Transcriptional Control during Quorum Sensing by LuxR and LuxR Homologues

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ABSTRACT

Quorum sensing is a mechanism used by many proteobacteria to regulate expression of target genes in a population-dependent manner. The quorum sensing system of *Vibrio fischeri* activates the luminescence (*lux*) operon when the autoinducer signaling molecule (N-3-oxohexanoyl homoserine lactone) is recognized and bound by the activator protein LuxR. LuxR subsequently binds to the *lux* box centered at –42.5 bp upstream of the transcription initiation site and activates transcription from the *lux* operon promoter, resulting in the emission of light at high cell densities. LuxR consists of 250 amino acids arranged into an N-terminal (regulatory) domain and a C-terminal (activation) domain, and is thought to function as an ambidextrous activator capable of making multiple contacts with the alpha and sigma subunits of RNA polymerase (RNAP). Published work describing the results of alanine scanning mutagenesis performed on the C-terminal domain of LuxR (residues 190-250) has identified residues (K198, W201 and I206) that appear to play a role in positive control of transcription initiation. Additional mutagenesis of residues 180-189 has been undertaken via a three-primer or four-primer PCR-based method in this study. Variants of LuxR were screened for their ability to activate luciferase production and to repress transcription from an artificial promoter, and production of full-length LuxR was measured, in an attempt to identify additional positive control variants. No additional positive control variants were found in this study. Work has also been undertaken to identify intergenic suppressors between positive control variants of LuxR and the RNAP alpha subunit, RpoA. Starting with a recombinant *Escherichia coli* strain encoding the *lux* operon and LuxR variant

I206E, a random chemical mutagenesis was performed on a vector encoding RpoA. Following transformation of the mutated plasmids encoding RpoA, high throughput luminescence assays were used to identify isolates with phenotypes brighter than the control. Isolation of an intergenic suppressor will confirm the existence of protein-protein interactions between LuxR and RpoA within the transcription initiation complex. The ability of other LuxR family members to establish productive protein-protein interactions with RNAP necessary for transcription initiation was also examined. LuxR homologues EsaR of *Pantoea stewarti* ssp. *stewartii*, a repressor of known function, and ExpR of *Erwinia carotovora* subsp. *carotovora* were also analyzed for their ability to activate the *lux* operon, as well as to repress transcription from an artificial promoter containing the *lux* box.

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My parents have always encouraged me and guided me to independence, never trying to limit my aspirations. I am grateful to them and amazed at their generosity. I couldn't have done it without you! Most importantly I thank my fiancé David O'Brien, who has shown untiring patience and support, reminding me of my priorities and keeping things in perspective. I look forward to our new life as we start it together. You are my everything!

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CHAPTER ONE
LITERATURE REVIEW

Quorum Sensing:

Many bacteria have the ability to regulate expression of specific target genes in a cell population-dependent manner. This mechanism of gene regulation is termed quorum sensing and is seen in Gram-positive as well as in Gram-negative bacteria. This process depends on signaling molecules produced by the cells. In Gram-negative bacteria, these signals are acyl-homoserine lactones and are commonly termed autoinducers. Upon reaching a threshold at a critical concentration, the autoinducer is able to stimulate the activation and/or repression of target genes (reviews: Miller and Bassler, 2001; Fuqua, et al., 2001; Withers et al., 2001).

In the Gram-negative marine bacterium, *Vibrio fischeri*, the quorum sensing model has been extensively studied using the luminescence or the *lux* operon and its ability to produce light (Nealson et al, 1970). *V. fischeri* is capable of free-living in seawater, as well as living in symbiotic relationships within the light organs of the Hawaiian squid, *Euprymna scolopes,* and with other fishes (Visick et al., 2000). In order for the system to work, the *Vibrio fischeri* autoinducer, produced by the autoinducer synthase (LuxI), and the autoinducer receptor protein, LuxR (an activator), form a complex. It has been proven in *V. fischeri* that the autoinducers can freely diffuse across membranes (Kaplan and Greenberg, 1985). At a high concentration, the autoinducer-LuxR complex binds to the promoter region at the *lux* box of the *lux* operon, and activates transcription, (Figure 1). Transcriptional activation of the operon leads to the

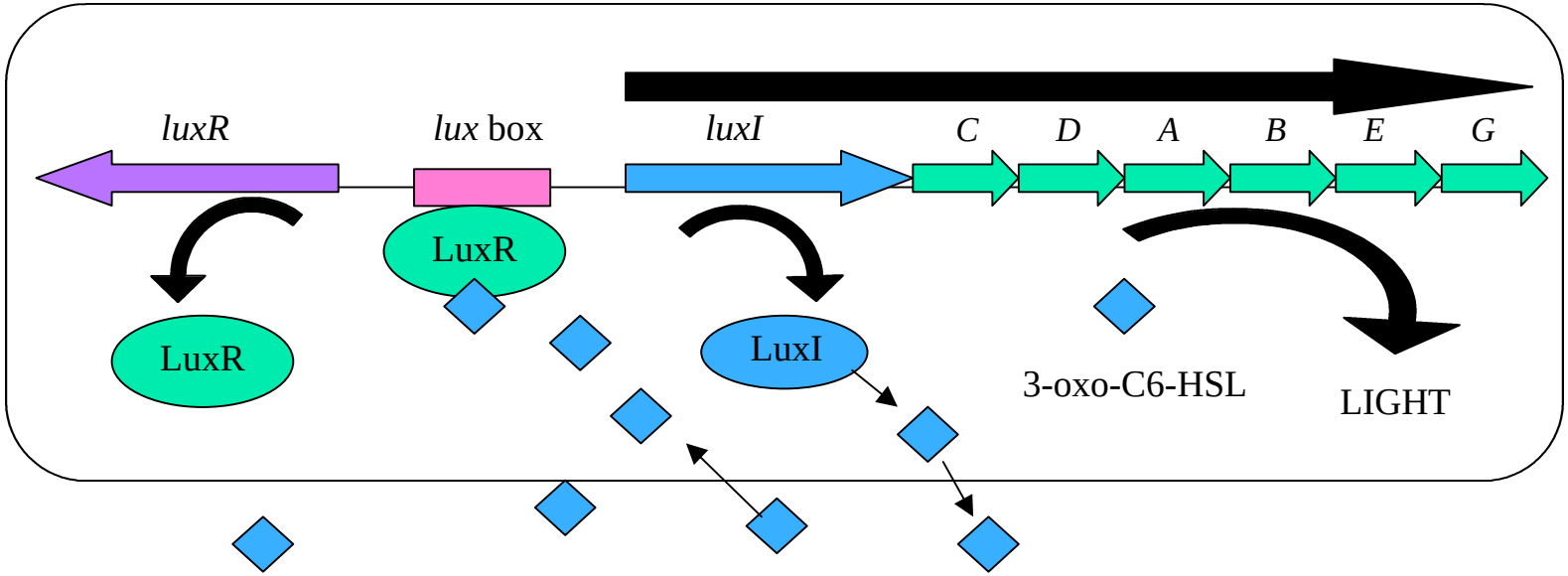

Figure 1: Model of quorum sensing in *Vibrio fischeri* at high cell densities
At high cell densities, a critical concentration of autoinducer (blue diamonds) is reached. When complexes of LuxR and autoinducer form they bind at the lux box, and together with RNAP, activate transcription thereby producing bioluminescence.

production of proteins Lux I, C, D, A, B, E and G. LuxC, D, and E are involved in the synthesis of the aldehyde substrate for luciferase; LuxA and B are the α and β subunits of the luciferase enzyme, and LuxG has no known function corresponding to bioluminescence. A positive feedback loop occurs as the LuxI protein is produced; LuxI synthesizes the autoinducer, N-3-oxohexanoyl homoserine lactone (3-oxo-C6-HSL). As the *V. fischeri* autoinducer (VAI) accumulates, the molecules form complexes with LuxR, the transcriptional activator of the system, which binds at the *lux* box and increases light production accordingly (Fuqua et al., 1996; Eberl et al. 1999; Withers et al., 2001). Thus, activation of this operon occurs only at high cell density. Within the light organ crypts, a high cell density is reached and activation of the operon is possible (Nyholm and McFall-Ngai, 1998). However, in the low nutrient environment of the open ocean, the low cell density of *V. fischeri* cells prevents activation of the *lux* operon. At low cell densities, there is always a basal level of autoinducer being produced, but the signal molecules do not reach a sufficient concentration to efficiently form complexes with LuxR. Transcription of the *lux* operon is not activated, and bioluminescence does not occur. This autoinduction system allows *V. fischeri* to chemically communicate and respond to changing environmental conditions. Whether the cells are in a high cell density/nutrient rich environment or in a low density/nutrient free-living state, *V. fischeri* cells have the capacity to make this distinction and either expend the energy needed for bioluminescence or conserve the energy for other essential functions (McFall-Ngai and Ruby, 1991; Ruby and McFall-Ngai, 1992). This type of communication is seen in other

bacteria, where a measurement of self population-density is important to their survival. However, *V. fischeri* remains the best understood quorum sensing model system.

LuxR: The Activator Protein

Studies of *luxR* mutations in recombinant *Escherichia coli* have suggested that LuxR is a two-domain polypeptide consisting of 250 amino acids (Figure 2; reviewed in Stevens and Greenberg, 1999). The domains are defined as the N-terminal regulatory domain (NTD) and the C-terminal activation domain (CTD). The NTD functions to bind autoinducer, and modulates the ability of the CTD to bind to the DNA. Between residues 116 and 160 of the CTD, multimerization is thought to occur as LuxR is hypothesized to function as a homodimer (Choi & Greenberg, 1992). Between residues 200 and 224, a helix-turn-helix (HTH) motif is seen which was the first indication that the CTD played a role in DNA binding.

In order for transcriptional activation to occur, LuxR must make specific protein-protein contacts with RNA polymerase, as well as the autoinducer, as it is bound to the *lux* box. The *lux* box is a 20 base-pair (bp) region with dyad symmetry, centered at the -42.5 position from the *luxI* transcriptional start site and is important for transcriptional regulation of *luxR* and *luxI*, (Figure 3). When LuxR is bound to the *lux* box, it is positioned to potentially make contacts with the RNA polymerase on both its proximal and distal surfaces (Figure 4). To investigate which specific amino acid residues of LuxR are required for making such contacts with the RNA polymerase and activating transcription, site-directed alanine mutagenesis was previously performed (Egland and

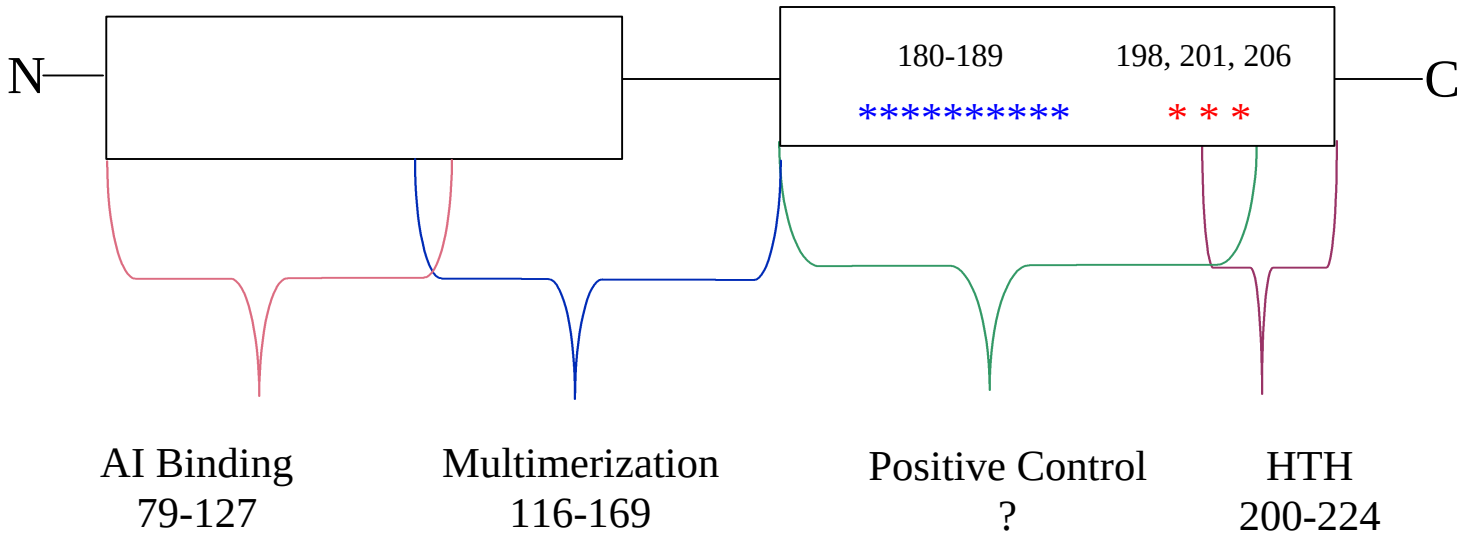

Figure 2: Cartoon Model of the Two Domain Structure of LuxR

LuxR is a 250 amino acid polypeptide. The N-terminal domain functions in autoinducer binding and it modulates the activity of the C-terminal domain. The C-terminal domain functions in DNA binding/positive control

* = LuxR variants with alanine substitutions at residues 198, 201, 206 proved to be positive control mutants (capable of binding to the DNA, but not in activating transcription) (Egland and Greenberg, 2001).

* = The role of LuxR residues 180-189 were analyzed in the current study Constructs were generated via a PCR-based site directed mutagenesis protocol.

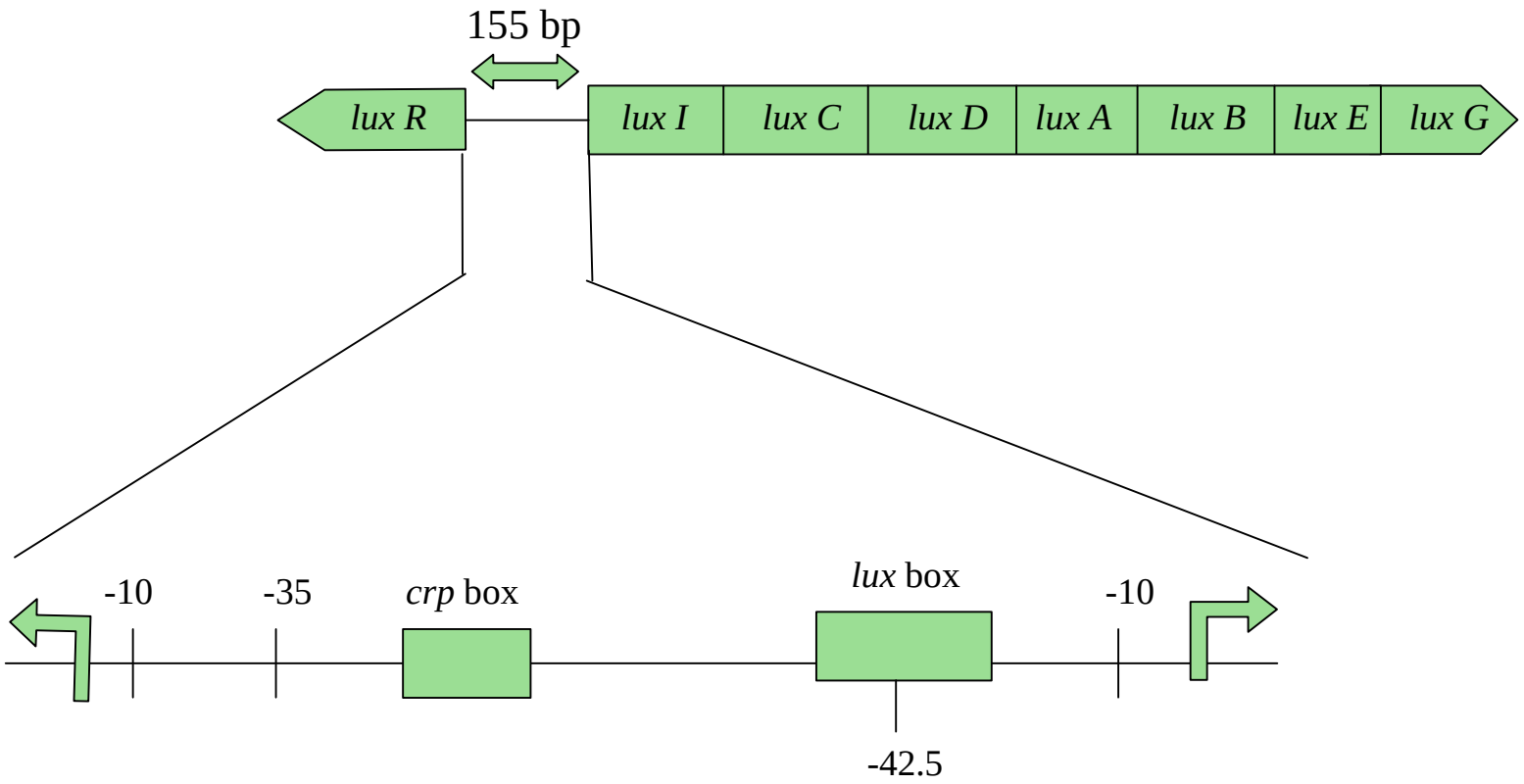

Figure 3: Arrangement of the *lux* operon genes
Based on Figure 1 Stevens et al., 1994. See text for details.

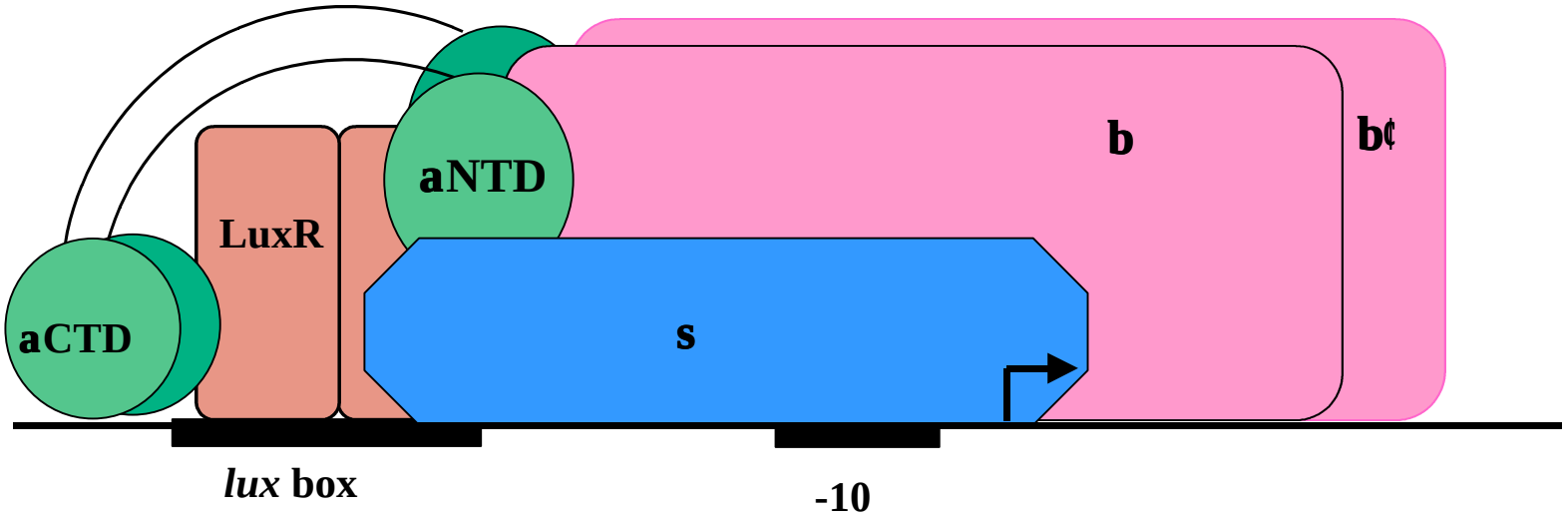

Figure 4: Cartoon model of the five subunit structure of *Escherichia coli* RNA polymerase (RNAP) holoenzyme and its interaction with LuxR bound to the DNA at the *luxI* promoter of the lux operon
(See text for details).

Greenberg, 2001; Trott and Stevens, 2001). These studies resulted in the generation and analysis of LuxR alanine substitution variants from amino acids 190-250. Originally, it had been hypothesized that the extreme C-terminal 40 residues of LuxR were involved in positive control of transcription activation (Choi and Greenberg, 1991). However, none of the variants between 210 and 250 were found to be involved in activation of transcription without affecting the ability of LuxR to bind at the *lux* box (Trott and Stevens, 2001). This phenotype demonstrated that the C-terminal amino acids of LuxR are not involved in transcriptional activation, but are involved in positioning the HTH for DNA binding. Conversely, of the variants generated between residues 190 and 224, three were found to be involved in transcriptional activation and were termed positive control variants (Egland and Greenberg, 2001). The positive control mutants, (198A, 201A, and 206A), have the ability to bind *lux* regulatory DNA but fail to activate transcription of the *lux* operon. These specific residues are hypothesized to be essential for making contacts with the RNA polymerase and facilitating the initiation of transcription (Figure 4).

Other activator proteins, homologous to LuxR, such as NarL and TraR, have been crystallized and their structures analyzed. In alignment with NarL, LuxR is seen to have sequence similarities of the C-terminal domain, or activation domain. Exposure studies of surface areas in specific NarL residues have been conducted and the accessibility to the solvent has been determined (Baikalov et al., 1996). Positive control variants of LuxR, (Egland and Greenberg, 2000), correlate to some of these exposed residues in the eighth α helix as well as areas between this helix and the seventh helix of the C-terminal domain of NarL (Figure 5). It is possible that other LuxR amino acid residues,

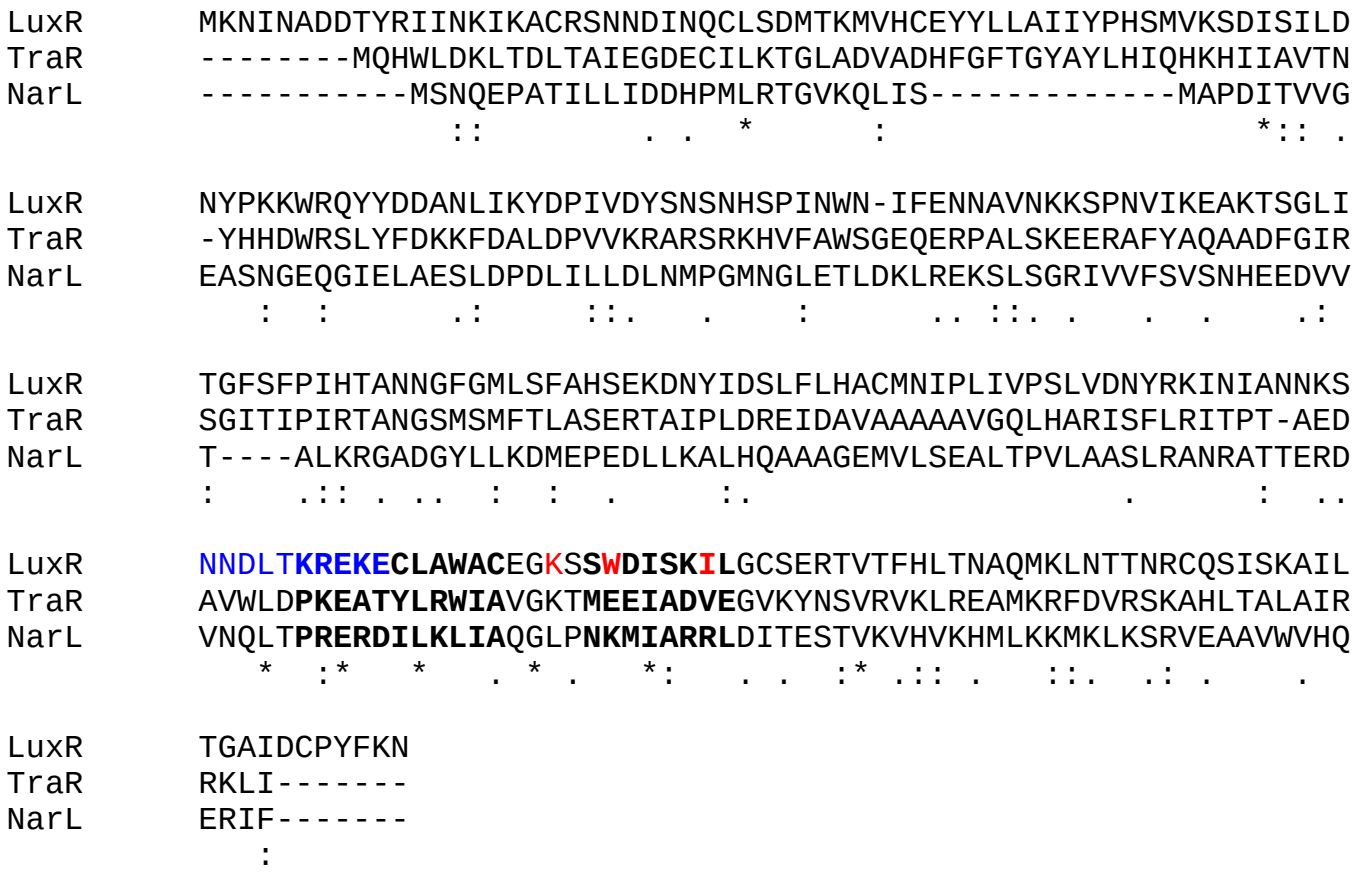

Figure 5: Amino Acid Sequence Alignment between LuxR and LuxR homologues TraR and NarL

***** indicates single, fully conserved residue
: indicates strong conservation
. indicates weak conservation
indicates no conservation

180A -189A (LuxR alanine substitution variants)
198A, 201A, 206A (LuxR positive control mutants)
Amino acid residues in bold: seventh and eighth surface-exposed helices of NarL

corresponding to those that have their surface areas exposed in NarL when substituted, could also yield positive control variants for transcriptional activation during quorum sensing in *V. fischeri*. This hypothesis has been investigated during this thesis project (Chapter 2).

Interestingly, two positive control variants in TraR have been identified. Unlike the residues found in LuxR, they are located in the N-terminal domain (Luo and Farrand, 1999). Though TraR is in the family of LuxR transcriptional activators, and has sequence similarities present throughout the protein, it nonetheless appears to function differently in some regards. An N-terminus deletion of LuxR still allows for activation of the *lux* operon (Choi and Greenberg, 1991; Stevens et al., 1994) while in TraR this deletion prevents activation of its corresponding operon (S. Winans, Personal Communication). Therefore, it seems unlikely that positive control variants in the NTD of TraR could correspond to any in LuxR. However positive control variants of LuxR map to a surface-exposed region on the crystal structure of TraR (Zhang et al., 2002).

LuxR Homologues:

Quorum sensing systems homologous to the LuxI-LuxR system of *V. fischeri* have been identified and studied extensively in other Gram-negative bacteria where they regulate various cellular processes such as plasmid conjugal transfer, motility, biofilm formation, antibiotic biosynthesis, and virulence factor production in plant and animal pathogens (reviews: Williams et al., 2000; Vannini et al., 2002; Miller and Bassler,

2001). For years, *V. fischeri* has served as the model for such systems of quorum sensing.

In *Pseudomonas aeruginosa*, quorum sensing is used to regulate means to invade and overtake a complex eukaryotic host (Parsek and Greenberg, 2000). The virulence of *P. aeruginosa* stems from its production of extracellular virulence factors such as exotoxins and proteases. The activation of the virulence genes is due to two quorum sensing systems homologous to the LuxI-LuxR system, called the Las and Rhl systems (Fuqua et al., 1994; Beatson et al. 2002). The autoinducer of the Las system is N-(3-oxododecanoyl)-homoserine lactone; it has a longer acyl side chain on the molecule than does the primary autoinducer of *V. fischeri.* This autoinducer forms complexes with LasR and regulates transcription of *lasI*, *rhlR*, and four other genes encoding key virulence factors (Beatson, et al., 2002). RhlR, complexed with its cognate autoinducer, N-butyryl-L-HSL regulates transcription of *rhlI*, *rhlA*, *lasB*, and *phzA* (additional key virulence factors). Even within these regulatory systems of quorum sensing, a hierarchy regulating virulence factors exists with the Las system being upstream of the Rhl system.

As *P. aeruginosa* invades a host, it is to its advantage to wait until the bacterial community is at a high cell density to express the virulence genes. The bacteria may evade the host's immune system until they are at an overpowering density, to only then attack when they are at their strongest, so as to defeat the host (Greenberg, 1997). Quorum sensing is activated when *P. aeruginosa* forms microcolonies and biofilms. By using quorum sensing as a way to monitor the army's strength, *P. aeruginosa* will only express its virulence genes when there is a high cell density; this eliminates the chance

for the host to detect the bacteria and overcome the infection. This system of invasion can be found in many bacteria (Koiv and Mäe, 2001; Mäe et al., 2001; Whitehead, et al. 2001).

Another example of this type of regulation during pathogenesis is seen in the quorum sensing system of *Pantoea stewarti* ssp. *stewartii* (*P. stewartii*). A *P. stewartii* plant infection causes Stewart's wilt disease in sweetcorn and leaf blight in maize (Pataky et al., 2000). Unlike LuxR, the transcriptional regulator of this system, EsaR, is thought to function as a repressor, instead of an activator, controlling capsular polysaccharide synthesis (CPS) through quorum sensing (Minogue, et al., 2002; Miller and Bassler, 2001). A high level production of CPS blocks the corn xylem vessels and hence causes necrotic lesions. The disease symptoms are thus a result of the function of the quorum sensing LuxR/LuxI homologues, EsaR and EsaI. EsaI, the autoinducer synthase of the system, produces the diffusible signal N-(3-oxo-hexanoyl)-L-homoserine lactone, which is identical to the *V. fischeri* autoinducer. A mutated or nonfunctional signal synthase leads to a loss in signal production, as well as a loss in CPS synthesis and bacterial virulence. In contrast, a mutated *esaR* gene results in an excessive production of CPS independent of the levels of signal present (von Bodman et al., 1998). Therefore, EsaR acts as a repressor, and only derepresses the system in the presence of inducing levels of signal.

A *lux* box-like palindromic sequence, called the *esaR* box, coincides with the putative -10 element of the *esaR* promoter. This suggests a negative autoregulatory function for EsaR since in the absence of an inducing level of signal, EsaR will repress

the *esaR* gene and prevent transcription. In contrast, LuxR functions as an activator when it associates with its cognate autoinducer, 3-oxohexanoyl HSL and thus binds at the *lux* box, which is -42 bp upstream from the transcriptional start site and recruits RNA polymerase to the promoter.

In the presence of inducing levels of signal, EsaR will bind the signal and hence derepress the system, leading to CPS synthesis. Derepressed *esaR* strains produce CPS constitutively at low cell densities, and are significantly less virulent than when compared to a wild-type parent (von Bodman et al., 1998). This phenotype suggests that the quorum sensing system of *P. stewartii* may work to delay the expression of CPS during the early stages of infection, and therefore reduce the interference with other mechanisms of pathogenesis (von Bodman et al., 1998).

A previous study demonstrated that the LuxR homologue, LasR from *Pseudomonas aeruginosa*, is able to recognize, bind and activate transcription of the *lux* operon from the palindromic *lux* box (Gray et al., 1994) while in the presence of its cognate autoinducer signal. In the presence of 3-oxohexanoyl HSL, LuxR is capable of weakly activating the *lasB* gene that encodes for elastase by recognizing, and binding the 20 bp palindrome upstream of *lasB*. Both regulatory proteins, LuxR and LasR, were unable to activate transcription from *lasB* and *luxR* respectively, without their cognate autoinducer present; the heterologous autoinducer was not recognized. The palindromic sequence reported upstream from *lasB* shows sequence identity at 10 of the 17 conserved sites of the *V. fischeri* sequences (Gray et al., 1994). It is predicted that bacteria possessing autoinducible genes might contain similar palindromes at the appropriate

positions for transcriptional activation/repression and hence may be recognized by other bacteria capable of quorum sensing (Welch et al., 2000). A consensus sequence for comparing these cis-acting regulatory elements based on DNA sequences from *V. fischeri*, *P. aeruginosa*, *Agrobacterium tumefaciens*, among others has been deduced (Gray et al., 1994).

Interestingly, the palindromic sequences found upstream from *luxR* (Egland and Greenberg, 2000) and *esaR* (Minogue et al., 2002) are similar. In the current study (Chapter 3), EsaR was tested to determine if it could recognize and bind the *lux* box, and in so doing, activate transcription from the luminescence operon. In *P. stewartii*, EsaR allows for derepression, and hence transcriptional activation solely in the presence of its cognate autoinducer. To more directly measure potential function as an activator, *E. coli* λ reporter strains were used to determine/analyze the ability of EsaR to increase the rate of transcription of the *lux* operon promoter and to bind to the DNA and repress transcription at an artificial promoter (Personal communication M. Urbanowski). Another LuxR homologue, ExpR, negatively regulates extracellular enzyme production in *Erwinia carotovora* ssp. *carotovora* (*E. carotovora*) and has significant amino acid homology to EsaR (Andersson et al., 2000) and LuxR (Figure 6). This LuxR homologue was also included in the activation/ binding analysis along with EsaR.

Unlike LuxR, ExpR is convergently transcribed from its quorum sensing counterpart, ExpI, and does not transcriptionally regulate ExpI. The expression of virulence determinants, such as plant cell wall-degrading enzymes, in *Erwinia carotovora* ssp. *carotovora* depends on a critical concentration of autoinducer. In *E. carotovora*, this

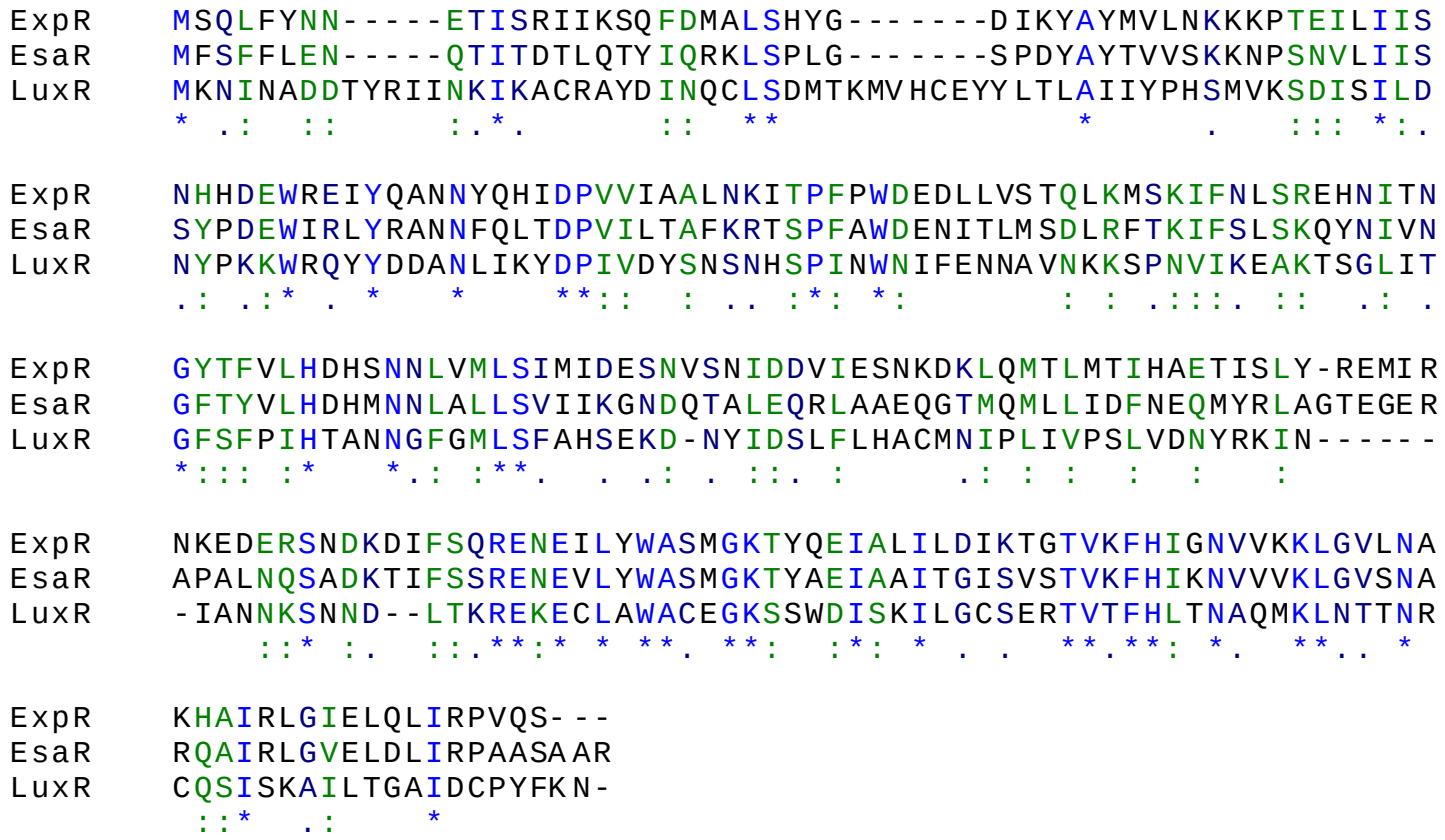

Figure 6: Amino Acid Sequence Alignment between LuxR and LuxR homologues EsaR and ExpR

Consensus key:

* - single, fully conserved residue

: - conservation of strong groups

. - conservation of weak groups

- no consensus

signal molecule is N-(3-oxohexanoyl)-L-homoserine lactone (Jones et al., 1993; Pirhonen, et al., 1993), which is identical to that of *V. fischeri,* and *P. stewartii.* In the absence of autoinducer, or without the ability to synthesize/express autoinducer as in the case of *E. carotovora expI* mutants, these strains are avirulent (Jones et al., 1993). Thus, quorum sensing systems found in *E. carotovora* and in *P. stewartii,* regulated by the proteins ExpR/ExpI and EsaR/EsaI respectively, are additional examples of microbial pathogens that regulate virulence determinants via quorum sensing.

Work described in this thesis examines questions related to the mechanism of transcriptional activation used during quorum sensing. In Chapter 2, the effects of alanine substitution mutations in the CTD of the activator protein LuxR were analyzed. The ability of these variant forms of LuxR to activate transcription of the *lux* operon at mid-exponential log phase was determined in the presence and absence of autoinducer. The interactions between LuxR and RNAP as they bind in complex at the *lux* box and initiate transcription was also analyzed in an intergenic suppression study. By examining the ability of positive control variants of LuxR to activate the *lux* operon in the presence of mutated forms of the RNAP alpha subunit, putative intergenic suppressor mutations were discovered. Future analysis of the nature of the genetic change in the suppressing forms of alpha may reveal essential amino acid interactions between RNAP and LuxR at the *lux* box. In Chapter 3, the ability of LuxR homologues EsaR and ExpR to recognize/bind the *lux* box, and activate transcription from the *lux* operon was examined. Though these proteins function differently from LuxR, their capacity to recognize and activate transcription from a non-native promoter in the presence and absence of

autoinducer was determined. This analysis has increased our understanding of the versatility and flexibility of the LuxR family of proteins to function as activators of transcription. Finally, Chapter 4 focuses on contributions made to the *Fischeri* Intrinsic Annotation Team (FIAT) of Integrated Genomics, Inc. (Chicago, IL) in a multi-lab effort to annotate the *Vibrio fischeri* genome. These efforts will lead to a better understanding of functions encoded in the genome that are necessary for establishment of a successful symbiosis between *V. fischeri* and its eukaryotic host and will also permit a complete analysis of the regulon controlled by LuxR during quorum sensing.

CHAPTER 2

Amino Acid Residues Involved in Protein-Protein Interactions Between RNA Polymerase and the Quorum Sensing Activator LuxR

INTRODUCTION:

To investigate which specific amino acid residues of LuxR are required for making contacts with RNA polymerase that lead to an activation of transcription initiation, alanine scanning site-directed mutagenesis was performed on LuxR. Each amino acid starting at residue 189 and moving upstream, until amino acid 180, toward the N-terminus of the CTD was separately mutated. Alanine-substituted LuxR variants were subsequently tested for their ability to activate transcription at the *lux* operon, bind at the *lux* box and produce the LuxR protein in an effort to identify additional positive control mutants.

Phenotypes of the previously identified LuxR positive control variants 198A, 201A, and 206A, revealed that these variants were unable to interact with *E. coli* RNAP in the appropriate manner to activate transcription of the *lux* operon (Egland and Greenberg, 2001). The specific amino acid residues of RNAP contacting these positive control residues of LuxR have not been elucidated. An intergenic suppressor study was undertaken to answer this question. Amino acid residue 206 was changed to glutamate, giving this residue an overall negative charge, rather than being nonpolar as the original amino acid, isoleucine. A dramatic change in overall charge may allow for a compensatory substitution to form in the RNAP α or σ subunits, hence reversing the dark phenotype of I206E, and allowing for the detection of a bioluminescent phenotype. The

α subunit of RNAP was chosen for this mutagenesis based on genetic evidence that specific, single amino acids were essential to initiate LuxR-dependent transcription (Finney et al., 2002). The σ subunit was not initially chosen since prior research involving this RNAP subunit did not give evidence of individual residues in σ being essential for LuxR-dependent transcription (D. Johnson and A. Stevens, Personal Communication).

MATERIALS AND METHODS

PCR-Three Primer Method:

A three primer method of PCR (Michael, 1994) was employed for the alanine scanning site-directed mutagenesis of LuxR. Mutagenic (internal) primers (Sigma-Genosys, The Woodlands, TX) were designed to change one specific amino acid to alanine, and to add/delete a restriction endonuclease site for screening purposes (Table 1). External primers (Sigma-Genosys) flanking the target sequence were also designed (Table 2). Mutagenic primers (Table 1) for creating LuxR amino acids E189A, E187A, and R186A, and I206E (for the intergenic suppression analysis) were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) before their addition to the PCR reactions. Components of the 30 µL phosphorylation reaction included 250 pmol primer, 1X T4 polynucleotide kinase buffer, 1.7 mM ATP, and 10 units T4 polynucleotide kinase. The reaction mixture was incubated at 37°C for 30 minutes, heat inactivated at 65°C for 20 minutes, then the entire phosphorylation mixture was added to the master mixture for the 100 µL PCR reaction. All other mutagenic primers subsequently used have been 5' phosphorylated during synthesis by the manufacturer (Sigma-Genosys) and added to the PCR reaction at a concentration of 3 µM.

The PCR master mixture contained the following final concentrations of reagents: 2 µM XBA200 primer (Sigma-Genosys), 2 µM PVU200 primer (Sigma-Genosys),

Table 1: Mutagenic Primers for *luxR* 5' → 3'

LuxR Amino Acid Residues	Sequence[a]	Restriction Site Change
180AB	CGAAAAATAAATATTGCAAATAATAAATCA**GCA**AACGATTTAAC	Adds *Ssp*I site
180AC	GTTAAATCGTT**TGC**TGATTTATTATTTGCAATATTTATTTTTCG	Adds *Ssp*I site
181AB	CGAAAAATAAATATTGCAAATAATAAATCAAAC**GCA**GATTTAAC	Adds *Ssp*I site
181AC	GTTAAATC**TGC**GTTTGATTTATTATTTGCAATATTTATTTTTCG	Adds *Ssp*I site
182AB	CGAAAAATAAATATTGCAAATAATAAATCAAACAAC**GCT**TTAAC	Adds *Ssp*I site
182AC	GTTAA**AGC**GTTGTTTGATTTATTATTTGCAATATTTATTTTTCG	Adds *Ssp*I site
183AB	CGAAAAATAAATATTGCAAATAATAAATCAAACAACGAT**GCA**AC	Adds *Ssp*I site
183AC	GT**TGC**ATCGTTGTTTGATTTATTATTTGCAATATTTATTTTTCG	Adds *Ssp*I site
184AB	TAAATATTGCAAATAATAAATCAAACAACGATTTA**GCA**AAAAG	Adds *Ssp*I site
184AC	CTTTT**TGC**TAAATCGTTGTTTGATTTATTATTTGC	----------
185AB	ATATTGCAAATAATAAATCAAACAACGATTTAACC**GCA**AGAG	Adds *Ssp*I site
185AC	CTCT**TGC**GGTTAAATCGTTGTTTGATTTATTATTTGC	----------
186A	CAAA**GCA**GAAAAAGAATGTTTAGCGTGGGCATGTG	Loses *Sph*I site
187A	CAAAAGA**GCA**AAAGAATGTTTAGCGTGGGCATGTG	Loses *Sph*I site
188AB	CAAAAGAGAA**GCA**GAATGTTTAGCGTGGGCATGTGAAG	Loses *Sph*I site
188AC	CTTCACATGCCCACGCTAAACATTC**TGC**TTCTCTTTTG	Loses *Sph*I site
189A	AAA**GCA**TGTTTAGCGTGGGCATGTG	Loses *Sph*I site
206E	GGGATATTTCGAAA**GA**ATTAGGCTGCAGTGAG	Adds *BstB*I site

a. Bold indicates changes leading to incorporation of alanine (or glutamate for 206E), underline indicates restriction site changes

Table 2: Amplification primers for *luxR*

XBA200	5'-CGTATAATGTGTGGAATTGTGAGCG
PVU200	5'-GAAGTGGTCCTGCAACTTTATCC

Table 3: Sequencing Primers for *luxR*

SEQINT2	5'-ATGTAATTAAAGAAGCGAAAAC
SEQINT	5'-GTTGTCTTTTTCTGAATGTGC
SEQPRO	5'-GTATGGCTGTGCAGGTCGTAAATC
SEQVEC	5'-GCTGAAAATCTTCTCTCATCC

Table 4: Sequencing Primers for *rpoA*

RLG1412	5'-CATTGCGTTCACGTCGTTGCTC
MAF1486	5'-CTAATAGACGCGTTCTCATCGGTC
MAF1487	5'-GTCGAAATCGTCAAGCCGCAGCACG
RLG1489	5'-CGTCGTGCGGCAACCATTCTG
MAF1490	5'-GGCTGACGTACATCACGTAAGTCAAG

0.2 mM dNTPs (Promega, Madison, WI), 2.5 units *Taq*2000 Polymerase (Stratagene, La Jolla, CA), 0.7 X *Taq*2000 reaction buffer (Stratagene), 40 units *Taq* DNA Ligase (New England Biolabs), 2 mM $MgSO_4$ (Fisher, Springfield, NJ), and 100 ng of linearized pSC300 template (Figure 7) (Choi and Greenberg, 1991). The template DNA, pSC300, was prepared from *E. coli* using the QIAprep miniprep kit (Qiagen, Valencia, CA) or by the alkaline-lysis miniprep procedure (Sambrook et al., 1989). Purified template was linearized with the restriction endonuclease *Pvu*II according to the manufacturer's directions (New England Biolabs). A Sprint thermal cycler (Hybaid, Middlesex, UK) program was used. The program for all PCR reactions was one cycle at 94°C for 2 minutes; 30 cycles: 94°C for one minute, 45°C for one minute, and 72°C for 2 minutes; one cycle at 72°C for 10 minutes.

PCR-Four Primer Method:

The technique of Overlap-PCR (Higuchi et al., 1988; Ho et al., 1989) was used in the mutagenesis of LuxR residues F180A, F181A, D182A, L183A, T184A, K185A, and K188A. This procedure proved to be much more useful and reliable than the previously described three-primer method. Two overlapping mutagenic primers were synthesized (Sigma Genosys) for each residue (Table 1). Each mutagenic primer encoded the alanine substitution, but only one required the addition or deletion of the new restriction site for screening purposes. The external primers used and the technique of purifying the template for the PCR reaction remained unchanged from the three primer method.

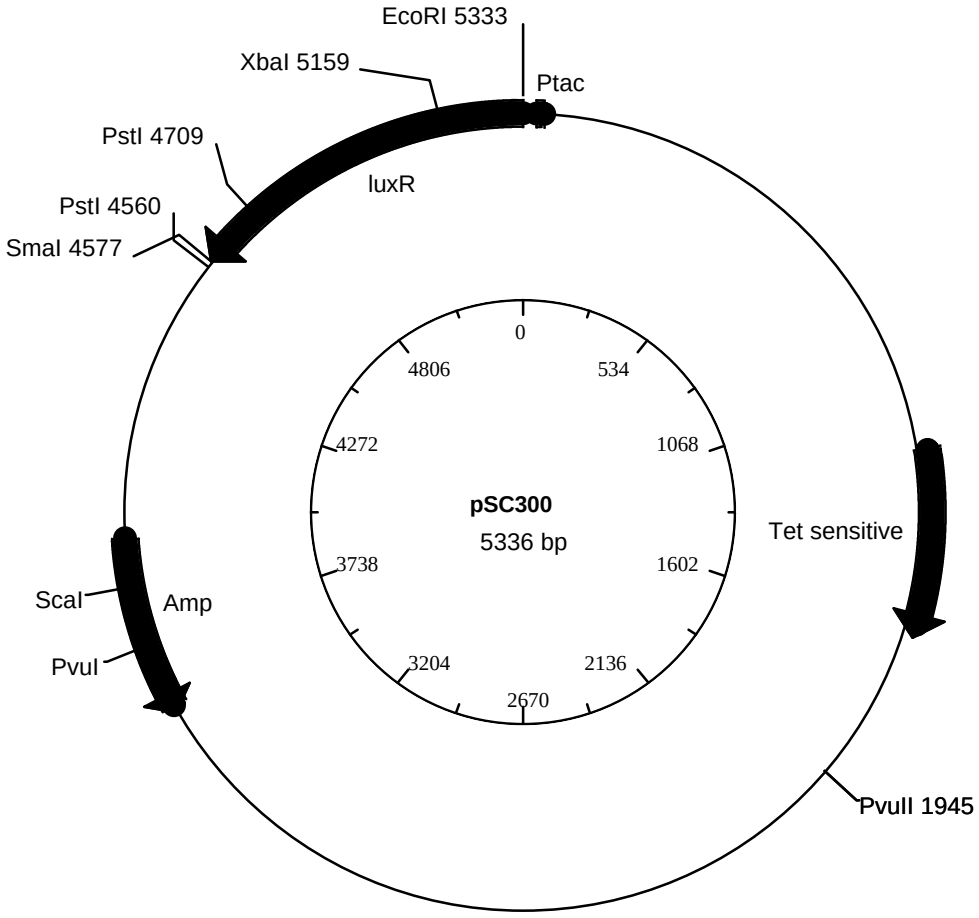

Figure 7: Map of expression vector pSC300
Relevant characteristics of pSC300 are depicted. Restriction endonuclease sites for alanine scanning site directed mutagenesis and cloning purposes are included.

After the template was linearized using either *Pvu*II or *Nde*I (New England Biolabs), two separate PCR reactions were set up each containing one external and one mutagenic primer for each residue to be mutated. The thermal cycler was programmed as follows: 94°C for 2 minutes; 30 cycles: 94°C for 30 seconds, 45°C, for one minute, and 72°C for 2 minutes; one cycle at 72°C for 10 minutes. The reaction was purified using the QIAquick PCR purification kit (Qiagen) and the concentrations determined before the second PCR reaction was set up. In the second reaction, the products from the first reactions were used as both the template and primer at a 1:1 ratio. The concentrations of the PCR products used were estimated by visualization after running the products in a 0.8% agarose gel. External primers at a concentration of 2 μM were included to gain even more full-length product. The final PCR products were again purified using a Qiagen purification kit.

Cloning:

Purified PCR products were digested with *Sma*I or *PvuI*, depending on the primers used, at room temperature for 2-4 hours, and *Xba*I (New England Biolabs) at 37°C for 2-4 hours. The expression vector, pSC300 (Choi and Greenberg, 1991) (Figure 7), was also digested with either *Sma*I or *Pvu*I, and *XbaI* using the same protocol. Digested products were analyzed by electrophoresis in a 0.8% agarose gel (BioRad, Hercules, CA). The pSC300 vector and the insert, containing the mutated DNA were individually extracted from the gel matrix and purified using the QIAquick gel extraction kit (Qiagen). Ligation reactions using T4 DNA Ligase (New England Biolabs) were

carried out at 16°C overnight as per the manufacturer's directions. Transformation into *E. coli* JM109 was achieved by heat shock treatment of the competent cells/hybrid DNA molecules in 0.1M $CaCl_2$ (J.T. Baker Chemical Co., Phillipsburg, NJ) at 37°C for 2 minutes. After incubation on ice for 5 minutes, the cells were grown in Luria-Bertani (LB) broth for one hour at 37°C and plated on LB agar medium containing 100 µg/mL ampicillin.

Transformed strains were subcultured in Luria-Bertani broth plus ampicillin (100 µg/mL), and grown overnight. Plasmid preparation was performed using the alkaline-lysis miniprep procedure. Depending on the restriction site change in the DNA sequence, the plasmids were digested, and compared to that of wild-type pSC300 cut with the same restriction endonuclease. A change in banding pattern after gel electrophoresis was taken as an indicator that the alanine mutation or in the case of I206E, the glutamate mutation, was present. Plasmid DNA from the correct constructs was prepared using the QIAprep miniprep kit (Qiagen), eluting the DNA from the column with dH_2O, followed by nucleotide sequencing at the Core DNA Sequencing Facility at Virginia's Bioinformatics Institute, Virginia Tech, Blacksburg, VA (CSF-VBI). Sequencing of the *luxR* gene and most of the promoter region of the constructs was performed to confirm the presence of the alanine or glutamate mutation and rule out any second site mutations that may have occurred during construction of the desired plasmid. The sequencing primers used in these reactions were: SEQINT2, SEQINT, SEQVEC, SEQPRO (Table 3). LuxR variants 180A-189A having the correct alanine substitutions were subsequently called the pMAF series.

Cloning LuxR Positive Control Variants 201A and 206A into Expression Vector pSC300:

Plasmids containing the LuxR positive control residues 201A and 206A (Egland and Greenberg, 2001) were digested with *Xba*I and *Pvu*I as per the manufacturer's directions. The expression vector pSC300 was digested in the same manner. Products from the restriction digests were run in a 0.8% agarose gel and the *luxR* insert band and the vector band from pSC300 were extracted, and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified DNA was then ligated and transformed into *E. coli* JM109. Transformants were selected on LB agar medium containing ampicillin (100 μg/mL).

Luminescence Assays:

Luminescence production was measured from strains of *E. coli* JM109 (Yannisch-Perron et al., 1985) transformed with pJR551 (Dunlap and Ray, 1989). Conferring chloramphenicol resistance of 30 μg/mL, plasmid pJR551 encoded the *lux* operon. A second plasmid present in the strains was pSC300, that encoded the alanine substitution variants of LuxR and conferred resistance to ampicillin at 100 μg/mL resistance gene. The racemic mixture of 3-oxohexanoyl-DL-HSL, needed for full transcriptional activation of the operon, was suspended in acidified ethyl acetate (10 μg/mL). The correct amount needed for the assays was added to an empty flask and the ethyl acetate was allowed to evaporate in the hood, leaving the dried autoinducer behind. The autoinducer was then resuspended at 200 nM in Luria-Bertani (LB) broth containing the

appropriate antibiotics. Strains were grown overnight at 30°C to an OD_{600} of 0.2-1.0, subcultured to an OD_{600} of 0.025, and subsequently grown to a final OD_{600} of 0.5 ± 0.02 (mid-exponential log phase growth). Luminescence was measured in relative light units, using a Turner 20/20 luminometer (Turner Designs, Sunnyvale, CA), over a 4-second integration period using 10 μL of culture. The luminescence assay was performed on cultures grown in duplicate with assays from each sample performed in triplicate. Cell aliquots of 0.5 mL were also pelleted from each strain; the supernatant was carefully removed and the relatively dry pellet was frozen at -80°C for use in luciferase assays and western immunoblotting.

Luminescence assays were also conducted in the absence of exogenous autoinducer to conclude if any of the alanine substitution mutations resulted in a form of LuxR that was capable of activating transcription of the *lux* operon independent of autoinducer (data not shown). The autoinducer synthase gene, *luxI*, of the *lux* operon encoded on plasmid pJR551, contains a temperature sensitive mutation rendering the enzyme nonfunctional at 31°C, therefore that was the growth temperature used for these experiments.

Luciferase Assays:

A procedure based on that of Dunlap and Greenberg (1985) was used to measure luciferase levels in cell extracts. Frozen cell pellets obtained during luminescence assays as described above were resuspended in 1mL of lysis buffer (prepared fresh) containing 10 mM KPO_4 pH 7.0, 10 mM ethylenediaminetetraacetic acid pH 8.0 (EDTA), 1 mM

dithiothreitol (DTT), 0.1% bovine serum albumin (BSA), H_2O, and 50 μg/mL lyzozyme. The resuspended pellet was frozen for one hour at -80°C, allowed to thaw at room temperature, briefly vortexed again, and then kept on ice until needed. An assay buffer was prepared at the same time as the lysis buffer, and contained identical ingredients except no EDTA or lyzozyme was added. The substrate for the luciferase assay was prepared by sonicating a 1:1000 dilution of the n-decyl aldehyde (decanal) (Sigma-Aldrich, St. Louis, Missouri) for 3 minutes, with resting periods every 30 seconds; the tube was stored on ice throughout the assay. The reduced coenzyme $FMNH_2$, was prepared from stocks of 0.5 mL (5 mM) flavin mononucleotide (FMN) that were added to 49.5 mL H_2O in an anaerobic bottle. Approximately 10 pellets of platinum-coated alumina were added to the bottle, which was then sealed with a rubber stopper. The bottle was purged of oxygen, pressurized with hydrogen gas, and shaken rapidly until the FMN was reduced, leaving the solution almost clear in color. For each reaction, 90 μL of assay buffer, 10 μL decanal, 100 μL $FMNH_2$, and 10 μL cell extract was added to a luminometer tube; luminescence was measured over a 30 second integration period with triplicate readings measured for each sample in relative light units.

DNA Binding/Repression Assays:

The effect of alanine substitutions in LuxR on DNA binding and repression at an artificial promoter construct containing the *lux* box was measured from *E. coli* JM109 strains containing p35LB10 (Egland and Greenberg, 2000) and plasmids encoding each of the pMAF encoded LuxR variants individually. The p35LB10 reporter construct

consists of a *lacZ* gene under its own promoter, however, the *lux* box has been inserted between and partially overlapping the -10 and -35 consensus sequences. If a LuxR variant binds at the *lux* box, *lacZ* expression is down-regulated. RNA polymerase has the ability to bind and initiate transcription of the *lacZ* gene only when LuxR or a variant of LuxR is not present at the *lux* box.

Strains were grown overnight at 30°C in LB broth containing the appropriate antibiotics to an OD_{600} of 0.2-1.0. The strains were subcultured into two sets (one with 200 nM 3-oxohexanoyl-DL-HSL and the other with none to an OD_{600} of 0.025 in LB broth containing the same antibiotics as the overnight cultures, and subsequently grown to a final OD_{600} of 0.5 ± 0.02 (mid-exponential log-phase growth). At this point, the cells were stored on ice. When all samples had reached an OD_{600} of 0.5, 5 μL of cells were diluted with 995 μL Z Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) with dithiothreitol (DTT) added at a concentration of 400 μM. Chloroform (50 μL) was added to the diluted cells and then the suspension was briefly vortexed to cause lysis. After lysis, 10 μL of the cell extract was added to luminometer tubes and incubated for one hour with 100 μL of room temperature reaction buffer and substrate from the chemiluminescent reporter assay kit (Tropix, Bedford, MA) mixed at a ratio of 100:1, respectively. After the one-hour incubation, 150 μL of room temperature accelerator was added to each tube with the same timing used to add the substrate. Light output was measured in the Turner 20/20 luminometer over a 4 second integration period. Strains with each variant were grown up in duplicate and assays were performed in triplicate, with light output measured in relative light units.

SDS-PAGE and Western Immunoblotting:

To confirm that the dark phenotypes of LuxR variants 183A, 184A, 186A, and 187A were due to a defect in recognition/binding of the *lux* box, and not due to the cells' inability to express the variant LuxR proteins, western immunoblotting and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Cell pellets (from the 0.5 mL luminescence assay aliquots) were removed from the -80°C freezer and allowed to thaw on the benchtop. The thawed pellets were resuspended in 100 μL of 5X sample buffer (1M Tris pH 6.8, 0.2 g SDS, 0.625 g glycerol, 0.5 mL β-mercaptoethanol, trace bromphenol blue (BPB), and boiled for three minutes prior to loading 20 μL of sample in to the 12% polyacrylamide gels. Two gels were run at the same time; one was stained for protein expression using GelCode™ Blue Stain Reagent (Pierce, Rockford, IL), while the proteins from the other polyacrylamide gel were transferred to a nitrocellulose membrane for 1 hour at 150V using The Bandit™ Tank Electroblotter (Owl Scientific, Inc., Portsmouth, NH; Model VEP-III). The nitrocellulose membrane was then treated with 1X NET buffer (150 mM NaCl, 5 mM EDTA, 54.6 mM Tris-HCl, 8 mM Tris-Base, 500 μL Triton X-100 per liter, containing 0.025% gelatin (Sigma 300 bloom) as the blocking agent, and incubated with anti-LuxR antibody (Babco 1) (Slock et al., 1990) at a working dilution of 1:1500 then incubated for one hour with gentle rocking. The 1 antibody was removed, the blot washed in fresh 1X NET buffer containing 0.025% gelatin and the horseradish peroxidase (HRP)-conjugated goat IgG fraction to rabbit IgG (ICN, Inc., Aurora, OH) was added at a 1:2000 dilution. After a two-hour incubation, the 2 antibody solution was discarded and the blot was rinsed in

excess 50 mM Tris, pH 7.5, for 15 minutes. The color development reagents (4-chloro-1-naphthol dissolved in 20 mL MeOH, along with 83 μL 3% hydrogen peroxide diluted in 50 mM Tris, pH 7.5) were then prepared fresh, mixed, and poured over the blot. The color development of the membrane was complete within 20 minutes, and a picture of the blot was captured using the Alphaimager 2000 (Alpha Innotech Corp., San Leandro, CA).

Intergenic Suppression Analysis:

Detection of intergenic suppressor mutations in *E. coli* between the α subunit of RNAP and the *luxR* variant I206E required the construction of three-plasmid strains. Competent *E. coli* JM109 cells were transformed through heat shock with pAMS129 (Stevens, et al., 1999), encoding the *lux* operon; the resulting strain was then made competent. Each strain containing the *lux* operon on pAMS129 was subsequently transformed with either the wild-type LuxR encoded on pAMS121 (Stevens, et al., 1999) or the I206E variant encoded on pSUP102 (Simon, et al., 1989), and the wild-type alpha encoded on pREIIα (Blatter et al., 1994) or the randomly, chemically mutated form of pREIIα.

The negative control strain (dark phenotype) therefore possessed genes encoding the *lux* operon (pAMS129) conferring kanamycin resistance at 100 μg/mL (Kn^{100}), the wild-type α subunit of RNAP on pREIIα conferring ampicillin resistance at 100 μg/mL (Ap^{100}), and the *luxR* variant I206E, resistant to chloramphenicol at 20 μg/mL (Cm^{20}). The positive control strain (bright phenotype) on the other hand, encodes the *lux* operon, the wild-type α subunit of RNAP, and the wild-type *luxR* gene. Test strains were

constructed by transforming the *E. coli* JM109 strain containing pAMS129 with the *luxR* variant I206E on the pSUP102 expression vector. This resulting strain was then made competent in order for the chemically mutated plasmid DNA (encoding pREIIα) to then be transformed, and subsequently tested for a renewed bright phenotype through luminescence readings.

The random, chemical mutagenesis of pREIIα was accomplished using hydroxylamine (Sigma-Aldrich). Hydroxylamine causes GC to AT transitions in the DNA and therefore, there should not be any frameshifts or large deletions as would be seen with other mutagenesis protocols. This protocol (Slock et al. 1990) needed to be optimized in order to determine the correct exposure time of the plasmid DNA to the hydroxylamine solution. Excessive exposure would be deleterious to plasmids, while an insignificant exposure time would only leave wild-type plasmids intact prohibiting any results. The optimization was accomplished by adding 35 μL of the mutagenic solution (5 mM Tris pH 6.0, 0.5 mM EDTA pH 8, and 0.5 M hydroxylamine), having a final pH of approximately 3.3, to 5 μg of pREIIα in a 35μL suspension (for a total volume of 70 μL), and incubating at 37°C.

At predetermined time points, aliquots of 5 μL were taken and added to 95 μL of 100 mM $CaCl_2$ in order to stop the reaction. After 24 hours, all of the aliquots were transformed into the experimental strain described previously and grown overnight at 37°C. A decrease in transformation efficiency from the aliquot taken at zero hours (when plasmid DNA is not mutated) up until the 24 hour time-point indicated that the mutagenesis was working properly. Plasmids mutated in the ampicillin resistance gene

or in the origin of replication would not be able to produce viable transformants. Colonies were subsequently picked and transferred to 96 well plates; each well was filled with 250 μL of LB agar containing $Kn^{100}Cm^{20}Ap^{100}$ and 1 mM IPTG (isopropylthiogalactosidase), for induction of the pTAC promoter present on the plasmid(s) encoding LuxR, and allowed to grow at 30°C for 24 hours. Luminescent readings over a 1 second integration time were taken using a Lucy microtiter dish luminometer (Anthos, Wals, Austria) after 12, 18, and 24 hours of growth. Maximum luminescence from the positive control strain was produced at 12 hours and was approximately 50,000 relative light units (RLU) as compared to the negative control, usually generating about 70 RLU.

After this optimization period, pREIIα DNA was exposed to the hydroxylamine solution for 18-24 hours at 37°C. Transformation into the experimental strain followed, the resulting colonies were picked, and then transferred to the 96 well plates. Luminescent readings were always taken after 12 hours of growth at 30°C.

Once possible suppressor mutants were discovered, quantitative luminescence assays, as previously described for testing the pMAF series of LuxR variants, were conducted, but in the presence of 200 nM 3-oxohexanoyl-DL-HSL and 1 mM IPTG for induction. Luminescent readings were recorded using a Turner 20/20 luminometer over a 4 second integration period. Positive results (luminescent phenotypes) compared to the wild-type strain containing wild-type *luxR*, pREIIα, and the *lux* operon, confirmed the luminescence readings exhibited during the high through-put screening process using the 96 well plates.

The hydroxylamine-treated pREIIα plasmids were then isolated from the three plasmid strains also containing pAMS129 and *luxR* variant 206E in pSUP102. Plasmid minipreps were performed using the QIAprep miniprep kit (Qiagen). The plasmid DNA was then serially diluted, transformed into *E. coli* JM109 competent cells, and selected for on Ap^{100} plates. Cells containing only the hydroxylamine treated pREIIα plasmids were Ap^{100} resistant but Kn^{100} and Cm^{20} sensitive. The *rpo*A sequence in the pREIIα plasmids were then sequenced at the CSF-VBI sequencing facility at Virginia Tech using primers RLG 1412, 1486, 1487, 1489, and 1490 (Table 4).

The hydroxylamine treated pREIIα plasmids, exhibiting a bright phenotype, were then transformed into *E. coli* JM109 competent cells containing pAMS121(encoding the wild-type *lux*R gene) and pAMS129 (encoding the *lux* operon) to confirm that the mutations, by themselves, would decrease light output when in the presence of wild-type LuxR. These luminescence assays were conducted as previously described using the Turner 20/20 luminometer.

RESULTS AND DISCUSSION

Identifying positive control variants involved in LuxR-dependent transcriptional activation of the *lux* operon

The effects of single alanine substitutions in LuxR were assessed by performing bioluminescence assays. These in vivo luminescence experiments were conducted using strains containing the plasmid reporter pJR551 with the pMAF series of LuxR variants. Assays were performed twice with triplicate readings for each sample. LuxR variants L183A, T184A, R186A, and E187A exhibited dark phenotypes indicating that transcriptional activation of the *lux* operon was not initiated (Figure 8). These variants may be defective in binding the *lux* box DNA. In the absence of exogenous autoinducer, LuxR variants 180A-189A did not produce any light (data not shown) and therefore none of the variants can activate transcription independent of 3-oxohexanoyl HSL.

By performing luciferase assays, it was quantitatively possible to more directly measure the enzyme production/expression from the *lux* operon. Luciferase assays were conducted in duplicate, with each sample tested in triplicate. The results from the luciferase assays confirmed those found in the luminescence assays. Variants L183A, T184A, R186A, and E187A were not able to activate the *lux* operon, and so therefore virtually no detectable luciferase enzyme was being produced (Figure 9). Although it

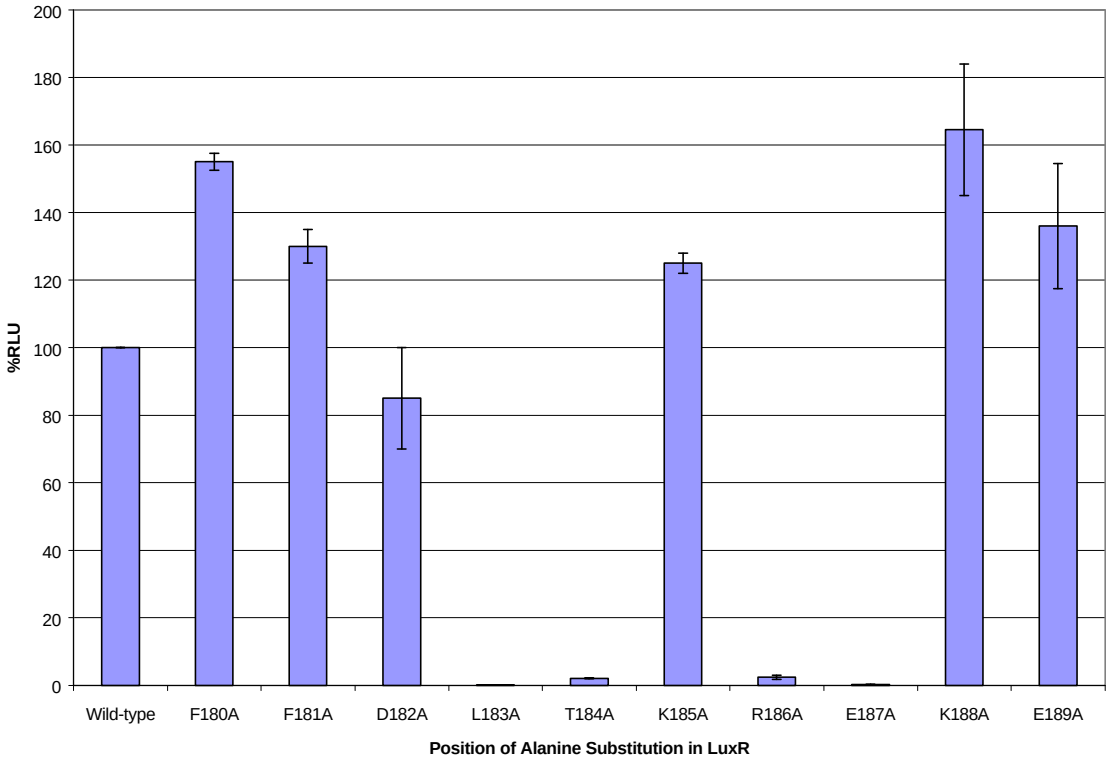

Figure 8: Luminescence Assays for LuxR Variants
The effect of alanine substitution on LuxR-dependent cellular luminescence determined in recombinant *Escherichia coli* in the presence of 3-oxohexanoyl HSL. Average value for 100%= 3950 RLU.

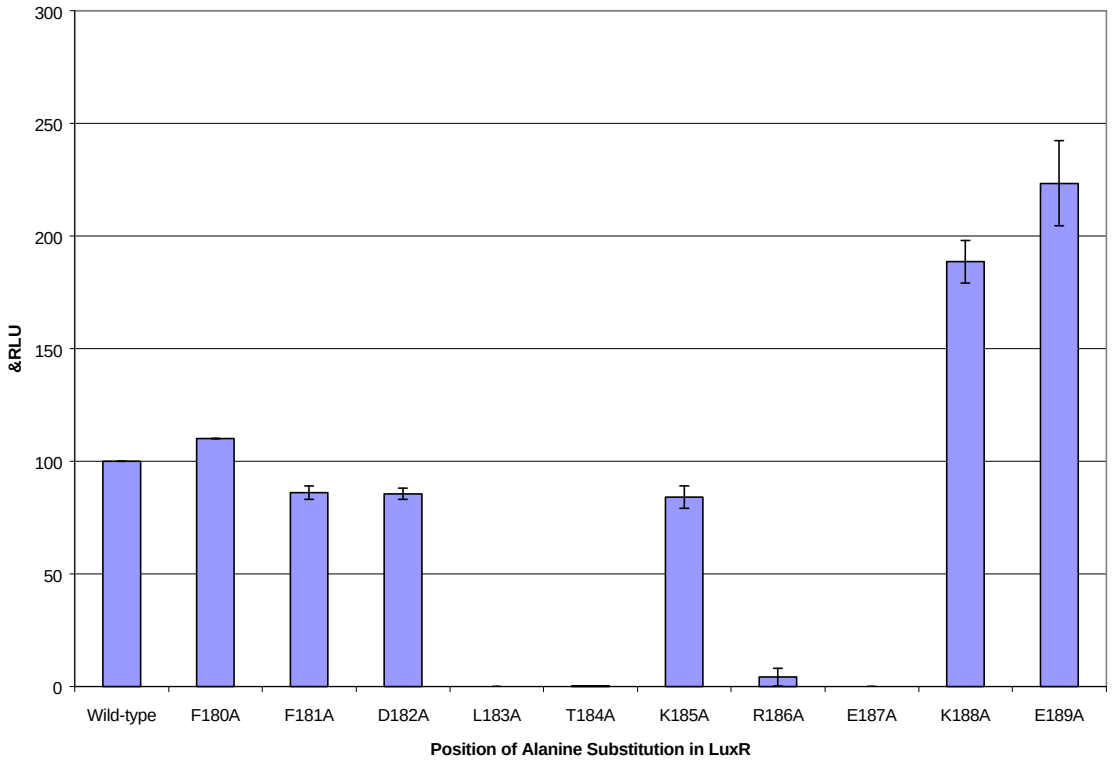

Figure 9: Luciferase Assays for LuxR Variants

The effect of alanine substitution on luciferase levels in extracts of recombinant *Escherichia coli* determined in the presence of 3-oxohexanoyl HSL. Average value for 100% = 1225 RLU.

was proven that LuxR variants 180A-189A were unable to activate transcription from the *lux* operon, it was unclear as to the nature of the defect providing this dark phenotype. To determine if the problem was in DNA recognition/binding the *lux* box DNA or in making the appropriate connections with RNAP, DNA binding/repression assays were conducted.

Determining the ability of LuxR alanine substitution variants to recognize/bind to the *lux* box DNA

The effects of alanine substitutions on DNA binding and repression were determined through in vivo experiments using strains containing the plasmid reporter p35LB10 in conjunction with the pMAF series of LuxR variants. Out of the 10 LuxR variants analyzed, 8 were defective in recognizing/binding the *lux* box DNA (Figure 10). Variants F181A and D182A exhibited wild-type phenotypes. Variants L183A, T184A, R186A, and E187A, exhibiting dark phenotypes in the luminescence and luciferase assays, but proved to be unable to recognize/bind the *lux* box and so did not repress transcription of the reporter, as did the wild-type strain. The other four variants, being bright in the luminescence and luciferase assays, were unable to repress transcription of the reporter, and so were only able to bind/activate at the promoter of the *lux* operon in the presence of *E. coli* RNAP. In the absence of an appropriately positioned RNAP binding site, these variants (F180A, K185A, K188A, and E189A) are unable to bind presumably due to lack of protein-protein contacts with RNAP.

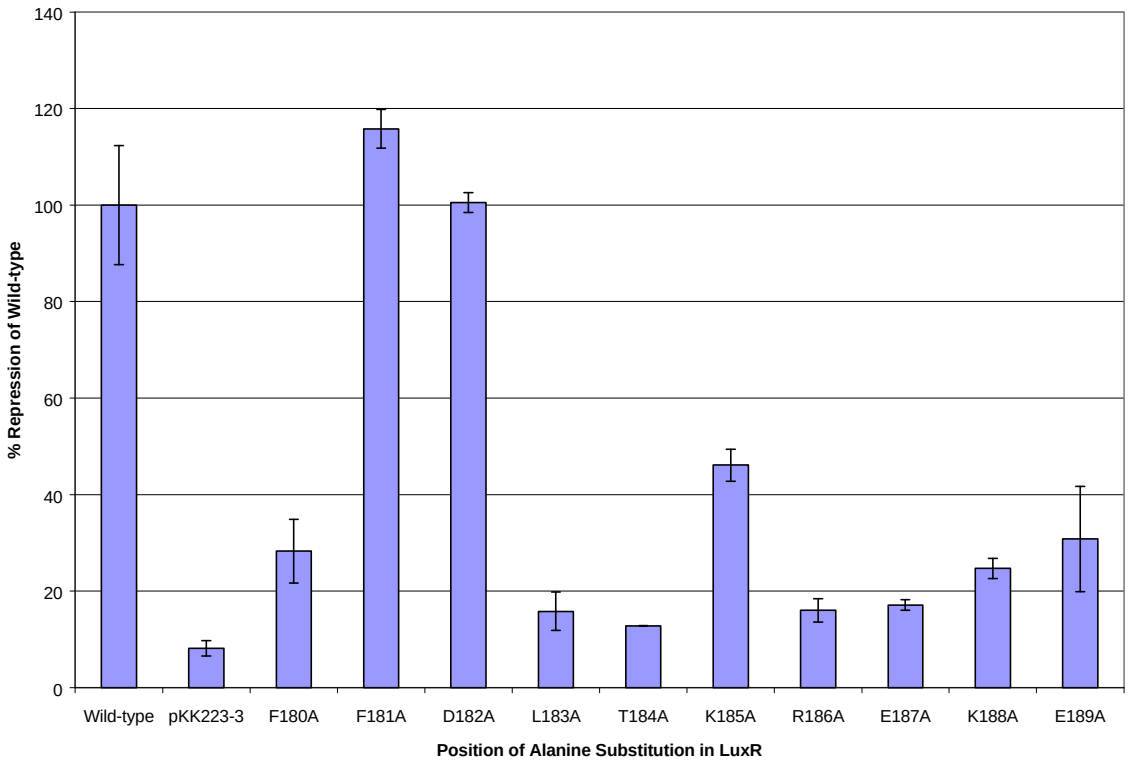

Figure 10: DNA Binding/Repression Assays for LuxR Variants
The effects of alanine substitution on DNA binding and repression at an artificial promoter construct containing the *lux* box was measured (Egland and Greenberg, 2000). Average value for 100% = 400 RLU.

Verification of variant LuxR protein production

SDS-PAGE and western immunoblotting experiments were performed to confirm that the variant forms of LuxR were being produced from the host strain, *E. coli* JM109. The western immunoblots confirmed production of variant LuxR (Figure 11). An equal amount of cellular extract was loaded on the gel, subsequently transferred to the nitrocellulose membrane and then treated with primary and secondary antibodies as described in the Materials and Methods, Chapter 2. An altered mobility was seen with variant E187A. The faster mobility may indicate a truncated protein but the DNA sequence of this variant, along with the other nine LuxR alanine substitution variants produced, was analyzed and proven to be of the correct size and nucleotide sequence.

Determination of intergenic suppression between amino acid residues of the α subunit of RNAP and LuxR positive control residue I206E

To investigate which residue(s) of the RNAP α subunit are associating with the LuxR positive control residue 206, an *E. coli* strain was transformed with three separate plasmids encoding the *lux* operon (pAMS129), the 206E variant of *luxR* (p206E), and a randomly, chemically mutated form of the RNAP α subunit (pREIIα). Light should be produced and detected from these cells with the occurrence of a compensatory mutation in the α subunit. Elucidation of the amino acid residue responsible for the interaction may be concluded after isolation of the variant pREIIα plasmid followed by sequencing of *rpoA.* Out of 2500 transformants screened via high throughput luminescence assays, two showed a possible revertant phenotype in that they produced luminescence at a level

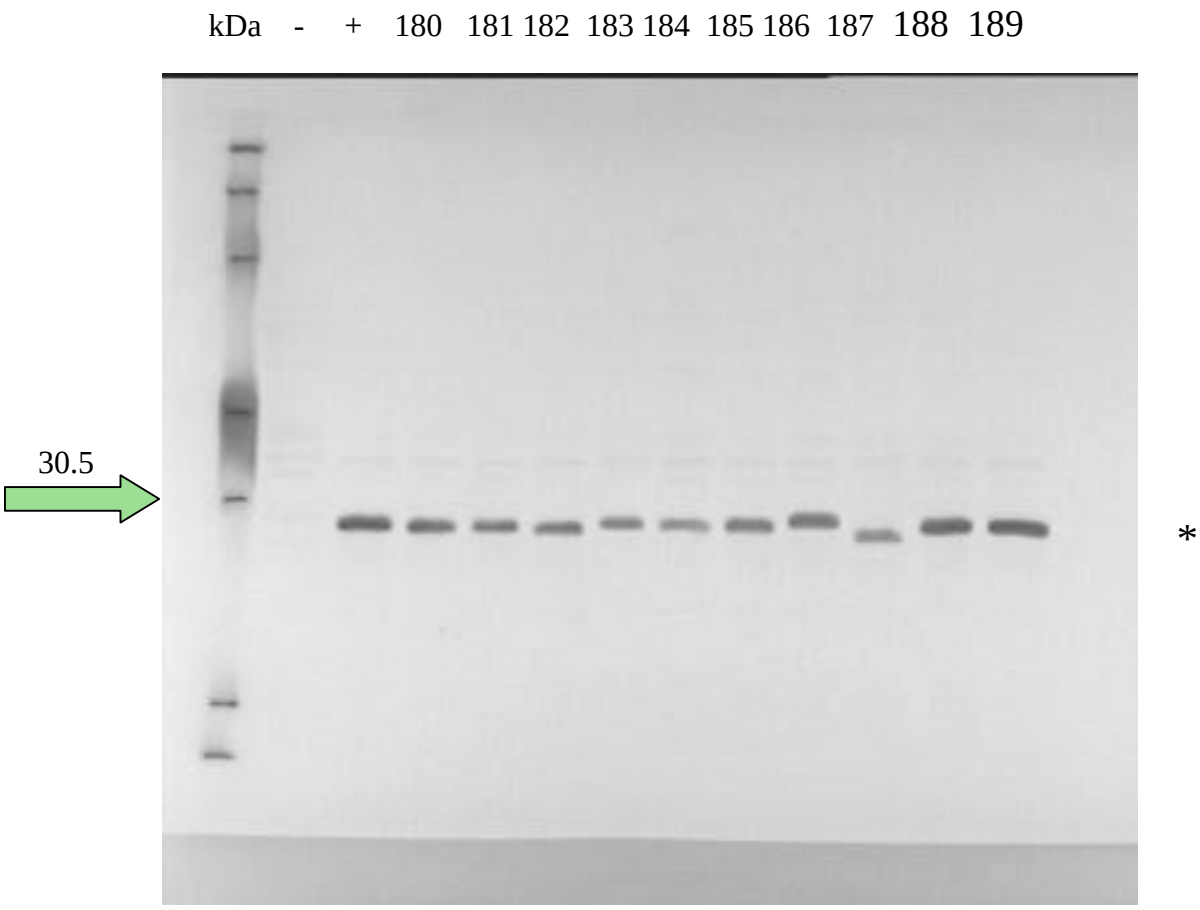

Figure 11: Western Immunoblot of LuxR Alanine Substitution Variants
Cell extracts from E. *coli* strains expressing the individual variants were exposed to LuxR antiserum to confirm LuxR protein production. The LuxR band is highlighted with an asterisk.

intermediate between the negative and positive controls (data not shown). These two possible intergenic suppressor mutants and were called IGS1 and IGS2 (intergenic suppressor 1 and 2).

Luminescence assays were conducted in the presence of 200 nM 3-oxohexanoyl HSL and 1 mM IPTG for induction to quantitate the level of luminescence produced from IGS1 and IGS2. These assays confirmed the high throughput assays conducted in the 96 well plates. Bioluminescence levels exhibited from IGS1 and IGS2 were in fact stronger than the wild-type levels (Figure 12). This finding indicated that a compensatory mutation had occurred in the alpha subunit of RNAP and had suppressed the effect from the mutation produced in amino acid residue 206 of LuxR.

To verify that IGS1 and IGS2 actually are intergenic suppressors, the hydroxylamine treated plasmids encoding them were transformed into *E. coli* strains containing the wild-type *luxR* gene, and the *lux* operon. Luminescence assays were again conducted and the results showed a significant decrease in light production as compared to the wild-type strain (Figure 13). Based on this information and the preliminary nucleotide sequence analysis of these plasmids encoding the hydroxylamine mutagenized DNA of the RNAP α subunit, it appears that amino acid residue 206 in LuxR is not interacting with the α CTD. Over 70% of *rpoA* has been sequenced in IGS1 and IGS2.

Results from the sequencing indicate that no mutations were incorporated into the CTD of *rpoA*; both IGS1 and IGS2 appear to have the wild-type sequence. Further investigation and sequencing of the NTD of *rpoA* should reveal the amino acid(s) responsible for the suppression of I206E in LuxR.

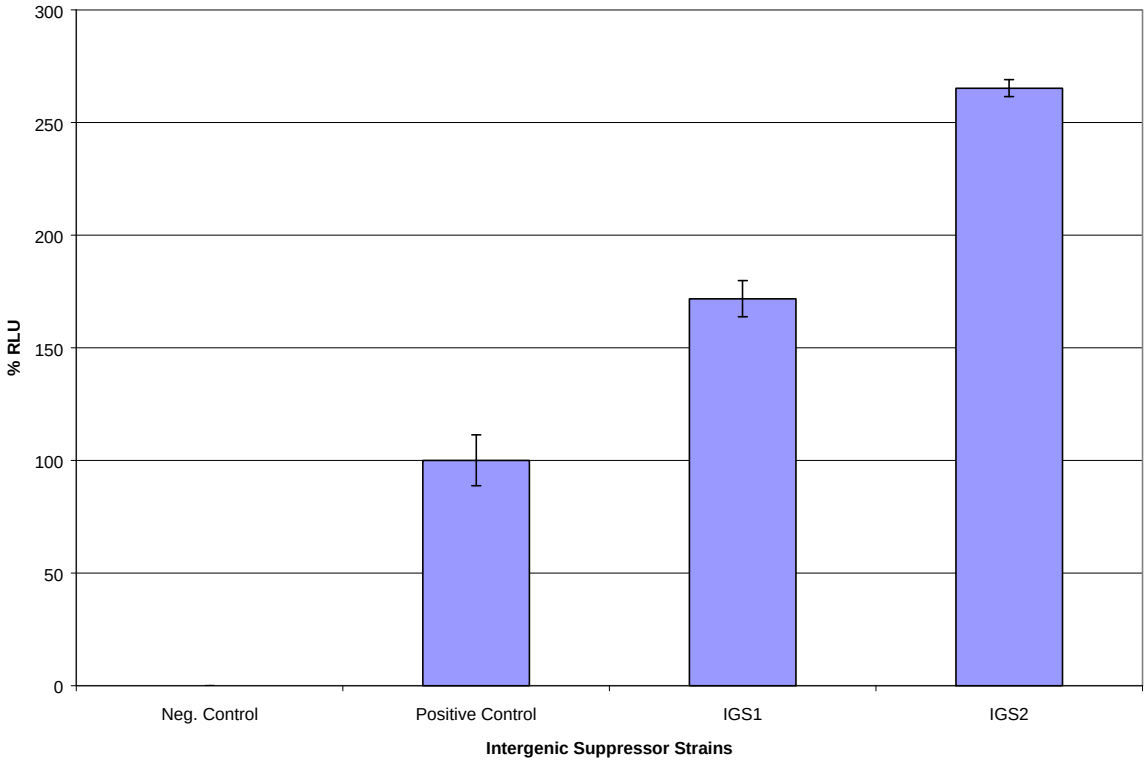

Figure 12: Intergenic Suppression Results for IGS1 and IGS2
All strains contain pAMS129 encoding the *lux* operon.

Neg. Control: p206E + pREIIα
Positive Control: pAMS121 + pREIIα
IGS1 (Suppressor #1): p206E + hydroxylamine treated pREIIα
IGS2 (Suppressor #2): p206E + hydroxylamine treated pREIIα

Average value for 100% = 1800 RLU.

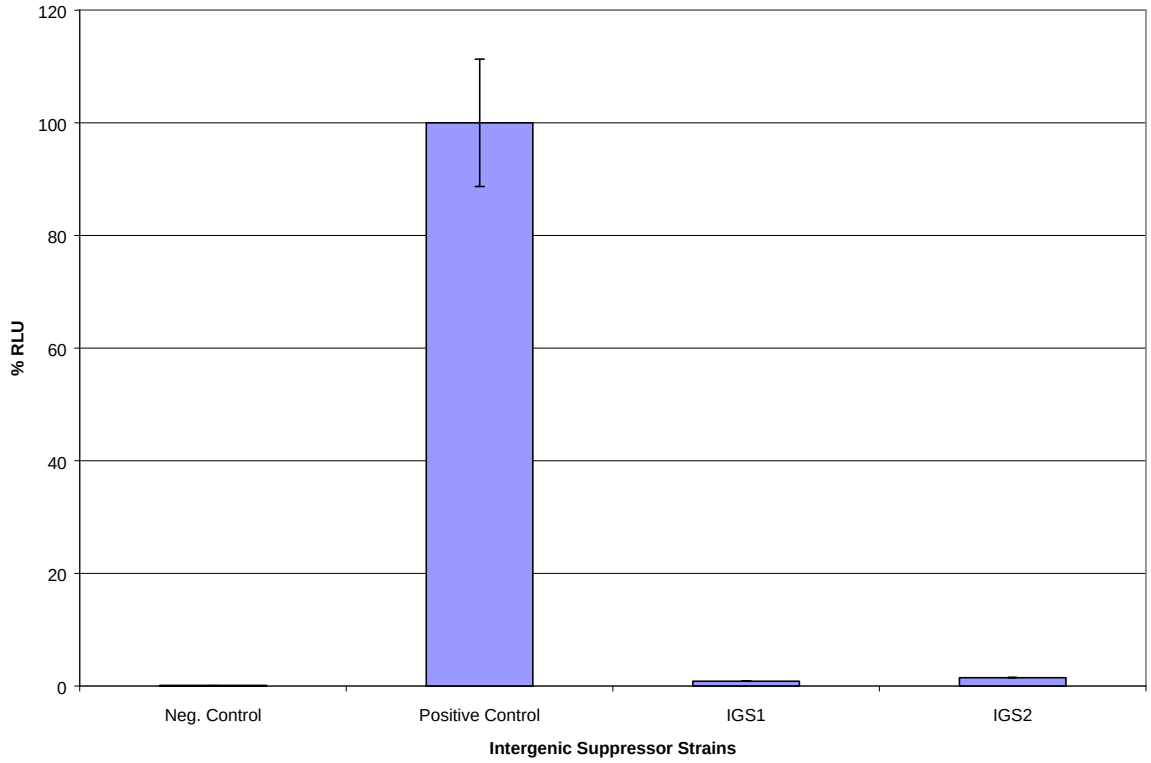

Figure 13: Complementation Analysis for Intergenic Suppressors
All strains contain pAMS129 encoding the *lux* operon.

Neg. Control: p206E + pREIIα
Positive Control: pAMS121 + pREIIα
IGS1 (Suppressor #1): pAMS121 + hydroxylamine treated pREIIα
IGS2 (Suppressor #2): pAMS121 + hydroxylamine treated pREIIα

Average value for 100% = 1800 RLU.

CONCLUSIONS:

No additional positive control variants were identified through the experimental analysis of the pMAF encoded LuxR variants. Though eight out of the ten were defective in binding the *lux* box DNA, these alanine substitutions could not be clearly shown to have any effect on making protein-protein interactions with RNAP and hence transcriptional activation of the *lux* operon. Based on these data, the previously identified positive control variants of LuxR (198A, 201A, and 206A), are the only single amino acid residues known to be critical for making these contacts and interacting with RNAP for transcription initiation. The two potential intergenic suppressor mutants discovered in this study, IGS1 and IGS2, contain amino acid substitution(s) responsible for suppressing the effect of 206E in LuxR. Interesting preliminary sequence analysis of IGS1 and IGS2 indicates that these mutations are not in the α CTD. Ongoing sequence analysis of the α NTD is underway, and should indicate the amino acid residue(s) responsible for suppression of the phenotype conferred by the 206E LuxR variant.

CHAPTER 3

Ability of EsaR and ExpR, two LuxR Homologues, to Bind to the *lux* Box and Activate the *lux* Operon

INTRODUCTION

LuxR homologue, ExpR, found in *Erwinia carotovora*, appears to be involved in negatively regulating the expression of extracellular products required for virulence determinants in this plant pathogen (Andersson et al., 2000). Interestingly, EsaR is a transcriptional repressor found in *Pantoea stewartii*, another plant pathogen that controls the production of extracellular polysaccharide (EPS) capsule that hinders the passage of water through the xylem (von Bodman et al., 1995). The goal of this study was to determine the ability of EsaR and ExpR to bind to the *lux* box and activate the *lux* operon. Molecular cloning techniques were employed to clone *luxR*, *esaR*, and *expR* into the expression vector pBAD22. β-galactosidase assays were used to determine the ability of EsaR and ExpR to activate the *lux* operon using two new lambda reporter constructs. The Top10λ*lux-lacZ Escherichia coli* strain tests whether or not EsaR and ExpR activated transcription from the *lux* operon like the positive control LuxR. The Top10λ35LB10 *E. coli* strain was used as a reporter to measure the ability of EsaR and ExpR to bind to the *lux* box. In this strain, the *lux* box is placed in between the -35 and -10 sites, so if the protein bound to the *lux* box it will repress transcription. If a difference in expression is observed in the activation assay, this second assay demonstrates if the defect was at the level of DNA recognition versus the ability of the EsaR and ExpR to communicate with the RNA polymerase when bound to the *lux* box.

MATERIALS AND METHODS

Cloning *luxR* into the expression vector pBAD22:

The plasmid, pJE202 (Engebracht, 1983) encoding the *luxR* gene, was isolated using the QIAprep Spin Miniprep Kit (Qiagen) from an overnight culture of *E. coli* JM109. After isolating the template DNA, the polymerase chain reaction (PCR) was used to amplify the *luxR* gene. The PCR 'master mix' contained the following final concentrations of reagents: 3 pmol/μL NLB primer (Table 5), 3 pmol/μL CLB primer (Table 5), 0.2 mM dNTP's (Promega), 2.5 units *Taq*2000 Polymerase (Stratagene), 1X *Taq*2000 reaction buffer (Stratagene), 1.3 mM $MgSO_4$ (Fisher, Springfield, NJ), and 100 ng of pJE202 template. The program for the PCR reaction was one cycle at 95°C for 2 minutes; 30 cycles: 94°C for 30 seconds, 45°C, for 30 seconds, and 72°C for 2 minutes; one cycle at 72°C for 10 minutes. The formation of the PCR products was confirmed by electrophoresis in a 0.8% agarose gel. The QIAquick PCR Purification Kit (Qiagen) was used to purify the *luxR* insert.

The purified *luxR* insert was then ligated into pGEM using the pGEM®-T easy vector system (Promega) allowing for blue-white screening after transformation. Ligation contents were introduced into DH5α competent cells and transformants selected on plates containing 0.2 mM IPTG, 40 μg/mL X-Gal, and 100 μg/mL ampicillin. White colonies from the overnight 37°C incubation were subcultured into LB broth containing

Table 5: Primers for *luxR* amplification

NLB	5’-CCGGAATTCACCATGAAAAACATAAATGCCGACGAC
CLB	5’-TCCCCCGGGCTATTAATTTTTAAAGTATGGGCA
XBA200	5’-CGTATAATGTGTGGAATTGTGAGCG
PVU200	5’-GAAGTGGTCCTGCAACTTTATCC

Table 6: Sequencing primers for pBAD-LuxR, EsaR, and ExpR

BADF	5’-TCGCAACTCTCTACTGTTC
BADR	5’-CTTCTCTCATCCGCCAAAAC

Table 7: Primers for *esaR* amplification

EsaRF2	5’-GGAATTCACCATGTTTTCTTTTTTCCTTG
EsaRR2	5’-CTCTAGATCACTACCTGGCCGCTGAC

Table 8: Primers for *expR* amplification

ExpRF2	5’-GGAATTCACCATGTCGCAGTTATTCTACAAC
ExpRR2	5’-CTCTAGATCACTATGACTGAACCGGTCGG

Table 9: Mutagenic primers for *expR*

FixExpR1	5'-CCATCTCTCT**G**TA<u>C</u>AGAGAGATG
FixExpR2	5'-CATCTCTCT<u>G</u>TA**C**AGAGAGATGG

Bold indicates silent mutation to incorporate an *Rsa*I site for screening
Underline indicates base change to incorporate original wild-type base

100 µg/mL ampicillin. Sequencing reactions using primers T7 and SP6 (Promega), specific for the pGEM-T (Promega), were performed at the CSF-VBI at Virginia Tech and confirmed the *luxR* insert was of the correct nucleotide sequence and was devoid of any second site mutations.

Purified pGEM plasmid DNA containing the *luxR* insert was then subjected to a sequential digest using *Eco*RI followed by an ethanol precipitation and addition of *Sma*I. After incubation at 37°C for 2.5 hours, the digested products were run in a 0.8% gel, the *luxR* insert was extracted and purified using the QIAquick Extraction Kit (Qiagen). The expression vector pBAD22 was isolated in the same manner as pJE202, described previously, and a sequential restriction digest was carried out using *Eco*RI and *Sma*I. The resulting bands were extracted from a 0.8% agarose gel after electrophoresis and purified using the QIAquick Extraction Kit (Qiagen). The *luxR* insert along with the vector from pBAD22 were ligated using T4 DNA Ligase (New England Biolabs) overnight at 16°C. Transformation into *E. coli* DH5α (Hanahan, D., 1983) was achieved by heat shocking the competent cells/hybrid DNA molecules in 0.1 M $CaCl_2$ (J.T. Baker Chemical Co., Phillipsburg, NJ) at 37°C for 2 minutes. After incubation on ice for 5 minutes, the cells were grown in LB broth for one hour at 37°C and spread on plates containing 100 µg/mL ampicillin. Subsequent colonies were picked and subcultured into LB broth containing 100 µg/mL ampicillin. Purification of the plasmids via the alkaline-lysis method followed overnight growth at 37°C.

The purified plasmid DNA was confirmed to carry the *luxR* insert based on the banding pattern present in the agarose gel when the DNA was cut with *Eco*RI and *Sma*I

as compared to the expression vector, pBAD22, without insert present. After this confirmation, the plasmids were sequenced to ensure that no second site mutations had arisen during the PCR or cloning steps. Sequencing was performed by CSF-VBI at Virginia Tech using sequencing primers BADF and BADR (Table 6). The resulting pBAD22-*luxR* plasmid, of correct sequence, was named pBAD-LuxR (pJKB1-1.5) (Figure 14).

Cloning *esaR* into pBAD22 :

The *E. coli* DH5α strain containing pSVB5-18 (von Bodman, 1995) which encodes EsaR was obtained from Susanne B. von Bodman. PCR was used to amplify the *esaR* gene in the same manner as that used for the *luxR* gene previously described, but primers EsaRF2 and EsaRR2 (Table 7) were employed for the formation of PCR product. The PCR products were again purified using the QIAquick PCR Purification Kit (Qiagen) and ligated into the pGEM T-easy vector (Promega). After transformation into DH5α, colonies containing the insert were isolated. The plasmid DNA was purified via the alkaline lysis method and subjected to restriction digestion with *Eco*RI and *Xba*I for subsequent cloning into pBAD22. Due to the inefficiency of *Xba*I digestion, *Eco*RI was exclusively used for extracting *esaR* from the pGEM T-easy vector since two *Eco*RI sites were present flanking the *esaR* gene. The expression vector pBAD22 was linearized using *Eco*RI, and the *esaR* gene with approximately 15 base pairs of the multiple cloning site from the pGEM T-easy vector was ligated into it. The extra base pairs at the end of the *esaR* gene are thought to not interfere with transcription of the gene since they are

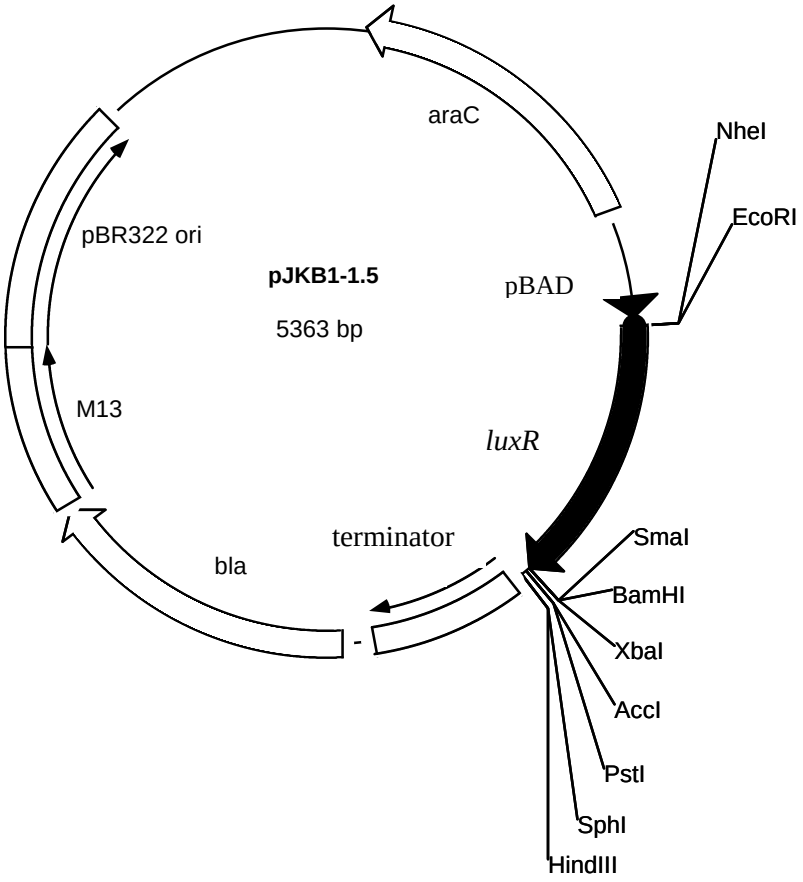

Figure 14: Plasmid Map of pBAD-LuxR (pJKB1-1.5)
Constructed by J. K. Ball

located after the translation termination site. To ensure that the *esaR* gene was correctly oriented in the expression vector, restriction digestion was carried out at the conveniently located *Kpn*I sites. The correct construct was named pBAD-EsaR (pJKB2-11.11) (Figure 15).

Correcting the Nucleotide Sequence of *expR* and Subsequent Cloning into pBAD22:

Initial cloning of *expR* followed the same protocol as that for *esaR* as described above. After PCR amplification, purification, ligation, and transformation of the gene into the pGEM T-easy vector, the *expR* insert was sequenced at the CSF-VBI and the PCR product, as well as the original plasmid template pSA018 (Andersson et al., 2000), were found to contain a stop codon approximately three quarters of the way into the coding sequence. Site directed mutagenesis was employed to change the amino acid residue back to its original codon. Primers were designed for the amplification (Table 8) and mutagenesis (Table 9) of *expR* using overlap PCR (Chapter 2) for incorporating the amino acid residue change as well as the addition of a *Rsa*I site for screening purposes. After PCR, the plasmid DNA was purified and ligated into the pGEM T-easy vector as described above. After *Eco*RI digestion of the pGEM T-easy vector and pBAD22, the *expR* insert was ligated into the expression vector and transformed into *E. coli* DH5α. Since there were not any convenient restriction sites in *expR* for determining the orientation of the gene orientation of the gene in the vector, PCR was again employed. Primers BADF and ExpRR2 (Tables 6 & 8) were used to amplify the gene and check the size of the resulting PCR product. Clones having the correct insert were sent to CLF-VBI

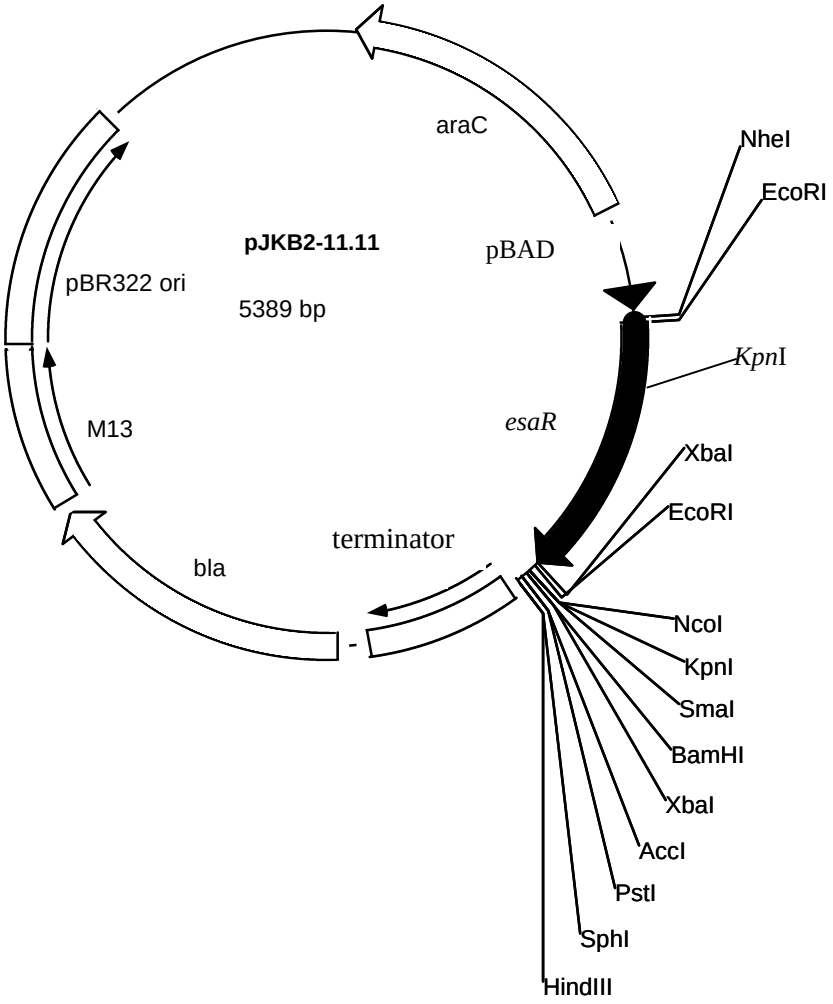

Figure 15: Plasmid Map of pBAD-EsaR (pJKB2-11.11)
Relevant characteristics shown; see text for details.
Constructed by J. K. Ball

for sequencing with BADF and BADR primers (Table 6) to ensure the correct sequence. The correct construct was subsequently named pBAD-ExpR (pJKB3-1.2) (Figure 16).

b-galactosidase Assays:

The *E. coli* Top10 activation (λ*lux-lacZ*) and repression (λ35LB10) strains (Grant et al., 1990; M. Urbanowski, personal communication) used for the assays were genotypically araBAD$^-$, and therefore did not have the ability to metabolize arabinose. Strains were grown overnight and subcultured into media without arabinose to prevent leaky expression of the pBAD promoter. The strains containing pBAD22, pBAD-LuxR, pBAD-EsaR, and pBAD-ExpR were grown overnight at 30°C in RM minimal media (2% casamino acids, 1X M9 salts (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl), 0.4% glucose, and 0.1 M $MgCl_2$) containing 100 μg/mL ampicillin to an OD_{600} of 0.2-1.0. Strains were then subcultured into four sets of RM minimal media (2% casamino acids, 1X M9 salts (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl), 0.4% glucose, and 0.1 M $MgCl_2$ with ampicillin (100 μg/mL) broth containing: (1) no addition (2) 0.2% arabinose (3) 1 μM 3-oxohexanoyl-DL-HSL, (4) 1 μM 3-oxohexanoyl-DL-HSL and 0.2% arabinose to an OD_{600} of 0.025, and subsequently grown to a final OD_{600} of 0.5 (mid-exponential log-phase growth). At this point, the cells were stored on ice. When all samples had reached an OD_{600} of 0.5, 5 μL of cells were diluted with 995 μL Z Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) with added DTT at a concentration of 400 μM. Chloroform (50 μL) was added to the cells and then the suspension was briefly

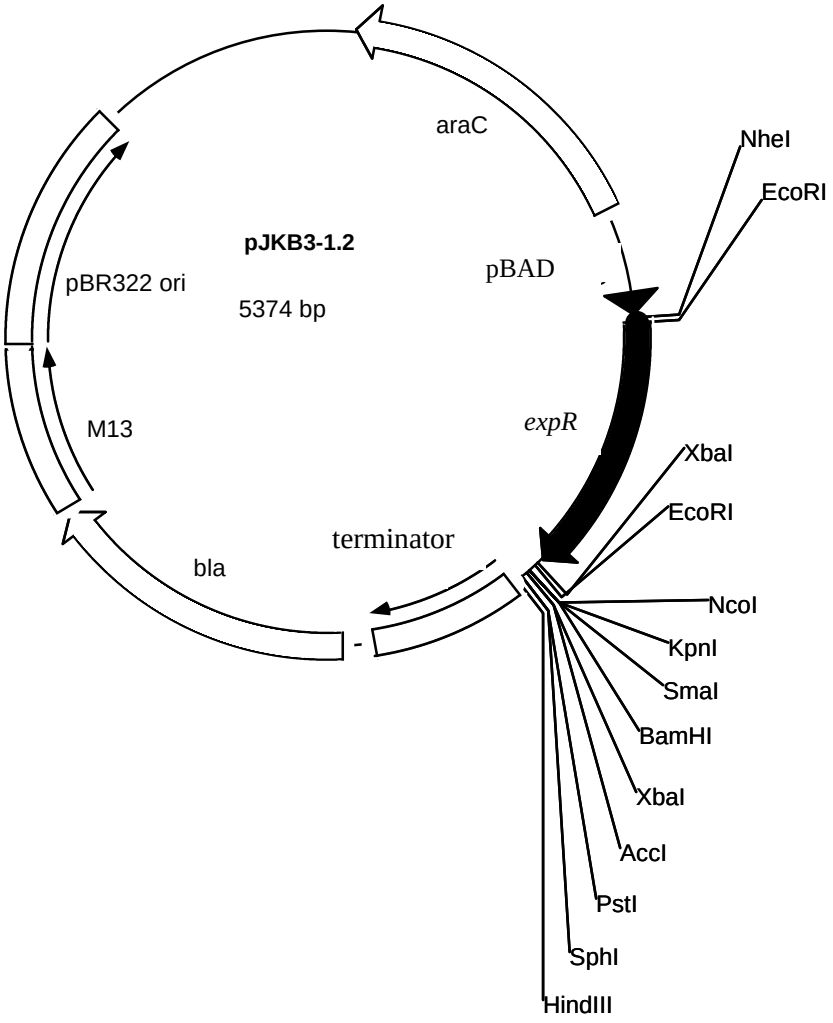

Figure 16: Plasmid Map of pBAD-ExpR (pJKB3-1.2)
Construction was assisted by J.K. Ball

vortexed to cause lysis. After lysis, 10 μL of the cell extract was added to wells of a Lucy microtiter dish plate and incubated for one hour with 100 μL of room temperature reaction buffer and substrate from the chemiluminescent reporter assay kit (Tropix, Bedford, MA) mixed at a ratio of 100:1, respectively. After the one-hour incubation, all of the samples were tested in the same order they were set up with reaction buffer mixed with substrate. The accelerator was brought to room temperature and 150 μL was added to each tube prior to being placed in the Lucy 1 measured for light output over a 20 second integration period. Each sample was tested in triplicate, and light output was measured in relative light units.

RESULTS AND DISCUSSION

The activation assay results (Table 10) using the reporter strain *E. coli* Top10λ*lux-lacZ* revealed that EsaR is capable of activating transcription from the *lux* operon promoter. EsaR activated transcription of the reporter roughly 5 fold while the positive control activated transcription at a higher rate (roughly 15 fold). In the presence of autoinducer, activation from EsaR was blocked. The test strain expressing ExpR produced low levels of β-galactosidase, less than two fold above background and therefore, the ExpR protein is barely able to activate transcription from the *lux* operon promoter.

Using the reporter strain *E. coli* Top10λ35LB10, the repression assay results (Table 11) demonstrate that EsaR can weakly repress transcription of the *lacZ* gene though not at the level of the control strain (roughly six fold repression) expressing LuxR from the pBAD-LuxR plasmid. The strain containing pBAD-ExpR, did not have the ability to repress transcription; the level of β-galactosidase produced from this strain was actually higher than that of the negative control strain containing the expression vector, pBAD22.

Table 10: Activation Assays for LuxR, EsaR and ExpR

Plasmid + Inducer	Relative Light Units
pBAD	1.70
pBAD + Ara	1.77
pBAD + VAI	1.77
pBAD + VAI + Ara	1.81
pBAD-LuxR	1.83
pBAD-LuxR + Ara	1.86
pBAD-LuxR+ VAI	3.31
pBAD-LuxR + VAI + Ara	33.5
pBAD-EsaR	2.17
pBAD-EsaR + Ara	9.70
pBAD-EsaR + VAI	2.01
pBAD-EsaR + VAI + Ara	2.96
pBAD-ExpR	2.07
pBAD-ExpR + Ara	3.59
pBAD-ExpR + VAI	2.08
pBAD-ExpR + VAI + Ara	3.32

Ara = arabinose at 0.2%

VAI = *Vibrio fischeri* autoinducer at 1μM

Table 11: Repression Assays for LuxR, EsaR and ExpR

Plasmid + Inducer	Relative Light Units
pBAD	115
pBAD + Ara	107
pBAD + VAI	114
pBAD + VAI + Ara	106
pBAD-LuxR	138
pBAD-LuxR + Ara	120
pBAD-LuxR+ VAI	125
pBAD-LuxR + VAI + Ara	26.1
pBAD-EsaR	123
pBAD-EsaR + Ara	71.7
pBAD-EsaR + VAI	133
pBAD-EsaR + VAI + Ara	97.9
pBAD-ExpR	152
pBAD-ExpR + Ara	146
pBAD-ExpR + VAI	147
pBAD-ExpR + VAI + Ara	146

Ara = arabinose at 0.2%

VAI = *Vibrio fischeri* autoinducer at 1μM

CONCLUSIONS:

It has been demonstrated that the LuxR homologue EsaR, previously shown to function as a repressor, has retained an ability to function as an activator of transcription by RNAP. Furthermore, it can bind and recognize the *lux* box but at a lower affinity than LuxR. On the other hand, ExpR activates transcription at rates just a little higher than background and seems to be unable to recognize the *lux* box. Interestingly, as previously proposed, EsaR is inactivated by the presence of 3-oxohexanoyl HSL (von Bodman et al., 1998). Addition of the VAI at 1 μM did not fully inactivate EsaR hence further experiments at a higher concentration of 3-oxohexanoyl HSL (100 μM as per Minogue et al., 2002) are warranted.

By performing the assays again under this set of conditions, it is possible that the background activity levels from the repression assays would be lowered, allowing for clearer results. Also, by subculturing the test strains at a much lower optical density, the amount of LacZ produced within the cells during the overnight culturing would be considerably diluted with a few extra generations. This would also lower the background of LacZ seen in the assays.

CHAPTER 4

Annotating the *Vibrio fischeri* Genome

FIAT TEAM

In conjunction with the Chicago-based company Integrated Genomics, Inc. (IG), work has been performed as part of a multi-lab team of researchers known as the FIAT (*Fischeri* Intrinsic Annotation Team) to help annotate the *Vibrio fischeri* genome. This company provides full genomic services including high throughput DNA sequencing, assembly, annotation, and metabolic reconstruction for industrial and academic clients. Originally the company focused on bacterial genomes, gathering and analyzing enough information to produce a database encompassing over 280 genomes and more than 3,500 biochemical pathways.

Within the IG website, the system termed ERGO™ is based on and allows for cross-genome analysis. By appointing different contigs to separate labs, it is possible for the analysis and cross referencing that is needed for assigning functions to these stretches of DNA, i.e., annotating the genome. The new gene assignments permitted by using the ERGO suite are reevaluated by the 'master' computer and may ultimately be tested as well in a biochemical lab, leading to the identification of potential targets for new antibiotics or for strain engineering for improved industrial fermentation, crop production, and drug discovery.

OVERALL CONTRIBUTIONS

To date, 326 open reading frames of the *V. fischeri* IG30 strain have been annotated through Ann Stevens' Lab; I have personally annotated 216 open reading frames. The annotation is conducted through homology searches, BLAST searches, comparing preserved operons among different organisms, investigating protein denaturation temperatures, membrane spanning helices and domains, as well as other relevant characteristic features of the amino acid sequences and/or operons. It was possible to determine the function of many of the open reading frames and submit the suggestion to the databank. As the project started, the DNA contigs were separated and disorganized. Through work from a number of labs around the country, the contigs should soon be fully merged. Annotations will proceed until all of the open reading frames of *V. fischeri* have been assigned by IG, Inc. and annotated. The anticipated completion date falls late this year or next. By completing the annotation of the genome, it will be possible to better understand the quorum sensing systems in *V. fischeri*, and possibly discover new regulons within the genome.

CHAPTER 5

REFERENCES

Andersson, R. A., Eriksson, A. R. B., Heikinheimo, R., Mäe A., Pirhonen, M., Koiv V., Hyytiainen, H., Tuikkata, A., and Palva, E. T. (2000). Quorum Sensing in Plant Pathogen *Erwinia carotovora* subsp. *carotovora*: The Role of $expR_{ECC}$. Molecular Plant-Microbe Interactions 13: 384-393.

Baikalov, I., Schroder, I., Kaczor-Grzeskowiak, M., Grzeskowiak, K., Gunsalus, and R.P., Dickerson, R.E. (1996). Structure of the *Escherichia coli* response regulator NarL. Biochemistry 35:11053-11061.

Beatson, S.A., Whitchurch, C.B., Sargent, J.L, Levesque, R.C., and Mattick, J.S. (2002). Differential Regulation of Twitching Motility and Elastase Production by Vfr in *Pseudomonas aeruginosa.* J. Bacteriol. 184: 3605-3613.

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

Beck von Bodman, S., Majerczak, D.R., and Coplin, D. L. (1998). A negative regulator mediates quorum sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. Proc. Natl. Acad. Sci. USA 95:7687-7692.

Blatter, E.E., Ross, W., Tang, H., Gourse, R.L., and Ebright, R.H. (1994). Domain organization of RNA polymerase α subunit: C-terminal 85 amino acids constitute an independently folded domain capable of dimerization and DNA binding. Cell 78:889-896.

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

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

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

Dunlap, P.V., and Ray, J.M. (1989). Requirement for autoinducer in transcriptional negative autoregulation of the *Vibrio fischeri luxR* gene in *Escherichia coli.* J. Bacteriol. 171:3549-3552.

Eberl, L. (1999). N-acyl homoserine lactone-mediated gene regulation in Gram negative bacteria. System. Appl. Microbiol. 22: 493-506.

Egland, K.A. and Greenberg, **E.P.** (2000). Conversion of the *Vibrio fischeri* Transcriptional Activator, LuxR, to a Repressor. J. Bacteriol. 182: 805-811.

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

Engebrecht, J., Nealson, K., and Silverman, M. (1983). Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from *Vibrio fischeri*. Cell 32:773-781.

Finney, A.H., Blick,R.J., Murakami, K., Ishihama, A., and Stevens, A.M. (2000). Role of C terminal Domain of the Alpha Subunit of RNA Polymerase in LuxR-Dependent Transcriptional Activation of the *lux* Operon during Quorum Sensing. J. Bacteriol. 184:4520-4528.

Fuqua, C., Winans, S.C., and Greenberg, E. P. (1994). Quorum Sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. 176: 269-275.

Fuqua, C., Winans, S.C., and Greenberg, E. P. (1996). Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. 50: 727-751.

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

Grant, S.G., Jessee, J., Bloom, F.R., and Hanahan, D., (1990). Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA 87: 4645-4649

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

Greenberg, E.P. (1997). Quorum Sensing in Gram-negative bacteria. ASM News 63: 371-377.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J.Mol.Biol. 166:557-580.

Higuchi, R., Krummel, B., and Saiki R.K. (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucl. Acids. Res. 16: 7351-7367.

Ho, S.N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77: 51.

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

Koiv V. and Mäe A. (2001). Quorum sensing controls the synthesis of virulence factors by modulating *rsmA* gene expression in *Erwinia carotovora* subsp *carotovora.* Molecular Genetics and Genomics 265: 287-292.

Jones, S., Yu, B., Bainton, N.J., Birdsall, M., Bycroft, B.W., Chhabra, K., Salmond, G.P.C., Stewart, G.S.A.B., and Williams, P. (1993). The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. EMBO J. 12: 2477-2482.

Luo, Z. Q. and Farrand, S. K. (1999). Signal-dependent DNA binding and functional domains of the quorum-sensing activator TraR as identified by repressor activity. Proc. Natl. Acad. Sci. USA. 96: 9009-9014.

Mäe, A., Montesano, M., Koiv, V., and Palva, E.T. (2001). Transgenic plants producing the bacterial pheromone N-acyl-homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora.* Molecular Plant-Microbe Interactions 14: 1035-1042.

McFall-Ngai, M. J., and Ruby, E. G. (1991). Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science 254: 1491-1494.

Michael, S. F. (1994). Mutagenesis by incorporation of a phosphorylated oligo during PCR amplification. BioTechniques 16:410-412.

Miller, M.B. and Bassler, B.L. (2001). Quorum sensing in bacteria. Annu. Rev. Microbiol. 55: 169-199.

Minogue, T. M., Wehland-von Trebra, M., Bernhard, F., and von Bodman, S. B. (2002). The autoregulatory role of EsaR, A quorum sensing regulator in *Pantoea stewartii* subsp. *stewartii*: evidence for a repressor function. *Molecular Microbiology* 44:1625-1635.

Nealson, K.H., Platt, T., and Hastings, J.W. (1970) Cellular control of the synthesis and activity of the bacterial luminescence system. J. Bacteriol. 104: 313-322.

Nyholm, S.V. and McFall-Ngai, M.J. (1998). Sampling the light-organ microenvironment of *Euprymna scolopes*: description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri*. Biol. Bull. 195:89–97.

Parsek, M. R. and Greenberg, E. P. (2000). Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. USA. 97: 8789-8793.

Pataky, J. K., Michener, P. M., Freeman, N. D., Weinzierl, R. A., and Teyker, R. H. (2000). Control of Stewart's wilt in sweet corn with seed treatment insecticides. Plant Dis. 84:1104-1108.

Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H., and Greenberg, E.P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA. 91: 197-201.

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

Ruby E.G. and McFall-Ngai, M.J. (1992). A squid that glows in the night: Development of an animal-bacteria mutualism. J. Bacteriol. 174: 4865-4870.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Simon, R., Quandt, J., and Klipp, W. (1989). New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bactetria. Gene 80: 131-169.

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

Stevens, A.M., Dolan, K.M., and Greenberg, E.P. (1994). 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.

Stevens, A.M., Fujita, N., Ishihama, A., and Greenberg, E.P. (1999). Involvement of the α subunit of RNA polymerase in LuxR-dependent activation of the luminescence genes during quorum sensing. J. Bacteriol. 181:4704-4707.

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

Vannini, A., Volpari, C., Gargiolo, C., Muraglia, E., Cortese, R., and DeFranco, R. (2002). The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. EMBO J. 21: 4393-4401.

Visick, K. L., Foster, J., Doino, J., McFall-Ngai, M., and Ruby. E. G. (2000). *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J. Bacteriol. 182: 4578-4586.

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

Whitehead, N.A., Barnard, A.M.L., Slater, H., Simpson, N.J.L., and Salmond, G.P.C. (2001). Quorum sensing in Gram negative bacteria. FEMS Micro. Reviews 25: 365-404.

Williams, P.W., Camara, M., Hartman, A., Swift, S., Milton, D., Hope, V.J., Winzer, K., Middleton, B., Pritchard D.I., and Bycroft B. W. (2000). Quorum sensing and the population-dependent control of virulence. Phil. Trans. R. Soc. London B pp667- 680.

Withers, H., Swift, S., and Williams, P. (2001). Quorum Sensing as an integral component of gene regulatory networks in Gram-negative bacteria. Curr. Opin. Micro. 4: 186-193.

Yanich-Perron, C., Viera, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

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

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M.S. in Microbiology, Virginia Polytechnic Institute & State University
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B.S. in Biochemistry, Virginia Polytechnic Institute & State University, 1999
Assoc. in Chemistry, Raritan Valley Community College, 1997

Employment History:

1/01-6/03 **Graduate Assistantship** at Virginia Tech., Blacksburg, VA
Performed alanine-scanning site directed mutagenesis via overlap-PCR on the quorum sensing activator protein, LuxR, of *Vibrio fischeri*
Research included luciferase, luminescence, DNA binding/repression assays, western immunoblotting and intergenic suppression studies involving *Escherichia coli* RNAP
Member of the *Vibrio fischeri* genome annotation team; annotated 216 ORF's
Mentored Lauren Senty, an undergraduate research student 8/01-12/02
Participated in microbiology departmental lab meetings and journal club

10/99-1/00 **Quality Control technician** at Jacobus Pharmaceuticals Inc., Plainsboro, NJ
6/00-8/00 Sampled and tested bulk/finished drug products; approved or denied products
Performed dissolution, thin-layer chromatography, titrations, chemical tests

12/98-1/99 **Lab Technician** at Biotechnology Training Institute, Lebanon, NJ
Performed experiments in manual peptide synthesis
8/99 Cell culture experiments: growing, splitting, freezing mammalian cell lines

6/98-8/98, **Research Assistant** of Dr. Stanley Katz at Rutgers University, NJ
Assisted in the design of experiments on antibiotic incidence in NJ milk supply
Performed zone of inhibition assays; publication waiting

Teaching Experience:

Graduate Teaching Assistant: General Biology Laboratory, Spring 2001 (BIOL 1016, 1116)

Presentations:

10/3/01 Microbiology Departmental Seminar, Virginia Tech, "Bacteria-host Interactions Involving Quorum Sensing"
8/20-8/25/02 Poster Presentation: Cold Spring Harbor Laboratory Phage & Genetics Meeting, Cold Spring Harbor, NY, "Amino Acid Residues Involved in Protein-Protein Interactions Between RNA Polymerase and the Quorum Sensing Activator LuxR"
5/18-5/23/03 Poster Presentation: ASM 103rd General Meeting, Washington D.C, "The Amino Acid Residues Involved in Protein-Protein Interactions between RNA Polymerase and the Quorum Sensing Activator LuxR"