DIRECTED EVOLUTION OF THE TRANSCRIPTIONAL ACTIVATOR LUXR

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ABSTRACT

LuxR-type proteins are responsible for density-dependent transcriptional regulation in quorum-sensing systems that employ acyl-homoserine lactones (acyl-HSLs) as signal molecules. The *Vibrio fischeri lux* operon, which includes LuxR, has been well studied and shown to function in *E. coli.* The components of the *lux* operon have recently been used to engineer *de novo* genetic circuits because they provide a versatile intercellular communications system. We have used directed evolution to engineer LuxR to expand the "toolbox" of components available for engineering synthetic circuits with a wide range of communications functions and to explore the evolution of LuxR specificity and response.

We developed and validated a screening system to identify mutants of LuxR that activate gene expression with noncognate acyl-HSLs, based on LuxR-mediated gene expression of GFP. We screened libraries of *luxR* mutants for variants exhibiting increased gene activation in response to octanoyl-HSL (C8HSL), with which wild-type LuxR interacts only weakly. Eight LuxR variants were identified that showed a 100-fold increase in sensitivity to C8HSL; these variants also displayed increased sensitivities to a broadened range of acyl-HSLs, including straight-chain acyl-HSLs, while maintaining a wild-type or greater response to LuxR's cognate acyl-HSL, 3-oxo-hexanoyl-homoserine lactone (3OC6HSL).

LuxR and its homologs respond to their cognate acyl-HSL signals with exquisite specificity. To generate a LuxR with a new signaling specificity, we used a dual selection system to identify LuxR variants that either activate gene expression (ON) or do not (OFF) under desired sets of conditions. The dual selection system was evaluated prior to its use, and a 490-fold enrichment in functional clones per round of ON/OFF selection was observed. We subsequently used the dual selection system to identify a new LuxR variant that retains the response to straight-chain acyl-HSLs attained by screening for activation with C8HSL, but no longer responds to 3OC6HSL. A single mutation in the N-terminal signal-binding domain reduces the response to acyl-HSLs having a carbonyl substituent at the third carbon of the acyl chain. The specificity-enhancing mutation does not affect LuxR response to straight-chain acyl-HSLs and would not have been identified by positive selection alone.

We also used the dual selection system to identify LuxR variants that activate transcription upon binding to a promoter containing a mutated operator site, at which LuxR is unable to activate transcription. An amino acid position critical for modulating the DNA-binding specificity of LuxR for the new DNA target was identified. The dual selection system provides a rapid and reliable method for identifying LuxR variants that have the desired response, or lack thereof, to a given set of acyl-HSL signals or DNA targets. LuxR variants with new specificities identified in this work are potentially useful components for constructing artificial cell-cell communication systems that program population-level behaviors.

THESIS SUMMARY

Many species of bacteria use the synthesis and recognition of small molecules to assess changes in their local population density and alter the expression of key genes accordingly. The components of these systems have been harnessed for engineering synthetic genetic circuits that include elements of intercellular communication. We have used directed evolution to engineer LuxR, a transcriptional activator from *Vibrio fischeri* responsible for density-dependent signal recognition and modulation of gene expression. Both the signal binding and signal binding specificities of LuxR have been engineered in order to expand the 'toolbox' of components available for engineering synthetic circuits with a wide range of communications functions and to explore the evolution of LuxR specificity and response.

Chapter 1 of this thesis provides an introduction to LuxR and its homologs and to the methods used in the studies described in this thesis. The general mechanism and components required for acyl-homoserine lactone (acyl-HSL)-based quorum sensing (QS) will be presented using the *Vibrio fischeri lux* regulon as a model system. Similar QS systems have been identified within the Gram-negative phylum *Proteobacteria* and the synthesis and recognition of diverse acyl-HSLs provides different bacterial species with their own chemical languages. Engineering of the QS transcriptional regulator LuxR is the subject of this work and the studies previously used to characterize this protein will be reviewed in detail. The diversity of LuxR homologs found in species of

α-, β-, and γ-*Proteobacteria* will also be introduced. Previous efforts to engineer LuxR, its homologs, and other transcription factors will also be discussed.

The *lux* system has been used to engineer *de novo* regulatory circuits requiring intercellular communication. LuxR homologs have been investigated, but these can show significant crosstalk and often also behave differently from LuxR in other ways. For applications in synthetic biology, it would be useful to have a set of 'standardized parts'—for example, a series of LuxR variants that respond to different chemical signals or activate gene expression at different promoters from which new networks can be assembled.

A postdoc in our laboratory, Yohei Yokobayashi, initiated the work towards applying the techniques of directed evolution to the engineering of transcription factors and synthetic genetic circuits. He showed that a detuned regulatory network, wherein the λ repressor CI was ubiquitously preventing expression at a target promoter, could be converted to a functional signal-dependent inverter through directed evolution of the CI protein. In the process, a library of genetic devices, with a range of new behaviors, was generated. I decided to apply a similar approach to the engineering of LuxR and initiated the project by targeting its response to a non-cognate acyl-HSL, octanoyl-HSL (C8HSL). LuxR requires 200-fold higher concentrations of C8HSL to achieve levels of activation observed with LuxR's cognate signal, 3-oxohexanoyl-homoserine lactone, 3OC6HSL.

Chapter 2 describes the implementation of a LuxR-dependent screen and the identification of LuxR variants with increased sensitivity to C8HSL. A LuxR reporter system in which gene activation leads to the production of green fluorescent protein (GFP) was constructed wherein high-throughput screening of LuxR-expressing colonies is achieved by exciting a plate of colonies with ultraviolet light and visually inspecting colonies for emission of green light. A library of *luxR* mutants was generated by random mutagenesis and screened for *gfp* activation with 200 nM C8HSL. Wild-type LuxR requires approximately 2 μM C8HSL to achieve the level of gene activation obtained with 10 nM 3OC6HSL. Six C8HSL-responsive LuxR variants were identified from a library of approximately 20,000 clones. These mutants were recombined and a library of approximately 1200 clones was screened for their ability to activate gene expression with 50 nM C8HSL. Eight LuxR variants were identified that showed a 100-fold increase in sensitivity to C8HSL; these variants also displayed increased sensitivities to pentanoyl-HSL and tetradecanoyl-HSL, while maintaining a wild-type or greater response to 3OC6HSL. The most sensitive variants activated transcription as strongly with C8HSL as the wild type did with 3OC6HSL. Three 'hypersensitive' variants were also identified that showed increased activation with 3OC6HSL.

All of the C8HSL-responsive LuxR variants identified showed broadened acyl-HSL specificity profiles and all responded to 3OC6HSL as well, or better, than wild type. We wanted to continue the process of engineering LuxR variants by increasing its specificity for a new acyl-HSL signal. To do this we needed to be able to identify LuxR variants

that both activate gene expression with the new target and do not respond to 3OC6HSL. Chapter 3 describes the implementation and evaluation of a dual selection system capable of identifying LuxR variants that either activate gene expression (positive selection, ON) or do not (negative selection, OFF) under desired sets of conditions. The ON/OFF selection system was adapted from a system developed by Y. Yokobayashi for use with the λ CI repressor. Modification of the dual selection system for use with an activator was required. To test the modified ON/OFF selection system we selected a plasmid encoding wild-type LuxR from a background of nonfunctional clones and showed that a single round of ON/OFF selection yields a 490-fold enrichment of functional clones. This result, which suggests that a functional LuxR variant can be identified from a library of 100 million in only three rounds of ON/OFF selection, demonstrated the potential of our dual selection system and led us to begin selecting libraries for LuxR variants exhibiting new acyl-HSL specificities with confidence that we could identify the desired variant if it existed in the library.

The parent used for the evolution of acyl-HSL specificity was LuxR-G2E, which activates gene expression upon binding a broadened range of acyl-HSLs, including C10HSL. The application of the ON/OFF selection system to identify a LuxR variant that retains the increase in sensitivity to C10HSL achieved by LuxR-G2E, but that has lost its ability to recognize and respond to LuxR's cognate signal 3OC6HSL is presented in chapter 4. A library was generated by random mutagenesis and LuxR variants were selected for the ability to survive ON rounds of selection with C10HSL and OFF rounds

of selection with 3OC6HSL. A single mutation, arginine 67 to methionine in the N-terminal signal-binding domain, reduces LuxR-G2E's response to acyl-HSLs having a carbonyl substituent at the third carbon of the acyl chain. This mutation has a similar effect when introduced into a LuxR homolog that responds to a different 3-oxo-HSL (3OC12HSL), LasR from *Pseudomonas aeruginosa*. This specificity-enhancing mutation does not affect LuxR response to straight-chain acyl-HSLs and would not have been identified by positive selection for activation by these signaling molecules. This work demonstrates that accumulation of a small number of point mutations is sufficient to switch specificity from one signal to another, passing through a broadly-specific intermediate. We expect that this dual selection system could be used to further refocus the specificity towards a single acyl-HSL by selecting for the absence of gene activation with multiple acyl-HSLs during OFF rounds of selection.

While LuxR and its homologs specifically recognize very diverse acyl-HSL signal molecules, they can often activate gene expression at each other's promoters. LuxR variants with new DNA binding specificities would be a powerful addition to the components available to synthetic biologists. The ability to use a single cell type to recognize and specifically respond to different acyl-HSLs by activating gene expression at different promoters would increase the capabilities of current communications systems. Also, by generating the components in the lab from a well-characterized, well-behaved starting point, one can target the desired properties from the beginning. To address this challenge, we applied our dual selection system to modify the DNA-binding specificity

of LuxR. Chapter 5 describes the selection of LuxR variants that activate gene expression at a mutated *lux* promoter at which LuxR does not activate gene expression. We generated a library by random mutagenesis and selected mutants activate gene expression at the new promoter. We also selected for decreased activation at the wild-type *lux* promoter. The two LuxR variants identified contain a mutation at amino acid position 212, from an arginine to either a histidine or a cysteine. The R212C mutation was accompanied by a second mutation, Q232H, and these two mutations are shown to act synergistically to confer the desired change in DNA-binding specificity. The promoter specificity of LuxR-R212C/Q232H was broadened while LuxR-R212H showed a preference for the new promoter. Both variants required approximately 100-fold higher concentrations of 3OC6HSL to achieve levels of activation observed with wild-type LuxR and the *lux* promoter. We have generated the first LuxR variants that respond to a new promoter and shown that random mutagenesis can be used to identify residues critical for DNA-binding specificity.

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ABBREVIATIONS

QS	Quorum sensing
Tc	Tetracycline
Cm	Chloramphenicol
Cr	Carbenicillin
bp	Base pairs
GFP	Green fluorescent protein
HTH	α-helix-turn-α-helix
IPTG	Isopropyl-β-D-thiogalactopyranoside
Acyl-HSL	Acyl-homoserine lactone
C4HSL	Butanoyl-homoserine lactone
C5HSL	Pentanoyl-homoserine lactone
C6HSL	Hexanoyl-homoserine lactone
3OC6HSL	3-Oxo-hexanoyl-homoserine lactone
C8HSL	Octanoyl-homoserine lactone
C10HSL	Deconoyl-homoserine lactone
C12HSL	Dodecanoyl-homoserine lactone
3OC12HSL	3-Oxo-dodecanoyl-homoserine lactone
C14HSL	Tetradecanoyl-homoserine lactone
PCR	Polymerase chain reaction

CHAPTER 1

Introduction

A. Genetic circuit engineering and intercellular communication

The ability to sense and respond to chemical stimuli is essential for the survival of all living organisms. Recognition of a chemical signal leads to a response that, hopefully, allows an organism to adapt and thus increase its probability of survival. Often, changes in gene expression occur following recognition of a specific chemical signal. Regulation of gene expression is crucial for interactions with the environment and for complex processes including development, apoptosis, circadian rhythms and biofilm formation. Pieces of the regulatory machinery required for modulating gene expression, including promoters, repressors and activators, have been borrowed from these complex regulatory networks in order to build simple networks commonly called 'genetic circuits.' Constructing genetic circuits *de novo* provides new opportunities to understand the governing principles and design strategies inherent in functional regulatory systems [1, 2]. By combining regulatory components from unrelated systems and generating simple regulatory circuits, researchers can gain an understanding of how more complex natural systems function in a controlled manner where perturbing a single component or condition leads to more easily interpreted results.

Initial work towards engineering synthetic genetic circuits has combined regulatory elements to produce simple circuits including toggle switches and a genetic oscillators [2-5]. Combining regulatory elements and generating a new, functional circuit, however, is not a trivial endeavor. These circuits are assembled from unrelated elements that have

not been optimized to work in the new configuration. An important challenge in engineering genetic circuits is to tune the components to function optimally in the new network. One way to address this problem is to apply what is known about the regulatory components and network design from previous biochemical, genetic and systems analyses to rationally adjust the parameters of the circuit components [6, 7]. A complenentary approach is to use directed evolution, the iterative process of generating genetic diversity and screening or selecting for a desired function, to identify functional circuits [8].

As circuit engineers become more competent at generating functional systems, increasingly complex circuits will become more feasible design goals [9]. Engineering increasingly complex behaviors, such as those that mimic developmental processed like pattern formation and tissue development or assembly of microbial communities such as biofilms, become a more attainable goal with the addition of elements of intercellular communication systems [10]. Bacterial 'quorum sensing' components can be used to send and receive chemical signals from one cell to another. Quorum sensing (QS) refers to the ability of a bacterium to perceive and respond to fluctuations in cell-population density. In general, bacteria produce and release small chemical signal molecules that increase in concentration as a function of cell density [11, 12]. The detection of these signal molecules leads to changes in gene expression that presumably increase the fitness of the host organism.

QS systems found in bacteria from the Gram-negative phylum *Proteobacteria* use the synthesis and detection of acyl-homoserine lactones (acyl-HSLs) [11, 13, 14]. In these systems an acyl-HSL synthase produces the diffusible signaling molecule and an acyl-HSL-responsive transcription factor activates transcription following signal binding [12]. The levels of acyl-HSL synthesis in these organisms are sufficiently low to prevent accumulation of the signals at low cell densities. At high cell densities, acyl-HSL accumulates in the cell and is bound by the transcriptional activator leading to changes in gene regulation. The potential for these components to be used in synthetic intercellular communications circuits was demonstrated by Weiss and Knight, who expressed the acyl-HSL-responsive activator in *E. coli* to produce 'receiver' cells that would respond to signals produced by 'sender' cells synthesizing the acyl-HSL signal [15].

The components used by Weiss and coworkers are from the *lux* QS system found in *Vibrio fischeri*. The acyl-HSL synthase found in *V. fisheri*, LuxI, produces 3-oxo-hexanoyl-homoserine lactone (3OC6HSL), which is recognized by the 3OC6HSL-dependent transcriptional activator LuxR. We chose to target the *lux* system as the starting point for engineering novel intercellular signaling components. The *lux* system was first discovered in 1970 and has since become one of the best characterized QS systems [11, 13, 16]. By evolving the quorum-sensing signal response regulator, LuxR, we hoped to explore the evolution of signal specificity and response in acyl-HSL-based QS systems and to develop generally applicable tools for the identification of transcriptional response regulators that are optimized to function in a new context.

The components of the *lux* QS system have been successfully incorporated into synthetic genetic circuits requiring cell-cell communication [15, 17-20]. One of these examples used a LuxR variant identified in this work [17]. Using 'receiver' cells containing a LuxR-based band-detect circuit that express a fluorescent protein over a limited range of signaling molecule concentrations, Basu *et al.* [17] demonstrated that an undifferentiated lawn of the 'receiver' cells generated a ring of gene expression around a central colony of 'sender' cells producing 3OC6HSL. A second band-detect circuit was generated by replacing wild-type LuxR with a 'hypersensitive' LuxR variant that requires lower concentrations of 3OC6HSL to activate gene expression. Plating a mixture of two strains containing the two band-detect circuits, each controlling the expression of different fluorescent reporter, led to the production of a bull's-eye pattern around a colony of sender cells (see appended publication [17]). The band of fluorescence observed with the evolved LuxR variant was further away from the sender cells, and therefore at lower concentrations of 3OC6HSL, than the band of fluorescence from the cells containing wild-type LuxR. This work demonstrated both the feasibility of using bacterial communication components to engineer systems that emulate complex processes such as pattern formation, and that LuxR variants with new behaviors are useful additions to the toolbox of components available for engineering genetic circuits.

In this chapter, I will briefly review the components involved in acyl-HSL-dependent QS and previous work towards engineering these systems and other transcription factors.

Particular emphasis will be placed on LuxR and its homologs, specifically their signal and DNA-binding specificities.

B. Acyl-homoserine lactone dependent transcriptional activation

The *lux* system from *Vibrio fischeri* was the acyl-HSL-based QS system discovered [16]. *V. fisheri* is a bioluminescent marine bacterium that colonizes the light organs of a variety of marine fish and squid, where it is found at very high cell densities (10^{10} cells/mL) and produces light. Nealson *et al.* were the first to report the role of a chemical signal molecule in the regulation of the light-producing enzyme luciferase [16]. This signal was later determined to be 3-oxo-hexanoyl-L-homoserine lactone (3OC6HSL) by ^{1}H NMR spectroscopy [21].

B.1. Mechanism of QS activation in V. fischeri

In *V. fischeri*, the 3OC6HSL signal is synthesized by the acyl-HSL synthase, LuxI. In environments of low cell density, the amphipathic acyl-HSL diffuses out of the cell through the membrane [22]. As the local environment becomes more crowded, the acyl-HSLs accumulate in the cell, where they bind to the acyl-HSL-dependent transcriptional activator, LuxR [23, 24]. Upon signal binding, LuxR activates transcription at the *lux* promoter leading to light production through the transcription of the luciferase (*lux*)

genes. Transcription of the *lux* genes is essential for *V. fisheri's* symbiotic relationship with marine eukaryotes [25]. In exchange for light production, *Euprymna scolopes*, the Hawaiian bobtail squid, provides *V. fischeri* with branch chain amino acids to support dense populations and luminescence.

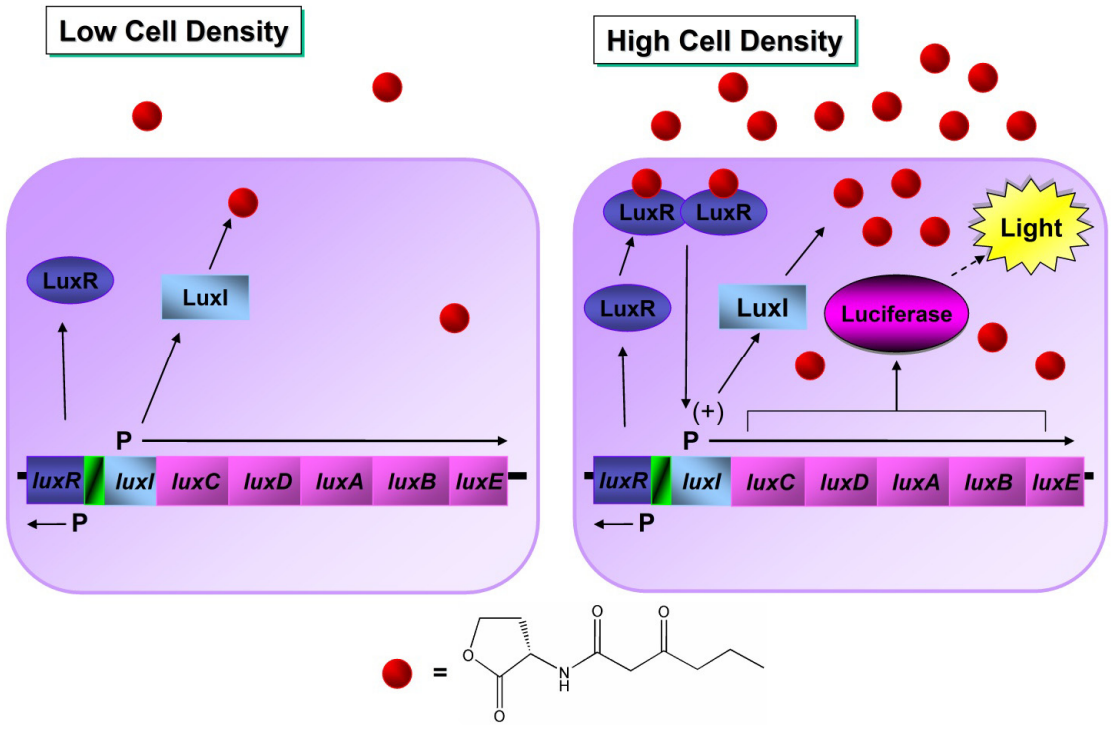

Figure 1.1. Schematic of the *Vibrio fischeri* quorum sensing system. The left panel depicts the regulatory state at low cell density. Low, basal expression of LuxI produces a small amount of 3OC6HSL, which diffuses out of the cell. The right panel shows that at high cell density accumulation of 3OC6HSL leads to its binding by LuxR. LuxR-3OC6HSL complexes dimerize and activate gene expression at the lux promoter leading to increased expression of the *lux* genes required for light production, *luxCDABE*, as well as *luxI* and *luxR*.

B.2. Homologous acyl-HSL dependent QS systems from the α-,β- and γ-Proteobacteria

Pairs of LuxI/LuxR homologs have been identified in many species of Gram-negative *Proteobacteria* (Fig. 1.2) [26, 27] where they regulate genes with diverse functions in response to changes in population density [28-33]. Different species of *Proteobacteria* synthesize and respond to acyl-HSLs with acyl chains of varying lengths and chemical modifications [34, 35] (Fig. 1.3). Due to the diversity of acyl-HSL signals used by different bacterial species and the high degree of specificity of both acyl-HSL production by LuxI homologs and acyl-HSL response by LuxR homologs, this type of QS has been postulated to be species specific [11].

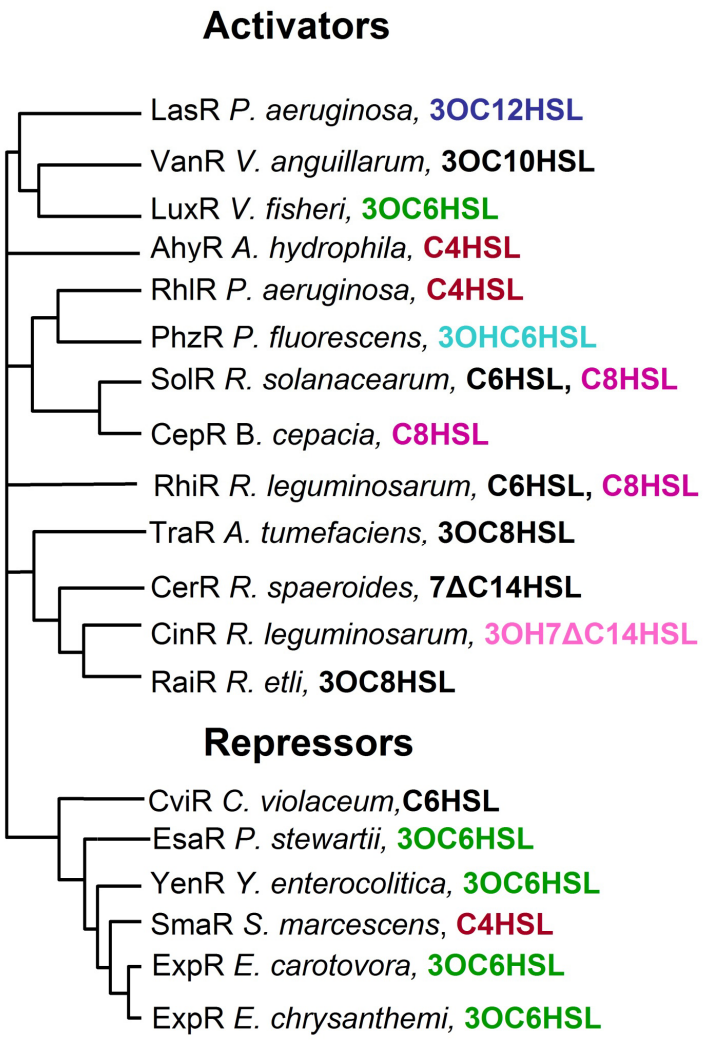

Figure 1.2. Diversity of LuxR homologs and acyl-HSLs found in Nature. Illustration of the divergence of selected LuxR homologs. Homologs are listed with both species and cognate acyl-HSL signal molecule. The tree was adapted from Gray and Garey, and Lerat and Moran [26, 27].

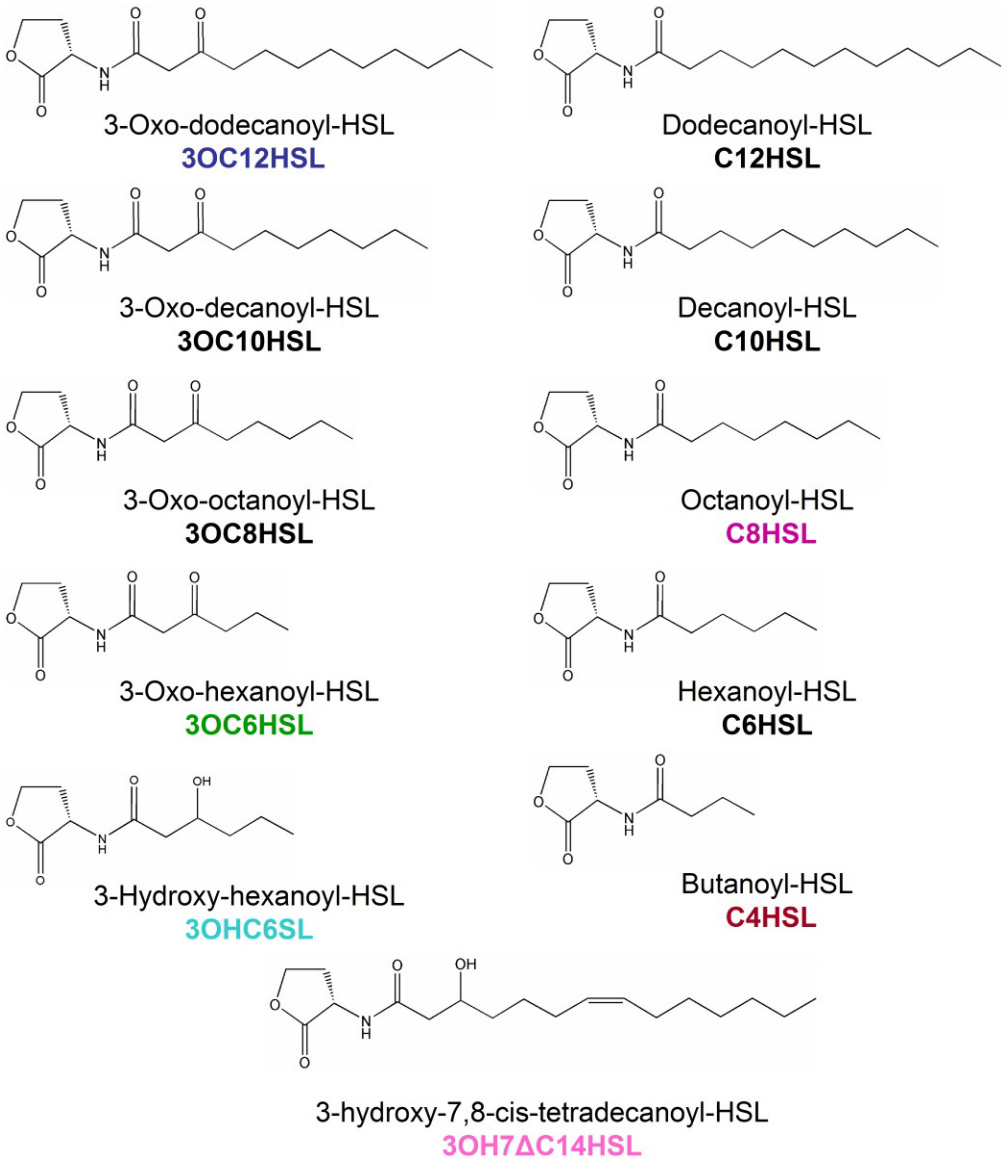

Figure 1.3. Structures, non-IUPAC names and abbreviations of the acyl-HSLs that activate the homologs listed in Fig. 1.2., as well as the acyl-HSLs used in these studies, are shown.

C. Elements of acyl-homoserine lactone-dependent quorum sensing systems

The essential elements for acyl-HSL-dependent quorum sensing are an acyl-HSL synthase, an acyl-HSL-responsive transcriptional regulator and a target promoter. A LuxR homolog typically responds to an acyl-HSL synthesized by its partner LuxI homolog. The precise arrangement and regulation of these QS systems vary from organism to organism, and there appear to be exceptions to every rule. We will focus on the *lux* system to introduce these essential elements. Important work regarding homologous systems will also be presented.

C.1. The lux operon

The *lux* operon was first characterized following its cloning into *E. coli* [36]. As shown in Figure 1.4, the *lux* genes are arranged into two divergently transcribed promoters separated by 155 base pairs (bp). One promoter, P_{luxR}, contains *luxR* and the other, P_{luxI}, contains *luxI* and the six other *lux* genes, *luxCDABEG*, required for light production. Gene expression from P_{luxI} is activated by LuxR and requires the presence of 3OC6HSL. Expression of *luxR* was shown to depend on cyclic-AMP receptor protein (CRP) [37, 38], and a CRP-binding site was subsequently identified between *luxI* and *luxR* [39]. LuxR also appears to regulate its own expression, both positively and negatively, and depends on cellular LuxR concentrations and a downstream element in the DNA encoding *luxD*

[37, 40-42]. The precise mechanisms of *luxR* autoregulation have not been fully elucidated.

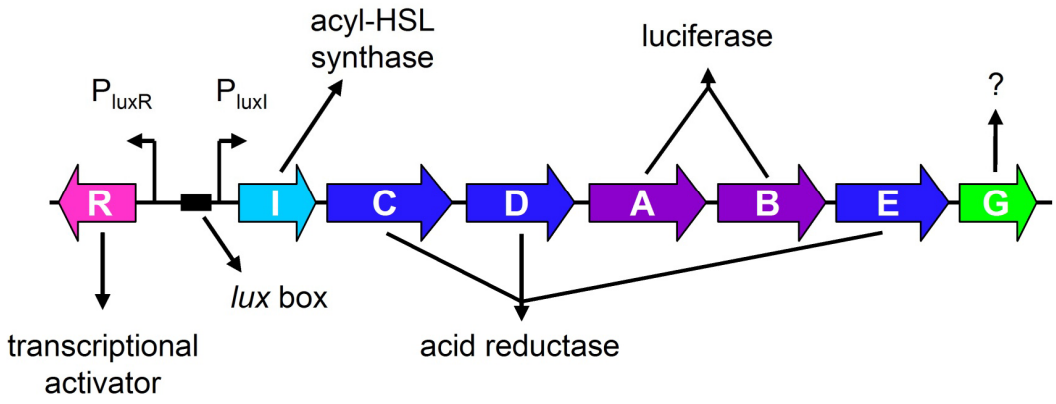

Figure 1.4. *Vibrio fischeri lux* gene organization. The *luxR* gene encoding the acyl-HSL-dependent transcriptional activator is transcribed from P_{luxR}. P_{luxI} contains *luxI*, which encodes the acyl-HSL synthase, followed by the five lux genes required for light production, *luxCDABE*, and an additional gene of unknown function *luxG*. The *luxA* and *luxB* genes encode the α- and β-components of luciferase. The *luxC*, *luxD* and *luxE* genes encode an acid reductase required for the production of the luciferase substrate, tetradecanal, from tetradecanoic acid.

LuxR activation of P_{luxI} requires a 20 bp inverted repeat centered 42.5 bp upstream of the P_{luxI} transcriptional start site called the *lux* box [43, 44]. Both halves of the *lux* box's inverted repeat are required for LuxR-mediated gene activation, and the precise positioning of the *lux* box relative to the transcriptional start site is also imperative [44]. The *lux* box was first implicated as the LuxR binding site by the demonstration that point

mutations in the *lux* box could eliminate P_{luxI} activation in *E. coli* [43]. Similar nucleotide sequences have been identified upstream of many promoters regulated by LuxR homologs. TraR, the LuxR homolog from *Agrobacterium tumefaciens*, was the first homolog shown to directly bind to the *tra* box in DNAseI protection assays [45]. Recently, purified LuxR was shown to directly protect the *lux* box *in vitro* [46]. LuxR homologs TraR, LasR, QscR and CepR have also been shown to bind to lux-type boxes *in vitro* [47-50].

C.2. Acyl-HSL synthesis by LuxI

LuxI catalyzes acyl-HSL formation from S-adenosyl-L-methionine (SAM) and acyl-acyl-carrier protein (acyl-ACP) [51], both of which are readily available in the cell. SAM is acylated by acyl-ACP and its methionine moiety lactonized to produce acyl-HSLs (Fig. 1.5). LuxI homologs synthesize acyl-HSLs with acyl chains from 4 to 16 carbons in length [35, 52]. The third carbon of the acyl chain is often modified to a carbonyl or, less frequently, a hydroxyl group [52]. Acyl-HSLs containing a single degree of unsaturation have also been identified [53]. The formation of acyl-HSLs with specific acyl chains by LuxI proteins is a result of their ability to specifically recognize only ACPs charged with the "correct" acyl moieties. The crystal structure of EsaI, the LuxI homolog from *Pantoea stewartii* that produces 3OC6HSL, identified a hydrophobic cavity that recognizes the acyl chain of the acyl-ACP [54]. The size of this pocket was hypothesized to confer EsaI's preference for acyl chains six carbons in length. In contrast, the crystal

structure of LasI, the LuxI homolog from *Pseudomonas aeruginosa* responsible for 3-oxo-dodecanoyl-homoserine lactone (3OC12HSL) production, contains a hydrophobic tunnel for acyl chain recognition that places no apparent restriction on acyl chain length [55]. Molecular modeling and mutagenesis studies indicate that the recognition of the ACP may play a role in recognizing acyl-ACPs with longer acyl chains. Gould *et al*. [55] hypothesize that, because longer acyl chains will have intrinsically higher binding affinities for the hydrophobic tunnel than shorter acyl chains, the required contribution from the ACP for binding by the enzyme is lower. Recognition of acyl-ACPs by LuxI homologs that make acyl-HSLs with shorter acyl chains likely requires the ACP to make a larger contribution to binding affinity. However, binding of acyl-ACPs carrying long acyl chains are sterically precluded from binding.

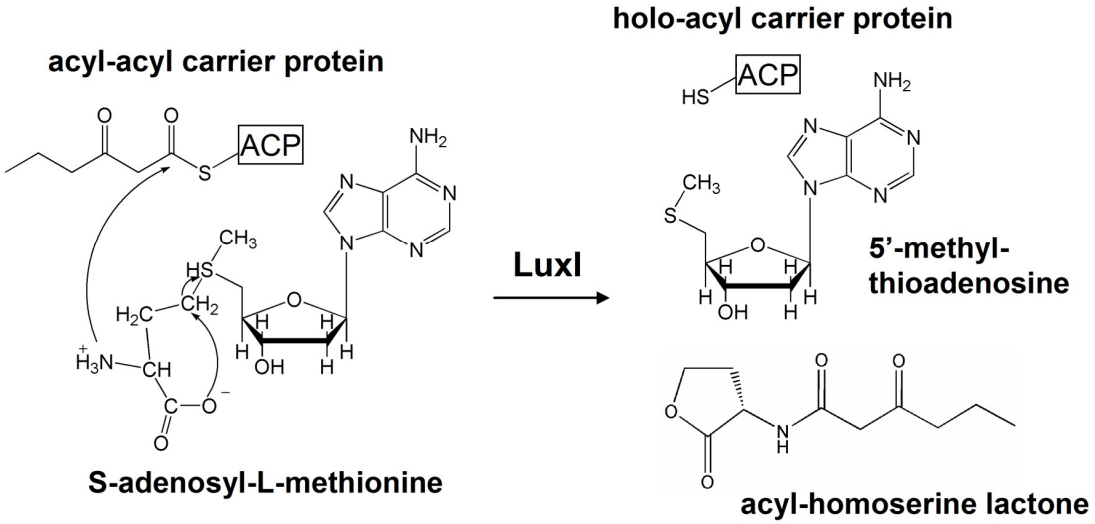

Figure 1.5. Schematic of acyl-HSL synthesis by LuxI from acyl-acyl-carrier protein and S-adenosyl-L-methionine.

A LuxI homolog was recently engineered to produce an acyl-HSL with a longer acyl chain [56]. The sequences of closely related LuxI homologs from *Erwinia* strains producing either 3OC6HSL or 3OC8HSL were examined to identify amino acids that differed between the two groups. Two mutations were shown to be sufficient to change a 3OC6HSL synthase to a 3OC8HSL synthase. An alignment with the crystal structure of EsaI indicates that the two changes may increase the size of the binding pocket, however, the precise molecular mechanisms that control the specificity of acyl-HSL production remain elusive. Furthermore, attempts at switching the specificity from 3OC8HSL to 3OC6HSL by targeting the same residues produced nonfunctional enzymes.

C.3. Acyl-HSL-mediated gene activation by LuxR homologs

LuxR recognizes the acyl-HSL synthesized by LuxI, 3OC6HSL, and activates gene expression by increasing the affinity of RNA polymerase for the *lux* promoter [57]. Early mutagenesis studies showed that the N-terminal domain of LuxR is responsible for acyl-HSL binding while its C-terminal domain is responsible for DNA binding and activation [24, 58-60]. The mutations critical for LuxR function clustered in two regions, between residues 79 to 127 and residues 184 to 230 [24, 60]. Some of the LuxR variants containing mutations to the N-terminal domain were shown to be capable of activating gene expression with elevated levels of 3OC6HSL and were hypothesized to be involved in acyl-HSL binding. Subsequent studies verified the role of the C-terminal domain in

DNA binding and activation [58, 59]. A region between residues 116 and 160 was implicated in multimerization [61].

C.3.1. Acyl-HSL response

Following the identification of LuxR's cognate signal molecule as N-3-oxo-hexanoyl-L-homoserine lactone [21], similar chemical structures were used to probe the specificity of the response. Analogs were tested with *V. fischeri* for their inducing activity and for their ability to inhibit LuxR's ability to respond to 3OC6HSL [62]. Gene activation by LuxR was greatest in the presence of 3OC6HSL. However, acyl-HSLs with similar structures were also able to stimulate LuxR-mediated activation. The ability to study recombinant LuxR in *E. coli* led to a second set of studies that examined both binding and activation by LuxR with a set of acyl-HSL analogs [34]. Binding studies measured the amount of tritium-labeled 3OC6HSL (^{3}H-3OC6HSL) that remained associated with the *E. coli* cells, and presumably bound by LuxR, following incubation with the signal and washing to remove any unbound or loosely bound acyl-HSLs [63]. Acyl-HSL analogs were assessed for their ability to prevent the accumulation of ^{3}H-3OC6HSL in LuxR-expressing cells. ^{3}H-3OC6HSL accumulation was observed to decrease as the acyl-chain length became increasingly shorter or longer than six carbons [34]. A preference for acyl-HSLs containing a 3-oxo moiety was also observed. Compounds with substitutions in the homoserine lactone ring were not found to bind to LuxR, with the exception of

compounds with a homocysteine thiolactone ring. Compounds observed to bind to LuxR were tested for their ability to stimulate gene transcription by LuxR. None of the analogs tested was as active as 3OC6HSL. Compounds with acyl side chains between five and nine carbons in length have slight activity, while compounds with 10 or more carbon atoms showed little or no activity. Comparisons of gene activation by acyl-HSLs with or without the 3-oxo moiety showed that 3-oxo-containing acyl-HSLs are much more potent inducers. Notably, a few of the acyl-HSLs tested did not stimulate gene expression despite being able to decrease ^{3}H-3OC6HSL accumulation. The analogs shown to bind to LuxR but do not stimulate gene activation were shown to inhibit LuxR-mediated gene expression in the presence of 3OC6HSL.

The acyl-HSL binding and response specificities of TraR, LasR, CarR and CepR have also been investigated [49, 64-66]. Similar observations were made in each study: acyl chain length, and the presence or absence of the 3-oxo group, are the major determinants of specificity.

The precise mechanism of 3OC6HSL binding and the conformational changes that occur prior to activation are only beginning to be elucidated. It has been consistently observed that LuxR and its homologs accumulate *in vivo* as functional proteins only in the presence of their target acyl-HSL [45, 46, 48, 49]. Overexpression of these proteins in the absence of acyl-HSL leads to the formation of insoluble inclusion bodies [45, 48, 49, 67]. Acy-HSL binding by purified LuxR has been shown to be reversible, whereas LasR and TraR

appear to bind the acyl-HSL sufficiently tightly to be considered irreversible. Each of these proteins requires nanomolar concentrations of acyl-HSL to activate gene expression indicating that there may be a difference in the way these proteins bind their cognate signals.

The crystal structure of TraR has been determined and the residues that make contact with the acyl-HSL have been identified [68, 69]. Mutations of the amino acid residues predicted to form hydrogen bonds with 3OC8HSL were shown to be essential for wild-type protein function [70]. The amino acid residues responsible for recognition of specific acyl-HSLs have been more difficult to predict. Residues of TraR predicted to interact with the carbonyl moiety of 3OC8HSL were mutated with the goal of stimulating the response of this protein to an analog, octanoyl-HSL (C8HSL) [70]. Rather than showing improved sensitivity to C8HSL, these variants lost the strong response that wild-type TraR displays towards 3OC8HSL. The attempts of Chai and Winans to shift the specificity of TraR towards 3OC6HSL by introducing bulkier hydrophobic amino acids, and thereby reduce the size of the acyl binding site, also led, in all but one case, to variants with greatly decreased acyl-HSL affinities.

C.3.2. DNA binding and activation

The C-terminal domain of LuxR is responsible for DNA binding and gene activation. When the N-terminal, regulatory domain is absent, it is also capable of activating gene expression in the absence of 3OC6HSL [58]. This positive-control mutant, LuxRΔN, requires RNA polymerase (RNAP) for DNA binding. LuxRΔN and σ^{70}-bound RNAP were subsequently shown to be the only proteins required for transcription from P_{luxI} [71], indicating that LuxR activates gene expression by recruiting RNAP to P_{luxI}. Initiation of transcription by LuxRΔN has since been shown to require the C-terminal domain (CTD) of RNAP *in vitro* and activation by both LuxRΔN and full-length LuxR was drastically decreased by the overexpression of a truncated RNAP lacking the CTD [72]. Therefore, LuxR activates gene expression by recruiting RNAP to P_{luxI} through direct binding to its CTD.

Amino acid sequence analysis identified a helix-turn-helix (HTH) motif within the C-terminal domain of LuxR [73, 74]. Two alanine scanning mutagenesis experiments have identified residues involved in DNA binding and activation by LuxR [75, 76]. Several mutants unable to bind DNA or activate transcription were identified, including alanine substitutions at positions 191, 193, 212, 217, 225, 229, 230, 238, and 240. DNA binding was assessed indirectly by measuring the ability of each variant to repress a lac promoter containing a *lux* box [77]. It is impossible to determine from these results which of the mutations yields folded proteins that are unable to bind DNA and which mutations

prevent activation by affecting other properties such as protein stability, folding or dimerization. The DNA-binding HTH motif in LuxR is predicted to occur between residues 200 and 224, suggesting that several of the residues identified may be directly involved in DNA binding. Alanine substitutions at positions 201 and 206 affect transcriptional activation but not DNA binding. These residues are hypothesized to make direct contact with RNA polymerase.

The DNA sequence requirements for specific DNA-binding by LuxR have not been assessed as thoroughly as acyl-HSL specificity. The observation that LuxR and LasR are capable of activating gene expression at each other's cognate promoter suggests that the DNA-binding specificities of these proteins have not significantly diverged [78]. This is not entirely surprising because the ability to respond to species-specific signals occurs at the level of acyl-HSL binding. The ability to bind a different DNA target than another LuxR homolog becomes important when more than one LuxR homolog is present in the same cell. *P. aeruginosa*, for example, has three LuxR homologs, RhlR, LasR and QscR. Each of these homologs has been shown to bind to different target sites, although all of the sites are considered *lux*-type boxes [47, 48, 79]. Substitutions at positions 8 and -8 of the *lux*-type boxes recognized by LasR and QscR are sufficient to switch their responses between the two sites [47]. LasR is the only LuxR homolog whose DNA-binding specificity has been investigated with several potential target sites [48]. LasR was shown to bind several promoters that had previously been identified *las*-responsive. LasR did not bind to all of the predicted sites and was shown to bind at unexpected sites.

Interestingly, the sites that were bound by LasR did not show a large degree of overall sequence similarity and did not require dyad symmetry.

Prior to this work, LuxR had not been engineered to respond to new acyl-HSLs or to activate gene expression at a new promoter. A lac promoter had been modified to include a *lux* box, converting LuxR from an activator to a repressor. However, LuxR itself was not modified. As discussed above, an attempt to engineer TraR to recognize a new autoinducer based on structural information yielded proteins that showed broadened specificity but were very poor activators.

D. Engineering bacterial transcription factors

Some of the most successful examples of transcription factor engineering have come from engineering eukaryotic zinc-finger (Zn-finger) proteins [80, 81]. The modularity of Zn-fingers has made these DNA-binding proteins good targets for engineering experiments. Each finger is responsible for binding three consecutive bp of the DNA target and they can be "strung" together to bind longer DNA sequences. The functionality of Zn-fingers is also modular in that an activating domain or repressor domain need only be attached to the protein to confer its function at the target DNA site. Small molecule binding to the effector domain then affects the rate of transcription from the target promoter. In contrast, many bacterial transcription factors, including HTH-

containing proteins like LuxR, are switched ON and OFF by conformational changes that affect the whole protein. This means that engineered proteins must maintain the mechanism of signal transduction from regulatory region to DNA-binding domain and may render these proteins more challenging targets with regards to engineering new functions. Whether targeting a Zn-finger or a bacterial HTH protein, the available strategies are similar and are introduced in the following section. Examples of bacterial transcription factor engineering are then discussed.

D.1. Directed evolution and rational design

Directed evolution is an iterative process of mutation or recombination and selection or screening. It mimics the process of natural evolution by creating genetic diversity and then identifying genes encoding the "fittest" proteins based on the goals of the project. Rational design, on the other hand, uses structure and sequence information, which can be analyzed via computational methods, to identify mutations that are likely to confer the desired change in function.

In general, sequence diversity is generated for directed evolution experiments by random mutagenesis or by recombining multiple parental sequences. Libraries of mutant genes produced by random mutagenesis are especially useful when there is little or no structural information available for a target protein. We chose to use random mutagenesis to target

the acyl-HSL specificity of LuxR because, at the time, there were no structures of LuxR homologs available and, more importantly, because the sequence identity between LuxR and its homologs is very low (<25%), identifying amino acids responsible for modulating acyl-HSL specificity was not feasible from sequence information alone.

A semi-rational approach is commonly used for engineering transcription factors [82-85]. In general, structural or functional information is used to identify residues predicted to modulate the target activity, and libraries that randomize these residues are screened or selected for the desired behavior.

D.2. Screening and selection

Identification of proteins that exhibit the desired behavior from a library of variants requires the ability to relate the desired protein function to a measurable output. In a screen, a functional protein yields an easily measured product. Directed enzyme evolution experiments have often relied on the use of surrogate substrates, i.e., substrates that are chemically similar to the substrate of interest and whose products can be easily quantified colorometrically, or the use of additional enzymes that selectively convert the desired product to a readily detectable compound. One of the advantages of working with transcription factors is that protein function can easily be tied to the production of a reporter protein that generates a measurable output. Commonly used reporter proteins

include β-galactosidase, luciferase and fluorescent proteins such as green fluorescent protein (GFP) and red fluorescent protein (RFP).

Selections differ from screens in that they tie the survival of the host cell to the desired protein function. It is often the production of an essential metabolite or an increase in antibiotic resistance that leads to survival. The upregulation of a gene conferring antibiotic resistance is commonly used to identify transcription factors that are on or off under a specific set of conditions. Most antibiotic resistance proteins chemically inactivate the antibiotic through enzymatic cleavage or derivatization. For example, β-lactamase (Bla) catalyzes the opening and hydrolysis of the lactam ring of β-lactam antibiotics [86] and chloramphenicol acetyl transferase (CAT) inactivates chloramphenicol by acetylating the antibiotic [87]. TetA, on the other hand, is a pump and confers tetracycline resistance by exporting the antibiotic from the cell [88].

The major advantage to using a selection instead of a screen is potential library size. Selections can easily cover libraries containing $>10^6$ variants, while screens are often laborious and can limit library size to $\sim 10^4$. Screens, however, remain the more popular method because selections are often plagued by false positives. While false positives occur regardless of the choice between screening and selection, they occur more frequently when cells are placed under selective pressure. Any solution that leads to survival will be selected, even if it is not the solution that has been targeted by the experimenter. As will be discussed in Chapter 3, a successful selection requires an

approach that ensures the surviving bacteria express mutant proteins with the targeted properties. Another advantage to using a screen is that the output is often measured, quantitatively or qualitatively, such that mutants can be easily ranked. The graded output, as opposed to simply being alive or dead, makes it easier to identify the best variant from a pool of variants that perform the desired function.

The use of negative screening or selection also figures prominently into this work. The screens and selections described above are generally used for identifying gain-of-function mutations. As will be discussed in Chapters 3 through 5, identifying variants with loss-of-function mutations is essential for evolving specificity [89]. The absence of reporter production can be used in screens but one runs the risk of identifying nonfunctional proteins. By screening for proteins that confer a desired function prior to screening for the absence of function under a different set of conditions, one can limit the prevalence of the nonfunctional proteins. Selecting for loss-of-function mutations is more complicated because it is impossible to retrieve DNA encoding a protein of interest from a dead bacterium. The most common way to implement negative selections is through the use of a repressor protein as an inverter. In this case, gene activation leads to the production of a repressor, which prevents the production of an antibiotic resistance gene, leading to antibiotic sensitivity. Toxic protein production can be employed in negative selections. In general, the basal level of expression of most toxic proteins leads to a prevalence of false positives and the use of a repressor to invert the signal provides an additional target at which the selection system can be undermined. Yokobayashi and Arnold have

developed a dual selection system that employs an inhibitor of Bla to directly target the protein that confers the ability to survive with β-lactam antibiotics [90].

D.3. Engineering signal binding

The tetracycline repressor (TetR) is a well-studied bacterial transcription factor that has been engineered to both recognize new small molecules and to bind to new DNA sequences [83, 85, 91, 92]. TetR is responsible for repressing the expression of TetA [93]. Because overexpression of TetA is toxic to cells, TetR has evolved a very high affinity for a DNA site located upstream of the *tetA* gene to prevent its expression. TetR also has a high affinity for tetracycline (tc, Fig. 1.5) and releases its DNA target to allow *tetA* expression before tc concentrations become toxic.

Variants of TetR that bind to an analog of tc that does not affect wild-type TetR, 4-dedimethylamino-6-demethyl-6-deoxy-tetracycline (cmt3, Fig.1.6), were identified by screening libraries generated by random mutagenesis for cmt3-dependent expression of β-galactosidase [85]. Recombination by DNA shuffling and another round of screening identified a single variant with improved cmt3-inducibility. Almost half of the mutations found in the best clones from the first round were located close to or in the tc-binding region. A new library targeting amino acid positions shown to be close to the sites that differ between tc and cmt3 was generated, using the wild type as parent, and screened for

cmt3-inducibility. A single mutation was identified that was partially inducible with cmt3 but was still showed a preference for tc. Recombination with a mutation identified by random mutagenesis identified a relaxed specificity variant that is induced by cmt3 at levels comparable to wild-type TetR with tc, and that shows decreased induction with tc. A final round screened a library, generated by randomizing the same amino acids as the previous round but with the best double-mutant as parent, for a decrease in response to a potent tc analog. Variants exhibiting the desired improvements in specificity were identified.

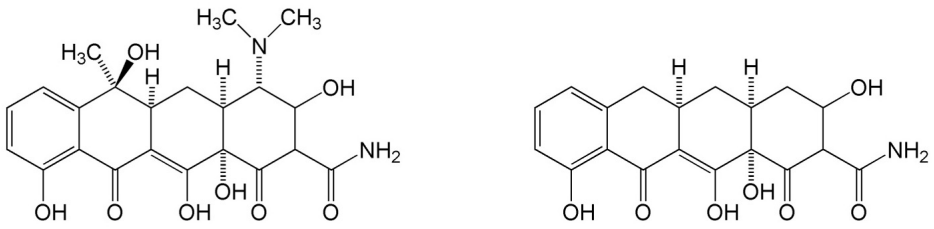

Figure 1.6. Chemical structures of tetracycline derivatives used in the directed evolution of TetR.

Engineering TetR to respond to cmt3 successfully incorporated both random and semi-rational library design strategies. Mutations critical for modulating TetR's response to tc and its cmt3 analog were identified. The relaxed specificity variant identified while screening for cmt3 was subsequently used as a parent to identify a TetR mutant inducible

by a different tc analog [92]. Two residues were targeted for randomization due to their proximity to the moiety that differs between tc and the new analog in the TetR. A single mutation was identified that conferred a 200-fold switch in specificity.

A eukaryotic activation domain has been fused to TetR to generate a hybrid tc-dependent transcriptional activator (tTA) that functions in mammalian cells [94]. This system is widely used as an inducible expression system in eukaryotes ranging from yeast to mice [95]. Furthermore, a set of eukaryotic, TetR-based transactivators that respond to cmt3 but not tc were developed from the cmt3-specific TetR variants to allow selective regulation of two genes by the different inducer molecules [96].

The majority of studies for the modification of signal-dependent transcription factors have altered the binding specificity of eukaryotic activation domains [97]. Further examples of engineering signal binding by bacterial transcription factors are unavailable.

D.4. Engineering DNA-binding interactions

The HTH DNA-binding motif, which is found in LuxR, is commonly found in prokaryotic transcriptional regulators. Examples of HTH engineering in the literature are scarce and the examples that are available have relied on structural information regarding the interactions between the protein and its target site [82, 84, 98-106]. Many published HTH engineering experiments have used solutions already present in Nature, including

swapping an entire DNA recognition helix [104] or replacing all of the amino acids that contact a specific DNA target recognized by a close homolog [105]. This strategy is not always successful [98] and does not generate DNA-binding proteins that recognize novel DNA sequences. Attempts at engineering HTH proteins that bind to previously unrecognized sequences have generally relied on libraries that randomize the amino acids shown to make specific contacts with DNA [82-84, 91]. In most cases, modest gains can be accomplished in this manner, however, new specificities are rare. Occasionally, mutations at residues that are not targeted have arisen adventitiously and are critical for binding the new target [91].

One of the successful strategies for engineering HTH proteins is to engineer single-chain proteins. Single-chain proteins that are covalent dimers of the DNA-binding domains from different transcription factors have also been shown to bind to hybrid operators [107-110]. Single-chain proteins that bind to new asymmetric DNA targets have been identified by screening [111] and phage display [102]. More recently, Liang et al. [112] constructed several single-chain heterodimers using engineered 434 repressor DNA-binding domains (identified in previous rational design and selection experiments), which also bound operators made up of half-sites bound by the individual domains. To achieve high DNA-binding affinities (K_d = $10^{-10\text{-}11}$ M), the single-chain proteins developed by Liang and colleagues require that the six base pair (bp) sequence between the two half-sites contains mostly A and T nucleotides.

The most successful efforts towards engineering the DNA-binding specificities of prokaryotic proteins have switched the DNA-binding specificity of Cre and Flp recombinases. Work from two independent laboratories used screens incorporating positive and negative selection pressure to identify variants that recombine only at a new target site [113-115]. In one of these experiments, directed evolution of the DNA-binding specificity of a Cre recombinase using positive screening alone and a combination of positive and negative screening showed that both positive and negative selective pressure were required to switch Cre's DNA-binding specificity [113].

E. References

1. Benner, S.A. & Sismour, A.M. Synthetic biology. *Nat. Rev. Genet.* **6**, 533-543 (2005).

2. Elowitz, M.B. & Leibler, S. A synthetic oscillatory network of transcriptional regulators. *Nature* **403**, 335-338 (2000).

3. Atkinson, M.R., Savageau, M.A., Myers, J.T. & Ninfa, A.J. Development of genetic circuitry exhibiting toggle switch or oscillatory behavior in *Escherichia coli*. *Cell* **113**, 597-607 (2003).

4. Fung, E., Wong, W.W., Suen, J.K., Bulter, T., Lee, S.G. & Liao, J.C. A synthetic gene-metabolic oscillator. *Nature* **435**, 118-122 (2005).

5. Gardner, T.S., Cantor, C.R. & Collins, J.J. Construction of a genetic toggle switch in *Escherichia coli*. *Nature* **403**, 339-342 (2000).

6. Weiss, R. Ph.D. Thesis, Massachusetts Institute of Technology (2001).

7. Weiss, R. & Basu, S. in *First Workshop on Non-Silicon Computing (www.hpcaconf.org/hpca8)* (2002).

8. Yokobayashi, Y., Weiss, R. & Arnold, F.H. Directed evolution of a genetic circuit. *Proc. Natl. Acad. Sci. USA* **99**, 16587-16591 (2002).

9. Sprinzak, D. & Elowitz, M.B. Reconstruction of genetic circuits. *Nature* **438**, 443-448 (2005).

10. Weiss, R. Challenges and opportunities in programming living cells. *The Bridge* **32**, 39-46 (2004).

11. Taga, M.E. & Bassler, B.L. Chemical communication among bacteria. *Proc. Natl. Acad. Sci. USA* **100**, Suppl. 2, 14549-14554 (2003).

12. Whitehead, N.A., Barnard, A.M.L., Slater, H., Simpson, N.J.L. & Salmond, G.P.C. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol. Rev.* **25**, 365-404 (2001).

13. Fuqua, C. & Greenberg, E.P. Listening in on bacteria: Acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 685-695 (2002).

14. Greenberg, E.P. Acyl-homoserine lactone quorum sensing in bacteria. *J. Microbiol.* **38**, 117-121 (2000).

15. Weiss, R. & Knight, T.F. Engineered communications for microbial robotics. *DNA6: Sixth International Meeting on DNA Based Computers*, 1-15 (2000).

16. Nealson, K.H., Platt, T. & Hastings, J.W. Cellular control of synthesis and activity of bacterial luminescent system. *J. Bacteriol.* **104**, 313-322 (1970).

17. Basu, S., Gerchman, Y., Collins, C.H., Arnold, F.H. & Weiss, R. A synthetic multicellular system for programmed pattern formation. *Nature* **434**, 1130-1134 (2005).

18. Basu, S., Mehreja, R., Thiberge, S., Chen, M.T. & Weiss, R. Spatiotemporal control of gene expression with pulse-generating networks. *Proc. Natl. Acad. Sci. USA* **101**, 6355-6360 (2004).

19. Kobayashi, H., Kaern, M., Araki, M., Chung, K., Gardner, T.S., Cantor, C.R. & Collins, J.J. Programmable cells: Interfacing natural and engineered gene networks. *Proc. Natl. Acad. Sci. USA* **101**, 8414-8419 (2004).

20. You, L.C., Cox, R.S., Weiss, R. & Arnold, F.H. Programmed population control by cell-cell communication and regulated killing. *Nature* **428**, 868-871 (2004).

21. Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. & Oppenheimer, N.J. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**, 2444-2449 (1981).

22. Kaplan, H.B. & Greenberg, E.P. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**, 1210-1214 (1985).

23. Engebrecht, J. & Silverman, M. Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **81**, 4154-4158 (1984).

24. Slock, J., Vanriet, D., Kolibachuk, D. & Greenberg, E.P. Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. *J. Bacteriol.* **172**, 3974-3979 (1990).

25. Ruby, E.G. Lessons from a cooperative, bacterial-animal association: The *Vibrio fischeri Euprymna scolopes* light organ symbiosis. *Annu. Rev. Microbiol.* **50**, 591-624 (1996).

26. Gray, K.M. & Garey, J.R. The evolution of bacterial LuxI and LuxR quorum sensing regulators. *Microbiology-(UK)* **147**, 2379-2387 (2001).

27. Lerat, E. & Moran, N.A. The evolutionary history of quorum-sensing systems in bacteria. *Mol. Biol. Evol.* **21**, 903-913 (2004).

28. Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. & Greenberg, E.P. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**, 295-298 (1998).

29. Lewenza, S., Conway, B., Greenberg, E.P. & Sokol, P.A. Quorum sensing in *Burkholderia cepacia*: Identification of the LuxRI homologs CepRI. *J. Bacteriol.* **181**, 748-756 (1999).

30. Passador, L., Cook, J.M., Gambello, M.J., Rust, L. & Iglewski, B.H. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127-1130 (1993).

31. Pirhonen, M., Flego, D., Heikinheimo, R. & Palva, E.T. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO J.* **12**, 2467-2476 (1993).

32. Valade, E., Thibault, F.M., Gauthier, Y.P., Palencia, M., Popoff, M.Y. & Vidal, D.R. The PmlI-PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease. *J. Bacteriol.* **186**, 2288-2294 (2004).

33. Zhang, L.H., Murphy, P.J., Kerr, A. & Tate, M.E. Agrobacterium conjugation and gene-regulation by N-acyl-L-homoserine lactones. *Nature* **362**, 446-448 (1993).

34. Schaefer, A.L., Hanzelka, B.L., Eberhard, A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Probing autoinducer-LuxR interactions with autoinducer analogs. *J. Bacteriol.* **178**, 2897-2901 (1996).

35. Schaefer, A.L., Taylor, T.A., Beatty, J.T. & Greenberg, E.P. Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *J. Bacteriol.* **184**, 6515-6521 (2002).

36. Engebrecht, J., Nealson, K. & Silverman, M. Bacterial bioluminescence - Isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* **32**, 773-781 (1983).

37. Dunlap, P.V. & Greenberg, E.P. Control of *Vibrio fischeri lux* gene-Transcription by a cyclic-AMP receptor protein LuxR protein regulatory circuit. *J. Bacteriol.* **170**, 4040-4046 (1988).

38. Dunlap, P.V. & Greenberg, E.P. Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic-AMP and cyclic-AMP receptor protein. *J. Bacteriol.* **164**, 45-50 (1985).

39. Shadel, G.S., Devine, J.H. & Baldwin, T.O. Control of the *lux* regulon of *Vibrio fischeri*. *J. Biolumin. Chemilumin.* **5**, 99-106 (1990).

40. Shadel, G.S. & Baldwin, T.O. The *Vibrio fischeri* LuxR protein is capable of bidirectional stimulation of transcription and both positive and negative regulation of the *luxR* gene. *J. Bacteriol.* **173**, 568-574 (1991).

41. Shadel, G.S. & Baldwin, T.O. Identification of a distantly located regulatory element in the *luxD* gene required for negative autoregulation of the *Vibrio fischeri luxR* gene. *J. Biol. Chem.* **267**, 7690-7695 (1992).

42. Shadel, G.S. & Baldwin, T.O. Positive autoregulation of the *Vibrio fischeri luxR* gene-LuxR and autoinducer activate cAMP-catabolite gene activator protein complex-independent and complex-dependent LuxR rranscription. *J. Biol. Chem.* **267**, 7696-7702 (1992).

43. Devine, J.H., Shadel, G.S. & Baldwin, T.O. Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC7744. *Proc. Natl. Acad. Sci. USA* **86**, 5688-5692 (1989).

44. Egland, K.A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: elements of the *luxl* promoter. *Mol. Microbiol.* **31**, 1197-1204 (1999).

45. Zhu, J. & Winans, S.C. Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters in vitro and decreases TraR turnover rates in whole cells. *Proc. Natl. Acad. Sci. USA* **96**, 4832-4837 (1999).

46. Urbanowski, A.L., Lostroh, C.P. & Greenberg, E.P. Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **186**, 631-637 (2004).

47. Lee, J.-H., Lequette, Y. & Greenberg, E.P. Activity of QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Mol. Microbiol.*, doi: 10.1111/j.1365-2958.2005.04960.x (2005).

48. Schuster, M., Urbanowski, M.L. & Greenberg, E.P. Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc. Natl. Acad. Sci. USA* **101**, 15833-15839 (2004).

49. Weingart, C.L., White, C.E., Liu, S.P., Chai, Y.R., Cho, H., Tsai, C.S., Wei, Y.P., Delay, N.R., Gronquist, M.R., Eberhard, A. & Winans, S.C. Direct binding of the quorum sensing regulator CepR of *Burkholderia cenocepacia* to two target promoters in vitro. *Mol. Microbiol.* **57**, 452-467 (2005).

50. Zhu, J. & Winans, S.C. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc. Natl. Acad. Sci. USA* **98**, 1507-1512 (2001).

51. Schaefer, A.L., Val, D.L., Hanzelka, B.L., Cronan, J.E. & Greenberg, E.P. Generation of cell-to-cell signals in quorum sensing: Acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc. Natl. Acad. Sci. USA* **93**, 9505-9509 (1996).

52. Schaefer, A.L., Hanzelka, B.L., Parsek, M.R. & Greenberg, E.P. Detection, purification, and structural elucidation of the acyl-homoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Methods Enzymol.* **305**, 288-301 (2000).

53. Gray, K.M., Pearson, J.P., Downie, J.A., Boboye, B.E.A. & Greenberg, E.P. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium Rhizobium leguminosarum: Autoinduction of a stationary phase and rhizosphere-expressed genes. *J. Bacteriol.* **178**, 372-376 (1996).

54. Watson, W.T., Minogue, T.D., Val, D.L., von Bodman, S.B. & Churchill, M.E.A. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Mol. Cell* **9**, 685-694 (2002).

55. Gould, T.A., Schweizer, H.P. & Churchill, M.E.A. Structure of the *Pseudomonas aeruginosa* acyl-homoserinelactone synthase LasI. *Mol. Microbiol.* **53**, 1135-1146 (2004).

56. Brader, G., Sjoblom, S., Hyytiainen, H., Sims-Huopaniemi, K. & Palva, E.T. Altering substrate chain length specificity of an acyl-homoserine lactone synthase in bacterial communication. *J. Biol. Chem.* **280**, 10403-10409 (2005).

57. Stevens, A.M. & Greenberg, E.P. Transcriptional activation by LuxR. in *Cell-Cell Signaling in Bacteria* (eds. Dunny, G.M. & Winans, S.C.) 231-42 (ASM Press, Washington, DC, 1999).

58. Choi, S.H. & Greenberg, E.P. The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. *Proc. Natl. Acad. Sci. USA* **88**, 11115-11119 (1991).

59. Choi, S.H. & Greenberg, E.P. Genetic dissection of DNA-binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **174**, 4064-4069 (1992).

60. Shadel, G.S., Young, R. & Baldwin, T.O. Use of regulated cell-lysis in a lethal genetic selection in *Escherichia coli*-identification of the autoinducer-binding region of the LuxR protein from Vibrio fischeri ATCC-7744. *J. Bacteriol.* **172**, 3980-3987 (1990).

61. Choi, S.H. & Greenberg, E.P. Genetic evidence for multimerization of LuxR, the transcriptional activator of *Vibrio fischeri* luminescence. *Mol. Mar. Biol. Biotechnol.* **1**, 408-413 (1992).

62. Eberhard, A., Widrig, C.A., McBath, P. & Schineller, J.B. Analogs of the autoinducer of bioluminescence in *Vibrio fischeri*. *Arch. Microbiol.* **146**, 35-40 (1986).

63. Hanzelka, B.L. & Greenberg, E.P. Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. *J. Bacteriol.* **177**, 815-817 (1995).

64. Passador, L., Tucker, K.D., Guertin, K.R., Journet, M.P., Kende, A.S. & Iglewski, B.H. Functional analysis of the *Pseudomonas aeruginosa* autoinducer PAI. *J. Bacteriol.* **178**, 5995-6000 (1996).

65. Welch, M., Todd, D.E., Whitehead, N.A., McGowan, S.J., Bycroft, B.W. & Salmond, G.P.C. N-acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in *Erwinia*. *EMBO J.* **19**, 631-641 (2000).

66. Zhu, J., Beaber, J.W., More, M.I., Fuqua, C., Eberhard, A. & Winans, S.C. Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. *J. Bacteriol.* **180**, 5398-5405 (1998).

67. Kaplan, H.B. & Greenberg, E.P. Overproduction and purification of the *luxR* gene-product-transcriptional activator of the *Vibrio fischeri* luminescence system. *Proc. Natl. Acad. Sci. USA* **84**, 6639-6643 (1987).

68. Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., Neddermann, P. & Di Marco, S. The crystal structure of the quorum sensing

protein TraR bound to its autoinducer and target DNA. *EMBO J.* **21**, 4393-4401 (2002).

69. Zhang, R.G., Pappas, T., Brace, J.L., Miller, P.C., Oulmassov, T., Molyneauz, J.M., Anderson, J.C., Bashkin, J.K., Winans, S.C. & Joachimiak, A. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**, 971-974 (2002).

70. Chai, Y.R. & Winans, S.C. Site-directed mutagenesis of a LuxR-type quorum-sensing transcription factor: Alteration of autoinducer specificity. *Mol. Microbiol.* **51**, 765-776 (2004).

71. Stevens, A.M. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Essential elements for activation of the luminescence genes. *J. Bacteriol.* **179**, 557-562 (1997).

72. Stevens, A.M., Fujita, N., Ishihama, A. & Greenberg, E.P. Involvement of the RNA polymerase alpha-subunit C-terminal domain in LuxR-dependent activation of the *Vibrio fischeri* luminescence genes. *J. Bacteriol.* **181**, 4704-4707 (1999).

73. Henikoff, S., Wallace, J.C. & Brown, J.P. Finding protein similarities with nucleotide sequence databases. *Methods Enzymol.* **183**, 111-132 (1990).

74. Kahn, D. & Ditta, G. Modular structure of FixJ - Homology of the transcriptional activator domain with the -35 binding domain of sigma factors. *Mol. Microbiol.* **5**, 987-997 (1991).

75. Egland, K.A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Analysis of the LuxR DNA binding region by alanine-scanning mutagenesis. *J. Bacteriol.* **183**, 382-386 (2001).

76. Trott, A.E. & Stevens, A.M. Amino acid residues in LuxR critical for its mechanism of transcriptional activation during quorum sensing in *Vibrio fischeri*. *J. Bacteriol.* **183**, 387-392 (2001).

77. Egland, K.A. & Greenberg, E.P. Conversion of the *Vibrio fischeri* transcriptional activator, LuxR, to a repressor. *J. Bacteriol.* **182**, 805-811 (2000).

78. Gray, K.M., Passador, L., Iglewski, B.H. & Greenberg, E.P. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. *J. Bacteriol.* **176**, 3076-3080 (1994).

79. Medina, G., Juarez, K., Valderrama, B. & Soberon-Chavez, G. Mechanism of *Pseudomonas aeruginosa* RhlR transcriptional regulation of the rhlAB promoter. *J. Bacteriol.* **185**, 5976-5983 (2003).

80. Beerli, R.R. & Barbas, C.F. Engineering polydactyl zinc-finger transcription factors. *Nat. Biotechnol.* **20**, 135-141 (2002).

81. Jantz, D., Amann, B.T., Gatto, G.J. & Berg, J.M. The design of functional DNA-binding proteins based on zinc finger domains. *Chemical Reviews* **104**, 789-799 (2004).

82. Fromknecht, K., Vogel, P.D. & Wise, J.G. Combinatorial redesign of the DNA binding specificity of a prokaryotic helix-turn-helix repressor. *J. Bacteriol.* **185**, 475-481 (2003).

83. Helbl, V., Tiebel, B. & Hillen, W. Stepwise selection of TetR variants recognizing tet operator 6C with high affinity and specificity. *J. Mol. Biol.* **276**, 319-324 (1998).

84. Huang, L.X., Sera, T. & Schultz, P.G. A permutational approach toward protein-DNA recognition. *Proc. Natl. Acad. Sci. USA* **91**, 3969-3973 (1994).

85. Scholz, O., Kostner, M., Reich, M., Gastiger, S. & Hillen, W. Teaching TetR to recognize a new inducer. *J. Mol. Biol.* **329**, 217-227 (2003).

86. Matagne, A., Lamotte-Brasseur, J. & Frere, J.M. Catalytic properties of class A beta-lactamases: efficiency and diversity. *Biochem. J.* **330**, 581-598 (1998).

87. Shaw, W.V. & Leslie, A.G.W. Chloramphenicol acetyltransferase. *Annu. Rev. Biophys. Biophys. Chem.* **20**, 363-386 (1991).

88. Paulsen, I.T., Brown, M.H. & Skurray, R.A. Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60**, 575-& (1996).

89. Varadarajan, N., Gam, J., Olsen, M.J., Georgiou, G. & Iverson, B.L. Engineering of protease variants exhibiting high catalytic activity and exquisite substrate selectivity. *Proc. Natl. Acad. Sci. USA* **102**, 6855-6860 (2005).

90. Yokobayashi, Y. & Arnold, F.H. A dual selection module for directed evolution of genetic circuits. *Natural Computing* **4**, 245-254 (2005).

91. Helbl, V. & Hillen, W. Stepwise selection of TetR variants recognizing tet operator 4C with high affinity and specificity. *J. Mol. Biol.* **276**, 313-318 (1998).

92. Henssler, E.M., Scholz, O., Lochner, S., Gmeiner, P. & Hillen, W. Structure-based design of Tet repressor to optimize a new inducer specificity. *Biochemistry* **43**, 9512-9518 (2004).

93. Ramos, J.L., Martinez-Bueno, M., Molina-Henares, A.J., Teran, W., Watanabe, K., Zhang, X.D., Gallegos, M.T., Brennan, R. & Tobes, R. The TetR family of transcriptional repressors. *Microbiol. Mol. Biol. Rev.* **69**, 326-356 (2005).

94. Gossen, M. & Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89**, 5547-5551 (1992).

95. Ryding, A.D.S., Sharp, M.G.F. & Mullins, J.J. Conditional transgenic technologies. *J. Endocrinol.* **171**, 1-14 (2001).

96. Krueger, C., Schmidt, A., Danke, C., Hillen, W. & Berens, C. Transactivator mutants with altered effector specificity allow selective regulation of two genes by tetracycline variants. *Gene* **331**, 125-131 (2004).

97. Buskirk, A.R. & Liu, D.R. Creating small-molecule dependent switches to modulate biological functions. *Chem. Biol.* **12**, 151-161 (2005).

98. Backes, H., Berens, C., Helbl, V., Walter, S., Schmid, F.X. & Hillen, W. Combinations of the alpha-helix-turn-alpha-helix motif of TetR with respective residues from LacI or 434Cro: DNA recognition, inducer binding, and urea-dependent denaturation. *Biochemistry* **36**, 5311-5322 (1997).

99. Ebright, R.H., Cossart, P., Gicquelsanzey, B. & Beckwith, J. Mutations that alter the DNA-sequence specificity of the catabolite gene activator protein of *Escherichia coli*. *Nature* **311**, 232-235 (1984).

100. Helbl, V. & Hillen, W. Stepwise selection of TetR variants recognizing tet operator 4C with high affinity and specificity. *J Mol Biol* **276**, 313-318 (1998).

101. Helbl, V., Tiebel, B. & Hillen, W. Stepwise selection of TetR variants recognizing tet operator 6C with high affinity and specificity. *J Mol Biol* **276**, 319-324 (1998).

102. Nilsson, M.T.I., Mossing, M.C. & Widersten, M. Functional expression and affinity selection of single-chain Cro by phage display: Isolation of novel DNA-binding proteins. *Protein Eng.* **13**, 519-526 (2000).

103. Simoncsits, A., Tjornhammar, M.L., Wang, S. & Pongor, S. Single-chain 434 repressors with altered DNA-binding specificities - Isolation of mutant single-

chain repressors by phenotypic screening of combinatorial mutant libraries. *Genetica* **106**, 85-92 (1999).

104. Wharton, R.P., Brown, E.L. & Ptashne, M. Substituting an alpha-helix switches the sequence-specific DNA interactions of a repressor. *Cell* **38**, 361-369 (1984).

105. Wharton, R.P. & Ptashne, M. Changing the binding-specificity of a repressor by redesigning an alpha-helix. *Nature* **316**, 601-605 (1985).

106. Wharton, R.P. & Ptashne, M. A new-specificity mutant of 434 repressor that defines an amino-acid base pair contact. *Nature* **326**, 888-891 (1987).

107. Jana, R., Hazbun, T.R., Fields, J.D. & Mossing, M.C. Single-chain lambda Cro repressors confirm high intrinsic dimer-DNA affinity. *Biochemistry* **37**, 6446-6455 (1998).

108. Percipalle, P., Simoncsits, A., Zakhariev, S., Guarnaccia, C., Sanchez, R. & Pongor, S. Rationally designed helix-turn-helix proteins and their conformational changes upon DNA binding. *EMBO J.* **14**, 3200-3205 (1995).

109. Robinson, C.R. & Sauer, R.T. Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. *Proc. Natl. Acad. Sci. USA* **95**, 5929-5934 (1998).

110. Simoncsits, A., Chen, J.Q., Percipalle, P., Wang, S.L., Toro, I. & Pongor, S. Single-chain repressors containing engineered DNA-binding domains of the phage 434 repressor recognize symmetric or asymmetric DNA operators. *J. Mol. Biol.* **267**, 118-131 (1997).

111. Simoncsits, A., Tjornhammar, M.L., Wang, S.L. & Pongor, S. Isolation of altered specificity mutants of the single-chain 434 repressor that recognize asymmetric DNA sequences containing the TTAA and TTAC subsites. *Nucleic Acids Res.* **27**, 3474-3480 (1999).

112. Liang, T.B., Chen, J.Q., Tjornhammar, M.L., Pongor, S. & Simoncsits, A. Modular construction of extended DNA recognition surfaces: mutant DNA-binding domains of the 434 repressor as building blocks. *Protein Eng.* **14**, 591-599 (2001).

113. Santoro, S.W. & Schultz, P.G. Directed evolution of the site specificity of Cre recombinase. *Proc. Natl. Acad. Sci. USA* **99**, 4185-4190 (2002).

114. Voziyanov, Y., Konieczka, J.H., Stewart, A.F. & Jayaram, M. Stepwise manipulation of DNA specificity in Flp recombinase: Progressively adapting Flp

to individual and combinatorial mutations in its target site. *J. Mol. Biol.* **326**, 65-76 (2003).

115. Voziyanov, Y., Stewart, A.F. & Jayaram, M. A dual reporter screening system identifies the amino acid at position 82 in Flp site-specific recombinase as a determinant for target specificity. *Nucleic Acids Res.* **30**, 1656-1663 (2002).

CHAPTER 2

Directed Evolution of *Vibrio fischeri* LuxR for Increased Sensitivity to a Broad Spectrum of Acyl-homoserine Lactones

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A. Abstract

LuxR-type transcriptional regulators play key roles in quorum-sensing systems that employ acyl-homoserine lactones (acyl-HSLs) as signal molecules. These proteins mediate quorum control by changing their interactions with RNA polymerase and DNA in response to binding their cognate acyl-HSL. The evolutionarily-related LuxR-type proteins exhibit considerable diversity in primary sequence and in their response to acyl-HSLs having acyl groups of differing length and composition. Little is known about which residues determine acyl-HSL specificity, and less about the evolutionary time scales required to forge new ones. To begin to examine such issues, we have focused on the LuxR protein from *Vibrio fischeri*, which activates gene transcription in response to binding its cognate quorum signal, 3-oxohexanoyl-homoserine lactone (3OC6HSL). Libraries of *lux*R mutants were screened for variants exhibiting increased gene activation in response to octanoyl-HSL (C8HSL), with which wild-type LuxR interacts only weakly. Eight LuxR variants were identified that showed a 100-fold increase in sensitivity to C8HSL; these variants also displayed increased sensitivities to pentanoyl-HSL and tetradecanoyl-HSL, while maintaining a wild-type or greater response to 3OC6HSL. The most sensitive variants activated gene transcription as strongly with C8HSL as the wild type did with 3OC6HSL. With one exception, the amino acid residues involved were restricted to the N-terminal, "signal-binding" domain of LuxR. These residue positions differed from critical positions previously identified via "loss-of-function" mutagenesis. We have demonstrated that acyl-HSL-dependent quorum-sensing

systems can evolve rapidly to respond to new acyl-HSLs, suggesting that there may be an evolutionary advantage to maintaining such plasticity.

B. Introduction

Several mechanisms have evolved to allow diverse bacterial species to detect changes in their local population density, and to modulate their gene expression accordingly [1, 2]. An opinion that quorum sensing is actually a form of diffusion sensing has recently been articulated [3], however, it is the view of many that the two stances are not necessarily mutually exclusive. The population-density-based control of gene expression in bacteria has been termed "quorum sensing" [4]. A paradigm of the quorum-sensing control of gene regulation within the Gram-negative phylum *Proteobacteria* has been the luminescence (*lux*) operon in *Vibrio fischeri*. In addition to the luciferase genes required for light production, this operon encodes LuxR, an acyl-homoserine lactone (acyl-HSL)-dependent transcriptional activator, and LuxI, an acyl-HSL synthase that catalyzes the production of 3-oxohexanoyl-homoserine lactone (3OC6HSL) [5]. Each bacterium expresses the Lux proteins at low basal levels throughout its entire lifecycle. At low cell densities, the small amounts of the amphipathic 3OC6HSL signal that are produced diffuse away from the cells [6, 7]. However, as a local population increases in density, 3OC6HSL concentrations increase. This results in a shift of the LuxR equilibrium towards its 3OC6HSL-bound, active state. Acyl-HSL binding leads to dimerization of LuxR and binding to the *lux* box, a 20-base pair inverted repeat located in the P_{lux}

promoter [8]. There the acyl-HSL-bound LuxR dimer activates expression of the *lux* genes after the recruitment of RNA polymerase [9, 10].

To date, genes encoding more than 50 LuxI/LuxR pairs have been identified in diverse species belonging to the α-, β-, and γ-subdivisions of the *Proteobacteria* [11]. While the basic quorum-sensing mechanism has been fairly well conserved in most species studied, the physiological functions controlled by acyl-HSL-based quorum-sensing systems are varied and include virulence [12, 13], antibiotic synthesis [14, 15], and biofilm formation [16, 17]. A LuxR homolog typically responds to an acyl-HSL synthesized by its partner LuxI homolog. Known quorum-sensing systems are tuned to preferentially synthesize and respond to a specific acyl-HSL having an acyl side group that may range in length from four to 16 carbons, may contain a degree of unsaturation, and may be modified with a carbonyl or hydroxyl functional group at carbon position 3 [18, 19].

Are LuxR homologs evolutionarily pliable, i.e., able to move into new signaling niches on short time scales? We are interested in this issue and in identifying how the specificity of acyl-HSL response is determined. The ability of LuxR to regulate gene expression in response to an acyl-HSL depends on signal recognition and binding; it may also include acyl-HSL-specific dimerization and DNA-binding mechanisms. Because LuxR homologs generally share low amino acid sequence identities (≤25%), however, it is difficult to determine precisely how they discriminate between various acyl-HSL molecules, or how this discrimination evolves and changes as a result of varying selective

pressures. In a recent study on acyl-HSL-binding specificity, residues of TraR (a LuxR homolog) predicted to interact with the carbonyl moiety of its cognate signal, 3-oxooctanoyl-HSL (3OC8HSL) were mutated with the goal of stimulating the response of this protein to an analog, octanoyl-HSL (C8HSL) [20]. Rather than showing improved sensitivity to C8HSL, these variants lost the strong response that wild-type TraR displays towards 3OC8HSL. The attempts of Chai and Winans to shift the specificity of TraR towards 3OC6HSL by introducing bulkier hydrophobic amino acids, and thereby reduce the size of the acyl binding site, also led, in all but one case, to variants with greatly decreased acyl-HSL affinities. Thus, alternative approaches may be more successful at altering the acyl-HSL specificities of LuxR or its homologs. To identify LuxR variants with altered acyl-HSL specificity, and to identify key residues which modulate this property, we have developed and implemented a system to direct the evolution of LuxR-mediated gene activation. Variants of LuxR were generated and screened to identify those that are highly responsive to acyl-HSL signals to which this transcriptional activator does not normally respond.

C. Results

C.1. Cloning vectors and screening tools

Modifications to a commercial two-plasmid system were made to allow for rapid cloning of *lux*R mutant libraries and screening for gene activation in response to exogenous signal molecules. The first plasmid, pLuxR, contains wild-type *lux*R under the control of a $P_{lac/ara-1}$ hybrid promoter. The *luxR* allele is constitutively expressed when placed in *E. coli* strains lacking *lacI* and *araC*. The second plasmid, p*lux*GFPuv, contains the gene *gfpuv* placed under the control of the P_{luxI} promoter. The *gfpuv* gene encodes a green fluorescent protein variant, GFPuv, which becomes visible when excited with standard, long-wavelength ultraviolet light [21]. GFPuv expression from the P_{luxI} promoter of p*lux*GFPuv is dependent upon the degree of activation that occurs as a result of a functional LuxR variant binding to a specific acyl-HSL signal molecule. The relative levels of gene activation observed with wild-type LuxR and the acyl-HSLs used in this study were similar to those previously reported in *V. fischeri* [22] and *E. coli* [23].

C.2. First-generation laboratory evolution—error-prone PCR

We used the two-plasmid system to identify LuxR variants that showed an increased ability to activate gene transcription in response to C8HSL. Past studies have shown that low-micromolar concentrations of C8HSL were required to achieve the same level of gene expression achieved by 5 nM of 3OC6HSL, the cognate signal of LuxR [22]. In the

current system, a C8HSL concentration of approximately 2 μM was required to achieve the level of gene activation obtained with 10 nM 3OC6HSL. Random mutations were introduced into wild-type *luxR* by error-prone PCR. *E. coli* hosts containing p*lux*GFPuv were transformed with pLuxR vectors containing the mutagenized PCR products. Transformants were initially plated onto LB-agar amended with 50 nM C8HSL and antibiotics as appropriate. However, initial screening with this concentration of acyl-HSL yielded only one mutant, LuxR-G1A. Subsequently, the library was screened for activation with 200 nM C8HSL. Colonies were screened under illumination with 365 nm ultraviolet light. Most colonies appeared dark under such illumination, indicating the expression of LuxR variants having either wild-type or non-functional properties. Of the ~20,000 colonies that were screened, nine fluorescent colonies were identified. These nine colonies were isolated and verified by re-screening, which identified one as a false positive. The remaining eight alleles were amplified and cloned into fresh background plasmids and strains to ensure that the observed phenotype was due to changes within *luxR*. An additional false positive was identified by doing so.

Of the seven remaining alleles, two were shown to be identical by DNA sequence analysis. The six LuxR variants from this first generation of laboratory evolution were designated LuxR-G1A through LuxR-G1F (Fig. 2.1). Quantitative characterization of the response of these mutants to 3OC6HSL and C8HSL was performed in liquid-phase assays by monitoring fluorescence levels using a microtiterplate spectrofluorimeter. As shown in Table 2.1, all six variants showed increased gene expression in the presence of

100 nM C8HSL as compared to wild-type LuxR. Amino acid substitutions identified in the first generation are shown in Figure 2.1.

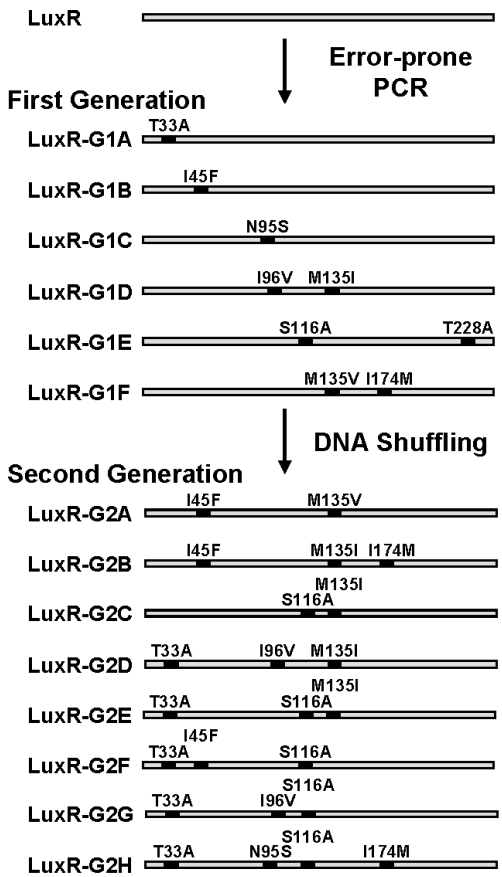


Figure 2.1. Amino acid sequence changes to first- and second-generation LuxR variants showing increased gene activation in response to C8HSL. The six first-generation variants were identified from a library of approximately 20,000 clones generated by error-prone PCR. Second-generation variants were generated by DNA shuffling of alleles from the first generation. Eight second-generation variants showing additional increases in gene activation in response to C8HSL were identified after screening 1,200 clones. The positions of amino acid substitutions within the 250 amino acid LuxR protein are indicated as black rectangles (refer to Table 2.1 for nucleotide sequence changes).

Table 2.1. Nucleotide and amino acid changes in the recovered luxR/LuxR mutants

***luxR* plasmid**	***luxR* gene** base and substitution	**LuxR protein** amino acid residue and substitution
First Generation		
pLuxR-G1A	A97→G A159→G	Thr33→Ala Synonymous
pLuxR-G1B	A133→T T501→C	Ile45→Phe Synonymous
pLuxR-G1C	A284→G A414→C	Asn95→Ser Synonymous
pLuxR-G1D	A286→G G405→A	Ile96→Val Met135→Ile
pLuxR-G1E	T346→G A683→G	Ser116→Ala Thr228→Ala
pLuxR-G1F	T162→A A403→G A522→G	Synonymous Met135→Val Ile174→Met
Second Generation		
pLuxR-G2A	A133→T A403→G	Ile45→Phe Met135→Val
pLuxR-G2B	A133→T G405→A A522→G	Ile45→Phe Met135→Ile Ile174→Met
pLuxR-G2C	T346→G G405→A	Ser116→Ala Met135→Ile
pLuxR-G2D	A97→G T162→A A286→G G405→A A414→C	Thr33→Ala Synonymous Ile96→Val Met135→Ile Synonymous
pLuxR-G2E	A97→G T162→A T346→G G405→A	Thr33→Ala Synonymous Ser116→Ala Met135→Ile
pLuxR-G2F	A97→G A133→T T162→A T346→G	Thr33→Ala Ile45→Phe Synonymous Ser116→Ala
pLuxR-G2G	A97→G A286→G T346→G	Thr33→Ala Ile96→Val Ser116→Ala
pLuxR-G2H	A97→G A284→G T346→G A522→G	Thr33→Ala Asn95→Ser Ser116→Ala Ile174→Met

Table 2.2. Activation of *gfpuv* gene expression by LuxR variants with four different acyl-HSLs

LuxR type	Fluorescence (AU)[a]						
	No acyl-HSL	3OC6HSL		C8HSL		C5HSL	C14HSL
		10 nM	100 nM	10 nM	100 nM	100 nM	500 nM
LuxR wt	*<50*	*1400*	*3000*	*<50*	*100*	*<50*	*<50*
First Generation							
LuxR-G1A	<50	2200	3300	<50	800	100	<50
LuxR-G1B	<50	2600	3200	<50	800	500	<50
LuxR-G1C	<50	2100	3000	<50	700	<50	<50
LuxR-G1D	<50	400	2000	100	1500	700	200
LuxR-G1E	<50	2200	3200	<50	500	<50	100
LuxR-G1F	<50	600	2700	<50	700	600	100
Second Generation							
LuxR-G2A	<50	1600	3200	200	1700	2200	100
LuxR-G2B	<50	2400	3500	900	2900	3000	400
LuxR-G2C	<50	500	1900	200	1900	1000	700
LuxR-G2D	<50	1300	3200	800	3100	2300	1300
LuxR-G2E	<50	1400	3200	1100	3600	3200	2200
LuxR-G2F	<50	3100	2900	800	2900	2600	900
LuxR-G2G	<50	3300	3500	500	2800	1400	1500
LuxR-G2H	<50	3300	3400	900	2500	1200	1500

[a]Units of fluorescence represent the fluorescence due to GFPuv production obtained with a given LuxR variant and p*lux*GFPuv. Fluorescence values were corrected with the background fluorescence obtained without a LuxR expressing plasmid. Both values were normalized to their cell densities prior to correction. All values are the mean of triplicate measurements. Variation was less than ± 15% for all reported values.

C.3. Second-generation laboratory evolution—DNA shuffling of mutant luxR alleles

To investigate whether the mutations could be combined to yield further improvements in C8HSL response, a new library of *luxR* alleles was generated via DNA shuffling of the six from the first generation, under conditions designed to minimize new point mutations [24]. As with the first generation, transformants were screened for GFPuv production on LB-agar, in this case amended with 50 nM C8HSL. Of 1200 colonies transformed with this library, 102 exhibited fluorescence. To identify those variants most sensitive to C8HSL, these 102 strains were screened using 10 and 20 nM C8HSL. Ten exhibited fluorescence under these conditions and were picked for further characterization. One false positive was identified after re-amplification and re-cloning of the alleles into fresh background materials. Sequence analysis revealed two of the alleles to be identical. Quantitative liquid-phase characterization of the eight remaining mutants showed that all exhibited remarkable increases in fluorescence output in response to not only 100 nM but also 10 nM C8HSL, as compared to either wild-type LuxR or any of the first-generation LuxR variants (Table 2.2). Sequencing revealed that no additional residue positions had been changed; the second-generation sequences were all recombinants of the parent sequences (Fig. 2.1).

C.4. C8HSL and 3OC6HSL dose responses

We compared the influence of a range of 3OC6HSL and C8HSL concentrations on transcriptional activation by wild-type LuxR and each of the second-generation variants (Fig. 2.2). Wild-type LuxR required approximately 2 μM C8HSL to elicit half the maximum GFPuv fluorescence observed using saturating amounts of 3OC6HSL, whereas only 10 nM 3OC6HSL was required for half-maximal activation (Fig. 2.2A). In comparison, all second-generation LuxR variants showed an increased sensitivity to C8HSL, requiring only 15 to 50 nM to achieve the half-maximal 3OC6HSL-induced fluorescence of the wild-type. Thus, C8HSL sensitivity and response by these LuxR variants increased by 20- to 100-fold. Variants LuxR-G2D and LuxR-G2E responded as sensitively and strongly to C8HSL as wild-type LuxR did to 3OC6HSL (Fig. 2.2E, F). The acquisition of this trait had little or no deleterious impact on the sensitivity of these variants to 3OC6HSL. Three variants, LuxR-G2A, LuxR-G2D and LuxR-G2E (Fig. 2.2B, E, F), showed responses to 3OC6HSL indistinguishable from that of wild-type LuxR. Variants LuxR-G2F, LuxR-G2G and LuxR-G2H (Fig. 2.2G–I) showed 5-fold increased sensitivity to 3OC6HSL, exhibiting significant levels of fluorescence with concentrations as low as 1 nM.

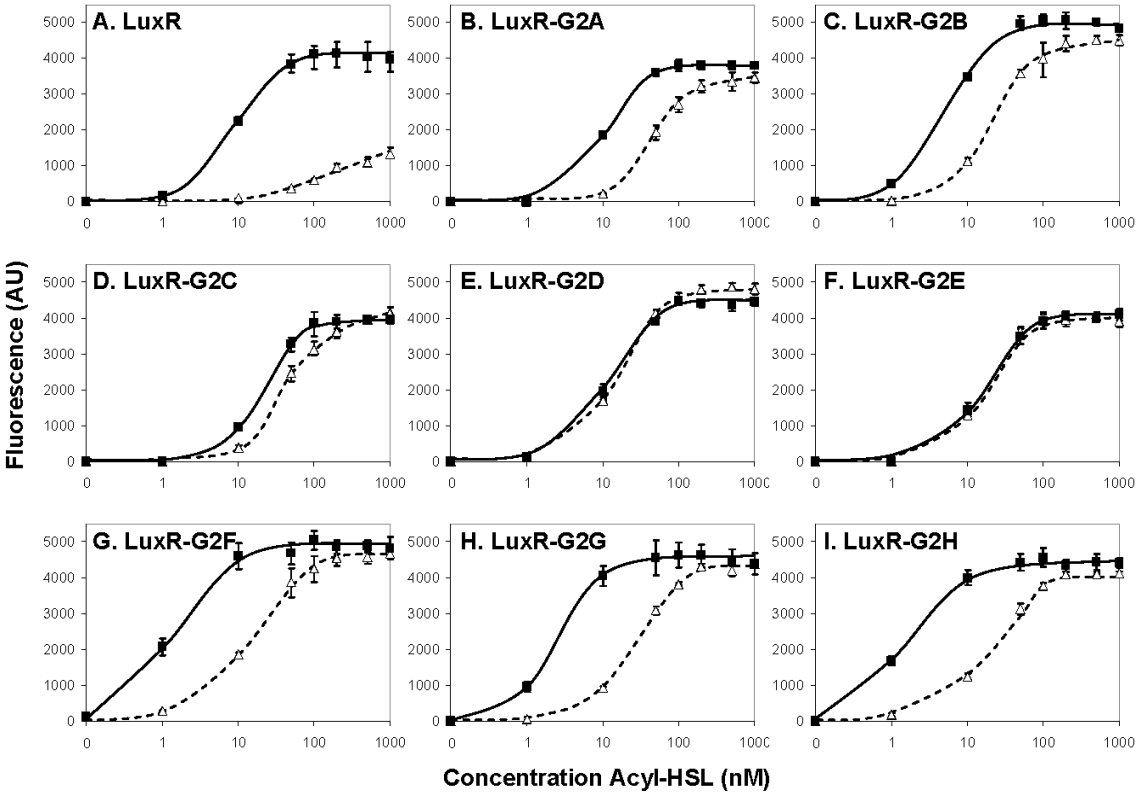


Figure 2.2. Detection of C8HSL and 3OC6HSL by wild-type LuxR and eight second-generation LuxR variants. For each panel and variant (**A** through **I**), data for 3OC6HSL are represented by closed squares (■), data for C8HSL are represented by open triangles (Δ). Fluorescence data reported are normalized to optical density, and corrected by subtracting background fluorescence from a control strain carrying pPROLar.A122 with p*lux*GFPuv. All measurements were performed in triplicate. Error bars indicate either the range of values or the standard deviation, whichever was greater.

C.5. Increased sensitivity to 3OC6HSL can be attributed to increases in LuxR protein concentration

To investigate whether increases in expression were responsible for observed increases in gene activation in response to 3OC6HSL, concentrations of the variant LuxR proteins were measured. Fusions of the *c-myc* epitope to the C-termini of wild-type LuxR and each of the second-generation variants were constructed. All *c-myc* LuxR variants functioned well, i.e., exhibited responses to acyl-HSLs which were similar to the variant lacking the *c-myc* epitope. Protein concentrations were monitored under different incubation conditions by Western immunoblot assay and compared.

We tested the accumulation of wild-type LuxR and the second-generation variant proteins after incubation in the absence or presence of saturating amounts of 3OC6HSL. In the absence of acyl-HSL, the amount of protein detected by Western immunoblot for each of the variants differed by less than two-fold from wild-type LuxR; however, in the presence of 1 μM 3OC6HSL, the four variants with increased responses to 3OC6HSL, LuxR-G2B, -G2F, -G2G and -G2H, also showed increased amounts of protein as compared to wild type (Fig. 2.3). Thus mutations leading to higher LuxR levels may lead to the increased sensitivities of these variants to 3OC6HSL and, in part, C8HSL.

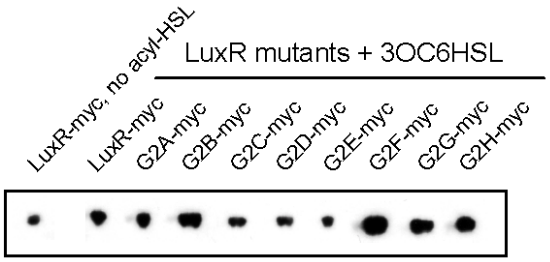


Figure 2.3. Protein concentrations of second-generation LuxR variants. The immunoblot was performed on a SDS-PAGE display of the total proteins harvested from strains expressing *c-myc* tagged second generation LuxR variants incubated in the presence of 1μM 3OC6HSL. *Myc*-coupled fluorescence for each of the variants varied by less than 10% in the presence and absence of acyl-HSL (latter not presented). In the absence of acyl-HSL, the amount of LuxR protein detected for each variants was within two-fold of that for the wild-type LuxR-*myc* fusion.

The amount of wild-type LuxR detected in the presence of 3OC6HSL was approximately two-fold more than was detected in the absence of signal. The increase in protein levels observed with the addition of 3OC6HSL is similar to that observed for TraR [20], consistent with the notion that acyl-HSL binding enhances the folding and/or stability of LuxR. If so, acyl-HSL binding and protein stability are inextricably linked.

C.6. Response of LuxR variants to acyl-HSLs with long and short acyl chains

The ability of the C8HSL-responsive LuxR variants to activate gene expression with other acyl-HSLs was also investigated. The response of each variant was examined using 100 nM pentanoyl-homoserine lactone (C5HSL) and 1 μM tetradecanoyl-homoserine lactone (C14HSL), two acyl-HSLs to which wild-type LuxR exhibits essentially no response (Table 2.2). Four first-generation variants (LuxR-G1A, -G1B, -G1D and -G1F)

showed small, yet significant, responses to C5HSL, as did three (LuxR-G1D, -G1E and -G1F) to C14HSL.

The second-generation variants all showed marked improvements in their sensitivity and response to both C5HSL and C14HSL. LuxR-G2A showed a three-fold and LuxR-G2B a >four-fold improvement in sensing C5HSL when compared to the best of the first-generation mutants. LuxR-G2G and LuxR-G2H showed a >seven-fold increase in gene activation in the presence of C14HSL when compared to the best of the first-generation mutants. LuxR-G2D, -G2E, and -G2F all showed broadened acyl-HSL specificity, exhibiting strong responses to both C5HSL and C14HSL. The most responsive mutant, LuxR-G2E, showed an ~five-fold increase in fluorescence with C5HSL and an 11-fold increase with C14HSL, while maintaining a wild-type level of response to 3OC6HSL.

C.7. Acyl-HSL specificity of the second-generation variants

The ratio of the 3OC6HSL and C8HSL concentrations required to attain half-maximal gene expression, termed the specificity, S, was used to assess changes in acyl-HSL responses (Table 2.3). While wild-type LuxR has an S of 200, indicating that it requires 200-fold more C8HSL than 3OC6HSL to reach half-maximal fluorescence levels, all of the second-generation mutants have $S < 20$. The variant with the largest increase in response to C8HSL, LuxR-G2E, has $S = 1$: it responds equally to both HSLs. This variant is also sensitive to C5HSL and C14HSL (see above). The variants which showed

increased protein concentration (LuxR-G2F, -G2G and -G2H) also exhibited a more than 10-fold decrease in S as compared to wild type. Whereas increases in protein concentration can increase response to all acyl-HSLs, they can not account for changes in specificity. Acyl-HSL specificity has been significantly altered in the all of the variants.

Table 2.3. Specificity of second-generation LuxR variants

LuxR type	**$[3OC6HSL]_{50}$ (nM)[a]**	**$[C8HSL]_{50}$ (nM)[a]**	**S[b]**
LuxR *wt*	10	2000	200
LuxR-G2A	10	50	5.0
LuxR-G2B	5	20	4.0
LuxR-G2C	20	35	1.8
LuxR-G2D	10	15	1.5
LuxR-G2E	20	20	1.0
LuxR-G2F	1.5	20	13.3
LuxR-G2G	2.5	30	12.0
LuxR-G2H	1.5	25	16.7

[a]$[3OC6HSL]_{50}$ and $[C8HSL]_{50}$ are the concentrations of these acyl-HSLs required to reach half-maximal gene activation as measured from the fluorescence output of GFPuv.
[b]The specificity constant, S, corresponds to the ratio of the concentrations of 3OC6HSL and C8HSL required to achieve half-maximal gene activation, or $[C8HSL]_{50}/[3OC6HSL]_{50}$.

C.8. Single mutants identify six mutations that increase C8HSL response by LuxR

Because every first-generation *luxR* mutant contained more than one nucleotide substitution, further information is required to determine which lead to increased response to C8HSL. Single mutants with each of the 13 nucleotide substitutions identified in the six first-generation variants, including four synonymous mutations, were prepared using site-directed mutagenesis. Quantitative liquid-phase characterization showed that amino acid substitutions at positions 33, 45, 95, 116, 135 and 174 confer increases in sensitivity to C8HSL (Table 2.4). Fluorescence levels similar to those obtained with wild-type LuxR were found for substitutions at amino acid position 228 and synonymous mutations at nucleotide positions 162, 414 and 501. Two mutations, one leading to substitution of I96 by valine and the synonymous mutation at nucleotide position 159, are slightly deleterious, causing small decreases in gene activation. The single-mutant containing only the T33 to alanine substitution was more sensitive to C8HSL than LuxR-G1A which contains both the T33A substitution and the synonymous mutation at position 159. However, the response of LuxR-G1D, which contains both the I96V and M135I substitutions, was indistinguishable from the single-mutant containing only the M135I mutation, suggesting that the deleterious effects of the I96V mutation may be masked by the presence of a beneficial substitution.

Table 2.4. Activation of *gfpuv* gene expression by *luxR* single mutants

		Fluorescence (AU)[a]				
Nucleotide substitution	**Amino acid substitution**	**No acyl-HSL**	**3OC6HSL**		**C8HSL**	
			10 nM	100 nM	10 nM	100 nM
luxR wt	LuxR *wt*	<50	*1700*	*3000*	<50	*200*
A97→G	Thr33→Ala	<50	2500	3000	200	1200
A133→T	Ile45→Phe	<50	3100	3100	300	1400
A159→G	Syn[b]	<50	300	1000	<50	<50
T162→A	Syn	<50	2000	2800	100	500
A284→G	Asn95→Ser	<50	2300	2600	200	1000
A286→G	Ile96→Val	<50	900	1200	<50	200
A346→G	Ser116→Ala	<50	2600	3000	200	700
A403→G	Met135→Val	<50	900	2100	100	700
G405→A	Met135→Ile	<50	700	1800	200	1100
A414→C	Syn	<50	1300	2700	<50	200
T501→C	Syn	<50	2000	2600	100	400
A522→G	Ile174→Met	<50	2300	2800	100	700
A683→G	Thr228→Ala	<50	1800	2400	100	300

[a]Units of fluorescence represent the fluorescence due to GFPuv production obtained with a given LuxR variant and p*lux*GFPuv as described for Table 2.2.
[b] Syn denotes a synonymous mutation, i.e. a nucleotide change which does not encode an amino acid change.

D. Discussion

The results of this study indicate that LuxR can evolve rapidly to sense and respond to a diversity of acyl-HSL signal molecules. By screening for changes in gene activation in the presence of C8HSL, we were able to identify variants of this signal-dependent transcriptional activator that respond strongly to this molecule and two additional acyl-HSLs. Only two generations and three or fewer residue changes were required to generate LuxR variants that activate gene expression in response to C8HSL with sensitivity equal to its response to the natural signal, 3OC6HSL.

The variants identified in this study may be altered in more than one of a number of properties and processes involved in gene activation by LuxR. LuxR-mediated gene activation involves key events of signal binding, dimerization, conformational changes, DNA binding, and transcriptional activation via interactions with RNA polymerase and other proteins [1]. Changes to LuxR stability (in the presence and absence of a competent acyl-HSL molecule), folding, solubility, and expression could also affect its ability to activate genes. Indeed, the stability of TraR (a LuxR homolog) in the cytosol has been shown to increase dramatically upon binding a competent acyl-HSL [25, 26]. The increase in LuxR concentration observed in the presence of 3OC6HSL, as compared to its concentration without signal, indicates that, like TraR, the stability of LuxR is affected by the presence of acyl-HSL. General improvements in response to various acyl-HSLs could result from changes in many of these properties. Acyl-HSL-specific response, however, can be extricated from these other properties by looking at S, the ratio

of responses, i.e. between the cognate and another acyl-HSL. Using this measure we have shown that the response to C8HSL was enhanced in all of the variants relative to the changes in response to 3OC6HSL, suggesting key changes had occurred beyond general concentration effects, and likely at the level of acyl-HSL binding.

Past studies involving the mutagenesis of *luxR* have been instrumental in constructing a structural and functional map of LuxR [reviewed in [27]]. Regions of LuxR that are essential for its function were identified by screening random point mutations within *luxR* [28, 29]. Mutations that led to nonfunctional LuxR variants clustered between residues 79-127 and 184-230. Reversal of the loss-of-function phenotype for several mutations within the N-terminal critical region was achieved by adding high concentrations of 3OC6HSL, suggesting that such mutations weaken the interaction between the sensor protein and its cognate signal [28]. None of the residue positions identified here were shown to be critical for function in the previous studies. Four of the mutations (T33A, I44F, M135I, and M135V) are located outside of the region previously postulated to be involved in acyl-HSL binding, albeit well within the predicted N-terminal domain [27]. Thus, by evolving LuxR to respond to an acyl-HSL molecule to which it is normally insensitive, we have identified residues involved in acyl-HSL specificity and response that are distinct from those revealed during loss-of-function mutant studies (see Fig. 2.4). This was not entirely unexpected, as many laboratory evolution studies have demonstrated that amino acids involved in modulated function are generally distinct from, and more tolerant to change, than those required for folding or function [30].

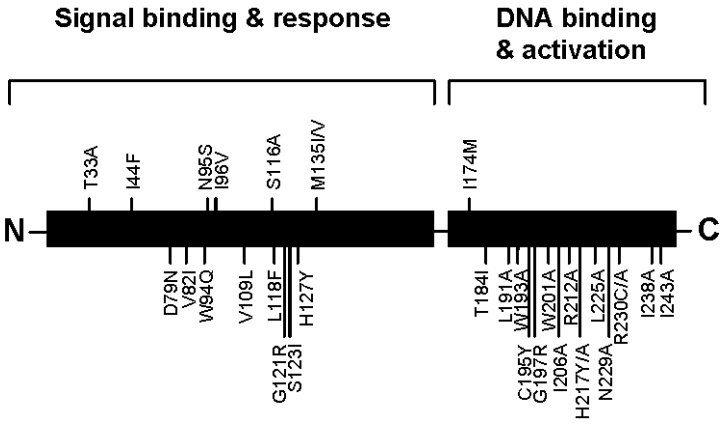


Figure 2.4. Mutations in LuxR responsible for altering acyl-HSL specificity differ from those which result in a loss of function. The 250-residue LuxR protein is composed of an N-terminal acyl-HSL-binding and regulation domain and a C-terminal DNA-binding and activation domain [27]. Amino acid substitutions identified in this study are shown above the bar, and amino acid substitutions previously shown to lead to a loss of acyl-HSL-mediated LuxR activation are shown below [28, 29, 31, 32].

D.1. The T33A, I45F, N95S, S116A, M135I and I174M mutations increase C8HSL response by LuxR

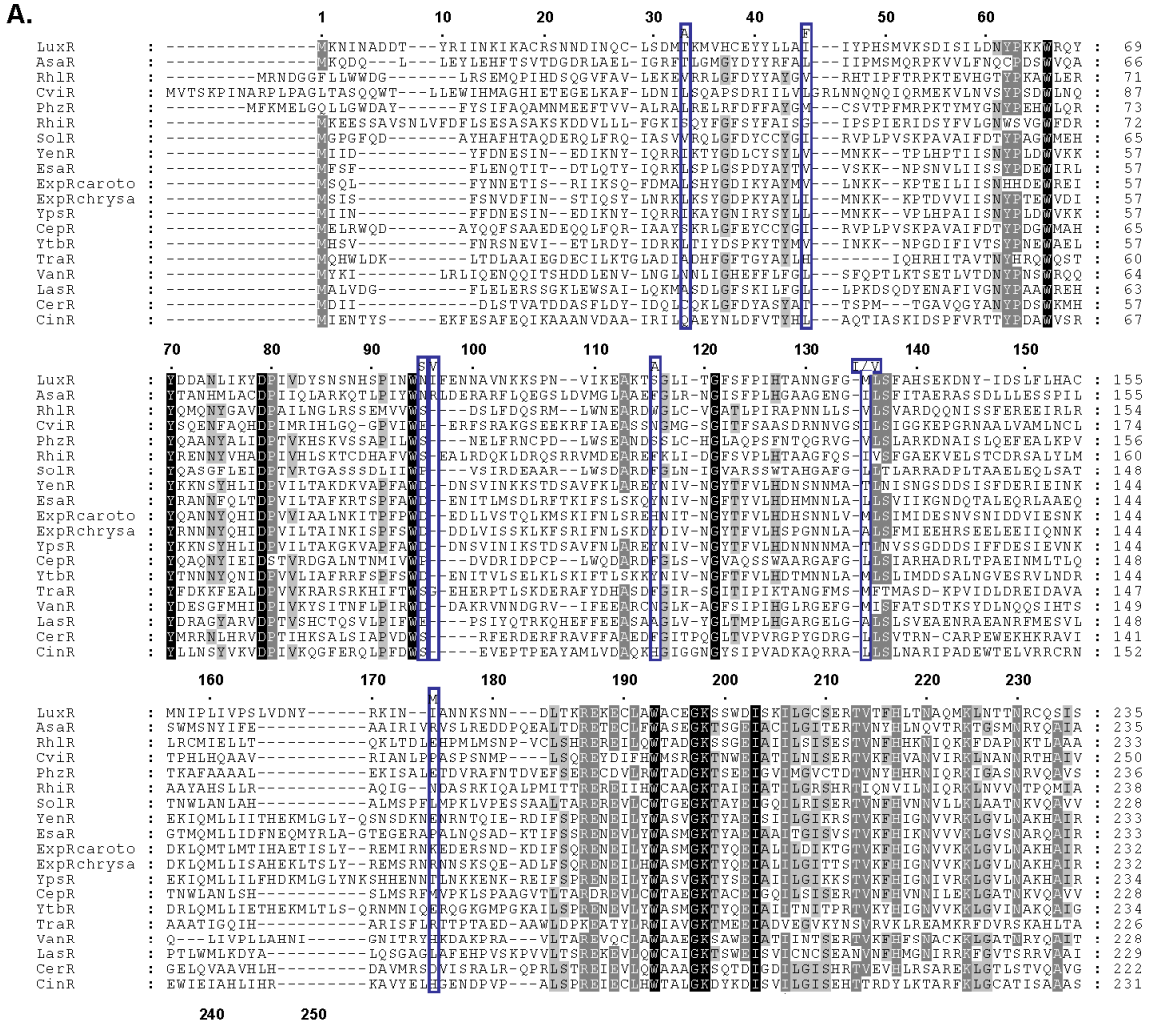
Analysis of the thirteen single-site mutants showed that a single mutation from each of the first-generation variants was responsible for the observed increase in C8HSL response (Table 2.4), with the exception of LuxR-G1F, in which both the M135V and I174M mutations increase C8HSL sensitivity. Recombining these mutations led to LuxR variants with further increases in their ability to activate gene expression with C8HSL but with varying responses to 3OC6HSL, C5HSL and C14HSL. The absence of N95S in the

second generation may be due to random chance, bias in the construction of the library, or its inability to confer any additional advantage when combined with the other mutations.

D.2.Substitutions at position 135 likely stabilize interactions with straight-chain acyl HSLs

The variants which showed increased response to C8HSL with minimal changes in their response to 3OC6HSL (LuxR-G2A, -G2B, -G2C, -G2D and -G2E) all contain a substitution at position 135 (Fig. 2.2 and Table 2.2). Mutation of M135 to isoleucine or valine appears to stabilize interactions between LuxR and C8HSL without affecting the sensor's recognition of its cognate signal, 3OC6HSL. Alignments of LuxR with a number of its homologs revealed that proteins that respond to acyl-HSLs containing a carbonyl group at the third carbon of their acyl chain often have a methionine residue at this position, whereas those that recognize straight-chain acyl-HSLs usually contain an isoleucine or a valine residue (see Fig. 2.5). Thus natural and laboratory evolution appear to converge to the same answer. The predominant preference at this position in all LuxR homologs seems to be for a hydrophobic residue (isoleucine, valine, leucine or methionine). Alignment to the published structure of TraR shows that 135 is only two

A.



B.

LuxR homolog	Organism	Acyl-HSL
LuxR	*Vibrio fischeri*	3OC6HSL
AsaR	*Aeromonas salmonicida*	C4HSL
RhlR	*Pseudomonas aeruginosa*	C4HSL
CviR	*Chromobacterium violaceum*	C6HSL
PhzR	*Pseudomonas aerofaciens*	C6HSL
RhiR	*Rhizobium leguminosarum*	C6HSL
SolR	*Ralstonia solanacearum*	C6HSL, C8HSL
YenR	*Yersinia enterocolitica*	C6HSL, 3OC6HSL
EsaR	*Erwinia stewartii*	3OC6HSL
ExpR	*Erwinia carotovora*	3OC6HSL
ExpR	*Erwinia chrysanthemi*	3OC6HSL
YpsR	*Yersinia pseudotuberculosis*	3OC6HSL
CepR	*Burkholderia cepacia*	C8HSL
YtbR	*Yersinia pseudotuberculosis*	C8HSL
TraR	*Agrobacterium tumefaciens*	3OC8HSL
VanR	*Vibrio anguillarum*	3OC10HSL
LasR	*Pseudomonas aeruginosa*	3OC12HSL
CerR	*Rhodobacter sphaeroides*	7,8-*cis*-C14HSL
CinR	*Rhizobium leguminosarum*	3O-7,8-*cis*-C14HSL

Figure 2.5. A. Multiple sequence alignments of 19 members of the LuxR family of transcriptional regulators. LuxR residue numbering is shown above the alignment. Conserved residues are denoted by shading with black (100% conservation), dark grey with white letters (80-99% conservation), and light grey with black letters (60-79% conservation). Site numbering for residue changes identified in this study are shown above the aligned column bounded by a blue vertical box. **B.** List of LuxR homologs used in the multiple sequence alignment, including the source species and the acyl-HSLs to which they are reported to respond preferentially and strongly.

positions away from an amino acid required for recognition of the 3-oxo moiety of 3OC8HSL in TraR [20], and could potentially be involved inmodulating the response to acyl-HSLs lacking a 3-oxo moiety. It also remains possible that residue 135 does make contact with the acyl-HSL molecule: the structure of LuxR has not yet been determined and may differ significantly from that of its distantly-related homolog, TraR [33].

D.3. Acyl-HSL specificity is influenced by mutations at LuxR positions 33 and 45

Each of the second-generation variants which exhibited the greatest increases in gene expression in response to C14HSL (LuxR-G2D to -G2H) contained the T33A mutation (Table 2.2). The T33A mutation may help LuxR to accommodate acyl-HSLs with acyl chains more than six carbons in length. TraR and LasR, which respond to acyl-HSLs having acyl side chains eight and twelve carbons in length, both contain an alanine residue at this position [34, 35]. Substitution of I45 by phenylalanine, on the other hand, led to an increase in GFPuv production in response to C5HSL in each of the three

second-generation variants, LuxR-G2A, -G2B and -G2F, in which it was identified. Therefore, I45F might improve interactions with short-chain acyl-HSLs such as C5HSL.

The identification of four LuxR variants showing increased sensitivity to 3OC6HSL was unexpected, in as much as wild-type already responds strongly to low nanomolar concentrations of this, its cognate signal. Increases in variant protein levels, as observed in the Western blots (Fig. 2.3), due to increases in expression, stability or solubility are the most likely explanation. The crystal structure of TraR was used to map the hypothetical positions of the amino acid substitutions identified in this study (Fig. 2.6). Curiously, the TraR residues which align with LuxR positions I45 and I174 are located at the interface between its signal- and DNA-binding domains. These mutations might act by stabilizing interactions between these two domains required for LuxR's switch-like behavior, thereby conferring increases in gene activation.

The acyl-HSL sensitivity of LuxR homologs may not have been maximized during natural evolution. LuxR-type proteins may be tunable over short evolutionary periods to meet the physical and chemical nature of the habitats in which the cells encoding them are active. Indeed, the amount of acyl-HSLs that must accumulate for a given quorum-sensing species to exhibit a quorum response can vary widely from low nanomolar to micromolar concentrations [5, 12, 22, 34, 36, 37], and likely reflects the particular physiological ecology of the organism.

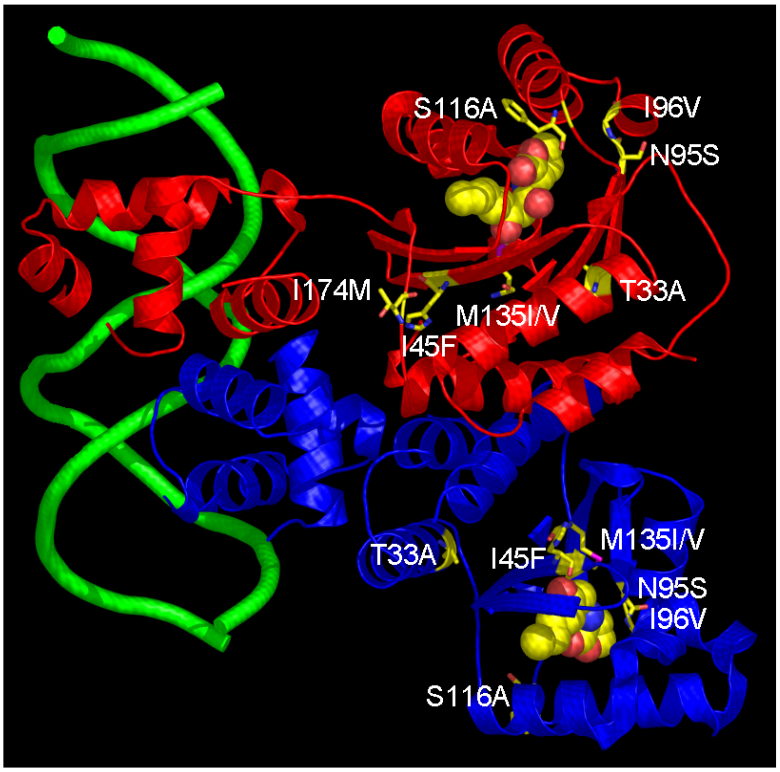


Figure 2.6. Hypothetical positions of residues in TraR corresponding to those found to modulate acyl-HSL specificity in LuxR. The crystal structure of the LuxR homolog TraR (PDB 1L3L) has been determined [26]. The two peptides comprising a TraR dimer are shown in red and blue. The double-stranded DNA to which TraR binds is shown in green. The two 3-oxooctanoyl-homoserine lactone (3OC8HSL) molecules are shown using space-filling spherical atoms (yellow represents carbon, red represents nitrogen, and blue represents oxygen). The amino acid residues which align with those identified in the LuxR variants exhibiting altered acyl-HSL responses are shown as sticks with coloring similar to that used for 3OC8HSL. The amino acids identified occur predominantly within the N-terminal domain and do not appear to make direct contact with the signal molecule. Because LuxR and TraR share less than 20% sequence identity at the amino acid level, these assignments are purely hypothetical. However, the overall structures of the two homologs are expected to be in large part similar. Their alignment can provide a useful first approximation of residue location within the three-dimensional structure of any LuxR-type protein.

While this study indicates that LuxR can rapidly evolve to respond to a broad range of acyl-HSL molecules, it did not reveal which or how many evolutionary changes are required to refocus it to a new signal molecule. Our findings indicate that the easiest evolutionary solution to generating a protein that responds to a new signaling molecule is by broadening its specificity. This is consistent with other directed evolution work that has shown that refocusing binding to shift, rather than broaden, specificity often requires additional evolutionary work [38]. Natural evolution of signal and substrate specificity has also been shown to fluctuate between specialized states through broad-specificity intermediates, allowing for the acquisition of new functions by expanding the capabilities of "old" proteins [39-43]. Laboratory evolution experiments which have successfully refocused binding or substrate specificity to a new target have specifically included screening for activity with a new substrate and the absence of activity with the enzyme's natural substrate [44, 45]. In future laboratory evolution studies with LuxR, we hope to understand the basis for acyl-HSL sensing as well as how diverse acyl-HSL-based intercellular communications systems have evolved and continue to evolve.

E. Materials and Methods

E.1. Bacterial strains, media and growth conditions

The bacterial strains used in this study were: *Escherichia coli* strain DH5α (F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1) and *E. coli* DH5α containing p*lux*GFPuv (DH5α(p*lux*GFPuv)), a pPROTet.E133-derived LuxR/acyl-HSL-inducible GFPuv expression vector encoding chloramphenicol resistance. Competent DH5α and DH5α(p*lux*GFPuv) cells were prepared by using the Z-competent *Escherichia coli* Transformation Kit (Zymo Research, Orange, CA). *E. coli* strains were cultured at 37 ^oC in LB medium or on LB agar plates. Antibiotics were added at the following concentrations: 100 $\mu g \cdot mL^{-1}$ chloramphenicol, or 20 $\mu g \cdot mL^{-1}$ kanamycin. Acyl-HSL stock solutions of 1 and 10 mM were prepared by dissolving appropriate amounts in ethyl acetate acidified with glacial acetic acid (0.01% v/v), and stored at –20 ^oC. Acyl-HSLs used in these studies were: 3-oxohexanoyl-DL-homoserine lactone (3OC6HSL; Sigma Aldrich, St. Louis, MO), pentanoyl-L-homoserine lactone (C5HSL; B. Hauer), octanoyl-DL-homoserine lactone (C8HSL; Fluka, St. Louis, MO), and tetradecanoyl-DL-homoserine lactone (C14HSL, Fluka). For screening experiments, C8HSL was amended to LB agar media prior to solidification to ensure complete mixing. For liquid-phase experiments, acyl-HSL was dispensed into sterile tubes, the ethyl acetate was evaporated under a stream of air, and sterile medium was added to the dried acyl-HSL. All liquid media containing acyl-HSL were prepared immediately prior to use.

E.2. Plasmid construction

All synthetic oligonucleotide primers used in this chapter are listed in Table 2.5. We constructed the LuxR expression vector, pLuxR, and the signal response screening plasmid, p*lux*GFPuv, using the two-plasmid pPROLar.A122 and pPROTet.E133 system from BD Biosciences Clontech (Palo Alto, CA) as plasmid backbones. Plasmid pLuxR encodes LuxR under the control of the hybrid $P_{lac/ara-1}$ promoter and was constructed by cloning PCR-amplified *luxR* from pKE705 [31], using primers 5-LuxR and 3-LuxR, into *Kpn*I and *Bam*HI digested pPROLar.A122. The sequence of pLuxR, including the promoter and the *luxR* gene, was verified by sequencing using the upstream primer 5-LarSeq2 and internal *luxR* primer LuxRSeq(int). The pLuxR plasmid contains a kanamycin resistance gene and the p15A replication origin. Plasmid p*lux*GFPuv encodes a variant of green fluorescent protein (GFPuv from pGFPuv, BD Biosciences Clontech, Palo Alto, CA) under the control of the P_{luxI} promoter. P_{luxI} was PCR-amplified from pKE555 [8] using the primers 5-pluxI and 3-pluxI and *gfpuv* was PCR-amplified from pGFPuv using 5-GFPuv and 3-GFPuv. These P_{luxI} and *gfpuv* PCR products were assembled and amplified by PCR with 5-pluxI and 3-GFPuv and cloned into pPROTet.E133 between its *Aat*II and *Hin*dIII sites. The sequence of the entire promoter region and *gfpuv* gene on p*lux*GFPuv was verified by DNA sequencing using the primers 5-pPROTetSeq and GFPuvSeq(int). This plasmid contains a chloramphenicol resistance gene and the ColE1 replication origin. The plasmid encoding the C-terminal fusions of *c-myc* with LuxR was cloned using the primers 5-LuxR and 3-LuxR-myc with pLuxR as

template. The fusion was cloned into *Kpn*I and *Bam*HI digested pPROLar.A122 to make pLuxR-myc. Versions of pLuxR-myc encoding the second-generation LuxR variants fused to *c-myc* were cloned similarly to pLuxR-myc using the appropriate pLuxR plasmid encoding the mutant *luxR* as template. *Bam*HI, *Hin*dIII and *Kpn*I were purchased from Roche Applied Science (Indianapolis, IN) and *Aat*II was purchased from New England Biolabs (Beverly, MA).

Table 2.5. Oligonucleotide primers used in Chapter 2

Primer	Sequence
5-LuxR	5-CGAACGGGGTACCCATGAAAAACATAAATGCCGACGACAC-3
3-LuxR	5-CGTTCGCGGATCCCGTACTTAATTTTTAAAGTATGGG CAATC-3
5-LarSeq2	5-CCTGAGCAATCACCTATGAACTGTC-3
LuxRSeq(int)	5-CGAAAACATCAGGTCTTATCACTGGG-3
5-pluxI	5-CGAACGCGACGTCAGTCCTTTGATTCTAATAAATTGGA TTTTTGTCAC-3
3-pluxI	5-CTTCTCCTTTACTCATACCAACCTCCCTTGCGTTTATTC-3
5-GFPuv	5-GGGAGGTTGGTATGAGTAAAGGAGAAGAACTTTTCACT-3
3-GFPuv	5-GTACCCAAGCTTTTATTTGTAGAGCTCATCCATGCCATG-3
5-pPROTetSeq	5-CCTCTTACGTGCCGATCAACGTC-3
GFPuvSeq(int)	5-CGAAGGTTATGTACAGGAACGCAC-3
3-LuxR-myc	5-CGTTCGCGGATCCTTACAGATCCTCTTCGCTGATCAGTTTCT GTTCAGCTCCACCATTTTTAAAGTATGGGCAATCAATTG-3

E.3. Library construction and screening

Error-prone PCR reactions were performed using *AmpliTaq* DNA polymerase (Applied Biosystems, Foster City, CA) and 50 μM $MnCl_2$ to increase the mutation rate as described [46]. The primers 5-luxR and 3-luxR were used to amplify the *luxR* gene using pLuxR as the template. The library was constructed by ligating *Kpn*I and *Bam*HI digested pPROLar.A122 with the products of error-prone PCR using T4 DNA ligase (Invitrogen, Carlsbad, CA). Vent DNA polymerase (New England Biolabs, Beverly, MA) was used to amplify wild-type *luxR,* which was digested and ligated into pPROLar.A122 for use as a control. The ligation mixtures were transformed into competent DH5α cells harboring p*lux*GFPuv and plated onto LB agar containing the appropriate antibiotics and 50 or 200 nM C8HSL. The plates were incubated at 37 ^{o}C for 18 hours prior to screening. To identify clones of interest, the agar plates were placed over a UV-transilluminator (VWR Scientific, West Chester, PA) at 365 nm and visually inspected for GFPuv fluorescence. The plates were stored at 4 ^{o}C and reexamined every 2-3 hours until colonies on a control plate expressing wild-type LuxR became visibly fluorescent.

Second-generation libraries were constructed by DNA shuffling as described [24]. The primers 5-luxR and 3-luxR were used to amplify the mutant *luxR* genes from six first-generation mutants using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). After purification and quantification, equal amounts of parent amplification products were mixed and subjected to a DNAseI digestion. The 100 μL digest contained ca. 4 μg of the parental mix, 10 μL of 0.5 M Tris-HCl (pH 7.4), 5 μL of 0.2 M manganese chloride and

0.14 units of DNAse I. After one minute of digestion at 15°C, the reaction was stopped by adding 5 μL of 1 M EDTA and by placing the mixture immediately on ice. The QIAEXII gel extraction kit (Qiagen, Valencia, CA) was used to purify fragments ranging from 50 to 150 bp in length. Fragments were randomly reassembled in a 50 μL reaction. Full-length *luxR* genes were synthesized by diluting the reassembly reaction 50- to 500-fold and amplified using *Pfu* Turbo DNA polymerase and the primers 5-luxR and 3-luxR. Ligations and transformations were performed similarly to the first generation and plated onto LB-agar containing 50 nM C8HSL. All 102 colonies that fluoresced after 18 hours were picked, purified, and inoculated into 1 mL LB containing the appropriate antibiotics. After 24 hours at 37 °C, the cultures were diluted 10-fold into fresh LB, and replica-stamped using a 96-well pin replicator onto solid media containing 10, 20, 50, 100 and 200 nM C8HSL. Ten mutants that fluoresced in response to 10 or 20 nM C8HSL were identified for further characterization.

E.4. Re-cloning and DNA sequencing

All mutants identified during the first- and second- generation screens were re-cloned into fresh background plasmids and strains to eliminate secondary-site effects. For each mutant, the *lux*R allele was amplified using Pfu Turbo polymerase and treated with *Dpn*I. The PCR products were digested and ligated into pPROLar.A122 (as above) and transformed into competent DH5α cells containing p*lux*GFPuv. The promoter and *luxR*

gene from all mutants of interest were sequenced using the upstream primer 5-LarSeq2 and internal *luxR* primer LuxRSeq(int).

E.5. Site-directed mutagenesis

DNA fragments containing each single point mutation of interest were generated using standard methods [47]. In the first step, the 5-LuxR primer and a reverse primer containing the desired mutation were used to amplify *luxR* upstream of the mutation; the 3-LuxR primer and a forward primer also containing the mutation were used to amplify luxR downstream of the mutation. In the second step, the two overlapping fragments were assembled to yield a full length *luxR* containing a single nucleotide alteration by PCR using the products of step one as template and the 5-LuxR and 3-LuxR primer set. These PCR products were digested and ligated into *Kpn*I and *Bam*HI digested pLuxR to replace the wild-type *luxR*. Sequences of each site-directed mutant were verified by DNA sequencing.

E.6. Quantitative characterization of LuxR-mediated gene expression of GFPuv

The assay used to measure LuxR-mediated gene activation was adapted from a light-based bioassay protocol [18]. Cells were first grown from single colonies or glycerol stocks in LB overnight, then diluted 200-fold into 100 mL of fresh LB medium

containing 5 mM potassium phosphate buffer, pH 6.5, and the appropriate antibiotics. Such cultures were incubated with shaking at 37 ^{o}C until they reached an OD_{600} of 0.5, and then harvested by centrifugation. Cell pellets were washed and resuspended to an OD_{600} of 0.6 in bioassay medium (0.05% w/v tryptone, 0.03% v/v glycerol, 100 mM sodium chloride, 50 mM magnesium sulfate and 5 mM potassium phosphate buffer, pH 6.5, containing antibiotics. The suspension was subsequently transferred into 48-well plates (VWR International) containing 0.5 mL bioassay medium with acyl-HSL, to a total volume of 2.5 mL per well. Thereafter, the 48-well plates were shaken at 37^{o}C for 4 hours. From each well, 200 μL was transferred to wells of a white 96-well microplate with a clear bottom. GFPuv fluorescence (395 nm excitation, 509 nm emission, 495 nm cutoff) was measured using a fluorescence microtiterplate reader (Molecular Devices, SpectraMAX Gemini XS); cell densities were measured using a microtiterplate reader at 600 nm. Fluorescence by cell suspensions was normalized to optical density. The fluorescence output of pPROLar.A122 with p*lux*GFPuv was used to determine the background fluorescence without LuxR, and this background fluorescence value was subtracted from all fluorescence measurements obtained with wild-type pLuxR and all mutants to determine fluorescence due only to LuxR-dependent gene activation. For a given variant, the same batch of cells was used for comparing responses to (1) different concentrations of a given acyl-HSL, or (2) different acyl-HSLs.

E.7. Assays of LuxR abundance in vivo by Western immunodetection

To estimate the relative concentrations of LuxR in uninduced and fully induced second-generation variants, we constructed C-terminal LuxR fusions to the amino acid sequence GGAEQKLISEEDL, i.e. the *c-myc* epitope tag with an N-terminal GGA linker. pLuxR-myc was transformed into DH5α(p*lux*GFPuv), the protein it encodes was determined to function similarly to its parent LuxR without the tag. Strains expressing the C-terminal *c-myc* fusions of wild-type LuxR and the second-generation LuxR variants were grown, harvested, resuspended, and incubated in the absence of acyl-HSL or with 1 μM 3OC6HSL in a similar manner as during the quantitative characterization of LuxR-mediated gene expression of GFPuv (see above). After incubation in the presence or absence of acyl-HSL and after monitoring OD_{600} and fluorescence: 1 mL of each cell suspension of interest was resuspended in 50 μL SDS loading buffer. Samples were boiled for 5 min and centrifuged for 10 min before the proteins in the clarified fluid were size fractionated via standard SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes (Invitrogen). After an 8 hour blocking step using tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) buffer containing 5% non-fat dried milk, membranes were incubated for 8 h in TBS buffer containing 1% non-fat dried milk and 4% bovine serum albumin (BSA, Sigma) with anti-*myc* mouse antisera (Invitrogen). Antibody-protein complexes were visualized using horseradish peroxidase-coupled anti-murine second antibody (Invitrogen) and SuperSignal West Pico Substrate (Pierce, Rockford, IL). Chemiluminescence was detected using Kodak BioMax Light Film (Rochester, NY). The developed film was subsequently scanned using a Hewlett Packard Scanjet 4400c and HP Precisionscan Pro software (version 3.1). Protein

concentrations, relative to wild-type LuxR, were determined from the scanned image via densitometry analysis using Quantity One software (Bio-rad, version 4.1.1).

F. References

1. Fuqua, C., Parsek, M.R. & Greenberg, E.P. Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**, 439-468 (2001).

2. Miller, M.B. & Bassler, B.L. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**, 165-199 (2001).

3. Redfield, R.J. Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol.* **10**, 365-370 (2002).

4. Fuqua, W.C., Winans, S.C. & Greenberg, E.P. Quorum sensing in bacteria - the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269-275 (1994).

5. Engebrecht, J. & Silverman, M. Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **81**, 4154-4158 (1984).

6. Kaplan, H.B. & Greenberg, E.P. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**, 1210-1214 (1985).

7. Pearson, J.P., Van Delden, C. & Iglewski, B.H. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**, 1203-1210 (1999).

8. Egland, K.A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: elements of the *luxl* promoter. *Mol. Microbiol.* **31**, 1197-1204 (1999).

9. Stevens, A.M., Fujita, N., Ishihama, A. & Greenberg, E.P. Involvement of the RNA polymerase alpha-subunit C-terminal domain in LuxR-dependent activation of the *Vibrio fischeri* luminescence genes. *J. Bacteriol.* **181**, 4704-4707 (1999).

10. Stevens, A.M. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Essential elements for activation of the luminescence genes. *J. Bacteriol.* **179**, 557-562 (1997).

11. Lerat, E. & Moran, N.A. The evolutionary history of quorum-sensing systems in bacteria. *Mol. Biol. Evol.* **21**, 903-913 (2004).

12. von Bodman, S.B. & Farrand, S.K. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an N-acyl-homoserine lactone autoinducer. *J. Bacteriol.* **177**, 5000-5008 (1995).

13. Passador, L., Cook, J.M., Gambello, M.J., Rust, L. & Iglewski, B.H. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127-1130 (1993).

14. Bainton, N.J., Stead, P., Chhabra, S.R., Bycroft, B.W., Salmond, G.P.C., Stewart, G. & Williams, P. N-(3-Oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. *Biochem. J.* **288**, 997-1004 (1992).

15. Pierson, L.S., Keppenne, V.D. & Wood, D.W. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J. Bacteriol.* **176**, 3966-3974 (1994).

16. Parsek, M.R. & Greenberg, E.P. Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. *Methods Enzymol.* **310**, 43-55 (1999).

17. Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. & Greenberg, E.P. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**, 295-298 (1998).

18. Schaefer, A.L., Hanzelka, B.L., Parsek, M.R. & Greenberg, E.P. Detection, purification, and structural elucidation of the acyl-homoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Methods Enzymol.* **305**, 288-301 (2000).

19. Schaefer, A.L., Taylor, T.A., Beatty, J.T. & Greenberg, E.P. Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *J. Bacteriol.* **184**, 6515-6521 (2002).

20. Chai, Y.R. & Winans, S.C. Site-directed mutagenesis of a LuxR-type quorum-sensing transcription factor: Alteration of autoinducer specificity. *Mol. Microbiol.* **51**, 765-776 (2004).

21. Crameri, A., Whitehorn, E.A., Tate, E. & Stemmer, W.P.C. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* **14**, 315-319 (1996).

22. Eberhard, A., Widrig, C.A., McBath, P. & Schineller, J.B. Analogs of the autoinducer of bioluminescence in *Vibrio fischeri*. *Arch. Microbiol.* **146**, 35-40 (1986).

23. Schaefer, A.L., Hanzelka, B.L., Eberhard, A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Probing autoinducer-LuxR interactions with autoinducer analogs. *J. Bacteriol.* **178**, 2897-2901 (1996).

24. Joern, J.M. DNA Shuffling. in *Directed Evolution Library Creation* (eds. Arnold, F.H. & Georgiou, G.) 85-89 (Humana Press, Totawa, NJ, 2003).

25. Zhu, J. & Winans, S.C. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc. Natl. Acad. Sci. USA* **98**, 1507-1512 (2001).

26. Zhang, R.G., Pappas, T., Brace, J.L., Miller, P.C., Oulmassov, T., Molyneauz, J.M., Anderson, J.C., Bashkin, J.K., Winans, S.C. & Joachimiak, A. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**, 971-974 (2002).

27. Stevens, A.M. & Greenberg, E.P. Transcriptional activation by LuxR. in *Cell-Cell Signaling in Bacteria* (eds. Dunny, G.M. & Winans, S.C.) 231-42 (ASM Press, Washington, DC, 1999).

28. Shadel, G.S., Young, R. & Baldwin, T.O. Use of regulated cell-lysis in a lethal genetic selection in *Escherichia coli*-identification of the autoinducer-binding region of the LuxR protein from Vibrio fischeri ATCC-7744. *J. Bacteriol.* **172**, 3980-3987 (1990).

29. Slock, J., Vanriet, D., Kolibachuk, D. & Greenberg, E.P. Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. *J. Bacteriol.* **172**, 3974-3979 (1990).

30. Voigt, C.A., Mayo, S.L., Arnold, F.H. & Wang, Z.G. Computational method to reduce the search space for directed protein evolution. *Proc. Natl. Acad. Sci. USA* **98**, 3778-3783 (2001).

31. Egland, K.A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Analysis of the LuxR DNA binding region by alanine-scanning mutagenesis. *J. Bacteriol.* **183**, 382-386 (2001).

32. Trott, A.E. & Stevens, A.M. Amino acid residues in LuxR critical for its mechanism of transcriptional activation during quorum sensing in *Vibrio fischeri*. *J. Bacteriol.* **183**, 387-392 (2001).

33. Urbanowski, A.L., Lostroh, C.P. & Greenberg, E.P. Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **186**, 631-637 (2004).

34. Fuqua, W.C. & Winans, S.C. A LuxR-LuxI type regulatory system activates agrobacterium Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J. Bacteriol.* **176**, 2796-2806 (1994).

35. Gambello, M.J. & Iglewski, B.H. Cloning and characterization of the *Pseudomonas aeruginosa* LasR gene, a transcriptional activator of elastase expression. *J. Bacteriol.* **173**, 3000-3009 (1991).

36. Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C. & Greenberg, E.P. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **98**, 2752-2757 (2001).

37. Whiteley, M., Lee, K.M. & Greenberg, E.P. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **96**, 13904-13909 (1999).

38. Matsumura, I. & Ellington, A.D. *In vitro* evolution of b-glucuronidase into a b-galactosidase proceeds through non-specific intermediates. *J. Mol. Biol.* **305**, 331-339 (2001).

39. Jenson, R.A. Enzyme recruitment in evolution of new function. *Annu. Rev. Microbiol.* **30**, 409-425 (1976).

40. Kacser, H. & Beeby, R. Evolution of catalytic proteins or on the origin of enzyme species by means of natural selection. *J. Mol. Evol.* **20**, 38-51 (1984).

41. Lazcano, A., Diazvillagomez, E., Mills, T. & Oro, J. On the levels of enzymatic substrate-specificity - implications for the early evolution of metabolic pathways. *Advan. Space Res.* **15**, 345-356 (1994).

42. Waley, S.G. Some aspects of evolution of metabolic pathways. *Comp. Biochem. Physiol.* **30**, 1-11 (1969).

43. Ycas, M. On earlier states of the biochemical system. *J. Theor. Biol.* **44**, 145-160 (1974).

44. Santoro, S.W. & Schultz, P.G. Directed evolution of the site specificity of Cre recombinase. *Proc. Natl. Acad. Sci. USA* **99**, 4185-4190 (2002).

45. Voziyanov, Y., Stewart, A.F. & Jayaram, M. A dual reporter screening system identifies the amino acid at position 82 in Flp site-specific recombinase as a determinant for target specificity. *Nucleic Acids Res.* **30**, 1656-1663 (2002).

46. Cirino, P.C., Mayer, K.M. & Umeno, D. Generating libraries using error-prone PCR. in *Directed Evolution Library Creation* (eds. Arnold, F.H. & G., G.) 3-9 (Humana Press, Totawa, NJ, 2003).

47. Horton, R.M., Ho, S.N., Pullen, J.K., Hunt, H.D., Cai, Z.L. & Pease, L.R. Gene-splicing by overlap extension. *Methods Enzymol.* **217**, 270-279 (1993).

CHAPTER 3

Design and Evaluation of a Dual Selection System for Identifying LuxR Variants with New Specificities

A. Abstract

To continue to our directed evolution of LuxR towards variants with new specificities, we constructed a selection system capable of both positive (ON) and negative (OFF) selection. An ON/OFF selection system is required to identify specific proteins because positive selection alone generally leads to variants with broadened specificities. A dual selection module developed previously was adapted to incorporate the P_{luxI} promoter and was shown to enrich by 490-fold per round of ON/OFF selection. Additional rounds of selection were necessary to completely eliminate the presence of nonfunctional plasmids. This work both demonstrates the potential of this dual selection system to enrich for functional LuxR variants from very large libraries ($>10^6$) and suggests controls that can be used to decrease the probability of identifying false positives.

B. Introduction

The previous chapter described the directed evolution of LuxR variants that respond to a broad range of acyl-homoserine lactones (acyl-HSLs). To identify an evolved LuxR variant that retains (or increases) its newly-acquired ability to respond to a non-natural signal molecule and decreases its ability to respond to LuxR's cognate signal, 3-oxohexahoyl-homoserine lactone (3OC6HSL), a selection system capable of both positive and negative selection was developed.

Gain-of-function mutants are identified by positive selection. While it is possible for a single amino acid substitution to both increase a protein's ability to recognize a new target and decrease, or prevent, the recognition of its natural target [1-3], it has been observed that this occurs infrequently in directed evolution experiments targeting new functions [4-6]. Instead, initial rounds of directed evolution often produce variants that exhibit broadened specificity [2, 4-11]. Matsumura and Ellington noted that their β-glucoronidase variants were active with substrates that were very dissimilar to both the native and target substrates [5]. Similar observations led Aharoni *et al.,* to postulate that the observed broadening of specificity is a common feature of protein evolution [4]. They also noted that it is evolutionarily advantageous for a protein to be able to increase its affinity for a new target without affecting its affinity for its native target molecule.

But what happens when it is necessary to refocus specificity? Matsumura and Ellington used repeated rounds of screening for increases in activity with their target substrate and

then characterized several mutants to find the desired inversion of specificity [5]. A more direct approach uses screening or selecting for both an increased response to a new target and a loss of response to the native target. The addition of negative rounds of selection or screening can be used to identify variants with new specificities without having to sift through a large number of variants identified by positive rounds and hoping that a variant with decreased affinity for the native molecule is present.

Santoro and Schulz compared positive screening alone to a combination of positive and negative screening in their directed evolution experiments targeting Cre recombinase variants capable of recognizing a new DNA target [12]. They showed that positive screening yielded variants capable of recombining at either the wild-type or mutated DNA target. The combination of both positive and negative screening led to the identification of a variant with an increase in recombination at the new target site and a decrease in its ability to act at its native site. The directed evolution of Cre recombinase illustrates the importance of including rounds of both positive and negative selective pressure in order to identify variants with an explicit specificity profile. Positive and negative screens have also been successfully implemented to identify Cre recombinases that recognize a different DNA site [13], tRNA-synthetases [14] and the endopeptidase OmpT [3].

Physical models have also alluded to the importance of including both positive and negative "pressure" in order to target specificity [1, 15, 16]. In designing specific

protein-protein or protein-DNA interactions, positive design is used to stabilize interactions with a desired target while negative design destabilizes undesired interactions. A physical model of DNA binding by zinc fingers showed that these proteins are optimized for specificity rather than affinity [1]. This result implies a trade-off between specificity and affinity may exist and, more importantly, it reinforces the experimental evidence that looking only for increases in affinity for a new target will infrequently yield variants with refocused specificities.

Before searching libraries for LuxR variants with new specificities, it was imperative that we both design a dual selection system and evaluate its ability to identify functional clones from a pool of nonfunctional "garbage." We have implemented an ON/OFF selection system for the directed evolution of LuxR based on the dual selection module previously developed by Yokobayashi and Arnold [17] for the identification of a functional repressor. Their selection system uses the expression of the tetracycline resistance gene, *tetA*, to confer survival during ON rounds of selection. OFF rounds of selection use the production of β-lactamase inhibitory protein (Bli) to confer sensitivity to β-lactam antibiotics when unwanted transcription occurs. β-lactamase (bla), which is constitutively expressed from the selection plasmid, is inhibited when bound by Bli. The *bli* gene was extensively modified by Yokobayashi and Arnold [17] to lower the GC content of the gene and to decrease the homology of the periplasmic leader sequences used by *bli* and *bla*. The reduced homology between the two leader sequences lowers the

probability of recombination events that could eliminate *bli* and undermine the selection process by producing false positives.

The primary advantage of using a selection system, as opposed to a screen, is an increase in potential library size to more than 10^6. While screens can be cumbersome and limit library size, selections are often limited by the prevalence of false positives that occur when bacteria (or other organisms) are under selective pressure. By employing a dual selection system, which requires proteins to be ON or OFF under different environmental conditions, the frequency of false positives is expected to decrease. We have implemented and evaluated a dual selection system for directed evolution of the transcriptional activator LuxR and its cognate promoter, P_{luxI}, and have demonstrated that it can enrich a functional activator approximately 490-fold per ON/OFF round of selection.

C. Results

C.1. Modification of a dual selection system for LuxR-mediated gene activation

Our goal was to adapt the generic selection module developed by Yokobayashi and Arnold [17] to LuxR-mediated transcriptional activation, such that the ability of LuxR to activate gene expression at P_{luxI}, under a given set of conditions, would correspond to the ability of a host bacterium to survive in the presence of different antibiotics. While the original dual selection system of Yokobayashi and Arnold [17] encodes both the positive (TetA) and negative (Bli) elements on a single selection plasmid, replacement of the repressed promoter with the *lux* promoter did not lead to simultaneous LuxR-mediated expression of both elements. We observed that the amount of basal gene expression of the second gene (*tetA*) in the operon always generated an ON output, irrespective of LuxR. Switching the order of the genes and replacing *tetA* with *cat* did not alleviate this problem. In general, promoters that require an activator to recruit RNA polymerase have higher levels of basal gene expression than promoters that are repressed by transcription factors blocking RNA polymerase binding [18]. Instead of trying to tune the output levels of the ON and OFF selection genes simultaneously, we separated the components into ON and OFF selection plasmids (Fig. 3.1). This requires that the selection plasmid be inactivated and replaced following each round of selection. However, separate ON and OFF selection plasmids act to lower the probability of propagating mutated selection plasmids that confer antibiotic resistance without LuxR, i.e. false positives, from one round to the next.

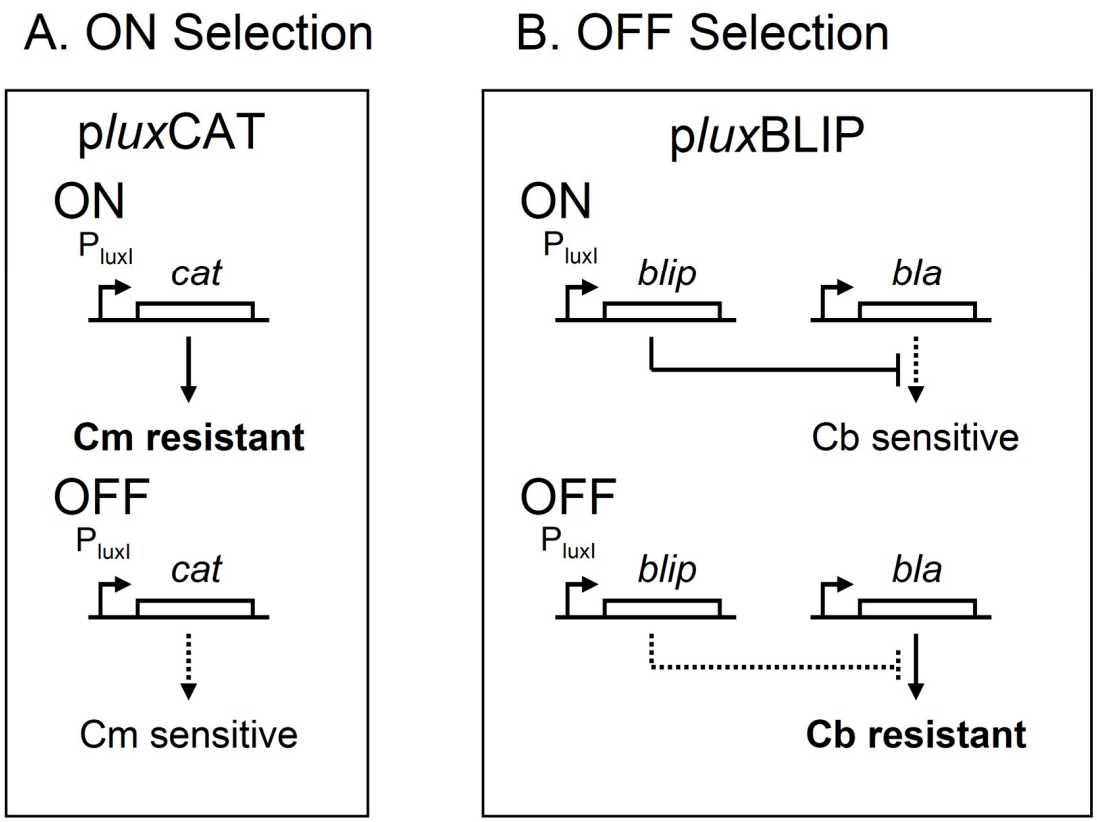


Figure 3.1. Schematic of the dual selection system for the directed evolution of LuxR variants with altered specificity. During rounds of ON selection, LuxR variants that are active under the desired conditions are identified by their ability to survive in the presence of chloramphenicol (Cm) due to LuxR-mediated activation of the chloramphenicol acetyl-transferase (*cat*) gene from the PluxI promoter. During rounds of OFF selection, LuxR variants which are inactive are identified by their ability to survive in the presence of carbenicillin (Cb) due to a lack of LuxR-mediated expression of the β-lactamase inhibitory protein (*bli*) gene. During OFF rounds of selection, β-lactamase is constitutively expressed in the cells and is inhibited by Bli, rendering the cells sensitive to carbenicillin.

The ON selection plasmid, pluxCAT, confers chloramphenicol resistance in the presence of LuxR-mediated transcriptional activation of the *cat* gene from the P_{luxI} promoter. The

OFF plasmid, pluxBLIP, renders bacteria in which LuxR is active sensitive to carbenicillin by producing Blip, which inactivates the constitutively expressed Bla. Thus an absence of LuxR-mediated gene activation is detected by survival in the presence of carbenicillin with the OFF selection plasmid.

C.2. Evalutaion of the dual selection system

To test our modified selection module, we selected a plasmid expressing *luxR* (pLuxR, Fig. 3.2B) from a background of nonfunctional plasmids. The nonfunctional plasmids either lack the *luxR* gene (pPROLar.A122, Fig. 3.2A) or constitutively express both *cat* and *bli* (pBLIPCAT, Fig. 3.2C). While pLuxR confers survival with the ON selection plasmid in the presence of 3OC6HSL and survival with the OFF selection plasmid in the absence of 3OC6HSL, pPROLar.A122 should survive only OFF selection rounds while pBLIPCAT should survive only ON rounds of selection.

The three plasmids, pLuxR, pPROLar.A122 and pBLIPCAT, were mixed with a 1:500:500 molar ratio and transformed into DH5α cells harboring the ON selection plasmid p*lux*CAT. The transformed cells were plated on chloramphenicol-containing LB plates with 100 nM 3OC6HSL to select for cells in the ON state (pLuxR or pBLIPCAT). Cells were recovered by overlaying liquid LB medium and the DNA from these cells was isolated by miniprep. To inactivate the ON selection plasmid, the recovered plasmid

DNA was digested with *Apa*LI, which cleaves pluxCAT and pluxBLIP but does not cleave pLuxR, pPROLar.A122 or pBLIPCAT.

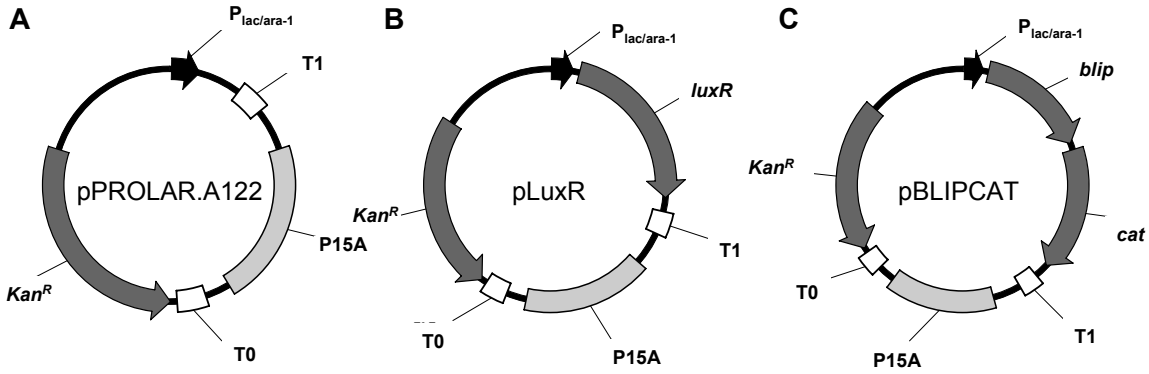


Figure 3.2. Plasmid maps of plasmids used to test dual selection system. **A.** pPROLar.A122 is an "empty" vector. It does not encode a functional *luxR* gene and P_{luxI} is never activated in cells containing this plasmid, **B.** pLuxR encodes the wild-type luxR gene under the control of a $P_{lac/ara-1}$. LuxR is constitutively expressed in DH5α, which does not contain *lacI* or *araC*. LuxR activates gene expression from P_{luxI} only in the presence of 3OC6HSL. **C.** pBLIPCAT constitutively expresses both of the selection proteins, CAT and Bli. CAT and Bli are expressed from this plasmid regardless of 3OC6HSL concentration.

The *Apa*LI-digested DNA from Round 1-ON was used to transform DH5α cells harboring the OFF selection plasmid, pluxBLIP. Cells were spread on plates containing carbenicillin without 3OC6HSL to select for cells in the OFF state (pLuxR or pPROLar.A122). Round 1-OFF cells were recovered, and the DNA was isolated and digested as following Round 1-ON. The purified DNA was transformed into DH5α containing pluxCAT to initiate Round 2 of ON/OFF selection.

From each round of selection, 45 colonies were randomly picked and tested for the presence of pLuxR, pPROLar.A122 or pBLIPCAT by colony PCR. Because these plasmids each contain the same backbone, PCR with primers that bind outside of the inserted region amplifies a section of differing length from each of the three plasmids. Amplification of pLuxR via PCR yields a 1100 base pair (bp) fragment, while pPROLar.A122 yields a 400 bp fragment and pBLIPCAT a 1650 bp fragment, allowing the three plasmids to be easily distinguished from one another.

Following Round 1-ON, 45 of the 45 (100%) colonies picked contained the pBLIPCAT plasmid (Fig. 3.3). This was expected due to the 500-fold excess of this plasmid over pLuxR. Twenty-two of the 45 (49%) colonies from Round 1-OFF contained pLuxR (Fig. 3.3). The other 23 colonies contained pPROLar.A122. A 490-fold enrichment of pLuxR was achieved in the first round of ON/OFF selection.

As shown in Fig. 3.3, 44 of 45 colonies contained pLuxR after Round 2-ON. The remaining colony harbored pPROLar.A122. Round 2-OFF returned exactly the same ratio of pLuxR to pPROLar.A122 as the second ON selection because both plasmids confer the same probability of survival OFF rounds. A third ON round yielded 100% of colonies containing pLuxR

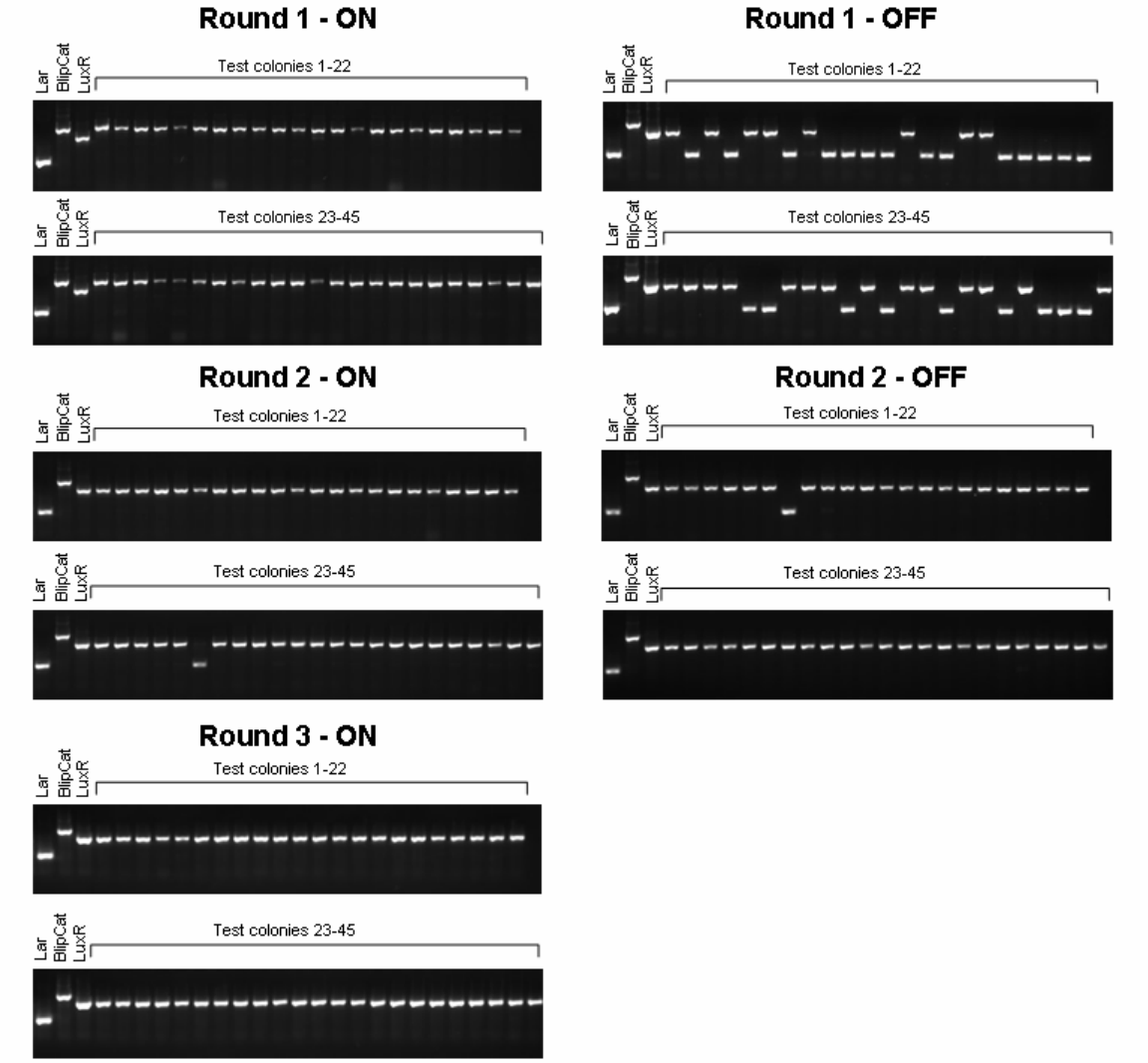


Figure 3.3. Agarose gel of colony PCR products used to determine the composition of the plasmid mixture after each round of positive and negative selection. Forty-five colonies were randomly picked and colony PCR with primers that bind to the common plasmid backbone of each of the three test plasmids was used to identify if test colonies contained pPROLar.A122 (Lar), pLuxR (LuxR) or pBLIPCAT (BLIPCAT). Controls of each plasmid are shown on the left three lanes of each gel.

D. Discussion

Implementation of an ON/OFF selection system for identifying LuxR variants required modifying the dual selection system developed by Yokobayashi and Arnold for identifying functional repressors [17]. Using a single selection plasmid for both ON and OFF rounds of selection was undermined by an excessive basal level of protein production from the second gene in the operon. The reason for the observed increase at only the second gene of the operon is unknown, however, an overall increase in the basal level of gene expression is not unexpected at a promoter that is modulated by an activator instead of a repressor [18]. Activators increase RNA polymerase's affinity for a given promoter and a low level of transcription is expected because the polymerase will occasionally bind to the promoter without being recruited by the activator. On the other hand, repressors physically block RNA polymerase from binding to the promoter so basal levels of transcription from these promoters are significantly lower. The modifications made here are likely applicable to the evolution of other activator proteins using similar dual selection modules.

The use of two separate selection plasmids required their inactivation following each round of selection. This was accomplished by selective digestion with an endonuclease that cleaves the selection plasmids but leaves the circuit plasmids intact. While this step increases the labor required for each ON/OFF round of selection, it ensures that any selection plasmids that have mutated and confer resistance without LuxR are not propagated. Due to this control, the frequency of false positives that arise due to mutated

selection plasmids should be consistent from round to round while the frequency of functional clones should increase exponentially. This reduces the probability of identifying false positives at the end of a directed evolution experiment.

The background level of nonfunctional plasmids in Round 2 indicates that the ON rounds of selection could have been more stringent; however, an extra round of selection was shown to eliminate the nonfunctional plasmids. To ensure that only functional variants are picked for further characterization, the isolated clones could be screened for their ability to activate the expression of a non-lethal reporter protein, such as the GFP variant used in Chapter 2 [7]. A low frequency of nonfunctional clones would not prevent the identification of functional clones with a final screen and could potentially decrease the number of selection rounds required because it would be unnecessary to ensure that all nonfunctional clones had been eliminated. Furthermore, a screen would add the ability rapidly identify the "best" variants from a pool that meet the minimum requirements imposed by the ON/OFF conditions used with dual selection system.

The 490-fold enrichment of functional LuxR variants per round of selection suggests that three rounds of ON/OFF selection should allow a functional LuxR variant to be identified from a library of more than 100 million. The proportional increase in functional variants in each round of selection is limited by the frequency of false positives that arise each round. Decreasing regions of high sequence similarity can be used to decrease the probability that a recombination event will lead to a false positive [17]. If false positives

were plaguing the selection system, it would be worthwhile to investigate other mechanisms that were allowing the bacteria to survive without the desired gene activation, or absence thereof.

With our new selection system for identifying LuxR variantd, the 490-fold enrichment is likely sufficient for identifying LuxR variants from the size of libraries that are possible to generate. The removal of the selection plasmids after each round of selection is important for decreasing the number of false positives identified. The following chapters describe the use of this dual selection system for identifying LuxR variants that respond to a non-cognate acyl-HSL but that have lost their ability to respond to 3OC6HSL and LuxR variants that activate gene expression at a mutated *lux* promoter.

E. Materials and Methods

E.1. Bacterial strains, media and growth conditions.

The bacterial strains used in this study were: *Escherichia coli* strain DH5α (F-ϕ80dlacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1) and *E. coli* DH5α containing pluxCAT (DH5α(pluxCAT)) or pluxblip (DH5α(pluxBLIP)). Competent cells were prepared by using the Z-competent *Escherichia coli* Transformation Kit (Zymo Research, Orange, CA). *E. coli* strains were cultured at 37 ^{o}C in LB medium or on LB agar plates. Antibiotics were added at the following concentrations: 150 μg/mL chloramphenicol, 100 μg/mL carbenicillin, and 50 μg/mL kanamycin. 3-Oxo-hexanoyl-homoserine lactone (3OC6HSL; Sigma Aldrich, St. Louis, Mo) stock solutions of 1 and 10 mM were prepared by dissolving appropriate amounts in ethyl acetate acidified with glacial acetic acid (0.01% v/v), and stored at -20 ^{o}C. For solid-phase experiments, 3OC6HSL was added to LB agar media prior to solidification to ensure complete mixing.

E.2. Plasmid construction

The LuxR expression vector, pLuxR, and the signal response plasmid, pluxGFPuv, have been described previously [7]. Plasmids pluxCAT and pluxBLIP are similar to pluxGFPuv but encode chloramphenicol acetyltransferase (CAT) or β-lactamase inhibitory protein (Bli) under the control of the P_{luxI} promoter and contains the β-

lactamase gene (*bla*). The pBPROTet plasmid was constructed by replacing the SacI-AatII *cat* fragment in pPROTet.E133 with the β-lactamase (*bla*) encoding SacI-AatII fragment from $p\lambda_{PRO12}$Select[17]. P_{luxI} was PCR-amplified from pluxGFPuv using the primers 5-pluxI [7] and 3-lux(CAT) or 3-lux(blip). *cat* was PCR-amplified from pPROTet.E133 using 5-CAT(lux) and 3-CAT(BamHI) (see Table 3.1 for oligonucleotide sequences). *bli* was PCR-amplified from $p\lambda_{PRO12}$Select using 5-BLIP(lux) and 3-BLIP(HindIII). The P_{luxI} and *cat* or *bli* PCR products were assembled and amplified by PCR with 5-pluxI and 3-CAT(BamHI) or 3-BLIP(HindIII) and cloned into pBPROTet between its *Aat*II and *BamHI or Hin*dIII sites.

The pBLIPCAT plasmid was constructed by PCR-amplifying *bli* from $p\lambda_{PRO12}$Select using 5-BLIP(KpnI) and 3-BLIP(HindIII) and *cat* from pPROTet.E133 with 5-CAT(BLIP) and 3-CAT(BamHI). These *bli* and *cat* PCR products were assembled and amplified by PCR with 5-BLIP(KpnI) and 3-CAT(BamHI) and cloned into pPROLar.A122 between its *KpnI* and *BamHI* sites.

Table 3.1. Oligonucleotide primers used in Chapter 3

Oligonucleotide	Sequence
5-pluxI	5-CGAACGCGACGTCAGTCCTTTGAT TCTAATAAATTGGATTTTTGTCAC-3
3-pluxI	5-CTTCTCCTTTACTCATACCAACCTCCCTTGCGTTTATTC-3
5-LarSeq2	5-CCTGAGCAATCACCTATGAACTGTC-3
3-lux(CAT)	5-GATTTTTTTCTCCATACCAACCTCCCTTGCGTTTATTC-3
3-lux(BLIP)	5-ACTTTTATATAAAAGCATACCAACCTCCCTTGCGTTTATTC-3
5-CAT(lux)	5-GGGAGGTTGGTATGGAGAAAAAAATCACTGGATATACC-3
3-CAT(BamHI)	5-GTTAGCGGACCAGCGAGCTCGATATCAAATTACGCC-3
5-BLIP(lux)	5-GGGAGGTTGGTATGCTTTTATATAAAATGTGTGACAATCA-3
3-BLIP(HindIII)	5-GTTAGCAAGCTTTTATACAAGGTCCC-3
5-BLIP(KpnI)	5–CGAACGGGGTACCCATGCTTTTATATAAAATGTGTGACAATCA-3
5-CAT(BLIP)	5-GGGACCTTGTATAAAAGCTTGCTAACCGAATAAACGC AAGGGAGGTTGGTATGGAGAAAAAAATCACTGGATATACC-3
3-LuxR+200	5-GTGAGCGAGGAAGCGGAATATATCC-3

E.3. Selection protocol

For positive selection, circuit plasmids were transformed into DH5α cells harboring the ON selection plasmid (pluxCAT) and plated on LB agar plates containing 100 nM 3OC6HSL, kanamycin and chloramphenicol. For negative selection, circuit plasmids were transformed into DH5α cells harboring the OFF selection plasmid (pluxBLIP) and plated on LB agar plates containing kanamycin and carbenicillin. Plates were incubated for 18 hours at 37^{o}C. Selected cells were recovered by overlaying liquid LB medium over the agar plates. Plasmid DNA from the cells was recovered by miniprep (QIAGEN). To inactivate the selection plasmids, the DNA was digested with *Apa*LI and purified. The digested DNA was subsequently used to transform the next round of selection.

E.4. Plasmid identification by colony PCR

Colony PCR was performed to identify each of the functional plasmids after each round of selection. The Colony Fast-Screen Kit (Epicentre) was used according to instructions, with two primers: 5-LarSeq2 and 3-LuxR+200. These primers bind to the backbone common to each of the three plasmids in the starting mix and allow the specific identification of the plasmids present in each colony. Positive controls of each of the three starting plasmids were run simultaneously.

F. References

1. Havranek, J.J., Duarte, C.M. & Baker, D. A simple physical model for the prediction and design of protein-DNA interactions. *J. Mol. Biol.* **344**, 59-70 (2004).

2. May, O., Nguyen, P.T. & Arnold, F.H. Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine. *Nat. Biotechnol.* **18**, 317-320 (2000).

3. Varadarajan, N., Gam, J., Olsen, M.J., Georgiou, G. & Iverson, B.L. Engineering of protease variants exhibiting high catalytic activity and exquisite substrate selectivity. *Proc. Natl. Acad. Sci. USA* **102**, 6855-6860 (2005).

4. Aharoni, A., Gaidukov, L., Khersonsky, O., Gould, S.M., Roodveldt, C. & Tawfik, D.S. The 'evolvability' of promiscuous protein functions. *Nat. Genet.* **37**, 73-76 (2005).

5. Matsumura, I. & Ellington, A.D. *In vitro* evolution of b-glucuronidase into a b-galactosidase proceeds through non-specific intermediates. *J. Mol. Biol.* **305**, 331-339 (2001).

6. Yano, T., Oue, S. & Kagamiyama, H. Directed evolution of an aspartate aminotransferase with new substrate specificities. *Proc. Natl. Acad. Sci. USA* **95**, 5511-5515 (1998).

7. Collins, C.H., Arnold, F.H. & Leadbetter, J.R. Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. *Mol. Microbiol.* **55**, 712-723 (2005).

8. Gould, S.M. & Tawfik, D.S. Directed evolution of the promiscuous esterase activity of carbonic anhydrase II. *Biochemistry* **44**, 5444-5452 (2005).

9. Graham, L.D., Haggett, K.D., Jennings, P.A., Lebrocque, D.S., Whittaker, R.G. & Schober, P.A. Random mutagenesis of the substrate-binding site of a serine-protease can generate enzymes with increased activities and altered primary specificities. *Biochemistry* **32**, 6250-6258 (1993).

10. Roodveldt, C. & Tawfik, D.S. Shared promiscuous activities and evolutionary features in various members of the amidohydrolase superfamily. *Biochemistry* **44**, 12728-12736 (2005).

11. Zhang, J.H., Dawes, G. & Stemmer, W.P.C. Directed evolution of a fucosidase from a galactosidase by DNA shuffling and screening. *Proc. Natl. Acad. Sci. USA* **94**, 4504-4509 (1997).

12. Santoro, S.W. & Schultz, P.G. Directed evolution of the site specificity of Cre recombinase. *Proc. Natl. Acad. Sci. USA* **99**, 4185-4190 (2002).

13. Voziyanov, Y., Stewart, A.F. & Jayaram, M. A dual reporter screening system identifies the amino acid at position 82 in Flp site-specific recombinase as a determinant for target specificity. *Nucleic Acids Res.* **30**, 1656-1663 (2002).

14. Santoro, S.W., Wang, L., Herberich, B., King, D.S. & Schultz, P.G. An efficient system for the evolution of aminoacyl-tRNA synthetase specificity. *Nat. Biotechnol.* **20**, 1044-1048 (2002).

15. Bolon, D.N., Grant, R.A., Baker, T.A. & Sauer, R.T. Specificity versus stability in computational protein design. *Proc. Natl. Acad. Sci. USA* **102**, 12724-12729 (2005).

16. Havranek, J.J. & Harbury, P.B. Automated design of specificity in molecular recognition. *Nat. Struct. Biol.* **10**, 45-52 (2003).

17. Yokobayashi, Y. & Arnold, F.H. A dual selection module for directed evolution of genetic circuits. *Natural Computing* **4**, 245-254 (2005).

18. Ptashne, M. Regulation of transcription: from lambda to eukaryotes. *Trends Biochem. Sci.* **30**, 275-279 (2005).

CHAPTER 4

Dual Selection Creates a New Signaling Specificity in the Quorum-sensing Transcriptional Activator LuxR

A. Abstract

The LuxR transcription factor from *Vibrio fischeri* activates gene expression in response to binding the signaling molecule 3-oxo-hexanoyl-homoserine lactone (3OC6HSL). LuxR homologs respond to acyl-homoserine lactones (acyl-HSLs) with diverse chemical structures. We previously described a LuxR variant, LuxR-G2E, that activates gene expression upon binding a broadened range of acyl-HSLs, including straight-chain acyl-HSLs. Here, we use an ON/OFF dual selection system to identify a new LuxR variant that retains the response to straight-chain acyl-HSLs, but no longer responds to LuxR's cognate signal, 3OC6HSL. A single mutation, arginine 67 to methionine in the N-terminal signal-binding domain, reduces LuxR-G2E's response to acyl-HSLs having a carbonyl substituent at the third carbon of the acyl chain. This mutation has a similar effect when introduced into a LuxR homolog that responds to a different 3-oxo-HSL (3OC12HSL), LasR from *Pseudomonas aeruginosa*. This specificity-enhancing mutation does not affect LuxR response to straight-chain acyl-HSLs and would not have been identified by positive selection for activation by these signaling molecules. The dual selection system provides a rapid and reliable method for identifying LuxR variants that have the desired response, or lack thereof, to a given set of acyl-HSL signals. This work demonstrates that accumulation of a small number of point mutations is sufficient to switch specificity from one signal to another, passing through a broadly-specific intermediate. LuxR variants with new signaling specificities will be useful components

for constructing artificial cell-cell communication systems that program population-level behaviors such as pattern formation.

B. Introduction

Quorum sensing (QS) is the process by which bacteria sense their local population density and modify the expression of key genes accordingly [1, 2]. While many QS signals have been identified (reviewed by Taga and Bassler [1]), one of the most prevalent and well-studied QS systems employs acyl-homoserine lactones (acyl-HSLs) [3]. In *V. fischeri*, the 3-oxo-hexanoyl-homoserine lactone (3OC6HSL, Fig. 4.1) signal is synthesized by LuxI [4]. In environments of low cell density, the amphipathic acyl-HSL diffuses out of the cell through the membrane [5]. As the local environment becomes more crowded, the acyl-HSLs accumulate in the cell, where they bind to the acyl-HSL-dependent transcriptional activator, LuxR [6, 7]. Upon signal binding, LuxR dimerizes [8] and activates transcription at the *lux* promoter by recruiting RNA polymerase [9]. Pairs of LuxI/LuxR homologs have been identified in many species of Gram-negative *Proteobacteria* [10, 11], where they regulate genes with diverse functions in response to changes in population density [1, 2]. LuxR homologs respond to acyl-HSLs with acyl chains of varying lengths and chemical modifications [12, 13] (Fig. 4.1).

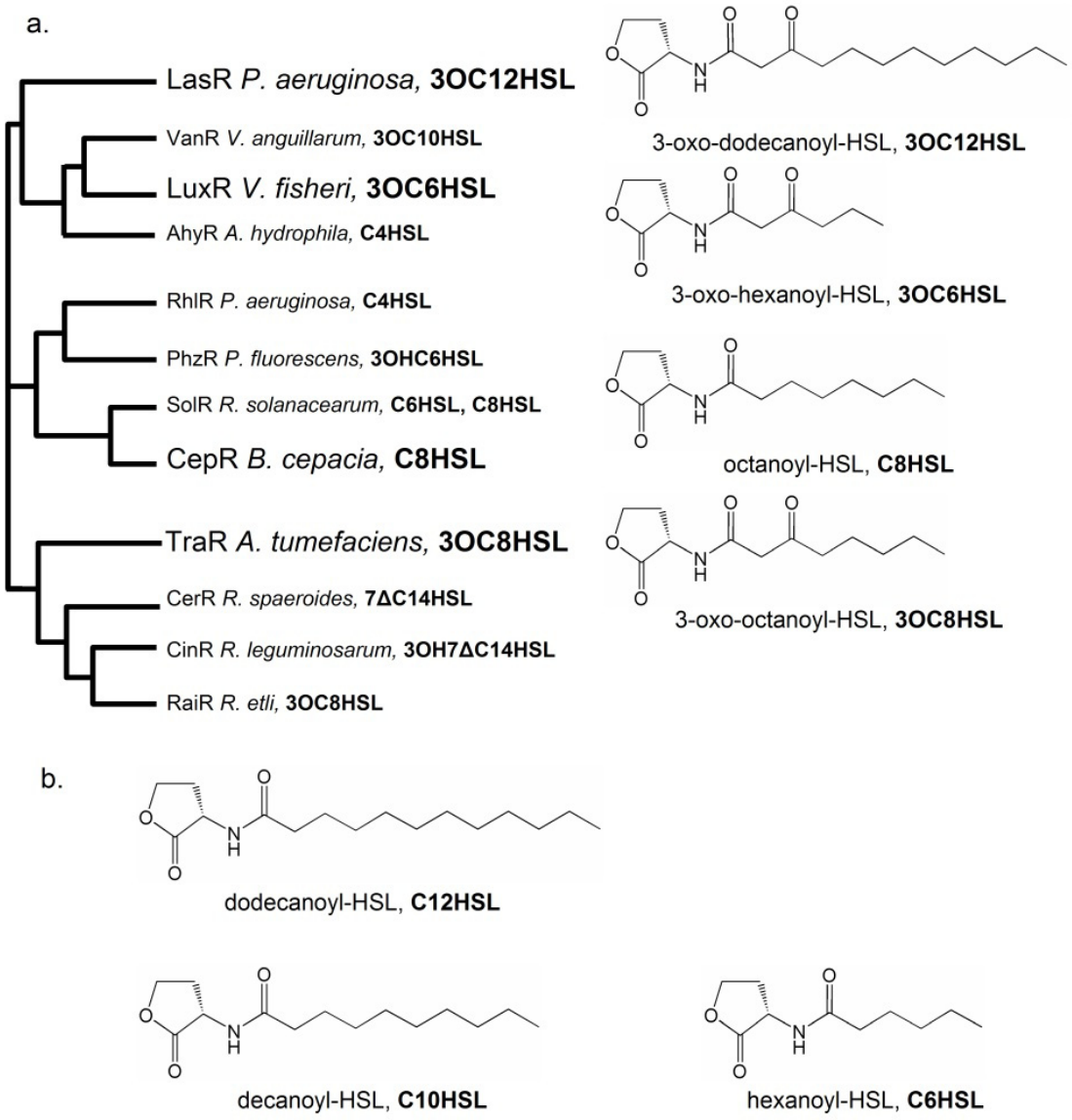


Figure 4.1. Diversity of LuxR homologs and acyl-HSLs. a. Illustration of the divergence of selected LuxR homologs. Homologs are listed with both species and cognate acyl-HSL signal molecule. Homologs discussed in this work are highlighted and the structure of their cognate acyl-HSL signal molecules shown. The tree was adapted from Gray and Garey [10], and Lerat and Moran [11]. b. Structures, names and abbreviations of non-cognate acyl-HSLs used in this study.

LuxR/LuxI and their homologs have been adopted by biological engineers to effect programmed intercellular communication and coordinate the behaviors of cell populations [14-18]. In an early demonstration of engineered cell-cell communication, Weiss and coworkers expressed LuxR in *E. coli* to produce 'receiver' cells that would respond to signals produced by 'sender' cells that expressed LuxI [17]. More recently, You et al. described a circuit that employs Lux QS components to sense and control the density of a bacterial population by expressing a killer gene, *ccdB*, from the *lux* promoter [18]. Kobayashi *et al.* described input/output modules based on the Lux system that coupled to a genetic 'toggle switch' and programmed density-dependent protein production in *E. coli* [16]. Lux QS components have also been used to build a variety of functional modules, including a chemical pulse generator [15] and chemical band-detect network [14]. Synthetic systems exhibiting complex behaviors have exploited these modules, using variants of LuxR that respond to different concentrations of the acyl-HSL signal. For example, using 'receiver' cells containing LuxR-based band-detect circuits that express fluorescent proteins over different concentration ranges of the signaling molecule, Basu *et al.* [14] demonstrated that an undifferentiated lawn of the 'receiver' cells generated a bulls-eye pattern of gene expression around a central colony of 'sender' cells producing 3OC6HSL.

LuxR/I and their homologs can serve a wide range of communication functions in synthetic networks. Applications at the next level of complexity, for example, will implement two-way communications between cellular populations [19, 20]. These

applications, however, require a high degree of specificity in the LuxR receiver functions, since crosstalk among chemical signals can undermine the function of the entire network. LuxR homologs have been investigated, but these can show significant crosstalk [21] and often also behave differently from LuxR in other ways. For applications in synthetic biology, it would be useful to have a set of 'standardized parts'—for example, a series of LuxR variants which respond to different chemical signals at different levels and specificities—from which new networks can be assembled.

Engineering protein specificity is challenging because it requires both stabilizing a desired interaction (positive design) and destabilizing undesired interactions (negative design) [22]. Directed evolution experiments have also shown that specificity rarely arises as a result of positive selection alone [23]. We previously used directed evolution to create LuxR variants exhibiting broadened acyl-HSL specificity and increased response sensitivity [24]. Random-mutant libraries were screened for increased response to octanoyl-homoserine lactone (C8HSL). All the C8HSL-responsive LuxR variants showed broad response to acyl-HSLs having acyl chains five to fourteen carbons in length; none were specific for the new signal. To generate a LuxR with a new signaling specificity, we modified the dual selection system recently described by Yokobayashi and Arnold [25] to select for LuxR variants that either activate gene expression (ON) or do not (OFF) under desired sets of conditions.

C. Results

C.1. A dual selection system for evolution of LuxR signaling specificity

To identify LuxR variants that respond specifically to a new signal, we adapted a dual selection system developed previously to select for functional genetic networks that properly transition gene expression from ON to OFF under desired conditions[25]. The selection scheme was outlined in Figure 3.1. To select for LuxR variants that activate gene expression under a given set of conditions, i.e., in the presence of a new acyl-HSL, a library of mutagenized *luxR* genes is selected with the ON selection plasmid pluxCAT, which yields chloramphenicol resistance with LuxR-dependent transcriptional activation. Identification of variants that show a lack of gene activation (i.e., with the wild-type cognate signal) is achieved by transformation with the OFF selection plasmid pluxBLIP, which encodes β-lactamase inhibitory protein (Bli) under the control of the *lux* promoter and β-lactamase (Bla) under the control of a constitutive promoter. Bli inactivates Bla and renders cells with active LuxR alleles sensitive to β-lactam antibiotics.

C.2. Directed evolution of LuxR-G2E to respond to C10HSL but not 3OC6HSL

To obtain the new LuxR signaling specificity, we started with a broad-specificity variant identified previously, LuxR-G2E (Table 4.1) [24]. To compare gene activation by LuxR and our evolved variants with exogenously added acyl-HSLs, we used a reporter plasmid, pluxGFPuv, that contains *gfpuv* under the control of the *lux* promoter. Liquid-phase

gfpuv bioassays quantitatively measure fluorescence output due to LuxR-mediated GFPuv production [24]. LuxR-G2E responds to DL-decanoyl-homoserine lactone (DL-C10HSL), while wild-type LuxR does not respond at concentrations to 10 μM. Wild-type LuxR and LuxR-G2E respond similarly to 3OC6HSL (Fig. 4.2).

Using error-prone PCR, we created a library of *luxR-G2E* genes encoding, on average, one to two amino acid substitutions in the N-terminal 160 amino acids of LuxR, shown to be responsible for acyl-HSL binding [26]. Two rounds of ON/OFF selection were used to enrich for LuxR variants that activate gene expression with C10HSL but not 3OC6HSL.

The LuxR expression vectors obtained from colonies surviving Round 2 ON/OFF selection were used as templates to amplify the mutant *luxR* genes by PCR with a high-fidelity polymerase. These genes were ligated into LuxR expression vectors and used for a final OFF/ON round of selection. The LuxR expression plasmids obtained from colonies surviving Round 3 selection were transformed with pluxGFPuv and screened for their ability to activate gene expression with 100 nM C10HSL. Eighty of the most fluorescent clones were transferred to a 96-well plate and grown overnight. These clones were further screened for the inability to activate GFPuv expression with 3OC6HSL on solid media.

We sequenced 12 of the 80 fluorescent clones and found four unique LuxR variants that exhibited the desired retention of C10HSL response and loss of 3OC6HSL response. All had the substitution of an arginine at position 67 for methionine, along with up to five additional new mutations. One, LuxR-G2E-R67M, has only the R67M mutation in addition to the three amino acid substitutions of its parent, LuxR-G2E (Table 4.1). All four unique variants exhibited identical responses with 3OC6HSL, C10HSL, C6HSL and C8HSL, indicating that the substitutions other than R67M are likely neutral (data not shown). We therefore focused on LuxR-G2E-R67M for all further characterization.

Table 4.1. Nucleotide and amino acid changes in *luxR*/LuxR and lasR/LasR mutants

***luxR* plasmid**	***luxR* gene** base substitution	**LuxR protein** amino acid substitution
Parent (2nd generation C8HSL-responder)		
pLuxR-G2E	A97→G	Thr33→Ala
	T162→A	
	T346→G	Ser116→Ala
	G405→A	Met135→Ile
Specificity mutant from selection		
pLuxR-G2E-R67M	A97→G	Thr33→Ala
	T162→A	
	G200→T	Arg67→Met
	T346→G	Ser116→Ala
	G405→A	Met135→Ile
	T498→C	
	T510→C	
R67M mutation in wild-type LuxR		
pLuxR-R67M	G200→T	Arg67→Met
R61M mutation in wild-type LasR		
pLasR-R61M	GCT181→ATG	Arg61→Met

C.3. Gene activation by LuxR, LuxR-G2E and LuxR-G2E-R67M with acyl-HSL signals

The *gfpuv* gene expression bioassay was used to quantitatively compare the influence of 3OC6HSL and C10HSL on transcriptional activation by wild-type LuxR, LuxR-G2E and LuxR-G2E-R67M (Fig. 4.2). LuxR and LuxR-G2E exhibited similar responses to 3OC6HSL; gene activation by LuxR-G2E-R67M, however, was barely above background even at concentrations as high as 10 μM 3OC6HSL. Responses to C10HSL showed the opposite trend: wild-type LuxR showed no response, while LuxR-G2E and LuxR-G2E-R67M showed similarly high levels of fluorescent protein production.

We then characterized the ability of LuxR-G2E-R67M to activate gene expression with four acyl-HSLs not used during directed evolution: C6HSL, C8HSL, dodecanoyl-homoserine lactone (C12HSL) and 3-oxo-dodecanoyl-homoserine lactone (3OC12HSL) (Fig. 4.2). LuxR-G2E-R67M has retained the increased ability to respond to straight-chained acyl-HSLs C6HSL, C8HSL and C12HSL shown by LuxR-G2E; its response to 3OC12HSL, however, was more than 50-fold weaker than LuxR-G2E's. Thus, it appears that the R67M mutation disrupts interaction with the 3-oxo group.

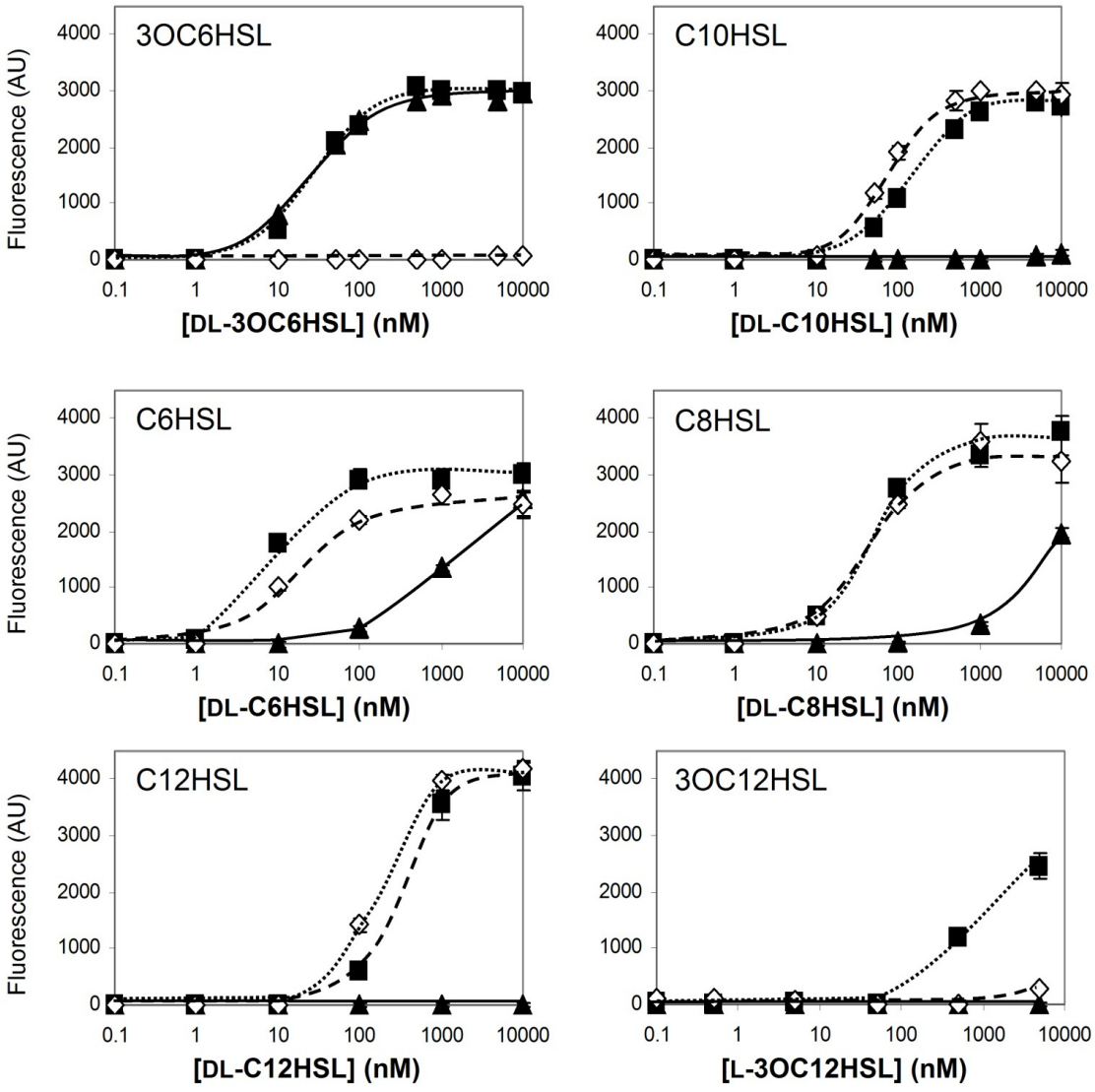


Figure 4.2. Activation of *gfpuv* transcription with 3OC6HSL, C10HSL, C6HSL, C8HSL, C12HSL and 3OC12HSL by wild-type LuxR (▲), LuxR-G2E (■) and LuxR-G2E-R67M (◊). Shown are units of fluorescence due to GFPuv production in *E. coli* containing pluxGFPuv and pLuxR, pLuxR-G2E or pLuxR-G2E-R67M. The data are normalized to optical density and corrected by subtracting background fluorescence from a control strain containing pluxGFPuv and pPROLar.A122.

C.4. Effect of the R67M substitution in wild-type LuxR and LuxR homolog LasR

To further assess the role of the R67M mutation in 3-oxo recognition, we introduced it into wild-type LuxR and compared LuxR-R67M's ability activate gene expression with 3OC6HSL and its straight-chain counterpart, C6HSL. Fig. 4.4 shows that introducing a methionine at position 67 of LuxR eliminates its ability to respond to 3OC6HSL but has little effect on response to C6HSL. Thus, the R67M substitution appears not to significantly affect binding to straight-chain acyl-HSLs.

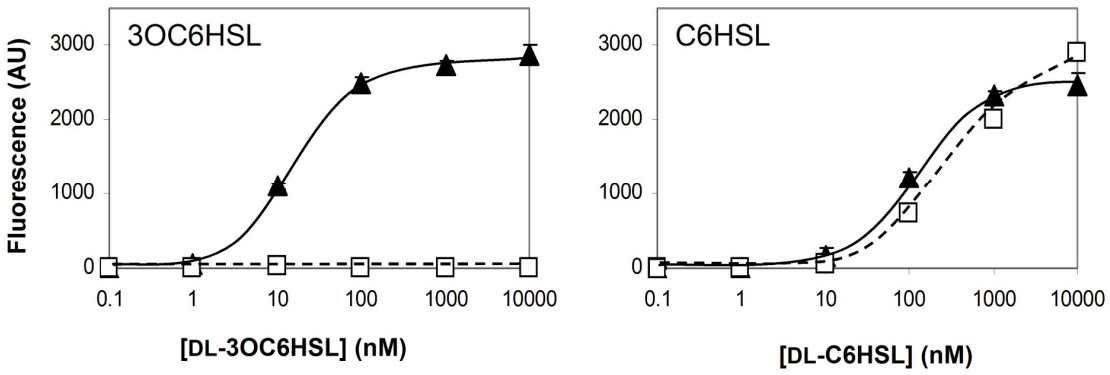


Figure 4.3. Activation of *gfpuv* transcription with 3OC6HSL and C6HSL by wild-type LuxR (▲) and LuxR-R67M (□). Shown are units of fluorescence due to GFPuv production in *E. coli* containing pluxGFPuv and pLuxR or pLuxR-R67M. The data are normalized to optical density and corrected by subtracting background fluorescence from a control strain containing pluxGFPuv and pPROLar.A122.

LasR is a LuxR homolog from *Pseudomonas aeruginosa* that responds to 3OC12HSL. It has only 20% identity to LuxR in the N-terminal signal-binding domain. LasR contains an arginine at residue 61, equivalent to R67 in LuxR. Because LasR also activates gene expression at the *lux* promoter [27], we used the same *gfpuv* gene expression bioassay to characterize its ability to activate gene expression in the presence of acyl-HSLs. If R67M in LuxR eliminates an interaction with the 3-oxo group, then the R61M substitution in LasR might reduce LasR's ability to respond to 3OC12HSL. Figure 4.4 shows that LasR-R61M in fact has decreased response to 3OC12HSL, while its ability to respond to C12HSL is largely unaffected.

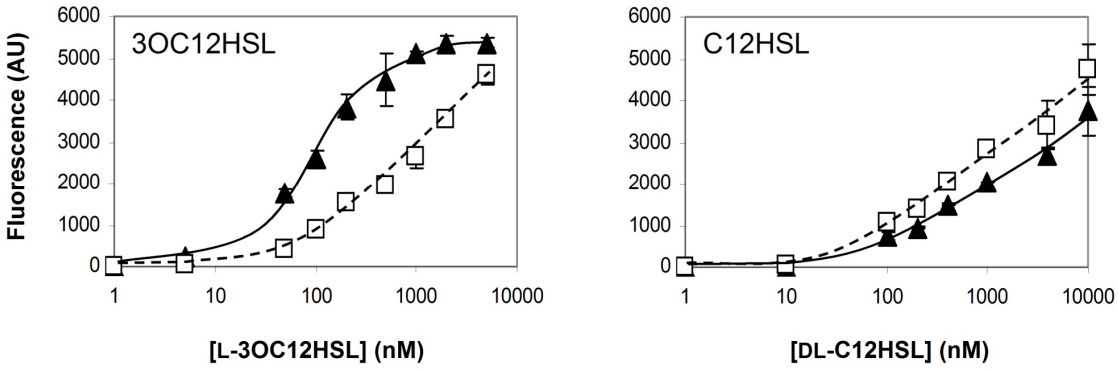


Figure 4.4. Activation of *gfpuv* transcription with 3OC12HSL and C12HSL by wild-type LasR (▲) and LasR-R61M (□). Shown are units of fluorescence due to GFPuv production in *E. coli* containing pluxGFPuv and pLasR or pLasR-R61M. The data are normalized to optical density and corrected by subtracting background fluorescence from a control strain containing pluxGFPuv and pPROLar.A122.

C.5. Gel mobility shift assays of acyl-HSL stimulated DNA binding

We used gel mobility shift assays to measure the ability of different acyl-HSLs to stimulate DNA binding by purified LuxR, LuxR-G2E and LuxR-G2E-R67M (Fig. 4.5). In the presence of 3OC6HSL, LuxR binds a 20 base pair (bp) pseudopalindromic DNA target known as the *lux* box, which is centered 42.5 bp upstream of the transcriptional start site in the *lux* promoter [28, 29]. Wild-type LuxR was able to bind a DNA probe containing the *lux* promoter in the presence of 3OC6HSL but not C10HSL, while LuxR-G2E could bind the same probe with 3OC6HSL or C10HSL (Fig. 4.5a). LuxR-G2E-R67M bound the DNA only in the presence of C10HSL. A titration of 3OC6HSL in the presence of 3.5 nM LuxR revealed that 100 nM of L-3OC6HSL was required to shift 50% of the specific probe (Fig. 4.5b), in agreement with published results [29]. Approximately 2 μM of L-3OC6HSL was required to shift 50% of the DNA with LuxR-G2E under similar conditions. LuxR-G2E-R67M was not observed to shift the DNA with up to 12.5 μM L-3OC6HSL. Wild-type LuxR, on the other hand, did not shift with the highest concentration of C10HSL, 12.5 μM. LuxR-G2E required approximately 1 μM C10HSL to shift 50% of the probe and LuxR-G2E-R67M achieved 50% binding with approximately 10 μM C10HSL. Hill coefficients determined for each interaction where binding was detected range from 0.7 to 1.0 (data not shown), similar to the value of 0.9 for wild-type LuxR with 3OC6HSL published by Urbanowski *et al*. [29].

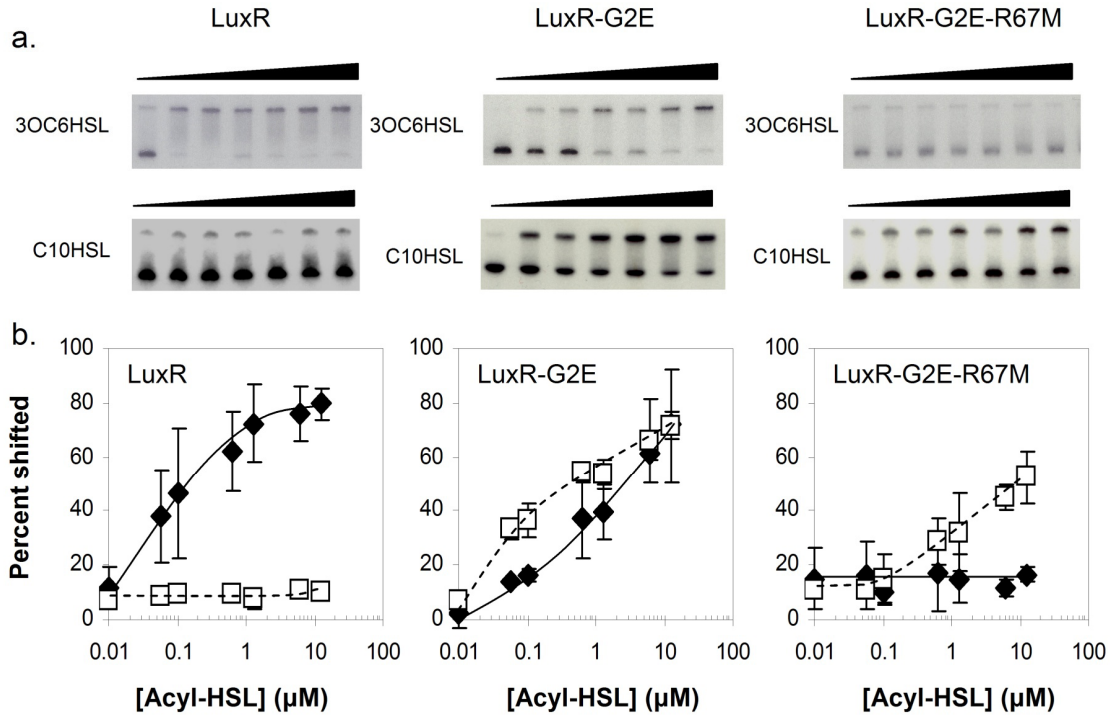


Figure 4.5. Gel mobility shift assays of LuxR, LuxR-G2E and LuxR-G2E-R67M in the presence of 3OC6HSL and C10HSL. a. Gel shift assay of binding to *lux* promoter DNA. All lanes contain approximately 1 fmol of DNA and 3.5 nM LuxR, LuxR-G2E or LuxR-G2E-R67M. Acyl-HSL was added to a final concentration of 0.01 μM, 0.05 μM, 0.10μM, 0.64 μM, 1.26 μM, 6.26μM and 12.5 μM to each lane from left to right. b. Data for 3OC6HSL are represented by closed diamonds (♦), and C10HSL by open squares (□). The amount of the *lux* promoter probe DNA shifted was determined at each concentration of acyl-HSL and averaged. Error bars represent standard deviation of the mean based on at least three measurements.

Western immunoblots further demonstrate the new acyl-HSL specificity of LuxR-G2E-R67M. While wild-type LuxR accumulates only in the presence of its cognate signal, 3OC6HSL, and LuxR-G2E accumulates with either 3OC6HSL or C10HSL, an increase in LuxR-G2E-R67M concentration was detected with the addition of C10HSL, but not 3OC6HSL, reflecting the new signal-binding specificity (Fig. 4.6).

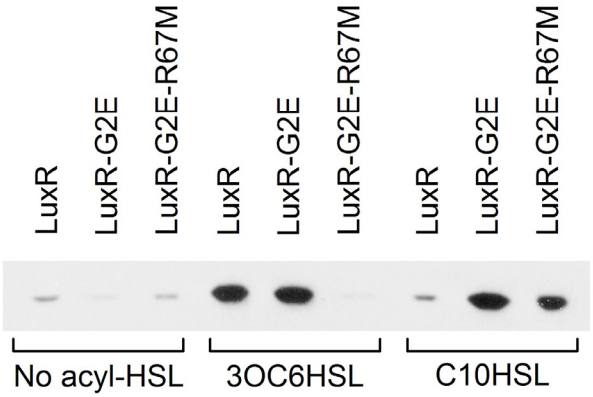


Figure 4.6. Protein accumulation is dependent upon acyl-HSL binding. Shown is a representative Western immunoblot of *c-myc* tagged LuxR, LuxR-G2E and LuxR-G2E-R67M in the absence of acyl-HSL or with 1 μM 3OC6HSL or C10HSL. Western immunoblots were performed as described [24].

C.6. Solid-phase test for crosstalk between LuxR and LuxR-G2E-R67M acyl-HSL response

A solid-phase assay with two fluorescent cell strains was used to determine whether any crosstalk occurred between LuxR and LuxR-G2E-R67M when cells were spread on plates containing 50 nM 3OC6HSL, 200 nM C10HSL or both. Two strains, one containing *gfpuv* under the control of LuxR and the other *gfpuv* under the control of LuxR-G2E-R67M, were plated on separate sides of a Petri dish. In the presence of 3OC6HSL, GFPuv production was observed as a result of LuxR-mediated gene activation (Fig. 4.7a). No fluorescence was observed from the second strain containing LuxR-G2E-R67M. Only LuxR-G2E-R67M-mediated GFPuv expression was observed with C10HSL (Fig. 4.7b). In the presence of 3OC6HSL and C10HSL, both sides of the

plates fluoresced (Fig. 4.7c). This finding indicates that LuxR and LuxR-G2E-R67M are sufficiently specific for their respective acyl-HSL signals to be used in synthetic genetic circuits requiring more than one acyl-HSL response system.

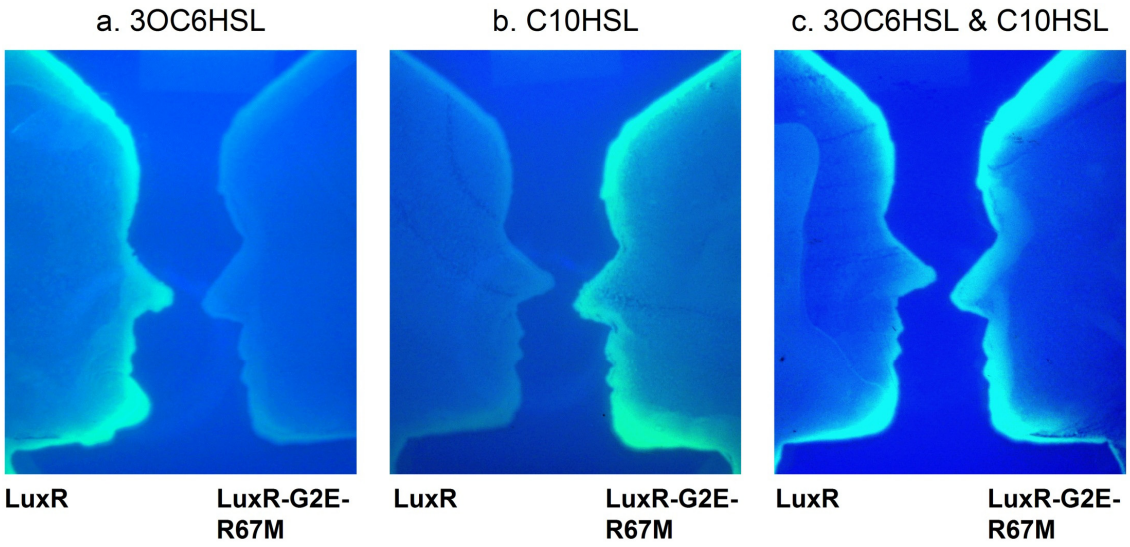


Figure 4.7. Solid-phase assays show minimal crosstalk between LuxR and LuxR-G2E-R67M. Cells on the left contain pLuxR and pluxGFPuv; cells on the right contain pLuxR-G2E-R67M and pluxGFPuv. a. Plate containing 3OC6HSL shows fluorescence only in LuxR-containing cells b. Plate containing C10HSL shows fluorescence only in LuxR-G2E-R67M-containing cells. c. Plate containing 3OC6HSL and C10HSL shows fluorescence from both LuxR and LuxR-G2E-R67M.

D. Discussion

To evolve a new signaling specificity, the selection scheme must identify variants that respond to the new chemical signal (positive selection) but also have decreased response to the cognate signal(s) (negative selection). Variants of the Tet repressor with altered specificity were identified after multiple rounds of positive screening produced several variants capable of recognizing a new effector molecule [30]. Subsequent testing of the variants by screening with different effectors identified a mutant that no longer responded to tectracycline or several tetracycline analogs. Screening and selection systems that directly incorporate both positive and negative selection have been used to modify the specificities of enzymes, including Cre recombinase [31, 32], tRNA synthetases [33] and the endopeptidase OmpT [34]. Both the tRNA synthetase and Cre recombinase experiments used production of a reporter protein (GFP, RFP or β-galactosidase) for rounds of positive selection. The tRNA synthetase experiments relied on a lack of reporter expression during rounds of negative screening. In contrast, the absence of Cre activity was determined by a second reporter gene that was disrupted by site-specific recombination, in a negative screening step. The dual selection system used here also requires a specific output (as opposed to a lack thereof) for both positive and negative rounds of selection.

Using the dual selection system of Fig. 4.2, we discovered a single arginine to methionine substitution at residue 67 that decreased LuxR-G2E's response to the LuxR cognate signal, 3OC6HSL while maintaining its acquired response to C10HSL. Changes in

specificity can be estimated by comparing the ratio of 3OC6HSL and C10HSL concentrations required to attain half-maximal levels of gene activation for the different LuxR variants. (In cases where no activation was observed, the highest concentration of acyl-HSL measured was used to obtain a lower bound for the specificity change.) A 100-fold change in specificity was attained by LuxR-G2E through its increase in C10HSL sensitivity. An additional change in specificity of at least 500-fold was acquired upon going from LuxR-G2E to LuxR-G2E-R67M. Overall, a more than 50,000-fold change in specificity of the acyl-HSL response was obtained by evolving wild-type LuxR into LuxR-G2E-R67M. Introducing the R67M mutation into wild-type LuxR generated a 1000-fold change in the ratio of 3OC6HSL to C6HSL response, while the R61M mutation in LasR yielded a more conservative 50-fold change in the ratio of 3OC12HSL to C12HSL response.

In LuxR, LuxR-G2E and LasR, this R→M substitution decreases binding to acyl-HSLs with the 3-oxo group, without affecting the recognition of straight-chain acyl-HSLs. Positive selection for response to the new straight-chain C10HSL signal would not have uncovered this specificity-enhancing mutation. A conserved methionine occurs at this position in a group of C8HSL-responsive homologs, including CepR, from the β-*Proteobacteria*. Thus this particular amino acid substitution, identified during laboratory evolution, was previously discovered during the natural evolution of these transcriptional activators to respond to different signaling molecules. Due to the low sequence identity between LuxR and its homologs, however, identification of this residue

as a key determinant of acyl-HSL specificity would have been difficult from sequence comparisons alone. The location of this residue in the crystal structure of TraR, the LuxR homolog from *Agrobacterium tumefaciens* and the only LuxR homolog for which a structure is available, indicates that it makes van der Waals contacts with the acyl-HSL but does not make direct contact with the 3-oxo moiety [35]. Furthermore, an attempt to target 3-oxo binding by TraR based on structural information did not yield any variants with new specificities[36]. A recent homology model of LuxR that suggests that the acyl side chain is flipped in the LuxR/acyl-HSL complex, as compared to the TraR/acyl-HSL complex, also failed to implicate residue 67 in 3-oxo recognition [37].

Gel mobility shift assays demonstrated that LuxR and the evolved variants bind DNA only in the presence of the specific acyl-HSLs that activate gene expression. A 250-fold change in the ratio of 3OC6HSL and C10HSL concentrations required to shift 50% of the DNA probe was observed with LuxR and LuxR-G2E. While LuxR and LuxR-G2E have similar dose-response curves with 3OC6HSL, LuxR-G2E required approximately ten-fold more signal to achieve 50% binding. LuxR-G2E-R67M also has a lower affinity for C10HSL than its parent, LuxR-G2E, although the activation by these two proteins is very similar. These results show that a decrease in signal binding *in vitro* does not necessarily yield a decrease in gene activation *in vivo* and supports the use of selection systems that directly measure signal-dependent gene expression, rather than DNA or signal binding, to generate variants with the precise output required for a given application.

Switching from a protein capable of recognizing 3OC6HSL but not C10HSL (LuxR) to one that recognizes C10HSL but no longer responds to 3OC6HSL (LuxR-G2E-R67M) was achieved via a series of single mutations. The three amino acid substitutions in LuxR-G2E each increase LuxR's response to C8HSL and have additive effects when recombined by DNA shuffling [24]. Thus, these three mutations could accumulate in any order under selective pressure for increased response to the straight-chain acyl-HSL. The R67M mutation, on the other hand, could only be detected as beneficial after significant increases in straight-chain acyl-HSL response had been achieved, because LuxR does not respond to C10HSL.

The directed evolution of a LuxR variant with a new specificity therefore involved the acquisition of 'promiscuous' functions [24] and subsequent 'respecialization.' It has been postulated that broad-specificity proteins are evolutionary intermediates between more specialized states and that they are important for the evolution of proteins with new functions [23, 38]. These new functions can provide selective advantages to organisms as they adapt to changing environments. Results from many directed evolution experiments indicate that a single mutation is much more likely to confer a new function (e.g., the ability to recognize a new signal or substrate) without abolishing the old function [32, 39-41]. If a promiscuous interaction adversely affects the fitness of the organism, negative selective pressure will lead to the accumulation of mutations that minimize the deleterious interaction and lead to an increasingly specialized protein. We have emulated this process with LuxR and its interactions with 3OC6HSL.

This work has also established the use of the dual selection system for the directed evolution of a transcriptional activator and suggests it can serve generally for engineering transcriptional regulators with specific ON/OFF responses. The use of evolved LuxR variants in designer genetic circuits has been established [14], and will likely increase as new variants are engineered. Figure 4.7 illustrates the lack of crosstalk between wild-type LuxR and LuxR-G2E-R67M. This mutant will be a useful component for intercellular communication circuits that require multiple chemical 'wires' between cell strains. Its evolution demonstrates that the acyl-HSL binding specificity of LuxR can be tuned using the ON/OFF selection to suit a particular application. Engineers targeting applications that involve engineered cell-cell communication, including biofabrication, tissue engineering and targeted gene therapies, may find these proteins to be valuable additions to their 'toolbox' of circuit components.

E. Materials and Methods

E.1. Bacterial strains, media and growth conditions

The bacterial strains used in this study are *Escherichia coli* strains DH5α (F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 and BL21 (F^- *dcm*+ Hte *ompT hsdS*(r_{B-} m_{B-}) *gal endA* Tet^r). *E. coli* strains were cultured at 37°C in LB medium or on LB agar plates. Acyl-

HSLs used in these studies were: 3-oxohexanoyl-DL-homoserine lactone (3OC6HSL; Sigma Aldrich, St. Louis, MO), hexanoyl-DL-homoserine lactone (C6HSL; Fluka, St. Louis, MO), octanoyl-DL-homoserine lactone (C8HSL; Fluka), decanoyl-DL-homoserine lactone (C10HSL, Fluka), dodecanoyl-DL-homoserine lactone (C12HSL; Fluka) and 3-oxododecanoyl-L-homoserine lactone (3OC12HSL; Quorum Science, Corville, IA). Antibiotics were added at the following concentrations to maintain the plasmids: 100 μg/mL carbenicillin, 20 μg/mL kanamycin, and 100 μg/mL chloramphenicol. During selection experiments antibiotics were added as follows: 100 μg/mL carbenicillin, 50 μg/mL kanamycin, and 150 μg/mL chloramphenicol.

E.2. Plasmid construction

The LuxR expression vector, pLuxR, and the signal response plasmid, pluxGFPuv, have been described previously[24]. Plasmids pluxCAT and pluxBLIP are described in Chapter 3.

pLuxR(SalI) is similar to pLuxR except that the *Sal*I endonuclease site upstream of the $P_{lac/ara-1}$ promoter was removed and new a *Sal*I endonuclease site was engineered into *luxR* at nucleotide positions 493-498 (amino acid positions 164-165) such that no amino acid changes were introduced. pluxRSalI was constructed by synthetic overlap extension PCR.

E.3. Library construction and selection

Error-prone PCR reactions were performed using *AmpliTaq* DNA polymerase (Applied Biosystems, Foster City, CA) and 300 μM $MnCl_2$ to increase the mutation rate as described [42]. The primers 5-luxRv2 (5-AGAGGAGAAAGGTACCCATGAAAAACA-3) and LuxR(SalI)-r2 (5-GCTATATTTATTTTCGATAATTGTCGACTAGAGAAGGAAC-3) were used to amplify the first 500 bp of the *luxR-G2E* gene using pLuxR-G2E as the template. The library was constructed by ligating *Kpn*I and *Sal*I digested pLuxRSalI with the products of error-prone PCR using T4 DNA ligase (Invitrogen, Carlsbad, CA). For the first ON round of selection (Round 1-ON), the ligation mixtures were transformed into competent DH5α cells harboring pluxCAT (DH5α(pluxCAT))and plated onto LB agar plates containing 50 μg/mL kanamycin, 150 μg/mL chloramphenicol and 100 nM C10HSL. Similar plates were used for subsequent ON rounds. Plates used for OFF rounds contained 50 μg/mL kanamycin, 100 μg/mL carbenicillin and 100 nM 3OC6HSL. For each round, cells were also plated with kanamycin and carbenicillin to estimate the library size by quantifying the number of colonies that survive under non-selective conditions. For both ON and OFF selections, LB agar plates were incubated at 37 ^{o}C for 14 hours and then harvested with LB. The plasmid DNA was recovered via miniprep (QIAGEN, Valencia, CA), digested with *Sca*I to inactivate pluxCAT or pluxBLIP, purified by spin column (Zymo Research, Orange, CA), and used to transform the competent cells for the next round. The plasmid DNA recovered from Round 2-ON was

used as the template for recloning to remove any false positives in which changes had occurred outside *luxR-G2E*. The *lux*R-G2E alleles were amplified using Pfu Turbo polymerase (Stratagene) and treated with *Dpn*I. The PCR products were digested and ligated into pLuxRSalI (as above) and transformed into competent DH5α cells containing pluxBLIP and subjected to a third round of OFF selection (Round 3-OFF). The cells were harvested and a final ON round of selection (Round 3-ON) was performed as described above. The DNA purified from Round 3-ON was digested with *Sca*I and used with pluxGFPuv to transform DH5α. The transformation was plated onto LB agar containing kanamycin, chloramphenicol and 100 nM C10HSL. Fluorescent colonies were identified as described previously [24] and grown in 1 mL cultures in deep-well microplates overnight at 37 ^{o}C.

E.4. Characterization of LuxR variants

Quantitative characterization of LuxR-mediated gene expression of GFPuv was performed as reported previously [24].

LuxR, LuxR-G2E and LuxR-G2E-R67M were purified as described [29] with slight modification: all buffers contained a lower concentration of 0.05% Tween-20. LuxR-G2E-R67M was purified in the presence of 5 μM DL-C10HSL. Gel shift experiments were also performed similarly to those described by Urbanowsi *et al*. [29]. The DNA probes were PCR products 173 bp in length from amplification of pluxGFPuv with 5-

pluxI and 3-pluxI [24]. Probes were generated by end-labeling the PCR products using [γ^{32}-P]-ATP plus T4 polynuceotide kinase. Protein-DNA binding reactions contained approximately 1 fmol of the DNA probe in a final volume of 16 µL of DNA binding buffer (20 mM Tris-HCL, [pH 7.4], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 µg bovine serum albumin/mL, and 5% ficoll 400) with 100 µg/mL poly(dI-dC)·poly(dI-dC) (GE Healthcare) competitor DNA. Purified protein and acyl-HSL were added as indicated and incubated for 25 min at 25 ^{o}C. The reaction mixtures were run on a native 5% Tris-glycine-EDTA gel at 4 ^{o}C. Following electrophoresis, the probes were detected using Kodak BioMax XAR Film (Rochester, NY) and quantified from the scanned image via densitometry analysis using AlphaEaseFc image analysis software (Alpha Innotech).

E.5. Solid-phase fluorescence assays

For the solid-phase experiment, separate cultures of *E. coli* DH5α containing either pLuxR and pluxGFPuv or pLuxR-G2E-R67M and pluxGFPuv were grown to stationary phase, diluted four-fold and blotted on top of LB-agar plates with a sterile sponge. Plates were incubated at 37 ^{o}C. Fluorescence images were captured with a digital camera while the plates were illuminated by a 360 nm ultraviolet light source.

F. References

1. Taga, M.E. & Bassler, B.L. Chemical communication among bacteria. *Proc. Natl. Acad. Sci. USA* **100**, Suppl. 2, 14549-14554 (2003).

2. Whitehead, N.A., Barnard, A.M.L., Slater, H., Simpson, N.J.L. & Salmond, G.P.C. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol. Rev.* **25**, 365-404 (2001).

3. Fuqua, C. & Greenberg, E.P. Listening in on bacteria: Acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 685-695 (2002).

4. Schaefer, A.L., Val, D.L., Hanzelka, B.L., Cronan, J.E. & Greenberg, E.P. Generation of cell-to-cell signals in quorum sensing: Acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc. Natl. Acad. Sci. USA* **93**, 9505-9509 (1996).

5. Kaplan, H.B. & Greenberg, E.P. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**, 1210-1214 (1985).

6. Engebrecht, J. & Silverman, M. Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **81**, 4154-4158 (1984).

7. Slock, J., Vanriet, D., Kolibachuk, D. & Greenberg, E.P. Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. *J. Bacteriol.* **172**, 3974-3979 (1990).

8. Choi, S.H. & Greenberg, E.P. Genetic evidence for multimerization of LuxR, the transcriptional activator of *Vibrio fischeri* luminescence. *Mol. Mar. Biol. Biotechnol.* **1**, 408-413 (1992).

9. Stevens, A.M., Fujita, N., Ishihama, A. & Greenberg, E.P. Involvement of the RNA polymerase alpha-subunit C-terminal domain in LuxR-dependent activation of the *Vibrio fischeri* luminescence genes. *J. Bacteriol.* **181**, 4704-4707 (1999).

10. Gray, K.M. & Garey, J.R. The evolution of bacterial LuxI and LuxR quorum sensing regulators. *Microbiology-(UK)* **147**, 2379-2387 (2001).

11. Lerat, E. & Moran, N.A. The evolutionary history of quorum-sensing systems in bacteria. *Mol. Biol. Evol.* **21**, 903-913 (2004).

12. Schaefer, A.L., Hanzelka, B.L., Eberhard, A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Probing autoinducer-LuxR interactions with autoinducer analogs. *J. Bacteriol.* **178**, 2897-2901 (1996).

13. Schaefer, A.L., Taylor, T.A., Beatty, J.T. & Greenberg, E.P. Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *J. Bacteriol.* **184**, 6515-6521 (2002).

14. Basu, S., Gerchman, Y., Collins, C.H., Arnold, F.H. & Weiss, R. A synthetic multicellular system for programmed pattern formation. *Nature* **434**, 1130-1134 (2005).

15. Basu, S., Mehreja, R., Thiberge, S., Chen, M.T. & Weiss, R. Spatiotemporal control of gene expression with pulse-generating networks. *Proc. Natl. Acad. Sci. USA* **101**, 6355-6360 (2004).

16. Kobayashi, H., Kaern, M., Araki, M., Chung, K., Gardner, T.S., Cantor, C.R. & Collins, J.J. Programmable cells: Interfacing natural and engineered gene networks. *Proc. Natl. Acad. Sci. USA* **101**, 8414-8419 (2004).

17. Weiss, R. & Knight, T.F. Engineered communications for microbial robotics. *DNA6: Sixth International Meeting on DNA Based Computers*, 1-15 (2000).

18. You, L.C., Cox, R.S., Weiss, R. & Arnold, F.H. Programmed population control by cell-cell communication and regulated killing. *Nature* **428**, 868-871 (2004).

19. Hsu, A., Vijayan, V., Fomundam, L., Gerchman, Y., Basu, S., Karig, D., Hooshangi, S. & Weiss, R. Dynamic control in a coordinated multi-cellular maze solving system. *2005 American Controls Conference* (2005).

20. Weiss, R. Challenges and opportunities in programming living cells. *The Bridge* **32**, 39-46 (2004).

21. Karig, D. & Weiss, R. Signal-amplifying genetic enables in vivo observation circuit of weak promoter activation in the RhI quorum sensing system. *Biotechnol. Bioeng.* **89**, 709-718 (2005).

22. Havranek, J.J. & Harbury, P.B. Automated design of specificity in molecular recognition. *Nat. Struct. Biol.* **10**, 45-52 (2003).

23. Matsumura, I. & Ellington, A.D. *In vitro* evolution of b-glucuronidase into a b-galactosidase proceeds through non-specific intermediates. *J. Mol. Biol.* **305**, 331-339 (2001).

24. Collins, C.H., Arnold, F.H. & Leadbetter, J.R. Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. *Mol. Microbiol.* **55**, 712-723 (2005).

25. Yokobayashi, Y. & Arnold, F.H. A dual selection module for directed evolution of genetic circuits. *Natural Computing* **4**, 245-254 (2005).

26. Shadel, G.S., Young, R. & Baldwin, T.O. Use of regulated cell-lysis in a lethal genetic selection in *Escherichia coli*-identification of the autoinducer-binding region of the LuxR protein from Vibrio fischeri ATCC-7744. *J. Bacteriol.* **172**, 3980-3987 (1990).

27. Gray, K.M., Passador, L., Iglewski, B.H. & Greenberg, E.P. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. *J. Bacteriol.* **176**, 3076-3080 (1994).

28. Egland, K.A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: elements of the *luxl* promoter. *Mol. Microbiol.* **31**, 1197-1204 (1999).

29. Urbanowski, A.L., Lostroh, C.P. & Greenberg, E.P. Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **186**, 631-637 (2004).

30. Scholz, O., Kostner, M., Reich, M., Gastiger, S. & Hillen, W. Teaching TetR to recognize a new inducer. *J. Mol. Biol.* **329**, 217-227 (2003).

31. Santoro, S.W. & Schultz, P.G. Directed evolution of the site specificity of Cre recombinase. *Proc. Natl. Acad. Sci. USA* **99**, 4185-4190 (2002).

32. Voziyanov, Y., Stewart, A.F. & Jayaram, M. A dual reporter screening system identifies the amino acid at position 82 in Flp site-specific recombinase as a determinant for target specificity. *Nucleic Acids Res.* **30**, 1656-1663 (2002).

33. Santoro, S.W., Wang, L., Herberich, B., King, D.S. & Schultz, P.G. An efficient system for the evolution of aminoacyl-tRNA synthetase specificity. *Nat. Biotechnol.* **20**, 1044-1048 (2002).

34. Varadarajan, N., Gam, J., Olsen, M.J., Georgiou, G. & Iverson, B.L. Engineering of protease variants exhibiting high catalytic activity and exquisite substrate selectivity. *Proc. Natl. Acad. Sci. USA* **102**, 6855-6860 (2005).

35. Zhang, R.G., Pappas, T., Brace, J.L., Miller, P.C., Oulmassov, T., Molyneauz, J.M., Anderson, J.C., Bashkin, J.K., Winans, S.C. & Joachimiak, A. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**, 971-974 (2002).

36. Chai, Y.R. & Winans, S.C. Site-directed mutagenesis of a LuxR-type quorum-sensing transcription factor: Alteration of autoinducer specificity. *Mol. Microbiol.* **51**, 765-776 (2004).

37. Koch, B., Liljefors, T., Persson, T., Nielsen, J., Kjelleberg, S. & Givskov, M. The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. *Microbiology-(UK)* **151**, 3589-3602 (2005).

38. Aharoni, A., Gaidukov, L., Khersonsky, O., Gould, S.M., Roodveldt, C. & Tawfik, D.S. The 'evolvability' of promiscuous protein functions. *Nat. Genet.* **37**, 73-76 (2005).

39. Gould, S.M. & Tawfik, D.S. Directed evolution of the promiscuous esterase activity of carbonic anhydrase II. *Biochemistry* **44**, 5444-5452 (2005).

40. Bloom, J.D., Meyer, M.M., Meinhold, P., Otey, C.R., MacMillan, D. & Arnold, F.H. Evolving strategies for enzyme engineering. *Curr. Opin. Struct. Bio.* **15**, 447-452 (2005).

41. Parikh, M.R. & Matsumura, I. Site-saturation mutagenesis is more efficient than DNA shuffling for the directed evolution of beta-fucosidase from beta-galactosidase. *J. Mol. Biol.* **352**, 621-628 (2005).

42. Cirino, P.C., Mayer, K.M. & Umeno, D. Generating libraries using error-prone PCR. in *Directed Evolution Library Creation* (eds. Arnold, F.H. & G., G.) 3-9 (Humana Press, Totawa, NJ, 2003).

CHAPTER 5

ON/OFF Selection for LuxR Variants with Altered DNA-binding Specificities

A. Abstract

Variants of the acyl-homoserine lactone-dependent transcriptional activator LuxR with altered DNA-binding specificities were identified using a directed evolution scheme incorporating both positive (ON) and negative (OFF) selection. LuxR variants that activate gene expression at new promoters are powerful additions to the regulatory components available to genetic circuit engineers. Currently, the use of more than one LuxR homolog in a single cell is limited by the crosstalk between these systems at the level of DNA-binding specificity. To identify LuxR variants that activate gene expression at a mutant promoter, $P_{luxG5A.}$, a library of *luxR* variants was selected for antibiotic resistance due to gene activation. Surviving clones were selected for decreased activation at the wild-type *lux* promoter. Two variants, LuxR-R212H and R212C/Q232H, were identified and *in vivo* bioassays showed that the promoter specificity of LuxR-R212C/Q232H was broadened to include P_{luxG5A} as well as P_{luxI} while LuxR-R212H showed a 10-fold preference for P_{luxG5A}, a 50,000-fold change in promoter specificity was achieved by the R212H mutation. The R212C and Q232H mutations were shown to be synergistic; the R212C mutation likely confers the desired change in DNA-binding affinity but destabilizes the protein, and the Q232H mutation appears to compensate for this defect. Selection of a second-generation library did not identify mutants with increased specificity for P_{luxG5A}, indicating new library design strategies are required to engineer LuxR variants with inverted promoter specificity.

B. Introduction

LuxR is a 28 kDa acyl-homoserine lactone (acyl-HSL)-dependent transcriptional activator from *Vibrio fischeri* [1-3]. LuxR recognizes a diffusible, quorum-sensing signal molecule, 3-oxo-hexanoyl-homoserine lactone (3OC6HSL) [4, 5], and activates gene expression at the *lux* promoter (P_{luxI}) by recruiting RNA polymerase [6, 7]. Gene activation by LuxR requires a 20 base pair (bp) operator site within P_{luxI} known as the *lux* box (Fig 5.1a,b) [6, 8]. The *lux* box is centered 42.5 bp upstream of the P_{luxI} transcriptional start site and its position has been shown to be essential for gene activation [6]. Sequence analyses have predicted that LuxR binds to its DNA target via an α-helix-turn-α-helix (HTH) motif between amino acid residues 200 and 224 [9]. The HTH motif is a common DNA-binding fold found in a myriad of bacterial activators and repressors [10, 11].

Alanine scanning mutagenesis of the C-terminal domain of LuxR was used to identify residues involved in DNA binding and activation [9, 12]. Several mutants unable to bind DNA or activate transcription were identified, including alanine substitutions at positions 191, 193, 212, 217, 225, 229, 230, 238 and 240. Alanine substitutions at positions 201 and 206 affect transcriptional activation but not DNA binding.

a.

Organism	Promoter	LuxR homolog	Sequence of *lux*-type box
Vibrio fischeri	P_{luxI}	LuxR	ACCT**G**TAGGATCGTA**C**AGGT
Pseudomonas aeruginosa	P_{lasB}	LasR/RhlR	ACCT**G**CCAGTTCTGG**C**AGGT
Pseudomonas aeruginosa	P_{lasI}	LasR	ATCT**A**TCTCATTTGC**T**AGTT
Pseudomonas aeruginosa	P_{phzA}	LasR/RhlR	ACCT**A**CCAGATCTTG**T**AGTT
Pseudomonas aeruginosa	P_{rhlAB}	RhlR	TCCT**G**TGAAATCTGG**C**AGTT
Pseudomonas aeruginosa	P_{PA1897}	QscR	ACCT**G**CCCGGAAGGG**C**AGGT
Agrobacterium tumefaciens	P_{traA}	TraR	ATGT**G**CA-GATC-TG**C**ACAT
Burkholderia cenocepacia	P_{cepI}	CepR	CT**G**TAAGAGTTAC**C**AG
Burkholderia cenocepacia	P_{aidA}	CepR	CT**G**TTTACTTTTA**C**AG
Ralstonia solanacearum	P_{solI}	SolR	CT**G**TCAATCCTGA**C**AG
Burkholderia pseudomallei	P_{bpsR}	BpsR	CGCT**G**TCATACTTGC**T**AGGT
Burkholderia pseudomallei	P_{bpsI}	BpsR	CCCT**G**TAAGGGTTAA**C**AGTT
Pantoea stewartii	P_{esaR}	EsaR	GCCT**G**TACTATAGTG**C**AGG
Pseudomonas fluorescens 2-79	P_{phzA}	PhzR	CACT**A**CAAGATCTGG**T**AGTT

b.

	1	2	3	4	5	6	7	8	9	10	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1
lux box	A	C	C	T	**G**	T	A	G	G	A	T	C	G	T	A	**C**	A	G	G	T
	T	G	G	A	**C**	A	T	C	C	T	A	G	C	A	T	**G**	T	C	C	A
lux_{G5A} box	A	C	C	T	**A**	T	A	G	G	A	T	C	G	T	A	**T**	A	G	G	T
	T	G	G	A	**T**	A	T	C	C	T	A	G	C	A	T	**A**	T	C	C	A

Figure 5.1. a. List of LuxR homologs and their cognate DNA targets. Organism, promoter, LuxR homolog name and DNA sequences of the *lux*-type boxes are shown. The 5 and -5 positions of the *lux*-type boxes are underlined and bold. b. Sequences of the wild-type *lux* box and the mutant, lux_{G5A}, used in this study. A transition was made at a single bp in each half site of the operator. The 5 and -5 positions mutated in the lux_{G5A} box are highlighted.

To date, more than 50 LuxR homologs have been identified within the Gram-negative phylum *Proteobacteria* [13]. Each LuxR homolog modulates gene expression following acyl-homoserine lactone (acyl-HSL) binding. All acyl-HSL signals contain a homoserine lactone ring; varied acyl side chain moieties allow species-specific signal recognition. While LuxR homologs have evolved to specifically respond to different acyl-HSL

signals, homologs from different species have been shown to interchangeably activate gene expression at other *lux*-type promoters. Both LuxR and LasR, a homolog from *Pseudomonas aeruginosa*, can activate gene expression at the other's target promoter in *Escherichia coli* [14]. The *lux*-type boxes identified from a range of species show significant sequence diversity (Fig. 5.1a). However, elements within these sites, including highly conserved nucleotides at positions 3, 4, 5, -3, -4, and -5, appear to be sufficient for DNA binding by most LuxR homologs. As LuxR and its homologs are purified, their specific DNA-binding specificities are being investigated [15, 16]. Precisely which sequences are recognized by LuxR and its homologs is not known.

LuxR and its homologs have been used in several synthetic genetic circuits to confer intercellular communication capabilities [17-20]. As the desired circuits become increasingly complex, the ability to use of more than one acyl-HSL response regulator in a single cell type will be advantageous. While it may be possible to identify LuxR homologs that do not show any crosstalk with respect to promoter specificity, an alternative approach is to use directed evolution to generate variants of LuxR that activate gene expression at a mutant promoter. A benefit of this approach is the production of a set of 'standardized' parts for engineering intercellular communications circuits.

We have previously identified a LuxR variant with a new acyl-HSL binding specificity (Chapter 4). LuxR variants with switched acyl-HSL specificities were targeted using a dual selection system that allows for ON/OFF selection. We sought to continue this work

by targeting the DNA-binding specificity of LuxR to identify variants that recognize a new DNA target and that have decreased affinities for the wild-type *lux* box. Directed evolution of the DNA-binding specificity of a Cre recombinase using positive screening alone and a combination of positive and negative screening showed that both positive and negative selective pressure were required to switch Cre's DNA-binding specificity. We hypothesized that a similar approach, using our dual selection system, would allow us to identify LuxR variants with novel DNA-binding specificities. LuxR variants that show increased gene activation at a mutated *lux* box and decreased activation at the wild-type *lux* box were identified using our ON/OFF selection system. Both variants with broadened DNA-binding specificity and variants with a preference for the new target were identified.

C. Results

C.1. Dual selection for evolution of LuxR promoter specificity

We targeted the 5 and -5 positions of the *lux* box for generating a mutated DNA target. This position is highly conserved in *lux*-type boxes. In constructing the lux_{G5A} box, we chose to maintain the palindrome and replace these residues in a conservative manner, purine for purine and pyrimidine for pyrimidine (Fig. 5.1b). A transition is less likely to cause large structural differences than either of the possible transversions. Liquid-phase bioassays measuring fluorescence due to LuxR-mediated GFPuv production showed

wild-type LuxR was unable to activate gene expression at a *lux* promoter containing the *lux*$_{G5A}$ box (P_{luxG5A}) with up to 50 μM 3OC6HSL (Fig. 5.3).

To select for LuxR variants capable of recognizing the *lux*$_{G5A}$ box and activating gene expression at P_{luxG5A}, we used a gain-of-function (ON) selection plasmid encoding chloramphenicol acetyl-transferase (CAT) under the control of P_{luxG5A} (Fig. 5.2). Selection of functional clones relies on LuxR-dependent activation of *cat* leading to chloramphenicol resistance. To select for mutants with decreased affinities for wild-type P_{luxI}, we used a loss-of-function (OFF) selection plasmid encoding the β-lactamase inhibitory protein (Bli) under the control of P_{luxI} and a constitutively expressed β-lactamase (*bla*) gene (Fig. 5.2). Selection of clones which are unable to activate gene expression relies on the absence of LuxR-dependent activation of Bli, which inactivates Bla and confers carbenicillin sensitivity.

A library generated by random mutagenesis of the DNA encoding the C-terminal third of LuxR, amino acid residues 166-250, was generated by error-prone PCR. The C-terminal domain was targeted because it has been shown to contain the DNA-binding and activation domains [21-23]. Clones transformed with this library were selected for chloramphenicol resistance due to gene activation at P_{luxG5A} with 5 μM 3OC6HSL. The plasmid DNA was recovered from the surviving cells and subjected to digestion with *Sca*I to selectively inactivate the selection plasmid, plux$_{G5A}$CAT. In a second ON round of selection, several clones survived with a 10-fold lower concentration of 3OC6HSL

(500 nM) and reduced incubation time of 14 hours. All subsequent rounds of ON selection used this set of more stringent conditions.

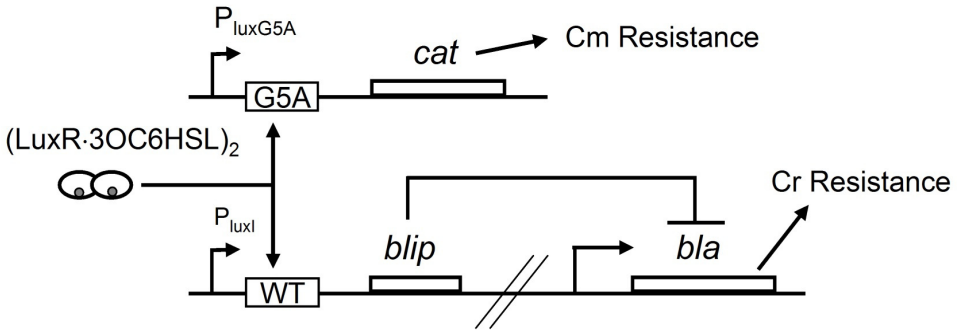


Figure 5.2. Schematic of the dual selection system for the directed evolution of LuxR variants with altered DNA-binding specificity. During positive rounds of selection, LuxR-mediated activation at a *lux* promoter containing the mutated *luxG5A* box, P_{luxG5A}, leads to chloramphenicol acetyl-transferase (CAT) expression and chloramphenicol resistance. Variants with decreased activation at the wild-type *lux* promoter are identified during rounds of negative selection by their ability to survive with carbenicillin. Unwanted gene activation by LuxR leads to carbenicillin sensitivity due to β-lactamase inhibitory protein (Blip) production and its inhibition of constitutively expressed β-lactamase (Bla) protein.

We transformed the *luxR* mutant-encoding plasmids recovered after Round 2-ON with the OFF selection plasmid, pluxBLIP, and selected colonies surviving with carbenicillin and 10 nM 3OC6HSL. Cells expressing wild-type LuxR with this concentration of acyl-HSL are not viable. DNA recovered from the surviving Round 2-ON cells was used for a third ON round of selection. At this point, approximately 25% of the library survived with chloramphenicol and 500 nM 3OC6HSL.

To ensure that the observed resistance was a result of changes within *luxR*, we used the DNA recovered from Round 3-ON and PCR-amplified the *luxR* genes with a high-fidelity polymerase. The amplified *luxR* genes were subjected to a final round of ON selection. At this point, more than two-thirds of the cells survived in the presence of chloramphenicol and 500 nM 3OC6HSL. To identify the best P_{luxG5A}-activating LuxR variants, *luxR* expression plasmids isolated from colonies surviving the final round of selection were transformed with a plasmid encoding GFPuv under the control of P_{luxG5A}, $plux_{G5A}$GFPuv. More than 60% of the colonies fluoresced with 500 nM 3OC6HSL. Fifteen strains that fluoresced with 100 nM 3OC6HSL were isolated.

DNA sequencing of the 15 clones identified two unique *luxR* mutants. All of the mutants contained a mutation at residue 212 of LuxR. Three of the genes identified contained two mutations: R212→C and Q232→H. The other 12 genes identified all

encode a single R212→H substitution. This result suggests that the 212 position is critical for recognition of the *lux*$_{G5A}$ box.

Because error-prone PCR cannot access all of the possible amino acids at a given position, we targeted position 212 for saturation mutagenesis. The NNN saturation library was transformed with plux$_{G5A}$GFPuv and plated with 100 nM 3OC6HSL. Ten of the most fluorescent clones were isolated and their *luxR* genes sequenced. Of the ten, seven encoded R212→H and three encoded R212→S.

C.2. Second-generation library selection

A second-generation library was generated by error-prone PCR of the C-terminal domain encoding region of *luxR-R212H*. The library was determined to have an average mutation rate of approximately 4.5 nucleotide substitutions per *luxR-R212H* gene. Selections were carried out as described above with concentrations of 100 nM 3OC6HSL added for ON rounds of selection and 50 nM 3OC6HSL for OFF rounds. More than 200,000 clones were selected under these conditions. No positive clones were identified.

C.3. Characterization of luxR mutants

To compare transcriptional activation at P_{luxI} and P_{luxG5A} by wild-type LuxR and the evolved LuxR variants, we used a liquid-phase bioassay and measured fluorescence due to GFPuv production with a range of concentrations of 3OC6HSL (Fig. 5.3). The two variants identified via ON/OFF selection, LuxR-R212C/Q232H and LuxR-R212H, showed similar dose-responses with P_{luxG5A}. Levels of LuxR-R212C/Q232H-mediated fluorescence at P_{luxI} and P_{luxG5A} were indistinguishable. LuxR-R212H showed a preference for $P_{luxG5A,}$ requiring approximately ten-fold more 3OC6HSL to achieve similar levels of activation at P_{luxI}. LuxR-R212S also showed a subtle preference for P_{luxG5A} (~five-fold) but required a two-fold higher 3OC6HSL concentration to activate gene expression at both promoters compared to LuxR-R212H.

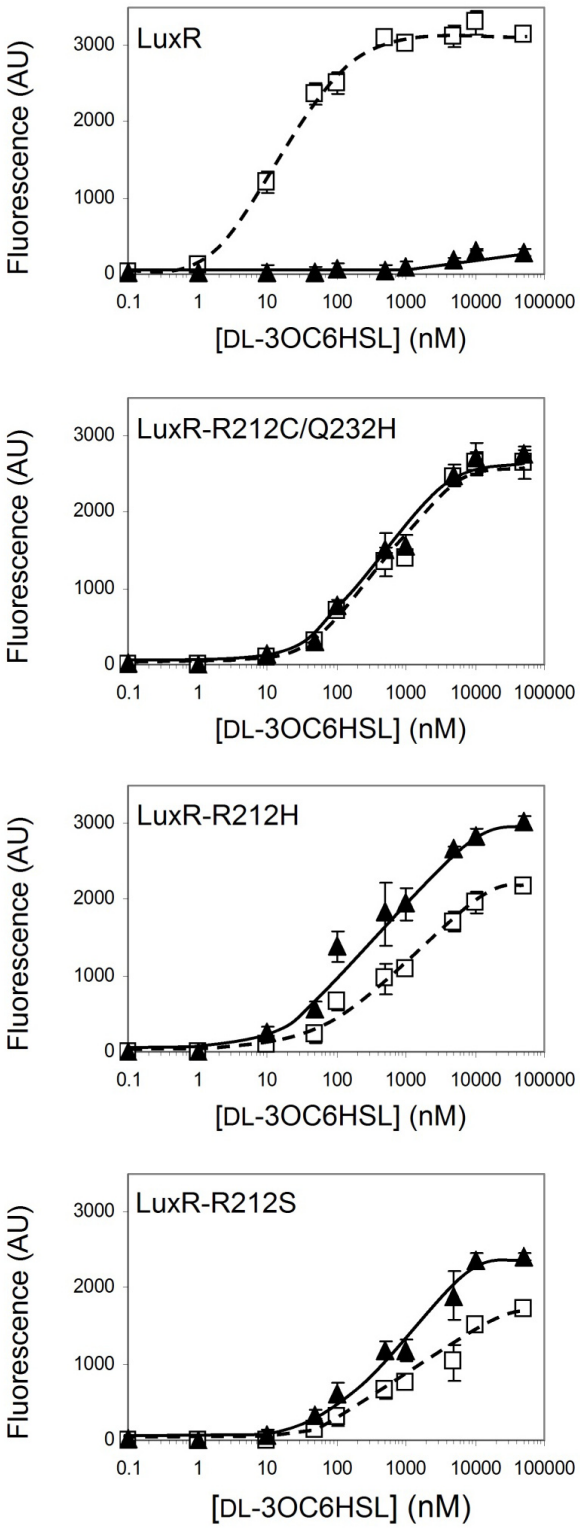


Figure 5.3. Activation of *gfpuv* transcription from P_{luxI} (□) and P_{luxG5A} (▲) by wild-type LuxR, LuxR-R212C/Q232H, LuxR-R212H and LuxR-R212S with 3OC6HSL. Shown are units of fluorescence due to GFPuv production in *E. coli* containing pluxGFPuv and pLuxR, pLuxR-R212C/Q232H, LuxR-R212H or pLuxR-R212S. The data are normalized to optical density and corrected by subtracting background fluorescence from a control strain containing pluxGFPuv and pPROLar.A122.

C.4. LuxR-R212C/Q232H activation at P_{luxG5A} requires both mutations

To assess the role of the individual mutations in LuxR-R212C/Q232H, the single mutants R212C and Q232H were constructed by site-directed mutagenesis. Quantitative liquid-phase characterization showed that the LuxR-R212C single mutant does not activate gene expression at P_{luxI} or P_{luxG5A} with up to 50 μM 3OC6HSL (Fig. 5.4). LuxR-Q232H showed increased levels of activation at both the wild-type and the mutated *lux* promoter, however, activation at P_{luxG5A} required more than 100-fold more 3OC6HSL than the double mutant, LuxR-R212C/Q232H. These two mutations must work synergistically to confer the increased response at P_{luxG5A}.

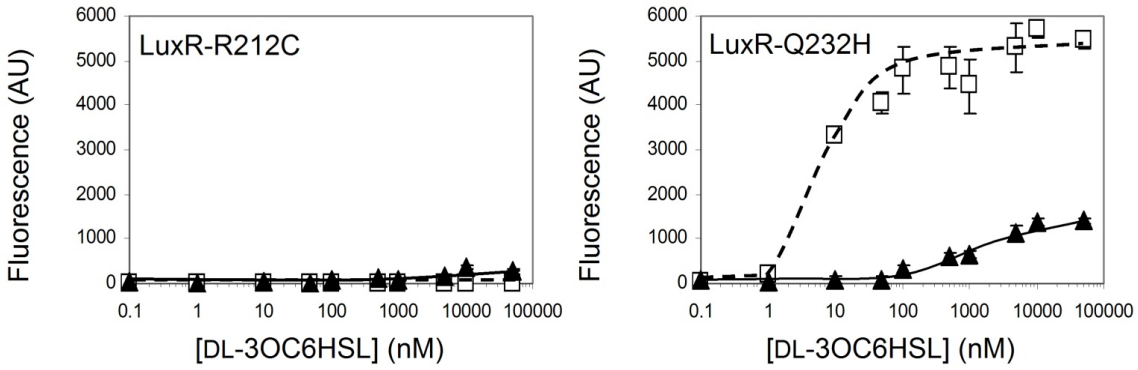


Figure 5.4. Activation of *gfpuv* transcription from P_{luxI} (□) and P_{luxG5A} (▲) by single mutants LuxR-R212C and LuxR-Q232H with 3OC6HSL. Shown are units of fluorescence due to GFPuv production in *E. coli* containing pLuxR-R212C or pLuxR-Q232H. The data are normalized to optical density and corrected by subtracting background fluorescence from a control strain containing pluxGFPuv and pPROLar.A122.

C.5. Accumulation of LuxR variants in vivo

Each of the LuxR variants was tagged with a *c-myc* epitope and assayed for its abundance *in vivo* using Western immunoblots. In the absence of 3OC6HSL, minimal levels of protein were observed for LuxR and each of the variants (data not shown). Accumulation of wild-type LuxR, LuxR-R212H, LuxR-R212C/Q232H, LuxR-R212S and LuxR-Q232H increased with the addition of 3OC6HSL (Fig. 5.5). LuxR-R212C did not increase in abundance with the addition of 3OC6HSL. The concentrations of LuxR-R212H, LuxR-R212C/Q232H and LuxR-R212S were lower than wild-type LuxR. LuxR-R212H showed the largest decrease in protein expression, to <10% of wild-type levels. LuxR-R212C/Q232H and LuxR-R212S accumulate to approximately 50% of wild-type levels. In contrast, the amount of LuxR-Q232H detected with 3OC6HSL was at more than five-fold higher than wild-type LuxR, indicating this mutation stabilizes LuxR.

Figure 5.5. Protein accumulation is dependent upon acyl-HSL binding. Shown is a representative Western immunoblot of *c-myc* tagged LuxR (WT), evolved DNA-binding variants and single-mutants with 10 μM 3OC6HSL. Minimal protein accumulation, similar to the amount shown for LuxR-R212C with 3OC6HSL, was observed for each of the proteins in the absence of acyl-HSL.

C.6. DNA-binding experiments

Wild-type LuxR, LuxR-R212C/Q232H, LuxR-R212H and LuxR-R212S were purified, and their ability to bind DNA *in vitro* was assayed using electromobility shift assays (EMSAs). As expected from the *in vivo* bioassay data, all of the variants showed lower affinities for DNA probes containing either the P_{luxI} or P_{luxG5A} promoter than wild-type LuxR with P_{luxI}. To observe DNA binding by the evolved variants, micromolar protein concentrations were required. Due to the elevated protein concentrations it was necessary to increase the amount of nonspecific DNA used in these studies to ensure the observed shifts were due to specific DNA-protein interactions. Under these stringent conditions, wild-type LuxR has a K_D of approximately 75 nM (Fig. 5.6), approximately 150-fold higher than the previously reported value of 0.5 nM [3]. LuxR did not bind to a DNA target containing the lux_{G5A} box at concentrations up to 2 μM. The three DNA-binding variants showed similar affinities for the lux_{G5A} box. Approximately 15% of the probe was bound with 2 μM protein (Fig. 5.6). Further increases in protein concentration led to excessive levels of non-specific binding and precipitation of the protein. The K_D of the mutants for the new site was estimated to be more than 1000-fold higher than LuxR's dissociation constant with its cognate DNA target. All three variants also showed a preference for the lux_{G5A} box. LuxR-R212C/Q232H did not detectably bind the wild-type *lux* box and LuxR-R212H reproducibly bound 1-2% of the wild-type probe at the highest protein concentration used in this study. Approximately 6% of the wild-type DNA probe

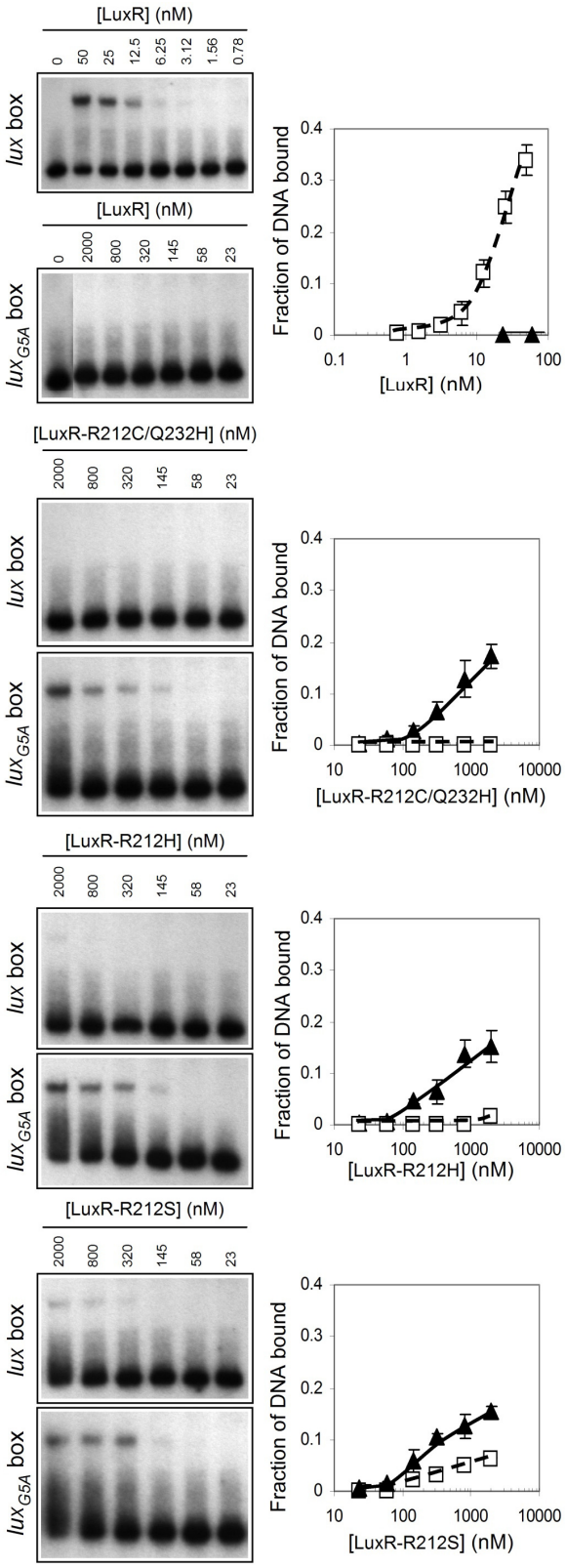


Figure 5.6. Electromobility shift assays of LuxR and the evolved variants with P_{luxI} and P_{luxG5A}. Gel shift assays are shown on the left and quantitative plots of the fraction of shifted DNA versus protein concentration are shown on the right. All lanes contained approximately 5 fmol of a 176 bp DNA fragment containing wither P_{luxI} or P_{luxG5A}, the indicated concentration of LuxR, LuxR-R212C/Q232H, LuxR-R212H and LuxR-R212S and 25 μM DL-3OC6HSL.

was bound by 2 μM LuxR-R212S. With only a two-fold preference for the mutant lux_{G5A} box, LuxR-R212S has the most broadened DNA-binding specificity.

C.7. RhlR-mediated gene activation at P_{luxI} and P_{luxG5A}

An alignment of LuxR homologs showed that a LuxR homolog from *P. aeruginosa*, RhlR, has a serine residue at the amino acid position that aligns with 212 in LuxR. RhlR-mediated GFPuv production from P_{luxI} and P_{luxG5A} was measured using the same bioassay as LuxR. As shown in Figure 5.7, RhlR activated gene expression at both promoters. Approximately five-fold higher concentrations of RhlR's cognate acyl-HSL, butanoyl-homoserine lactone (C4HSL), were required to achieve similar levels of gene activation at the wild-type *lux* promoter compared to P_{luxG5A}. This result indicates that a serine residue at this position may confer a broadening of DNA-binding specificity in both RhlR and LuxR.

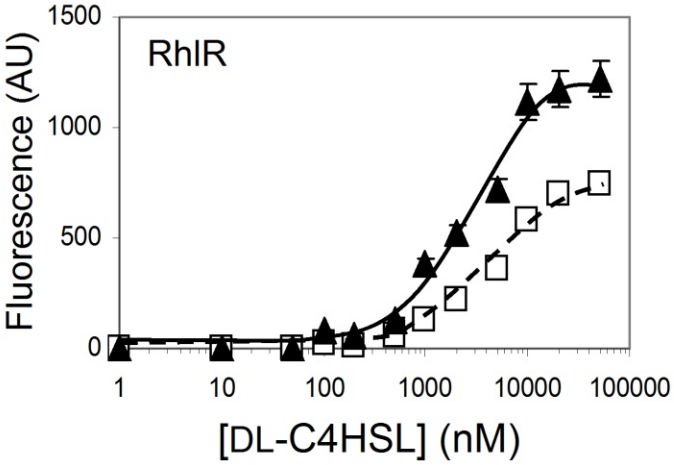


Figure 5.7. RhlR-mediated GFPuv gene expression at P_{luxI} and P_{luxG5A}. Shown are units of fluorescence due to GFPuv production in *E. coli* containing pRhlR with pluxGFPuv or plux$_{G5A}$GFPuv. The data are normalized to optical density and corrected by subtracting background fluorescence from a control strain containing pluxGFPuv and pPROLar.A122.

D. Discussion

Examples of HTH engineering for new DNA-binding specificity in the literature are scarce, and the examples that are available have relied on structural information regarding the interactions between the protein and its target DNA site [24-34]. A crystal structure of LuxR is not available, and although LuxR and its homologs share higher sequence identity in their DNA-binding domains than their acyl-HSL binding domains, the sequence similarity between the C-terminal domains of LuxR and TraR, the only LuxR homolog for which a complete crystal structure is available, is very low (<20%). This is likely due to the fact that *tra* box contains an internal deletion on each side of the inverted repeat, as compared to the *lux* box (Fig. 5.1a) [14].

We generated a library of *luxR* mutants via error-prone PCR and used an ON/OFF selection system to identify LuxR variants that both activate gene expression at a promoter containing a mutated *lux* box, P_{luxG5A}, and that show decreased gene activation at the wild-type *lux* promoter. The dual selection system was employed to prevent the identification of LuxR variants with increased gene activation at both P_{luxI} and P_{luxG5A}. Our selection identified two variants, LuxR-R212H and LuxR-R212C/Q232H. The conditions used for OFF rounds of selection were not sufficiently stringent to require the variants identified to be specific for the P_{luxG5A} promoter, and both LuxR variants exhibit broadened promoter specificity. However, a 50-fold increase in 3OC6HSL concentration required to reach half-maximal gene expression at P_{luxI} was observed with LuxR-R212C/Q232H and a 500-fold increase was observed with LuxR-R212H. Therefore, the

dual selection system successfully identified variants with decreased gene activation at P_{luxI}. A third variant, LuxR-R212S, was identified by screening a library that randomized codon 212. These results indicate that residue 212 is a modulator of specific interactions with positions 5 and -5 of the *lux* box. Mutating this residue to an alanine was previously shown to inactivate LuxR [9, 12] and an alignment of this residue with the published structure of TraR shows that the residue may make direct contact with the phosphate group between nucleotides 4 and 5 of the *lux* box (Fig. 5.8). This residue has not been previously implicated in affecting the DNA-binding specificity of any LuxR homolog.

Lux-type boxes containing the A/T bp targeted in this work have only been identified in *Pseudomonas* strains. LasR has been shown to bind and activate gene expression at promoters containing either an A/T or G/C bp at this position, although it contains an alanine residue at position 212 [16, 35]. The presence of a serine residue in RhlR at the amino acid position that aligns with residue 212 in LuxR prompted us to test its ability to activate gene expression at both P_{luxI} and P_{luxG5A}. As with LuxR-R212S, RhlR showed a preference for $P_{luxG5A,}$ requiring approximately five-fold more 3OC6HSL to achieve similar levels of activation at P_{luxI}. The finding that RhlR and LasR have relaxed DNA-binding specificities with regards to positions 5, -5 is interesting in light of the fact that the presence of three acyl-HSL response regulators coexist in this bacterial species. Intuitively, one might expect three regulators to have higher DNA-binding specificity to avoid activating gene expression at the 'wrong' promoter. The ability to distinguish the different target promoters of the three LuxR homologs must arise from differences at

other nucleotide positions. A recent study showed that two of the LuxR homologs found in *P. aeruginosa*, LasR and QscR, identify their respective promoters by recognizing bp 8 and -8 [36].

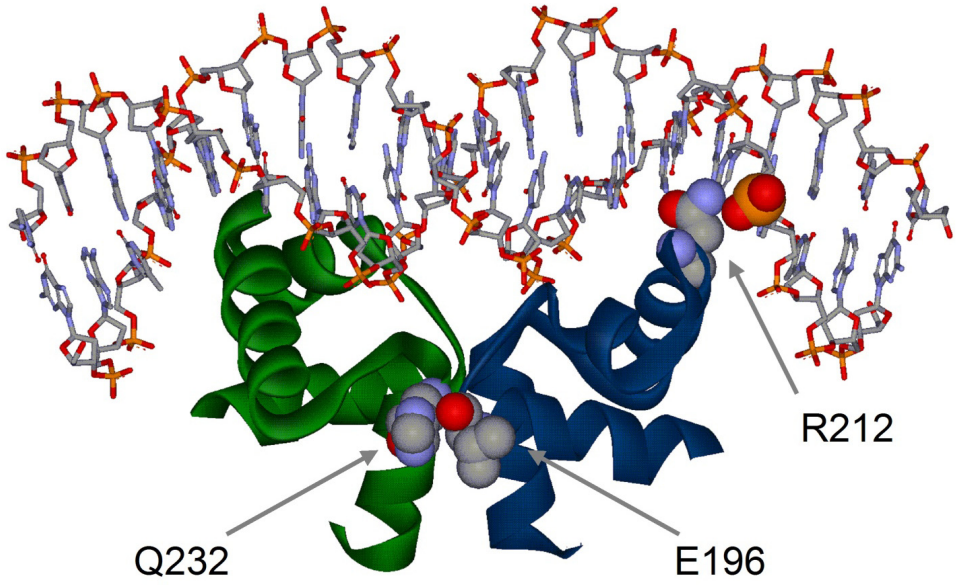


Figure 5.8. Positions of residues in TraR corresponding to residues 212 and 232 in LuxR. The crystal structure of the LuxR homologue TraR (PDB 1L3L) has been determined [11]. The *tra* box DNA target and the C-terminal domains of two TraR peptides are shown. The two peptides comprising a TraR dimer are shown in green and blue. The *tra* box is shown as sticks (grey represents carbon, red represents nitrogen, blue represents oxygen and orange represents phosphorus). The amino acid residues which align with R212 and Q232 are shown space-filling spheres with coloring similar to that used for DNA. The phosphate group between positions 4 and 5 of the lux box is shown as space filling spheres. The glutamic acid (E196) residue located proximally to Q232 is also shown.

Changes in specificity can be estimated by comparing the ratio of 3OC6HSL concentrations required to attain half-maximal levels of gene activation at the P_{luxI} and P_{luxG5A} promoters for the different LuxR variants. A 5,000-fold change in specificity was attained by LuxR-R212C/Q232H. Both LuxR-R212H and LuxR-R212S showed a more than 50,000-fold change in promoter specificity.

The observation from the gel shift experiments that each of the evolved variants has a higher DNA-binding affinity for P_{luxG5A} that P_{luxI} was unexpected. Our bioassays showed that LuxR-R212C/Q232H activates gene expression equally well at either promoter but it did not bind P_{luxI} *in vitro*. This discrepancy indicates that the conformational changes or interactions with RNA polymerase required for activation may also affect DNA binding. The finding that a truncated LuxR consisting only of the C-terminal domain required RNA polymerase to bind DNA demonstrated that LuxR's interactions with RNA polymerase can influence its ability to bind the *lux* box [23]. This finding suggests that selection or screening systems that directly measure the desired output, i.e., gene activation, may be better for optimizing genetic regulatory networks than assays targeting DNA binding directly.

The finding that the R212C single-mutant is nonfunctional and that the Q232H mutation does not confer altered promoter specificity on its own indicates that the R→C mutation promotes recognition of the lux_{G5A} box, but that it is also deleterious with regards to

activating gene expression. The observation that LuxR-R212C does not accumulate *in vivo* indicates that its inability to activate gene expression is due to decreased protein stability or misfolding. LuxR-R212C/Q232H, LuxR-R212H, and LuxR-R212S were also shown to accumulate to lower protein concentrations. In general, mutations at position 212 negatively affect protein stability and/or folding, and positively affect P_{luxG5A} recognition. The Q232H mutation likely compensates for the defect caused by the R212C mutation. The Q232H mutation could confer the observed increase in activation in one or more ways including increasing protein stability or solubility, stabilizing interactions with RNA polymerase, and increasing DNA affinity. The five-fold higher protein concentration observed with LuxR-Q232H, compared to wild type, suggests that the increase in gene activation observed with LuxR-Q232H is a result of increased protein stability. Alignment to the published structure of TraR suggests that the Q232H mutation may stabilize the dimeric form of the protein through an electrostatic interaction with glutamic acid 196 on the adjacent polypeptide chain (Fig. 5.8).

The majority of published HTH engineering experiments have used solutions already present in Nature, including swapping an entire DNA recognition helix or replacing all of the amino acids that contact a specific DNA target recognized by a close homolog. This strategy is not always successful [24], and does not generate DNA-binding proteins that recognize novel DNA sequences. Attempts at engineering HTH proteins that bind to previously unrecognized sequences have previously relied solely on libraries that randomize the amino acids shown to make contacts with the DNA. In most cases,

modest gains can be accomplished in this manner. New specificities, however, are rare. Our results suggest that DNA-binding proteins that recognize new DNA targets can be identified from libraries generated by random mutagenesis. This strategy is especially useful when structural information is unavailable. Furthermore, the identification of mutations at residues not targeted in site-saturation libraries, which have arisen adventitiously [37], are critical for binding the new target suggests that screening or selecting a library generated by random mutagenesis is a viable complementary approach for identifying DNA-binding mutants even when a crystal structure is available.

The identification of a LuxR variant with two synergistic mutations that increase activation at P_{luxG5A} indicates that multiple mutations may be required to switch DNA-binding specificity. We used our best first-generation mutant as parent and selected more than 200,000 clones transformed with a *luxR-R212H* library for additional improvements in promoter specificity. We did not identify any positive clones from these experiments. This suggests that either switching LuxR's specificity from the G/C bp to an A/T bp at positions 5 and -5 is not chemically possible, or that a LuxR variant meeting the criteria determined by the selection conditions was not present in the second-generation library. The second, more plausible explanation indicates that a new library design is necessary. Alanine scanning mutagenesis of a 29 residue "miniature" DNA-binding protein demonstrated the importance of a well-packed hydrophobic core for DNA binding and specificity [38]. Therefore, making further gains in engineering the DNA-binding specificity with HTH motifs may require the generation of libraries that target a large

number of residues, including both those that interact directly with the DNA and those that form the core of the protein. The dual selection system is ideal for this use because libraries of more than 10^8 unique clones could be selected for variants with new DNA-binding specificities. If, like the R212C mutation, residues that alter the DNA-binding specificity frequently destabilize the protein, an alternative library design strategy is to start with a stabilized parent [39]. We have previously identified LuxR variants with increased sensitivities to 3OC6HSL that exhibit much higher protein concentrations than wild-type LuxR in the presence of acyl-HSL. One of these stabilized variants may provide the necessary advantage required to identify new LuxR variants with switched DNA-binding specificities.

E. Materials and Methods

E.1. Bacterial strains, media and growth conditions

The bacterial strains used in this study are *Escherichia coli* strains DH5α (F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 and BL21 (F^- *dcm*+ Hte *ompT hsdS*(r_{B-} m_{B-}) *gal endA* Tet^r). *E. coli* strains were cultured at 37 ^oC in LB medium or on LB agar plates. Antibiotics were added at the following concentrations to maintain the plasmids: 100 μg/mL carbenicillin, 20 μg/mL kanamycin, and 100 μg/mL chloramphenicol. During selection experiments antibiotics were added as follows: 100 μg/mL carbenicillin, 50 μg/mL kanamycin, and 150 μg/mL chloramphenicol. 3-Oxohexanoyl-DL-homoserine lactone (3OC6HSL; Sigma Aldrich, St. Louis, MO) stock solutions were prepared by dissolving appropriate amounts in ethyl acetate acidified with glacial acetic acid (0.01% v/v), and stored at -20 ^oC.

E.2. Plasmid construction

The positive selection and GFPuv reporter plasmids containing P_{luxG5A}, $plux_{G5A}CAT$ $plux_{G5A}GFPuv$, were constructed from pluxGFPuv [40] and pluxCAT (described in Chapter 3) using standard site-directed mutagenesis techniques. The oligonucleotides used were: luxBoxB-f (5-CTTAACATAAGCACCTATAGGATCGTATAGGTTTAC GCAAGAAAATGG-3) and its reverse complement, luxBoxB-r.

E.3. Mutagenesis of luxR

Mutagenesis of the region of *luxR* encoding the C-terminal third of the protein was carried out using error-prone PCR [41] to yield a mutation frequency of approximately 2 nucleotide substitutions per *luxR* gene. The LuxRSalI-f (see above) and 3-LuxR [40] primers were used to amplify the desired region. The pool of mutagenized *luxR* genes was cloned into pLuxRSalI between its SalI and BamHI sites.

The forward and reverse primers used for saturation mutagenesis at position 212 were: 5-LuxRSat212 (5-GGGATATTTCAAAAATATTAGGCTGCAGTGAGNNNACTGTC ACTTTCC-3), and 3-LuxRSat(int) (5-CTGCAGCCTAATATTTTTGAAATATCCC-3). The 3' end of luxR containing the randomized 212 codon was amplified from pLuxSalI with LuxRSat212 and 3-LuxR+200 (5-GTGAGCGAGGAAGCGGAATATATCC-3). The 5' end of the gene was amplified with 5-LuxR [40] and 3-LuxRSat(int). Overlap extension PCR using the PCR products from the two reactions described above, 5-LuxR and 3-LuxR was used to construct the entire *luxR* gene containing the randomized 212 codon. The library was ligated into pLuxRSalI following digestion with *Kpn*I and *Bam*HI.

LuxR single-mutants containing the R212C and Q232H mutations were constructed using standard site-directed methods.

E.4. Selection of LuxR variants with new DNA-binding properties

In Round 1-ON, competent cells harboring plux$_{G5A}$CAT were transformed with a pool of mutagenized pLuxRSalI and plated on LB-agar amended with 5 μM 3OC6HSL, 150 μg/mL chloramphenicol and 50 μg/mL kanamycin. Surviving colonies were harvested after 20 h at 37^{o}C by overlaying LB medium. pLuxSalI plasmids were recovered from the selected cells via miniprep (QIAGEN) and endonuclease digestion of plux$_{G5A}$CAT with *Sca*I. The recovered DNA was transformed into DH5α cells harboring the plux$_{G5A}$CAT plasmid for Round 2-ON. In Round 2-ON (and all subsequent ON selections), the LB-agar contained 500 nM 3OC6HSL. Surviving colonies were harvested after 14 h at 37 ^{o}C. The plasmid DNA recovered and plux$_{G5A}$CAT inactivated via digestion. The *luxR* expression plasmids recovered after Round 2-ON were transformed into DH5α cells harboring plux$_{WT}$Blip and plated with 10 nM 3OC6HSL, 100 μg/mL carbenicillin and 50 μg/mL kanamycin (Round 2-OFF). Cells surviving after 14 h at 37^{o}C were harvested and treated similarly to after Round 1-ON and Round 2-ON. Re-cloning of the library following Round 3-ON was carried out with a proofreading polymerase (Pfu, Stratagene), 5-LuxR and 3-LuxR. PCR products were ligated into pLuxRSalI following digestion with *Kpn*I and *Bam*HI. pLuxRSalI plasmids recovered after the Round 4-ON were transformed into DH5α with plux$_{G5A}$GFPuv and plated with 100 nM 3OC6HSL, 100 μg/mL chloramphenicol and 50 μg/mL kanamycin. Eighty

colonies were picked and plated with a titration of 3OC6HSL. The 15 best colonies, based on fluorescence, were chosen for subsequent characterization.

Plasmids encoding the selected LuxR variants were isolated by transforming miniprep DNA into plasmid-free DH5α and plating onto LB-plates supplemented with kanamycin. The plasmids encoding the LuxR variants of interest were also transformed with pluxGFPuv so that their ability to activate gene expression at the wild-type *lux* promoter could be assessed.

E.5. Characterization of selected LuxR variants

Bioassays to quantitatively characterize LuxR-mediated gene expression of GFPuv from both P_{luxI} and P_{luxG5A} were performed as described [40]. Western blots to assay the abundance of the LuxR variants in vivo as described [40].

LuxR variants were purified as described [3]. Modifications to the published procedure include: the concentration of tween-20 in all buffers was lowered to 0.05% v/v.

Gel mobility shift assays were performed similarly to the methods described as described in Chapter 4. The DNA probes containing either the wt lux box or lux_{G5A} box were 173 bp in length and amplified from pluxGFPuv or $plux_{G5A}$GFPuv with 5-LuxI and 3-LuxI

[40]. Probes were generated by end-labeling the PCR products using [γ^{32}-P]-ATP plus T4 polynuceotide kinase. Protein-DNA binding reactions contained approximately 5 fmol of the DNA probe, 200 μg/mL poly(dI-dC)•poly(dI-dC) and 250 μg/mL sonicated salmon sperm DNA in a final volume of 10 μL of DNA binding buffer (20 mM Tris-HCl, [pH 7.4], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μg bovine serum albumin/mL, and 5% ficoll 400). Purified protein and acyl-HSL were added as indicated and incubated for 25 min at 25 ^{o}C. The reaction mixtures were run on a native 5% Tris-glycine-EDTA gel at 4 ^{o}C. Following electrophoresis, the probes were detected using Kodak BioMax XAR Film (Rochester, NY) and quantified from the scanned image via densitometry analysis using AlphaEaseFc image analysis software (Alpha Innotech).

F. References

1. Fuqua, C., Parsek, M.R. & Greenberg, E.P. Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**, 439-468 (2001).

2. Greenberg, E.P. Acyl-homoserine lactone quorum sensing in bacteria. *J. Microbiol.* **38**, 117-121 (2000).

3. Urbanowski, A.L., Lostroh, C.P. & Greenberg, E.P. Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **186**, 631-637 (2004).

4. Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H. & Oppenheimer, N.J. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**, 2444-2449 (1981).

5. Kaplan, H.B. & Greenberg, E.P. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**, 1210-1214 (1985).

6. Egland, K.A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: elements of the *luxI* promoter. *Mol. Microbiol.* **31**, 1197-1204 (1999).

7. Stevens, A.M., Fujita, N., Ishihama, A. & Greenberg, E.P. Involvement of the RNA polymerase alpha-subunit C-terminal domain in LuxR-dependent activation of the *Vibrio fischeri* luminescence genes. *J. Bacteriol.* **181**, 4704-4707 (1999).

8. Devine, J.H., Shadel, G.S. & Baldwin, T.O. Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC7744. *Proc. Natl. Acad. Sci. USA* **86**, 5688-5692 (1989).

9. Trott, A.E. & Stevens, A.M. Amino acid residues in LuxR critical for its mechanism of transcriptional activation during quorum sensing in *Vibrio fischeri*. *J. Bacteriol.* **183**, 387-392 (2001).

10. Stevens, A.M. & Greenberg, E.P. Transcriptional activation by LuxR. in *Cell-Cell Signaling in Bacteria* (eds. Dunny, G.M. & Winans, S.C.) 231-42 (ASM Press, Washington, DC, 1999).

11. Zhang, R.G., Pappas, T., Brace, J.L., Miller, P.C., Oulmassov, T., Molyneauz, J.M., Anderson, J.C., Bashkin, J.K., Winans, S.C. & Joachimiak, A. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**, 971-974 (2002).

12. Egland, K.A. & Greenberg, E.P. Quorum sensing in *Vibrio fischeri*: Analysis of the LuxR DNA binding region by alanine-scanning mutagenesis. *J. Bacteriol.* **183**, 382-386 (2001).

13. Lerat, E. & Moran, N.A. The evolutionary history of quorum-sensing systems in bacteria. *Mol. Biol. Evol.* **21**, 903-913 (2004).

14. Gray, K.M., Passador, L., Iglewski, B.H. & Greenberg, E.P. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. *J. Bacteriol.* **176**, 3076-3080 (1994).

15. Lee, J.-H., Lequette, Y. & Greenberg, E.P. Activity of QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Mol. Microbiol.* (2005).

16. Schuster, M., Urbanowski, M.L. & Greenberg, E.P. Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc. Natl. Acad. Sci. USA* **101**, 15833-15839 (2004).

17. Basu, S., Gerchman, Y., Collins, C.H., Arnold, F.H. & Weiss, R. A synthetic multicellular system for programmed pattern formation. *Nature* **434**, 1130-1134 (2005).

18. Kobayashi, H., Kaern, M., Araki, M., Chung, K., Gardner, T.S., Cantor, C.R. & Collins, J.J. Programmable cells: Interfacing natural and engineered gene networks. *Proc. Natl. Acad. Sci. USA* **101**, 8414-8419 (2004).

19. Weiss, R. & Knight, T.F. Engineered communications for microbial robotics. *DNA6: Sixth International Meeting on DNA Based Computers*, 1-15 (2000).

20. You, L.C., Cox, R.S., Weiss, R. & Arnold, F.H. Programmed population control by cell-cell communication and regulated killing. *Nature* **428**, 868-871 (2004).

21. Choi, S.H. & Greenberg, E.P. The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. *Proc. Natl. Acad. Sci. USA* **88**, 11115-11119 (1991).

22. Choi, S.H. & Greenberg, E.P. Genetic dissection of DNA-binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **174**, 4064-4069 (1992).

23. Stevens, A.M., Dolan, K.M. & Greenberg, E.P. Synergistic binding of the *Vibrio fischeri* LuxR transcriptional activator domain and RNA polymerase to the *lux* promoter region. *Proc. Natl. Acad. Sci. USA* **91**, 12619-12623 (1994).

24. Backes, H., Berens, C., Helbl, V., Walter, S., Schmid, F.X. & Hillen, W. Combinations of the alpha-helix-turn-alpha-helix motif of TetR with respective residues from LacI or 434Cro: DNA recognition, inducer binding, and urea-dependent denaturation. *Biochemistry* **36**, 5311-5322 (1997).

25. Ebright, R.H., Cossart, P., Gicquelsanzey, B. & Beckwith, J. Mutations that alter the DNA-sequence specificity of the catabolite gene activator protein of *Escherichia coli*. *Nature* **311**, 232-235 (1984).

26. Fromknecht, K., Vogel, P.D. & Wise, J.G. Combinatorial redesign of the DNA binding specificity of a prokaryotic helix-turn-helix repressor. *J. Bacteriol.* **185**, 475-481 (2003).

27. Helbl, V. & Hillen, W. Stepwise selection of TetR variants recognizing tet operator 4C with high affinity and specificity. *J Mol Biol* **276**, 313-318 (1998).

28. Helbl, V., Tiebel, B. & Hillen, W. Stepwise selection of TetR variants recognizing tet operator 6C with high affinity and specificity. *J Mol Biol* **276**, 319-324 (1998).

29. Huang, L.X., Sera, T. & Schultz, P.G. A permutational approach toward protein-DNA recognition. *Proc. Natl. Acad. Sci. USA* **91**, 3969-3973 (1994).

30. Nilsson, M.T.I., Mossing, M.C. & Widersten, M. Functional expression and affinity selection of single-chain Cro by phage display: Isolation of novel DNA-binding proteins. *Protein Eng.* **13**, 519-526 (2000).

31. Simoncsits, A., Tjornhammar, M.L., Wang, S. & Pongor, S. Single-chain 434 repressors with altered DNA-binding specificities - Isolation of mutant single-chain repressors by phenotypic screening of combinatorial mutant libraries. *Genetica* **106**, 85-92 (1999).

32. Wharton, R.P., Brown, E.L. & Ptashne, M. Substituting an alpha-helix switches the sequence-specific DNA interactions of a repressor. *Cell* **38**, 361-369 (1984).

33. Wharton, R.P. & Ptashne, M. Changing the binding-specificity of a repressor by redesigning an alpha-helix. *Nature* **316**, 601-605 (1985).

34. Wharton, R.P. & Ptashne, M. A new-specificity mutant of 434 repressor that defines an amino-acid base pair contact. *Nature* **326**, 888-891 (1987).

35. Whiteley, M. & Greenberg, E.P. Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. *J. Bacteriol.* **183**, 5529-5534 (2001).

36. Lee, J.-H., Lequette, Y. & Greenberg, E.P. Activity of QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Mol. Microbiol.*, doi: 10.1111/j.1365-2958.2005.04960.x (2005).

37. Helbl, V. & Hillen, W. Stepwise selection of TetR variants recognizing tet operator 4C with high affinity and specificity. *J. Mol. Biol.* **276**, 313-318 (1998).

38. Yang, L. & Schepartz, A. Relationship between folding and function in a sequence-specific miniature DNA-binding protein. *Biochemistry* **44**, 7469-7478 (2005).

39. Bloom, J.D., Labthavikul, S.T., Otey, C.R. & Arnold, F.H. Protein stability promotes evolvability. *(submitted)* (2005).

40. Collins, C.H., Arnold, F.H. & Leadbetter, J.R. Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. *Mol. Microbiol.* **55**, 712-723 (2005).

41. Cirino, P.C., Mayer, K.M. & Umeno, D. Generating libraries using error-prone PCR. in *Directed Evolution Library Creation* (eds. Arnold, F.H. & G., G.) 3-9 (Humana Press, Totawa, NJ, 2003).

CHAPTER 6

Conclusions

We have generated variants of the quorum sensing transcriptional regulator LuxR with new signal and DNA-binding specificities for use in synthetic genetic circuits. One of our hypersensitive variants has already been used as a component in a multicellular band-detect network that generates programmed pattern formation on a solid surface. We anticipate that our new LuxR variants will be the cornerstones of future efforts to construct more complex intercellular communications systems. Furthermore, laboratory evolution of specific signal- and DNA-binding interactions has provided insight into how these systems have evolved in Nature and the biochemical mechanisms involved in distinguishing between chemically similar targets.

Through the directed evolution of LuxR, we have identified several amino acid residues that modulate acyl-HSL and DNA-binding specificity that had not been identified previously. It will be interesting to reassess these mutations as more structural details become available. Would we have been able to identify these sites as specificity determinants if a crystal structure was available? While this scenario is unlikely, perhaps the structures of wild-type LuxR and variants from this work will provide some insight into how these mutations confer the observed change in LuxR-mediated gene activation.

The generation of transcriptional activators with new functions in the absence of structural information demonstrates that directed evolution is a valid design strategy for producing new regulatory components. These evolved variants add new functionalities to make a ‘standardized’ set of components that share many biochemical properties but respond to

different signals or regulate gene expression at different promoters with a range of sensitivities. The wide range of circuit parameters attainable with these new components will aid in the generation of increasingly complex synthetic genetic circuits.

The dual selection system, which successfully identified both acyl-HSL and DNA-binding LuxR variants, is a generally applicable technology and can easily be modified for the directed evolution of other transcription factors. The potential to select libraries of 10^8 or more may enable, for the first time, the directed evolution of an entire genetic circuit by simultaneously randomizing multiple components and selecting for the desired output.

APPENDIX A

Plasmid, gene and protein sequences

A. Sequences of wild-type LuxR, LuxR homologs, evolved LuxR variants and the pLuxR expression vector

A.1. General information

LuxR (Genbank number AAD48473) is composed of 250 amino acids (750 bp). The N-terminal domain of the protein, residues 1 to ~160 residues, is involved in acyl-HSL binding. The C-terminal domain, residues ~180-250, is involved in DNA-binding and activation. The LuxR expression vector, pLuxR, was derived from the commercially available pPROLar.A122 plasmid and contains *luxR* under the control of a $P_{lac/ara-1}$ hybrid promoter. This plasmid was used for all cloning procedures as well as for protein expression.

Two synonymous mutations, T498→C and T510→C, were used to introduce a *Sal*I restriction site into the *luxR* gene between the N- and C-terminal domains so that each domain could be targeted independently. The pLuxR plasmid was also modified to remove a *Sal*I site upstream of the $P_{lac/ara-1}$ promoter. All *luxR* mutants presented in Chapters 4 and 5 contain the internal *Sal*I restriction site. In random mutagenesis experiments targeting the acyl-HSL binding specificity of LuxR, error-prone PCR was used to amplify the region stretching from the *Kpn*I site to the *Sal*I site to include the first 167 amino acids of LuxR. In random mutagenesis experiments targeting the DNA-binding specificity of LuxR, error-prone PCR was used to amplify the region stretching from the *Sal*I site to the *Bam*HI site.

Figure A.1. lists the nucleotide sequence of full-length, wild-type luxR.

Figure A.2. lists the amino acid sequence of full length, wild-type LuxR.

Figure A.3. lists the nucleotide sequence of the pLuxR vector containing wild-type *luxR*.

Figure A.4. shows the plasmid map of the pLuxR vector.

Figure A.5. lists the nucleotide sequences of the *luxR* homologs used in this work. The wild-type *luxR* gene is substituted by the wild-type *lasR* gene in pLasR. The wild-type *rhlR* gene replaces *luxR* in pRhlR. A *Bam*HI site within the *rhlR* gene prevented the use of this site when cloning the RhlR expression vector. A *Hind*III site directly upstream of the *Bam*HI site in pPROLar.A122 is the 3' restriction site in pRhlR.

Figure A.6. lists the amino acid sequence of full length, wild-type LasR and RhlR.

Table A.1. lists the sequences of the *luxR* mutants generated by random mutagenesis in Chapter 2.

Table A.2. lists the sequences of the *luxR* single-mutants constructed in Chapter 2.

Table A.3. lists nucleotide and amino acid substitutions of *luxR*/LuxR and *lasR*/LasR mutants described in Chapter 4.

Table A.4. lists nucleotide and amino acid substitutions of *luxR*/LuxR mutants described in Chapter 5.

Figure A.1. Nucleotide sequence of full-length, wild-type *luxR*.

```
1   ATGAAAAACA TAAATGCCGA CGACACATAC AGAATAATTA ATAAAATTAA
51  AGCTTGTAGA AGCAATAATG ATATTAATCA ATGCTTATCT GATATGACTA
101 AAATGGTACA TTGTGAATAT TATTTACTCG CGATCATTTA TCCTCATTCT
151 ATGGTTAAAT CTGATATTTC AATTCTAGAT AATTACCCTA AAAAATGGAG
201 GCAATATTAT GATGACGCTA ATTTAATAAA ATATGATCCT ATAGTAGATT
251 ATTCTAACTC CAATCATTCA CCAATTAATT GGAATATATT TGAAAACAAT
301 GCTGTAAATA AAAAATCTCC AAATGTAATT AAAGAAGCGA AAACATCAGG
351 TCTTATCACT GGGTTTAGTT TCCCTATTCA TACGGCTAAC AATGGCTTCG
401 GAATGCTTAG TTTTGCACAT TCAGAAAAAG ACAACTATAT AGATAGTTTA
451 TTTTTACATG CGTGTATGAA CATACCATTA ATTGTTCCTT CTCTAGTTGA
501 TAATTATCGA AAAATAAATA TAGCAAATAA TAAATCAAAC AACGATTTAA
551 CCAAAAGAGA AAAAGAATGT TTAGCGTGGG CATGCGAAGG AAAAAGCTCT
601 TGGGATATTT CAAAAATATT AGGCTGCAGT GAGCGTACTG TCACTTTCCA
651 TTTAACCAAT GCGCAAATGA AACTCAATAC AACAAACCGC TGCCAAAGTA
701 TTTCTAAAGC AATTTTAACA GGAGCAATTG ATTGCCCATA CTTTAAAAAT
```

Figure A.2. Amino acid sequence of full-length, wild-type LuxR.

```
1   MKNINADDTY RIINKIKACR SNNDINQCLS DMTKMVHCEY YLLAIIYPHS
50  MVKSDISILD NYPKKWRQYY DDANLIKYDP IVDYSNSNHS PINWNIFENN
100 AVNKKSPNVI KEAKTSGLIT GFSFPIHTAN NGFGMLSFAH SEKDNYIDSL
150 FLHACMNIPL IVPSLVDNYR KINIANNKSN NDLTKREKEC LAWACEGKSS
200 WDISKILGCS ERTVTFHLTN AQMKLNTTNR CQSISKAILT GAIDCPYFKN
```

Figure A.3. Nucleotide sequence of the pLuxR vector containing wild-type *luxR*.

```
1    GTCGACTCGA GCATAGCATT TTTATCCATA AGATTAGCGG ATCTAACCTT
51   TACAATTGTG AGCGCTCACA ATTATGATAG ATTCAATTGT GAGCGGATAA
101  CAATTTCACA CAGAATTCAT TAAAGAGGAG AAAGGTACCC ATGAAAAACA
151  TAAATGCCGA CGACACATAC AGAATAATTA ATAAAATTAA AGCTTGTAGA
201  AGCAATAATG ATATTAATCA ATGCTTATCT GATATGACTA AAATGGTACA
251  TTGTGAATAT TATTTACTCG CGATCATTTA TCCTCATTCT ATGGTTAAAT
301  CTGATATTTC AATTCTAGAT AATTACCCTA AAAAATGGAG GCAATATTAT
351  GATGACGCTA ATTTAATAAA ATATGATCCT ATAGTAGATT ATTCTAACTC
401  CAATCATTCA CCAATTAATT GGAATATATT TGAAAACAAT GCTGTAAATA
451  AAAAATCTCC AAATGTAATT AAAGAAGCGA AAACATCAGG TCTTATCACT
501  GGGTTTAGTT TCCCTATTCA TACGGCTAAC AATGGCTTCG GAATGCTTAG
551  TTTTGCACAT TCAGAAAAAG ACAACTATAT AGATAGTTTA TTTTTACATG
601  CGTGTATGAA CATACCATTA ATTGTTCCTT CTCTAGTTGA TAATTATCGA
651  AAAATAAATA TAGCAAATAA TAAATCAAAC AACGATTTAA CCAAAAGAGA
701  AAAAGAATGT TTAGCGTGGG CATGCGAAGG AAAAAGCTCT TGGGATATTT
751  CAAAAATATT AGGCTGCAGT GAGCGTACTG TCACTTTCCA TTTAACCAAT
801  GCGCAAATGA AACTCAATAC AACAAACCGC TGCCAAAGTA TTTCTAAAGC
851  AATTTTAACA GGAGCAATTG ATTGCCCATA CTTTAAAAAT TAAGTACGGG
901  ATCCCTGCAG GCCTCAGGGC CCGATCGATG CGGCCGCTTA ATTAATTAAT
951  CTAGAGGCAT CAAATAAAAC GAAAGGCTCA GTCGAAAGAC TGGGCCTTTC
1001 GTTTTATCTG TTGTTTGTCG GTGAACGCTC TCCTGAGTAG GACAAATCCG
1051 CCGCCCTAGA CCTAGGGGAT ATATTCCGCT TCCTCGCTCA CTGACTCGCT
1101 ACGCTCGGTC GTTCGACTGC GGCGAGCGGA AATGGCTTAC GAACGGGGCG
1151 GAGATTTCCT GGAAGATGCC AGGAAGATAC TTAACAGGGA AGTGAGAGGG
1201 CCGCGGCAAA GCCGTTTTTC CATAGGCTCC GCCCCCCTGA CAAGCATCAC
1251 GAAATCTGAC GCTCAAATCA GTGGTGGCGA AACCCGACAG GACTATAAAG
1301 ATACCAGGCG TTTCCCCCTG GCGGCTCCCT CGTGCGCTCT CCTGTTCCTG
1351 CCTTTCGGTT TACCGGTGTC ATTCCGCTGT TATGGCCGCG TTTGTCTCAT
1401 TCCACGCCTG ACACTCAGTT CCGGGTAGGC AGTTCGCTCC AAGCTGGACT
1451 GTATGCACGA ACCCCCCGTT CAGTCCGACC GCTGCGCCTT ATCCGGTAAC
1501 TATCGTCTTG AGTCCAACCC GGAAAGACAT GCAAAAGCAC CACTGGCAGC
1551 AGCCACTGGT AATTGATTTA GAGGAGTTAG TCTTGAAGTC ATGCGCCGGT
1601 TAAGGCTAAA CTGAAAGGAC AAGTTTTGGT GACTGCGCTC CTCCAAGCCA
1651 GTTACCTCGG TTCAAAGAGT TGGTAGCTCA GAGAACCTTC GAAAAACCGC
1701 CCTGCAAGGC GGTTTTTTCG TTTTCAGAGC AAGAGATTAC GCGCAGACCA
1751 AAACGATCTC AAGAAGATCA TCTTATTAAT CAGATAAAAT ATTACTAGAT
1801 TTCAGTGCAA TTTATCTCTT CAAATGTAGC ACCTGAAGTC AGCCCCATAC
1851 GATATAAGTT GTTACTAGTG CTTGGATTCT CACCAATAAA AAACGCCCGG
1901 CGGCAACCGA GCGTTCTGAA CAAATCCAGA TGGAGTTCTG AGGTCATTAC
1951 TGGATCTATC AACAGGAGTC CAAGCGAGCT CTCGAACCCC AGAGTCCCGC
2001 TCAGAAGAAC TCGTCAAGAA GGCGATAGAA GGCGATGCGC TGCGAATCGG
```

2051 GAGCGGCGAT ACCGTAAAGC ACGAGGAAGC GGTCAGCCCA TTCGCCGCCA
2101 AGCTCTTCAG CAATATCACG GGTAGCCAAC GCTATGTCCT GATAGCGGTC
2151 CGCCACACCC AGCCGGCCAC AGTCGATGAA TCCAGAAAAG CGGCCATTTT
2201 CCACCATGAT ATTCGGCAAG CAGGCATCGC CATGGGTCAC GACGAGATCC
2251 TCGCCGTCGG GCATGCGCGC CTTGAGCCTG GCGAACAGTT CGGCTGGCGC
2301 GAGCCCCTGA TGCTCTTCGT CCAGATCATC CTGATCGACA AGACCGGCTT
2351 CCATCCGAGT ACGTGCTCGC TCGATGCGAT GTTTCGCTTG GTGGTCGAAT
2401 GGGCAGGTAG CCGGATCAAG CGTATGCAGC CGCCGCATTG CATCAGCCAT
2451 GATGGATACT TTCTCGGCAG GAGCAAGGTG AGATGACAGG AGATCCTGCC
2501 CCGGCACTTC GCCCAATAGC AGCCAGTCCC TTCCCGCTTC AGTGACAACG
2551 TCGAGCACAG CTGCGCAAGG AACGCCCGTC GTGGCCAGCC ACGATAGCCG
2601 CGCTGCCTCG TCCTGCAGTT CATTCAGGGC ACCGGACAGG TCGGTCTTGA
2651 CAAAAAGAAC CGGGCGCCCC TGCGCTGACA GCCGGAACAC GGCGGCATCA
2701 GAGCAGCCGA TTGTCTGTTG TGCCCAGTCA TAGCCGAATA GCCTCTCCAC
2751 CCAAGCGGCC GGAGAACCTG CGTGCAATCC ATCTTGTTCA ATCATGCGAA
2801 ACGATCCTCA TCCTGTCTCT TGATCAGATC TTGATCCCCT GCGCCATCAG
2851 ATCCTTGGCG GCAAGAAAGC CATCCAGTTT ACTTTGCAGG GCTTCCCAAC
2901 CTTACCAGAG GGCGCCCCAG CTGGCAATTC CGACGTCTGT GTGGAATTGT
2951 GAGCGGATAA CAATTTCACA CAGGGCCCTC GGACACCGAG GAGAATGTCA
3001 AGAGGCGAAC ACACAACGTC TTGGAGCGCC AGAGGAGGAA CGAGCTAAAA
3051 CGGAGCTTTT TTGCCCTGCG TGACCAGATC CCGGAGTTGG AAAACAATGA
3101 AAAGGCCCCC AAGGTAGTTA TCCTTAAAAA AGCCACAGCA TACATCCTGT
3151 CCGTCCAAGC AGAGGAGCAA AAGCTCATTT CTGAAGAGGA CTTGTTGCGG
3201 AAACGACGAG AACAGTTGAA ACACAAACTT GAACAGCTAC GGAACTCTTG
3251 TGCGTAAGGA AAAGTAAGGA AAACGATTCC TTCTAACAGA AATGTCCTGA
3301 GCAATCACCT ATGAACT

Figure A.4. Plasmid map of the pLuxR vector containing wild-type *lux.*

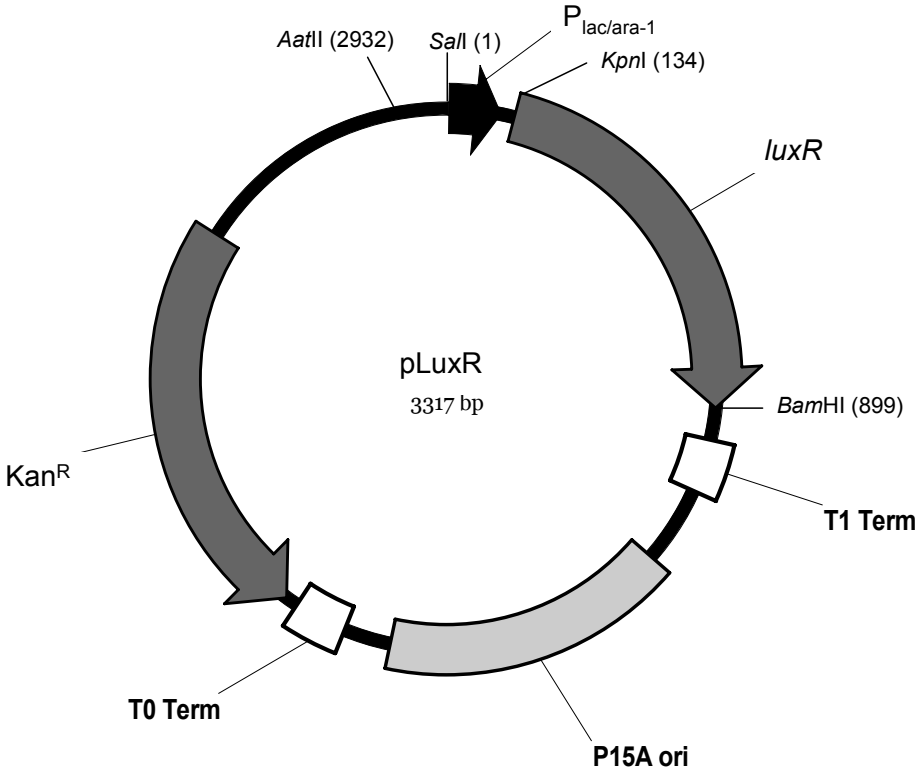


Figure A.5. Nucleotide sequences of the *luxR* homologs used in this work

lasR

```
1    ATGGCCTTGG TTGACGGTTT TCTTGAGCTG GAACGCTCAA GTGGAAAATT
51   GGAGTGGAGC GCCATCCTGC AGAAGATGGC GAGCGACCTT GGATTCTCGA
101  AGATCCTGTT CGGCCTGTTG CCTAAGGACA GCCAGGACTA CGAGAACGCC
151  TTCATCGTCG GCAACTACCC GGCCGCCTGG CGCGAGCATT ACGACCGGGC
201  TGGCTACGCG CGGGTCGACC CGACGGTCAG TCACTGTACC CAGAGCGTAC
251  TGCCGATTTT CTGGGAACCG TCCATCTACC AGACGCGAAA GCAGCACGAG
301  TTCTTCGAGG AAGCCTCGGC CGCCGGCCTG GTGTATGGGC TGACCATGCC
351  GCTGCATGGT GCTCGCGGCG AACTCGGCGC GCTGAGCCTC AGCGTGGAAG
401  CGGAAAACCG GGCCGAGGCC AACCGTTTCA TAGAGTCGGT CCTGCCGACC
451  CTGTGGATGC TCAAGGACTA CGCACTGCAG AGCGGTGCCG GACTGGCCTT
501  CGAACATCCG GTCAGCAAAC CGGTGGTTCT GACCAGCCGG GAGAAGGAAG
551  TGTTGCAGTG GTGCGCCATC GGCAAGACCA GTTGGGAGAT ATCGGTTATC
601  TGCAACTGCT CGGAAGCCAA TGTGAACTTC CATATGGGAA ATATTCGGCG
651  GAAGTTCGGT GTGACCTCCC GCCGCGTAGC GGCCATTATG GCCGTTAATT
701  TGGGTCTTAT TACTCTCTGA
```

rhlR

```
1    ATGAGGAATG ACGGAGGCTT TTTGCTGTGG TGGGACGGTT TGCGTAGCGA
51   GATGCAGCCG ATCCACGACA GCCAGGGCGT GTTCGCCGTC CTGGAAAAGG
101  AAGTGCGGCG CCTGGGCTTC GATTACTACG CCTATGGCGT GCGCCACACG
151  ATTCCCTTCA CCCGGCCGAA GACCGAGGTC CATGGCACCT ATCCCAAGGC
201  CTGGCTGGAG CGATACCAGA TGCAGAACTA CGGGGCCGTG GATCCGGCGA
251  TCCTCAACGG CCTGCGCTCC TCGGAAATGG TGGTCTGGAG CGACAGCCTG
301  TTCGACCAGA GCCGGATGCT CTGGAACGAG GCTCGCGATT GGGGCCTCTG
351  TGTCGGCGCG ACCTTGCCGA TCCGCGCGCC GAACAATTTG CTCAGCGTGC
401  TTTCCGTGGC GCGCGACCAG CAGAACATCT CCAGCTTCGA GCGCGAGGAA
451  ATCCGCCTGC GGCTGCGTTG CATGATCGAG TTGCTGACCC AGAAGCTGAC
501  CGACCTGGAG CATCCGATGC TGATGTCCAA CCCGGTCTGC CTGAGCCATC
551  GCGAACGCGA GATCCTGCAA TGGACCGCCG ACGGCAAGAG TTCCGGGGAA
601  ATCGCCATCA TCCTGAGCAT CTCCGAGAGC ACGGTGAACT TCCACCACAA
651  GAACATCCAG AAGAAGTTCG ACGCGCCGAA CAAGACGCTG GCTGCCGCCT
701  ACGCCGCGGC GCTGGGTCTC ATCTGA
```

Figure A.6. Amino acid sequences of the LuxR homologs used in this work

LasR

```
1   MALVDGFLEL ERSSGKLEWS AILQKMASDL GFSKILFGLL PKDSQDYENA
51  FIVGNYPAAW REHYDRAGYA RVDPTVSHCT QSVLPIFWEP SIYQTRKQHE
101 FFEEASAAGL VYGLTMPLHG ARGELGALSL SVEAENRAEA NRFIESVLPT
151 LWMLKDYALQ SGAGLAFEHP VSKPVVLTSR EKEVLQWCAI GKTSWEISVI
201 CNCSEANVNF HMGNIRRKFG VTSRRVAAIM AVNLGLITL
```

RhlR

```
1   MRNDGGFLLW WDGLRSEMQP IHDSQGVFAV LEKEVRRLGF DYYAYGVRHT
51  IPFTRPKTEV HGTYPKAWLE RYQMQNYGAV DPAILNGLRS SEMVVWSDSL
101 FDQSRMLWNE ARDWGLCVGA TLPIRAPNNL LSVLSVARDQ QNISSFEREE
151 IRLRLRCMIE LLTQKLTDLE HPMLMSNPVC LSHREREILQ WTADGKSSGE
201 IAIILSISES TVNFHHKNIQ KKFDAPNKTL AAAYAAALGL I
```

Table A.1. Nucleotide and amino acid changes in the recovered *luxR*/LuxR mutants described in Chapter 2

luxR	***luxR* gene** base and substitution	**LuxR protein** amino acid residue and substitution
First Generation		
pLuxR-G1A	A97→G	Thr33→Ala
	A159→G	Synonymous
pLuxR-G1B	A133→T	Ile45→Phe
	T501→C	Synonymous
pLuxR-G1C	A284→G	Asn95→Ser
	A414→C	Synonymous
pLuxR-G1D	A286→G	Ile96→Val
	G405→A	Met135→Ile
pLuxR-G1E	T346→G	Ser116→Ala
	A683→G	Thr228→Ala
pLuxR-G1F	T162→A	Synonymous
	A403→G	Met135→Val
	A522→G	Ile174→Met
Second Generation		
pLuxR-G2A	A133→T	Ile45→Phe
	A403→G	Met135→Val
pLuxR-G2B	A133→T	Ile45→Phe
	G405→A	Met135→Ile
	A522→G	Ile174→Met
pLuxR-G2C	T346→G	Ser116→Ala
	G405→A	Met135→Ile
pLuxR-G2D	A97→G	Thr33→Ala
	T162→A	Synonymous
	A286→G	Ile96→Val
	G405→A	Met135→Ile
	A414→C	Synonymous
pLuxR-G2E	A97→G	Thr33→Ala
	T162→A	Synonymous
	T346→G	Ser116→Ala
	G405→A	Met135→Ile
pLuxR-G2F	A97→G	Thr33→Ala
	A133→T	Ile45→Phe
	T162→A	Synonymous
	T346→G	Ser116→Ala
pLuxR-G2G	A97→G	Thr33→Ala
	A286→G	Ile96→Val
	T346→G	Ser116→Ala
pLuxR-G2H	A97→G	Thr33→Ala
	A284→G	Asn95→Ser
	T346→G	Ser116→Ala
	A522→G	Ile174→Met

Table A.2. Single-mutants mutants described in Chapter 2

***luxR* gene** base and substitution	**LuxR** amino acid residue and substitution
A97→G	Thr33→Ala
A133→T	Ile45→Phe
A159→G	Synonymous
T162→A	Synonymous
A284→G	Asn95→Ser
A286→G	Ile96→Val
T346→G	Ser116→Ala
A403→G	Met135→Val
G405→A	Met135→Ile
A414→C	Synonymous
T501→C	Synonymous
A522→G	Ile174→Met
A683→G	Thr228→Ala

Table A.3. Nucleotide and amino acid changes in *luxR*/LuxR and *lasR*/LasR mutants described in Chapter 4

luxR	***luxR* gene** base and substitution	**LuxR protein** amino acid residue and substitution
Parent (2nd generation C8HSL-responder)		
pLuxR-G2E	A97→G	Thr33→Ala
	T162→A	Synonymous
	T346→G	Ser116→Ala
	G405→A	Met135→Ile
Specificity mutants from selections		
pLuxR-G2E-R67M	A97→G	Thr33→Ala
(recovered 7/12)	T162→A	Synonymous
	G200→T	Arg67→Met
	T346→G	Ser116→Ala
	G405→A	Met135→Ile
pLuxR-C10Sp1	A97→G	Thr33→Ala
	T114→C	Synonymous
	T162→A	Synonymous
	A172→G	Ile58→Val
	G200→T	Arg67→Met
	T346→G	Ser116→Ala
	G405→A	Met135→Ile
	A423→G	Synonymous
pLuxR-C10Sp2	A34→G	Ile12→Val
	A97→G	Thr33→Ala
	A101→G	Lys34→Arg
	T162→A	Synonymous
	G200→T	Arg67→Met
	T255→A	Synonymous
	A298→C	Asn100→His
	T346→G	Ser116→Ala
	A355→G	Ile119→Val
	G405→A	Met135→Ile
pLuxR-C10Sp_8	A97→G	Thr33→Ala
	T162→A	Synonymous
	G200→T	Arg67→Met
	A314→G	Lys105→Arg
	T346→G	Ser116→Ala
	G405→A	Met135→Ile
	A470→G	Asn157→Ser
	T498→C	Synonymous (*Sal*I)
	T510→C	Synonymous (*Sal*I)
Introduction of R67M mutation into wild-type LuxR		
pLuxR-R67M	G200→T	Arg67→Met
Introduction of R61M (aligns with R67M of LuxR) mutation into wild-type LasR		
pLasR-R61M	GCT181→ATG	Arg61→Met

Table A.4. Nucleotide and amino acid changes in *luxR*/LuxR variants described in Chapter 5

luxR	***luxR* gene** base and substitution	**LuxR protein** amino acid residue and substitution
Parent		
pLuxR(SalI)	T498→C	Synonymous (*Sal*I)
	T501→C	Synonymous (*Sal*I)
DNA-binding mutants from selections		
pLuxR-R212C/Q232H	C634→T	R212→C
	A695→T	Q232→H
pLuxR-R212H	G635→A	R212→H
DNA-binding mutants from saturation of residue 212		
pLuxR-R212S	C634→A	R212→S
Single-mutants		
pLuxR-R212C	C634→T	R212→C
pLuxR-Q232H	A695→T	Q232→H

B. Sequences of the fluorescent reporters used for screening and characterization of LuxR variants.

B.1. General information

The pluxGFPuv plasmid was constructed for screening LuxR variants that activate gene expression in the presence of non-cognate signal molecules, based on LuxR-mediated expression of a green fluorescent protein (GFP) variant. GFPuv (Clontech) was used because it can be excited by standard long-wavelength ultraviolet light such that a plate of colonies can be easily screened for GFPuv production by illuminating the plate with in a standard gel box and visually inspecting for green colonies.

A variant of pluxGFPuv containing a mutated *lux* promoter was constructed for the final round of screening used to identify the best variants from the pool of variants selected for their ability to activate gene expression at the new promoter. The promoter differs from P_{luxI} at two positions: the 5 and -5 position of the *lux* box were altered simultaneously to maintain the inverted repeat structure.

A liquid-phase bioassay system was developed that assesses LuxR-mediated gene activation by quantitatively determining the level of fluorescence due to GFPuv.

Reporter proteins containing two other fluorescent reporters were constructed for use with microscopes. The GFPmut3 variant has an excitation maximum at 489. The DsRed

construct was used to show a lack of crosstalk between LuxR and the evolved acyl-HSL specificity mutant, LuxR-G2E-R67M.

Figure A.7. lists the nucleotide sequence of the wild-type P_{luxI} promoter.

Figure A.8. lists the nucleotide sequences of the wild-type lux box and mutant lux_{G5A} box.

Figure A.9. lists the nucleotide sequences of the fluorescent reporters, *gfpuv,gfpmut3* and *dsred-express*.

Figure A.10. lists the amino acid sequences of the fluorescent reporters, GFPuv, GFPmut3 and DsRed-express.

Figure A.11. lists the nucleotide sequence of the pluxGFPuv reporter plasmid.

Figure A.12. shows the plasmid map of pluxGFPuv.

Figure A.7. Nucleotide sequence of the wild-type P_{luxI} promoter.

```
1   AGTCCTTTGA TTCTAATAAA TTGGATTTTT GTCACACTAT TGTATCGCTG
51  GGAATACAAT TACTTAACAT AAGCACCTGT AGGATCGTAC AGGTTTACGC
101 AAGAAAATGG TTTGTTATAG TCGAATAAAC GCAAGGGAGG TTGGT
```

Figure A.8. Nucleotide sequences of the wild-type *lux* box and mutant lux_{G5A} box.

lux box

```
1   ACCTGTAGGA TCGTACAGGT
```

lux_{G5A} *box*

```
1   ACCTATAGGA TCGTATAGGT
```

Figure A.9. Nucleotide sequences of the fluorescent reporters, gfpuv,gfpmut3 and *dsred-express*, used in this study.

gfpuv

```
1   ATGAGTAAAG GAGAAGAACT TTTCACTGGA GTTGTCCCAA TTCTTGTTGA
51  ATTAGATGGT GATGTTAATG GGCACAAATT TTCTGTCAGT GGAGAGGGTG
101 AAGGTGATGC AACATACGGA AAACTTACCC TTAAATTTAT TTGCACTACT
151 GGAAAACTAC CTGTTCCATG GCCAACACTT GTCACTACTT TCTCTTATGG
201 TGTTCAATGC TTTTCCCGTT ATCCGGATCA TATGAAACGG CATGACTTTT
251 TCAAGAGTGC CATGCCCGAA GGTTATGTAC AGGAACGCAC TATATCTTTC
301 AAAGATGACG GGAACTACAA GACGCGTGCT GAAGTCAAGT TTGAAGGTGA
351 TACCCTTGTT AATCGTATCG AGTTAAAAGG TATTGATTTT AAAGAAGATG
401 GAAACATTCT CGGACACAAA CTCGAGTACA ACTATAACTC ACACAATGTA
451 TACATCACGG CAGACAAACA AAAGAATGGA ATCAAAGCTA ACTTCAAAAT
501 TCGCCACAAC ATTGAAGATG GATCCGTTCA ACTAGCAGAC CATTATCAAC
551 AAAATACTCC AATTGGCGAT GGCCCTGTCC TTTTACCAGA CAACCATTAC
601 CTGTCGACAC AATCTGCCCT TTCGAAAGAT CCCAACGAAA AGCGTGACCA
651 CATGGTCCTT CTTGAGTTTG TAACTGCTGC TGGGATTACA CATGGCATGG
701 ATGAGCTCTA CAAATAA
```

gfpmut3

```
1   ATGCGTAAAG GAGAAGAACT TTTCACTGGA GTTGTCCCAA TTCTTGTTGA
51  ATTAGATGGT GATGTTAATG GGCACAAATT TTCTGTCAGT GGAGAGGGTG
101 AAGGTGATGC AACATACGGA AAACTTACCC TTAAATTTAT TTGCACTACT
151 GGAAAACTAC CTGTTCCATG GCCAACACTT GTCACTACTT TCGGTTATGG
201 TGTTCAATGC TTTGCGAGAT ACCCAGATCA TATGAAACAG CATGACTTTT
251 TCAAGAGTGC CATGCCCGAA GGTTATGTAC AGGAAAGAAC TATATTTTTC
301 AAAGATGACG GGAACTACAA GACACGTGCT GAAGTCAAGT TTGAAGGTGA
351 TACCCTTGTT AATAGAATCG AGTTAAAAGG TATTGATTTT AAAGAAGATG
401 GAAACATTCT TGGACACAAA TTGGAATACA ACTATAACTC ACACAATGTA
451 TACATCATGG CAGACAAACA AAAGAATGGA ATCAAAGTTA ACTTCAAAAT
501 TAGACACAAC ATTGAAGATG GAAGCGTTCA ACTAGCAGAC CATTATCAAC
551 AAAATACTCC AATTGGCGAT GGCCCTGTCC TTTTACCAGA CAACCATTAC
601 CTGTCCACAC AATCTGCCCT TTCGAAAGAT CCCAACGAAA AGAGAGACCA
651 CATGGTCCTT CTTGAGTTTG TAACAGCTGC TGGGATTACA CATGGCATGG
701 ATGAACTATA CAAATAA
```

dsred-express

```
1   ATGGCCTCCT CCGAGGACGT CATCAAGGAG TTCATGCGCT TCAAGGTGCG
51  CATGGAGGGC TCCGTGAACG GCCACGAGTT CGAGATCGAG GGCGAGGGCG
101 AGGGCCGCCC CTACGAGGGC ACCCAGACCG CCAAGCTGAA GGTGACCAAG
151 GGCGGCCCCC TGCCCTTCGC CTGGGACATC CTGTCCCCCC AGTTCCAGTA
201 CGGCTCCAAG GTGTACGTGA AGCACCCCGC CGACATCCCC GACTACAAGA
251 AGCTGTCCTT CCCCGAGGGC TTCAAGTGGG AGCGCGTGAT GAACTTCGAG
301 GACGGCGGCG TGGTGACCGT GACCCAGGAC TCCTCCCTGC AGGACGGCTC
351 CTTCATCTAC AAGGTGAAGT TCATCGGCGT GAACTTCCCC TCCGACGGCC
401 CCGTAATGCA GAAGAAGACT ATGGGCTGGG AGGCCTCCAC CGAGCGCCTG
451 TACCCCCGCG ACGGCGTGCT GAAGGGCGAG ATCCACAAGG CCCTGAAGCT
501 GAAGGACGGC GGCCACTACC TGGTGGAGTT CAAGTCCATC TACATGGCCA
551 AGAAGCCCGT GCAGCTGCCC GGCTACTACT ACGTGGACTC CAAGCTGGAC
601 ATCACCTCCC ACAACGAGGA CTACACCATC GTGGAGCAGT ACGAGCGCGC
651 CGAGGGCCGC CACCACCTGT TCCTG
```

Figure A.10. Amino acid sequence of the fluorescent reporters GFPuv, GFPmut3 and DsRed-express, used in this study.

GFPuv

```
1   MSKGEELFTG VVPILVELDG DVNGHKFSVS GEGEGDATYG KLTLKFICTT
51  GKLPVPWPTL VTTFSYGVQC FSRYPDHMKR HDFFKSAMPE GYVQERTISF
101 KDDGNYKTRA EVKFEGDTLV NRIELKGIDF KEDGNILGHK LEYNYNSHNV
151 YITADKQKNG IKANFKIRHN IEDGSVQLAD HYQQNTPIGD GPVLLPDNHY
201 LSTQSALSKD PNEKRDHMVL LEFVTAAGIT HGMDELYK
```

GFPmut3

```
1   MSKGEELFTG VVPILVELDG DVNGHKFSVS GEGEGDATYG KLTLKFICTT
51  GKLPVPWPTL VTTFGYGVQC FARYPDHMKQ HDFFKSAMPE GYVQERTIFF
101 KDDGNYKTRA EVKFEGDTLV NRIELKGIDF KEDGNILGHK LEYNYNSHNV
151 YIMADKQKNG IKVNFKIRHN IEDGSVQLAD HYQQNTPIGD GPVLLPDNHY
201 LSTQSALSKD PNEKRDHMVL LEFVTAAGIT HGMDELYK
```

DsRed-express

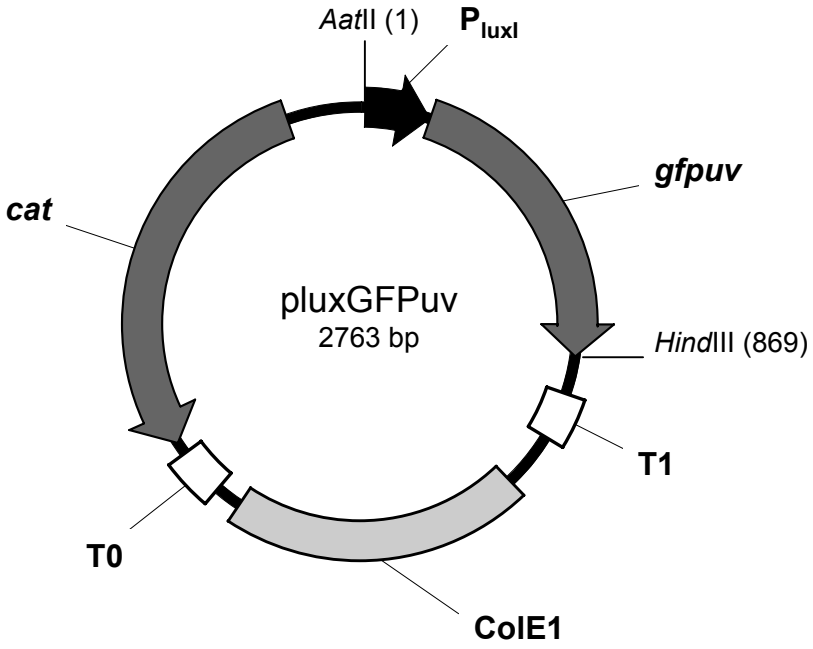
```
1   MASSEDVIKE FMRFKVRMEG SVNGHEFEIE GEGEGRPYEG TQTAKLKVTK
51  GGPLPFAWDI LSPQFQYGSK VYVKHPADIP DYKKLSFPEG FKWERVMNFE
101 DGGVVTVTQD SSLQDGSFIY KVKFIGVNFP SDGPVMQKKT MGWEASTERL
151 YPRDGVLKGE IHKALKLKDG GHYLVEFKSI YMAKKPVQLP GYYYVDSKLD
201 ITSHNEDYTI VEQYERAEGR HHLFL
```

Figure A.11. Nucleotide sequence of the pluxGFPuv reporter plasmid.

```
1    GACGTCAGTC CTTTGATTCT AATAAATTGG ATTTTTGTCA CACTATTGTA
51   TCGCTGGGAA TACAATTACT TAACATAAGC ACCTGTAGGA TCGTACAGGT
101  TTACGCAAGA AAATGGTTTG TTATAGTCGA ATAAACGCAA GGGAGGTTGG
151  TATGAGTAAA GGAGAAGAAC TTTTCACTGG AGTTGTCCCA ATTCTTGTTG
201  AATTAGATGG TGATGTTAAT GGGCACAAAT TTTCTGTCAG TGGAGAGGGT
251  GAAGGTGATG CAACATACGG AAAACTTACC CTTAAATTTA TTTGCACTAC
301  TGGAAAACTA CCTGTTCCAT GGCCAACACT TGTCACTACT TTCTCTTATG
351  GTGTTCAATG CTTTTCCCGT TATCCGGATC ATATGAAACG GCATGACTTT
401  TTCAAGAGTG CCATGCCCGA AGGTTATGTA CAGGAACGCA CTATATCTTT
451  CAAAGATGAC GGGAACTACA AGACGCGTGC TGAAGTCAAG TTTGAAGGTG
501  ATACCCTTGT TAATCGTATC GAGTTAAAAG GTATTGATTT TAAAGAAGAT
551  GGAAACATTC TCGGACACAA ACTCGAGTAC AACTATAACT CACACAATGT
601  ATACATCACG GCAGACAAAC AAAAGAATGG AATCAAAGCT AACTTCAAAA
651  TTCGCCACAA CATTGAAGAT GGATCCGTTC AACTAGCAGA CCATTATCAA
701  CAAAATACTC CAATTGGCGA TGGCCCTGTC CTTTTACCAG ACAACCATTA
751  CCTGTCGACA CAATCTGCCC TTTCGAAAGA TCCCAACGAA AAGCGTGACC
801  ACATGGTCCT TCTTGAGTTT GTAACTGCTG CTGGGATTAC ACATGGCATG
851  GATGAGCTCT ACAAATAAAA GCTTGGATCC CTGCAGGCCT CAGGGCCCGA
901  TCGATGCGGC CGCTTAATTA ATTAATCTAG AGGCATCAAA TAAAACGAAA
951  GGCTCAGTCG AAAGACTGGG CCTTTCGTTT TATCTGTTGT TTGTCGGTGA
1001 ACGCTCTCCT GAGTAGGACA AATCCGCCGC CCTAGACCTA GGCGTTCGGC
1051 TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA
1101 GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA
1151 GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC
1201 GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA
1251 AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT
1301 CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT
1351 TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT
1401 CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC
1451 CCCCGTTCAG CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT
1501 CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC
1551 AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG
1601 GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC
1651 TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC
1701 AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT
1751 TACGCGCAGA AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG
1801 GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG
1851 ACTAGTGCTT GGATTCTCAC CAATAAAAAA CGCCCGGCGG CAACCGAGCG
1901 TTCTGAACAA ATCCAGATGG AGTTCTGAGG TCATTACTGG ATCTATCAAC
1951 AGGAGTCCAA GCGAGCTCGA TATCAAATTA CGCCCCGCCC TGCCACTCAT
2001 CGCAGTACTG TTGTAATTCA TTAAGCATTC TGCCGACATG GAAGCCATCA
2051 CAGACGGCAT GATGAACCTG AATCGCCAGC GGCATCAGCA CCTTGTCGCC
```

```
2101 TTGCGTATAA TATTTGCCCA TGGTGAAAAC GGGGGCGAAG AAGTTGTCCA
2151 TATTGGCCAC GTTTAAATCA AAACTGGTGA AACTCACCCA GGGATTGGCT
2201 GAGACGAAAA ACATATTCTC AATAAACCCT TTAGGGAAAT AGGCCAGGTT
2251 TTCACCGTAA CACGCCACAT CTTGCGAATA TATGTGTAGA AACTGCCGGA
2301 AATCGTCGTG GTATTCACTC CAGAGCGATG AAAACGTTTC AGTTTGCTCA
2351 TGGAAAACGG TGTAACAAGG GTGAACACTA TCCCATATCA CCAGCTCACC
2401 GTCTTTCATT GCCATACGAA ATTCCGGATG AGCATTCATC AGGCGGGCAA
2451 GAATGTGAAT AAAGGCCGGA TAAAACTTGT GCTTATTTTT CTTTACGGTC
2501 TTTAAAAAGG CCGTAATATC CAGCTGAACG GTCTGGTTAT AGGTACATTG
2551 AGCAACTGAC TGAAATGCCT CAAAATGTTC TTTACGATGC CATTGGGATA
2601 TATCAACGGT GGTATATCCA GTGATTTTTT TCTCCATTTT AGCTTCCTTA
2651 GCTCCTGAAA ATCTCGATAA CTCAAAAAAT ACGCCCGGTA GTGATCTTAT
2701 TTCATTATGG TGAAAGTTGG AACCTCTTAC GTGCCGATCA ACGTCTCATT
2751 TTCGCCAGAT ATC
```

Figure A.12. Plasmid map of the pluxGFPuv reporter plasmid.



C. Sequences of plasmids, genes and proteins comprising the dual selection system for the directed evolution of LuxR.

C.1. General information

Two separate selection plasmids were constructed for ON and OFF round of selection using the dual selection system. The ON selection plasmid, pluxCAT, yields chloramphenicol resistance with LuxR-dependent transcriptional activation. Chloramphenicol acetyl-transferase (*cat*) is expressed when LuxR activates gene expression at P_{luxI}.

The OFF selection plasmid, pluxBLIP, encodes β-lactamase inhibitory protein (Bli) under the control of the *lux* promoter and β-lactamase (Bla) under the control of a constitutive promoter. Bli inactivates Bla and renders cells with active LuxR alleles sensitive to β-lactam antibiotics.

Both pluxCAT and pluxBLIP contain the ColE1 replication origin and the *bla* antibiotic resistance gene.

The pBLIPCAT plasmid encodes both *bli* and *cat* under the control of the $P_{lac/ara-1}$ promoter. In DH5α, both the positive and negative selection elements are constitutively expressed, such that cells are always resistant to chloramphenicol and sensitive to

carbenicillin. This plasmid confers an "always ON" phenotype and was used in the characterization of the dual selection system.

Figure A.13. lists nucleotide sequence of the genes used in the dual selection system

Figure A.14. lists the amino acid sequences encoded by the *cat*, *bla* and *bli* genes.

Figure A.15. lists the nucleotide sequence of the positive (ON) selection plasmid, pluxCAT, containing the wild-type P_{luxI} promoter.

Figure A.16. shows the plasmid map of the positive (ON) selection plasmid, pluxCAT, containing the wild-type P_{luxI} promoter.

Figure A.17. lists the nucleotide sequence of the negative (OFF) selection plasmid, pluxBLIP, containing the wild-type P_{luxI} promoter.

Figure A.18. shows the plasmid map of the negative (OFF) selection plasmid, pluxBLIP, containing the wild-type P_{luxI} promoter.

Figure A.19. lists the nucleotide sequence of pBLIPCAT.

Figure A.20. shows the plasmid map of pBLIPCAT.

Figure A.13. Nucleotide sequence of the genes used in the dual selection system.

cat

```
1    ATGGAGAAAA AAATCACTGG ATATACCACC GTTGATATAT CCCAATGGCA
51   TCGTAAAGAA CATTTTGAGG CATTTCAGTC AGTTGCTCAA TGTACCTATA
101  ACCAGACCGT TCAGCTGGAT ATTACGGCCT TTTTAAAGAC CGTAAAGAAA
151  AATAAGCACA AGTTTTATCC GGCCTTTATT CACATTCTTG CCCGCCTGAT
201  GAATGCTCAT CCGGAATTTC GTATGGCAAT GAAAGACGGT GAGCTGGTGA
251  TATGGGATAG TGTTCACCCT TGTTACACCG TTTTCCATGA GCAAACTGAA
301  ACGTTTTCAT CGCTCTGGAG TGAATACCAC GACGATTTCC GGCAGTTTCT
351  ACACATATAT TCGCAAGATG TGGCGTGTTA CGGTGAAAAC CTGGCCTATT
401  TCCCTAAAGG GTTTATTGAG AATATGTTTT TCGTCTCAGC CAATCCCTGG
451  GTGAGTTTCA CCAGTTTTGA TTTAAACGTG GCCAATATGG ACAACTTCTT
501  CGCCCCCGTT TTCACCATGG GCAAATATTA TACGCAAGGC GACAAGGTGC
551  TGATGCCGCT GGCGATTCAG GTTCATCATG CCGTCTGTGA TGGCTTCCAT
601  GTCGGCAGAA TGCTTAATGA ATTACAACAG TACTGCGATG AGTGGCAGGG
651  CGGGGCGTAA
```

bla

```
1    ATGAGTATTC AACATTTCCG TGTCGCCCTT ATTCCCTTTT TTGCGGCATT
51   TTGCCTTCCT GTTTTTGCTC ACCCAGAAAC GCTGGTGAAA GTAAAAGATG
101  CTGAAGATCA GTTGGGTGCA CGAGTGGGTT ACATCGAACT GGATCTCAAC
151  AGCGGTAAGA TCCTTGAGAG TTTTCGCCCC GAAGAACGTT TTCCAATGAT
201  GAGCACTTTT AAAGTTCTGC TATGTGGCGC GGTATTATCC CGTATTGACG
251  CCGGGCAAGA GCAACTCGGT CGCCGCATAC ACTATTCTCA GAATGACTTG
301  GTTGAGTACT CACCAGTCAC AGAAAAGCAT CTTACGGATG GCATGACAGT
351  AAGAGAATTA TGCAGTGCTG CCATAACCAT GAGTGATAAC ACTGCGGCCA
401  ACTTACTTCT GACAACGATC GGAGGACCGA AGGAGCTAAC CGCTTTTTTG
451  CACAACATGG GGGATCATGT AACTCGCCTT GATCGTTGGG AACCGGAGCT
501  GAATGAAGCC ATACCAAACG ACGAGCGTGA CACCACGATG CCTGTAGCAA
551  TGGCAACAAC GTTGCGCAAA CTATTAACTG GCGAACTACT TACTCTAGCT
601  TCCCGGCAAC AATTAATAGA CTGGATGGAG GCGGATAAAG TTGCAGGACC
651  ACTTCTGCGC TCGGCCCTTC CGGCTGGCTG GTTTATTGCT GATAAATCTG
701  GAGCCGGTGA GCGTGGGTCT CGCGGTATCA TTGCAGCACT GGGGCCAGAT
751  GGTAAGCCCT CCCGTATCGT AGTTATCTAC ACGACGGGGA GTCAGGCAAC
801  TATGGATGAA CGAAATAGAC AGATCGCTGA GATAGGTGCC TCACTGATTA
851  AGCATTGGTA A
```

bli

```
1    ATGCTTTTAT ATAAAATGTG TGACAATCAA AATTATGGGG TTACTTACAT
51   GAAGTTTTTA TTGGCATTTT CGCTTTTAAT ACCATCCGTG GTTTTTGCAA
101  GTAGTGCAGG TGTTATGACA GGAGCAAAAT TCACGCAGAT CCAGTTTGGT
151  ATGACACGTC AGCAGGTCCT CGACATAGCA GGTGCTGAGA ACTGTGAGAC
201  TGGTGGATCG TTCGGTGACA GCATCCATTG TCGTGGACAT GCAGCAGGAG
251  ACTATTATGC ATACGCAACC TTCGGCTTCA CCAGCGCAGC TGCAGACGCA
301  AAGGTGGATT CGAAAAGCCA GGAAAAACTG CTTGCACCAA GCGCACCAAC
351  TCTTACTCTT GCTAAGTTCA ACCAAGTCAC TGTTGGTATG ACTAGAGCAC
401  AAGTACTTGC TACCGTCGGA CAGGGTTCTT GTACCACTTG GAGTGAGTAC
451  TATCCAGCAT ATCCATCGAC GGCAGGAGTG ACTCTCAGCC TGTCCTGCTT
501  CGATGTGGAC GGTTACTCGT CGACGGGGTT CTACCGAGGC TCGGCGCACC
551  TCTGGTTCAC GGACGGGGTG CTTCAGGGCA AGCGGCAGTG GGACCTTGTA
```

Figure A.14. Amino acid sequence of the proteins used in the dual selection system.

CAT

```
1    MEKKITGYTT VDISQWHRKE HFEAFQSVAQ CTYNQTVQLD ITAFLKTVKK
51   NKHKFYPAFI HILARLMNAH PEFRMAMKDG ELVIWDSVHP CYTVFHEQTE
101  TFSSLWSEYH DDFRQFLHIY SQDVACYGEN LAYFPKGFIE NMFFVSANPW
151  VSFTSFDLNV ANMDNFFAPV FTMGKYYTQG DKVLMPLAIQ VHHAVCDGFH
201  VGRMLNELQQ YCDEWQGGA
```

Bla

```
1    MSIQHFRVAL IPFFAAFCLP VFAHPETLVK VKDAEDQLGA RVGYIELDLN
51   SGKILESFRP EERFPMMSTF KVLLCGAVLS RIDAGQEQLG RRIHYSQNDL
101  VEYSPVTEKH LTDGMTVREL CSAAITMSDN TAANLLLTTI GGPKELTAFL
151  HNMGDHVTRL DRWEPELNEA IPNDERDTTM PVAMATTLRK LLTGELLTLA
201  SRQQLIDWME ADKVAGPLLR SALPAGWFIA DKSGAGERGS RGIIAALGPD
251  GKPSRIVVIY TTGSQATMDE RNRQIAEIGA SLIKHW
```

Bli

```
1    MLLYKMCDNQ NYGVTYMKFL LAFSLLIPSV VFASSAGVMT GAKFTQIQFG
51   MTRQQVLDIA GAENCETGGS FGDSIHCRGH AAGDYYAYAT FGFTSAAADA
101  KVDSKSQEKL LAPSAPTLTL AKFNQVTVGM TRAQVLATVG QGSCTTWSEY
151  YPAYPSTAGV TLSLSCFDVD GYSSTGFYRG SAHLWFTDGV LQGKRQWDLV
```

Figure A.15. Nucleotide sequence of the positive (ON) selection plasmid, pluxCAT, containing the wild-type P_{luxI} promoter.

```
1    GACGTCAGTC CTTTGATTCT AATAAATTGG ATTTTTGTCA CACTATTGTA
51   TCGCTGGGAA TACAATTACT TAACATAAGC ACCTGTAGGA TCGTACAGGT
101  TTACGCAAGA AAATGGTTTG TTATAGTCGA ATAAACGCAA GGGAGGTTGG
151  TATGGAGAAA AAAATCACTG GATATACCAC CGTTGATATA TCCCAATGGC
201  ATCGTAAAGA ACATTTTGAG GCATTTCAGT CAGTTGCTCA ATGTACCTAT
251  AACCAGACCG TTCAGCTGGA TATTACGGCC TTTTTAAAGA CCGTAAAGAA
301  AAATAAGCAC AAGTTTTATC CGGCCTTTAT TCACATTCTT GCCCGCCTGA
351  TGAATGCTCA TCCGGAATTT CGTATGGCAA TGAAAGACGG TGAGCTGGTG
401  ATATGGGATA GTGTTCACCC TTGTTACACC GTTTTCCATG AGCAAACTGA
451  AACGTTTTCA TCGCTCTGGA GTGAATACCA CGACGATTTC CGGCAGTTTC
501  TACACATATA TTCGCAAGAT GTGGCGTGTT ACGGTGAAAA CCTGGCCTAT
551  TTCCCTAAAG GGTTTATTGA GAATATGTTT TTCGTCTCAG CCAATCCCTG
601  GGTGAGTTTC ACCAGTTTTG ATTTAAACGT GGCCAATATG GACAACTTCT
651  TCGCCCCCGT TTTCACCATG GGCAAATATT ATACGCAAGG CGACAAGGTG
701  CTGATGCCGC TGGCGATTCA GGTTCATCAT GCCGTCTGTG ATGGCTTCCA
751  TGTCGGCAGA ATGCTTAATG AATTACAACA GTACTGCGAT GAGTGGCAGG
801  GCGGGGCGTA ATTTGATATC GAGCTCGCTT GGAAGCTTGG ATCCCTGCAG
851  GCCTCAGGGC CCGATCGATG CGGCCGCTTA ATTAATTAAT CTAGAGGCAT
901  CAAATAAAAC GAAAGGCTCA GTCGAAAGAC TGGGCCTTTC GTTTTATCTG
951  TTGTTTGTCG GTGAACGCTC TCCTGAGTAG GACAAATCCG CCGCCCTAGA
1001 CCTAGGCGTT CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA
1051 TACGGTTATC CACAGAATCA GGGGATAACG CAGGAAAGAA CATGTGAGCA
1101 AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT
1151 TTTCCATAGG CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA
1201 GTCAGAGGTG GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC
1251 CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG
1301 ATACCTGTCC GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT TCTCAATGCT
1351 CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC
1401 TGTGTGCACG AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA
1451 CTATCGTCTT GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG
1501 CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA
1551 GAGTTCTTGA AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT
1601 TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA
1651 GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT
1701 TGCAAGCAGC AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT
1751 GATCTTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG
1801 GGATTTTGGT CATGACTAGT GCTTGGATTC TCACCAATAA AAAACGCCCG
1851 GCGGCAACCG AGCGTTCTGA ACAAATCCAG ATGGAGTTCT GAGGTCATTA
1901 CTGGATCTAT CAACAGGAGT CCAAGCGAGC TCAAACTTGG TCTGACAGTT
1951 ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG TCTATTTCGT
2001 TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA
2051 GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT
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2101 CACCGGCTCC AGATTTATCA GCAATAAACC AGCCAGCCGG AAGGGCCGAG
2151 CGCAGAAGTG GTCCTGCAAC TTTATCCGCC TCCATCCAGT CTATTAATTG
2201 TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC AGTTAATAGT TTGCGCAACG
2251 TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG
2301 GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC
2351 CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA
2401 GAAGTAAGTT GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT
2451 AATTCTCTTA CTGTCATGCC ATCCGTAAGA TGCTTTTCTG TGACTGGTGA
2501 GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA CCGAGTTGCT
2551 CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA
2601 AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT
2651 CTTACCGCTG TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT
2701 GATCTTCAGC ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA
2751 GGAAGGCAAA ATGCCGCAAA AAAGGGAATA AGGGCGACAC GGAAATGTTG
2801 AATACTCATA CTCTTCCTTT TTCAATATTA TTGAAGCATT TATCAGGGTT
2851 ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA
2901 ATAGGGGTTC CGCGCACATT TCCCCGAAAA GTGCCACCT
```

Note: plux$_{G5A}$CAT, a version of this plasmid containing the *lux$_{G5A}$* box (see A.2.2 for sequence information), was utilized for the directed evolution of DNA-binding specificity by LuxR.

Figure A.16. Plasmid map of the pluxCAT selection plasmid containing the wild-type P_{luxI} promoter.

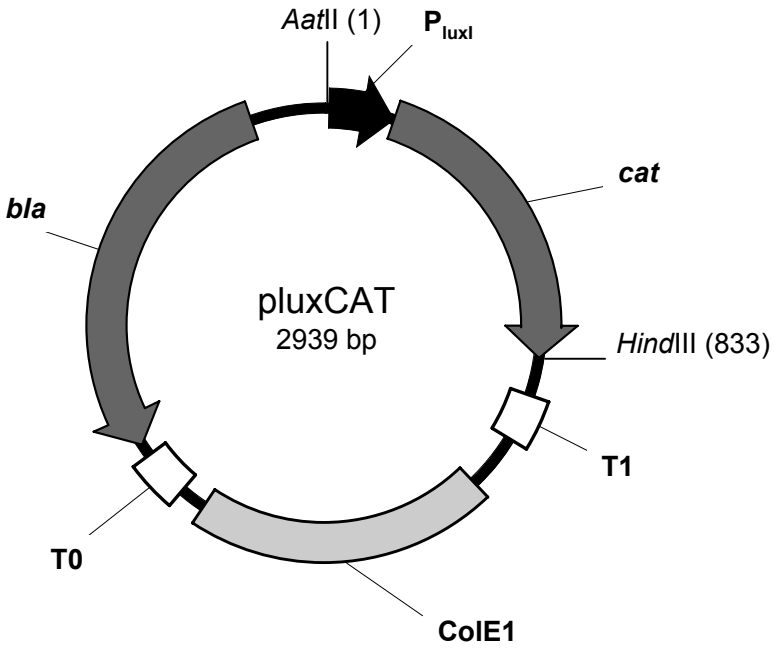

Figure A.17. Nucleotide sequence of the negative (OFF) selection plasmid, pluxBLIP, containing the wild-type P_{luxI} promoter.

```
1    GACGTCAGTC CTTTGATTCT AATAAATTGG ATTTTTGTCA CACTATTGTA
51   TCGCTGGGAA TACAATTACT TAACATAAGC ACCTGTAGGA TCGTACAGGT
101  TTACGCAAGA AAATGGTTTG TTATAGTCGA ATAAACGCAA GGGAGGTTGG
151  TATGCTTTTA TATAAAATGT GTGACAATCA AAATTATGGG GTTACTTACA
201  TGAAGTTTTT ATTGGCATTT TCGCTTTTAA TACCATCCGT GGTTTTTGCA
251  AGTAGTGCAG GTGTTATGAC AGGAGCAAAA TTCACGCAGA TCCAGTTTGG
301  TATGACACGT CAGCAGGTCC TCGACATAGC AGGTGCTGAG AACTGTGAGA
351  CTGGTGGATC GTTCGGTGAC AGCATCCATT GTCGTGGACA TGCAGCAGGA
401  GACTATTATG CATACGCAAC CTTCGGCTTC ACCAGCGCAG CTGCAGACGC
451  AAAGGTGGAT TCGAAAAGCC AGGAAAAACT GCTTGCACCA AGCGCACCAA
501  CTCTTACTCT TGCTAAGTTC AACCAAGTCA CTGTTGGTAT GACTAGAGCA
551  CAAGTACTTG CTACCGTCGG ACAGGGTTCT TGTACCACTT GGAGTGAGTA
601  CTATCCAGCA TATCCATCGA CGGCAGGAGT GACTCTCAGC CTGTCCTGCT
651  TCGATGTGGA CGGTTACTCG TCGACGGGGT TCTACCGAGG CTCGGCGCAC
701  CTCTGGTTCA CGGACGGGGT GCTTCAGGGC AAGCGGCAGT GGGACCTTGT
751  ATAAAAGCTT GGATCCCTGC AGGCCTCAGG GCCCGATCGA TGCGGCCGCT
801  TAATTAATTA ATCTAGAGGC ATCAAATAAA ACGAAAGGCT CAGTCGAAAG
851  ACTGGGCCTT TCGTTTTATC TGTTGTTTGT CGGTGAACGC TCTCCTGAGT
901  AGGACAAATC CGCCGCCCTA GACCTAGGCG TTCGGCTGCG GCGAGCGGTA
951  TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA
1001 CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA
1051 AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC CCCTGACGAG
1101 CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT
1151 ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG
1201 TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA
1251 AGCGTGGCGC TTTCTCAATG CTCACGCTGT AGGTATCTCA GTTCGGTGTA
1301 GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG
1351 ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA
1401 CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC
1451 GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG
1501 GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT
1551 ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC
1601 TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA
1651 AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG
1701 TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGACTA GTGCTTGGAT
1751 TCTCACCAAT AAAAAACGCC CGGCGGCAAC CGAGCGTTCT GAACAAATCC
1801 AGATGGAGTT CTGAGGTCAT TACTGGATCT ATCAACAGGA GTCCAAGCGA
1851 GCTCAAACTT GGTCTGACAG TTACCAATGC TTAATCAGTG AGGCACCTAT
1901 CTCAGCGATC TGTCTATTTC GTTCATCCAT AGTTGCCTGA CTCCCCGTCG
1951 TGTAGATAAC TACGATACGG GAGGGCTTAC CATCTGGCCC CAGTGCTGCA
2001 ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT CAGCAATAAA
2051 CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA ACTTTATCCG
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2101 CCTCCATCCA GTCTATTAAT TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG
2151 CCAGTTAATA GTTTGCGCAA CGTTGTTGCC ATTGCTACAG GCATCGTGGT
2201 GTCACGCTCG TCGTTTGGTA TGGCTTCATT CAGCTCCGGT TCCCAACGAT
2251 CAAGGCGAGT TACATGATCC CCCATGTTGT GCAAAAAAGC GGTTAGCTCC
2301 TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG TGTTATCACT
2351 CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG CCATCCGTAA
2401 GATGCTTTTC TGTGACTGGT GAGTACTCAA CCAAGTCATT CTGAGAATAG
2451 TGTATGCGGC GACCGAGTTG CTCTTGCCCG GCGTCAATAC GGGATAATAC
2501 CGCGCCACAT AGCAGAACTT TAAAAGTGCT CATCATTGGA AAACGTTCTT
2551 CGGGGCGAAA ACTCTCAAGG ATCTTACCGC TGTTGAGATC CAGTTCGATG
2601 TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA CTTTCACCAG
2651 CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA AAAAAGGGAA
2701 TAAGGGCGAC ACGGAAATGT TGAATACTCA TACTCTTCCT TTTTCAATAT
2751 TATTGAAGCA TTTATCAGGG TTATTGTCTC ATGAGCGGAT ACATATTTGA
2801 ATGTATTTAG AAAAATAAAC AAATAGGGGT TCCGCGCACA TTTCCCCGAA
2851 AAGTGCCACC T
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Figure A.18. Plasmid map of the pluxBLIP selection plasmid containing the wild-type P_{luxI} promoter.

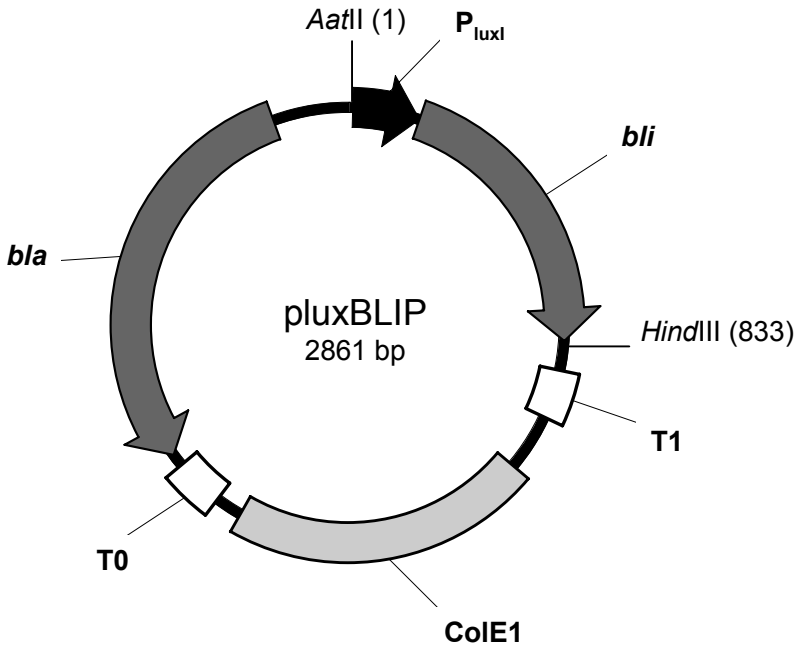

Figure A.19. Nucleotide sequence of the selection test plasmid pBLIPCAT.

```
1    GTCGACTCGA GCATAGCATT TTTATCCATA AGATTAGCGG ATCTAACCTT
51   TACAATTGTG AGCGCTCACA ATTATGATAG ATTCAATTGT GAGCGGATAA
101  CAATTTCACA CAGAATTCAT TAAAGAGGAG AAAGGTACCC ATGCTTTTAT
151  ATAAAATGTG TGACAATCAA AATTATGGGG TTACTTACAT GAAGTTTTTA
201  TTGGCATTTT CGCTTTTAAT ACCATCCGTG GTTTTTGCAA GTAGTGCAGG
251  TGTTATGACA GGAGCAAAAT TCACGCAGAT CCAGTTTGGT ATGACACGTC
301  AGCAGGTCCT CGACATAGCA GGTGCTGAGA ACTGTGAGAC TGGTGGATCG
351  TTCGGTGACA GCATCCATTG TCGTGGACAT GCAGCAGGAG ACTATTATGC
401  ATACGCAACC TTCGGCTTCA CCAGCGCAGC TGCAGACGCA AAGGTGGATT
451  CGAAAAGCCA GGAAAAACTG CTTGCACCAA GCGCACCAAC TCTTACTCTT
501  GCTAAGTTCA ACCAAGTCAC TGTTGGTATG ACTAGAGCAC AAGTACTTGC
551  TACCGTCGGA CAGGGTTCTT GTACCACTTG GAGTGAGTAC TATCCAGCAT
601  ATCCATCGAC GGCAGGAGTG ACTCTCAGCC TGTCCTGCTT CGATGTGGAC
651  GGTTACTCGT CGACGGGGTT CTACCGAGGC TCGGCGCACC TCTGGTTCAC
701  GGACGGGGTG CTTCAGGGCA AGCGGCAGTG GGACCTTGTA TAAAAGCTTG
751  CTAACCGAAT AAACGCAAGG GAGGTTGGTA TGGAGAAAAA AATCACTGGA
801  TATACCACCG TTGATATATC CCAATGGCAT CGTAAAGAAC ATTTTGAGGC
851  ATTTCAGTCA GTTGCTCAAT GTACCTATAA CCAGACCGTT CAGCTGGATA
901  TTACGGCCTT TTTAAAGACC GTAAAGAAAA ATAAGCACAA GTTTTATCCG
951  GCCTTTATTC ACATTCTTGC CCGCCTGATG AATGCTCATC CGGAATTTCG
1001 TATGGCAATG AAAGACGGTG AGCTGGTGAT ATGGGATAGT GTTCACCCTT
1051 GTTACACCGT TTTCCATGAG CAAACTGAAA CGTTTTCATC GCTCTGGAGT
1101 GAATACCACG ACGATTTCCG GCAGTTTCTA CACATATATT CGCAAGATGT
1151 GGCGTGTTAC GGTGAAAACC TGGCCTATTT CCCTAAAGGG TTTATTGAGA
1201 ATATGTTTTT CGTCTCAGCC AATCCCTGGG TGAGTTTCAC CAGTTTTGAT
1251 TTAAACGTGG CCAATATGGA CAACTTCTTC GCCCCCGTTT TCACCATGGG
1301 CAAATATTAT ACGCAAGGCG ACAAGGTGCT GATGCCGCTG GCGATTCAGG
1351 TTCATCATGC CGTCTGTGAT GGCTTCCATG TCGGCAGAAT GCTTAATGAA
1401 TTACAACAGT ACTGCGATGA GTGGCAGGGC GGGGCGTAAT TTGATATCGA
1451 GCTCGCTGGA TCCCTGCAGG CCTCAGGGCC CGATCGATGC GGCCGCTTAA
1501 TTAATTAATC TAGAGGCATC AAATAAAACG AAAGGCTCAG TCGAAAGACT
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1751 GTGAGAGGGC CGCGGCAAAG CCGTTTTTCC ATAGGCTCCG CCCCCCTGAC
1801 AAGCATCACG AAATCTGACG CTCAAATCAG TGGTGGCGAA ACCCGACAGG
1851 ACTATAAAGA TACCAGGCGT TTCCCCCTGG CGGCTCCCTC GTGCGCTCTC
1901 CTGTTCCTGC CTTTCGGTTT ACCGGTGTCA TTCCGCTGTT ATGGCCGCGT
1951 TTGTCTCATT CCACGCCTGA CACTCAGTTC CGGGTAGGCA GTTCGCTCCA
2001 AGCTGGACTG TATGCACGAA CCCCCCGTTC AGTCCGACCG CTGCGCCTTA
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2101 ACTGGCAGCA GCCACTGGTA ATTGATTTAG AGGAGTTAGT CTTGAAGTCA
2151 TGCGCCGGTT AAGGCTAAAC TGAAAGGACA AGTTTTGGTG ACTGCGCTCC
2201 TCCAAGCCAG TTACCTCGGT TCAAAGAGTT GGTAGCTCAG AGAACCTTCG
2251 AAAAACCGCC CTGCAAGGCG GTTTTTTCGT TTTCAGAGCA AGAGATTACG
2301 CGCAGACCAA AACGATCTCA AGAAGATCAT CTTATTAATC AGATAAAATA
2351 TTACTAGATT TCAGTGCAAT TTATCTCTTC AAATGTAGCA CCTGAAGTCA
2401 GCCCCATACG ATATAAGTTG TTACTAGTGC TTGGATTCTC ACCAATAAAA
2451 AACGCCCGGC GGCAACCGAG CGTTCTGAAC AAATCCAGAT GGAGTTCTGA
2501 GGTCATTACT GGATCTATCA ACAGGAGTCC AAGCGAGCTC TCGAACCCCA
2551 GAGTCCCGCT CAGAAGAACT CGTCAAGAAG GCGATAGAAG GCGATGCGCT
2601 GCGAATCGGG AGCGGCGATA CCGTAAAGCA CGAGGAAGCG GTCAGCCCAT
2651 TCGCCGCCAA GCTCTTCAGC AATATCACGG GTAGCCAACG CTATGTCCTG
2701 ATAGCGGTCC GCCACACCCA GCCGGCCACA GTCGATGAAT CCAGAAAAGC
2751 GGCCATTTTC CACCATGATA TTCGGCAAGC AGGCATCGCC ATGGGTCACG
2801 ACGAGATCCT CGCCGTCGGG CATGCGCGCC TTGAGCCTGG CGAACAGTTC
2851 GGCTGGCGCG AGCCCCTGAT GCTCTTCGTC CAGATCATCC TGATCGACAA
2901 GACCGGCTTC CATCCGAGTA CGTGCTCGCT CGATGCGATG TTTCGCTTGG
2951 TGGTCGAATG GGCAGGTAGC CGGATCAAGC GTATGCAGCC GCCGCATTGC
3001 ATCAGCCATG ATGGATACTT TCTCGGCAGG AGCAAGGTGA GATGACAGGA
3051 GATCCTGCCC CGGCACTTCG CCCAATAGCA GCCAGTCCCT TCCCGCTTCA
3101 GTGACAACGT CGAGCACAGC TGCGCAAGGA ACGCCCGTCG TGGCCAGCCA
3151 CGATAGCCGC GCTGCCTCGT CCTGCAGTTC ATTCAGGGCA CCGGACAGGT
3201 CGGTCTTGAC AAAAAGAACC GGGCGCCCCT GCGCTGACAG CCGGAACACG
3251 GCGGCATCAG AGCAGCCGAT TGTCTGTTGT GCCCAGTCAT AGCCGAATAG
3301 CCTCTCCACC CAAGCGGCCG GAGAACCTGC GTGCAATCCA TCTTGTTCAA
3351 TCATGCGAAA CGATCCTCAT CCTGTCTCTT GATCAGATCT TGATCCCCTG
3401 CGCCATCAGA TCCTTGGCGG CAAGAAAGCC ATCCAGTTTA CTTTGCAGGG
3451 CTTCCCAACC TTACCAGAGG GCGCCCCAGC TGGCAATTCC GACGTCTGTG
3501 TGGAATTGTG AGCGGATAAC AATTTCACAC AGGGCCCTCG GACACCGAGG
3551 AGAATGTCAA GAGGCGAACA CACAACGTCT TGGAGCGCCA GAGGAGGAAC
3601 GAGCTAAAAC GGAGCTTTTT TGCCCTGCGT GACCAGATCC CGGAGTTGGA
3651 AAACAATGAA AAGGCCCCCA AGGTAGTTAT CCTTAAAAAA GCCACAGCAT
3701 ACATCCTGTC CGTCCAAGCA GAGGAGCAAA AGCTCATTTC TGAAGAGGAC
3751 TTGTTGCGGA AACGACGAGA ACAGTTGAAA CACAAACTTG AACAGCTACG
3801 GAACTCTTGT GCGTAAGGAA AAGTAAGGAA AACGATTCCT TCTAACAGAA
3851 ATGTCCTGAG CAATCACCTA TGAACT

Figure A.20. Plasmid map of the selection test plasmid pBLIPCAT.