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Substrate Specificity and Function of the Pheromone Receptor AinR in *Vibrio fischeri* ES114

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Two distinct but interrelated pheromone-signaling systems, LuxI/LuxR and AinS/AinR, positively control bioluminescence in *Vibrio fischeri*. Although each system generates an acyl-homoserine lactone (AHL) signal, the protein sequences of LuxI/LuxR and AinS/AinR are unrelated. AinS and LuxI generate the pheromones *N*-octanoyl-AHL (C_8-AHL) and *N*-3-oxo-hexanoyl-AHL ($3OC_6$-AHL), respectively. LuxR is a transcriptional activator that responds to $3OC_6$-AHL, and to a lesser extent to C_8-AHL. AinR is hypothesized to respond to C_8-AHL and, based on homology to *Vibrio harveyi* LuxN, to mediate the repression of a Qrr regulatory RNA. However, a Δ*ainR* mutation decreased luminescence, which was not predicted based on *V. harveyi* LuxN, raising the possibility of a distinct regulatory mechanism for AinR. Here we show that *ainR* can complement a *luxN* mutant, suggesting functional similarity. Moreover, in *V. fischeri*, we observed *ainR*-dependent repression of a P_{qrr}-*lacZ* transcriptional reporter in the presence of C_8-AHL, consistent with its hypothesized regulatory role. The system appears quite sensitive, with a half-maximal effect on a P_{qrr} reporter at 140 pM C_8-AHL. Several other AHLs with substituted and unsubstituted acyl chains between 6 and 10 carbons also displayed an AinR-dependent effect on P_{qrr}-*lacZ*; however, AHLs with acyl chains of four carbons or 12 or more carbons lacked activity. Interestingly, $3OC_6$-AHL also affected expression from the *qrr* promoter, but this effect was largely *luxR* dependent, indicating a previously unknown connection between these systems. Finally, we propose a preliminary explanation for the unexpected luminescence phenotype of the Δ*ainR* mutant.

Vibrio fischeri is a valuable model for studying pheromone signaling (PS), and its PS-mediated control of luminescence was a fundamental discovery in the field of bacterial cell-cell communication (1). *V. fischeri* uses three entwined PS systems to control the *luxICDABEG* luminescence operon (2), which is induced during infection of symbiotic hosts and serves as a colonization factor (3–5). One of these PS systems is the widespread AI-2 signaling system (6, 7), underpinned by the LuxS pheromone synthase along with receptors LuxQ and LuxP; however, AI-2 exerts relatively little effect on bioluminescence under the conditions tested (8). The two major PS controls of luminescence use acylhomoserine lactone (AHL) signals. The first AHL system discovered was LuxI/LuxR (9, 10). LuxI is a signal synthase that generates *N*-3-oxo-hexanoyl-homoserine lactone ($3OC_6$-AHL) (11). At a threshold concentration, this membrane-permeative autoinducer AHL binds to LuxR (9, 12, 13). $3OC_6$-AHL-LuxR then binds the *lux* box located within the intergenic region between *luxR* and *luxI* and activates *luxICDABEG* transcription.

It was later discovered that *V. fischeri* has a second AHL system comprised of AinS and AinR, whose protein sequences bear no resemblance to those of LuxI/LuxR (8, 14–17). AinS generates *N*-octanoyl-homoserine lactone (C_8-AHL), which can bind LuxR directly, although it is a weaker activator than $3OC_6$-AHL and can inhibit $3OC_6$-AHL-mediated activation (17, 18). It is thought that a major role for C_8-AHL involves sensing by AinR, which then converges with AI-2 signaling, acting via LuxU, LuxO, Hfq, and the regulatory RNA Qrr to increase levels of the transcriptional regulator LitR (17, 19–21).

Homologs of LitR are widespread in the *Vibrionaceae* and are similarly controlled via pheromone receptors and Qrr regulatory RNAs. Such LitR homologs include HapR in *Vibrio cholerae*, OpaR in *Vibrio parahaemolyticus*, and SmcR in *Vibrio vulnificus*. The LitR homolog in *Vibrio harveyi* is LuxR, and unlike LitR, it directly regulates bioluminescence. Through an unfortunately confusing twist of nomenclature, *V. harveyi* LuxR bears no structural similarity to *V. fischeri* LuxR. Indeed, the organisms above generally lack AHL systems similar to *V. fischeri* LuxI/LuxR. LitR homologs are often called PS "master regulators," but in *V. fischeri*, LuxI/LuxR is the PS system that directly controls bioluminescence, with LitR playing a role by activating transcription of *luxR* (19).

The proposed function of AinR in the *V. fischeri* signaling cascade (Fig. 1) has been inferred largely by homology to *V. harveyi* LuxN (22–25). LuxN phosphorylates and dephosphorylates LuxU, and LuxU-P initiates a cascade involving LuxO, resulting in activation of Qrr regulatory RNAs, which posttranscriptionally repress expression of the PS master regulator. LuxN′s cognate pheromone *N*-D-3-hydroxybutanoyl homoserine lactone (AI-1) decreases its kinase activity, initiating a shift toward the unphosphorylated form of LuxU, less Qrr, and more master regulator (24). This system has the noteworthy trait that a signal receptor mutant (e.g., Δ*luxN*) does not mimic the lack of signal and in fact has the opposite effect. This perhaps counterintuitive effect is illustrated with respect to the predicted model of AinR function in Fig. 1. Interestingly, a Δ*ainR* mutation resulted in dimmer luminescence (26), which is the opposite of that predicted by the current model. However, this observation alone does not rule out AinR's having the predicted effect on Qrr.

Signal cross talk might further entwine the Ain and Lux systems. Given that AinS's product, C_8-AHL, is recognized by LuxR,

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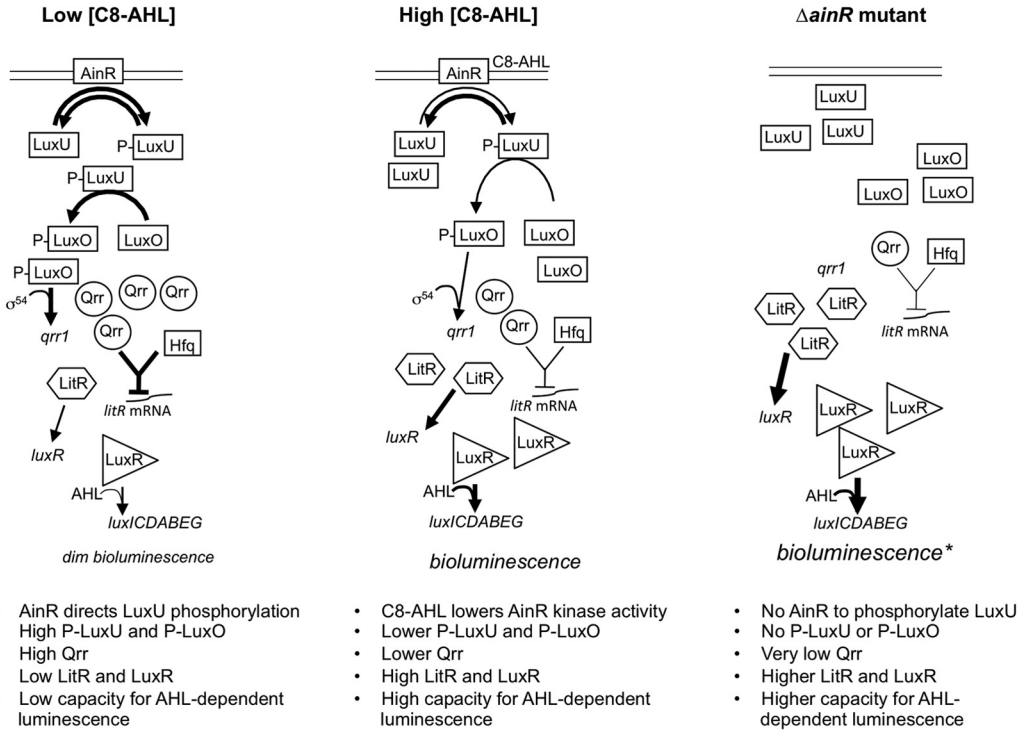

FIG 1 Model of AinR-mediated pheromone signaling in *V. fischeri*. This model is drawn largely based on the function of the homolog LuxN in *V. harveyi*, although in that bacterium, the homolog of LitR is the direct regulator of luminescence. Differences in font size, arrow thickness, and numbers of gene products shown are meant to reflect differences in activation or abundance. An asterisk highlights the fact that a Δ*ainR* mutant actually displays dimmer bioluminescence than the wild type (26). This simplified model omits any input from AI-2 and LuxQ into the shared regulatory cascade.

and that C_8-AHL's net effect depends on the availability of $3OC_6$-AHL, we were interested in whether these two AHLs might also have interrelated effects on AinR-mediated signaling. Moreover, because AinR apparently shares a signaling pathway with the widespread pheromone AI-2, we wondered whether AinR would similarly sense AHL signals from a broad array of bacteria or have more a specific AHL range, like LuxR (18).

Given the central position of AinR in our current model of the *V. fischeri* PS circuitry and the lack of experimental evidence for its function, the goals of this study were (i) to determine whether C_8-AHL signals through AinR to direct the predicted net decrease of transcription from the *qrr* promoter, (ii) to test the range of AHL pheromones to which AinR directs this response, and (iii) to measure the relative sensitivity of AinR for these cognate AHLs.

MATERIALS AND METHODS

Bacteria, growth media, and reagents. Bacterial strains are listed and briefly described in Table 1. *V. fischeri* ES114 was the wild-type strain used throughout (27). Plasmids were transformed into *Escherichia coli* strain DH5α (28) or, in the case of plasmids with the R6K origin of replication, into strain DH5αλ*pir* (29). *E. coli* was grown in LB medium (30), and *V. fischeri* was grown in LBS medium (31) or SWTO medium (32). Solid media were prepared with 15 mg ml^{-1} agar. For selection of *E. coli*, chloramphenicol (Cam) and kanamycin (Kan) were added to LB at final concentrations of 20 and 40 μg ml^{-1}, respectively. For selection of *V. fischeri* on LBS, Cam, erythromycin (Erm), and Kan were used at concentrations of 2, 5, and 100 μg ml^{-1}, respectively. $3OC_6$-AHL and C_8-AHL were obtained from Sigma-Aldrich (St. Louis, MO), and *N*-butanoyl (C_4)-AHL, *N*-3-hydroxy-butanoyl ($3OHC_4$)-AHL, *N*-hexanoyl (C_6)-AHL, *N*-heptanoyl (C_7)-AHL, *N*-3-hydroxy-octanoyl ($3OH\text{-}C_8$)-AHL, *N*-3-oxo-octanoyl ($3OC_8$)-AHL, *N*-decanoyl (C_{10})-AHL, *N*-dodecanoyl (C_{12})-AHL, *N*-tetradecanoyl (C_{14})-AHL, *N*-oxo-tetradecanoyl ($3OC_{14}$)-AHL, *N-cis*-tetradec-9Z-enoyl (*cis*-C_{14}-9Z-enoyl)-AHL, and *N*-octadecanoyl (C_{18})-AHL were obtained from Cayman Chemical (Ann Arbor, MI). Ethyl acetate-dissolved AHLs were added in defined amounts to flasks, and the solvent was evaporated overnight. AHLs were then redissolved to specific concentrations in SWTO medium and diluted as necessary.

Molecular genetics and sequence analyses. Plasmids were constructed using standard techniques and are described briefly in Table 1. DNA ligase and restriction enzymes were obtained from New England BioLabs (Beverly, MA). Oligonucleotides used for PCR and cloning are listed in Table 1 and were synthesized by Integrated DNA Technologies (Coralville, IA). PCR was conducted with Phusion high-fidelity DNA polymerase (Finnzymes, Finland) in an iCycler (Bio-Rad Laboratories, Hercules, CA). Plasmids used for cloning were isolated with the GenElute plasmid miniprep kit (Sigma-Aldrich, Inc., St. Louis, MO). DNA was repurified between cloning steps with the DNA Clean and Concentrator-5 kit (Zymo Research, Orange, CA). Cloned PCR products were sequenced at the University of Michigan DNA Sequencing Core Facility, and sequences were analyzed using the Lasergene Core suite (DNASTAR, Madison, WI).

To generate the P_{qrr}-*lacZ* transcriptional reporter plasmid pHK20, 450 bp upstream of *V. fischeri qrr* was PCR amplified using primers pr_HK25 and pr_HK26. The resulting amplicon was digested with SphI and NheI and cloned into the SphI and NheI sites in the promoterless-*lacZ* vector parent pAKD701 (33). To generate the *ainR*-containing shuttle vector pAS61, *ainR* was PCR amplified from ES114 using primers ASAinRF and ASAinRR, and the amplicon was digested with AvrII and ligated into XbaI-digested pVSV105.

Mutant construction. Mutant alleles were transferred from *E. coli* into *V. fischeri* on plasmids by triparental matings using the conjugative helper

TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence[a]	Source or reference
E. coli strains		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	34
DH5α	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	28
DH5αλ*pir*	DH5α lysogenized with λ*pir*	29
V. harveyi TL183	Δ*luxN* Δ*luxQ* Δ*cqsS*	B. Bassler
V. fischeri strains		
DC22	C_8-AHL bioreporter: ES114 Δ*ainS* Δ*luxR-luxI*, mutant *luxR* (MJ1 T33A R67M S116A M135I), P_{luxI}-*luxCDABEG*	D. Colton
ES114	Wild-type isolate from *E. scolopes*	27
JB18	ES114 *litR*::*ermR*	This study
JHK001	ES114 Δ*ainSR luxI*	This study
JHK003	ES114 Δ*ainR*	This study
JHK007	ES114 Δ*ainS* Δ*luxIR* P_{luxI}-*luxCDABEG*	This study
JHK008	ES114 Δ*ainR litR*::*ermR*	This study
JHK009	ES114 Δ*ainS luxI* Δ*luxQ*	This study
JHK010	ES114 Δ*ainSR* Δ*luxIR* P_{luxI}-*luxCDABEG*	This study
NL55	ES114 Δ*ainSR*	38
NL60	ES114 Δ*ainS*	38
NL63	ES114 Δ*ainS luxI*	This study
VCW2G7	ES114 *luxI* (frameshift mutation)	17
Plasmids[b]		
pAKD701	Promoterless *lacZ*, pES213, R6Kγ $oriT_{RP4}$, *kanR*	33
pAS61	*ainR* in pVSV105; pES213, R6Kγ $oriT_{RP4}$, *camR*	This study
pDC44	Δ*luxIR* P_{luxI}-*luxCDABEG* allele; ColE1 R6Kγ $oriT_{RP4}$, *camR*	D. Colton
pEVS104	Conjugative helper plasmid; R6Kγ $oriT_{RP4}$ *kanR*	34
pHK20	P_{qrr}-*lacZ* reporter in pAKD701; pES213, R6Kγ $oriT_{RP4}$ *kanR*	This study
pVCW2A6	*luxI* frameshift allele; ColE1 $oriT_{RP4}$ *camR*	17
pJLB123	P_{orf1}-*luxR* in pVSV104; pES213, $oriT_{RP4}$ R6Kγ *kanR*	32
pNL62	**Δ***ainS* allele; ColE1 R6Kγ $oriT_{RP4}$ *kanR*, *ermR*	N. Lyell
pTM268	P_{qrr1}-*gfp*, mCherry, pES213, R6Kγ $oriT_{RP4}$ *camR*	21
pVAR29	Δ*luxQ* allele; R6Kγ $oriT_{RP4}$ *camR ccdB araC*	26
pVAR62	Δ*ainR* allele; R6Kγ $oriT_{RP4}$ *camR ccdB araC*	26
pVSV104	pES213, R6Kγ $oriT_{RP4}$ *kanR lacZ*α	47
pVSV105	pES213, R6Kγ $oriT_{RP4}$ *camR lacZ*α	47
Oligonucleotides[c]		
ASAinRF	TACCTAGGATGTTAACTACTTTACCTAAAG	This study
ASAinRR	ATCCTAGGAATAAATTATCGAAGTAGCC	This study
pr_HK25	GGGGGCATGCGGCTTCTACTGCAGCATCAATTGA	This study
pr_HK26	GGGGGCTAGCAAAGGGTCAATATACCTATTGCAGGG	This study

[a] Drug resistance genes: *camR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*).
[b] All alleles cloned in this study are from *V. fischeri* strain ES114. The replication origin(s) of each vector is listed as R6Kγ, ColE1, and/or pES213. Plasmids based on pES213 are stable and do not require antibiotic selection for maintenance (47).
[c] All oligonucleotides are shown 5′ to 3′. Underlined regions are restriction enzyme recognition sites.

plasmid pEVS104 (34) in strain CC118λ*pir* (35). Recombinational insertion and marker exchange were identified by screening for antibiotic resistance, and putative mutants were tested by PCR. Marker exchange using plasmids pVAR29 and pVAR62 was facilitated by an arabinose-inducible toxin system on the vector sequence as previously described (26, 36). Resulting mutants are listed in Table 1. The *litR*::*ermR* mutant JB18 is phenotypically indistinguishable from the *litR*::*ermR* mutant JB19 (32) and was constructed in the same way except that the *ermR* cassette was in the opposite orientation. To construct the Δ*ainSR luxI* mutant JHK001, plasmid pVCW2A6 (17) bearing *luxI* with the frame-shifting 4-bp insertion allele originally on pHV200I$^-$ (37) was exchanged into the Δ*ainSR* strain NL55 (38). The Δ*ainS* allele in pNL62 was exchanged into VCW2G7 (*luxI*) to generate strain NL63. To add *luxR* mutations to NL63 (Δ*ainS luxI*) and JHK001 (Δ*ainSR luxI*), the Δ*luxRI*::P_{luxI}-*luxC* allele on pDC44 was exchanged into these parent strains to generate JHK007 and JHK010, respectively. To generate the Δ*ainS luxI* Δ*luxQ* mutant JHK009, the Δ*luxQ* allele on pVAR29 (26) was exchanged into NL63 (Δ*ainS luxI*). The Δ*ainR* allele in pVAR62 was exchanged into wild-type strain ES114 and JB18 (*litR*::*ermR*) to generate JHK003 and JHK008, respectively.

Luminescence and fluorescence measurements. Overnight *V. fischeri* cultures were diluted 1:1,000 in 25 ml of SWTO in 125-ml flasks and then incubated at 24°C with shaking (200 rpm). Samples (500 μl) were removed at various times, and optical density at 595 nm (OD_{595}) was measured with a BioPhotometer (Brinkman Instruments, Westbury, NY). Samples were aerated by rapid shaking, and relative luminescence was measured immediately with a Glomax TD-20/20 luminometer (Promega, Madison, WI) (3). Specific luminescence reported is the relative luminescence per OD_{595} unit.

Fluorescence expressed from the *gfp*-derived *qrr* reporter on plasmid pTM268 was measured with a Synergy 2 plate reader (Biotek, Winooski, VT). Overnight cultures were diluted 1:100 in clear-bottomed, black-walled microtiter plates containing SWTO, with or without AHL, and measured at regular intervals for green fluorescent protein (GFP) using an excitation/emission wavelength pair of 485 nm/540 nm. Green fluorescence from the P_{qrr}-*gfp* reporter on pTM268 was normalized against red fluorescence from the constitutive P_{tet}-mCherry gene, also on the plasmid, to arrive at the specific fluorescence reported (GFP/mCherry). The EC_{50}, defined as the AHL concentration at which the effect on the P_{qrr}-*gfp* reporter was half maximal, was determined based on curve fitting using SigmaPlot's "ligand binding" function (Systat Software, San Jose, CA).

Transcriptional *lacZ* reporter assays. *V. fischeri* strains harboring the P_{qrr}-*lacZ* transcriptional reporter plasmid pHK20 or the promoterless-*lacZ* vector parent pAKD701 were grown overnight in LBS and subcultured 1:300 into 24-well microtiter plates containing 1.5 ml fresh SWTO per well, with or without AHL, and incubated at 24°C with shaking at 200 rpm. Cells were collected at an OD_{595} of ~2.5 by centrifugation, the supernatant was discarded, and cell pellets were stored overnight at −80°C. β-Galactosidase assays were performed as previously described (3). EC_{50}s were determined with the P_{qrr}-*lacZ* reporter based on curve fitting using SigmaPlot's "ligand binding" function (Systat Software).

C_8-AHL bioassays. C_8-AHL accumulation was assessed for strains grown at 24°C with shaking in 125-ml flasks containing 15 ml of SWTO. The Δ*ainS* mutant NL60 was included as a negative control. At an OD_{595} of ~2.5, cells were pelleted, supernatants were filter sterilized, and an equal volume of acidified ethyl acetate (1:1,000 glacial acetic acid/ethyl acetate) was added. The mixture was incubated on a rotary shaker at 55 rpm for 30 min, 3 ml of the organic phase were removed and added to sterile glass beakers, the ethyl acetate was evaporated, and extracts were dissolved in 3 ml SWTO. A 200-μl portion of extract dissolved in SWTO was added to a 96-well microplate. Wells were then inoculated with the C_8-AHL bioreporter strain DC22, which lacks the *luxI* and *ainS* pheromone synthase genes and in which expression of *luxCDABEG* is activated by a mutant LuxR ($LuxR_{MJ1}$ T33A R67M S116A M135I) that is responsive to C_8-AHL but not to $3OC_6$-AHL (39). C_8-AHL concentration was determined by measuring the luminescence of DC22 in a Synergy 2 plate reader (BioTek) and comparing it to that of C_8-AHL standards.

RESULTS

To begin testing the prediction that *V. fischeri* AinR functions similarly to LuxN from *V. harveyi*, we first complemented a *V. harveyi luxN* mutant with *ainR*. We used *V. harveyi* strain TL183 (Δ*luxN* Δ*luxQ* Δ*cqsS*), which has mutations in all three known pheromone receptors that act on LuxU in this bacterium, to minimize any chance of AinR-independent signaling in the transconjugants. The introduction of *V. fischeri ainR* led to decreased luminescence in this Δ*luxN* (and Δ*luxQ* Δ*cqsS*) *V. harveyi* mutant, but brighter luminescence was restored by adding C_8-AHL (Fig. 2). The parental vector lacking *ainR* did not have these effects. Thus, *ainR* appeared to functionally replace *luxN* in *V. harveyi* but responded to the C_8-AHL signal generated by AinS in *V. fischeri*.

To more directly test whether AinR has the predicted effect on transcription of *qrr* in *V. fischeri* (Fig. 1), we generated a P_{qrr}-*lacZ* transcriptional reporter using the sequence upstream of *qrr* (also called *qrr1*) from *V. fischeri* as the source of the promoter and assayed its activity under different conditions. Experiments were performed in a genetic background lacking both *luxI* and *ainS*, to eliminate endogenous AHL production and allow us to control which potential signals were present. We found that in the absence of AHL, the reporter showed lower activity in the *ainR* mutant, and that in the presence of *ainR*, adding 1 μM C_8-AHL, $3OC_6$-AHL, or both lowered P_{qrr}-*lacZ* reporter activity (Fig. 3A). Providing *ainR in trans* to the Δ*ainR* mutant restored higher levels of P_{qrr}-*lacZ* reporter expression in the absence of AHL, and this effect could be reversed by the addition of C_8-AHL (Fig. 3B). However, as discussed below, when *ainR* was added in *trans* to the Δ*ainR* mutant, the effect of $3OC_6$-AHL on the *qrr* reporter was not fully complemented (Fig. 3B).

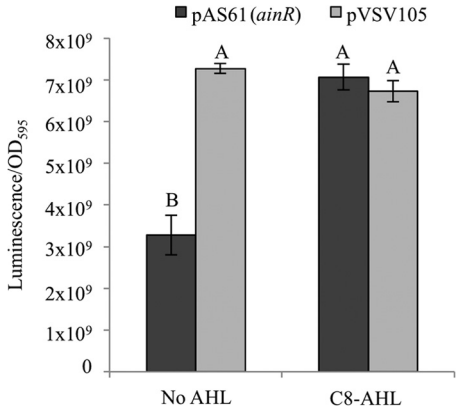

FIG 2 Complementation of a *V. harveyi luxN* mutant with *ainR*. Peak specific luminescence of the *V. harveyi* pheromone sensor mutant TL183 (Δ*cqsS* Δ*luxN* Δ*luxQ*), grown in SWTO, with or without 1 μM C_8-AHL, and either bearing *ainR* from *V. fischeri* on plasmid pAS61 (dark gray) or carrying the parental shuttle vector pVSV105 (light gray) as a control is shown. Peak specific luminescence was observed at an OD_{595} of ~1. Uppercase letters shared between bars indicate no statistically significant difference ($P > 0.5$), whereas different letters indicate significant difference ($P < 0.0005$), based on a one-way analysis of variance (ANOVA) and *post hoc* testing using Tukey's honestly significant difference test. Data are from one representative experiment of three, each with three biological replicates. Error bars represent standard errors ($n = 3$).

The *ainR*- and AHL-dependent effects on the *qrr* reporter were reproducible and statistically significant ($P < 0.02$), but they represented relatively modest 2- to 3-fold changes in LacZ activity. We considered the possibility that AI-2-mediated signaling through LuxQ might dampen or obscure AinR-mediated effects on *qrr*, because LuxQ- and AinR-mediated signaling are thought to converge at LuxU. However, we found no difference in the magnitude of such AHL-driven control of the P_{qrr}-*lacZ* reporter in a Δ*luxQ* mutant background (Fig. 3C). Thus, for our purpose of detecting *ainR*-dependent changes in *qrr*, there seemed to be no advantage in introducing the Δ*luxQ* allele.

The data above are consistent with the model of AinR function presented in Fig. 1, but the 1 μM C_8-AHL used was at the upper end of what might be considered physiologically relevant. We therefore tested the response of the P_{qrr}-*gfp* reporter on pTM268 to different doses of C_8-AHL. C_8-AHL concentrations from 1 μM to 50 pM were sufficient to decrease P_{qrr}-*gfp* activity, while concentrations below 50 pM were not significantly different than the negative control lacking added AHL (Fig. 4). The EC_{50} for C_8-AHL, defined as the concentration at which its effect on P_{qrr}-*gfp* was half maximal, was estimated at 140 pM for the data in Fig. 4. Similar EC_{50}s were determined in repetitions of this experiment.

Having established *ainR*-dependent effects on P_{qrr}-*lacZ* by both the C_8-AHL and $3OC_6$-AHL pheromones produced by *V. fischeri*, we next tested a variety of other AHL pheromones in this assay. AHLs with acyl chain lengths deviating from C_8-AHL by ±2 carbons, regardless of substitution at the third carbon of the acyl chain (unsubstituted or oxo- or hydroxy-substituted), signifi-

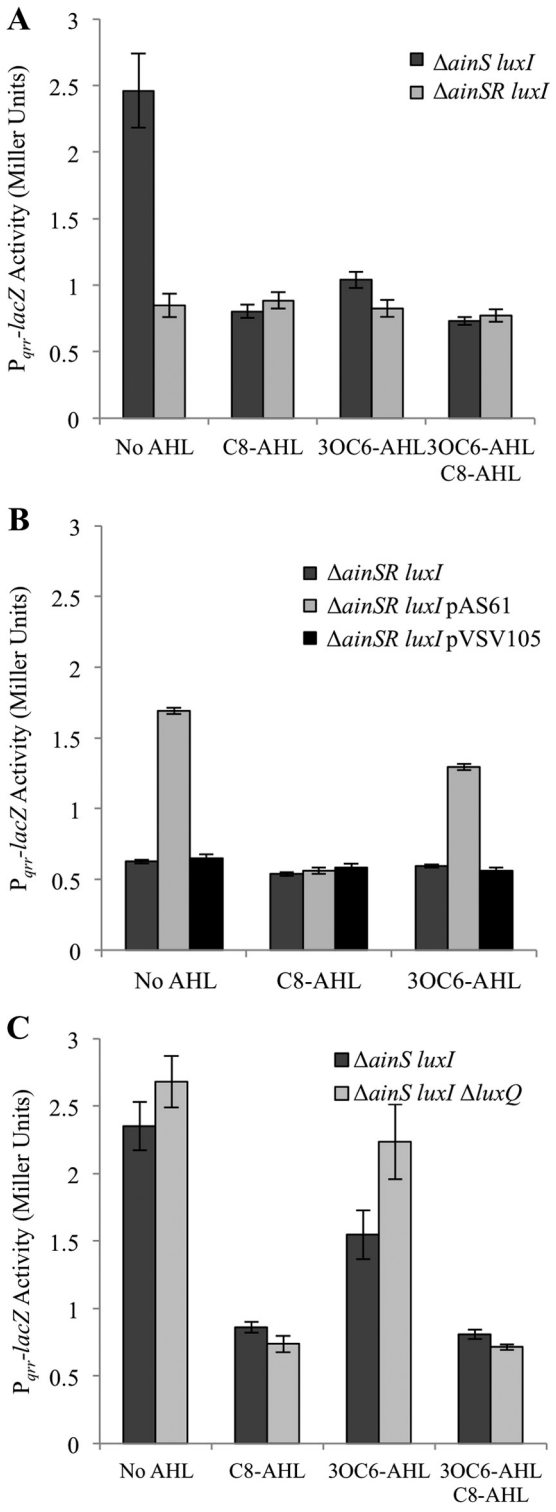

FIG 3 *ainR*-dependent and AHL-dependent effects on a *qrr* transcriptional reporter. Each panel shows β-galactosidase activity expressed from the P$_{qrr}$-*lacZ* transcriptional reporter in cultures grown on SWTO with a 1 μM concentration of each AHL indicated. (A) Reporter activity in mutants NL63 (Δ*ainS luxI*) or JHK001 (Δ*ainSR luxI*). (B) Reporter activity in JHK001 (Δ*ainSR luxI*) with or without pAS61 (*ainR*) or its empty parent vector pVSV105. (C) Reporter activity in NL63 (Δ*ainS luxI*) or JHK009 (Δ*ainS luxI* Δ*luxQ*) in response to different AHL treatments. Data in each panel are from a single representative from among three independent experiments, and bars represent standard errors (A, $n = 4$; B and C, $n = 3$).

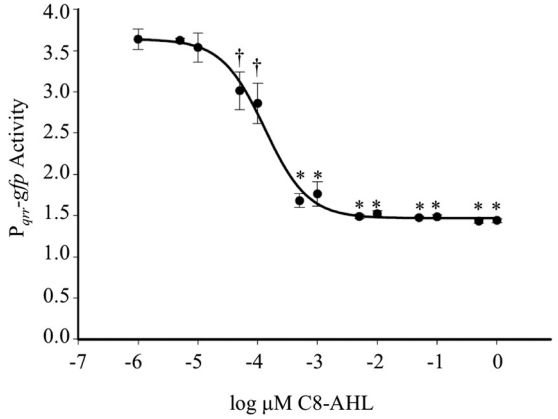

FIG 4 Effect of C_8-AHL dose on *qrr* transcriptional reporter. The AHL pheromone synthase mutant NL63 (Δ*ainS luxI*) harboring the P$_{qrr}$-*gfp* reporter plasmid pTM268 was exposed to C_8-AHL concentrations ranging from 0 μM to 1 μM. GFP expression was normalized to the red fluorescence expressed from a constitutive mCherry gene on the same plasmid. Curve fitting and associated EC_{50} calculation were performed using SigmaPlot (EC_{50} = 140 pM; $R^2 = 0.9582$). Statistically significant decreases in reporter activity are indicated by daggers ($P < 0.05$) and asterisks ($P < 0.01$).

cantly depressed reporter activity in an *ainR*-dependent manner (Fig. 5) ($P < 0.01$). AHLs with 4-carbon acyl chains or with acyl chains 12 carbons or longer did not affect reporter activity, regardless of substitution.

Although C_6-AHL, 3OC_6-AHL, C_7-AHL, 3OHC_8-AHL, and C_{10}-AHL each displayed *ainR*-dependent depression of P$_{qrr}$-lacZ activity, for the results shown in Fig. 5, these AHLs had been tested at the relatively high concentration of 1 μM. By testing the reporter response as a function of AHL dose, over a series of serial 10-fold dilutions, we found that C_6-AHL and C_{10}-AHL were the least active, eliciting a response only at 1 μM (Table 2). 3OC_6-AHL was the next weakest in activity and significantly affected the reporter only down to 100 nM (Table 2). C_7-AHL was active at 10 nM, while 3OC_8-AHL and 3OHC_8-AHL were active down to 1 nM (Table 2). The EC_{50}s for these AHLs followed similar trends, although by this measure 3OHC_8-AHL was more active than 3OC_8-AHL (Table 2). None of these AHLs was as active as C_8-AHL in this reporter system; however, those with the chain lengths most similar to that of C_8-AHL were closest in activity.

We next tested whether any of the noninducing or relatively weakly inducing AHLs could interfere with AinR-dependent C_8-AHL-mediated signaling, as is the case in some other AHL-dependent PS systems. In these experiments we tested the effect of adding other AHLs along with 1 nM C_8-AHL, a relatively low C_8-AHL concentration that nonetheless influenced the *qrr* reporter (Fig. 4). Under these conditions, adding a 1 μM concentration of the noninducing AHLs, which included C_4-AHL, 3OHC_4-AHL, C_{12}-AHL, 3OC_{14}-AHL, *cis*-C_{14}-9Z-enoyl-AHL, and C_{18}-AHL, had no discernible effect on C_8-AHL-mediated signaling (data not shown). Thus, even in a 1,000-fold molar excess, these noninducers did not interfere with signaling by the cognate pheromone C_8-AHL. For relatively weakly inducing AHLs, including C_6-AHL, 3OC_6-AHL, C_7-AHL, 3OHC_8-AHL, and C_{10}-AHL, we added different concentrations both at and just below the threshold where they individually displayed an effect on the P$_{qrr}$-*lacZ* reporter (Table 2). We reasoned that these AHLs probably bind AinR at or below

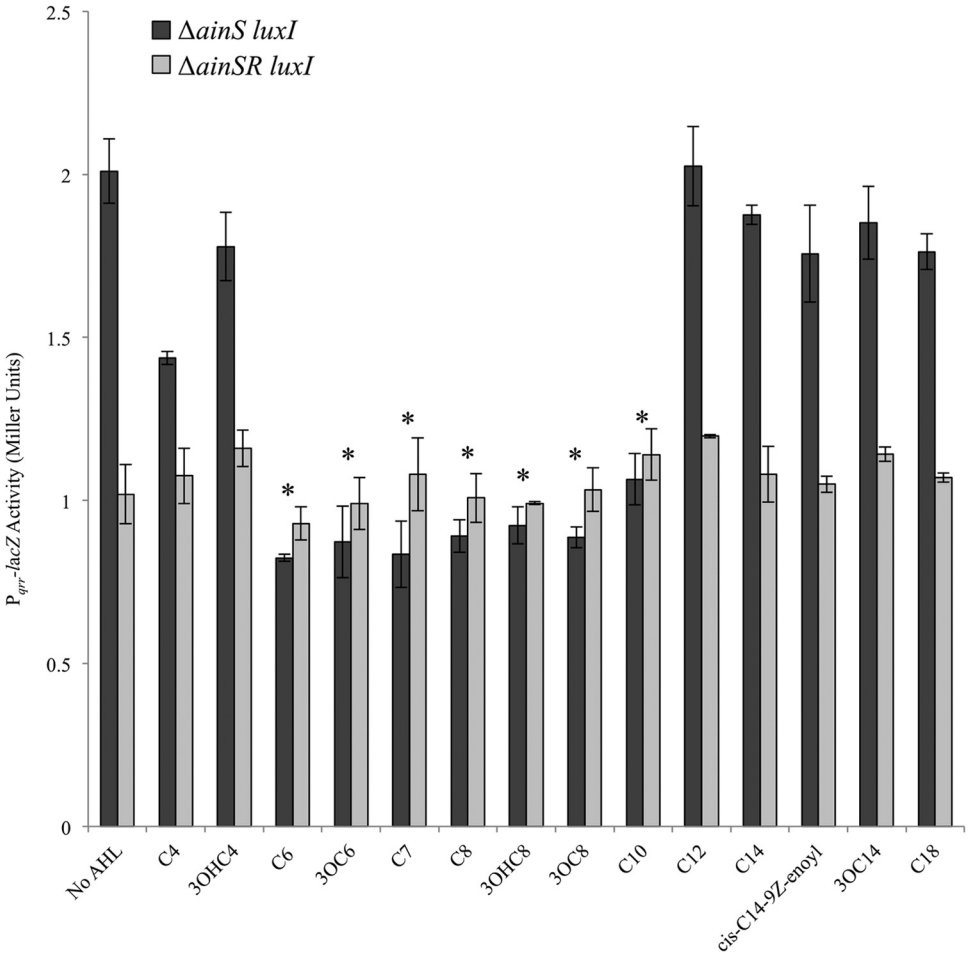

FIG 5 Effects of a variety of AHL molecules on *ainR*-dependent signaling. β-Galactosidase activity expressed from the P$_{qrr}$-*lacZ* transcriptional reporter on pHK20, harbored by NL63 (Δ*ainS luxI*) or JHK001 (Δ*ainSR luxI*), is shown. Cultures were grown in SWTO with 1 μM dissolved AHL. Data from one representative experiment of three independent experiments are shown. Asterisks indicate statistically significantly lower activity compared to the "No AHL" treatment as determined by Student's *t* test ($P < 0.01$). Error bars represent standard errors ($n = 3$).

the concentration at which they detectably affect the *qrr* reporter, and if they bind AinR well but are poor at influencing AinR's kinase activity, they might effectively interfere with C_8-AHL signaling at concentrations where they themselves have a modest or undetectable effect on the *qrr* reporter. However, we again saw no inhibition of C_8-AHL-mediated signaling (data not shown).

TABLE 2 Sensitivity of *ainR*-dependent effects on the P$_{qrr}$-*lacZ* reporter to different AHL molecules

AHL	AHL concn (nM)	
	EC_{50}[a]	Minimum stimulatory dose[b]
C_6-AHL	114	1,000
$3OC_6$-AHL	29	100
C_7-AHL	11	10
$3OC_8$-AHL	8	1
$3OHC_8$-AHL	0.8	1
C_{10}-AHL	476	1,000

[a] EC_{50}s were generated from dose-response assays using NL63 (Δ*ainS luxI*) harboring the P$_{qrr}$-*lacZ* reporter pHK20 grown in SWTO. AHL was supplemented in 10-fold steps over a range of 1 μM to 1 pM. Data are from a single representative experiment of three independent experiments.
[b] Lowest tested AHL concentration that elicited a significantly lower ($P < 0.05$) P$_{qrr}$-*lacZ* activity than the treatment lacking AHL.

The results above show an *ainR*-dependent effect of a variety of AHLs on P$_{qrr}$-*lacZ* expression, and we speculated that our data reflected AinR acting as a receptor for these AHLs. We were especially intrigued that AinR might recognize the LuxI product, $3OC_6$-AHL; however, the assays described above were done in genetic backgrounds that retained the AHL-dependent activator LuxR, which is the cognate $3OC_6$-AHL receptor. We therefore tested all the AHLs that were active in our assays (Fig. 5 and Table 2) in a *luxR* mutant background. A 1 μM concentration of each of these AHLs was again sufficient to decrease *qrr* reporter activity in the presence of *luxR* (Fig. 6A, dark bars). However, deletion of *luxR* eliminated the effect of $3OC_6$-AHL on *qrr* reporter activity, while the effects of all other AHLs were the same regardless of the presence or absence of *luxR* (Fig. 6A, light bars; also data not shown). Complementing the *luxR* mutant with *luxR in trans* on pJLB123 restored $3OC_6$-AHL-dependent reduction of P$_{qrr}$ reporter activity, whereas the vector alone did not (data not shown). Due to the shared antibiotic resistance markers of pJLB123 (*luxR*), pVSV104 (parent of pJLB123), and the P$_{qrr}$-*lacZ* transcriptional

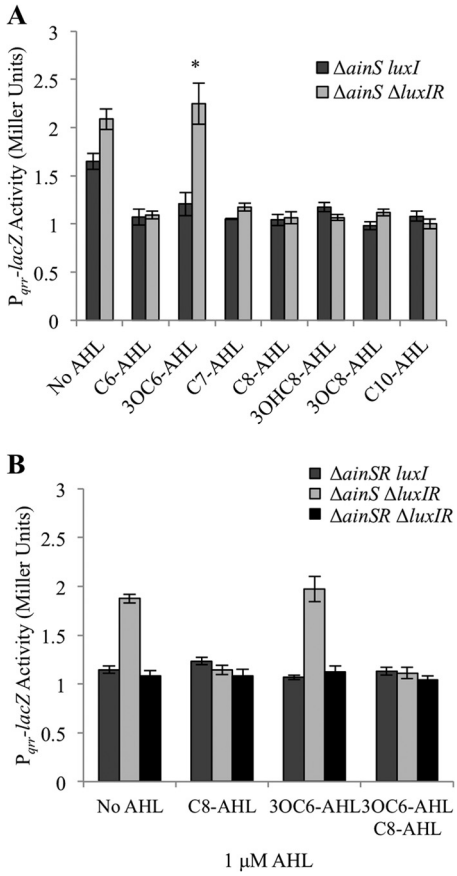

FIG 6 The effect of $3OC_6$-AHL on P$_{qrr}$-*lacZ* activity is dependent on *luxR*. β-Galactosidase activity expressed from the P$_{qrr}$-*lacZ* reporter on pHK20, from cultures grown in SWTO with a 1 μM concentration of each AHL, is indicated. Data in each panel are from a single representative experiment of three independent experiments, and error bars represent standard errors ($n = 3$). (A) Reporter activity in NL63 (Δ*ainS luxI*) and JHK007 (Δ*ainS* Δ*luxIR*). The asterisk indicates a statistically significant difference between the two strains ($P < 0.02$). (B) Reporter activity in mutants JHK001 (Δ*ainSR luxI*), JHK007 (Δ*ainS* Δ*luxIR*), and JHK010 (Δ*ainSR* Δ*luxIR*).

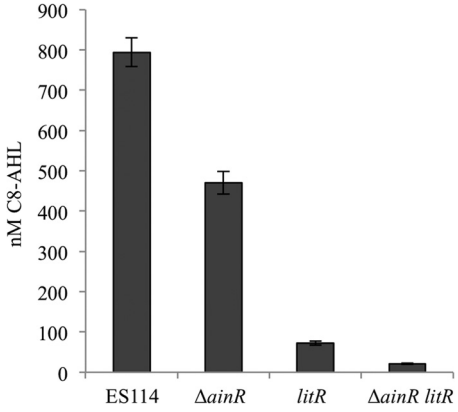

FIG 7 C_8-AHL accumulation is reduced in Δ*ainR* mutants. C_8-AHL was extracted and its concentration estimated from cultures of ES114, the Δ*ainR* mutant JHK003, the *litR* mutant JB18, or the *litR* Δ*ainR* mutant JHK008. The C_8-AHL synthase mutant NL60 (Δ*ainS*) served as a negative control and had no detectable activity (data not shown). Cultures were grown to an OD_{595} of ~2.5 in SWTO, and C_8-AHL levels were determined by bioassays of ethyl acetate-extracted cultures. Error bars indicate standard errors ($n = 3$).

reporter plasmid pHK20, which was used for Fig. 6A, for complementation we used the P$_{qrr}$-*gfp* reporter pTM268, which was used in generating the data for Fig. 4 with results similar to those obtained with the P$_{qrr}$-*lacZ* reporter.

We were curious whether $3OC_6$-AHL-LuxR may act on *qrr* independently of a $3OC_6$-AHL-AinR-dependent effect (Fig. 3A and B). If there were two different mechanisms by which $3OC_6$-AHL affected *qrr*, one that is AinR dependent and another that is LuxR dependent, then we might find an additive reduction in *qrr* reporter activity if the *luxR* and *ainR* mutations were combined in a single strain. However, this was not the case, as deletion of both *ainR* and *luxR* resulted in reporter activity that was not significantly different ($P > 0.05$) from that of the *ainR* mutant (Fig. 6B). Thus, these data are consistent with AinR and LuxR acting in the same pathway to affect *qrr* in response to $3OC_6$-AHL.

Finally, we sought to investigate why the Δ*ainR* mutation leads to dimmer luminescence (26), when (i) the opposite might reasonably be predicted by the model in Fig. 1, (ii) our data above support the predicted relationship between *ainR* and *qrr*, including lower *qrr* transcription in the *ainR* mutant, and (iii) Miyashiro et al. already confirmed that loss of *qrr* results in brighter (not dimmer) luminescence (21). Ray and Visick (26) observed that the Δ*ainR* mutation did not affect luminescence when excess C_8-AHL was added, and we therefore tested C_8-AHL accumulation by Δ*ainR* mutants. Evidence suggests a LitR-mediated positive-feedback mechanism for the AinS/AinR system (8), so we tested C_8-AHL accumulation in both wild-type and *litR* mutant backgrounds. Consistent with the previous report that a transcriptional reporter showed LitR activation of *ainSR* (8), C_8-AHL levels were reduced in a *litR* mutant (Fig. 7). More importantly, we found that an in-frame deletion of *ainR* modestly but significantly ($P < 0.05$) reduced C_8-AHL accumulation in the wild-type and *litR* mutant backgrounds (Fig. 7). As discussed further below, these results, together with the C_8-AHL amendment experiment of Ray and Visick (26), suggest that the Δ*ainR* mutation results in dimness because this allele causes decreased C_8-AHL levels.

DISCUSSION

Many bacteria possess multiple PS systems, which may allow distinct population density-dependent regulons or hierarchical activation of pheromone-dependent phenotypes. *V. fischeri* possesses three PS systems, and it primarily uses two distinct and structurally unrelated AHL-dependent systems, LuxI/LuxR and AinS/AinR, to control the induction of luminescence in its mutualistic light-organ symbiosis with the squid *Euprymna scolopes*. While LuxI, LuxR, and AinS have been studied in some detail, the role of AinR has been largely speculative, based on its homology to the *V. harveyi* AHL receptor LuxN. Unlike *V. fischeri*, *V. harveyi* is not known to enter monospecies light organ symbioses, it lacks LuxI/LuxR homologs, and it contains only one AHL system. Given that the activities of AinR and LuxN may be shaped differently by the distinct lifestyles and PS circuitry of these bacteria, we investigated the function of AinR. Our results indicate that AinR does mediate control over the Qrr regulatory RNA in *V. fischeri*, as previously predicted (Fig. 1). Thus, the unexpected luminescence phenotype of a Δ*ainR* mutant does not reflect a fundamental difference in AinR's placement in the PS regulatory circuitry. Interestingly, we found that AinR-dependent control responds to a range of AHLs

and is exquisitely sensitive to C_8-AHL, and we discovered a new regulatory connection between the Lux and Ain systems.

High sensitivity of AinR toward C_8-AHL. The sensitivity of AinR to C_8-AHL is unusual among characterized AHL sensor proteins. The *ainR*-dependent effect on a P_{qrr} reporter was half-maximal (EC_{50}) at ~140 pM, while concentrations approaching 50 pM were still sufficient to decrease reporter activity (Fig. 4). TraR from *Agrobacterium tumefaciens* can elicit responses to similar subnanomolar levels of AHL, at least when overexpressed (40, 41), but sensitivity in the 10 nM range or higher appears to be more common for AHL receptors, including LuxR's response to $3OC_6$-AHL (42). In *V. harveyi*, the EC_{50} for a LuxN-dependent response to its cognate pheromone AI-1 was 23 nM (23), although the response measured was downstream of Qrr, rather than a *qrr* reporter itself as we used. Thus, the difference in EC_{50} may reflect the phenotype and/or its position in the PS circuitry. However, the two studies suggest that AinR is a hundredfold more sensitive to its cognate pheromone than is LuxN. Interestingly, Swem et al. showed that a S184N mutation made LuxN more sensitive to AI-1, reducing the EC_{50} for AI-1 to 11 nM (23), and an alignment of LuxN and AinR indicated that the native AinR has an N at that residue.

Relatively low specificity for C_8-AHL. The high sensitivity of AinR toward C_8-AHL may come at a cost of low specificity. We found that AHLs with substituted or unsubstituted acyl chains of 6 to 10 carbons affected P_{qrr} reporter activity (Fig. 5), and inactivity was seen only with acyl chains of four carbons or more than 12 carbons. In general, the further the deviation from the C_8 chain length, the less effective the AHL in our assay (Table 2). Although C_8-AHL was the most active pheromone in our assays, other AHLs with seven or eight carbons showed activity at low concentrations, in the 1 to 10 nM range. In comparison, LuxR seems more selective and less sensitive than AinR (18). LuxN is also regarded as selective for AI-1, and its sensitivity and selectivity for other AHLs should be of interest, affording an opportunity for comparison with AinR.

Alternative models to explain the evolution of AinR and its properties. It is interesting to consider what selective pressure(s) might have driven AinR evolution toward high sensitivity and low specificity. Although LuxI/LuxR appears important for persistence within the host, initial activation of luminescence and other colonization factors is under the control of AinS and C_8-AHL (17). These observations led Lupp et al. to propose a model of sequential PS systems, with Ain functioning at moderate cell densities and jump starting the Lux system to function at higher densities (17). In this model, AinR could serve as a sentinel receptor, and accordingly it may have evolved a high sensitivity to detect small amounts of C_8-AHL as cells approach moderate density. Other traits of AinR, including the relatively broad range of signals detected and the surprising lack of apparent inhibition by noncognate AHLs, may be a coincidental consequence of evolution toward high sensitivity.

Alternatively, AinR might have evolved to detect a broad array of AHLs, including those from other bacteria. Such signaling could be important in environments for *V. fischeri* outside the light organ, where AinR may serve as a multisignal receptor, funneling information about the bacterial community into the PS network. This hypothesis is consistent with the current thinking that the Ain system converges with the widespread bacterial signal AI-2. The AI-2 and Ain systems control the LitR regulon, which is distinct from the LuxR regulon, providing an opportunity for distinct responses by *V. fischeri* either to a community or to itself. On the other hand, LuxN also converges with AI-2 signaling in *V. harveyi*, yet LuxN is generally thought of as a self-specific receptor. Moreover, in *V. fischeri* there is significant cross talk between the Lux and Ain systems, which does not suggest such distinct functions. AinR has evolved rapidly between *V. fischeri* strains (43), and we may gain insight into the selective pressures that have shaped it by evaluating a reconstructed ancestral AinR or AinR in isolates from environments with no known light organ hosts.

Cross talk between Lux and Ain. One motivation behind this study was the potential to discover new interconnections between Lux and Ain. C_8-AHL/LuxR cross talk had been established, but reciprocal signaling between LuxI-synthesized $3OC_6$-AHL and AinR would further entwine these systems. We initially suspected that AinR detected $3OC_6$-AHL, because $3OC_6$-AHL led to decreased P_{qrr} reporter activity in an *ainR*-dependent manner (Fig. 3). However, we subsequently found that this effect was also dependent on *luxR*, which encodes the $3OC_6$-AHL receptor (Fig. 6). Although we cannot rule out perception of $3OC_6$-AHL by AinR, based on our data we speculate that $3OC_6$-AHL-LuxR mediates repression of P_{qrr} transcription in a manner that requires the presence of *ainR*.

The mechanism underlying this additional layer of PS cross talk is unknown, but it could involve direct or indirect LuxR-mediated regulation of *ainSR*. Such a mechanism would be consistent with our observation that deleting both *ainR* and *luxR* did not additively reduce P_{qrr} activity (Fig. 6). One simple model would be that $3OC_6$-AHL-LuxR acts as a repressor of the *ainSR* promoter. Although LuxR is best known as a transcriptional activator, it can act as a repressor (44). Moreover, Gilson et al. identified a putative *lux* box binding site for LuxR upstream of *ainS* and suggested that it overlaps a −35 promoter element (45), consistent with LuxR acting as a repressor. Subsequently, Antunes et al. did not identify *ainSR* as a member of the LuxR regulon during a screen for up- and downregulation in response to $3OC_6$-AHL addition (46); however, unlike the experiments of Antunes et al., our study was conducted in a *luxI ainS* mutant background lacking endogenous AHL production, which might reveal effects on *ainSR* regulation that would be obscured by C_8-AHL and positive feedback in the Ain system. It may be relevant to note that when a plasmid-borne copy of *ainR* was provided in *trans* to the Δ*ainR* mutant, the effect of C_8-AHL on the *qrr* reporter was complemented fully, while the effect of $3OC_6$-AHL was not (Fig. 3B). Perhaps when *ainR* is expressed from a nonnative promoter in multicopy, repression of *ain* by $3OC_6$-AHL-LuxR is lost. Alternative mechanisms are possible, too, including $3OC_6$-AHL-LuxR-mediated repression of *luxU* or *luxO*, the effects of which would be dependent on the presence of AinR to initiate the cascade that phosphorylates LuxU and LuxO.

Insight into the dim luminescence of the Δ*ainR* mutant. Although it was not the main focus of our study, our results help explain the apparently paradoxical observation that *luxN* and *ainR* mutants in *V. harveyi* and *V. fischeri*, respectively, have similar effects on Qrr but opposite effects on luminescence. The luminescence defect in an *ainR* strain was rescued by adding C_8-AHL (26), and we have now shown that the Δ*ainR* allele results in decreased C_8-AHL output, in either wild-type or *litR* mutant backgrounds. Consistent with the idea that the Δ*ainR* allele causes decreased luminescence because of lowered C_8-AHL levels, in an

ainS mutant background lacking C_8-AHL, an additional *ainR* mutation has the effect predicted in Fig. 1 of enhancing luminescence (data not shown). The reason C_8-AHL affects luminescence even without its AinR receptor probably relates to LuxR and its activation by C_8-AHL. Ultimately, bioluminescence in *V. fischeri* is activated by AHL-LuxR, and previous studies overwhelmingly suggest that in strain ES114, C_8-AHL drives LuxR-dependent activation, especially in broth cultures (5, 16, 17, 19). Although C_8-AHL is a weaker activator of LuxR, in ES114 it accumulates to much higher levels than does $3OC_6$-AHL in broth culture (2). In *V. harveyi*, the homolog of *V. fischeri* LitR is the direct regulator of luminescence, and there is no analogous additional layer of AHL-dependent regulation. It seems likely that the dimness of the Δ*ainR* mutant is due to a lack of C_8-AHL-LuxR activation of the *lux* genes, a mechanism for which there is no parallel in a *V. harveyi luxN* mutant.

Although a mechanism awaits investigation, we speculate that the in-frame deletion of *ainR* may destabilize the *ainS* message. The *ainS* and *ainR* genes are adjacent on the same strand and separated by 11 bp, with no obvious terminator between them, suggesting that they may be transcriptionally linked, resulting in one polycistronic mRNA. Moreover, the lack of *ainR* does not result in dimmer luminescence when either *ainS* or *ainR* is expressed ectopically from a plasmid, so this effect is evident only when the two are in *cis* (data not shown). Interestingly, there is a large inverted repeat within *ainR*, which we further speculate may be responsible for stabilizing the *ainS* portion of the transcript, perhaps by blocking 3′-5′ exonuclease digestion. If this is correct, such a mechanism could allow cotranscription of *ainSR* but subsequently change the stoichiometry of the partners favoring the AinS synthase over the AinR receptor.

Areas for future research. Although AinR fits in a regulatory circuit similar to that of LuxN, it seems that as an interface between the AHL signal and LuxU there may be significant differences between LuxN and AinR. Other researchers have made great strides in dissecting the structure and function of LuxN, and we believe that comparative analyses including AinR will be enlightening. Similarly, we are intrigued by the divergence of AinR between *V. fischeri* isolates from different environments (43), and further investigation could elucidate selective pressures on AinS/AinR for *V. fischeri* in different hosts. Finally, we are interested in the regulatory control of AinS/AinR expression, and our results have opened new research avenues, particularly with respect to our discovery of LuxR-mediated regulation and the potential for posttranscriptional control.

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