CONTROL OF THE AINS/AINR PHEROMONE-SIGNALING SYSTEM IN VIBRIO FISCHERI ES114

by

JOHN HENRY KIMBROUGH

(Under the Direction of Eric V. Stabb)

ABSTRACT

Many bacteria use small pheromone molecules to regulate group behaviors. The marine bacterium *Vibrio fischeri* uses two distinct but integrated acyl-homoserine lactone (AHL) pheromone-signaling systems (PSS) to regulate behaviors during colonization of its host squid *Euprymna scolopes*, including induction of symbiotic bioluminescence. One *V. fischeri* PSS, consisting of LuxI and LuxR, was the first AHL-based system discovered and remains the archetype for similar AHL signaling found broadly throughout the phylum *Proteobacteria*. However, LuxI/LuxR itself is regulated along with several other genes by a second structurally unrelated AHL-based PSS that is specific to the *Vibrionaceae* family. This PSS, which is underpinned by AinS and AinR, is responsible for regulating behaviors associated with initiating the symbiosis with *E. scolopes*, and it represents part of a conserved "core" PSS within the *Vibrionaceae*. Despite its importance in *V. fischeri*, many aspects of AinS function are not well-understood. In this dissertation, I describe aspects of AinS regulation, AinR's role in signal perception, and previously unknown mechanisms of cross-talk between AinS/AinR and LuxI/LuxR. I provide evidence that AinR has high sensitivity and low selectivity for a range of AHL pheromones. Additionally, I show that

the presence of *ainR in cis* affects regulation of *ainS*, which appears related to a large inverted repeat in *ainR*. I also describe how spontaneous mutations affecting a PSS regulator downstream of AinS/AinR, LuxO, result in decreased *ainSR* expression and increased survivability when *V. fischeri* is grown in static culture. Finally, I explore additional levels of cross-talk between the Ain and Lux PSSs. Together, these results show how previously unknown connections between AinS/AinR, LuxI/LuxR, and the core *Vibrio* PSS finely tune regulation of symbiotic factors in response to changing environmental conditions. This work extends our knowledge of bacterial communication and its role in host-microbe and microbe-microbe interactions.

INDEX WORDS: *Aliivibrio*, *Photobacterium*, quorum sensing, *ain*

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DEDICATION

To 16-year old Hank, for making the best decision you could have ever possibly made.

ACKNOWLEDGEMENTS

"Look on my works, ye Mighty…"

In retrospect, I probably should have been an actuary. I spend most of my time thinking about risk, mostly how to minimize it. Whether the risk the Microbiology Department and the Graduate School took accepting me, or that my boss took when he let me join the lab, or that I took moving 900 miles to a cultural bizzaro-world was a successful one will hopefully be determined by the end of this story. My estimation of my own abilities is always skewed in one direction or the other, so I leave it to you, dear reader, to judge me as best as your abilities will allow you.

My favorite part of writing is working on the discussion section, for two reasons. First, it's generally the final section to assemble, which means you're almost done. Second, it offers a kind of catharsis, allowing you to step back and look at the work you've done and realize you haven't just been spinning your wheels for the last year(s). This is exceptionally important, from a professional and personal/psychological standpoint. The discussion allows for some speculation, some hand-waving, and some interpretation of the facts you have before you. Writing the discussion before the work is done is pointless, because it will change, in spite of how smart you think yourself to be. In this discussion, I could have tried to condense the last five years into a Greatest Hits of the people and experiences that have shaped me, personally and professionally, into the person I am now. But this would have be pointless, because the work isn't done.

I iterated this section about a dozen ways in my head before laying finger to key. I debated using an introspective approach versus a glib one, sweeping versus succinct. At one point I considered making a table with the names of people and their individual relative acknowledgement quotient (IRAQ). IRAQ would have measured a person's impact on me over the last five years and attempted to distill circumstances and events into a single number used to determine the success or failure of my graduate career. However, developing a system to measure such contributions quickly got out of hand. Some of the most negative experiences drove me to work harder; some positive ones held me back. Bad approaches rarely reveal themselves before it's too late, and IRAQ was relegated to the freezer to sit among other dead ends and wait for a new Hesiodic player to open the box.

Some people use this section to name names or dust off their Bartlett's or wax philosophical, but I that's not what I wanted to do. The people who helped get me to this point know who they are; they know what they've done for me. I've measured out my life with Petri dishes, but most graduate students won't because it's too difficult. The reasons we do what we do are often less noble than we would like to admit to anyone, ourselves included. I've tried to keep my life in perspective. I've grown up and grown old here. I've tried to be honest with myself, tried to do the best I could with what I had. Whether or not that was a successful risk remains to be seen.

If this section has made sense to you, you're lying. It only makes a bit of sense to me, but it's my catharsis all the same. In reality, though, it's not even mine. There are only two things in this world a man truly owns, and this dissertation isn't one of them. It belongs to the lab, the Department, the National Science Foundation, Science, and Nature.

But most importantly it belongs to you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview of pheromone signaling in *V. fischeri*

Bacteria employ diverse strategies to carve out ecological niches and increase their fitness. Some behaviors represent large resource sinks for individual cells but provide a fitness benefit for a population under specific conditions. Regulation of such energy-intensive group behaviors is often accomplished through the use of bacterial pheromones, which are small diffusible molecules that mediate cell-cell signaling (1-3). Bacterial pheromone-regulated behaviors have traditionally been couched in terms of response to high cell density, leading the process of pheromone signaling to be called quorum sensing (QS) (4). While cell density appears to be a key unifying characteristic of such pheromone-signaling systems (PSS) (reviewed in 5), there is growing appreciation that high cell density is necessary but not always sufficient to induce pheromone-regulated behaviors (6, 7). Beyond the simple QS model, biotic and abiotic factors often influence regulation of the signaling system and/or synthesis of the signal itself (8). Additionally, a wide variety of bacteria possess multiple independent PSSs suggesting functional roles beyondQS and underscoring the importance of finely tuned regulation of pheromone-mediated behaviors (9-11).

The marine γ-proteobacterium *Vibrio fischeri* was one of the first bacteria discovered to use pheromones in controlling a density-dependent group behavior, in this

case bioluminescence (2, 12). Since that time, *V. fischeri* has become an excellent model for studying several aspects of pheromone signaling, including the integration of environmentally responsive regulators into its PSSs (13), the integration of and cross-talk between signal circuits in the same bacterium (14-16), and system evolution across strains from distinct environments (17). *V. fischeri* uses three interconnected PSSs to control bioluminescence, which in many strains is induced upon colonization of specific hosts. In *V. fischeri* ES114, bioluminescence and other PSS-controlled behaviors help it maintain its mutualistic relationship with the Hawaiian bobtail squid, *Euprymna scolopes* (18-20). The importance of *V. fischeri*'s signaling to successful colonization of the host and the ability to study this ecologically relevant symbiotic context experimentally have contributed to the interest in studying its PSSs.

The *luxI/luxR* system was the first PSS discovered in *V. fischeri* and became the archetype acyl homoserine lactone (AHL) pheromone system. LuxI synthesizes the pheromone *N*-3-oxo-hexanoyl AHL (3OC6-AHL; Fig. 1.1A), which can freely diffuse in and out of the cell (21, 22). Upon reaching a threshold concentration, 3OC6-AHL binds its cognate receptor LuxR and activates transcription of the *luxICDABEG* operon (as well as other genes; 23-25). Induction of LuxA, LuxB, LuxC, LuxD, LuxE, and LuxG results in bioluminescence, while LuxI generates more 3OC6-AHL, which constitutes a positive feedback loop (26, 27). LuxI/LuxR-type PSSs are distributed broadly among the α-, β-, and γ-proteobacteria and utilize a diverse array of AHLs and similar molecules to control a variety of behaviors (Fig. 1.1B; 28-30).

It later became apparent that *V. fischeri* contains a second, structurally distinct AHL-based PSS composed of AinS and AinR. AinS synthesizes the pheromone *N*-octanoyl

AHL (C8-AHL, Fig. 1.1A), which was presumed to be detected by AinR. This PSS affects luminescence through a C8-AHL-LuxR interaction and through a conserved PSS cascade discussed below that ultimately controls LuxR expression (14, 31-33).

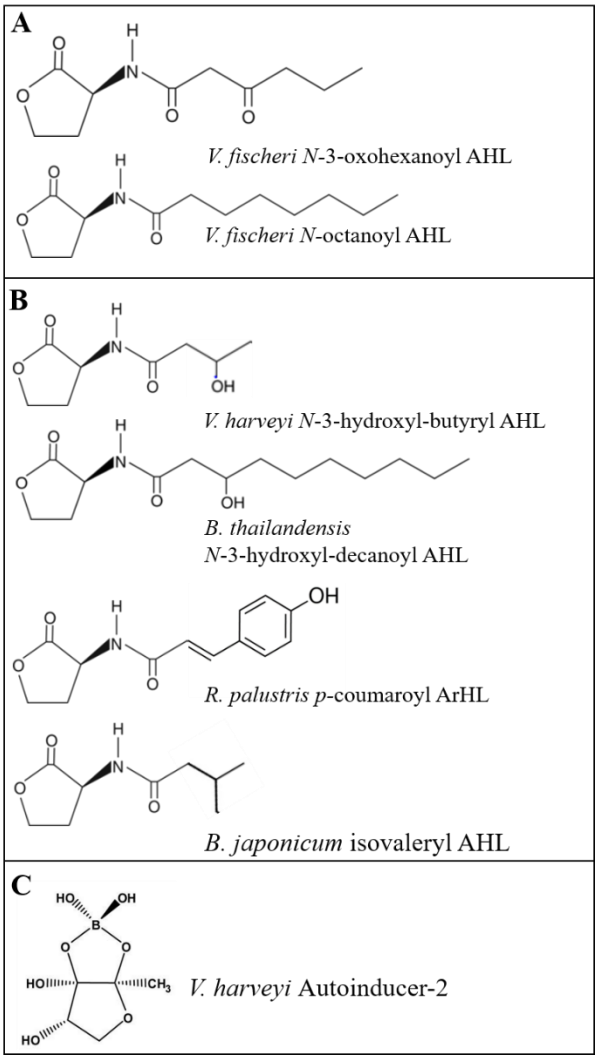

Figure 1.1. Chemically diverse structures of Gram-negative signaling molecules. A) Acyl-homoserine lactone (AHL) molecules used in *V. fischeri* pheromone signaling. B) AHL and arylated AHLs (ArHL) containing different chemical modifications and side chains from the γ-proteobacterium *V. harveyi*, the β-proteobacterium *Burkholderia thailandensis* and the α-proteobacteria *Rhodopseudomonas palustris* and *Bradyrhizobium japonicum* (28-30). C) Chemical structure of Autoinducer-2 (AI-2) from *V. harveyi* (34).

A third PSS in *V. fischeri* uses a signal called AI-2 (Fig. 1.1C), which has been found in diverse bacterial groups (35). AI-2 is generated through LuxS activity, and in the *Vibrionaceae* AI-2 is bound by LuxP, which then modulates LuxQ's signal transducing activity (36, 37). In *V. fischeri* the AI-2 PSS is thought to weakly modulate the core signal transduction circuit shared with AinS/AinR, but AI-2 contributes little to luminescence regulation under the conditions tested, and therefore it has not been studied in depth in this bacterium (15). By contrast, the shared core signaling circuit is strongly influenced by the AinS/AinR system, which is the subject of this dissertation.

AinS/AinR was first described in a derivative of the bright *V. fischeri* strain MJ1, which was isolated from the light organ of the pinecone fish *Monocentris japonica* (31). C8-AHL was found to interfere with 3OC6-AHL-LuxR activity, leading to initial speculation that C8-AHL might function to keep the *luxI/luxR* system from premature activation (32). However, the co-discovery of a putative receptor for C8-AHL (AinR) encoded adjacent to *ainS*, and the similarity of *ainS/ainR* to the *luxM/luxN* PSS of *V. harveyi*, suggested a more complex role (31-33).

Because *V. fischeri*'s symbiosis with *M. japonica* is experimentally intractable, most research on this bacterium and its signaling systems switched to using strain ES114, a wild-type isolate from *E. scolopes*. Based on the pioneering work with strain MJ1 and similar strains, LuxI/LuxR is typically considered the *de facto* regulator of bioluminescence. However, under most laboratory growth conditions ES114 actually produces comparatively little 3OC6-AHL (38), and its luminescence is largely driven by the C8-AHL produced by AinS (16). Work by Ruby and his coworkers has expanded the known role of the AinS/AinR system and illuminated its governance of critical aspects of

the life history of *V. fischeri* ES114 (16, 72, 91). This chapter will provide an overview of the AinS/AinR PSS with respect its regulatory function, its role in the symbiosis between *V. fischeri* and *E. scolopes*, and the systems with homology to it in other *Vibrio* species.

Mechanistic underpinnings of AinS/AinR and homologous systems

Although AinS/AinR is an AHL-dependent system, and AinS synthesizes C8-AHL in a reaction similar to that catalyzed by LuxI, AinS is evolutionarily unrelated to LuxI-type enzymes and belongs to a distinct class of AHL synthase (31, 39). Furthermore, unlike the widely distributed LuxI/LuxR-type PSSs, systems similar to AinS/AinR have thus far only been found in the *Vibrionaceae* family (Fig. 1.2), and have only been described in depth in *V. harveyi* (LuxM/LuxN) and *V. anguillarum* (VanM/VanN; 33, 40, 41). Despite interspecific variation in AHL synthase activity and signal receptor sensitivity, AHL-based PSSs like AinS/AinR functionally converge with diverse non-AHL signaling systems, including those based on AI-2 and cholera autoinducer (10, 42). Thus, in many *Vibrio* species multiple PSSs feed information from chemically distinct signals into a conserved phosphorylation cascade described below.

Activity of the conserved "core" PSS is determined by the phosphorylation state of pathway intermediates, which is influenced by pheromone levels. This process is best understood in the AinR homolog LuxN, leading to the model in Figure 1.3. At low pheromone concentrations (Fig. 1.3A), LuxN autophosphorylates, catalyzes an internal phosphotransfer reaction, and subsequently shuttles this phosphoryl-group to the phosphotransfer protein LuxU (43-45). Based on homology and mutant phenotypes, AinR is thought to act on LuxU in a similar fashion. LuxU then phosphorylates a conserved residue on the σ^{54}-dependent activator LuxO (46). LuxO-P activates transcription of *qrr* in

V. fischeri, or up to five *qrr* genes in other vibrios (47-49). Qrr is a sRNA that binds to the transcript of the master regulator of the system, called *litR* in *V. fischeri*, and targets this mRNA for degradation. LitR activates *ainSR* and *luxR*, so a decrease in LitR results in

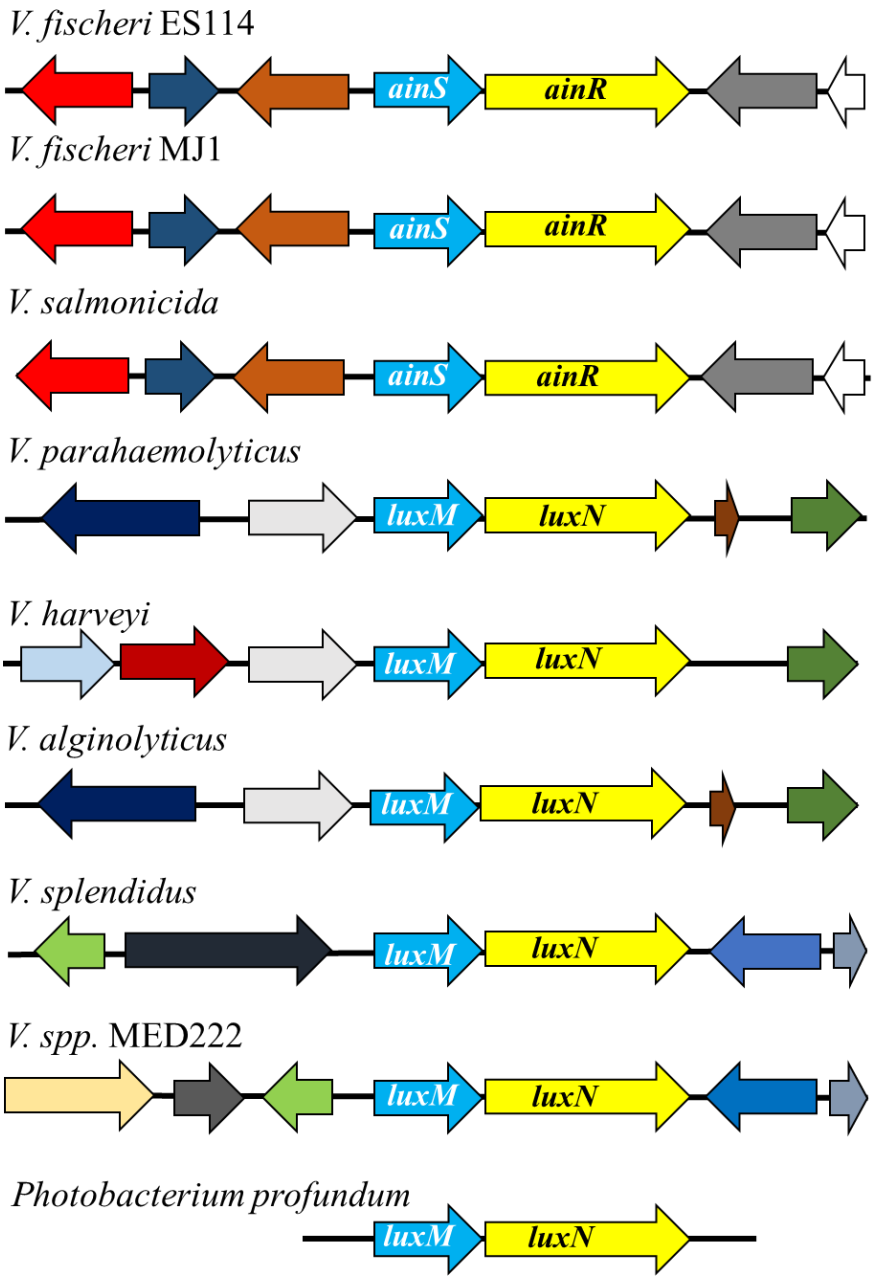

Figure 1.2. Distribution and arrangement of *ainS/ainR* and homologous systems in the *Vibrionaceae* family. Aligned sequences are from *V. fischeri* ES114 and MJ11, *V. salmonicidia* LF1238, *V. parahaemolyticus* RMID2210633, *V. harveyi* ATCC BAA-1116, *V. alginolyticus* 12G01, *V. splendius* 12B01, *Vibrio spp.* MED222, and *Photobacterium profundum* SS9. The 10-kb region surrounding *V. fischeri* ES114 *ainS* was used to query the University of Chicago SEED (50).

lower bioluminescence, as there is less C8-AHL and LuxR (15, 51). As pheromone levels rise (Fig. 1.3B), C8-AHL interaction with AinR depresses its autokinase activity allowing the protein's phosphatase activity, which is unaffected by pheromone levels, to dominate (43, 44). This shifts the phosphorylation equilibrium resulting in less LuxO-P, less Qrr, more LitR, and increased bioluminescence.

As noted above, AinS produces the C8-AHL signal that presumably feeds into this core PSS through AinR. Like LuxI-type AHL synthases, AinS and its homologs show system-specific substrate preferences (39). C8-AHL is preferentially synthesized by AinS using *N*-octanoyl-acyl carrier protein (C8-ACP) and *S*-adenosylmethionine (SAM). Unlike LuxI, however, AinS can generate C8-AHL *in vitro* using acylated coenzyme A (CoA) as an acyl-donor, although the use of the C8-CoA substrate reduces C8-AHL production to ~14% of that produced using C8-ACP (39, 52). AinS was similarly shown to use acyl-ACP substrates of acyl chain lengths between 6 and 10 carbons, but at lower efficiency than when using C8-ACP. Furthermore, more recent studies using highly sensitive and specific chemical analysis for the production of AHL pheromones other than 3OC6-AHL and C8-AHL in *V. fischeri* ES114 revealed growth phase-dependent production of *N*-hexanoyl AHL (C6-AHL) and *N*-heptanoyl AHL (C7-AHL) in addition to C8-AHL(53). Whether C6- and C7-AHL have a real biological function in this system or are simply superfluous byproducts is uncertain, although at high enough concentration they can influence LuxR activity in transgenic *Escherichia coli* (14, 33). Such promiscuity in AHL signal generation is not unique to *V. fischeri*, as the AinS-homolog VanM from *V. anguillarum* produces both *N*-3-hydroxy-C6-AHL (3OHC6-AHL) and C6-AHL, although the former is made in >30-fold excess to the latter (40). By contrast, LuxM from *V. harveyi* has only been

reported to synthesize *N*-3-hydroxy-butanoyl AHL (3OHC4-AHL), and this specificity may stem from a smaller acyl chain binding pocket incapable of large deviations from the native ligand (54).

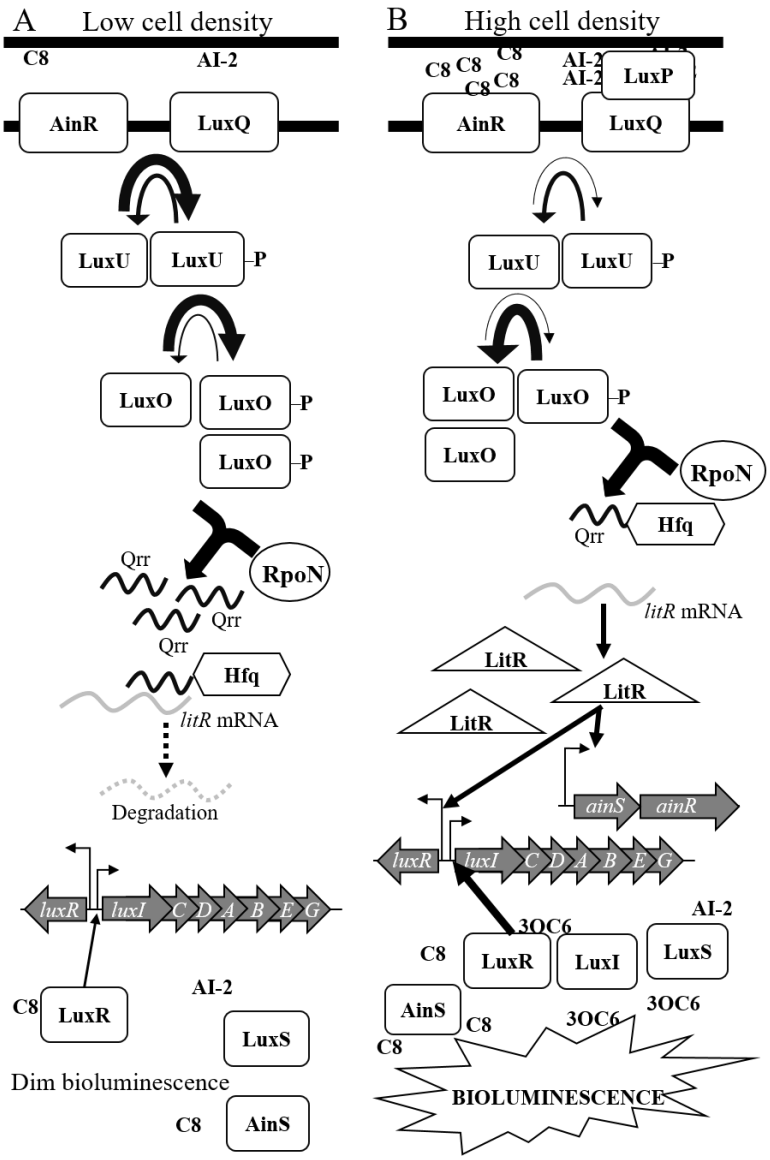

Figure 1.3. Pheromone-signaling architecture in *V. fischeri* ES114. Symbiotic luminescence is driven by LuxI producing 3OC6-AHL, which initiates a positive feedback loop through LuxR activation of the *lux* operon. The pheromone receptors AinR and LuxQ phosphorylate and dephosphorylate LuxU, which phosphorylates LuxO, activating transcription of *qrr*. Qrr represses *litR* expression. A) At low pheromone levels, AinR and LuxQ kinase activity dominates, resulting in more active LuxO-P, higher levels of Qrr, low *litR* expression, and low luminescence that is driven by AinS-synthesized C8-AHL interacting with LuxR. B) Higher pheromone levels depress kinase activity of AinR and LuxQ, shifting the equilibrium in favor of inactive LuxO, less Qrr, more LitR, and bright luminescence that is driven by 3OC6-AHL-LuxR.

The signal-receptor of these PSSs has been studied in greater depth than the signal synthases, and much of what we know about AinR is derived from studies of *V. harveyi*'s LuxN. Both proteins similarly function to pass information about the local pheromone concentration into the core PSS, but there are key differences in LuxN and AinR's structure that may account for differences in their respective substrate specificities and selectiveness. AinR and LuxN belong to a family of hybrid sensor kinase-response regulators with dual kinase-phosphatase functionality, and as noted above LuxN's relative kinase/phosphatase activity depends on the concentration of its cognate signal (43, 44). When pheromone levels are low, LuxN's kinase activity predominates, and it autophosphorylates a conserved histidine residue (His471) that is subsequently transferred to a conserved aspartate residue (Asp771) (44). This phosphoryl group is then shuttled to the phosphotransfer protein LuxU at His58 (43, 44, 46). As pheromone levels increase, the kinase activity is depressed, which allows the phosphatase activity to dominate, resulting in less phosphorylated LuxU (and LuxO). LuxN's, phosphatase activity requires Asp771 but not His471, and it is unaffected by pheromone levels (43, 44).

The structure-function relationship of *V. harveyi* LuxN has been examined extensively. LuxN is an inner-membrane protein and consists of nine transmembrane helices, four periplasmic loops, and four cytoplasmic loops (55). The predicted site of signal binding involves helices 4 to 7 and periplasmic loops 2 and 3, with residues in helices 5 (Leu166) and 6 (His210) dictating acyl-chain length and C3-substitution parameters, respectively, creating a highly-selective internal environment specific to the native 3OHC4-AHL signal. The concentration for half-maximal activation of LuxN by this signal is ~20 nM (56, 57). AinR differs from LuxN at these sites, corresponding to substitutions

of L166A and H210Q. These residues may alter the shape of the pheromone binding pocket in AinR, allowing longer-tailed AHLs to modulate its activity (57).

While the range of AHL detection by AinR could be broader than that of *V. harveyi* LuxN, potentially including other *Vibrio*-synthesized signals (40, 58), a recent comparative study of LuxN and AinR suggested that the S203 residue in AinR might discriminate against short-chain AHLs, like the *V. harveyi* signal 3OHC4-AHL (57). Moreover, the differences between LuxN and AinR may suggest the latter has heightened signal sensitivity, broader signal-recognition capacity, and greater resistance to antagonism by non-cognate signals (56, 57). Mutant analysis of LuxN revealed a number of residues important for increasing signal sensitivity or biasing LuxN towards its "kinase-off" state, and several of these mutations reside outside of the highly conserved regions common to all LuxN/AinR-type sensors, yet these mutant LuxN alleles are found naturally in AinR.

Differences in the specificity and selectivity of AinR and LuxN may suggest that they have diverged into distinct niche-specific roles (57). LuxN's restricted binding pocket potentially favors a stringent self-recognition role, while AinR's more open pocket could be used to broadly survey the population of medium-length AHL producers nearby. Although the environment inside the *E. scolopes* light organ is a monoculture of *V. fischeri*, this bacterium also resides mixed communities in sediment and in the guts of fishes (59). While promiscuity in signal recognition seems a poor strategy for the regulation of production of public goods for the benefit of nearby *V. fischeri* cells, it may provide *V. fischeri* with a way of coping with mixed-species communities by using a highly sensitive, non-selective sensor. Such eavesdropping has been previously reported in the case of the promiscuous LuxR-type regulators CviR in *Chromobacterium violaceum* and BraR in

Burkholderia phymatum (60, 61), but monitoring population-wide pheromone production through a non-LuxR-type AHL receptor would be novel.

In general, most evidence suggests that AinR's role in the central pheromone circuitry parallels that of LuxN in *V. harveyi* but with one notable discrepancy. Specifically, a Δ*luxN* mutant is brighter than wild-type, owing to a lack of LuxU (and LuxO) phosphorylation, less Qrr, and less repression of a master regulator; however, a Δ*ainR* mutant actually displays a small *decrease* in luminescence (62). We speculate that some sequence in *ainR* might have a regulatory role with respect to *ainS* at the nucleic acid level, and that its deletion in the Δ*ainR* mutant influenced luminescence in some way unrelated to loss of AinR. While it is unknown if *ainS/ainR* constitutes a single mRNA transcript, differential expression of genes in an operon has been observed and in some cases relies on the presence of an inverted repeat (IR) to differentially stabilize distinct transcript elements (63, 64).

Circuit integration and cross-talk between *luxI/luxR* and *ainS/ainR*

Integrated and hierarchical control of PSS is common among species possessing multiple signaling systems, and this is true in several AHL-based PSSs (65). For example, in *Pseudomonas aeruginosa*, the Las PSS stimulates the Rhl PSS (66). Activation of one PSS by another in the same bacterium can be followed by the second PSS subsequently repressing its activator. Such regulatory circuitry exists between the Cci and Cep systems in the opportunistic human pathogen *Burkholderia cenocepacia*, and a similar scheme underlies the interaction between ExpR and SinR/SinI in *Sinorhizobium meliloti* (67-70). In both cases, the activated system represses the activating system to tune signal

production, although the underlying significance of this regulation has yet to be fully determined.

Although much remains to be learned about coordination between the two AHL-based PSSs in *V. fischeri*, the *luxI/luxR* and *ainS/ainR* systems appear sequentially stimulated and may be integrated at multiple levels. The *ainS/ainR* PSS is the first system activated in culture and during colonization of the squid host. As noted above it primes *luxI/luxR* in two ways (16). First, C8-AHL interacts with LuxR to induce *lux* operon transcription while 3OC6-AHL levels remain low (14, 71). Second, C8-AHL perception by AinR shifts the core PSS to produce more LitR, which activates LuxR expression through an unknown mechanism (15, 51).

In weighing these two mechanisms, it is notable that *ainS* mutants are transiently dimmer than *litR* mutants (72), which still synthesize C8-AHL, suggesting that C8-AHL stimulation of LuxR is a more important influence on luminescence than its effect on LitR levels. However, once the *luxI/luxR* system has been fully activated, C8-AHL becomes a competitive inhibitor of 3OC6-AHL. This phenomenon is illustrated by the observations that *ainS* mutants are dimmer than wild-type when 3OC6-AHL is low or absent, yet these same mutants are brighter than wild-type when 3OC6-AHL is present at stimulatory levels (e.g. once cell density is very high; 13, 16, 31, 32). These results have recently been expanded upon using computational modeling and a more controlled experimental approach, wherein LuxR was expressed constitutively, not under the influence C8-AHL, and luminescence output was measured over a range of defined combinations of C8- and 3OC6-AHL concentration. The results further indicated the ability of C8-AHL to inhibit 3OC6-AHL-mediated stimulation and suggested that differences in responses to the two

AHLs were driven not just by their affinity for LuxR but also by differential cooperativities of AHL-LuxR complexes, which must multimerize to be active (73). Interestingly, a comparison of LuxR variants showed that both natural and artificial evolution can lead to different relative responses to these signals (73-75). In other words, LuxR can be more or less sensitive to each of the two signals, and be more or less specific, suggesting the influence of the AinS product on LuxR in ES114 is not unavoidable.

In contrast to the documented effects of Ain on the Lux system, it remains unclear if and how Lux regulates Ain. Indeed, beyond the effects of LitR and CRP discussed below, little is known about expression of the *ain* locus. The arrangement and spacing of the genes suggest that *ainS* and *ainR* comprise a small operon, but this possibility has not been tested. Interestingly, a potential LuxR binding site or "*lux* box" overlapping a putative -35 promoter element upstream of *ainS* suggests LuxR-3OC6-AHL may repress *ainSR* (31). No evidence for such regulation was found in a microarray analysis of the LuxR regulon in *V. fischeri* ES114 (23), although the experimental setup, wherein 3OC6-AHL was added to a background of endogenously produced signals, primarily C8-AHL, could have obscured some regulated genes. Similarly, Qin *et al.* concluded *ainS* is not part of the LuxR regulon in *V. fischeri* MJ1 based on reporter assays conducted in transgenic *E. coli* (24), although a subsequent study found that in transgenic *E. coli*, the effect of CRP on an *ainS* reporter was dependent on also introducing *litR* (76).

It is interesting to speculate how cross-talk between the *ain* and *lux* systems might relate to successful symbiotic colonization of the squid host *E. scolopes* by *V. fischeri*. As noted above, *ain* is the first system activated in lab culture and during symbiotic infection, and it primes expression of the *lux* system and the initiation of luminescence, which is a

colonization factor that contributes to symbiotic persistence (16, 77-79). However, C8-AHL ultimately becomes a competitive inhibitor of 3OC6-AHL-mediated activation when 3OC6-AHL builds up at very high cell densities, such as those found in the light organ. LuxR-3OC6-AHL repression of *ainSR* might therefore make sense for the bacterium, to avoid too much interference with the *luxIR* system once it has been induced.

Evolution of AinS and AinR

While much attention has been paid to the evolution of the regulatory components of the *luxI/luxR* system, less attention has been given to the divergence of the *ainS/ainR* system among different wild-type *V. fischeri* strains. Bose *et al*. reported a higher than average divergence of AinS/AinR between ES114 and the bright fish-symbiont MJ11, compared to the other components of the core PSS in these strains (17). This observation may reflect functional differences between their respective AinS enzymes, explaining the lower C8-AHL levels produced by MJ11, although there could also be distinct regulatory differences between *V. fischeri* wild-type strains (18). Additionally, AinS has diverged significantly from its LuxM and VanM homologs (40). This divergence may provide an explanation for AinS's substrate preference of saturated acyl-ACP over the hydroxylated acyl-ACP donors favored by LuxM and VanM.

From an evolutionary perspective, it is also worth noting that the function of the AinS/AinR system relies on its connection to a core signaling pathway that is shared by AI-2 signaling and, in other species, by other non-AHL PSSs as well. This signaling-circuit architecture stands in contrast to LuxI/LuxR type systems, which can operate essentially as standalone systems and appear to have moved horizontally across bacterial taxa where they regulate diverse and distinct behaviors. The reliance on several other

unlinked genes (e.g. *luxU*) may explain in part why systems like AinS/AinR are restricted to the *Vibrionaceae*, and Ain's relationship to a conserved core regulatory cascade may suggest that its regulatory function is likewise more conserved across the *Vibrionaceae*.

Metabolic integration of AinS/AinR

Changes in cell density have been a long-recognized condition governing PSS regulation in different bacteria (2). However, work in our lab and others has demonstrated the importance of environmentally-responsive regulatory systems integrated into these pheromone signaling cascades. Much of the work in *V. fischeri* has been directed at the *luxI/luxR* system and has revealed key contributors to *luxI/luxR* regulation, which are associated with cellular responses to diverse environmental conditions (13, 76, 80, 81). Identifying cell density-independent factors influencing *ainS/ainR* expression may reveal conditions similar to those encountered by the bacterium during the first stages of host colonization, as this system is responsible for regulating many of *V. fischeri*'s early colonization genes (72).

The availability of specific carbon sources, notably glucose, is as important determinant of *ainS* regulation. The *ainS* promoter is regulated by the cAMP-responsive regulator CRP (76), a known regulator of PSSs across a phylogenetically diverse set of bacteria (82-84). In many bacteria, cAMP-CRP activity is highest when glucose is absent, and cAMP-CRP activates genes involved in the catabolism of non-glucose carbon sources, as well as other genes such as those encoding virulence factors and PSS's. Thus, in many bacteria, glucose represses pheromone signaling.

Glucose-mediated repression of bioluminescence was demonstrated in MJ1 and in transgenic *E. coli* harboring the MJ1 *lux* operon over thirty years ago (85, 86). Only

recently however was *ain* regulation by CRP described and the previously observed activation of *luxR* by CRP confirmed in ES114 (85, 86). Lyell *et al.* showed a cAMP-CRP-dependent increase in *ainS* transcript levels in *V. fischeri* ES114 as well as cAMP-CRP binding to the *ainS* promoter region (76). Furthermore, Lyell and colleagues described increased activation of a P_{ainS}-*lacZ* reporter when expressed in *E. coli* in the presence of *litR* and *crp,* but when *litR* was absent the reporter showed decreased expression and, surprisingly, *crp*-dependent repression. Taken together, the results of their work corroborated evidence that LitR activates *ainS* expression while also suggesting that in transgenic *E. coli* lacking *litR* there is an additional, and perhaps artifactual, effect of CRP repressing *ainS.* As predicted, the C8-AHL in ES114 cultures was decreased when the medium was supplemented with glucose; however, under these conditions the C8-AHL synthesized by a Δ*crp* mutant unexpectedly exceeded that of the wild-type ES114. These results suggest that CRP may affect C8-AHL accumulation via an unknown mechanism.

Although the connection between PSS and a common central metabolite like glucose may seem unexpected, pheromone-signaling systems are capable of global regulation in bacteria, affecting the expression of more than 10% of the genes in a bacterium, and often including genes underlying central metabolic processes (87). As is the case in the core PSS of other vibrios (88-90), AinS/AinR regulates genes related to the metabolism of various nutrients, including sulfur, histidine, and folate (72). The most obvious metabolic perturbation of *ainS* mutants though is that during growth in an unbuffered rich medium, *ainS* mutants achieved only 75% growth yield compared to their wild-type parent strains owing to persistent acidification of the medium, consistent with a defective acetate switch (16, 91, 92). The acetate switch occurs in batch cultures when

acetate is produced leading to a drop in pH, followed by a reuptake and reassimilation of the acetate with a concomitant pH increase (92). The acetate switch in *V. fischeri* is regulated by AinS through *litR*-dependent activation of gene for acetyl-coA synthetase, *acs* (91). Interestingly, lower activity of a P_{acs}-*lacZ* reporter was observed during growth in media supplemented with glucose, suggesting CRP may be responsible for activating *acs* expression during glucose-starvation, and several potential CRP-binding sites were identified in the *acs* promoter region. This observation adds redundancy to the system, as CRP would have a positive regulatory impact on two components of the *acs* system, direct *acs* regulation and *ainS*-dependent *acs* regulation (76).

The role of AinS and AinR in the *V. fischeri*-*E. scolopes* symbiosis

The *V. fischeri*- *E. scolopes* symbiosis is an excellent model for the study of host-microbe relationships and a remarkable example of coevolution between host and symbiont. During the colonization process, *V. fischeri* is enriched from a minor constituent of the total microbial population in the local marine environment to the sole colonist of the squid light organ, successfully navigating a series of host-imposed checkpoints along the way (93). The AinS/AinR system is essential for the establishment of this relationship and regulates key aspects of symbiotic initiation and persistence within *E. scolopes* (15, 16, 72).

Motility is essential for initiating successful colonization and must be tightly regulated, as both non-motile and hypermotile mutants suffer severe colonization defects (94). AinS/AinR, negatively regulates the expression of fourteen motility genes through its effect on the core PSS, and *ainS* mutants are hypermotile (51, 72, 95). However, *litR* mutants are similarly hypermotile, yet they are capable of full colonization and persistence

within the host and outcompete their wild-type parent when in mixed competition (51). Thus, the symbiotic attenuation of *ainS* mutants apparently cannot be explained by their effect on *litR* and motility.

In addition to regulating motility, AinS/AinR also affects chemotaxis by repressing expression of an amino acid-responsive methyl-accepting chemotaxis protein, VfcA, which is responsive to serine, threonine, alanine, and cysteine (72, 96). Like motility, chemotaxis is an important factor in initiating and maintaining the symbiosis (97, 98). However, while repression of *ain* may be important for a chemotactic response to these amino acids under certain conditions, there is no evidence that VfcA-mediated chemotaxis, or the regulation of VfcA by AinS/AinR affects symbiotic competence. It appears instead that the key chemoattractants are chitin-derived oligosaccharides (98, 99).

Luminescence is also controlled by AinS/AinR, as described above, and is an essential factor for persistence of *V. fischeri* within the light organ (77-79). Animals colonized by *ainS* mutants are less luminous than those of wild type-colonized animals, likely owing to a lack of LuxR-C8-AHL activation of the *lux* operon during initiation (16). Similarly, *ainS* mutant do not persist well within the light organ, although the introduction of a *luxO* mutation relieved the *ainS* mutant of its luminescence and persistence defects after 72 hr of symbiosis. Fine analysis of the stages of symbiosis revealed *luxO* mutants bear similar initiation defects to *ainS* strains (72). Surprisingly, while a *litR* mutant is capable of outcompeting its wild-type parent in the host, it displays a similar initiation defect to an *ainS* mutant after 12 hr of colonization (51, 72). These results demonstrate the necessity for a properly regulated PSS cascade to undergo normal colonization of the host. Even small changes in the network that would hypothetically bias the cell to a more

favorable state for colonization can negatively impact symbiosis establishment, probably reflecting the interconnected nature of the signaling pathways with their associated regulatory networks as well as the importance of coordination, timing and magnitude of gene regulation.

Rationale for the study

Regulation of the *luxI/luxR* PSS in *V. fischeri* has been studied extensively over the last forty years, likely owing to its obvious and direct role in governing an exciting and readily apparent phenotype; bioluminescence. However, despite regulating key aspects of symbiosis initiation including the *luxI/luxR* PSS, comparatively little is known about the regulation of the *ainS/ainR* PSS. Furthermore, while the role of the core *Vibrio* PSS has been studied in great detail in *V. harveyi*, its role in *V. fischeri* has largely been ignored or thought to be similar to that in other vibrios. Recently, however, reports revealed unique connections between the *V. fischeri* core PSS and other systems (62, 100), and Ray *et al.* found that bioluminescence was not enhanced in an Δ*ainR* mutant, as predicted by our understanding of the *V. harveyi* system, but was decreased instead (62). Because AinS/AinR regulates known colonization factors in a model symbiosis, and because it represents a system distributed among a variety of agriculturally and medically relevant organisms, the *Vibrionaceae*, it merits further study.

The goal of this work was to examine the factors involved in the function and regulation of the *ainS*/*ainR* PSS. Specifically, I focused on two intertwining questions 1) How is AinR's function similar to or different from that of LuxN, and 2) What regulates expression of AinS/AinR?

Determining AinR's role in *V. fischeri* pheromone signaling

AinR was originally identified as a potential pheromone receptor adjacent to the C8-AHL synthase AinS (31). Owing to AinS/AinR's homology to the well-characterized LuxM/LuxN system from *V. harveyi*, AinR's function as a pheromone sensor and component of the core *Vibrio* PSS was assumed to be like that of LuxN. However, *V. fischeri*'s ecological niche as a specific light-organ symbiont, and the presence of a second AHL-type PSS in *V. fischeri* distinguish it from *V. harveyi* and may have selected for functional divergence between AinS/AinR and LuxM/LuxN. Moreover, the report of an unexpected luminescence phenotype in a Δ*ainR* mutant suggested AinR and LuxN might have somewhat different functions (62). These observations, along with AinR/AinS's documented role in symbiotic infection led me to ask two basic questions about AinR 1) Does it sense C8-AHL and 2) Does it fit into the core PSS like LuxN?

In Chapter 2, I provide evidence that AinR does respond to C8-AHL and feeds into the core *Vibrio* PSS to modulate activity of the sRNA Qrr as predicted in Fig. 1.3. Consistent with Ain's proposed role as the first of the two AHL systems induced in *V. fischeri*, AinR is supremely sensitive to C8-AHL, and elicits responses at much lower pheromone concentrations than LuxN. I further demonstrate that AinR also senses a variety of AHL pheromones ranging in size from six to ten carbon units with various substitutions at the 3' position. This observation takes on added importance because of the discovery that *V. fischeri* produces C6-AHL, which has been known since the discovery of AinS, and C7-AHL in a growth phase-dependent manner (53), suggesting these molecules may serve a true biological role in *V. fischeri* cells (33).

Identifying cell density-independent regulators of *ainS/ainR*

Given AinS/AinR's position atop the core PSS and its role in controlling other systems required for symbiosis initiation and persistence, determining the regulators of this system could provide insight into the environment *V. fischeri* encounters during early stages of symbiosis. Thus, a key question about the *ainS/ainR* PSS that has not been systematically explored is what, if any, environmental factors influence its activity. Previous work has shown the *ain* and *lux* PSSs are both under the positive control of CRP, and depend on carbon source availability (76). The only other known regulator of the *ainS/ainR* system is the activator LitR, but its mechanism of regulation is not well understood and may differ from orthologous regulators in other vibrios (15, 101).

In Chapter 3, I attempt to use a transposon mutant library composed of *V. fischeri* strains with mutations in non-essential genes to identify regulators of the system. While we discovered three mutants in this library with decreased AinS activity, I eventually traced the source of these phenotypes to spontaneous mutations conferring LuxU-independent hyperactivity of the LuxO regulator. These mutants arise spontaneously when *V. fischeri* is grown in static culture and appear to exploit an as-yet uncharacterized part of the Qrr regulon conferring greater survivability in broth culture but at the cost of decreased symbiotic competence.

Despite an initially unsuccessful screen for mutants affecting *ainS*-dependent phenotypes, my data do point to LuxR-3OC6-AHL-mediated repression of the *ain* system, which was first posited in Chapter 2 and explored further in Chapter 4. Additionally, I demonstrate in Chapter 2 the luminescence defect in a Δ*ainR* mutant is the result of decreased C8-AHL synthesis, independent of LitR. In Chapter 4, I provide evidence that

this C8-AHL deficiency correlates with an inverted repeat element located in the *ainR* sequence. This inverted repeat is absent in *V. harveyi luxN* and may represent a novel method of intra-system regulation for an AHL-based PSS.

In the final chapter of this dissertation I discuss how this research has furthered our understanding of the *ainS/ainR* and core *Vibrio* PSS in *V. fischeri*. I also provide an outline of future directions for the study of this system's role in intracellular communication, species interactions, and the evolution of the system in diverse wild-type strains.

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CHAPTER 2

SUBSTRATE SPECIFICITY AND FUNCTION OF THE PHEROMONE RECEPTOR AINR IN VIBRIO FISCHERI ES114[1]

[1] John H. Kimbrough and Eric V. Stabb. 2013. Journal of Bacteriology. **195**(22): 5223-32. Reprinted here with permission of publisher.

Abstract

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 (C8-AHL) and *N*-3-oxo-hexanoyl-AHL (3OC6-AHL), respectively. LuxR is a transcriptional activator that responds to 3OC6-AHL, and to a lesser extent to C8-AHL. AinR is hypothesized to respond to C8-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 C8-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 C8-AHL. Several other AHLs with substituted and unsubstituted acyl chains between six and ten carbons also displayed an AinR-dependent effect on P_{qrr}-*lacZ*; however, AHL's with acyl chains of four carbons or twelve or more carbons lacked activity. Interestingly, 3OC6-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.

Introduction

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 acyl-homoserine lactone (AHL) signals. The first AHL system discovered was LuxI/LuxR (9, 10). LuxI is a signal synthase that generates *N*-3-oxo-hexanoyl-AHL (3OC6-AHL) (11). At a threshold concentration this membrane-permeable "autoinducer" AHL binds to LuxR (9, 12, 13). 3OC6-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 LuxI/LuxR (8, 14-17). AinS generates *N*-octanoyl-AHL (C8-AHL), which can bind LuxR directly, although it is a weaker activator than 3OC6-AHL and can inhibit 3OC6-AHL-mediated activation (17, 18). It is thought that a major role for C8-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 signaling cascade (Fig. 2.1) has been inferred largely by homology to *V. harveyi* LuxN (22-25). LuxN phosphorylates and dephosphorlyates LuxU, and LuxU-P initiates a cascade involving LuxO, resulting in activation of Qrr regulatory RNAs, which post-transcriptionally 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 Figure 2.1. 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 having the predicted effect on Qrr.

Signal cross-talk might further entwine the Ain and Lux systems. Given that AinS's product, C8-AHL, is recognized by LuxR, and that C8-AHL's net effect depends on the availability of 3OC6-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 C8-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.

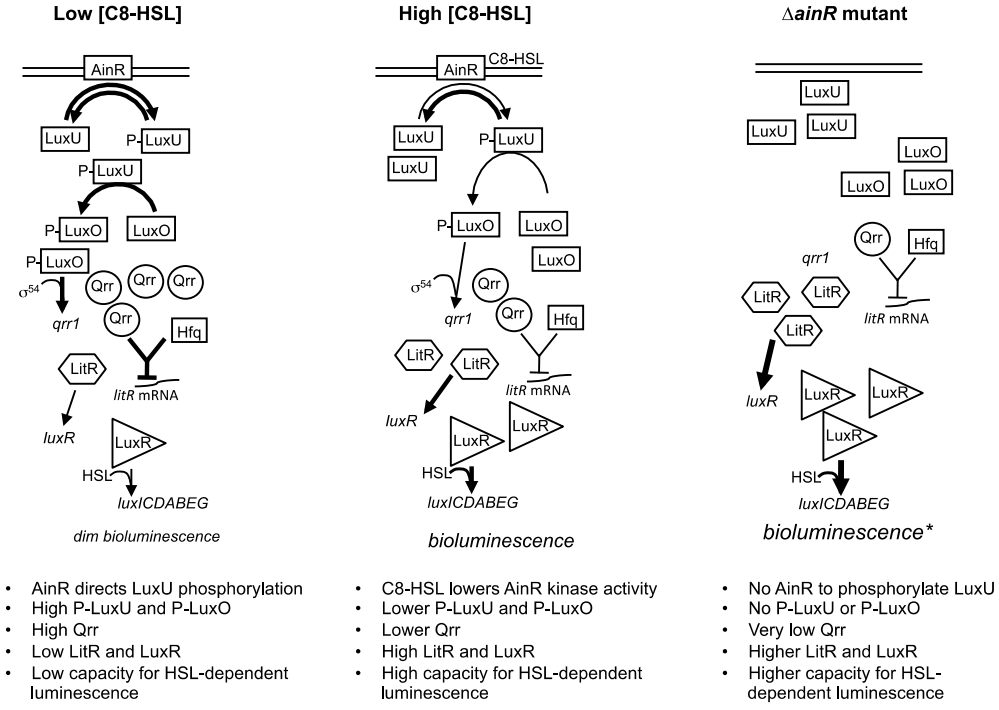

Figure 2.1. Model of AinR-mediated pheromone signaling in *V. fischeri*. This model is drawn largely based on the function of the homolog LuxN in *V. harveyi*, although in that bacterium the homolog of LitR is the direct regulator of luminescence. Differences in font size, arrow thickness, and numbers of gene products shown are meant to reflect relative changes in activation or abundance. An asterisk highlights that a Δ*ainR* mutant actually displays dimmer bioluminescence than wild type (26). This simplified model omits any input from AI-2 and LuxQ into the shared regulatory cascade.

Materials and Methods

Bacteria, growth media, and reagents

Bacterial strains are listed and briefly described in Table 2.1. *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 DH5αλpir (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. 3OC6-AHL and C8-AHL were obtained from Sigma-Aldrich (St. Louis, MO), and *N*-butanoyl (C4)-AHL, *N*-3-hydroxy-butanoyl (3OHC4)-AHL, *N*-hexanoyl (C6)-AHL, *N*-heptanoyl (C7)-AHL *N*-3-hydroxy-octanoyl (3OH-C8)-AHL, *N*-3-oxo-octanoyl (3OC8)-AHL, *N*-decanoyl (C10)-AHL, *N*-dodecanoyl (C12)-AHL, *N*-tetradecanoyl (C14)-AHL, *N*-oxo-tetradecanoyl (3OC14)-AHL, *N-cis*-tetradec-9Z-enoyl (cis-C14-9Z-enoyl)-AHL, and *N*-octadecanoyl (C18)-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 2.1. DNA ligase and restriction enzymes were obtained from New England Biolabs

Table 2.1: Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
E. coli		
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		
DC22	C8-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, R6K**γ**, $oriT_{RP4}$, *camR*	D. Colton

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
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* frame-shift allele; ColE1, *oriT*$_{RP4}$, *camR*	17
pJLB123	P_{orf1}-*luxR* in pVSV104; pES213, *oriT*$_{RP4}$, R6Kγ, *kanR*	32
pNL62	Δ*ainS* allele; ColE1, R6K**γ**, *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	43
pVSV105	pES213, R6Kγ, *oriT*$_{RP4}$, *camR*, *lacZ*α	43
Oligonucleotides[c]		
ASAinRF	TACCTAGGATGTTAACTACTTTACCTAAAG	This study
ASAinRR	ATCCTAGGAATAAATTATCGAAGTAGCC	This study
pr_HK25	GGGGGCATGCGGCTTCTACTGCAGCATCAATTGA	This study
pr_HK26	GGGGGCTAGCAAAGGGTCAATATACCTATTGCAGGG	This study

[a] Drug resistance abbreviations used: *camR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*);
[b] All alleles cloned in this study are from *V. fischeri* strain ES114. Replication origin(s) of each vector are 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 highlight restriction enzyme recognition sites.

(Beverly, MA). Oligonucleotides used for PCR and cloning are listed in Table 2.1 and were synthesized by Integrated DNA Technologies (Coralville, IA). PCR was conducted with Phusion high-fidelity DNA polymerase (Finnzymes, Finland) in an iCycler (BioRad Laboratories, Hercules, CA). Plasmids used for cloning were isolated with the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Inc., St. Louis, MO). DNA was re-purified 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 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 conjugative helper 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 2.1. 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 Δ*ainSR* strain NL55 (38). The Δ*ainS* allele in pNL62

was exchanged into VCW2G7 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 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). 500-µl samples were removed at indicated 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}.

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 GFP using an excitation/emission 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* also on the plasmid to arrive at the specific fluorescence reported (GFP/MCherry). The EC_{50} value, 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 24ºC with shaking at 200 rpm. Cells were collected at OD_{595} ~2.5 by centrifugation, the supernatant was discarded, and cell pellets were stored overnight at -80°C. ß-galactosidase assays were performed as previously described (3).

C8-AHL bioassays

C8-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 mix 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. 200 µL of extract dissolved in SWTO were added to a 96-well microplate. Wells were then inoculated with the C8-AHL bioreporter strain DC22, which lacks the *luxI* and *ainS* pheromone synthase genes and wherein expression of *luxCDABEG* is activated by a mutant LuxR ($LuxR_{MJ1}$ T33A, R67M, S116A, M135I) that is responsive to C8-AHL but not to 3OC6-AHL (39). C8-AHL concentration was determined by measuring luminescence of DC22 in a Synergy 2 plate reader (BioTek) and comparing to C8-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 C8-AHL (Fig. 2.2). The parental vector lacking *ainR* did not have these effects. Thus, *ainR* appeared to functionally replace *luxN* in *V. harveyi* but responded to the C8-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. 2.1), we generated a P_{qrr}-*lacZ* transcriptional reporter using the sequence upstream of *qrr* (also *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 of C8-AHL, 3OC6-AHL, or both lowered P_{qrr}-*lacZ* reporter activity (Fig. 2.3A). 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 C8-AHL (Fig. 2.3B). However, as discussed below, when *ainR* was added *in trans* to the

Δ*ainR* mutant, the effect of 3OC6-AHL on the *qrr* reporter was not fully complemented (Fig. 2.3B).

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

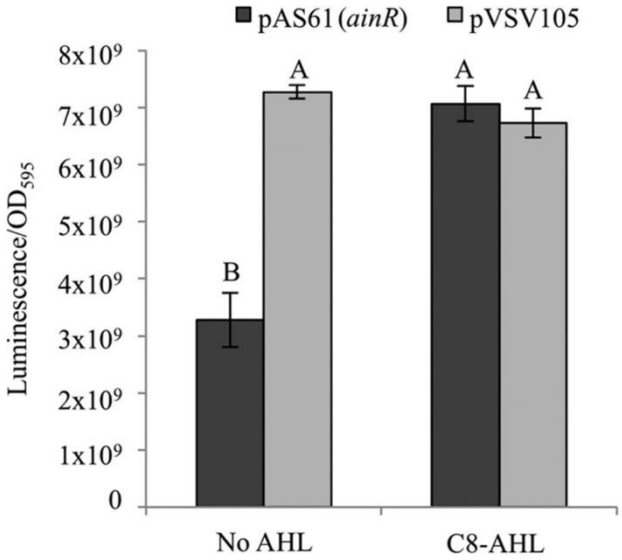

Figure 2.2. Complementation of a *V. harveyi luxN* mutant with *ainR*. Peak specific luminescence of the *V. harveyi* pheromone-sensor mutant TL183 (Δ*cqsS* Δ*luxN* Δ*luxQ*), grown in SWTO, with or without 1 μM C8-AHL, and either bearing *ainR* from *V. fischeri* on plasmid pAS61 (dark gray) or carrying the parental shuttle vector pVSV105 (light gray) as a control. Upper case 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 ANOVA and post hoc testing using Tukey's honestly significant difference test. Data is shown from one representative experiment of three, each with three biological replicates. Error bars represent standard error ($n = 3$).

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*

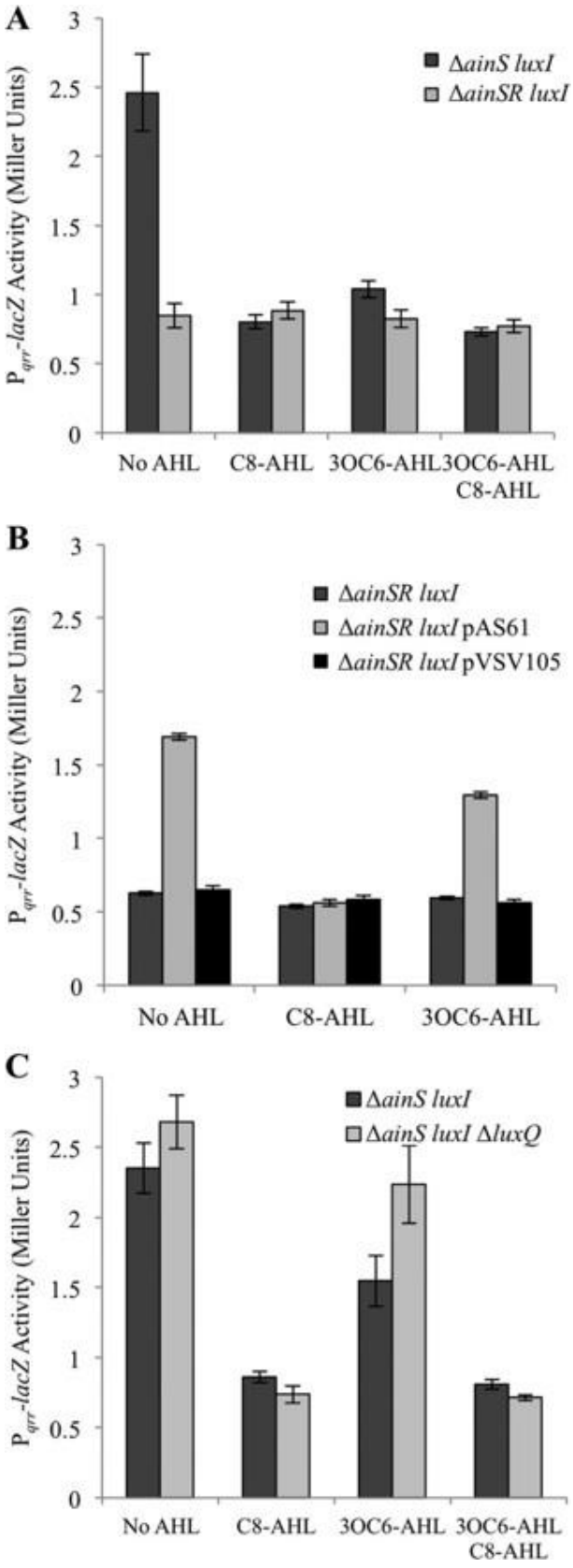

Figure 2.3. *ainR*-dependent and AHL-dependent effects on a *qrr* transcriptional reporter. Each panel shows β-galactosidase activity expressed from the P_{qrr}-*lacZ* transcriptional reporter in cultures grown on SWTO with 1 μM of each AHL indicated. (A) Reporter activity in mutants NL63 (Δ*ainS luxI*) 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-C, *n*=3).

mutant background (Fig. 2.3C). 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 Figure 2.1, but the 1 μM C8-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 C8-AHL. C8-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. 2.4). The EC_{50} value for C8-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 Figure 2.4. Similar EC_{50} values were determined in repetitions of this experiment.

Having established *ainR*-dependent effects on P_{qrr}-*lacZ* by both the C8-AHL and 3OC6-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 C8-AHL by ±2 carbons, regardless of 3′ substitution (unsubstituted, -oxo-, or –hydroxy-), significantly depressed reporter activity in an *ainR*-dependent manner (Fig. 2.5, $P<0.01$). AHLs with 4-carbon acyl chains or acyl chains twelve carbons or longer did not affect reporter activity, regardless of substitution. Although in Figure 2.5 it appears that C4-AHL reduced reporter activity, this result was anomalous and did not repeat in other experimental replicates. By contrast, the *ainR*-dependent effects of C6-AHL, 3OC6-AHL, C7-AHL, 3OHC8-AHL, and C10-AHL shown in Figure 2.5 were reproduced and statistically

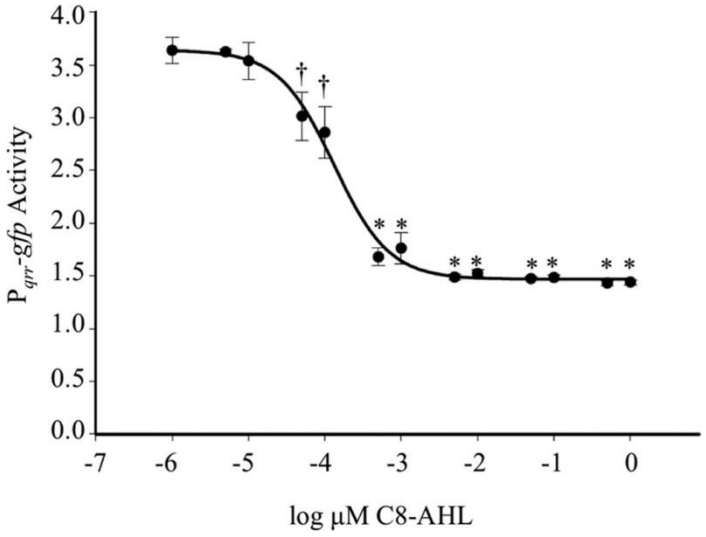

Figure 2.4. Effect of C8-AHL dose on *qrr* transcriptional reporter. The AHL pheromone synthase mutant NL63 (Δ*ainS luxI*) harboring the P_{qrr}-*gfp* reporter plasmid pTM268 was exposed to C8-AHL concentrations ranging from 0 μM to 1 μM. GFP expression was normalized to the red fluorescence expressed from a constitutive *mcherry* on the same plasmid. Curve fitting and associated EC_{50} calculation was performed using SigmaPlot (EC_{50} = 140 pM, R^2 = 0.9582).

significant in three other repetitions of this experiment.

Although C6-AHL, 3OC6-AHL, C7-AHL, 3OHC8-AHL, and C10-AHL each displayed *ainR*-dependent depression of P_{qrr}-*lacZ* activity, in Figure 2.5 these AHLs had

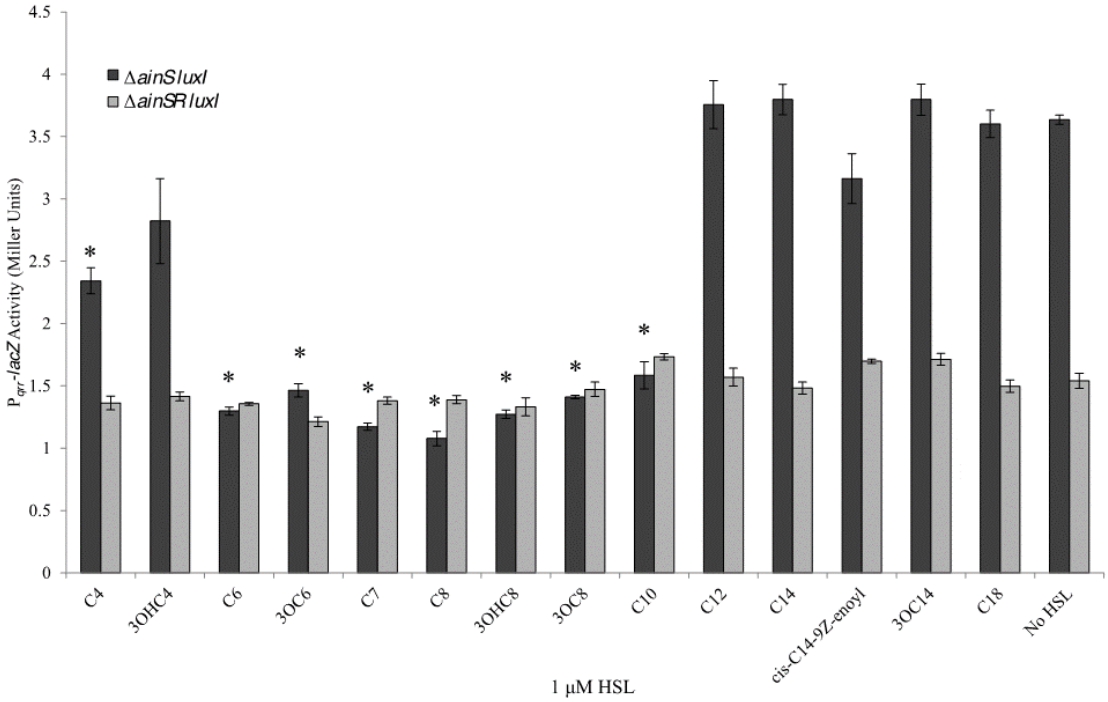

Figure 2.5. Effects of a variety of AHL molecules on *ainR*-dependent signaling. β-galactosidase activity expressed from the P_{qrr}-*lacZ* transcriptional reporter on pHK20, harbored by NL63 (Δ*ainS luxI*, dark bars) or JHK001 (Δ*ainSR luxI*, light bars), respectively. Cultures were grown in SWTO with 1 μM dissolved AHL. Data from one representative experiment of three independent experiments is shown. Asterisks indicate statistically significantly lower activity compared to the "No AHL" treatment as determined by Student's *t*-test ($P<0.01$). Bars represent standard errors ($n=3$).

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 ten-fold dilutions, we found that C6-AHL and C10-AHL were the least active, only eliciting a response at 1 μM (Table 2.2). 3OC6-AHL was the next weakest in activity, and only significantly affected the reporter down to 100 nM (Table 2.2). At a significance of $P < 0.05$, C7-AHL was active at 10 nM, while 3OC8-AHL and 3OHC8-AHL were active down to 1 nM (Table 2.2). None of these AHLs

were as active as C8-AHL in this reporter system; however, those with the most similar chain lengths to C8-AHL were closest in activity.

Table 2.2. Sensitivity of *ainR*-mediated signaling to different AHL molecules

AHL	EC_{50} (nM AHL)[a]	Minimum Stimulatory Dose (nM AHL)[b]
C6-HSL	114	1000
3OC6-HSL	29	100
C7-HSL	11	10
3OC8-HSL	8	1
3OHC8-HSL	0.8	1
C10-HSL	476	1000

[a] EC_{50} values 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 ten-fold dilutions over a range of 1μM to 1 pM. Data shown are from a single representative experiment of three independent experiments.
[b] Minimum stimulatory dose indicates the lowest tested AHL concentration that elicited a significantly lower ($P < 0.05$) P_{qrr}-*lacZ* activity than the treatment lacking AHL.

We next tested whether any of the non- or relatively weakly inducing AHLs could interfere with AinR-dependent C8-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 C8-AHL, a relatively low C8-AHL concentration that nonetheless influenced the *qrr* reporter (Fig. 2.4). Under these conditions, adding 1 μM of the non-inducing AHLs, which included C4-AHL, 3OHC4-AHL, C12-AHL, 3OC14-AHL, *cis*-C14-9Z-enoyl-AHL, and C18-AHL had no discernible effect on C8-AHL-mediated signaling (data not shown). Thus, even in one thousand-fold molar excess, these non-inducers did not interfere with signaling by the cognate pheromone C8-AHL. For relatively weakly inducing AHLs, including C6-AHL, 3OC6-AHL, C7-AHL, 3OHC8-AHL, and C10-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 2.2). We reasoned that these AHLs probably bind AinR at or below 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 C8-AHL signaling at concentrations where they themselves have a modest or non-detectable effect on the *qrr* reporter. However, we again saw no inhibition of C8-AHL-mediated signaling (data not shown).

The results above show an *ainR*-dependent effect of a variety of AHL's on P_{qrr}-*lacZ* expression, and we speculated that our data reflected AinR acting as a receptor for these AHL's. We were especially intrigued that AinR might recognize the LuxI product, 3OC6-AHL; however, the assays above were done in genetic backgrounds that retained the AHL-dependent activator LuxR, which is the cognate 3OC6-AHL receptor. We therefore tested all the AHL's that were active in our assays (Fig. 2.5 and Table 2.2) in a *luxR* mutant background. 1 μM of each of these AHLs was again sufficient to decrease *qrr* reporter activity in the presence of *luxR* (Fig. 2.6A, dark bars). However, deletion of *luxR* eliminated the effect of 3OC6-AHL on *qrr* reporter activity, while the effects of all other AHLs were the same regardless of the presence or absence of *luxR* (Fig 2.6A, light bars, and data not shown). Complementing the *luxR* mutant with *luxR in trans* on pJLB123 restored 3OC6-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, pVSV104, and the P_{qrr}-*lacZ* transcriptional reporter plasmid pHK20, which was used in

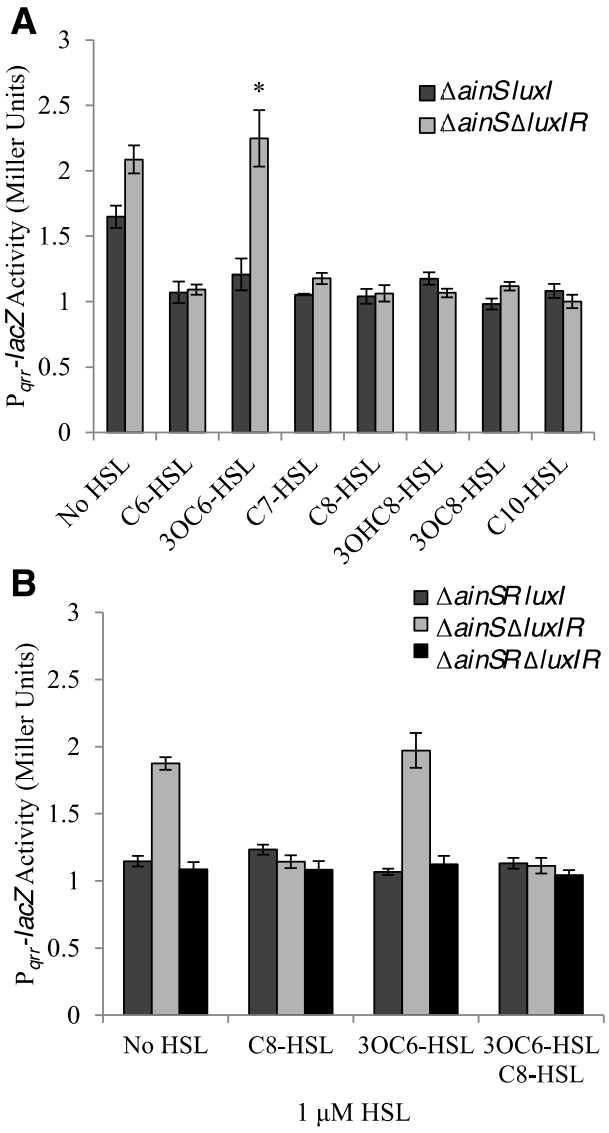

Figure 2.6. The effect of 3OC6-AHL on P_{qrr}-*lacZ* activity is dependent on *luxR*. β-galactosidase activity expressed from the P_{qrr}-*lacZ* reporter on pHK20, from cultures grown in SWTO with 1 μM of each AHL indicated. Data in each panel are from a single representative experiment of three independent experiments, and bars represent standard errors (n = 3). (A) Reporter activity in NL63 (Δ*ainS luxI*, dark bars) and JHK007 (Δ*ainS* Δ*luxIR*, light bars). Asterisk indicates statistically significant difference between the two strains (P < 0.02). (B) Reporter activity in mutants JHK001 (Δ*ainSR luxI*), JHK010 (Δ*ainSR* Δ*luxIR*), and JHK001 (Δ*ainSR luxI*).

Figure 2.7A, for complementation we used the P_{qrr}-*gfp* reporter pTM268, which was used in generating the data for Figure 2.4 with similar results to the P_{qrr}-*lacZ* reporter.

We were curious whether 3OC6-AHL-LuxR may be acting on *qrr* independently of a 3OC6-AHL-AinR dependent effect (Fig. 2.3, panels A and B). If there were two different mechanisms by which 3OC6-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) than that of the *ainR* mutant (Fig.

2.6B). Thus, these data are consistent with AinR and LuxR acting in the same pathway to affect *qrr* in response to 3OC6-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 Figure 2.1, and (ii) our data above support the predicted relationship between *ainR* and *qrr*. Ray and Visick (26) observed that Δ*ainR* mutation did not affect luminescence when excess C8-AHL was added, and we therefore tested C8-AHL accumulation by Δ*ainR* mutants. Evidence suggests a LitR-mediated positive feedback mechanism for the AinS/AinR system (8), so we tested C8-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), C8-AHL levels were reduced in a *litR* mutant. Moreover, we found that an in-frame deletion of *ainR* modestly but significantly (P <0.05) reduced C8-AHL accumulation in the wild-type or *litR* mutant backgrounds (Fig. 2.7). As discussed further below, these results, together with the C8-AHL amendment experiment of Ray and Visick (26), suggest that the Δ*ainR*

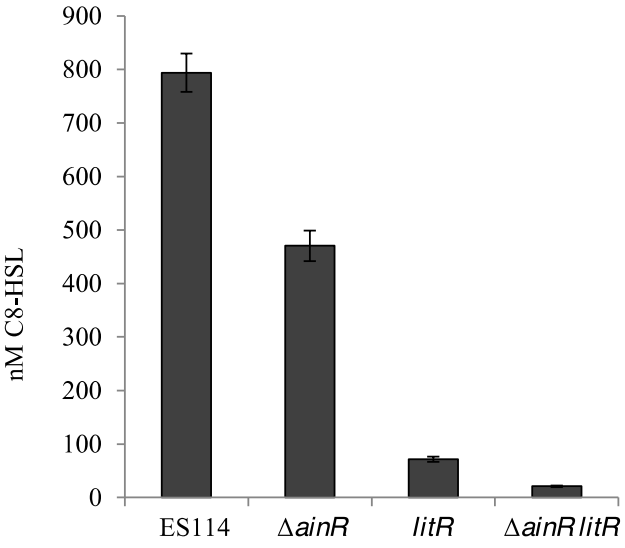

Figure 2.7. C8-AHL accumulation is reduced in Δ*ainR* mutants. C8-AHL was extracted and its concentration estimated from cultures of ES114, the Δa*inR* mutant JHK003, the *litR* mutant JB18, or the *litR* Δ*ainR* mutant JHK008. The C8-AHL synthase mutant NL60 (Δ*ainS*) served as a negative control and had no detectable activity (data not shown). Cultures were grown to OD_{595} ~2.5 in SWTO, and C8-AHL levels were determined by bioassays of ethyl acetate-extracted cultures. Bars indicate standard errors (n= 3).

mutation results in dimness because this allele causes decreased C8-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 *E. 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 mono-species 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. 2.1). 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 C8-AHL, and we discovered a new regulatory connection between the Lux and Ain systems.

High sensitivity of AinR toward C8-AHL

The sensitivity of AinR toward C8-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. 2.4). TraR from *Agrobacterium tumefaciens* can elicit responses to similar sub-nM levels of AHL, at least when over-expressed (40, 41), but sensitivity in the 10 nM range or higher appears to be more common for AHL receptors, including LuxR's response to 3OC6-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 hundred fold more sensitive for its cognate pheromone than is LuxN. Interestingly, Swem *et al*. showed a S184N mutation made LuxN more sensitive to AI-1, reducing the EC_{50} toward 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 C8-AHL

The high sensitivity of AinR toward C8-AHL may come at a cost of low specificity. We found that AHLs with substituted or unsubstituted acyl chains of six to ten carbons affected P_{qrr} reporter activity (Fig. 2.5), and inactivity was only seen with acyl chains of four carbons or greater than twelve carbons. In general, the further the deviation from the C8 chain length, the less effective the AHL in our assay (Table 2.2). Although C8-AHL was the most active pheromone in our assays, other AHL's 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 AHL's 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 C8-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 high sensitivity to detect small amounts of C8-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 non-cognate 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 multi-signal 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. C8-AHL/LuxR cross-talk had been established, but reciprocal signaling between LuxI-synthesized 3OC6-AHL and AinR would further entwine these systems. We initially suspected AinR detected 3OC6-AHL, because 3OC6-AHL led to decreased P_{qrr} reporter activity in an *ainR*-dependent manner (Fig. 2.3). However, we subsequently found this effect was also dependent on *luxR*, which encodes the 3OC6-AHL receptor (Fig. 2.6). Although we cannot rule out perception of 3OC6-AHL by AinR, based on our data we speculate that 3OC6-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-talkis unknown, but 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. 2.6). One simple model would be that 3OC6-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 down-regulation in response to 3OC6-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 C8-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 C8-AHL on the *qrr* reporter was complemented fully, while the effect of 3OC6-AHL was not (Fig. 2.3B). Perhaps when *ainR* is expressed from a non-native promoter in multicopy, repression of *ain* by 3OC6-AHL-LuxR is lost. Alternative mechanisms are possible too, including 3OC6-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 ΔainR *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 C8-AHL (26), and we have now shown that the Δ*ainR* allele results in decreased C8-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 C8-AHL levels, in an *ainS* mutant background lacking C8-AHL, an additional *ainR* mutation has the effect predicted in Figure 2.1 of enhancing luminescence (data not shown). The reason C8-AHL affects luminescence even without its AinR receptor probably relates to LuxR and its activation by C8-AHL. Ultimately, bioluminescence in *V. fischeri* is activated by AHL-LuxR, and previous studies overwhelmingly suggest that in strain ES114, C8-AHL drives LuxR-dependent activation,

especially in broth cultures (5, 16, 17, 19). Although C8-AHL is a weaker activator of LuxR, in ES114 it accumulates to much higher levels than does 3OC6-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 C8-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 only evident 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 correct, such a mechanism could allow co-transcription 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 similar regulatory circuit as LuxN, it seems that as an interface between 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 post-transcriptional control.

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CHAPTER 3

ANTISOCIAL LUXO MUTANTS PROVIDE A STATIONARY-PHASE SURVIVAL ADVANTAGE IN VIBRIO FISCHERI ES114[2]

[2] John H. Kimbrough and Eric V. Stabb. 2016.
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Abstract

The squid light-organ symbiont *Vibrio fischeri* controls bioluminescence using two acyl-homoserine lactone pheromone-signaling (PS) systems. The first of these systems to be activated during host colonization, AinS/AinR, produces and responds to *N*-octanoyl homoserine lactone (C8-AHL). We screened activity of a P_{ainS}-*lacZ* transcriptional reporter in a transposon-mutant library and found three mutants with decreased reporter activity, low C8-AHL output, and other traits consistent with low *ainS* expression. However, the transposon insertions were unrelated to these phenotypes, and genome resequencing revealed that each mutant had a distinct point mutation in *luxO*. In wild type, LuxO is phosphorylated by LuxU and then activates transcription of the sRNA Qrr, which represses *ainS* indirectly by repressing its activator LitR. The *luxO* mutants identified here encode LuxU-independent, constitutively active "LuxO*" proteins. The repeated appearance of these *luxO* mutants suggested that they had some fitness advantage during construction and/or storage of the transposon-mutant library, and we found that *luxO** mutants survived better and outcompeted wild type in prolonged stationary-phase cultures. From such cultures we isolated additional *luxO** mutants. In all, we isolated LuxO* allelic variants P41L, A91D, F94C, P98L, P98Q, V106A, V106G, T107R, V108G, R114P, L205F, H319R, H324R, and T335I. Based on the current model of the *V. fischeri* PS circuit, *litR* knock-out mutants should resemble *luxO** mutants; however, *luxO** mutants outcompeted *litR* mutants in prolonged culture and had much poorer host-colonization competitiveness than is reported for *litR* mutants, illustrating additional complexities in this regulatory circuit.

Importance

Our results provide novel insight into the function of LuxO, which is a key component of pheromone-signaling (PS) cascades in several members of the *Vibrionaceae*. Our results also contribute to an increasingly appreciated aspect of bacterial behavior and evolution whereby mutants that do not respond to a signal from like cells have a selective advantage. In this case, although “antisocial" mutants locked in the PS signal-off mode can outcompete parents, their survival advantage does not require wild-type cells to exploit. Finally, this work strikes a note of caution for those conducting or interpreting experiments in *V. fischeri*, as it illustrates how pleiotropic mutants could easily and inadvertently be enriched in this bacterium during prolonged culturing.

Introduction

Many bacteria use pheromones to regulate group behaviors (1). These pheromone-signaling (PS) systems generally require sufficiently high cell density for pheromone accumulation and are also regulated in response to the environment (2-6). Accordingly, PS outputs depend on both population density and an appropriate context. The importance of such control is easily rationalized, given the energetic cost of many PS-activated processes, such as bioluminescence (7, 8). This cost of inducing PS-controlled systems is underscored by the observation that spontaneous signal-blind “antisocial” PS-negative mutants often appear in populations, and it is thought that these mutants are enriched as “cheaters” if their lack of participation in group behavior minimizes their own costs while exploiting the presence of PS-positive relatives (9-11).

Vibrio fischeri is an excellent model for studying PS and was central to the discovery of cell-cell communication in bacteria (12). *V. fischeri* is a bioluminescent light-organ symbiont that that controls luminescence and other phenotypes using three distinct but interconnected PS systems, with signal synthase/receptor combinations LuxI/LuxR, AinS/AinR, and LuxS/LuxPQ (13-18). Luminescence is induced largely by LuxI/LuxR which produces and responds to *N*-3-oxo-hexanoyl homoserine lactone (HSL). However, *luxR* itself is controlled in part by the other two systems, and LuxR can be activated by the AinS-produced pheromone *N*-octanoyl-HSL (C8-AHL). AinS/AinR also controls motility and genes important for initiating colonization of *V. fischeri*'s squid host, *Euprymna scolopes* (18, 19). The LuxS/LuxPQ system, which synthesizes and responds to Autoinducer-2 (AI-2) (20, 21), uses the same core signal-transduction pathway as AinS/AinR. Because LuxS/AI-2 has only modest effects in *V. fischeri* under the conditions tested (17), we have focused more on AinS/AinR.

AinS/AinR control *luxR* and other genes through a core PS circuit (Fig. 3.1) that is conserved in the *Vibrionaceae*. Much of this PS cascade was elucidated in *Vibrio harveyi*, and recent studies have verified parallel functions with subtle differences in *V. fischeri* (17-19, 22-26). In the model that has emerged (Fig. 3.1A-B), at low pheromone concentrations (Fig. 3.1A) AinR phosphorylates LuxU, which in turn phosphorylates the σ^{54}-dependent activator LuxO. LuxO-P activates transcription of a sRNA, Qrr, which post-transcriptionally represses the PS "master regulator" *litR* (24, 26). By contrast, when C8-AHL accumulates to higher levels, its binding to AinR is thought to decrease AinR's kinase activity, allowing AinR's phosphatase activity to dominate, resulting in relatively more unphosphorylated LuxO, deactivation of *qrr*, and induction of the LitR regulon, which

includes *ainSR* and *luxR* (Fig. 3.1B). LitR homologs are widespread "master regulators" of the PS-dependent phenotypes in other members of the *Vibrionaceae* (17, 22, 23). Spontaneous mutations in the *Vibrio cholerae hapR* and *V. parahaemolyticus opaR*, which encode their respective PS master regulators, have been enriched under some conditions (11, 27-29), but no parallel to these observations has been reported in *V. fischeri*.

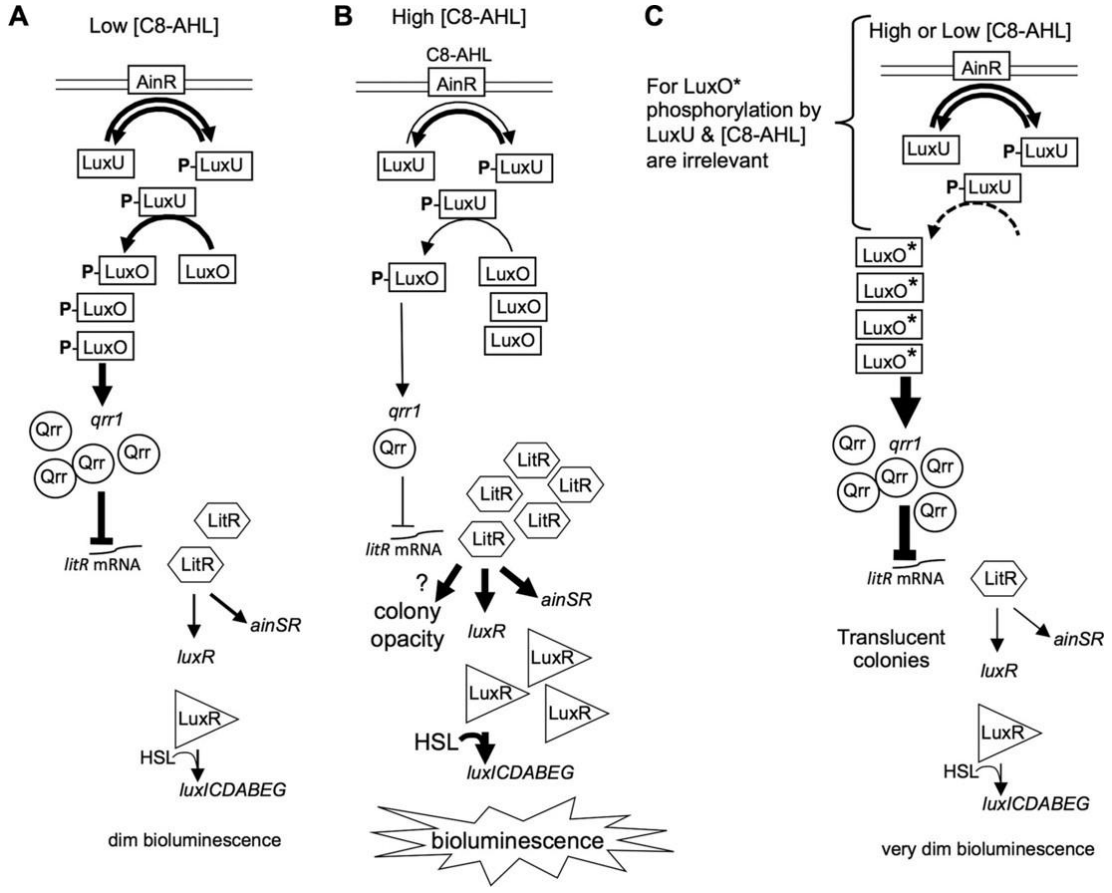

Figure 3.1. Model showing roles of Ain and LuxO in pheromone regulatory circuit. The pheromone receptor AinR phosphorylates and de-phosphorylates LuxU, which phosphorylates LuxO, stimulating it to activate transcription of *qrr*. The regulatory sRNA Qrr represses expression of the "master regulator" LitR. At low C8-AHL levels (panel A), LuxO-P ultimately leads to relatively low LitR levels. At high C8-AHL levels, (panel B), binding of C8-AHL to AinR reduces its kinase activity, reducing the relative amounts of phosphorylated LuxU and LuxO, resulting in more LitR and induction of the LitR regulon including *luxR*, bioluminescence, and *ainSR*. On plates, this induction also results in colony opacity through an unknown mechanism. The activity of LuxO* (panel C) does not require phosphorylation, although prior to this study the effects of LuxU were untested. LuxO* essentially locks this circuit "off", and phenotypes resemble those at low C8-AHL. The colonies of *luxO** mutants on plates remain translucent despite their high cell density.

The *V. fischeri* AinS/AinR system is activated early during colonization of its symbiotic host squid and is responsible for "priming" LuxI/LuxR-based symbiotic luminescence (18). Given that luminescence is only weakly induced outside the host, and that AinS/AinR apparently sits atop the PS signaling hierarchy early in infection, regulatory controls over *ainSR* may reveal important elements of the host environment encountered during symbiosis establishment. Only CRP and LitR are known to activate *ainSR* (17, 22, 30), and the goal of this study was to discover new regulators of *ainSR*. However, we unwittingly isolated spontaneous mutants in the core PS circuitry, providing insight into its function and revealing conditions under which such mutants can be enriched.

Materials and Methods

*Bacteria, growth media, and reagents***.** Bacterial strains are listed and briefly described in Table 3.1. *V. fischeri* ES114 was the wild-type strain used throughout (31). Plasmids were transformed into *Escherichia coli* strain DH5α (32) or DH5α*λpir* (33) in the case of plasmids with the R6K origin of replication. *E. coli* was grown in LB medium (34) or brain heart infusion (BHI) medium (Bacto), and *V. fischeri* was grown in LBS (35), SWTO (36), or FMM (4) medium. Solid media were prepared with 15 g L^{-1} agar. For selection of *E. coli*, chloramphenicol (Cam) and kanamycin (Kan) were added to LB at final concentrations of 20 and 100 μg ml^{-1}, respectively, and erythromycin (Erm) was added to BHI at a final concentration of 150 μg ml^{-1}. For selection of *V. fischeri* on LBS, the concentrations of Cam, Erm, and Kan used were 2, 5, and 100 μg ml^{-1}, respectively. For colorimetric screening of β-galactosidase activity, 5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside (X-gal) was added to LBS at 100 μg ml^{-1}. C8-AHL was obtained from Sigma-Aldrich (St. Louis, MO).

Table 3.1. Bacterial strains, plasmids, and oligonucleotides used in this study.

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
E. coli		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE(Am) recA λpir*	41
DH5α	F⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	32
DH5α λ*pir*	DH5α lysogenized with λ*pir*	33
V. fischeri		
AKD100	ES114 Tn*7*-*ermR*	47
AKD200	ES114 Tn*7*-*camR*	48
CG103	Wild-type isolate from *Cleidopus gloriamaris*	84
CL59	ES114 *luxO* (D47E)	19
DC22	C8-AHL bioreporter: ES114 Δ*ainS* Δ*luxR-luxI*, mutant *luxR* (MJ1-T33A, R67M, S116A, M135I), P_{luxI}-*luxCDABEG*	22
DM131	ES114 *flaJ*::*kanR*	48
EM17	Wild-type isolate from *Euprymna morsei*	85
ES114	Wild-type isolate from *E. scolopes*	31
ES12	Wild-type isolate from *E. scolopes*	86
ES213	Wild-type isolate from *E. scolopes*	86
ES401	Wild-type isolate from *E. scolopes*	85
ET101	Wild-type isolate from *Euprymna tasmanica*	87
H905	Planktonic isolate, Hawaii	76
KV1319	ES114 2-bp insertion in *celG*	51
KV5005	ES114 Δ*rpoN*	43
JB18	ES114 *litR*::*ermR*	22
JHK057	ES114 *luxO* (V106A)	This study
JHK061	ES114 *luxO* (T335I)	This study
JHK062	ES114 *luxO* (V106A) Δ*rpoN*	This study
JHK063	ES114 *luxO* (T335I) Δ*rpoN*	This study
JHK065	ES114 *luxO* (V106A) *luxU*::pEVS122	This study
JHK066	ES114 *luxO* (T335I) *luxU*::pEVS122	This study
JHK068	ES114 *luxU*::pEVS122	This study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
JHK069	ES114 *luxO* (D47E) *luxU*::pEVS122	This study
JHK070	ES114 *luxO* (V106G)	This study
JHK073	ES114 *luxO* (V106A) *litR*::*ermR*	This study
JHK074	ES114 *luxO* (A91D)	This study
JHK075	ES114 *luxO* (P41L)	This study
JHK076	ES114 *luxO* (A91D) *luxU*::pEVS122	This study
JHK087	ES114 *luxO* (V106G) *luxU*::pEVS122	This study
JHK105	ES114 *luxO* (T335I) Δ*qrr1*	This study
NL60	ES114 Δ*ainS*	4
PMF8	ES114 *litR*::*kanR*	23
PP3	Planktonic isolate, Hawaii	76
TIM305	ES114 Δ*qrr1*	24
TIM306	ES114 Δ*luxO*	24
TME014F5	ES114 *mtsA::*miniTn*5*, *ermR*	This study
TME014B6	ES114 *znuC2::*miniTn*5*, *ermR*	This study
TME021D9	ES114 *yfhD::*miniTn*5*, *ermR*	This study
VFS002F6	ES114 *vf0295*::miniTn*5*, *ermR*	C. Whistler
VFS002F6-T[b]	ES114 *vf0295*::miniTn*5 luxO* (A91D), *ermR*	C. Whistler
VFS012E9	ES114 *yfbQ*::miniTn*5*, *ermR*	C. Whistler
VFS012E9-T	ES114 *yfbQ*::miniTn*5 luxO* (P41L), *ermR*	C. Whistler
VFS014F5-T	ES114 *mtsA*::miniTn*5 luxO* (V106A), *ermR*	C. Whistler
VFS014B6	ES114 *znuC2*::miniTn*5*, *ermR*	C. Whistler
VFS014B6-T	ES114 *znuC2*::miniTn*5 luxO* (V106G), *ermR*	C. Whistler
VFS021D9-T[c]	ES114 *yfhD*::miniTn*5 luxO* (T3315), *ermR*	C. Whistler
VLS2	Wild-type isolate from *E. scolopes*	84
WH1	Planktonic isolate, Massachusetts	84
WTTG0	ES114 *luxO* (T107R)	This study
WTTG1	ES114 *luxO* (F94C)	This study
WTTG2	ES114 *luxO* (R114P)	This study
WTTG3	ES114 *luxO* (H324R)	This study
WTTG4	ES114 *luxO* (P98Q)	This study
WTTG5	ES114 *luxO* (R113L)	This study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
WTTG12	ES114 *luxO* (P98L)	This study
WTTG17	ES114 *luxO* (V108G)	This study
WTTG20	ES114 *luxO* (V106G)	This study
WTTG21	ES114 *luxO* (V106G)	This study
WTTG22	ES114 *luxO* (H319R)	This study
WTTG24	ES114 *luxO* (L205F)	This study
Plasmids[d]		
pAKD701	Promoterless *lacZ*, pES213, R6Kγ $oriT_{RP4}$, *kanR*	37
pEVS79	pBC SK (+) *oriT*, *camR*	40
pEVS104	Conjugative helper plasmid; R6Kγ $oriT_{RP4}$ *kanR*	40
pEVS122	R6Kγ, $oriT_{RP4}$, *ermR*, *lacZ*α	33
pEVS170	mini-Tn*5*-*ermR*, R6Kγ $oriT_{RP4}$ *kanR*	50
pHK10	P_{ainS}-*lacZ* reporter in pAKD701; pES213, R6Kγ $oriT_{RP4}$ *kanR*	This study
pHK11	*mtsA* in pVSV104, *kanR*	This study
pHK12	P_{ainS}-*gfp* P_{con}-*mCherry* in pJLS27; pES213, R6Kγ $oriT_{RP4}$ *kanR, camR*	This study
pHK20	P_{qrr}-*lacZ* reporter in pAKD701; pES213, R6Kγ $oriT_{RP4}$ *kanR*	22
pHK29	*znuC2* in pVSV104, *kanR*	This study
pHK45	ES114 *luxO* in pVSV105, *camR*	This study
pHK70	*luxO* (V106A) in pVSV105, *camR*	This study
pHK71	*luxO* (D47E) in pVSV105, *camR*	This study
pHK73	*luxO* (T335I) in pVSV105, *camR*	This study
pHK77	*luxO* (V106A) and flanking sequence in pEVS79, *camR*	This study
pHK78	*luxO* (T335I) and 1.5 kbp flanking sequence in pEVS79, *camR*	This study
pHK79	228-bp *luxU* fragment in pEVS122, *ermR*	This study
pHK80	*luxO* (V106G) and flanking sequence in pEVS79, *camR*	This study
pHK82	*luxO* (V106G) in pVSV105, *camR*	This study
pHK83	*luxO* (A91D) in pVSV105, *camR*	This study
pHK84	*luxO* (P41L) in pVSV105, *camR*	This study
pHK85	*luxO* (P41L) and flanking sequence in pEVS79, *camR*	This study
pHK86	*luxO* (A91D) and flanking sequence in pEVS79, *camR*	This study
pHK87	*luxO* (T335I) and 1.1 kbp flanking sequence in pEVS79, *camR*	This study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
pJLS27	Promoterless *gfp*, P_{con}-*mCherry* pES213, R6Kγ *oriT*$_{RP4}$, *kanR*	38
pLosTfoX	*tfoX* in pEVS79, *camR*	42
pMSM28	Δ*rpoN* allele, R6Kγ *oriT*$_{RP4}$, *camR*	43
pJLB95	*litR*::*ermR* (opposite), ColE1, *camR*	This study
pMulTfoX	*tfoX* in pVSV104, *kanR*	42
pTM267	*kan gfp* P_{tetA}-*mCherry* in pVSV105, *camR*	24
pTM268	P_{qrr}-*gfp* P_{tetA}-*mCherry* in pVSV105, *camR*	24
pVSV104	Shuttle vector; pES213, R6Kγ, *oriT*$_{RP4}$, *kanR*, *lacZ*α	39
pVSV105	Shuttle vector; pES213, R6Kγ, *oriT*$_{RP4}$, *camR*, *lacZ*α	39
Oligonucleotides[e]		
pr_HK03	GGGGCATGCAGAACCAAGACCTGCTCGTGCTAA	This study
pr_HK04	GGCGCTAGCCATCAGTTGTTGAAGTAAATTAAAATTCTGCG	This study
pr_HK05	CATGGTACCATATAGCCGTCTAGATGTAAACATTTCCAAACCG	This study
pr_HK06	CATCCTAGGTTATGGCGCCTCTGTTAAATTAGTACTTTGTTTT	This study
pr_HK07	CATGGTACCAAAATTAATTGTTATGTTATAACATAACAATTAAATAGCC	This study
pr_HK08	CATCCTAGGCATCCAACATTCAGTACACTCCC	This study
pr_HK29	GGCTCTAGACATCAGTTGTTGAAGTAAATTAAAATTCTGCG	This study
pr_HK73	CATGGCATGCATATACCTATTGCAGGGAGCGTGC	This study
pr_HK74	CATGGGTACCCAGCGATTTGATTAACATACTGACTCACGATAG	This study
pr_HK93	CATGGGATCCAGACTATTTATGTCTCAGCCACACC	This study
pr_HK94	CATGGGTACCATCGCCAAATCATGATTGG	This study
pr_HK95	CATGGGATCCTCGGAAGTTGCAGAAGAAGG	This study
pr_HK96	CATGGGTACCTGGTTCCACAGGCCGTATAC	This study
pr_HK101	CATGGGATCCGGGACTATCGTGAGTCAGTA	This study
pr_HK102	CATGGGATCCTGCCATTGTTGCAAGCTTATCT	This study
pr_HK110	CATGGGATCCTGCAAATTGCGTTTTGCG	This study
pr_HK111	CATGGGTACCGCTGTCAAACAAGCGGATTTAATA	This study

[a] Drug resistance abbreviations used: *camR*, chloramphenicol resistance; *ermR*, erythromycin resistance; and *kanR*, kanamycin resistance (*aph*).
[b] Strains designated "VFSXXXXX-T" were isolated from a mapped-transposon mutant library and represent the translucent (-T) *luxO**-bearing derivatives of the original transposon-mutant parent. Strains designated "WTTG" were isolated in a series of independent static culture survival experiments.
[c]VFS021D9-T lacked the Kan-resistance gene from the transposon delivery vector but displayed some Kan resistance.
[d] All alleles cloned in this study are from *V. fischeri* strain ES114. Replication origin(s) of each vector are listed as R6Kγ, ColE1, *oriV* and/or pES213. Plasmids based on pES213 are stable and do not require antibiotic selection for maintenance (39).
[e] All oligonucleotides are shown 5' to 3'. Restriction enzyme recognition sequences are underlined.

*Molecular genetics and sequence analysis***.** Oligonucleotides and plasmids are listed in Table 1, and the latter were constructed using standard techniques and materials as described previously (22). Genomic DNA for genome resequencing and natural transformation was purified using the Easy-DNA gDNA Purification kit (Life Technologies, Grand Island, NY).

The P_{ainS}-*lacZ* transcriptional reporter plasmid pHK10 was generated by PCR amplifying 428 bp upstream of *ainS* using primers pr_HK03 and pr_HK04, digesting the resulting amplicon with SphI and NheI, and cloning this fragment between the SphI and NheI sites of pAKD701 (37). To generate the P_{ainS}-*gfp* transcriptional reporter, pHK12, the same promoter region used in pHK10 was amplified using primers pr_HK03 and pr_HK29, digested with SphI and XbaI, and ligated into similarly digested pJLS27 (38). To generate pHK45, pHK70, pHK71, pHK73, pHK82, pHK83, and pHK84, *luxO* was amplified from ES114, VFS014F5-T, CL59 (19), VFS021D9-T, VFS014B6-T, VFS002F6-T, and VFS012E9-T, respectively, using primers pr_HK73 and pr_HK74. The resulting amplicons were digested with SphI and KpnI and ligated into SphI- and KpnI-digested pVSV105 (39). To generate pHK11, *mtsA* was amplified from ES114 using primers

pr_HK05 and pr_HK06. The resulting amplicon was digested with KpnI and AvrII and ligated into KpnI- and AvrII-digested pVSV104 (39). To generate pHK29, *znuC2* was amplified from ES114 using primers pr_HK07 and pr_HK08. The resulting amplicon was digested with KpnI and AvrII and ligated into KpnI- and AvrII-digested pVSV104.

Mutant alleles were transferred from *E. coli* into *V. fischeri* on plasmids by triparental matings using the conjugative helper strain CC118λ*pir* pEVS104 (40, 41). Recombination and marker exchange were identified by screening for antibiotic resistance, and putative mutants were tested by PCR. Transposon insertions were placed in new strain backgrounds using competence induced by overexpression of *tfoX* from pMulTfoX or pLosTfoX, followed by plasmid curing, as previously described (42). Strains TME014F5, TME014B6, and TME021D9 were generated using DNA from transposon mutants VFS014F5-T, VFS014B6-T, and VFS021D9-T, respectively, using *tfoX*-mediated transformation (42). To place spontaneous *luxO* mutations in fresh strain backgrounds, 1.5-kbp regions flanking the mutation sites were PCR amplified using primer sets pr_HK93 and pr_HK94 (P41L, A91D, V106A, and V106G alleles) or pr_HK95 and pr_HK96 (T335I allele), respectively. Amplicons were digested with KpnI and BamHI and ligated into similarly digested pEVS79 (40) to create pHK77 (*luxO* V106A), pHK78 (*luxO* T335I), pHK80 (*luxO* V106G), pHK85 (*luxO* P41L), and pHK86 (*luxO* A91D). Strains JHK057, JHK061, JHK070, JHK074, and JHK075 were generated by exchanging the mutated *luxO* variants on pHK77, pHK78, pHK80, pHK85, and pHK86, respectively, into ES114. To generate the *luxO** Δ*qrr1* double mutant, 1.1-kbp regions flanking the mutation site of *luxO** (T335I) were PCR amplified using pr_HK110 and pr_HK111. This amplicon was digested with KpnI and BamHI and ligated into similarly digested pEVS79 to create

pHK87. The *luxO* allele on pHK87 is smaller than that of pHK78 and does not overlap the *qrr1* sequence. Strain JHK105 was generated by exchanging the mutated *luxO* variant on pHK87 in the Δ*qrr1* strain TIM305.

To generate *luxU* mutants, an internal 228-bp fragment of *luxU* was amplified using primers pr_HK101 and pr_HK102. The resulting amplicon was digested with BamHI and ligated into BamHI-digested pEVS122 (33) to generate pHK79. The *luxU*::pEVS122 allele of pHK79 was introduced into JHK057, JHK061, ES114, CL59, JHK070, JHK074, and JHK075 to generate strains JHK065, JHK066, JHK068, JHK069, JHK087, JHK076, and JHK077, respectively. To generate *rpoN* mutants, the Δ*rpoN* allele on plasmid pMSM28 (43) was introduced into strains JHK057 and JHK061 to generate strains JHK062 and JHK063, respectively. To generate the *luxO* litR*::*erm* double mutant, the *litR*::*erm* allele on pJLB95 (22) was introduced into JHK057 to generate strain JHK073.

*Genome resequencing***.** 10 μg gDNA was fragmented by sonication in iced TE buffer five times for 1 minute with 1 sec pulses at 40% duty using a W-380 ultrasonic processor (Heat System-Ultrasonics, Inc.) to produce ~200-bp fragments for Illumina library construction following the Illumina Tru-seq manufacturer's protocol (San Diego, CA) except that genomic DNA adaptors were diluted 1:100 prior to ligation. Sequencing was performed using an Illumina Hi-Seq 2000 Genome Analyzer at the University of Missouri DNA Core Laboratory. Assembled reads were compared to the resequenced and published ES114 genomes (44, 45) as references using the Integrative Genomics Viewer (46).

*Luminescence measurements***.** Overnight *V. fischeri* cultures were diluted 1:1000 in 25 ml SWTO in 125-ml flasks and incubated with shaking (200 rpm) at 24°C. At regular

intervals, the optical density at 595 nm (OD_{595}) was measured for 500-µl samples using a BioPhotometer (Brinkman Instruments, Westbury, NY). Relative luminescence was measured with a TD-20/20 luminomenter (Turner Designs, Sunnyvale, CA) immediately following shaking to aerate the sample. Specific luminescence was calculated as the luminescence per OD_{595}.

lacZ *and* gfp *reporter assays.* Strains harboring the P_{ainS}-*lacZ* or P_{qrr}-*lacZ* reporter plasmids pHK10 and pHK20 (22), respectively, or the promoterless parent vector pAKD701, were grown overnight in LBS, subcultured 1:300 into 1.5 ml of SWTO in 24-well microtiter plates and incubated with shaking (200 rpm) at 24°C. Cells were collected at OD_{595} ~2.5 by centrifugation, the supernatant was discarded, and the cell pellet was stored overnight at -80°C. β-galactosidase assays were then performed as previously described (7). Strains harboring the P_{ainS}-*gfp* or P_{qrr}-*gfp* reporter plasmids pHK12 and pTM268 (24), respectively, or the promoterless parent vectors pJLS27 and pTM267 were grown overnight in LBS and subcultured 1:1000 into flasks containing 25 ml SWTO and incubated with shaking (200 rpm) at 24°C. At regular intervals, 200-µl samples were aliquoted into clear-bottomed, black-walled, 96-well plates, where green fluorescence and OD_{595} were measured using a Synergy 2 plate reader (BioTek).

Motility assays. Motility was determined by diluting overnight cultures 1:1000 in SWTO and growing to an OD_{595} ~0.5. Five-µl aliquots of culture were then spotted on the surface of 0.25% agar FMM plates containing 2.2 mM *N*-acetylglucosamine (19). The diameters of areas visibly covered by swimming cells were measured after 24 hr of incubation at 28°C. The Erm-resistant ES114-derivative AKD100 and ES114 *flaJ*::*aph* strain DM131 were used as positive and negative motility controls, respectively (47, 48).

*C8-AHL bioassays***.** C8-AHL accumulation was assessed as previously described (22). Briefly, culture supernatants were extracted with acidified ethyl acetate, extracts were dried and resuspended in SWTO, and C8-AHL levels were determined by comparison to standards using the bioassay strain DC22 (22, 49)

*Transposition frequency***.** The frequency of recovering transposon mutants following conjugative introduction of pEVS170 (50) was determined for ES114 and *luxO** mutant JHK057, each mixed 1:1 with the *celG* mutant KV1319, which is phenotypically wild type except that it can be readily distinguished as white colonies on LBS plates containing 10 mM D-cellobiose and X-gal (51). Performing Tn mutagenesis on 1:1 mixtures of ES114:KV1319 or JHK057:KV1319 enabled us to normalize values to transposition frequency in KV1319 and to test whether a *luxO** mutant such as JHK057 could be enriched from a mixed population during transposon mutagenesis. Conjugation spots were incubated at 28°C for six hours before being resuspended in 500 µL Instant Ocean (Aquarium Systems, Mentor, OH), dilution-plated to determine recipient strain ratios, and then stored overnight at -80º C in glycerol stocks. Stocked conjugations were thawed at room temperature and plated on LBS containing D-cellobiose, X-gal, and Erm to select transposon-containing strains and to use blue/white screening to distinguish each mutant's parent. Erm-resistant colonies were patched on Kan plates to eliminate mutants with the Kan marker outside the transposon on pEVS170.

*Viability in static culture***.** To monitor survival rates of *V. fischeri* strains singly, overnight *V. fischeri* cultures were inoculated into LBS and grown to OD_{595} ~2.5 to simulate the inoculum density present in the construction of the transposon mutant library. These cultures were then diluted 1:1,000 in fresh LBS and grown statically in 24-well

microtitre plates at 28°C. CFU were determined for the initial inoculum and after 24, 48 and 96 hrs. To determine mixed-culture viability, strains were grown separately as described above, and at OD_{595} ~2.0, cultures were mixed in ratios of 1:1, 10:1, or 100:1 and co-cultured until OD_{595} ~2.5, at which point they were subcultured, grown and sampled as described above. Strains were differentiated by colony opacity (ES114 vs. *luxO** or *litR*::*ermR*), Erm resistance (*luxO** vs. *litR*::*ermR* or *luxO* litR*::*erm*), or Cam resistance (ES114 *camR* vs. *luxO** or *luxO**Δ*qrr1*).

*Squid colonization***.** Competitive colonization of *E. scolopes* hatchlings was determined as previously described (51). Briefly, hatchlings were exposed to a ~1:1 mixture of strains for 12 hours, and the ratio of colonizing strains was determined 48 hours after initial exposure to the inoculum. The relative competitive index (RCI) was determined by dividing the final mutant to wild type ratio by the ratio of the strains in the inoculum. Unmarked *luxO** mutants were competed against the CamR-marked *V. fischeri* strain AKD200, which has wild-type colonization competitiveness (48, 52).

Results

In an attempt to identify regulators of *ainSR*, we used blue/white screening on X-gal plates to test activity of a P_{ainS}-*lacZ* reporter in a library of *V. fischeri* transposon mutants. Collectively, this library has representative mutants with insertions in approximately 2100 distinct genes (Randi Foxall and Cheryl Whistler, personal communication). Mutants VFS021D9-T, VFS014B6-T, and VFS014F5-T, with Tn insertions in *yfhD* (VF_A0984), *znuC2* (VF_2367), and *mtsA* (VF_1566), respectively, had

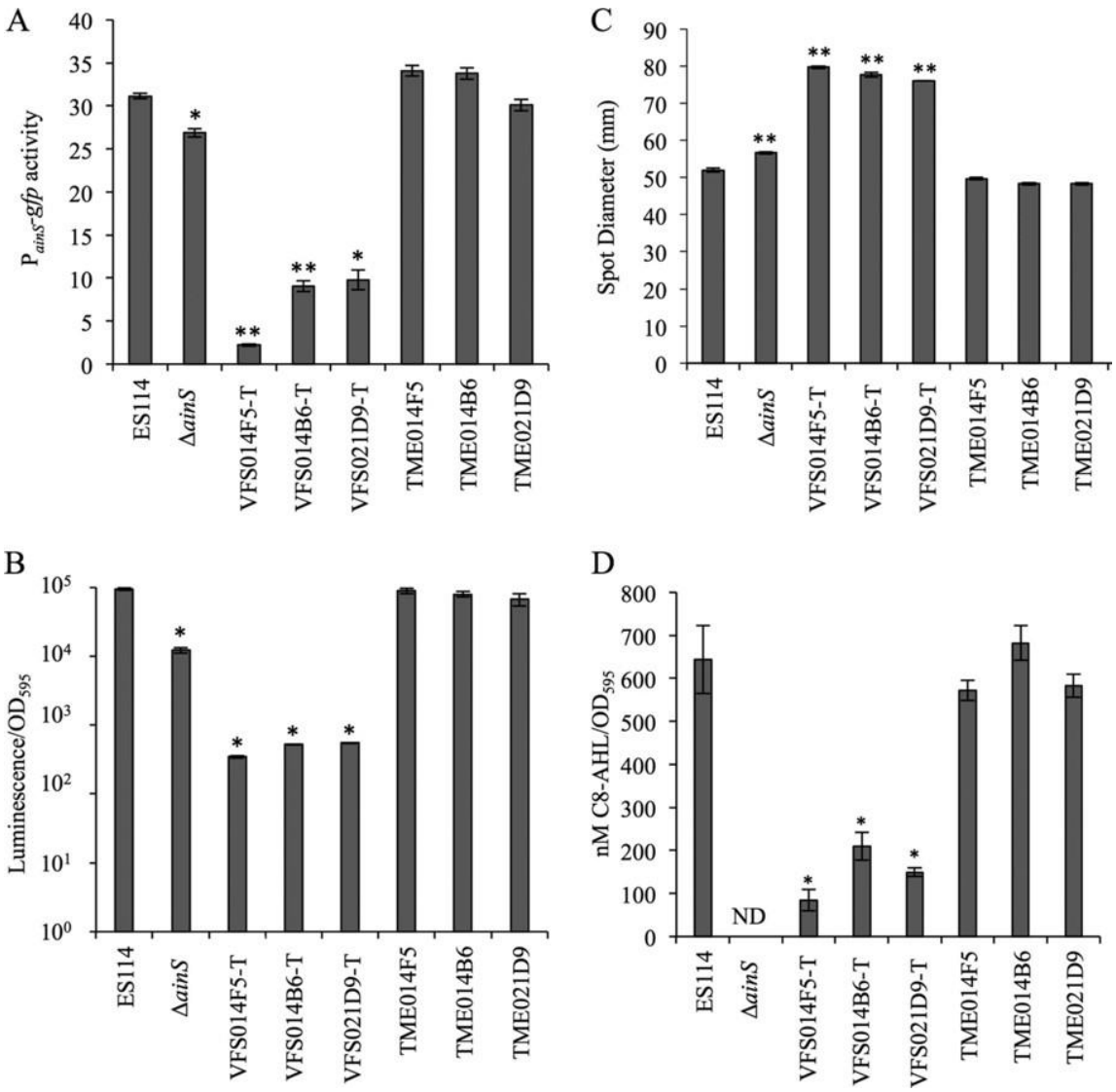

Figure 3.2. Transposon library mutants (VFS strains), but not their backcrossed-Tn counterparts (TME strains), have phenotypes consistent with deficient *ainS* pheromone signaling. (A) P_{ainS}-*gfp* transcriptional reporter activity in cultures grown in SWTO to OD_{595} ~2.5. (B) Peak specific luminescence of cultures grown in SWTO. (C) Strain motility as measured by increase in spot diameter over 24 hr. (D) C8-AHL extracted and bioassayed from cultures grown in SWTO. ND, none detected. In each panel, data are from single representative experiments of three independent experiments. Error bars on all panels indicate standard errors (n = 3). Asterisks represent significant differences from ES114 (* $P < 0.01$, ** $P < 0.001$).

markedly lower P_{ainS}-*lacZ* reporter activity. The Tn insertions from these three mutants were reintroduced into the ES114 wild-type background and compared to the original mutants, ES114, and Δ*ainS* mutant NL60, with respect to P_{ainS}-*gfp* reporter activity and three other phenotypes associated with loss of *ainS*; (i) decreased luminescence in broth,

(ii) increased swimming motility, and (iii) lowered production of C8-AHL (18, 19). VFS021D9-T, VFS014B6-T, and VFS014F5-T displayed decreased P_{ainS}-*gfp* expression (Fig. 3.2A), relatively dim luminescence (Fig. 3.2B), hypermotility (Fig. 3.2C), and low C8-AHL output (Fig. 3.2D); however, none of these phenotypes were associated with the Tn insertions backcrossed into ES114 (Fig. 3.2). Moreover, wild-type copies of *mtsA* and *znuC2* provided *in trans* failed to complement the mutant phenotypes of strains VFS014F5-T and VFS014B6-T (data not shown), further indicating that the Tn insertions in these mutants were not causal to the *ainS*-related phenotypes.

The genome sequences of VFS014F5-T, VFS014B6-T, and VFS021D9-T confirmed their respective transposon locations and also revealed that each mutant has a unique point mutation in *luxO,* resulting in V106A, V106G, and T335I LuxO variants (Table 2.2). No other deviations from the wild-type sequence were discovered in the mutants. Given the current model of PS signaling (Fig. 3.1), the phenotypes of these mutants would be consistent with constitutive LuxO activity, leading to high Qrr expression, low LitR levels, and decreased activation of *ainSR*. A *luxO* mutant with a D47E allele that effectively mimics phosphorylated LuxO shows similar phenotypes (17, 53). The mutants also displayed translucent colony morphology similar to that of *litR* mutants, and we ultimately appended their strain names with the suffix "-T" to indicate "Translucent" variants. As discussed below, we eventually discovered that there were corresponding Tn-library mutants that were not translucent, and adopting this stain nomenclature

Table 3.2: Summary of *luxO** mutants described in this study

Strain	*luxO** mutation	LuxO* amino acid substitution	*luxO** crossed into ES114	*luxO** function assessed in *trans*[a]
VFS002F6-T	C272A	A91D	X	X
VFS012E9-T	C122T	P41L	X	X
VFS014F5-T	T317C	V106A	X	X
VFS014B6-T	T317G	V106G	X	X
VFS021D9-T	C1004T	T335I	X	X
WTTG0	C320G	T107R		
WTTG1	T281G	F94C		
WTTG2	G341C	R114P		
WTTG3	A971G	H324R		
WTTG4	C293A	P98Q		
WTTG5	G338T	R113L		
WTTG12	C293T	P98L		
WTTG17	T323G	V108G		
WTTG20	T317G	V106G		
WTTG21	T317G	V106G		
WTTG22	A956G	H319R		
WTTG24	C613T	L205F		

[a] *luxO** genes were cloned into the low-copy vector pVSV105, and their ability to affect *ainS*-mediated phenotypes in the Δ*luxO* strain TIM306 was confirmed (24).

distinguishes between, for example, original library mutant VFS014F5 (*mtsA*::Tn) and what is presumably its derivative VFS014F5-T (*mtsA*::Tn *luxO* V106A).

To test whether the *luxO* alleles have the predicted downstream effect on the core PS circuit, we cloned the spontaneous *luxO* point mutations from the Tn-mutant backgrounds, moved them into ES114 by allelic exchange, and compared P_{qrr}-*gfp* activitiesin the resulting strains and the original mutants. Consistent with the model in Fig. 3.1, the mutants encoding V106A, V106G, or T335I LuxO variants, as well as a previously described mutant encoding the LuxO D47E variant, yielded higher P_{qrr}-*gfp* expression than wild type (Fig. 3.3). By contrast, strains where the Tn insertions had been backcrossed into ES114, with wild-type *luxO*, displayed wild-type expression of P_{qrr}-*gfp* (Fig. 3.3).

Thus, the *luxO* mutations and not the Tn insertions were causal to this mutant phenotype. Below, mutant alleles that apparently encode constitutively active LuxO are designated *luxO**, consistent with the nomenclature in previous reports of similar alleles in other vibrios (54). We explored whether LuxO* variants bypass elements of the PS regulatory circuitry (Fig. 3.1), first by testing whether the *luxO** mutants were still sensitive to C8-AHL. As shown in Figure 3.1, transcription of *qrr* should decrease as C8-AHL levels increase. To test whether *luxO** mutants also responded to C8-AHL, we assayed the activity of a P_{qrr}-*gfp* transcriptional reporter in wild type and five *luxO** mutants (some of which were isolated in experiments described below). Supplementation of cultures with C8-AHL resulted in a significant decrease in P_{qrr}-*gfp* activity in ES114 but not in any of the *luxO** mutants (Fig. 3.4), demonstrating that they are blind to addition of the C8-AHL signal.

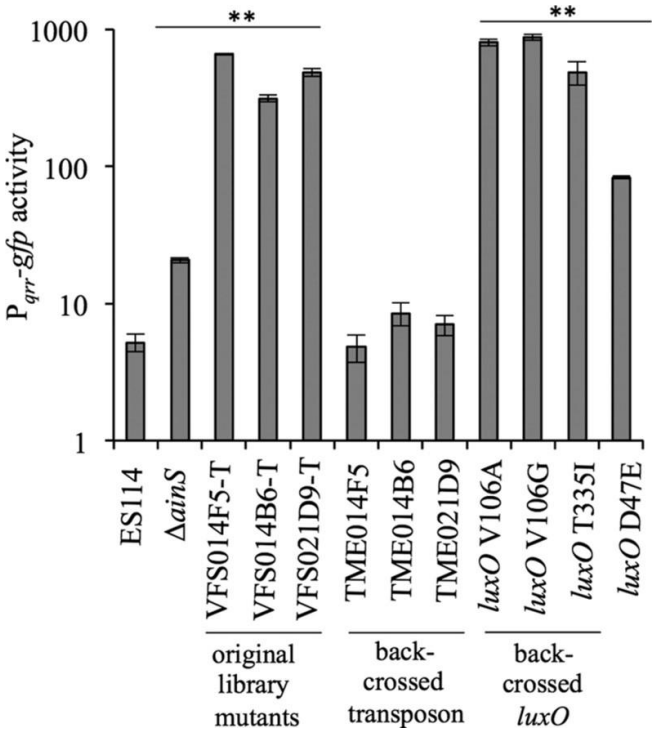

Figure 3.3. P_{qrr}-*gfp* transcriptional reporter activity is elevated in strains with *luxO** mutations. GFP activity expressed from strains harboring the P_{qrr}-*gfp* reporter on pTM268, grown in SWTO, and assayed at OD_{595} ~2.5. Data are from a single representative experiment of three independent experiments. Error bars indicate standard error (n = 3). ** indicates strains were significantly different from wild-type ES114 (P< 0.001).

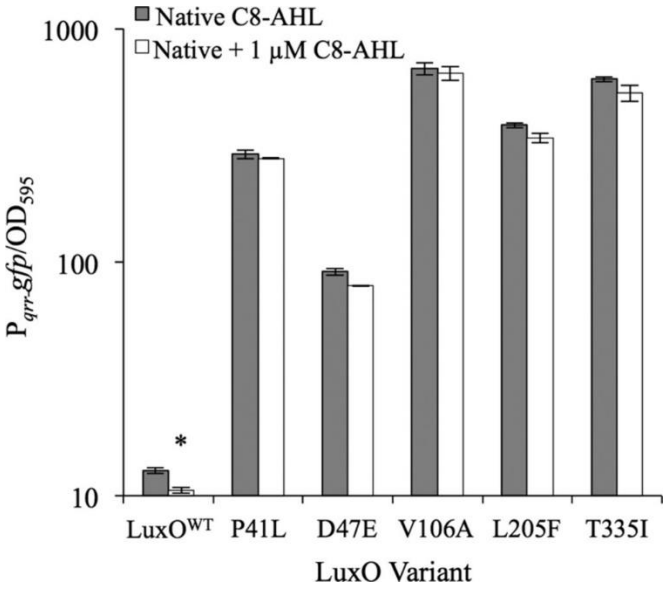

Figure 3.4. Strains bearing constitutively active LuxO* variants are insensitive to C8-AHL. GFP expression in strains ES114, JHK075 (LuxO P41L), CL59 (LuxO D47E), JHK057 (LuxO V106A), WTTG24 (LuxO L205F), and JHK061 (LuxO T335I) harboring the P_{qrr}-*gfp* reporter plasmid pTM268, grown in SWTO with and without 1 µM added C8-AHL, assayed at OD_{595} ~2.0. Data are from a single representative experiment of three independent experiments. Error bars indicate standard error (n = 2). Asterisks indicate significantly different reporter activity for a particular strain when C8-AHL is added, as determined using Student's t-test. * P< 0.05.

We further examined whether LuxO* variants are independent of the core PS circuitry (Fig. 3.1) by examining whether their activity required *luxU* or *rpoN*, which encode LuxU and σ^{54}, respectively. LuxU is the only known phosphoryl-donor for LuxO, which in turn activates *qrr* as a σ^{54} enhancer (Fig. 3.1). To test the role of *luxU*, we introduced a *luxU*::pEVS122 allele that inactivated LuxU function, as indicated by the bright luminescence of the ES114-derived *luxU*::pEVS122 strain JHK068 (data not shown). Even with *luxU* disrupted, the three *luxO** mutations described above, as well as the D47E mutant described previously, increased P_{qrr}-*gfp* expression (Fig. 3.5) and decreased P_{ainS}-*gfp* expression (data not shown). By contrast, deletion of *rpoN* eliminated the effect of the *luxO** V106A and T335I variants and phenocopied the ES114 Δ*rpoN* strain KV5005 (data not shown; 43). Thus, as discussed below, at least for the *luxO** alleles tested, their activity is independent of LuxU but remains dependent on σ^{54}.

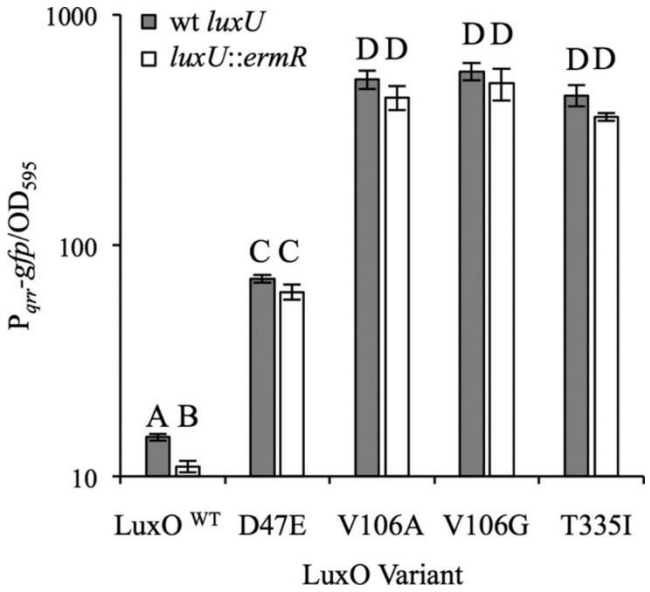

Figure 3.5. The inactivation of *luxU* does not eliminate the effect of different *luxO* variants on P_{qrr}-*gfp* reporter activity. GFP expression in strains harboring the P_{qrr}-*gfp* transcriptional reporter pTM268, grown in SWTO, and assayed at OD_{595} ~2.5. Uppercase letters shared between bars indicate no statistically significant difference ($P > 0.05$), whereas different letters indicate significant difference ($P < 0.01$), based on a one-way analysis of variance (ANOVA) and *post hoc* testing using Student's *t*-test. Data are from a single representative experiment of three independent experiments. Error bars indicate standard errors ($n = 3$).

After evaluating the characteristics of the newly discovered *luxO** variants, we were curious about their relatively high representation in the transposon mutant library. Gain-of-function mutations resulting in constitutively active LuxO were recovered from three mutants in a library of less than three thousand, which seems remarkably frequent. Moreover, such *luxO** mutations presumably ought to happen far less frequently than simple loss-of-function mutations in *litR*, yet based on our simplified working model (Fig. 3.1) both should theoretically have similar phenotypic characteristics. We therefore hypothesized that *luxO** mutations provided some benefit during the Tn library construction, storage, and/or propagation, thereby resulting in their enrichment. Furthermore, we propose that this *luxO** phenotype must be different than that of *litR* loss-of-function mutants.

To test this hypothesis, we assessed the *luxO** V106A strain JHK057 for its ability to out-compete wild type under conditions mimicking library development. For example, we tested relative viability after freezing overnight at -80º C, and the frequency of transposon insertion following conjugative transfer of pEVS170 in a mixed culture. Only in the latter assay did we see a difference in the *luxO** mutant (Fig. 3.6); however, this difference was relatively minor, and our results actually suggest that *luxO** mutants are somewhat poorer recipients for conjugation-based Tn mutagenesis, and if anything should be modestly enriched against as parents for Tn mutagenesis.

We discovered evidence that the spontaneous *luxO** mutations occurred after Tn mutagenesis. The well occupied by VFS014B6 in the 96-well plates housing the mutant library contained a mix of translucent and opaque colony forming units. PCR and sequencing revealed that all colonies from this well contained the Tn insertion, but only the translucent colonies carried the *luxO** mutation. Two other Tn mutants, VFS002F6 (*luxO** A91D) and VFS012E9 (*luxO** P41L), were similarly stocked as a mix of *luxO** and wild-type *luxO* strains in a uniform Tn-mutant background. Each of these *luxO** mutants (Table 3.2) possesses phenotypes similar to those described above for the initial *luxO** mutants, and these *luxO** alleles are similarly independent of *luxU* (data not shown). Additionally, a mixture of opaque and translucent colonies in the well containing VFS014F5, but not the other Tn mutants, was discovered in the master Tn library, a copy of which was sent to our lab (Randi Foxall and Cheryl Whistler, personal communication). Taken together our results suggested the *luxO** mutants had arisen after transposon insertion, that freezing did not favor these mutants, and that the enrichment of *luxO** mutants relative to their parent strains likely resulted during library outgrowth.

To test whether *luxO** mutants could have been enriched during growth and storage of the Tn-mutant library, we grew ES114 and JHK057 (*luxO* V106A) during prolonged incubation in static cultures of LBS medium. Each strain initially grew similarly and reached cell numbers (CFU/ml) at 24 hours that were not significantly different from one another ($P > 0.05$); however, from 24 to 96 hours ES114 suffered greater decreases in CFU (data not shown). Moreover, translucent colonies began to appear in ES114-inoculated wells after 48 hrs and eventually dominated ES114-inoculated cultures. Most of these new spontaneous translucent mutants harbored *luxO** alleles (see strains with "WTTG" prefix, Table 3.2); however, a small subset possessed characteristics similar to *luxO** mutants while having wild-type *luxO* sequences. Interestingly, another subset of translucent mutants maintained wild-type P_{qrr}-*gfp* activity, suggesting there are multiple paths to translucent colony morphology and static-culture survival, including at least one independent of the central PS network.

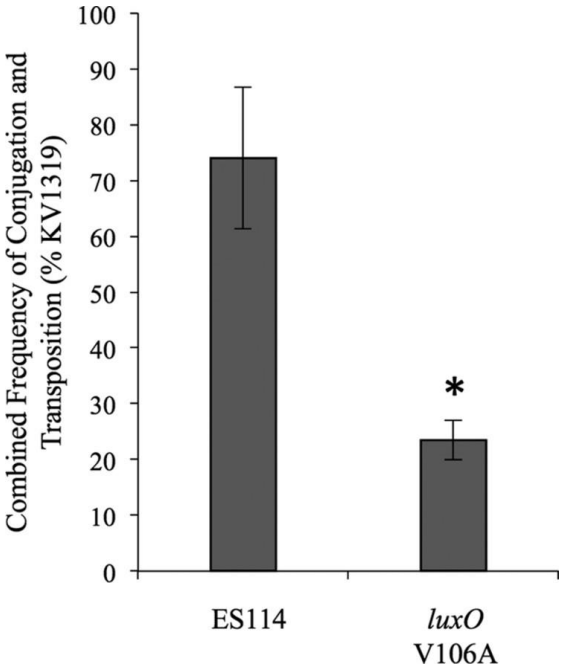

Figure 3.6. A *luxO** mutation does not enhance frequency of conjugation-based transposon mutagenesis. The transposon-containing vector pEVS170 was conjugated into mixed recipient cultures of KV1319 (*celG*) combined ~1:1 with either JHK057 (*luxO* V106A) or wild type. Following conjugation, non-selective plating determined the ratio of recipient strains, and plating on Erm was used to determine the number of Tn mutants. By including 10 mM D-cellobiose and X-gal, we differentiated recipients as either blue (JHK057 or ES114) or white (KV1319). Data are from one representative experiment of three independent experiments. Error bars indicate standard error ($n = 5$). * indicates a significant difference in the recovery of Tn mutants relative to KV1319 ($P < 0.01$).

We next competed strains to approximate the conditions under which a *luxO** mutant might be enriched after arising in a wild-type culture. When JHK057 (*luxO* V106A)

was grown with ES114 in static co-culture at initial ES114:mutant ratios of 1:1, 10:1, or 100:1, the ratio of JHK057 to ES114 had increased as much as 10-fold after 24 hrs (Fig. 3.7 A-D). This change in strain ratios was most dramatic in cultures where JHK057 had initially been the most outnumbered (Fig. 3.7 A-D). Moreover, the CFU/mL of the *luxO** mutant actually increased even during periods when the CFU/mL of ES114 dropped. In each case, the final strain ratio reflected a similar small numerical advantage for the *luxO** mutant, regardless of how outnumbered it was in the initial inoculum (Fig. 3.7D). We observed a similar *luxO**-dependent advantage for JHK057 and JHK061 (*luxO* T335I) when these strains were competed against the Cam-marked ES114-derivative AKD200 (data not shown).

The advantage of *luxO** mutants appeared to be dependent on prolonged stationary-phase culturing. When cultures were kept in log phase ($OD_{595} < 0.5$) for thirty generations by repeated subculturing, translucent derivatives did not appear in clonal wild-type cultures, and in mixed competitions the *luxO** mutant was not enriched relative to the wild type (data not shown).

Given that *luxO** mutants should lead to strong repression of *litR* (Fig. 3.1), and that both *litR* and *luxO** mutants share a common translucent colony phenotype, we considered the possibility that *litR* mutants would have the same advantage relative to ES114 in mixed cultures; however, this was not the case (data not shown). Although translucent colonies began to dominate mixed cultures of ES114 and the *litR* mutant, upon closer examination most translucent colonies lacked the erythromycin resistance of the *litR*::*ermR* mutant and were instead spontaneous mutants of ES114. These results, along with those demonstrating differences in growth and luminescence in shaking culture and

P_{qrr}-*gfp* reporter activity (data not shown), indicated *luxO** and *litR* mutants do not phenocopy.

To further explore the difference between *luxO** and *litR* mutants, we competed JB18 (*litR*::*ermR*) and JHK057 (*luxO* V106A) in static cultures. Here too, the *luxO** mutant dominated when mixed with *litR* mutants (Fig. 3.7 E-H). Similar to its competition with ES114, JHK057 outcompeted the *litR* mutant at all three initial strain ratios, and the experiments ended at relatively similar strain ratios, with a slight dominance by the *luxO** mutant, regardless of the starting ratio (Fig. 3.7 D and H). Similarly, the *luxO** *litR* mutant JHK073 outcompeted the *litR* mutant PMF8 (data not shown) (23). Taken together, the data indicated that in prolonged static broth LBS culture, a *luxO** mutant can outcompete a *litR* mutant, and that a competitive advantage for a *luxO** mutant does not require *litR*. We next tested the *litR* mutant JB18 and the Δ*qrr1* mutant TIM305 for their abilities to give rise to spontaneous *luxO** mutants when grown in static culture. We grew strains carrying the P_{qrr}-*lacZ* reporter pHK20 in static culture for up to 96 hr, periodically plating on LBS containing X-gal, and screening for blue colonies, but were unable to isolate any mutants with increased P_{qrr}-*lacZ* activity, indicating that *litR* and *qrr* are required for cells carrying a *luxO** mutation to arise and dominate cultures. In the case of *litR*, this result contrasts with the observation above that a *luxO** *litR* mutant could outcompete a *litR*

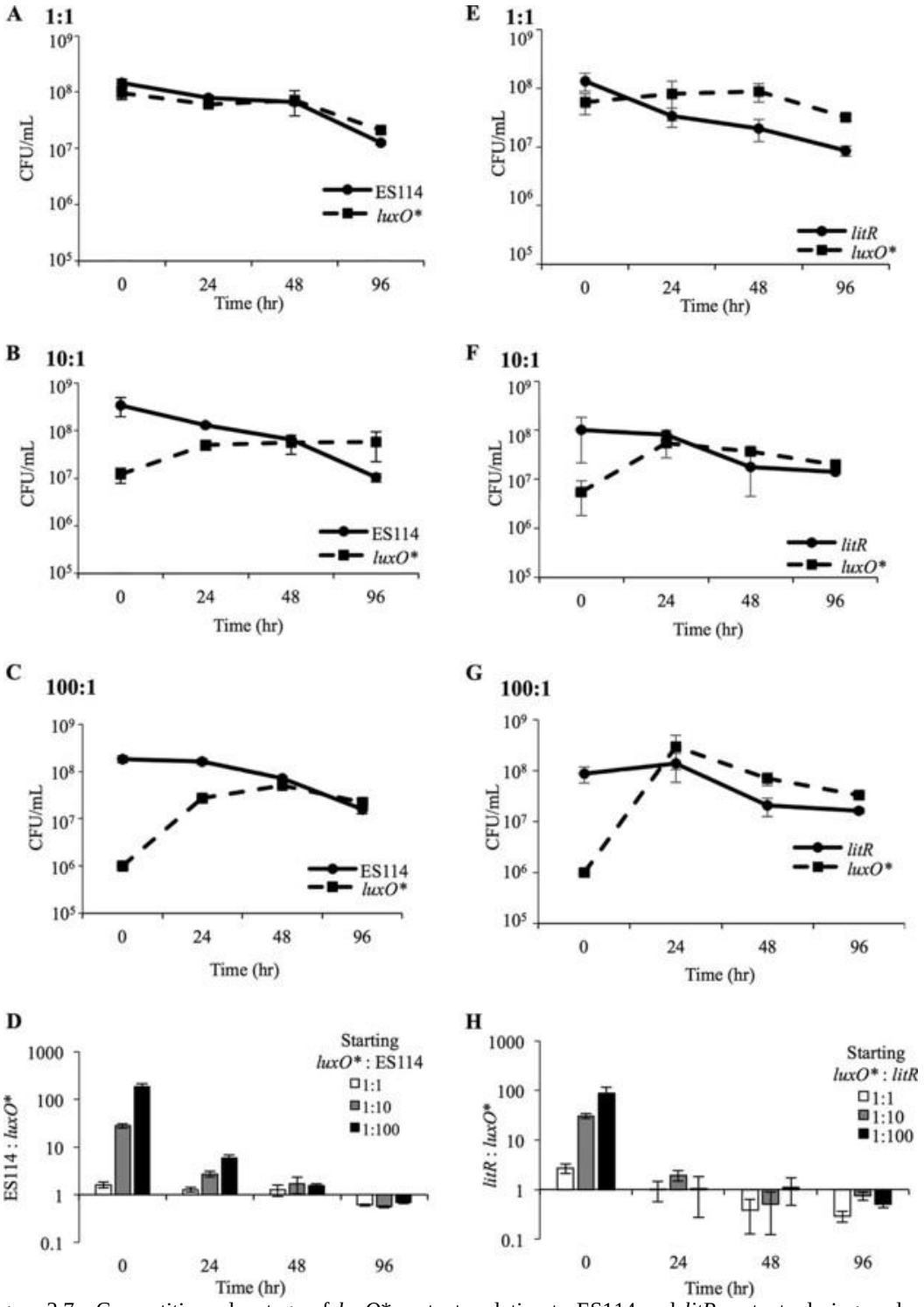

Figure 3.7. Competitive advantage of *luxO** mutants relative to ES114 and *litR* mutants during prolonged co-culture. ES114 (panels A-D) or *litR* mutant JB18 (panels E-H) were mixed with JHK057 (*luxO* V106A) in ratios of 1:1, 10:1, or 100:1 with JHK057 always at a disadvantage. Mixes were diluted in 24-well microtitre plates and grown statically for 96 hr. At intervals of 0, 24, 48, and 96 hr, wells were thoroughly mixed and dilution plated to determine the ratios of viable CFU for each strain in the mixture based on translucence (JHK057 vs. ES114) or Erm resistance (JHK057 vs. JB18). Data are from a single representative experiment of three independent experiments. Error bars indicate standard error (n = 3).

mutant when they were co-inoculated (data not shown). However, we were interested in whether the *luxO** survival advantage acts through Qrr, as *qrr* is the only described regulatory target of LuxO. We competed AKD200 against JHK105 (*luxO* T335I Δ*qrr1*) and found that the initial strain ratios were maintained through 96 hr (Fig. 3.8). As in other experiments, translucent mutants began to appear within 48 hr, but were solely derived of AKD200 (data not shown).

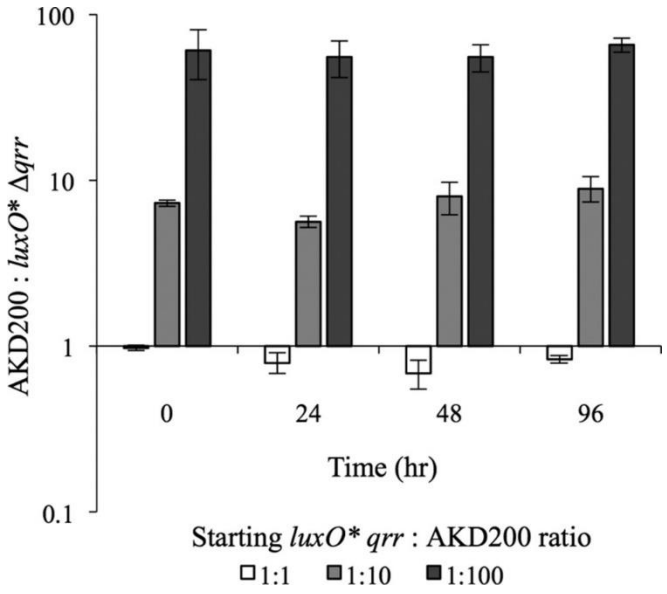

Figure 3.8. Qrr is required for a *luxO**-mediated survival advantage. Cam-marked ES114 (AKD200) and JHK105 (*luxO* T335I Δ*qrr*) were grown overnight and subcultured into fresh LBS medium until reaching an OD_{595} ~2.0 at which time they were mixed in ratios of 1:1, 1:10, or 1:100 with JHK061 always at a disadvantage. Mixes were outgrown to OD_{595} ~2.5 and then diluted 1:1000 in fresh LBS medium in 24-well microtitre plates grown statically for 96 hr. At intervals of 0, 24, 48, and 96 hr, wells were thoroughly mixed and dilution plated to determine the ratios of viable cells of each mixture by Cam resistance. Data are from a single representative experiment of two independent experiments. Error bars indicate standard error (n = 3).

Mutant JHK061 (*luxO** T335I) also does not phenocopy with a *litR* mutant with respect to symbiotic colonization. Whereas *litR* mutants outcompete wild-type during colonization of *E. scolopes* (23), JHK061 was significantly (P < 0.001) outcompeted by the Cam-marked ES114-derivative AKD200 during host infection and colonization (Fig. 3.9). This competitive defect of the *luxO** mutant does not stem from differential survival of the inoculum in artificial seawater (data not shown). These results are consistent with the attenuated colonization previously reported for the *luxO* D47E mutant CL59 (19), but

appear to be more severe, potentially owing to the higher relative activity of the *luxO** T335I allele in JHK061 compared to the *luxO** D47E allele in CL59 (Fig. 3.3).

Given the identification of *luxO** alleles other than D47E, we were curious if certain sites were more prone to mutation than others and/or if additional allelic variants were possible. Using the strategy of static-culture *luxO** enrichment, we grew twenty-six independent cultures for 48 hrs, dilution plated, and picked translucent colonies for *luxO* sequencing. In all, ten more *luxO** alleles were identified (F94C, P98L, P98Q, T107R, V108G, R113L, R114P, L205F, H319R, and H324R) and *luxO** V106G was recovered two more times in our screen (Fig. 3.10). Taken together, alleles were distributed across LuxO, with the exception of the DNA-binding domain. Interestingly, the screen also yielded translucent mutants with wild-type *luxO*. Two such translucent mutants had elevated P_{qrr}-*gfp* activity, while

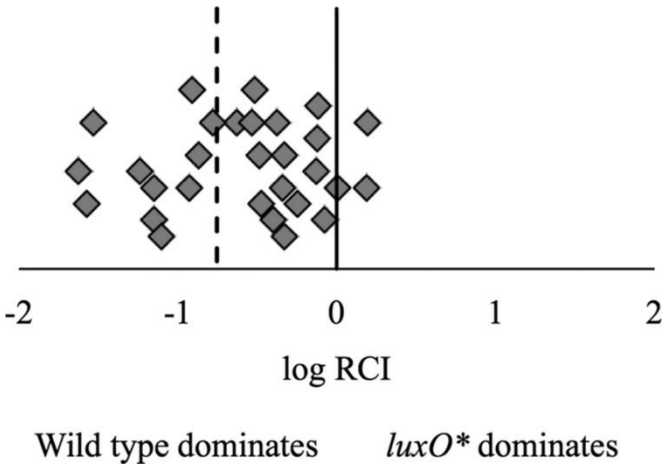

Figure 3.9. The *luxO** strain JHK061 shows a defect when competed 1:1 against the wild-type ES114 derivative AKD200 during colonization of *E. scolopes*. Newly hatched squid (n = 29) were exposed to a mixed inoculum of JHK061 (*luxO** T335I) and AKD200 (~2000 CFU) in filter-sterilized Instant Ocean for 12 hr with subsequent water change at 24 hr. After 48 hr, squid were homogenized, dilution plated on LBS agar, and patched to determine Cam resistance. The log relative competitive index (RCI) was calculated as the log of the ratio of JHK061 to AKD200 in the host homogenate to the ratio of the two strains in the inoculum. The solid line represents an equal ability to colonize the host; the dashed line represents the mean log RCI of -0.61. A single representative experiment of three independent experiments is shown. Statistical significance was determined using a one-sample Student's *t*-test where the null hypothesis is a mean log RCI of 0. Each diamond represents the log RCI in one hatchling.

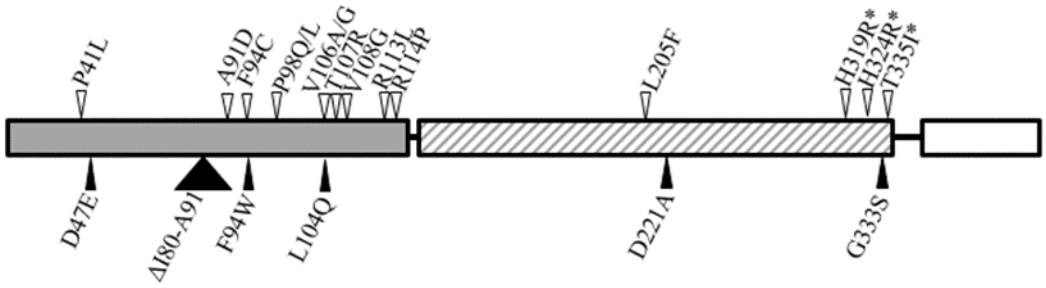

Figure 3.10. Distribution of *luxO** variants obtained in this study (open triangles) and those from previous studies in other vibrios aligned to the *V. fischeri* sequence (filled triangles) (53, 54, 57, 58, 60). Rectangles represent LuxO domains, including the regulatory (grey), AAA+/RpoN-interaction (hatched), and DNA-binding (white) domains. * indicates potential interaction site with LuxU (78).

nine mutants had wild-type levels of P_{qrr}-*gfp* activity and luminescence but clumped in static broth culture (data not shown).

Finally, given that some components of the PS circuitry, including *ainSR* and *luxIR*, have diverged between *V. fischeri* lineages (55), we wondered whether spontaneous *luxO** mutants, or other mutants that up-regulate *qrr*, would be similarly enriched during prolonged growth in static culture of other wild-type strains besides ES114. We tested wild-type *V. fischeri* isolates CG103, ES12, ES114, ES213, ES401, EM17, ET101, H905, PP3, VLS2, and WH1 (Table 3.1), by growing strains carrying the P_{qrr}-*lacZ* reporter pHK20 in static culture for up to 96 hr, as described above. We further tested blue colonies by displacing pHK20 with the P_{qrr}-*gfp* reporter pTM268 and screening for high fluorescence to ensure that mutants had genomic changes that increased *qrr* reporter expression. Strains ES12, ES114, ES213, ES401, EM17, and ET101 gave rise to mutants with increased *qrr* expression.

Discussion

In this study we sought to identify regulators of the *ainSR* PS system using a library of Tn-insertion mutants; however, the effects we observed on *ainSR* expression were

unrelated to the Tn insertions and were instead due to spontaneous mutations in the core PS-signaling circuit (Fig. 3.1C). The most notable mutants had constitutively active variants of the regulator LuxO, referred to as *luxO** mutants (54), which outcompeted wild-type during prolonged static co-culture in LBS medium (e.g., Fig. 3.7).

Our data suggest that these *luxO** mutations arose after Tn mutagenesis, and that mutants were probably enriched in subsequent steps. Although the 96-hr duration of our competition experiments is longer than any incubations used during library construction and propagation, >10-fold enrichment of *luxO** mutants were observed in as little as 24 hrs (Fig. 3.7) and three successive subculturings after 12-hr static culture incubations was sufficient to generate translucent mutants (data not shown). Moreover, overnight incubations in LBS were used in stocking the original Tn mutants prior to sequencing, in generating the defined master library, and finally to propagate the library prior to introducing the P_{ainS}-*lacZ* reporter. Knowing now that *luxO** (or phenotypically similar) mutants are prevalent in most cultures of ES114 after 48 hrs of incubation, and that *luxO** mutants are significantly enriched by 24 hrs, it seems plausible that during construction and handling of the Tn-mutant library, three in approximately two thousand wells became significantly contaminated by a *luxO** derivative. In a similar vein, Petrun and Lostroh reported a growth advantage during stationary phase for seven-day-old cultures of *V. fischeri* over one-day-old cultures (56), and it would be interesting to assess such cultures for the presence of *luxO** variants. Overall, our findings serve as a cautionary tale, encouraging the prompt use of cultures and the verification of phenotypes using complementation and/or backcrossing. In this circumstance, examining the opacity or

translucence of newly made strains also provides a convenient screen against these unexpected mutations.

The prevalence of luxO* *in the* Vibrionaceae.

LuxO is part of a PS network conserved throughout the *Vibrionaceae* that has been well studied over the last three decades. Other *luxO** mutants have been reported in the *Vibrionaceae*, isolated both from natural populations and during laboratory experiments (54, 57-61). Recently, phenotypic discrepancies between two Δ*luxU* mutants of *V. cholerae* were discovered to be due to one strain harboring a previously undetected *luxO** mutation (58, 59), which is reminiscent of our own discovery of spontaneous *luxO** causing phenotypes we initially attributed to defined transposon insertions. In another parallel to our study, *luxO** mutants were recovered in strains subjected to transposon mutagenesis (54, 58, 62, 63); however, we found that *luxO** mutants of *V. fischeri* were not enriched by this procedure specifically (Fig. 3.6), and *luxO** mutants were readily recovered from strains that had not been genetically manipulated or subjected to Tn mutagenesis. If our observations in *V. fischeri* hold true in other *Vibrio* species, it may simply be that rounds of growth into stationary phase during genetic manipulations can give rise to *luxO** mutants. In this regard, if we consider Keynan and Hastings's (64) and Silverman *et al.*'s (65) observations of "luminescence variation" in *V. harveyi* resulting in genetically stable dim and dark mutants appearing in old, statically grown cultures, it is tempting to speculate that at least some of these may have been *luxO** mutants. One such dark mutant was used in the first description of the *luxO* locus (66), raising the possibility that a spontaneous *luxO** mutant may have contributed to the discovery of *luxO* almost thirty years ago (65).

Are LuxO mutants "cheaters"?*

One area of recent interest in the study of bacterial PS and group behaviors is the appearance of "cheaters" in populations. These cheaters are PS-negative mutants that benefit from the PS-induced behaviors of nearby cells while minimizing their own costs, and thus are enriched in the population (9-11). Given that *luxO** mutants make little C8-AHL and remain locked in a non-induced state (e.g. dim; Fig. 3.1C, 3.2), but can also take over mixed cultures with wild type (Fig. 3.7), such mutants might fit the description of PS "cheaters". Although such cheating mutants have been described in a variety of bacterial systems, to our knowledge this would be the first description of cheaters arising spontaneously in *V. fischeri*, and may shed light on a more general method of survival among vibrios and the importance of tight regulation of the pheromone systems of *V. fischeri* in its natural habitats.

On the other hand, *luxO** mutants did show some survival benefits even in the absence of wild type (data not shown), and they may simply be better adapted to broth culture environments without needing to exploit a nearby wild-type population. In this sense, it seems possible that these mutants, and perhaps others, are "antisocial" without being "cheaters". In situations where social behaviors have costs without benefits, being antisocial could simply be viewed as "prudent" or "efficient". The terminal or master regulators of LuxO-containing PS circuits (e.g. LitR in *V. fischeri*) control a diverse array of phenotypes including opacity (67), swarming (68), protease production (29), biofilm formation (27), symbiosis (23), and luminescence (69). These behaviors may present fitness costs without compensatory benefits under certain circumstances, and *luxO** mutants would have the effect of dampening expression of these costly systems.

If *luxO** mutations are advantageous due to their repression of PS "master regulators" like *litR*, this begs the question of why presumably more rare gain-of-function mutations in *luxO* should dominate instead of loss-of-function mutations in *litR*. In fact, such loss-of-function mutations to these regulators have repeatedly been observed in other members of the *Vibrionaceae*. For example, in *V. cholerae*, mutants disrupted in the *litR* homolog *hapR* account for cheaters arising in populations of that bacterium and have been frequently isolated (11, 27, 29). One reason *luxO** mutants may be enriched rather than *litR*-minus mutants may be that, in some instances, fitness is maximized by decreasing the master regulator without completely eliminating it. At least in *V. fischeri*, in terms of static culture-survival, *luxO** mutants outcompete either their wild-type parents or *litR* mutants (Fig. 3.7). Thus, while *luxO** mutations may be more rare, in this circumstance they confer greater fitness. Others have suggested that PS systems may have evolved in ways that make the appearance of cheaters difficult (70, 71) , and perhaps the *V. fischeri* system has similarly evolved such that the simple loss of *litR* is not advantageous.

Insights into the LuxO-Qrr regulatory module.

Based on a simple model of the LuxO-containing PS regulatory circuit (Fig. 3.1A-B), *luxO** mutants and *litR* loss-of-function mutants should have a similar phenotype, with the caveat noted above that LuxO* should diminish but may not eliminate LitR. In addition to showing that *luxO** mutants outcompeting *litR* mutants (Fig. 3.7), prolonged growth in stationary phase selected for *luxO** mutants but not *litR* mutants, and a *luxO** *litR* double mutant outcompeted a *litR* mutant (data not shown). Furthermore, *luxO** and *litR* mutants do not phenocopy with respect to their ability to compete with wild type in host colonization (Fig. 3.9), growth and luminescence in broth culture (data not shown), and

P_{qrr}-*gfp* reporter activity (data not shown). Moreover, while *luxO** mutations confer a survival advantage in a variety of strain backgrounds, this advantage is Qrr-dependent (Fig. 3.8). Given these observations, we speculate that the Qrr regulon extends beyond *litR*. There is precedent for such regulation, as Qrr has multiple targets in other *Vibrio* species (72, 73), although there are also usually multiple semi-redundant copies of *qrr*, whereas *V. fischeri* has only one *qrr* gene (24). Our data seem consistent with a model where Qrr regulates an unknown target that affects survival and competitiveness during prolonged culturing.

Our results also point to mutants that could help reveal alternative targets for Qrr and perhaps novel inputs to *qrr* control. As noted above, prolonged culturing of ES114 yields not only *luxO** mutants but also other translucent-colony mutants with wild-type *luxO*. These mutants fell into two classes. One set was relatively abundant, quickly settled in clumps out of static broth culture, and had wild-type P_{qrr}-*gfp* activity. Analyses of these mutants might provide insight into the key targets for stationary-phase survival downstream of Qrr. The second set was less common and had high P_{qrr}-*gfp* activity similar to *luxO** mutants. These mutants might reveal other regulatory inputs into *qrr* control. While we have shown our *luxO** mutants act independently of their only known phospho-donor, LuxU (Fig. 3.5), this does not preclude the existence of some hitherto unknown input into the system acting at or downstream of LuxU. Alternatives inputs into LuxU have been shown in *V. harveyi* and *V. cholerae* through HqsK and VpsS, respectively, and perhaps there exist similar pheromone-independent inputs phosphorylating LuxO (74, 75).

Symbiotic phenotypes of luxO* *and* litR *mutants.*

Our data are also consistent with previous reports that suggest *luxO** and *litR* mutants have different symbiotic phenotypes. Fidopiastis *et al.* showed a *litR* mutant outcompeted its wild-type parent (23); however, Lupp and Ruby reported a colonization defect at 12 hr post-inoculation for a *luxO* D47E mutant (19). Understanding the role of each gene was further complicated by findings that minor competition defects for *luxO* and *qrr* mutants are not rescued by the addition of a *litR* mutation (24). This observation suggested a requirement for *luxO* and *qrr* for full colonization of the host. Our results are consistent with those of Lupp and Ruby, and it also seems possible that the more active the *luxO** allele (e.g. Fig. 3.3) the greater the competitive defect.

Taken together, the results suggest increased *qrr* expression may be detrimental to *V. fischeri*'s ability to colonize *E. scolopes*. In that regard, *litR* mutants did not display increased *qrr* reporter expression (data not shown), despite LitR feedback regulating the *ainSR* pathway (17, 22, 30). Given the symbiotic disadvantage of *luxO** mutations, it seems unlikely that they would be enriched in natural symbiotic populations of *V. fischeri*, although they might be found in free-living populations or in mixed-species communities in the guts of fishes. At least in the shallow sandy reefs of Hawaii, where symbiosis apparently enhances *V. fischeri* populations (76, 77), symbiotic fitness presumably filters out *luxO** mutants.

New insights into LuxO structure and function.

The many different *luxO** alleles that we found (Fig. 3.10) indicate that there are multiple paths to LuxO* activity. Classical D47E mutations alter the amino acid (D47) that is usually phosphorylated to activate the protein, and presumably the longer side chain

at this site in some way mimics the conformational changes imposed by phosphorylation at this residue. This study contributes to a body of previously described mutations showing that less intuitive mechanisms of generating LuxO* variants are possible (Fig. 3.10). These mechanisms could include deactivation of negative autoregulation by the N-terminal receiver domain, modulation of the predicted site of interaction with the phosphor-donor LuxU (78), promotion of new interactions with other phosphate donors, or other mechanisms. Previous studies of *luxO* and other σ^{54}-dependent regulators have described similar effects of mutations in approximately the same positions within the protein as we isolated (53, 79). LuxO belongs to a subclass of σ^{54}-dependent regulators whereby the N-terminal receiver domain represses the catalytic activity of the central AAA^{+} domain, and deletion of the receiver domain results in constitutive activity of the protein (53, 79). Furthermore, the majority of the mutant variants we isolated were clustered within a putative helix that, when LuxO dimerizes, serves to stabilize the "off"-state conformation in other systems (79-81). Phosphorylation of the conserved aspartate residue or targeted mutagenesis within these helices destabilize the interaction biasing LuxO to an ATP-hydrolyzing, oligomerization-promoting active state (79-82). It is noteworthy, and perhaps surprising, that each of the alleles we tested appeared to be active independent of *luxU* and was more active than a D47E variant (Fig. 3.5). However, allowing these mutations to arise under natural selective pressures resulted in only mutations that provide a competitive advantage for the bacterium and may serve as a more powerful tool for examining protein function than targeted mutagenesis alone.

A dearth of ainSR *regulators.*

We initially sought to identify novel controls of *ainSR*, and the fact that we did not identify any unknown regulators of *ainS* begs the question, are there any such regulators of *ainSR* beyond what is known for CRP and LitR (17, 22, 30), other than the possible regulation of *ainSR* by LuxR (22, 83)? Our lack of success in identifying new regulators might reflect limitations of our original P_{ainS}-*lacZ* reporter-based screen. Moreover, the transposon library is not comprehensive and will also inherently miss any regulators of the system that are essential for *V. fischeri*'s survival. In this regard, it is worth noting that while not essential, neither *crp* nor *litR* are among the genes disrupted in this transposon mutant library. Finally, regulators may only become apparent using different conditions (e.g. media) or other approaches. It seems likely that the regulation of *ainSR* is far less complex than that of *luxI* and *luxR*, which could reflect the function of the former being more general and the latter more context dependent.

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CHAPTER 4

REGULATION OF THE AINS/AINR PHEROMONE-SIGNALING SYSTEM IN VIBRIO FISCHERI ES114[3]

[3] For submission to *Journal of Bacteriology*

Abstract

Vibrio fischeri uses the AinS/AinR acyl-homoserine lactone (AHL) pheromone-signaling system to control bioluminescence and other symbiotic colonization factors. The Ain system is thought to initiate cell-cell signaling at moderate cell densities and prime the other AHL-based signaling system comprised of LuxI and LuxR. Given its role in initiating and controlling a symbiotic signaling cascade, we became interested in the regulation of the Ain system. Here we used bioinformatics analyses and comparisons of the genomic sequences from two *V. fischeri* strains and a *Vibrio salmonicida* strain to explore expression and possible mechanisms of regulation at the *ain* locus. The *ainS* and *ainR* genes were predicted to constitute an operon, which we corroborated using RT-PCR. Comparisons of the *ainSR* locus illustrated the sharp divergence between strains that was previously reported but also revealed a stark area of conservation across the *ainS-ainR* junction, including a large inverted repeat in *ainR*. We found that this inverted repeat *in cis* can affect AinS AHL synthase activity, which could account for the previously unexplained low-AHL phenotype of a Δ*ainR* mutant. We also extend a previous observation of a possible "*lux* box" LuxR binding site upstream of *ainS*, by showing the conservation of this site as well as a second putative *lux* box in strain ES114. Importantly, we show that LuxR can mediate repression of *ainS*, providing a negative feedback mechanism from the second induced AHL system onto the first. Our results provide new insights into the regulation, expression, and evolution of *ainSR*.

Introduction

The light-organ symbiont *Vibrio fischeri* ES114 uses pheromone signaling (PS) to regulate behaviors essential for colonizing its host squid, *Euprymna scolopes* (1-3). One of these behaviors, bioluminescence, was among the first phenotypes discovered to be regulated by PS, and its examination over the last fifty years has been fundamental to understanding cell-cell communication in bacteria (4). Of *V. fischeri*'s three integrated PS systems (Fig. 4.1), two acyl-homoserine lactone (AHL) based systems are primarily responsible for regulating bioluminescence and other colonization factors, while the Autoinducer-2 (AI-2) signal, which is conserved across many bacteria, only modestly influences these phenotypes under the conditions tested (2).

The two AHL-based PS systems in *V. fischeri* that regulate bioluminescence and other symbiotic factors are comprised of the signal synthase/receptor pairs LuxI/LuxR and AinS/AinR (1, 5, 6). Each of these systems produce AHL molecules that can diffuse through membranes and mediate cell-cell signaling once they reach stimulatory concentrations (7). LuxI synthesizes the pheromone *N*-3-oxohexanoyl AHL (3OC6-AHL) (8, 9), which binds LuxR, promoting LuxR dimerization and association with a regulatory element upstream of *luxI* called the "*lux* box" (7, 10). AHL-LuxR complexes activate transcription of the *luxICDABEG* operon, which results in more LuxI, more 3OC6-AHL, and bioluminescence. LuxI and LuxR are well studied and are the archetype for similar PS systems throughout the Proteobacteria.

Although less well known and understood, the structurally distinct AinS/AinR PS system also uses an AHL signal and can play a key role in luminescence induction and symbiotic competence (3). AinS synthesizes the pheromone *N*-octanoyl AHL (C8-AHL),

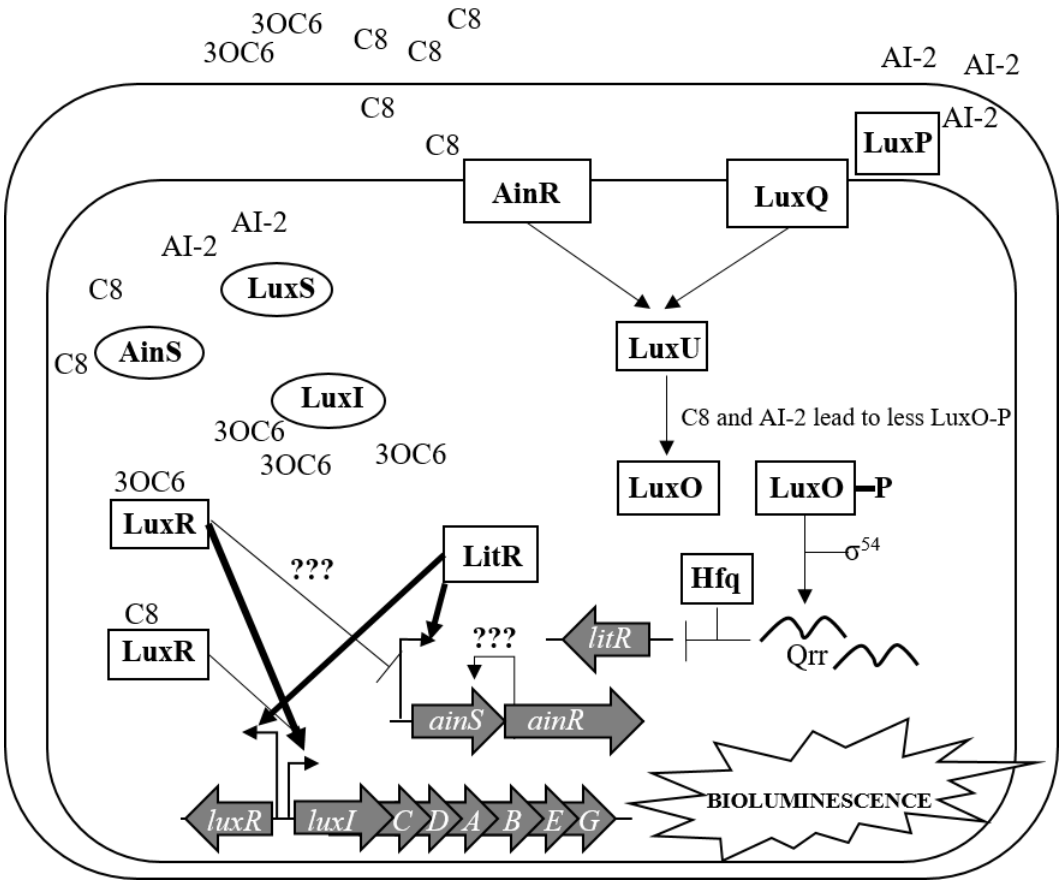

FIGURE 4.1. Model of *V. fischeri* pheromone-signaling systems. The pheromone receptors AinR and LuxQ phosphorylate and de-phosphorylate LuxU, which phosphorylates LuxO, stimulating it to activate transcription the regulatory sRNA of *qrr*. Qrr represses expression of the "master regulator" LitR. As C8-AHL and AI-2 levels increase, the equilibrium of the system shifts to less LuxO-P, less Qrr, and more LitR. High C8-AHL and LitR levels increase luminescence through LuxR. Differences in font size, arrow thickness, and numbers of gene products shown are meant to reflect relative differences in activation or abundance. Question marks indicate potential methods of regulation discussed in this study.

which is sensed by AinR (11, 12) but can also bind and activate LuxR (13, 14). Information about local C8-AHL levels is funneled by AinR into a core PS system conserved among the *Vibrionaceae* family, converging with the AI-2 system, to affect bioluminescence through a regulatory cascade comprised of LuxU, LuxO, the sRNA Qrr, and the terminal regulator of the system, which is called LitR in *V. fischeri* (Figure 1; 15-18). LitR activates LuxR expression and as noted above C8-AHL can activate LuxR, albeit more weakly than 3OC6-AHL. Thus, in more than one way the Ain system serves to activate *lux* expression

and "prime" 3OC6-AHL-based signaling (3, 13-15). However, at certain AHL ratios, C8-AHL can also inhibit 3OC6-AHL-based activation (13, 19-21).

In *V. fischeri* strain ES114, which was isolated from the light organ of *E. scolopes*, the Ain system is critical for induction of luminescence in broth culture and underlies regulation of symbiotic colonization factors. Despite its importance during symbiotic initiation, little is known about the regulation of *ainS/ainR*. LitR activates the system, closing a positive feedback loop (2, 11), and CRP-cAMP was recently identified as an activator of both *ainS/ainR* and *luxI/luxR* (22). However, given the complex regulation of *luxI* and *luxR* (19, 23, 24), we predict that other regulatory mechanisms await discovery. Indeed, studies have suggested that the presence of *ainR in cis* somehow affects AinS activity (11, 16) and that a feedback loop exists between LuxR and the *ain* system (11).

We previously reported that both the *lux* and *ain* loci have evolved rapidly and diverged between *V. fischeri* strains more than most housekeeping genes (25). Moreover, we found that comparison of the *luxIR* intergenic region between strains provided insight into conserved regulatory sequences (25). In this study we similarly used bioinformatic comparisons and targeted experimentation to gain insight into expression of the *ain* locus.

Materials and Methods

Bacteria, growth media, and reagents. Bacterial strains are listed and briefly described in Table 4.1. *V. fischeri* ES114 was the wild-type strain used throughout (26). Plasmids were transformed into *Escherichia coli* strain DH5α (27) or DH5α*λpir* (28) in the case of plasmids with the R6K origin of replication. *E. coli* strain MG1655 (29) and its derivatives were used as recipients for plasmids expressing different *ainR* alleles as described below. *E. coli* was grown in LB medium (30) or brain heart infusion (BHI)

medium (Bacto), and *V. fischeri* was grown in LBS (31) or SWTO (23). Solid media were prepared with 15 g L^{-1} agar. For selection of *E. coli*, chloramphenicol (Cam) and kanamycin (Kan) were added to LB at final concentrations of 20 and 100 μg ml^{-1}, respectively, and erythromycin (Erm) was added to BHI at a final concentration of 150 μg ml^{-1}. For selection of *V. fischeri* on LBS, the concentrations of Cam, Erm, and Kan used were 2, 5, and 100 μg ml^{-1}, respectively. 3OC6-AHL and C8-AHL were obtained from Sigma-Aldrich (St. Louis, MO).

Table 4.1. Bacterial strains and plasmids used in this study.

Strain, plasmid, or oligonucleotide	**Relevant characteristics[a]**	**Source or reference**
E. coli		
CC118λpir	Δ(*ara-leu*) *araD ΔlacZ74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	33
DH5α	F− φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	27
DH5α λpir	DH5α lysogenized with λ*pir*	28
JW1644-5	Δ(*araD-araB*)*567* Δ(*lacZ4787*(::*rrnB-3*)) λ^- Δ(*rnt-730*::*kanR*) *rph-1* F^- Δ(*rhaD-rhaB*)*568 hsd514*	44
JW1793-1	Δ(*araD-araB*)*567* Δ(*lacZ4787*(::*rrnB-3*)) λ^- Δ(*rnd-729*::*kanR*) *rph-1* F^- Δ(*rhaD-rhaB*)*568 hsd514*	43
JW3618-2	Δ(*araD-araB*)*567* Δ(*lacZ4787*(::*rrnB-3*)) λ^- Δ(*rph-749*::*kanR*) *rph-1* F^- Δ(*rhaD-rhaB*)*568 hsd514*	43
JW5741-1	Δ(*araD-araB*)*567* Δ(*lacZ4787*(::*rrnB-3*)) λ^- Δ(*rnr-729*::*kanR*) *rph-1* F^- Δ(*rhaD-rhaB*)*568 hsd514*	44
MG1655	F^- λ^- *ilvG*$^-$ *rfb-50 rph-1*	29
SK2595	*araD139 galE15 galK16* Δ(*ara-leu*)*7697 spoT1* λ^- *hsdR2* Δ(*codB-lacI*)*3 mcrA0 relA1 rpsL150 mcrB9999* Δ(*rnz-500*::*kanR*) Δ(*elaC500*::*kanR*)	45

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
V. fischeri		
DC22	C8-AHL bioreporter: ES114 Δ*ainS* Δ*luxR-luxI*, mutant *luxR* (MJ1-T33A, R67M, S116A, M135I), P_{luxI}-*luxCDABEG*	22
DC43	ES114 Δ*ainS* Δ*luxI* P_{con}-$luxR_{MJ1}$ P_{luxI}-*luxCDABEG*	20
DJ01	ES114 Δ*ainS* Δ*luxI* P_{con}-$luxR_{ES114}$ P_{luxI}-*luxCDABEG*	20
ES114	Wild-type isolate from *E. scolopes*	26
JB18	ES114 *litR*::*ermR*	11
JHK003	ES114 Δ*ainR*	11
JHK007	ES114 Δ*ainS* Δ*luxIR* P_{luxI}-*luxCDABEG*	11
JHK045	ES114 Δ*ainS* Δ*luxI* P_{con}-$luxR^{ES114}$ P_{luxI}-*luxCDABEG litR*::*ermR*	This study
JHK046	ES114 Δ*ainS* Δ*luxIR* P_{luxI}-*luxCDABEG litR*::*ermR*	This study
JHK055	ES114 $ainR_{trunc}$ (Δ30-815)	This study
JHK056	ES114 $ainR_{nat}$ (Δ36-815)	This study
JHK091	ES114 Δ*luxI* P_{con}-$luxR^{MJ1}$ P_{luxI}-*luxCDABEG litR*::*ermR*	This study
JHK099	ES114 Δ*ainS* Δ*luxI* P_{con}-$luxR^{MJ1}$ P_{luxI}-*luxCDABEG litR*::*ermR*	This study
JHK107	ES114 *ainS*::*lacZ* chromosomal transcriptional fusion	This study
JHK114	ES114 Δ*ainR*	This study
JHK115	ES114 $ainR_{scar}$	This study
JHK118	ES114 *ainS*::*lacZ luxR*::*ermR*	This study
JHK119	ES114 $ainR_{scar\text{-}repaired}$	This study
JHK120	ES114 $ainR_{mut}$	This study
NL55	ES114 Δ*ainSR*	19
NL60	ES114 Δ*ainS*	19
NL63	ES114 Δ*ainS luxI*	11
Plasmids[b]		
pAKD701	Promoterless *lacZ*, pES213, R6Kγ $oriT_{RP4}$, *kanR*	32
pCL149	*luxR*::*ermR* allele; *oriT camR ermR*	2

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
pCR-Blunt	PCR product cloning vector; ColE1 *oriV kanR*	Invitrogen
pCR-Blunt II TOPO	PCR product cloning vector; ColE1 *oriV kanR*	Invitrogen
pDJ01	P_{con}-*luxR*$_{ES114}$ P_{luxI}-*luxCDABEG ColE1*, R6K*γ, oriT*$_{RP4}$, *kanR, camR*	20
pEVS104	Conjugative helper plasmid; R6Kγ *oriT*$_{RP4}$ *kanR*	34
pEVS118	Suicide vector; R6Kγ, *oriT*$_{RP4}$, *camR*	28
pEVS122	Suicide vector; R6Kγ, *oriT*$_{RP4}$, *ermR, lacZα*	28
pHK12	P_{ainS}-*gfp* P_{con}-*mCherry* in pJLS27; pES213, R6Kγ *oriT*$_{RP4}$ *kanR, camR*	35
pHK34	*ainR*$_{nat}$ (Δ30-815) in pCR-Blunt; ColE1 *oriV kanR*	This study
pHK37	*ainR*$_{nat}$ (Δ30-815) allele; ColE1 R6Kγ *oriV oriT*$_{RP4}$ *camR kanR*	This study
pHK75	1500-bp *ainR* downstream in pEVS122; R6Kγ *oriT*$_{RP4}$ *ermR lacZα*	This study
pHK76	*ainR*$_{nat}$ (Δ36-815) allele; R6Kγ *oriT*$_{RP4}$ *ermR lacZα*	This study
pHK93	P_{ainS}-*ainR*$_{trunc}$ (Δ36-815) in pCR-Blunt; ColE1 *oriV kanR*	This study
pHK94	P_{ainS}-*ainR*$_{nat}$ (Δ30-815) in pCR-Blunt; ColE1 *oriV kanR*	This study
pHK95	P_{ainS}-*ainR* in pCR-Blunt; ColE1 *oriV kanR*	This study
pHK102	P_{ainS}-*ainR*$_{trunc}$ (Δ30-815) allele; ColE1 R6Kγ *oriV oriT*$_{RP4}$ *kanR camR*	This study
pHK103	P_{ainS}-*ainR*$_{nat}$ (Δ36-815) allele; ColE1 R6Kγ *oriV oriT*$_{RP4}$ *kanR camR*	This study
pHK104	P_{ainS}-*ainR* allele; ColE1 R6Kγ *oriV oriT*$_{RP4}$ *kanR camR*	This study
pHK128	Δ*ainS* allele in pCR-Blunt; ColE1 *oriV kanR*	This study
pHK129	*ainSR* ClaI-NdeI fragment with mutated IR1 (*ainR*_IR_conAA) in pCR-Blunt; ColE1 *oriV kanR*	This study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
pHK131	Transcriptional *ainS*::*lacZ* vector in pCR-Blunt; ColE1 *oriV kanR*	This study
pHK132	Transcriptional *ainS*::*lacZ* allele; ColE1 R6Kγ *oriV* $oriT_{RP4}$ *kanR camR*	This study
pHK135	1,350-bp upstream of *ainR* in pCR-Blunt; ColE1 *oriV kanR*	This study
pHK136	Δ*ainR*, 1,480-bp downstream *ainR* in pHK135; ColE1 *oriV kanR*	This study
pHK137	$ainR_{scar}$ in pHK136; ColE1 *oriV kanR*	This study
pHK138	Δ*ainR* allele; ColE1 R6Kγ $oriT_{RP4}$ *kanR camR*	This study
pHK139	$ainR_{scar}$ allele; ColE1 R6Kγ $oriT_{RP4}$ *kanR camR*	This study
pHK147	*ainSR* cotranscript fragment in pCR-Blunt, ColE1 *oriV kanR*	This study
pHK152	$ainR_{mut}$ in pHK95; ColE1 *oriV kanR*	This study
pHK153	$ainR_{mut}$ allele; ColE1 R6Kγ $oriT_{RP4}$ *kanR camR*	This study
pJLS27	Promoterless *gfp*, P_{con}-*mCherry* pES213, R6Kγ $oriT_{RP4}$ *kanR*	36
pJLB95	*litR*::*ermR* (opposite) allele; ColE1 *camR ermR*	11
Oligonucleotides[c]		
pr_HK01	GGATCTGGCTTTTAAAAAATGCATCATCTGC	This study
pr_HK13	AGCGCCCAATACGCAAACC	This study
pr_HK14	CCGGCGTGTCAATAATATCACTCTGTACA	This study
pr_HK17.2	CATGGGATCCTAGAGAGCGGATAAAATACCC TACCCAA	This study
pr_HK19	GGGGCTAGCAATGCTGACTCTGGG	This study
pr_HK20	GGGGCTAGCGGGACCCGCAGACAT	This study
pr_HK27.3	CATGGGTACCAGAACCAAGACCTGCTCGTGCT AA	This study
pr_HK28.2	CATGGGATCCTAAGGGTTTACCTTTGTCCGCT CTCTA	This study
pr_HK40.1	CATGGGATCCATAAGTGGTTATAACACCGATA AAAAAATAGCC	This study

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
pr_HK41.4	CATGGCATGCTGAAGGTGCTTGCTATTACTGATCA	This study
pr_HK126	CATGGGATCCTGAAGGTGCTTGCTATTACTGATC	This study
pr_HK136	CATGGCTAGCTTAACTACTTTACCTAAAGTTTATTTAC	This study
pr_HK137	CATGGCTAGCTAACCACTTATCTACGACCT	This study
pr_HK146	AAAGTACTCATAACACCACTACC	This study
pr_NL28.3	GGGCCTAGGCATTTATATAAAACTCACTGATTTCGAAGTTT	19
pr_NL29	GGGGCCTAGGTAACACCGATAAAAAAATAGCCAGAAC	19
pr_NL62	GGCGCTTTACCGTTTGGTGAAAACTTACTT	19
pr_NL79	CCTCGACTAATCGAATAGATATAGAACTTTTAT	22
pr_NL89	AAATCTAAGGGTTTACCTTTGTCCGCTCTC	22

[a] Drug resistance abbreviations used: *camR*, chloramphenicol resistance; *ermR*, erythromycin resistance; and *kanR*, kanamycin resistance (*aph*).
[b] All alleles cloned in this study are from *V. fischeri* strain ES114. Replication origin(s) of each vector are listed as R6Kγ, ColE1, *oriV* and/or pES213. Plasmids based on pES213 are stable and do not require antibiotic selection for maintenance.
[e] All oligonucleotides are shown 5' to 3'. Restriction enzyme recognition sequences are underlined.

Molecular genetic techniques. Oligonucleotides and plasmids are listed in Table 4.1 and the latter were constructed using standard techniques and materials as described previously (11).

To generate the Δ*ainR* deletion allele on allelic exchange vector pHK138, a 1,350-bp fragment upstream of *ainR* including the start codon was PCR amplified using primers pr_HK146 and pr_NL28.3 and cloned into pCR-Blunt to generate pHK135. The 1,519-bp region downstream of *ainR* including the stop codon was PCR amplified using primers pr_NL29 and pr_HK126, digested with BamH1 and AvrII, and ligated into BamHI- and

AvrII-digested pHK135 to create pHK136. This plasmid contains a unique AvrII restriction site between the *ainR* start and stop codons along with sequences flanking *ainR*. To reintroduce different *ainR* variants into Δ*ainR* mutants, wild-type *ainR* was PCR amplified using primers pr_HK136 and pr_HK137 and the resulting amplicon was digested with NheI and ligated into AvrII-digested pHK136 to generate pHK137. This plasmid contains an additional six nucleotides flanking the *ainR* coding sequence following the start codon and preceding the stop codon and is referred to below as $ainR_{scar}$. The mobilizable Δ*ainR* and $ainR_{scar}$ allelic exchange vectors were generated by digesting pH136 and pHK137 with BamHI and ligating them with BamHI-digested pEVS118 (28) generating pHK138 and pHK139, respectively.

To generate the $ainR_{trunc}$ allele on allelic exchange vector pHK37, in which *ainR* is truncated after the first 16-bp of the inverted repeat IR1 (Fig. 4.2), 428-bp upstream of *ainS* through the first half of the *ainR* inverted repeat was PCR amplified using primers pr_HK17.2 and pr_HK28.2. The 1,500-bp region comprising the final 15 bp of *ainR* through the sequence downstream of *ainR* was PCR-amplified using primers pr_HK40.1 and pr_HK41.4. The two amplicons were digested with BamHI, ligated together, and blunt-end cloned into pCR-Blunt II TOPO to generate pHK34. This plasmid was then digested with KpnI and ligated to KpnI-digested pEVS118 to generate pHK37. To generate the $ainR_{nat}$ allele on allelic exchange vector pHK76, in which *ainR* is truncated after IR1, 1,500-bp downstream of *ainR* was again amplified using primer pair pr_HK40.1 and pr_HK41.4. This amplicon was digested with BamHI and SphI and ligated with BamHI and SphI-digested pEVS122 (28) to generate pHK75. The fragment from 428-bp upstream of *ainS* through IR1 was PCR amplified with primers pr_HK27.3 and pr_HK28.2. The

resulting amplicon was digested with BamHI and KpnI and ligated into similarly digested pHK75 to generate the *ainR*$_{nat}$ allele on pHK76.

To generate alleles expressing *ainS* and variants of *ainR,* the fragment containing the 428-bp upstream of *ainS* through 1,500-bp downstream of the *ainR* stop codon in strains ES114, JHK055, and JHK056 (described below) were PCR-amplified using primers pr_HK27.3 and pr_HK41.4 and cloned into pCR-Blunt II TOPO in identical orientations generating pHK95, pHK93 and pHK94, respectively. To add a selectable marker compatible with kanamycin-resistant *E. coli* RNase mutants, each plasmid was then digested using KpnI and SpeI and ligated with similarly digested pEVS118, which encodes resistance to chloramphenicol, to generate pHK104, pHK102 and pHK103, respectively.

To assess the function of an inverted repeat (IR1) in *ainR*, a synthetic DNA fragment (Integrated DNA Technologies, Coralville, IA) was designed to preserve the amino acid sequence of AinR while also disrupting the mirror symmetry of IR1 in *ainR*. This sequence, *ainR*_IR_conAA, is contained in an 844-bp ClaI to NdeI fragment. The synthetic fragment was cloned into pCR-Blunt to generate pHK129, and PCR-amplified using primers pr_HK13 and pr_HK14. The resulting amplicon was ClaI- and NdeI-digested and ligated into similarly digested pHK95 to replace the native *ainR* sequence and generate pHK152. This plasmid was then digested with KpnI and SpeI and ligated with similarly digested pEVS118 to generate the mobilizable allelic exchange vector pHK153.

To generate an *ainS*::*lacZ* transcriptional fusion allele, the 3-kb region flanking the *ainS* deletion site in strain NL60 was PCR amplified using primers pr_NL62 (19) and pr_NL79 (22) and blunt cloned into pCR-Blunt to form pHK128. The *lacZ* allele from pAKD701 (32) was PCR amplified using primers pr_HK19 and pr_HK20. The resulting

amplicon was digested using NheI and ligated into AvrII-digested pHK128 to generate pHK131, which was digested with XhoI and XbaI and ligated into similarly digested pEVS118 to generate the mobilizable allelic exchange vector pHK132.

Mutant alleles were transferred from *E. coli* into *V. fischeri* on plasmids by triparental matings using the conjugative helper strain CC118λ*pir* pEVS104 (33, 34). Recombination and marker exchange were identified by screening for antibiotic resistance, and putative mutants were tested by PCR. In this way, the *ainS*::*lacZ* allele on pHK132 was introduced into the Δ*ainS* strain NL60 (19) to generate JHK107, and the *luxR*::*ermR* allele on pCL149 (2) was introduced into JHK107 to generate strain JHK118. To generate *litR* mutants, the allele on pJLB95 (11) was introduced into DC43, DJ01, and JHK007 to generate JHK099, JHK045, and JHK046, respectively. To generate strains with different *ainR* variants, the alleles on pHK36, pHK76, pHK138, or pHK153 were introduced into ES114 to generate strains JHK055, JHK056, JHK114, and JHK120, respectively. JHK114 was subsequently used as the parent strain for the reintroduction of the $ainR_{scar}$ allele on pHK139, thus generating JHK115. This strain was then recombinationally repaired using the native *ainR* locus on pHK104, generating strain JHK119, which barring unknown spontaneous mutations is genetically identical to ES114. The P_{con}-*luxR* P_{luxI}-*luxCDABEG* Δ*luxI* locus on pDJ1 was introduced into the *litR*::*ermR* strain JB18 (11) to generate strain JHK091.

Luminescence measurements. Overnight *V. fischeri* cultures were diluted 1:1,000 in 25 ml SWTO in 125-ml flasks and incubated with shaking (200 rpm) at 24°C. At regular intervals, the optical density at 595 nm (OD_{595}) was measured for 500-µl samples using a BioPhotometer (Brinkman Instruments, Westbury, NY). Relative luminescence was

measured with a TD-20/20 luminomenter (Turner Designs, Sunnyvale, CA) immediately following shaking to aerate the sample. Specific luminescence was calculated as the luminescence per OD_{595}.

Transcriptional reporter assays. Strains harboring the P_{ainS}-*gfp* reporter plasmid pHK12 (35) or the promoterless parent vector pJLS27 (36) were grown overnight in LBS and subcultured 1:1,000 into flasks containing 25 ml SWTO with or without 3OC6-AHL or C8-AHLand incubated with shaking (200 rpm) at 24°C. At regular intervals, 200-µl samples were aliquoted into clear-bottomed, black-walled, 96-well plates, where green fluorescence and OD_{595} were measured using a Synergy 2 plate reader (BioTek).

For assays using the *ainS*::*lacZ* transcriptional fusions strains JHK107 and JHK118, strains were grown overnight in LBS and subcultured 1:1,000 in 125-mL flasks containing 25 mL fresh SWTO, with or without 3OC6-AHL, and incubated at 24°C with shaking at 200 rpm. Cells were collected at OD_{595} ~2.5 by centrifugation, the supernatant was discarded, and cell pellets were stored overnight at -80°C. ß-galactosidase assays were performed as previously described (6).

C8-AHL bioassays. C8-AHL accumulation was assessed as previously described (22). Briefly, culture supernatants were extracted with acidified ethyl acetate, extracts were dried and resuspended in SWTO, and C8-AHL levels were determined by comparison to standards using the bioassay strain DC22. For C8-AHL bioassays of *E. coli* harboring *ainS*, test strains were grown in LB medium at 37°C.

Co-transcript assessment. Overnight cultures of *V. fischeri* ES114, JHK003 (11), NL55 (19), and NL60 (19) were diluted 1:1,000 in SWTO and grown to an OD_{595} ~0.5, at which point total RNA was extracted using the RNASnap method of Stead *et al.* (37),

followed by sodium acetate-ethanol precipitation. RQ1 DNase (Promega, Madison, WI) was used to remove genomic DNA from samples according to the manufacturer's protocol. 50 ng of DNA-free RNA was used as template for reverse transcription using the Superscript III First Strand cDNA synthesis kit (Invitrogen, Orange, CA) with random hexamers according to the manufacturer's protocol. The resulting cDNA was diluted 1/10 in a PCR reaction using the primers pr_HK01 and pr_NL89 (22), which encompass a 236-bp fragment spanning the junction of *ainS* and *ainR*. The resulting amplicon was cloned and sequenced to confirm its identity.

Results

To gain insight into the regulation of *ainS* and *ainR*, we examined and compared the *ain* locus in three strains; *V. fischeri* ES114, a dim strain characteristic of other isolates from *E. scolopes*, *V. fischeri* MJ11, which is a bright isolate from the Japanese pinecone fish *Monocentris japonica*, and *Vibrio salmonicida* LFI1238, which was isolated from a diseased cod. ES114 and MJ11 represent different clades of *V. fischeri* (25, 38), while *V. salmonicda* is a closely related fish pathogen, and genome sequences are available for all three strains (38-40). As previously reported (25), the *ainS* and *ainR* genes have diverged more between ES114 and MJ11 than have most orthologs in these strains (e.g. *rluB*), and this trend was also evident in a comparison of ES114 and *V. salmonicida* (Fig. 4.2A). In all three strains there is an 11-bp gap between the stop codon of *ainS* and the start codon of *ainR*, and the DOOR operon-prediction database predicted that *ainSR* functioned as a single transcriptional unit (41). RT-PCR indicated that *ainS* and *ainR* sequences can be found on the same RNA (Fig. 4.3), which was confirmed by sequencing the amplicon (data not shown). Using ARNold, we identified a putative Rho-independent transcriptional

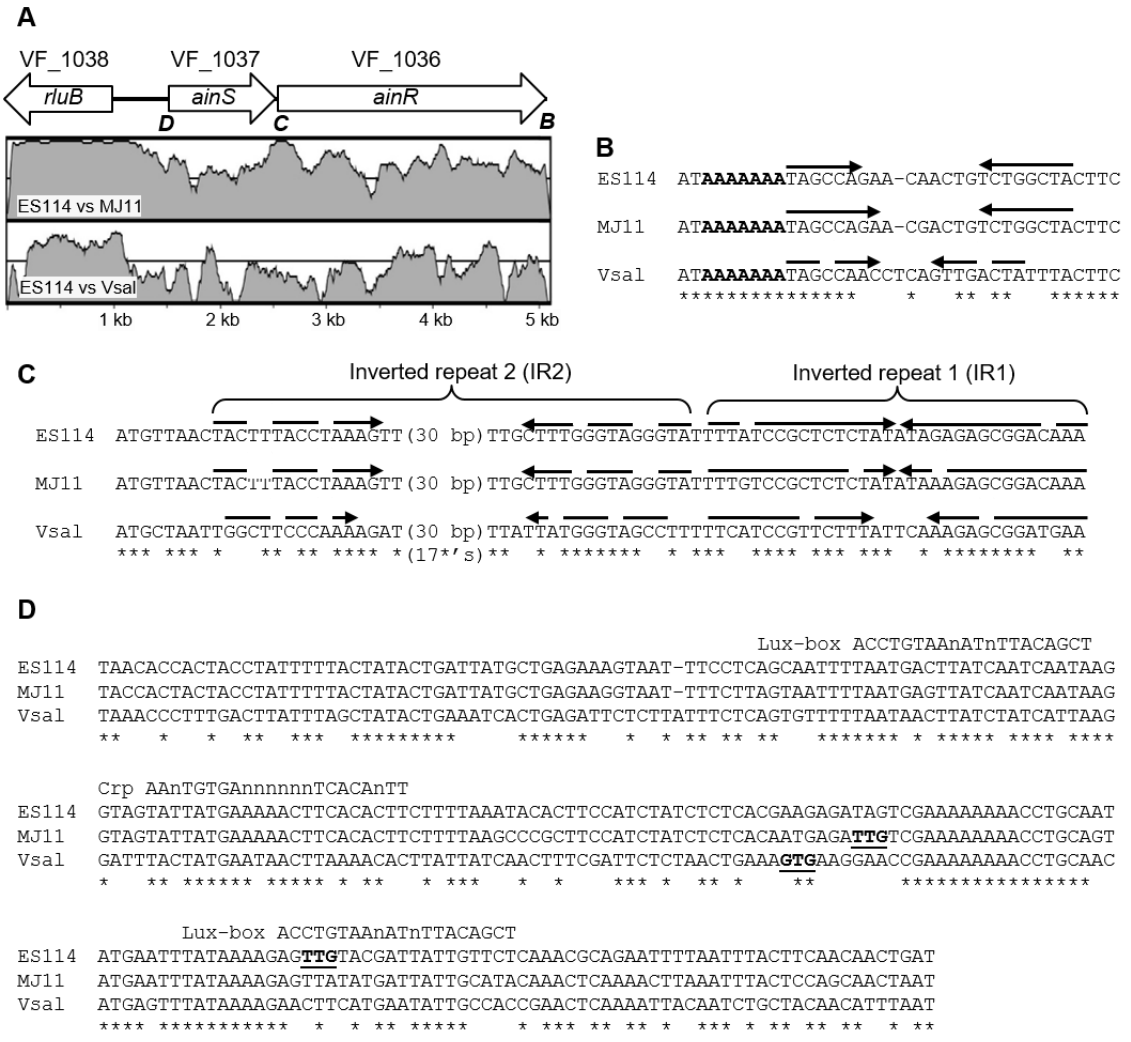

Figure 4.2. Comparison of the *ainSR* locus in divergent strains. The sequence between the stop codons of VF_1038 and VF_1035, which is convergent with *ainR*, in *V. fischeri* ES114 was compared to the orthologous regions from *V. fischeri* MJ11 and *V. salmonicida* LFI1238. In panel A, arrows show the arrangement of the three complete genes at this locus, which extends to the stop codon of VF_1035 on the right. Bold and italicized letters under the arrows indicate regions for which DNA sequence is shown in the corresponding panels. Homology between ES114 and MJ11 or *V. salmonicida* is shown for corresponding regions in shaded plots that range from fifty to one hundred percent identity within a 100-bp window as determined by VISTA (57) with the LAGAN (58) alignment function and default settings. The grey line denoted seventy-five percent identity. Panel B shows putative Rho-independent transcriptional terminators downstream of VF_1035 that were identified by ARNold (59), with arrows indicating inverted repeat stems, with stem mismatches indicated by gaps on the arrows. A bold-lettered run of A nucleotides indicates the canonical string of U's following a stem loop structure on the reverse strand, which would represent transcript for VF_1035. Panel C shows sequences aligned from the start codon of *ainR*, with arrows indicating inverted repeats and mismatches indicated by gaps on the arrows. Panel D includes sequences upstream of *ainS*, with start codons predicted by the SEED (60) indicated with bold and underlined letters. A CRP binding site (22) and possible "*lux* box" LuxR-binding sequences are highlighted by alignment underneath the respective consensus binding sequence. In panels B-D, asterisks indicate bases conserved in all three strains.

terminator downstream of *ainR*, between it and VF_1035 (Fig. 4.2B), although it appears

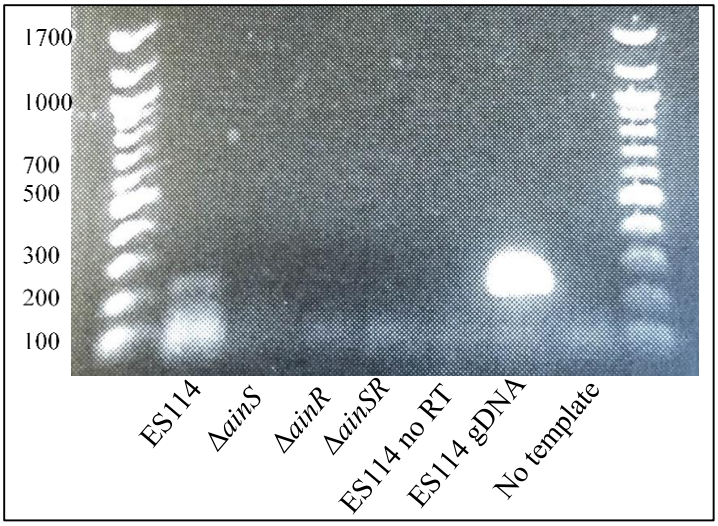

Figure 4.3. *ainS* and *ainR* are found on the same RNA. RNA was extracted from cultures of ES114, NL60 (Δ*ainS*), JHK003 (Δ*ainR*), NL55 (Δ*ainSR*). Reverse transcription reactions were performed using 50 ng RNA, and the resulting cDNA pools were probed for the 236-bp region spanning *ainS* and *ainR*. No reverse transcriptase, ES114 genomic DNA, and no template controls are also shown. Molecular size standards, in base pairs, are listed on the left-hand side.

more likely to terminate the VF_1035 transcript than that of *ainSR*, based on orientation.

Despite the relatively low homology across *ainSR* between ES114 and MJ11, there is a short stretch of high sequence identity spanning the downstream end of *ainS* and the beginning of *ainR* (Fig 4.2A and C). A striking feature within this portion of *ainR* is a nearly perfect 32-bp inverted repeat element designated IR1 (Fig. 4.2C). Moreover, although the *V. salmonicida* sequence has diverged from that of the *V. fischeri* strains, IR1 is also evident in this species. Later, based on RNA-folding predictions, another inverted repeat designated IR2 became evident, although there are more mismatches in the repeat and the region between the repeats is over 30 bp (Fig 4.2C).

To look for possible clues related to *ainS* regulation, we examined the region preceding the putative *ainS* start codon, which is annotated as a non-canonical (non-ATG) start codon in each of the three strains, although the position of the predicted start is different in each strain (Fig 4.2D). In keeping with previous reports, we identified a CRP bind-site (22) and a putative *lux* box just upstream of the CRP site (42), which are reasonably well conserved across strains. We also identified a second putative *lux* box overlapping the predicted *ainS* translational start site in ES114 (Fig. 4.2D). No other

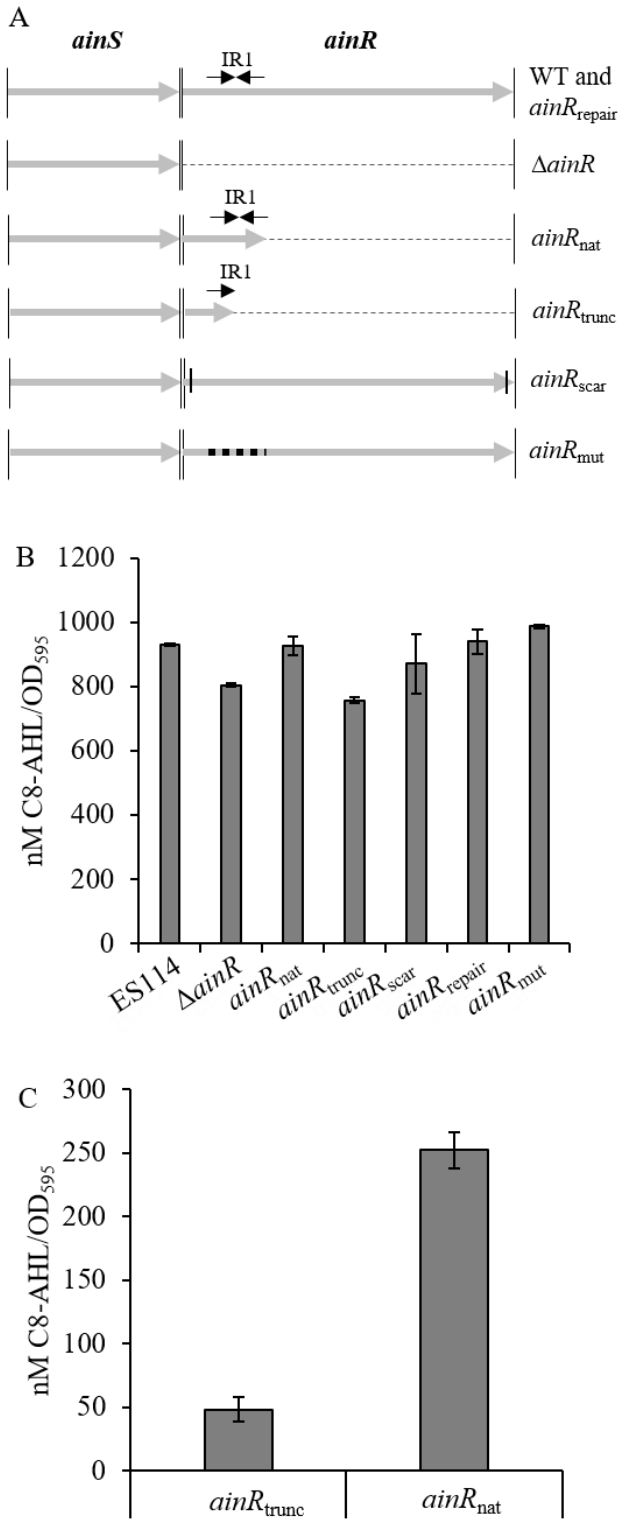

Figure 4.4. Disruption of IR1 sequence and spacing from *ainS* affect C8-AHL accumulation. A) Summary of *ainR* alleles used in this study. Thin vertical bars delineate the ends of *ainS* and *ainR*, shown as thick grey arrows. Black dotted lines represent deleted portions of *ainR*. IR1 and variants are shown as thin, black arrows. Thick horizontal bars indicate restriction enzyme scars. Alternating black and grey boxes represent a mutated IR1 in which the wild-type amino acid sequence is conserved but the symmetry of IR1 has been removed. B) *V. fischeri* strains ES114, JHK003 (Δ*ainR*), JHK055 ($ainR_{nat}$), JHK055 ($ainR_{trunc}$), JHK115 ($ainR_{scar}$), JHK119 ($ainR_{repair}$), JHK120 ($ainR_{mut}$) were grown shaking in SWTO medium to an OD_{595} ~1.5 and C8-AHL was extracted and quantified. C) The $ainR_{trunc}$ and $ainR_{nat}$ alleles on pHK102 and pHK103, respectively, harbored by *E. coli* MG1655 were grown shaking in LB medium to OD_{595} ~1.5 and C8-AHL was extracted and quantified. In panels B and C, a single representative experiment of three independent experiments is shown and bars indicate standard error (n = 2).

potential regulatory sequences were immediately apparent, and we chose to focus on IR1 and the potential *lux* box elements for their potential role in *ainSR* control.

The discovery of IR1 in *ainR* was of particular interest, because we showed in an earlier study that C8-AHL, the product of AinS activity, was decreased when *ainR* was not present *in cis* (11). We therefore hypothesized that IR1 in *ainR* might stabilize the *ainS* transcript, resulting in more C8-

AHL production, thus accounting for the decreased C8-AHL production of a Δ*ainR* mutant. To begin testing the correlation between IR1 and C8-AHL, we constructed *ainR* mutant variants with truncations after IR1 in strain JHK056 ($ainR_{nat}$) or within IR1 in strain JHK055 ($ainR_{trunc}$) (Fig. 4.4A). We found that truncation within IR1 resulted in similar C8-AHL production as that of the full Δ*ainR* mutant; however, truncation of *ainR* after IR1 yielded wild-type levels of C8-AHL (Fig 4.4B).

The above experiments with ES114 and the Δ*ainR*, $ainR_{nat}$, and $ainR_{trunc}$ mutants showed a simple correlation between the presence or absence of the full IR1 and higher or lower C8-AHL, respectively; however, two additional experiments did not support this simple correlation. First, we reintroduced *ainR* into the Δ*ainR* mutant by cloning *ainR* on an NheI-digested fragment into an AvrII restriction site within the Δ*ainR* allele. Although this process re-introduced the complete *ainR* and IR1, it also introduced a 6-bp restriction site scar immediately after the *ainR* start codon, generating a new allele that we designated $ainR_{scar}$ (Fig. 4.4A). Re-introducing IR1 on the $ainR_{scar}$ allele did not restore wild-type C8-AHL levels (Fig 4.4B). To test whether a second-site mutation in the $ainR_{scar}$ mutant was responsible for its unexpected phenotype, we crossed the wild-type *ainR* sequence into the $ainR_{scar}$ mutant ($ainR_{repair}$ – Fig 4.4A), which restored C8-AHL to wild-type levels. Thus, it appears that the effect of IR1 is influenced by context and can be largely reversed by a 6-bp insertion near the *ainR* start.

In the second experiment inconsistent with a simple correlation between the IR and C8-AHL levels, we created strain JHK120 containing an $ainR_{mut}$ allele (Fig. 4.4A), in which the symmetry of IR1's DNA sequences was destroyed while preserving the amino

acid sequence of AinR. Despite lacking IR1, this strain produced wild-type levels of C8-AHL (Fig 4.4B).

We considered the possibility that IR1 stabilizes *ainS* mRNA by inhibiting 3' RNA exonuclease activity from reaching and degrading the *ainS* coding part of the *ainSR* transcript, and we hoped to test this by exploiting defined RNase mutants in *E. coli*. When we placed constructs expressing $ainR_{trunc}$ and $ainR_{nat}$ into *E. coli* MG1655, we observed a similar pattern of C8-AHL accumulation as we saw for these alleles in *V. fischeri* (Fig. 4.4C). However, when we screened *E. coli* mutants with disruptions in the genes coding for RNases D (JW1793-1), PH (JW3618-2), T (JW1644-5), R (JW5741-1), or Z (SK2595), none of the mutants affected the relative C8-AHL output from these two *ainR* alleles (data not shown; 43-45).

In addition to investigating IR1 in *ainR*, we sought to clarify the role, if any, of LuxR in regulating *ainSR*. Others have identified a putative *lux* box upstream of *ainS* (42), and we previously reported a LuxR-dependent decrease in P_{qrr}-*lacZ* reporter activity, which our evidence suggested might be due to LuxR repression of the *ainSR* promoter (11). On the other hand, *ainS* was not identified as part of the LuxR regulon (46, 47), although as discussed below those studies are arguably not definitive on this point. Testing the effect of LuxR on *ainSR* is potentially complicated by the role of the Ain system in regulating LuxR, e.g. via LitR. We therefore utilized a set of engineered strains with *luxR* expression under the control of a constitutive promoter and lacking both AHL synthases and *litR* (20). Using these engineered strains, we found that addition of 500 nM 3OC6-AHL significantly decreased activity of a P_{ainS}-*gfp* reporter only when *luxR* was present (Fig. 4.5A). Furthermore, no decrease in reporter activity was elicited in strains expressing LuxR with

no exogenous 3OC6-AHL or with addition of 500 nM C8-AHL (Fig. 4.5A). The activation of the *lux* operon by 3OC6-AHL and LuxR can be attenuated by adding C8-AHL (13, 14, 20), and we likewise found that adding C8-AHL along with 3OC6-AHL lessened the effect of the latter on repressing *ainS* reporter activity (Fig. 4.5A).

We extended this analysis of LuxR's ability to repress *ainS* first by comparing the repression of the P_{ainS}-*gfp* reporter by LuxR from ES114 with LuxR from MJ1, which is more similar to MJ11, over a physiologically relevant range of 3OC6-HSL concentrations. Both $LuxR^{ES114}$ and $LuxR^{MJ1}$ repressed reporter activity in a dose-dependent manner (Fig. 4.6). The response curve of $LuxR^{MJ1}$ showed that slightly higher concentrations of 3OC6-AHL are required to decrease reporter activity (Fig. 4.6), although it should be noted that the *ainS* promoter target

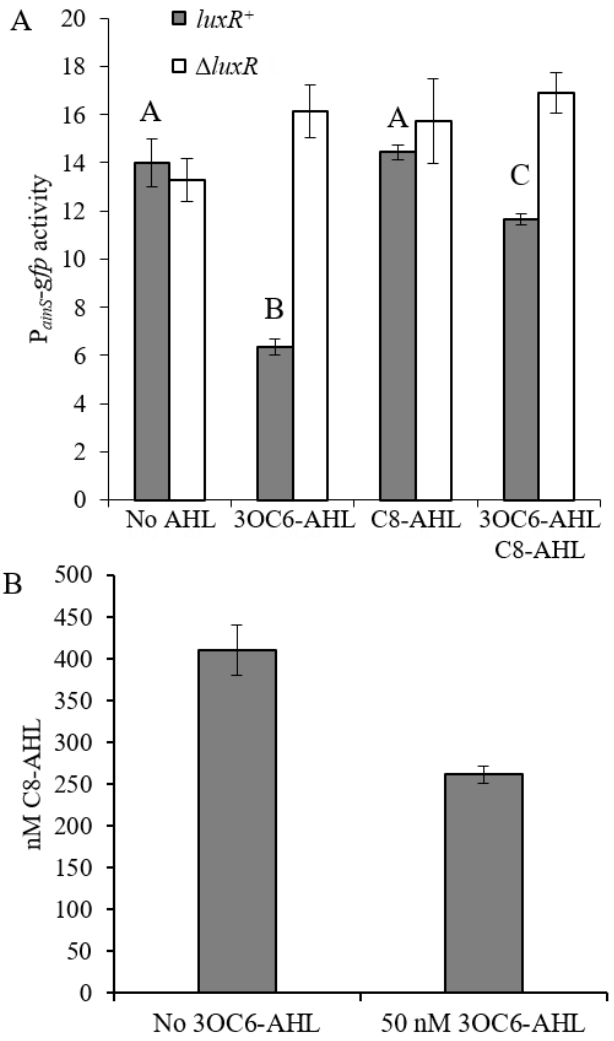

Figure 4.5. LuxR-3OC6-AHL represses *ainS* promoter activity and C8-AHL synthesis. A) Strains with (JHK045, grey bars) or without (JHK046, white bars) *luxR* harboring the P_{ainS}-*gfp* reporter plasmid pHK12were grown shaking in SWTO medium supplemented with different pheromone regimes of either 500 nM 3OC6-AHL or C8-AHL or a mix of both pheromones. GFP expression was measured at OD_{595} ~2.5. Letters indicate significant differences in GFP activity as determined by one-way ANOVA (n = 3). B) C8-AHL accumulation in culture was determined using strain JHK091 grown in SWTO medium with either 0 nM or 50 nM exogenous 3OC6-AHL. * $P < 0.05$. Error bars indicate standard error (n = 2).

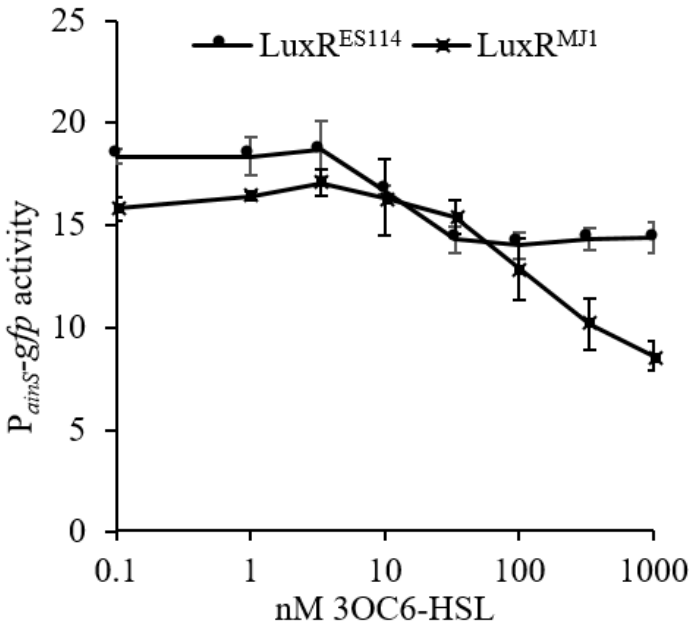

Figure 4.6. LuxR from *V. fischeri* ES114 and MJ1 each affect *ainS* promoter activity. Strains expressing *luxR* from *V. fischeri* ES114 (JHK045, circles) or MJ1 (JHK099, squares harboring the P_{ainS}-*gfp* reporter plasmid pHK12 were grown shaking in SWTO medium with increasing concentrations of 3OC6-AHL, and green fluorescence was measured at OD_{595} ~2.5. Bars indicate standard error (n = 3).

is from ES114. The concentrations of 3OC6-HSL required to achieve half-maximal repression of the reporter were 125 nM and 24 nM for $LuxR^{MJ1}$ and $LuxR^{ES114}$, respectively. We also tested the effect of LuxR-3OC6-AHL at the functional level of C8-AHL synthesis. We again used a strain where *luxR* is disconnected from native *ain*-influenced regulation, but in this case *ainS* was present. When this strain was grown with added 3OC6-AHL, it produced significantly less C8-AHL (Fig. 4.5B). Thus, our data show that LuxR can repress transcription from the *ainS* promoter and diminish output of C8-AHL, which is the product of AinS.

As noted above, others have highlighted a potential *lux* box near the CRP binding site (42), and we found a second putative *lux* box overlapping the predicted AinS translational start site specific to ES114. As our plasmid-based reporter was generated from ES114 sequence including both these sites, we cannot distinguish which, if either, of them is involved in the LuxR-dependent repression described above. However, we also generated a chromosomal *ainS*::*lacZ* reporter strain, in which the putative *lux* box overlapping the TTG start codon is absent. In this reporter background we see no evidence

of *luxR*-dependent repression, with or without 3OC6-AHL (Fig. 4.7), which suggests this site may be required for LuxR-mediated regulation of *ainS*.

Discussion

V. fischeri uses the *luxIR* and *ainSR* AHL-based PS systems to control symbiotic phenotypes (1, 3, 48), with the *ain* system being the first of these to be activated and priming the *luxIR* system through a signaling cascade conserved amongst members of the *Vibrionaceae* family. Despite the placement of *ainSR* atop this signaling cascade, only recently have we begun to understand the regulation of this system (2, 11, 22, 49). In this study we expanded upon those findings, using bioinformatic and comparative genomic analyses (Fig. 4.2) to confirm the prediction that *ainSR* are co-transcribed and identify possible regulators of *ainSR*.

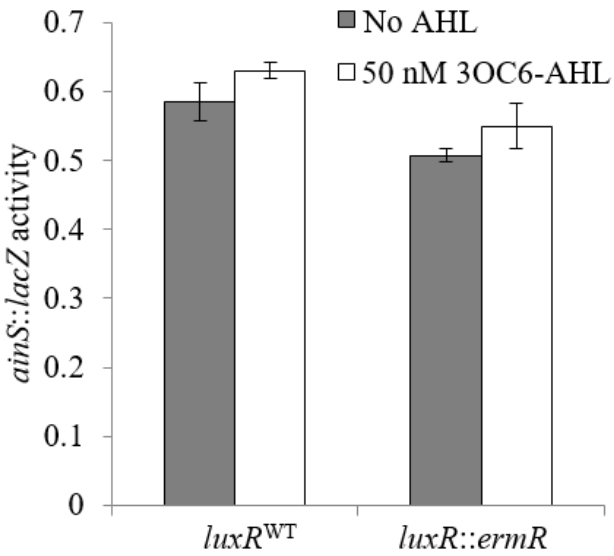

Figure 4.7. Removal of the "*lux* box" overlapping the predicted *ainS* translational start site eliminates LuxR-3OC6-AHL-dependent *ainS* repression. Strains JHK107 (*luxR*) and JHK118 (*luxR*::*ermR*) were grown shaking in SWTO medium with and without 50 nM 3OC6-AHL to an OD_{595} ~2.5 and β-galactosidase activity was measured as previously described (6). A single representative experiment of three independent experiments is shown and bars indicate standard error (n = 3).

LuxR-mediated repression of ainSR produces a negative feedback loop

Hierarchical activation of PS systems, such as the jump-starting of LuxIR by AinSR, is a common feature of bacteria possessing multiple PS systems (50, 51). We have now shown a negative feedback loop also exists between the second system and the first in *V. fischeri* ES114. LuxR represses the *ainS* promoter, and this activity responds to 3OC6-

and C8-AHL in much the same way that these signals activate (3OC6-AHL) or antagonize (C8-AHL in the presence of 3OC6-AHL) activation of the *luxI* promoter (Fig. 4.5A). Moreover, the repressive effect of LuxR on the *ainS* promoter occurred with 3OC6-AHL at physiologically relevant concentrations (Figs. 4.5B and 4.6), suggesting it has a real function for the bacterium. In the host, LuxR repression of *ainS* could lower the concentration of C8-AHL while *luxIR* expression increases during establishment and progression of the symbiosis, but the two systems might reach an equilibrium state where each system controls its respective regulon without further interference from the other. Monitoring expression of each pheromone synthase within the host, either by using two-color fluorescent reporters or by measuring transcript levels, could be valuable in discerning the dynamics between these systems in real time.

Negative feedback loops are known in other bacterial cell-cell signaling systems, although the relationship between Ain and Lux suggests a novel inter-system circuitry. In *Pseudomonas aeruginosa*, RsaL serves a negative regulator of the *lasIR* PS system, binding its activator LasR and preventing it from activating expression of the *lasI* AHL synthase, thus maintaining signal levels during growth (52, 53). In a similar but more complicated scheme, *Sinorhizobium meliloti* ExpR activates expression of the AHL-synthase *sinI* and represses expression of a second *sinI* activator, *sinR* (54), thus, like RsaL, ultimately repressing the expression of its cognate-signal synthase. Perhaps a closer parallel to LuxR repression of Ain would be in *Burkholderia cenocepacia*, which contains CepI/CepR and CciI/CciR AHL-based PS systems. In *B. cenocepacia* CepR is required for *cciIR* induction, but CciR represses *cepI* (55), forming an inter-system negative feedback loop. As more

bacteria with multiple PS systems are investigated, such inter-circuit feedback loops may become more apparent.

Based on previous research, identification of this feedback regulation of *ainSR* by LuxR can be considered both surprising and unsurprising. When the *ain* locus was discovered (in strain MJ1), Gilson and colleagues (42) reported a putative "*lux* box" element in upstream of *ainS*, suggesting either a positive or negative feedback loop might exist from *lux* to *ain*. However, several studies have probed the LuxR regulon in different *V. fischeri* strains since then, yet *ainSR* was never identified as a target of LuxR regulation until now (46, 47, 56). This begs the question how was *ainS* repression by *lux* so elusive to escape detection by multiple studies? In this regard it is worth noting that the effect of LuxR repression is small, although the direct effect of LuxR might be amplified by the positive feedback between AinS/AinR and LitR in the core signal transduction pathway it controls. A proteomic analysis of the LuxR regulon, might have missed a small change in AinS levels (47). Qin and colleagues examined a variety of known LuxR-regulated genes for changes in promoter activity in transgenic *E. coli*, and they included an *ainS* reporter, which did not appear regulated (56). However, we subsequently found that regulation of *ainS* by CRP in *E. coli* is dependent on the presence of *litR*, and the absence of that gene might have likewise obscured a regulatory effect of LuxR in earlier study (56). Each of the aforementioned studies (47, 56), also used strain MJ1 as a genetic background, and our bioinformatic analyses suggest the possibility that ES114, but not MJ1, has a *lux* box overlapping the AinS translational start (Fig 4.2D). Removal of this second *lux* box was sufficient to relieve LuxR-3OC6-AHL-dependent repression of *ainS* (Fig. 4.7), although further analysis, for example using EMSAs with purified LuxR to examine binding at one

or the other possible *lux* box upstream upstream of *ainS*, should be performed before concluding the role of this *lux* box (if any) *in vivo*.

Perhaps the hardest data to reconcile with our own are that of Antunes *et al.* (46), who performed a microarray screen of the LuxR regulon by adding 3OC6-HSL to ES114 and measuring transcriptome changes in ES114. That study was performed in a strain producing some 3OC6-AHL and large amounts of C8-AHL, which can accumulate to levels inhibitory to even large concentrations of 3OC6-AHL, thus affecting its activity, as we and others have shown (Fig. 4.5A; 11, 13, 14, 20). Given the number of positive and negative interactions in the two AHL PS systems, as well as their conditionally dependent regulation, it is not hard to imagine that while the feedback loop we described here is real, it may have been obscured in the conditions used by Antunes *et al.* (46). Whether or not this regulation is important in wild-type under ecologically relevant growth conditions remains to be determined, although it seems unlikely that it would have evolved by chance and not due to a fitness advantage conferred in some situation(s).

Intra-system regulation promotes C8-AHL accumulation

One of the more striking findings of our analysis of the *ainSR* locus was the presence of a small region of relatively high conservation between ES114 and MJ11, including two inverted repeat elements, IR1 and IR2, within the 5' part of *ainR* (Fig. 4.2A and C). The sequences of AinS and AinR have diverged more than other components of the core *Vibrio* PS system (i.e. LuxU, LuxO, LitR) (25), but this IR-containing region was almost completely conserved.

We believe that these IR elements may relate to previously unexplained observations about the importance of *ainR* for C8-AHL accumulation, although the

underlying mechanism remains elusive. Previously, Ray and Visick (16) reported a luminescence defect in a Δ*ainR* mutant, and we subsequently showed less C8-AHL accumulation in Δ*ainR* strains, independent of the positive feedback in the Ain system mediated by LitR (11). Furthermore, we found *ainR* must be present *in cis* with *ainS* to alleviate this defect, and we have now shown that *ainS* and *ainR* are cotranscribed (Fig. 4.3). We speculated that loss of the more perfect of the two inverted repeats, IR1, may play a role in this observed depression in C8-AHL synthesis in a Δ*ainR* mutant. While we showed elimination of half of IR1 mimicked a full *ainR* deletion, destroying the symmetry of the repeat while maintaining the amino acid sequence actually resulted in higher C8-AHL accumulation (Fig. 4.4B). Moreover, the effect of IR1 was lost when a 6-bp insertion was placed just after the start codon (Fig. 4.4B), and predictions of mRNA folding suggested this sequence would disrupt structures formed by IR1 and IR2 (data not shown). These results indicate the mechanism by which *ainR* regulates *ainS* and C8-AHL production is more complex than the simple mechanism that we initially proposed (11). Further examination of the transcript levels, and transcript half-lives, using these IR1 mutants may reveal clues to how *ainR* sequence affects C8-AHL production. Whatever the mechanism, it may allow *ainSR* to be co-transcribed while AinS and AinR are expressed in different stoichiometries.

Sequence differences between ainSR *loci require further examination*

We have used a sequence-based approach to identify two previously unknown levels of regulation of the *ainSR* PS system; however, new puzzling questions have been revealed and remain unanswered. Genome annotation of the three strains places the start codon, all of which are non-ATG codons, at three separate locations (Fig. 4.2D).

Interestingly, a region of nearly complete conservation arises starkly between the predicted translational start sites of *V. fischeri* MJ11 and ES114, and its possible function warrants further investigation. Mapping the translational and transcriptional start sites for *ainS* is ongoing and will be useful in further exploring the regulation of LuxR.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this dissertation was to examine the function and control of the *ainSR* pheromone-signaling system (PSS) in *Vibrio fischeri* ES114. PSSs are traditionally couched as cell-density sensing systems, but it is clear they are also regulated by other factors. While the cell density-independent factors regulating the *lux* PSS have been extensively characterized, the regulatory factors influencing the activity of the *ain* PSS remain largely unknown. Moreover, it seems likely that some mechanisms underlying the integration between the *lux* and *ain* circuits remain to be elucidated. In this chapter, I will discuss how my work has uncovered further complexities in this system and will suggest future avenues of investigation of the *ainSR* PSS.

***ainR* encodes C8-AHL-dependent receptor**

Previous work examined the substrate specificity and selectivity of *V. harveyi* LuxN (1), and these observations were generally assumed to apply to *V. fischeri*'s orthologous receptor AinR. Subsequent work suggested *ainR*'s position in the core PSS was either incorrect or its role more complex than previously assumed (2). In Chapter 2, I outlined the signaling parameters of AinR (3). I showed AinR is more sensitive and more promiscuous than LuxN, responding to sub-nanomolar concentrations of its cognate signal, C8-AHL (Fig. 2.4), but also to a broad array of other AHLs (Fig. 2.5). Such differences in the signal-perception of AinR and LuxN were later attributed to variations in specific

amino acid residues around the pheromone-binding pocket and suggest niche-specific evolution of these sensors (4). Examining AinR in a variety of *V. fischeri* strains and other closely-related species may shed light on the ancestral function of AinR, whether it was originally a "self-only" sensor and evolved to recognize a broader range of AHL signals to exploit other AHL-producing bacteria within mixed-species communities outside the host, or if LuxN's substrate preferences may have narrowed to tightly control its PSSs. Alternatively, *V. fischeri*'s niche in a single-species light organ may have made the need to distinguish "non-self" unimportant, and enabled AinR to sacrifice selectivity to gain sensitivity. Comparison of *V. fischeri* and its relatives from different sources may help distinguish the possibilities.

ainR functions as a regulator of C8-AHL synthesis

AinR also functions as a regulator promoting C8-AHL synthesis, as shown in Chapter 2. AinR influenced *ainSR* regulation through the regulatory cascade involving Qrr and LitR, as was previously hypothesized (Fig. 2.3); however, the presence of *ainR in cis* with *ainS* also affects C8-AHL output. Surprisingly, deletion of *ainR* decreases C8-AHL accumulation (Fig. 2.7; 3). In Chapter 4, I presented evidence that this regulation may involve a conserved inverted repeat (IR1, Fig. 4.2C) in the upstream portion of *ainR.* Partial deletion of IR1 was sufficient to decrease C8-AHL accumulation to levels of the Δ*ainR* mutant (Fig. 4.4A), and introducing a 6-bp restriction enzyme scar used to restore a wild-type *ainR* to the Δ*ainR* mutant also disrupted the effect of *ainR* on C8-AHL synthesis, although it remains unclear through what mechanism this occurs. Puzzlingly, eliminating the symmetry of IR1 while maintaining the amino acid sequence resulted in a small but reproducible increase in C8-AHL production (Fig. 4.4A).

Based on my results, I speculate that IR1 is responsible for stabilizing the *ainS* portion of the *ainSR* transcript, although the underlying mechanism now seems more complex than we previously hypothesized and may rely on several characteristics of the *ainSR* locus. Introduction of even six nucleotides may be sufficient to decrease the folding energy of the *ainSR* transcript to favor a smaller IR incapable of protecting *ainS* (data not shown). RNA secondary structures have been shown to control pheromone synthesis in *Sinorhizobium meliloti*, albeit within the 5' UTR of the AHL synthase SinI (5). Similarly, post-transcriptional regulation via inverted repeat-mediated stabilization of operon components, resulting in differential expression levels, has been described in other non-pheromone systems (6, 7). However, IR1 affecting expression of a cell-cell signaling system would be novel. Further work must be completed to determine the mechanism by which the IR promotes C8-AHL synthesis including measurements of transcript abundance, its size and processing, and its half-life, which could be assessed through such methods as Northern blotting, qPCR, and RNA-seq.

Differential post-transcriptional regulation may enable the cell to cotranscribe *ainS* and *ainR* (Fig. 4.3) while modulating the ratio of their encoded proteins. This may be especially useful given AinR's sensitivity to C8-AHL, demonstrated in Chapter 2 (Fig. 2.4), which potentially decreases the number of sensors required to mount a response at an appropriate pheromone concentration and thus decreasing the need for many large integral membrane proteins. Fluorescent-tagged AinR could be useful in determining the number and position of these sensors around the cell.

Extensive cross-talk between *ain* and *lux* fine tunes signaling

Cross-talk between the *ain* and *lux* systems has been studied before (8-10), but I have shown that the interconnectedness of these systems is more extensive than previously known. In Chapter 2, I presented data suggesting a role for 3OC6-AHL-LuxR in repressing the *ain* system (Fig. 2.6; 3). The effect of LuxR on *ain* activity did not appear to work through any known targets of LuxR regulation (data not shown; 11), and the presence of a putative *lux* box upstream of the predicted AinS start codon suggested LuxR might directly repress *ain* (12), although *ainSR* was absent from previous screens of LuxR-regulated genes (11, 13). In Chapter 4, I expanded the work of Chapter 2 and showed that LuxR represses *ain* promoter activity in a 3OC6-AHL-dependent manner and also decreased the amount of C8-AHL synthesized in broth culture. Interestingly, this effect was conserved across LuxR proteins from different *V. fischeri* wild-type strains, but differed in its sensitivity to 3OC6-AHL and interference from C8-AHL. Thus, the role of 3OC6-LuxR in regulating *ain* expression may be somewhat strain specific, owing to the different affinities and 3OC6-AHL production capabilities of diverse *V. fischeri* strains.

LuxR is generally considered an activator, although transcription of one ORF, VF_1615, was found to be repressed by 3OC6-AHL-LuxR (11). LuxR also acted as a repressor in an engineered system in *E. coli* where a *lux* box was positioned over the -35 or -10 elements upstream of a *lacZ* reporter (14). It is interesting to note that the putative *lux* box upstream of *ainS* overlaps a potential -35 element, and a second putative *lux* box overlaps the predicted translational start site. The *ainS* genes from *V. fischeri* ES114 and MJ11 and the fish pathogen *V. salmonicida* are predicted to begin at different locations, and all three possess non-ATG start codons. Determining the *ainS* transcriptional start site

and the *ainS* start codon through N-terminal protein sequencing would help define the elements controlling *ainS,* and potentially provide insight to as yet unknown regulators of the system. Direct promoter-binding of LuxR to either of the putative *lux* boxes upstream of *ainS* could definitively answer the question of whether or not, and where, LuxR acts to repress *ain.*

New AHL signals expand AinS's role in LuxR control

When I showed in Chapter 2 that C6- and C7-AHL could signal through AinR, this observation initially seemed superfluous with respect to signaling during light organ infections. In this monospecific infection, only 3OC6- and C8-AHL were thought to be present and relevant. But work performed by May *et al.* demonstrated growth phase-dependent, AinS-mediated synthesis of these signals, with accumulating concentrations within the range of AinR's sensitivity (3, 15). I show in Appendix A that the C6- and C7-AHL signals act as agonists (alone) and antagonists (in the presence of 3OC6-AHL) of LuxR-mediated *lux* activation. Modulation of LuxR activity by non-cognate signals has been reported previously in *V. fischeri* and in a transgenic *Escherichia coli* model (9, 10), but these studies were performed without the current understanding of all of the PSSs present in the cell or (in transgenic *E. coli*) without the complex interacting components within the various PSSs. Using a variety of engineered *V. fischeri* strains (16), I showed that the degree to which these non-cognate signals, and C8-AHL, affect 3OC6-AHL-LuxR signaling depends on the source of the LuxR protein, the signal itself, and the presence of *ainR*.

The presence of C6- and C7-AHL in concentrations bioactive to AinR and LuxR adds an additional layer of complexity such that AinS-synthesized signals other than C8-

AHL could interfere with LuxR activity, or in some cases promote it, depending on the strain and substrate availability. This may be relevant if there are circumstances where AinS's substrate preferences, which differ than those of LuxI (17), allow it to affect LuxR's activity. UTRs upstream of LuxR-regulated genes have broad deviations from the consensus *lux* box element (11, 18), and it would be interesting to determine if these non-cognate signals alter LuxR activity in some Lux-box-sequence specific manner. If so, such studies could elucidate novel biological functions of these pheromones. Future studies using naturally and experimentally evolved LuxR variants might show how the relationship of these sensor/signal pairs dictates subsequent protein-protein and protein-DNA interactions. Furthermore, the discovery of these molecules and the known effect of C8-AHL on LuxR-3OC6-AHL-mediated regulation raises the question of the completeness of previous studies of the LuxR regulon (11). Small but meaningful changes in regulation may be obscured by the presence of inhibitory signals, and reexamining the LuxR regulon could provide new information on the extent of its control in the cell.

Unlike 3OC6-AHL (19), nothing is known about the amount of C8-AHL or the other AinS-produced acyl-HSLs present in the light organ. Reliance on bioassays to detect these pheromones has biased surveys to known molecules and could be subject to interference from unknown molecules, resulting in flawed estimates of total pheromone levels. Rapid and sensitive detection and quantification of a wide range of pheromone molecules and the substrates from which they are synthesized is now possible using high-throughput analytical tools (20). Such techniques have already been used to discover C6- and C7-AHL in *V. fischeri* and could provide valuable information about the identities and amounts of different pheromones and pheromone precursors in the light organ (15).

An antisocial pheromone-signaling network promotes survival in lab culture

One of the outstanding questions is what environment-responsive regulators, if any, govern *ain* activity. Previous research in our lab identified CRP as an activator of the *ain* system (21), as is the terminal regulator of the core PSS, LitR (8). However, comparatively little is known about how *ain* responds to external cues other than carbon source (which governs CRP) and C8-AHL availability. In Chapter 3, I described an attempt to elucidate such regulators, but inadvertently enriched for constitutively active variants of the regulator LuxO, which activates expression of the luminescence-repressing small RNA Qrr (22). These LuxO* mutants arise during prolonged growth into stationary phase in static broth cultures in *V. harveyi* (23) and among several *V. fischeri* wild-type strains.

Unlike ES114, some wild-type strains do not generate mutations resulting in increased *qrr* expression (e.g. *luxO**), at least in our screen. While AinS and AinR have diverged more than other components of the core PSS in ES114 and MJ1, including LuxO (24), the absence of apparent LuxO* variants enriched under these conditions suggests alternative routes for static-culture survival among these strains, potentially influenced by differing selective pressures on internal components or regulatory targets of their PSSs. For example, strains that do not generate *luxO** alleles may undergo spontaneous mutations in *litR,* which we did not observe in *V. fischeri* ES114 but is more typical in other vibrios under some circumstances (25, 26). Alternatively, survival-promoting regulators may fall under *litR*-independent, Qrr-mediated control, as appears to be the root of *V. fischeri* ES114's survival advantage.

The results presented in Chapter 3 point to Qrr as a branch point in the core PSS, and elucidation of its regulatory targets will provide insight into the factors governing

culture survival and host colonization. Qrr modulates expression of a variety of PSS components in other vibrios, including LuxO, HapR, AphA, and LuxMN, in addition to several non-PSS-related genes, many of which have no known function (27-29). In light of its single Qrr, compared to four or five in other vibrios, *V. fischeri* may represent a useful model system for dissecting Qrr-mediated regulation without the noise of overlapping function among multiple Qrr species in the same cell. Moreover, determining the Qrr and LitR regulons would further our understanding of complex regulatory networks controlling symbiosis, which cannot always be explained using mutant analysis of known core PSS components. For example, a *litR* mutant has a competitive advantage in host colonization over its wild-type parent, whereas a *qrr, luxO,* and *luxO** strains are outcompeted (8, 22, 30). Furthermore, introduction of a *litR* mutation into *qrr* or *luxO* mutant backgrounds fails to restore symbiotic competitiveness, illustrating the requirement for a fully-functional core PSS, and some unknown symbiosis-relevant branches therefrom, to control symbiosis establishment (30).

In light of the tractability of *V. fischeri* and the diversity of strains from a variety of ecological niches, this system offers an opportunity to examine the evolutionary strategies employed by different strains due to selective pressures governing their lifestyles. For example, obligate free-living strains may experience less pressure to maintain their core PS system than symbiotic strains, or the targets of Qrr underlying stationary phase survival may differ across strains from different host. *V. fischeri* could serve as a useful model in the burgeoning field of bacterial sociality, particularly because of its propensity to become "antisocial", through enrichment of strains with LuxO*, rather than default to a "cheater" state as is common in *V. cholerae* (31). These antisocial mutants do not appear to benefit

from the presence of their cooperative wild-type parents, and thus the root of their survival advantage remains mysterious.

A fundamental question of *V. fischeri*'s life history is the utility of bioluminescence. While luminescence is required for persistence within the light organ (32, 33), it represents a drain on cellular resources resulting in depressed growth rate when maximally induced (33). Presumably, luminescence existed in an ancestor of the present symbiotic *V. fischeri*, but served some other purpose. Because the luminescence reaction consumes oxygen, some hypothesize this process lowers local oxygen concentrations and reduces exposure to reactive oxygen species (34, 35). Therefore, the appearance of LuxO* mutants in *V. fischeri* could qualify them as traditional cheater mutants, benefitting from the protective effects of luminescence by cooperative cells while making a smaller investment in AHL production and luminescence. The appearance of LuxO* mutants could be discouraged under conditions where the probability of finding a host would be high, and selected against further through host-mediated policing within the light organ through an unidentified mechanism that culls dark mutants (32). Alternatively, *luxO** mutants may be more metabolically streamlined, allowing them to divert resources to survival rather than maintaining factors that promote symbiosis with a hypothetical host, amounting to some form of "bet hedging." It is possible that the puzzling lack of secondary *litR* mutations arising in *luxO** strains is due to selection against these mutants under culture conditions but could act as a work around should *luxO** strains move back into areas with squid hosts.

Defining the regulators of *ainS* expression

Despite identifying environment-induced generation of *luxO** alleles as an indirect regulator of *ainS*, the question remains whether there are regulators of *ainSR* beyond CRP,

LuxR, and LitR. I have begun a preliminary mutagenesis-based search for *ain* regulators and have identified known regulators of the system including *litR* and several genes involved in iron acquisition, *iutA* and *iucA*, which presumably act on *litR* through Fur, as described previously (36). Additionally, *hns* was identified as an *ain* repressor, and it similarly represses *lux* expression (37). I have identified several novel mutants with transposon insertions affecting *ainS*::*lacZ* reporter activity, although there is no singular characteristic uniting these mutants. Based on my screen, *spoT* ((p)ppGpp synthetase), *fbp* (fructose 1,6-bisphosphatase), *aspC* (aromatic amino acid transaminase), waaL (O-antigen ligase), VF0149 (glycosyltransferase), and *ptmA* (flagellin modification protein) repress the system and *mnmG* (tRNA modification enzyme) and *rpoQ* (quorum-regulated σ-factor) activate *ainS*. Several of these mutants, including *spoT, fbp, aspC,* and *mnmG* have been complemented *in trans* and have had their *ainS*::*lacZ* activities restored to those of the parent strain. The mechanisms used by these newly identified contributors to *ain* expression remain undiscovered.

Summary

The work presented herein provides an expanded view of the *ainSR* PSS in *V. fischeri*. I have confirmed the position of the sensor AinR within the core PSS network and have shown *ainR* exerts a novel mechanism of intra-system *in cis* regulation over *ainS*, potentially by stabilizing the *ainS* portion of the co-transcript. I have also uncovered new levels of cross-talk between the AHL PSSs. I have further demonstrated enrichment for *V. fischeri* that forego the use of the core PSS altogether to better survive during prolonged stationary phase. Most of these enriched mutants had constitutively active variants of the regulator LuxO, which enhanced survival via an as yet uncharacterized genes controlled

by the luminescence-repressing small RNA Qrr. These results illustrate several ways in which *V. fischeri* controls the activity of its PSSs irrespective of cell-density and provides a number of avenues for future research in this model bacterial signaling system.

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Appendix A

ASSESSING THE EFFECT OF N-HEXANOYL- AND N-HEPTANOYL-HOMOSERINE LACTONE ON THE ACTIVITY OF LUXR IN VIBRIO FISCHERI

Introduction

Vibrio fischeri ES114 produces additional acyl-homoserine lactone (AHL) molecules beyond the canonical *N*-3-oxohexanoyl AHL (3OC6-AHL) and *N*-octanoyl AHL (C8-AHL) signals produced by LuxI and AinS, respectively, although the functional significance of these other AHLs is uncertain. Recently, *N*-hexanoyl AHL (C6-AHL) and *N*-heptanoyl AHL (C7-AHL) were discovered in ES114 supernatants by using highly sensitive and discriminatory liquid chromatography tandem mass spectrometry analyses (Shawn Campagna, personal communication and 1, 2), corroborating and extending a previous observation of LuxI-dependent C6-AHL synthesis by *V. fischeri* MJ1 (3).

An important question is whether C6- and C7-AHL have significant effects on *V. fischeri* signaling or are simply unavoidable byproducts of 3OC6-AHL and/or C8-AHL biosynthesis that do not accumulate enough to matter. In Chapter 2, I demonstrated AinR was sensitive to C6- and C7-AHL (4), with the two half-maximal effects on a P_{qrr} reporter of 114 and 11 nM, respectively, and C6- and C7-AHL accumulated in ES114 supernatants C6- and C7-AHL to concentrations >100 nM (2), suggesting they could function as signals through AinR at physiologically relevant concentrations. However, the more potent signal for AinR, C8-AHL, was always present at far greater concentrations than C6- or C7-AHL (2), and no antagonism was found between the AHLs (4), indicating that the effect of C6- or C7-AHL on AinR likely amounts to an insignificant augmentation of C8-AHL-based signaling.

Another possibility is that C6- and C7-AHL might affect LuxR. Under some conditions 3OC6-AHL accumulation by strain ES114 is undetectable, although C6-, C7- and C8-AHL are present, and even when 3OC6-AHL has begun to accumulate its activity

toward LuxR can be dampened by other AHLs (5). A previous study found that LuxR from strain MJ1 can respond to C6- and C7-AHL, at least in transgenic *Escherichia coli* (6). C6- and C7-AHL also stimulated luminescence, and an ability to inhibit 3OC6-AHL stimulation of luminescence, in *V. fischeri* strain B61, although that work was presumably performed in a background of endogenous C8-AHL and without consideration of how the Ain signaling pathway affected luminescence, as it had not yet been discovered (7). Thus, despite this evidence, the function of C6- and C7-AHL as LuxR agonists, or antagonists of 3OC6-AHL activity, in *V. fischeri* remains uncertain. Moreover, recent work has demonstrated that LuxR proteins derived from *V. fischeri* strains MJ1 and ES114 differ in their sensitivity to activation and inhibition by 3OC6- and C8-AHL (5).

If C6- or C7-AHL do affect LuxR, such results could shed light not only on native *V. fischeri* signaling but also on LuxR-based bioassays. The analytical chemical methods employed by Campagna and his co-workers directly assessed AHLs and underscore the pitfalls of traditional AHL-detection bioassays, whose results could be misinterpreted owing to the complicating biological effects of AHL mixtures. In short, LuxR-based bioassays generally compare luminescence output induced by an experimental standard to that of a single-AHL standard curve (e.g. 3OC6-AHL), with the assumption that non-cognate AHLs (e.g. C6- or C7-AHL) are absent or inconsequential. Knowing that C6- and C7-AHL are present raises the possibility that they are influencing bioassays.

In this Appendix I explore the generation of C6- and C7-AHL by *V. fischeri* ES114 as well as the effects of these AHLs on LuxR. I show that C6-AHL is synthesized by LuxI and AinS, and that C6-, C7-, and C8-AHL affect *lux* activation to varying degrees when assayed directly and against different LuxR variants.

Materials and Methods

Bacteria, growth media, and reagents. Bacterial strains are listed and briefly described in Table A.1. *V. fischeri* ES114 was the wild-type strain used throughout (8). *V. fischeri* was grown in LBS (9) or SWTO (10) medium. Solid media were prepared with 15 g L^{-1} agar. 3OC6-AHL and C8-AHL were obtained from Sigma-Aldrich (St. Louis, MO). C6-AHL and C7-AHL were obtained from Cayman Chemical (Ann Arbor, MI). Deuterated (D_2) AHL standards were synthesized using previously published protocols and were generously provided by S. Campagna (1).

Table A.1. Bacterial strains used in this study.

Strain	Relevant characteristics	Source or reference
V. fischeri		
DC43	P_{con}-*luxR*^{MJ1} P_{luxR}-*luxCDABEG* Δ*ainS* Δ*luxI*	5
DC62	P_{con}-*luxR*^{ES114} P_{luxR}-*luxCDABEG* Δ*ainSR* Δ*luxI*	5
DC64	P_{con}-*luxR*^{MJ1} P_{luxR}-*luxCDABEG* Δ*ainSR* Δ*luxI*	5
DJ01	P_{con}-*luxR*^{ES114} P_{luxR}-*luxCDABEG* Δ*ainS* Δ*luxI*	5
ES114	Wild-type strain	8
JHK007	P_{con}-empty P_{luxR}-*luxCDABEG* Δ*ainS* Δ*luxIR*	4
NL60	ES114 Δ*ainS*	11
NL63	ES114 Δ*ainS luxI* (point mutant)	4
VCW2G7	*luxI* (point mutant)	12

Pheromone extraction and quantification. Overnight *V. fischeri* cultures were diluted 1:1,000 in 25 ml SWTO in 125-ml flasks and incubated with shaking (200 rpm) at 24°C. At regular intervals, the optical density at 595 nm (OD_{595}) was measured for 500-µl samples using a BioPhotometer (Brinkman Instruments, Westbury, NY). Pheromones were extracted from culture supernatants using acidified ethyl acetate containing 50 nM D_2-AHL standards.

Absolute quantitation was performed by isotope dilution mass spectrometry using a Thermo TSQ Quantum Discovery Max equipped with a Surveyor MS Pump Plus and Acella autosampler. Absolute concentrations of C6- and C7-AHL were calculated by taking the peak area of the unlabeled AHL divided by the peak of the standard multiplied by the concentration of the standard. High-performance liquid chromatography and mass spectrometry conditions were identical to previous reports (1).

Luminescence measurements. Overnight *V. fischeri* cultures grown in LBS were diluted 1:100 in 200 µl SWTO medium in clear-walled, black-bottom 96-well plates and incubated with shaking (200 rpm) at 24°C. Cultures were supplemented with known concentrations of AHL standards. At regular intervals, OD_{595} and luminescence were measured using a Synergy 2 plate reader (BioTek). For all experiments, luminescence is reported at OD_{595} ~1.0.

Results and Discussion

Endogenous production of C6- and C7-AHL could imply that these signals serve some purpose relevant to *V. fischeri*'s life history. Elucidating the mechanisms behind their synthesis and potential functions will further our understanding of the complex pheromone-signaling systems in this model bacterium. With this in mind, we asked two questions: 1) Which AHL synthase is responsible for C6- and/or C7-AHL synthesis? and 2) How do these two signals affect *V. fischeri'*s other AHL receptor, LuxR?

Competing reports have ascribed the synthesis of C6-AHL to LuxI or AinS (Shawn Campagna, personal communication and 3). In order to determine which of these synthases is responsible for C6-AHL synthesis, we measured the C6-AHL production of the Δ*ainS* mutant NL60 (11), the *luxI* frameshift mutant VCW2G7 (12), and the Δ*ainS luxI* double

mutant NL63 (4) grown in SWTO medium (Fig. A.1). C6-AHL production peaked around 15 hr (data not shown) and pheromone accumulation in the culture of the single mutants agrees closely with previous reports, ranging from ~100-200 nM (2). However, the Δ*ainS luxI* NL63 failed to accumulate C6-AHL above the limit of detection, demonstrating that both synthases are capable of producing C6-AHL. Furthermore, neither of these mutants nor ES114 produced detectable C7-AHL, even after 18 hr of growth in SWTO (data not shown). Campagna and co-workers detected C7-AHL more than twelve hours after subculturing, later in growth than other AHLs appeared, and their cultures were grown in LM medium further into stationary phase than our experiments (2). Taken together, the results of these different studies suggest specific AHL production, particularly C7-AHL, may depend on particular growth media or conditions. The dependence of C7-AHL synthesis on particular growth conditions could in turn indicate that a seven-carbon acyl substrate for AinS is not always available.

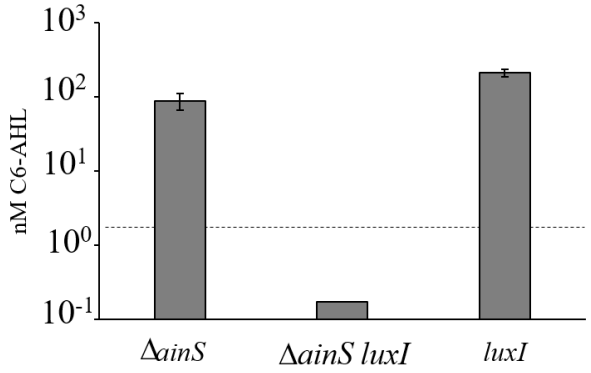

Figure A.1. Accumulation of C6-AHL in different AHL-synthase mutants. The AHL synthase mutants NL60 (*ainS*), NL63 (*ainS luxI*), and VCW2G7 (*luxI*) were grown at 24°C at 200 rpm shaking for 15 hr, total pheromone was extracted using acidified ethyl acetate containing 50 nM D_2-C6-AHL internal standard, and AHL was quantified using liquid chromatography tandem mass spectrometry. Dotted line, limit of detection, 1.97 nM.

We next explored whether C6- and C7-AHL could affect the ability of LuxR to activate transcription of the *lux* operon or interfere with *lux* activation via 3OC6-AHL-LuxR. To accomplish this goal, we utilized a previously described set of engineered *V.*

fischeri strains, using ES114 as the parent, and carrying *luxR* cloned from the dim strain ES114 or from the bright fish light organ isolate MJ1 (4, 5). In our engineered strains, *luxR* is expressed from a constitutive promoter and activates *luxCDABEG* from a LuxR-dependent fragment of the *luxI* promoter. Endogenous AHL production is eliminated by the deletion of *luxI* and *ainS*, allowing us to define pheromone concentrations in these assays. We also assayed strains with (DJ01 and DC43) or without (DC62 and DC64) *ainR*, because a previous study (5) suggested AinR might have an unanticipated AHL-dependent effect on luminescence output in this setup.

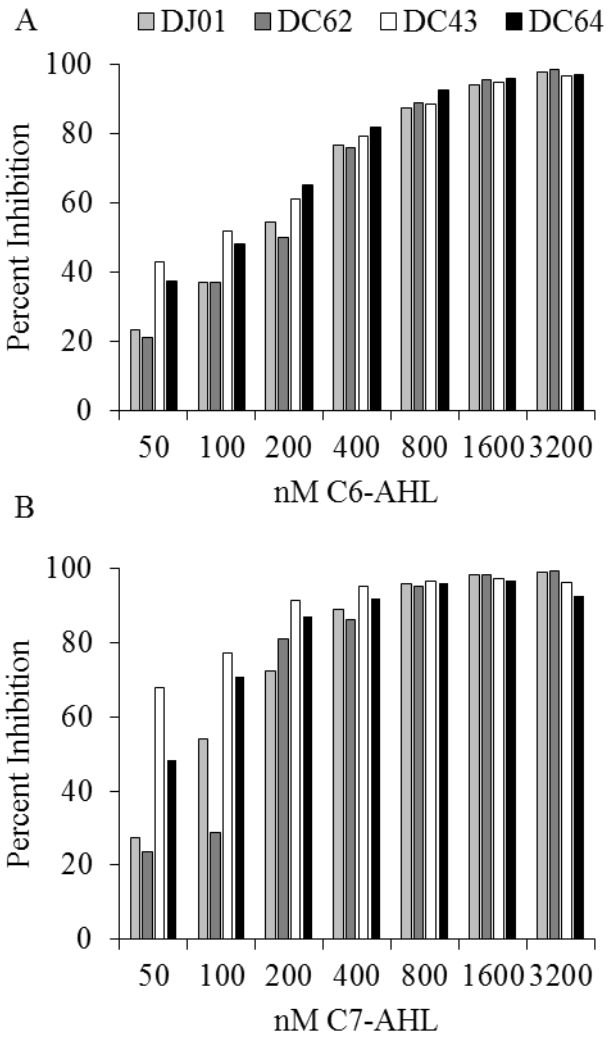

Figure A.2. C6-AHL and C7-AHL inhibit 3OC6-AHL-LuxR activation of *lux*. Strains DJ01 (P_{con}-$luxR^{ES114}$ P_{luxR}-*luxCDABEG* Δ*ainS* Δ*luxI* light grey bars), DC62 (P_{con}-$luxR^{ES114}$ P_{luxR}-*luxCDABEG* Δ*ainSR* Δ*luxI*; dark grey bars), DC43 (P_{con}-$luxR^{MJ1}$ P_{luxR}-*luxCDABEG* Δ*ainS* Δ*luxI*; white bars), and DC64 (P_{con}-$luxR^{MJ1}$ P_{luxR}-*luxCDABEG* Δ*ainSR* Δ*luxI*; black bars) were grown in 96-well plates with SWTO containing 320 nM 3OC6-AHL and a gradient of A) C6-AHL or B) C7-AHL and assessed for luminescence at OD_{595} ~1.0. Percent inhibition was calculated as the decrease in luminescence compared to luminescence of strains lacking competitor AHL. The strain JHK007 (not shown), which lacks *luxR*, served as a negative control and showed no luminescence in the presence of any AHL.

We tested each *luxR* variant with increasing concentrations of C6-, C7-AHL with or without 320 µM 3OC6-AHL (Figs. A.2 and A.3). We first focused on the ability of C6- or C7-AHL to inhibit

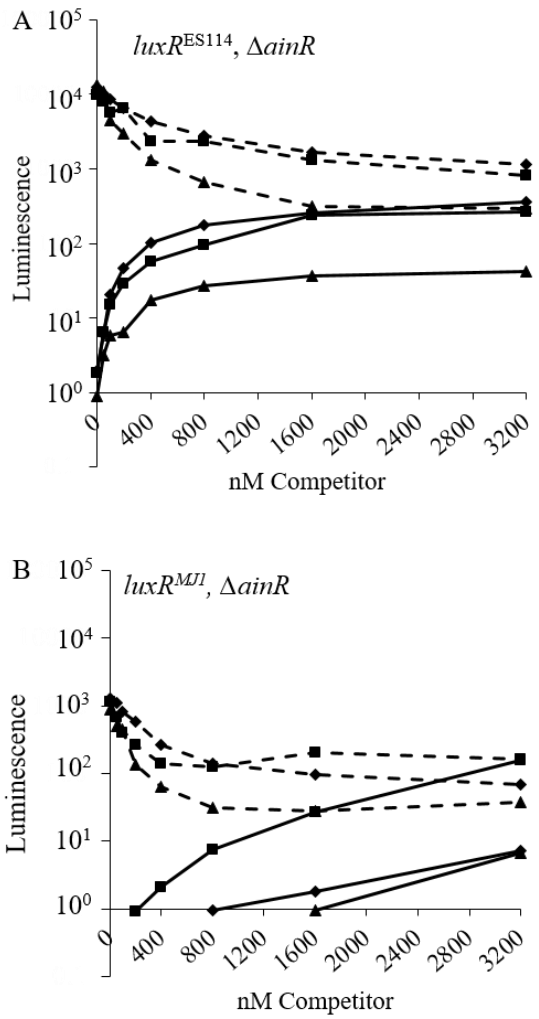

Figure A.3. C6-AHL, C7-AHL, and C8-AHL exert different effects on inhibition and activation of LuxR from *V. fischeri* ES114 (A) or MJ1 (B). In both strains, *luxI* and *ainSR* have been deleted and *luxR* expression is controlled by a constitutive *luxI*-based reporter. Strains were grown in 96-well plates in SWTO medium supplemented with 0 nM (solid lines) or 320 nM (dashed lines) of 3OC6-AHL and a gradient of either C6-AHL (diamonds), C7-AHL (triangles), or C8-AHL (squares). Luminescence was measured at OD_{595} ~1.0.

3OC6-AHL-mediated activation of luminescence, in the presence or absence of *ainR* (Fig. A.2). We found that luminescence induced by 3OC6-AHL was >90% reduced when more than 800 nM C6-AHL or more than 400 nM C7-AHL were added (Fig. A.2). Within the physiological range of C6- or C7-AHL production (50 to 200 nM), 3OC6-AHL-mediated activation of luminescence was inhibited to a greater degree for $LuxR^{MJ1}$ than for $LuxR^{ES114}$, and in general C7-AHL was slightly more inhibitory than C6-AHL (Fig A.2).

Interestingly, the deletion of *ainR* in strains expressing either *luxR* variant resulted in less inhibition (more luminescence output) at C7-AHL concentrations of 50 and 100 nM (Fig. A.2). By contrast, the presence of *ainR* did not change the effect of C6- or C8-AHL on luminescence (data not shown). At these relatively low concentrations where we observe an *ainR*-dependent effect for C7-AHL but not C6-AHL, C7-AHL is also a stronger agonist of AinR-mediated signaling than is C6-AHL (4). Nonetheless, the mechanism underlying this effect is mysterious. AinR-mediated signaling is known to

affect *luxR* transcription in wild type, but in this setup *luxR* is expressed from a constitutive non-native promoter. Possible explanations include the AinR-controlled regulon controlling something that affects turnover of LuxR protein or AHLs. Alternatively, perhaps AinR itself competes with LuxR for C7-AHL. Experiments that introduce a *qrr* or *litR* mutation would help distinguish between a direct role of AinR protein or a regulatory effect from its signaling effects mediated via Qrr and LitR.

We next examined the ability of C6- and C7-AHL to direct LuxR-mediated activation, compared this ability to the above-described inhibition of 3OC6-AHL mediated stimulation, and included C8-AHL for perspective (Fig. A.3). All three of these non-cognate AHLs directed LuxR-mediated activation. For $LuxR^{ES114}$, C6- and C7-AHL were stronger activators than C8-AHL, whereas for $LuxR^{MJ1}$, C7-AHL was a more potent activator than either C6 or C8-AHL (Fig. A.3). On the other hand, with respect to inhibition of 3OC6-AHL-mediated activation, both *luxR* variants were more strongly affected by C8-AHL than C6- or C7-AHL. These results extend earlier observations that $LuxR^{ES114}$ and $LuxR^{MJ1}$ are functionally distinct, and we now show that there is not a simple relationship between strength of an AHL as a LuxR agonist and its ability to inhibit 3OC6-AHL mediated activation.

The discovery of AinS-synthesized C6- and C7-AHL, two pheromones previously shown to have some bioactivity to AinR and LuxR, was an exciting development in an already complex scheme of pheromone signaling in *V. fischeri.* AinS/AinR modulates the activity of the LuxI/LuxR pheromone system directly through C8-AHL-LuxR activation of the *lux* operon (6, 7, 12) and through the core *Vibrio* pheromone-signaling circuit via LitR (13). The presence of two other AinS-synthesized LuxR-bioactive compounds

suggests more extensive cross-talk that may be driven by the metabolic state of the cell or by environmental conditions. Importantly, our results now show that physiologically relevant C6- and C7-AHL concentrations can influence signaling by $LuxR^{ES114}$. Their ability to activate the *lux* operon in the absence of 3OC6-AHL should result in more LuxI and could prime the system, leading to LuxI-mediated positive feedback and jump-starting the accumulation of 3OC6-AHL. On the other hand, they can also dampen the effects of the cognate signal. The dynamic interactions of these different AHLs warrants further investigation.

Our results also show that the similarity of the non-3OC6-AHL signal (i.e. chain length) does not necessarily dictate the efficacy with which it interacts with LuxR, adding further complexities to the relationship between chemical signals and strain-specific receptors. These results may inform future work on the mechanisms by which different AHL signaling molecules interact with their target receptors and their subsequent role in controlling transcription activation.

Finally our results underscore the pitfalls of using traditional LuxR-based bioassays to assess 3OC6-AHL. In light of our data (Figs. A.2 and A.3), it seems likely that in previous studies C6- and C7-AHL may have been present in sufficient quantity to influence luminescence output in bioassays. Most bioassays of 3OC6-AHL are based on $LuxR^{MJ1}$, and would have been particularly sensitive to activation by C7-AHL or inhibition by C8-AHL (Fig. A.3), although C6-, C7-, or C8-AHL can each affect output. This shortcoming would be more pronounced when assessing strains like ES114 that produce relatively little 3OC6-AHL in culture. Bioassays will remain valuable tools, but consideration of AHL

mixes would be prudent. Moreover, the techniques of analytical chemistry employed to identify C6- and C7-AHL should augment the use of bioassays in the future.

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