Structure/Function Analysis of the Quorum-sensing Regulator EsaR from the Plant Pathogen *Pantoea stewartii*

Daniel J. Schu

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Ann M. Stevens, Committee Chair
John G. Jelesko, Committee Member
David L. Popham, Committee Member
Florian D. Schubot, Committee Member
Boris A. Vinatzer, Committee Member

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Abstract

*Pantoea stewartii* subsp. *stewartii* is the causative agent of Stewart’s wilt disease in maize. Disease symptoms develop after the bacteria grow to high cell densities in the plant xylem and secrete an abundance of exopolysaccharide (EPS). EPS production is regulated by quorum sensing. Two regulatory proteins are key to the process of quorum sensing, the LuxI and LuxR homologues EsaI and EsaR. Most LuxR homologues function as activators of transcription in the presence of their cognate acylated homoserine lactone signal (AHL). EsaR utilizes an AHL-response opposite of the majority of the LuxR homologues. EsaR represses EPS production at low cell densities. However, at high cell densities when high concentrations of AHL are present, EsaR is inactivated and derepression of EPS production occurs. The mechanism that enables EsaR to respond to AHL in a manner opposite to that of most LuxR homologues remains elusive. A comparative study of EsaR and the well characterized quorum-sensing regulators LuxR from *Vibrio fischeri* and TraR from *Agrobacterium tumefaciens* was initiated. Previous studies demonstrated that in the absence of AHL, EsaR retains the ability to function as a weak activator of the lux operon in recombinant *Escherichia coli*. This thesis research further characterized the role of EsaR as an activator. Variant forms of EsaR with deletions or single residue substitutions were generated and their ability to regulate transcription was examined *in vivo*. Furthermore, a native EsaR-activated promoter has been identified, which controls expression of a putative regulatory sRNA in *P. stewartii*. 
It is apparent that EsaR functions as a transcription factor at low concentrations of AHL as demonstrated by its ability to inhibit EPS production. At high concentrations, the AHL appears to bind and cause a conformational shift in the protein leading to its inactivation. The second goal of this study was to further elucidate the mechanism by which AHL regulates EsaR. Pulse-chase experiments demonstrated that EsaR is resistant to proteases with or without AHL \textit{in vivo}. Limited proteolytic digestions \textit{in vitro} suggest that the protein does undergo conformational changes in response to AHL. Gel filtration chromatography, sucrose gradient ultracentrifugation, and cross-linking experiments proved that this conformational change does not impact the multimeric state of EsaR.

To better understand the mechanism of regulation by AHL, the final goal of this project was to examine the interactions which result in EsaR-responsiveness to AHL. Several individual amino acid substitutions were identified that cause EsaR to function in an AHL-independent manner, by which variants retain the ability to bind and block gene expression in the presence of AHL. These residues have been mapped onto a homology model of EsaR and their role has been examined \textit{in vitro}. The ability of these EsaR* variants to bind AHL and an analysis of the effects individual mutations have on the overall conformation of the protein was performed.

Overall this study has revealed several unique aspects of the quorum-sensing system in \textit{P. stewartii} whereby gene expression is regulated at both low and high cell density. Studies were also initiated to examine the mechanism of AHL-responsiveness of EsaR. The mechanism by which AHL modulates most LuxR homologues remains elusive. The ability to purify EsaR +/- its cognate AHL may prove critical in elucidating this mechanism.
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# Table of Contents

## Chapter One: Literature review
- Quorum sensing in Gram-negative proteobacteria: 2
- LuxI/LuxR-type quorum sensing: 3
- The quorum-sensing system of *P. stewartii*: regulation at both low and high cell density: 4
- Synthesis and diversity of AHL signals: 6
- The LuxR protein family: 7
- Interactions of LuxR homologues with RNAP: 10
- Quorum quenching: 13
- Research Plan: 14
- References: 16

## Chapter Two: Structure/Function Analysis of the *Pantoea stewartii* Quorum-sensing Regulator EsaR as an Activator of Transcription
- Abstract: 29
- Introduction: 30
- Materials and Methods: 32
  - Bacterial strains and growth conditions: 32
  - Plasmid construction: 32
  - EsaR activation of promoter constructs *in vivo*: 35
  - Assay for repression by EsaR variants *in vivo*: 36
  - Northern blot analysis: 36
  - Primer extension analysis: 37
- Results and Discussion: 38
  - Ability of EsaR to activate promoters with the *esaR* box versus the *lux* box: 38
  - Effect of deletions in EsaR on its ability to regulate transcription: 39
  - Role of individual amino acid residues in positive control by EsaR: 40
  - EsaR-dependent activation of a native promoter in *P. stewartii*: 42
- Acknowledgements: 43
- References: 44

## Chapter Three: Effects of Autoinducer Binding on Structural Properties of the Quorum-sensing Regulator EsaR from *Pantoea stewartii*
- Abstract: 57
- Introduction: 58
- Materials and Methods: 60
  - Measurements of EsaR stability in recombinant
Chapter Four: Characterization of Essential Residues Involved in EsaR AHL-Responsiveness

Abstract 84
Introduction 85
Materials and Methods 88
Purification of histidine (His)$_6$-maltose-binding protein-GLY$_5$- EsaR (HMGE$^*$) proteins 88
Partial in vitro proteolysis 89
In vitro AI binding assays 89
Results and Discussion 92
Selection and purification of EsaR$^*$ variants for in vitro analysis 92
Ability of EsaR$^*$ variants to bind AHL 92
Conformational analysis of EsaR variants via limited proteolytic digestion 93
Concluding remarks 95
Acknowledgements 96
References 97

Chapter Five: Overall Conclusions 104
References 109
# List of Figures

## Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Model of quorum sensing in <em>Vibrio fischeri</em> at high cell densities</td>
<td>22</td>
</tr>
<tr>
<td>1.2</td>
<td>Model of EPS regulation in <em>P. stewartii</em></td>
<td>23</td>
</tr>
<tr>
<td>1.3</td>
<td>Transcriptional regulation during quorum sensing</td>
<td>24</td>
</tr>
<tr>
<td>1.4</td>
<td>EsaR homology model</td>
<td>25</td>
</tr>
<tr>
<td>1.5</td>
<td>The Class II promoter of the <em>V. fischeri lux</em> operon</td>
<td>26</td>
</tr>
<tr>
<td>1.6</td>
<td>A clustal alignment of LasR, LuxR, EsaR, and TraR</td>
<td>27</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Promoter constructs and activation assays</td>
<td>52</td>
</tr>
<tr>
<td>2.2</td>
<td>Expression and activity of EsaR deletion variants</td>
<td>53</td>
</tr>
<tr>
<td>2.3</td>
<td>Examining positive control variants of EsaR</td>
<td>54</td>
</tr>
<tr>
<td>2.4</td>
<td>Analysis on the sRNA divergently transcribed from <em>esaR</em></td>
<td>55</td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>EsaR accumulation and stability in recombinant <em>E. coli</em> SG22163</td>
<td>76</td>
</tr>
<tr>
<td>3.2</td>
<td>Limited <em>in vitro</em> digestion of EsaR by thermolysin</td>
<td>77</td>
</tr>
<tr>
<td>3.3</td>
<td>Limited <em>in vitro</em> digestion of EsaR by trypsin</td>
<td>78</td>
</tr>
<tr>
<td>3.4</td>
<td>Gel filtration chromatography of EsaR + AHL</td>
<td>79</td>
</tr>
<tr>
<td>3.5</td>
<td>Sucrose gradient ultracentrifugation of EsaR + AHL</td>
<td>80</td>
</tr>
<tr>
<td>3.6</td>
<td>Gel filtration chromatography of EsaR without AHL</td>
<td>81</td>
</tr>
<tr>
<td>3.7</td>
<td>Analysis of the oligomeric state of EsaR +/- AHL</td>
<td>82</td>
</tr>
</tbody>
</table>

## Chapter 4

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Position of substitutions in EsaR* variants mapped on the homology model of the N-terminal domain of EsaR</td>
<td>101</td>
</tr>
<tr>
<td>4.2</td>
<td>Ability of EsaR* variants to bind AHL <em>in vitro</em></td>
<td>102</td>
</tr>
<tr>
<td>4.3</td>
<td>Limited proteolytic digestion of EsaR* variants by thermolysin</td>
<td>103</td>
</tr>
</tbody>
</table>
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Strains and plasmids</td>
<td>47</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Primers</td>
<td>49</td>
</tr>
<tr>
<td>Table 2.3 :</td>
<td>Stability and activity of deletion variants of EsaR</td>
<td>51</td>
</tr>
<tr>
<td>Table 4.1:</td>
<td>Characterization of AI-insensitive variants</td>
<td>98</td>
</tr>
<tr>
<td>Table 4.2:</td>
<td>Strains and plasmids used in this study</td>
<td>99</td>
</tr>
</tbody>
</table>
Chapter One

Literature Review
Quorum sensing is a form of bacterial cell-cell communication that allows for a population to function in unison, as a multicellular unit, to initiate a concerted effort. This phenomenon is associated with the regulation of many different activities across a broad spectrum of both Gram-negative and Gram-positive bacteria. This cooperative behavior is coordinated by a small signal molecule termed autoinducer. There is a direct correlation between the density of the population and the concentration of this autoinducer molecule. It is only at a critical concentration that the bacteria will respond in a cooperative manner. Activities under quorum-sensing control include bioluminescence, conjugal plasmid transfer, biofilm maturation, and virulence in numerous bacterial genera (37, 81).

Quorum sensing in Gram-negative proteobacteria

Quorum sensing was first discovered over 25 years ago in two luminous marine Gram-negative proteobacteria, *Vibrio fischeri* and *Vibrio harveyi*. Initially, the majority of research on bioluminescence regulation focused on *V. fischeri* (51). This bacterium colonizes the light organs of certain marine fishes and squids, where it grows to very high cell densities and has the ability to produce light. *V. fischeri* uses a signaling molecule termed autoinducer, which is an acyl homoserine lactone (AHL) (15), to measure the bacterial population density, and at high concentrations induce the light-producing enzyme luciferase (21). Subsequent identification of other AHL-based quorum-sensing systems from the opportunistic human pathogen *Pseudomonas aeruginosa* and the plant pathogens *Erwinia carotovora* and *Agrobacterium tumefaciens* revealed that quorum sensing exists in many species of bacteria (25, 57, 58). To date over 50 species have been recognized to produce AHLs as a means to regulate many processes (76).
**LuxI/LuxR-type quorum sensing**

Two regulatory components are present in the *V. fischeri* quorum-sensing system. Specifically, a protein called LuxI is responsible for the production of the AHL autoinducer signal molecule. A protein called LuxR, which senses and binds the autoinducer, acts as an autoinducer-dependent transcriptional activator of the luciferase or lux operon (21, 67).

As an autoinducer-producing population of *V. fischeri* grows the concentration of autoinducer, which diffuses out of the cell into the environment, increases as a function of cell population density. When the autoinducer reaches a certain level, it interacts with LuxR, and the LuxR-autoinducer complex can then bind to the lux operon promoter and activate transcription of the lux operon (67).

The luminescence structural and regulatory genes in *V. fischeri* are localized to a 9 kb stretch of DNA that consists of two separate transcriptional units, luxR and the luxICDABEG operon (20). The luxI gene encodes the autoinducer synthase that has been shown to be the only *V. fischeri* gene required for the synthesis of 3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) in recombinant *Escherichia coli*. The rest of the genes in the lux operon are required for the emission of bioluminescence. The luxC, luxD, and luxE genes encode proteins involved in the synthesis of the aldehyde substrate for luciferase. luxA and luxB encode the α and β subunits of luciferase. The function of luxG is that of a flavin reductase, supplying reduced flavin mononucleotide (FMN) to the system (53, 68). The luxR gene codes for the LuxR protein, which binds and forms a complex with the autoinducer and subsequently activates the transcription of the lux operon. This creates a positive feedback for autoinducer synthesis and leads to a rapid increase in luminescence normally at the mid-exponential phase of growth in batch culture (20).
A crucial element of the mechanism of activation by LuxR is a 20-bp region of DNA, centered at the -42.5 from the luxI transcription start site. This sequence of DNA has been termed the lux box (11, 24, 29). It has been shown that mutations in the lux box can eliminate the activation of the luxI promoter by LuxR in E. coli (11) (Fig. 1.1).

The quorum-sensing system of *Pantoea stewartii*: regulation at both low and high cell density

In *Pantoea stewartii* subspecies *stewartii*, quorum sensing is involved in the control of virulence. This known plant pathogen is the causative agent of Stewart’s wilt disease in sweetcorn and leaf blight in maize (1). *P. stewartii* is transmitted to the plant by the corn flea beetle, *Chaetocnema pulicaria*. Beetles that harbor these pathogens feed on emerging corn seedlings and introduce the bacteria into the leaves of the plant (56). The bacteria then migrate through the intercellular spaces of the leaves and make their way to the xylem. Once the bacteria reach the xylem, they are capable of growing to high cell density and secrete an abundance of exopolysaccharide (EPS). Production of EPS blocks the corn xylem vessels, leading to vascular collapse and plant wilt (40, 54, 56).

EPS production is under direct control by a two-component system, which is in turn regulated by a cell density-dependent quorum-sensing system. In regard to the two-component system, *P. stewartii* utilizes a multicomponent phosphorelay system to control the function of a response regulator, which is required for activation of genes involved in EPS production. This system initially responds to an unknown external signal by stimulating an outer membrane lipoprotein, RcsF (44). RcsF plays an essential role in transmitting a signal to the membrane-bound sensor kinase, RcsC. Initial experiments on this system suggested that RcsC then directly
phosphorylated a response regulator, but it has now been demonstrated that RcsC transfers the phosphoryl group to an intermediate membrane bound protein known as RcsD. RcsD is responsible for transferring a phosphoryl group to the response regulator, RcsB. RcsB exists within the cytoplasm of *P. stewartii*, and upon phosphorylation a conformation shift occurs within the protein, which allows for interaction with another regulator protein known as RcsA. Once this heterodimer forms, the complex binds to various promoters, which leads to activation of genes involved in EPS production (43).

RcsA is regulated by a cell density-dependent quorum-sensing system that utilizes homologs of the LuxI and LuxR proteins designated EsaI and EsaR (1). At low cell density, which correlates with a low concentration of AHL, EsaR autoregulates itself by binding to a region within its own promoter. It also binds within the promoter of *rcsA* and blocks transcription. At high cell density AHL production reaches a critical concentration allowing for EsaR-AHL complexes to form, which leads to dissociation of EsaR from DNA and expression of both *esaR* and *rcsA*. (6, 43, 46, 47) (Fig. 1.2). A deletion of *esaI* leads to loss of AHL and EPS production, and virulence. A deletion in *esaR* results in an increase in EPS production, independent of AHL concentrations. Therefore, EsaR is a repressor of EPS production at low cell density, but in the presence of certain levels of AHL EPS production is derepressed. Interestingly, strains with an inactivated *esaR* gene are capable of synthesizing EPS at low cell density and appear to be less virulent than the wild-type parent. This suggests that *P. stewartii* uses quorum sensing to delay the expression of EPS during the early stages of infection so that it does not interfere with other mechanisms of pathogenesis (75). Thus in *P. stewartii* AHL production is instrumental to pathogenesis by controlling the timing of the quorum-sensing response.
Synthesis and diversity of AHL signals

The AHL signal molecules are currently the most predominantly identified and studied group of the quorum-sensing signal molecules. Their production is carried out by the AHL synthases, which have been divided into three different families: the LuxI family, the AinS family, and the HdtS family (50). Of these, the LuxI family of synthases appears to be the most prevalent. They have been found in more than 50 species of various proteobacteria (41). AHLs consist of two chemical groups: a homoserine lactone ring, which is conserved among this class of signal molecules, and an acyl side group, which confers specificity of recognition to various bacterial species. The AHL synthase utilizes S-adenosyl-methionine (SAM) to synthesize the homoserine lactone ring, whereas the acyl side chain is generated from lipid metabolism, and donated to the reaction by acyl-carrier proteins (ACP) (55, 62). AHL synthesis is initiated by the binding of SAM to the synthase followed by the acylated ACP, which allows for the generation of an amide bond, and subsequent release of the ACP. Upon release, the homoserine lactone ring is formed and the AHL signal molecule dissociates from the synthase (48).

Structural studies have been performed on two LuxI-homologues, EsaI and LasI, which synthesize 3-oxo-C6-homoserine lactone and 3-oxo-C12-homoserine lactone, respectively (28, 77). In the case of LasI, the binding pocket in the protein allows for a longer acyl chain to be added to the homoserine lactone. In the case of EsaI this binding pocket is blocked by various residues, and therefore the addition of longer acyl groups is prevented. These structural studies therefore demonstrated that variation in acyl side chain length among the LuxI family of synthase corresponds to the size of the binding pocket of the AHL synthase (28, 50, 77). It is this variation in the acyl side chain length and the substituents on it (i.e. keto groups, hydroxyl
groups, and double bonds) that permits recognition by the native LuxR homologue of the quorum-sensing system.

**The LuxR protein family**

The AHL-responsive LuxR homologues are all about 250 amino acids in length, and are composed of two functional domains. The N-terminal domain is involved in AHL-binding, whereas the C-terminal domain contains a helix-turn-helix (HTH) DNA-binding motif that is required for transcription regulation (50). This family of proteins binds to DNA as a dimer. The DNA binding region for these proteins has a dyad symmetry and is around 20 bp in length (11, 19, 23, 80). The LuxR family of proteins can be divided into three classes. The first two classes of proteins are characterized as activators that require AHL in order to bind DNA. RNA polymerase upon binding DNA is recruited to the promoter and the regulated genes are transcribed (38, 50, 60, 64, 85) (Fig. 1.3). The third class of proteins consists of LuxR-homologues that function as repressors. They are also unique in that they bind DNA in the absence of AHL. It is only when AHL associates with this class of proteins that they lose their affinity for DNA (30, 47, 49, 63) (Fig. 1.3).

The class I members of this family are unique in that they require AHL to be present during translation and folding of the protein, in order for the protein to take on a stable and functional conformation. An example of a class I member is TraR from *Agrobacterium tumefaciens* (30). This class of proteins has been characterized as intrinsically unstructured proteins that require ligand binding for a disorder-to-order transition to occur. It has been suggested that a protein which exists in this state may allow for increased ligand binding rates and thereby a faster response to the stimulus, by increasing the likelihood that initial long-range
interactions will occur (65). It has also been suggested that ligand binding during translation and folding of the protein may optimize the specificity of the protein-ligand interaction (14). In the case of TraR, it binds 3-oxo-octanoyl homoserine lactone (3-oxo-C8-HSL), synthesized by TraI, in order to take on a stable and active conformation in vivo (74). Initial experiments examining the stability of TraR demonstrated that the protein was expressed at low levels in recombinant E. coli because it did not accumulate in the absence of its cognate AHL due to degradation by cytoplasmic proteases (85). This was further confirmed with pulse-chase experiments that demonstrated that the Clp and Lon proteases rapidly degraded TraR in the absence of AHL. It was only in the presence of AHL that the protein accumulated in vivo (85). In vitro experiments utilizing trypsin also showed that the purified apo-TraR is rapidly degraded by trypsin, whereas purified TraR-3-oxo-C8 HSL was significantly more resistant to degradation (79, 85). These experiments therefore support the idea that TraR exists in an unfolded and protease-susceptible conformation in the absence of AHL. It is only during folding that its cognate AHL can interact with the protein, which allows for a transition into a stable and functional conformation. Consequently, its crystal structure has been solved only in the presence of its cognate autoinducer, 3-oxo-C8-HSL, and target DNA by two laboratories (72, 83). This structure confirms that TraR functions as a dimer, has a two-domain structure, and that each monomer of TraR binds one molecule of 3-oxo-C8-HSL. Interestingly, in contrast to LuxR, the 3-oxo-C8-HSL was found to bind to TraR almost irreversibly (84, 85).

The class II members of the LuxR family of proteins are unique in that they appear to take on a stable conformation both in the absence and presence of their cognate AHL. However, it is only in the presence of AHL that they can bind DNA. The V. fischeri LuxR protein represents an example of a class II member of the family. It is one of the most studied members
of the family of transcriptional regulators involved in acyl-homoserine lactone-mediated quorum sensing. Current evidence supports the view that the autoinducer binds to the N-terminal domain, which in turn causes activation of the C-terminal domain. Once the autoinducer is bound by the N-terminal domain, LuxR forms a homodimer and its C-terminal domain can then interact with the lux box to activate transcription \(67, 71\). The N-terminal domain may be associated with the inner leaflet of the cytoplasmic membrane in its inactive form \(39\). Until LuxR was finally purified, a fragment of LuxR (LuxR\(\Delta N\)) that contains only the C-terminal domain was used for the first in vitro studies of transcription. If purified LuxR\(\Delta N\) is allowed to interact alone in solution with lux DNA, it binds upstream of the lux box. \(\sigma^{70}\)-RNA polymerase (RNAP) by itself appears to associate with the -10 region of the lux promoter, but does not bind to the lux box or the -35 region of the luxI promoter. However, together they bind to the lux box and the luxI promoter \(66\). LuxR protein has now been shown to form a homodimer after interaction with the autoinducer \(17, 71\). In its full length purified form, LuxR, along with N-(3-oxo-hexanoyl) homoserine lactone (3-oxo-C6-HSL), has been shown to bind specifically and with high affinity to the lux box. From these studies involving purified LuxR, it has been shown that the LuxR-3-oxo-C6-HSL complex can be reversibly inactivated by dilution, which suggests that 3-oxo-C6-HSL is not tightly bound to LuxR \(71\). In certain quorum-sensing model systems, such as \textit{V. fischeri}, this may be representative of a response to a decrease in signal concentrations due to a rapid drop in population density \(71\).

There are over 50 proteins which make up the family of LuxR homologues. The majority of these proteins function as activators similar to TraR and LuxR, but a subset of them has been found to act primarily as repressors. This third class of the protein family is unique in that its members can exist in an active conformation in the absence of their cognate AHL. It is believed
that, in contrast to the class I and class II members of the family, the protein is in a dimeric form in a AHL-free state and assumes a conformation capable of binding DNA. Interestingly, this subclass of proteins contains two unique regions in comparison to the other LuxR homologous: (i) an extended linker region between the AHL-binding N-terminal domain and C-terminal DNA-binding domain and (ii) extra residues at the C-terminus (70) (Fig. 1.4).

One of the best characterized members of the class III of proteins is EsaR from the plant pathogen P. stewartii. As described above, EsaR regulates its own expression and the expression of rcsA, which in turn regulates EPS production. The promoter regions of these two genes contain a sequence of DNA with dyad symmetry showing sequence homology to the lux box centered around a predicted -10 region (6, 47). Using promoter-reporter fusion constructs in vivo, it was revealed that in the absence of the cognate AHL, there were elevated levels of expression from these promoters. In order to confirm binding by EsaR in the absence of AHL, gel retardation assays were performed in which purified EsaR dimerized and bound to DNA without AHL. Surface plasmon resonance (SPR) was then utilized to demonstrate that AHL induces structural changes in EsaR that neutralize its DNA binding affinity (47). It is with these results in mind that a reverse mechanism of AHL regulation has been proposed for the class III LuxR family members. Although EsaR was initially identified as a repressor, it has been shown that it retains an ability to activate transcription by RNA polymerase (RNAP) (73).

**Interactions of LuxR homologues with RNAP**

In the LuxI/LuxR-type system of quorum sensing, in order for transcription to be carried out correctly, LuxR must bind to the lux box centered at the -42.5 position of the luxI promoter,
where it is in a position to make multiple contacts with RNAP (22, 36) (Fig. 1.4). Thus LuxR is an ambidextrous activator at this class II promoter (4).

RNA polymerase has the subunit composition α₂ββ′σ (3, 7) (Fig. 1.5). The α subunits are responsible for interactions with upstream promoter elements and trans-acting factors including a large subset of activators of transcription (4, 16, 33). The α subunit consists of two domains, a N-terminal domain (αNTD) and a C-terminal domain (αCTD). At a class II promoter, the αNTD contains the residues necessary for interaction with the remainder of the RNA polymerase and as well as for interactions with upstream activators on the proximal side (Nui et al., 1996). The αCTD contains residues, which are involved in interactions with the DNA and with activators on their distal side (2, 52). At a class I promoter, the primary interactions between RNAP and the activator occur through the distal interface of the αCTD (34, 35). The β and β’ subunits are responsible for the catalytic activity of RNA polymerase (45). The σ subunit is responsible for recognition and binding of the -35 and -10 sites (31), but can also make interactions with some activators.

An analysis of the protein-protein interactions occurring between RNA polymerase and LuxR was conducted in recombinant E. coli. The σ subunit has two specific regions, region 2 and 4, which are highly conserved and consist of very basic amino acids, supporting the fact that they are involved in DNA binding (5, 12). During activation, region 4 is positioned so that it can interact with upstream transcriptional activators (61). Region 4 of the σ⁷₀ subunit from E. coli was shown to be involved in LuxR-dependent activation of the luxI promoter (36). Furthermore, σ mutants of RNAP illustrated that some individual residues in region 4 have increased and decreased effects on the mechanism of LuxR-dependent transcription initiation (36). It has been hypothesized that these residues may have an effect on the RNA polymerase to
form a complex with the activator, the ability of the complex to bind to the promoter, or the ability of the complex to initiate transcription of the operon (36).

Similar studies were also done on the role of the α subunit C-terminal domain (αCTD) of RNA polymerase in LuxR-dependent transcriptional activation of the lux operon. Through the analysis of RNAP mutants, individual residues in the αCTD were tested to determine if they played a role in activation by significantly decreasing or increasing the rates of transcription as compared to the wild-type. Several amino acids were discovered to have a significant effect on Lux-dependent transcription. Residues 262, 265, 295 and 296 likely play roles in α-LuxR interactions, while residues 290 and 314 are thought to play a role in DNA recognition.

In order to further understand the interactions occurring between the LuxR family of activators and RNAP, a thorough analysis was performed on LuxR and TraR to identify critical residues required for activation. LuxR analysis revealed that when three specific amino acids were changed (K198, W201, I204) to an alanine in the C-terminal domain, these substitutions had a negative effect on its ability to activate transcription (18). At this point no amino acids in the N-terminal domain of LuxR have been reported to interact with RNA polymerase and affect its ability to activate. Indeed, just the CTD of LuxR, LuxRΔN, is capable of activating transcription of the lux operon in an AI-independent manner (8). These results suggest that in the case of LuxR, only the C-terminal domain is required for interactions with RNA polymerase for activation to occur. Alanine-screening mutagenesis performed on TraR revealed that single substitutions at positions W184, V187, K189, E193, V197, and D217 in the C-terminal domain were shown to have a negative effect on its ability to activate transcription (78). In TraR, it has also been reported that two specific amino acids (N10, G123) in the N-terminal region are required for activation (59). Therefore, both the C-terminal and N-terminal
domains are required to for interactions with RNA polymerase. These studies reveal that there is some divergence in regards to the mechanism of activation among the LuxR family of proteins. With this in mind, it is important that other LuxR family members be analyzed to better understand these mechanisms, which are essential for a bacterium to utilize a quorum-sensing response.

**Quorum quenching**

Because quorum-sensing systems are prevalent across a broad spectrum of bacteria, there is increasing interest in determining if methods can be developed to manipulate the bacterial cell-cell communication. Ideally, chemical signals would be added into an environment so that beneficial functions of bacteria might be enhanced or undesired ones eliminated (82). Some naturally occurring cases of organisms with the ability to interfere with or stimulate bacterial cell-cell communication have already been identified (10, 32, 42, 69). The marine red algae, *Delisea pulchra*, produces halogenated furanone signals that prevent bacteria from colonizing its surfaces, presumably due to an inhibition of the quorum-sensing response in the bacteria (27). The Aii proteins found in *Bacillus* species encode AHL inactivating enzymes (13, 26). Some lung epithelial cells and several types of mammalian sera have the capacity to degrade AHL signals (9). However, in order to artificially alter the QS response of bacteria through intelligently designed chemicals/drugs, there must be a complete understanding of the molecular interactions that occur between the autoinducers, regulatory proteins and target DNA within one organism and during cross talk between organisms. The goal of this thesis research is to better understand the mode of action of the quorum-sensing regulator EsaR from *P. stewartii*. Insights gained are likely to reveal possible avenues to control its activity in the future.
Research Plan

The quorum-sensing regulator EsaR from *P. stewartii* is a unique member of the LuxR family of proteins. EsaR responds to AHL in a reverse mechanism compared to the majority of LuxR homologues. EsaR is functional in the absence of AHL, and was originally characterized as a repressor. Recent evidence showed that EsaR has retained the ability to also activate transcription in the absence of AHL (73). If EsaR has dual functions in the cell at low cell density, it would suggest a more complex quorum sensing network in *P. stewartii*, in which two different sets of genes are expressed both at low and high cell density. The goal of the studies described in Chapter Two was to further characterize EsaR as an activator of transcription. The activator function of LuxR and TraR from *Agrobacterium tumefaciens* have been thoroughly studied. Critical residues, required for interaction with RNAP for activation to occur, were identified in these proteins. In the case of LuxR several interacting residues were found solely in the C-terminal domain as compared to TraR, which was found to contain critical residues in both its N-terminal and its C-terminal domains (18, 78). Through the use of sequence alignment with LuxR and homology modeling of TraR to EsaR (23), several of these positive control variants (PC variants) were mapped on EsaR (Fig 1.6). A combination of deletion and site-directed mutagenesis strategies were employed to identify critical residues of EsaR, which are required for transcriptional activation. The relevance of EsaR as an activator has been established through the identification of a native promoter of *P. stewartii* that controls expression of a sRNA.

Because EsaR is functional in the absence of AHL, it was hypothesized that EsaR would also be post-translationally regulated in a reverse manner compared to other LuxR homologues. It was believed that binding of AHL would cause the protein to become more susceptible to
proteolytic processing. The evidence presented in Chapter Three contradicts this hypothesis by demonstrating that the protein is equally stable under conditions with and without its cognate AHL. Therefore, the regulation by AHL was further probed, and it was revealed that AHL binding leads to conformational changes within the protein, as opposed to changes in oligomeric state, which contribute to dissociation from DNA.

The last objective of this work, described in Chapter Four, was to further probe the mechanism by which EsaR responds to AHL. EsaR is unique to the well characterized LuxR homologues in that it can be analyzed in both its ligand-free and ligand-associated forms. Structural models predict subtle variations between the different classes of LuxR family members. In order to uncover mechanistic differences, genetic and biochemical methods were utilized to assess the effects of substitutions in EsaR* variants that would allow EsaR to bind DNA and regulate transcription in the presence of AHL. Two classes of variants were obtained in this study: (i) those that were deficient in AHL binding and (ii) those that retained the ability to bind AHL, but lost the ability undergo conformational changes. These initial results have allowed for the identification of residues required for AHL responsiveness (Fig 1.6). Overall this study will shift accepted paradigms about quorum-sensing regulators, and also offer further insight into the manner by which bacteria sense and coordinate changes in gene expression.
REFERENCES


Thode, A., D. Donham, and M. Churchill. 2006. EsaR homology model based on the autoinducer associated TraR dimer-DNA crystal structure, Personal communication.


Figure 1.1. Model of quorum sensing in *Vibrio fischeri* at high cell densities. At high cell densities, the concentration of autoinducer (blue circles) reaches a critical level, at which time LuxR and autoinducer come together and bind the *lux* box. Together with RNAP, the complex activates the transcription of the *lux* operon, thereby producing bioluminescence. See text for further details.
Figure 1.2. Model of EPS regulation in *P. stewartii*. The converging Esal/EsaR quorum-sensing and Rcs environmental stimuli-sensing regulatory networks coordinately regulate EPS production. See text for further details.
Figure 1.3. **Transcriptional regulation during quorum sensing.** (Top-left) Low concentrations of autoinducer, Class I and II regulators will not bind to promoter regions. (Top-right) Autoinducer must reach a critical concentration and form complexes with Class I and II regulators in order to initiate transcription. (Bottom-left) Class III regulators act as repressors of transcription. At low concentrations of autoinducer, regulators bind to the -10 site in the promoter region and block RNA polymerase from initiating transcription. (Bottom-right) It is only at a critical concentration of autoinducer that this class of proteins is bound and released from the promoter region, allowing transcription to occur.
Figure 1.4. EsaR homology model. This is based on the autoinducer associated TraR dimer-DNA structure and used with the permission of A. Thode, D. Donham and M. Churchill. Cyan colored regions were generated from a threaded model. Red colored regions are repressor specific, residues 170-178 are located in the extended interdomain linker region and residues 237-249 consist of the extended C-terminus of EsaR, as labeled above. Both the AHL and DNA are shown as ball and stick.
Figure 1.5. The Class II promoter of the *V. fischeri lux* operon. Cartoon model of the five essential subunits of *Escherichia coli* RNA polymerase (RNAP) holoenzyme and its interaction with LuxR bound to the DNA at the *lux* operon promoter (See text for details).
Figure 1.6. A clustal alignment of LasR, LuxR, EsaR, and TraR. Sequences were aligned using Biology Workbench, with modifications as appropriate according to literature (Stevens and Greenberg, 1999; Bottomley et al. 2007). The red residues are residues involved in positive control, required for interaction with RNA polymerase for activation of transcription to occur. To date no residues associated with positive control have been characterized in LasR. Asterisks indicate residues involved in AHL binding by LasR (above) and TraR (below). The yellow residues are critical for the EsaR* phenotype. Green residues indicate conserved, functionally similar residues; blue indicates invariant residues across the LuxR homologues. Numbering is for EsaR.
Chapter Two

Structure/Function Analysis of the Pantoea stewartii Quorum-sensing Regulator EsaR as an Activator of Transcription

Daniel J. Schu¹, Aurelien L. Carlier², Katherine P. Jamison¹, Susanne von Bodman²,

and Ann M. Stevens¹*

¹Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061, USA
²Department of Plant Science, University of Connecticut, Storrs CT 06269, USA

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* Corresponding author. Mailing address: 219 Life Sciences 1 (0910), Washington St., Blacksburg, VA 24061, USA; Phone: (540) 231-9378; Fax: (540) 231-4043; E-mail: ams@vt.edu
ABSTRACT:

In *Pantoea stewartii* subsp. *stewartii*, two regulatory proteins are key to the process of cell-cell communication known as quorum sensing, the LuxI and LuxR homologues EsaI and EsaR. Most LuxR homologues function as activators of transcription in the presence of their cognate acylated homoserine lactone signal (AHL). However, EsaR was initially found to function as a repressor in the absence of AHL. Previous studies demonstrated that in the absence of AHL, EsaR retains the ability to function as a weak activator of the *lux* operon in recombinant *Escherichia coli*. Here it is shown that both the N-terminal and C-terminal domains of EsaR are necessary for positive regulation. A site-directed mutagenesis study, guided by homology modeling to LuxR and TraR, has revealed three critical residues in EsaR that are involved in activation of RNA polymerase. In addition, a native EsaR-activated promoter has been identified, which controls expression of a putative regulatory sRNA in *P. stewartii*. 
**INTRODUCTION:**

Quorum sensing is a population-wide behavioral response that has been identified in a variety of prokaryotes. The term quorum sensing is used to describe the ability of a microorganism to sense and initiate a response to a self-produced intercellular signaling molecule commonly known as autoinducer. Several important bacterial processes are regulated through quorum sensing including antibiotic production, release of exoenzymes, production of virulence factors, induction of genetic competency, conjugative plasmid transfer, biofilm formation and bioluminescence (11, 20, 37, 38).

In Gram-negative proteobacteria, the quorum-sensing systems typically contain a LuxI homologue that functions as an autoinducer synthase producing an acylated homoserine lactone (AHL) signal and a LuxR homologue that responds to AHL and controls gene expression. Of the over 50 LuxR homologues identified, the majority function as AHL-dependent activators similar to LuxR (11, 20, 37). However, a subset of them, including EsaR from *Pantoea stewartii* subsp. *stewartii*, have been found to regulate expression of specific target genes by repression and AHL-dependent derepression (2, 6, 23, 34).

In *P. stewartii*, quorum sensing is involved in the control of virulence. This plant pathogen is the causative agent of Stewart’s wilt disease in sweet corn and leaf blight in maize. Disease is initiated when the bacterium begins producing large amounts of an exo/capsular polysaccharide (EPS), which blocks the corn xylem vessels and induces necrotic lesions (7). EPS production is under the control of a multi-tiered regulatory cascade (4, 29). EsaI/R reside at the top of this regulatory hierarchy. The LuxI homologue, EsaI, synthesizes AHL, which at high enough concentrations can induce the
production of EPS through the AHL-dependent inactivation of EsaR (4, 34). EsaR functions in the absence of AHL by binding to DNA as a dimer, and blocking transcription of genes involved in EPS production (22, 25). Like LuxR, which requires the *lux* box for DNA binding, EsaR also requires a DNA binding site known as the *esaR* box. Unlike the *lux* box, which is centered around the -42.5 region of the promoter in a class II promoter orientation (10, 18), the *esaR* box is centered around the -10 site and blocks transcription by RNA polymerase at the *esaR* and *rcsA* promoters (5, 22). The *lux* box and the P_{esaR} *esaR* box sequences differ by 5 bases.

A previous study demonstrated that EsaR retains the ability to function as a weak activator of the *lux* operon in recombinant *Escherichia coli* (33). The goal of this study was to further characterize EsaR as an activator of transcription. Studies on LuxR and TraR from *Agrobacterium tumefaciens*, identified critical residues necessary for interaction with RNA polymerase (RNAP) for activation to occur (9, 24, 36). In the case of LuxR several interacting residues were found solely in the C-terminal domain, whereas critical residues were found in both the N-terminal and C-terminal domains of TraR (9, 24, 36). Through the use of sequence alignment with LuxR and homology modeling of TraR to EsaR (28), several of these previously characterized positive control (PC) variants were mapped on EsaR. A combination of deletion and site-directed mutagenesis strategies were employed to identify critical residues of EsaR, which are required for activation. The relevance of EsaR as an activator has been established through the identification of a native promoter of *P. stewartii* that controls expression of a sRNA.
MATERIALS AND METHODS:

Bacterial strains and growth conditions

Strains and plasmids used in this study are described in Table 2.1. The *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth (LB) or RM minimal medium (RM) (2% casamino acids, M9 salts [12.8 g of Na₂HPO₄ (7H₂O), 3 g of KH₂PO₄, 0.5 g of NaCl, and 1 g of NH₄Cl per liter], 0.4% glucose, and 1 mM MgCl₂), and supplemented, where indicated below, with 100 μg/ml ampicillin (Ap), 50 μg/ml kanamycin (Kn), 10 μM N-(β-ketocaproyl)-L-homoserine lactone (3-oxo-C6-HSL) (AHL) (Sigma, St. Louis, MO), 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), or 4 μg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Sigma). The *P. stewartii* strains were grown at 28°C in LB in the presence of 30 μg/ml nalidixic acid (NA).

Plasmid construction

To construct variants of the luxI promoter containing the esaR box, overlap-PCR (16, 17) was performed with the template *p*luxI-*lacZ* (33), which contained the lux operon promoter fused to a lacZ reporter. Two sets of overlapping mutagenic primers, EBUP/EBDOWN and EBUP2/EBDOWN2, were synthesized (Table 2.2) that contained the esaR box in two different orientations. They were used in two separate PCR reactions with an upstream external primer, ECORI, containing an EcoRI site, and the downstream external primer, BAMHI, containing a BamHI site (Table 2.2). A second round of PCR was then performed to generate the desired 400 bp products, which were cloned into pGEM-T (Promega, Madison, WI) and sequenced to confirm their integrity (Virginia Bioinformatics Institute Core Laboratory (VBI), Virginia Tech, Blacksburg).
intermediate pGEM-T constructs and *luxI-lacZ*, were digested with *Eco*RI and *Bam*HI and the promoter regions from pGEM-T were ligated into *luxI-lacZ* replacing the *lux* operon promoter with the newly constructed *esaR* box containing promoters. The constructs containing the promoters with the *lux* or *esaR* box fused to *lacZ* were digested with *Eco*RI and *Mfe*I, and subcloned into pEXT22 (pEXT-*luxI*, -*esab1* or -*esab2*) (26), which replicates at 1-1.5 copies/cell.

To measure the activity of EsaR as a repressor, a new low copy number reporter was constructed using primers NPesaF and NPesaR (Table 2.2) to amplify the *P*esaR via PCR with pSVB5-18 as a template (4). The promoter region was then fused to *lacZ*, and cloned into pEXT22 (pRNP-*lacZ*) using *Eco*RI and *Mfe*I sites as described above.

A series of deletion variants of EsaR was also constructed using PCR to amplify the regions of interest, followed by subsequent subcloning into pBAD22. Two constructs, pBAD-161EsaRΔN and pBAD-179EsaRΔN, were generated with the primers ESAR161 and EsaR179 in combination with ESARRC (Table 2.2) to create N-terminal domain deletions of amino acids 162-252 and 180-252 respectively (Table 2.1 and 2.3). An inframe deletion of a portion of the autoinducer-binding domain (residues 65-107) from *esaR* was created by performing a set of sequential digestions. First, pBAD-EsaR was digested with *Pvu*II and *Hind*III. In the second reaction pBAD-EsaR was digested with *Hpa*I and *Hind*III. A 450bp fragment was recovered from the second reaction and was ligated to a 6 kb fragment from the first reaction to generate pBAD-ΔABD. A deletion of the linking loop between the N-terminal and C-terminal domains corresponding to residues 171-178 was accomplished by amplifying the C-terminus with primers C-termfwd and T7rev and the N-terminus with primers T7 and N-termrev (Table
2.2) from template pET28b::H6esaR. The two products were then used in another round of PCR to obtain a 1kb product that was subcloned using NcoI and XhoI sites into pET28b (pET28b::H6-esaRD1). Other deletions were created where the C-terminus of EsaR (residues 237-249) was removed or both the linking loop (residues 171-178) and the C-terminus (residues 237-249) were removed. Primers T7 and CTDELR (Table 2.2) were used in these PCR reactions with either pET28b::H6esaR or pET28b::H6-esaRD1 as templates. The two products were cloned into pET28b (pET28b::H6-esaRD2 and pET28b::H6-esaRD3). The three His-tagged constructs along with pBAD-EsaR (contains non His-tagged esaR) were then digested with KpnI and HindIII, and the truncated esaR genes were individually subcloned into pBAD-EsaR creating pBAD-EsaR Δ171-8, pBAD-EsaRΔCT and pBAD-EsaR ΔΔ. (Table 2.1 and 2.3).

Overlap-PCR was also used for site-directed mutagenesis of esaR in pBAD-EsaR to generate possible positive control (PC) variants. Two overlapping mutagenic (internal) primers (Table 2.2) were designed to change one specific amino acid (E7A, N119A, K196A, A199W, I204A), and to add a restriction endonuclease site (NdeI) to constructs K196A and I204A for screening purposes. In all cases, except for the E7A construct, external primers (BADR, ESARF2) (Table 2.2) were also used in amplifying the gene. In the case of the E7A construct the BADVF primer was used in the amplification of the N-terminal domain. The second round PCR products were ligated into pGEM-T. The pGEM-T constructs along with pBAD-EsaR were digested with HindIII and Hpal and the region of esaR containing the corresponding residues changes were ligated into the pBAD vector.
pPesaR-AC::gfp (Table 2.1) was constructed by amplifying a 253bp fragment from the chromosome of DC283 (wild-type \textit{P. stewartii}) (7) by PCR using primers PesaRbamfwd and PesaRbamrev (Table 2.2). The PCR product was ligated into Topo vector pCR2.1 (Invitrogen) to generate pCR-PesaR. To generate a transcriptional fusion of the promoter region to \textit{gfp}, pCR-PesaR was digested with \textit{Bam}HI and subcloned into the \textit{Bam}HI restriction site of pFPV25 (31). The orientation of the promoter fusion was verified by PCR and DNA sequencing.

\textbf{EsaR activation of promoter constructs in vivo}

\textit{E. coli} Top 10 strains containing pBAD-EsaR, pBAD-LuxR, or pBAD constructs encoding deletion or PC variants were cotransformed with either \textit{luxI}-\textit{lacZ}, pEXT-esab1 or pEXT-esab2. Cells were grown as previously described (33) except they were induced at an \textit{OD}_{600} of 0.25 and harvested at an \textit{OD}_{600} of 1.0. 5 μl of cells were stored at -70°C prior to analysis of LacZ expression via chemiluminescent β-galactosidase assays (Tropix, Bedford, MA).

A \textit{P. stewartii} strain ESN10 containing pPesaR-AC::gfp was grown overnight in LB containing Ap and NA. Cells were subcultured to 0.05 \textit{OD}_{600} with or without 20 μM AI, and grown to an \textit{OD}_{600} of 1. 200 μL of each culture was then placed in a 96-well optical bottom microtiter plate for the analysis of both fluorescence output (excitation and emission wavelengths of 485 and 535 nm, respectively) and \textit{OD}_{590} on a Tecan SpectraFluor Plus plate-reader (Tecan, Mannedorf/Zurich, Switzerland). The output was normalized by dividing the RFU by the OD. Assays were performed as two independent triplicate sets.
Assay for repression by EsaR variants in vivo

pBAD constructs encoding wild-type EsaR, deletion variants, or PC variants were also cotransformed with pRNP-lacZ and assayed under the following conditions to determine the ability of a variant to repress transcription. Overnight cultures were subcultured to an $OD_{600}$ of 0.05 in 5 mL of RM under two different conditions: (1) no addition or (2) plus 0.2% arabinose. Cells were harvested at an $OD_{600}$ of 0.5 and 5 μL aliquots were stored in the -70°C freezer prior to analysis of LacZ expression via chemiluminescent β-galactosidase assays (Tropix, Bedford, MA).

Northern blot analysis

An esal- strain (ESN51) was grown up overnight in LB at 30°C and subcultured into 25 mL of LB to an $OD_{600}$ of 0.025. Specific volumes of culture were harvested for each strain as follows: $OD_{600}$ of 0.2 (10 mL), 0.5 (5 mL), 1.0 (1 mL), and 2.0 (0.5 mL). The pellets were thawed at room temperature, and lysed by adding 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA). The RNA was extracted according to the manufacturer’s protocol. 15 – 20 μg of RNA was precipitated, and electrophoresed on a 1% MOPS agarose gel. The RNA was then transferred to a nitrocellulose membrane through the use of Turboblotter (Whatman, GE Healthcare), according to their protocol. Primers ESSRF and ESSRR (Table 2.2) were used to amplify a 500 bp region, downstream of the hypothesized promoter region in P. stewartii, from chromosomal DNA to be used as a probe in the identification of a transcript from a northern blot. Random Primed $^{32}$P-labeled probes were made from the PCR products using the Boehringer Mannheim Kit (Roche Applied Science, Indianapolis, IN). The $^{32}$P-labeled probes were hybridized to the membrane with QuickHyb according to the manufacturer’s
protocol (Stratagene, La Jolla, CA). The membrane was then air dried and exposed to a Storm phosphorimager (General Electric Company).

**Primer extension analysis**

RNA was purified by the Trizol method described above from *P. stewartii* ESN51 (esaI- strain), which constitutively produces the transcript, grown to an OD$_{600}$ of 0.5. Primer PESR (Table 2.2) was created, which contained a fluorophore (5’ 6-FAM) on the 5’end. The primer was diluted to 10 nmol/mL and 2 μL was used in extension reactions consisting of 10 μg of sample RNA, 2 μL of primer, and 5 μL of the 2X AMV Primer Extension Buffer (Promega). The mixture was heated at 57 °C for 20 minutes to anneal the primer. It was then cooled to ambient temperature for 10 minutes. RT extension mix was added (AMV primer extension 5 μL 2X Buffer, 1.4 μL sodium pyrophosphate, 1μL AMV RT, and 1.5 μL nuclease-free dH$_2$O) to the previous reaction and incubated at 42 °C for 30 minutes. The reaction was then stored at -20 °C prior to fragment analysis at VBI.
RESULTS AND DISCUSSION:

Ability of EsaR to activate promoters with the esaR box versus the lux box

The quorum-sensing regulator EsaR from *P. stewartii* was initially identified and characterized as a repressor of transcription, versus the majority of its homologues from the LuxR family of proteins, which function as activators of transcription. Recent studies on EsaR in recombinant *E. coli* demonstrated that EsaR retains the ability to also activate transcription of the *V. fischeri luxI* promoter, which was fused to a *lacZ* reporter. EsaR activated the reporter at a level roughly four-fold lower than LuxR (33). Taken together with EMSA analysis, which suggested that EsaR has an approximately four-fold weaker affinity for the *lux* box (33), it appeared that this variation in levels of expression between the two homologues could be attributed to their differential affinity for the DNA. Nevertheless, it was demonstrated that EsaR is capable of binding to the *lux* box, albeit with reduced affinity, and making appropriate interactions with *E.coli* RNAP to activate transcription from the *luxI* promoter.

The *lux* box is a 20 bp palindrome, which differs by 5 bp from the native DNA binding site of EsaR at *P.esaR*, the *esaR* box. Thus two new reporters were constructed that contained the *esaR* box in two separate orientations, in place of the *lux* box in the *luxI* promoter to establish whether or not this would enhance DNA binding by EsaR in vivo (Fig. 2.1A). Incorporating the native DNA binding site of EsaR into the reporter did indeed allow for a stronger interaction between EsaR and its native target DNA. EsaR in the presence of the *esaR1* box expressed LacZ at levels comparable to those generated when LuxR drove expression of the reporter in presence of the *lux* box and 3-oxo-C6-HSL. The reporter under the expression of the promoter containing the *esaR2* box in the
second orientation had lower levels of expression (Fig. 1B). Hence the *esaR* box is in the preferred orientation for EsaR to activate transcription, providing some information about preferred base recognition.

**Effect of deletions in EsaR on its ability to regulate transcription**

A comparative analysis of EsaR to LuxR and TraR was initiated, with regard to domain stability and function. LuxR from *V. fischeri* is stable and functional in the absence of its N-terminal domain (27). In comparison, TraR from *A. tumefaciens* requires both the N- and C-terminal domains for the protein to remain stable and functional (36). Several deletion variants of EsaR were analyzed with respect to their stability and ability to regulate transcription. Two truncated variants were constructed that lacked portions of the N-terminal domain (Δ2-160 residues) and (Δ2-178 residues) (Table 2.3). Western immunoblots from recombinant *E. coli* cells overexpressing these constructs revealed that the variants were unstable (data not shown). These results would suggest that in regards to stability of the C-terminal domain, EsaR more closely behaves like TraR.

The subset of the LuxR protein family originally identified as repressors, like EsaR (3, 12), which functions in the absence of the AHL, contains two unique regions in comparison to the majority of the LuxR protein family. These two regions consist of (1) an extended linker region between the AHL binding N-terminal domain and DNA binding C-terminal domain and (residues 171-178), and (2) an extension at the C-terminus (residues 237-249) (Table 2.3) (28). Deletion variants in which these two regions were removed from EsaR singly and in combination were constructed (Δ171-178, Δ237-249 and double Δ) (Table 2.3) to reveal the possible roles these unique regions
have in protein stability and/or function. Another deletion made at residues 65-107 (ΔABD) resulted in removal of a large portion of the hypothesized 3-oxo-C6-HL binding region in the N-terminal domain. Western blot analysis on these variants confirmed that all four variants were expressed, and remained stable within the cells (Fig. 2.2A). However, none of the deletion variants were capable of binding to the esaR box and repressing transcription (Fig. 2.2B). Therefore, the deletions rendered EsaR nonfunctional as a transcriptional regulator.

**Role of individual amino acid residues in positive control by EsaR**

A further comparison of EsaR, LuxR and TraR was conducted to examine the specific protein-protein interactions that occur between EsaR and RNAP during transcriptional activation. A large number of residues in both the N- and C-terminal domains of TraR (D10, G123, W184, V187, K189, E193, V197, and D217) play critical roles in activation (19, 36). Previous work on LuxR revealed three specific residues (K200, W203, I208) in the C-terminal domain that were required for activation of transcription at the luxI promoter (9). Interestingly, three residues from TraR (K189, E193, and V197) align closely with the positive control (PC) variants of LuxR. Through sequence alignments and homology modeling of EsaR (based on the structure of TraR) (28), five amino acids (E7 and N119 in the N-terminal domain, and K196, A199, and I204 in the C-terminal domain) were selected for examination as PC control variants of EsaR.

In order to characterize the PC variants of EsaR, repression assays were used to differentiate between variants that lost their affinity for DNA as compared to those that retained it. Several of the variants (E7A, N119A, K196A, A199W) retained their ability
to block transcription from the *esaR* promoter by binding the *esaR* box, which is centered around the -10 site. One variant, I204A, had higher levels of LacZ expression compared to wild-type suggesting that this variant lost some affinity for the DNA (Fig. 2.3B).

The newly developed promoter construct containing the *esaRL* box centered at -42.5 of the *luxR* promoter was used in activation assays to test the putative PC variants. Activation assays preformed on the C-terminal domain variants K196A and A199W yielded a four-fold decrease in activation as compared to the wild-type protein. The I204A variant also had decreased levels of activation, but this is attributed to its decreased affinity to the DNA (Fig. 2.3C). Activation assays were also performed on two N-terminal domain variants of EsaR. The E7A variant activated transcription at a four-fold lower level than wild-type EsaR. Assays performed on the N119A variant showed no significant decrease in activation (Fig. 2.3C).

Overall results from these assays revealed that the E7A, K196A, and A199W variants lost the ability to activate transcription, but were still capable of binding to the DNA at levels comparable to wild-type EsaR based on standard repression assays. This result was surprising for the E7A variant as it is not as stable as wild-type EsaR based on western immunoblot analysis. This mutant protein may be capable of binding to the DNA with higher affinity, but this should not have influenced the activation assay results. The I204A variant appears to have a decreased affinity for DNA as is demonstrated by the increased levels of β-galactosidase activity in the repression assay. Taken together these results demonstrate that like TraR and unlike LuxR, both the NTD (E7A) and CTD (K196A and A199W) of EsaR appear to play key roles in transcriptional activation.
**EsaR-dependent activation of a native promoter in P. stewartii**

The ability of EsaR to function as an activator was examined in the heterologous host, *E. coli*, using primarily artificial promoter constructs. However, a native promoter activated by EsaR in *P. stewartii* has now been identified. The EsaR-activated promoter is divergently transcribed from the *esaR* promoter. A *gfp* fusion to this promoter, which extends 60 bp downstream from the center of the *esaR* box, was downregulated 35-fold by the addition of AHL in the presence of EsaR in *P. stewartii* (41189 +/- 615 RLU in the absence of AHL and 1177 +/- 55 RLU in the presence of AHL). This was a surprising result, as the construct had been made to serve as a negative control for EsaR-dependent transcription.

Analysis of the sequence downstream of the EsaR-activated promoter suggested that the closest open reading frame was 564 bp away, with homology to a glutathionylspermidine synthase. Therefore, northern blot analysis was performed to determine the size of the transcript produced from this EsaR activated promoter. A single band was visualized at around 100 bases (Fig. 2.4). There are no obvious rho-independent terminators at the end of the transcript region. Primer extension determined that the +1 start site of the transcript was a guanosine, located 91 bp downstream from the center of the *esaR* box. This is ~30 bases downstream of where the +1 site would be predicted based on the fusion construct where the *esaR* box was in a position near -60 as is found in Class I activated promoters. This suggests that the transcript may be processed at the 5’end. Because of the relative small size of the transcript and the fact that no clear open reading frame is present, it is hypothesized that the transcript may function as a small regulatory RNA within *P. stewartii* and it has been tentatively named EsaS.
Not unexpectedly, there are no obvious targets for the sRNA in the *P. stewartii* genome based on complementary nucleotide sequences. Efforts to establish its function within the cell are on-going. Interestingly, in the homologous YenR/I quorum-sensing system from *Yersinia enterocolitica*, a YenR-activated promoter controls expression of a putative sRNA divergently transcribed from *yenR* (35). Multiple cases of coupled regulation of a divergently encoded small regulatory RNA and its transcriptional regulators have also been characterized in *E. coli*. Examples of this type of organization included SgrR/S (32), OxyR/S (1), and GcvA/B (30).

Identification of a transcript activated by EsaR in *P. stewartii* establishes that this regulatory function has been retained in the native host. Therefore EsaR can function as both an activator and repressor at low cell density with accumulation of AHL leading to inactivation and derepression of certain transcripts at high cell density. This dual-level control may afford an advantage to *P. stewartii* as it progresses from its initial colonization of the host to expression of tissue destructive virulence factors.

**ACKNOWLEDGEMENTS**

We thank K. Michel for technical assistance. We also thank A. Thode, D. Donham and M. Churchill for generating and sharing their homology model of EsaR with us and A. Levchenko for his support of this project.

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*Underlined sequences correspond to unique restriction site utilized for cloning*
Table 2.3. Stability and activity of deletion variants of EsaR

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*AI Binding = , DNA Binding = , extended regions = *

*Y=Yes N=No*
Figure 2.1. Promoter constructs and activation assays. A) Sequences are shown for constructs with the native binding sites for LuxR, the lux box, and EsaR, the esaR box which were cloned into reporter constructs at a position centered around -42.5. The esaR box was subcloned in two different orientations. B) β-galactosidase assays were performed on strains with these pEXT22-based promoter constructs and either pBAD-LuxR or pBAD-EsaR. Constructs and growth conditions are as indicated. Raw data values in terms of relative light units are reported from samples obtained in two independent trials that were tested in triplicate. Error bars represent the standard deviation from the mean.
Figure 2.2. Expression and activity of EsaR deletion variants. A) Western immunoblot illustrate the expression levels of deletion variants of EsaR with lanes (1) and (5) wild-type EsaR, (2) Δ171-178, (3) Δ237-249, (4) ΔΔ and (6) Δ65-107 (ABD). B) Repression assays of strains expressing EsaR deletion variants as indicated. The strain containing the pBAD22 vector was used as a control to determine maximum levels of expression from the constitutively expressed promoter. Raw data values in terms of relative light units are reported from samples obtained in two independent trials that were tested in triplicate. Error bars represent the standard deviation from the mean.
Figure 2.3. Examining positive control variants of EsaR  A) Western immunoblot illustrating the expression levels of positive control variants of EsaR with lanes (1) wild-type EsaR, (2) E7A, (3) N119A, (4) K196A, (5) A199W and (6) I204A.  B) Repression assays of strains expressing EsaR point variants as indicated. Raw data values in terms of relative light units are reported from samples obtained in two independent trials that were tested in triplicate. Error bars represent the standard deviation from the mean. C) Ability of EsaR point variants to activate transcription. Constructs and growth conditions are as indicated. Raw data values in terms of relative light units are reported from samples obtained in two independent trials that were tested in triplicate. Error bars represent the standard deviation from the mean.
Figure 2.4. Analysis on the sRNA divergently transcribed from esaR. Expression of the EsaR-dependent sRNA in a esal- strain, ESN51 of P. stewartii. Size standards in bases are indicated to the left.
Chapter Three

Effects of Autoinducer Binding on Structural Properties of the Quorum-sensing Regulator EsaR from \textit{Pantoea stewartii}
ABSTRACT:

Production of exo/capsular polysaccharide (EPS) is naturally repressed at low cell densities via the quorum-sensing regulator EsaR in the corn pathogen *Pantoea stewartii* subsp. *stewartii*. However, at high cell densities when high concentrations of its cognate acylated homoserine lactone (AHL) signal are present, EsaR is inactivated and derepression of EPS production occurs. The mechanism that enables EsaR to respond to AHL in a manner opposite to that of most LuxR homologues remains elusive. In fact AHL is required for the purification of most LuxR family members, therefore no *in vitro* comparative analysis has been performed on the ligand-free ad ligand-associated forms of any LuxR family protein. This type of analysis is possible with EsaR. In comparison to the majority of LuxR family members, EsaR has two unique regions, (i) an extended linker region between the AHL-binding N-terminal domain and the DNA binding C-terminal domain, and (ii) an extended C-terminus. Biochemical methods have been used to examine the mechanistic differences between EsaR and other LuxR homologues. Pulse-chase experiments have demonstrated that EsaR is equally resistant to protease degradation *in vivo* in the absence or presence of AHL. However, limited *in vitro* proteolytic digestions suggest that the protein does undergo conformational changes in response to AHL. Gel filtration chromatography and sucrose gradient ultracentrifugation proved that this conformational change does not impact the multimeric state of the protein.
INTRODUCTION:

The ability of a bacterium to survive or even thrive in a particular environment is dependent upon its ability to sense and respond to various environmental parameters, which may include osmotic activity, pH, temperature, concentration of nutrients, or even the presence of other bacteria. Cell-cell communication or quorum sensing has been characterized in a subset of Gram-negative proteobacteria, which produce and respond to freely diffusible signal molecules termed autoinducers. In the well studied *Vibrio fischeri* quorum-sensing system the autoinducer (3-oxo-hexanoyl-L-homoserine lactone) (AHL) is produced by the LuxI autoinducer synthase protein. This ligand is then recognized by a receptor protein known as LuxR within the cytoplasm. Binding of the autoinducer molecule permits LuxR to take on a functional conformation that enables it to bind to DNA, and thereby activate genes involved in the production of bioluminescence (8, 14, 25, 26).

Over 50 different species of Gram-negative proteobacteria have been shown to carry out a similar type of signaling and recognition. For the majority of the over fifty LuxR homologues identified in different bacterial species, a model has been proposed, whereby AHL binding induces dimerization of the protein, leading to stabilization and DNA binding (19, 20, 30). In the case of TraR, *in vivo* experiments demonstrated that the half-life of TraR is significantly shortened in the absence of its cognate AHL (3-oxo-C8-HSL). More specifically, two different families of proteases, Clp and Lon, play a direct role in regulating the levels of TraR within the cell (3, 20, 30, 31).
It has been proposed that the LuxR family of proteins can be divided into three classes (12). The majority of the LuxR family members of proteins are activators that become functional after interacting with AHL cotranslationally like TraR (class I) or posttranslationally like LuxR (class II) (12). The third class has been described as repressors, which take on a functional conformation and bind DNA in the absence of the cognate ligand. Examples of these homologues are EsaR from *Pantoea stewartii*, ExpR from *Erwinia chrysanthemi*, and YpsR from *Yersinia pseudotuberculosis* (1, 2). EsaR is the most extensively characterized of this subfamily of LuxR homologues. EsaR regulates its own expression and expression of *rcsA*, which regulates EPS production in the plant pathogen, by repression and AHL-mediated derepression (5, 16). However, little biochemical information is available about the mechanism of AHL regulation of the EsaR protein subfamily.

A simple explanation for the AHL-dependent inactivation of EsaR would be post-translational regulation by AHL, which causes the protein to become more susceptible to proteolytic processing. Hence, the stability of EsaR with and without its cognate AHL was examined. Surprisingly, EsaR was equally stable under both conditions. With these results in mind, the mechanism of regulation by AHL was further probed, and it was revealed that AHL binding does indeed lead to conformation changes within the protein. However, these conformation changes did not affect the oligomeric state of the protein.
MATERIAL AND METHODS:

Measurements of EsaR stability in recombinant *Escherichia coli*

EsaR stability was examined in *E. coli* strain SG22163 (malP::lacI<sup>0</sup>) (9), which is protease proficient. EsaR was expressed from pKK-EsaR, which contains a P<sub>lac</sub>-esaR fusion. pKK-EsaR was constructed by digesting both pKK223-3 (4) and pBAD-EsaR (24) with EcoRI. Standard cloning protocol was used to ligate *esaR* into the pKK223-3 vector. The product was screened for directionality of cloning by digesting with *SmaI* and *AatII* to insure that the P<sub>lac</sub> promoter was upstream of *esaR*. The strain carrying the plasmid was grown up at 30°C in LB broth containing ampicillin (Ap) at 100 μg/ml and 1 mM IPTG in the absence or presence of 10 μM AHL to an OD<sub>600</sub> of 1.0. The cells were pelleted by centrifugation, placed at -70°C, and subjected to western immunoblotting with a 1:500 dilution of polyclonal antiserum generated against EsaR (24).

To measure the half-life of EsaR in *E. coli* SG22163, two plasmids, pT7-esaR and pJZ410 (31), were introduced into the strain. Plasmid pT7-esaR, which contains a P<sub>T7</sub>-esaR fusion, was constructed as follows. The esaR-coding region was amplified by PCR with pBAD-EsaR as the template. The reverse primer (BAMESR1) (5’ GGATCCTTACTACCTGGCCGCTGACGCTG 3’) included a BamHI site; the upstream primer (PCIES1) (5’-ACATGTTTTTCTTTTCTTTTGTGAAATCAACAATAAACGG-3’) contained a PciI site centered on the esaR ATG start codon. The PCR product was cloned into pGEM-T (Promega, Madison, WI) and sequenced. A 760-bp PciI-BamHI fragment was digested from the pGEM construct, and ligated into NcoI-BamHI-digested pMLU115 (23). In the resulting plasmid, pT7-esaR, esaR expression is controlled by the phage T7 promoter. Plasmid pJZ410 (31) has a gene encoding T7 RNA polymerase.
under the control of a heat inducible promoter. Cells were cultured in LB containing 100 μg/ml Ap and 20 μg/ml gentamycin (Gm) at 28°C to an OD$_{600}$ = 0.4. They were then shocked at 45°C for 20 min to induce expression of T7 RNA polymerase, and treated with 200 μg/ml rifampicin (Rif) to inhibit the host RNA polymerase. After 20 min at 45°C, the cultures were shifted to 30°C for 30 min, after which $[^{35}S]$methionine was added to a final concentration of 5 μCi/ml. 10 μM AHL was also added to one set of cultures. Radiolabeling was terminated after 3 min by adding nonlabeled methionine (5 mM). Aliquots were taken at various time points, up to 60 min, and cell pellets were placed at -20°C after centrifugation. The samples were analyzed using 12% SDS-PAGE and a Storm phosphorimager (General Electric Company).

**Purification of histidine (His)$_6$-maltose-binding protein-GLY$_5$- EsaR (HMGE) protein**

A His-MBP-EsaR (HMGE) fusion protein was constructed through two successive rounds of PCR. The primers in the first round were used to amplify the esaR gene from pKK-EsaR. The forward primer TEVESAR2 (5’-GAGAACCTGTACTTCCA GGGTGGTGGTGGTGGTATGTTTTCTTTTTTTCTTGAAAAATC-3’) contained a TEV proteolytic site and the N-terminal portion of the esaR gene separated by 15 nucleotides coding for 5 glycine residues. The reverse primer ATTBR (5’GGGGACAACTTTGTACAAGAAAGTTGCAATTACTACCTGGCCGCTGACGCTG C-3’) contained an attB site downstream of the C-terminal end of the esaR gene. An 800 bp product was recovered from PCR and used as the template in the second round of PCR. The forward primer ATTBTEV (5’-GGGGACAACTTTGTACAAGAAAGTTGCAATTACTACCTGGCCGCTGACGCTG C-3’) contained a TEV proteolytic site and the N-terminal portion of the esaR gene separated by 15 nucleotides coding for 5 glycine residues. This product was used as the template for the second PCR round.
GGAGAACCTGTACTTCCAG-3') containing the attB site upstream of the TEV proteolytic site, and the reverse primer from the 1st round PCR were used in the second round of PCR. An 850 bp DNA fragment was recovered containing attB-TEV-GLY₅-
esaR-attB. This DNA fragment was then used in a BP reaction following the vendor’s protocol (Invitrogen) with the plasmid pDONR201 (Invitrogen). pDONR201 confers kanamycin resistance (Kn) at 50 μg/ml, and is an entry vector in the Gateway System (Invitrogen). pDONR201 containing the fragment with esaR was recovered and screened by PCR with primers TEVESAR2 and ATTBR. Using the vendor’s protocol, a LR reaction was performed with the vector pDEST-HISMBP (18) in which the vector contains a Pₗₐₑ promoter controlling the expression of a His₆-MBP-TEV-GLY₅-EsaR protein (pHMGE). Recovered plasmid DNA was again screened with primers TEVESAR2 and ATTBR and sequenced to confirm integrity (Virginia Bioinformatics Institute Core Laboratory (VBI), Virginia Tech, Blacksburg). E. coli Top10 (11) with pHMGE was cultured at 30°C in LB broth containing 100 μg/ml Ap, in the presence or absence of 10 μM AHL to an OD₆₀₀ = 0.6, at which point 0.5 mM IPTG was added, and the culture was then incubated overnight at 19°C. Cells were concentrated by centrifugation at 4°C for 10 mins, and resuspended in HNIG buffer (20 mM HEPES, 300 mM NaCl, 20 mM imidazole, 10% glycerol (pH 7.4)). The resuspension was lysed using a French press, and the lysates were cleared by ultracentrifugation at 40,000 r.p.m with a Ti 70 rotor at 4°C for 1 hr. The fusion protein was then purified using FPLC chromatography at 4°C (AKTAPrime plus, GE Health Sciences, Pittsburgh, PA) and a 5 mL column containing Ni²⁺ resin (GE Health Sciences). A linear gradient elution with
resuspension buffer containing from 20 mM to 500 mM imidazole was used to elute the HMGE from the column.

**Partial *in vitro* proteolysis**

Reactions with a total volume of 50 µL contained 13.5 µM purified HMGE fusion protein, which was incubated with 0.25 mg/mL of thermolysin in the absence or presence of 67.5 µM (5X concentration to HMGE) AHL for 1 hr at 37°C in the following buffer: 2mM CaCl₂, 5% glycerol, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0. Reactions with thermolysin were stopped by the addition of 2 µL of 0.5 M EDTA (pH 8.0). Similarly, the fusion protein was also separately exposed to 0.25 mg/ml trypsin for 1hr at room temperature with and without a concentration of 67.5 µM (5X) AHL in the following buffer: 20 mM MgSO₄, 20 mM Tris-HCl (pH 7.5), 10 mM CaCl₂. 12.5 µL of sample buffer (0.624 mL 1 M Tris (pH 6.8), 0.2 g SDS, 1.04 mL glycerol, 0.5 mL β-mercaptoethanol, trace bromophenol blue) was added to each reaction and visualized by 12% SDS-PAGE.

**Gel filtration chromatography**

A XK 16 column (16cm x 26cm) was packed with Superdex S-200HR (GE Health Sciences), and was equilibrated with 20 mM Hepes, 150 NaCl, 10% glycerol (pH 7.4), and 67.5 µM (5X concentration to HMGE) AHL with a 0.7 ml/min flow rate at 4°C. These same conditions were used for calibration and experimental runs. The void volume (V₀) was 52 mL, determined by the elution of Blue Dextran. For calibration of the column, the following proteins obtained from Sigma (St. Louis, MO) were used as standards: chymotrypsinogen, albumin, β-amylase, and apoferritin. The Stokes radius of EsaR in the presence of AHL was determined by interpolation using a calibration curve
constructed by plotting the Stokes radii of the standard proteins versus the V_e of each protein. For the assays, 0.5 mL of 13.5 µM His-MBP-EsaR was loaded into the column.

**Sucrose gradient ultracentrifugation**

Albumin (BSA) (66 kD), yeast alcohol dehydrogenase (YAD) (150 kD), β-amylase (200 kD) (Sigma), and purified EsaR fusion protein were resuspended to 25 µM in 20 mM Hepes, 150 mM NaCl, 10% glycerol, 20 mM imidazole, and 125 µM (5X concentration of HMGE) AHL (pH 7.4). 100 µL was loaded on top of a 10 mL isokinetic 0-20% sucrose gradient. Samples were ultracentrifuged for 36 hr at 28,000 r.p.m. in a SW41 rotor at 4ºC. 0.5 mL fractions were collected from the bottom of the tube and analyzed with 12% SDS-PAGE (20 fractions in total). Densitometry was performed with a Molecular Imager Gel Doc XR (Bio-Rad, Hercules, CA) on the bands from each gel to determine the average point of distribution in the fractions. The sedimentation coefficient for EsaR fusion protein was determined by interpolation using a calibration curve by plotting the sedimentation coefficient values versus the average fraction for BSA, YAD, and β-amylase.

**Estimation of molecular weight**

The values for Stokes radius (R_s, in nanometers) and sedimentation coefficient (S, in Svedberg units) obtained experimentally were used to estimate the molecular weight (MW) of EsaR fusion protein in the presence of AHL with the equation MW=4205S x R_s in Daltons.

**Crosslinking of EsaR**

EsaR fusion protein was cross-linked with BS^3 crosslinker (Thermo Scientific, Rockford, IL). EsaR was used at a final concentration of 10 µM and BS^3 crosslinker was
added to a final concentration of 100 µM (1:10 ratio). There were two assay conditions, one reaction contained 50 µM (5X concentration of HMGE) AHL and the other had no AHL. The reactions were carried out in 20 mM Heps, 150 mM NaCl, 10% glycerol (pH 7.4) at 37ºC. 20 µL aliquots were removed from each reaction at given time points and the reaction was stopped by the addition of 1µL of 1M Tris (pH 7.5) and 5 µL of sample buffer (0.624 mL 1 M Tris (pH 6.8), 0.2 g SDS, 1.04 mL glycerol, 0.5 mL β-mercaptoethanol, trace bromophenol blue). The aliquots were then visualized by 8% SDS-PAGE.
RESULTS AND DISCUSSION:

EsaR expressed in recombinant *E. coli* is stable in both the absence and presence of AHL

Very little work has focused on how any of the LuxR-homologues are regulated post-translationally by AHL, since the majority of the LuxR family members are unstable/less stable in the absence of AHL. TraR from *Agrobacterium tumefaciens* has a much shorter half-life in the absence as compared to in the presence of AHL. Experiments in protease-deficient *E. coli* strains revealed that the Clp and Lon proteases were responsible for processing TraR at a much faster rate when AHL was not present versus when AHL was permitted to complex with the protein during translation (31). With these results in mind, it was hypothesized that EsaR from *P. stewartii* is regulated by cytoplasmic proteases in a reverse manner, whereby in the absence of AHL, EsaR takes on a more stable and resistant conformation. It was proposed that binding of AHL would cause a conformational change to occur within EsaR, which would expose proteolytic cleavage sites and thereby destabilize the protein.

The *in vivo* stability of EsaR in the presence or absence of AHL was examined in recombinant *E. coli* SG22163 (10). The accumulation of EsaR in the absence and presence of AHL was measured through western immunoblots. EsaR was present in similar quantities as demonstrated by their intensities as visualized on the western immunoblot under both conditions (Fig. 3.1A). Thus, its mechanism of control by AHL is not simply the reverse of that found in TraR (31). Pulse-chase experiments were subsequently performed to compare turnover rates of EsaR in the presence or absence of AHL in a more quantitative manner. EsaR was stable under both conditions up to 60 min
after synthesis of the protein (Fig. 3.1B). Therefore, EsaR appears to be in an equally stable conformation in either the absence or presence of AHL, and is not targeted for rapid proteolysis within the cell. This finding complements analysis of EsaR in the absence or presence of AHL via circular dichroism spectropolarimetry which demonstrated that AHL increases rather than decreases the Tm of the protein (16).

**Partial in vitro proteolysis to examine conformational changes in EsaR**

Experiments on TraR from *Agrobacterium tumefaciens* confirmed that binding of AHL stabilized the protein such that it takes on a more resistant conformation in the presence of proteases (31). An *in vitro* assay involving trypsin was used to look at these conformational changes. In the absence of AHL, apoTraR appeared to undergo a significant amount of processing by trypsin. When AHL was present, the two domains of TraR appeared to be stabilized and resistant to this processing. It was suggested that the protein may exist in an unfolded or monomeric state in the absence of AHL, which leaves hydrophobic residues exposed, and allows for the protein to be targeted for proteolysis (31).

Similar limited *in vitro* proteolytic digestions with purified His-MBP-EsaR (HMGE) fusion protein were performed to gain further insights into the effects AHL binding has on EsaR. The fusion protein was purified in both the absence and presence of 10 µM AHL, with the AHL added co-translationally and post-translationally. Two different proteases (thermolysin and trypsin) were used to produce cleavage patterns of EsaR that were analyzed via SDS-PAGE. Assays with thermolysin revealed bands produced on the SDS-PAGE gels in the two samples with EsaR exposed to AHL either co- or post-translationally that were not present in the absence of AHL (Fig. 3.2) Hence
AHL can elicit effects on EsaR post-translationally, as had been expected. The bands were extracted and their sequence was determined via MALDI-TOF (Virginia Tech Mass Spectrometry Incubator core facility). The bands produced from the thermolysin cleavage reactions were comprised of residues 1-160 (the NTD of EsaR) and 23-74 (a region close to the putative AHL binding pocket). It appears the HMGE was more resistant to thermolysin digestion in the presence versus the absence of AHL, as bands attributed to EsaR are more intense in the presence of AHL (Fig. 3.2).

Similar assays with trypsin revealed a unique band present in both fractions containing AHL. In the case of the differential band patterns in the presence of trypsin, it is not as clear if AHL increases or decreases susceptibility to trypsin. However, AHL was demonstrated to illicit conformational changes post-translationally. The band was again extracted, and through mass spectrometry determined to correspond to residues 130-213 of EsaR (Figure 3.3). This region includes the linker region between the NTD and CTD. This suggests that interdomain conformational changes are occurring in response to AHL binding, a phenomenon that has been speculated to occur amongst all LuxR family members, but has never before been biochemically demonstrated.

These in vitro assays can also be regarded as powerful biochemical tools for future analysis of the protein. First, these assays revealed AHL binding by EsaR indirectly. Secondly, in the case of the thermolysin assay, binding by AHL protected the N-terminal domain from proteolysis. This may prove to be an important tool for NMR-based structural studies since previous attempts to remove the affinity tag from the N-terminal domain of the protein have been unsuccessful.
**EsaR is dimeric in the presence of AHL**

Because EsaR was equally resistant to proteolytic digestion *in vivo* both in the presence and absence of AHL, alternative mechanisms of AHL inactivation under physiological conditions were considered. It is known that AHL binding drives dimerization of TraR and LuxR (23, 31), and that the majority of the dimer-stabilizing molecular interactions occur between the α6 helices of LasR (3). This corresponds to EsaR residues 132-161. EsaR is known to bind to DNA as a dimer in the absence of AHL (16), but its quaternary structure in the presence of AHL has never been well defined. The effect of addition of AHL on the multimeric state of EsaR was therefore determined through a combination of gel filtration chromatography, sucrose gradient ultracentrifugation, and cross-linking experiments. During gel filtration experiments, EsaR fusion protein exposed to AHL eluted with a single peak (79.56 mL) (Fig. 3.4A). The position of the peak in regards to the standard curve suggested that the EsaR fusion protein is dimeric in the presence of AHL. Through the use of known standards (7, 21), a Stokes radius of 5.02 nm was calculated for the fusion protein (Fig. 3.4B). However, to more accurately determine the molecular weight of the fusion protein in the presence of AHL, a sedimentation coefficient was established through the use of sucrose gradient ultracentrifugation (Fig. 3.5A). Sedimentation coefficients have previously been established for the protein standards (13, 27). The predicted sedimentation coefficient for HMGE is 6.94 S, which is determined by interpolation using a calibration curve generated by plotting the sedimentation coefficient values versus the average fraction for each protein (Fig. 3.5B). In combination with the Stokes radius, the molecular weight was estimated to be 146 kD. A monomer of the fusion protein is 73.3 kD, therefore these
experiments suggest that the fusion protein is dimeric in the presence of AHL. Gel filtration chromatography was also performed on the HMGE fusion protein in the absence of AHL. The protein eluted with a single peak (74.88 mL) (Fig. 3.6). The protein eluted much earlier in comparison to the protein in the presence of AHL. It appears that in the absence of AHL, HMGE exists in a greater oligomeric state than that of a dimer. Previous studies on EsaR demonstrated that in the absence of AHL, the protein binds DNA as a dimer (16). It was also noted that higher order oligomeric complexes were formed by EsaR; these complexes were only detected when EsaR protein concentrations exceeded 200 nM (16). Because concentrations of HMGE exceeded this concentration in the gel filtration experiments, it is believed that a similar scenario may be observed with HMGE in the absence of AHL.

To further verify the finding that EsaR is dimeric in the presence of AHL, crosslinking experiments were performed in both the absence and presence of AHL. Maltose-binding protein (MBP) was used as a negative control since it is a monomeric protein, which should not be cross-linked by BS$^3$ (Fig. 3.7B). Therefore any cross-linking observed in HMGE should be due to the interactions between EsaR. Over a 30 min period, the majority of HMGE was crosslinked at comparable rates, plus or minus AHL (Fig. 3.7A). EsaR is therefore not dissociating into a monomeric state in the presence of AHL.

**Concluding remarks**

EsaR is the best understood member of this unique subclass of the LuxR family of proteins whose members include ExpR from *Erwinia chrysanthemi* (6), YpsR from *Yersinia pseudotuberculosis* (2), and EanR from *Pantoea ananatis* (17). All of these
proteins have an extended interdomain linker region and an extended CTD in comparison to the majority of LuxR homologues (22). They regulate gene expression in a reverse mechanism whereby in the absence of AHL they are capable of binding DNA, but take on an inactive conformation in the presence of AHL (6, 15, 16). Structurally, EsaR deviates from most LuxR homologues because it is stable and exists as a dimer in both the absence and presence of its cognate AHL. Furthermore EsaR is not post-translationally regulated by proteases in vivo. The in vitro proteolysis assays revealed AHL causes conformational changes in the N-terminal domain of the protein which lead to subsequent changes in the C-terminal domain. These changes in the C-terminal domain of the protein have been attributed to the loss of DNA binding. The thermal stability of EsaR was previously examined with circular dichroism spectroscopy. In the presence of AHL, EsaR was shown to be more stable (16). Taken with the results presented here, it can be speculated that binding of AHL by EsaR causes a conformational shift in the protein, in which the protein takes on a more rigid conformation. By losing flexibility, EsaR may no longer be able to make appropriate interactions with DNA, leading to dissociation. Currently, the only detailed structural information obtained for the LuxR protein family is from crystal structures of TraR and LasR (3, 29), and also NMR analysis of SdiA from E. coli (28). The mechanism by which AHL binds to the NTD of LuxR homologues, and modulates the activity of the CTD is an unresolved issue in the quorum-sensing field. Future structural studies of EsaR would provide valuable insights on the precise nature of the mechanism whereby AHL modulates the activity of EsaR.
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REFERENCES


Figure 3.1. EsaR accumulation and stability in recombinant *E. coli* SG22163. (A) Depiction of *in vivo* experiments, in which EsaR accumulation is measured through western immunoblots in the absence and presence of AHL. (B) Pulse-chase experiments, in which EsaR was labeled in *E. coli* SG22163 in the absence or presence of 10 µM AHL. Stability was examined at various time points up to 60 min. The image is representative of experiments performed in duplicate.
Figure 3.2. Limited *in vitro* digestion of EsaR by thermolysin

A) SDS-PAGE analysis of resistance of EsaR to thermolysin +/- AHL. Lane 1) His-MBP-EsaR (HMGE) without thermolysin  2) HMGE (0.25 mg/ml thermolysin)  3) HMGE purified with AHL (0.25 mg/ml thermolysin)  4) HMGE with post-translationally added AHL (0.25 mg/ml thermolysin) Arrows indicate bands of interest.  

B) Amino acid sequence of EsaR. Band indicated by upper arrow is composed of highlighted residues.  

C) Amino acid sequence of EsaR. Band indicated by lower arrow is composed of highlighted residues. The image is representative of experiments performed in duplicate.
Figure 3.3. **Limited in vitro digestion of EsaR by trypsin.** A) SDS-PAGE analysis of resistance of EsaR to trypsin +/- AHL. Lane 1) His-MBP-EsaR (HMGE) without trypsin 2) HMGE (0.25 mg/ml trypsin) 3) HMGE purified with AHL (0.25 mg/ml trypsin) 4) HMGE with post-translationally added AHL (0.25 mg/ml trypsin). Arrow indicates band of interest. B) Amino acid sequence of EsaR. Band indicated by arrow is composed of highlighted residues. The image is representative of experiments performed in duplicate.
Figure 3.4. Gel filtration chromatography of EsaR + AHL. A) Purified His-MBP-EsaR (HMGE) was sized fractionated by gel filtration. Its peak of elution was at 79.56 mL. B) The Stokes radius of the EsaR fusion protein in the presence of AHL was determined by interpolation using a calibration curve constructed by plotting the Stokes radii of the standard proteins versus the $V_e$ of each protein ($y = -0.1269x + 14.981$). For calibration of the column, the following proteins were used as standards (with their Stokes radii and $V_e$, elution volumes): chymotrypsinogen (2.24 nm and 101.48 mL), albumin (3.55 nm and 87.59 mL), β-amylase (5.4 nm and 76.74 mL), and apoferritin (6.1 nm and 70.06).
Figure 3.5. Sucrose gradient ultracentrifugation of EsaR + AHL. A) The location of His-MBP-EsaR (HMGE) protein in the 10 mL sucrose gradient was determined by analyzing 0.5 mL fractions (from bottom to top) via 12% SDS-PAGE. The image is representative of experiments performed in duplicate. B) The sedimentation coefficient for EsaR fusion protein was determined by interpolation using a calibration curve by plotting the sedimentation coefficient values versus the average fraction for protein standards (y=-0.5435x + 12.922). The protein standards sedimentation coefficient values and average fraction were: BSA (4.6 S and 15), YAD (7.6 S and 10.5), and β-amylase (8.9 S and 7).
Figure 3.6. Gel filtration chromatography of EsaR without AHL. A) Purified His-MBP-EsaR (HMGE) was sized fractionated by gel filtration. Its peak of elution was at 74.88 mL.
Figure 3.7. Analysis of the oligomeric state of EsaR +/- AHL. A) Cross-linking of HMGE by BS³ in the absence (-) or presence (+) of AHL over thirty minutes, as indicated. Mobility of HMGE without exposure to BS³ is shown in the first lane. B) Control demonstrating that maltose binding protein (MBP) is not crosslinked by BS³ under the same conditions. The size of the MBP remained 43 kD, with no bands of higher mobility in the presence of BS³. The images are representative of experiments performed in duplicate.
Chapter Four

Characterization of Essential Residues Involved in EsaR AHL-Responsiveness
ABSTRACT:

During quorum sensing in the plant pathogen Pantoea stewartii subsp. stewartii, EsaI, an acylated homoserine lactone (AHL) synthase and EsaR coordinately control capsular polysaccharide production. The capsule is expressed only at high cell density when AHL levels are high, leading to inactivation of EsaR. In lieu of detailed structural information, the precise mechanism whereby EsaR recognizes AHL and responds to it in a manner opposite to that of most other LuxR homologues is unresolved. Hence, a random mutagenesis genetic approach was designed to isolate EsaR* variants that are immune to the effects of AHL. Error-prone PCR was used to generate the desired mutants, which were subsequently screened for their ability to repress transcription in the presence of AHL. Following sequencing, site-directed mutagenesis was used to generate all possible mutations of interest as single, rather than multiple amino acid substitutions. Several individual amino acids playing a critical role in the AHL-insensitive phenotype have been identified and mapped onto a homology model of EsaR. The role of these has now been examined in vitro. The ability of EsaR* variants to bind AHL and an analysis of the effect individual mutations have on the overall conformation of the protein was performed.
INTRODUCTION:

*Pantoea stewartii* subsp. *stewartii* (syn. *Erwinia stewartii*) is an enterobacterial pathogen that causes Stewart’s vascular wilt and leaf blight of maize (3). The bacterium also colonizes an insect vector, the corn flea beetle *Chaetocnema pulicaria*, in which the bacteria survive harsh winter temperatures (10). The beetles emerge from hibernation in spring and feed on young maize seedlings thereby depositing the bacterial inoculum directly into the host tissue (10). The bacteria preferentially colonize the xylem of the host where they grow to high cell densities and produce an abundance of Stewartan exo/capsular polysaccharides (EPS) (5). This condition obstructs the free flow of xylem fluid, leading to seedling wilt, and the chlorotic and necrotic parallel streaking characteristic of Stewart’s wilt disease in mature plants (10). The production of EPS is regulated by the EsaR/EsaI quorum-sensing system. EsaI encodes an AHL synthase that produces 3-oxo-C6-HSL (AHL) constitutively. Unlike LuxR from *Vibrio fischeri* and many of its homologues, which function as activators, EsaR was initially characterized as a repressor (1). It functions by binding to target promoters (i.e. *esaR* and *rcsA*) in the absence of AHL as a homodimer in a position to block transcription (4, 8). Derepression occurs presumably because AHL causes EsaR to dissociate from the DNA.

The main objective of the study was to characterize interactions between AHL and EsaR, and through these interactions interpret what happens to the protein upon AHL binding. How the binding of AHL to the N-terminal domain modulates the activity of the DNA binding C-terminal domain is an unresolved issue in the quorum-sensing field. Multiple efforts to obtain a detailed crystal structure of EsaR have been unsuccessful primarily due to protein solubility issues. Hence, in lieu of a detailed structure for EsaR to guide further site-directed mutagenesis, error-prone PCR mutagenesis (11) was used to gain additional insights into the conformation of EsaR.
in the presence and absence of AHL. A blue/white genetic screen was developed to isolate variants of EsaR that repress transcription in the presence of AHL. After this initial screening, quantitative β-galactosidase assays were also performed on strains of interest in order to confirm the AHL-independent phenotype of the EsaR* variants. After multiple rounds of mutagenesis and screening, a total of 14 EsaR* variants were obtained that appeared to be non-responsive to AHL, but were still able to bind to DNA (7).

All of these variants proved to be stable as determined by western immunoblotting and were produced in approximately the same concentration as the wild-type EsaR (7). Genes encoding the EsaR* variants were sequenced so that the nature of the amino acid substitutions could be determined. While some of the variants contained single amino acid substitutions, many contained more than one. Hence the sites of mutation in the genes encoding the EsaR* variants with multiple substitutions were dissected using site-directed mutagenesis to generate only a single substitution per gene. These single amino acid substitutions were examined for their AHL responsiveness via the *in vivo* assay system and 8 individual amino acids were found to be responsible for the EsaR* phenotype (Table 4.1) (7).

The location of the genetic changes in *esaR* producing the EsaR* phenotype have proven to be useful to begin developing models about the mechanism of AHL inactivation of EsaR. The key amino acid substitutions have been mapped onto a homology model of EsaR using PyMOL (Fig. 4.1). The 8 individual residues key to the EsaR* phenotype were mapped near the predicted site of AHL binding. 4 of the 8 EsaR residues, 32, 98, 101, and 106, are predicted to be involved in AHL recognition through studies of TraR and LasR (2, 13), but this remains to be confirmed. The modes of action of the other 4 are unknown. It was anticipated that two primary classes of EsaR* variants would be identified from the screening process; variants that (i) no
longer bind AHL or (ii) bind AHL but are no longer responsive to it so they can still bind DNA. The DNA binding ability of this second class of variants may be due to resistance to changes in conformation. Two in vitro assays were developed to help distinguish between these two classes of variants.
MATERIAL AND METHODS:

Purification of histidine (His)₆-maltose-binding protein -GLY₅-EsaR* (HMGE*) proteins

HMGE* fusion proteins were constructed through two successive rounds of PCR. The primers in the first round were used to amplify the esaR gene from pBAD-ESAR* constructs. The forward primer TEVESAR2 (5’-GAGAACCTGTACTTCCAGGGTGTTGGTGTTGTTGATGTTTTCTTTTTTCCCTTGAATCTC-3’) contained a TEV proteolytic site and the N-terminal portion of the esaR gene separated by 15 nucleotides coding for 5 glycine residues. The reverse primer ATTBR (5’-GGGGACAACTTTGTACAAGAAAGTTGCATTACTACCTGGCCGCTGACGCTGC-3’) contained an attB site downstream of the C-terminal end of the esaR gene. An 800 bp product was recovered from PCR and used as the template in the second round of PCR. The forward primer ATTBTEV (5’-GGGGACAACTTTGTACAAAAAAGTTGTGGAGATGTACTTCCAG-3’) containing the attB site upstream of the TEV proteolytic site, and the reverse primer from the 1st round PCR were used in the second round of PCR. An 850 bp DNA fragment was recovered containing attB-TEV-GLY₅-esar*-attB. This DNA fragment was then used in a BP reaction following the vendor’s protocol (Invitrogen) with the plasmid pDONR201 (Invitrogen). pDONR201 confers kanamycin resistance (Kn) at 50 μg/ml, and is an entry vector in the Gateway System (Invitrogen). pDONR201 containing the fragment with esaR* was recovered and screened by PCR with primers TEVESAR2 and ATTBR. Using the vendor’s protocol, a LR reaction was performed with the vector pDEST-HISMBP (9) in which the vector contains a P₇₅₈ promoter controlling the expression of a His₆-MBP-TEV-GLY₅-EsaR* protein (pHMGE*) (Table 4.2). Recovered plasmid DNA was again screened with primers TEVESAR2 and ATTBR and sequenced to confirm integrity (Virginia Bioinformatics Institute Core Laboratory (VBI), Virginia Tech, Blacksburg). E. coli Top10 (6) with the eight pHMGE*
constructs was cultured at 30°C in LB broth containing 100 μg/ml Ap, in the presence or absence of 10 μM AHL to an OD$_{600}$ = 0.6, at which point 0.5 mM IPTG was added, and the culture was then incubated overnight at 19°C. Cells were concentrated by centrifugation and resuspended in HNIG buffer (20 mM HEPES, 300 mM NaCl, 20 mM imidazole, 10% glycerol (pH 7.4)). The resuspension was lysed using a French press, and the lysates were cleared by ultracentrifugation at 40,000 r.p.m at 4°C with a Ti 70 rotor. The eight fusion proteins were then purified separately using FPLC chromatography (AKTAprime plus, GE Health Sciences, Pittsburgh, PA) and a 5 mL column containing Ni$^{2+}$ resin (GE Health Sciences). A linear gradient elution with HNIG resuspension buffer containing from 20 mM to 500 mM imidazole was used to elute the HMGE* variants from the column.

**Partial in vitro proteolysis**

Reactions with a total volume of 50 μL contained 13.5 μM of the variant HMGE* fusion protein, which was separately incubated with 0.25 mg/mL of thermolysin in the absence or presence of 67.5 μM (5X in regards to protein concentration) AHL for 1 hr at 37°C in the following buffer: 2 mM CaCl$_2$, 5% glycerol, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0. Reactions with thermolysin were stopped by the addition of 2 μL of 0.5 M EDTA (pH 8.0). 12.5 μL of sample buffer (0.624 mL 1 M Tris (pH 6.8), 0.2 g SDS, 1.04 mL glycerol, 0.5 mL β-mercaptoethanol, trace bromophenol blue) was added to each reaction and visualized by 12% SDS-PAGE.

**In vitro AI binding assays**

Wild-type and EsaR* variants were resuspended in 1 mL HNG buffer (20 mM HEPES, 300 mM NaCl, 10% glycerol (pH 7.4)) to a final concentration of 2 μM with an equal
concentration of AHL. The protein was exposed to 100 µL of Ni-NTA resin (Qiagen, Valencia, CA) with gentle rocking for 30 min at 4°C. The protein-bound resin was loaded into a column which was subsequently washed with 1 mL of HNG buffer containing 20 mM imidazole. The protein was eluted with a single step gradient with buffer containing 500 mM imidazole. 1 mL of elution buffer was also exposed to resin by itself and eluted to be used as a blank in quantifying protein concentrations in later steps. The concentration of wild-type EsaR and EsaR* variants was calculated with OD_{280} readings along with a predicted extinction coefficient for each protein. The protein samples were diluted to a concentration of 92 nM in 1mL of HNG buffer. AHL was extracted from the protein by exposing the sample to 1 mL of acidified ethyl acetate two times, removing the top layer in each case. Three 500 µL aliquots of the extracted AHL in acidified ethyl acetate, for each protein, were placed in separate test tubes, and the acidified ethyl acetate was evaporated from the AHL.

_E. coli_ strain MG1655 (wild type (CGSC no. 7740)) harboring pJW01S (12) (_luxR_ divergently transcribed from _P_ _luxI_ fused to _gfp_) was grown up overnight at 30°C in RM minimal medium (2% casamino acids, 1x M9 salts (12.8 g Na_{2}HPO_{4} 7H_{2}O, 3 g KH_{2}PO_{4}, 0.5 g NaCl and 1 g NH_{4}Cl/liter), and 1 mM MgCl_{2}) with 0.4% succinate and 50 µg/mL kanamycin (Kn). The strain was then subcultured to an OD_{600} of 0.05 in 200 mL of the same medium, and grown at 30°C to an OD_{600} of 0.25. 5 mL was transferred to test tubes containing AHL extracted from wild-type EsaR and EsaR* variants. 5 mL of the culture was also transferred to three separate test tubes to act as a control for no AHL. The cultures were grown to an OD_{600} of 0.5. 200 µL of culture was placed in a 96-well optical bottom microtiter plate for the analysis of both fluorescence output (excitation and emission wavelengths of 485 and 535 nm, respectively) and OD_{590} on a Tecan SpectraFluor Plus plate-reader (Tecan, Mannedorf/Zurich, Switzerland).
Fluorescence values were standardized by dividing by the final OD$_{600}$ for each sample. The assays were performed as two independent triplicate sets.
RESULTS AND DISCUSSION:

Selection and purification of EsaR* variants for in vitro analysis

Several variants of EsaR (EsaR*) that are AHL-insensitive have previously been identified (7). These variants each have single amino acid substitutions, and their stability was confirmed through western immunoblotting. Quantitative β-galactosidase assays were used to determine the degree of sensitivity to AHL. Variants able to repress transcription in the presence of AHL at least roughly 2-fold more than the wild-type control, were chosen for further investigation (7).

Eight EsaR* variants (A32V, A81T, D83E, F94Y, F98Y, S101P, Y104D, I106F) (Table 4.1) were chosen for use in in vitro experiments. N-terminal His-MBP affinity tags were placed on the eight variants to facilitate purification. Assays were developed to differentiate between two potential classes of variants. The first class represents variants with amino acid substitutions that inhibit binding of AHL. The second class represents variants that have retained the ability to bind AHL. This second class is seen as the more intriguing subset because residue changes may result in the inability of the protein to undergo normal conformational changes in response to the AHL ligand.

Ability of EsaR* variants to bind AHL

A newly developed in vitro AHL binding assay allowed for direct comparative measure of the ability of the EsaR* variants to bind AHL. In the assay purified HMGE and HMGE* variants are exposed to AHL at a 1:1 ratio in regards to protein concentration. The proteins are repurified, and bound AHL is extracted. The extracted AHL is then exposed to recombinant E. coli harboring a plasmid, which expresses LuxR and contains a gfp reporter gene under the control of P_{lux}. The in vitro AHL binding experiments revealed that two of the eight variants,
D83E and F94Y, retained the ability to bind AHL. The six remaining variants appeared to be deficient in AHL binding (Fig. 4.2). Based on amino acid sequence alignments (Chapter One) and homology modeling, four of the six residues 32, 98, 101, 106 identified during the screen, were predicted to be involved in AHL binding based on amino acid alignments and homology models to LasR and TraR (2, 13). When the position of these residues is modeled onto a homology model of EsaR, they cluster around the predicted AHL binding pocket (Fig. 4.1). Therefore, the AHL binding assays suggested that the amino acid substitutions at six of the residues (A32V, A81T, F98Y, S101P, Y104D, I106F) directly or indirectly interfered with AHL binding.

**Conformational analysis of EsaR variants via limited proteolytic digestion**

An *in vitro* proteolytic assay utilizing the protease thermolysin gives differential digestion pattern of wild-type EsaR in the presence and absence of AHL (Chapter Three). Thermolysin preferentially cleaves sites with bulky hydrophobic residues such as isolucine, valine, alanine, methionine, and phenylalanine. This assay was used to examine structural changes resulting from amino acid substitutions in EsaR.

The *in vitro* proteolytic assays gave some further insight into structural changes occurring within the protein. In the case of assays performed in the absence of AHL, variants A32V, F98Y, and S101P gave a banding pattern similar to the pattern seen with wild type in the presence of AHL (Fig. 4.3 A & B). In the case of the S101P variant, replacing a serine residue with a more bulky residue such as proline may introduce steric hinderance or cause the protein refolding, thereby inhibiting binding by AHL and protecting the hydrophobic region from proteolysis by thermolysin. In the case of the A32V and F98Y variants, it can be suggested that these substitutions disrupt the AHL binding pocket, whereby the appropriate residues required
for cleavage by thermolysin are not as freely exposed (Fig. 4.3 A & B). After examining the predicted location of these residues on the homology model of EsaR (Fig. 4.1), it can be suggested that these residues exist on the surface of the protein. A32V has a bulkier side chain at residue 32 than wild-type EsaR, and appears to be locked in a conformational state that indirectly antagonizes AHL binding. In the case of the F98Y variant, there is a change in polarity. Thus, by introducing a polar residue, interactions between the pocket and AHL may be indirectly disrupted. As suggested from the homology model of EsaR, residues 32 and 98 appear closer to the surface of the protein as compared to residues 81, 101, 104 and 106. By disrupting the opening of the AHL binding pocket, hydrophobic residues within the binding pocket may no longer be freely accessible to thermolysin in comparison to the wild-type AHL binding pocket.

Assays revealed that three of the variants (A81T, Y104D, and I106F) also gave similar banding patterns both in the presence and absence of the ligand (Fig. 4.3 A & B). This pattern was the same as that produced by wild-type EsaR in the absence of AHL. The A81T variant creates a change in polarity. In regards to the homology model, residue 81 exists within the binding pocket of AHL and may be required for some critical interaction with AHL. By making substitutions at this residue, the overall hydrophobicity of the AHL binding pocket may have changed, leading to inhibition of AHL binding. The Y104D variant is distinct because there is a charge difference between the residues. By introducing a negatively charged residue within the binding pocket, it may repel AHL from binding. Finally, the I106F variant has a bulky side group substituted, which may create steric hinderance within the binding pocket and block AHL from binding. Overall, these assays in combination suggest that residues 32, 81, 98, 101, 104, and 106 may play a direct or indirect role in binding of AHL by EsaR.
The final two residues, D83E and F94Y, were unique in that they retained the ability to bind AHL in the *in vitro* binding assays. This phenotype was confirmed with the thermolysin assay, in which the variants demonstrated similar banding patterns compared to wild type +/-AHL suggesting that the variants were capable of interacting with AHL. In the case of the D83E variant, results from both assays suggest that it has a slightly decreased affinity for AHL in comparison to wild type as shown by the levels of Gfp expression in the *in vitro* binding assays (Fig. 4.2), and the intensity of the resistant band with regards to the thermolysin assay (Fig. 4.3B). However, residues 83 and 94 are unique because they appear to play a role in the mechanism by which EsaR changes conformations in response to AHL.

**Concluding remarks**

Two different classes of AHL-insensitive EsaR* variants were identified in this study. The first set of variants (A32V, A81T, F98Y, S101P, Y104D, and I106F) has an effect on AHL binding. The alignment of these residues with LasR and TraR suggest that the three proteins have similar, but not identical structural configurations (Chapter One). It appears that AHL associates with a hydrophobic region within EsaR, and by altering residues 32, 98, 104, and 106, which have been shown to be important in TraR and LasR for binding, a conformational change is introduced within the N-terminal domain such that the AHL cannot make a stable interaction. An alternative explanation is that substitutions at these positions may be causing refolding of the protein in such a way that critical amino acids for binding are not readily available to AHL. The second set of variants, D83E and F94Y, retain the ability to bind AHL, therefore the proteins are deficient in the mechanism by which a conformational change is conferred upon EsaR. These residues are intriguing and will require further analysis to help uncover the mechanism of AHL-responsiveness utilized by EsaR and its homologues.
The majority of work on the LuxR family of proteins has pertained to activators, which require the native AHL for purification. EsaR presents a unique opportunity to study the mechanism of AHL-responsiveness used by the LuxR protein family, as it can be purified under conditions of both in the presence and absence of AHL. Current studies rely heavily on homology models to LasR and TraR to gain insight into the structure, but the predicted structure of EsaR is unique to the structures of LuxR-type activators as members of this subfamily have an extended linker region and C-terminus. Therefore, future structural studies of EsaR will be required to fully characterize its mechanism of AHL-responsiveness.

ACKNOWLEDGEMENTS

We thank Jessica (Koziski) Scruggs for work on the initial screening and characterization of the EsaR* variants and Andre Levchenko for his support of this project. This work was funded by a National Institutes of Health GM0066786 subcontract.
REFERENCES


Table 4.1. Characterization of AI-insensitive variants

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Figure 4.1. Position of substitutions in EsaR* variants mapped on the homology model of the N-terminal domain of EsaR. Using PyMOL, the side chains of the critical amino acids, 32, 81, 83, 94, 98, 101, 104, and 106, are highlighted in: (Red) D83E and F94Y (suggested involvement in mechanism of conformational change) (Yellow) A32V, A81T, F98Y, S101P, Y104D, and I106F (residues suggested to make direct or indirect interactions with AHL); (Green) AHL.
Figure 4.2. Ability of EsaR* variants to bind AHL in vitro.  *E. coli* strain MG1655 (Wild type harboring pJW0lS (*luxR* divergently transcribed from *P*~*luxI* fused to *gfp*) was grown in the presence of AHL extracted from purified wild type and EsaR* variant proteins as indicated.  The control was *E. coli* strain MG1655 harboring pJW0lS grown under similar assays conditions in the absence of AHL.  Fluorescence values were standardized by dividing by the final OD$_{600}$ for each sample. The assays were performed as two independent triplicate sets.
Figure 4.3. Limited proteolytic digestion of EsaR* variants by thermolysin. A) Patterns of thermolysin cleavage of EsaR* variants in the absence of AHL. B) Patterns of thermolysin cleavage of EsaR* variants in presence of AHL. Arrows indicate bands of interest corresponding to the N-terminal domain of EsaR. The images are representative of experiments performed in duplicate.
Chapter Five
Overall Conclusions
Bacterial cell-cell communication, termed quorum sensing, was originally identified in the Gram-negative marine organisms *Vibrio fischeri* and *Vibrio harveyii* (8). Two key regulatory components are present in the *V. fischeri* quorum-sensing system. Specifically, a protein called LuxI is responsible for the production of the acyl-HSL (AHL) signal molecule. A protein called LuxR, which senses and binds the AHL, acts as an AHL-dependent transcriptional activator of the *lux* operon, necessary for production of bioluminescence (3, 4, 11).

It has now become clear that this process of bacterial cell-cell communication is well-conserved across a broad spectrum of both Gram-negative and Gram-positive bacteria, although the precise molecular basis of it can vary. With the identification and subsequent characterization of over 50 different LuxI/R homologous systems, it is evident that numerous bacterial phenotypes can be affected by quorum sensing. Many of these phenotypes are associated with symbiotic relationships as is the case with *V. fischeri*, but more often these systems appear to play a role in some host-pathogen interaction. This is the case with *Pantoea stewartii* subsp. *stewartii*, this species quorum-sensing system participates in the control of virulence (1). This plant pathogen is the causative agent of Stewart’s wilt disease and leaf blight in maize. *P. stewartii* is transmitted to the plant by the corn flea beetle, *Chaetocnema pulicaria*. Beetles that harbor these pathogens feed on emerging corn seedlings and introduce the bacteria into the leaves of the plant (10). The bacteria then migrate through the intercellular spaces of the leaves and make their way to the xylem. Once the bacteria reach the xylem, they are capable of growing to high cell density and secrete an abundance of exopolysaccharide (EPS). Production of EPS blocks the corn xylem vessels, leading to vascular collapse and plant wilt (5, 9, 10).

In *P. stewartii*, two regulatory proteins are key to the process of quorum sensing, the LuxI and LuxR homologues EsaI and EsaR (1). Most LuxR homologues function as activators of
transcription in the presence of their cognate acylated homoserine lactone signal (AHL). EsaR has an opposite response to AHL in comparison to the majority of the LuxR homologues. At low cell densities in the absence of AHL, EsaR functions by binding DNA and repressing its target promoters. At high cell density, which corresponds to high concentrations of AHL, EsaR associates with the signal molecule leading to derepression (6, 7). Currently, very little is understood about this reverse mechanism of control by AHL.

Therefore, a comparative study to two well characterized quorum-sensing regulators LuxR from *Vibrio fischeri* and TraR from *Agrobacterium tumefaciens* was initiated to determine the level of functional homology that is retained among the LuxR family of proteins. EsaR retains the ability to function as a weak activator of the *lux* operon in recombinant *Escherichia coli* in the absence of AHL (12). Using a structural and functional analysis, EsaR has now been further characterized as an activator. As is the case with TraR, it was revealed that both domains of EsaR are necessary for positive regulation. A site-directed mutagenesis study, guided by homology modeling to LuxR and TraR, has revealed three critical residues in EsaR that are involved in activation of RNA polymerase. In addition, a native EsaR-activated promoter has been identified, which controls expression of a putative regulatory sRNA in *P. stewartii*. Identification of a transcript directly activated by EsaR establishes that this regulatory function has been maintained in the native host and therefore has physiological relevance. Quorum sensing in *P. stewartii* is therefore unique in that the quorum sensing system regulates genes at both low and high cell density: (i) at low cell density EsaR can function as both an activator and repressor thereby controlling the expression of one set of genes and (ii) at high cell density, the accumulation of AHL leads to inactivation and derepression of genes regulated by EsaR, which allows for differential control of a second set of genes. Future identification of quorum-sensing
controlled functions at the different stages of the infection process might permit for successful
disease intervention strategies to be developed. A combination of bioinformatic and
experimental methods could be employed to more fully characterize the EsaR regulon.

Very little is understood about the mechanism whereby AHL binding results in the
subsequent release of EsaR from DNA. In fact, no in vitro comparative analysis has been
performed on the ligand-free and ligand-associated forms of any LuxR family protein. Hence,
the second focus of this study was to further elucidate the mechanism by which AHL regulates
EsaR. It has clearly been demonstrated that EsaR is equally resistant to protease degradation in
live cells in the presence or absence of AHL. Further, AHL does not impact the oligomeric state
of EsaR, but it does have more subtle impacts on the overall conformation of the protein.

To reach a more complete understanding of the mechanism of regulation by AHL, the
final goal of the study was to more closely examine the role of individual amino acid residues in
AHL-responsiveness. A random mutagenesis genetic approach was designed to isolate EsaR* variants that are immune to the effects of AHL. Eight individual amino acids playing a critical
role in the AHL-insensitive phenotype have been identified. Six of these residues either directly
or indirectly impact AHL binding, while two appear to play a role in the conformational shift
that occurs in response to AHL.

There is limited detailed structural information about LuxR homologues. LasR from
Pseudomonas aeruginosa and TraR require AHL binding for purification (2, 13). This has
therefore restricted the ability to examine the mechanism of AHL-responsiveness of these
proteins. EsaR can be purified in the presence or absence of its cognate AHL. Therefore, future
structural studies on AHL interaction with EsaR have the potential to significantly impact the quorum-sensing field.
REFERENCES


