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Attenuation of Virulence in Pathogenic Bacteria Using Synthetic Quorum-Sensing Modulators under Native Conditions on Plant Hosts

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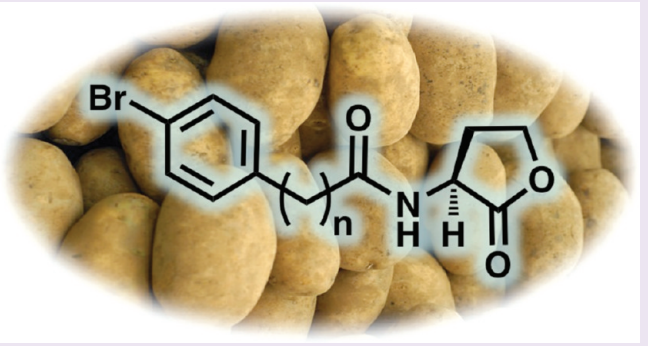
Supporting Information

ABSTRACT: Quorum sensing (QS) is often critical in both pathogenic and mutualistic relationships between bacteria and their eukaryotic hosts. Gram-negative bacteria typically use *N*-acylated L-homoserine lactone (AHL) signals for QS. We have identified a number of synthetic AHL analogues that are able to strongly modulate QS in culture-based, reporter gene assays. While informative, these assays represent idealized systems, and their relevance to QS under native conditions is often unclear. As one of our goals is to utilize synthetic QS modulators to study bacterial communication under native conditions, identifying robust host—bacteria model systems for their evaluation is crucial. We reasoned that the host—pathogen interaction between *Solanum tuberosum* (potato) and the Gram-negative pathogen *Pectobacterium carotovora* would be ideal for such studies as we have identified several potent, synthetic QS modulators for this pathogen, and infection assays in potato are facile. Herein, we report on our development of this host—pathogen system, and another in *Phaseolus vulgaris* (green bean), as a means for monitoring the ability of abiotic AHLs to modulate QS-regulated virulence in host infection assays. Our assays confirmed that QS modulators previously identified through culture-based assays largely retained their activity profiles when introduced into the plant host. However, inhibition of virulence in wild-type infections was highly dependent on the timing of compound dosing. This study is the first to demonstrate that our AHL analogues are active in wild-type bacteria in their native eukaryotic hosts and provides compelling evidence for the application of these molecules as probes to study QS in a range of organisms and environments.



Bacteria have the ability to assess their local population densities and switch from a unicellular to a multicellular lifestyle once they achieve a threshold cell number. This process is widespread in bacteria and has been termed quorum sensing (QS).[1−3] QS is regulated by the production of small molecule signals, known as autoinducers, and their detection by cognate receptor proteins.[4] The concentration of autoinducer defines whether a bacterial population has reached a sufficient density (or quorum), and therefore environmental factors (such as the area available for diffusion) also play a critical role in this signaling process.[5−7] QS phenotypes include, but are not limited to, virulence factor production, biofilm formation, bioluminescence, sporulation, and conjugation, many of which benefit from occurring only at high cell densities.[8−11] For example, by restricting virulence factor production to high population densities, bacteria are able to evade host defenses until sufficient numbers are present to successfully withstand and overwhelm host defenses.[12,13]

In the Gram-negative bacteria, the most common class of autoinducers are the *N*-acylated L-homoserine lactones (AHLs).[3,14,15] QS was discovered in the marine symbiont *Vibrio fischeri*, which produces the *N*-(3-oxo)-hexanoyl L-HL (OHHL) to regulate bioluminescence.[16,17] This signal is produced by the AHL synthase LuxI and is perceived by the cytoplasmic receptor protein LuxR.[3] This synthase:receptor pairing has been found subsequently in most other AHL circuits and appears to be the minimal functional unit for QS in Gram-negative bacteria. The AHL signals can diffuse across cell membranes (some may be actively pumped out[18]), and as cell density increases and environmental conditions permit, a threshold intercellular AHL concentration is reached, resulting in AHL binding to its cognate LuxR-type receptor. Typically, the AHL:LuxR-type receptor complexes then dimerize and associate with QS-specific promoter sites, initiating the expression of genes responsible for QS phenotypes.[14,15,19] In some instances, such as in the plant pathogen *Pectobacterium carotovora*, this mechanism is reversed (*i.e.*, dissociative as opposed to associative), with the apo-receptor homodimer binding the QS-specific promoter site, but then dissociating from DNA upon AHL binding, and thereby relieving the repression of the QS phenotype.[20]

As many QS-regulated phenotypes have significant impacts on human health and agricultural yields, there is considerable interest in the development of strategies to attenuate QS signaling.[21−26] The use of synthetic AHL analogues capable of intercepting

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native AHL:LuxR-type receptor binding has been widely explored as an approach to regulating QS.[15,27,28] Our group and others have previously identified a number of potent, abiotic agonists and antagonists of LuxR-type receptors in a range of bacteria, including *V. fischeri* (LuxR),[29] the plant pathogen *Agrobacterium tumefaciens* (TraR),[30,31] the opportunistic pathogen *Pseudomonas aeruginosa* (LasR and QscR),[31−33] and the soil bacterium *Chromobacterium violacium* (CviR).[34] More recently, we demonstrated that the activity of the non-native AHLs identified in these model QS reporter systems are generally conserved across LuxR-type receptors that utilize degenerate (*i.e.*, the same) AHL signals, which significantly broadens the utility of such analogues as probes to study QS.[35] For example, agonists and antagonists of LuxR in *V. fischeri* exhibit similar activity profiles in the two LuxR-type receptors in the plant pathogen *Pectobacterium carotovora* (ExpR1/ExpR2), and both of these bacteria utilize OHHL for QS.

In general, small molecules are screened for LuxR-type receptor activity in culture-based assays using bacterial reporter strains that lack AHL synthases and contain well-established reporters (such as GFP or β-galactosidase) for the LuxR-type receptor.[27] In some instances, these QS reporter circuits have even been transferred to *Escherichia coli* to further simplify these systems.[36] Such idealized culture-based assays permit the quantitative evaluation of both agonism and antagonism of a specific LuxR-type receptor, while facilitating the rapid identification of potential QS modulators from large libraries of compounds. However, a better understanding of how synthetic ligands modulate QS under native conditions (*i.e.*, in wild-type bacteria on their native eukaryotic host) is necessary in order to further develop them as chemical probes of this process. We sought to develop a straightforward assay that would permit such an analysis, using both wild-type bacteria and their natural host organisms, and this constitutes the major goal of the present study.

Other host−pathogen model systems have previously been used to evaluate the role of specific virulence factors or endogenous regulatory elements, including QS, on host infections. For example, Ausubel and co-workers have successfully used the model nematode *Caenorhabditis elegans* to monitor QS-regulated virulence during host infections by the Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis*.[37,38] These prior studies have focused on the use of classical genetic approaches to selectively eliminate the activity of a specific target gene in the bacteria prior to host inoculation. Our approach differs in that we are modulating the expression of the gene products of interest (*e.g.*, QS-regulated virulence factors) through a small-molecule-based (or "chemical genetic") approach.[39] This strategy is dependent on the binding of our ligands to LuxR-type receptors and offers the opportunity for temporal and spatial regulation of QS-regulated gene expression throughout the host infection process.

We reasoned that the naturally occurring host−pathogen interaction between *P. carotovora* Ecc71 and its host *Solanum tuberosum* (potato) would be ideal for our studies as virulence in *P. carotovora* is QS-dependent, potato tubers are readily available and easily inoculated, and symptoms of infection can be observed and readily quantified.[40,41] Further, as we have previously identified agonists and antagonists of *P. carotovora* (see above), a collection of QS modulators was immediately available for evaluation under native conditions.[35]

Herein, we report our development of the *S. tuberosum*−*P. carotovora* host−pathogen system as a model for evaluating the efficacy of non-native AHL QS modulation under native conditions. Our studies confirm that QS modulators previously identified through culture-based assays with reporter strains largely retain their activity when introduced into hosts. However, inhibition of wild-type virulence was highly dependent upon the dosage and timing of addition of the synthetic inhibitor. A model to explain this divergent response between wild-type and reporter strains of *P. carotovora* is presented. In order to probe the generality of this plant pathogen-host plant assay strategy, we also evaluated the activity of our ligands as modulators of QS-regulated virulence in infection assays of *Phaseolus vulgaris* (green beans) by the Gram-negative pathogen *Pseudomonas syringae* B278A.[42] In addition to confirming the exportability of our strategy, these assays represent, to our knowledge, the first analysis of *P. syringae* responses to non-native AHLs. Several novel QS agonists and antagonists were discovered for this pathogen. The results of this study are significant, as they indicate that our AHL analogues can modulate QS in wild-type bacteria under native conditions. This finding should facilitate their application as chemical tools to study the role of bacterial QS in a range of important, biologically relevant environments.

RESULTS AND DISCUSSION

In *P. carotovora* Ecc71, OHHL binds to two homologous LuxR-type receptors, ExpR1 and ExpR2, initiating the production of a variety of plant cell wall degrading enzymes (virulence factors) such as cellulase and pectate lyase.[40,41,43] In our previous culture-based studies of this strain,[35] we evaluated the responses of native and non-native AHLs to both of these receptors in isolation in *P. carotovora* mutants in which ExpR1 or ExpR2 was retained but both the AHL synthase (ExpI) and the other receptor (ExpR1 or ExpR2) were rendered nonfunctional.[40,44] Our results revealed a range of potent AHL agonists and antagonists that were active in both receptors. A subset of these active AHLs was selected for the studies herein (see below).

Selection of Host and Virulence Assay for *P. carotovora*. In host plant infections, the effects of virulence factor production by *P. carotovora* manifest themselves in the soft rot (or maceration) of plant tissue. This macerated material is straightforward to separate from uninfected tissue and weigh, which allows for the quantification of *P. carotovora* virulence in infected host plants. *Solanum tuberosum* (potato) is a natural host for *P. carotovora* and represents an excellent model plant for assaying virulence, as the tuber can be infected in a spatially addressed manner and the macerated tissue can be readily observed in a relatively short period of time (<72 h). In addition, *S. tuberosum* is an inexpensive, abundant, and agriculturally important crop. We therefore chose the tuber of *S. tuberosum* as the host plant tissue for our *P. carotovora* infection assays in the presence of AHLs.

Modulation of *P. carotovora* (Δ*expI*) Virulence *in vivo*. Although our ultimate goal was to evaluate QS under native conditions with wild-type bacteria, we performed our preliminary studies in the potato host using a *P. carotovora* (Δ*expI*) mutant strain (AC5094).[43] This strain lacks a functional OHHL synthase (ExpI) yet retains both ExpR1 and ExpR2, and virulence is dependent on the exogenous addition of the native QS ligand (OHHL), thereby providing us with a highly controlled system for developing our potato infection protocol. We conducted several studies to optimize the potato−*P. carotovora* (Δ*expI*) infection assay, including varying the innocula volume, the number of bacteria relative to OHHL concentration, as well as the postinoculation incubation time (data not shown). These results

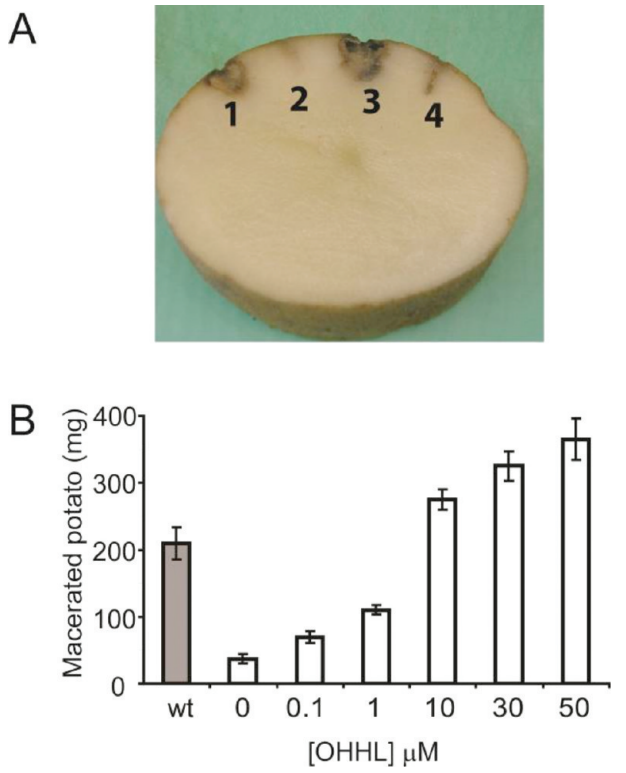

Figure 1. *S. tuberosum* (potato) maceration by *P. carotovora*. Potato tubers were inoculated with 10 μL of overnight cultures (~1 × 10^5 CFU/mL), of *P. carotovora* Ecc71 (wild-type or Δ*expI*) and incubated for 48 h (see Methods). (A) Image of potato inoculated at multiple injection sites with (left to right): (1) wild-type *P. carotovora*, (2) buffer only, (3) *P. carotovora* (Δ*expI*) with 50 μM OHHL (**5**), or (4) *P. carotovora* (Δ*expI*) without AHL. (B) Maceration of potato with wild-type *P. carotovora* (gray) or *P. carotovora* (Δ*expI*) (white) using varying concentrations of OHHL (**5**). Results are expressed as the average macerated mass ± the standard error of the mean (n = 6).

established that injection of a 10-μL inoculum (1 × 10^5 CFU/mL at 50 μM OHHL) into the potato, followed by a 48 h incubation time yielded the most consistent potato maceration quality and quantity between samples (see Methods). Lower concentrations of bacteria (10^2–10^4 CFU/mL) required significantly longer infection times, and the resulting maceration was highly variable. At higher concentrations of bacteria (10^6–10^9 CFU/mL), infection proceeded more rapidly, but in many cases harvesting the macerated material was difficult as it became increasingly watery. Using our optimized assay methods, potato maceration was clearly observed at sites inoculated with wild-type *P. carotovora*, yet was dependent on the addition of OHHL in a concentration-dependent manner at sites inoculated with *P. carotovora* (Δ*expI*) (Figure 1). As expected, inoculation of potatoes with OHHL in the absence of bacteria induced no maceration (data not shown).

Next, we selected a 23-member focused library of synthetic AHLs from our in-house AHL collection for analysis in potato that we had previously evaluated in culture-based *P. carotovora* (Δ*expI*) reporter assays (Figure 2).[35] These compounds all retained the native L-HL headgroup, yet contained different acyl groups, and exhibited a wide range of agonistic and antagonistic activities in *P. carotovora* (Δ*expI*). Several naturally occurring AHLs were included in the library, including OHHL (**5**) from

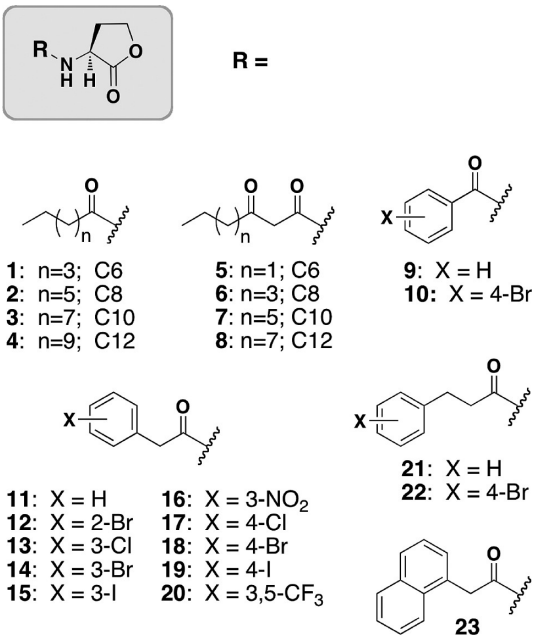

Figure 2. Structures of the AHL library evaluated in this study. Alkyl chain carbon number is shown for AHLs **1**–**8** for clarity. Alternate nomenclature for selected native AHLs used in this study: AHL **5** = *N*-(3-oxo)-hexanoyl L-HL (OHHL), AHL **6** = *N*-(3-oxo)-octanoyl L-HL (OOHL), and AHL **8** = *N*-(3-oxo)-dodecanoyl L-HL (OdDHL).

P. carotovora, *V. fischeri*, and *Pseudomonas syringae*; OOHL (**6**) from *A. tumefaciens*; and OdDHL (**8**) from *P. aeruginosa*. The AHL library also contained a number of non-native AHLs (**9**–**23**) with carbon spacers of different length between an acyl-chain aromatic group and the L-HL ring. Among these compounds were several *N*-phenylacetanoyl L-homoserine lactones (PHLs) (**11**–**20**), a number of which we have previously shown to be highly potent non-native agonists and antagonists of a range of LuxR-type receptors.[29,33,45]

We evaluated the activity of the AHL library members as both agonists and antagonists of *P. carotovora* (Δ*expI*) virulence in the potato infection assay and compared these results to culture-based cellulase assays with *P. carotovora* (Δ*expI*) for the same compounds. Cellulase is one of the primary virulence factors of *P. carotovora*, and its activity can be correlated with its production as a marker of QS-controlled virulence. We utilized our previously developed cellulase assay protocol to obtain these data in the *P. carotovora* AC5094 (Δ*expI*) strain (see Methods).[35] Trends between the culture-based cellulase assays and the potato maceration assays for the non-native AHLs were very well conserved for both agonism and antagonism (see Table 1 and Supplementary Figure 1). These results support the utility of *P. carotovora* culture-based assays as preliminary screens for identifying ligands with potential activity under native conditions in a plant host.

Modulation of Wild-Type *P. carotovora* Virulence *in vivo*. We selected four AHL antagonists (OdDHL (**8**), **10**, **18**, and **22**; Figure 2) to further evaluate in potato infection assays with wild-type *P. carotovora* Ecc71. OdDHL (**8**), *p*-bromo PHL (**18**) and *p*-bromo phenylpropanoyl HL (**22**) were selected as they were identified as the three most potent antagonists (72–84% inhibition) in both our potato maceration and culture-based assays with *P. carotovora* (Δ*expI*). The shorter homologue of **18** and **22**,

Table 1. Agonism and Antagonism Data for the AHL Library in Cellulase and Potato Maceration Assays in *P. carotovora* (Δ*expI*)

Compound	Cellulase activity[a,b]		Potato maceration[a,c]	
	% Agonism[d]	% Antagonism[e]	% Agonism[f]	% Antagonism[g]
1	92	14	97	-4
2	72	46	79	29
3	39	83	25	71
4	9	80	11	67
5: OHHL	100	0	100	0
6: OOHL	74	19	87	18
7	27	68	39	48
8: OdDHL	7	85	15	77
9	5	19	6	31
10	8	47	7	56
11	6	3	7	4
12	21	42	5	32
13	66	36	69	47
14	88	37	94	28
15	55	41	72	49
16	112	-6	108	-8
17	24	55	13	41
18	27	78	15	84
19	22	72	21	64
20	12	11	15	10
21	9	29	12	16
22	7	79	19	72
23	92	-2	78	7

[a] Assays performed with *P. carotovora* (Δ*expI*). [b] Results reported as the average of three replicates relative to the activity of the native ligand OHHL (**5**). [c] Results reported as the average of six replicates relative to the activity of the native ligand OHHL (**5**). [d] Agonism assays performed at 200 μM AHL. [e] Antagonism assays performed at a 1:1 ratio of **5** and the indicated AHL with each at 5 μM. [f] Agonism assays performed at 50 μM AHL. [g] Antagonism assays performed at a 1:1 ratio of **5** (OHHL) and the indicated AHL with each at 50 μM. Error did not exceed 10% in any of these assays. Compounds selected for further study in wild-type *P. carotovora*-potato maceration assays are indicated in bold.

p-bromo phenyl HL (**10**), was selected to evaluate the dynamic range of the antagonism assay, as it was a less potent antagonist (55%) in *P. carotovora* (Δ*expI*). We tested these AHLs using the optimized host-infection assay conditions developed above (50 μM compound) with wild-type *P. carotovora*; however, no significant reduction of maceration was observed with any of these compounds in infected potatoes (Figure 3A). A dose−response study with **18** showed that higher doses (50−200 μM) were also largely ineffective at inhibiting maceration by wild-type *P. carotovora*; these higher concentrations of **18** were strongly inhibitory in the *P. carotovora* (Δ*expI*)−potato assays, however (Figure 3B).

Our initial hypothesis was that the AHL antagonist was diffusing away from the inoculation site in the potato over the time course of the assay and thus reducing its availability to inhibit QS in wild-type *P. carotovora*. In infection assays with the *P. carotovora* (Δ*expI*) mutant, both compounds (OHHL (**5**) and the antagonist AHL) would diffuse away from the site of inoculation and mask this effect. In contrast, OHHL (**5**) is continually produced in wild-type *P. carotovora* and could potentially outcompete the antagonist over the 48 h assay. However, AHLs **8**, **10**, **18**, and **22** were also equally inactive in culture-based cellulase assays with wild-type *P. carotovora* (data not shown), where AHL concentrations should be homogeneous, arguing against this diffusion explanation for the potato assay data.

We next considered ligand stability. It is important to note that hydrolysis of the lactone in both native and our non-native AHLs

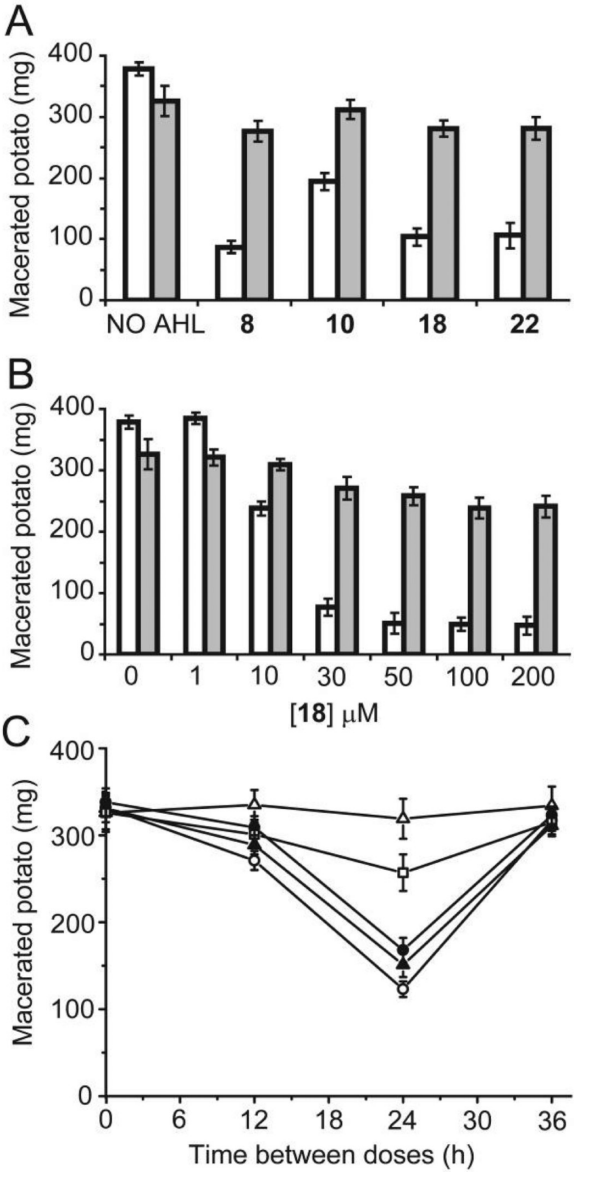

Figure 3. Inhibition of *P. carotovora* virulence in potato by non-native AHLs. (A) Potato maceration assay data at 48 h postinoculation (with compound and bacteria) with 50 μM of the indicated AHL or (B) increasing concentrations of AHL **18**: (white bars) *P. carotovora* (Δ*expI*), (gray bars) wild-type *P. carotovora*. (C) Potato maceration assay data after a second dose (50 μM) of the indicated AHL at the indicated time for wild-type *P. carotovora*: (Δ) Buffer only, (○) **8**, (□) **10**, (▲) **18**, and (●) **22**. Mass of macerated potato was determined after 48 h. Results are expressed as the average macerated mass ± the standard error of the mean ($n = 6$).

renders an inactive compound.[46,47] As the half-life of the lactone in these AHLs is ∼24−48 h in aqueous media, and the potato infection assays were performed over a 48 h period, some ligand degradation theoretically would have occurred. Again, in the *P. carotovora* (Δ*expI*) mutant, both OHHL (**5**) and non-native AHL would hydrolyze at approximately similar rates, and this effect would not be apparent in the potato assays. In the wild-type strain, however, OHHL (**5**) would be continuously replenished while the non-native AHL was degraded, and the antagonists would therefore exhibit reduced activities over time. This

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reasoning would explain the lack of activity for AHLs **8**, **10**, **18**, and **22** in both the potato and cellulase assays in wild-type *P. carotovora*. Lactone hydrolysis may be further accelerated in the potato host, as previous studies with soft rot bacteria, such as *P. carotovora* and *Erwinia chrysanthemi,* suggest that these pathogens induce a rapid influx of H^+ into plant cells, which subsequently stimulates an increase in the pH of the plant apoplastic fluid (to pH > 8.0, which will favor lactone hydrolysis).[48,49]

If our ligand instability hypothesis was correct, we anticipated that wild-type *P. carotovora* virulence inhibition in potato could be restored by additional doses of AHL antagonist. We conducted a series of experiments in which a second dose of AHLs **8**, **10**, **18**, and **22** (at 50 μM) was introduced at set time intervals to infection sites in inoculated potatoes (12, 24, or 36 h). As shown in Figure 3C, an additional dose of antagonist at 24 h postinoculation resulted in significant maceration inhibition at 48 h, with trends for **8**, **10**, **18**, and **22** matching those observed in the *P. carotovora* (Δ*expI*)−potato assays (albeit ∼10−20% less active overall). In contrast, additional AHL doses applied at either 12 or 36 h still showed significant maceration at 48 h. We note that the total concentration of AHL present during these assays is well below the range at which toxic effects are observed in culture -based assays (>200 μM) of *P. carotovora*, arguing against the inhibitory effect displayed by dosing arising from compromised *P. carotovora* viability. Also, the inhibition of virulence was only temporary in samples in which a second dose was added at 24 h, as a substantial increase in maceration was observed at 72 h postinoculation (relative to 48 h; data not shown). Subsequent studies aimed at defining the appropriate timing for the first dose of antagonist demonstrated that only treatments at 0−6 h were effective at attenuating virulence in *P. carotovora*−potato assays; later treatments were markedly less effective (see Supplementary Figure 2).

We propose that the observed time dependence in the antagonist dosing studies (Figure 3C) is consistent with the ligand instability hypothesis. In this model, a second AHL dose applied at 12 h is introduced too early and hydrolyzes over the remaining 36 h, and limited antagonistic activity is observed at the 48 h end point. In turn, waiting until 36 h to apply the second dose is too late, as by then the initial concentration of antagonist has dropped below its threshold for activity, virulence factor production is initiated, and potato maceration has begun. Again, in both of these scenarios, OHHL (**5**) is continually replenished, and its concentration will significantly increase as QS is initiated (*i.e.,* autoinduction[3]) and thereby further demote the activity of the AHL antagonist. Dosing a second time at 24 h appears to be optimal for this assay, providing a sufficiently high level of AHL antagonist to prevent significant virulence factor production over the 48 h infection assay. We postulate that the reduced maceration inhibition at 72 h is also due to AHL hydrolysis and high levels of endogenous OHHL (**5**) overwhelming the antagonist (when dosed at 50 μM). The continued inhibition of QS beyond 72 h will likely require further refinement of our dosage protocol (both in timing and AHL concentration).

In order to test our hypothesis that AHL degradation was occurring in the inoculated tuber, we developed a reporter assay utilizing the *V. fischeri* ES114 (Δ*luxI*) strain,[50] which reports LuxR activity *via* luminescence (*lux*). We selected 4-Br PHL (**18**) for testing, as this compound is a potent LuxR and ExpR inhibitor.[31,35] Briefly, we inoculated potatoes with a solution of **18** for 6−48 h. We then excised these sites at specific time points, homogenized the material, added media containing the *V. fischeri* (Δ*luxI*) reporter, and monitored luminescence (see Supporting Information for assay details). The assay data revealed that the LuxR antagonistic activity of AHL **18** was inversely correlated with incubation time in tuber, with an almost complete loss of antagonism after 18 h (see Supplementary Figure 3). This loss of activity is likely due to AHL hydrolysis, as LuxR antagonism by **18** could be restored by acidification of homogenized potato material at 48 h after AHL exposure, conditions that should promote recyclization of the hydrolyzed AHL. These findings are consistent with our model that AHL hydrolysis is a primary mechanism by which antagonists are rendered inactive in *S. tuberosum* infections.

Collectively, these results indicate that our AHLs are indeed active in wild-type *P. carotovora* on its native plant host, but the timing of compound addition, as well as the overall assay time, are critical features for consideration in the general development of QS assays using AHL modulators in wild-type bacteria. The dosing data also indicate that the inhibitory activities of our AHLs are reversible. Furthermore, and perhaps of broader interest, these assay data provide insight into the timing of virulence initiation and thus QS, in *P. carotovora* under native conditions on a plant host. It is such insights that chemical probes of QS should have the ability to provide.

Development of an *in vivo* Assay for Evaluating QS in *P. syringae*. The ability to study the effects of non-native AHLs on QS in a range of native pathogen−host systems would be valuable. As a test case, we explored the exportability of the *P. carotovora*−potato assay format developed above to another plant pathogen−host system: *P. syringae* B278A and its host plant *Phaseolus vulgaris* (green beans). *P. syringae* B278A, like *P. carotovora* Ecc71, uses OHHL (**5**) as its native QS signal. OHHL is synthesized by AhlI and perceived by its cognate receptor (AhlR), initiating the production of proteases and alginate that are essential for plant infection.[42] While prior studies have confirmed diminished virulence in *P. vulgaris* infected with a *P. syringae* synthase knockout (Δ*ahlI*), the sensitivity of *P. syringae* to non-native AHLs remained unknown.[42] Our earlier studies of non-native AHLs in *P. carotovora* indicated that bacteria utilizing the same native AHL for QS are generally responsive to similar non-native AHL agonists and antagonists (see above).[35] We therefore reasoned that the AHL library evaluated in the potato maceration studies above (Figure 2) would retain its activity profile in *P. syringae*, and we selected these compounds for study in green beans.

We developed a slightly modified variant of the potato infection assay above to evaluate *P. syringae* virulence in green beans (see Methods). Previous studies have confirmed that the direct inoculation of *P. syringae* into green beans results in the formation of water-soaked lesions marked by a brown and/or yellow "halo".[42,51] To examine the extent to which the lesions occur in infections with a *P. syringae* QS mutant, we inoculated green beans with either wild-type *P. syringae* B278A, *P. syringae* (Δ*ahlI*), or sterile phosphate buffer and examined bean maceration after 72 h (Figure 4A). The results confirmed a significantly reduced "halo" in sites infected with *P. syringae* (Δ*ahlI*) that could be differentiated from wild-type infection lesions. Similar to the *P. carotovora*−potato infection assays, the contents of these lesions could be collected and readily quantified as a measure of virulence. As expected, the exogenous addition of OHHL (**5**) to sites on green beans infected with *P. syringae* (Δ*ahlI*) restored virulence in a concentration-dependent manner (Figure 4B).

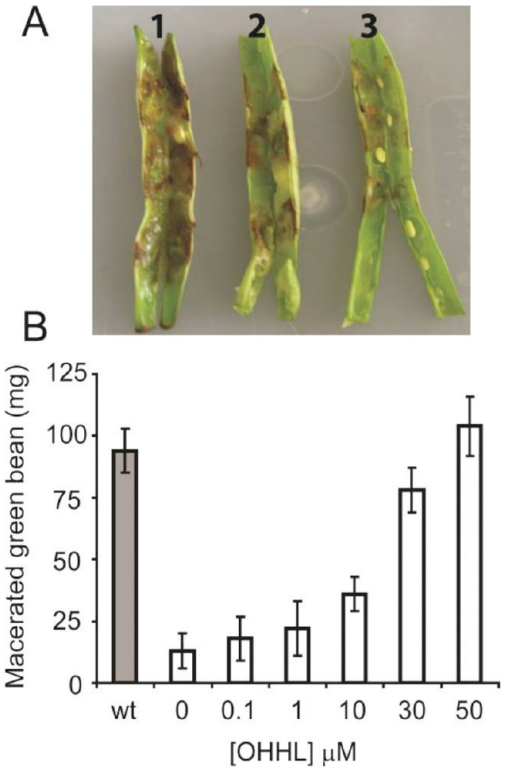

Figure 4. *P. vulgaris* (green bean) maceration by *P. syringae*. Green beans were inoculated with 10 μL of an overnight culture ($\sim 1 \times 10^5$ CFU/mL) of wild-type *P. syringae* or *P. syringae* (Δ*ahlI*) and the indicated concentration of AHL and incubated for 72 h (see Methods). (A) Image of green beans inoculated with (left to right): (1) wild-type *P. syringae*, (2) *P. syringae* (Δ*ahlI*), (3) buffer alone. (B) Maceration of green bean with wild-type *P. syringae* (gray bar) or with *P. syringae* (Δ*ahlI*) and varying concentrations of OHHL (**5**) (white bars). Results are expressed as the average macerated mass ± the standard error of the mean (n = 9).

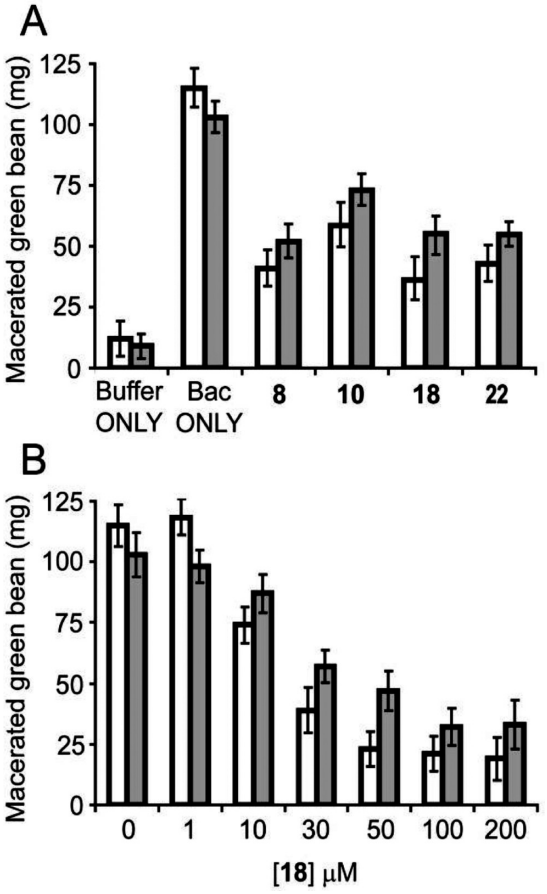

Figure 5. Inhibition of *P. syringae* virulence in green bean by non-native AHLs **8**, **10**, **18**, and **22**. (A) Green bean maceration assay data at 72 h postinoculation (with compound and bacteria) with 30 μM of the indicated AHL or (B) increasing concentrations of AHL **18**: *P. syringae* (Δ*ahlI*) (white bars); wild-type *P. syringae* (gray bars). Results are expressed as the average macerated mass ± the standard error of the mean (n = 9). BAC = bacteria.

Modulation of *P. syringae* Virulence *in Vivo*. We utilized the green bean maceration assay to examine the activity of the 23-member AHL library from above on *P. syringae* (Δ*ahlI*) virulence at 30 μM. Several highly active non-native AHL agonists and antagonists of virulence in *P. syringae* were identified (see Supplementary Table 2 and Supplementary Figure 4) and represent the first non-native AHL modulators of AhlR to be reported. In general, the activity profiles for these compounds in AhlR, based on the results of the host infection assays, was comparable to those in LuxR in *V. fischeri*[29] and in ExpR1/ExpR2 in *P. carotovora*.[35] For brevity, a detailed structure−activity analysis for the 23-member AHL library is provided in the Supporting Information. Similar to results in *P. carotovora*, AHLs **8**, **10**, **18**, and **22** were moderate to potent antagonists in these preliminary screens in *P. syringae* (Δ*ahlI*) (Figure 2), and we thus selected these four AHLs for testing in wild-type *P. syringae* infections of green beans.

The wild-type *P. syringae*-green bean assays with AHLs **8**, **10**, **18**, and **22** provided surprising results. We found that a single 30 μM dose of any of the AHL antagonists, coadministered with the bacterial innoculum, was sufficient to attenuate green bean maceration at 72 h (Figure 5A). This is in sharp contrast to the wild-type *P. carotovora*−potato assays above in which a single dose of the AHL antagonists was insufficient to attenuate potato maceration at 48 h. Dose−response studies with AHL **18** confirmed that the attenuation of virulence inhibition in *P. syringae* was concentration-dependent (Figure 5B). We believe that the variation in dosing requirements between the green bean and potato assays may arise from differences between *P. syringae* and *P. carotovora* in AHL production and AHL sensitivity, as well as environmental factors such as moisture and oxygen content and pH, which may influence AHL stability and bacterial metabolism.[52,53] These variable factors are likely to play an important role in controlling the bacterial "quorum" size and thus the amount of compound necessary for effective inhibition. However, similar to the wild-type *P. carotovora* infection assays, *P. syringae* virulence inhibition by AHL **18** was only temporary, as an increase in green bean maceration was observed at 96 h postinoculation (data not shown). This is most likely due to a loss of QS inhibition arising from hydrolysis of the AHL antagonist and the continued accumulation of OHHL (**5**), as we propose above for wild-type *P. carotovora*.

The results of these *P. syringae*:green bean assays are important, as they support the general exportability of our strategy for the evaluation of small molecule modulators of QS under native conditions in the context of a plant host. Moreover, the general conservation in the activity of these four AHL antagonists in two different wild-type pathogens and in two vastly different host tissues is striking and underscores the potential utility of our

non-native AHL modulators as chemical probes of QS in diverse, biologically relevant environments.

Summary and Outlook. Numerous AHL analogues have been shown to both strongly activate and inhibit QS in culture-based studies of LuxR-type reporter strains.[27,28] However, expanding the future utility of these synthetic analogues is dependent, in part, on transitioning from culture-based assays with reporter strains to assays measuring native QS phenotypes, ideally with wild-type bacteria, in conjunction with their eukaryotic hosts. It is under these latter conditions that many exciting questions can be asked about the role and mechanisms of bacterial QS in biologically relevant environments. As such, identifying robust host−bacteria model systems for their evaluation is essential and was the motivation for this study.

Herein, we have tested the host−pathogen interaction between *Solanum tuberosum* (potato) and the Gram-negative pathogen *Pectobacterium carotovora* as a means for monitoring the ability of synthetic AHLs to modulate QS-regulated virulence in host infection assays using both reporter strains and wild-type bacteria. We developed a straightforward potato tuber infection assay for monitoring QS regulated virulence in *P. carotovora* (Δ*expI*) reporter and *P. carotovora* wild-type strains and used this assay to screen a focused library of synthetic AHLs. Our assays confirmed that QS modulators previously identified through *P. carotovora* culture-based assays largely retained their agonistic and antagonistic activity profiles when introduced into the potato host. However, inhibition of virulence in wild-type *P. carotovora* infections was highly dependent on the timing of compound dosing. Additional dosages of AHL antagonist were required at specific times in potato infection to observe maximal virulence inhibitory effects and offer a window into the timing of the *P. carotovora* infection process. Small molecule probes can offer significant advantages for the study of biological processes that are time-dependent, and these results with AHL antagonists in *P. carotovora*−potato infection provide such an example. This dosage dependence also highlights the potential value of biologically compatible materials or devices that would facilitate the controlled release of AHL-derived QS modulators over time; toward this end, we recently reported an example of a polymer film for the controlled release of AHLs that showed sustained activity over a 4−5 day period.[54]

We also demonstrated that our host−pathogen assay strategy is not limited to potato and *P. carotovora* by testing the AHL library in a second host−pathogen system, *P. vulgaris* (green bean) and *P. syringae*. A similar plant maceration assay was readily developed, and a set of AHLs were identified that could inhibit wild-type *P. syringae* infections in green bean. These AHLs are notable in their own right, as they represent to our knowledge the first non-native QS modulators to be reported in *P. syringae*. The most potent AHL antagonists in *P. syringae* were identical to those found in *P. carotovora*, and this overlap corroborates our observations for other Gram-negative bacteria that utilize degenerate native AHLs for QS (*e.g.*, OHHL (**5**)). However, the conservation in the activity of four AHLs (**8**, **10**, **18**, and **22**) between infections of two distinct plant tissue types (pod and tuber) by these two distinct plant pathogens is remarkable. Interestingly, unlike in potato, no additional compound doses were required in green beans to observe maximal virulence inhibition, indicating that, not unexpectedly, compound-dosing strategies for QS modulators will most likely be host- and pathogen-dependent. Together, the results of this study are the first to demonstrate that our AHL analogues are active in wild-type bacteria in their native eukaryotic hosts and provide strong support for their broader use as chemical probes of QS in both mutualistic and pathogenic bacteria−host interactions.

METHODS

Compounds and Materials. DMSO stock solutions of AHLs (10 mM) were prepared from previously synthesized solid stocks.[31,45] DMSO did not exceed 2% (by volume) in any of the assays performed in this study. Fresh russet potatoes (*S. tuberosum*) and green beans (*P. vulgaris*) were purchased from local sources and used within 72 h.

Strains and Growth Conditions. Wild-type *P. carotovora* Ecc71 and *P. carotovora* AC5094 (Δ*expI*) were provided by Prof. Arun Chatterjee (U. Missouri) and maintained on Luria−Bertani (LB) plates (supplemented with kanamycin for AC5094).[40] Overnight cultures of *P. carotovora* were grown in salts-yeast extract-glycerol (SYG) medium as previously described.[35] Wild-type *P. syringae* B278A and *P. syringae* BHSL(Δ*ahlI*) were provided by Prof. David Willis (UW−Madison) and maintained on LB plates supplemented with 2% (v/v) glycerol. Overnight liquid cultures were also grown in LB medium.[51] In culture-based experiments, the AHL doses examined in this study displayed no toxic effects on bacterial growth (data not shown).

Cellulase Assays. Culture-based cellulase assays were performed as previously described with *P. carotovora* AC5094 (Δ*expI*) grown in SYG medium supplemented with celery extract.[35]

Plant Maceration Assays. Potato tubers or green beans were surface sterilized by washing in a 5% commercial bleach solution for 15 min. The potatoes or beans were then rinsed 3× with an equal volume of sterile ddH_2O and allowed to air-dry under sterile conditions. Overnight cultures of *P. carotovora* or *P. syringae* strains were pelleted and resuspended in an equal volume of 50 mM phosphate buffer (pH 7.0). This process was repeated 3× to remove any pre-existing virulence factors and/or AHLs from the culture. The cultures were then diluted 1:100 in phosphate buffer to achieve a final cell density of ∼1×10^5 CFU/mL. AHLs were added to the bacterial cultures from DMSO stock solutions to achieve the desired concentrations.

Maceration assays were performed using a previously reported protocol with slight modifications.[44] Infection sites in potatoes were prepared by puncturing the surface of the intact potato with a sterile 1 mL micropipet tip. Infection sites in intact green beans were made with sterile razor blades and were approximately 1 cm in length. A 10-μL aliquot of the appropriate bacteria−AHL mixture was directly inoculated into these sites. Following inoculation, potatoes were wrapped in parafilm, placed in shallow dishes lined with moistened filter paper, and incubated at 30 °C for 48 h. Green beans were placed in sterile Erlenmeyer flasks with 10 mL of ddH_2O, sealed with aluminum foil, and incubated at 30 °C for 72 h. Following incubation, infected sites were exposed using a sterile knife, the macerated mass of the infected area was collected by gently scraping with a sterile metal spatula, and the mass of macerated vegetable material was measured immediately using an analytical balance. Using this procedure, macerated material was easily distinguished and recoverable from uninfected vegetable tissue. Potato results were expressed as an average of six infection sites ($n = 6$), and green bean results were expressed as an average of nine infection sites ($n = 9$).

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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