

ILLUMINATING EVOLUTION: EXPLORING THE VARIABILITY AND FUNCTIONAL
DIVERSITY OF *VIBRIO FISCHERI LUXR*

by

KATHRYN ANN BELLISSIMO

(Under the Direction of Eric V. Stabb)

ABSTRACT

The regulation of bioluminescence in *Vibrio fischeri* relies on the transcriptional regulator LuxR to activate *lux* genes in response to two main pheromone signals: *N*-3-oxo-hexanoyl-L-homoserine lactone (3OC6-HSL) and *N*-octanoyl-L-homoserine lactone (C8-HSL). Related pheromone-signaling (PS) systems are commonly found in Pseudomonadota (formerly Proteobacteria) and are responsible for regulating processes like biofilm formation, virulence, and other community-based behaviors relevant to agriculture, industry, and healthcare. *V. fischeri* bioluminescence serves as an excellent model system for studying LuxR-family transcriptional regulators due to its genetic tractability and the easily measurable nature of luminescence as an output. In this dissertation I explore the notable genetic and functional divergence between *luxR* from twenty-one strains of *V. fischeri* and close relatives. I first tested the HSL responsiveness of eight *V. fischeri luxR* variants using an otherwise isogenic *V. fischeri* background engineered to be disconnected from other PS regulation, to have constitutive *luxR* expression, and to lack endogenous HSL synthesis. Strains expressing each LuxR variant produced a unique response to HSL signals added singly and in combination. I then explored whether the differences in *luxR* coding sequences impact its mRNA or LuxR protein levels and determined that these levels varied

by *luxR* sequence and by the presence of HSL. These differences correlated with the predicted stability of *luxR* mRNA 5' secondary structures, which in other genes is known to affect translation initiation efficiency. LuxR variants also differed in the extent to which HSL increased LuxR protein stability. Finally, I tested the HSL responsiveness of an additional four *luxR* sequences, specifically *luxR1* and *luxR2* from the *V. fischeri* relatives *Vibrio logei* and *Vibrio salmonicida*. These *luxR* sequences also exhibited substantial evolutionary divergence, and they could not effectively induce bioluminescence in our engineered *V. fischeri* background. However, when the LuxR-binding “*lux* box” sequence was swapped for one from *V. salmonicida*, that bacterium’s LuxR2 was able to activate a small amount of bioluminescence. These results highlight species-specific regulatory adaptations and coevolution occurring between *luxR* and the *lux* box. Taken together, our findings expand our understanding of *luxR* evolution and regulation of the PS systems in *V. fischeri*.

INDEX WORDS: *Aliivibrio*, *Photobacterium*, Quorum sensing, Pheromone-signaling, Bioluminescence, LuxR

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DEDICATION

I dedicate this dissertation to my parents, Leslie and Richard Bellissimo. Thank you for being a never-ending source of support and encouragement throughout not only this PhD, but my entire life. Thank you for sending me to DNA camp, buying me my first microscope, and always believing I would be the first Dr. Bellissimo. You never told me I couldn't do this, and for that I will always be grateful.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview

Though bacteria exist as individual cells, they have developed systems for communicating with one another to convey information about their environment and coordinate group behaviors. This communication occurs via pheromone signals, sometimes called “autoinducers” (AI), between cells in a local population (3). Such behaviors are cell-density dependent; at low population densities, cells release AIs into the environment, but the AI concentration remains too low to induce a response. However, when the cells reach a high enough density (or a “quorum”), AIs build up in the environment triggering the individuals to respond. This process is called bacterial pheromone signaling (PS), or “quorum sensing” (3).

AI molecules were initially thought to be merely census-taking molecules, however it is now understood that these signaling systems represent more sophisticated information-processing networks (4-6). A quorum may be necessary for cells to communicate by PS, yet a quorum alone may not be sufficient to induce a response, and pheromone concentrations do not strictly correlate with cell density (1, 5-9). For example, pheromone synthases are often controlled by environmentally responsive regulators, and even with a necessary quorum, a lack of specific environmental factors can prevent a population-wide response (1, 6-9). In addition, many diverse PS systems incorporate positive-feedback regulation, which can lead to hysteresis (6, 10). Furthermore, bacteria can employ several distinct PS systems at once, some with cross-talk between systems (11). If AI signals are purely to communicate a quorum, it is unclear what the

purpose of environmental regulation, positive-feedback, and multiple PS systems in one organism would be. It seems clear that in at least some cases, PS systems are “quorum dependent” but have not evolved simply to “quorum sense”, and several other functional explanations for PS have been proposed (5, 6). In this dissertation, I contribute to our understanding of the complex evolution of PS systems by investigating a key pheromone responsive regulator, LuxR, in the model PS system of *Vibrio fischeri*.

***Vibrio fischeri* as a model of pheromone signaling (PS)**

PS was originally discovered in *V. fischeri*, a bacterium that uses homoserine lactone-based pheromones to coordinate induction of bioluminescence during symbiosis with marine animals (12-14). At the time of its discovery, and for two decades after, this HSL-based form of cell-cell signaling was believed to be a quirk of marine bioluminescent bacteria (4, 5). Peptide-based PS was found almost concurrently in Gram-positive bacteria, where it underpinned DNA transfer, but again only a narrow range of bacteria were thought to employ it. However, starting in the 1990's PS systems have since been discovered in numerous other bacteria (4, 5, 15, 16). These systems, some of which are homologous to the PS systems in *V. fischeri*, are now implicated in regulation of processes other than bioluminescence, such as antibiotic production, biofilm formation, and virulence (16-20). *V. fischeri* remains a valuable model system for studying PS with multiple useful properties underlying its experimental tractability. *V. fischeri* is non-pathogenic, has a rapid growth rate in culture, a large genetic toolbox, and complex interconnected PS systems that model key elements of PS systems in other bacteria (11, 21, 22). Thus, studying the complexities of the *V. fischeri* PS system helps us better understand how information flows through PS systems, many of which are relevant to industry, agriculture, and human health (16-20).

A more unique property of *V. fischeri* further enhances its value as a model organism; it has natural symbioses that researchers can reconstitute in lab (11, 21-23). *V. fischeri* emits light from specialized organs of both fish and squid, and some of the squid have proven amenable for laboratory study. In particular, the symbiosis between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes*, has been adopted by several research groups (24, 25). The contribution of the bacteria to this mutualism is their bioluminescence, which as noted above is a PS-controlled behavior. Thus, while most bacterial PS systems are studied exclusively (or nearly so) in culture or artificial infection systems, the *V. fischeri* PS system has been examined in an ecologically relevant context: a natural organ in a co-evolved host (23-25).

In addition to its contributions to understanding PS, the relationship between *V. fischeri* and *E. scolopes* serves broadly as a valuable model for understanding host-microbe interactions (23, 25). These bobtail squid possess a specialized “light organ” for housing their bioluminescent symbionts (24-26). Exclusively colonized by *V. fischeri*, the light organ provides the bacteria with an environment that supports robust growth and division, while granting the squid a significant survival advantage. Via a process called counterillumination, the squid harness the bacterial bioluminescence to glow through their ventral side and match the intensity of moonlight during their nocturnal activities (27, 28). It is thought that this adaptive camouflage masks the squid's silhouette from below, enabling it to evade predators beneath it in the water column and to approach prey more stealthily (Fig. 1.1A).

E. scolopes squid hatch aposymbiotically, but within hours *V. fischeri* begins rapidly colonizing the light organ, typically achieving a bioluminescent infection in a day (24, 25, 29). Upon hatching, juvenile squid have two sets of three pores leading to the inner crypts of the light organ. Each set of pores is at the base of two ciliated epithelial appendages (CEA) responsible for

moving seawater and particles across the surface of the light organ, gathering bacteria for potential colonization (Fig. 1.1B) (24, 25, 30). *V. fischeri* initially colonizes by forming a biofilm near the pore openings, then navigates through a pore into one of the light organ's crypts (23, 30). After successful colonization, the symbionts trigger the CEA to regress, preventing further infection (24, 25, 31). Within the light organ, the *V. fischeri* population increases and the cells initiate luminescence once they perceive host-related environmental signals (described below) and reach a quorum.

The dynamics of the symbiotic infection and the

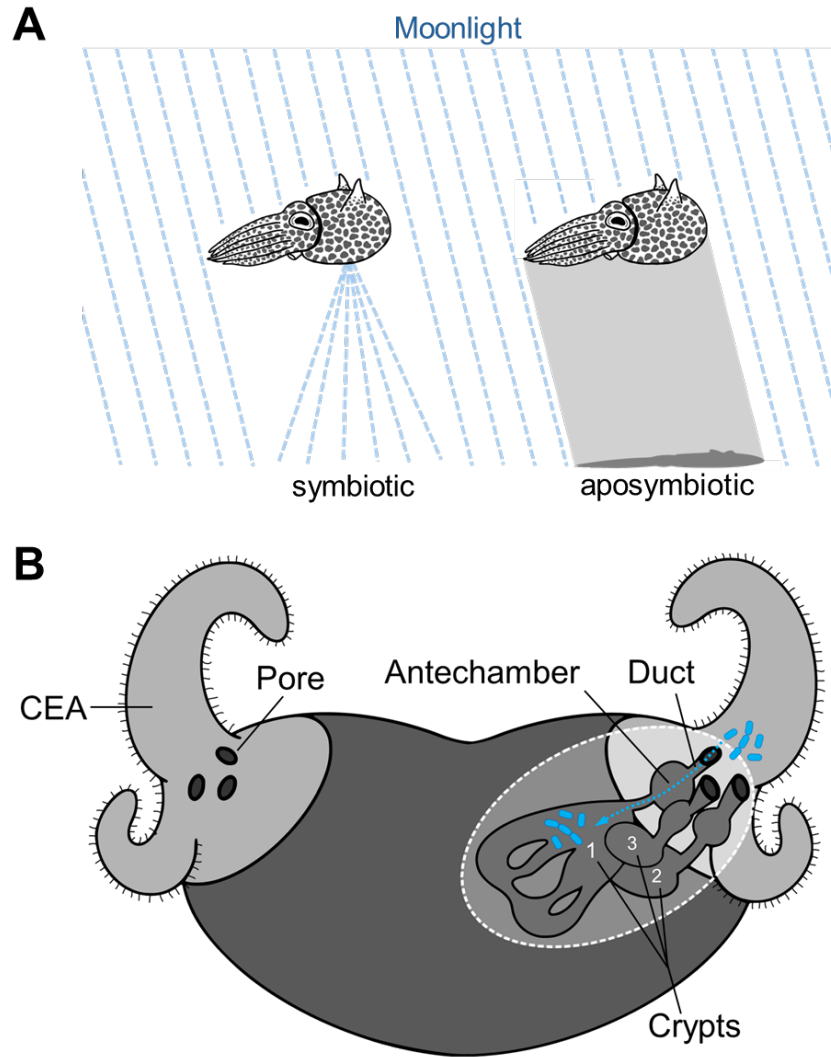


Figure 1.1. The Squid-*Vibrio* symbiosis. (A) *E. scolopes* harnessing *V. fischeri* luminescence for counterillumination. Aposymbiotic squid cast a shadow in the moonlight making their silhouette visible from below, while symbiotic squid shed light to match the moonlight and mask their silhouette. (B) Light organ of juvenile *E. scolopes*. Arrows designate the major structures of the light organ. Upon hatching, each lobe of the light organ is equipped with a pair of ciliated epithelial appendages (CEA). Positioned at the base of these appendages are three pores, each connected to a duct, followed by an antechamber, and ultimately an epithelium-lined crypt. The number of each crypt corresponds to the order that they mature during development. The blue cells and dotted arrow depict how *V. fischeri* gather at the pores and travel to the crypts, where they will ultimately replicate, reach a quorum, and bioluminesce.

bacterial attributes necessary for colonization have been the subject of many studies. A diurnal rhythm in the symbiosis is responsible for seeding the environment with *V. fischeri* cells to infect the next generation of squid. Each morning, the squid expels 90% of the *V. fischeri* colonizing the light organ into the environment (24, 25, 29, 32). These vented cells remain bright for a time but eventually become dim as they are removed from both the quorum and luminescence-triggering cues in the host. During the day, the remaining bacteria in the light organ replenish the population, restarting the cycle and achieving full bioluminescence again by nightfall (24, 25, 29, 32). Essential genes for effective light organ colonization, including those for biofilm formation, motility, and bioluminescence, are termed colonization factors. Specific colonization factors may be important at different points in the symbiosis (23, 33). For example, bioluminescence is dispensable for initial colonization but required for persistence (34-36). My research focuses on the bacterial genetics governing bioluminescence regulation and the communication systems facilitating information processing and response.

Multiple PS systems in *V. fischeri*

The PS network in *V. fischeri* involves at least three pheromone signal-receptor combinations, two signal-transduction systems, and cross-talk between these systems (Fig. 1.2) (11, 22). The primary system with respect to bioluminescence regulation is the *luxICDABEG* (*lux*) operon along with the divergent gene *luxR*, which together encode the key proteins required for luminescence and its regulation (37, 38). LuxA and LuxB encode the α and β subunits of a heterodimeric luciferase, which catalyzes a redox reaction that produces light as a byproduct (Fig. 1.3) (37, 38). LuxC, LuxD, LuxE, and LuxG generate the substrates used in the luminescence reaction: a long-chain aliphatic aldehyde and a reduced flavin mononucleotide (Fig. 1.3) (1, 37-40). LuxI produces the acyl-homoserine lactone (HSL) pheromone *N*-3-oxo-hexanoyl-L-

homoserine lactone (3OC6-HSL), the main AI for this system (Fig. 1.2) (41). 3OC6-HSL combines with the transcriptional activator LuxR, which allows LuxR to multimerize and bind to a 20-bp imperfect inverted repeat directly adjacent to the *lux* promoter (centered at -42.5) called the “*lux* box” (Fig. 1.2) (42, 43). This bound LuxR-HSL complex interacts with RNA polymerase (RNAP), compensating for a weak -35 promoter element, and activates transcription of the *lux* operon (19, 22, 42, 43). Increased *lux* operon transcription yields more 3OC6-HSL production, generating a positive-feedback loop. At high cell-density, high concentrations of 3OC6-HSL build up in the growth medium, triggering this positive feedback loop in neighboring cells.

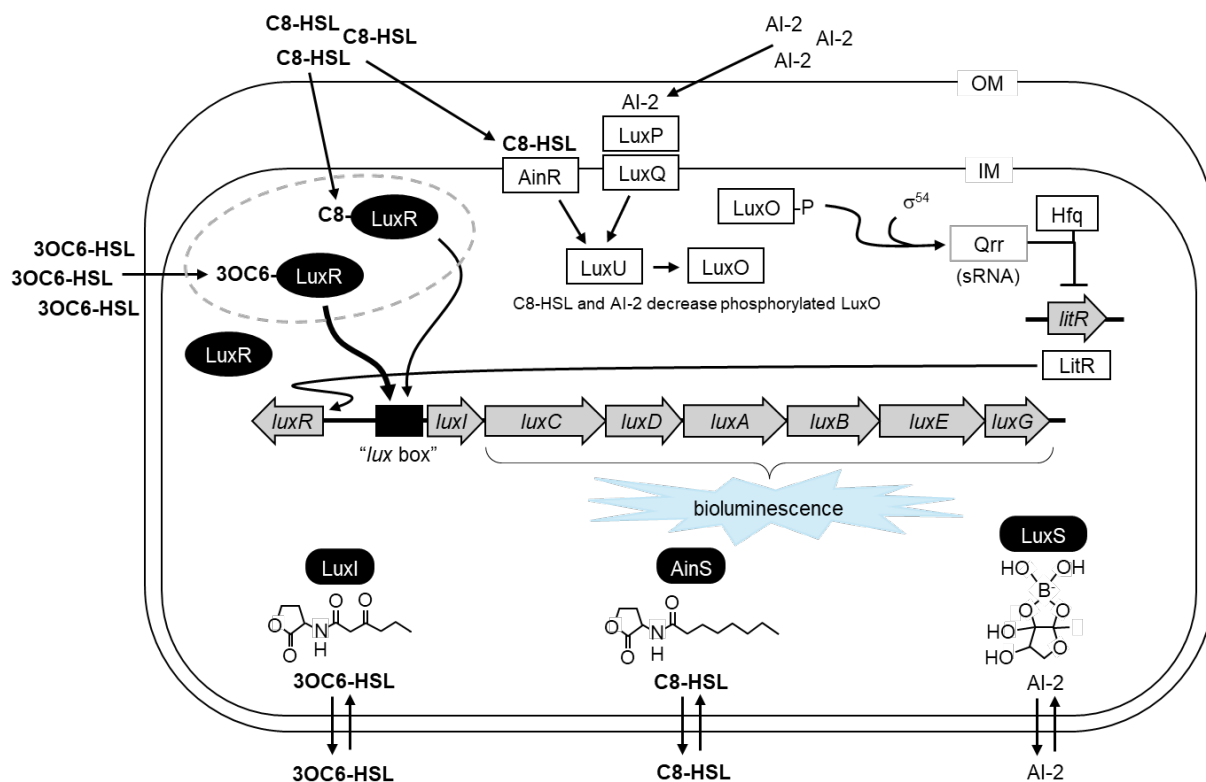
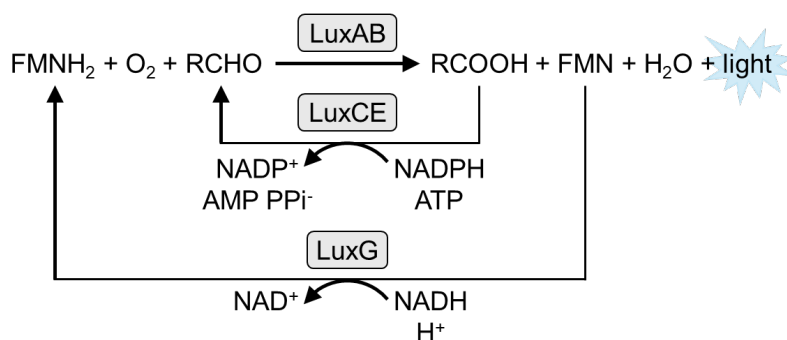


Figure 1.2. Model of HSL-mediated regulation of the *lux* operon in *V. fischeri*. Arrows illustrate the relative positions of the genes of the *lux* operon: *luxICDABEG* and the divergent gene *luxR*. LuxI produces 3OC6-HSL which forms a complex with LuxR and activates transcription of the *lux* operon by binding the “*lux* box” sequence of the *lux* promoter. C8-HSL (produced by AinS) indirectly increases *luxR* transcription through the pictured signal cascade but can also form a complex with LuxR and directly activate *lux* operon transcription. Dotted circle indicates crosstalk that is of interest to us for this study. LuxS produces AI-2, which feeds into the same signal cascade as C8-HSL via LuxPQ. Autoinducers are shown freely diffusing through the inner membrane (IM) and outer membrane (OM).

Figure 1.3. Biochemistry of Lux-mediated light production in *V. fischeri*. LuxD (not shown) contributes to RCOOH synthesis. Figure adapted from Bose *et al.* Fig. 1 (1).



LuxI/LuxR signaling systems and their homologs are prevalent throughout the Pseudomonadota (16, 19, 20) and various other bacterial species use these systems to coordinate the expression of not just one but multiple genetic loci (44, 45). This observation led researchers to explore whether *V. fischeri* LuxR possesses additional gene targets beyond the initially identified regulation of bioluminescence. Their investigations revealed that LuxR regulates approximately eighteen additional genes outside of the *lux* operon, the functions of which remain largely unknown, with only a small subset having been characterized (46-48). Further investigation into the LuxR regulon needs to be conducted to elucidate how these additional genes influence *V. fischeri* metabolism within the PS network.

Though the LuxI/LuxR system has the strongest control over luminescence in *V. fischeri*, another structurally unrelated system, AinS/AinR, also plays an important role (22). AinS, an HSL synthase distinct from LuxI, produces *N*-octanoyl-L-homoserine lactone (C8-HSL), another HSL AI that binds its own receptor AinR (Fig. 1.2) (49-51). AinR sits atop a phosphorelay cascade, and in the absence of C8-HSL, AinR tends toward phosphorylating LuxU, which in turn phosphorylates LuxO (52, 53). Phosphorylated LuxO activates expression of the small RNA (sRNA) Qrr, an Hfq chaperone-dependent sRNA that post-transcriptionally represses LitR (Fig. 1.2) (39, 54). LitR is a positive regulator of LuxR and homologous to *Vibrio cholerae* HapR, *Vibrio parahaemolyticus* OpaR, and *Vibrio harveyi* LuxR (which is confusingly *not* related to *V. fischeri* LuxR) (55). LitR, HapR, OpaR, and further homologs are considered PS “master regulators” and

they along with the LuxU, LuxO, Qrr signaling system are largely conserved within the *Vibrionaceae* family. The respective regulons for these systems are large, but for the purpose of this dissertation I will focus on LitR's influence on bioluminescence.

When C8-HSL binds to AinR, it decreases the amount of phosphorylated LuxU and LuxO and ultimately de-represses LitR, thus activating LuxR and affecting *lux* operon regulation (Fig. 1.2) (53, 56). Evidence suggests that AinS/AinR and C8-HSL are primarily responsible for luminescence at low/intermediate cell densities before sufficient levels of 3OC6-HSL have accumulated (57). It is hypothesized that C8-HSL acts to jumpstart *luxR* and *lux* operon expression, which then initiates the positive-feedback loop necessary for 3OC6-HSL accumulation and high luminescence output at higher cell density. C8-HSL can also bind directly to LuxR and activate transcription of the *lux* operon as a LuxR-HSL complex however, C8-HSL does not activate LuxR as effectively as 3OC6-HSL (Fig. 1.2) (58-60).

A third signaling molecule, historically called Autoinducer 2 (AI-2), also affects luminescence through the same phosphorelay pathway as C8-HSL (Fig. 1.2). Based on the structure solved in *V. harveyi*, AI-2 is a furanosyl borate diester structurally unrelated to HSLs (61). LuxS activity is responsible for AI-2, among other molecules, and LuxP and LuxQ serve as receptors for AI-2, with LuxQ functioning similarly to AinR. LuxQ connects to the signaling cascade with LuxU, LuxO, Qrr, and LitR much like AinR (Fig. 1.2); however, AI-2 cannot bind directly to LuxR, and its effects on luminescence are minor under the conditions tested to date (56). Therefore, I will not discuss AI-2 further.

Environmental regulation of the *lux* operon

As mentioned previously, bacterial pheromone-signaling communicates more about the surrounding environment than just whether there is a quorum of neighboring cells. Both the *lux*

operon and LuxR have additional regulators (outside of LitR and LuxR itself) that change their expression based on responses to environmental factors. Factors such as glucose levels, redox conditions, oxygen availability, phosphate levels, and iron concentration are sensed by systems that can directly or indirectly regulate luminescence (1, 7, 9, 34, 35, 62, 63) (Fig. 1.4A). Why these systems are involved in *lux* regulation is not entirely understood, but it is likely a mechanism for the cells to detect if they are in seawater or symbiosis, and/or whether conditions are favorable for expending the significant amount of energy required to produce luminescence. Importantly, environmental regulators do not simply co-regulate luminescence along with AI's, the environmental regulators actually control the levels of AI pheromones. Accordingly, pheromone levels do not simply correlate with cell density but also with environmental cues like glucose, redox, phosphate, and iron.

Glucose levels control luminescence via CRP-mediated regulation. When environmental glucose levels are low, cyclic AMP (cAMP) concentrations increase, leading to its interaction with the cAMP receptor protein (CRP). The resulting cAMP-CRP complex binds to DNA sequences across the genome, thereby controlling transcription from many promoters and orchestrating a regulatory response to low glucose levels (Fig. 1.4A). Notably, there is a CRP site in the intergenic region between *luxR* and *luxI*, where CRP acts as an activator of LuxR through a Class I-type promoter interaction (Fig. 1.4B) (8, 64, 65). There is also evidence of a CRP-binding site upstream of *ainS* where CRP appears to regulate *ainS* expression in a mechanism that involves LitR (8). cAMP and CRP are both required for luminescence induction due to their important role in jumpstarting *luxR* expression (62, 66-68). CRP may also exert direct regulation on the *lux* operon, but conflicting reports leave uncertainty regarding whether it activates or represses *lux* expression

(8, 69). Overall, cAMP and CRP are important for stimulating the PS systems of *V. fischeri* in response to glucose-limited conditions, such as those within the host during symbiosis (60, 70-72).

Redox conditions are another environmental factor that feeds directly into both luminescence and HSL signal synthesis. The *lux* repressor ArcA is a part of the ArcAB two-component regulatory system that responds to redox conditions (1, 73). Oxidizing conditions

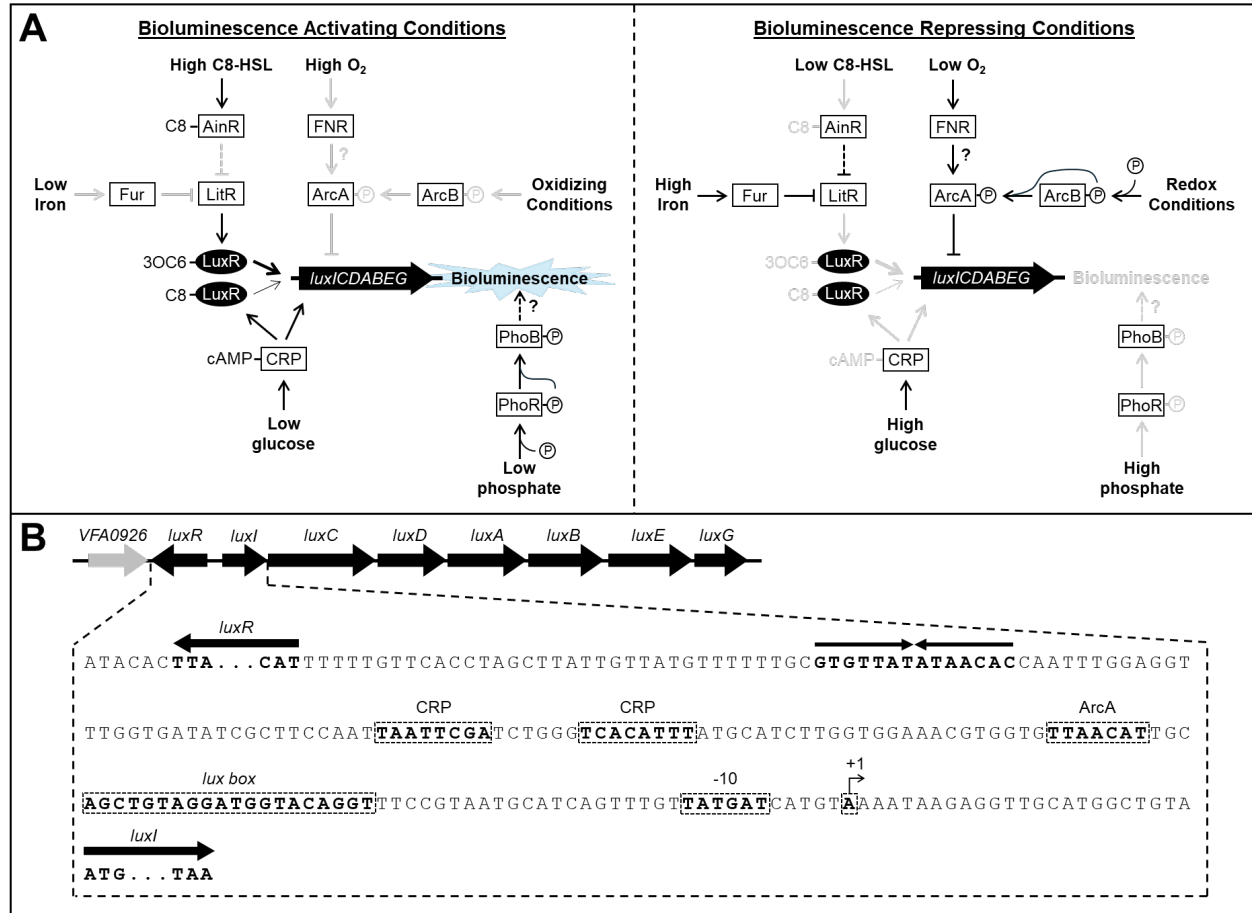


Figure 1.4. Additional regulation of luminescence in *V. fischeri*. (A) Environmental regulation of bioluminescence. This diagram illustrates how both *luxR* and *luxICDABEG* are regulated in response to environmental conditions that stimulate bioluminescence (left) or not (right). Arrowhead lines indicate activation and flat ended lines indicate repression. Grey lines indicate functions that are inactive under those conditions. Dashed lines represent intermediate steps that are not shown. Question marks indicate the mechanism of that step is not well understood. This is not an exhaustive diagram and several conditions that regulate luminescence by unknown methods were not included. (B) The *luxRI* intergenic region. Illustrates the organization of the *lux* operon in *V. fischeri* ES114 (WT) with sequence for the *luxRI* intergenic region specified below. Sequence indicates ES114 *luxR* and *luxI* (arrows), direct regulation binding sites (CRP and ArcA), the *lux* box, and the placement of the -10 and +1 of the *lux* promoter.

repress this system, but under more reducing conditions the sensor kinase ArcB phosphorylates ArcA through a phosphorelay (Fig. 1.4A). Phosphorylated ArcA binds to a site in the *lux* promoter close to the *lux* box and is thought to block LuxR binding, thereby repressing *lux* transcription (Fig. 1.4A and B) (1, 73). This repression has a dramatic impact on *lux* expression and can decrease luminescence 100- to 1000-fold (1). Importantly, though there is regulation of the *lux* operon that is aeration-dependent, the redox conditions that the ArcAB system responds to is driven by availability of any respiratory electron acceptor, not O₂ *per se*, and a separate system is responsible for regulation based on aeration (9).

Symbiosis with the host induces anaerobic respiration in *V. fischeri*, but luciferase requires oxygen for the luminescence reaction, leading to the prediction that the light organ is a microaerobic environment (7, 74, 75). Consistent with this connection between activation of bioluminescence and a change in respiratory pathways, a common activator of genes responsible for switching to anaerobic respiration in bacteria, FNR, was predicted to be a *lux* regulator and was commonly cited as such (7, 76-79). FNR (named in *Escherichia coli* for its role in fumarate and nitrate reduction) works by binding a specific DNA sequence (5'-TTGAT(N₄)ATCAA-3') and either activating or repressing transcription depending on the location of binding relative to a promoter (80-83). Though it was originally predicted to directly bind a putative FNR-site upstream of the *lux* promoter in *V. fischeri* MJ1 and activate luminescence, *fnr* and *arcA* deletion mutants in both MJ1 and ES114 led to the conclusion that FNR instead indirectly represses the *lux* operon, likely through activation of ArcA (Fig. 1.4A) (7). It seems FNR regulation represses *lux* expression if the environment becomes too anaerobic, likely because without oxygen luciferase cannot generate any light (7). Despite this indirect repression, FNR expression does not impact *V. fischeri*'s ability to colonize the squid light organ (7).

Phosphate levels in the environment also play a role in luminescence, and presumably differences in phosphate availability between seawater and the light organ are a cue the bacteria use to recognize the host environment. In a similar fashion to ArcA, PhoB is a response regulator in a two-component regulatory system. When environmental phosphate is low the sensor kinase PhoR phosphorylates PhoB which in turn binds to a “*pho* box” sequence and activates transcription of the adjacent promoter (84-88). When *V. fischeri* experiences low phosphate levels, this PhoR/PhoB regulatory system causes a significant increase in luminescence, however there is no obvious *pho* box sequence near the *lux* promoter (9). Instead PhoB is likely activating luminescence indirectly, but the exact mechanism of this regulation is not yet known (Fig. 1.4A). Though PhoB regulation on luminescence is indirect, *V. fischeri phoB* mutants have an impaired ability to colonize the light organ, making it an important colonization factor (9, 34, 35). Moreover, a fluorescent PhoB-dependent reporter revealed that the PhoB regulon is induced in symbionts in some, but not all, areas of the light organ.

Through another indirect mechanism, a low-iron environment increases luminescence expression via the ferric uptake regulator, Fur (63, 89). Fur binds to a specific Fur binding site in the promoter for LitR and represses *litR* expression (Fig. 1.4A). In iron-limiting conditions, Fur derepresses LitR, allowing LitR to resume its role as a LuxR activator and increase luminescence output (63, 89). Iron availability is often limiting for growth of host-colonizing bacteria, and multiple lines of evidence suggest symbiotic *V. fischeri* cells induce a response to low iron. For example, a heme-scavenging system in *V. fischeri* is repressed by Fur under iron-replete conditions but is induced in the light organ (90).

Several additional environmental factors influence luminescence expression, however, we do not yet fully understand the regulatory mechanisms. For example, Mg^{2+} levels influence

luminescence through multiple mechanisms. The PhoPQ two-component regulatory system responds to low Mg^{2+} in other species of bacteria (91-93), and evidence suggests that PhoQ increases luminescence in response to low Mg^{2+} in *V. fischeri* (9). However, Mg^{2+} still effects luminescence output in *phoQ* mutants, suggesting additional ways this factor feeds into *lux* expression (9).

HSL structure, function, and synthesis

HSLs, sometimes called AHLs (short for acyl homoserine lactones) or acyl-HSLs, are small neutral molecules consisting of a homoserine lactone ring and acyl chain four to eighteen carbons in length. These chains are occasionally modified with an oxo- or hydroxy-group on the third carbon, a terminal methyl branch, or unsaturated carbons (94). The combination of hydrophobicity and polarity in HSL molecules allows them to cross membranes but also diffuse in aqueous environments. The acyl chains typically have an even number of carbons, though odd-numbered HSLs are also produced (16, 95). At least 150 species of Pseudomonadota produce HSLs, and they are the best-known signaling molecule used for cell-cell signaling by Gram-negative bacteria (94, 96).

There are three enzyme families responsible for HSL synthesis: LuxI, LuxM, and HdtS (32). LuxI-family HSL synthases were discovered in *V. fischeri* and are now considered the most common type of HSL synthase, found in over 90 species of Pseudomonadota (94). Using the substrates S-adenosyl-L-methionine (SAM) and acyl-acyl carrier protein (acyl-ACP), LuxI-type proteins catalyze acylation and lactonization reactions to produce a variety of HSLs from four to 18 carbons long (94, 97). LuxI and its homologs have been extensively studied and their function is the best understood of the HSL synthases. LuxM-family HSL synthases were initially discovered in *V. harveyi* and are found in some *Vibrio* species, though they have not been found in any other

genus (49, 98-100). *V. fischeri* AinS belongs to the LuxM family of synthases, which catalyze the synthesis of HSLs from SAM and either acyl-ACP or acyl-CoA (50). The last type of HSL synthase, HdtS, was discovered producing HSLs in *Pseudomonas fluorescens* but actually belongs to a family of lysophosphatidic acid acyltransferases ubiquitous throughout both bacteria and eukaryotes (32, 94, 101). The HSL synthesis capabilities of HdtS remain uncharacterized and homologs have also been found in species that cannot produce HSLs; however, it appears these proteins can function as both lysophosphatidic acid acyltransferases and HSL synthases (32, 94).

V. fischeri contains both LuxI, responsible for producing 3OC6-HSL, and AinS, which produces C8-HSL. However recently it was determined that *V. fischeri* produces a variety of HSLs, not just 3OC6- and C8-HSL (102). HSLs were measured from five strains of *V. fischeri*, and twelve different HSLs were found among the strains, with seven that all strains had in common. Between the strains, the LuxI amino-acid sequences were identical, but there was variation in the AinS and LuxR sequences. The authors suggested that the varying HSLs produced by the strains were likely the result of the differences in AinS, and that LuxR could be evolving to receive the changing signals. My doctoral work aims to better understand the sensitivity and specificity of LuxR to the various signals that it is exposed to *in vivo*.

Structure and function of LuxR

In *V. fischeri*, the transcriptional activator LuxR is an approximately 250-amino-acid protein that has yet to be crystalized or isolated apart from 3OC6-HSL, although some truncated LuxR proteins have been purified and tested experimentally (42, 103, 104). Unbound to HSL, LuxR forms inclusion bodies when over-expressed in *E. coli*, which has created difficulties for isolating the correctly folded protein that can reliably bind DNA and HSL *in vitro* (105). Studies on the LuxR-family protein TraR from *Agrobacterium tumefaciens* found that purification of TraR

requires the presence of its cognate signal 3OC8-HSL to ensure protein stability and solubility (106, 107). When similar purification techniques were used with LuxR and 3OC6-HSL, a LuxR-3OC6-HSL complex that specifically bound *lux* box DNA *in vitro* was purified (108). However, the need for 3OC6-HSL in order to purify LuxR complexes makes it difficult to compare LuxR with and without various HSLs, and this insolubility in the absence of HSL has hampered the ability to study HSL binding *in vitro*. As noted above, no one has yet determined the crystal structure of *V. fischeri* LuxR or purified it unbound to HSL.

Though not having access to purified LuxR or a crystal structure has limited the study of LuxR, some functional domains have been approximated by analyzing truncated LuxR proteins, *luxR* mutants, and crystal structures of other LuxR-family proteins (103, 104, 109-111). These studies have been primarily conducted with LuxR from the *V. fischeri* strain MJ1 (LuxR^{MJ1}), so although the length of LuxR can vary by strain, the numbering of residues used here follows the sequence of LuxR^{MJ1} (Fig. 1.5). The N-terminal two-thirds of LuxR, spanning approximately the first one hundred and fifty-seven residues of the protein, is responsible for autoinducer binding, for blocking the C-terminal domain prior to HSL binding, and possibly for LuxR autorepression (110). Residues involved in HSL-binding are scattered throughout the N-terminal portion of LuxR, and based on mutational analysis, *in silico* predictions, and comparisons to TraR, the most critical HSL binding residues of LuxR are Y62, W66, and D79 which are predicted to form hydrogen bonds with 3OC6-HSL (112, 113). Other residues (Fig. 1.5, Table 1.1, “HSL Binding”) are predicted to be involved in HSL binding either by interacting with the ligand directly or by influencing the shape of the binding pocket (112-114). I will discuss the potential role of the N-terminal in C-terminal blocking further below, however the final responsibility of this region, LuxR autoregulation, will not be discussed in great detail. Early studies showed that LuxR loses

Table 1.1. Function of specific amino-acid residues of LuxR. Previous studies identified mutations affecting these locations and predicted the function of each residue based on the phenotypes of the mutants. The predicted functions in this table are generalized due to the variety of different phenotypes each study measured for their different LuxR mutants. Studies were conducted on *V. fischeri* MJ1 LuxR and amino-acid numbers reflect the locations in that specific sequence.

AA#	Predicted Function	Source	AA#	Predicted Function	Source
T9	HSL dependency	(115)	G197	DNA binding (non-essential)	(111, 116, 117)
T33	HSL binding (non-essential)	(59, 118)	K198	<i>lux</i> activation/RNAP interaction	(117)
L42	HSL binding (non-essential)	(112)	W201	<i>lux</i> activation/RNAP interaction	(116, 117)
I45	HSL binding (non-essential)	(59, 113, 118)	D202	Other	(111, 116, 117)
I46	HSL binding (non-essential)	(112)	I203	DNA binding (non-essential)	(117)
I58	HSL binding (non-essential)	(119)	I206	<i>lux</i> activation/RNAP interaction	(117)
Y62	HSL binding (C8 essential)	(112, 119)	L207	DNA binding (non-essential)	(117)
W66	HSL binding (3OC6 essential)	(112)	C209	DNA binding (non-essential)	(117)
R67	HSL binding (3OC6 essential)	(114)	R212	DNA binding (essential)	(117, 120)
D72	HSL binding (non-essential)	(121)	T213	DNA binding (non-essential)	(117, 120)
D79	HSL binding (non-essential)	(110-112)	V214	DNA binding (non-essential)	(117, 120)
V82	HSL binding (non-essential)	(110, 111)	F216	DNA binding (essential)	(117, 120)
N86	HSL binding (non-essential)	(122)	H217	DNA binding (essential)	(110, 116, 117)
W94	HSL binding (3OC6 essential)	(111, 112)	L218	DNA binding (non-essential)	(117, 120)
N95	HSL binding (non-essential)	(59)	T219	Other	(117, 123)
I96	HSL binding (non-essential)	(59)	N220	DNA binding (non-essential)	(117, 120)
V102	HSL binding (non-essential)	(121)	A221	HSL dependency	(123, 124)
V109	HSL binding (3OC6 & C8 essential)	(110, 112, 116)	M223	HSL dependency	(115, 117, 120)
I110	HSL binding (3OC6 & C8 essential)	(112)	K224	DNA binding (non-essential)	(117, 120)
S116	HSL binding (non-essential)	(59, 118)	L225	DNA binding (essential)	(120)
L118	HSL binding (3OC6 essential)	(110)	N229	DNA binding (essential)	(120)
G121	HSL binding (3OC6 essential)	(103, 111, 124)	R230	DNA binding (essential)	(120)
S123	HSL binding (3OC6 essential)	(110)	S233	DNA binding (non-essential)	(120)
H127	HSL binding (non-essential)	(111)	K236	DNA binding (non-essential)	(120)
M135	HSL binding (non-essential)	(59, 112, 118, 121)	I238	DNA binding (essential)	(120)
S149	HSL dependency	(115)	L239	DNA binding (non-essential)	(120)
S164	HSL dependency	(115)	T240	DNA binding (non-essential)	(120)
I174	Other	(59)	G241	DNA binding (non-essential)	(120)
D182	DNA binding (non-essential)	(123)	I243	DNA binding (essential)	(120)
T184	Other	(111, 116)	D244	DNA binding (non-essential)	(123)
L191	DNA binding (essential)	(117)	C245	Other	(122)
W193	DNA binding (essential)	(117)	P246	HSL dependency	(115, 120)
C195	DNA binding (non-essential)	(116, 117, 123)	F248	DNA binding (non-essential)	(120)
E196	DNA binding (non-essential)	(117)			

Predicted Function Key	Details
HSL binding (3OC6 essential)	Mutations at this location make LuxR unable to respond to 3OC6-HSL
HSL binding (C8 essential)	Mutations at this location make LuxR unable to respond to C8-HSL
HSL binding (non-essential)	Mutations at this location affect HSL response but do not eliminate it
HSL dependency	Mutations at this location allow LuxR to activate the <i>lux</i> operon without added HSL
DNA binding (essential)	Mutations at this location make LuxR unable to bind DNA/non-functional (dark mutants)
DNA binding (non-essential)	Mutations at this location affect DNA binding but do not eliminate it (some require RNAP for binding)
<i>lux</i> activation/RNAP interaction	Mutations at this location affect <i>lux</i> activation but do not affect DNA binding
Other	Mutations at this location had an effect on LuxR function but the exact role is unknown



Figure 1.5. Important residues of LuxR amino acid sequence. The 250 amino acid sequence of *V. fischeri* LuxR^{MJ1} with important residues highlighted. A list of these residues and their predicted functions can be found in Table 1.1, along with a highlighting color key.

the ability to regulate its own expression when residues 10-20 are deleted, so this region was deemed critical for autorepression (79, 125). However, these findings were never reproduced, and no follow-up study of LuxR autorepression has been completed since, leading to skepticism in the community.

The C-terminal domain makes up the final third of the protein (residues 158-250, approx.) and is responsible for DNA binding and transcriptional activation. The C-terminal domain contains a helix-turn-helix (HTH) domain (residues 200-224), a feature common among transcriptional activators and other DNA-binding proteins (19, 126). Residues critical for binding and recognition of the *lux* box sequence of the *lux* promoter exist both in the HTH and throughout the rest of the C-terminal domain (Fig. 1.5, Table 1.1, “DNA Binding”), the most important being L191, W193, R212, F216, H217, L225, N229, R230, L238, and I243 (Fig. 1.5, Table 1.1, “DNA Binding (essential)”) (110, 116, 117, 120). When mutated at these residues LuxR loses its DNA binding capacity, indicating they are either involved in directly contacting DNA or essential to the configuration of the binding domain (117, 120). The C-terminal domain also contains residues that interact with RNA polymerase (RNAP) and/or are critical for transcriptional activation in some

capacity (Fig. 1.5, Table 1.1, “*lux* activation/RNAP interaction”) (104, 111). Changes to these residues render the protein unable to activate the *lux* operon but still able to bind the *lux* box DNA (104, 111).

In the absence of bound HSL, LuxR folds in such a way that a region somewhere between amino acids 20-162 blocks the C-terminal domain. This folding prevents LuxR from binding the *lux* box and masks LuxRs transcriptional activator function (104). Once LuxR binds to an HSL, a conformational change occurs that unblocks the C-terminal and allows the HTH domain of the protein to bind to the *lux* box (42). When the N-terminal 162 residues are deleted, the truncated LuxR is even more active than the wild-type protein and no longer dependent on HSL for this activity (104). Some mutations, such as S149N, S164F/W/Y, A221V, M223I, and P246A/L also cause this HSL independence and allow LuxR to activate the *lux* operon without ligand binding (Fig. 1.5, Table 1.1, “HSL dependency”) (103, 115, 120, 124). These amino acids likely have important roles in determining a conformation whereby the N-terminus blocks the C-terminus prior to HSL binding and their disruption frees the DNA binding site to interact with the *lux* box.

There is also evidence that HSL binding causes LuxR to multimerize, with each LuxR subunit bound to an individual HSL molecule (19, 127). Two hydrophobic regions in LuxR, one between residues 117 to 139 and the other from 149 to 171, are hypothesized to play an important role in this multimerization (127). This prediction of LuxR structure and function aligns with what we know about TraR (2, 109). TraR and other LuxR-homologs form dimers when bound to their respective HSLs, so it is considered likely that LuxR also forms a dimer (2, 128). TraR exists as an unstable monomer until introduced to 3OC8-HSL at which point the HSL binds to TraR and the protein dimerizes (128). Each TraR in this dimer is bound to an HSL, and the protein is only active in this conformation (2, 128). The TraR dimer is asymmetrical, with each monomer folded

slightly differently which makes it more difficult to estimate a dimer structure for LuxR using predictive software like AlphaFold (2, 109). The residues involved in multimerization of both TraR and LuxR are scattered throughout the protein due to the multiple contact points between each monomer, so there is no singular “multimerization domain”.

LuxR homologs and evolution

We can further examine the function and evolution of LuxR by observing the significant variation in LuxR among *V. fischeri* strains. Take, for example, the LuxR ortholog sequences of the strains ES114 and MJ11, which are dim and bright, respectively, when grown in rich media. While most proteins share 94-100% amino acid identity between these two strains, the LuxR orthologs only share about 75% identity (129). The differences in the luminescence between *V. fischeri* strains could be a result of this rapid evolution of *luxR*, *luxICDABEG*, and the *luxR-luxI* intergenic regions as a means of adapting to different hosts/environments (129). The differences between naturally evolved variants of *V. fischeri* may impact luminescence by changing the way LuxR interacts with HSLs, however this has not yet been studied. My work aims to further characterize the function of multiple variants of *V. fischeri* LuxR within the limitations of not having purified protein for HSL-binding experiments or a known crystal structure for any of the variants.

Using computational modeling to better understand LuxR function

A previous student from our research group, Deanna Colton, developed a simplified model of LuxR’s interaction with 3OC6- and C8-HSLs in collaboration with the Hagen Lab from the University of Florida (60). To test this model, they grew engineered *V. fischeri* strains in defined mixtures of 3OC6- and C8-HSL and measured their luminescence response. In each strain, they replaced the native *luxR* with one of four variants: *luxR*^{ES114}, *luxR*^{MJ1}, *luxR*^{MJ1-A}, or *luxR*^{MJ1-B}. Each

of these *luxR* genes was placed under the control of a constitutive promoter to disconnect it from regulatory systems, including AinR (Fig. 1.2), which would otherwise affect *luxR* expression. The HSL synthase genes *luxI* and *ainS* were deleted to eliminate endogenous HSL production. Each LuxR variant responded differently to the HSLs as predicted (60). The two naturally evolved *luxR* genes (*luxR*^{ES114} and *luxR*^{MJ1}) conveyed strong responses to 3OC6-HSL and weak responses to C8-HSL. The other two *luxR* variants, *luxR*^{MJ1-A} and *luxR*^{MJ1-B}, were experimentally evolved from *luxR*^{MJ1} (59, 114). *luxR*^{MJ1-A} conveyed a strong luminescence response to both HSLs, while *luxR*^{MJ1-B} resulted in a weak response to 3OC6-HSL and a strong response to C8-HSL (59, 114). These findings fit the model well and also suggested that variations in LuxR can modulate signal sensitivity not only by changing its binding affinity for different HSLs (K_1 and K_2 in Colton et. al Fig.1), but also by changing the cooperativity of LuxR-HSL multimer formation (m and n in Colton et. al Fig.1) (60).

Despite encouraging results using computational modeling, the model is still simplified, makes several assumptions, and leaves many unanswered questions. For example, though we know LuxR can form complexes with 3OC6- and C8-HSL, we do not yet know whether 3OC6- and C8-HSL can both be bound to the same complex. This modeling approach is helpful, but limited in the number of parameters that can be introduced before it becomes too complex for computational analysis to settle on a single best fit model. Parameters were added based on knowledge of the underlying biology in an attempt to explain data that did not fit well with the simplified model. However, with more parameters added to the model, multiple combinations of values for parameter provided equally good fits to the experimental data. Therefore, as we increasingly came to understand the complexity of LuxR function, this particular method of modeling was not expanded

upon further. However, we continued investigating how variants of LuxR have evolved different responses to mixtures of 3OC6- and C8-HSL.

Goals of this study

The overarching goal of this study is to develop a deeper understanding of the function and evolution of the PS systems in *V. fischeri*. I will contribute toward this goal by focusing on how LuxR has evolved and how it interacts with and responds to multiple signaling molecules. I aim to explore the signaling abilities of naturally evolved variants of *luxR* by collecting multiple *luxR* sequences from different isolates of *V. fischeri* and determining how each LuxR variant responds to HSL signals. This information will allow us to develop a better understanding of LuxR function and in doing so better understand the crosstalk between the structurally unrelated LuxI/LuxR and AinS/AinR systems.

CHAPTER 2

CONVERGENCE OF NATURAL AND DIRECTED-EXPERIMENTAL EVOLUTION IN

*VIBRIO FISCHERI LUXR*¹

¹ Kathryn A. Bellissimo, Stephen J. Hagen, and Eric V. Stabb. 2024. To be submitted to *mBio*.

Abstract

Bioluminescence in *Vibrio fischeri* is controlled by a pheromone-signaling (PS) system underpinned by LuxR, which activates transcription in response to a cognate signal, *N*-3-oxo-hexanoyl-L-homoserine lactone (3OC6-HSL), produced by LuxI. *V. fischeri* produces other HSLs and may encounter HSLs produced by other bacteria, making the evolution of LuxR signal selectivity an important determinant of its function in cell-cell communication. LuxR has diverged notably between *V. fischeri* strains ES114 and MJ11, but it is unclear if such divergence extends to other *V. fischeri* strains or how LuxR variants differ functionally. In this study, we analyzed the sequences of *luxR* from seventeen *V. fischeri* strains revealing twelve unique genes encoding nine LuxR variants. The C-terminal DNA-binding region of LuxR was most conserved, while regions thought to be involved in HSL binding and LuxR dimerization were more variable. Interestingly, some of the naturally evolved LuxR variations paralleled mutations previously recovered in experimental evolution aimed at altering LuxR's HSL repertoire. We engineered otherwise isogenic *V. fischeri* strains with eight natural *luxR* variants under constitutive expression disconnected from transcriptional PS feedback regulation, in a background lacking endogenous HSL synthesis. The resulting strains, differing only in their *luxR* sequence, had wide variation in their responses to different added HSLs. Surprisingly, the *luxR* gene from strain H905 stimulated bioluminescence even without added HSL, and it contained an allelic variation previously reported in a genetic screen for HSL-independent mutants. Our results illuminate the natural evolution of LuxR and should inform future synthetic engineering of LuxR as an information processor.

Importance

The LuxI/LuxR system from *V. fischeri* is an archetype “quorum sensing” PS system. Since this system’s discovery decades ago many similar HSL-based signaling systems have been discovered in diverse Pseudomonadota (formerly Proteobacteria), where they direct important group behaviors such as biofilm formation and host colonization. The pheromone-signal receptor and transcriptional activator LuxR has served both as a model for understanding PS in Pseudomonadota and as an easily triggered activator in synthetic regulatory circuits. Our findings extend the observation of rapid divergence of LuxR between *V. fischeri* strains and indicate the functional consequences of this evolution. Our examination of *luxR*’s natural evolution complements laboratory-based directed-evolutionary experiments and provides new insights into the signal sensitivity and specificity in this PS system.

Introduction

Bioluminescence in *Vibrio fischeri* is regulated by two structurally and functionally distinct homoserine lactone- (HSL-) based pheromone-signaling (PS) systems (130). One of these, the LuxI/LuxR system, is encoded at the *lux* locus (37, 38) and is the major regulator of the *lux* operon underlying bioluminescence. LuxI produces *N*-3-oxo-hexanoyl-L-homoserine lactone (3OC6-HSL) (131), which diffuses in and out of cells (132) and, when it reaches a critical concentration, binds LuxR (108), stimulating it to multimerize, bind a “*lux* box” sequence, and activate transcription of the *lux* operon (42). In a second HSL-based system, AinS produces *N*-octanoyl-L-homoserine lactone (C8-HSL) which acts through its cognate receptor AinR to modulate a core signaling cascade involving LuxU, LuxO, and the regulatory RNA Qrr to control LitR (49-51, 133). LitR is a homolog of PS “master regulators” found in other members of the

Vibrionaceae, including HapR in *Vibrio cholerae* and (confusingly) *Vibrio harveyi* LuxR, which bears no similarity to *V. fischeri* LuxR. In *V. fischeri*, LitR activates transcription of *luxR*, among other genes (39, 51, 52, 55-57, 134). In addition to working through this signal cascade, C8-HSL can also bind and activate LuxR, albeit less effectively than 3OC6-HSL (58-60).

LuxR's structure is not defined, but much can be inferred from bioinformatic analyses, functional studies of LuxR fragments, and the structure of *Agrobacterium tumefaciens* TraR, which is a LuxR homolog (2, 109). TraR and other LuxR homologs function as asymmetrical dimers, with each monomer binding to its own HSL molecule (2, 109, 128, 135). Analyses of LuxR from *V. fischeri* strain MJ1 (LuxR^{MJ1}) indicated that the N-terminal two-thirds of the protein is important for HSL binding (16, 103, 110, 111, 135), and the C-terminal third is important for DNA binding and interactions with RNA polymerase, with a helix-turn-helix DNA-binding domain between amino acids 200-224 (16, 58, 111, 117, 120, 125, 135-137). Contacts involved in dimerization were predicted near amino acids 118-161, and there appear to be additional dimerization contacts distributed throughout the protein (2, 109, 127, 128, 138).

It seems likely that *luxR* was acquired along with the *lux* operon by an ancestor of *V. fischeri* that already contained the core *Vibrio* signaling cascade (LuxU, LuxO, Qrr, and LitR) (69, 139). This combination of LuxI/LuxR and AinS/AinR in the same cell would have placed an unusual evolutionary pressure on the two distinct PS systems. Although LuxI/LuxR and AinS/AinR are unrelated proteins, the chemical similarity of their respective signals might have selected for or against crosstalk between the two systems. It is not uncommon for a Pseudomonadota to harbor multiple LuxI/LuxR systems (20, 135, 140), which might produce selective pressure for or against heteromultimer formation by the distinct LuxR homologs, in

addition to pressures associated with signal crosstalk. *V. fischeri* is unusual in that its two HSL-based systems are similar only in the structure of their respective cognate pheromones.

Genomic comparisons of two *V. fischeri* strains (141, 142), revealed the *luxI/luxR* and *ainS/ainR* systems diverged more than most genes had. Whereas most orthologs between strains ES114 and MJ11 share over 95% amino acid identity, LuxR is only 75% conserved (129). The functional implication of such LuxR variation is unknown, but precedent for LuxR adapting to different HSL signals was illustrated in a series of directed-evolution studies resulting in LuxR variants with increased responsiveness to C8-HSL (59, 122), increased responsiveness to short-chain HSLs (118), decreased responsiveness to 3OC6-HSL (114), specificities for either 3OC6- or 3OC8-HSL (119), or HSL-independence (115). Previously, we reported that LuxR^{ES114} and LuxR^{MJ11} behave differently with respect to C8-HSL and 3OC6-HSL (60). Here, we compared *luxR* from diverse *V. fischeri* strains and characterized functional differences with respect to HSL responses in an isogenic background to highlight LuxR activity.

Results

LuxR varies significantly between *V. fischeri* strains

To extend the comparison of naturally evolved *luxR* alleles, we collected *luxR* sequences from seventeen *V. fischeri* strains, including twelve *luxR* sequences first reported in this study and another five *luxR* sequences from extant databases (Table 2.1). Those seventeen *luxR* genes represented twelve unique sequences that encode nine distinct proteins. Non-identical *luxR* genes from strains H905 and VLS2 encoded identical LuxR proteins, and similarly the *luxR* genes from MJ11, CG103, SA1, and SR5 encode the same protein, although only the latter two have identical *luxR* genes. The *luxR* in widely used wild-type strain ES114, which was isolated from the light organ of *Euprymna scolopes* in Hawaii, encoded a LuxR identical to that in other Hawaiian strains

ES12, ES213, and ES401, as well as to the LuxR in Japanese isolate EM17. Table 2.1 describes strain origins and indicates those that encoded identical LuxR proteins.

Table 2.1. Wild-type *V. fischeri* strains referenced in this study.

<i>V. fischeri</i> strain	<i>luxR</i> sequence source	Accession number	Isolate location and host species	Reference
CG101	This study	PP751035	Australia, <i>Cleidopus gloriamaris</i> 🐟	(143)
CG103 ¹	This study	PP751036	Australia, <i>Cleidopus gloriamaris</i> 🐟	(144)
EM17 ²	This study	PP751037	Japan, <i>Euprymna morsei</i> 🐙	(143)
*ES114 ²	NCBI	CP000021.2	Hawaii, US, <i>Euprymna scolopes</i> 🐙	(145)
ES12 ²	This study	PP751038	Hawaii, US, <i>Euprymna scolopes</i> 🐙	(146)
ES213 ²	NCBI	NZ_LTYU00000000.1	Hawaii, US, <i>Euprymna scolopes</i> 🐙	(146)
ES401 ²	This study	PP751039	Hawaii, US, <i>Euprymna scolopes</i> 🐙	(143)
*ET101	This study	PP751040	Australia, <i>Euprymna tasmanica</i> 🐙	(147)
*ET401	This study	PP751041	Australia, <i>Euprymna tasmanica</i> 🐙	(148)
*H905 ³	This study	PP751042	Hawaii, US, planktonic 🐙	(149)
*MJ1	NCBI	M19039.1	Japan, <i>Monocentris japonica</i> 🐟	(150)
*MJ11 ¹	NCBI	CP001133.1	Japan, <i>Monocentris japonica</i> 🐟	(143)
*PP3	This study	PP751043	Hawaii, US, planktonic 🐙	(149)
SA1 ¹	This study	PP751044	Mediterranean, <i>Sepiolo affinis</i> 🐙	(151)
SR5 ¹	NCBI	NZ_CM001401.1	Mediterranean, <i>Sepiolo robusta</i> 🐙	(151)
VLS2 ³	This study	PP751045	Hawaii, US, <i>Euprymna scolopes</i> 🐙	(145)
*WH1	This study	PP751046	Massachusetts, US, planktonic 🐙	(145)
¹ Encode identical LuxR proteins (CG103, MJ11, SA1, SR5)				
² Encode identical LuxR proteins (EM17, ES114, ES12, ES213, ES401)				
³ Encode identical LuxR proteins (H905, VLS2)				
* <i>luxR</i> cloned into engineered <i>V. fischeri</i> ES114 derivative for analysis in isogenic background				
Host type: 🐟 = fish, 🐙 = squid, 🐙 = none				

To put the divergence of these *luxR* alleles in perspective, we compared the relatedness of the *luxR* sequences (Fig. 2.1A) with that of housekeeping genes *recA*, *mdh*, *katA*, and *pyrC* from the same strains (Fig. 2.1B). Overall, the housekeeping genes share a much higher identity with one another, whereas the *luxR* alleles were more divergent (Fig. 2.1). The lowest pairwise identity among the housekeeping genes was 94.1%, while *luxR* nucleotide identities were as low as 79.9%. The *V. fischeri* *luxR* alleles cluster into three groups (Fig. 2.1B), and this topology roughly mirrors

that seen previously for the *luxI-luxR* intergenic region (129). Moreover, these groupings correlate with phenotypic differences that are described below.

For the most part, the phylogenetic relatedness of strains implied by the housekeeping genes resembled the clustering of *luxR* sequences, i.e., the trees assembled from sequences of *luxR* or housekeeping genes showed similar patterns in Figures 2.1A and 2.1B; however, there were exceptions. For example, strains EM17 and MJ1 were nearest neighbors with respect to housekeeping genes (Fig. 2.1B), which shared 94.6% identity, but they fell in different clusters

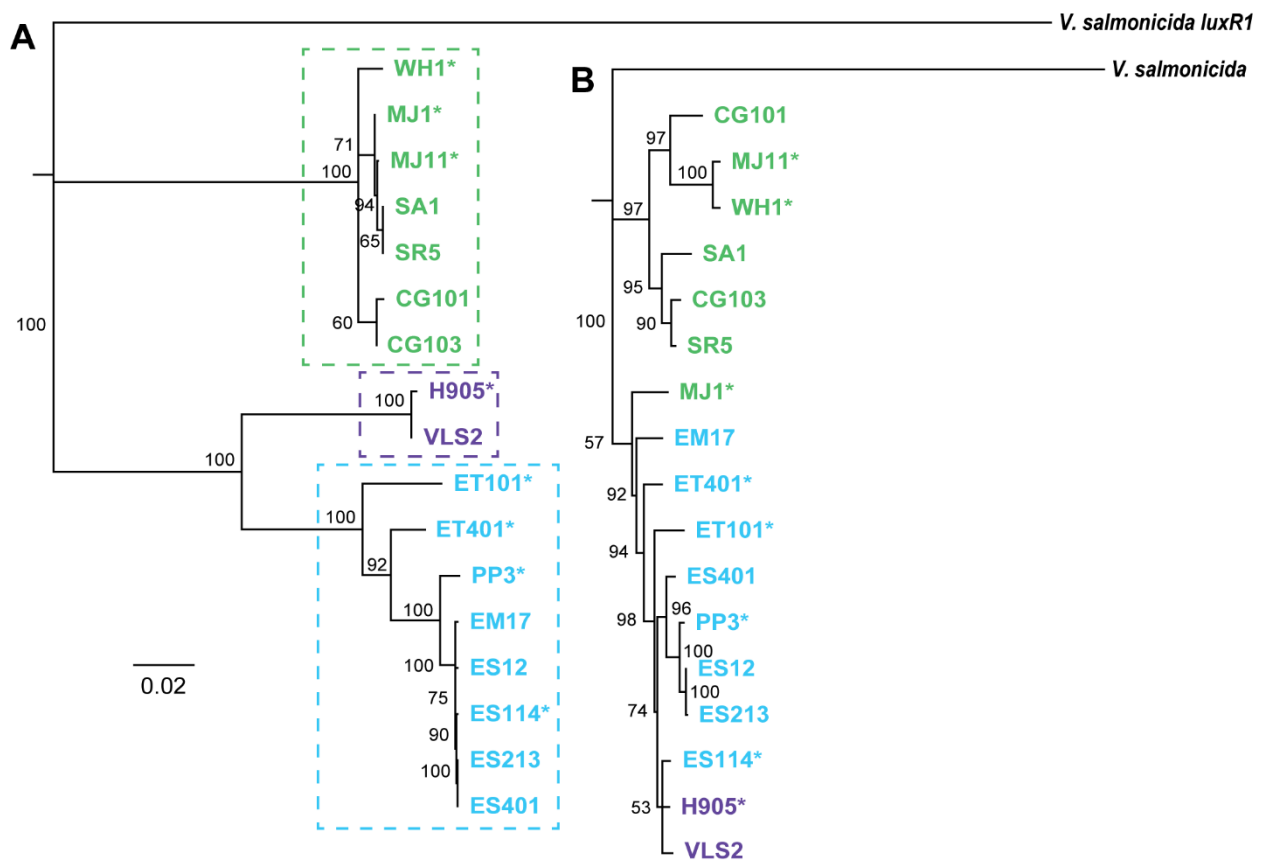


Figure 2.1. Relatedness of *luxR* and housekeeping genes from *V. fischeri* strains analyzed in this study. Consensus trees were generated based on Clustal Omega alignments for (A) *luxR* genes from seventeen *V. fischeri* strains and (B) concatenated housekeeping genes *recA-mdh-katA-pyrC* for the same strains. In both trees *Vibrio salmonicida* sequences serve as outgroups. The scale is common to both panels (scale bar = 0.02 nucleotide substitutions per site). Branch labels indicate consensus support (%). *luxR* genes from starred (*) strains were cloned into an isogenic background and tested as described in the text. Each of three clusters is highlighted in a different color: Green = “MJ1 cluster”, purple = “H905 cluster”, blue = “ES114 cluster”.

with respect to *luxR*. EM17 and MJ1 were isolated from sympatric squid and fish, respectively, collected in Japan (Table 2.1). We also found that five strains (EM17, ES12, ES114, ES213, and ES401) that contained identical *luxR* genes had diverged somewhat with respect to housekeeping genes (Fig. 2.1), as they do with respect to the *luxI-luxR* intergenic region (129).

Alignments of LuxR and TraR reveal regions of conservation and variation

We selected eight *V. fischeri luxR* allelic variants (from ES114, PP3, ET401, ET101, H905, MJ1, MJ11, and WH1) to study in greater depth (marked with * in Fig. 2.1). The LuxR protein sequences from these eight strains were aligned along with the LuxR homolog TraR, which is the most closely related homolog to LuxR with a defined crystal structure (2, 109). In Figure 2.2, important domains and residues are labeled in and below TraR's sequence, so that the positions of these domains and residues can be compared in alignment to LuxR. Colored bars above sequences in Figure 2.2 illustrate what residues in LuxR are most conserved (green) or variable (red), based on the number of allelic variants at each position in the dataset. The DNA-binding domain remains well conserved among the LuxR variants, while there is more variation in the N-terminal HSL-binding domain. However, the three residues forming hydrogen bonds between TraR and its HSL, *N*-3-oxo-octanoyl-L-homoserine lactone (3OC8-HSL), are conserved among the LuxRs tested in this study (Fig. 2.2, triangles) (2, 109).

Previous studies have analyzed LuxR (typically LuxR^{MJ1}) and identified residues that may be important for LuxR activity (58, 59, 103, 110-124, 136, 137). Of particular interest are several directed-evolution experiments that altered LuxR^{MJ1} responses to HSLs (59, 114, 115, 118, 119, 122). Interestingly, several of the experimentally evolved mutations from these studies are reflected in naturally evolved *luxR* alleles (Fig. 2.2; daggers above alignment and residues in red). The relevant residues found in those studies and their resulting phenotypes are listed in Table 2.2.

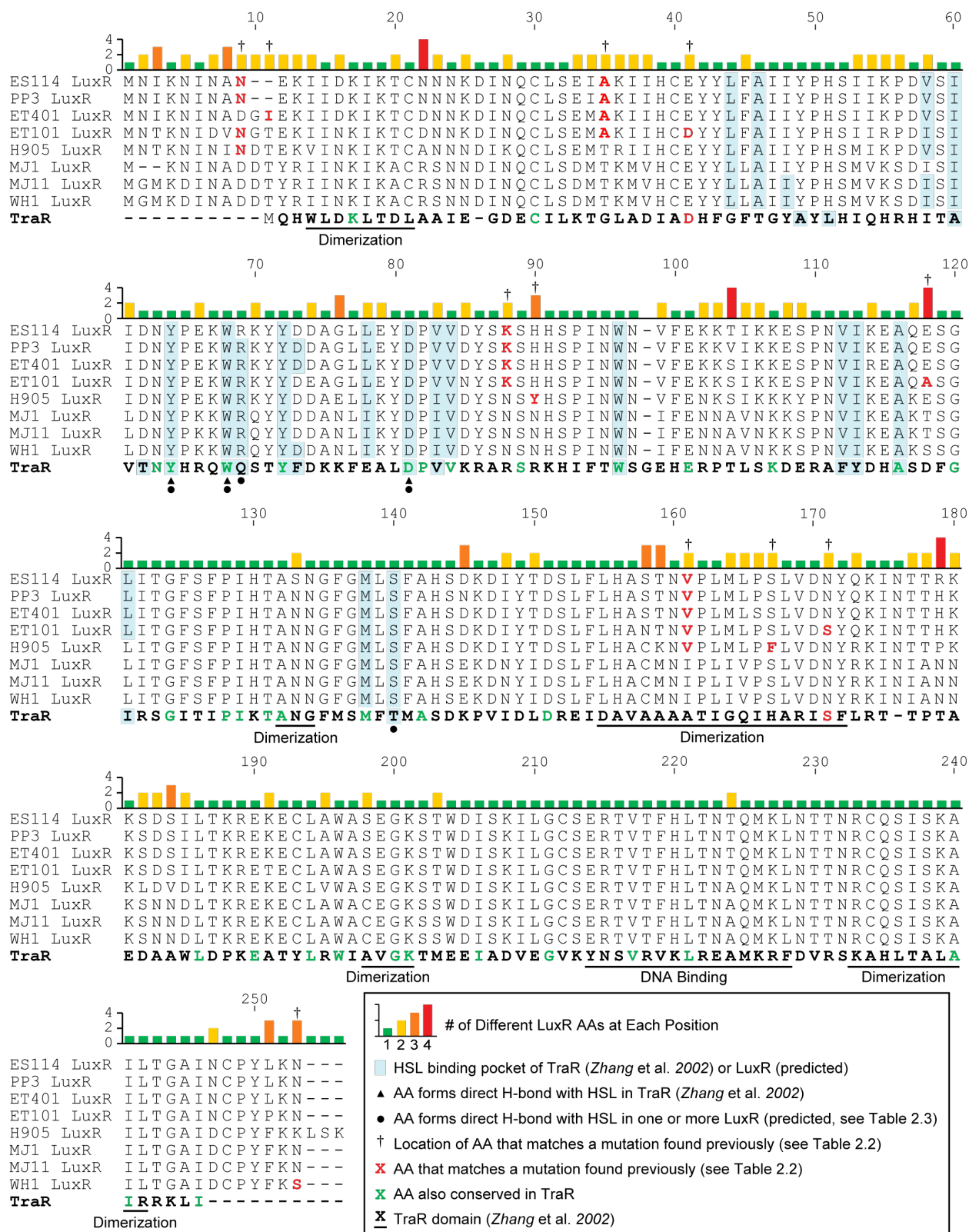


Figure 2.2. Alignment of LuxR amino acid sequences (see previous page). Clustal Omega alignment of the eight *V. fischeri* LuxR variants analyzed in this study. The colored bars above each residue indicate the number of different LuxR amino acids at that location (green, yellow, orange, and red bars indicate one, two, three, or four amino acids). TraR is aligned beneath the LuxRs, and residues conserved across TraR and LuxR are indicated with green letters in the TraR sequence. Residues that belong to the TraR HSL binding pocket (2) or that are predicted by PrankWeb3 to be part of the LuxR HSL binding pocket are highlighted in blue. Daggers (†) above the alignment mark the location of residues that match a mutation found in previous experiments (matching residues colored red, see Table 2.2 for details). Triangles (▲) indicate TraR amino acids known to hydrogen bond with 3OC8-HSL (2). Circles (●) indicate amino acids predicted to form hydrogen bonds with one or more LuxR (see Table 2.3). TraR regions involved in DNA binding or dimerization are indicated with labeled lines under the alignment (2).

Table 2.2. Relevant LuxR variants from directed evolution studies.

AA substitution relative to MJ1	Position Fig. 2.2	Description Summary	Wild-type <i>V. fischeri</i> strain(s) with this residue in LuxR
Sitnikov et al. 1996 (115)			
T9I	11	HSL signal-independent activity	ET401
S164F	167	HSL signal-independent activity	H905 ²
Collins et al. 2005 (59)			
T33A ³	35	Increased response to C8-HSL, enhanced in combination with S116A and M135I	ES114 ⁴ , PP3, ET401, ET101
S116A ³	119	Increased response to C8-HSL, enhanced in combination with T33A and M135I	none
M135I ³	138	Increased response to C8-HSL, enhanced in combination with T33A and S116A. Moderately decreased response to 3OC6-HSL	none
Collins et al. 2006 (114)			
R67M	69	Selectivity against 3OC6- and 3OC12-HSL in T33A, S116A, M135I background	none
Hawkins et al. 2007 (118)			
I45F	47	Increased response to C4-HSL in T33A, S116A, M135I background	none
E39D	41	Increased response to C4-HSL in T33A, S116A, M135I, T115A background	ET101
I158V	161	Increased response to C4- and 3OC6-HSL in T33A, S116A, M135I, T115A background	ES114 ⁴ , PP3, ET401, ET101, H905 ¹
Tashiro et al. 2016 (119)			
Y62H	64	Selectivity against C6-, C8-, and 3OC8-HSL, reduced sensitivity to 3OC6-HSL	none
D7N	9	Increased response to 3OC6-HSL in Y62H background in combination with N250S	ES114 ⁴ , PP3, ET101, H905 ²
N88Y	90	Increased response to 3OC6-HSL in Y62H background	H905 ²
T115A	118	Increased response to 3OC6-HSL in Y62H background	ET101
N250S	253	Increased response to 3OC6-HSL in Y62H background in combination with D7N	WH1
Kimura et al. 2016 (122)			
N86K	88	Increased response to C6-, 3OC6-, C8-, and 3OC8-HSL	ES114 ³ , PP3, ET401, ET101

¹ LuxR sequence not cloned into engineered background or displayed in Fig. 2.2
² VLS2 encodes identical LuxR protein
³ Hawkins et al. 2007 (118) subsequently showed T33A, S116A, M135I in combination (allele LuxR-G2E) increased responsiveness to a range of signals from C5- to C14-HSL
⁴ EM17, ES12, ES213, and ES401 encode identical LuxR proteins

Table 2.3. AutoDock Vina predictions of LuxR residues H-bonding to 3OC6- or C8-HSL.

HSL	LuxR	Y64*	W68*	R69*	D81*	S140*
3OC6	ES114	x	x	x	x	
	PP3		x	x	x	x
	ET401	x	x	x	x	x
	ET101		x	x	x	x
	H905	x	x	x	x	x
	MJ1		x	x	x	x
	MJ11		x	x	x	x
	WH1		x	x	x	x
C8	ES114	x	x		x	
	PP3	x	x		x	x
	ET401	x	x		x	
	ET101	x	x		x	x
	H905	x	x		x	x
	MJ1	x	x		x	
	MJ11	x	x		x	
	WH1	x	x		x	x

* Residue designation from LuxR alignment Figure 2.2.

Computational predictions of LuxR variant structures

We next used AlphaFold2 to predict structures for the LuxR variants and compared those structures with the verified TraR structure (2, 109, 152). Overall, AlphaFold2 predicted similar structures for the different LuxR variants. A structural alignment of LuxR^{ES114} with the TraR crystal structure is shown in Figure 2.3A. Though the aligned structures are similar, the HSL

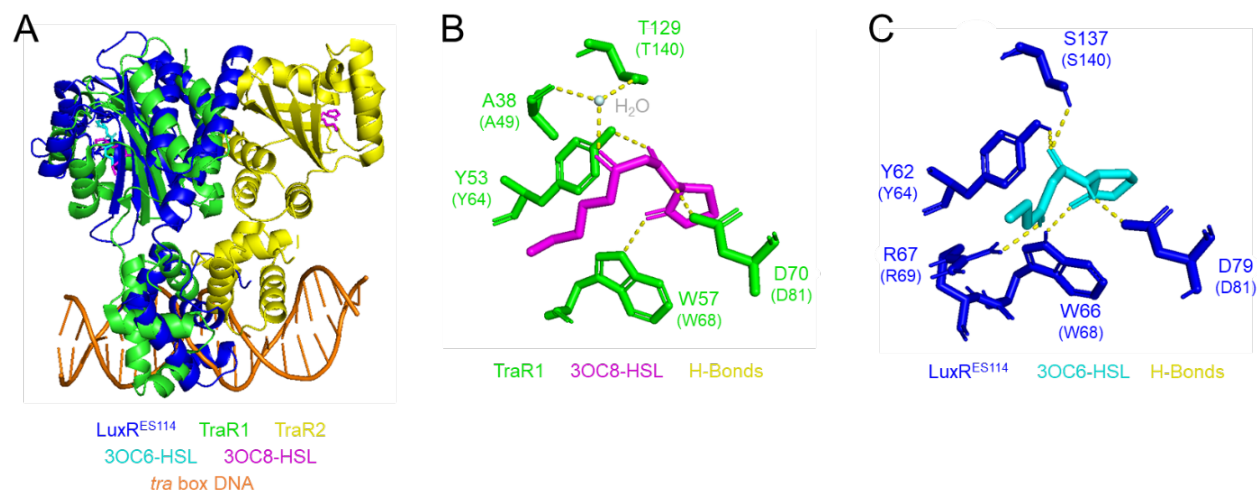


Figure 2.3. LuxR structures predicted with AlphaFold2. (A) ES114 LuxR structure predicted by AlphaFold2 aligned with the crystal structure of the TraR dimer bound to its autoinducer (3OC8-HSL) and *tra* box DNA. Due to the asymmetrical nature of the TraR dimer, LuxR aligns better with one TraR monomer than the other (second structural alignment not shown). (B) Close up of the HSL binding sites of TraR and (C) ES114 LuxR. Molecular structures are shown for TraR amino acids responsible for 3OC8-HSL binding and LuxR amino acids predicted to be involved in HSL binding (AutoDock Vina). Amino acid labels in parentheses indicate position on the alignment in Fig. 2.2.

binding sites of TraR and LuxR do not align well by this methodology (Fig. 2.3A). To gain more insight into HSL-LuxR interactions, predicted structures were run through the program PrankWeb3 to predict residues that may be important for ligand binding in LuxR (Fig. 2.2, blue highlighting on LuxR sequences) (153-155). These predicted residues correlate closely with TraR's known HSL binding site (Fig. 2.2, blue highlighting on TraR sequence), and most of the predictions for HSL binding were common across the LuxR variants (Fig. 2.2). Many but not all of these residues lie within the roughly defined HSL binding domain proposed previously (103, 110, 111, 125).

The predicted LuxR structures were also analyzed using the molecular docking software AutoDock Vina to predict the locations where 3OC6-HSL or C8-HSL would bind within the protein (Table 2.3) (156, 157). In this analysis, the HSL binding pockets of TraR (Fig. 2.3B) and LuxR (Fig. 2.3C) aligned more clearly than when the full-length structure of LuxR predicted by AlphaFold was aligned to the TraR structure (Fig. 2.3A). For each LuxR-HSL pair, the AutoDock Vina predictions independently placed the HSLs in roughly the same position, hydrogen bonded to at least three of five possible residues (Table 2.3 and Fig. 2.2, circles below sequence). All LuxR variants had predicted hydrogen bonds to 3OC6-HSL at W68, R69, and D81 though in some Y64 and/or S140 were also predicted (Table 2.3) (residue positions numbered as in Fig. 2.2). For C8-HSL, all LuxRs were predicted to hydrogen bond at Y64, W68, and D81 though some also bound at S140 (Table 2.3). Each of these five ligand binding residues flagged by AutoDock Vina either align with residues involved in TraR binding its cognate HSL (Fig. 2.2, triangles below sequence) and/or were also predicted to have a role in LuxR's HSL binding using PrankWeb3 (Fig. 2.2, blue highlighting).

LuxR-family activators typically form dimers, so we attempted to predict LuxR dimerization domains based on the sequence and structural alignments discussed above. We were able to generate predicted dimer structures for the LuxRs using AlphaFold Multimer (not shown), but LuxR does not align well with the TraR complex possibly due to asymmetry in TraR

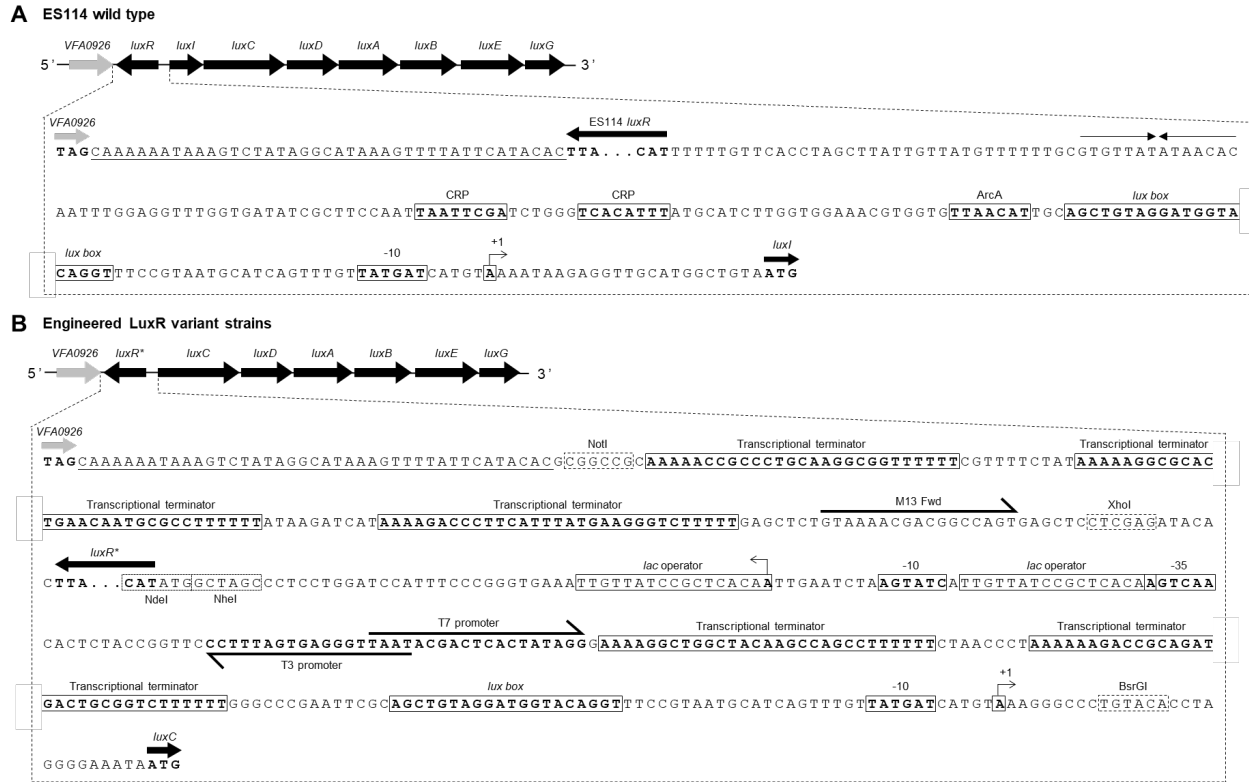


Figure 2.4. Genetic organization of the *lux* locus in *V. fischeri* ES114 and the strains engineered for this study. (A) Genetic organization of the *lux* locus in *V. fischeri* ES114 (WT) with the specific sequence for the ES114 *VFA0926-luxR* and *luxR-I* intergenic regions specified underneath. Sequence includes labels for the start and/or stop codons for *VFA0926*, *luxR*, and *luxI* (arrows), binding sites for regulators (CRP and ArcA), a palindromic repeat of unknown function (convergent arrows), the *lux box*, and the placement of the -10 and +1 of the *lux* promoter. (B) Illustrates the organization of the *lux* operon in strains engineered for this study to assay the activity of *luxR* variants, with sequence for the *VFA0926-luxR* and *luxR-C* intergenic regions specified underneath. The native *luxRI* was deleted and replaced with an engineered construct between *VFA0926* and *luxC*. The engineered constructs replace the native *luxR* with any of the *luxR* variants (*luxR**) under the control of a constitutive promoter, removes the non-LuxR regulation sites, adds bi-directional transcriptional terminators up- and down-stream of *luxR*, and replaces *luxI* with a BsrGI site used for cloning. Lac operators engineered to overlap the promoter can be used to control expression in strains containing *lacI^q*.

dimerization (2, 109, 158). The LuxR complex could be similarly asymmetrical, but if so, we were unable to predict that using AlphaFold Multimer.

***luxR* variants confer different responses to HSL signals in a common isogenic background**

To test whether the eight *luxR* alleles described above confer different phenotypic outputs, we cloned the *luxR* variants from strains ES114, PP3, ET401, ET101, H905, MJ1, MJ11, and WH1 into an engineered isogenic *V. fischeri* background. In this genetic background, we used ES114 as a parent and deleted *luxI* and *ainS*, which encode HSL synthases for 3OC6-HSL and C8-HSL, respectively, to prevent endogenous signal production thereby allowing experimental control over signal concentration. The intergenic region between *luxR* and the *lux* operon was also altered to prevent known non-Lux regulatory pathways from affecting luminescence output (Fig. 2.4). Specifically, we placed *luxR* under control of an artificial constitutive promoter, added bi-directional transcriptional terminators between *luxR* and the *lux* operon, and trimmed the *lux* operon promoter of regulatory sites while maintaining the native ES114 sequence from the *lux* box to the +1 of the *lux* transcriptional start (Fig. 2.4). We also added restriction and primer sites for use in cloning and sequencing (Fig. 2.4). The intergenic region replacement allows *luxR* expression without native exogenous regulation (e.g., by LitR or CRP) and disconnects *lux* operon expression from regulation other than LuxR (e.g., by ArcA). These engineered strains are isogenic except for the varying *luxR* coding sequences, allowing us to compare how differences in the *luxR* sequence affect luminescence response.

Each of the engineered *luxR* variants was tested for its response to exogenously added 3OC6-HSL and C8-HSL singly and in combination. These experiments measured luminescence and OD₆₀₀ at sixty-four different combinations of the two HSLs, each ranging from 0 to 3200 nM.

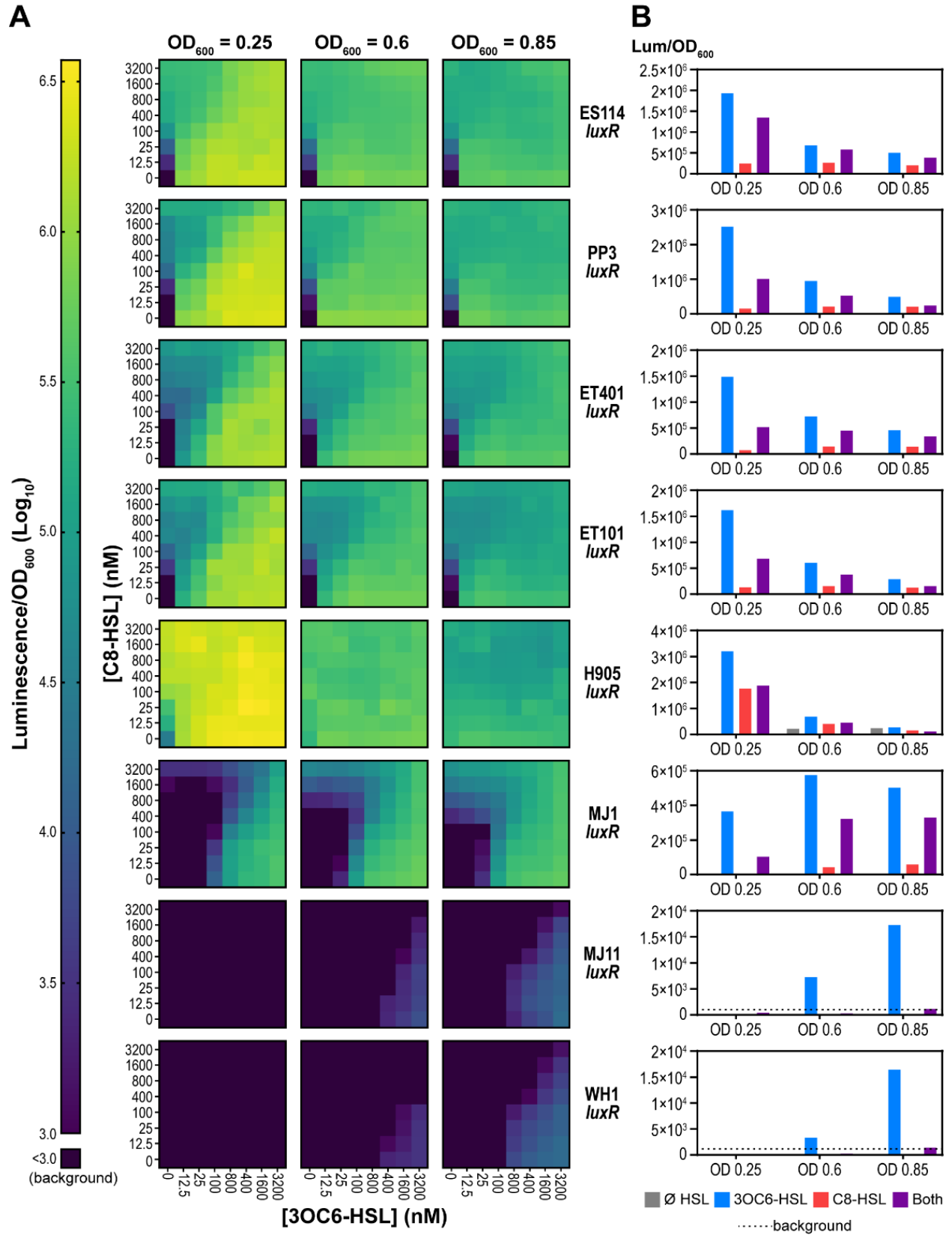


Figure 2.5. 3OC6-HSL and C8-HSL responses singly and in combination (see previous page).

(A) Heatmaps showing luminescence/OD responses on a colorimetric log scale. Each heatmap displays an eight-by-eight grid of HSL concentrations with 3OC6-HSL on the X-axis and C8-HSL on the Y-axis. All responses lower than 10^3 were considered below background. Each *luxR* variants response is displayed at three different OD₆₀₀ values. (B) Bar graphs representing luminescence/OD for each of the four corners of the heatmaps. Bottom left: $\emptyset$ HSL, bottom right: 3200 nM 3OC6-HSL, top left: 3200 nM C8-HSL, top right: 3200 nM of both 3OC6- and C8-HSL. Dotted lines indicate background (10^3). Note that the Y-axis scales on the bar graphs differ for each *luxR*.

We performed experiments in triplicate, but the heatmaps in Figure 2.5 display representative luminescence response data for each *luxR* variant strain at three different OD₆₀₀ levels in a single experiment. The unanticipated importance of OD₆₀₀ in these experiments is discussed below. For all eight LuxR variants, 3OC6-HSL response always elicited the brightest response (Fig. 2.5). The combination of 3OC6- and C8-HSL does not have an additive effect, but rather the weaker activator (C8-HSL) can have a repressive effect on luminescence output in the presence of 3OC6-HSL (Fig. 2.5), consistent with earlier studies (60). However, the level at which C8-HSL represses the combined response varied for different *luxR* alleles (Fig. 2.5).

Unexpectedly, differences in *luxR* sequence impacted the OD₆₀₀ at which the luminescence response was highest. When the sum of all sixty-four wells (i.e., all HSL combinations) is considered, expression of LuxR^{ES114}, LuxR^{PP3}, LuxR^{ET401}, LuxR^{ET101}, and LuxR^{H905} resulted in peak luminescence around an OD₆₀₀ of 0.25, while the strain expressing LuxR^{MJ1} was brightest at an OD₆₀₀ of 0.6, and strains with LuxR^{MJ11} and LuxR^{WH1} were brightest around OD₆₀₀ of 0.85 (Fig. 2.5). Although we planned to analyze and report luminescence data at a common OD₆₀₀, doing so would lose the complexity of the responses. The strain expressing LuxR^{MJ11} for example does not show detectable luminescence to any HSLs at low OD₆₀₀ but does have a measurable 3OC6-HSL response later in growth, but by that OD₆₀₀ the luminescence response of LuxR^{H905} has faded (Fig. 2.5). When considered at their respective brightest OD₆₀₀, the value of the peak luminescence

responses differs by *luxR* allele. LuxR^{H905} elicited the brightest response, followed by LuxR^{ES114}, LuxR^{PP3}, LuxR^{ET401}, and LuxR^{ET101}. LuxR^{MJ1} elicited the next brightest response, followed by the very dim response elicited by LuxR^{MJ11} and LuxR^{WH1}. The importance of OD₆₀₀ was puzzling, considering the strains were isogenic other than with respect to *luxR*, and HSL was added at the beginning of the experiment rather than accumulating as OD₆₀₀ increased. As described below, our data suggest the relationship of OD₆₀₀ and peak luminescence is not specific to signal-LuxR interactions or native *lux* regulation.

Each of the LuxR variants had a unique response to 3OC6-HSL and C8-HSL, though they formed phenotypically similar groups that tended to correlate with sequence similarity (Fig. 2.1 and 2.5). The closely related LuxR^{ES114}, LuxR^{PP3}, LuxR^{ET401}, and LuxR^{ET101} (Fig. 2.1) elicited similar responses to 3OC6- and C8-HSL; although LuxR^{ES114} produced a slightly higher response to C8-HSL (or a 1:1 mix of the two HSLs) relative to activation by 3OC6-HSL than the other three LuxRs in this group (Fig. 2.5). Another grouping similar both in sequence and function was LuxR^{MJ11} and LuxR^{WH1}, which only differ by three amino acids (Fig. 2.5). LuxR^{MJ11} and LuxR^{WH1} had the weakest 3OC6-HSL responses, and they both failed to elicit a response to C8-HSL above background (Fig. 2.5). LuxR^{MJ1} is also similar in sequence to LuxR^{MJ11} and LuxR^{WH1} (Fig. 2.1), but the latter two had distinct responses from that of LuxR^{MJ1} (Fig. 2.5). LuxR^{MJ1} and LuxR^{MJ11} differ by only three amino acids near the N-terminus (Fig. 2.2), but the former elicited a stronger response to 3OC6-HSL and a detectable response to C8-HSL (Fig. 2.5). Finally, the sequence of LuxR^{H905} was distinct from that of the other seven LuxRs (Fig. 2.1), and it elicited a unique luminescence response (Fig. 2.5), being less discriminating between C8- and 3OC6-HSL, and even stimulating luminescence without HSL. Taken together, the eight LuxR variants expressed can be

reduced to four general phenotypic types represented by $\text{LuxR}^{\text{ES114}}$, $\text{LuxR}^{\text{H905}}$, LuxR^{MJ1} , and $\text{LuxR}^{\text{MJ11}}$.

Figure 2.6 compares the responses elicited by these four representatives ($\text{LuxR}^{\text{ES114}}$, $\text{LuxR}^{\text{H905}}$, LuxR^{MJ1} , and $\text{LuxR}^{\text{MJ11}}$) using a subset of the data in Figure 2.5 plotted such that quantitative responses to HSL dose can be visualized. In Figure 2.6, lines plot luminescence output on a log scale with HSL concentration on a linear axis, and output is given in the presence of 3OC6-HSL, C8-HSL, or a 1:1 mix of the two. Figure 2.6 highlights the concentrations at which HSL dose-dependent responses plateau as well as the quantitative differences in responsiveness. This perspective emphasizes the effect C8-HSL has on dampening the response to 3OC6-HSL. For $\text{LuxR}^{\text{MJ11}}$, many HSL responses are near or below the limit of reliable detection (Fig. 2.6,

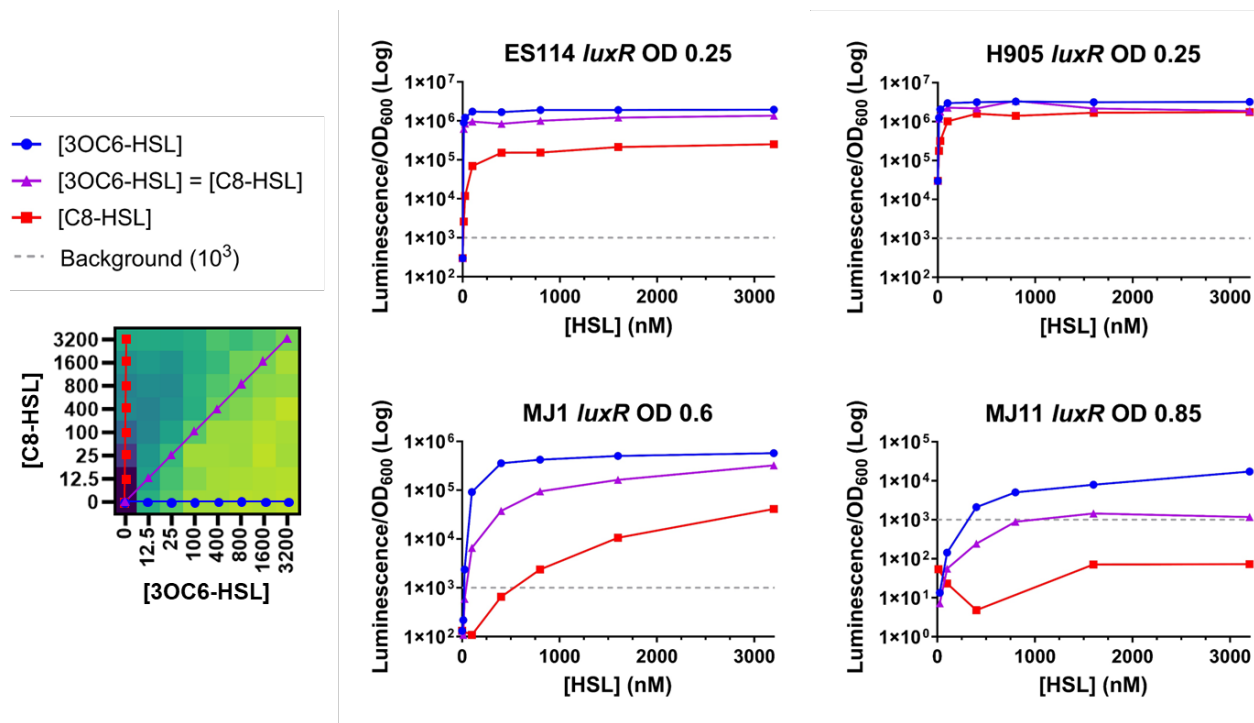


Figure 2.6. *LuxR*-HSL dose response curves. Luminescence responses with four representative *luxR* variants (from ES114, H905, MJ1, and MJ11) across increasing concentrations of 3OC6-HSL, C8-HSL, or an equal amount of each. Each *luxR* is in an isogenic background (Fig. 2.4B), and each graph displays data from the OD₆₀₀ at which that variant was brightest. Luminescence/OD₆₀₀ displayed on a log scale. Data below the dashed line is considered below background (10³). Note that the Y-axis scales differ for each *luxR*.

dotted lines), and the response elicited by 3OC6-HSL is not saturated even once doses exceed μM levels. Comparisons of responses elicited by $\text{LuxR}^{\text{ES114}}$, $\text{LuxR}^{\text{H905}}$, and LuxR^{MJ1} show differences in both maximal response and the HSL concentration at which such activation is achieved. For example, expression of $\text{LuxR}^{\text{ES114}}$ results in a C8-HSL response that is saturated by $\sim 400 \text{ nM}$ and at close to 10% of the luminescence elicited by 3OC6-HSL, whereas the strain expressing LuxR^{MJ1} responds much more weakly to C8-HSL, with a response orders of magnitude below that elicited by 3OC6-HSL and failing to saturate even at $3.2 \mu\text{M}$ C8-HSL (Fig. 2.6). In most instances, the response to a 1:1 mix of the two HSLs is between the two individual dose-response curves, illustrating the repressive effect of C8-HSL on activation by 3OC6-HSL. The exception is $\text{LuxR}^{\text{H905}}$, which has similar enough HSL responses that the dose curves essentially overlap.

Maximum luminescence negatively correlates with the OD_{600} at which that maximum occurs

As shown in Figure 2.5, when isogenic strains differing only by their *luxR* variants are exposed to varying combinations of 3OC6- and C8-HSL, the OD_{600} at which the luminescence response is brightest varies. Although we have no explanation for this unexpected phenomenon, we did observe a striking pattern of negative correlation between the maximum luminescence/ OD_{600} of a given strain-HSL combination and the OD_{600} value at which that maximum occurs (Fig. 2.7). When the data from each well (i.e., each strain-HSL combination) is plotted as luminescence/ OD_{600} vs OD_{600} , the plotted shapes are remarkably consistent when grouped by luminescence output. The brightest wells peaked at lower OD_{600} (Fig. 2.7A), wells that achieved an intermediate maximum luminescence showed a more drawn-out response across intermediate OD_{600} values (Fig. 2.7B and C) and the wells that displayed the dimmest maximal luminescence peaked at later OD_{600} (Fig. 2.7D). Moreover, on average, wells with intermediate maximal luminescence (Fig. 2.7B and C), seemed to showed patterns resembling dual peaks, or a

peak and a shoulder, with a plateau in between and aligning with the two peaks of bright (Fig. 2.7A) and dim (Fig. 2.7D) strain-HSL combinations. These patterns do not appear driven by either LuxR variant or HSL but rather show remarkable consistency across LuxR-HSL combinations based on maximal luminescence output (Fig. 2.7E).

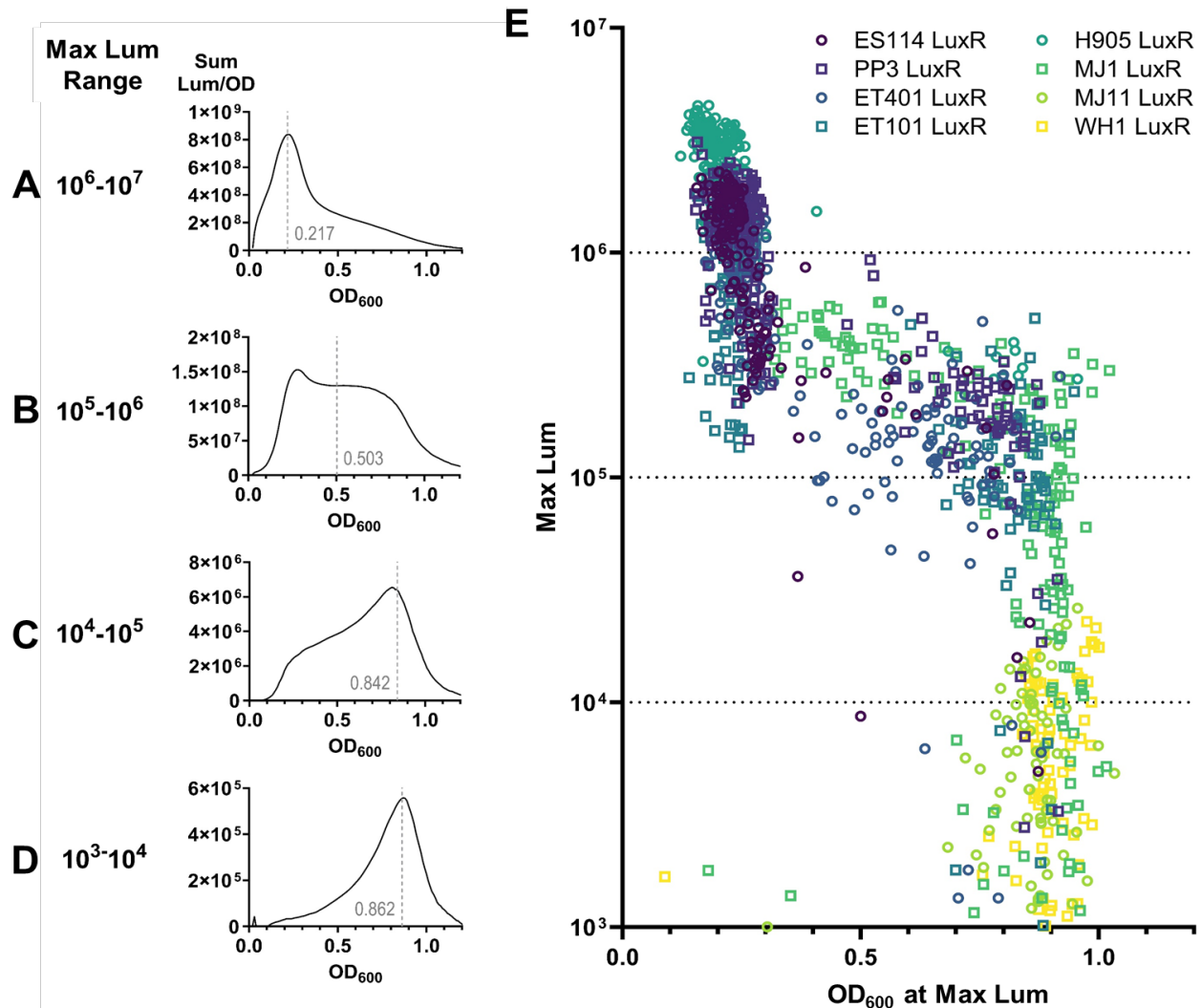


Figure 2.7. Maximum Luminescence/OD₆₀₀ as a function of OD₆₀₀. (A-D) Graphs displaying the sum of the luminescence/OD₆₀₀ vs OD₆₀₀ for all wells grouped into one of four maximum luminescence (Max Lum) ranges (10^3 - 10^4 , 10^4 - 10^5 , 10^5 - 10^6 , or $>10^6$ luminescence/OD₆₀₀). The average OD₆₀₀ at which Max Lum occurs for wells that fall in each luminescence range is displayed with a vertical dashed line. (E) The Max Lum value and the OD₆₀₀ at which that maximum occurred were plotted for each well of the sixty-four-well (sixty-four C8- and 30C6-HSL combinations) experiments with isogenic strains each engineered with one of eight *luxR* variants. Data points are colored and shaped based on the corresponding *luxR* variant. Max Lum values below 10^3 were considered below background and excluded.

We considered the possibility that this unexpected pattern (Fig. 2.7) might be somehow related to accumulation or degradation of a non-HSL metabolite that affected LuxR-based signaling. To test this possibility, we measured the luminescence and OD₆₀₀ of *V. fischeri* JB22, a strain with *lux* operon induction disconnected from LuxR. In JB22, the *luxCDABEG* genes are controlled by LacI^q and the P_{A1/34} promoter, allowing us to directly control *lux* expression and luminescence with isopropyl β-D-1-thiogalactoside (IPTG) (35). Figure 2.8 shows that controlling luminescence of JB22 with IPTG yielded similar, if not identical, patterns of OD₆₀₀ dependence on peak luminescence to that observed in Figure 2.7. IPTG levels inducing intermediate luminescence expression produce a dual-peak response across intermediate OD₆₀₀ values (Fig. 2.8B), similar to those in Figures 2.7B and C. At high IPTG levels bright luminescence/OD₆₀₀ peaks at lower OD₆₀₀ (Fig. 2.8C), similar to what we observed in Figure 2.7A. Leaky low-level luminescence in the absence of IPTG did not shift peak luminescence to high OD (Fig. 2.8A) as was observed in Figure 2.7D. However, taken together the data in Figure 2.8 strongly suggest that factors unrelated to LuxR-based *lux* operon activation influence the phenomenon illustrated in Figure 2.7.

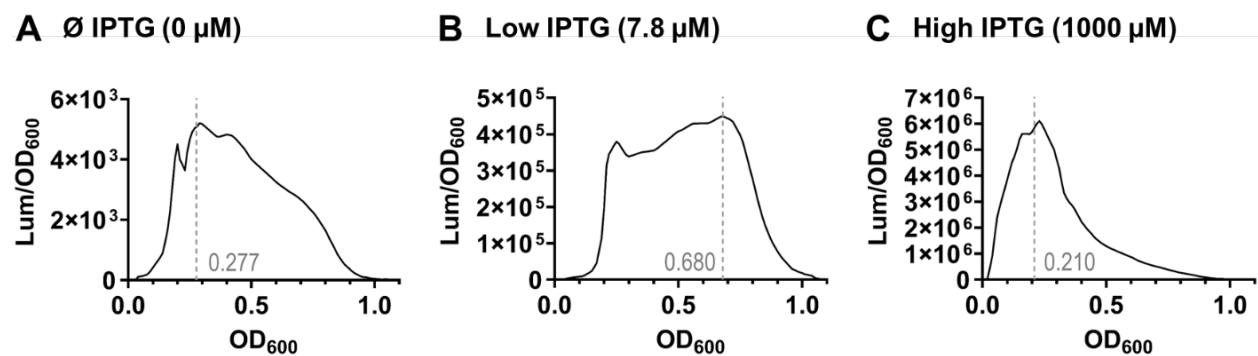


Figure 2.8. Maximum Luminescence/OD₆₀₀ as a function of OD₆₀₀ with an IPTG-controlled *lux* operon. Luminescence/OD₆₀₀ vs OD₆₀₀ data for *V. fischeri* JB22 grown in three different concentrations of IPTG. The *lux* operon in JB22 produces luminescence, is controlled by LacI^q-IPTG, and is independent of the LuxI/LuxR system. Each graph is the sum of three sets of interpolated data. Luminescence is plotted for cultures with no (∅) IPTG (A), low IPTG (B), or high IPTG (C).

luxR variants elicit different responses to HSLs of various chain lengths

In addition to testing responses to 3OC6-HSL and C8-HSL, we also tested how the engineered *luxR* variants responded to a variety of other HSLs. Luminescence and OD₆₀₀ were measured for all eight engineered *luxR* variants as well as a no-LuxR control ($\Delta luxR$) with 3200 nM C4-, C6-, C8-, C10-, C12-, 3OC6-, 3OC8-HSL or no HSL ($\emptyset$ HSL). We observed similar response groups as before, with LuxR^{ES114}, LuxR^{PP3}, LuxR^{ET401}, and LuxR^{ET101} eliciting similar responses, and LuxR^{MJ11} and LuxR^{WH1} eliciting similar responses (data not shown). We again selected four representative strains (expressing LuxR^{ES114}, LuxR^{H905}, LuxR^{MJ1}, and LuxR^{MJ11}) to compare maximum luminescence along with the no-LuxR control in a simplified figure (Fig. 2.9). Strains expressing LuxR^{ES114} and LuxR^{MJ1} responded most strongly to HSLs six to eight carbons in length, with the luminescence response getting weaker as the carbon chain gets longer than eight

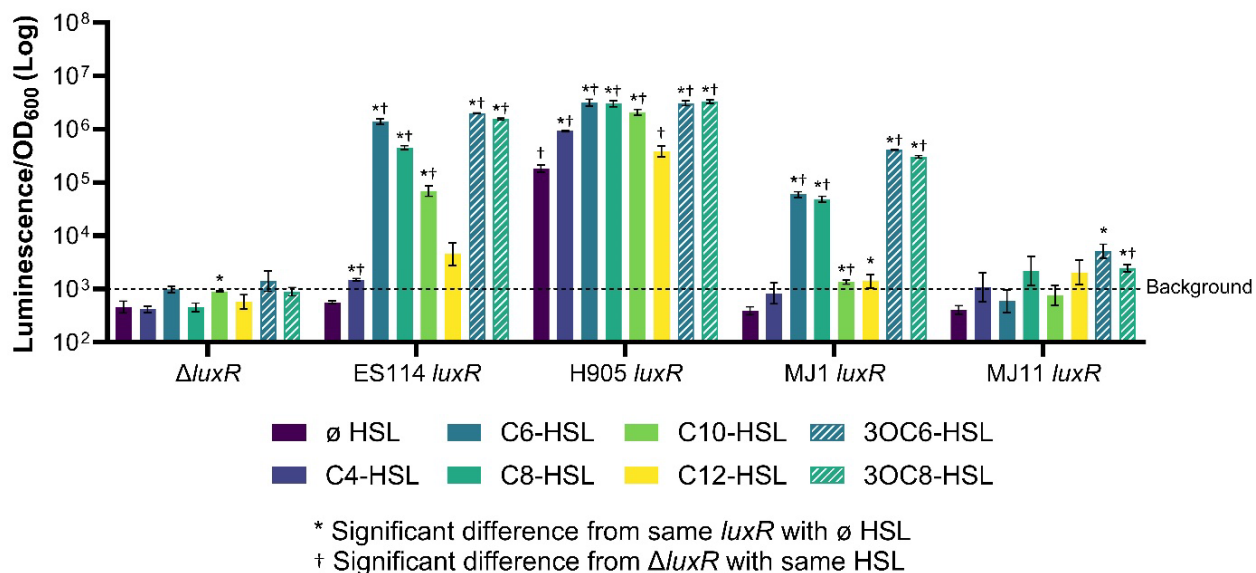


Figure 2.9. Luminescence responses to varied HSLs. Each engineered strain carrying a different *luxR* variant was grown with one of seven HSLs or a no-HSL control ($\emptyset$ HSL), and the maximum luminescence/OD₆₀₀ is reported on a log scale. Values below 10³ (horizontal dashed line) are considered below background. Error bars indicate standard error of the mean (n=3). Each bar was compared to the bar from the same *luxR* with no HSL added to determine if the HSL produced a significant response (*). Each bar was also compared to the $\Delta luxR$ control (KB15) with the same HSL added to determine if the *luxR* variant was causing a significant impact on the response (†) Significance determined with one-way ANOVA, $p < 0.05$.

or shorter than six (Fig. 2.9). This pattern is most evident for LuxR^{MJ1}, as there is a sharp decrease in luminescence response for HSLs other than six or eight carbons long. The strain expressing LuxR^{MJ1} also responds more strongly to HSLs containing an oxo-group on the third carbon, as evidenced by its strongest responses being 3OC6- and 3OC8-HSL (Fig. 2.9). The strain expressing LuxR^{MJ11} only significantly responds to 3OC6- and 3OC8-HSL and even then, its response was relatively weak (Fig. 2.9).

Uniquely, the strain expressing LuxR^{H905} activated luminescence in every condition we tested, including with no added HSL (Fig. 2.9). To our knowledge, this is the only published evidence of a wild-type LuxR that can activate the *lux* operon without HSLs present. Though LuxR^{H905} may appear to respond to every HSL, its response to C12-HSL was not significantly different from the luminescence produced with no HSL. It responded roughly equivalently to C6-, 3OC6-, C8-, and 3OC8-HSL (Fig. 2.9).

Discussion

The results of this study indicate that considerable *luxR* variation extends across several *V. fischeri* strains (Fig. 2.1 and 2.2) and distinct *luxR* alleles encode functional differences that can affect outputs in response to cognate and non-cognate HSLs (Fig. 2.5, 2.6, and 2.9). We observed these differences in LuxR-dependent activation of the *lux* operon in engineered strains that were isogenic except for the *luxR* coding sequence. These strains both disconnected *luxR* from its native transcriptional regulation (Fig. 2.4) and lacked endogenous signal production, enabling us to control HSL levels without the complications of positive feedback. Although we cannot rule out the possibility that the observed differences between the various *luxR* alleles could be related to differing concentrations of LuxR protein we can regardless conclude that something in the *luxR*

coding sequence itself is responsible. Perhaps the most striking result was the finding of natural allelic variations that genetically and phenotypically paralleled the results of previous studies aimed at directed evolution of LuxR (Table 2.2).

Several mutations that have appeared in directed evolution experiments aimed at changing the signal repertoire of LuxR are also present in this set of naturally evolved LuxR sequences (Fig. 2.2 and Table 2.2). Most directed evolution studies of *luxR* have begun with the MJ1 allele and enriched or screened for mutants with altered HSL responsiveness (59, 114, 115, 118, 119, 122). For clarity, we will discuss the involved residues with respect to their position in the alignment presented in Figure 2.2 rather than their position in LuxR^{MJ1}. In one example of directed and natural evolution converging, a T35A variation was recovered in a screen for increased responsiveness to C8-HSL (59), and this variation also appears in LuxR^{ES114}, LuxR^{PP3}, LuxR^{ET401}, and LuxR^{ET101} (Table 2.2 and Fig. 2.2), which in our engineered background similarly correlated with increased responsiveness to C8-HSL (Fig. 2.5 and 2.6). The same four natural strains also contain *luxR* encoding a N88K difference relative to LuxR^{MJ1}, and Kimura *et al.* (122) found this variation in a directed evolution experiment which also led to increased responsiveness to C8-HSL. LuxR^{ES114} is identical to the LuxR proteins from strains EM17, ES12, ES213, and ES401, meaning that both the T35A and N88K alleles were found in most *Euprymna* isolates, and from squid species as geographically dispersed as Hawaii, Japan, and Australia.

Natural *luxR* variants also parallel an experiment by Sitnikov *et al.*, who screened for HSL-independent activity, finding mutations underlying T11I and S167F variations in LuxR (115). These mutants were luminescent in the absence of HSL, although they still responded to HSL (115). The I11 and F167 residues associated with acquiring LuxR-independent activity are found naturally in strains ET401 and H905, respectively (Fig. 2.2 and Table 2.2). ET401 is among the

brightest squid isolates reported by Bose *et al.* (129), although LuxR^{ET401} in our engineered background did not elicit especially bright luminescence and required HSL for activity (Fig. 2.5 and 2.9). The LuxR encoded by H905 is identical to that in VLS2, and these two strains share the unusual property of being visibly luminescent strains from Hawaii, where they were isolated from seawater and a squid light organ, respectively (145, 149). Interestingly, in our engineered background, LuxR^{H905} was the only variant that directed HSL-independent *lux* activation (Fig. 2.5 and 2.9).

Some directed evolution studies layered mutations in combinations (Table 2.2) (59, 114, 118, 119). For example, E41D found by Hawkins *et al.* (118) increased responsiveness to short-chain HSL in a background that contained the T35A variation along with two other amino acid substitutions in LuxR^{MJ1}. We found that LuxR^{ET101} contained D41 along with A35 (Fig. 2.2 and Table 2.2). An I161V variation from the same study similarly conferred increased responsiveness to C4-HSL and 3OC6-HSL in a background that included four other changes to LuxR^{MJ1}. V161 appears naturally in LuxR^{H905} among others (Table 2.2), but the strain expressing LuxR^{H905} was the only one tested that showed a significant response to C4-HSL (Fig. 2.9).

In another example of layering modifications, Tashiro *et al.* (119) first used directed evolution to find a LuxR^{MJ1} variant with increased selectivity against some non-cognate HSLs but also decreased sensitivity to 3OC6-HSL. Then, in this background, they found additional mutations that would ameliorate the decreased sensitivity to 3OC6-HSL (119). Several natural LuxR variants contain the same differences from LuxR^{MJ1} that increased responsiveness to 3OC6-HSL in this background, but these were never found in “MJ1 cluster” LuxRs (LuxR^{MJ1}, LuxR^{MJ11}, and LuxR^{WH1}, Fig. 2.2). LuxRs that contained one or more of these residues, when expressed in our engineered background, elicited brighter luminescence than any of the “MJ1 cluster” LuxR

variants (Fig. 2.5 and 2.9). Conversely, Slock *et al.* (111) determined that a mutation encoding a C198F change to LuxR^{MJ1} resulted in loss of LuxR activity and lack of bioluminescence, yet LuxR^{CG101} contains F198 (not shown). Given its close similarity to LuxR^{CG103} and LuxR^{MJ11} (Fig. 2.1 and Table 2.1), we did not examine LuxR^{CG101} in detail, but strain CG101 and CG103 are similarly brightly luminescent (129). It seems likely LuxR^{CG101} is active and that other differences between it and LuxR^{MJ1} influence the effect of an F198 residue.

Previous studies exploring the directed evolution of LuxR suggest that certain phenotypic effects of a particular variation may depend on the context of other amino acid residues in the protein. This perhaps unsurprising observation highlights an important note of caution in interpreting our study of LuxRs with many differences. Given that a S167F change to LuxR^{MJ1} caused HSL-independent activation, it is tempting to speculate that the natural F167 present in LuxR^{H905} is the reason why expression of this LuxR in our engineered background causes HSL-independent activation. However, given the many differences between LuxR^{MJ1} and LuxR^{H905}, and the context-dependent effect of amino acid variations, such conclusions cannot be made firmly. Nonetheless, the LuxR variations and resulting phenotypes seem to show parallels between directed and natural evolution.

It is important to note that the luminescence in this study measures how responsive each *luxR* is to controlled levels of HSL in an isogenic background, which is quite different from how bright each *luxR* parent strain can get. In fact, the luminescence output of engineered *luxR* variant strains did not correlate well with the brightness of the parent strains (129). Despite LuxR^{MJ11} having a very poor HSL response in this study (Fig. 2.5) MJ11 is among the brighter strains observed in culture (129). Indeed, MJ11 is much brighter than ES114, even though in our engineered background LuxR^{ES114} has brighter luminescence than LuxR^{MJ11}. The fact that EM17,

ES12, ES114, ES213, and ES401 all have identical *luxR* sequences, but the brightness of their parent strains vary also highlights how *luxR* sequence is not predictive of parent strain luminescence phenotype (129). Both the DNA binding domains of the *luxR* alleles and the *lux* boxes are highly conserved across strains, but variation of other elements of the complex luminescence regulatory circuit may drive strain differences in luminescence output.

A comparison of strain origins (Table 2.1), *luxR* sequences (Fig. 2.1A) and housekeeping gene sequences (Fig. 2.1B) provides new insights into *luxR* evolution. We found that *luxR* sequence does not always correspond with the strain's geographic origin or the relatedness of housekeeping genes. EM17 and MJ1 are closely related strains based on housekeeping genes (Fig. 2.1B), and both were isolated from hosts in Japan, however, their *luxR* sequences are divergent (Table 2.1, Fig. 2.1). Instead, *luxR*^{EM17} is identical to *luxR*^{ES12}, *luxR*^{ES114}, *luxR*^{ES213}, and *luxR*^{ES401}, each of Hawaiian origin, with the commonality being a squid host. Unlike Hawaii, Japan has both sympatric fish and squid hosts for *V. fischeri*, and our observations are consistent with horizontal acquisition of *luxR* being important for evolution of EM17 as a squid, rather than fish, colonizer where both host types reside. In contrast to EM17 and MJ1, *luxR* can be highly conserved in strains from widely different geographic origins. For example, *luxR*^{MJ11} and *luxR*^{WH1} are closely related and underpinned similar phenotypes, yet MJ11 is a fish isolate from Japan and WH1 a seawater isolate from Massachusetts, USA (Table 2.1, Fig. 2.1 and 2.5). Finally, a noteworthy observation in Figure 2.1 was the finding that the housekeeping genes of H905 and VL2 suggest they are the closest relatives in the collection to ES114 (Fig. 2.1B), but their *luxR* sequences formed a cluster unlike any others (Fig. 2.1A). Overall, the results in Figure 2.1 and Table 2.1 suggest both varied selective pressures and genetic recombination at the *luxR* locus.

For the most part, phenotypic variation in our engineered strains appeared to correlate with the genetic distance between their respective *luxR* alleles, but there were some significant differences observed between strains with very similar *luxR* sequences. These differences could help pinpoint interesting areas of the sequence not typically considered important for function. For example, LuxR^{MJ1} and LuxR^{MJ11} only differ by three N- terminal amino acids, (Fig. 2.2 locations 2, 3, and 5) but these changes drastically affected function. Previous studies suggested the first 20 residues of LuxR^{MJ1} may play a role in autoinducer binding, but why or which amino acids are significant have not been elucidated (58, 103, 136). In the future, introducing point mutations at these locations could help assess the impact of these changes near the start of LuxR.

More broadly, the genetic and phenotypic datasets here could inform future studies predicting the relationship between LuxR sequence and function. One way to further this goal would be to make chimeras of these LuxR variants by swapping domains between LuxRs from different groups. Alternatively, computational approaches like machine learning could be applied to design hybrid *luxR* genes predicted to elicit specific functional outputs, and these could be synthesized and tested. Either approach could be iterative, leading to a progressively more accurate predictive model of function based on *luxR* sequence. Understanding signal specificity, e.g., how LuxR^{H905} accommodates an expanded collection of HSLs (Fig. 2.9), could allow for the creation of a universal HSL-biosensor or provide insights towards creating more specific-HSL LuxR-biosensors (113, 121, 159-161).

Given that a crystal structure of LuxR is not available, the alignments and bioinformatic analyses here provide valuable insights (Fig. 2.2 and 2.3). The three amino acids involved in direct TraR-HSL H-bonds (Fig. 2.2, triangles under sequence) are conserved in every LuxR, and this observation is corroborated by the predicted LuxR-HSL binding domains generated by PrankWeb3

and AutoDock Vina, which independently identified those same amino acids as composing the HSL binding pocket (Table 2.3, Fig. 2.2 and 2.3C). Mutations at those locations have been previously identified to have significant impacts on LuxR function. Tashiro *et al.* found that a Y64H mutation reduced HSL responses, and hypothesized that the mutation eliminates a H-bond with the ring carbonyl group of HSLs (119). Another study by Koch *et al.* observed a complete deactivation of LuxR activity with a W68H mutation, and similarly concluded that the mutation likely disrupts a H-bond with the ligand (112). LuxR^{MJ1} D81 has also been deemed important for LuxR-HSL binding by mutational analysis, and changing it results in dim or dark phenotypes (110-112). Also of note is the predicted H-bond at location R69 (Fig. 2.2, Fig. 2.3C, and Table 2.3) predicted to form between all LuxRs and 3OC6-HSL, but never C8-HSL. AutoDock Vina predicted that this H-bond forms with the oxo-group of the third carbon on 3OC6-HSL (Fig. 2.3C), which C8-HSL lacks. This residue is not conserved in TraR, but was identified as important for LuxR-HSL binding when Collins *et al.* determined that an R69M mutation abolishes LuxR's ability to bind oxo-HSLs, consistent with our findings (114). The strong conservation of Y64, W68, R69, and D81 is perhaps an unsurprising indication of evolutionary pressure to conserve responsiveness to the cognate 3OC6-HSL signal (Fig. 2.2 and Table 2.2).

V. fischeri LuxR serves as an excellent model for understanding HSL-based signaling systems in Pseudomonadota. In other organisms, these systems regulate important group behaviors such as biofilm formation or production of virulence factors, but the regulation of luminescence in *V. fischeri* allows us to study the mechanisms behind these systems with an easily measurable output. Regulators like LuxR can also serve as activators in synthetic regulatory circuits, and the better we understand LuxR structure and function the more precisely and predictably we can manipulate these circuits to respond to different signals. The sequence diversity within the LuxR-

type regulator family both implies a wide variety of purposes that these regulators could be used for and complicates their study. Our examination of *luxR* evolution draws connections between what we know LuxR can be artificially made to do and what it is evolving to do naturally.

Table 2.4. Mutant *V. fischeri* strains and plasmids used in this study.

Name	Relevant Characteristics	Reference
<i>E. coli</i>		
DH5 α	$\phi 80dlacZ \Delta M15 \Delta(lacZYA-argF) U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1$	(164)
CC118 λ pir	$\Delta(ara-leu) araD \Delta lac74 galE galK phoA20 thi-1 rpsE rpsB argE$ (Am) <i>recA</i> λ pir	(165)
<i>V. fischeri</i>		
DC36	ES114 $\Delta ainS lacI^q$ P _{A1/34} - <i>luxCDABEG</i>	(60)
JB22	ES114 <i>lacI</i> ^q P _{A1/34} - <i>luxCDABEG</i>	(35)
KB15	ES114 $\Delta ainS \Delta luxR-luxI$, $\Delta luxR::kanR$, P _{luxI} - <i>luxCDABEG</i>	This study
KB16	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{ES114} , P _{luxI} - <i>luxCDABEG</i>	This study
KB18	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{MJ1} , P _{luxI} - <i>luxCDABEG</i>	This study
KB21	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{PP3} , P _{luxI} - <i>luxCDABEG</i>	This study
KB22	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{ET401} , P _{luxI} - <i>luxCDABEG</i>	This study
KB23	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{ET101} , P _{luxI} - <i>luxCDABEG</i>	This study
KB24	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{H905} , P _{luxI} - <i>luxCDABEG</i>	This study
KB25	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{MJ11} , P _{luxI} - <i>luxCDABEG</i>	This study
KB26	ES114 $\Delta ainS \Delta luxR-luxI$, <i>luxR</i> ^{WH1} , P _{luxI} - <i>luxCDABEG</i>	This study
Plasmids		
pCR-BluntII-TOPO	PCR product cloning vector; ColE1, <i>kanR</i>	Invitrogen
pDC55	$\Delta luxRI$ replacement LuxR-dependent bioreporter; <i>luxR</i> ^{MJ1}	(60)
pEVS104	Conjugative helper plasmid; R6K γ , <i>oriT</i> _{RP4} , <i>kanR</i>	(162)
pKAB14	<i>luxR</i> ^{ES114} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB15	<i>luxR</i> ^{ES12} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB16	<i>luxR</i> ^{EM17} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB17	<i>luxR</i> ^{ES401} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB18	<i>luxR</i> ^{ET401} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB19	<i>luxR</i> ^{PP3} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB26	<i>luxR</i> ^{CG101} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB27	<i>luxR</i> ^{CG103} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB28	<i>luxR</i> ^{ET101} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB29	<i>luxR</i> ^{H905} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB30	<i>luxR</i> ^{SA1} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB31	<i>luxR</i> ^{VL52} in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pKAB36	$\Delta luxR::kanR$, P _{luxI} - <i>luxCDABEG</i> , <i>cmR</i>	This study

pKAB38	$\Delta luxR-luxI$, $luxR^{ES114}$, $P_{luxI}-luxCDABEG$, cmR	This study
pKAB39	$luxR^{WH1}$ in pCR-BluntII-TOPO; ColE1, $kanR$	This study
pKAB43	$\Delta luxR-luxI$, $luxR^{MJ1}$, $P_{luxI}-luxCDABEG$, cmR	This study
pKAB46	$\Delta luxR-luxI$, $luxR^{PP3}$, $P_{luxI}-luxCDABEG$, cmR	This study
pKAB47	$\Delta luxR-luxI$, $luxR^{ET401}$, $P_{luxI}-luxCDABEG$, cmR	This study
pKAB48	$\Delta luxR-luxI$, $luxR^{ET101}$, $P_{luxI}-luxCDABEG$, cmR	This study
pKAB49	$\Delta luxR-luxI$, $luxR^{H905}$, $P_{luxI}-luxCDABEG$, cmR	This study
pKAB50	$\Delta luxR-luxI$, $luxR^{MJ11}$, $P_{luxI}-luxCDABEG$, cmR	This study
pKAB51	$\Delta luxR-luxI$, $luxR^{WH1}$, $P_{luxI}-luxCDABEG$, cmR	This study

Table 2.5. Oligonucleotides used in this study.

Oligo	Sequence*	Use
KAB18	GAATACCTCTATACTCCTCTGATGG	<i>luxR</i> PCR amplification and sequencing (based on ES114 <i>luxI</i>)
KAB19	CTCATTTAGAGCAACTTATTCGCCAAG	<i>luxR</i> PCR amplification and sequencing (based on ES114 <i>VF_A0926</i>)
KAB22	GAATACCTTTATACTCCTCCGATGG	<i>luxR</i> PCR amplification and sequencing (based on MJ11 <i>luxI</i>)
KAB23	CAGCTTATTCGTCAAGATTTTCATGG	<i>luxR</i> PCR amplification and sequencing primer (based on MJ11 <i>VF MJ11_A1044</i>)
KAB26	CTAGCTCGAGATACACTTAATTTTTTAAGGTAT	ES114, PP3, & ET401 <i>luxR</i> cloning primer (Rev)
KAB27	CTAGCATATGAACATTAAAAATATAAATGCTAA	ES114 & PP3 <i>luxR</i> cloning primer (Fwd)
KAB36	CTAGCATATGAAAAACATAAATGCCGACGACA	MJ1 <i>luxR</i> cloning primer (Fwd)
KAB37	CTAGCTCGAGTTAATTTTTTAAAGTATGGGCAATCA	MJ1 & MJ11 <i>luxR</i> cloning primer (Rev)
KAB38	CTAGCATATGAACATTAAAAATATAAATGCTGA	ET401 <i>luxR</i> cloning primer (Fwd)
KAB39	CTAGCATATGAATACTAAAAATATAGATGTTAATGG	ET101 <i>luxR</i> cloning primer (Fwd)
KAB40	CTAGCTCGAGATACACTTAATTTTTTAGGGTAT	ET101 <i>luxR</i> cloning primer (Rev)
KAB41	CTAGCATATGAATACTAAAAACATAAATATTAATGA	H905 <i>luxR</i> cloning primer (Fwd)
KAB42	CTAGCTCGAGCTCATTTACTTAATTTTTTAAAGTAT	H905 <i>luxR</i> cloning primer (Rev)
KAB43	CTAGCATATGGGTATGAAAGACATAAATGCCG	MJ11 & WH1 <i>luxR</i> cloning primer (Fwd)
KAB44	CTAGCTCGAGTTAACTTTTTAAAGTATGGGCAATC	WH1 <i>luxR</i> cloning primer (Rev)

* Sequences in 5' to 3' orientation; underlined sequences indicate restriction-enzyme recognition sites added to facilitate cloning; bold sequences indicate *luxR* start or stop codons in Fwd or Rev primers, respectively.

Materials and Methods

Strains and Media. The strains used in this study are listed in Tables 2.1 and 2.3. KB15 was used as parent strain into which the rest of the *luxR* variants were introduced. To make strain KB15, the strain DC36 (60) (derived from *Vibrio fischeri* wild-type ES114) was engineered with the *luxI-luxR* intergenic region described in Figure 2.4 and a *kanR* cassette in place of *luxR* to facilitate future *luxR* insertions. *Escherichia coli* strain DH5 α was used for cloning. *V. fischeri* cultures were grown in either LBS medium (162) at 28°C or SWTO medium (1) at 24°C with final antibiotic concentrations of 2 μ g/mL for chloramphenicol and 100 μ g/mL for kanamycin as needed. *E. coli* cultures were grown in lysogeny broth (LB) medium (163) at 37°C with final concentrations of 20 μ g/mL for chloramphenicol and 40 μ g/mL for kanamycin. Agar was added at a final concentration of 1.5 % for solid media. HSLs were obtained from Sigma-Aldrich and dissolved in acidified ethyl acetate (1:1000 glacial acetic acid: ethyl acetate) to create stock solutions. HSL stock solution was aliquoted into a sterile container and the solvent was evaporated before adding media. SWTO medium was added to achieve the appropriate HSL concentration (e.g., 3200 μ M), and this solution was further diluted with SWTO to achieve the reported HSL concentrations.

Sequencing *luxR* variants. The *luxR* sequences for five *V. fischeri* strains (ES114, MJ11, MJ1, SR5, and ES213) were already available (accession numbers Table 2.1). For the remaining twelve *V. fischeri* strains, *luxR* was PCR amplified and TOPO™ cloned with the Invitrogen™ Zero Blunt™ TOPO™ PCR Cloning Kit and the resulting cloned inserts were sequenced. Primers KAB18 and KAB19 (Table 2.5) were designed based on the ES114 genomic sequence and used to amplify *luxR* from ES114, ES12, EM17, ES401, ET401, PP3, H905 and VLS2 genomes to make TOPO plasmids pKAB14, pKAB15, pKAB16, pKAB17, pKAB18, pKAB19, pKAB29, and pKAB31, respectively. Primers KAB22 and KAB23 (Table 2.5) were designed based on the MJ11

genome and used to amplify *luxR* from CG101, CG103, ET101, SA1, and WH1 genomes to make TOPO plasmids pKAB26, pKAB27, pKAB28, pKAB30, and pKAB39, respectively. Alignments and trees comparing LuxR sequences were generated with Geneious Prime version 2024.0.5 (<https://www.geneious.com>).

LuxR Structural Predictions. LuxR protein structures were predicted with AlphaFold v2.3.2. using the online AlphaFold.ipynb Google Collab Notebook. The TraR sequence and structure were downloaded from UniProt (Accession number P33905). Once structures were generated, we used the PrankWEB: Ligand Binding Site Prediction website (153-155) to predict the LuxR amino acids within the HSL binding pocket. These LuxR structures were imported into the program PyRx along with the molecular structures of 3OC6- and C8-HSL (PubChem CID 688505 and 6914579, respectively) where we predicted how the HSLs would bind each LuxR structure using the AutoDock Vina plugin (156, 157). Structural alignments of LuxR and TraR were created using The PyMOL Molecular Graphics System, Version 2.5.2 Schrödinger, LLC (Fig. 2.3). PyMOL was also used to predict possible H-bonds between LuxR and 3OC6-HSL by using the find>polar contacts function on the LuxR-HSL ligand binding model generated by AutoDock Vina.

Genetic manipulations. Eight of the *V. fischeri luxR* variants (ES114, PP3, ET401, ET101, H905, MJ1, MJ11, and WH1) were engineered into an isogenic *V. fischeri* background. To clone each *luxR*, forward and reverse primer pairs (Table 2.5) were used to PCR amplify the target *luxR* from their respective TOPO plasmids (ES114, PP3, ET401, ET101, H905, and WH1), genomic DNA (MJ11), or the previously constructed plasmid pDC55 (MJ1) (60). PCR products were digested with NdeI and XhoI and cloned into NdeI- and XhoI-digested pKAB36, with transformants screened for loss of the *kanR* marker, which was replaced by *luxR*. In this way, *luxR* alleles from ES114, PP3, ET401, ET101, H905, MJ1, MJ11, and WH1 were cloned into pKAB36 to make

pKAB38, pKAB46, pKAB47, pKAB48, pKAB49, pKAB43, pKAB50, and pKAB51, respectively. These eight plasmids were each conjugated into *V. fischeri* KB15, using *E. coli* CC118 λ pir with pEVS104 as a conjugative helper (162), enabling allelic exchange to replace the *kanR* on the chromosome of KB15 with each respective *luxR* variant resulting in strains KB16, KB21, KB22, KB23, KB24, KB18, KB25, and KB26, respectively. Tables 2.4 and 2.5 list key *V. fischeri* strains, plasmids, and oligonucleotides. Details of cloning intermediates used to create pKAB36 are available on request. Each *luxR* variant was verified by sequencing the plasmid used for allelic exchange.

LuxR variant HSL response experiments. Mixed 3OC6-HSL and C8-HSL experiments were set up as described in Colton *et al.* 2015 (60). HSLs stocks in ethyl-acetate were added to tubes and the solvent was allowed to evaporate. SWTO medium was added to HSL-containing tubes to a concentration of 6400 nM HSL and was then serially diluted to the following concentrations: 3200 nM, 1600 nM, 800 nM, 400 nM 100 nM, 25 nM and 12.5 nM. An 8x8 grid of a 96 well plate was filled with 200 μ L of SWTO + HSL, increasing from 0 nM to 3200 nM with 3OC6-HSL increasing along one axis and C8-HSL increasing along the other. 2 μ L of an overnight *V. fischeri* culture was aliquoted into all wells. When testing *luxR* responses to multiple separate HSLs, SWTO medium was added to each evaporated HSL-containing tube to a concentration of 3200 nM HSL. 200 μ L of SWTO and 3200 nM HSL was aliquoted into wells and 2 μ L of an overnight culture was added. KB15 was used as a Δ *luxR* control to help identify the level of luminescence we would consider “background”.

JB22 luminescence. IPTG was added to SWTO media from a 500 mM IPTG stock to a concentration of 1000 μ M IPTG. This IPTG-amended SWTO medium was serially diluted 1:2 to create SWTO solutions of 500 μ M, 250 μ M, 125 μ M, 62.5 μ M, 31.25 μ M, 15.63 μ M, 7.81 μ M,

3.91 μM , 1.95 μM , and 0.98 μM IPTG. 200 μL of SWTO at each IPTG concentration was distributed into a 96-well plate in triplicate along with three wells of SWTO without IPTG. 2 μL of an overnight *V. fischeri* JB22 (35) culture was inoculated into each well.

Growth and luminescence measurement. Luminescence experiments were all completed in black, clear-bottom 96-well plates and grown at 24°C shaking in a Biotek Cytation 1 plate reader with a Peltier Cooling Module used to maintain a consistent temperature. Luminescence and OD₆₀₀ measurements were taken every 30 minutes for 18 hours. Although wells were filled with equivalent volume, OD₆₀₀ was not pathlength-corrected to any standard in our analysis.

Maximum Luminescence/OD₆₀₀ (Max Lum). Data from three replicates of luminescence experiments were organized by Luminescence/OD₆₀₀ (Lum/OD₆₀₀) versus OD₆₀₀. Interpolation was used on the Lum/OD₆₀₀ versus OD₆₀₀ data for each well to fill in the gaps of Lum/OD₆₀₀ values for all OD₆₀₀ values between 0 and 1.40 (by increments of 0.01). The Maximum Luminescence/OD₆₀₀ (Max Lum) and the OD₆₀₀ at which that maximum occurred were identified for each well. LuxR variant data was organized into groups by Max Lum range: values between 10^3 to 10^4 , 10^4 to 10^5 , 10^5 to 10^6 , and values $>10^6$. Max Lum values below 10^3 were considered background and excluded from analysis. The interpolated Lum/OD₆₀₀ data for each well were added together within the four Max Lum range groups to produce the summed Lum/OD₆₀₀ versus OD₆₀₀ curves for each Max Lum range (Fig. 2.7). JB22 data was interpolated and analyzed similarly, but graphs were produced for each IPTG concentration rather than Max Lum ranges (Fig. 2.8).

Acknowledgements

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CHAPTER 3

ALLELIC VARIATION IN THE *LUXR* CODING SEQUENCE CAN AFFECT *LUXR* MRNA AND LUXR PROTEIN LEVELS²

² Kathryn A. Bellissimo and Eric V. Stabb. 2024. To be submitted to *Journal of Bacteriology*.

Abstract

The transcriptional regulator LuxR is responsible for activating bioluminescence genes in *Vibrio fischeri* in response to two main pheromone signals: *N*-3-oxo-hexanoyl-L-homoserine lactone (3OC6-HSL) and *N*-octanoyl-L-homoserine lactone (C8-HSL). LuxR's specificity for and response to these signals are critical for effective cell-to-cell communication, and LuxR has diverged in these respects between *V. fischeri* strains. Though changes to the *luxR* sequence have been examined for their functional impact on bioluminescence output, here we aimed to investigate whether variations in *luxR* coding sequence have significant impact on its transcript and protein levels. We found that naturally evolved *luxR* variants exhibit differential protein and mRNA transcript levels in an otherwise isogenic background. Notably, variations in the predicted stability of secondary structure in the 5' end of the *luxR* transcript strongly correlated with LuxR protein levels and bioluminescence output, consistent with models of 5' transcript sequences affecting the efficiency of translation initiation. Some LuxR protein variants also were stabilized in the presence of added 3OC6- and/or C8-HSL. Although the presence of more LuxR tended to correlate with more *lux* operon induction and brighter luminescence, the varied levels of LuxR variants could not fully account for observed differences in selectivity for specific HSLs. This study highlights a signal-dependent feedback mechanism on signal-receptor levels, as well as an apparent post-transcriptional regulatory mechanism that underscores the importance of considering the effects that changing the 5' CDS can have on expression.

Importance

Bioluminescence in *V. fischeri* was the first example of bacterial pheromone signaling (PS), or “quorum sensing”, discovered. Many similar PS systems have since been identified throughout the Pseudomonadota (formerly Proteobacteria) where they regulate a variety of collective behaviors such as biofilm formation or virulence. *V. fischeri*’s LuxI/LuxR system still serves as a valuable model of bacterial PS, which we can use to both enhance our understanding of PS and as a tool for developing synthetic regulatory circuits. This study focuses on the variation in *luxR* alleles, showing a PS feedback mechanism whereby different LuxR variants are differentially stabilized by cognate signal ligands, as well as a correlation between non-coding elements of *luxR* mRNA and LuxR protein levels. Our findings highlight the importance of considering 5’ end transcript folding dynamics and transcription initiation efficiency in both the evolution of gene regulation and when engineering changes into a gene’s coding sequence.

Introduction

LuxR and its homologs are responsible for regulating a variety of community-based behaviors throughout the phylum Pseudomonadota. These behaviors range from the bioluminescence generated by the mutualistic symbiont *Vibrio fischeri* to the production of virulence factors in pathogens (16-20). Such bacterial group behaviors can be controlled by pheromone signaling (PS) systems, which were first discovered in *V. fischeri* (12-14). In part due to its easily measurable and harmless output, *V. fischeri* bioluminescence has a longstanding history as an accessible model system for studying bacterial PS systems.

The regulation of bioluminescence in *V. fischeri* involves two distinct homoserine lactone- (HSL-) based PS systems (130). Primary regulatory control is exerted by the LuxI/LuxR system, which includes the *lux* operon (*luxICDABEG*) and the divergent gene *luxR*, encoding the genes

required for bioluminescence and the regulator for those genes, respectively (37, 38). When LuxR combines with the pheromone *N*-3-oxo-hexanoyl-L-homoserine lactone (3OC6-HSL), the complex binds a sequence in the *lux* operon promoter known as the “*lux* box” to activate transcription (42, 108). The first gene of the *lux* operon, *LuxI*, is an HSL synthase that produces 3OC6-HSL, causing a positive feedback loop (131). Another, unrelated HSL synthase *AinS* produces a chemically similar pheromone, *N*-octanoyl-L-homoserine lactone (C8-HSL). Upon binding its receptor *AinR*, C8-HSL modulates a signaling cascade involving *LuxU*, *LuxO*, and *Qrr* (sRNA) to de-repress *LitR*. *LitR*, a master regulator found throughout the *Vibrionaceae* family, activates *LuxR*, connecting the *AinS/AinR* system to the regulation of luminescence (39, 51, 52, 55-57, 134). However, C8-HSL can also bind directly to *LuxR*, creating a *LuxR*-C8 complex that for most natural *LuxR* variants is less effective at activating the *lux* operon than the *LuxR*-3OC6-HSL complex (58-60).

The presence of these two HSL signaling molecules that can each activate *LuxR* creates crosstalk between the *LuxI/LuxR* and *AinS/AinR* signaling systems. It is not uncommon for *Pseudomonadota* to contain multiple *LuxI/LuxR* type systems (20, 135, 140); however, it is rare for a single species to have two genetically and structurally unrelated HSL-based signaling systems. This mix of chemically similar signals places an unusual evolutionary pressure on *LuxR* to select for or against the crosstalk with the *Ain* system, and both *luxR* and the *lux* operon have diverged more between *V. fischeri* strains than is typical for orthologs (141, 142). For example, *luxR* sequenced from seventeen *V. fischeri* strains had nucleotide identities as low as 79.9% while housekeeping genes showed >94% conservation (Chapter 2).

The differences in natural *luxR* genes led to diverse HSL-dependent outputs in a *V. fischeri* background genetically engineered to compare *luxR* constitutively expressed in a controlled

comparison. For example, isogenic strains with different *luxR* variants displayed a range of luminescence outputs and had different relative responses to 3OC6- and C8-HSL (Chapter 2). Interestingly, several natural LuxR variations paralleled those recovered from studies aimed at artificially evolving LuxR responsiveness to HSLs (Chapter 2). Among the most interesting genetic variants was *luxR*^{H905}, which has evolved similar responses to 3OC6- and C8-HSL as well as HSL-independent activation of the *lux* operon, while encoding a LuxR with an amino acid variation that was recovered based on a directed search for HSL-independent activation (115).

Given that the strains engineered for comparative analysis were isogenic aside from *luxR*, the phenotypes observed in Chapter 2 must be caused by differences in the *luxR* coding sequence. Moreover, native *luxR* regulation by CRP, LitR, and known regulatory sites located in the LuxIR intergenic region (55, 64, 66, 166, 167) were removed in the engineered background, which was intended to express *luxR* constitutively. However, this genetic setup would not prevent regulatory mechanisms encoded within *luxR* from affecting LuxR levels. For example, LonA-dependent proteolysis of LuxR might vary (168, 169), and there is precedent for a post-translational positive-feedback mechanism whereby HSL stabilizes the LuxR homolog TraR from turnover (106). Here, we aimed to expand on our previous study by determining whether variations in *luxR* result in altered LuxR protein and/or *luxR* mRNA levels, and if so whether these are affected by HSLs.

Results

Tagging LuxR protein for analysis

In our previous study of LuxR evolution, we identified differences in HSL response between LuxRs taken from different strains of *V. fischeri* (Chapter 2). This phenotypic diversity was observed in an engineered background with no HSL synthases, constitutive *luxR* transcription,

and no known *lux* operon regulatory sites other than the *lux* box (see Chapter 2 Fig. 2.4 for detailed description of the engineered background). We selected four LuxR representatives from that study to investigate further by measuring LuxR expression in the engineered background. The variability of LuxR made it difficult to generate useful antibodies against an epitope conserved across all natural LuxR proteins. Therefore, we instead tried several tags on both the C- and N-terminus of LuxR and found a C-terminal myc tag that had previously been used on LuxR (59) was the least detrimental to LuxR function (data not shown). Each LuxR-myc luminescence response was slightly attenuated relative to the response with the respective LuxR, but the trends with respect to comparing different HSLs or different LuxR variants were not noticeably different (data not shown). We therefore used LuxR-myc versions of representative LuxR variants for further study.

LuxR protein levels are affected by *luxR* sequence and HSL

We grew our four engineered LuxR-myc variant strains in shake flasks with either no HSL, 3200 nM 3OC6-HSL, or 3200 nM C8-HSL to an OD₆₀₀ of approximately 1, then measured luminescence and took samples for protein extraction. The scale of the OD₆₀₀ values in this study do not relate to the OD₆₀₀ values reported in Chapter 2 as these were pathlength corrected differently, but overall trends can be compared. The luminescence/OD₆₀₀ trends aligned with what we reported previously (Chapter 2), with both LuxR type and HSL affecting luminescence output (Fig. 3.1A). In general, the strain expressing LuxR-myc^{H905} is brightest, followed by strains engineered with LuxR-myc^{ES114}, LuxR-myc^{MJ1}, and LuxR-myc^{MJ11}, respectively (Fig. 3.1A). The 3OC6-HSL responses also tend to be stronger than those elicited with C8-HSL (Fig. 3.1A). Only the strain expressing LuxR-myc^{H905} produced significant luminescence in the absence of HSL ($p < 0.01$), paralleling the HSL-independent induction seen with untagged LuxR^{H905} (Chapter 2).

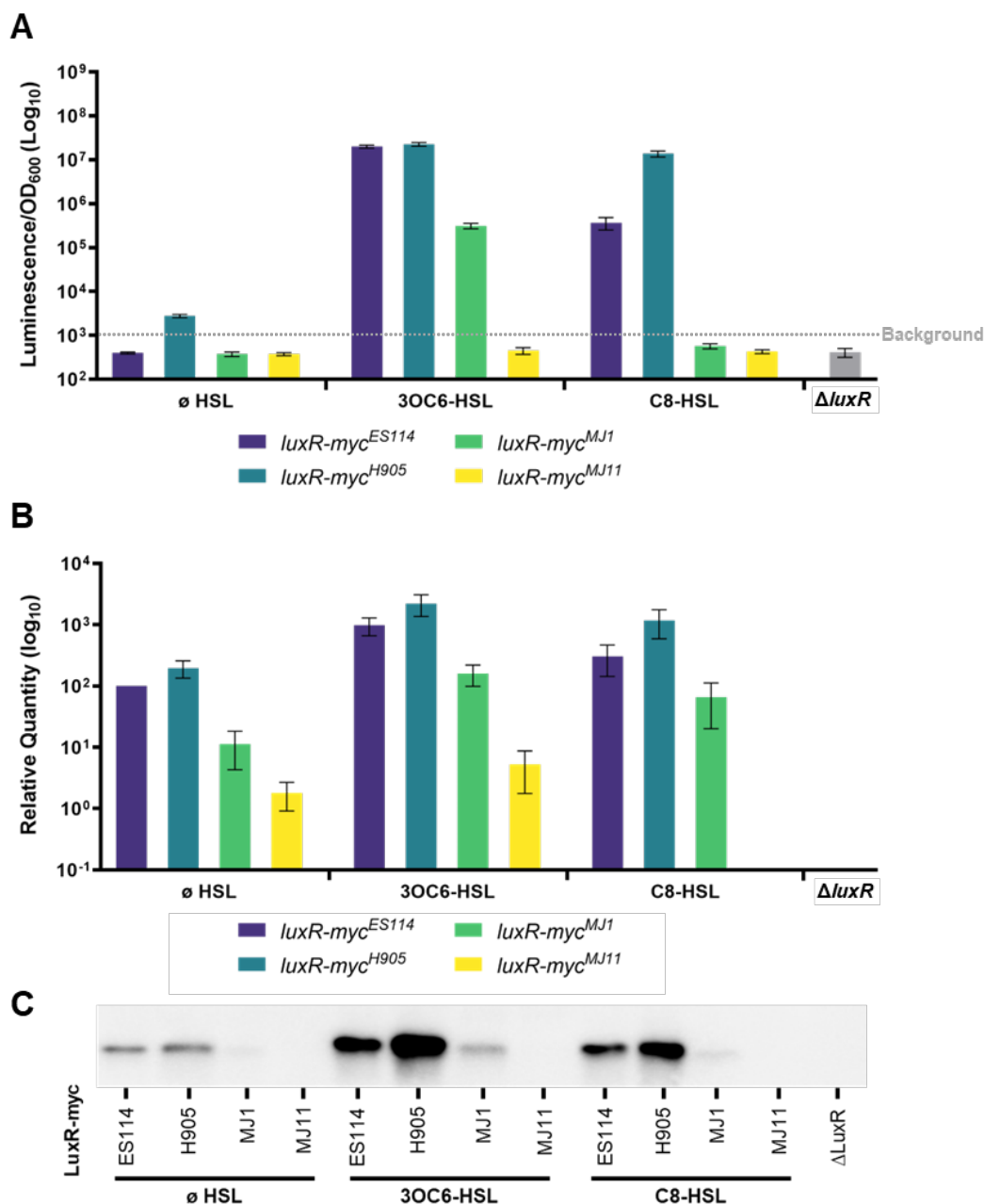


Figure 3.1. LuxR variants have different luminescence responses and LuxR protein levels. (A) Luminescence/OD₆₀₀ (log₁₀) and (B) normalized quantified Western blot data (log₁₀) for engineered LuxR-myc variants with no added HSL, 3200 nM 3OC6-HSL, or 3200 nM C8-HSL. Luminescence was measured at an OD₆₀₀ of approximately 1, then whole-cell protein was extracted from the same cultures for Western blot. Bars represent the average of three experiments. Error bars represent standard error of the mean. (C) Representative Western blot from one of the three replicates. Blots were quantified using Bio-Rad Image Lab software, normalized to total lane protein, then scaled such that the ES114 LuxR-myc ø HSL band equaled 100 before averaging across blots. Dotted line in (A) represents the threshold of background luminescence.

Because of the attenuation associated with the tag, the response of LuxR-myc^{MJ1} with C8-HSL and response of LuxR-myc^{MJ11} to either HSL were below background.

Proteins from the samples used for Luminescence and OD₆₀₀ measurements in Figure 3.1A were extracted and LuxR was quantified using Western blots (Fig. 3.1B and C). The amount of LuxR tended to correlate with the total amount of luminescence, following the same order from most to least LuxR or luminescence. LuxR-myc^{H905} was most abundant, followed by LuxR-myc^{ES114}, LuxR-myc^{MJ1}, and LuxR-myc^{MJ11}, respectively (Fig. 3.1B). Addition of 3OC6-HSL and to a lesser extent C8-HSL appeared to result in more LuxR present (Fig. 3.1B and C), and this effect was explored further when examining LuxR half-life. Bands for LuxR-myc^{MJ11} were either extremely faint or absent entirely, limiting our ability to determine whether HSL had an impact on levels of this LuxR variant (Fig. 3.1B and C). As noted above, the only variation between these strains is *luxR*, which implies either intragenic effects on transcription or post-transcriptional or post-translational regulatory mechanisms.

Allele-specific variation in LuxR levels is not dependent on LonA

Given reports that LonA (also simply Lon) protease acts on LuxR (168, 169), and the finding that *lonA* mutants are brighter than wild type (9), we speculated that different susceptibility to LonA could underlie the different amounts of LuxR protein for each variant. To test this possibility, we introduced a transposon-insertion *lonA*::Tn allele (170) into our engineered strains. Consistent with previous results, *lonA*::Tn caused increases in luminescence; however, disrupting *lonA* did not change the relative pattern of LuxR differences for the LuxR variants. Thus, differential susceptibility to LonA was not the cause of the relative differences between variants (Fig. 3.2).

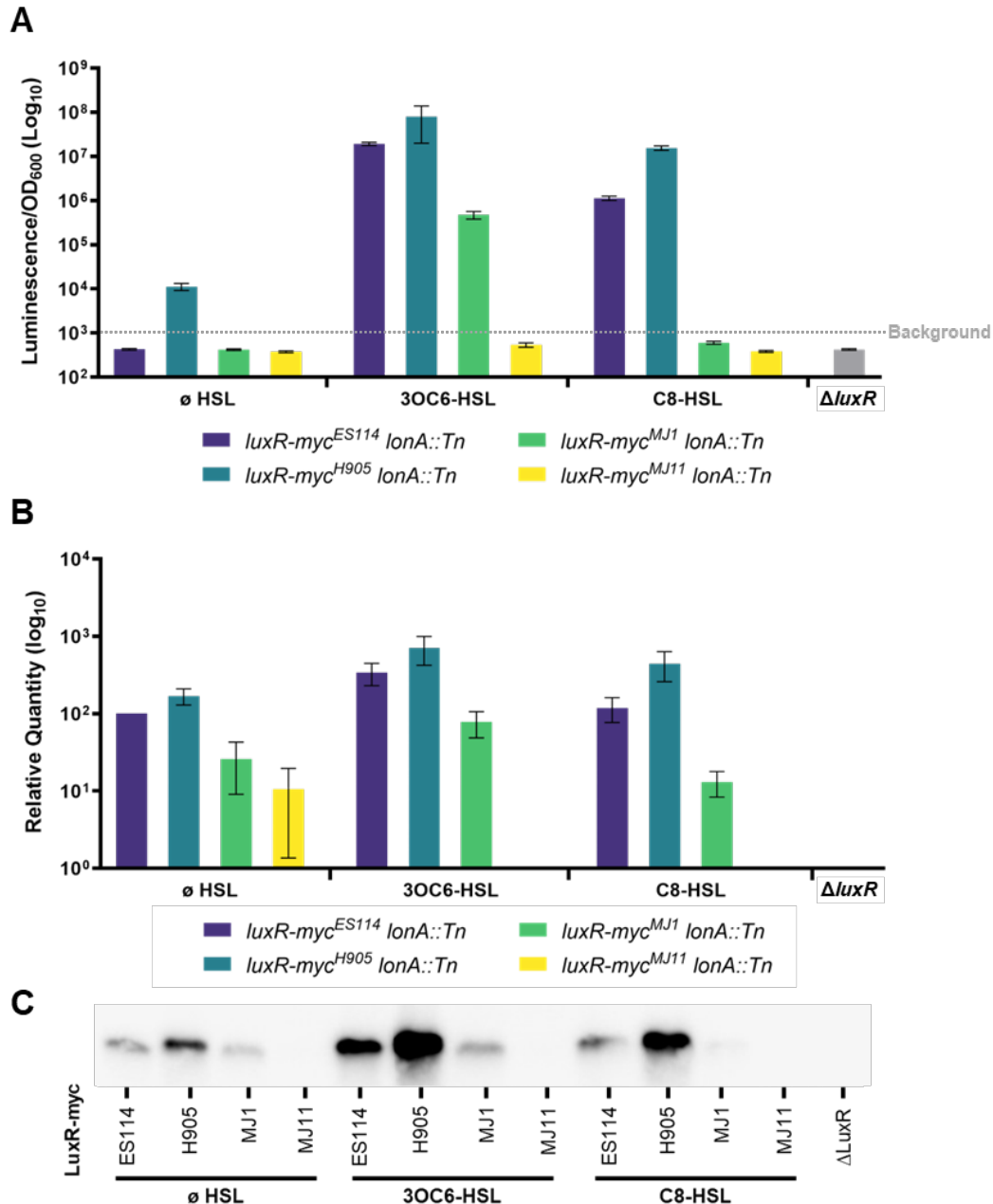


Figure 3.2. *lonA*::Tn LuxR variant strains still have different luminescence responses and LuxR protein levels. (A) Luminescence/OD₆₀₀ (log₁₀) and (B) normalized quantified Western blot data (log₁₀) for engineered LuxR-myc with a Tn insert in *lonA* in no added HSL, 3200 nM 3OC6-HSL, or 3200 nM C8-HSL. Luminescence was measured at an OD₆₀₀ of approximately 1, then whole-cell protein was extracted from the same cultures for Western blot. Bars represent the average of three experiments. Error bars represent standard error of the mean. (C) Representative Western blot from one of the three replicates. Blots were quantified using Bio-Rad Image Lab software, normalized to total lane protein, then scaled such that the ES114 LuxR-myc *lonA*::Tn ø HSL band equaled 100 before averaging across blots. Dotted line in (A) represents the threshold to be considered luminescent above background.

Half-life of LuxR variants

To further investigate whether there are differences in protein stability between the LuxR variants, we assessed the half-life of LuxR-myc. The engineered *luxR* promoter contains two lac operators (Chapter 2) and we made it inducible with isopropyl β -D-1-thiogalactopyranoside (IPTG) by introducing *lacI^q* elsewhere on the chromosome. With this system, we can grow the LuxR-myc variants in the presence of IPTG to stimulate *luxR* transcription, then wash away the IPTG (thus turning off *luxR* transcription) and measure how LuxR-myc levels change over time. Similar control of expression was used previously to measure the half-life of GFP variants in *V. fischeri* (171). In a series of Western blots, we measured LuxR-myc for each LuxR variant immediately after washing away IPTG and then over time, up to 24 hours, calculating what percentage of the original LuxR remained over the first six hours while cells were actively growing (Fig. 3.3A and B). By fitting these data to exponential decay curves, we approximated half-life values for LuxR-myc^{ES114}, LuxR-myc^{H905}, and LuxR-myc^{MJ1} with no added HSL, 3OC6-HSL, and C8-HSL (Fig. 3.3C). Because LuxR-myc^{MJ11} protein levels are too low to measure degradation with any certainty (Fig. 3.1C), it was excluded from this analysis.

We found that LuxR-myc^{H905} degrades more slowly when 3OC6- or C8-HSL is present, with a half-life approximately three times longer in the presence of 3OC6-HSL (Fig. 3.3B and C). The remaining LuxR-HSL combinations have much more similar degradation rates, however, the predicted half-life of LuxR-myc^{ES114} with added 3OC6-HSL was significantly longer than when C8-HSL or no HSL is present ($P < 0.05$), indicating the 3OC6-LuxR complex was more stable. LuxR-myc^{MJ1} similarly appears to have a slightly longer half-life when in the presence of 3OC6-HSL, but the difference was not statistically significant (Fig. 3.3B and C).

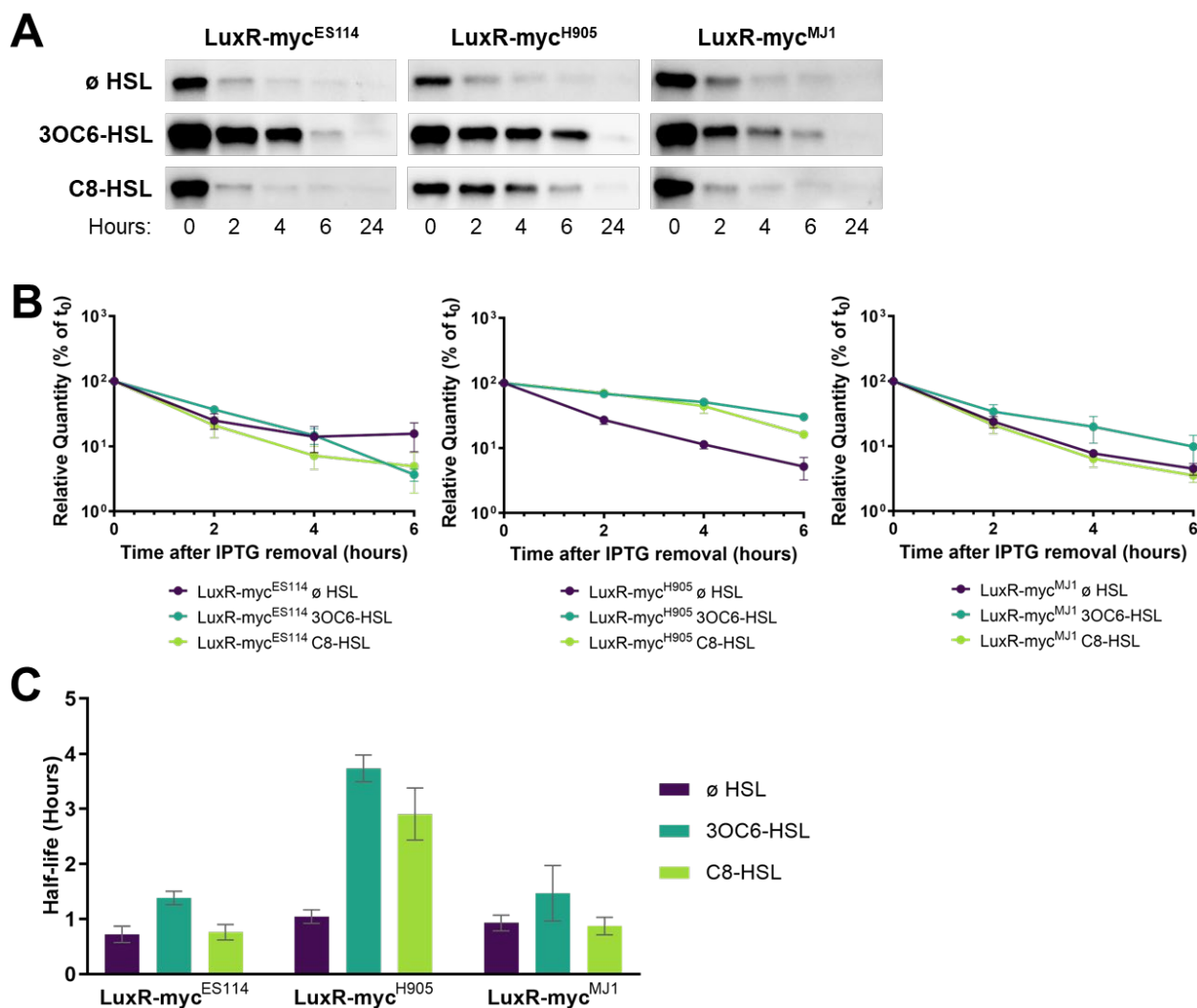


Figure 3.3. LuxR protein degradation. (A) Representative Western blots from one of three replicates for each condition. (B) Quantified Western blot data plotted as the percentage of protein remaining as compared to time zero (t_0) for each condition versus the time after IPTG was removed from the culture ($\log^{10}$ Y-axis). (C) Half-life was predicted for LuxR in each condition by fitting exponential decay curves to the data in from B. Error bars represent standard error of the mean.

Variation in *luxR* mRNA levels for different *luxR* alleles

Given the differences in LuxR protein levels observed for different *luxR* alleles, we tested if *luxR* mRNA levels varied similarly. Although each *luxR* is transcribed from the same constitutive promoter, we observed differences in *luxR* mRNA that largely paralleled quantification of LuxR protein; the mRNA from *luxR-myc^{H905}* was most abundant, followed by

luxR-myc^{ES114}, *luxR-myc^{MJ1}*, and *luxR-myc^{MJ11}*, respectively (Fig. 3.4). As with *LuxR-myc^{MJ11}*, *luxR-myc^{MJ11}* mRNA was near the limit of detection. We unexpectedly observed more *luxR-myc^{H905}* transcript present with 3OC6-HSL ($P < 0.05$) (Fig. 3.4). We hypothesized that the sequence differences could lead to different mRNA structures with varied stability, though this would not explain why 3OC6-HSL would affect *luxR-myc^{H905}* transcript levels.

Predicted stability of *luxR* mRNA 5' secondary structures correlate with LuxR and Luminescence

Differences in transcript secondary structure at the 5' end, particularly the 5' untranslated region (UTR) through the first eleven codons of the coding sequence (CDS), can affect the rate of translation initiation, thereby affecting how much protein is produced (172-177). Lower rates of translation can in turn impact mRNA levels, because actively translated mRNA is protected from

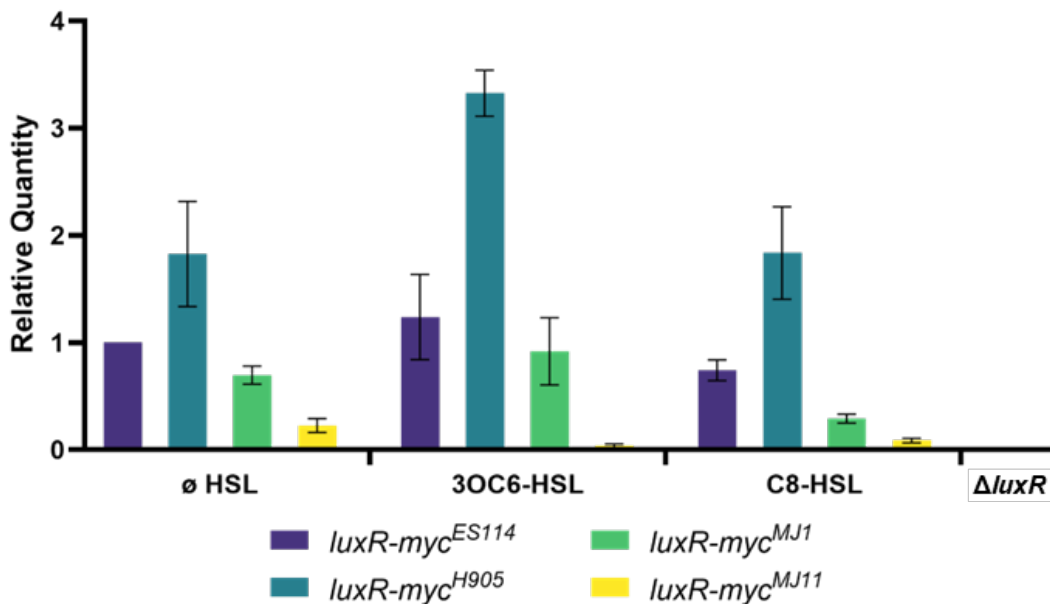


Figure 3.4. Relative Quantity of *luxR* mRNA. RNA was extracted from *LuxR-myc* variant strains after growth to an OD₆₀₀ of approximately 1 with either no added HSL, 3200 nM 3OC6-HSL, or 3200 nM C8-HSL. RT-PCR was completed and the quantity of *luxR* transcript was calculated relative to *lpxA*. Data was normalized to *LuxR-myc^{ES114}* with no HSL equal to 1. Error bars represent standard error of the mean.

RNase activity by the ribosome (177-180). Predicting the secondary structure of the entire mRNA transcript for each *luxR* variant did not yield any significant differences in stability (data not shown) however there were differences when considering just the 5' end of the transcript sequence. We used the Turner 2004 energy model to predict the minimum free energies (MFE) of each *luxR* variant from the transcriptional start of the engineered *luxR* promoter through the first eleven codons of *luxR* (Fig. 3.5D, “engineered +1”) and plotted those values against the Luminescence/OD₆₀₀ data (Fig. 3.5A). We found a negative exponential correlation between the predicted stability of secondary structures in the 5' end of *luxR* transcripts and the luminescence output of the corresponding strain (Fig. 3.5A). More stable secondary structure in the 5' end of transcripts corresponded to lower luminescence. Similar trends were observed with Western blotting and RT-PCR data, which showed higher protein and mRNA levels as the predicted stability of secondary structure in 5' transcript decreased (Fig. 3.5 B and C).

To determine if the differences in 5' transcript MFE was an artifact of the engineered *luxR* promoter or if these differences could also be observed in the wild-type strains, we again used the Turner 2004 energy model to predict the MFE's of each *luxR* variant and their native promoters. The exact transcriptional start site for *luxR* is unknown, but Shadel and Baldwin (166) identified three possible +1 sites for *luxR*^{MJI}, and we modeled mRNA for each of these three possibilities. The MFE values we predicted for these possible native *luxR* transcripts were lower than those of the engineered promoter due to the shorter length of these transcripts, but they followed a similar pattern to what we observed with the engineered promoter (Fig. 3.5D). The MFEs always order $luxR^{MJ11} \leq luxR^{MJ1} < luxR^{ES114} < luxR^{H905}$, from most to least stable, indicating that the differences in predicted 5' transcript secondary structure are not exclusively an artifact of the engineered promoter in our variant strains.

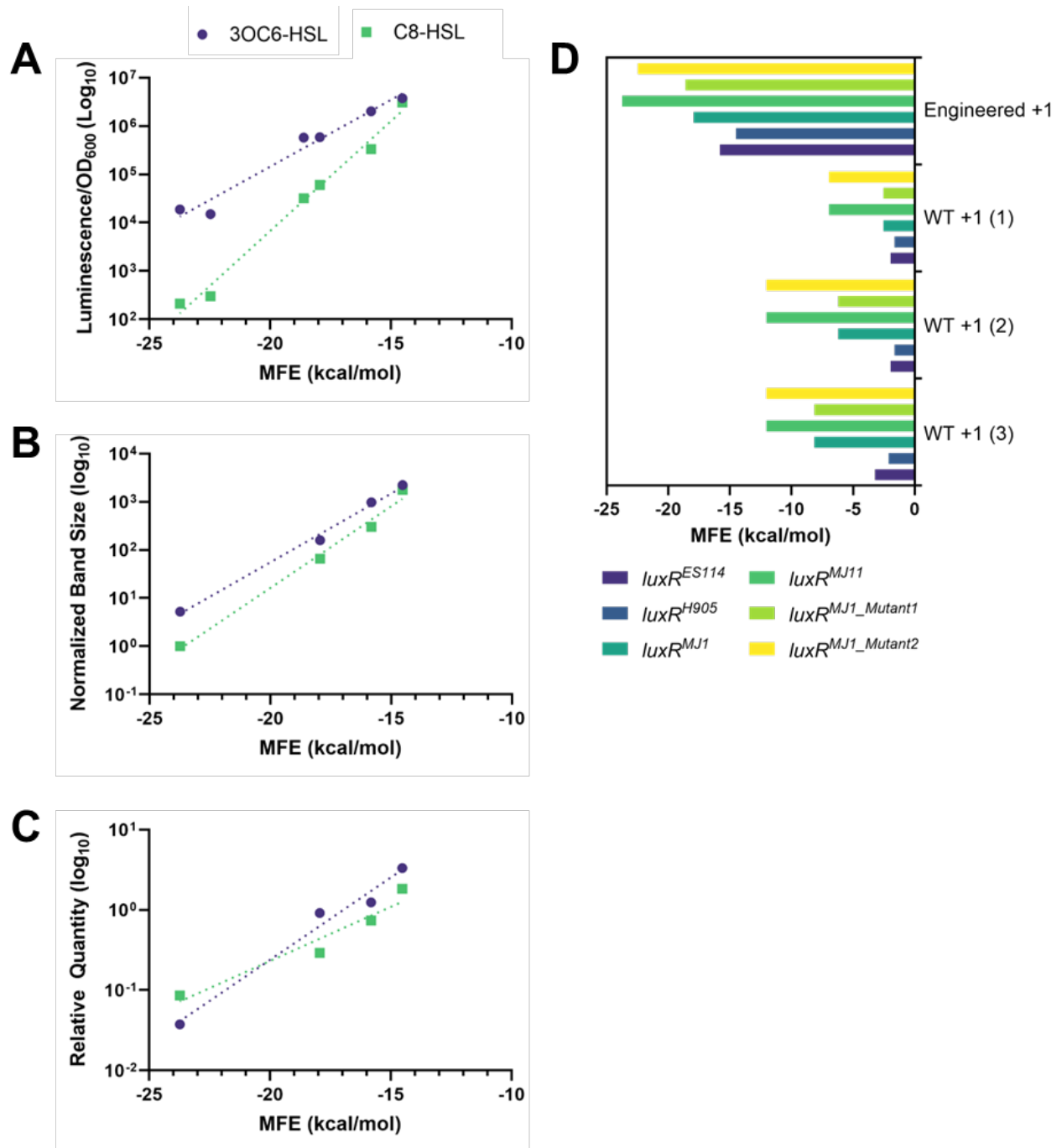


Figure 3.5. Minimum free energy of RNA secondary structure for +1 through first 11 codons of *luxR* sequences. Turner 2004 energy model minimum free energy (MFE) was calculated for each engineered *luxR* 5' transcript and plotted against its (A) Maximum Luminescence/OD₆₀₀, (B) Normalized Band Size from Western blotting, and (C) Relative Quantity of mRNA from RT-PCR. (D) Bar graph of the *luxR* variant MFE's using either the engineered promoter +1, or each *luxR*'s native promoter starting from three possible +1 locations.

5' end transcript stability could explain the phenotypic differences between LuxR^{MJ1} and $\text{LuxR}^{\text{MJ11}}$, caused by a seemingly small sequence variation (Fig. 3.6A). There are three differences between these *luxR* sequences: a six base-pair insertion after the start codon of $\text{luxR}^{\text{MJ11}}$ that encodes two more amino acids than luxR^{MJ1} , a nonsynonymous point mutation in $\text{luxR}^{\text{MJ11}}$ that encodes an asparagine rather than an aspartic acid like luxR^{MJ1} , and a synonymous mutation of an ACA in luxR^{MJ1} to an ACT in $\text{luxR}^{\text{MJ11}}$. To further examine the differences between these LuxRs, we engineered two luxR^{MJ1} mutants that have sequences in-between luxR^{MJ1} and $\text{luxR}^{\text{MJ11}}$; one carrying the six-bp insertion (Mutant 1), and the other with the nonsynonymous point mutation that changes asparagine to aspartic acid (Mutant 2) (Fig. 3.6A). We measured luminescence/OD₆₀₀

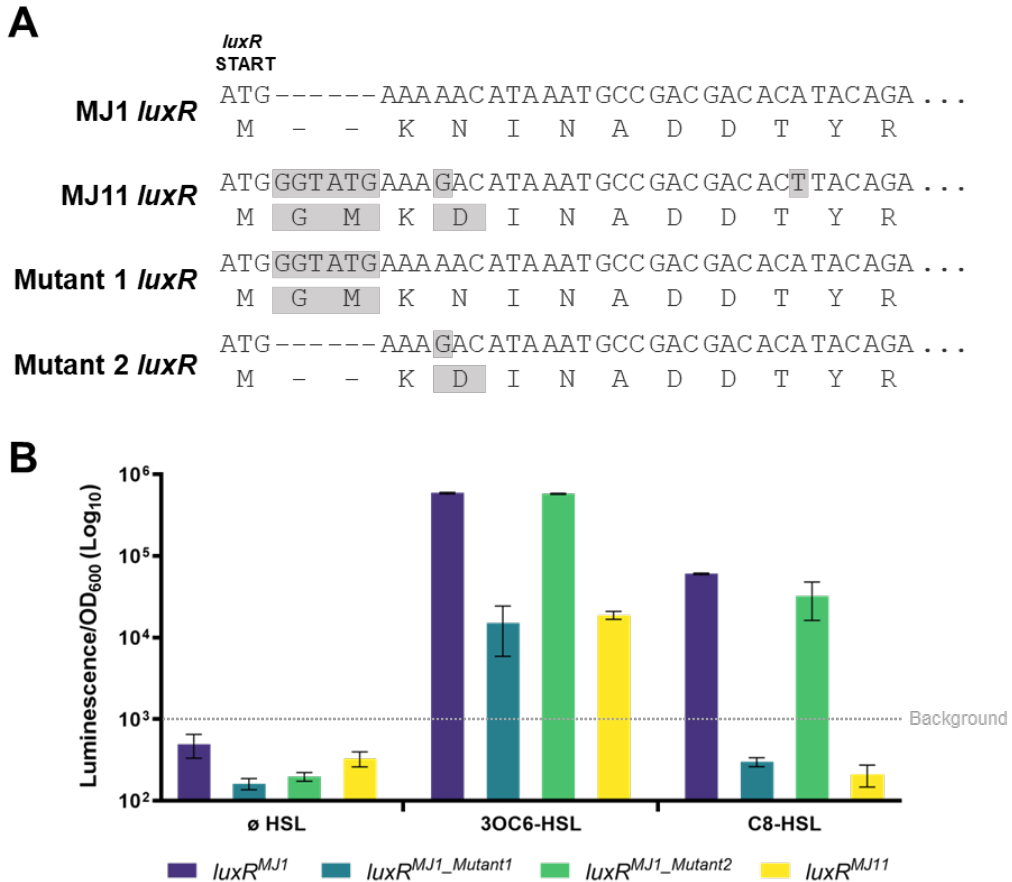


Figure 3.6. LuxR^{MJ1} versus $\text{LuxR}^{\text{MJ11}}$. (A) Sequence alignment of the first 11 codons of luxR^{MJ1} , $\text{luxR}^{\text{MJ11}}$, $\text{luxR}^{\text{MJ1_Mutant1}}$, and $\text{luxR}^{\text{MJ1_Mutant2}}$ with their translations. Differences from luxR^{MJ1} are highlighted grey. (B) Maximum Luminescence/OD₆₀₀ (log₁₀) for MJ LuxR-myc variants with no added HSL, 3200 nM 3OC6-HSL, or 3200 nM C8-HSL.

for these two mutants and found that the majority of the phenotypic difference was caused by the change present in $\text{LuxR}^{\text{MJ1_Mutant1}}$, which had a similar 3OC6- and C8- response to $\text{LuxR}^{\text{MJ11}}$ (Fig. 3.6B). $\text{LuxR}^{\text{MJ1_Mutant2}}$ had a slightly attenuated C8-response compared to LuxR^{MJ1} but responded equally well to 3OC6-HSL (Fig. 3.6B). These mutant LuxRs also had predicted MFE values in-between LuxR^{MJ1} and $\text{LuxR}^{\text{MJ11}}$ (Fig. 3.5D), which fit in with the exponential correlation in Figure 3.5A, further supporting our hypothesis that 5' end transcript stability affects the LuxR phenotype.

LuxR protein levels does not explain all the phenotypic differences between the LuxRs

Though it appears that protein and 5' end transcript stability help explain the differences in overall brightness that we observed, they do not explain the differences in HSL selectivity observed between the LuxR variants. For example, $\text{LuxR}^{\text{H905}}$ responds to 3OC6- and C8-HSL much more similarly than the other variants we tested. At an OD_{600} of 1, $\text{LuxR}^{\text{ES114}}$ responds to 3OC6-HSL 63-fold more strongly than C8-HSL, while $\text{LuxR}^{\text{H905}}$ responds to 3OC6-HSL only 1.7-fold more strongly than C8-HSL (Fig. 3.1A). This effect is exaggerated because these two responses reach peak brightness at different OD_{600} values in $\text{LuxR}^{\text{ES114}}$ (see Chapter 2 for details), but even when comparing maximum luminescence/ OD_{600} responses for 3OC6- and C8-HSL regardless of OD_{600} , $\text{LuxR}^{\text{ES114}}$ responds to 3OC6-HSL about 5-fold more strongly than C8-HSL, while the 3OC6-HSL response remains only about 2-fold stronger for $\text{LuxR}^{\text{H905}}$. To determine if this ratio can be affected by LuxR expression, we used the IPTG inducible LuxR variant strains to change the transcription level of $\text{luxR}^{\text{H905}}$ and see if the differences between the two HSL responses change. When induced with 15.6 μM IPTG, $\text{LuxR}^{\text{H905}}$ levels were similar to or lower than the levels of $\text{LuxR}^{\text{ES114}}$ induced with 1000 μM IPTG (data not shown), so we tested $\text{LuxR}^{\text{H905}}$ with 1000, 125, and 15.6 μM IPTG to encompass a range of $\text{LuxR}^{\text{H905}}$ levels from its natural transcription down to $\text{LuxR}^{\text{ES114}}$ transcription levels. We found that though the overall brightness

of each LuxR^{H905} response decreased the difference between the 3OC6- and C8-HSL responses did not change significantly with lowered IPTG concentration (Fig. 3.7). The LuxR^{H905} 3OC6-HSL response remained about twice as strong as C8-HSL, indicating that the difference in HSL selectivity between LuxR^{ES114} and LuxR^{H905} is caused by something other than their different protein/mRNA levels, such as protein structure/function.

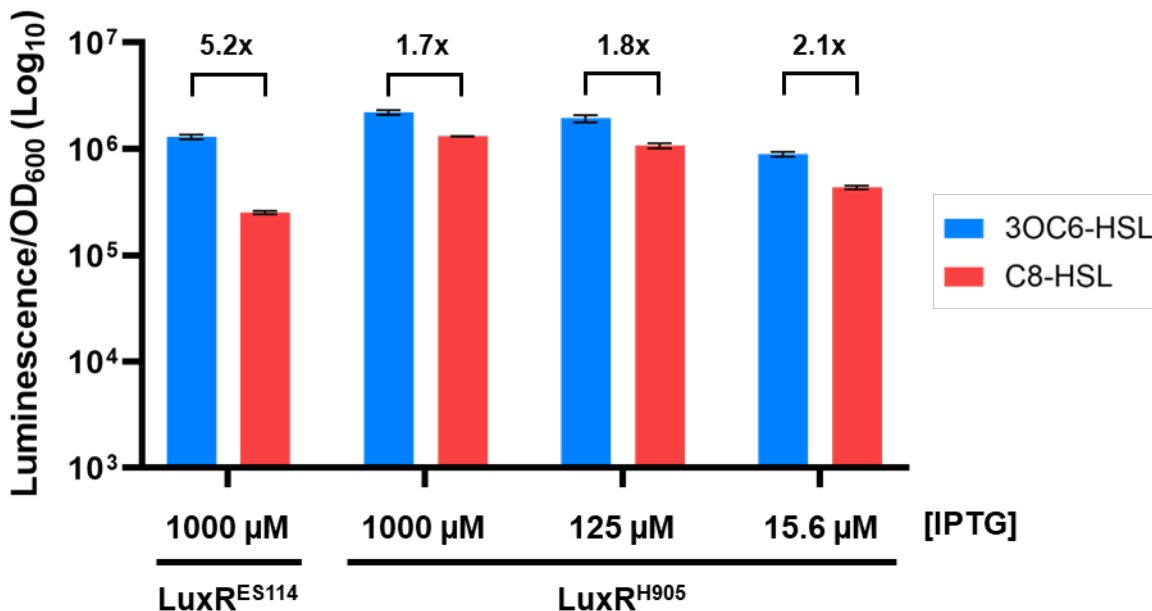


Figure 3.7. 3OC6 response vs C8 response for LuxR^{ES114} and LuxR^{H905}. Luminescence/OD₆₀₀ was measured for LuxR with added 3OC6- and C8-HSL at varying IPTG concentrations. For each condition, the maximum luminescence/OD₆₀₀ of the 3OC6 response was divided by the maximum luminescence/OD₆₀₀ of the C8 response to calculate the fold-difference, which is noted above each pair of bars. Error bars indicate standard error of the mean for three experiments.

Discussion

Our previous work showed that *luxR* alleles taken from different strains of *V. fischeri* underpin different responses to 3OC6- and C8-HSL in an engineered and otherwise isogenic background (Chapter 2). In this study we determined that these engineered strains also produce different amounts of LuxR protein and mRNA transcript, despite differing only in the *luxR* coding sequences. In a wild-type background, LuxR expression is regulated by a variety of activators and repressors including LitR, CRP, ArcA, and potentially autoregulation by LuxR itself (1, 55, 62,

64, 166, 167). However, the binding sites for these regulators are absent in our engineered background, and a constitutive (or IPTG-controlled) promoter controls *luxR* transcription (see Fig. 2.4 in Chapter 2). We expected that *luxR* mRNA levels would be the same for the variants and that LuxR concentration likely would be as well; however, we detected surprising variation in both. The determination that the LuxR variant strains were, in fact, producing different amounts of LuxR protein and *luxR* mRNA prompted us to investigate how the *luxR* sequences caused these effects.

Our results suggest that a major contributor to the discrepancies in *luxR* transcript and protein levels between the *luxR* variants is the secondary structure of the 5' transcript end. When we predicted the mRNA secondary structure of the engineered 5' UTR through the first eleven codons of the *luxR* CDS and calculated the MFE for these structures, we found the differences between these values had a negative correlation with luminescence, LuxR protein, and *luxR* mRNA levels for each variant strain (Fig. 3.5A-C). *luxR* variants with 5' transcript ends that formed more stable secondary structures had weaker luminescence responses and lower mRNA and protein levels. These effects may be the result of restricted ability of ribosomes to initiate translation, for example if the ribosome binding site (RBS) is within the secondary structure of the transcript. There is ample evidence that the stability of RNA structure near the RBS is a major determinant of translation-initiation efficiency (172-177). Highly expressed genes tend to have weaker RNA folding around the RBS, allowing for more efficient ribosome binding. Actively translating ribosomes protect mRNA from endonucleolytic RNases, so poorly translated transcripts not only produce less protein, but these transcripts are also degraded more quickly (177-180).

Although we examined *luxR* alleles in a background with a non-native promoter, including an artificial 5' UTR, the difference of predicted 5' transcript-end stability was not simply an artifact of the engineered 5' UTR. When we performed the same mRNA analysis using predicted

transcriptional starts for the native *luxR* promoters, we found a similar pattern of MFE values for the different *luxR* alleles relative to each other (Fig. 3.5D). This observation suggests that the 5' *luxR* sequence could also be influencing translation efficiency in the wild-type strains, a phenomenon that has never been explored.

The importance of the 5' CDS in translation initiation provides an explanation for why two of our *luxR* variants, *luxR^{MJI}* and *luxR^{MJI1}*, had such different phenotypes despite so few differences between their sequences. The two *luxR* alleles we designed to be intermediates between the *luxR^{MJI}* and *luxR^{MJI1}* sequences (Mutant 1 and Mutant 2) likewise showed intermediate phenotypes and MFE values, suggesting that 5' transcript structure is affected by the differences between *luxR^{MJI}* and *luxR^{MJI1}* sequences enough to elicit large differences in LuxR and luminescence output. The large difference in luminescence between strains carrying these naturally evolved *luxR* variants was perplexing when only considering the effects of the LuxR structure, because the sequence differences were not in any predicted functional domain. It now seems likely the root cause was related to mRNA folding dynamics, which serves as an important reminder that the specific sequence and codon usage, especially at the 5' end, is important to consider when comparing homologs and engineering new proteins. The various ways in which codon usage and nonsynonymous mutations can affect expression is often overlooked but can have drastic consequences. One study in *E. coli* created a variety of nonsynonymous GFP mutations and found that, despite encoding the same protein, there were up to 250-fold differences in expression between them, largely due to changes close to the start codon (173). It is possible that cells use simple changes to the 5' transcript as a method of evolving different translational regulation without effecting protein function or requiring large-scale changes to regulators, and that could be what we are observing in the differences between *luxR^{MJI}* and *luxR^{MJI1}* (175).

Our data also indicate that the presence of a cognate-signal HSL has an impact on LuxR levels. LuxR-myc^{ES114} degrades more slowly when 3OC6-HSL is present, and LuxR-myc^{H905} degrades more slowly when either 3OC6- or C8-HSL are added (Fig. 3.3), leading to increased levels of LuxR in the presence of these cognate signals (Fig. 3.1 and 3.2). The well-studied LuxR homolog TraR from *Agrobacterium tumefaciens* changes conformation when bound to HSL, allowing not only for dimerization and DNA binding, but also providing protection from proteases (107). Previous studies have predicted that LuxR-HSL binding results in a similar conformation change (69, 136, 181), and we hypothesize that this is what is allowed the LuxR to remain more stable with HSL present. Although LonA (also “Lon”) is reported to be a LuxR protease LonA did not affect the relative pattern of differences between the LuxR variants (Fig. 3.1 and 3.2). This observation could be evidence that LonA is not responsible for the majority of LuxR turnover or that LonA activity does not distinguish between LuxR with and without HSL (168-170). TraR is primarily degraded by Clp and Lon, and previous studies have found there are not noticeable differences in TraR levels until genes for both proteases are deleted (107). It would be interesting to see if the same is true for LuxR, and future studies aimed at understanding LuxR degradation should consider whether a combination of protease deletions can change how much HSL impacts LuxR protein levels.

Though the levels of LuxR for variants of this protein largely correlate with overall luminescence output in the strains expressing them, LuxR levels alone do not entirely explain the differences in HSL specificity we observed for the LuxR variants. For example, we show that LuxR^{H905} responds much more evenly to 3OC6- and C8-HSLs than LuxR^{ES114} and this response ratio is not dependent on expression (Fig. 3.7). It appears that HSL specificity is still best explained by functional differences in the resulting LuxR protein structures. Other phenotypes, such as the

HSL independence of LuxR^{H905} examined in Chapter 2, are also better explained by protein function rather than by expression levels. In fact, LuxR-myc^{ES114} and LuxR-myc^{H905} protein levels are similar in the absence of HSL (Fig. 3.1B and C) yet LuxR-myc^{H905} is luminescent above background while LuxR-myc^{ES114} is not (Fig. 3.1A). Taken together, the observations in this study do not overwrite the results from Chapter 2 but instead show that there are multiple factors at work to create the diverse phenotypes of the *luxR* variants.

Many studies have been done on LuxR that introduce changes to the sequence and consider how these changes affect protein function, but the impact on transcription or translation efficiency is rarely (if ever) considered. Ignoring this important factor could be disadvantageous to the expansion of our knowledge of *luxR* function. It seems that *luxR* is evolving autoregulatory function into its own coding sequence that was previously overlooked and could be important to consider in future experiments regarding LuxR-type regulators. This is especially important for those who wish to use LuxR as an engineered biosensor; it would be prudent to use a *luxR* sequence with an unstable 5' mRNA structure if you wanted to ensure high levels of expression. We also do not know much about *luxR* transcription or translation, other than which regulators affect its expression in wild-type backgrounds (1, 55, 62, 64, 166, 167). Expression studies in the LuxI/LuxR system typically focus on the LuxR regulon rather than LuxR itself (42, 46-48, 68, 77, 133, 135, 182). Early studies investigating *luxR* autoregulation and CRP's role in *luxR* activation estimated regions potentially important for *luxR* expression (64, 67, 166, 167), however, research on this topic has stalled and the *luxR* transcriptional start has never been expressly defined. It would be advantageous for future studies to define the *luxR* transcript as well as the position of the RBS if we wish to continue engineering *luxR* and its homologs.

Table 3.1. Strains and plasmids used in this study.

Name	Relevant Characteristics	Reference
<i>E. coli</i>		
CC118λpir	Δ(<i>ara-leu</i>) <i>araD</i> Δ <i>lac74 galE galK phoA20 thi-1 rpsE rpsB argE</i> (Am) <i>recA λpir</i>	(165)
DH5α	φ80 <i>dlacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) U169 <i>deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	(164)
<i>V. fischeri</i>		
ES114	Wild type	(145)
DC36	ES114 Δ <i>ainS lacI^q</i> P _{A1/34} - <i>luxCDABEG</i>	(60)
KB14	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{ES114} C-myc, P _{luxI} - <i>luxCDABEG</i>	This study
KB15	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> Δ <i>luxR::kan^R</i> , P _{luxI} - <i>luxCDABEG</i>	(Ch. 2)
KB18	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1} , P _{luxI} - <i>luxCDABEG</i>	(Ch. 2)
KB25	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ11} , P _{luxI} - <i>luxCDABEG</i>	(Ch. 2)
KB36	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1_Mutant2} , P _{luxI} - <i>luxCDABEG</i>	This study
KB37	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1_Mutant1} , P _{luxI} - <i>luxCDABEG</i>	This study
KB38	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{ES114} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>lonA::mini-Tn5-erm^R</i>	This study
KB47	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1} C-myc, P _{luxI} - <i>luxCDABEG</i>	This study
KB48	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{H905} C-myc, P _{luxI} - <i>luxCDABEG</i>	This study
KB49	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ11} C-myc, P _{luxI} - <i>luxCDABEG</i>	This study
KB74	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{ES114} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>lacI^q</i>	This study
KB75	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>lacI^q</i>	This study
KB76	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{H905} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>lacI^q</i>	This study
KB85	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>lonA::mini-Tn5-erm^R</i>	This study
KB86	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{H905} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>lonA::mini-Tn5-erm^R</i>	This study
KB87	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ11} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>lonA::mini-Tn5-erm^R</i>	This study
SLV39	ES114 <i>lonA::mini-Tn5-erm^R</i>	(170)
Plasmids		
pCR-BluntII-TOPO	PCR product cloning vector; ColE1, <i>kanR</i>	Invitrogen
pEV5104	Conjugative helper plasmid; R6Kγ, <i>oriTRP4</i> , <i>kan^R</i>	(183)
pJMO14	<i>lacI^q</i> , <i>cm^R</i>	(184)
pKAB34	Δ <i>luxR-luxI</i> , <i>luxR</i> ^{ES114} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>cm^R</i>	This study
pKAB36	Δ <i>luxR::kan^R</i> , P _{luxI} - <i>luxCDABEG</i> , <i>cm^R</i>	(Ch. 2)
pKAB65	Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1_Mutant2} , P _{luxI} - <i>luxCDABEG</i> , <i>cm^R</i>	This study
pKAB66	Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1_Mutant1} , P _{luxI} - <i>luxCDABEG</i> , <i>cm^R</i>	This study
pKAB73	Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ1} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>cm^R</i>	This study
pKAB74	Δ <i>luxR-luxI</i> , <i>luxR</i> ^{H905} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>cm^R</i>	This study
pKAB75	Δ <i>luxR-luxI</i> , <i>luxR</i> ^{MJ11} C-myc, P _{luxI} - <i>luxCDABEG</i> , <i>cm^R</i>	This study
pLostfoX	<i>tfoX^{VF}</i> , <i>cm^R</i>	(185)

Materials and Methods

Strains and Media. The strains used in this study are listed in Table 3.1. All parent strains used in this study were derived from wild-type *Vibrio fischeri* ES114. *Escherichia coli* strain DH5 α was used for cloning. *V. fischeri* cultures were grown in either LBS medium (162) at 28°C or SWTO medium (1) at 24°C with final antibiotic concentrations of 2 μ g/mL for chloramphenicol, 5 μ g/mL for erythromycin, and 100 μ g/mL for kanamycin, as needed. *E. coli* cultures were grown in lysogeny broth (LB) medium (163) at 37°C with final concentrations of 20 μ g/mL for chloramphenicol and 40 μ g/mL for kanamycin, as needed. HSLs were obtained from Sigma-

Table 3.2. Oligonucleotides used in this study.

Oligo	Sequence*	Use
KAB26	CTAGCTCGAGATACACTTAATTTTAAAGGTAT	<i>luxR^{ES114}</i> cloning primer (Rev)
KAB27	CTAGCATATGAACATTAAAAATATAAATGCTAA	<i>luxR^{ES114}</i> cloning primer (Fwd)
KAB36	CTAGCATATGAAAAACATAAATGCCGACGACA	<i>luxR^{MJ1}</i> cloning primer (Fwd)
KAB37	CTAGCTCGAGTTAATTTTAAAGTATGGGCAATCA	<i>luxR^{MJ1}</i> & <i>luxR^{MJ11}</i> cloning primer (Rev)
KAB41	CTAGCATATGAATACTAAAAACATAAATATTAATGA	<i>luxR^{H905}</i> cloning primer (Fwd)
KAB42	CTAGCTCGAGCTCATTTACTTAATTTTAAAGTAT	<i>luxR^{H905}</i> cloning primer (Rev)
KAB43	CTAGCATATGGGTATGAAAGACATAAATGCCG	<i>luxR^{MJ11}</i> cloning primer (Fwd)
KAB70	CTATGGGCCCTCAGAAGAACTCGTCAAGAAG	<i>kanR</i> cloning primer (Fwd)
KAB71	CTAGTGTACAATGATTGAACAAGATGGATTGCACG	<i>kanR</i> cloning primer (Rev)
KAB86	CTAGCATATGAAAGACATAAATGCCGACGAC	Primer used to make <i>luxR^{MJ1_Mutant2}</i> (Fwd)
KAB93	CCAGGAGGCATATGGGTATGAAAAACATAAATGCCGACGAC	Primer used to make <i>luxR^{MJ1_Mutant1}</i> (Fwd)
KAB103	GGCTGCAGTGAGCGTACTGT	<i>luxR</i> RT-PCR primer (Fwd)
KAB104	GCTTTAGAAATACTTTGGCAGCGGTT	<i>luxR</i> RT-PCR primer (Rev)
pr_NL94	CTGGTAATGTAACGATTGGCGAAGGTACGGAAGTG	<i>lpxA</i> RT-PCR primer (Fwd)
pr_NL95	CCGATAACAACTGTTGTTGCTTCACCACCATAT TTC	<i>lpxA</i> RT-PCR primer (Rev)

* Sequences in 5' to 3' orientation; underlined sequences indicate restriction-enzyme recognitions sites added to facilitate cloning; bold sequences indicate *luxR* start or stop codons in Fwd or Rev primers, respectively.

Aldrich and dissolved in acidified ethyl acetate (1:1000 glacial acetic acid: ethyl acetate) to create stock solutions. Each HSL-ethyl acetate solution was aliquoted into a sterile container and the solvent was evaporated before adding media. SWTO medium was added to the appropriate HSL concentration (e.g., 3200 μ M).

Genetic manipulations. Four of the *V. fischeri luxR* variants (ES114, H905, MJ1, and MJ11) from our previous study (Chapter 2) were tagged on their C-terminal end with a myc epitope (EQKLISEEDL) and engineered into a *V. fischeri* background originating from ES114 (Table 3.1). pKAB34 (*luxR*^{ES114}-myc) was conjugated into *V. fischeri* DC36 using *E. coli* CC118 λ pir with pEVS104 as a conjugative helper, to create KB14 by allelic exchange (162). To create a $\Delta luxR$ control and make future *luxR* engineering easier, we PCR amplified a *kan*^R cassette from the TOPO™ plasmid using primers KAB70 and KAB71. We then used NdeI and XhoI restriction sites to replace *luxR*^{ES114}-myc on pKAB34 with the *kan*^R cassette, creating pKAB36. Our $\Delta luxR$ control, KB15, was made by allelic exchange via conjugation of pKAB36 into DC36 (162). The rest of the *luxR*-myc sequences (with flanking NdeI and XhoI restriction sites) were ordered as gene blocks from IDT and cloned into pKAB36 (between NdeI and XhoI) in place of *kan*^R. The resulting plasmids (pKAB65, pKAB66, pKAB73, pKAB74, and pKAB75) were inserted into *V. fischeri* KB15 by allelic exchange via conjugation to make KB36, KB37, KB47, KB48, and KB49, respectively. The *lacI*^q strains were also made with allelic exchange via conjugation, inserting pJMO14 into KB14, KB47, and KB48 to make KB74, KB75, and KB76, respectively. SLV39 genomic DNA containing *lonA*::mini-Tn5-*erm*^R was transformed into KB14, KB47, KB48, and KB49 via pLostfoX natural transformation (185) to make KB38, KB85, KB86, and KB87, respectively. Tables 3.1 and 3.2 list key plasmids and oligonucleotides used to engineer mutant

strains and details of cloning intermediates are available on request. Each strain was verified by sequencing either the plasmid used for allelic exchange or a PCR fragment of the resultant genome.

Western and RT-PCR sample preparation. Strains were grown shaking overnight at 28°C in 5 mL LBS media. The next morning, subcultures were made in flasks of 20 mL SWTO media (with or without 3200 nM HSL added) and grown shaking at 24°C until an OD₆₀₀ of approximately 1. Luminescence was measured immediately following absorbance when applicable. At the appropriate OD₆₀₀, cells were pelleted by centrifugation, and the supernatant was removed. If the samples were for protein lysis (Western Blotting) the pellets were frozen at -80°C immediately. If the samples were for RNA extraction (RT-PCR), the pellets were first resuspended in 1 mL Invitrogen RNAlater™ Stabilization Solution before freezing. Cell pellets were stored at -80°C until further use.

LuxR Protein Degradation Assay. Strains were grown shaking overnight at 28°C in 5 mL LBS media. The next morning, subcultures were made in flasks of 20 mL SWTO media + 1000 µM IPTG (with or without 3200 nM HSL added) and grown shaking at 24°C. When they reached an OD₆₀₀ of approximately 1, each culture was pelleted and washed with SWTO to remove the IPTG, then resuspended in SWTO (with the same original HSL concentration) in a clean flask. Immediately after washing off the IPTG (0 hours), a sample of cells were pelleted, the supernatant was removed, and frozen at -80°C until further use. The sample flasks were left at room temperature and additional samples were removed, pelleted, and frozen after 2 hours, 4 hours, 6 hours, and 24 hours.

SDS-PAGE and Western Blotting. Cell pellets were removed from -80°C storage and defrosted at room temperature. Pellets were suspended in 100 µL B-PER™ Complete Bacterial Protein Extraction Reagent then incubated for 15 minutes at room temperature with gentle rocking. After

incubation, samples were centrifuged at 16000 x g for 20 minutes and the supernatant contained whole protein lysate. To measure the concentration of protein in the lysates we used the Thermo Scientific™ Pierce™ Rapid Gold BCA Protein Assay Kit.

Lysate and 2X Sample Buffer (4% SDS, 10% β-mercaptoethanol, 0.005% bromophenol blue, 20% glycerol, and 0.1 M Tris pH 7) were combined in a 1:1 ratio to a protein concentration of 2 µg/µL. 5 µL Precision Plus Protein™ WesternC™ Blotting Standard and 10 µL of each protein sample were run on 12% acrylamide SDS-PAGE gels (Bio-Rad Mini-PROTEAN TGX Stain Free Gels). Completed gels were imaged for total protein then transferred to nitrocellulose membranes. These blots were incubated with 1:1000 Anti-c-Myc Tag primary antibody then 1:10000 Anti-rabbit secondary antibody along with 1:10000 Anti-StrepTactin-HRP conjugate (for the ladder). Thermo Scientific™ SuperSignal™ West Pico PLUS Chemiluminescent Substrate kit was used to induce chemiluminescence. Gel and Western blots were imaged with the Bio-Rad ChemiDoc™ MP Imaging System and LuxR bands were quantified and normalized to total lane protein using Bio-Rad Image Lab software.

RT-PCR. Cells suspended in Invitrogen RNAlater™ Stabilization Solution were removed from -80°C storage and defrosted. Samples were centrifuged to remove RNAlater™ supernatant, and RNA was extracted from the now-pelleted samples using the Zymo Quick-RNA™ Miniprep Kit. Then reverse transcription was completed using the Quantabio qScript™ cDNA Synthesis Kit with 1 µg of RNA from each sample. RT-PCR was completed with 100 ng of cDNA in the Azure Cielo™ Real-Time PCR System using Quantabio PerfeCTa® SYBR® Green SuperMix Reaction Mix. (Primers KAB103 and KAB104 were used to amplify all *luxR* sequences while primers pr_NL94 and pr_NL95 were used to amplify *lpxA* as an internal control (Table 3.2). RT-PCR data analysis was done using Azure Cielo™ qPCR analysis software and Microsoft Excel.

Measuring maximum luminescence/OD₆₀₀ response. To measure the maximum luminescence/OD₆₀₀ across growth such as in Figures 3.6B and 3.7, we conducted experiments in black, clear-bottom 96-well plates in a Biotek Cytation 1 plate reader with a Peltier Cooling Module used to maintain a consistent temperature. Cells were inoculated into 200 μ L of SWTO media with appropriate HSL or IPTG concentrations added. Plates were grown in the reader at 24°C shaking and luminescence and OD₆₀₀ measurements were taken every 30 minutes for eighteen hours. Wells were filled with equivalent volume, but OD₆₀₀ was not pathlength-corrected to any standard for these kinetic experiments.

Sequence analysis. RNA sequence structure prediction was performed using the Vienna package RNAfold tool (186) through Geneious Prime® 2024.0.5 (<https://www.geneious.com>). The Turner 2004 energy model was used with temperature set to 24°C. The free energies of the optimal structures were reported in kcal/mol. The Clustal Omega alignment of 5' *luxR* sequences reported in Figure 3.6A was also completed in Geneious Prime® 2024.0.5.

Acknowledgements

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CHAPTER 4

GENETIC AND FUNCTIONAL DIVERGENCE OF *LUXR* IN CLOSE RELATIVES OF
VIBRIO FISCHERI ³

³ Kathryn A. Bellissimo and Eric V. Stabb. 2024. To be submitted to *Microbiology Spectrum*.

Abstract

Understanding the evolution of LuxR, the primary transcriptional activator responsible for bioluminescence in *Vibrio fischeri*, sheds light on the complex regulation of a pheromone-signaling system found throughout the Pseudomonadota (formerly Proteobacteria). In this study, we investigated the *luxR* sequences from *Vibrio logei* and *Vibrio salmonicida*, close relatives of *V. fischeri*, to better understand their genetic and functional divergence. *V. logei* and *V. salmonicida* each have two *luxR* genes at the *lux* locus, in contrast to the single *luxR* of *V. fischeri*. Through sequence analysis, we observed substantial differences between the *luxR* alleles and greater evolutionary divergence among *luxR* sequences than for most orthologs between the species. When introduced into an engineered *V. fischeri* background, neither *V. logei* nor *V. salmonicida* *luxR* variants could effectively induce the *V. fischeri* *lux* operon and luminesce. However, *V. salmonicida* LuxR2 exhibited modest activation when one of the *lux* box sequences from this species was exchanged for the *V. fischeri* *lux* box, highlighting species-specific regulatory adaptations. Additionally, analysis of *V. logei* and *V. salmonicida* LuxR proteins sequences revealed naturally evolved differences from *V. fischeri* that were previously identified in experimental evolution of *V. fischeri* LuxR, as well as extensive variation in predicted dimerization domains. Our findings underscore the interplay between *luxR* sequence variation and *lux* box recognition, providing insights into the co-evolution of LuxR and its regulatory elements in *Vibrio* species.

Importance

V. fischeri bioluminescence, which is regulated by the LuxI/LuxR pheromone signaling (PS) system, serves as a useful model of PS systems employed to regulate bacterial group behaviors. Here we compared the sequences and functional capacity of *V. fischeri luxR* along with *luxR* homologs *luxR1* and *luxR2* from *V. logei* and *V. salmonicida*, which despite significant sequence conservation cannot activate the *V. fischeri lux* operon. The lack of cross-species complementation appears to be due in part to species specificity in the *lux* box sequences on target promoters. Moreover, comparative sequence and functional analyses provide a useful dataset for predicting key residues defining activity of specific LuxR orthologs. Our findings emphasize the need for further research on the co-evolution of *luxR* and their *lux* box sequences, especially in species with multiple *luxR* homologs.

Introduction

Vibrio fischeri is a Gram-negative, bioluminescent bacterium that lives in light-organ symbioses with fish and squid, in host gut consortia, and outside hosts in the marine environment (12-14). *V. fischeri* bioluminescence is produced by the *lux* operon, *luxICDABEG*, which is regulated by the transcriptional activator LuxR (Fig. 4.1A) (37, 38, 130). Transcription of *luxR* is regulated by two pheromone signaling (PS) systems, and LuxR itself responds to pheromone signals. When bound to homoserine lactone (HSL) pheromone signals, LuxR binds to a sequence in the *lux* promoter known as the “*lux* box” and activates transcription of the *lux* operon (42). The first gene of the *lux* operon, *luxI*, is a HSL synthase that produces the cognate signal for LuxR, *N*-3-oxo-hexanoyl-L-homoserine lactone (3OC6-HSL), creating a positive-feedback loop (38, 41, 108, 131).

The psychrophilic luminescent symbiote *Vibrio logei* and cryptically bioluminescent fish pathogen *Vibrio salmonicida* are close relatives of *V. fischeri* that also contain *lux* operons regulated by PS (151, 187-189). However, the arrangement of their *lux* operons differ from that of

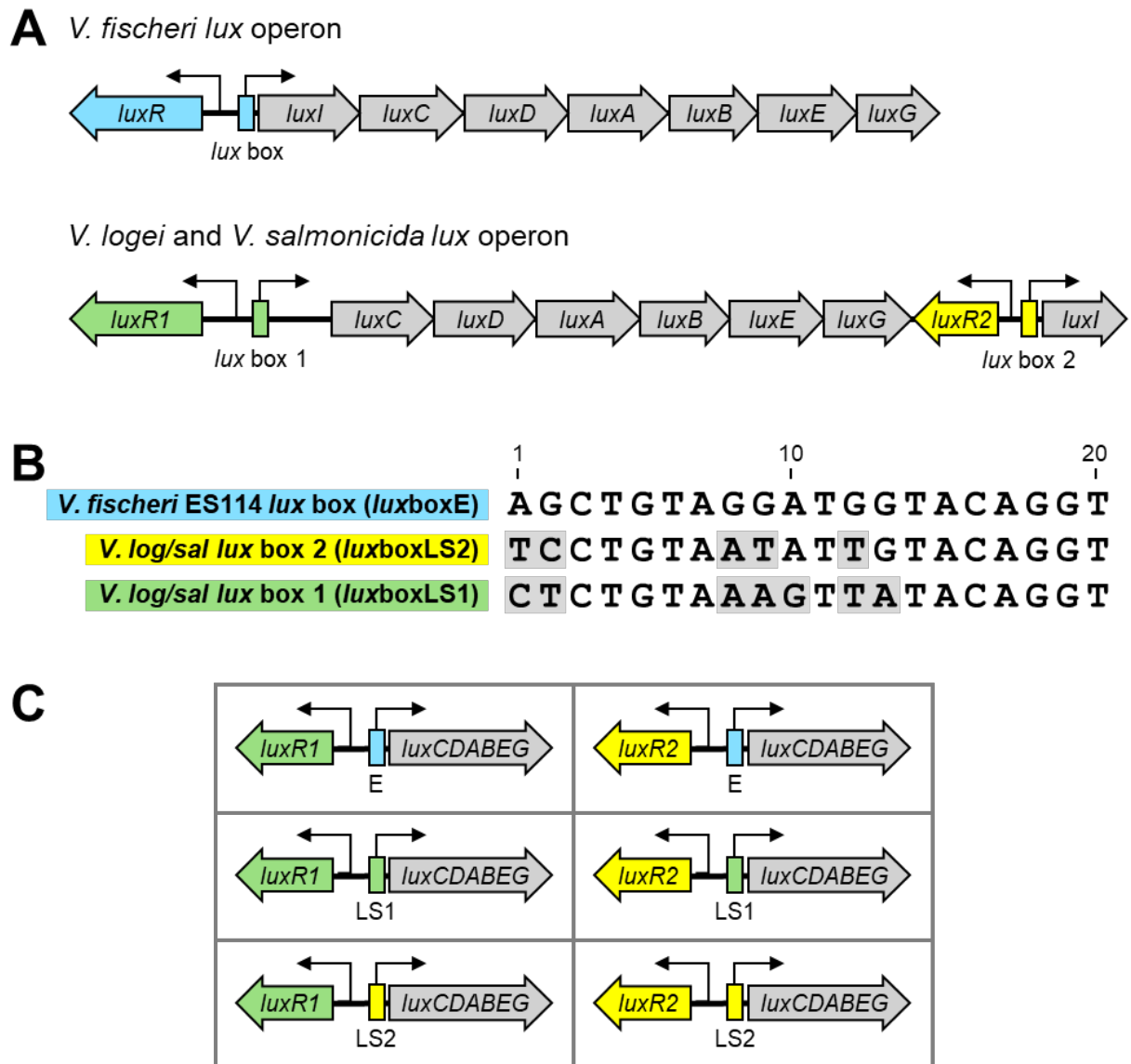


Figure 4.1. *lux* operon differences. (A) Organization of the *lux* operon genes in *V. fischeri* (top) and *V. logei* and *V. salmonicida* (bottom). (B) Alignment of the *V. fischeri* ES114 *lux* box with the two *lux* boxes found in *V. logei* and *V. salmonicida*. Grey highlighting indicates bases that are different from the *V. fischeri* ES114 *lux* box (*lux* box E) sequence. (C) Diagram of the six combinations of *luxR* and *lux* box engineered for this study. These combinations included *luxR1* and *luxR2* from both *V. logei* and *V. salmonicida*, making twelve engineered strains total represented by the six schematics in panel (C).

V. fischeri (190). In both *V. logei* and *V. salmonicida* genes *luxCDABEG* are under the control of a transcriptional promoter with one *lux* box and *luxI* is transcribed by a distinct promoter with its own *lux* box (Fig. 4.1A) (189-191). There are also two *luxR* genes, one located just upstream of the *lux* operon like in *V. fischeri* (*luxR1*), and the other between *luxG* and *luxI* (*luxR2*) (Fig. 4.1A) (189-191). Interestingly, *V. logei* and *V. fischeri* have been isolated from light organs of the same animals in Mediterranean squid populations, suggesting interspecies competition in mixed infections (151).

Each of these three *Vibrio* species also encode the AinS/AinR PS system, which is structurally distinct from LuxI/LuxR but produces a chemically similar signaling molecule, *N*-octanoyl-L-homoserine lactone (C8-HSL) (49-51, 133, 192-194). Though C8-HSL and the AinS/AinR systems work through a separate signal cascade to regulate *luxR* transcription, C8-HSL can also bind to LuxR like 3OC6-HSL and directly activate the *lux* operon (58-60). LuxR's ability to bind multiple signaling molecules creates crosstalk between the two HSL-based PS-systems, which may put unusual evolutionary pressure on LuxR to either select for or against signal specificity.

In our previous studies we found that even small sequence variations in *luxR* from different *V. fischeri* strains can significantly impact its function (Chapters 2 and 3). Here, we sought to examine how the larger differences in *luxR* between *V. fischeri*, *V. logei*, and *V. salmonicida*, could alter LuxR structure and function. The differences that have evolved in *V. logei* and *V. salmonicida* must not abolish LuxR function entirely, as they effectively control their *lux* operons in their native backgrounds (188, 190, 191, 193), but they could change aspects of LuxR HSL binding, DNA binding, or dimerization. To further expand our understanding of LuxR evolution, we compared the *luxR* sequences from *V. logei* and *V. salmonicida* to those of their *V. fischeri* relatives

then introduced these *luxR* sequences to an engineered *V. fischeri* background and measured how they induce luminescence of the *lux* operon in response to HSL signals.

Results

V. logei/salmonicida luxR1 and luxR2 form two groups distinct from V. fischeri luxR

To expand our understanding of *luxR* function and evolution, we examined *luxR* from *V. fischeri*'s close relatives *V. logei* and *V. salmonicida* to test how more divergent *luxR* sequences would affect *V. fischeri lux* operon activation. We first sequenced the *luxR1* and *luxR2* genes from *V. logei* SA6 (151) and *V. salmonicida* LFI1238 (187, 192) to confirm that the sequences of these strains matched those available on NCBI. We then aligned these four new *luxR* sequences with those of the seventeen available *V. fischeri luxR* sequences (see Chapter 2 Table 2.1 for accession numbers) and compared their relatedness (Fig. 4.2A). We also created a tree of concatenated housekeeping genes from seventeen *V. fischeri* strains along with *V. logei* SA6 and *V. salmonicida* LFI1238 to compare this phylogenetic analysis to the tree of their *luxR* sequences (Fig. 4.2B).

As observed previously (Chapter 2), the housekeeping genes of these strains are much more closely related than their *luxR* sequences (Fig. 4.2). Housekeeping genes of the *V. fischeri* strains are about 95% identical to one another and about 85% identical to *V. logei* or *V. salmonicida*, while *V. logei* and *V. salmonicida* are 87.3% identical to each other, and distances between strains on the tree shown in Figure 4.2B supports their designations in three related but distinct species. (Fig. 4.2B). In contrast, the *luxR* sequences from *V. logei* and *V. salmonicida* are much less similar to *V. fischeri luxR* sequences, at around 65% identity. Interestingly, the *luxR1* and *luxR2* sequences (Fig. 4.1A) are more closely related to their homolog from the other species than to the second

luxR locus in the same species (Fig. 4.2A). Moreover, the two *luxR* loci (*luxR1* and *luxR2*) in *V. logei* and *V. salmonicida* are more deeply branched apart from each other than are the most different *luxR* alleles within *V. fischeri*. The large sequence differences in *luxR* genes and much larger divergence of *luxR* than of housekeeping genes suggests possible functional variation, although the effects on function will naturally be determined by the location and nature of the differences.

Alignments of LuxR1, LuxR2, LuxR and TraR reveal regions of conservation and variation

We compared the four LuxR amino acid sequences from *V. logei* and *V. salmonicida* in an alignment with the well characterized *V. fischeri* LuxR^{MJ1} (59, 114, 115, 118, 119, 122, 123), and *Agrobacterium tumefaciens* TraR, which is a LuxR homolog with a defined crystal structure and known domains (2, 109). Unlike what we found when comparing different *V. fischeri* LuxR sequences in Chapter 2, there is much more variation spread throughout these LuxRs, however the three amino acids that TraR uses to hydrogen bond with its cognate HSL remain conserved in all four *V. logei* and *V. salmonicida* LuxR sequences (triangles below sequences, Fig. 4.3). We also compared these LuxR sequences to alleles recovered in directed evolution experiments performed with *V. fischeri* LuxR^{MJ1} and found several of the residues that induced phenotypes in those studies are naturally present in *V. logei* and *V. salmonicida* LuxR sequences (Table 4.1 and Fig. 4.3, daggers and red residues). Many of these residues fall within or near residues that align with TraR dimerization domains.

***V. logei* and *V. salmonicida* LuxRs cannot activate *V. fischeri* ES114 *lux* operon**

To determine how well the *V. logei* and *V. salmonicida* *luxR* sequences can activate luminescence compared to the *V. fischeri* *luxR* sequences tested previously in Chapter 2, we cloned the four *luxR* sequences into our engineered *V. fischeri* ES114 system. The engineered *V. fischeri*

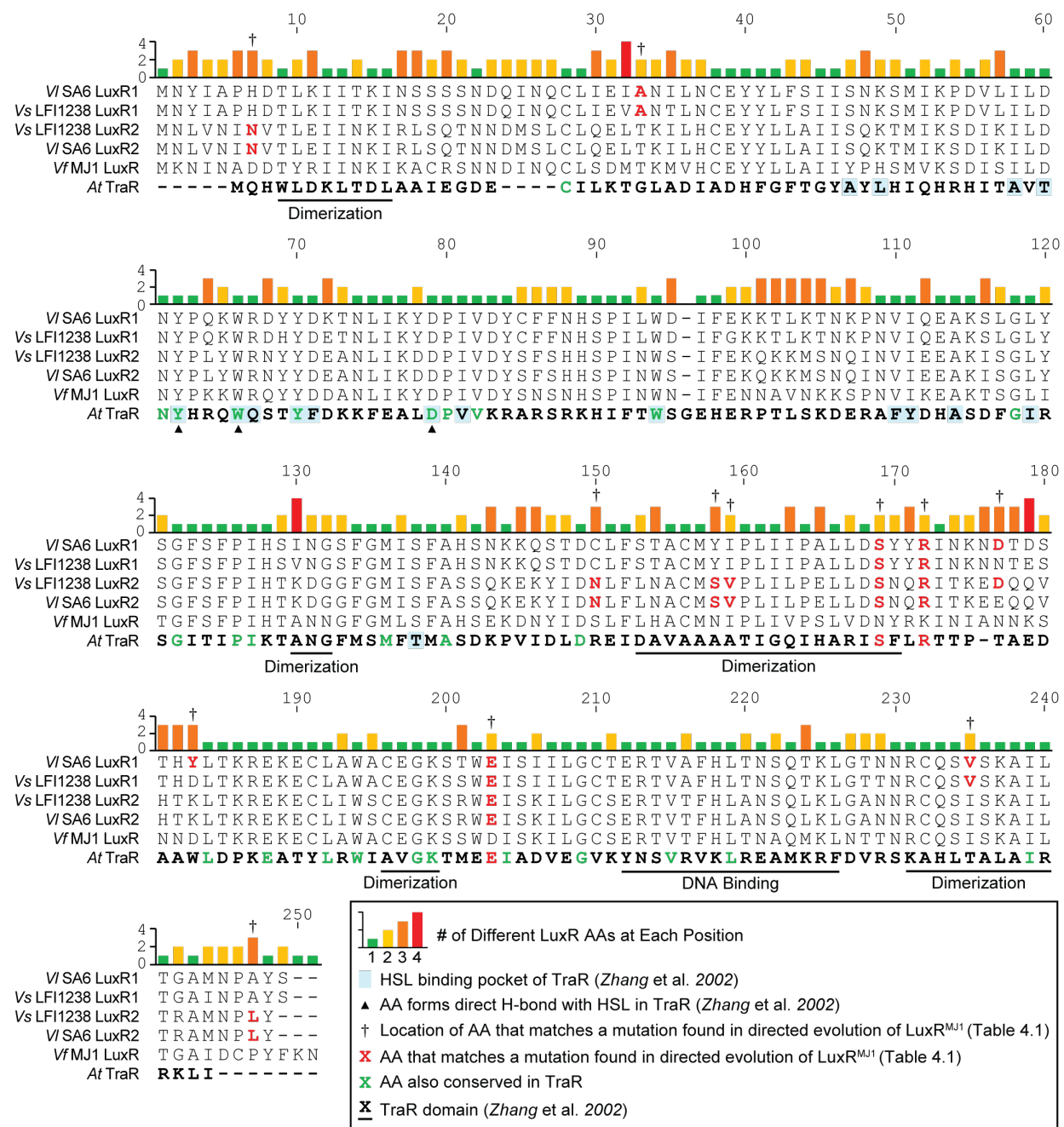


Table 4.1. Locations of mutations found via directed evolution of LuxR^{MJ1} in Figure 4.3.

AA substitution relative to <i>Vf</i> MJ1	Position Fig. 4.3	Description Summary	Wild-type strain(s) with this residue in LuxR
<i>Sitnikov et al. 1996 (115)</i>			
S149N	150	HSL signal-independent activity	<i>Vl</i> LuxR2, <i>Vs</i> LuxR2
P246L	247	HSL signal-independent activity	<i>Vl</i> LuxR2, <i>Vs</i> LuxR2
<i>Collins et al. 2005 (59)</i>			
T33A*	33	Increased response to C8-HSL, enhanced in combination with S116A and M135I	<i>Vl</i> LuxR1, <i>Vs</i> LuxR1
S116A*	117	Increased response to C8-HSL, enhanced in combination with T33A and M135I	none
M135I*	136	Increased response to C8-HSL, enhanced in combination with T33A and S116A. Moderately decreased response to 3OC6-HSL	none
<i>Collins et al. 2006 (114)</i>			
R67M	67	Selectivity against 3OC6 and 3OC12-HSL in T33A, S116A, M135I background	none
<i>Hawkins et al. 2007 (118)</i>			
I45F	45	Increased response to C4-HSL in T33A, S116A, M135I background	none
N157S	158	Increased response to C4-, C5-, and 3OC6-HSL in T33A, S116A, M135I, N168S background	<i>Vl</i> LuxR2, <i>Vs</i> LuxR2
I158V	159	Increased response to C4 and 3OC6-HSL in T33A, S116A, M135I, T115A background	<i>Vl</i> LuxR2, <i>Vs</i> LuxR2
N168S	169	Increased response to C4-, C5-, and 3OC6-HSL in T33A, S116A, M135I, I45F background	<i>Vl</i> LuxR1, <i>Vs</i> LuxR1, <i>Vl</i> LuxR2, <i>Vs</i> LuxR2
K171R	172	Increased response to C4-, C5-, and 3OC6-HSL in T33A, S116A, M135I, T115A background	<i>Vl</i> LuxR1, <i>Vs</i> LuxR1, <i>Vl</i> LuxR2, <i>Vs</i> LuxR2
N176D	177	Increased response to C4-, C5-, and 3OC6-HSL in T33A, S116A, M135I, I45F, T227A background	<i>Vl</i> LuxR1, <i>Vs</i> LuxR2
T227A	228	Increased response to C4-, C5-, and 3OC6-HSL in T33A, S116A, M135I, I45F, N176D background	none
<i>Tashiro et al. 2016 (119)</i>			
Y62H	62	Selectivity against C6, C8, and 3OC8-HSL, reduced sensitivity to 3OC6-HSL	none
D7N	7	Increased response to 3OC6-HSL in Y62H background in combination with N250S	<i>Vl</i> LuxR2, <i>Vs</i> LuxR2
T115A	116	Increased response to 3OC6-HSL in Y62H background	none
N250S	251	Increased response to 3OC6-HSL in Y62H background in combination with D7N	none
<i>Lu et al. 2016 (123)</i>			
D182Y	183	Activates mutant <i>lux</i> box A**	<i>Vl</i> LuxR1
D202E	203	Stronger activation of WT <i>lux</i> box and mutant <i>lux</i> box A** in I234V, D244H background. 2x as strong as D244H alone.	<i>Vl</i> LuxR1, <i>Vs</i> LuxR1, <i>Vl</i> LuxR2, <i>Vs</i> LuxR2
I234V	235	Stronger activation of WT <i>lux</i> box and mutant <i>lux</i> box A in D202E, D244H background. 2x as strong as D244H alone.	none
D244H	245	Activates mutant <i>lux</i> boxes A** and C***	none
* <i>Hawkins et al. 2007 (118)</i> subsequently showed T33A, S116A, M135I in combination (allele LuxR-G2E) increased responsiveness to a range of signals from C5- to C14-HSL			
** Mutant <i>lux</i> box A: AGCTCAGGATGGTACAGGT (123)			
*** Mutant <i>lux</i> box C: AGCTGAGGATGGTACACGT (123)			

background used in this study has no HSL synthase genes ($\Delta luxI$ and $\Delta ainS$), and the *luxI-luxR* intergenic region has a constitutive *luxR* promoter and no *luxR* or *lux* regulator binding sites, but still contains the native ES114 *lux* box and *lux* promoter (see Chapter 2, Fig. 2.4 for detailed sequence comparison between WT ES114 and the engineered strains). These changes allow us to observe *luxR* function in response to specific HSL concentrations added to the media, with no complicating factors such as transcriptional positive feedback loops or other known non-*luxR* mediated transcriptional regulation. We measured luminescence and OD₆₀₀ over the course of eighteen hours of growth and recorded the maximum luminescence/OD₆₀₀ for the four *V. logei* and *V. salmonicida luxR* variants and a $\Delta luxR$ control (KB15) using no HSL, C4-, C6-, C8-, C10, 3OC6-, and 3OC8-HSLs. None of the *luxR* variants were able to induce the *lux* operon to produce luminescence above background using the *V. fischeri* ES114 *lux* box in any HSL condition (Fig. 4.4A).

***V. salmonicida* LuxR2 activates *lux* box 2 (*luxI* promoter)**

We speculated that the *V. logei* and *V. salmonicida* LuxR proteins could be unable to recognize a *V. fischeri lux* box, which differs somewhat in sequence from *lux* boxes in *V. logei* and *V. salmonicida* (Fig. 4.1B), so we created versions of the engineered strains containing the two *lux* boxes present in both *V. logei* and *V. salmonicida* (Fig. 4.1C). The first *V. logei/V. salmonicida lux* box (*lux* box LS1) is adjacent to the *luxCDABEG* promoter and is seven base pairs different from the *V. fischeri* ES114 *lux* box (*lux* box E) (Fig. 4.1A and B). The other *V. logei/V. salmonicida lux* box (*lux* box LS2) is adjacent to the *luxI* promoter and is only five bases different from *lux* box E (Fig. 4.1A and B). None of the four *V. logei* and *V. salmonicida luxR* variants produced a response to any of the HSL conditions using *lux* box LS1 (Fig. 4.4B), however, *V. salmonicida luxR2* induced luminescence above background in response to 3OC6- or 3OC8-HSL in

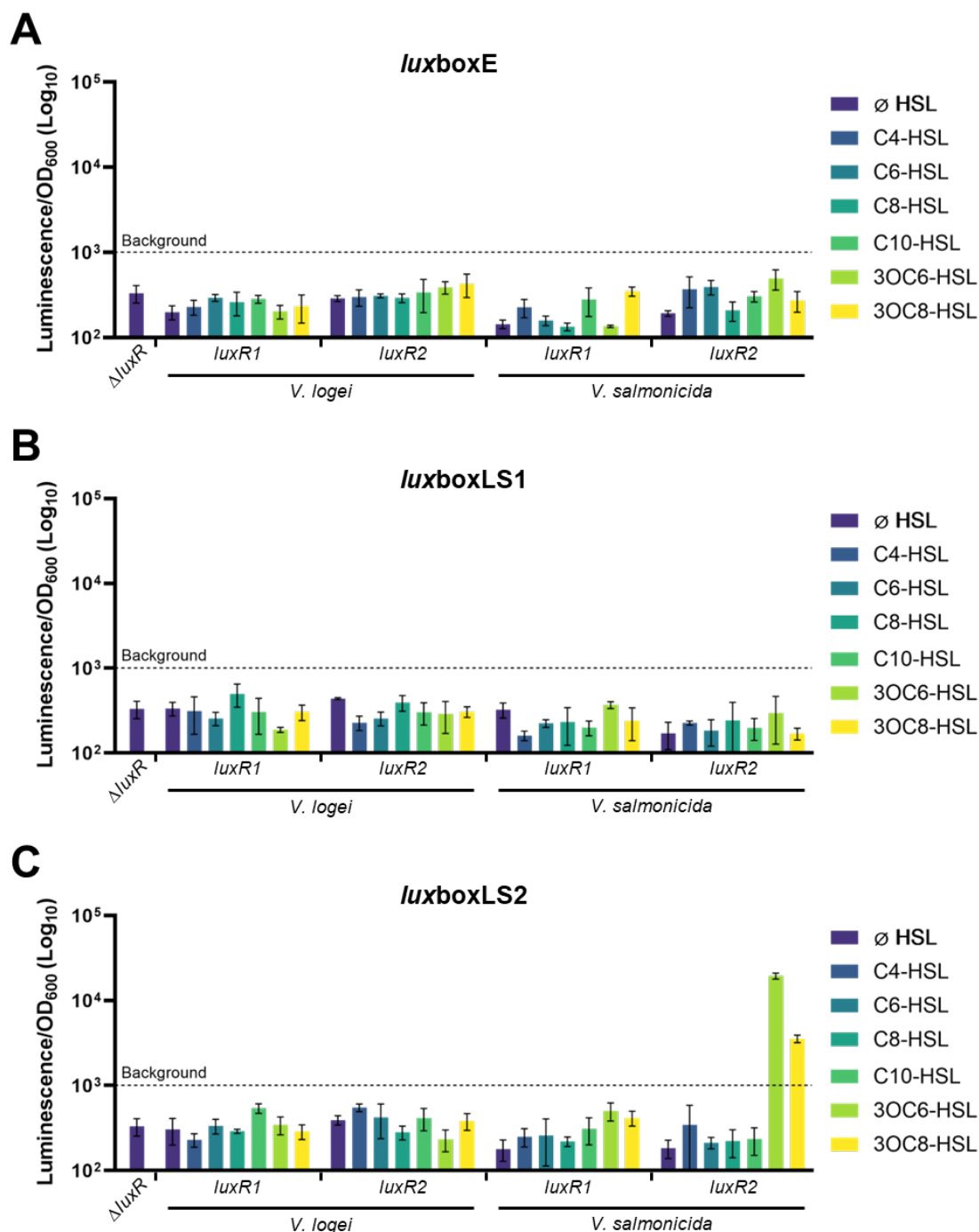


Figure 4.4. *V. salmonicida luxR2* responds to 3OC6- and 3OC8-HSL with *lux box 2* (*lux box LS2*) in the *lux* promoter. Maximum Luminescence/OD₆₀₀ data on a log scale indicating how bright each *luxR*-HSL combination became with (A) *V. fischeri* ES114 *lux box* (*lux box E*), (B) *V. logei*/*salmonicida lux box 1* (*lux box LS1*), or (C) *V. logei*/*salmonicida lux box 2* (*lux box LS2*). Cells were tested with 3.2 μ M C4-, C6-, C8-, C10-, 3OC6-, or 3OC8-HSL, as well as without HSL ($\emptyset$ HSL) in triplicate. Error bars indicate standard error of the mean. A *luxR* deletion strain ($\Delta luxR$) was included as a negative control. Only bars above the “background” line at 10^3 are considered luminescent.

combination with *luxboxSL2* (Fig. 4.4C). Even so, the level of luminescence produced by *V. salmonicida luxR2* was low. Interestingly, *V. logei luxR2* did not activate *lux box LS2*, even though it only differs from *V. salmonicida luxR2* by one amino acid (Fig. 4.3, position 177).

Discussion

The results of this study indicate that *luxR1* and *luxR2* from *V. logei* and *V. salmonicida* have diverged enough from *V. fischeri luxR* that they cannot activate the *V. fischeri lux* operon (Fig. 4.4A). It is possible that *luxR1* and *luxR2* induce a weak luminescence response that is below the limit of detection in these experiments, however, even if so, the result would indicate significant functional divergence of *luxR* alleles. We showed that this lack of cross-species complementation is due in part to variations that have evolved in the *lux* box sequences, as changing the *lux* box to native *V. logei/salmonicida lux* boxes enabled activation with the combination of *V. salmonicida luxR2* and *lux box LS2* (Fig. 4.4B and C). The *lux box LS2* more closely resembles the *lux box E* from *V. fischeri*, differing by five of twenty positions, whereas *lux box LS1* differs from the *V. fischeri lux box E* at seven of twenty positions (Fig. 4.1B). Moreover, divergence in the DNA binding domains of LuxR, LuxR1, and LuxR2, may underlie different affinities for specific *lux* box sequences. A comparison of LuxR from seventeen diverse strains revealed only one residue that varied in the fifteen-residue DNA-binding domain (Chapter 2), whereas each pairwise comparison of LuxR1, LuxR2 and LuxR over this same stretch reveals three differences (Fig. 4.3). In the future, computational modelling of these molecular structures may help test whether specific residue-base interactions underlie LuxR-*lux* box (in)compatibility in specific combinations of LuxR and target DNA.

Another possible explanation for the lack of activation in some *luxR* and *lux* box combinations (Fig. 4.4B and C) is that in *V. logei* and *V. salmonicida*, *luxR1* and *luxR2* may have evolved such that the two encoded proteins work as a heterodimer, with neither LuxR1 nor LuxR2 able to activate *lux* transcription effectively on its own. This possibility is supported by the literature; Hansen *et al.* speculated that *luxR1* and *luxR2* work as a heterodimer in *V. salmonicida* because both are required for optimal LuxI expression (193), while Konopleva *et al.* hypothesized *V. logei* *luxR1* and *luxR2* work as a heterodimer because the presence of *luxR1* can rescue *luxR2* from the negative effects of Lon protease on luminescence activity (195). The *lux* boxes are imperfect inverted repeats, with each repeat binding a LuxR monomer, so the dual possibility of LuxR1/LuxR2 heterodimers and different DNA binding specificity of each monomer also raises the question of whether *lux* box sequences might evolve to better accommodate homo- or heterodimers of the activator. Such a scenario would enable a substantially more complex PS system in *V. logei* and *V. salmonicida* than in *V. fischeri*. The scope of this study simply examined different *luxR* sequences individually, but in the future, it would be interesting to test the heterodimer hypothesis in our engineered background by introducing both *luxR1* and *luxR2* at the same time and with a greater variety of *lux* box sequences to see how that changes the luminescence output.

The possibility that heterodimer formation has evolved may also be reflected in the sequence analysis shown in Figure 4.3. There is a lot of variation in LuxR in regions that align with TraR's dimerization domains. It is possible that the LuxR1 and LuxR2 sequences co-evolved to form a heterodimer, perhaps at the expense of forming homodimers, adding complexity to the regulation of the *lux* operon in these species. Dimerization domains may hold more importance for LuxR function than has been previously appreciated. Directed evolution experiments aimed at altering the HSL responsiveness of LuxR^{MJ1} fall not in the ligand binding pocket but do align with

dimerization domains in the homolog TraR (Fig. 4.3). Some of these allelic changes in LuxR^{MJ1} are reflected naturally in LuxR1 or LuxR2, including residues S158, V159, S169, R172, and D177 (position numbers according to Fig. 4.3). Rather than improving HSL responses through improved HSL binding kinetics, they could instead be residues that impact LuxR dimerization (Table 4.1) (118).

The four total LuxR1 and LuxR2 sequences from *V. logei* and *V. salmonicida* also naturally contain other residues that correspond to mutations identified in LuxR^{MJ1} directed evolution experiments (Table 4.1, Fig. 4.3, red residues). An N7 residue that is present in both LuxR2 sequences aligns with a D7N change that was found to confer LuxR^{MJ1} with increased responsiveness to 3OC6-HSL in combination with Y62H (119) and our previous study found that this change close to the N-terminus could actually be impacting function through the nucleotide sequence near the 5' end of the mRNA affecting translation efficiency (Chapter 3). The LuxR1 sequences both had the residue A33 which increases LuxR^{MJ1} responsiveness to C8-HSL and was found in multiple *V. fischeri* LuxR variants in Chapter 2 (59). This variation could be conferring a similar C8-HSL responsiveness to *V. logei* and *V. salmonicida* LuxR1, but the lack of any activation by LuxR1 in our background prevents us from drawing any insights into its HSL selectivity. Two other residues that were present in both LuxR2 sequences, N150 and L247 (Fig. 4.3), conferred HSL signal-independent activity in LuxR^{MJ1} (115). We did not observe any HSL-independence from either LuxR2, however, we can speculate that these residues could again be related to dimerization. LuxR^{MJ1} typically requires HSL binding to dimerize and activate the *lux* box, but mutations at this location may have conferred HSL independence by allowing LuxR^{MJ1} to dimerize properly without the aid of HSL, which would mean that these residues are dimerization contacts. A final point of comparison worth highlighting is that LuxR2 from *V. logei*

and *V. salmonicida* have different activities (Fig. 4.4) but differ only in having E177 or D177 (Fig. 4.3), respectively, and this is residue near a putative dimerization domain was also recovered in a directed evolution study of LuxR^{MJ1} (Table 4.1).

One directed-evolution study of LuxR^{MJ1} altered the *lux* box to disrupt activation and then selected *luxR* mutants that restored activation (123), and some of the residues identified in this way are found naturally in *V. logei* and *V. salmonicida*. Specifically, residues Y183 and E203 allowed LuxR^{MJ1} to activate the mutant *lux* box sequences in an H245 background. Y183 is present in *V. logei* LuxR1 and E203 is found in all four *V. logei* and *V. salmonicida* LuxR sequences (Table 4.1, Fig. 4.3), although neither of the *V. logei* and *V. salmonicida* *lux* boxes harbor the sequences used in the aforementioned directed-evolution screen. It seems reasonable to postulate that *luxR* sequences have evolved alongside the *lux* box sequences to maintain the ability to bind that DNA, which is why we found differences in the DNA binding domains of *luxR* sequences from different species.

It may also be worth noting that *V. logei* and *V. salmonicida* LuxR1 also both contain an I163 residue, which corresponds to a mutation found in an experiment screening for LuxR^{MJ1} loss-of-function “dark mutants” (111). In this study, Slock *et al.* identified that LuxR^{MJ1} containing the three mutations H141P, V163I, and R231H (residue numbers correspond with Fig. 4.3) could not induce any luminescence (111). However, it was later determined that residue R231 is essential for DNA binding and can disrupt LuxR^{MJ1} function on its own (111, 120), so it is unclear whether V163I has any significant contribution to the phenotype. It is possible that I163 is responsible for LuxR1’s lack of *lux* activation, but we find the heterodimer hypothesis to be more compelling.

V. logei SA6 and *V. salmonicida* LFI1238 are both psychrophilic species that were isolated from marine hosts. *V. logei* SA6 was isolated from a symbiosis in the light organ of a *Sepiola*

affinis bobtail squid found off the coast of France in the Mediterranean Sea, along with four strains of *V. fischeri* and one other strain of *V. logei* taken from the same squid (151). *V. salmonicida* LFI1238 was isolated from a diseased Atlantic Cod (*Gadus morhua*) provided to researchers by a Norwegian fishery (187). Despite *V. logei* more closely resembling *V. fischeri* in terms of its niche, the structure of its *lux* locus and regulators is more like that of *V. salmonicida*. Perhaps the similarity of *V. logei* and *V. salmonicida* is connected to the colder seawater that these species live in, while the warm-adapted Hawaiian *V. fischeri* strains have diverged more.

This work has helped to further our understanding of LuxR evolution in *Vibrio* species. We found that *V. logei* and *V. salmonicida* *luxR* sequences are not capable of activating luminescence in a *V. fischeri* background and hypothesize that this occurred because heterodimers between *luxR1* and *luxR2* are necessary for effective *luxR* interactions with the *lux* box. When given access to its native *lux* boxes, *V. salmonicida* *luxR2* was able to slightly activate *lux* box LS2 with 3OC6- or 3OC8-HSLs. This points to intriguing co-evolution occurring not only between the *luxR1* and *luxR2* sequences, but also between the *luxR*s and their *lux* boxes. Future studies of *luxR* homologs or attempts at creating *luxR* biosensors should keep in mind the importance of pairing a *luxR* with its native *lux* box and how changing the *luxR* sequence could have drastic effects on its ability to bind its *lux* box or dimerize to form an active protein complex. This is especially important to consider in species that harbor multiple *luxR* homologs, as they could be forming heterodimers that work best when both sequences are being expressed at the same time.

Materials and Methods

Strains and Media. The strains used in this study are listed in Table 4.2. KB15, derived from wild-type *Vibrio fischeri* ES114, was used as a parent strain into which *luxR* variants were introduced. *Escherichia coli* strain DH5 α was used for cloning. *V. fischeri* cultures were grown in

either LBS medium (162) at 28°C or SWTO (1) medium at 24°C with final antibiotic concentrations of 2 µg/mL for chloramphenicol and 100 µg/mL for kanamycin as needed. *E. coli* cultures were grown in lysogeny broth (LB) medium (163) at 37°C with final concentrations of 20 µg/mL for chloramphenicol and 40 µg/mL for kanamycin. HSLs were obtained from Sigma-Aldrich and dissolved in acetified ethyl acetate (1:1000 glacial acetic acid: ethyl acetate) to create a stock solution. HSL stock solution was aliquoted into sterile containers and the solvent was allowed to evaporate before SWTO medium was added to an HSL concentration of 3200 µM.

Sequencing *luxR* variants. The *luxR* sequences for *V. salmonicida* and *V. logei* were available on NCBI. However, the *V. logei luxR* sequences available were from *V. logei* ATCC 35077, whereas we had *V. logei* strain SA6 available as a source for *luxR1* and *luxR2* cloning. We confirmed via Sanger sequencing that the *luxR* sequences we obtained from SA6 matched those available on NCBI for ATCC 35077. Alignments and trees were generated with Geneious Prime 2024.0.5 (<https://www.geneious.com>).

Genetic manipulations. Each *luxR* was PCR amplified off their respective genome using the forward and reverse primer pairs in Table 4.4. *luxR1* PCR products were digested with NdeI and XhoI and cloned into NdeI- and XhoI-digested pKAB36 while *luxR2* PCR products were digested with NheI and XhoI and cloned into NheI- and XhoI-digested pKAB36. *V. salmonicida luxR1*, *V. salmonicida luxR2*, *V. logei luxR1*, and *V. logei luxR2* replaced *kanR* on pKAB36, making pKAB54, pKAB55, pKAB58, and pKAB59, respectively. Transformants were screened for the loss of kanamycin resistance. These plasmids were conjugated into *V. fischeri* KB15 using *E. coli* CC118 λ pir with pEVS104 as a conjugative helper (162), enabling allelic exchange to replace the *kanR* on the chromosome of KB15 with each respective *luxR* variant resulting in KB27, KB28, KB29, and KB30, respectively.

To easily change the *V. fischeri* ES114 *lux* box (*lux* box E) to the *V. logei/salmonicida* *lux* boxes (*lux* box LS1 or *lux* box LS2), we ordered gene blocks from IDT containing the same *kanR* and *luxIR* intergenic region as pKAB36 but with different *lux* box sequences (See Fig. 4.1). These two gene blocks were digested with BsrGI and XhoI and cloned into a BsrGI- and XhoI-digested *luxR* variant plasmid (pKAB46) to create pKAB88 (*kanR*, *lux* box LS1) and pKAB89 (*kanR*, *lux* box LS2), selecting for transformants on kanamycin. These plasmids were identical to pKAB36 except for the *lux* box sequence. *luxR1* PCR products were digested with NdeI and XhoI and cloned into NdeI- and XhoI-digested pKAB88 and pKAB89 while *luxR2* PCR products were digested with NheI and XhoI and cloned into NheI- and XhoI-digested pKAB88 and pKAB89. *V. salmonicida luxR1*, *V. salmonicida luxR2*, *V. logei luxR1*, and *V. logei luxR2* replaced *kanR* on both pKAB88 and pKAB89, making pKAB90, pKAB91, pKAB94, and pKAB95, respectively with *lux* box LS1 and pKAB92, pKAB93, pKAB96, and pKAB97, respectively with *lux* box LS2. Transformants were screened for the loss of kanamycin resistance. These plasmids were conjugated into *V. fischeri* KB15 using *E. coli* CC118 λ pir with pEVS104 as a conjugative helper (162), enabling allelic exchange to replace the *kanR* through *lux* box on the chromosome of KB15 with each respective *luxR* variant. pKAB90-97 made KB65-72, respectively. The strains, plasmids, and relevant oligonucleotides used in this study are listed in Tables 4.2-4.4. Details of cloning intermediates used to create pKAB36 are available on request. Each strain was verified by sequencing the plasmid used for allelic exchange, or a PCR of the genome when necessary.

Luminescence response to HSL. Luminescence experiments were all completed in 96-well plates and grown at 24°C shaking in a Biotek Cytation 1 plate reader with a Peltier Cooling Module used to maintain a consistent temperature. Luminescence and OD₆₀₀ measurements were taken every 30 minutes for 18 hours. OD₆₀₀ was not pathlength corrected in our analysis. HSL was evaporated

in tubes and SWTO media was added to each to a concentration of 3200 nM HSL. 200 µL of SWTO + 3200 nM HSL was aliquoted into wells and 2 µL of an overnight culture was added. Experiments were completed with C4-, C6-, C8-, C10-, 3OC6-, and 3OC8-HSL.

Table 4.2. Bacterial strains used in this study.

Strain Name	Relevant Characteristics	Reference
<i>E. coli</i> CC118λpir	Δ(<i>ara-leu</i>) <i>araD</i> Δ <i>lac74 galE galK phoA20 thi-1 rpsE rpsB argE</i> (Am) <i>recA</i> λpir	(165)
<i>E. coli</i> DH5α	φ80dlacZ ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	(164)
<i>V. fischeri</i> ES114	Wild-type <i>V. fischeri</i> in this study	(145)
<i>V. fischeri</i> KB15	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> Δ <i>luxR::kan^R</i> , P _{luxI} - <i>luxCDABEG</i>	(Ch. 2)
<i>V. fischeri</i> KB27	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR1^{V. salmonicida} LF1123</i> , P _{luxI} - <i>luxCDABEG</i>	This study
<i>V. fischeri</i> KB28	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR2^{V. salmonicida} LF1123</i> , P _{luxI} - <i>luxCDABEG</i>	This study
<i>V. fischeri</i> KB29	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR1^{V. logei} ATCC35077</i> , P _{luxI} - <i>luxCDABEG</i>	This study
<i>V. fischeri</i> KB30	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR2^{V. logei} ATCC35077</i> , P _{luxI} - <i>luxCDABEG</i>	This study
<i>V. fischeri</i> KB65	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR1^{V. salmonicida} LF1123</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS1</i>	This study
<i>V. fischeri</i> KB66	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR2^{V. salmonicida} LF1123</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS1</i>	This study
<i>V. fischeri</i> KB67	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR1^{V. salmonicida} LF1123</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS2</i>	This study
<i>V. fischeri</i> KB68	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR2^{V. salmonicida} LF1123</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS2</i>	This study
<i>V. fischeri</i> KB69	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR1^{V. logei} ATCC35077</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS1</i>	This study
<i>V. fischeri</i> KB70	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR2^{V. logei} ATCC35077</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS1</i>	This study
<i>V. fischeri</i> KB71	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR1^{V. logei} ATCC35077</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS2</i>	This study
<i>V. fischeri</i> KB72	ES114 Δ <i>ainS</i> Δ <i>luxR-luxI</i> , <i>luxR2^{V. logei} ATCC35077</i> , P _{luxI} - <i>luxCDABEG</i> Δ <i>luxboxE::luxboxLS2</i>	This study
<i>V. logei</i> ATCC35077	Wild-type <i>V. logei</i> in this study	(151)
<i>V. salmonicida</i> LF11238	Wild-type <i>V. salmonicida</i> in this study	(192)

Table 4.3. Plasmids used in this study.

Plasmid Name	Relevant Characteristics	Reference
pEVS104	Conjugative helper plasmid; R6K γ , <i>oriT</i> _{RP4} , <i>kan</i> ^R	(183)
pKAB36	$\Delta luxR::kan^R$, P _{luxI} - <i>luxCDABEG</i> , <i>cm</i> ^R	(Ch. 2)
pKAB46	$\Delta luxR-luxI$, <i>luxR</i> ^{PP3} , P _{luxI} - <i>luxCDABEG</i> , <i>cm</i> ^R	(Ch. 2)
pKAB54	$\Delta luxR-luxI$, <i>luxR1</i> ^{V. salmonicida LF1123} , P _{luxI} - <i>luxCDABEG</i> , <i>cm</i> ^R	This study
pKAB55	$\Delta luxR-luxI$, <i>luxR2</i> ^{V. salmonicida LF1123} , P _{luxI} - <i>luxCDABEG</i> , <i>cm</i> ^R	This study
pKAB58	$\Delta luxR-luxI$, <i>luxR1</i> ^{V. logei ATCC35077} , P _{luxI} - <i>luxCDABEG</i> , <i>cm</i> ^R	This study
pKAB59	$\Delta luxR-luxI$, <i>luxR2</i> ^{V. logei ATCC35077} , P _{luxI} - <i>luxCDABEG</i> , <i>cm</i> ^R	This study
pKAB88	$\Delta luxR::kan^R$, P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS1$, <i>cm</i> ^R	This study
pKAB89	$\Delta luxR::kan^R$, P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS2$, <i>cm</i> ^R	This study
pKAB90	$\Delta luxR-luxI$, <i>luxR1</i> ^{V. salmonicida LF1123} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS1$, <i>cm</i> ^R	This study
pKAB91	$\Delta luxR-luxI$, <i>luxR2</i> ^{V. salmonicida LF1123} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS1$, <i>cm</i> ^R	This study
pKAB92	$\Delta luxR-luxI$, <i>luxR1</i> ^{V. salmonicida LF1123} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS2$, <i>cm</i> ^R	This study
pKAB93	$\Delta luxR-luxI$, <i>luxR2</i> ^{V. salmonicida LF1123} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS2$, <i>cm</i> ^R	This study
pKAB94	$\Delta luxR-luxI$, <i>luxR1</i> ^{V. logei ATCC35077} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS1$, <i>cm</i> ^R	This study
pKAB95	$\Delta luxR-luxI$, <i>luxR2</i> ^{V. logei ATCC35077} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS1$, <i>cm</i> ^R	This study
pKAB96	$\Delta luxR-luxI$, <i>luxR1</i> ^{V. logei ATCC35077} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS2$, <i>cm</i> ^R	This study
pKAB97	$\Delta luxR-luxI$, <i>luxR2</i> ^{V. logei ATCC35077} , P _{luxI} - <i>luxCDABEG</i> $\Delta luxboxE::luxboxLS2$, <i>cm</i> ^R	This study

Table 4.4. Oligonucleotides used in this study.

Oligo	Sequence	Relevant usage
KAB45	CTAGCATATGAACTATATCGCTCCTCATGACAC	<i>V. salmonicida</i> and <i>logei luxR1</i> cloning primer (Fwd)
KAB46	CTAGCTCGAGTTATGAATACGCTGGATTGATC	<i>V. salmonicida luxR1</i> cloning primer (Rev)
KAB47	CTAGGCTAGCGATTGTAACCTAGTCAACATAAA	<i>V. salmonicida</i> and <i>logei luxR2</i> cloning primer (Fwd)
KAB48	CTAGCTCGAGTTAATATAACGGGTTCATTGCTC	<i>V. salmonicida</i> and <i>logei luxR2</i> cloning primer (Rev)
KAB49	CTAGCTCGAGTTATGAATACGCTGGGTTCATC	<i>V. logei luxR1</i> cloning primer (Rev)

* Sequences in 5' to 3' orientation; underlined sequences indicate restriction-enzyme recognitions sites added to facilitate cloning; bold sequences indicate *luxR* start or stop codons in Fwd or Rev primers, respectively.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The primary goal of this dissertation was to enhance our understanding of how the pheromone-signaling (PS) systems in *V. fischeri* have evolved variation in information processing. PS systems like those in *V. fischeri* are common throughout the Pseudomonadota (formerly Proteobacteria), and they regulate bacterial processes that are relevant to human health, industry, and agriculture. *V. fischeri* is a non-pathogenic, fast-growing, genetically tractable species that uses PS to control bioluminescence – an easily measurable output. These traits make it an ideal model organism for studying the mechanisms behind PS in Gram-negative bacteria. In the previous chapters, I focused on examining the evolutionary and functional diversity of the transcriptional regulator LuxR, a critical activator of the *lux* genes responsible for bioluminescence in *V. fischeri*. As the receptor for the pheromone signals, establishing how *luxR* evolves to respond to these signals is a key component to our overall understanding of PS systems.

Through an investigation into the signal-response capabilities of naturally occurring *luxR* variants, I dove into the complexity of *luxR* evolution to improve our understanding of how its sequence impacts both protein function and expression. As described in Chapters 2 and 4, I collected over twenty naturally occurring variants of *luxR* from both *V. fischeri* strains and its close relatives *V. logei* and *V. salmonicida* and compared their sequences to each other and to *luxR* sequences that were artificially generated and identified through directed evolution experiments aimed at changing *luxR* signal specificity. Using an engineered background that allowed control

of autoinducer concentration and separated the *lux* genes from native transcriptional regulation, I also compared the functional responses of several *luxR* sequences to both cognate and non-cognate signals to see how they varied phenotypically. In doing so I measured the luminescence output of *V. fischeri* strains that varied only in their *luxR* coding sequences and identified how those differences affected their ability to respond to specific HSL signaling molecules.

In Chapter 2, I explored the variability that is evolving in the *luxR* sequence between different strains of *V. fischeri*. By comparing *luxR* sequences and concatenated housekeeping gene sequences, we determined that *luxR* has diverged far more than is typical for genes from these strains. We also compared eight LuxR variant amino acid sequences, and found that within the *V. fischeri* strains, there was considerable variability in the predicted HSL binding domain. However, all the LuxR sequences had complete conservation of three amino acids that are predicted to be critical for hydrogen bonding to HSL ligands. We created engineered variant strains containing *luxR* sequences from these eight *V. fischeri* strains and tested how *luxR* sequence affects luminescence response in the presence of cognate and noncognate HSL signals. Each LuxR variant exhibited a unique luminescence response, though they clustered into phenotypically similar groups that generally aligned with sequence similarity. We classified the *V. fischeri* LuxR responses into four phenotypic groups represented by LuxR^{ES114}, LuxR^{H905}, LuxR^{MJ1}, and LuxR^{MJ11}.

LuxR^{MJ11} was not a good activator of the *lux* operon in our engineered background, yet MJ11 is among the brightest strains of *V. fischeri* that has been isolated (129). In Chapter 3, we discuss how the poor activation by LuxR^{MJ11} is caused by a stable 5' end mRNA transcript secondary structure, which we hypothesize is preventing access to the ribosome binding site (RBS) and decreasing translation efficiency. Some other element(s) of MJ11 *lux* regulation must be

compensating for this decreased efficiency in the native strain, since LuxR^{MJ11} activates bright luminescence in the parent strain. This phenomenon was not only specific to LuxR^{MJ11}; for each *luxR* tested, parent strain brightness was not predictive of LuxR responsiveness in our engineered strains. Which begs the question: what elements of *lux* regulation could be responsible for this discrepancy? One possibility is that LuxR^{MJ11} is significantly better at activating its native *lux* box than ES114's, despite only varying by two bases. MJ11 could also somehow be activating LuxR expression more than ES114 does, either via changes to the *luxR* promoter or a LuxR regulator. These and other possibilities should be investigated for a fuller understanding of why activation by a strain's *luxR* in our isogenic engineered background doesn't correlate with the strain's overall brightness.

Chapter 2 also compares residues found in the naturally evolved LuxR sequences with residues identified in directed evolution experiments that screened for LuxR^{MJ1} mutants with altered HSL responsiveness. The predicted roles of the residues found in those studies sometimes aligned with phenotypes we observed in our LuxR variants. For example, residue F164 (using the residue number from the original study and not our alignment) was identified in Sitnikov *et al.* in a study screening for LuxR^{MJ1} mutants that could luminesce without added HSL (115). Interestingly this residue appeared in LuxR^{H905} which was able to elicit a response when no HSL was added. Though it is tempting to conclude that this residue is responsible for the phenotype, LuxR^{H905} has so many additional differences from LuxR^{MJ1} that we cannot connect this phenotype to that specific residue with certainty. However, we believe it is notable that it occurred naturally and think it warrants further examination.

A natural continuation of our examination of LuxR function would be to make additional changes to the various *luxR* sequences in the engineered background to see how those changes

affect luminescence output. One way to do so would be to focus on the residues from the directed evolution experiments that occurred naturally in LuxR variants (like F164), which we outlined in Chapter 2. The apparent convergence of natural and experimental evolution points to potential residues of interest in important functional areas of LuxR sequence. F164 would be an easy place to start, as a simple point mutation could test whether a change at that residue eliminates the LuxR^{H905} HSL independence phenotype. Larger scale changes to *luxR* could also be interesting, like swapping specific domains of the different *luxR* variants to make chimeras. Changing the HSL binding domain, DNA binding domain, or even the first eleven codons that we believe are important for translation efficiency could yield very interesting results that would certainly add to our understanding of *luxR* function and evolution. During my studies, we also predicted potential ancestors of the modern-day *luxR* sequences by working with Dr. Eric Gaucher from Georgia Tech, but never tested them. Continuing that project by cloning those predicted *V. fischeri luxR* ancestral sequences into the engineered background and observing their luminescence responses to HSL signals would be a great way to explore *luxR* evolution further.

Chapter 4 examined additional *luxR* variant phenotypes, by seeing how the *luxR1* and *luxR2* sequences from *V. logei* and *V. salmonicida* functioned in our engineered *V. fischeri* background. We found that none of the *V. logei* or *V. salmonicida* LuxRs elicited a response to any cognate or noncognate HSLs, however *V. salmonicida luxR2* was able to elicit weak responses to 3OC6- and 3OC8-HSL when we replaced the *V. fischeri lux* box with a native *V. salmonicida lux* box. The analyses of LuxR amino acid sequences in Chapters 2 and 4 tell us that the DNA binding domains of *V. fischeri* LuxRs are much more similar to one another than to those from *V. logei* and *V. salmonicida*, which we predict is due to coevolution between a given *luxR* and its *lux* box. Finding that the sequence of the *lux* box affected the ability of *V. salmonicida luxR2* to

activate the *lux* operon points to *luxR* specificity toward the *lux* box as being a more important factor than we had initially considered. Though we knew the *V. logei* and *V. salmonicida* *lux* boxes were not identical to the *lux* box upstream of the *lux* operon in *V. fischeri* ES114, there is considerable variability in *lux* boxes within ES114 (43), and we did not think those interspecies differences would be enough to fully prevent LuxR activity. However, now it seems it would be beneficial to dig deeper into *luxR-lux* box specificity in the future.

A simple first step toward testing *luxR-lux* box coevolution could be to test the reverse of the Chapter 4 strains, by trying *V. fischeri* *luxR* sequences with *V. logei/salmonicida* *lux* boxes. There are also several slightly different *lux* operon *lux* boxes even within the *V. fischeri* strains that we did not test (129). The engineered strains were an attempt to only have one variable changing (i.e., the *luxR* coding sequence) which is why we did not change the *lux* box to match the parent strain in every engineered variant. However, an interesting continuation of this work would be to replace the ES114 *lux* box with the native *lux* box of each *V. fischeri* *luxR* variant and see if the luminescence responses change. If there are differences, one could glean information about how *luxR* and the *lux* box are coevolving. There are also several other *lux* boxes found throughout the *V. fischeri* genome used by LuxR to regulate additional non-*lux* genes (43). Testing those *lux* boxes in the engineered background could provide new insights into the regulation of other genes in the LuxR regulon and further inform how LuxR-*lux* box specificity is evolving.

Evidence suggests *V. logei* and *V. salmonicida* *luxR1* and *luxR2* also form heterodimers in their native strains, further explaining why we saw such little luminescence in our single-*luxR* strains even with their native *lux* boxes. Not only has this hypothesis been suggested by previous studies (193, 195), but the *V. logei* and *V. salmonicida* LuxR sequences examined in Chapter 4 showed increased variation in regions predicted to be important for dimerization, supporting this

idea. This hypothesis should be examined in more detail, both to better understand the mechanisms of *lux* operon regulation in these species, and to gain insight into *luxR* dimerization. Little is known about the way LuxR dimerizes, other than comparisons to homologs like TraR. Because dimerization appears to be critical for LuxR to activate the *lux* operon, understanding both how it does so, and which residues are important would be advantageous towards the overall goal of detailing *luxR* function. A first step could be to expand upon the study described in Chapter 4, by adding both *V. logei* and *V. salmonicida luxR1* and *luxR2* sequences to the variant strains. I predict that strains engineered with both *luxR1* and *luxR2* will elicit stronger luminescence responses, more similar in overall brightness to the *V. fischeri luxR* responses from Chapter 2. If so, it could also be interesting to mix and match various pairs of *luxR* sequences in the engineered strain and see if the responses vary when a different *luxR* is offered up as a dimerization partner. Combined with a sequence analysis, there is potential to pinpoint amino acids that are important dimerization contacts using this method.

From both experimental analyses in Chapters 2 and 4 we determined that the variations evolving in *luxR* have profound effects on *luxR* signal-responsiveness and ability to activate the *lux* operon. Chapter 3 examined how differences in *luxR* sequence could also impact its mRNA and protein levels in addition to luminescence response. Using quantified Western blot and RT-PCR, we measured LuxR protein and *luxR* mRNA transcript in our engineered variant strains in the absence of HSL or presence of 3OC6- or C8-HSL. Unexpectedly, the amount of protein and mRNA varied by LuxR variant and by which HSL was added. These strains were isogenic except for the *luxR* coding sequence, so these variations had to be caused by the differences between the *luxR* variants.

We predicted the minimum free energy (MFE) of the 5' end mRNA secondary structure for each *luxR* variant and found that they differed substantially, and that these differences could explain why overall brightness, LuxR protein, and *luxR* transcript varies between the *luxR* variants. A more stable 5' end mRNA secondary structure decreases translation initiation efficiency by impeding access to the ribosome binding site (RBS) (172-177), and decreased protection from actively translating ribosomes leaves mRNA more susceptible to RNase degradation (177-180). We believe this is what caused the large differences we observed between LuxR^{MJ1} and LuxR^{MJ11} activity despite their similar sequences; LuxR^{MJ11} has a very stable 5' end mRNA secondary structure caused by the sequence of its first few codons. By measuring protein degradation and estimating LuxR half-life, we also determined that the differences in HSL selectivity could be explained by protein stability. We hypothesize that LuxR is stabilized or protected from degradation by the presence of HSLs, likely because HSL-binding affects its conformation and dimerization, like its homolog TraR. However, it is still possible that the additional differences between the *luxR* variants found throughout the rest of the sequence affect things like HSL or DNA binding specificity, which could also be impacting brightness. It is likely a combination of 5' end transcript stability, protein stability, and protein functionality working together determine the amount of luminescence activated by each *luxR*.

Chapter 3 brings attention to important factors of *luxR* regulation that have not been previously considered. LuxR appears to be evolving self-regulation via its 5' end secondary structure which will be important to consider in conjunction with the many other ways *luxR* regulation is controlled in *V. fischeri*. In addition, studies aiming to artificially engineer *luxR* sequence should take care when amending the first few codons, as they could accidentally decrease *luxR* translation efficiency and reduce LuxR's ability to activate the *lux* operon. It would be

interesting to see if this knowledge could instead be used to create a more highly expressed version of LuxR with more activation power by destabilizing the 5' mRNA end secondary structure, though caution should be used as translation efficiency dynamics can be complex and cause unintended consequences. We also know very little about *luxR* transcription at the present, and future studies should focus on identifying the transcriptional start of *luxR* as well as its overall transcript length. Though studies like the ones found in this dissertation are succeeding at advancing our understanding of LuxR function using a genetic approach, perfecting LuxR purification would be immensely helpful for measuring LuxR-HSL binding kinetics or DNA binding directly. A combined approach of genetic and biochemical analysis would be a robust way to elucidate LuxR function, and we hope to see advancements in that area in the future.

One of our original goals for this study was to update our model of *luxR* function using computational modeling, similar to what was done by Colton *et al.* (60), however this eventually proved impractical. The complex HSL responses described in Chapter 2 made it impossible to fit the model to the data without including more parameters, however the addition of more parameters made the modeling imprecise, with multiple combinations of quantitative parameter estimations fitting the experimental data equally well. In addition, LuxR expression is more complicated than we initially suspected, with things like 5' end transcript folding and LuxR stability playing a role in varying LuxR levels. It may still be possible to use computational modeling to predict specific aspects of LuxR function, like HSL selectivity, rather than a model that is descriptive of the entirety of LuxR activity. It could be beneficial to see what computational modeling or machine learning could tell us about *luxR* function, interrogating datasets for connections that human analysts, who must necessarily limit themselves to a feasible number of likely targets, might miss. These avenues should be explored further in the future.

Previous studies have worked on engineering artificial LuxR-type activators into specific HSL biosensors as a method for determining which (if any) HSLs are being produced by a given culture (1, 55, 62, 64, 166, 167). The work presented in this dissertation lends itself to the future development of more specific or more broadly responsive *luxR* biosensors by providing insights into important features and residues of the LuxR sequence and their impact on LuxR function. For example, if one wanted to engineer a LuxR biosensor that is good at responding to a wide variety of HSL signals, the LuxR^{H905} sequence could be a good place to start. However, it would be important to identify and change the part of the sequence that causes HSL-independence, as that could introduce false positives. It would also be wise for future LuxR biosensor designers to make sure the 5' end of the transcript does not form secondary structure that is too stable, as we determined this can drastically impact the amount of LuxR and thus its activation capabilities.

Final conclusions

Our examination of *luxR* sequence variation lends itself to future work interested in *luxR* evolution or unraveling the mysteries of *lux* operon regulation in *V. fischeri*. Our comparisons of *luxR* point to important residues and domains that affect LuxR function, while also determining that the *luxR* coding sequence unexpectedly affects *luxR* mRNA and LuxR protein levels. We have also contributed twelve new *V. fischeri luxR* sequences to the NCBI database, which will enable others to investigate *luxR* further. As a critical activator of the *lux* operon, *luxR* is an essential piece of the PS system puzzle in *V. fischeri*. By detailing the PS system in this useful model system, we provide a basis for studying PS systems in more dangerous or less genetically tractable bacterial species. In sum, the foundational work done in this dissertation will inform future efforts to engineer artificial regulatory circuits with designed *luxR* alleles, and it could eventually improve our understanding of bacterial processes that impact human health, industry, and agriculture.

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