follow the main research approaches used to develop LasR modulators over the past decade.

Group A includes natural and non-natural AHLs, with a focus on OdDHL analogues that have shown effective modulation of LasR and closely related homologues.[45] Because AHLs naturally derive their receptor specificity from variations in acyl tail structure, many laboratories (including our own)[16,21] have attempted to rationally extend these properties to new AHLs with non-native tails.[46]

Other research groups have taken a complementary approach to rationally designing LasR modulators by retaining (presumably) important ligand−receptor contacts in the 3-oxo-C12 acyl tail of OdDHL, while varying the structure of the cyclic headgroup. This approach can bypass the liabilities associated with the hydrolytically unstable homoserine lactone. **Group B** comprises such OdDHL mimics with alternative head groups.

Some laboratories have sought to combine the advantageous properties of Groups A and B by simultaneously altering both halves of the canonical AHL structure. **Group C** contains the most promising OdDHL mimics with non-natural head and tail groups.

Finally, **Group D** is made up of either lead compounds identified through high-throughput screens or natural product derivatives that strongly modulate LasR and/or QS-dependent phenotypes in *P. aeruginosa*. As opposed to the other three Groups, these Group D compounds have structures that significantly differ from native AHLs. Taken as a whole, this library serves as a representative subset of the most notable LasR modulators reported to date. (For a more detailed background for each compound and pertinent citations, see Supplementary Note S1 and Table S2.)

***P. aeruginosa* LasR Reporter Screens Reveal Potent Agonists and Antagonists.** To allow for direct comparisons of potency and efficacy across each class of LasR modulator, we first performed our studies in a single *P. aeruginosa* AHL synthase-null strain—PAO-JP2 (Δ*lasIrhlI*) harboring the LasR reporter plasmid p*lasI*-LVA*gfp*—under standardized growth and media conditions (see Experimental Section).[47] Given that the majority of the compounds in the library have been previously reported as LasR antagonists, we expected most compounds to effectively inhibit LasR activity in PAO-JP2; thus, we submitted all of the compounds to full dose−response analysis for competitive LasR antagonism (in the presence of OdDHL) in this *P. aeruginosa* strain (Table 1; for dose−response curves, see Figure S1). However, to perform a more thorough analysis of compound activity, we also evaluated each compound for LasR agonism in a single-concentration agonism screen (Table S3). Compounds showing significant LasR

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639

Table 1. IC_{50} Values for LasR Inhibition by Library Members in *P. aeruginosa* PAO-JP2 (p*lasI*-LVA*gfp*)[a]

compound	IC_{50} (μM)[b]	95% CI (μM)	max. inhibition (%)[c]
2 (OOHL)[d]	5.5	3.1−9.8	55
3 (OHHL)	40	26−61	80
4[e]	≥100	−	25
5	73	54−99	40
6	175	108−284	75
7	116	89−151	80
8[d]	12	3.9−34	60
9[d]	3	0.92−9.7	35
10 (CL)[d]	21	11−39	55
11 (ITC-12)	agonist	−	−
12[e]	9.7	6.3−15	70
13[e]	>200	−	55
14[e]	−	−	−
15[e]	≥100	−	45
16 (mBTL)	agonist	−	−
17 (C10-CPA)[e]	≥50	−	45
18 (V-06-018)[e]	5.2	3.7−7.3	85
19 (TP-1)	agonist	−	−
20 (TP-5)[e,f]	69	61−78	100
21 (C-30)[g]	no activity	−	−
22 (PD-12)	2.5	1.2−5.1	50

[a]Dose−response assays were performed for each compound in the presence of 150 nM OdDHL. [b]Compounds labeled "Agonist" showed LasR-modulatory activity only at levels ≥100% (LasR activation level of OdDHL at 150 nM). [c]Denotes the largest amount of LasR inhibition seen for each compound at any concentration tested. For the full inhibition trace, see Figure S1. [d]Dose−response exhibited nonmonotonic behavior. Concentrations at which LasR activity began to increase were excluded for calculation of IC_{50} values. [e]Compound exhibited limited solubility either in DMSO when preparing stock solutions or in media when performing the dose−response assay. Data obtained at these compound concentrations were excluded from the efficacy and potency analyses. See Note S5 for rationale of data exclusion and Figure S5 for absorbance data at 600 nm. [f]Compound exhibited a dose−response curve with a Hill slope ≠ 1. [g]Compound exhibited cytotoxicity at concentrations ≤1 mM. Data obtained at these compound concentrations were excluded from the efficacy and potency analyses.

activation were then submitted to agonism dose−response analysis (Table 2; for dose−response curves, see Figure S2).

Table 2. EC_{50} Values for LasR Activation by Library Members in *P. aeruginosa* PAO-JP2 (p*lasI*-LVA*gfp*)[a]

compound	EC_{50} (μM)	95% CI (μM)	max. activation (%)[b]
1 (OdDHL)	0.139	0.116−0.167	100
2	>200	−	75
8	>200	−	45
9	140	90−210	65
11	2.6	1.9−3.7	80
14	17	11−26	45
15	>200	−	15
16	4.2	2.5−7.3	90
19	0.071	0.044−0.11	100

[a]Determined by testing AHLs over a range of concentrations for ability to mediate LasR expression of *lasI*-LVA*gfp*. [b]Denotes the highest value of LasR activation seen for each compound at any concentration within the dose−response assay. For the full agonism trace, see Figure S2.

All of the Group A compounds elicited LasR activity in these *P. aeruginosa* dose−response studies. The most potent LasR antagonists in the group were the naturally occurring AHL **2** (OOHL) and the trifluoromethyl-substituted phenyl propionoyl HL (PPHL) **9**. Though both exhibited IC_{50} values in the single-digit micromolar range, their maximum LasR inhibition was modest (<60% relative to OdDHL). Interestingly, the isothiocyanate compound **11** (ITC-12) showed a different activity profile than that reported previously.[24] Meijler and co-workers designated **11** a partial LasR agonist with a maximum efficacy of ∼40%; additionally, they reported that **11** decreased production of the virulence factors elastase B and pyocyanin by approximately 50% in the wild-type *P. aeruginosa* strain PAO1. Our assays also revealed **11** to be a partial LasR agonist, but the compound's maximum efficacy was 2-fold higher (80%). Corroborating this strong LasR agonistic activity in the reporter assay, our later QS phenotypic assays (see below) showed that **11** can strongly increase elastase B production in both wild-type PAO1 and synthase-null PAO-JP2 strains of *P. aeruginosa*. The disparate activity profiles for **11** between our two laboratories is unclear, but likely may be due to the use of different reporter plasmids and/or initial cell densities in the *P. aeruginosa* reporter assays, and different media conditions in the phenotypic assays.[24]

Intriguingly, the AHLs that displayed the most potent antagonism of LasR (**2**, **8**, **9**, and **10**) in our assays also displayed a characteristic *inversion of activity to agonism* (i.e., nonmonotonic, or "paradoxical," dose−response behavior) at higher concentrations. We term these compounds with concentration-dependent bimodal activity "non-classical partial agonists", as their dose−response behavior differs significantly from "classical" partial agonists (e.g., **11** above), which display monotonic dose−response curves instead (see Figure 3A for an illustration of each dose−response type). This nonmonotonic behavior has been seen previously for AHL-derived antagonists evaluated in *E. coli* reporter strains that heterologously produce LasR,[21,23,48] but we have only recently observed such nonmonotonic dose−response behavior in *P. aeruginosa*.[35] As we observe this nonmonotonic AHL dose−response for LasR in both species, our data suggest that the behavior is not simply an artifact of using a heterologous reporter system. We return to the origins of this bimodal activity below (see Mechanistic Insight 1). Among this set of compounds, it is worth noting that **10** (CL) has also been reported to inhibit the related LuxR-type receptor, CviR, via displacement of its native AHL and stabilization of receptor in an inactive homodimer.[49] Examining if **10** has the similar ability to simultaneously stabilize and deactivate LasR (at least at lower concentrations) would certainly be of interest.

The Group B compounds generally suffered from lower solubilities in LB medium relative to the other Groups, precluding testing at high concentrations (Figure S5; Note S5). Nevertheless, within the soluble regime of these compounds, our aniline derivative **12**[37] was found to be an effective inhibitor of LasR in the PAO-JP2 reporter strain (IC_{50} = 9.7 μM; maximum inhibition = 70%). The phenol derivative **14** reported by Suga and co-workers[20] displayed no ability to antagonize LasR in PAO-JP2 in the presence of 150 nM OdDHL (the EC_{50} of the native ligand), corroborating previous assays by our laboratory.[21] Surprisingly, when we submitted the same compound to agonism dose−response analysis, we discovered that **14** was in fact a classical partial agonist of LasR, with a maximum efficacy of 50% (Table 2). This

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639

observation then explained our antagonism data: When high concentrations of **14** outcompete OdDHL present at a concentration also enabling 50% LasR activation, the antagonism dose–response curve shows no net change in LasR activity. These results illustrate how antagonism screens vs a native ligand present at its EC_{50}—analyzed in the absence of accompanying agonism assay data—can obscure the full activity profile of a particular compound. Testing for such partial agonism is certainly prudent, as LuxR-type receptor partial agonists have attracted some attention for their ability to tune receptor responses in ways inaccessible by traditional agonists or antagonists alone.[11,50]

The compounds in Groups C and D elicited a wide range of responses from the LasR receptor in PAO-JP2. The acylated thiolactone **16** of Bassler and co-workers,[11] previously reported to partially antagonize (and agonize) LasR in an *E. coli* reporter, displayed no antagonism of LasR under our conditions, and at concentrations ≥5 μM, it began to activate LasR to a greater extent than 150 nM OdDHL alone. The agonism dose–response analysis for **16** confirmed that this AHL analogue is a LasR classical partial agonist in our PAO-JP2 assay, with a maximal LasR activation of 90%. Compound **17** (C10-CPA) modestly inhibited LasR activity (~50% at 200 μM), though solubility in the assay medium was too low to test at higher concentrations (Figure S5). Compound **18** (V-06-018; uncovered by Greenberg and co-workers in a high throughput screen),[27] when dosed at single-digit micromolar concentrations, displayed the highest LasR inhibition efficacy (>80%) of any library compound dosed at similar concentrations.

Triphenyl compound **19** (TP-1P), also reported by the Greenberg lab,[27] was the only agonist (apart from the native ligand **1**) that maximally activated LasR. It was also the most potent non-native activator of LasR in these *P. aeruginosa* assays, displaying an EC_{50} of 71 nM (~2-fold lower than OdDHL). Notably, compound **19** is the only non-AHL derivative that has been shown via structural analyses to bind in the LasR ligand-binding site, making analogous contacts as OdDHL.[51] Interestingly, the structurally related TP analogue, **20** (TP-5), is a moderate LasR *inhibitor*. Moreover, it displays a LasR inhibition dose–response that was unique among all compounds tested herein: Complete inhibition of LasR occurred over a remarkably narrow concentration range, and after performing the dose–response assay at higher resolution, we found that the best-fit sigmoidal inhibition curve had a Hill slope of −3. We currently have two hypotheses for the mechanism by which **20** inhibits LasR. The Prinz laboratory has previously postulated that receptor denaturation through allosteric interactions of an antagonist with an unstable protein results in a steep dose–response curve.[52] Given that **20** has been shown to cause LasR instability and aggregation (precluding structural analysis),[51] denaturation through allosteric interactions may explain this behavior. Alternatively, the Shoichet laboratory has attributed such phenomena to the colloidal aggregation or precipitation of small-molecule modulators, followed by deactivation or sequestration of the target protein.[53] Because **20** inhibited LasR at concentrations (50–100 μM) approaching those that showed qualitative precipitation (>125 μM), this phase change mechanism may also contribute to the steep inhibition profile.[54,55]

Turning to the frequently cited natural product-derived QS modulator—halogenated furanone **21**[56]—we found this derivative was toxic to *P. aeruginosa* at concentrations ≥100 μM (Figure S5). At all lower concentrations, **21** elicited no inhibition of LasR activity in PAO-JP2. Though this result conflicts with a recent report by Liz-Marzán and co-workers,[57] we note that **21** showed very little LasR inhibition in their bioassay (<20%) at concentrations as high as 10 μM. Additionally, the concentration of **21** at which the authors saw significant LasR inhibition (100 μM) caused significant growth effects in our assay conditions (Figure S5).

The tetrazole **22** was the most potent inhibitor of LasR activity in our *P. aeruginosa* PAO-JP2 assays, with an IC_{50} of 2.5 μM. This potency value is significantly different from the IC_{50} of 30 nM reported by the Greenberg laboratory;[27] however, similar to this previous report, we found that the greatest magnitude of LasR inhibition at any concentration was about 50%.[58] The incongruity in potency for **22** between our study and Greenberg's work may be due to the use of a different LasR-regulated promoter or due to different growth and media conditions. Such discrepancies (also noted for compounds **11** and **21** above) underscore the necessity of using standardized reporters and assay conditions when comparing the dose–response profiles of different compound classes.

Together, the above-standardized LasR reporter assays in the native *P. aeruginosa* background allow for the first direct comparison of compound activity for the 22 chosen molecules. When taking into account both potency and maximum efficacy of LasR modulation, the two compounds that stand out as the most effective LasR modulators under these conditions are **18** (V-06-018) as an antagonist (IC_{50} = 5.2 μM; maximum inhibition = 85%) and **19** (TP-1) as an agonist (EC_{50} = 71 nM; maximum activation = 100%).

A Complementary Heterologous *E. coli* LasR Reporter Study Tests Compounds for Direct LasR Modulation. We next sought to determine if each compound in the LasR modulator library was acting directly on LasR; we thus submitted the library to antagonism and agonism dose–response analysis in an *E. coli* strain (JLD271) harboring LasR that reports on LasR activity via production of β-galactosidase (see Experimental Section).[59] In general, these compounds were more potent LasR modulators in this *E. coli* strain relative to the *P. aeruginosa* PAO-JP2 reporter (Tables 3 and 4). However, the overall shapes of the LasR antagonism dose–response curves for the Group A compounds were conserved between the two strains (see Figures S1 and S3 for full *P. aeruginosa* and *E. coli* curves, respectively). This result supports the common assertion that AHL-type ligands (i.e., ligands like those in Group A) modulate LasR activity directly. Additionally, the maximum percent LasR inhibition trends among highly soluble AHLs in this Group match well between the *P. aeruginosa* and *E. coli* reporters (i.e., **3**, **6**, **7** > **2**, **5**, **8**, **10** > **9**, **11**). Such closely matching trends in activity and dose–response behavior strongly support that the discrepancies in AHL potency between reporter strains are primarily due to mechanisms that affect intracellular availability of the compounds (e.g., active efflux),[35] as opposed to differences in the mechanisms of the LasR receptor–ligand interaction between *E. coli* and *P. aeruginosa* reporters (see Mechanistic Insight 2 below).

The non-AHL-derived compounds in Groups B, C, and D displayed far more varied and unexpected dose–response behaviors in the *E. coli* LasR reporter. OdDHL mimics **12** and **13**, which were LasR *antagonists* in the PAO-JP2 reporter, were found instead to partially agonize LasR in the *E. coli* background (Table 4). Moreover, the maximum LasR responses for partial agonists **14** and **15** were markedly

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626–14639

Table 3. IC_{50} Values for LasR Inhibition by Library Members in *E. coli* JLD271 (pJN105L, pSC11)[a]

compound	IC_{50} (μM)[b]	95% CI (μM)	max. inhibition (%)[c]
2 (OOHL)[d]	0.078	0.032−0.19	35
3 (OHHL)[d]	10.4	5.3−21	70
4[e]	2.8	1.1−6.8	65
5	2.8	1.3−6	65
6[d]	1.0	0.34−3.2	70
7[d]	3.5	2.6−4.8	75
8[d]	0.16	0.043−0.57	45
9	agonist	–	N/A
10 (CL)[d]	0.49	0.1−2.3	40
11 (ITC-12)	agonist	–	N/A
12	–	–	N/A
13	4.7	1.9−12	40
14	agonist	–	N/A
15	agonist	–	N/A
16 (mBTL)	agonist	–	N/A
17 (C10-CPA)	–	–	N/A
18 (V-06-018)[e]	2.3	0.89−6.1	50
19 (TP-1)	agonist	–	N/A
20 (TP-5)[e,f]	70	56−88	85
21 (C-30)[g]	–	–	N/A
22 (PD-12)	–	–	N/A

[a]Antagonism dose−response assays were performed for each compound in the presence of 2 nM OdDHL. [b]Compounds labeled as "agonist" showed LasR-modulatory activity only at levels ≥100% (the LasR activation level of OdDHL at 2 nM). [c]Denotes the largest amount of LasR inhibition seen for each compound at any concentration within the dose−response assay. For the full inhibition trace, see Figure S3. [d,e,f,g]See Table 1 footnotes.

Table 4. EC_{50} Values for LasR Activation by Library Members in *E. coli* JLD271 (pJN105L, pSC11)[a]

compound	EC_{50} (μM)	95% CI (μM)	max. activation (%)[b]
1	0.0018	0.0016−0.0021	100
2	4.5	3−6.7	95
3	>100	–	30
8	8.4	4.5−16	90
9	0.65	0.29−1.4	105
10	33	23−48	60
11	0.017	0.014−0.02	95
12	0.92	0.53−1.6	40
13	>100	–	15
14	0.096	0.06−0.15	85
15	0.24	0.16−0.35	90
16	0.013	0.0067−0.025	90
17	–	–	0
18	–	–	5
19	0.0078	0.0047−0.013	100

[a]Determined by testing AHLs over a range of concentrations for ability to mediate LasR expression of *lasI-lacZ*. [b]Denotes the highest value of LasR activation seen for each compound at any concentration within the dose−response assay. For the full agonism trace, see Figure S4.

increased in the *E. coli* reporter. Such significant alterations of LasR-modulatory ability between native strain reporters and heterologous reporters have been previously observed.[60,61] We further explore this phenomenon in Mechanistic Insight 3 below.

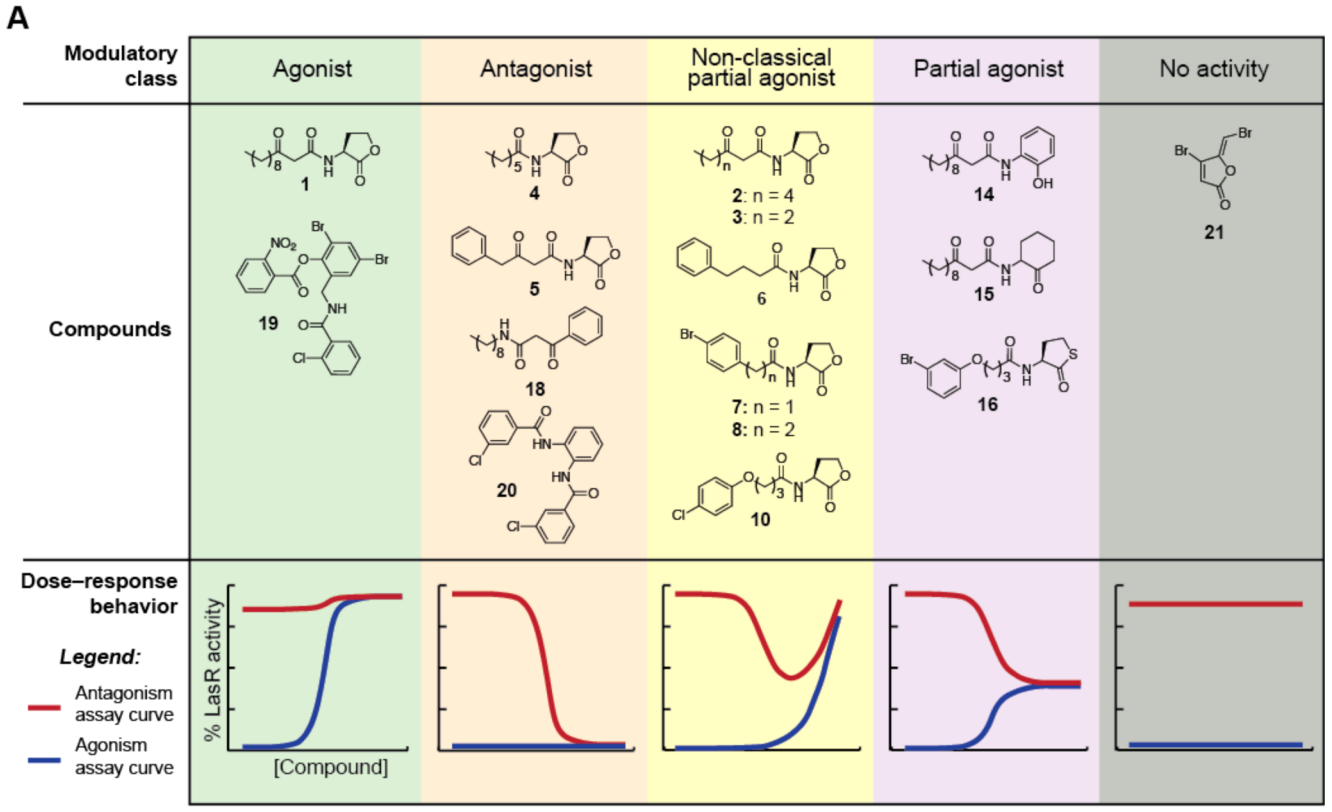
Compounds **17** (C10-CPA), **21** (C-30), and **22** (PD-12) were found to be completely inactive in the *E. coli* LasR reporter (Table 3). Compound **21** caused significant growth effects at concentrations ≥20 μM, and at lower concentrations, no LasR inhibition was observed, similar to the above experiments performed in *P. aeruginosa*. In turn, while compounds **17** and **22** had elicited weak to strong LasR inhibition in the *P. aeruginosa* reporter, these activities were abolished when LasR was isolated in the heterologous *E. coli* reporter, suggesting these two compounds modulate LasR in *P. aeruginosa* via an indirect mechanism.

In general, the LasR agonism activity trends for the library were largely conserved between the *E. coli* and *P. aeruginosa* reporters (Table 4), although compounds were anywhere from 10- to over 100-fold more potent in the *E. coli* background. Again, we believe this is due to increased intracellular availability in *E. coli* relative to *P. aeruginosa*. Compound **19** remained the most potent LasR agonist in the library, displaying the only single-digit nanomolar EC_{50} value (~8 nM).

Figure 3 summarizes all of the activity trends that we observed for the LasR modulator library using both the *E. coli* and *P. aeruginosa* LasR reporters. Combining data from the two sets of reporters, we were able to systematically classify the compounds as LasR agonists, antagonists, partial agonists, and nonclassical partial agonists. We confirmed that **18** (V-06-018) displays the best combination of efficacy and potency as a LasR antagonist, while the most potent LasR agonist was the triphenyl compound **19**. We were also able to exclude certain compounds from further analysis as LasR ligands as they act via indirect mechanisms. With these results in hand, we next sought to further our understanding of some of the unexpected activity profiles that we encountered in the course of our compound screening.

Mechanistic Insight 1: "Non-Classical" Partial Agonists Display Nonmonotonic Dose Curves Due to Two Discrete Binding Events—One Competitive and One Noncompetitive. As highlighted above, we identified seven compounds (**2**, **3**, **6**, and **7**−**10**) that displayed nonmonotonic dose response curves for LasR antagonism in either the *P. aeruginosa* or *E. coli* reporter assays. Our laboratory has previously noted the occurrence of such paradoxical dose−response curves for non-native AHL modulators of various LuxR-type receptors,[21,23,35,48,62] and we recently hypothesized that the bimodal activity observed during competitive antagonism assays may be due to formation of inactive mixed-ligand heterodimers of the receptor. Thus, at intermediate concentrations of non-native AHL, the formation of inactive heterodimers of receptors bound to native and non-native ligand is read out as antagonism, while at high concentrations of non-native ligand, the non-native ligand fully outcompetes the native ligand, resulting in the formation of active homodimers of the receptor that is read out as (typically weak) agonism.[37] This mechanism has been proposed for other receptor types that can function as dimers when bound to their cognate small molecule ligand, such as nuclear hormone receptors.[63,64] We sought to support or refute this hypothesis through additional experiments on LasR. Accordingly, we performed a converse dose−response study, where we dosed in varying concentrations of **1** (OdDHL) to outcompete a non-native ligand in the reporter strain. Presumably, for the mixed-ligand heterodimer hypothesis to hold, OdDHL would reach a concentration that would favor

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639



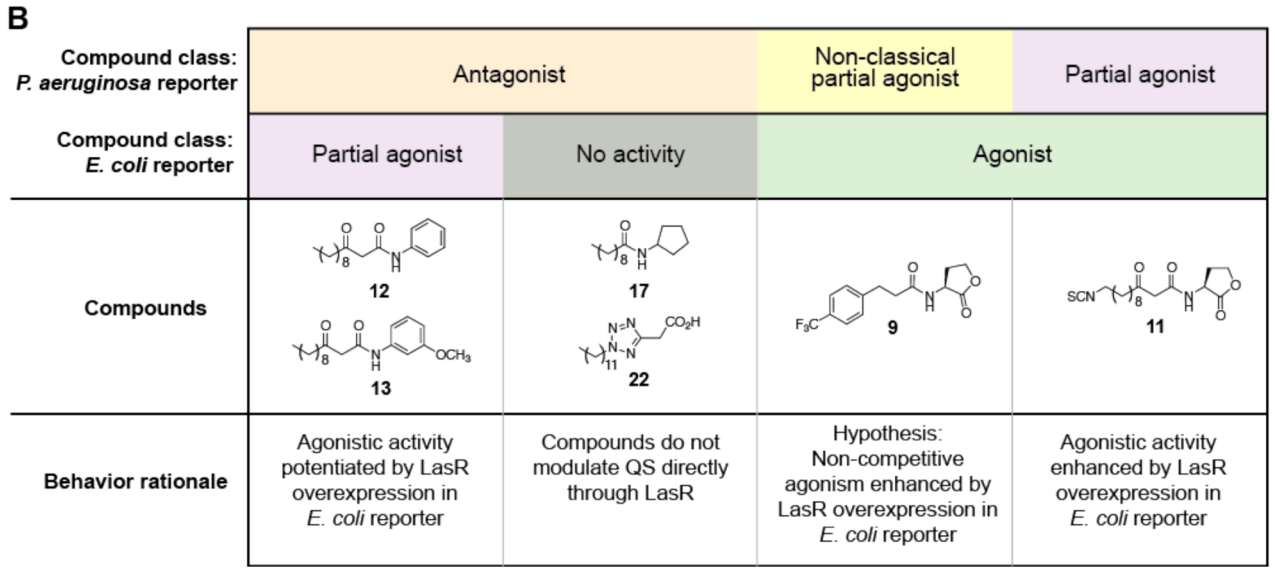


Figure 3. Activity trends of LasR modulators, classified by dose–response assay behavior. (A) Compounds with conserved activity across LasR reporters in *P. aeruginosa* and *E. coli*. (B) Compounds showing altered activity profiles between *P. aeruginosa* and *E. coli* reporter strains.

mixed-ligand heterodimer formation and thus elicit a similar nonmonotonic dose–response curve.

We chose to perform this experiment with brominated PPHL **8** due to its potency and strong bimodal activity in both the *E. coli* and *P. aeruginosa* LasR reporters (Figure 4); we used the *E. coli* LasR reporter since both **1** and **8** are more potent in this species. In contrast to the original antagonism dose–response (Figure 4, blue plot), which shows a nonmonotonic curve, the converse dose–response experiment (Figure 4, red plot) showed no bimodal activity that would be expected to accompany the formation of mixed-ligand LasR dimers at intermediate concentrations of OdDHL. Instead, the converse dose–response was entirely monotonic. This result effectively refutes the hypothesis that the bimodal activity is due to formation of inactive mixed-ligand heterodimers at concentration ranges that allow both ligands to bind to the LasR active site.

In view of these results, we needed to alter our hypothesis and next considered whether the bimodal activity of some AHLs may be due to two discrete binding events at two distinct small-molecule binding sites (on LasR or another target). To begin to investigate this possibility, we performed a two-

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626–14639

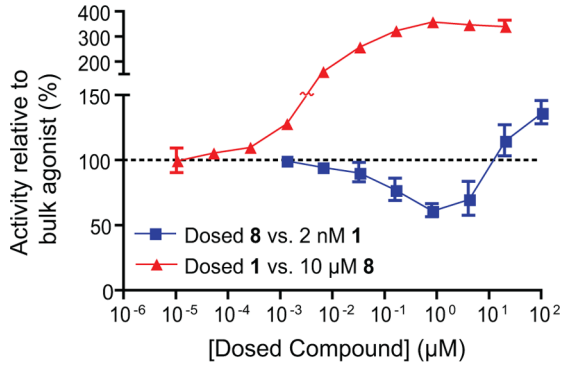


Figure 4. Converse dose−response experiments with LasR native ligand **1** (OdDHL) and nonclassical partial agonist **8** in *E. coli* LasR reporter JLD271 (pJN105L, pSC11). Blue squares (original dose−response with bimodal activity): Varying concentrations of **8** in the presence of **1** at its EC_{50} (2 nM). Red triangles (converse dose−response with monotonic activity): Varying concentrations of **1** in the presence of a bulk addition of 10 μM **8**. Error bars: SEM of $n = 3$ trials.

dimensional dose−response analysis of the nonclassical partial agonist **8** with native ligand **1** in the *E. coli* reporter (Figure 5).

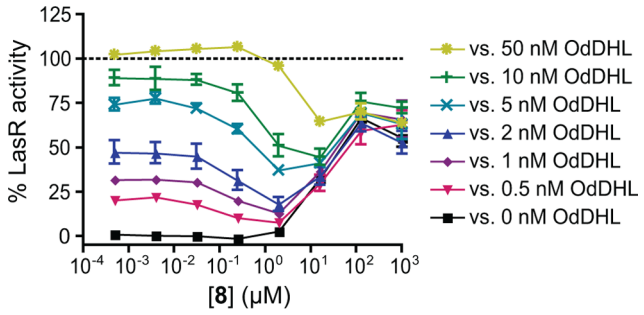


Figure 5. Nonclassical partial agonist behavior of compound **8** in a two-dimensional dose−response study with LasR native ligand **1** (OdDHL). Assay was performed using the *E. coli* LasR reporter JLD271 (pJN105L, pSC11). The antagonistic behavior (at concentrations <10 μM) is competitive with **1** and shifts to higher potency when competed against higher concentrations of **1**. The partial agonist behavior of **8** (at concentrations >10 μM), on the other hand, is insurmountable with increasing concentrations of **1**. Quantitative IC_{50} values from the antagonistic regime of each curve are shown in Figure S6. Error bars: SEM of $n = 3$ trials.

Interestingly, we observed that the inhibitory regime of the dose−response curve of **8** (at concentrations <10 μM) shifts to higher concentrations against increasing doses of OdDHL (**1**),[65] whereas the EC_{50} of the partial agonism regime (at concentrations >10 μM) exhibited no such shift. Thus, we can conclude that the partial agonism binding event occurring at high concentrations of **8** is noncompetitive with native ligand **1**. We confirmed that this behavior is replicated in the *P. aeruginosa* reporter (Figure S7) and, consequently, is not an artifact of the heterologous *E. coli* background. We additionally performed this two-dimensional dose−response assay with native ligand **1** and a different compound, **2** (OHHL), a naturally occurring AHL with a nonaromatic acyl tail that exhibited the same nonmonotonic activity profile. Despite the structural differences between compounds **2** and **8**, the two compounds displayed the same noncompetitive agonism at high concentrations (see Figure S8). Again, we note that this noncompetitive binding event may be allosteric on LasR or may involve a different distinct protein and/or other target(s); the cell-based reporter gene assay utilized here cannot distinguish between these possibilities. Additional experiments—for example, in vitro studies with purified LasR (or a related, more soluble homologue) and a target DNA sequence—are clearly needed to refine this hypothesis and are ongoing in our laboratory. Nevertheless, we believe this alternative ligand binding interaction may represent an interesting new target for the modulation of LasR (and most likely other LuxR-type receptor) activity, and is worthy of future study.

Mechanistic Insight 2: A *P. aeruginosa* Δ*mexAB-oprM* LasR Reporter Shows AHLs Are More Susceptible to Active Efflux than Non-AHLs. Our laboratory recently reported that the presence of the RND efflux pump MexAB-OprM in *P. aeruginosa* reduces the potency of QS modulators;[35] we concluded that these compounds (primarily AHL-type) were being pumped out of the cell, thereby reducing their intracellular concentration. We also showed via a nonspecific pump inhibitor that, despite the presence of multiple homologous pumps in *P. aeruginosa*, MexAB-OprM was the primary cause of compound potency reduction.[35] Now with access to a wider range of compound scaffolds (relative to our past study)[35] in our LasR modulator library, we sought to identify compounds that resisted efflux-induced losses in potency. Such an activity profile, even if resistance to efflux were only moderate, would mark a compound as a choice scaffold for further development. More broadly, we reasoned that screening the library would reveal structural features that either enhance or reduce compound efflux. To evaluate these properties, we performed LasR agonism and antagonism dose−response activity assays on the library using a *P. aeruginosa* mutant strain that lacked a functional MexAB-OprM pump (PAO-JG21) and harbored the LasR reporter plasmid *plasI*-LVA*gfp*. (Though this wider range of compounds may act as substrates of other homologous pumps in *P. aeruginosa*, the MexAB-OprM pump is the most likely cause of efflux.) We observed that for the majority of the compounds, trends in activity (dose−response curve shape, slope of sigmoidal curve, etc.) were conserved (see Figures S1 and S2), and only the potencies of compounds were shifted. Thus, the fold-change in compound IC_{50} (or EC_{50}) from the pump-active reporter to the pump mutant reporter served as the metric by which susceptibility to active efflux was evaluated (Table 5).

This study of efflux susceptibility revealed four clear trends that are directly dependent on compound structural class. First, AHLs with aromatic or long, aliphatic tails (≥8) were more susceptible to active efflux than those with shorter acyl tails (≤6), corroborating previous reports;[3,4,35] for example, compounds **1** (OdDHL) and **2** (OOHL) show 10-fold shifts in potency between pump-active and pump mutant *P. aeruginosa* reporters, while **3** (OHHL, with a six-carbon acyl tail) shows no discernible shift. Second, perhaps unsurprisingly, a covalent (i.e., "irreversible") binding mechanism for LasR modulation reduces susceptibility to active efflux—the isothiocyanate **11**, despite its close structural similarity to OdDHL, exhibits only a 2-fold shift in potency between pump-active and pump mutant agonism dose−response studies, presumably because (at least a percentage of) it is covalently linked to LasR.[24] Third, the presence of a homoserine lactone headgroup greatly increases recognition by MexAB-OprM; compounds with alternative head groups (i.e., **12**, **14**, and **18**) showed significant reduction in susceptibility to active efflux. Fourth, the triphenyl scaffold appears to not be strongly recognized by MexAB-OprM. For

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639

Table 5. Comparison of LasR Antagonist or Agonist Potency between Pump-Active (PAO-JP2) and Pump-Mutant (PAO-JG21) *P. aeruginosa* LasR Reporter Strains[a]

antagonism			
compound[b]	PAO-JP2 IC_{50} (μM)	PAO-JG21 IC_{50} (μM)	fold change[c]
2 (OOHL)	5.5	0.57	9.6
3 (OHHL)	40	41	**1.0**
5	73	8.9	8.2
6	175	20	8.8
7	116	8.2	14.1
8	12	1.5	8.0
9	3	0.42	7.1
10 (CL)	21	1.3	16.2
12	9.7	3.7	2.6
18 (V-06-018)	5.2	6.1	**0.9**
20 (TP-5)	69	63	**1.1**
22 (PD-12)	2.5	0.11	22.7
agonism			
compound	PAO-JP2 IC_{50} (μM)	PAO-JG21 IC_{50} (μM)	fold change[b]
1 (OdDHL)	0.14	0.019	7.4
2	>200	26	>7.7
8	>200	24	>8.3
9	140	8.6	16.3
11 (ITC-12)	2.6	1.3	2.0
14	17	15	**1.1**
16 (mBTL)	4.2	0.56	7.5
19 (TP-1)	0.071	0.036	2.0

[a]Both strains utilize the plasmid p*lasI*-LVA*gfp* to report compound ability to mediate LasR expression of *lasI*-LVA*gfp*. [b]Data for compounds with incalculable fold-changes in potency (due to incomplete dose−response curves) are listed in Table S4. [c]Compounds with statistically insignificant shifts in EC_{50} ($p > 0.1$) are shown in bold. For statistical analysis, see Table S4.

instance, the LasR agonist **19** exhibited only a 2-fold increase in potency in the absence of MexAB-OprM. Similarly, triphenyl-derived antagonist **20** only exhibited a 1.1-fold shift in potency, within statistical error of the assay. These four structure−activity trends should be strongly considered in the design of next-generation LasR (and likely other LuxR-type receptor) modulators. Namely, short-tail AHLs, AHL analogues with non-native head groups, and triphenyl ligands appear to be a worthwhile chemical space to further explore for potent, efflux-resistant LuxR-type QS modulators. The very recent report of novel, irreversible inhibitors of LasR based on compound **19** by Perez and co-workers provides additional support for the continued study of triphenyl scaffolds.[66]

We further expanded upon our prior study of AHL efflux in *P. aeruginosa* by next comparing compound potencies (see Table S5) in all three LasR reporter strains: pump-active *P. aeruginosa*, pump mutant *P. aeruginosa*, and *E. coli*. We observed that the trend of potency shifts between pump-active and pump mutant *P. aeruginosa* strains did not fully match the trend between the *E. coli* and pump-active *P. aeruginosa* strains; nonetheless, the compounds were almost all more potent in *E. coli* vs the *P. aeruginosa* pump mutant (the only exception being antagonist **20**, which was effectively equipotent in all three strains). These data suggest that, as we anticipated for the broader structural array of compounds studied herein, other factors beyond active efflux are likely contributing to the amplified potency shifts between the *E. coli* and *P. aeruginosa* LasR reporters, such as differential membrane permeability or susceptibility to enzymatic degradation.

Mechanistic Insight 3: Because of LasR Overexpression, Compound Activity Profiles Can Vary between *E. coli* and *P. aeruginosa* Reporters. As shown in Figure 3B, a subset of library compounds displayed LasR modulation profiles that significantly changed depending on whether the reporter was in a *P. aeruginosa* or an *E. coli* background. We reasoned that the two compounds displaying a complete loss of efficacy in *E. coli* (**17** and **22**) are likely modulating LasR in *P. aeruginosa* through some upstream interaction (see above). Harder to explain, however, were the compounds that still modulated LasR but had markedly altered activity profiles (e.g., compounds **9** and **11**−**13**; Figure 3B, columns 1, 3, and 4). In 1998, Winans and co-workers hypothesized that heterologous expression of LuxR-type receptors could cause substantial changes in efficacy due to the receptor being overexpressed in such systems relative to the native background; this proposition stemmed from their studies with the LasR-homologue TraR that showed compounds shift from antagonist to agonist upon TraR overexpression in *Agrobacterium tumefaciens*.[67] We sought to test this hypothesis by transforming the same LasR expression plasmid used in our *E. coli* reporter strain (pJN105L) into *P. aeruginosa* PAO-JP2 and performing analogous dose−response analyses while overexpressing LasR via addition of L-arabinose. Control experiments for the test compounds (i.e., **9** and **11**−**13**) in PAO-JP2 (i) in the absence of the LasR expression plasmid and presence of L-arabinose and (ii) in the presence of the LasR expression plasmid and the absence of L-arabinose indicated that neither the plasmid nor the inducer (L-arabinose) alone were influencing LasR activity (Figure S9).

We postulated that if the Winans laboratory hypothesis were correct for the test compounds, their dose−response behaviors in the PAO-JP2 reporter with LasR overexpressed via pJN105L would mimic their behaviors in the *E. coli* LasR reporter. For compounds **11** and **12**, we did indeed see the anticipated activity profile shifts (Figure 6): Compound **11** (a partial agonist in the PAO-JP2 LasR reporter strain) converted to a full agonist, and compound **12** (an antagonist in the PAO-JP2 LasR reporter strain) converted to a partial agonist. The potencies of both compounds in the *P. aeruginosa* LasR overexpression reporter were still less than those in the *E. coli* LasR reporter, likely due to the differences in active efflux and membrane permeability between *E. coli* and *P. aeruginosa* (as described above). We believe that this loss in potency in *P. aeruginosa* is also the reason behind compound **13** showing no partial agonism in the PAO-JP2 LasR overexpression reporter (Figure S10B). The data for compound **9**, however, refuted our hypothesis (Figure S10A); **9** retained its nonmonotonic dose−response when moving from the PAO-JP2 LasR native-expression reporter to the overexpression reporter (in contrast to its observed monotonic dose−response in the *E. coli* reporter; Figure S10A). We consequently speculate that LasR overexpression may not be the only factor causing the altered activity profile of **9** in *E. coli*. Namely, because the non-monotonic dose−response curves are likely produced from *two* (or more) discrete binding events (see above), we believe that, in the *E. coli* LasR reporter, the potency of the *agonistic* binding event for compound **9** may shift far more strongly than that of the *antagonistic* binding event, causing the agonistic event to subsume the antagonistic one.[68]

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639

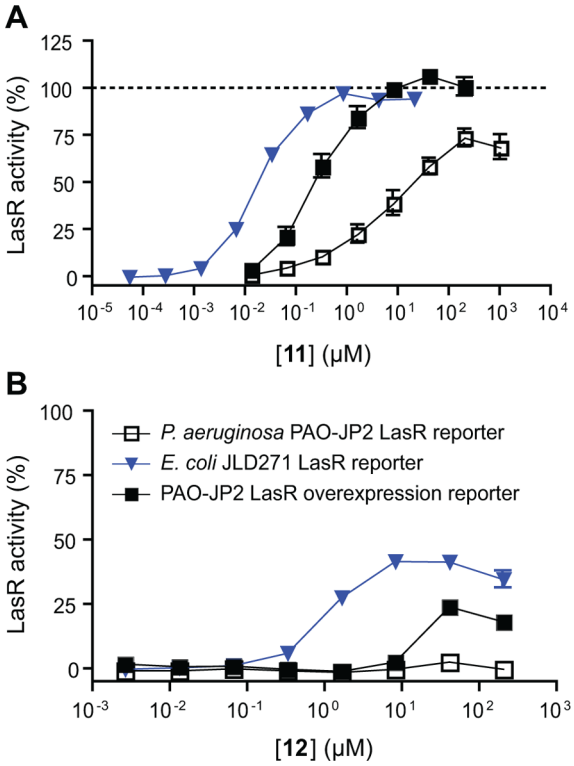


Figure 6. LasR overexpression alters dose–response behavior for some compounds. Dose–response assays using compounds **11** (A) and **12** (B) showed that the behavior of the *P. aeruginosa* reporter overexpressing LasR (filled squares) more closely matched that of the *E. coli* LasR reporter (blue triangles) than that of the *P. aeruginosa* native LasR expression reporter (empty squares). Error bars: SEM of $n = 3$ trials.

Although LasR activity profiles were significantly altered in *E. coli* reporters for only a few compounds tested herein, this incongruent behavior in heterologous strains (relative to native backgrounds) is common enough to have been noted by multiple other laboratories studying LuxR-type receptors.[60,61,69] Our results corroborate the claim of the Winans laboratory that heterologous reporters are prone to such anomalies, and ongoing work in our laboratory is focused on developing an *E. coli* LasR reporter and a set of assay conditions that better mimic LasR activity trends in *P. aeruginosa*.

Elastase Assays Confirm Compound Efficacy on LasR in Wild-Type *P. aeruginosa*. The comparative activity data for the LasR modulator library above, augmented with new mechanistic insights, allowed us to rigorously choose compounds that we believed would be effective in an assay directly measuring QS-dependent phenotype activity in *P. aeruginosa*. We elected to test the effects of these compounds on the production of the well-studied virulence factor elastase B (LasB). LasB is a metalloprotease that degrades immune components and causes tissue damage within infected hosts.[70] Critically, elastase B production is strongly regulated by the *las* QS circuit.[71] Recent studies have shown that while all phenotypic regulation by LasR is dependent on environmental factors and growth conditions,[72] the influence of the *las* system on elastase B production is much clearer and more direct than that on other prominent virulence phenotypes, for example, biofilm[73,74] or pyocyanin[12] production. We therefore reasoned it would be the most direct test of the compounds' ability to modulate LasR in wild-type *P. aeruginosa*.

To quantify elastase B production, we performed a colorimetric assay in the wild-type *P. aeruginosa* strain PAO1 using an elastin–Congo red substrate (see Experimental Section). We submitted a focused subset of compounds with definitive activity profiles and/or interesting structural features to this assay (Figure 7)—activators **11**, **16** (partial agonists with high LasR activation) and **19** (full agonist), along with inhibitors **7**, **8** (retention of AHL headgroup), **12**, **13** (retention of OdDHL tail), and **18**, **20**, and **22** (non-AHL scaffolds). As a key control, we used the *P. aeruginosa* Δ*lasIrhlI* mutant PAO-JP2 to mimic a fully QS-inhibited wild-type strain.

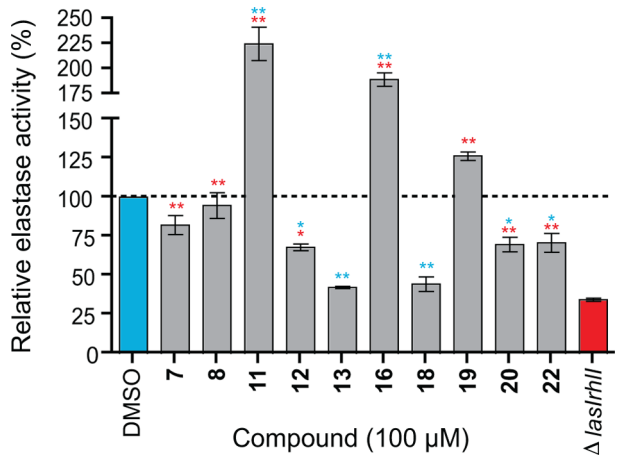


Figure 7. Elastase B activity in wild-type *P. aeruginosa* (PAO1) in the absence (DMSO; negative control; blue bar) or presence (gray bars) of 100 μM LasR modulator, and in Δ*lasIrhlI* mutant PAO-JP2 (full QS-dependent inhibition; positive control; red bar). Error bars: SEM of $n = 3$ trials. Red stars: significance from Δ*lasIrhlI* control; Blue stars: significance from DMSO control. * = $p < 0.05$, ** = $p < 0.01$; For full tabular and statistical data, see Table S6.

Activity trends were well conserved between the elastase B assays in wild-type PAO1 and the LasR reporter assays in PAO-JP2. Interestingly, the AHLs **7** and **8** showed only modest (<25%) elastase inhibition. Both were shown to be particularly susceptible to efflux by MexAB-OprM, and previous reports have shown that the homoserine lactone head is prone to hydrolysis,[75,76] so we believe that in the presence of continually replenished native ligand (in the wild-type strain), the AHLs are unable to effectively inhibit LasR over the 16 h growth span necessary for the assay. Nonlactone OdDHL mimics **12** and **13** were able to inhibit elastase by ≥50%, though we were surprised to see that **13** inhibited elastase more effectively than **12**, despite its lower potency in the PAO-JP2 LasR reporter. Notably, compound **18** (V-06-018), which displayed potent and efficacious LasR inhibition in all reporter assays, showed complete QS-dependent inhibition of elastase (no statistically significant difference from the Δ*lasIrhlI* mutant).

The results of these elastase assays show that our reporter bioassay experiments offer a largely predictive view of LasR-dependent phenotypic modulation under uniform growth conditions. Those compounds that showed resistance to active efflux and consistently potent LasR antagonism were highly effective at overcoming the common hurdles that make small-molecule modulation of *P. aeruginosa* QS phenotypes so challenging.

DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626–14639

CONCLUSIONS AND OUTLOOK

In summary, we report the assembly and comparative evaluation of a library of compounds that comprises some of the most potent and efficacious LasR modulators known. We submitted this focused library to standardized screening conditions allowing comparison of LasR modulatory ability across a variety of structural classes. Our biological assays allowed us to measure potency, efficacy, susceptibility to active efflux, and whether or not the modulators are directly targeting LasR. This systematic analysis of *P. aeruginosa* LasR modulators has revealed many salient points to consider when designing future compounds as research tools or for antivirulence applications.

First, we have shown that data obtained using the reporter constructs and assay conditions described herein are largely predictive for small-molecule modulation of QS-dependent virulence phenotypes—here, elastase B production—in wild-type *P. aeruginosa*. These reporters and assay protocols could be readily adopted as standard methods for assaying LasR ligands. We also demonstrate that the simultaneous analysis of LasR activity and active efflux susceptibility allows a very clear picture of compound efficacy in *P. aeruginosa* (at least when grown in the common bacterial growth medium LB).

Second, we have identified a possible alternative site/target for LasR modulation. We identified natural and non-natural AHLs that are ostensibly activating LasR through this noncompetitive site/target, and we believe that further research should be focused on characterizing and exploiting this phenomenon. Perhaps most notably in this regard, a non-competitive antagonist would bypass the challenges inherent in treating wild-type pathogens that are constitutively producing their native QS autoinducers.[36]

Third, our studies serve to highlight two compounds for their ability to strongly modulate LasR and influence QS-dependent phenotypes in wild-type *P. aeruginosa*: (i) triphenyl compound **19** (TP-1) as an agonist and (ii) compound **18** (V-06-018) as an antagonist. Compound **19** exhibits multiple desirable traits for a LasR modulator. We have shown that the triphenyl scaffold is less susceptible to active efflux, and **19** consistently ranks as the most potent LasR modulator in our reporter studies. Further, because **19** is known to bind the OdDHL binding site and makes similar molecular contacts to LasR as OdDHL,[51] we believe that analogues of **19** may have a propensity to mode switch between LasR activation and inhibition—similarly to non-native AHL analogues (indeed, this is already exemplified by the disparate activities of **19** and **20**).[21] A potent triphenyl inhibitor of LasR would circumvent the liabilities associated with the hydrolyzable homoserine lactone head and would likely maintain resistance to active efflux. Again, the recent work of Perez and co-workers on new derivatives of **19** is encouraging in this regard.[66] Finally, compound **18** (V-06-018) displayed consistently high efficacy and potency in all of the reporter and phenotypic assays in this study. Though it is similar in structure to the Group B compounds (which have received significant attention from groups that design LasR modulators),[7,46] it appears to be generally more potent. Consequently, efforts to further refine SAR around the features of **18** and enhance its solubility might result in a very powerful *P. aeruginosa* QS inhibitor.

To close, the past 20 years have seen enormous advances in understanding of the intricate social networks utilized by bacteria, and the chemical tools developed by research laboratories to target QS pathways are certainly contributing to this effort.[11,12,49,77] While these compounds can be uniquely valuable in the process of delineating QS circuits, many researchers have called attention to two particular shortcomings in the field: (i) the dearth of *directly comparative* QS modulator SAR data acquired with standardized screening conditions,[46,78] and (ii) the relative lack of small molecules capable of potently modulating QS-controlled phenotypes in wild-type bacterial strains.[79−81] Herein, we report experiments that now address both deficiencies through a comprehensive study of the QS receptor LasR in *P. aeruginosa*. Looking forward, our findings provide important context for the design of next-generation LasR ligands and effective antivirulence strategies in *P. aeruginosa*. Moreover, the mechanistic insights we gained are likely broadly applicable to small molecule ligand interactions with LuxR-type receptors beyond LasR. Accordingly, these structural features and mechanisms should be considered when designing synthetic modulators of any LuxR/LuxI-type QS network in Gram-negative bacteria.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b06728.

Bacterial strain and plasmid descriptions, reporter and elastase assay data, compound characterization data, and supplemental text. (PDF)

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The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support for this work was provided by the NIH (GM109403) and the Burroughs Wellcome Fund. J.D.M. was supported in part by the UW−Madison NIH Biotechnology Training Program (T32 GM08349). We gratefully acknowledge Professors Michael Meijler and Peter Greenberg for donation of test compounds, as well as Dr. Joseph Gerdt and Dr. Nora Eibergen for helpful discussions.

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DOI: 10.1021/jacs.5b06728
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DOI: 10.1021/jacs.5b06728
J. Am. Chem. Soc. 2015, 137, 14626−14639

APPENDIX THREE: Design, Synthesis, and Biochemical Characterization of Non-Native Antagonists of the *Pseudomonas aeruginosa* Quorum Sensing Receptor LasR with Nanomolar IC_{50} Values

D.E. Manson performed and designed experiments and synthesized compounds, M.C. O'Reilly synthesized compounds, K.E. Nyffeler performed reporter experiments, D.E. Manson and H.E. Blackwell wrote the text.

Manson, D.E.; O'Reilly, M.C.; Nyffeler, K.E.; Blackwell, H.E.; *ACS Infect.* **2020**, *6*(4), 649-661

https://doi.org/10.1021/acsinfecdis.9b00518

ACS Infectious Diseases

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Design, Synthesis, and Biochemical Characterization of Non-Native Antagonists of the *Pseudomonas aeruginosa* Quorum Sensing Receptor LasR with Nanomolar IC_{50} Values

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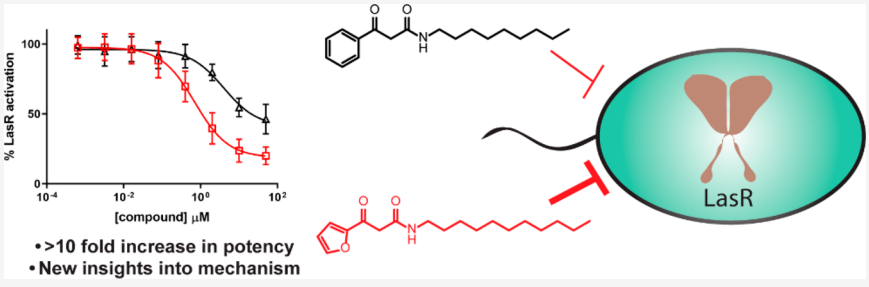
Cite This: *ACS Infect. Dis.* 2020, 6, 649–661 Read Online



ABSTRACT: Quorum sensing (QS), a bacterial cell-to-cell communication system mediated by small molecules and peptides, has received significant interest as a potential target to block infection. The common pathogen *Pseudomonas aeruginosa* uses QS to regulate many of its virulence phenotypes at high cell densities, and the LasR QS receptor plays a critical role in this process. Small molecule tools that inhibit LasR activity would serve to illuminate its role in *P. aeruginosa* virulence, but we currently lack highly potent and selective LasR antagonists, despite considerable research in this area. V-06-018, an abiotic small molecule discovered in a high-throughput screen, represents one of the most potent known LasR antagonists but has seen little study since its initial report. Herein, we report a systematic study of the structure–activity relationships (SARs) that govern LasR antagonism by V-06-018. We synthesized a focused library of V-06-018 derivatives and evaluated the library for bioactivity using a variety of cell-based LasR reporter systems. The SAR trends revealed by these experiments allowed us to design probes with 10-fold greater potency than that of V-06-018 and 100-fold greater potency than other commonly used *N*-acyl-L-homoserine lactone (AHL)-based LasR antagonists, along with high selectivities for LasR. Biochemical experiments to probe the mechanism of antagonism by V-06-018 and its analogues support these compounds interacting with the native ligand-binding site in LasR and, at least in part, stabilizing an inactive form of the protein. The compounds described herein are the most potent and efficacious antagonists of LasR known and represent robust probes both for characterizing the mechanisms of LuxR-type QS and for chemical biology research in general in the growing QS field.

KEYWORDS: *N-acyl-L-homoserine lactone, bacterial communication, inter-cellular signaling, LuxR-type receptor, small molecule probes, virulence*

Microbial resistance to antibiotics is emerging faster than new treatments are being developed, setting the stage for a public health crisis.[1,2] As traditional antibiotics become less effective, interest has arisen in attenuating virulence *via* interference with non-essential pathways.[3] Inhibition of quorum sensing (QS), a mode of bacterial communication dependent on the exchange of chemical signals, has been shown to reduce virulence phenotypes in multiple human pathogens without affecting cell viability.[4−6] Accordingly, it has attracted significant interest as a potential antivirulence strategy for combatting bacterial infections.[7,8] Our laboratory[9−11] and others[12−15] are interested in the development of small molecule and peptide probes to dissect the mechanisms of QS and their roles in infection.

The prototypical QS circuit in Gram-negative bacteria is the LuxI/LuxR synthase/receptor pair, first discovered in the marine symbiont *Vibrio fischeri*.[6] At low cell density, a LuxI-type enzyme synthesizes the QS signal, an *N*-acyl-L-homoserine lactone (AHL), at a low basal rate. These low-molecular weight molecules can freely diffuse out of the cell; although, in certain cases, they are also actively exported.[16] The concentration of AHL signal is largely proportional to cell density (and this correlation is highly dependent on the

Special Issue: Chemical Microbiology

Received: December 23, 2019
Published: February 9, 2020

https://dx.doi.org/10.1021/acsinfecdis.9b00518
ACS Infect. Dis. 2020, 6, 649–661

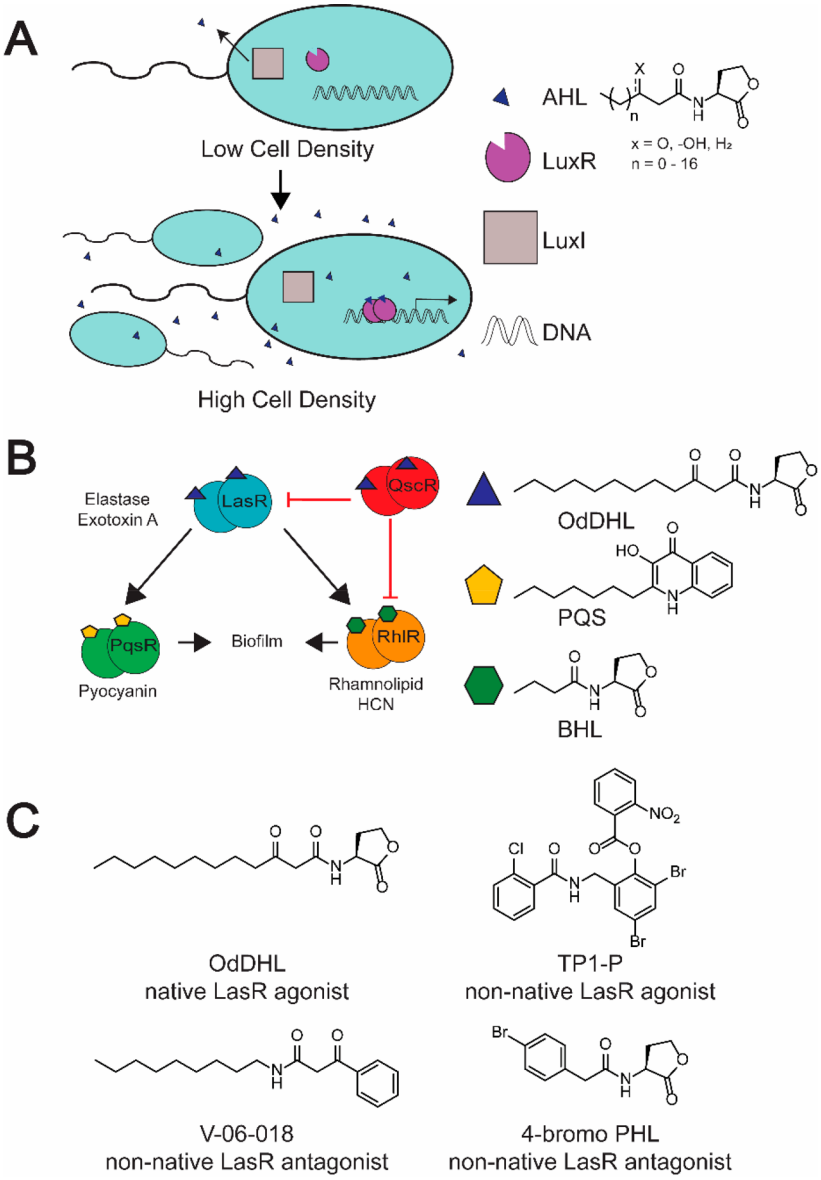


Figure 1. (A) General schematic of LuxI/LuxR-type quorum sensing (QS) in Gram-negative bacteria. (B) Simplified view of QS in *P. aeruginosa*. LasI/R and RhlI/R are LuxI/R homologues. QscR is an "orphan" LuxR-type receptor and responds to OdDHL. PqsR is a LysR-type receptor that responds to the Pseudomonas quinolone signal (PQS). AHL synthases are omitted for clarity. (C) Structures of native agonist OdDHL (EC_{50} = 139 nM), non-AHL antagonist V-06-018 (IC_{50} = 5.2 μM), non-AHL agonist TP1-P (EC_{50} = 71 nM), and representative, synthetic AHL antagonist 4-bromo PHL (IC_{50} = 116 μM); potency values all obtained in the same *P. aeruginosa* LasR reporter (from ref 30).[20]

environment), but as a bacterial community grows, the level of AHL signal in the local environment likewise increases (Figure 1A). At high cell densities, the intracellular AHL concentration is sufficient for productive binding of the AHL to its cognate LuxR-type receptor, a transcription factor. The activated receptor/ligand complex then typically dimerizes and binds to DNA, which subsequently alters gene expression levels to promote group-beneficial behaviors. In pathogenic bacteria, these behaviors can include the production of toxic virulence factors and biofilm. Typically, once a "quorum" is achieved, expression of the LuxI-type synthase is also increased, amplifying AHL production in a positive "autoinduction" feedback loop.[17]

Pseudomonas aeruginosa is an opportunistic pathogen that regulates many aspects of virulence using QS. This bacterium has a high rate of resistance to traditional antibiotics and causes infections that are especially dangerous for individuals with cystic fibrosis (CF), burn victims, and AIDS patients. The QS system in *P. aeruginosa* is relatively complex (Figure 1B),[18] consisting of two LuxI/LuxR pairs (LasI/LasR and RhlI/RhlR) along with an orphan LuxR-type receptor (QscR), which lacks a related synthase and native AHL signal. LasI synthesizes *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL), which targets LasR but also strongly activates QscR. RhlI synthesizes *N*-butyryl-L-homoserine lactone (BHL), which targets RhlR. Additionally, *P. aeruginosa* has a LysR-type receptor, PqsR, which is unrelated to LuxR-type receptors and uses 2-heptyl-3-hydroxy-4-(1*H*)-quinolone (i.e., the Pseudomonas quinolone signal (PQS)) as its ligand. These four QS systems are intimately linked and control different aspects of *P. aeruginosa* virulence that are highly dependent on the environment (Figure 1B).[9] LasR plays a central role in the QS hierarchy. For

https://dx.doi.org/10.1021/acsinfecdis.9b00518
ACS Infect. Dis. 2020, 6, 649−661

instance, LasR directly regulates the production of virulence factors such as elastase, alkaline protease, and exotoxin and regulates rhamnolipid, HCN, and pyocyanin production *via* control of the *rhl* and *pqs* systems.[18] Biofilm, a major virulence phenotype in *P. aeruginosa*, is also regulated by LasR *via* the *rhl* and *pqs* systems.[19] In turn, LasR and RhlR are repressed by QscR, which again is strongly activated *via* LasR's native signal, OdDHL.

The connection between QS and virulence in *P. aeruginosa*, and in other Gram-negative bacterial pathogens, has motivated the development of small molecules and macromolecules capable of inhibiting LuxI-type synthases,[21] destroying or sequestering AHL signals,[22] or blocking the binding of AHL signal to LuxR-type receptor.[23] The latter competitive inhibition strategy has seen the most study to date, with significant contributions by the Spring,[7] Bassler,[24] Greenberg,[25] and Meijler[15] laboratories, as well as our lab.[26] Due to its prominent position in the *P. aeruginosa* QS system (*vide supra*), much of the effort devoted to identifying small molecule modulators of QS in *P. aeruginosa* has focused on LasR. The majority of the known synthetic ligands that modulate LasR were identified by making systematic changes to the lactone "headgroup" and acyl "tailgroup" of OdDHL (e.g., 4-bromo PHL; Figure 1C).[27,28] However, these past efforts have failed to yield compounds that antagonize LasR with both high efficacies and potencies.[29] To our knowledge, none of these AHL analogues have lower than double-digit micromolar (μM) IC_{50} values in reporter gene assays of LasR activity in *P. aeruginosa*.[30] These IC_{50} values contrast with the nanomolar (nM) EC_{50} value of LasR's native ligand, OdDHL, and those of other non-native agonists (e.g., the triphenyl derivative TP1; Figure 1C).[25,26] The poor antagonism potencies for AHL analogues may be due, at least in part, to reliance on the AHL scaffold, which has several major liabilities for probe molecules. AHLs are susceptible to lactone hydrolysis, enzymatic degradation, and active efflux by *P. aeruginosa*.[16,31,32] These drawbacks make the development of non-AHL antagonists of LasR, and other LuxR-type receptors, highly desirable.[30] That said, conversion of non-AHL scaffolds known to strongly agonize LasR (e.g., TP1) into antagonists (i.e., "mode switching") has also not provided sub-μM LasR antagonists so far,[26] underscoring the challenges of this process.

High-throughput screens of small molecule libraries provide another pathway to identify non-AHL LasR antagonists.[33] One such screen by Greenberg and co-workers in 2006 revealed the compound V-06-018, a β-keto amide with a phenyl headgroup and a nine carbon tail (Figure 1C).[33] V-06-018 is a relatively potent LasR antagonist in both *E. coli* and *P. aeruginosa* LasR reporter strains (single-digit micromolar IC_{50}) and has been shown to inhibit genes and phenotypes related to virulence in *P. aeruginosa*.[9,33] The phenyl headgroup and aliphatic acyl tail of V-06-018 resemble that of the homoserine lactone headgroup and acyl tail of LasR's native ligand, OdDHL (Figure 1C). However, as V-06-018 lacks a lactone moiety, it is not susceptible to hydrolysis or enzymatic cleavage by AHL lactonases.[31,32] A prior study of ours also revealed that V-06-018 is not actively effluxed from *P. aeruginosa* by the promiscuous MexAB-OprM efflux pump, which is known to efflux both native and non-native AHLs with long acyl tails.[16] Despite these desirable qualities, V-06-018 has seen practically no scrutiny from a structure–function perspective and no substantive use as a chemical probe since its initial report over a decade ago.[30] We reasoned that the V-06-018 scaffold could provide entry into LasR antagonists with improved potencies along with robust physical properties, and in the current study we report our findings with regard to the first structure–function analysis of this scaffold. Our combined cell-based assays, synthesis, and iterative compound design revealed a set of new LasR antagonists based on V-06-018 with potencies, efficacies, and receptor selectivities in *P. aeruginosa* that, to our knowledge, surpass all known compounds reported to date. Follow on biochemical experiments on these compounds and V-06-018 support a mechanism of antagonism by which they interact with the OdDHL-binding site in LasR and, at least in part, stabilize an inactive form of the protein.

RESULTS AND DISCUSSION

V-06-018 is Selective for LasR over RhlR and QscR in *P. aeruginosa*. We began our study by exploring the selectivity of V-06-018 for LasR over the other two LuxR-type receptors (RhlR and QscR) in *P. aeruginosa* as, other than its antagonistic activity in LasR,[30] this profile was unknown. In view of the overlapping activities of these three receptors in *P. aeruginosa* (see Figure 1B), small molecule tools that are selective for LasR (or indeed any of these receptors) are of significant interest for use as mechanistic probes in this pathogen. We submitted V-06-018 to reporter gene assays in *E. coli* to examine its antagonistic activity (in competition with the receptors' native or preferred ligand) and agonistic activity (alone) in LasR, RhlR, and QscR, using our previously reported methods (see the Materials and Methods section). In these reporter assays in a heterologous background (i.e., *E. coli*), each of the receptors was examined in isolation from the others, allowing for clearer selectivity profiles to be defined relative to using analogous *P. aeruginosa* reporter systems. Receptor activity was monitored *via* β-galactosidase production. These experiments revealed V-06-018 was only an antagonist of LasR, displayed no activity (as either an antagonist or agonist) in RhlR, and was only a very weak antagonist QscR at the highest concentrations tested (see Figure S1). This high receptor selectivity profile rendered the V-06-018 scaffold even more compelling for new LasR antagonist development in *P. aeruginosa*.

An Efficient Synthesis of V-06-018 and Analogues. We next sought to devise a synthetic route to V-06-018 that was scalable and adaptable to analogue synthesis. The only previously reported synthesis of V-06-018 gave the molecule in 5% yield, albeit in one step.[24] That synthesis involved refluxing ethyl benzoyl acetate and nonylamine in ethanol. We reasoned the low yield for this reaction could be due to imine formation; therefore, we decided to protect the ketone in ethyl benzoyl acetate as a ketal (e.g., **2** → **3**; Scheme 1) and then saponified the ester to access the carboxylic acid (**4**). Standard carbodiimide-mediated amide bond coupling (*via* EDC) of the acid with nonylamine proceeded smoothly to yield amide **5**. Deprotection of the ketone furnished V-06-018 in 44% yield over four steps, in quantities typically greater than 100 mg. This synthetic route was advantageous as it could be easily modified to generate V-06-018 analogues with alternate tail groups (R′ in Scheme 1) through the coupling of different amines. In turn, alternate head groups could be incorporated by coupling different carboxylic acid building blocks (**4**), many of which are readily accessible from acylation reactions of substituted acetophenones using diethyl carbonate as an electrophile (e.g., **1**→ **2**; Scheme 1).[34] We introduced both

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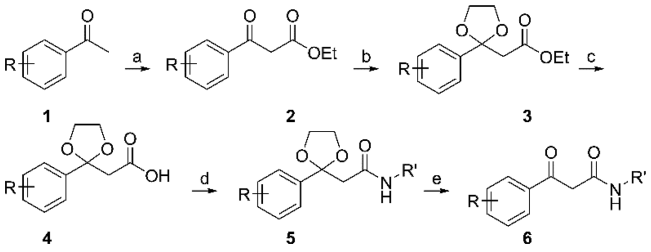
Scheme 1. Synthesis of V-06-018 and Related Analogues[a]



[a]Reagents over arrows: a = NaH, $(C_2H_5)_2CO_3$, THF, Δ; b = $C_2H_6O_2$, *p*-TsOH, benzene, Δ, Dean−Stark trap; c = 1:1 LiOH (1M, aq), THF; d = EDC·HCl, DMAP, H_2NR', CH_2Cl_2; e = *p*-TsOH, acetone. See the Materials and Methods section and Supporting Information (SI) for additional details.

modifications in our subsequent synthesis of a focused library of V-06-018 analogues.

Structure-Informed Design of a V-06-018 Analogue Library. We approached our design of V-06-018 analogues by first considering the binding mode of OdDHL to LasR (Figure 2). The reported X-ray structure of OdDHL bound to the LasR ligand-binding domain (LBD) indicates that the lactone, amide, and keto functionality in OdDHL can make several hydrogen bonds with residues in the LasR ligand-binding site (e.g., Tyr 56, Trp 60, Asp 73, and Ser 129).[35] In view of their structural similarity (see Figure 1C), it is not unreasonable to assume that V-06-018 could target the same binding site on LasR as OdDHL. We therefore were interested in synthesizing analogues that could either gain or lose the ability to make the same hydrogen-bonding contacts as OdDHL, to examine their effects on V-06-018 activity. As the phenyl headgroup of V-06-018 cannot engage in a hydrogen bond with LasR, we synthesized a series of analogues *via* Scheme 1 with alternate head groups (**8**, **12**, **13**, and **17**−**21**; Figure 3) that either place a heteroatom in a position to potentially accept or, in the case of phenols **17** and **18**, accept and/or donate a hydrogen bond.

To examine LasR's tolerance for increased steric bulk on V-06-018's headgroup, we synthesized naphthyl derivative **10**

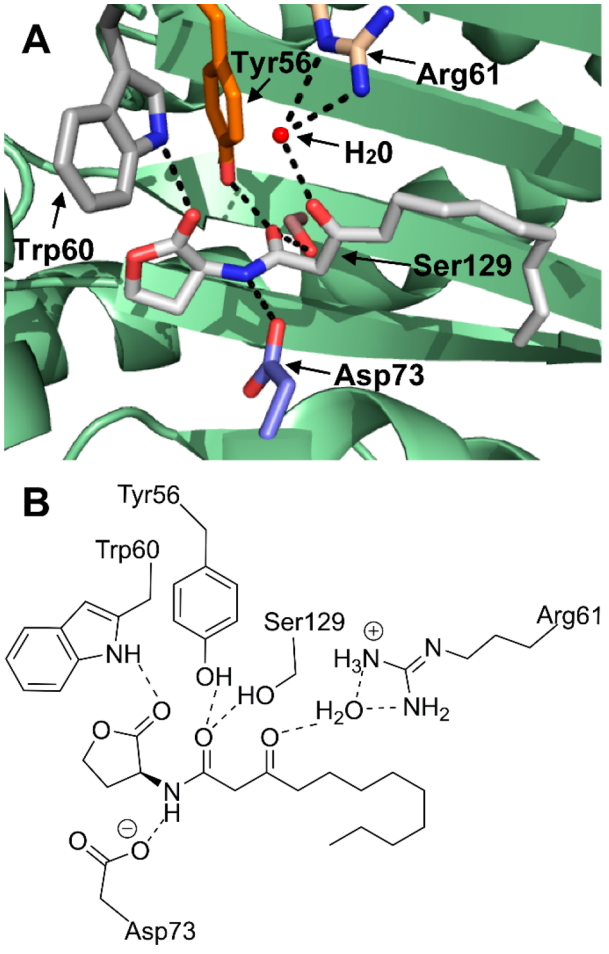


Figure 2. Three-dimensional (A) and two-dimensional (B) images of the OdDHL-binding site in the $[LasR\ LBD/OdDHL]_2$ cocrystal structure (PDB ID: 2UV0).[36] Dashed lines indicate putative hydrogen bonds between the labeled residues or water (shown as a red ball in part A) and OdDHL. OdDHL in part A is shown with carbon in gray, oxygen in red, and nitrogen in blue.

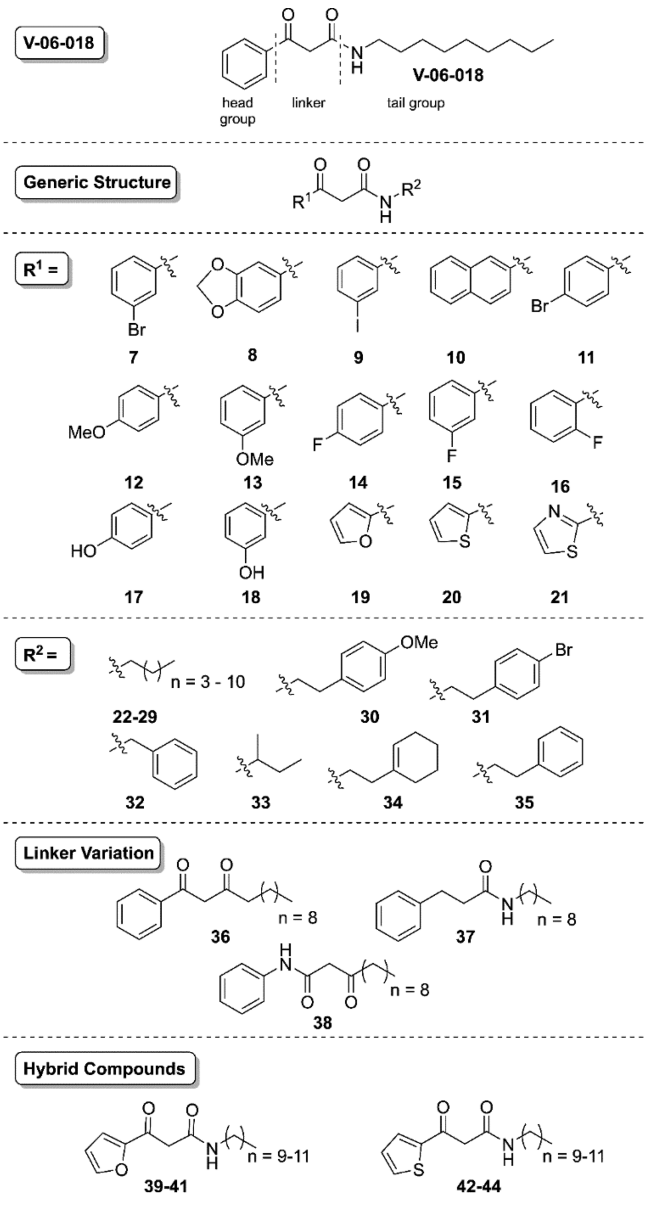


Figure 3. Library of V-06-018 analogues. Systematic changes were made to the head, tail, and linker regions of V-06-018 (see text). Compound **26** in this series, composed of a phenyl head and a nine carbon tail, is V-06-018.

https://dx.doi.org/10.1021/acsinfecdis.9b00518
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(Figure 3). We also synthesized a variety of analogues with halogenated aryl headgroups (**7**, **9**, **11**, and **14**−**16**) to explore electronic effects on activity. Within this set, compounds **9**, **12**, and **13** were also inspired by work reported by Spring and co-workers, who found that related molecules with these headgroups were efficacious inhibitors of the production of QS-regulated virulence factors in *P. aeruginosa*.[14] To alter the electronics and hydrogen-bonding ability of the V-06-018 headgroup without significantly increasing its size, we constructed a set of analogues with heterocyclic, aromatic headgroups (**19**−**21**).

Turning to the tailgroup of V-06-018, we again looked to OdDHL for guidance. The importance of hydrophobic contacts between ligands and the OdDHL acyl tail-binding pocket in LasR has been noted (i.e., at residues Ala 127 and Leu 130),[37,38] and AHL-based LasR agonists decrease in potency as their tails decrease from 12 carbons in length.[39] To examine the importance of tail length for V-06-018's antagonistic activity, we introduced 5−12 carbon tails *via* the amine coupling in Scheme 1, yielding compounds **22**−**29** (Figure 3; compound **26** is V-06-018). To mimic the molecular architecture of known AHL[30] and TP-type[26] antagonists of LasR, we included several derivatives with cyclic tail groups (**30**−**32**, **34**, and **35**). In addition, we examined an analogue with a *sec*-butyl tail (**33**, racemic) to evaluate LasR's tolerance for bulk at the position vicinal to the V-06-018 amide nitrogen. Lastly, to evaluate the importance of the heteroatoms in the "linker" region between the headgroup and tail, we synthesized diketone **36** and amide **37**. Compound **38**, a constitutional isomer of V-06-018, was reported previously by our lab;[40] we included it here for comparison and to further expand our SAR analyses.

Evaluation of the V-06-018 Library for LasR Antagonism. We examined the activity of the V-06-018 library for LasR antagonism using a *P. aeruginosa* mutant strain (PAO-JP2, Δ*lasIrhlI*) that lacks the ability to synthesize OdDHL (or BHL) and contains a green fluorescent protein (GFP) reporter plasmid to examine LasR activity.[16,41] We used a *P. aeruginosa* LasR reporter as opposed to the *E. coli* LasR reporter introduced above, as we were most interested in the activity of the compounds (and their eventual use as probes) in the native organism. Further, as we previously showed that V-06-018 is not subject to active efflux by the MexAB−OprM pump in *P. aeruginosa*,[16] we wanted to examine if these close analogues were also active in the presence of this pump. In this *P. aeruginosa* reporter system, compounds capable of LasR antagonism should reduce GFP production, and this loss can be quantitated by fluorescence (see the Methods and Materials section). To start, we screened the library for LasR antagonism at a concentration of 10 μM in competition against 150 nM OdDHL. Analogues with substituents on the headgroup were found to be generally less efficacious as LasR antagonists relative to V-06-018 (compounds **7**−**18**, Figure 4A), suggestive that bulkier V-06-018 analogues may not be as well accommodated in the AHL-binding site, regardless of their hydrogen-bonding ability. Decreasing the size of the headgroup and including a polar atom was more fruitful. Two of the analogues based on five-membered heterocycles, furan **19** and thiophene **20**, had equivalent efficacy to V-06-018 (~90% LasR antagonism). Not all heterocycles were effective as headgroups, however; thiazole **21** lost efficacy relative to V-06-018.

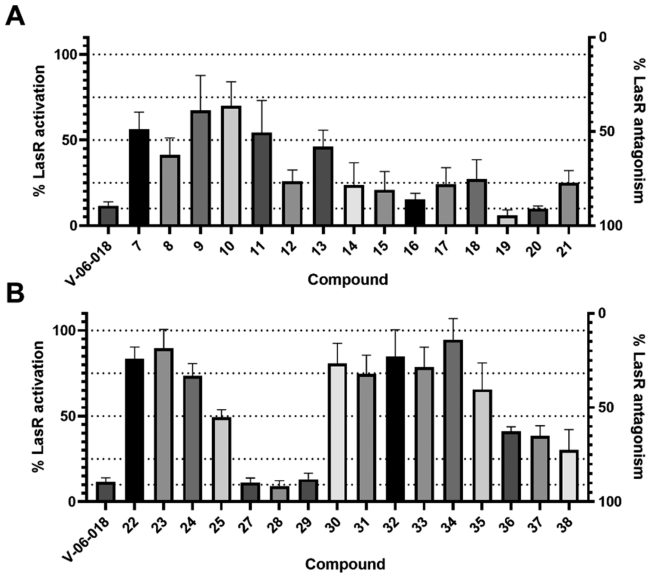

Figure 4. Primary LasR antagonism screening data in *P. aeruginosa* reporter PAO-JP2 for the (A) headgroup and (B) tailgroup and linker modified V-06-018 analogues. Compounds were screened at 10 μM in the presence of 150 nM OdDHL. Bacteria treated with 150 nM OdDHL only was defined as 100% LasR activity/0% LasR antagonism; conversely, bacteria treated with DMSO only (i.e., vehicle) was defined as 0% LasR activation/100% LasR antagonism. Error bars indicate SD of $n \geq 3$ trials.

Turning to the tailgroup modified V-06-018 analogues, we found that only compounds with unbranched, acyclic alkyl tails were efficacious LasR antagonists (e.g., **27**−**29**, Figure 4B). No compounds with cyclic moieties or branching (i.e., **30**−**35**) in their tails were capable of antagonizing LasR by more than 50%. The length of the tail was also important; analogues **27**−**29**, with 10- to 12-carbon tails, were equally as efficacious as V-06-018. The shorter tail analogues **22**−**25**, however, antagonized LasR by less than 50%. These data suggest that binding interactions between LasR and these truncated V-06-018 analogues may have been reduced due to the lack of hydrophobic contacts (again, shown to be important for LasR/OdDHL binding).[37,38] Modifications to the linker region also resulted in less active analogues. All three linker-modified compounds (**36**−**38**) lost efficacy relative to V-06-018, implicating the presence and position of the amide in V-06-018 as critical to LasR antagonism. Overall, these primary screening data indicated that only subtle alterations to the head- and tailgroups of V-06-018, and not the linker group, were tolerated for strong LasR antagonism.

Dose−Response Antagonism Analysis of Primary Screening Hits. To obtain a quantitative measure of compound potency, we performed dose−response analyses on the compounds that antagonized LasR ≥ 90% at 10 μM (**19**, **20**, and **27**−**29**) using the same *P. aeruginosa* LasR reporter strain and calculated their IC_{50} values (Table 1). We were excited to observe that each of these analogues was more potent than V-06-018. Increasing the length of the V-06-018 tail from 10 to 12 carbons (i.e., as in **27**−**29**) led to a ~ 3−4-fold increase in potency. The heterocyclic analogues were also stronger LasR antagonists than V-06-018; furan **19** was approximately 2-fold more potent than V-06-018, and thiophene **20** was closer to 5-fold.

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Table 1. Potency and Maximum LasR Inhibition (Efficacy) Data for Selected Compounds in *P. aeruginosa*[a]

compound	IC_{50} (μM)[b]	95% CI (μM)[c]	maximum inhibition (%)[d]
V-06-018 (**26**)	2.3	(1.7−3.1)	89
19	1.2	(0.8−1.8)	96
20	0.5	(0.3−0.6)	84
27	0.7	(0.5−0.9)	93
28	0.5	(0.4−0.7)	92
29	0.7	(0.5−1.0)	91
39	0.2	(0.2−0.3)	83
40	0.2	(0.2−0.3)	85
41	3.8	(2.0−7.1)	89
42	0.2[e]	(0.1−0.2)	91
43	0.2[e]	(0.1−0.2)	93
44	0.6	(0.5−0.8)	84

[a]For details of PAO-JP2 reporter strain, see the Methods and Materials section. [b]Antagonism experiments performed by competing the compounds against OdDHL (**1**) at its approximate EC_{50} (150 nM for PAO-JP2) and inhibitory activity was measured relative to receptor activation at this EC_{50}. IC_{50} values determined by testing compounds over a range of concentrations (0.64 nM−50 μM). All assays performed in triplicate. [c]CI = 95% confidence interval. [d]Denotes the best-fit value for the bottom of the computed dose−response curve. [e]Compound exhibited non-monotonic dose−response behavior. Reported IC_{50} corresponds to the antagonism portion of the curve. Full antagonism dose−response curves are shown in Figure S2.

Second-Generation V-06-108 Analogues and LasR Agonism Profiles. Encouraged by the antagonistic activity profiles of our initial set of compounds, we designed and synthesized a set of "hybrid" second-generation V-06-018 analogues that combined features of the most active compounds. These compounds were composed of a furan or thiophene headgroup united with 10, 11, or 12 carbon tails (compounds **39**−**44**; see Figure 2) and were synthesized and evaluated for LasR antagonism in *P. aeruginosa* as described above. The second-generation compounds displayed a variety of activities in the LasR antagonism assay (listed in Table 1). Notably, furan derivatives **39** and **40**, containing 10 or 11 carbon tails, respectively, were more potent than their parent compounds and were each 10-fold more potent than V-06-018. The 12-carbon furan analogue **41**, however, lost activity relative to its parent compounds.

We note that the thiophene analogues of **39** and **40**, compounds **42** and **43**, displayed non-monotonic partial agonism behavior in the LasR dose−response assays;[29,30] namely, at concentrations below 2 μM, these compounds antagonized LasR, while at concentrations above 2 μM they agonized LasR. We have reported this activity profile for a series of ligands in reporter assays of LuxR-type proteins to date.[27,28,30] The antagonist portions of their dose−response curves indicated that **42** and **43** were each highly potent at lower concentrations, with IC_{50} values 10-fold lower than that of V-06-018. Interestingly, thiophene analogue **44**, differing by only one methylene unit more than **43**, lacked observable non-monotonic activity.

The discovery that two of the hybrid compounds were non-monotonic partial LasR agonists prompted us to measure dose−response agonism curves for all our most potent compounds (Figure S3). V-06-018 and compounds **27**−**29**, composed of phenyl headgroups, did not activate LasR. We also screened our first-generation library for LasR agonism at a single concentration (100 μM) and found that none of the analogues with phenyl headgroups activated LasR; however, thiophene **20** weakly agonized LasR (to 20%; Figure S4). We found that furans **39** and **40** could very weakly agonize LasR (7% and 4%, respectively) at the highest concentration screened (50 μM). Relative to **39** and **40**, thiophenes **42** and **43** were stronger LasR agonists at 50 μM (30% and 22%, respectively), which matched their activity profile at this concentration in the dose−response antagonism analysis (as described above).

Activation in this cell-based reporter assay requires LasR to initiate transcription of *gfp*. This process requires LasR to adopt a conformation capable of homodimerization and productive DNA binding. Our results suggest that, at sufficiently high compound concentration, these furan and thiophene ligands can make contacts with LasR (either directly or indirectly *via* some other target) that promotes this process. However, contacts with just the headgroups of **39**, **40**, **42**, and **43** are presumably insufficient, as compounds **41** and **44**, composed of the same furan and thiophene headgroups, respectively, yet linked to a 12-carbon tail, failed to activate LasR even at high concentrations. These results suggest that contacts with the tail—specifically, a tail of 9−11 carbons—along with the headgroup are necessary for LasR agonism by this ligand class at high concentrations. Whether these ligands target the OdDHL-binding site or another site on LasR, or another factor altogether, to promote LasR activation at these concentrations remains to be determined.

***E. coli* Reporter Assays Indicate V-06-018 and Analogues Act Directly *via* LasR.** We next examined if our improved V-06-018 analogues elicit their antagonistic activity *via* acting directly on LasR using an *E. coli* LasR reporter system (see the Methods and Materials section).[42−44] As highlighted above, LasR is directly and indirectly regulated by other QS systems in *P. aeruginosa*, and thus activity profiles in the *P. aeruginosa* LasR reporter are a measure of this inter-regulated network. To address this question, we obtained dose−response curves for all of the compounds in Table 1 in an *E. coli* LasR reporter strain and found that their relative efficacies and potencies largely tracked between the *E. coli* and *P. aeruginosa* reporters (Figure S5, Table S2). This alignment between the *P. aeruginosa* and *E. coli* reporter data suggests that these compounds elicit their effects *via* direct interactions with LasR. We note that all of our antagonists were less efficacious and potent against LasR in the *E. coli* reporter relative to *P. aeruginosa*. For example, the lead compound **40** was only 4-fold more potent than V-06-018 in *E. coli* vs being 10-fold more potent in *P. aeruginosa*. This reduction in potency also obscured the non-monotonic effects observed above for compounds **42** and **43**. We postulate that this reduction in potency in *E. coli* is an artifact of differences in LasR expression levels between the two reporter systems (non-native level in *E. coli* vs native level in *P. aeruginosa*).[45] With more LasR present, higher concentrations of ligands are presumably required to inhibit LasR activity. Critically, the stronger efficacies and potencies of these V-06-018 derived antagonists in the native host background will increase their utility as probe molecules.

We were also curious to see if the new antagonists, like V-06-018, were selective for LasR over RhlR and QscR in *P. aeruginosa*. Screening representative compounds (**39** and **40**) in the *E. coli* RhlR and QscR reporter systems showed that **40** is highly LasR selective, with no observable activity in either RhlR or QscR (Figure S6). Compound **39** was found to be

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inactive in RhlR and, similar to V-06-018, only a weak QscR antagonist (~35% inhibition) at the very highest concentration tested. These results further underscore the receptor selectivity profile of the V-06-018 scaffold and the value of these compounds as chemical tools to study QS in *P. aeruginosa*.

***P. aeruginosa* Reporter Data Support a Competitive Mechanism of LasR Antagonism for V-06-018 and Related Compounds.** We were interested to determine if V-06-018 and our new lead antagonists were acting as competitive LasR antagonists, and we examined this question by testing them against OdDHL at varying concentrations in the *P. aeruginosa* LasR reporter assay. The observed potency of a competitive LasR antagonist should vary with OdDHL concentration, as both molecules are competing for space in the same ligand-binding site. We obtained antagonism dose–response curves for V-06-018 and one of our lead compounds (**40**, which did not display non-monotonic behavior) in competition with OdDHL at 150 nM, 1 μM, and 10 μM (Figure 5). We observed an OdDHL-concentration-dependent decrease in the potency of both compounds. The relative potency trends for V-06-018 and **40** were also maintained, with compound **40** significantly more potent than V-06-018 at 150 nM and 1 μM. Unlike V-06-018, compound **40** was still capable of antagonizing LasR (to 55%) even in the presence of 10 μM OdDHL. These results are supportive of the ability of V-06-018 and its close analogues to act as competitive antagonists of LasR.

Antagonists and Non-Classical Partial Agonist 42 Solubilize LasR. We sought to further characterize the interactions between V-06-018 and related analogues with LasR to understand how they engender receptor antagonism. Very little is known about the molecular mechanisms that lead to antagonism of LuxR-type receptors by small molecules, largely due to the instability of these proteins *in vitro* even in the presence of their native AHL ligand.[46] LasR requires OdDHL throughout the production and purification process to be isolated and has proven intractable to structural studies in full length form.[36,47,48] In principle, antagonists of LuxR-type proteins can operate by binding either in place of an AHL signal, or to a hypothetical, allosteric-binding site. Once bound, antagonists can then cause antagonism by further destabilizing the protein (as has been shown for QscR and LasR)[38,47,49] or by forming soluble complexes that are either incapable of dimerization or binding to DNA (as has been shown for CviR and LasR),[50−52] or presumably combinations of these mechanisms (and potentially others). We were curious to investigate whether soluble LasR could be isolated when it was produced in the presence of V-06-018 or our new antagonists, or if it was destabilized in their presence relative to OdDHL. To test these questions, we produced LasR in *E. coli* grown in the presence of no compound (DMSO control) or 50 μM OdDHL, V-06-018, **40**, or **42** (see the Methods and Materials section). After 16 h of protein production, we lysed the *E. coli* cells and separated the whole cell (WC) and soluble (S) lysate on an SDS-PAGE gel (Figure 6; quantitative analysis of the bands in the gel is provided in Table S3).

As expected, we did not obtain any LasR in the soluble fraction of cells grown without exogenous compound (DMSO), while we obtained soluble LasR in the culture grown with exogenous OdDHL (S band ~30% as intense as WC band; Figure 6). These data recapitulate the finding that LasR requires a ligand to be soluble *in vitro*.[53] We detected soluble bands for LasR produced in the presence of V-06-018

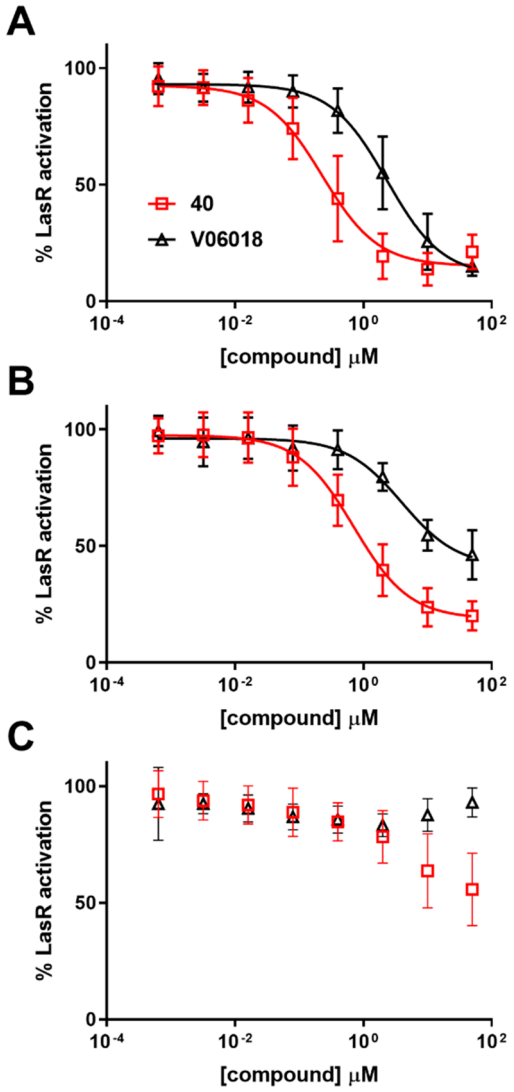

Figure 5. Dose–response LasR antagonism curves for V-06-018 and analogue **40** in *P. aeruginosa* PAO-JP2. Dose–response curves of V-06-018 (black triangles) and **40** (red squares) in competition with (A) 150 nM, (B) 1 μM, and (C) 10 μM OdDHL. V-06-018 has IC_{50} values of 2.3 and 3.9 μM vs 0.15 and 1 μM OdDHL, respectively; **40** has IC_{50} values of 0.2 and 0.7 μM vs 0.15 and 1 μM OdDHL, respectively. IC_{50} values could not be calculated for these compounds in competition with 10 μM OdDHL (curves in part C).

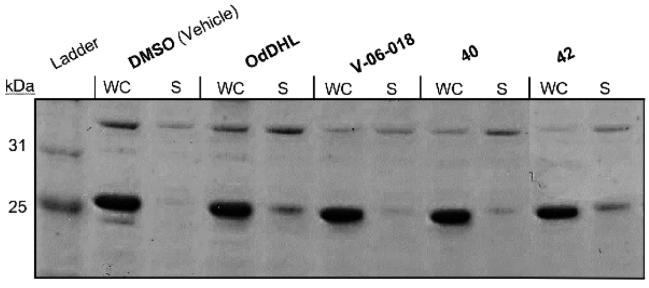

Figure 6. Characterization of LasR *via* SDS-PAGE gel in the presence of different ligands. Whole Cell (WC) and soluble (S) portions of *E. coli* cell lysates with LasR overexpressed in the presence of DMSO or 50 μM of OdDHL, V-06-018, **40**, or **42**. LasR has a mol wt of 27.9 kDa.

https://dx.doi.org/10.1021/acsinfecdis.9b00518
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and furan **40**. The bands were 4-fold smaller than that of OdDHL (~7% as intense as WC band, vs ~30% for OdDHL, Table S3), suggesting that these ligands do not solubilize LasR to the same extent as OdDHL. This result correlates with the previous report of Schneider and co-workers, demonstrating that certain synthetic AHL-type antagonists (along with the close V-06-018 analogue **38**) form soluble complexes with LasR, albeit in less amounts than OdDHL.[51] Schneider went on to show that these complexes were unable to bind to LasR's target DNA using electrophoretic mobility shift assays (EMSAs), which allows for the interpretation that these ligands can stabilize an inactive LasR complex (e.g., incapable of dimerization or DNA binding). We also observed thiophene **42** solubilized LasR. The soluble band for **42** was more intense than those observed for V-06-018 and **40** and comparable to that of OdDHL (~30%). We note that **42** has a non-monotonic activity profile in the *P. aeruginosa* reporter assay and is capable of weak LasR agonism at higher concentrations; the larger quantity of LasR isolated in this experiment relative to V-06-018 and **40** (at 50 μM concentration) is therefore interesting and could arise due to this agonistic activity profile. Collectively, these SDS-PAGE data support the hypothesis that V-06-018 and related analogues act at LasR antagonists, at least in part, *via* inducing a soluble but inactive conformation of LasR. The reduced amount of protein in these soluble fractions relative to OdDHL suggests that V-06-018 and **40** may also cause antagonism by promoting LasR unfolding (i.e., destabilizing the receptor); thus, more than one mechanism of antagonism is likely operative. Further biochemical (e.g., EMSAs) and structural experiments are required to test these mechanistic hypotheses and are ongoing in our laboratory.

LasR Mutants Reveal Residues Critical for Activation and Inhibition by Synthetic Ligands. The results of the competitive LasR antagonism dose–response assays, *E. coli* reporter assays, and protein production experiments outlined above suggest that V-06-018 and the lead analogues target LasR and interact with the OdDHL-binding site to cause antagonism. In view of our original compound design, we were curious as to whether the residues in LasR that are known to govern LasR/OdDHL interactions (Figure 2) were also important to LasR antagonism by the V-06-018 ligand class, and we applied a method utilized previously in our laboratory involving LasR mutants with modifications to the OdDHL-binding site.[35,36,54] In this past work, a set of LasR single-point mutants were generated in which residues implicated in hydrogen-bonding interactions with OdDHL were converted to residues incapable of hydrogen bonding but of approximately the same steric size (e.g., Tyr → Phe). The mutant LasR proteins were then tested for activity using a LasR reporter plasmid in an *E. coli* host background (analogous to the *E. coli* LasR reporter assay system above). Compounds showing reduced activity in these mutants relative to wild-type LasR then can be postulated to make a contact with LasR that depends on the mutated residue. We tested V-06-018 and furan **39** at 100 μM in three LasR mutants with modifications to residues that make hydrogen bonds to OdDHL (Tyr 56, Trp 60, and Ser 129; see Figure 2).[54] Notably, all of these single-point LasR mutants (Y56F, W60F, and S129A) are still functional in the reporter assay, but are less active than wild-type LasR (as measured *via* reduced OdDHL potencies; Figure S7), reflective of the importance of these LasR/OdDHL interactions for activation. (As noted above, antagonists display reduced efficacy in general in this heterologous background relative to the native (*P. aeruginosa*) reporter system.)

V-06-018 was found to antagonize all three LasR mutants to a significantly lesser extent than wild-type LasR (Figure 7A).

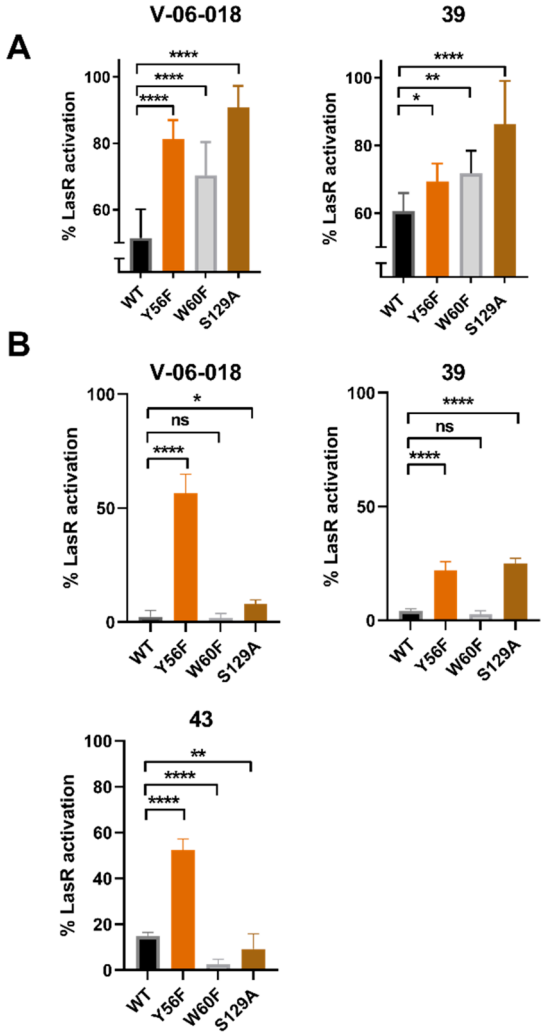

Figure 7. (A) LasR mutant antagonism data for V-06-018 and lead compound **39**. Compounds tested at 100 μM against OdDHL at its approximate EC_{50} value in the specific *E. coli* LasR reporter strain (as indicated on the *x* axis). (B) LasR mutant agonism data for V-06-018, **39**, and **43**. Compounds tested at 100 μM. For antagonism experiments, 100% is defined as the EC_{50} concentration of OdDHL in that specific LasR reporter strain (see Figure S6); for agonism experiments, 100% is defined as the activity of 100 μM OdDHL in that specific LasR reporter strain. Significance was assessed *via* a one-way ANOVA: **** = $p < 0.0001$; *** = $p < 0.001$; ** = $p < 0.01$; * = < 0.05. ns = no significant difference.

The same trend was true for furan **39**. Tyr 56 and Ser 129 are believed to form hydrogen bonds with the amide carbonyl of OdDHL (Figure 2), and they potentially could bind to one of the two linker carbonyl oxygens in V-06-018 and its analogues.[36] Trp 60 hydrogen bonds with the lactone carbonyl oxygen of OdDHL, and it may be capable of hydrogen bonding with the furan oxygen of **39**. An analogous hydrogen bond to the headgroup of V-06-018 is not possible, but the lower activity of V-06-018 in the W60F LasR mutant suggests that Trp 60 interacts in some other manner with V-06-018 to enforce antagonism. Further studies are necessary to pinpoint

https://dx.doi.org/10.1021/acsinfecdis.9b00518
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the specific molecular interactions that govern LasR antagonism by these two ligands. Nevertheless, these experiments with LasR mutants support V-06-018 and new antagonist **39** interacting with the OdDHL-binding site in LasR.

We also were curious to learn whether alterations to these LasR residues could impact the ability of our compounds to agonize LasR. Therefore, we examined the agonistic activities V-06-018, furan **39**, and thiophene **43** in the three LasR mutant reporter strains at 100 μM; thiophene **43** was included in these agonism assays due to its non-monotonic agonism profile (see above). We were surprised to find that all three compounds agonized the LasR Y56F mutant to a significantly greater extent than wild-type LasR. For example, V-06-018, which does not agonize wild-type LasR, activated LasR Y56F to ∼60% (relative to OdDHL) at 100 μM. In view of this unexpected result, we screened the remainder of our lead compounds in this LasR mutant reporter and found that they all were capable of activating the LasR Y56F mutant to some extent (from 9−56% at 100 μM; Figure S8). V-06-018 and **39** also agonized the LasR S129A mutant significantly more than wild-type LasR. These results suggest that removing the hydrogen bonds donated by Tyr 56 or Ser 129, or reducing sterics at these positions, may allow these V-06-018-type ligands more freedom to adjust their position in the LasR OdDHL-binding pocket and to adopt new contacts that engender LasR agonism as opposed to antagonism. None of our compounds were found to agonize the LasR W60F mutant; in fact, **43** lost agonistic activity in that mutant relative to wild-type LasR.

In our laboratory's prior mutational studies of LasR, we observed compound **38** (Figure 3), a LasR antagonist and constitutional isomer of V-06-018, could agonize both the LasR Y56F and W60F mutants. We termed this transition from antagonist to agonist "Janus" behavior (after the two-faced Roman god).[35] Here, we observed V-06-018 and compound **39** exhibit analogous "Janus" behavior in Y56F and S129A, but not in W60F (like **38**). These results suggest that chemical modification of either the ligand (*via* chemical synthesis; i.e., V-06-018 → **38** or **39**) or LasR (*via* mutagenesis of at least these three residues) is sufficient to alter contacts between the ligand and receptor to allow for either agonism or antagonism, or the degree thereof, and that these changes to molecular contacts are likely very subtle. The implications of these findings—specifically, that single-point mutations can convert potent LasR antagonists into agonists—on the propensity for resistance to arise in *P. aeruginosa* to LasR antagonists did not escape our attention. We do note that the agonistic activity of these compounds is quite low (relative to OdDHL in wild-type LasR). Additional experiments are required to explore the possibility of LasR mutants to arise naturally upon sustained treatment with V-06-018 or related analogues. However, our lab and others have shown previously that resistance to QS inhibitors, even if it was to develop, should be slow to spread through and not overtake a population of bacteria,[55,56] supporting the continued search for such compounds. Moreover, the ability of V-06-018, **38**, **39**, and **43** to agonize the LasR mutants suggests that structural studies of these LasR mutant/ligand complexes could be particularly noteworthy, as they could illuminate the mechanisms by which these ligands both agonize LasR mutants and antagonize wild-type LasR. The heightened stability of LasR/agonist complexes relative to LasR/antagonist complexes could significantly enable such structural studies.

SUMMARY AND CONCLUSIONS

The work reported herein was motivated by the need for chemical probes of a key QS receptor, LasR, in the opportunistic pathogen *P. aeruginosa*. Despite considerable research to date, antagonists with submicromolar potencies, high efficacies, and selectivities for LasR over the other QS circuits in *P. aeruginosa* have been elusive. We performed the first structure−function analysis of the small molecule V-06-018, a promising yet unstudied LasR antagonist emerging from a high-throughput screen reported over 10 years ago.[33] We developed a versatile and efficient synthetic route to V-06-018, produced a focused library of analogues using this route to explore the headgroup, linker, and tail portions of V-06-018, and evaluated the library for LasR modulatory ability using cell-based reporter systems. These screening data revealed stringent SARs for LasR antagonism by this ligand scaffold, including the requirement for a linear, alkyl tailgroup between 9−12 carbons in length, an amide in the linker, an intolerance for substitution on the aryl headgroup, and a tolerance of certain 5-membered heterocyclic headgroups. These SARs allowed us to design and synthesize second-generation LasR antagonists with nanomolar IC_{50} values in *P. aeruginosa* (e.g., **39** and **40**). These compounds represent, to our knowledge, the most potent and efficacious synthetic antagonists of LasR to be reported, with IC_{50} values in *P. aeruginosa* 10-fold lower than V-06-018 and at least 100-fold lower than other AHL-based ligands.[48] We note that we discovered these analogues after synthesizing fewer than 40 compounds; further development of the V-06-018 scaffold would likely yield even more potent compounds.

Our results indicate that the V-06-018 scaffold is quite selective for LasR over the other two LuxR-type receptors in *P. aeruginosa*, with **39**, **40**, and V-06-018 showing neither antagonistic nor agonistic activity in RhlR, **40** being inactive in QscR, and **39** and V-06-018 showing only modest antagonistic activity in QscR at the very highest concentrations tested. This activity profile is significant because the ability to selectively attenuate LasR activity in the midst of the highly inter-regulated QS system of this pathogen will facilitate mechanistic studies, and it highlights the value of these V-06-018 analogues as chemical tools to study QS in *P. aeruginosa*.

We also report herein our investigations into the mechanism by which V-06-018 and related compounds modulate LasR activity. In the course of these studies, certain analogues were found to display interesting dual activity profiles—capable of strong LasR antagonism at nanomolar levels, yet LasR agonism at micromolar levels (i.e., non-monotonic partial agonists)—and we were intrigued by their mechanisms of action as well. Examination of the lead compounds against OdDHL at various concentrations and in an *E. coli* LasR reporter support a mechanism by which they bind competitively with OdDHL and interact directly with LasR. V-06-018 and furan antagonist **39** were found to be significantly less efficacious in LasR mutants that lack key residues in the ligand-binding site shown to make hydrogen-bonding contacts with OdDHL. This result is congruent with these compounds binding in the same site on LasR as or near to OdDHL. Protein production studies of LasR in the presence of V-06-018, furan-based antagonist **40**, and thiophene-based antagonist **42** demonstrated that these compounds support folding of the protein into a soluble form, suggestive that they may stabilize an inactive form of the protein, analogous to the mechanism of CviR antagonism by

https://dx.doi.org/10.1021/acsinfecdis.9b00518
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the chlorolactone AHL analogue (CL).[50] V-06-018 and **40** also appear to reduce the amount of soluble LasR relative to **42** or the native agonist OdDHL, indicating that receptor destabilization could also contribute to the mechanism of inactivation by certain of these compounds. Finally, study of V-06-018 and furan-based antagonist **39** revealed that they were each capable of shifting from LasR antagonists to agonists in a LasR mutant lacking a single hydrogen-bonding motif in the ligand-binding site (e.g., Tyr 56→ Phe 56; removal of the Tyr hydroxyl). This finding indicates that subtle interactions of these ligands with LasR can have dramatic effects on receptor activity and suggests a novel route for exploring the mechanisms of this ligand class *via* structural studies of LasR mutant/ligand complexes. Overall, this study has provided a set of highly potent LasR antagonists that should find broad use as chemical probes of QS in *P. aeruginosa*, a robust chemical route to generate these compounds, and new insights into the mechanisms of LasR antagonism. These compounds and insights expand the understanding of LuxR-type QS in this important opportunistic pathogen.

MATERIALS AND METHODS

Chemistry. All chemicals were obtained from Sigma-Aldrich, Agros Organics, or TCI America. All reagents and solvents were used without further purification except for hexane, ethyl acetate, and dichloromethane, which were distilled prior to use. Analytical thin-layer chromatography (TLC) was performed on 250 μm glass backed silica plates with a F-254 fluorescent indicator from Silicycle. Visualization was performed using UV light and iodine. All new compounds were fully characterized for purity and identity; see SI for characterization data. Compound stock solutions were prepared in DMSO at appropriate concentrations and stored at −4 °C prior to use.

Representative Procedures for the Synthesis of V-06-018. *Synthesis of Ethyl 2-(2-phenyl-1,3-dioxolan-2-yl)-acetate (**3**; R = H).* Ethyl benzoyl acetate (1.92 mL, 10 mmol, 1 equiv), ethylene glycol (3.35 mL, 60 mmol, 6 equiv), and *p*-toluene sulfonic acid (192 mg, 1 mmol, 0.1 equiv) were added to a 250 mL round-bottom flask equipped with a Dean−Stark trap. The mixture was heated to reflux for approximately 24 h. The mixture was washed with saturated sodium bicarbonate (1 × 100 mL), water (1 × 100 mL), and saturated brine (1 × 100 mL). The organic portion was dried over magnesium sulfate and concentrated under reduced pressure. The crude material was purified by flash silica gel chromatography (20% ethyl acetate in hexane), and **3** was isolated as a colorless oil (1.87 g, 79% isolated yield).

*Synthesis of 2-(2-Phenyl-1,3-dioxolan-2-yl)acetic acid (**4**; R = H).* Compound **3** (287 mg, 1.2 mmol, 1 equiv) was dissolved in THF (12 mL, 0.1 M) in a 100 mL round-bottom flask, after which aqueous 1 M lithium hydroxide (12 mL, 12 mmol, 10 equiv) was added. The reaction mixture was heated to 70 °C, and reaction progress was monitored by TLC. Upon consumption of the starting material, the organic layer was washed with saturated sodium bicarbonate (20 mL). The combined aqueous layers were extracted with ethyl acetate (20 mL). The pH of the combined aqueous layers was acidified with 10% aq citric acid and then extracted with ethyl acetate (3 × 20 mL). These organic portions were combined, dried over magnesium sulfate, and concentrated under reduced pressure to yield **4** as a colorless, crystalline solid that was >95% pure by ^{1}H-NMR and used without further purification (226 mg, 90% crude yield).

*Synthesis of N-Nonyl-2-(2-phenyl-1,3-dioxolan-2-yl)-acetamide (**5**; R = H, R′= nonyl).* Acid **4** (226 mg, 1.08 mmol, 1 equiv), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC-HCl; 207 mg, 1.62 mmol, 1.5 equiv), 4-dimethylaminopyridine (DMAP; 20 mg, 0.162 mmol, 0.15 equiv), and nonylamine (238 μL, 1.3 mmol, 1.2 equiv) were dissolved in CH_2Cl_2 (10.8 mL, 0.1M), and the reaction mixture was stirred for ∼15 h at room temperature. The reaction mixture was diluted into diethyl ether and washed with 1 M HCl (2 × 30 mL), saturated sodium bicarbonate (2 × 30 mL), water (1 × 30 mL), and brine (1 × 30 mL). The organic portion was dried over magnesium sulfate and concentrated under reduced pressure to yield **5** as a colorless, crystalline solid that was >95% pure by ^{1}H-NMR and used without further purification (303 mg, 85% crude yield).

*N-Nonyl-3-oxo-3-phenylpropanamide (V-06-018, **26**).* Compound **5** (303 mg, 0.92 mmol, 1 equiv) and *p*-toluene sulfonic acid (175 mg, 0.92 mmol, 1 equiv) were dissolved in acetone (9.2 mL, 0.1 M) in a 25 mL round-bottom flask. The reaction mixture was stirred at room temperature for 24 h. The mixture was diluted in diethyl ether (20 mL) and washed with saturated sodium bicarbonate (1 × 30 mL), water (1 × 30 mL), and brine (1 × 30 mL) and then dried over magnesium sulfate and concentrated under reduced pressure. The resulting solid was purified by flash silica gel chromatography (20% ethyl acetate in hexanes) to give a V-06-018 (**26**) as a white solid (170 mg, 64% isolated yield).

Biology. A listing of all of the bacterial strains and plasmids used in this study is provided in Table S1. Bacteria were cultured in Luria−Bertani medium (LB) and grown at 37 °C. Growth was quantified by absorbance at 600 nm (OD_{600}). Absorbance and fluorescence measurements were made on a Biotek Synergy 2 plate reader running Gen 5 software (version 1.05). Buffers used in biological experiments included: Z buffer (60 mM Na_2HPO_4,40 mM NaH_2PO_4, 10 mM KCl, 1 mM H_2O), phosphate buffer (60 mM Na_2HPO_4,40 mM NaH_2PO_4), and phosphate buffered saline (137 mM NaCl, 2.68 mmol KCl, 10 mM Na_2HPO_4,1.8 mM KH_2PO_4). Dose−response curves were generated using GraphPad Prism software (version 8). Detailed descriptions of all biological experiments are provided in the SI.

***P. aeruginosa* Reporter Assay Protocol.** LasR reporter experiments in *P. aeruginosa* were performed as reported previously.[30] Briefly, a single colony of *P. aeruginosa* PAO-JP2[41] was grown overnight in LB medium containing 300 μg/mL carbenicillin. The culture was diluted 1:100 in fresh LB medium without antibiotic. The subculture was grown to OD_{600} = 0.25−0.3. A 2 μL aliquot of compound stock solution (in DMSO) was added to the interior wells of a black, clear-bottom 96-well plate. A 198 μL aliquot of bacterial culture was added to all compound-containing wells. For antagonism experiments, at least three wells were filled with 198 μL of grown subculture (i.e., untreated subculture); the remainder of the subculture was treated with exogenous OdDHL (i.e., treated subculture) at various concentrations (150 nM, 1 μM, or 10 μM) prior to dispensing. Plates were incubated without shaking (static) for 6 h, after which GFP production was read for each well using a plate reader (excitation at 500 nM, emission at 540 nM) and normalized to cell growth. Activity was reported relative to cells containing only OdDHL.

https://dx.doi.org/10.1021/acsinfecdis.9b00518
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***E. coli* Reporter Assay Protocol.** LasR, RhlR, and QscR assays in *E. coli* JLD271 (Δ*sdiA*) or DH5α utilized a β-galactosidase reporter and were conducted as previously reported.[26] A representative protocol for the LasR assay is provided here. Briefly, a single colony of *E. coli* strain JLD271 bearing plasmids pJN105-L[44] and pSC11-L[42] was grown in LB medium. Overnight the culture was diluted 1:10 in fresh LB medium with 100 μg/mL ampicillin and 10 μg/mL gentamicin and grown to an OD_{600} = 0.23−0.27. Once grown, arabinose was added to a final concentration of 4 mg/mL. A 2 μL aliquot of compound stock solution (in DMSO) or only DMSO (vehicle control) was added to the interior wells of a clear 96-well microtiter plate. For agonism assays, 198 μL aliquots of the subculture were dispensed into all internal wells. For antagonism assays, the subculture was dispensed into at least three wells containing only DMSO; the remainder of the subculture was treated with the appropriate concentration of OdDHL and dispensed into all remaining interior wells. Plates were incubated at 37 °C with shaking at 200 rpm for 4 h.

To measure resulting β-galactosidase production, each interior well of a chemical-resistant 96-well plate (Costar 3879) was filled with 200 μL of Z buffer, 8 μL of $CHCl_3$, and 4 μL of 0.1% aqueous SDS. After the incubation period, the OD_{600} of each well of the bacteria-containing plate was measured. A 50 μL aliquot of each well of the bacteria-containing plate was transferred to the lysis-buffer-containing chemical resistant plate, and the cells were lysed. A 100 μL aliquot from each well was transferred to a fresh clear-bottom 96-well plate. The Miller assay was started by adding 20 μL of the substrate *ortho*-nitrophenyl-β-galactoside (ONPG, 4 mg/mL in phosphate buffer) to each well. The plates were then incubated at 30 °C for 30 min, and absorbances at 420 and 550 nm were read. Miller units were calculated for each well (see SI for detailed description). Activity was reported relative to wells containing only OdDHL.

LasR Overexpression and SDS-PAGE Protocols. *E. coli* BL21-DE3 harboring the pET17b (LasR) plasmid was grown overnight in LB medium from a single colony. The overnight culture was diluted 1:80 into fresh LB medium buffered with 100 mM MOPS, adjusted to pH 7, and grown to an OD_{600} = 0.5. Protein expression was induced by the addition of 0.4 M isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was grown overnight at 17 °C. The next day, cells were pelleted by centrifugation. Whole cell and soluble portions of cell lysate were isolated and prepared *via* the Bacterial Protein Extraction Reagent (B-PER, ThermoFisher Scientific) according to package instructions. Cell lysates were run on a Biorad 10% SDS gel and stained with Coomassie. Band intensities were quantified using ImageJ (see Table S3).

■ ASSOCIATED CONTENT

Supporting Information

Full details of instrumentation, synthetic protocols, bioassay protocols, additional biological data, and characterization data for all new compounds are available online in the Supporting Information. . The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.9b00518.

Discussions about general instrumentation information, synthetic and reporter assay protocols, protein expression and gel methods, and characterization data, tables of bacterial strains and plasmids used, potency and efficacy of selected compounds, and band intensities, and figures of dose−response assay data, primary agonism assay data, activity profiles, single-concentration agonism assay data, and ^{1}H- and ^{13}C-NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support for this work was provided by the NIH (R01 GM109403 and R35 GM131817). M.C.O. acknowledges support from the Arnold and Mabel Beckman Foundation through an Arnold O. Beckman Postdoctoral Fellowship. K.E.N. was supported in part by the UW−Madison NIH Chemistry−Biology Interface Training Program (T32 GM008505). NMR facilities in the UW−Madison Department of Chemistry were supported by the NSF (CHE-0342998) and a gift from Paul J. Bender. MS facilities in the UW−Madison Department of Chemistry were supported by the NSF (CHE-9974839).

■ ABBREVIATIONS

QS, quorum sensing; AHL, *N*-acyl-L-homoserine lactone; SAR, structure−activity relationship; OdDHL, *N*-(3-oxo-dodecanoyl)-L-homoserine lactone

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