

Amino Acid Residues in LuxR Critical for its Mechanism of
Transcriptional Activation during Quorum Sensing

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(ABSTRACT)

Vibrio fischeri, a symbiotic bioluminescent bacterium, serves as one of the best understood model systems for a mechanism of cell-density dependent bacterial gene regulation known as quorum sensing. During quorum sensing in *V. fischeri*, an acylated homoserine chemical signal (autoinducer) is synthesized by the bacteria and used to sense their own species in a given environment. As the autoinducer levels rise, complexes form between the autoinducer and the N-terminal domain of a regulatory protein, LuxR. In response to autoinducer binding, LuxR is believed to undergo a conformational change that allows the C-terminal domain to activate transcription of the luminescence or *lux* operon. To further understand the mechanism of LuxR-dependent transcriptional activation of the *lux* operon, PCR-based site-directed mutagenesis procedures have been used to generate alanine-substitution mutants in the C-terminal forty-one amino acid residues of LuxR, a region that has been hypothesized to play a critical role in the activation process. An *in vivo* luminescence assay was first used to test the effects of the mutations on LuxR-dependent activation of the *lux* operon in recombinant *Escherichia coli*. Luciferase levels present in cell extracts obtained from these strains were also quantified and found to correlate with the luminescence results. Eight strains encoding altered forms of LuxR exhibited a “dark” phenotype with luminescence output less than 50% and luciferase levels less than 50% of the wildtype control strain. Western immunoblotting analysis with cell extracts from the luminescence and luciferase assays verified that the altered forms of LuxR were expressed at levels approximately equal to wildtype. Therefore, Low luminescence and luciferase levels could be the result of a mutation that either affects the ability of LuxR to

recognize and bind its DNA target (the *lux* box) or to establish associations with RNA polymerase (RNAP) at the *lux* operon promoter necessary for transcriptional initiation. A third *in vivo* assay was used to test the ability of the altered forms of LuxR to bind to the *lux* box (DNA binding assay/repression). All of the LuxR variants exhibiting the “dark” phenotype in the luminescence and luciferase assay were also found to be unable to bind to the *lux* box in the DNA binding assay. Therefore, it can be concluded that the alanine substitutions made at these positions affect the ability of LuxR to bind to the *lux* box in the presence and absence of RNA polymerase. Another class of mutants exhibited wildtype phenotypes in the luminescence and luciferase assays but were unable to bind to the *lux* box in the DNA binding assay. The alanine substitutions made at these amino acid residues may be making contacts with RNAP that are important for maintaining the stability of the DNA binding region of LuxR. Alanine substitutions made at these positions have a defect in DNA binding at the promoter of the *lux* operon only in the absence of RNAP. None of the alanine substitutions made in the C-terminal forty-one amino acids of LuxR were found to affect activation of transcription of the *lux* operon without also affecting DNA binding. Taken together, these results support the conclusion that the C-terminal forty-one amino acids of LuxR are important for DNA recognition and binding of the *lux* box rather than positive control of the process of transcription initiation.

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CHAPTER ONE

INTRODUCTION

Quorum sensing

Many bacteria regulate gene expression in response to the detection of molecular signals that are present in their environment. During quorum sensing, a particular cellular phenotype is expressed only at high cell densities of a bacterial species due to the accumulation of a molecular signal in the local environment. The bacteria produce and release a specific chemical signal, termed autoinducer, as a means of communication between members of their own species. The bioluminescent marine bacterium, *Vibrio fischeri*, was the first gram-negative bacterium found to use a quorum sensing mechanism of gene regulation. The *V. fischeri* autoinducer enters and leaves the cells through diffusion; therefore, it can accumulate to high concentrations at high cell densities in a confined environment and lead to activation of the expression of cellular luminescence (*lux*) genes. If instead, there are a low number of *V. fischeri* cells in a given environment, then the signal being produced will not reach a high enough concentration, to activate *lux* gene expression (Fuqua et al., 1996). A symbiotic relationship has evolved between *V. fischeri* and marine fish, allowing for high cell densities of the bacteria to be reached within specialized light organs. In this environment, high cell densities are achieved and the *lux* genes are activated by a transcription factor, LuxR, in the presence of accumulated *V. fischeri* autoinducer signal (N-(3-oxohexanoyl) homoserine lactone, Figure 1 and 2). The luminescence produced by the activation of the *lux* genes is then used by the animal host as a means of communication (Swift et al., 1996).

Although *V. fischeri* is the best understood model system for studying quorum sensing in gram-negative bacteria, in the past decade, many other bacteria have been found to regulate expression of specific genes in a homologous manner. Many of these other quorum sensing-regulated phenotypes are associated with the virulence of the microorganisms, such as genes that

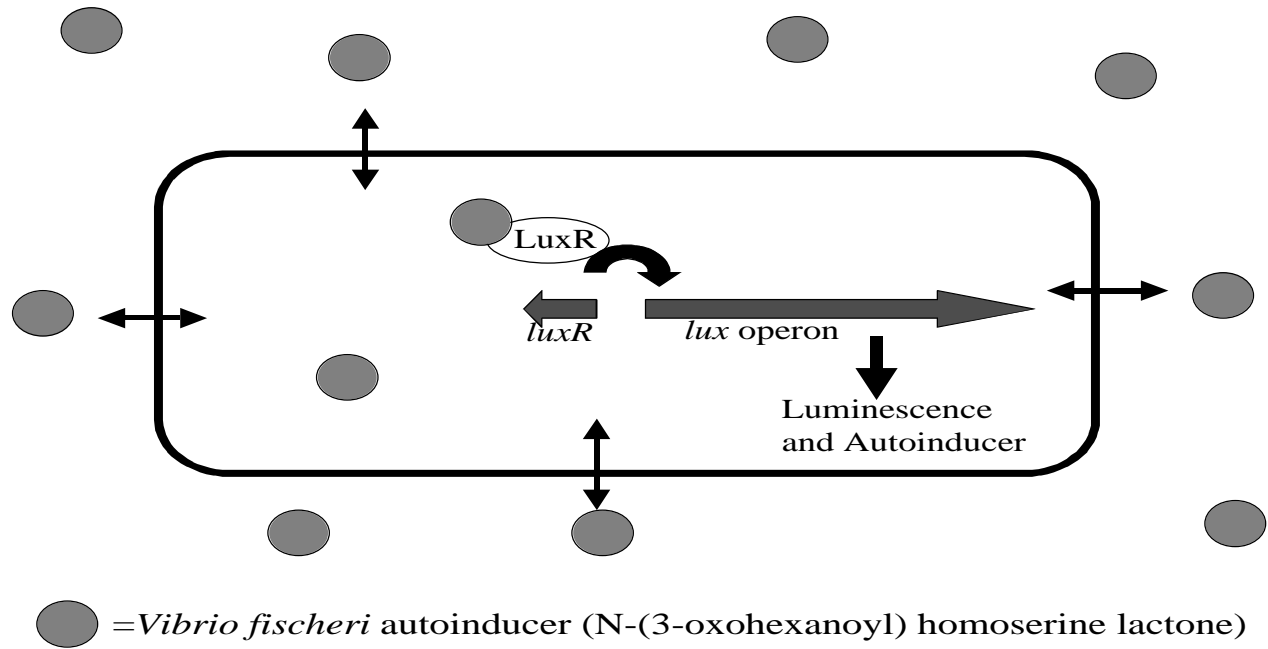


Figure 1: Model of quorum sensing in *Vibrio fischeri*.

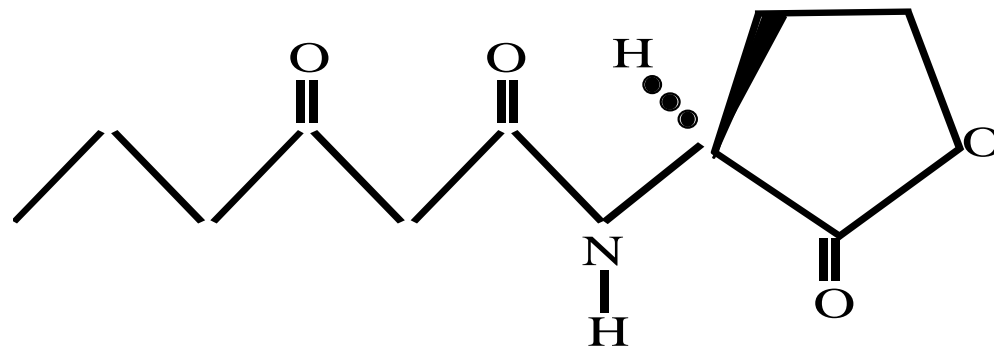


Figure 2: *Vibrio fischeri* autoinducer, N-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL or VAI).

code for antibiotics and enzymes important for pathogenesis. It is of interest to understand quorum sensing in *V. fischeri* at the molecular level such that the information may be applied to these other less understood systems. A better understanding of the mechanism of transcriptional activation employed at the promoter of the *lux* operon of *V. fischeri* may eventually lead to the development of ways to interfere with the transcriptional activation of the genes necessary for the virulence of these other medically and agriculturally important microorganisms (Fuqua et al., 1996).

P. aeruginosa is known to control expression of several of its virulence genes by quorum sensing, including an exotoxin, alkaline protease, and elastin-specific protease (Fuqua et al., 1994). The activation of these genes is dependent upon the binding of the autoinducer to a LuxR homologue, LasR. Therefore, only after a significant cell density of *P. aeruginosa* has been reached at the site of infection will these virulence genes be expressed. The advantage of regulating the virulence genes in this manner is the ability to avoid detection by the immune system of the host while cell numbers are low. It can be compared to an army waiting to show its true strength only after all the troops have arrived to the scene. By the time the host has realized the strength of the enemy, it is too late to completely defend itself (Greenberg, 1997). This type of control of the expression of virulence determinants can also be found in the process of the infection of plant tissues by *Erwinia carotovora*. When sufficient numbers of the bacteria have colonized, the plant tissue exoenzyme genes are turned on, allowing the cell wall of the plant to be degraded (Fuqua et al., 1994). By allowing a release of nutrients from the breakdown of the cell wall, an opportunity arises for nutritional competitors. It has been found that the *E. carotovora* responds by producing the antibiotic carbapenem. The gene for this antibiotic is under the control of the same autoinducer/LuxR homologue, ExpR, that activates the genes for the exoenzyme production. By producing the antibiotic in conjunction with the exoenzymes, competitors for the plant's nutrients can be reduced (Salmond et al., 1995).

The “working” molecular model of quorum sensing

In addition to *P. aeruginosa* and *E. carotovora*, many other bacteria have been found to express proteins that are similar in sequence and function to the LuxR/LuxI quorum sensing system used by *V. fischeri*. Similarities in amino acid sequence tend to be found in highly conserved regions of the homologous proteins. LuxR consists of 250 amino acid residues and has the primary functions of binding autoinducer and activating transcription of the *lux* operon (Figure 3; Fuqua et al., 1996). The region associated with transcriptional regulation is located in the C-terminal domain of the protein (Choi and Greenberg, 1991), while the region associated with autoinducer recognition is located at the N-terminal domain of the protein (Hanzelka and Greenberg, 1995). The N-terminal region of LuxR is responsible for modulating the activity of the C-terminal domain in response to the binding of autoinducer. Only when autoinducer has bound to the N-terminal domain of LuxR is the C-terminal module able to bind to the regulatory region of the *lux* DNA and activate transcription of the *lux* operon (Choi and Greenberg, 1991). A truncated form of LuxR consisting of the C-terminal domain alone is capable of binding the DNA and of activating the transcription of the appropriate genes in the absence of autoinducer (Stevens and Greenberg, 1997). Binding to the DNA has been thought to occur through a helix-turn-helix motif (residues 196 to 210), while the extreme C-terminal forty amino acid residues of LuxR have been shown to be necessary for the activation of the *lux* operon, and thus bioluminescence (Choi and Greenberg, 1992).

In *V. fischeri*, the primary autoinducer is N-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL or VAI) (Figure 2; Eberhard et al., 1981). The *V. fischeri* autoinducer is synthesized by LuxI, an autoinducer synthase. 3-oxo-hexanoyl-acyl carrier protein and S-adenosyl-methionine have been shown to be the substrates required by LuxI to make the autoinducer (Eberhard et al., 1981; Hanzelka and Greenberg, 1996). The autoinducer signals produced by LuxI homologues in other bacteria are of similar chemical structure (Swift et al., 1996); all have the form of an N-acyl-L-homoserine lactone with slight differences in their acyl sidechains. Not all bacteria

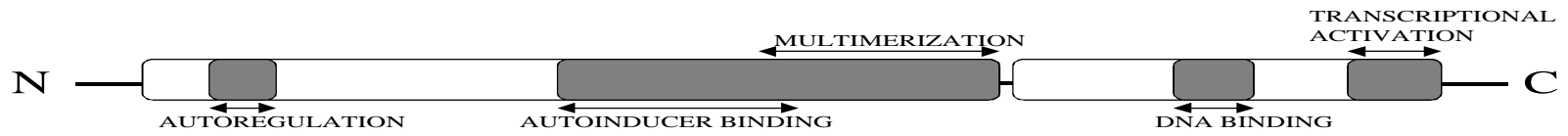


Figure 3: Model of the important functional regions of LuxR (based on Figure 2 in Stevens & Greenberg, 1999).

involved in quorum sensing produce a distinct N-acyl-L-homoserine lactone signal; many release signals that are structurally identical, but differ in the phenotype that they induce. Likewise, some bacteria may produce more than one structural type of autoinducer, with each signal affecting expression of a different phenotype (Swift et al., 1996). The amino terminal region of LuxR homologues can distinguish between their cognate autoinducers and the autoinducers of other bacteria that vary in chemical structure. It has been found that changes in either chain length or the homoserine lactone ring affect the activity of the LuxR protein (Fuqua et al., 1996; Schaefer et al., 1996).

The arrangement of the *lux* genes in *V. fischeri* is such that *luxI* and *luxR* are divergently transcribed (Figure 4). Although this is the most common gene arrangement in quorum sensing systems, other arrangements of the *luxR/luxI* homologues exist. For example, some systems have the 3' ends of these genes overlapping, while others are transcribed in the same direction (Fuqua et al., 1996). In *V. fischeri*, a region called the *lux* box has been found to be important for the regulation of the transcription of both *luxR* and *luxI*. Although full-length LuxR has not been purified, it has been possible to purify the C-terminal domain of LuxR, LuxR_N. The *lux* box region, centered at -42.5 bp from the transcription start site of *luxI*, is thought to be bound by LuxR in the presence of the *V. fischeri* autoinducer. In *in vitro* studies using the LuxR C-terminal domain, it was determined that LuxR_N and RNA polymerase must both be present to have DNaseI protection of this *lux* box site (Stevens et al., 1994). It has been suggested that LuxR may bind to RNA polymerase in solution, thus facilitating binding of the two proteins to the *lux* box and the *lux* promoter (Stevens and Greenberg, 1997). However, recent genetic evidence has demonstrated the ability of LuxR to bind specifically to the *lux* box while functioning as an artificial repressor of the transcription a downstream reporter gene (England and Greenberg, 2000).

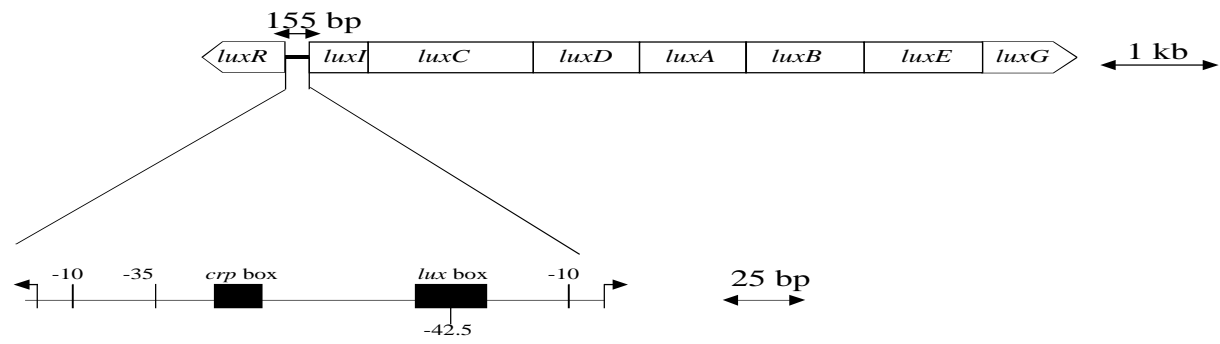


Figure 4: Arrangement of the *Vibrio fischeri* *lux* genes (based on Figure 1 in Stevens et al., 1994).

Analysis of the quorum sensing transcription factor LuxR

Previous studies in recombinant *Escherichia coli* involving a deletion mutagenesis analysis of the C-terminal domain of LuxR has identified a region of approximately forty amino acids critical for LuxR's ability to activate transcription of the *lux* operon. A truncated form of LuxR, containing a deletion of the C-terminal forty amino acids is unable to activate transcription of the *lux* operon. This form of LuxR is thought to still be capable of binding to the DNA since it retains its function as an autoregulator (Dunlap and Ray, 1989). Truncations larger than forty amino acids result in the loss of the autoregulatory phenotype and therefore presumably the ability to bind to the DNA. This result is supported by amino acid sequence analysis that has identified a helix-turn-helix motif (residues 196 to 210) adjacent to the C-terminal forty amino acid region believed to be critical for transcriptional activation (Choi and Greenberg, 1992). Information gathered from a random mutagenesis of LuxR likewise supports the hypothesis that the C-terminal domain contains regions important for DNA binding and/or transcriptional activation. Single amino acid point substitutions in the C-terminal domain have identified variant forms of LuxR that can be placed in two categories: mutations that affect LuxR's ability to activate transcription of the *lux* operon (Shadel et al., 1990; Slock et al., 1990) and mutations that result in a form of LuxR that is capable of autoinducer-independent activation of transcription of the *lux* operon (Poellinger et al., 1995; Sitnikov et al., 1996). Some mutations of both types fall in the 40 amino acid region speculated to be important for transcriptional activation.

The goal of this research project was to determine which amino acid residues in the C-terminal forty amino acid residues of LuxR are critical for transcriptional activation of the *lux* operon. A polymerase chain reaction (PCR)-based site-directed mutagenesis procedure was used to generate a set of thirty-eight alanine substitution mutants in the C-terminal forty-one amino acids of LuxR. To determine the effects of the mutations, *in vivo* assays were used to measure the ability of the variant forms of LuxR to activate transcription of the *lux* operon (luminescence

assay). Mutants unable to activate transcription of the *lux* operon resulted in cells emitting little or no light. The identification of mutants exhibiting this “dark” phenotype were verified directly through the measurement of the luciferase enzyme found within cell extracts of strains expressing the variant forms of LuxR (luciferase assay). Since a “dark” phenotype could be the result of a mutation that either affects an amino acid residue critical for DNA binding or formation of the complex necessary for transcriptional activation, a third assay (DNA binding/repression assay) was used to measure the ability of the variant forms of LuxR to bind to the region of DNA found at the promoter of the *lux* operon, termed the *lux* box. LuxR variants exhibiting the “dark” phenotype in the luminescence and luciferase assays that retain the ability to bind to the *lux* box have a mutation that affects an amino acid residue critical for formation of the transcriptional complex that activates expression of the *lux* operon. LuxR variants exhibiting the “dark” phenotype that are unable to bind to the *lux* box have a mutation that affects an amino acid residue critical for DNA binding/recognition at the promoter of the *lux* operon.

A better understanding of the interactions occurring between LuxR and RNA polymerase at the *lux* operon promoter will likely be applicable to the interactions seen in homologous quorum sensing systems. Furthermore, amino acid sequence alignments of LuxR with some of its homologues, have identified several positions in the C-terminal forty amino acids of LuxR that are identical or functionally similar to amino acids found at the same position in many of the other LuxR homologues (Stevens and Greenberg, 1999b). Since many of the LuxR homologues are responsible for the transcriptional activation of genes important for symbiotic or pathogenic interactions of microorganisms with plant or animal hosts, the knowledge gained from studying the mechanism of transcriptional activation of the *lux* operon in *V. fischeri* has potential medical and agricultural importance. Future research may focus on methods to disrupt or enhance the interactions of the LuxR homologues with RNA polymerase at the promoter region as a method of controlling the phenotypes expressed by microorganisms in these other quorum sensing systems.

CHAPTER TWO

MATERIALS AND METHODS

Bacterial strains and plasmids

See Table 1 for descriptions of bacterial strains and plasmids used in this study.

Primer design

See Tables 2 and 3 for descriptions of primer sequences used in this study (Sigma-Genosys, The Woodlands, TX).

Two-primer PCR

Each of the 100 μ l PCR reactions for the mutagenesis of amino acid residues 246-250 contained a final concentration of the following reagents: 2 μ M of one specific mutant primer, 2 μ M XBA200 primer (Sigma-Genosys, The Woodlands, TX), 2 mM dNTPs (Promega, Madison, WI), 2.5 units *Taq*2000 Polymerase (Stratagene, La Jolla, CA), 1X *Taq*2000 Reaction Buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin) (Stratagene, La Jolla, CA), 2 mM MgSO₄ (Fisher, Springfield, NJ), and 100 ng of linearized pSC300 (Figure 5) template. The PCR reaction for 215 was followed as described above with one modification; the PVU200 primer was substituted for the XBA200 primer. Template DNA for use in the PCR reactions was prepared using the QIAprep miniprep kit (Qiagen, Valencia, CA), followed by restriction enzyme endonuclease digestion with *Pvu*II (Promega, Madison, WI). The linearized DNA was subsequently electrophoresed on a 0.8% agarose gel (BioRad, Hercules, CA) and extracted from the agarose matrix using the QIAquick gel extraction kit (Qiagen, Valencia, CA). A Sprint thermal cycler (Hybaid, Middlesex, UK) was programmed as follows for all PCR reactions: one cycle: 94°C for two minutes; 30 cycles: 94°C for one minute, 46°C for one minute, and 72°C for two minutes; and one cycle: 72°C for ten minutes.

Table 1: Bacterial Strains and Plasmids

Strain or Plasmid	Relevant Characteristics	Source or Reference
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB) F' (traD36 proAB⁺ lacI^q lacZ M15)</i>	Yanisch-Perron et al., 1985
pKK223-3	Cloning vector, <i>ptac</i> , ColE1, Ap ^r	Pharmacia (Piscataway, NJ)
pSC300	<i>luxR</i> cloned into pKK223-3	Choi & Greenberg, 1991
pAT series	Mutant <i>luxR</i> genes cloned into pSC300	This study
pJR551	<i>lux</i> operon, Mu insertion in <i>luxI</i>	Dunlap & Ray, 1989
p35LB10	<i>lacZ</i> controlled by a <i>lux</i> box-containing promoter, Sm ^r /Sp ^r , Gm ^r	Egland & Greenberg, 2000

Table 2: Sequences of mutagenic primers

Residue of Alanine Substitution	Mutant Primer Sequence (5' to 3') ^a	Restriction Endonuclease Site Change
210	GGCTGT <u>G</u> CTGAGCGTACTGTCAC <u>T</u> TTCC	Loss of <i>Pst</i> I site
211	GGCTGTAGT <u>G</u> CGCTACTGTCAC <u>T</u> TTCC	Loss of <i>Pst</i> I site
212	GGCTGTAGT <u>G</u> AGGCTACTGTCAC <u>T</u> TTCC	Loss of <i>Pst</i> I site
213	GGCTGTAGT <u>G</u> AGCGT <u>G</u> CTGTCAC <u>T</u> TTCC	Loss of <i>Pst</i> I site
214	GGCTGTAGT <u>G</u> AGCGTACTG <u>C</u> GACTTTCC	Loss of <i>Pst</i> I site
215	TAGGCTGCAGT <u>G</u> AGCGTACTGTCG <u>C</u> TTTCC	No Change
216	GGCTGTAGT <u>G</u> AGCGTACTGTCAC <u>T</u> GCGCATTTAACC	Loss of <i>Pst</i> I site
217	GGCTGTAGT <u>G</u> AGCGTACTGTCAC <u>T</u> TCGCATTTAACC	Loss of <i>Pst</i> I site
218	GGCTGTAGT <u>G</u> AGCGTACTGTCAC <u>T</u> TCCATGCAACC	Loss of <i>Pst</i> I site
219	TTAGC <u>A</u> AATGCTCAAATGAAAC	Loss of <i>Fsp</i> I site
220	TTAACCG <u>C</u> AGCGCAAATGAAAC	Loss of <i>Fsp</i> I site
222	TTAACCAATGCG <u>G</u> CAATGAAAC	Loss of <i>Fsp</i> I site
223	TTAACCAATGCTCAAGC <u>G</u> AAAC	Loss of <i>Fsp</i> I site
224	TTAACCAATGCTCAAATG <u>G</u> CACTC	Loss of <i>Fsp</i> I site
225	GAAAGC <u>A</u> AATACAACAAACCGTTGCC	Loss of <i>Msp</i> AI site
226	GAAACTC <u>G</u> CTACAACAAACCGTTGCC	Loss of <i>Msp</i> AI site
227	GAAACTCAATGCAACAAACCGTTGCC	Loss of <i>Msp</i> AI site
228	CTCAATACAGCAAACCGTTGCC	Loss of <i>Msp</i> AI site
229	CTCAATACAACAG <u>C</u> GCGCTGCC	Loss of <i>Msp</i> AI site
230	CTCAATACAACAAAC <u>G</u> CTTGCC	Loss of <i>Msp</i> AI site
231	ACAAACCG <u>C</u> GCACAAAGTATTTTC	Loss of <i>Msp</i> AI site
232	ACAAACCGTTGCGCAAGTATTTTC	Loss of <i>Msp</i> AI site
233	ACCGTTGCCAAGCTATTTCTAAAGC	Loss of <i>Msp</i> AI site
234	ACAAACCGTTGCCAAAGTGCATCTAAAG	Loss of <i>Msp</i> AI site
235	ACAAACCGTTGCCAAAGTATTGCTAAAG	Loss of <i>Msp</i> AI site
236	ACCGTTGCCAAAGTATTTCTGCAGCAATTTTAAC	Loss of <i>Msp</i> AI site
238	TCTAAAGCAGCTTTAACAGGAGCAATCGATTGCCATA	Add a <i>Cla</i> I site
239	CAATTGCAACAGGAGCAATCGATTGCCATA	Add a <i>Cla</i> I site
240	CAATTTTAGCAGGAGCAATCGATTGCCATA	Add a <i>Cla</i> I site
241	CAATTTAACAGCAGCAATCGATTGCCATA	Add a <i>Cla</i> I site
243	TTAACAGGAGCAGCTGATTGCCATA	Add a <i>Pvu</i> II site
244	CAATTGCTTGCCATACTTCAAAAATT	Loss of <i>Dra</i> I site
245	CAATTGATGCGCCATACTTCAAAAATT	Loss of <i>Dra</i> I site
246	TCCCCGGGCTATTAATTTTAAAGTATGCGCAATC	No change
247	TCCCCGGGCTATTAATTTTAAAAGCTGGGC	No change
248	TCCCCGGGCTATTAATTTTAGCGTATGG	No change
249	TCCCCGGGCTATTAATTGCAAAGTATGG	No change
250	TCCCCGGGCTATTAAGCTTTAAAGTATGG	No change

^aUnderlined nucleotides are changes made in *luxR* to code for an alanine residue at the specified location. Boldfaced nucleotides are changes made in *luxR* for the addition or deletion of the indicated restriction site.

Table 3: Sequences of non-mutagenic primers

Primer Name	Primer Sequence (5' to 3')
PVU200	GAAGTGGTCCTGCAACTTTATCC
XBA200	CGTATAATGTGTGGAATTGTGAGCG
SEQVEC	GCTGAAAATCTTCTCTCATCC
SEQINT	GTTGTCTTTTTCTGAATGTGC
SEQPRO	GTATGGCTGTGCAGGTCGTAAATC
SEQINT2	ATGTAATTAAAGAAGCGAAAAC

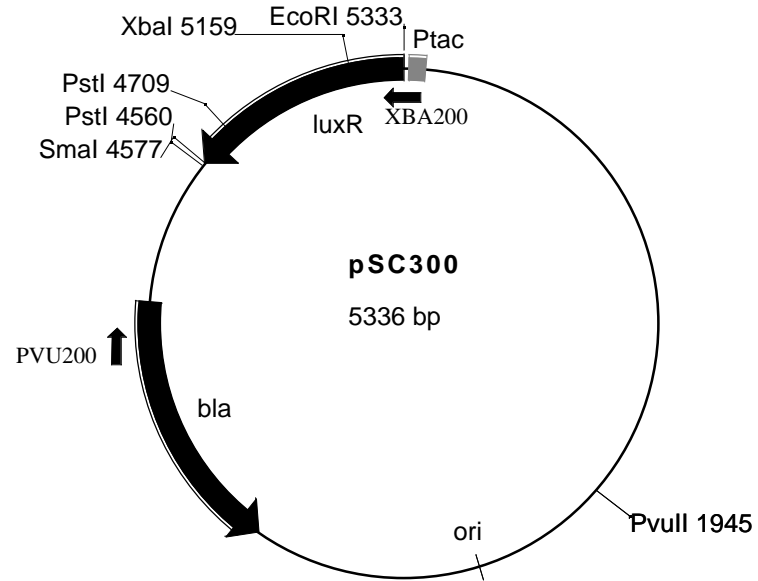


Figure 5: Diagram illustrating relevant characteristics of pSC300 (Choi and Greenberg, 1991) and the annealing sites of the PVU200 and XBA200 primers.

Three-primer PCR

The procedure followed was based on the method of Michael (1994). Each of the mutagenic primers designed for the mutagenesis of amino acid residues 210-214, 216-220, 222-236, 238-241, and 243-245 were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) prior to their addition to the PCR reactions. The phosphorylation reaction [200 pmole primer, 1X Reaction Buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol) 1.7 mM ATP, and 10 units T4 polynucleotide kinase (New England BioLabs, Beverly, MA)] was incubated at 37°C for 30 minutes, followed by heat inactivation of the kinase at 65°C for twenty minutes. The entire phosphorylation reaction was added directly to the 100 µl PCR reaction containing the final concentration of the following reagents: 2 µM XBA200 primer, 2 µM PVU200 primer, 2 mM dNTPs, 2.5 units *Taq*2000 Polymerase, 0.7 X *Taq*2000 Reaction Buffer, 40 units *Taq* DNA ligase (New England Biolabs, Beverly, MA), 2 mM MgSO₄, and 100 ng of linearized pSC300 template. Template preparation and thermal cycler conditions were identical to those used for the “Two-Primer PCR”.

Cloning

The PCR reactions for alanine substitutions at positions 210-214, 216-220, 222-236, 238-241, 243-250, and 246-250 were purified directly using the Wizard PCR purification kit (Promega, Madison, WI) and subsequently digested with the *Sma*I restriction endonuclease (New England Biolabs, Beverly, MA). After heat inactivation of the *Sma*I for twenty minutes at 65°C, the DNA was digested with *Xba*I (New England Biolabs, Beverly, MA). The digests were electrophoresed on a 0.8% agarose gel and the appropriate band was extracted using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The cloning vector, pSC300, was prepared using the QIAprep miniprep kit, followed by *Sma*I and *Xba*I digestion as described above. The digest was electrophoresed on a 0.8% agarose gel and the vector was extracted using the QIAquick gel extraction kit. Ligation of the PCR product into the cloning vector was completed using T4 DNA ligase (New England Biolabs, Beverly, MA) as per the manufacturer's

instructions. The cloning of the 215 PCR product to introduce the mutation at residue 215 into pSC300 was followed using the methods described above with the following changes. The *SmaI/XbaI* digestion for both pSC300 and the PCR product was replaced by a digestion with *PstI* (New England BioLabs, Beverly, MA). The vector was subsequently dephosphorylated with Calf Intestinal Phosphatase (New England Biolabs, Beverly, MA) prior to the gel extraction procedure.

Ligation reactions for all mutants were transformed into *E. coli* strain JM109 and plated on Luria-Bertani (LB) agar containing 100 µg/ml ampicillin. Plasmid DNA derived from both the two-primer PCR method and the three-primer PCR methods was prepared from the transformants using an alkaline-lysis miniprep procedure (Sambrook et al., 1989). Since not all clones of the three-primer method that contain the correct size insert contain the desired mutation, plasmid DNA from the potential mutant clones was screened on the basis of the specific restriction site addition/deletion incorporated by the mutant primer (Table 2). All clones of the two-primer method containing the insert of the correct size should contain the desired mutation, thus it was only necessary for plasmid DNA to be screened for the ligation of the correct size insert into pSC300. The first plasmid identified to contain the desired nucleotide changes to encode for an alanine residue at position 243 was named pAT243A. This method of naming the plasmids containing the mutant forms of *luxR* was followed for all the *luxR* clones obtained by the three-primer and two-primer methods.

DNA sequencing

Plasmid DNA of clones identified as containing the desired mutation was prepared using the QIAprep Miniprep kit. The entire *luxR* gene and promoter region was sequenced on both strands to verify that no other second site mutations were generated during the PCR and cloning steps of the mutagenesis procedure. All sequencing was completed at the Virginia Tech DNA Sequencing facility using the SEQVEC, SEQINT, SEQPRO, AND SEQINT2 sequencing primers (Table 3).

Second site mutations

Due to undesired second site mutations in *luxR* identified during the sequencing of pAT223A and pAT250A, the portion of *luxR* not containing the second site mutation was recloned into the *Pst*I sites of pSC300 and sequenced as described above.

Luminescence assays

Plasmids containing the correct *luxR* mutation were transformed into *E. coli* JM109 pJR551 (Dunlap and Greenberg, 1985) and selected on LB agar containing ampicillin (100 µg/ml) and chloramphenicol (30µg/ml). Strains were grown in triplicate using the following protocol. Overnight cultures were grown at 30°C in LB containing the appropriate antibiotics and 200 nM *Vibrio fischeri* autoinducer, N-(3-oxohexanoyl) homoserine lactone (VAI), (Sigma, St Louis, MO) to an optical density at 600 nm (OD₆₀₀) of 0.200-1.00. The overnight cultures were subcultured into media containing the appropriate antibiotics and VAI to an OD₆₀₀ of 0.025. These subcultures were subsequently grown at 30°C to a final OD₆₀₀ of 0.500. Luminescence output was measured over a four second integration period with a Turner 20/20 luminometer (Turner, Sunnyvale, CA) using 10 µl of culture. Cell pellets were obtained from the centrifugation of 0.5 ml aliquots of each culture and careful removal of the supernatant. The pellets to be used for the luciferase assays were stored at -70°C, while the pellets to be used for SDS-PAGE and Western immunoblotting were stored at -20°C. Luminescence assays were also performed in the absence of autoinducer to determine 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. Since the mutation in *luxI* encoded on pJR551 is a temperature sensitive mutation that will allow LuxI to synthesize autoinducer below 30°C, the strains were grown in duplicate at 31°C in the absence of exogenous autoinducer. All other conditions of the luminescence assay were performed as described above.

Luciferase assays

The procedure followed was based on the method of Dunlap and Greenberg (1985). One frozen cell pellet of each mutant, obtained as described above in “Luminescence Assays”, was resuspended in 0.5 ml of lysis buffer (10 mM KPO₄ pH 7.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.1% bovine serum albumin (BSA), 50 µg/ml lysozyme) and placed at -70°C for forty-five minutes. The pellets were allowed to thaw at room temperature and then immediately placed on ice. Ten milliliters of a 1:1000 dilution of the decanal substrate was prepared by sonication at 30 second intervals for a total pulse time of three minutes. Fifty milliliters of a 50 mM stock of flavin mononucleotide (FMN) was reduced in an anaerobic container using a combination of platinum pellets and hydrogen gas. Each luciferase reaction contained final volumes of the following reagents: 10 µl of the crude cell extract, 10 µl of decanal, 90 µl of assay buffer (10 mM KPO₄, 0.1% BSA, 1 mM DTT), and 100 µl of reduced FMNH₂. The FMNH₂ was added directly to the tube containing the other three items with a Hamilton syringe after the tube was placed within the chamber of the luminometer. The luminescence emitted from the reaction was measured (3 sec delay, 30 sec integration time) using a Turner 20/20 luminometer with a specially modified manual injection port. The data obtained for each mutant is a result of duplicate trials in which each trial consisted of individual luciferase reactions performed in quadruplicate.

DNA binding/repression assays

Plasmids encoding for the correct mutant *luxR* mutation were transformed into JM109 p35LB10 (Egland and Greenberg, 2000) and selected on media containing spectinomycin (100 µg/ml), ampicillin (100 µg/ml), and gentamycin (10 µg/ml). The data obtained for each mutant is a result of duplicate trials in which each trial consisted of individual β-galactosidase reactions performed in triplicate. The assay was performed based on conditions described in Egland and Greenberg (2000). To obtain cell extracts for the assay, overnight cultures were grown at 30°C in LB containing the appropriate antibiotic to an OD₆₀₀ of 0.200-1.00. Each overnight culture was

subcultured to an OD_{600} of 0.025 into two sets of LB media containing the appropriate antibiotics. One of the two sets of media also contained 200 nM VAI. Both sets of subcultures were grown at 30°C to a final OD_{600} of 0.500 and subsequently placed on ice. A small volume of each subculture (5 μ l) was diluted 1:200 in Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 400 nM DTT) and lysed using 50 μ l of chloroform. - galactosidase levels of each mutant was measured using the Tropix chemiluminescent reporter assay kit (Tropix, Bedford, MA) and a Lucy microtiter dish luminometer (Anthos, Wals, Austria) as described in Eglund and Greenberg (2000).

Western immunoblotting

Expression levels of the mutant forms of LuxR was measured using western immunoblotting techniques as described in Brahamsha and Greenberg (1988). Cell pellets for the procedure were obtained as described above in “Luminescence Assay”. The resolving gel contained a acrylamide concentration of 12%. LuxR primary rabbit antiserum was used at a dilution of 1:1000 (Slock et al., 1990) to probe a nitrocellulose blot using a colorimetric detection system.

CHAPTER THREE

RESULTS AND DISCUSSION

Identifying alanine substitution variants of LuxR defective in transcriptional activation of the *lux* operon

The effect of each of the alanine substitutions constructed for this study on the ability of LuxR to activate transcription of the *lux* operon was determined *in vivo* using the reporter plasmid pJR551 (Dunlap and Ray, 1989) and each of the plasmids in the pAT series in recombinant *Escherichia coli*. Of the 38 strains encoding the alanine substitution mutants tested in this study, seven (residues 212, 217, 225, 229, 230, 238, and 243) were found to emit 2% or less of the levels of luminescence observed with wildtype LuxR, and one (residue 216) was identified to emit approximately 30% of the wildtype levels of luminescence (Figure 6). Those mutant forms of LuxR found in this assay to stimulate cellular luminescence at levels less than 50% the wildtype control were considered to have a significant defect in transcriptional activation of the *lux* operon.

Since it has been shown that several factors such as glucose, iron, and oxygen levels can influence the amount of luminescence emitted from *V. fischeri* (Ruby and Nealson, 1976; Haygood and Nealson, 1985; Nealson and Hastings, 1977), the quantity of luciferase found in cell extracts obtained from the luminescence assay was directly measured via luciferase assays. Similar to measuring cellular β -galactosidase, the levels of luciferase found within the cell can be quantitated as a measure of transcriptional activation from the promoter of the *lux* operon. All seven of the mutants (residues 212, 217, 225, 229, 230, 238, 243) that emitted 2% or less of the wildtype level of luminescence in the luminescence assay were found to have 10% or less of the wildtype levels of luciferase (Figure 7). The one mutant (residue 216) found to emit 30% of the wildtype level of luminescence was found to have less than 40% the wildtype level of luciferase.

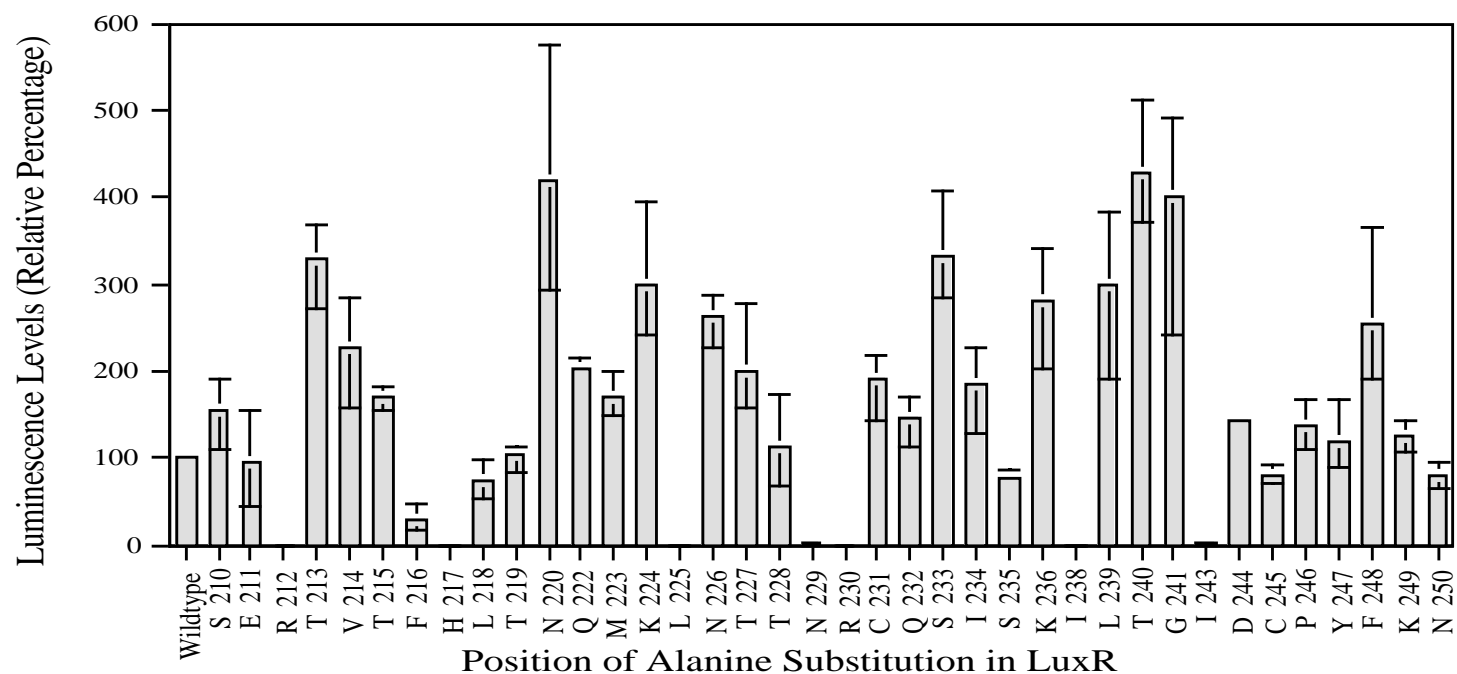


Figure 6: Effects of alanine substitutions on LuxR-dependent cellular luminescence in *E. coli*. The value for each alanine substitution mutant represents the average of three independent experiments. The error bars represent the range of the highest and lowest value from the mean. The wildtype LuxR (pSC300) value, per 10 μ l culture at an OD₆₀₀ of 0.500, was set at 100% for each experiment. The average value for the negative control, pKK223-3 (not shown), was less than 0.01% wildtype levels of luminescence. The letter preceding each alanine substitution position indicates an abbreviation for the amino acid residue at that position in the wildtype sequence.

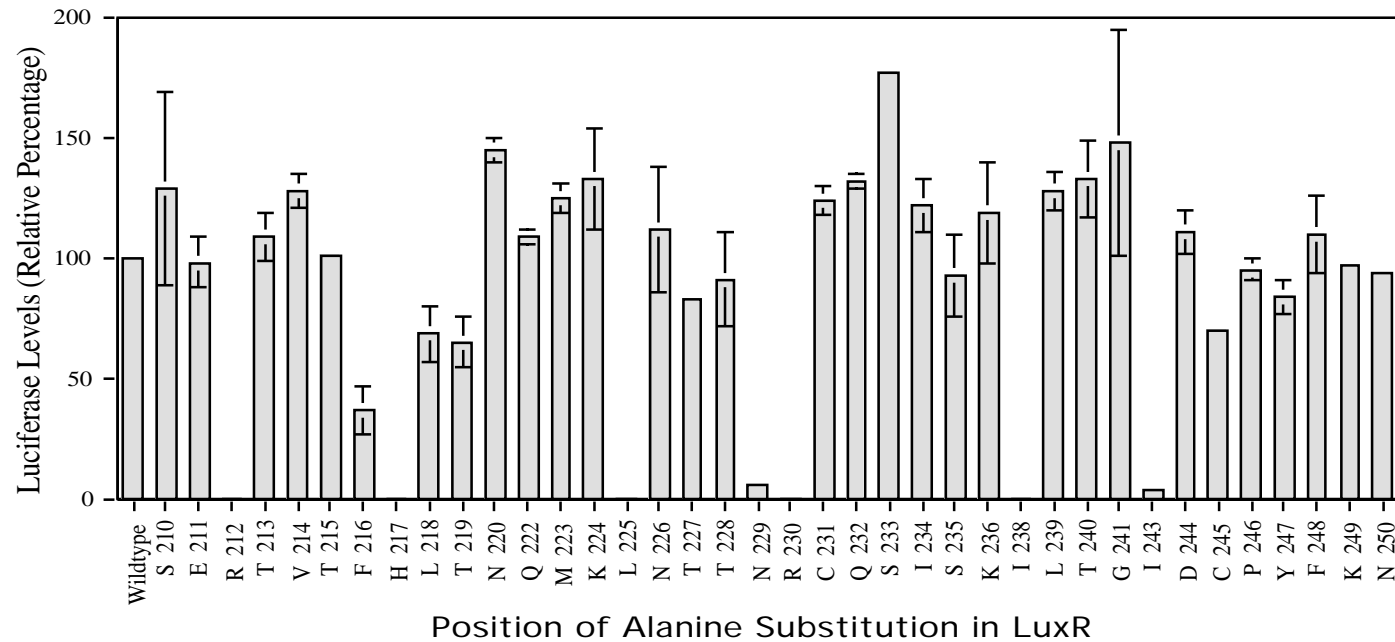


Figure 7: Effects of alanine substitutions on LuxR-dependent cellular luciferase levels in *E. coli*. The value for each alanine substitution mutant represents the average of two independent experiments each performed in triplicate. The error bars represent the range of each experiment from the mean. The wildtype LuxR (pSC300) value, per 10 μ l culture at an OD₆₀₀ of 0.500, was set at 100% for each experiment. The negative control, pKK223-3 (not shown), exhibited less than 0.3% wildtype levels of luciferase. The letter preceding each alanine substitution position indicates an abbreviation for the amino acid residue at that position in the wildtype sequence.

As in the luminescence assay, values of less than 50% the wildtype levels of luciferase were considered to be statistically significant. It can be concluded from the results of the luciferase assay that the alanine substitutions made at these eight positions (212, 216, 217, 225, 229, 230, 238, 243, 250) affect LuxR's ability to activate transcription of the *lux* operon and thus exhibit a "dark" phenotype. However, based on the luminescence and luciferase assays, it can not be determined if this "dark" phenotype is the result of a mutation affecting DNA binding/recognition or protein-protein interactions with RNA polymerase (RNAP) critical for transcriptional activation.

Do any of the alanine substitutions in LuxR result in an enhanced ability to activate transcription of the *lux* operon?

The luminescence assay also identified several strains encoding alanine substitution mutants that emitted luminescence greater than 200% that of wildtype (residues 213, 214, 220, 222, 224, 226, 233, 236, 239, 240, 241, and 248) (Figure 6). However, the levels of luciferase (Figure 7) for these same mutants indicated cellular luciferase levels less than 200% that of wildtype. The luciferase assay is a more accurate reflection of the levels of transcriptional activation at the promoter of the *lux* operon. The values obtained in this assay show a strong correlation to the luminescence assay, but are less than what we consider statistically significant (< 200%). Therefore, we conclude that none of the alanine substitutions made in LuxR result in an enhanced ability to activate transcription of the *lux* operon. We hypothesize that the levels of luminescence observed with the strains expressing these mutant forms of LuxR may have been affected by cellular processes that enhanced the luminescence reaction.

Do any of the alanine substitutions in LuxR result in its ability to activate transcription of the *lux* operon independent of autoinducer?

Previous random mutagenesis of the C-terminal domain of LuxR has identified single amino acid substitutions that allow LuxR to activate transcription of the *lux* operon independent of autoinducer (Poellinger et al., 1995; Sitnikov et al., 1996). Since several of these substitutions

are located within the C-terminal forty-one amino acid residues of LuxR, we tested all thirty-eight of the alanine substitution mutants for the autoinducer-independent phenotype in the luminescence assay. None of the alanine substitutions were shown to exhibit this phenotype (data not shown).

Are the altered forms of LuxR expressed at levels equivalent to the wildtype?

In order to verify that the results obtained in the luminescence and luciferase assay were not due to a significant difference in the levels of mutant LuxR expression versus the wildtype, western immunoblotting analysis was performed in triplicate for all thirty-eight LuxR mutants. The western immunoblot of the strains exhibiting the “dark” phenotype is shown in Figure 8. As quantified in one trial, expression levels for all the thirty-eight mutants were within approximately two-fold that of wildtype LuxR (data not shown). Therefore, the thirty-eight mutant forms of LuxR are expressed at stable levels within *E. coli*, and none have any apparent truncations.

Do the alanine substitutions in LuxR affect its ability to bind to the *lux* box?

The effect of the alanine substitution mutations on the ability of LuxR to bind to the *lux* box was determined *in vivo* using the reporter plasmid p35LB10 (Egland and Greenberg, 2000) and each of the thirty-eight plasmids in the pAT series in recombinant *E. coli*. The p35LB10 plasmid contains the *lacZ* gene fused to the *E. coli* consensus -10 and -35 sites. The sequence between these two sites has been replaced by the *lux* box sequence. Binding of wildtype LuxR to the *lux* box in the presence of autoinducer represses transcription of *lacZ*. If a mutation affects LuxR's ability to bind to the *lux* box, then this form of LuxR is unable to repress transcription of *lacZ*, resulting in high levels of cellular β -galactosidase. LuxR mutants exhibiting less than 50% wildtype levels of repression are considered to have an alanine substitution mutation that affects the ability of LuxR to bind to the *lux* box. Results of the DNA binding assay indicated that nineteen of the thirty-eight mutants had > 50% and < 200% wildtype levels of repression

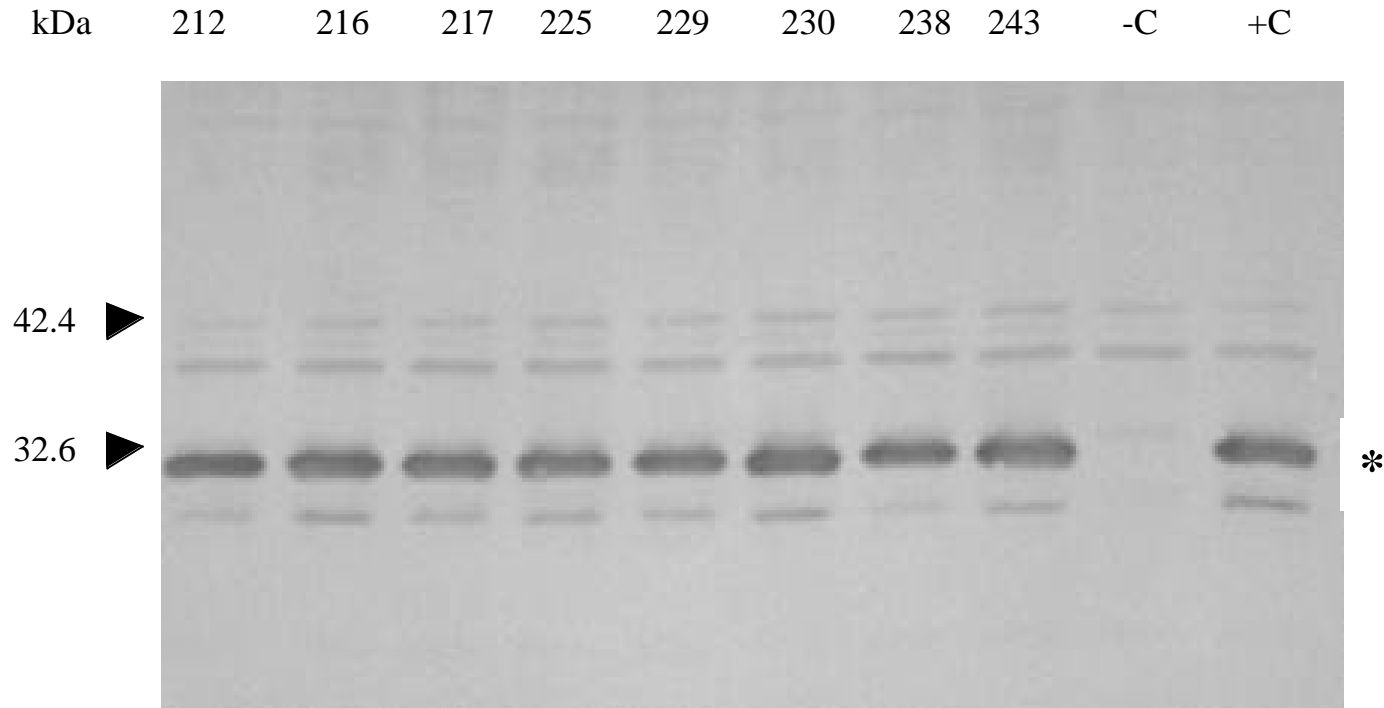


Figure 8: Western immunoblot of cell extracts from strains exhibiting the “dark” phenotype (< 50% luminescence/luciferase levels of wildtype). The LuxR band is highlighted with an asterisk (*) on the right. The mobility of molecular weight size standards is indicated by arrow heads. The position of the alanine substitution in LuxR is given at the top. The positive control “+C” lane illustrates wildtype LuxR expressed from pSC300. The negative control “-C” lane illustrates the negative/vector control (pKK223-3).

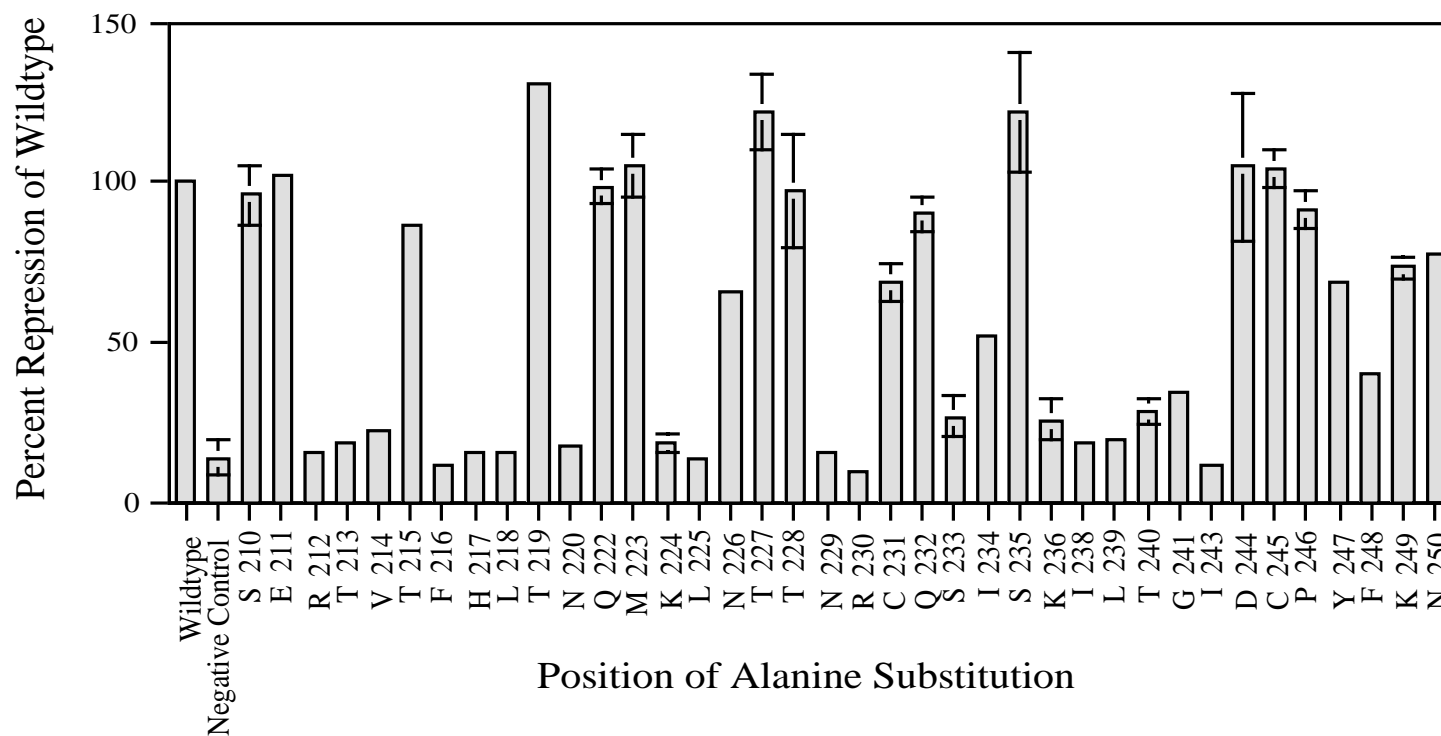


Figure 9: Effects of alanine substitutions on the ability of LuxR to bind to the *lux* box in *E. coli*. The value for each alanine substitution mutant represents the average of two independent experiments each performed in triplicate. The error bars represent the range of each experiment from the mean. The wildtype value (pSC300), per 5 μ l culture at an OD₆₀₀ of 0.500 diluted 1:200, was set at 100% for each experiment with the actual average being equivalent to 7.48 ± 0.75 fold repression for all experiments. The negative/vector control (pKK223-3) value shown in the graph is the average value from all experiments. The letter preceding each alanine substitution position indicates an abbreviation for the amino acid residue at that position in the wildtype sequence.

Table 4: Phenotypes observed in strains expressing the LuxR alanine substitutions in the luminescence/luciferase and DNA binding assays

Observed Phenotypes		Position of Alanine Substitution in LuxR
Luminescence & Luciferase Assays^a	DNA Binding/Repression Assay^b	
Luminescent	Repression	210, 211, 215, 219, 222, 223, 226, 227, 228, 231, 232, 234, 235, 244, 245, 246, 247, 249, 250
Luminescent	No Repression	213, 214, 218, 220, 224, 233, 236, 239, 240, 241, 248
Non-luminescent (Dark)	Repression	None
Non-luminescent (Dark)	No Repression	212, 216, 217, 225, 229, 230, 238, 243

^aluminescent (> 50% to < 200% wildtype levels); non-luminescent (< 50% wildtype levels)

^brepression (> 50% to < 200% wildtype levels); no repression (< 50% wildtype levels)

(Figure 9; Table 4). Since these nineteen mutants also exhibited the wildtype luminescent phenotype in the luminescence and luciferase assays, we conclude that the alanine substitutions made at these positions in LuxR do not affect the DNA binding or transcriptional activation functions of the protein. These mutant forms of LuxR are considered to be equivalent to wildtype.

All eight mutants exhibiting the “dark” phenotype in the luminescence and luciferase assays were found to have 20% or less wildtype levels of repression (Figure 9; Table 4). It can be concluded that the alanine substitutions made at these positions affect the ability of LuxR to bind to the *lux* box in the presence and absence of RNA polymerase (RNAP). Interestingly, eleven of the mutants that exhibited the wildtype luminescent phenotype in the luminescence and luciferase assay were also unable to bind to the *lux* box in the DNA binding assay (Figure 9; Table 4). It is hypothesized that these altered forms of LuxR have a defect in DNA binding that can be suppressed through interactions with RNA polymerase (RNAP) at the promoter of the *lux* operon, thus accounting for the observed wildtype luminescent phenotype. The alanine substitutions made at these amino acid residues may be making contacts with RNAP that are important for maintaining the stability of the DNA binding region. Egland and Greenberg (2000) found that LuxR_N, a truncated form of LuxR containing only the C-terminal domain of LuxR (Stevens and Greenberg, 1997), was unable to bind to the *lux* box in the *in vivo* DNA binding assay, even though luminescence and luciferase assays suggest that this truncated form of the protein is capable of transcriptional activation of the *lux* operon equivalent to wildtype LuxR (Choi and Greenberg, 1991). Since purified LuxR_N has been previously shown *in vitro* to require RNAP for binding to the *lux* box at the promoter of the *lux* operon (Stevens and Greenberg, 1997) it is hypothesized that this form of LuxR, as seen with eleven of the alanine substitution mutants in this study (Table 4), has a defect in DNA binding that can be stabilized by interactions with RNAP.

Since the generation of alanine substitutions in LuxR may affect the overall conformation of the protein, it is impossible to conclude from the results of the DNA binding assay whether a defect in DNA binding is truly due to the disruption of a specific DNA-amino acid interaction. The location of the helix-turn-helix (HTH) motif of NarL, a member of the FixJ-LuxR family of transcriptional activators, has been determined via analysis of its crystal structure (Baikalov et al., 1996). Based on this information, the HTH motif of LuxR is predicted to be located between residues 200 and 224 (as opposed to 196-210, Choi and Greenberg, 1992). Of the alanine substitutions made in LuxR that affect its ability to bind to the *lux* box, eight that are found within the predicted HTH motif of LuxR (residues 212, 213, 214, 216, 217, 218, 220, 224). It is possible that the amino acid residues at these positions in LuxR are making specific contacts with the DNA. Future work involving suppressor mutation analysis or *in vitro* studies of DNA binding may be able to confirm these interactions.

One unexpected finding from this work was that none of the alanine substitutions made in the C-terminal forty-one amino acids of LuxR affects its ability to activate transcription of the *lux* operon without affecting DNA binding (Figure 9; Table 4). The expected phenotype for such mutants would be “dark” in the luminescence and luciferase assays, but still capable of repression in the DNA binding assay. This result demonstrates that the C-terminal forty-one amino acids of LuxR do not directly interact with RNAP in a manner required exclusively for transcriptional activation of the *lux* operon via protein-protein interactions.

This is in contradiction to previous observation made by Choi and Greenberg (1992) that truncations of ten to forty amino acids from the C-terminal domain of LuxR results in a form of LuxR capable of binding to the DNA, but not activating transcription. Their findings were based on the assumption that LuxR employs the same mechanism to bind to the DNA at the promoter of the *lux* operon that it does at its own promoter for autorepression. It has been suggested by Sitnikov et al. (1996) that LuxR may bind to its own promoter region during autoregulation via a mechanism different from that used for transcriptional activation of the *lux* operon. *In vitro*

studies of the DNA binding properties of LuxR N also supports this hypothesis (Stevens and Greenberg, 1997). This may explain why the autorepression assay used in Choi and Greenberg (1992) did not identify the significance of the C-terminal forty amino acids in DNA binding at the *lux* operon promoter. It is possible that the C-terminal forty amino acids of LuxR are important for binding at the promoter of the *lux* operon, but not at the promoter of *luxR*. Recent studies testing the LuxR truncations of Choi and Greenberg (1992) in the DNA binding assay developed by Egland and Greenberg (2000) determined that truncations of more than ten amino acids from the C-terminus of LuxR results in the loss of the ability of LuxR to bind to the *lux* box (Egland and Greenberg, 2000). This result supports our finding that the C-terminal forty-one amino acids of LuxR are important for DNA binding at the promoter of the *lux* operon. Since previous research (Stevens and Greenberg, 1999a) has demonstrated a requirement of LuxR for the CTD of RNAP for transcriptional activation of the *lux* operon, we hypothesize that there may be a region upstream of the HTH motif involved in making these essential protein-protein interactions with RNAP. Future alanine-scanning mutagenesis in the C-terminal domain of LuxR may be able to define this region.

In summary, we conclude from this study that the C-terminal forty-amino acids of LuxR have an important role in DNA binding, but are not required exclusively for the protein-protein interactions with RNAP necessary for the positive control mechanism of transcriptional activation of the *lux* operon. Additional work will be necessary to identify the specific interactions between the amino acids in the C-terminal region of LuxR and the *lux* box sequence, as well as to locate alternative regions of the C-terminal domain of LuxR that may be important for protein-protein associations necessary for transcriptional activation. By identifying regions in LuxR that are making contacts with RNAP and the DNA, we hope to more accurately define the mechanism of transcriptional activation used by LuxR. The amino acid sequence similarity of the C-terminal domain of LuxR with its homologues indicates that information gained about

the mechanism of transcriptional activation used by LuxR will be applicable to other quorum sensing systems (Stevens and Greenberg, 1999b).

CHAPTER FOUR

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Virginia Polytechnic Institute and State University, Blacksburg, VA
Microbiology/Immunology option

Secondary Education, June 1993

Clover Hill High School, Chesterfield, VA

**PROFESSIONAL
EXPERIENCE**

Graduate Teaching Assistant

Spring 1999	General Microbiology Laboratory
Fall 1998	Pathogenic Bacteriology Laboratory
Spring 1998	General Microbiology Laboratory
Fall 1997	General Microbiology Laboratory

**HONORS &
AWARDS**

1997	Golden Key Honor Society
1996	Phi Sigma Psi Honor Society

**PROFESSIONAL
MEMBERSHIPS**

1998	American Society for Microbiology
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GRANTS

2000	Travel Fellowship (\$225)
1999	Sigma Xi, Grants-in-Aid of Research (\$678)

PRESENTATIONS

“Amino Acid Residues in LuxR Critical for its Mechanism of
Transcriptional Activation during Quorum Sensing”

Amy E. Trott and Ann M. Stevens

Estes Park, CO

The Wind River Conference on Prokaryotic Biology

June 8, 2000

“Amino Acid Residues in LuxR Critical for its Mechanism of
Transcriptional Activation during Quorum Sensing”

Amy E. Trott and Ann M. Stevens

Virginia Polytechnic Institute and State University, Blacksburg, VA

The Virginia Branch Meeting of the American Society for Microbiology

November 6, 1999

“Bacterial Gene Regulation of Quorum Sensing Systems”

Virginia Polytechnic Institute and State University, Blacksburg, VA

Amy E. Trott and Ann M. Stevens

Microbiology/Immunology Seminar Series

December 2, 1998

CONFERENCES

ATTENDED

The General Meeting of American Society for Microbiology, Atlanta, GA

May 1998

REFERENCES

Available upon request