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Quorum Sensing in Bacterial Species that Use Degenerate Autoinducers Can Be Tuned by Using Structurally Identical Non-native Ligands

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Abstract

Many bacteria use quorum sensing (QS) to regulate cell-density dependent phenotypes that play critical roles in the maintenance of their associations with eukaryotic hosts. In Gram-negative bacteria, QS is primarily controlled by N-acylated L-homoserine lactone (AHL) signals and their cognate LuxR-type receptors. AHL-LuxR-type receptor binding regulates the expression of target genes necessary for QS phenotypes. We recently identified a series of non-native AHLs capable of intercepting AHL-LuxR binding in the marine symbiont *Vibrio fischeri*, and thereby strongly promoting or inhibiting QS in this organism. *V. fischeri* utilizes N-(3-oxo)-hexanoyl L-HL (OHHL) as its primary QS signal, and OHHL is also used by several other bacterial species for QS. Such signal degeneracy is common among bacteria, and we sought to determine if our non-native LuxR agonists and antagonists, which are active in *V. fischeri*, would also modulate QS phenotypes in other bacteria that use OHHL. Herein, we report investigations into the activity of a set of synthetic LuxR modulators in the plant pathogen *Pectobacterium carotovora* subsp. *carotovora* Ecc71. This pathogen uses OHHL and two closely related LuxR-type receptors, ExpR1 and ExpR2, to control virulence, and we evaluated their responses to synthetic ligands by quantifying virulence factor production. Our results suggest an overall conservation in the activity trends of the ligands between the ExpR receptors in *P. carotovora* Ecc71 and LuxR in *V. fischeri*, and indicate that these compounds could be used as tools to study QS in an expanded set of bacteria. Notable differences in activity were apparent for certain compounds, however, and suggest that it might be possible to selectively regulate QS in bacteria that utilize degenerate AHLs.

Keywords

acylated homoserine lactone; cell recognition; chemical probes; quorum sensing; screening

Introduction

Quorum sensing (QS) is widespread in bacteria, and provides them with a mechanism by which to act as a multicellular group.^[1] This cell–cell signaling system is fundamentally a

chemical one, and is based on the production of small molecule signals, or autoinducers, and their detection by cognate protein receptors.^[2] The concentration of these signaling molecules in a given environment is proportional to the number of bacteria present (and, therefore, diffusion rates can also play a prominent role).^[3] When bacteria reach a sufficiently high population density, they can alter gene expression levels so as to carry out a range of processes that require the cooperation of a large number of cells, including virulence factor production, biofilm formation, bioluminescence, sporulation, and conjugation.^[4] These diverse processes have widespread effects on human health, agriculture, and the environment.^[3b, 5] A common, but not exclusive, theme among bacteria that exploit QS is their close interactions with eukaryotic hosts. In the case of pathogenic bacteria, QS allows the bacteria to amass in sufficiently high densities before launching a coordinated attack on the host and overwhelming its defenses.^[6] Symbiotic bacteria, in contrast, use QS to commence mutually beneficial relationships with their hosts at high cell densities.^[7]

The best characterized QS system is arguably in the Gram-negative Proteobacteria.^[8] These bacteria use N-acylated L-homoserine lactones (AHLs) as their primary QS signals (Scheme 1). The approximately 20 known AHLs generally differ only in the number of carbons and substitution on their acyl chains (e.g., mostly oxidation at the 3-position or a *cis*-alkene). The first AHL, *N*-(3-oxo)-hexanoyl L-HL (OHHL), was discovered in the marine symbiont *Vibrio fischeri*, which uses QS to control bioluminescence.^[9] The AHL signal is generated by a synthase enzyme (termed LuxI) and sensed with a ~25 kDa cytoplasmic receptor (termed LuxR); all subsequently characterized AHL-type QS circuits contain, at a minimum, homologues of these two regulatory proteins.^[8a] While many critical questions remain, research over the past 40 years has enabled the formulation of a general LuxI–LuxR-type QS mechanism.^[10] In brief, AHLs can diffuse into and out of the cell, and are produced at low levels by LuxI-type synthases at low cell densities. As the cells multiply, an intracellular threshold AHL concentration is reached, and the AHLs bind to their cognate LuxR-type receptors. Thereafter, the AHL–receptor complexes associate with (or in less common cases dissociate from) DNA sequences at specific QS regulated promoters, and regulate the expression of genes responsible for the observed QS phenotypes (Figure 1). X-ray crystallographic analyses of an AHL–LuxR-type protein complex (TraR from *Agrobacterium tumefaciens*) bound to its target DNA yielded the first molecular level view of this binding event in 2002,^[11] and provided critical support for this general QS mechanism.

Given the broad impact of QS on eukaryotic hosts, there is considerable interest in elucidating, and ultimately manipulating, these chemical dialogues between bacteria. This interest has grown more intense as QS is pursued as a potential anti-virulence strategy.^[12] Interception of AHL–LuxR-type receptor binding provides a straightforward approach for the development of new chemical tools to study QS and its role in both pathogenic and mutualistic interactions. Towards this goal, synthetic AHL analogues have been investigated as non-native modulators of QS in Gram-negative bacteria,^[13] and our laboratory has made recent contributions in this area.^[14] This work has been largely focused on the relatively well-characterized QS systems in *V. fischeri*, the plant pathogen *A. tumefaciens*, and the opportunistic pathogen *Pseudomonas aeruginosa*,^[15] which use OHHL, *N*-(3-oxo)-octanoyl L-HL (OOHL), and *N*-(3-oxo)-dodecanoyl L-HL (OdDHL) as their primary QS signals, respectively (Scheme 1). Several potent, non-native agonists and antagonists of QS in each of these species have emerged from this work, underscoring the potential for manipulating density dependent phenotypes in bacteria through the exogenous addition of non-native ligands. Furthermore, careful scrutiny of these AHLs has yielded structure–activity relationships that provide valuable insights into variations in the putative binding modes and affinities of each ligand for its target LuxR-type receptor.^[8c, 13, 14, 16] However, the utility of

non-native AHL analogues (and other compound classes)^[17] as general regulators of QS in bacteria beyond *V. fischeri*, *A. tumefaciens*, and *P. aeruginosa* remains largely unexplored.^[18]

We noted that the native AHL signals utilized by these three organisms appear to be the three most common QS signals used by Gram-negative bacteria,^[8] with OHHL being the most commonly used overall (utilized by at least ~10–15 species; Scheme 1). This degeneracy has raised questions about the possibility of bacteria using AHL signals for not only intraspecies, but also interspecies, sensing.^[19] Moreover, this signal degeneracy suggested that non-native compounds previously identified as modulators of specific LuxR-type receptors could be reappropriated to regulate QS in alternate bacteria that utilize the same native AHL. If correct, this approach would significantly expand the utility of the currently known, synthetic LuxR-type receptor agonists and antagonists to a variety of new bacterial strains. Differences in ligand activities between organisms could provide new insights into structural differences between their respective LuxR-type receptors. Such information would be valuable in view of the difficulties of manipulating LuxR-type proteins in vitro.^[20] We report our initial efforts to evaluate the feasibility of non-native ligand reappropriation between species.

This study describes our investigations into the activity of a set of synthetic ligands, previously identified as agonists and antagonists of LuxR in *V. fischeri*, in the plant pathogen *Pectobacterium carotovora* subsp. *carotovora* Ecc71, which uses OHHL to regulate virulence. *P. carotovora* Ecc71 is responsible for soft-rot diseases in a range of economically important crops, and the production of cell-wall degrading virulence factors associated with these diseases is driven by two LuxR-type receptors, ExpR1 and ExpR2, the latter of which is the primary regulator of virulence.^[21] Both ExpR1 and ExpR2 bind OHHL, resulting in their dissociation from DNA; this halts the transcription of *rsmA*, which codes for a virulence repressor protein (RsmA), and thereby initiates virulence factor production.^[21b] Notably, this dissociative process is in direct contrast to the associative QS mechanism of OHHL–LuxR in *V. fischeri* (Figure 1). We evaluated the responses of ExpR1 and ExpR2 in *P. carotovora* Ecc71 to our synthetic LuxR agonists and antagonists by quantifying exoenzyme production. The results suggest an overall conservation in the activity trends of the ligands between the ExpR receptors in *P. carotovora* and LuxR in *V. fischeri*; this supports our hypothesis that non-native ligands can similarly modulate receptors that utilize the same native AHL signal. However, some marked differences in activity were apparent for certain compounds, and agonists and antagonists were identified with activity exclusive to each strain. Furthermore, additional studies also confirmed differences in ligand activities between ExpR1 and ExpR2 in *P. carotovora*. These findings suggest that it could be possible to selectively regulate QS in bacteria that utilize degenerate AHLs, and have implications for the chemical interrogation of QS in mixed microbial environments where interspecies signaling could occur. In addition, as many bacteria beyond *P. carotovora* are believed to have more than one LuxR-type receptor that responds to the same AHL (notably, LasR and QscR in *P. aeruginosa*),^[22] our results highlight the possibility of dissecting such intraspecies LuxR-type receptor hierarchies with exogenous ligands.^[23]

Results and Discussion

AHL library selection

Earlier studies in our laboratory have yielded a library of approximately 150 synthetic native and non-native AHLs.^[14a, b, d, e] We selected a smaller, 38-member subset of these AHLs for screening in the current study, all of which have been previously evaluated for LuxR antagonistic and agonistic activities in *V. fischeri*, and are readily available in our laboratory.

These compounds share the native *L*-homoserine lactone (*L*-HL) head-group, yet contain different acyl group structures, and we subdivided them into three AHL sublibraries based on their acyl groups (Scheme 2A–C). Library A contained eleven AHLs with aliphatic acyl groups, either with or without 3-keto functionality. This library contained the native AHL for *P. carotovora* and *V. fischeri* (OHHL, **A1**), as well as the native AHLs for a number of other bacteria including *A. tumefaciens* (OOHL, **A2**), *P. aeruginosa* (C4 HL, **A4** and OdDHL, **A6**), and *Chromobacterium violaceum* (C6 HL, **A7**; Scheme 1).^[8a,c] Library B was comprised of 20 *N*-phenylacetanoyl *L*-HL derivatives (PHLs), several of which have previously been identified as potent agonists and antagonists of LuxR in *V. fischeri*.^[14d] The seven AHLs in library C were one-carbon shorter or one-carbon longer PHL homologues (benzoyl HLs (**C1–C2**) and phenylpropionyl HLs (PPHLs, **C3–C4**), or contained extended aromatic systems (**C5–C7**). We earlier determined PPHL **C4** and 1-naphthyl HL **C7** to be strong antagonists of LuxR.^[14a, b, e]

Overview of *P. carotovora* QS

The *P. carotovora* (formerly *Erwinia carotovora*) species have a somewhat more complex QS system compared to *V. fischeri*, with several differences apparent between strains. In general, these pathogens use either OHHL or OOHL to mediate the production of antibiotics (carbapenems) and cell-wall degrading virulence factors. Antibiotic production is regulated by CarR receptors, while exoenzyme production is typically regulated by ExpR (or VirR) receptors. Binding of either OHHL or OOHL to ExpR receptors results in their dissociation from DNA; this halts the production of the repressor RsmA, which prevents virulence factor production, and thereby initiates virulence.^[21b, 24] In the *P. carotovora* Ecc71 strain, neither a CarR receptor nor carbapenem production has been observed, and virulence factor production is primarily regulated by a single receptor ExpR2 and OHHL.^[21] Additional assays have confirmed the presence of a second LuxR-type protein in *P. carotovora* Ecc71, termed ExpR1, which plays an ancillary role in regulating virulence factor production and is also responsive to OHHL. ExpR1 and ExpR2 share approximately 60% primary sequence identity with each other and about 25% identity with LuxR in *V. fischeri*. OHHL–ExpR1 binding also results in the receptor's dissociation from DNA, thereby, reducing *rsmA* expression and stimulating exoenzyme production, albeit at significantly lower levels than ExpR2.^[21b] Such dissociative mechanisms, while less common than the associative type (Figure 1), are not unprecedented, and have been documented for LuxR-type receptors in other bacteria (e.g., EanR, SmaR, and EsaR).^[21c, 25] However, to our knowledge, studies of the effects of non-native AHLs on this class of LuxR-type receptors are yet to be reported. Therefore, any active ligands identified in the current study would be of additional interest from a more fundamental perspective.

Development of a *P. carotovora* QS assay

We required a straightforward, cell-based assay to assess ExpR1 and ExpR2 activity in *P. carotovora* Ecc71 in the presence of our AHL derivatives. *P. carotovora* Ecc71 is known to regulate the production of proteases, cellulases, and pectate lyase through both ExpR1 and ExpR2.^[21b, c] As assays for monitoring the activities of these exoenzymes are well developed,^[26] they serve as excellent reporters of QS activity. For this study, we selected to monitor ExpR1 and ExpR2 activities using the cellulase E.C. 3.2.1.4 from *P. carotovora* Ecc71. This enzyme catalyzes the hydrolysis of cellulosic 1,4- β -D-glycosidic linkages, and is actively excreted out of the cell. Enzymatic activity can be monitored by using a colorimetric cell-based assay with the cellulose analogue 4-nitrophenyl- β -D-cellobioside (4-NPC) as a substrate, and can be correlated to ExpR receptor activity (see the Experimental Section).^[21c, 26b] We utilized two *P. carotovora* mutant strains both derived from Ecc71 for the cellulase assays (Table 1); both lack the OHHL synthase (ExpI) and contain either ExpR1 alone (AC5117: $\Delta expI::\Delta expR2$) or ExpR2 alone (AC5099: $\Delta expI::\Delta expR1$).^[21b, c]

Introduction of exogenous OHHL (A1) into these strains, therefore, allows for activation of each receptor individually.

We performed cellulase assays using the two *P. carotovora* mutants at variable concentrations of OHHL, and established that the EC₅₀ values for OHHL in these assays ranged from 2–4 μM (Figure 2). These results also confirmed Expr2 as the principal regulator of cellulase production, with approximately double the activity of Expr1 under similar assay conditions. Interestingly, these EC₅₀ values are comparable to those for OHHL in an analogous LuxI mutant of *V. fischeri* (ES114: Δ*luxI*).^[14d] In view of these comparable EC₅₀ values, we reasoned that we could perform small molecule agonism and antagonism assays in *P. carotovora* at the same concentrations as our previous *V. fischeri* luminescence-based LuxR assays.^[14d] Thus, we performed agonism studies at 200 μM of AHL, and competitive antagonism studies at a 1:1 ratio of non-native AHL to OHHL (each at 5 μM, the approximate EC₅₀ value for OHHL). We note that these ligand concentrations could initially seem high; however, AHL concentrations in the confined environments in which these and related organisms utilize QS (e.g., in the wound site of a plant, the squid light organ, and biofilms) have been documented to reach comparable levels, and we, therefore, contend that they are biologically relevant.^[27]

Global comparison of AHL agonism and antagonism trends in *P. carotovora* and *V. fischeri*

We screened libraries A–C for agonistic and antagonistic activities in the *P. carotovora* Expr1 (AC5117) and Expr2 (AC5099) reporter strains using the cellulase assay and compound concentrations outlined above. In order to evaluate general trends in AHL-mediated LuxR-type receptor agonism and antagonism between *P. carotovora* and *V. fischeri*, we first plotted the observed compound activities in either *P. carotovora* Expr1 or Expr2 versus those for the same compounds in *V. fischeri* LuxR. We utilized our previously reported luminescence data^[14b] (supplemented with new data for compounds **B6**, **B12–14**, **C6**, and **C7**) for the LuxR data set. This initial comparative analysis of reporter strain screening data established that Expr1 agonism and antagonism trends for libraries A–C closely approximated those in LuxR, with limited exceptions (Figure 3A and B). However, Expr2 displayed a number of notable variations in compound activities relative to LuxR (Figure 3C and D). Detailed examinations of the receptor-specific agonistic and antagonistic trends observed in each sublibrary are outlined below.

AHL agonism trends in Expr1, Expr2, and LuxR

Agonism screening of library A yielded several interesting results, most notably for Expr2. Expr2 appeared to be less sensitive to AHL acyl group perturbations relative to Expr1 and LuxR (Table 2). First, Expr2 retained approximately 80% of its activity when the AHL acyl chain was expanded by two carbons relative to OHHL (**A1** vs. **A2**). This effect was in sharp contrast to Expr1 and LuxR, for which this ligand expansion reduced receptor activity to approximately 25%. Agonistic activity dropped off significantly in Expr2, however, as 3-keto aliphatic chain length extended past eight carbons. Second, unlike LuxR and Expr1, for which the 3-keto functionality appears critical for agonistic activity (e.g., **A1–A4** vs. **A6–A11**), the removal of this functional group had markedly less effect on agonism in Expr2. In general, our results corroborate earlier studies by Sjobolm et al. on a subset of these native AHLs in Expr2.^[21b, 24a] We note that Expr2 displays a particularly robust agonistic response to other naturally occurring AHLs (e.g., **A2** and **A7–A9**), and that this AHL tolerance is not unique to *P. carotovora*. Similar sensitivity to noncognate native AHLs has been observed in other soil-dwelling bacteria, such as *C. violaceum*,^[28] and highlights the potential for cross-species recognition of QS signals within the mixed bacterial populations endemic to soil.

Library B yielded many PHL activators of ExpR1 and ExpR2 that we had previously identified as potent agonists of LuxR (Table 2); this indicates that trends in PHL agonism was largely conserved between the three receptors.^[14b, d] The 3-Cl and 3-Br PHLs (**B8** and **B9**) proved to be moderate agonists of ExpR1, while the entire family of 3-halo PHLs (**B7–B10**) were effective agonists of ExpR2. The 3-Br PHL (**B9**) was the strongest 3-halo PHL activator of both receptors. Similarly conserved with LuxR was the “superactivator” ability of the 3-NO₂ PHL (**B11**), which displayed comparable or higher activity in ExpR1 and ExpR2 relative to their native ligand, OHHL (**A1**). However, PHLs with substitutions in either the 2- or 4-positions of the phenyl ring (**B2–B6** and **B15–B19**), or no substitution at all (**B1**), displayed relatively low agonistic activities (i.e., maximal agonism 30%) in ExpR1 and ExpR2; again this is consistent with our results in LuxR. The one outlier of this trend was the disubstituted 3,5-CF₃ PHL (**B20**), which is a very potent LuxR agonist (109%), yet displayed low agonistic activity in both ExpR1 (9%) and ExpR2 (15%).^[14e]

Screening library C for agonistic activity revealed that, analogous to LuxR, perturbations to either the acyl group spacer length (as in **C1–C4**) or degree of saturation (**C5**) in aryl HL analogues resulted in compounds with minimal agonistic activity in either ExpR1 or ExpR2 (Table 2).^[14b] However, substituting the PHL phenyl ring with a naphthyl group (**C6** and **C7**) yielded a surprising divergence in ligand activity. In ExpR2, the 2-naphthyl HL (**C6**) displayed nearly half maximal activity (56%), while the 1-naphthyl HL (**C7**) was comparable to OHHL (95%). These data are in sharp contrast to those for LuxR and ExpR1, in which both naphthyl HLs display no appreciable agonism,^[14e] and again suggest, in conjunction with the screening data for library A, that ExpR2 is more accepting of steric perturbations to the AHL acyl group compared to LuxR and ExpR1.

AHL antagonism trends in ExpR1, ExpR2, and LuxR

Comparisons between the antagonistic activities of libraries A–C in LuxR, ExpR1, and ExpR2 provided additional points of convergence and divergence for AHL recognition by the three receptors (Table 3). We highlight the most prominent features here. From library A, the 3-keto-free equivalent (**A7**) of the native ligand OHHL (**A1**) was a poor inhibitor of ExpR2 (12%), but displayed reasonably strong antagonistic activity in both LuxR and ExpR1 (~70%). In contrast, the 3-keto-free C10 AHL **A9** was a strong inhibitor of all three receptors (more so in ExpR1 than ExpR2), and constitutes the most active LuxR-type receptor antagonist found in these primary antagonism screens (76–96%). Interestingly, all three receptors were also significantly inhibited by the native AHL from *P. aeruginosa*, OdDHL (**A4**, ~80%). Since *P. carotovora* and *P. aeruginosa* can occupy the same soil environment, this finding is notable, as it—together with the above agonism assay data for ExpR2—could indicate a possible means of interspecies crosstalk by QS *inhibition* with AHL signals.

Similar to the agonism assay data for library B, the majority of the potent PHL antagonists of LuxR were also antagonists of ExpR1 and ExpR2, with the most overlap between LuxR and ExpR1 (Table 3). These antagonists were largely less potent in ExpR2 versus ExpR1. Nevertheless, library B yielded the highest percentage of ExpR antagonists in this study, further underscoring the value of the PHL scaffold as a privileged structure for LuxR-type receptor modulator design. For example, the 4-Cl, 4-Br, and 4-I PHLs (**B16–B18**) were moderate to strong antagonists in ExpR1 and ExpR2 (50–80%), which were largely comparable to their activities in LuxR. In turn, many of the 3-substituted PHLs displayed antagonistic activities against ExpR1 and ExpR2 that were either equivalent to or slightly surpassed the activities of the 4-substituted PHLs, with 3-I PHL **B10** and 3-SCH₃ PHL **B14** being the most potent PHL inhibitors overall (63–83%). Interestingly, the slightly weaker 3-halogenated PHL antagonists (e.g., **B8** and **B9**) are also appreciable *agonists* of all three receptors (at 200 μM; see agonism data above); this suggests that they are out-competing the

native ligand **A1** in these competitive antagonism assays. PHLs bearing halogens at the 2-position of the phenyl ring showed lower antagonistic activities overall, with maximal inhibitory activity being observed for the 2-I PHL (**B5**, ~60%). The unsubstituted PHL (**B1**) displayed the lowest antagonistic activity of any PHL in this study; this indicates that some functionality on the PHL phenyl ring, and preferably in the 3- or 4-position only, is critical for inhibition of LuxR, ExpR1, and ExpR2.

Paralleling library B, the majority of library C showed antagonistic activity against both ExpR1 and ExpR2 at levels comparable to that observed for LuxR (Table 3). Most notable, however, was the conservation of antagonistic activity displayed by 4-Br PPHL (**C4**) in LuxR, ExpR1, and ExpR2. PPHL **C4** has been previously shown to inhibit LuxR-type receptors in multiple other bacteria (most strongly against LasR in *P. aeruginosa*),^[14b] and these data for ExpR1 and ExpR2 serve to corroborate and further expand the utility of this nonnative AHL as a cross-species QS antagonist. Lastly, the naphthyl AHLs **C6** and **C7** displayed the most unique antagonism trend in library C. Both were potent antagonists of ExpR1 and LuxR (65–80%), yet failed to inhibit ExpR2 to any appreciable extent. Interestingly, these two naphthyl AHLs were moderate to highly potent *agonists* of ExpR2 instead (see above).

EC₅₀ and IC₅₀ values for selected AHLs in *P. carotovora* and *V. fischeri*

We performed dose-response studies on the most active AHL agonists and antagonists of ExpR1 and ExpR2 using *P. carotovora* cellulase assays to obtain more quantitative information about their relative activities. We focus our analysis here on the eleven AHLs most active in ExpR2, as this receptor is the primary regulator of virulence in *P. carotovora*.^[21b] The EC₅₀ and IC₅₀ for these AHLs in ExpR2, along with their respective values in ExpR1 and LuxR for comparison, are listed in Table 4. The C6 AHL (**A7**) agonist had a fourfold higher EC₅₀ in ExpR2 relative to **A1** (8 vs. 2 μM). However, **A7** was capable of only 25% maximal induction relative to **A1** in either LuxR or ExpR1. Likewise, the 3-Cl and 3-Br PHLs (**B8** and **B9**) and 1-naphthyl HL (**C7**) had about three- to greater than tenfold lower EC₅₀ values in ExpR2 relative to LuxR and ExpR1. In contrast, the EC₅₀ values for the 3-NO₂ PHL (**B11**) were comparable across *all three receptors* (~0.5 μM), and, more notably, were approximately ten-fold lower than the native ligand, **A1**. Few ligands with agonistic activities that surpass that of the native AHLs are known, and to our knowledge, 3-NO₂ PHL (**B11**) represents the first superactivator of ExpR1 and ExpR2 to be reported. These data are noteworthy, because they suggest, together with our earlier studies of **B11**'s superactivator ability in LuxR,^[14d] that PHL **B11** might behave as a universal activator of LuxR-type receptors that are selective for OHHL.

Unlike the ExpR2 agonists, dose-response analyses revealed that the most potent ExpR2 antagonists from the primary screens displayed IC₅₀ values that were largely analogous to those in ExpR1 and LuxR (Table 4). OdDHL (**A4**) and the decanoyl HL (**A9**) were the most active inhibitors overall (IC₅₀ ≈ 0.6 μM across all three receptors), which highlights their utility as naturally occurring inhibitors of OHHL-based QS circuits. The next most active was the non-native, 4-I PHL **B18**. This PHL is also a strong inhibitor of TraR in *A. tumefaciens* and LasR in *P. aeruginosa*,^[14a, b] and these data further extend its spectrum of activity as a general LuxR-type receptor inhibitor. While 4-Br PHL **B17** displayed a fourfold lower IC₅₀ value in ExpR2 relative to ExpR1 and LuxR (~0.9 vs. 4 μM), the IC₅₀ values for its one-carbon longer homologue PPHL **C4** was comparable between all three receptors (~2 μM). The most potent antagonist of the 3-substituted PHLs, 3-SCH₃ PHL (**B14**), had IC₅₀ values similar to **B17** and **C4** in ExpR1, ExpR2, and LuxR. Collectively, these dose-response data indicate that selective *inhibition* of ExpR2 over ExpR1 (and other LuxR-type receptors) with either native or non-native AHL is more challenging than selective *activation* of ExpR2 with similar AHL ligands.

AHL activators and inhibitors target QS in *P. carotovora*

We sought additional data to support whether the AHL activators and inhibitors identified in the cellulase assays elicited their activities through ExpR1 and ExpR2. Therefore, we examined the ability of a subset of these AHLs to modulate the production of another virulence factor in *P. carotovora* regulated by ExpR1 and ExpR2, the exoenzyme pectate lyase. Pectate lyase catalyzes the cleavage of internal α -1,4-linkages in polygalacturonic acid (PGA), and its activity can be readily monitored by measuring the accumulation of the unsaturated (1,4)- α -D-galacturonan product at 235 nm using established procedures (see the Experimental Section).^[26a] Thus, in analogy to cellulase, pectate lyase activity can be correlated with ExpR receptor activity. We utilized the same *P. carotovora* reporter strains for pectate lyase assays that were used in the cellulase assays above (i.e., the ExpR1 (AC5117) and ExpR2 (AC5099) reporters; Table 1).^[21b] As expected, dose-response analyses for OHHL in the pectate lyase assays revealed an EC₅₀ value in both reporters that was comparable to that observed in the cellulase assays ($\sim 4 \mu\text{M}$). More notably, however, the AHL agonists (**A7**, **B9**, **B11**, and **C7**) and the AHL antagonists (**A4**, **B18**, and **C4**) displayed trends in activity and absolute activity values in the pectate lyase assay that were largely identical to the cellulase assay data (Table S1 in the Supporting Information). This conservation in ligand activity between two different ExpR regulated phenotypes suggests that these synthetic AHLs act through ExpR1 and ExpR2 receptors to modulate QS in *P. carotovora*.

As mentioned above, OHHL binding to ExpR1 and ExpR2 in *P. carotovora* results in the dissociation of these receptors from DNA, and thereby reduces the expression of RsmA, a repressor of virulence factor production (Figure 1). As the levels of RsmA decrease, the production of virulence factors like cellulase and pectate lyase increases. Indeed, a triple knockout strain of *P. carotovora* (AC5118; $\Delta\text{expI}:\Delta\text{expR1}:\Delta\text{expR2}$, Table 1), lacking both a functional OHHL synthase and the two ExpR receptors, does not express appreciable levels of RsmA and is constitutively virulent.^[21b] We reasoned that if our AHLs regulate virulence factor production exclusively through ExpR1 and ExpR2 in *P. carotovora*, then the exogenous addition of these compounds to this *P. carotovora* triple knockout strain (AC5118) should have no effect on virulence factor production. To test this theory, we performed cellulase assays on selected active ExpR1 and ExpR2 modulators in the AC5118 strain. As expected, cellulase activity levels for these agonists (OHHL: **A1**, **A7**, **B9**, **B11**, and **C7**) or antagonists (**A4**, **B18**, and **C4**) were comparable to the untreated control (Figure S1 in the Supporting Information). The observed insensitivity of *P. carotovora* AC5118 to our agonists and antagonists provides further support for our model that these AHLs elicit their activities through the ExpR1:ExpR2 QS circuit. We note that these results, as well as those for cellulase and pectate lyase, do not preclude the possibility of our ligands modulating ExpR1 and ExpR2 indirectly through other receptors/pathways. While alternate AHL responsive receptors have not been identified in *P. carotovora* Ecc71 (such as CarR; see above), further experiments will be necessary to address this issue definitively.

Conclusions

There is widespread interest in the development of non-native small molecules that can attenuate QS pathways in bacteria, and this curiosity has driven a variety of synthetic and chemical biology efforts over the past three decades.^[13] The bulk of these efforts, however, have been focused on a relatively small subset of model QS circuits. Herein, we have confirmed the utility of one of these well-defined QS systems, the marine symbiont *V. fischeri*, as a predictive model for the discovery of agonists and antagonists that regulate QS in other Gram-negative bacterial species that utilize degenerate AHLs, effectively broadening the species activity range of our existing synthetic AHL libraries. Specifically, we demonstrated that a library of natural and non-native AHLs known to modulate the LuxR

receptor in *V. fischeri* is also capable of agonizing and antagonizing the ExpR1 and ExpR2 receptors in the plant pathogen *P. carotovora*. While the majority of the AHLs displayed similar trends in activity across ExpR1, ExpR2, and LuxR, there were several exceptional points of divergence in ligand response, with some AHLs being highly selective agonists or antagonists of one single receptor. These differences hint at the exciting potential for manipulating a QS circuit in one organism by using a non-native AHL signal within mixed bacterial populations. Moreover, the observed divergence in the responses of ExpR1 and ExpR2 to selected AHLs, such as **A3** or **C7**, suggests that it also might be possible to manipulate different LuxR-type QS circuits within the same organism. This latter prospect is particularly exciting given that numerous bacteria beyond *P. carotovora* are believed to have more than one LuxR-type receptor that responds to the same AHL.^[22-23]

The value of our strategy is perhaps more salient when one considers that AHL binding to LuxR or to either ExpR1 or ExpR2 elicits opposite effects, that is, through their association with or dissociation from DNA, respectively (Figure 1). Indeed, it is somewhat surprising that despite such functional differences only one AHL in the library, PHL **B20**, selectively modulates (agonizes) LuxR over ExpR1 or ExpR2. This selectivity suggests a mode of activation specific to receptors that associate with, as opposed to disassociate from, DNA upon AHL binding. The general conservation of activity between ExpR1 and LuxR, however, suggests that ligand specificity is generally conserved regardless of the mechanism of DNA binding, and that the substrate promiscuity of ExpR2 is not merely due to the dissociative mechanism. The identification of common antagonists for all three receptors, such as **A9** and **B14**, argues that these antagonists could function by association with the receptor AHL ligand binding site in a manner that competes with the native ligand, yet prevents the receptor from adopting a suitable conformation for either effective DNA association (LuxR) or disassociation (ExpR). In turn, the ability of the 3-NO₂ PHL **B11** to superactivate all three receptors suggests that binding to this PHL can stabilize the receptors in conformations that promote either DNA association or disassociation; the degree of stabilization by **B11** would then be greater relative to OHHL.

Finally, the results presented herein suggest that additional focused AHL libraries (~30-40 compounds) could be assembled based on the previously characterized responses of *A. tumefaciens* and *P. aeruginosa* to these ligands.^[14a, b] Such libraries then could be evaluated for QS agonism and antagonism in other bacterial species that utilize the same native AHLs (OOHL and OdDHL, Scheme 1).^[1b] Given our results in *P. carotovora*, it is reasonable to assume that study of these “prescreened” libraries of AHLs could substantially accelerate the identification of new QS regulators. Overall, by broadening the species range amenable to QS modulation with non-native AHL analogues, we anticipate that new insights into this important cell–cell signaling network could be achieved.

Experimental Section

Compounds

For all small molecule assays, fresh stock solutions of AHLs (10 mM) were prepared in DMSO from previously synthesized, solid stocks of compounds.^[14a, b] All other reagents were purchased from Sigma–Aldrich. DMSO did not exceed 2% (by volume) in all assays in this study. DMSO controls were included in all small molecule screens to account for the amount used for compound delivery.

Strains and media

V. fischeri ES114 ($\Delta luxI$) was provided by Edward Ruby^[29] and maintained on Luria–Bertani salt (LBS) medium. *P. carotovora* AC5117 ($\Delta expI:\Delta expR2$), AC5099 ($\Delta expI:$

$\Delta expR1$), and AC5118 ($\Delta expI:\Delta expR1:\Delta expR2$) were provided by Arun Chatterjee^[21b] and maintained on either LB or a modified salts/yeast/glucose (SYG) medium supplemented with celery extract.^[30] SYG media was prepared according to the recipe in ref. [30] and modified as follows (in w/v%): $MgSO_4$ (0.005%), $(NH_4)_2SO_4$ (0.1%), glycerol (1%), yeast extract (0.025%), sucrose (0.05%), KH_2PO_4 (0.35%), and K_2HPO_4 (0.85%) were dissolved in a solution of celery extract (10%; pH 7.0) in ddH₂O (90%; pH 7.0). Celery extract was prepared by autoclaving freshly chopped celery (250 g) in ddH₂O (100 mL). The resulting liquid was centrifuged for 30 min (8000g at 4°C), and the supernatant reserved and separated into aliquots (50 mL) prior to SYG media preparation.

Luminescence assay in *V. fischeri*

LuxR agonism and antagonism screens for AHLs **B6**, **B11–B16**, **B20**, **B21**, and **C1–C7** were performed as previously described in *V. fischeri* ES114.^[14d] IC₅₀ and EC₅₀ values were determined by performing this assay at variable ligand concentrations, and plotting the data by using the Graphpad Prism software package (v 5.0).

Cellulase assay in *P. carotovora*

The cellulase assay protocol was modified from the method of Deshpande et al.^[26b] Overnight cultures of *P. carotovora* were grown in SYG media, diluted 50-fold, and transferred in aliquots (200 μ L) to the wells of a 96-well microtiter plate containing the appropriate amount of AHL stock solution (200 μ M for agonism assays or 5 μ M AHL and 5 μ M OHHL for antagonism assays) to achieve the desired final AHL concentration. Plates were shaken (200 rpm) for ~4 h at 30°C, after which portions (50 μ L) of the substrate 4-NPC (4 mg mL⁻¹) in sodium acetate buffer (50 mM; pH 7.0) were added. The plates were shaken for an additional 2 h, and the reactions were quenched by the addition of NaOH (2 M; 50 μ L). Release of 4-nitrophenol was quantified by using a multimode plate reader (Biotek Synergy 2) and measuring absorbance at 410 nm. Cell density was normalized at 600 nm (OD₆₀₀) per well, and cellulase activity was corrected in untreated DMSO (AHL free) controls. IC₅₀ and EC₅₀ values were determined by performing this assay at variable ligand concentrations.

Pectate lyase assay in *P. carotovora*

The pectate lyase assay protocol was largely analogous to that of Collmer et al.,^[26a] yet adapted for use in 96-well microtiter plates. In brief, overnight cultures of *P. carotovora* AC5117 ($\Delta expI:\Delta expR2$) and AC5099 ($\Delta expI:\Delta expR1$) were grown in SYG media, diluted tenfold, and transferred in aliquots (200 μ L) to the wells of a 96-well microtiter plate containing the appropriate amount of AHL stock solution to achieve the desired final AHL concentration. Plates were shaken (200 rpm) for ~4 h at 30°C until cell density reached an OD₆₀₀ of approximately 0.6 (1.45×10^7 CFU mL⁻¹). Aliquots of cells from each well (100 μ L) were transferred to the wells of a quartz bottom 96-well plate, and portions (100 μ L) of pectate lyase buffer (375 mg Tris-HCl, 75 mg polygalacturonic acid, 50 mL ddH₂O; pH 10) were added. Pectate lyase activity was determined after 15 min by using a multimode plate reader by measuring the change in absorbance at 235 nm for PGA. Cell density was normalized at 600 nm (OD₆₀₀) per well, and pectate lyase activity was corrected in untreated DMSO (AHL free) controls. IC₅₀ and EC₅₀ values were determined by performing this assay at variable ligand concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of the associative and dissociative mechanisms of AHL-based QS as exemplified by *V. fischeri* and *P. carotovora*, respectively. Both bacteria utilize the same AHL (OHHL) signal for QS. Binding of OHHL to LuxR, ExpR1, or ExpR2 results in either association with or dissociation from DNA, and the subsequent transcriptional activation of QS genes.

Figure 2. OHHL (A1) dependent cellulase activity in two *P. carotovora* mutant strains that report the response of ExpR1 ($\blacktriangle$) and ExpR2 ($\bullet$). Enzymatic activity was assessed by measuring the absorbance of 4-nitrophenol at 410 nm; see the Experimental Section for details of strains and assay. Results are the average of triplicate assays; error bars reflect $\pm$ s.d.

Figure 3.

Global comparison of ligand activity between ExpR1, ExpR2, and LuxR. A), B) Cellulase assay data reporting ExpR1, and C), D) ExpR2 activity plotted versus luminescence assay data reporting LuxR, for AHL libraries A–C. Agonism (A, C) assays were performed at 200 μM AHL and antagonism (B, D) was analyzed at 5 μM A1+5 μM AHL. Luminescence assay data are taken from ref. [14b] except for compounds **B6**, **B11–B16**, **B20**, and **C1–C7**, which were evaluated in this study. Results are the average of triplicate assays; error did not exceed $\pm 10\%$ of the mean.

Scheme 1.

Three common AHLs and selected bacteria known to utilize them for QS. OHHL (shaded in gray) is one of the most prevalent AHLs. OHHL signaling in *Vibrio fischeri* and *P. carotovora* are studied herein.

Scheme 2.

Structures of the three focused libraries of synthetic AHLs evaluated in this study (libraries A–C). The number of carbons in the AHL acyl groups of library A is shown for clarity.

Table 1

Strains used in this study and relevant characteristics.

Strain ID	Relevant genes	Antibiotics ^a	Used to test
<i>P. carotovora</i> Ecc71 ^b			
AC5099	$\Delta \text{expI}; \Delta \text{expR1}$	Km:Spec	ExpR2
AC5117	$\Delta \text{expI}; \Delta \text{expR2}$	Spec:Gm	ExpR1
AC5118	$\Delta \text{expI}; \Delta \text{expR1}; \Delta \text{expR2}$	Km:Spec:Gm	AHL specificity for ExpR1 and ExpR2
<i>V. fischeri</i> ES114 ^c			
VCW2G7	ΔluxI		LuxR

^aKm: kanamycin, Spec: spectinomycin, Gm: gramicidin.

^bReported in ref. [21b].

^cReported in ref. [29]. See the Experimental Section for growth conditions.

Table 2

Comparative agonism assay data for AHLs in *V. fischeri* and *P. carotovora*.^a

Compound	Activation [%]			Compound	Activation [%]		
	<i>V. fischeri</i> LuxR ^{b,c}	<i>P. carotovora</i> ExpR1 ^{d,e}	<i>P. carotovora</i> ExpR2 ^{d,f}		<i>V. fischeri</i> LuxR ^{b,c}	<i>P. carotovora</i> ExpR1 ^{d,e}	<i>P. carotovora</i> ExpR2 ^{d,f}
A1 (OHHL)	100	100	100	A2	24	29	78
A3	2	6	48	A4	2	2	17
A5	4	1	9	A6	0	0	17
A7	2	5	94	A8	25	19	79
A9	12	6	44	A10	1	0	31
A11	2	0	11	B1	3	6	21
B2	2	8	24	B3	6	9	28
B4	12	9	32	B5	7	11	24
B6	4	9	19	B7	8	11	46
B8	61	49	81	B9	70	63	97
B10	28	32	61	B11	130	115	103
B12	5	6	15	B13	7	4	24
B14	4	0	13	B15	2	4	14
B16	3	5	29	B17	3	4	25
B18	4	6	19	B19	24	15	24
B20	107	9	15	C1	2	0	6
C2	2	2	7	C3	3	4	12
C4	2	3	8	C5	3	4	18
C6	2	8	56	C7	3	7	95

^a Assays were performed with 200 μM compound and normalized to OHHL. Results are the average of triplicate assays; errors did not exceed ±10% of the mean. Noteworthy activity trends between receptors are italicized; see the Experimental Section for details.

^b Strain: *V. fischeri* ES114 ($\Delta luxD$).

^c Measured by luminescence; luminescence assay data taken from ref. [14b except for compounds **B6**, **B11**–**B16**, and **B21**, which were evaluated in this study.

^d Measured by cellulase activity.

^e Strain: *P. carotovora* AC5117 ($\Delta expI$; $\Delta expR2$).

f Strain: *P. carotovora* AC5099 ($\Delta\text{expl}:\Delta\text{expRI}$).

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Table 3
Comparative antagonism assay data for AHLs in *V. fischeri* and *P. carotovora*.^a

Compound	Inhibition [%]		Compound		Inhibition [%]	
	<i>V. fischeri</i> LuxR ^b	<i>P. carotovora</i> ExpR1	<i>P. carotovora</i> ExpR2	<i>V. fischeri</i> LuxR ^b	<i>P. carotovora</i> ExpR1	<i>P. carotovora</i> ExpR2
A1 (OHHL)	0	0	0	A2	63	65
A3	47	42	33	A4	86	85
A5	5	4	7	A6	43	48
A7	69	67	12	A8	86	78
A9	96	91	76	A10	73	77
A11	27	4	7	B1	14	4
B2	49	44	27	B3	53	57
B4	47	49	32	B5	63	65
B6	23	27	3	B7	63	52
B8	65	63	54	B9	68	73
B10	78	83	63	B11	-18	-15
B12	78	74	62	B13	71	68
B14	88	79	71	B15	59	64
B16	75	78	48	B17	79	71
B18	85	73	68	B19	47	44
B20	0	4	16	C1	34	37
C2	40	29	13	C3	44	48
C4	80	78	66	C5	48	57
C6	83	58	5	C7	84	69
						- 6

^a Assays were performed by using 5 μM compound against 5 μM OHHL. Results are the average of triplicate assays; errors did not exceed ±10% of the mean. Noteworthy trends in activity between receptors are italicized. Negative inhibition values indicate that the compound activated at the tested concentration; see Table 2 for details of strains.

^b Assay data taken from ref. [14]b except for compounds **B6**, **B11–B16**, **B20**, and **C1–C7**, which were evaluated in this study.

Table 4

EC₅₀ and IC₅₀ values for AHL agonists and antagonists of LuxR, ExpR1, and ExpR2.^a

	EC ₅₀ [μM]			IC ₅₀ [μM]		
	LuxR ^b	ExpR1	ExpR2	LuxR ^b	ExpR1	ExpR2
A1 (OHHL)	4	3	2	A4	0.60	0.75
A7	>10 ^{c,d}	>10 ^d	8	A9	0.50	0.90
B8	>50	>50	20	B14	1.5	3.5
B9	>200	>200	35	B17	3.5	0.85
B11	0.3	0.7	0.5	B18	0.70	0.90
C7	>200 ^c	>200	80	C4	1.65	2.75

^a See Table 2 for details of strains and assay methods. EC₅₀ and IC₅₀ values determined by testing compounds over a range of concentrations (0.02–2×10⁵ nM) in each strain either alone or against OHHL (5 μM), respectively.

^b EC₅₀ and IC₅₀ values taken from ref. [14a].

^c Determined in this study.

^d Dose-response curve reached a plateau over the concentrations tested, yet the level of maximal induction did not exceed 25% OHHL; EC₅₀ value was calculated from this dose-response curve.