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GacA REGULATION OF LUMINESCENCE IN THE SYMBIOTIC BACTERIUM
Vibrio fischeri

BY

ALICIA EVE BALLOK

Molecular, Cellular and Developmental Biology, B.S., 2005

THESIS

Submitted to the University of New Hampshire

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In

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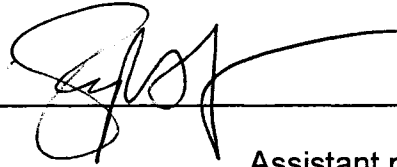
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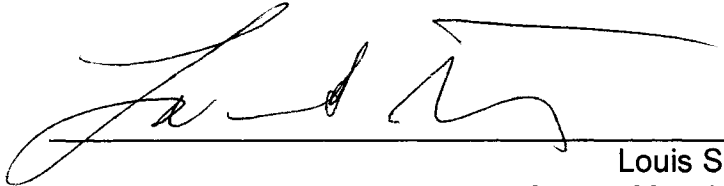
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ABSTRACT

GacA REGULATION OF LUMINESCENCE IN THE SYMBIOTIC BACTERIUM *Vibrio fischeri*

By

Alicia Eve Ballok

University of New Hampshire, December , 2007

The symbiosis of *Euprymna scolopes* and *Vibrio fischeri* depends on the ability of the bacterium to colonize the squid and produce light. GacA is an activator of symbiosis phenotypes including luminescence though it is not understood how GacA functions. My hypothesis, based on other bacterial models, is that GacA activates expression of CsrBs, functional RNAs, which sequester and repress a translational repressor, CsrA. I approached this question with mutagenesis and complementation techniques. We found mutation of CsrA or addition of a CsrB *in trans* could complement the GacA mutant for symbiosis and luminescence phenotypes. The inability of other luminescence regulators including a constitutively expressed *lux* operon to complement GacA Δ indicated that luminescence was regulated post-transcriptionally. A putative CsrA binding site found in the sequence of the *lux* mRNA increased light production when deleted. These data demonstrate that GacA regulates luminescence post-transcriptionally via CsrB/CsrA antagonism, supporting my hypothesis.

CHAPTER 1

INTRODUCTION

Symbiosis, when two organisms benefit from an association with the other (Lederberg, 2000), is a survival strategy employed by organisms in every domain and at every level of complexity. The most studied symbiotic associations occur between eukaryotes and microbes. Plants commonly benefit from an association with microbes in their root systems such as in the association of soybeans with *Bradyrhizobium japonicum* where bacteria fix nitrogen for the plant and the plant provides environmental protection and nutrients to the bacteria (Hennecke, 1990). Most animals, including humans, would be malnourished if they did not have symbiotic gut microorganisms to aid in digestion. These bacteria can also contribute to immunity and normal gut development (Hooper and Gordon, 2001; Hooper, 2004). Mice raised without gut bacteria have to eat 30% more to maintain normal weight (Sears, 2005). My research revolves around the highly specific symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the luminescent bacterium *Vibrio fischeri*.

The *E.scolopes/V.fischeri* symbiosis is beneficial for both organisms (reviewed in (Ruby and McFall-Ngai, 1992; Nyholm and McFall-Ngai, 2004; Visick and Ruby, 2006)). The nocturnal squid is believed to benefit from the

luminescence produced by its bacterial symbionts that are cultured and housed in a specialized organ. At night, when the squid hunts on the reef, it uses its series of lenses and reflectors to manipulate the bacterially-produced light to mimic the moonlight. This light may protect the squid from predatory fish, swimming along the sea floor, looking up for a shadow to pass across the moon (McFall-Ngai, 1990), a type of camouflage called counterillumination. The bacteria, though capable of existing in a free-living state, associate with the squid which provides nutrients and an environment for rapid population growth (McFall-Ngai, 1990). Each day at dawn, the squid vents (expels) 95% of the bacteria from its light organ and facilitates growth of a new culture during the day (Lee and Ruby, 1994). This daily culturing and purging results in a high number of *V. fischeri* in the Hawaiian reefs where squid live, whereas the surrounding tropical pacific waters have low *V. fischeri* populations (Lee and Ruby, 1994).

Establishing the Symbiosis

The squid hatches without its symbiont and therefore must collect it from the environment (Ruby and Lee, 1998). Recruiting one specific type of bacterium in the ocean is seemingly difficult given the large numbers of diverse microbes in the sea (about a million per ml) (Dolan, 2006). *E. scolopes* has developed a system that weeds out the undesirable bacteria and allows only the symbiotic strains of *V. fischeri* to enter the light organ (reviewed in Nyholm and McFall-Ngai, 2004). Concurrently, symbiotic *V. fischeri* coordinate gene expression in a way that permits entry into the light organ (Reviewed in Visick and Ruby, 2006).

The earliest discernable stage of bacterial association is the aggregation of bacterial cells in squid-derived mucus (Nyholm and McFall-Ngai, 2003). The squid hatches with ciliated appendages in a region of ciliation located near the pores that lead to the light organ (McFall-Ngai, 1998). Shortly after the squid hatches, the cilia on these appendages begin to beat and this motion collects many different kinds of bacteria from the surrounding seawater. Contact with these bacteria, in particular their peptidoglycan, initiates secretion of mucus from the ciliated appendages of the squid (Nyholm *et al.*, 2002). It is important to point out that this mucus shedding is indiscriminate and occurs in response to all bacterial membranes. Although Gram-positive bacteria trigger mucus shedding, they do not aggregate in the mucus (Nyholm and McFall-Ngai, 2003). In contrast, all Gram-negative bacteria are capable of aggregating in the mucus. However, *V. fischeri* tend to dominate in these aggregates (Nyholm and McFall-Ngai, 2003). This hierarchy of association exemplifies the first step in specifying the type of bacteria that enter the light organ.

Once in the mucus, the bacteria must move toward the light organ. It is believed that the *V. fischeri* move into the pores and then into the ducts of the light organ in response to a chemoattractant, N-acetylneuraminic acid, a component of squid light organ mucus (DeLoney-Marino *et al.*, 2003). Flagellar motility is very important in initiating the symbiosis. In fact, non-motile mutants are incapable of establishing the symbiosis and mutants in FlaA (a gene important in flagellin synthesis) are severely impaired in colonization (Graf *et al.*,

1994; Millikan and Ruby, 2004) and mutants of either FlrA or σ^{54} are incapable of initiating the symbiosis (Millikan and Ruby, 2004; Millikan and Ruby, 2003).

In the ducts leading to the light organ, symbiotic *V. fischeri* encounter and must overcome host imposed challenges often referred to as the gauntlet. First, the ducts are lined with cilia that beat away from the light organ and the bacteria must swim against this opposing current (McFall-Ngai, 1998). Second, the squid contains patrolling macrophage-like hemocytes (Nyholm and McFall-Ngai, 1998) which presumably phagocytose invading microbes. Third, there are toxic levels of an oxidative species, NO, generated by the squid enzyme nitric oxide synthase (Davidson *et al.*, 2004). *V. fischeri* is apparently able to overcome these defenses and enter the light organ. Even inside the light organ the bacteria face continued host-imposed stress. Within the deep crypt space cells of the light organ, there are transcripts encoding halide peroxidase which creates another deadly oxidative species, hypohalous acid (Small and McFall-Ngai, 1999). The oxidative species created in the light organ are detrimental and oxidative stress mutants of *V. fischeri* like those defective in catalase production are less competitive at colonization than the wild type (Visick and Ruby, 1998).

After overcoming the challenges of getting into the light organ, the bacteria must grow on host-provided nutrients (McFall-Ngai, 1990) and induce changes in the host. *V. fischeri* membrane components Lipid A as well as a subunit of peptidoglycan trachial cytotoxin (TCT) play important roles in these changes. TCT, a molecule found in other organisms, is required for virulence in pathogens like *Bordetella pertussis*. TCT appears to induce hemocyte movement within the

appendage tissue of the ciliated field of the light organ (Koropatnick *et al.*, 2004). Lipid A, a component of the LPS is responsible for inducing apoptosis and combined with TCT triggers regression of the ciliated field. Many other changes occur during symbiotic association: (1) The production of NO is irreversibly diminished in the ducts (Davidson *et al.*, 2004). (2) The ducts to the light organ constrict (Kimbell and McFall-Ngai, 2004). (3) The cells in the crypt spaces of the light organ change shape (Doino, 1998). (4) The density of the microvilli surface of the crypts increases possibly to either supply nutrients (Montgomery and McFall-Ngai, 1994) or to change the intimacy of the interaction by suspending the bacterial cells away from the underlying tissue (Lamarcq and McFall-Ngai, 1998) (5) Mucus secretion from the appendages outside the light organ is stopped while at the same time, mucus secretion within the light organ tissue increases (Nyholm and McFall-Ngai, 2004). Other than LPS and TCT, signals for these changes are still unknown; however, they play an important role in halting the permissiveness of the light organ to other bacterial symbionts (Whistler *et al.*, 2007).

Initiation of symbiosis is clearly tightly controlled by the host and symbiont, but the specificity does not end upon colonization. To maintain the symbiosis, the bacteria must produce light. Bacteria that do not make light, such as those defective in luciferase (LuxA) or activation of light production (LuxI), can establish the symbiosis but can not persist in the light organ (Visick *et al.*, 2000). Although the mechanisms by which the squid can discriminate light from dark symbionts is still unknown, Whistler and McFall-Ngai propose that the squid could use

photoreceptors to distinguish between light and impose sanctions upon dark bacteria perhaps selectively eliminating these cheaters (Stabb, 2005). Whether or not this hypothesis is true, it is clear that luminescence is a requirement imposed on the bacteria by the squid. Is there any benefit of luminescence for the bacteria directly? A number of theories have been proposed, two of which revolve around oxygen consumption by the luciferase reaction and sensitivity to reactive oxygen species. If the symbiotic *V. fischeri* utilize all available oxygen in the crypt spaces they can starve the squid cells of oxygen, thereby preventing production of reactive oxygen species. Alternatively, utilizing the oxygen will lower the intracellular oxygen levels and protect the oxygen sensitive enzymes (Ruby *et al.*, 2005). Another hypothesis is that within the constraints of the light organ where growth is limited, the luciferase reaction can serve as an alternative electron sink (Stabb, 2005). Finally, the light production may aid in DNA repair because it stimulates light-dependent photolyase-mediated DNA repair (Czyz *et al.*, 2003) however, photolyase has no significant effect on squid colonization, suggesting stimulation of DNA repair is not the role of bioluminescence in this symbiosis (Walker *et al.*, 2006) .

Luminescence and its Regulation

In *Vibrio fischeri*, luminescence is a product of a number of enzymes linked in an operon. The light is produced by the luciferase enzyme, made up of two protein subunits encoded by *luxA* and *B* (Meighen and Dunlap, 1993). Luciferase requires substrates that must be manufactured by the bacterium.

Genes also located on the same operon, *luxCDE*, encode proteins for synthesis of the substrates of the luciferase reaction (Meighen and Dunlap, 1993). There are other regulatory factors involved, such as activators and repressors that mediate light production at varying cell densities.

Luminescence is an important yet energy-expensive function and therefore must be tightly controlled (Stabb, 2005). *V. fischeri* has an elaborate system in place for regulating luminescence. Luminescence regulation involves three hierarchical quorum sensing circuits; LuxI/R, AinS/AinR, and LuxS/P/Q, as well as additional known activators and repressors that modulate these quorum sensing pathways (Engbrecht and Silverman, 1984; Lupp *et al.*, 2003).

At low cell density, as would be observed in free-living bacterial populations in the ocean, luminescence is repressed. In the current model of light regulation described by Lupp and colleagues, (Lupp *et al.*, 2003), at low cell densities there are no quorum sensing molecules for the transcriptional activator LuxR to bind, so it does not activate luminescence (Fig. 1) (Reviewed in Milton, 2006). When bound to the quorum sensing molecule, acyl homoserine lactone (AHL), LuxR dimerizes and activates transcription of the *lux* operon (Engbrecht *et al.*, 1983). *luxR* transcription is self-induced as well as induced by a transcriptional activator LitR (Fidopiastis *et al.*, 2002). At this low cell density LitR expression is repressed indirectly by a protein called LuxO (Miyamoto *et al.*, 2000).

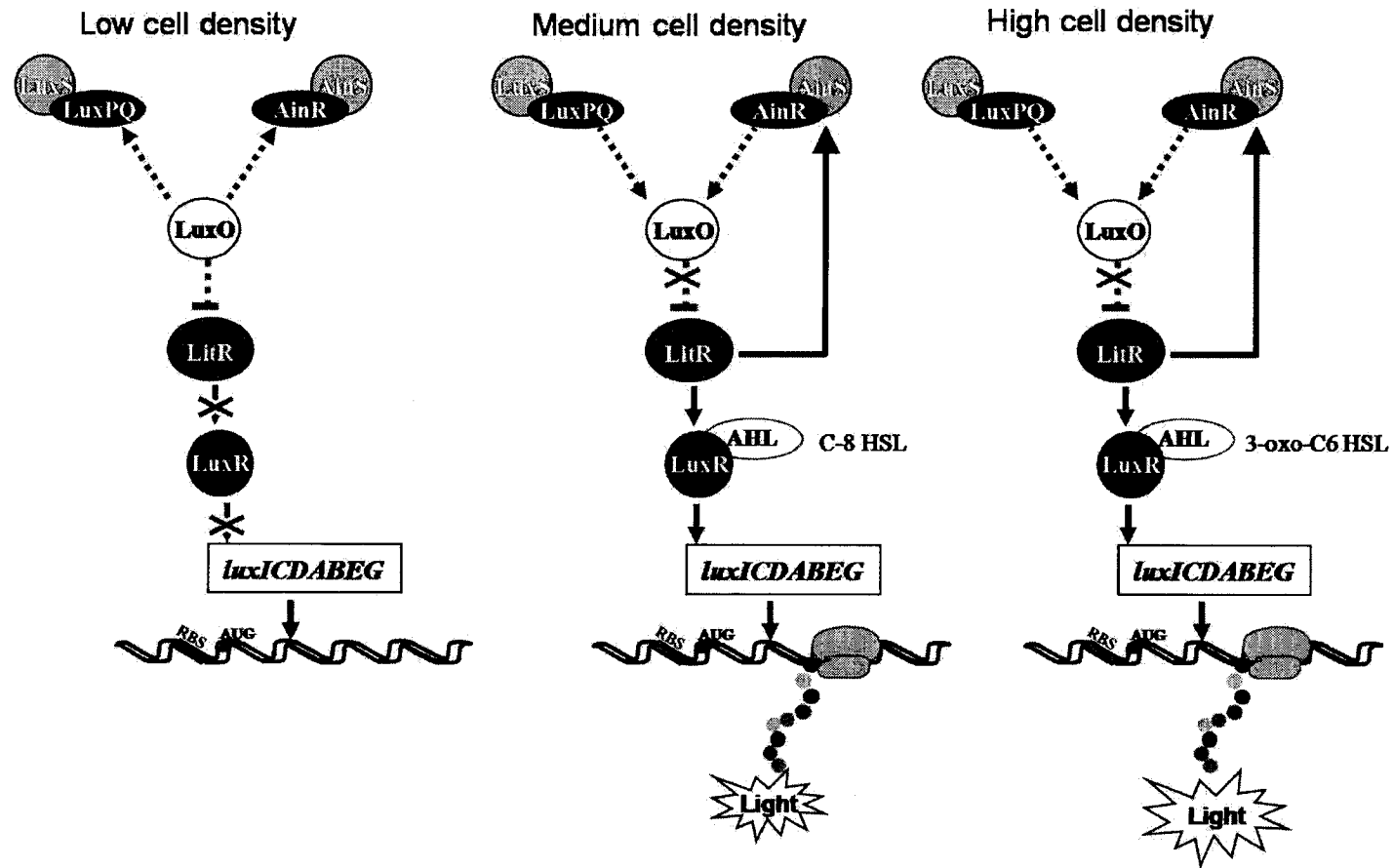


Fig. 1. Density-dependent luminescence expression At low cell density luminescence is repressed by LuxO. As the cell density increases, LuxO repression is relieved and C-8 HSL, the product of AinS, binds to LuxR and activates *lux* transcription. At high densities, 3-oxo-C6 HSL, the product of LuxI, activates luminescence to a high level (outlined in Lupp *et al.* 2003).

alleviated by the LuxS/PQ and AinS/R quorum sensing systems (Lupp and Ruby, 2004). The LuxS/PQ system is made up of three proteins; LuxS is the signal synthase which makes the AI₂, LuxP located in the periplasm, receives the AI₂ signal and transmits it to LuxQ which is the signal receptor kinase (Lupp and Ruby, 2004). AinS is a signal synthase that produces a C₈-autoinducer homoserine lactone (AHL) that is sensed by the AinR signal receptor (Gilson, 1995). These systems act together to dephosphorylate LuxO thereby inactivating it. AinS serves a second purpose at this cell density, in that it is the dominant quorum sensing molecule in culture and is capable of binding to LuxR and activating *lux* transcription to a low level (Lupp *et al.*, 2003).

At very high cell densities as is seen within the squid host, luminescence is activated to a very high level by 3-oxo-C₆-HSL (Lupp *et al.*, 2003; Visick *et al.*, 2000). LuxO is still repressed by AinS/R and LuxS/PQ but the AinS product is no longer the dominant AHL (Schaefer, unpublished). *luxI*, the first gene in the *lux* operon, is responsible for the 3-oxo-C₆-HSL production and thus activates its own expression. The response to varying levels of quorum sensing molecules seen in *V. fischeri* is reminiscent of the multiphasic *Vibrio harveyi* quorum sensing response (Waters and Bassler, 2006).

GacA/CsrA/CsrB

Another protein involved in luminescence regulation is GacA (Global activator A) (Reviewed in Heeb and Haas, 2001). This is a response regulator protein found in proteobacteria that along with its partner signal receptor protein

GacS. The GacS homolog was first identified in *Pseudomonas syringae* as required for pathogenicity on beans (Hrabak and Willis, 1992). GacA was first identified as an activator of antibiotic and cyanide production in *P. fluorescens* (Laville *et al.*, 1992). Since then, this two-component system has been found in a number of different bacteria and appears to have pleiotropic effects (Heeb and Haas, 2001). In *V. fischeri* mutants in GacA are deficient in light production in culture and this defect can not be complemented with either 3-oxo-C6 or C8 AHL (Whistler and Ruby, 2003). GacA also has little effect on abundance of AHL (Whistler and Ruby, 2003) which means that GacA does not act primarily through AHL regulation. This contrasts to GacA regulation of quorum sensing in *Pseudomonas* species (Bertani and Venturi, 2004; Chancey *et al.*, 1999). Since GacA does not regulate quorum sensing by a mechanism analogous to that previously described in *Pseudomonas*, just how GacA regulates luminescence in *V. fischeri* is still not known.

GacA not only regulates light production, but it also influences a suite of other important symbiotic traits. A GacA mutant is also deficient in; normal LPS, siderophore, normal motility, growth on simple sugars and a high growth yield (Whistler and Ruby, 2003). In terms of squid phenotypes, this mutant is a poor colonizer and once in the light organ, reaches a low density (about 2% of wild-type). It also fails to trigger normal host morphogenesis, leaving the light organ open to other symbionts (Whistler *et al.*, 2007).

GacA is important in host colonization and similar effects have been seen in other organisms. In *V. cholerae* VarA mutants (a GacA homolog) are

attenuated in virulence and colonization of the mouse model (Wong *et al.*, 1998). The GacA homolog, UvrY, is required for colonization of urinary tracts of monkeys by *E. coli*. *P. fluorescens* requires GacA for proper biocontrol and ecological fitness (Heeb and Haas, 2001). In these organisms, GacA activates expression of small non-coding RNAs called CsrBs (Carbon storage regulator) in most organisms or RsmBs (Regulator of secondary metabolism) in pseudomonads (Lenz *et al.*, 2005; Liu *et al.*, 1997; Johansson and Cossart, 2003) .

Work in *E. coli* identified CsrB function as a repressor of a repressor (Romeo, 1998). CsrBs are a class of small RNAs with a secondary structure containing several stem-loops with specific GGA motifs. These motifs interact with a protein called CsrA (RsmA in Pseudomonads), first discovered in *E. coli* as a regulator of gluconeogenesis (Romeo, 1998; Romeo *et al.*, 1993). One CsrB RNA is capable of binding to several CsrA proteins (Reviewed in Romeo, 1998; Babitzke and Romeo, 2007). CsrA is a small protein (60-65aa) that is made up of a series of five beta sheets followed by an alpha helix (Gutierrez *et al.*, 2005). This protein functions as a homodimer to bind mRNA transcripts, prevent their translation and sometimes signal their degradation (Gutierrez *et al.*, 2005; Romeo, 1998). There are two particular domains of significance in this protein, the regions formed by aminoacids 2-7 and 40-47 (Mercante *et al.*, 2006). In the homodimeric form, region 2-7 of one subunit is located next to region 40-47 of the other subunit, forming the RNA binding site (Mercante *et al.*, 2006). CsrA binds mRNA with multiple GGA motifs, usually in stem-loops, in the 5' UTR and including the ribosomal binding site (Dubey *et al.*, 2005). By binding at the

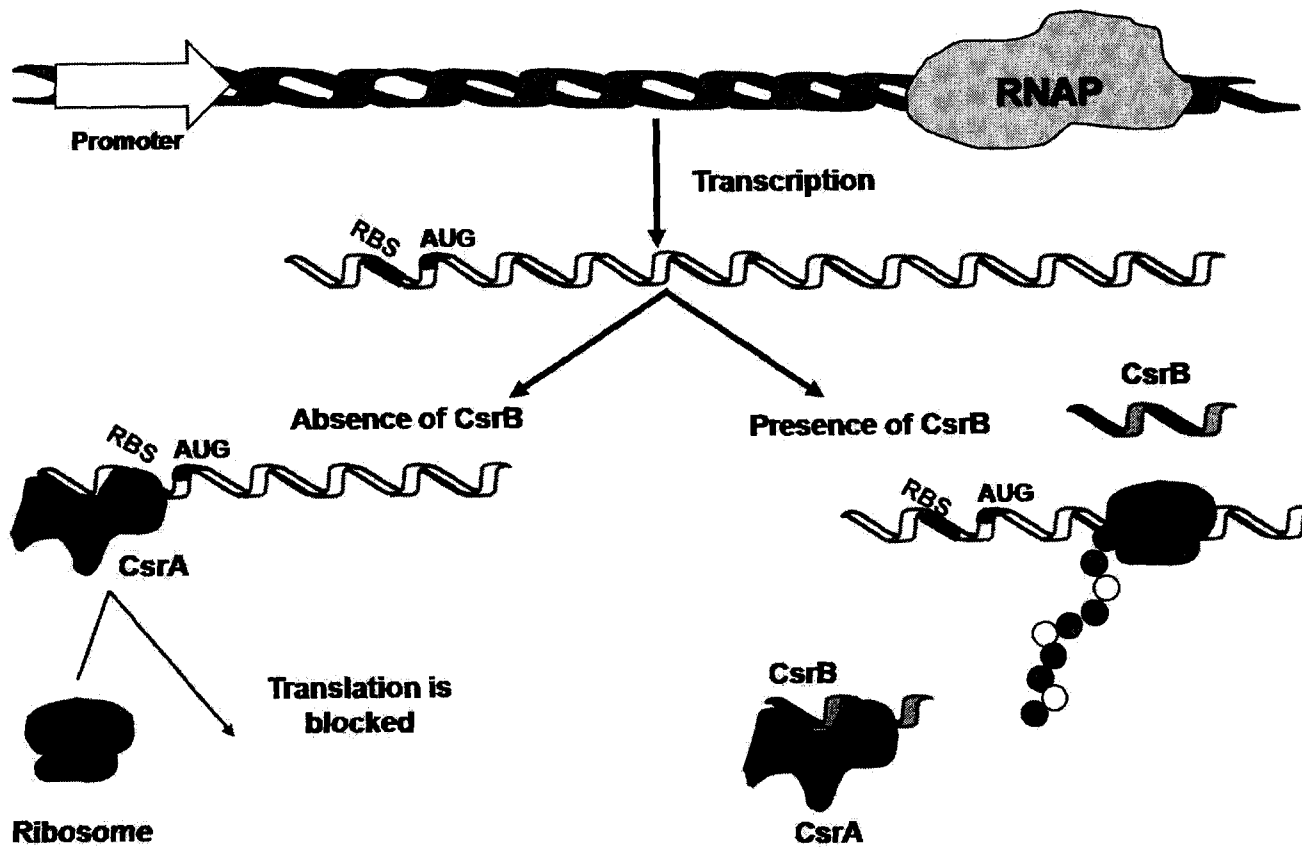


Fig.2. Post-transcriptional regulation by CsrA/CsrB. In the absence of CsrB, target transcripts are bound by CsrA, blocking the ribosomal binding site and translation. When CsrB is present, this small RNA sequesters CsrA, allowing ribosomal binding and translation.

ribosomal binding site, CsrA blocks the ribosome from binding and translating the mRNA into protein. If CsrBs are present, CsrA will preferentially bind the CsrBs, thereby sequestering the protein and allowing translation to occur (Romeo, 1998). CsrBs have many stem-loops therefore a single CsrB sRNA can bind many CsrA molecules.

The regulatory relationships of CsrA/CsrB and GacA known in other bacteria may be relevant to *V. fischeri* GacA regulation. Recently, an autoregulatory loop between UvrY and CsrA/CsrB has been identified in *E. coli* where CsrA upregulates expression of CsrB via activation of UvrY (Reviewed in Romeo and Babitzke, 2007). CsrA/CsrB regulate a number of phenotypes in other bacteria including growth, biofilm formation, virulence, carbon storage, motility and EPS (Romeo, 1998; Lenz *et al.*, 2003). Naturally these phenotypes are related to the phenotypes associated with GacA mutants because in these organisms, GacA activates expression of CsrBs. This relationship has not been shown in *V. fischeri* but we have evidence to support its existence. Sequences for CsrBs were recently identified by a bioinformatic approach in *V. fischeri* and tested for functionality in *E. coli* (Kulkarni *et al.*, 2006). These CsrBs are approximately 400 bases in length and contain 21 CsrA binding motifs, many of which are in stem loops showing great similarity to the other identified CsrA-sequestering RNAs (Kulkarni *et al.*, 2006). In species like *Legionella pneumophilla*, GacA seems to function solely through CsrBs as an antagonist of CsrA; however, in *E. coli* GacA regulates biofilms independently of CsrBs (Molofsky and Swanson, 2003; Suzuki *et al.*, 2002).

Research Objectives

My research focuses on elucidating the mechanism of GacA regulation of luminescence as well as other symbiotic phenotypes of *V. fischeri*. Using a genetic approach, I will address questions about how GacA exerts its positive influence on luminescence. AHL complementation and quantification data suggest that it is not through quorum sensing signal (AHL) production (Whistler and Ruby, 2003) but it could still affect sensing of these quorum signals. Previous studies raise the question of whether GacA even regulates luminescence by a transcriptional means since the AHLs have a direct effect on transcription, or alternatively whether it regulates light by a post-transcriptional mechanism. Based on the seemingly conserved regulatory relationship between GacA and CsrBs in other organisms, it is likely that GacA regulates the identified CsrBs. I specifically addressed the influence of GacA on the CsrBs and whether this explains regulation of luminescence and the other GacA-associated phenotypes. **I hypothesized that GacA activates symbiotic phenotypes of *Vibrio fischeri* via CsrB repression of CsrA.** Through mutagenesis and complementation I show that luminescence and other phenotypes of the GacA mutant can be complemented by CsrB1 or CsrB2, and that CsrA exerts a negative effect on luminescence post-transcriptionally, most likely by binding directly to the *lux* transcript.

CHAPTER 2

GacA REGULATION OF *lux* INDICATES A UNIQUE CIRCUITRY OF CsrA AND QUORUM SENSING PATHWAYS IN THE SYMBIONT *Vibrio fischeri*

Alicia E. Ballok, Alecia N. Septer, Eric V. Stabb, Cheryl A. Whistler

Introduction

During host association, bacteria use a number of regulators arranged in complex networks making for rapid, sensitive, and appropriate control during infection. Whether the outcome of the infection is beneficial or pathogenic, the regulators are often the same. For example, many bacterial species use multiple quorum sensing circuits that interface with central metabolic, motility, and virulence regulators (Reviewed in Miller and Bassler, 2001; Lazazzera, 2000). Similarly, GacA (Global Activator A) is a global activator of gene expression found in the γ proteobacteria that regulates host-association and quorum sensing in a number of organisms (Reviewed in (Heeb and Haas, 2001)).

For the bioluminescent light organ symbiont *Vibrio fischeri*, the regulator GacA coordinates expression of traits that mediate colonization of its host, the squid *Euprymna scolopes* (Whistler and Ruby, 2003). The squid hatches without the bacteria, and thus symbiotic *V. fischeri* have developed devices to surmount the gauntlet of host defenses. Once inside the light organ, *V. fischeri* use

quorum-sensing regulated luminescence to maintain the association (Nyholm and McFall-Ngai, 2004; Visick and Ruby, 2006). In culture, GacA regulates known colonization traits including motility, siderophore production, carbon utilization and normal LPS synthesis (Whistler and Ruby, 2003). GacA is also necessary for light production in culture. Interestingly, GacA has little effect on AHL-quorum sensing signal production, and the addition of exogenous AHL does not restore luminescence to the mutant (Whistler and Ruby, 2003). During squid colonization, GacA coordinates functions that allow the bacteria to aggregate in mucus secreted by the squid, move toward the light organ and bypass host defenses (Whistler *et al.*, 2007). Once bacteria reach the light organ, GacA allows growth to a high density and induction of host-morphological changes that limit entry of additional symbiotically competent bacteria (Whistler *et al.*, 2007). However, GacA is not necessary for light production *in vivo* suggesting additional unidentified players regulate light within the host.

In other organisms, GacA has much the same effect on host association and has been thoroughly studied in a number of species including *Pseudomonas spp.*, *Legionella pneumophila*, *Erwinia carotovora* as well as several *Vibrios* (Heeb and Haas, 2001; Whistler *et al.*, 1998; Hammer *et al.*, 2002; Eriksson *et al.*, 1998; Lenz *et al.*, 2005; Wong *et al.*, 1998). In the model organism *Escherichia coli* as in other organisms, GacA indirectly regulates mRNA translation via the antagonistic partners CsrA and CsrB (Romeo, 1998). CsrBs are small untranslated RNAs (sRNAs) with a secondary structure of stem-loops containing (AR)GGA motifs (Liu *et al.*, 1997). These same motifs appear in the

5' UTR of certain mRNAs, often overlapping with the ribosomal binding site (RBS). The CsrA protein recognizes and binds the stem-loop of regulated transcripts, preventing translation of the mRNA, or affecting stability of the transcript (Baker *et al.*, 2002). When present, each CsrB sRNA binds multiple CsrAs, sequestering them, and allowing translation to occur. GacA is a known activator of CsrBs in the above organisms thus functions primarily to relieve translational blockage by CsrA.

Recent work by Bassler and colleagues has defined the interaction of CsrA/CsrB with quorum sensing in *Vibrio cholerae* using the *V. harveyi lux* operon as an output for quorum sensing regulation (Fig.3) (Lenz, Miller, Zhu, Kulkarni, and Bassler, 2005). In *V. cholerae*, a relative of *V. fischeri*, the GacA homolog VarA activates expression of three sRNAs, CsrB, C, and D, which inhibit CsrA. CsrA has an indirect and positive effect on the AI-2 quorum-sensing regulated LuxO. At low cell density, LuxO is activated by LuxU, which is phosphorylated by the CqsA/S and AI-2 quorum sensing systems. LuxO along with σ_{54} activates two qrr RNAs and these along with the RNA chaperone Hfq destabilize HapR, a homolog of the LitR in *V. fischeri* and LuxR in *V. harveyi*, which activate luminescence (Lenz *et al.*, 2004; Lenz *et al.*, 2005). In *V. fischeri* some of the regulators are conserved including LuxO, LuxS, LuxPQ and the HapR homolog LitR (Milton, 2006; Lupp *et al.*, 2004; Fidopiastis *et al.*, 2002). However, there are additional regulators not present in *V. cholerae*, such as two AHL quorum sensing pathways, AinS/R and LuxI/R which act to sequentially induce luminescence (Lupp and Ruby, 2005). In the absence of quorum sensing

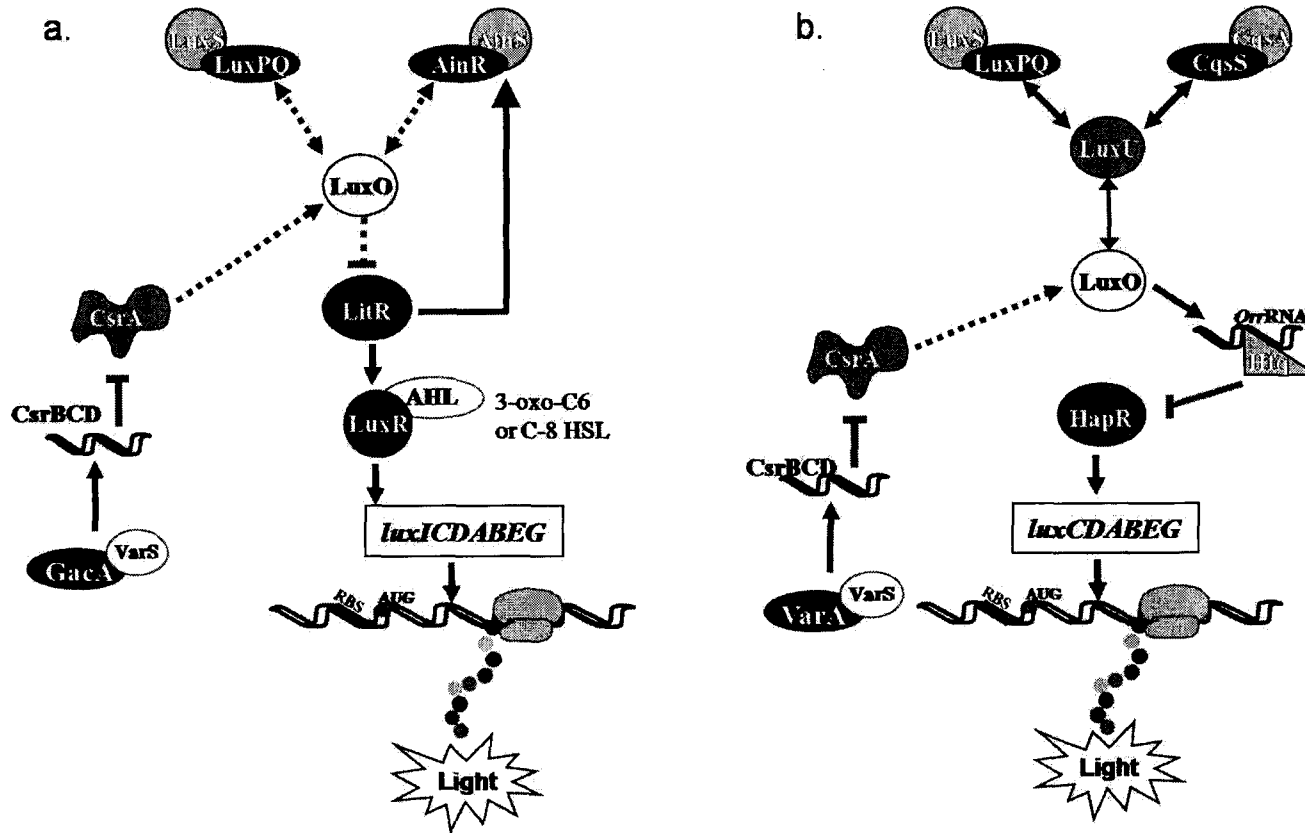


Fig. 3. *Vibrio fischeri* luminescence regulation (a.) based on the *Vibrio cholerae* model (b.). If luminescence is regulated in the same way that the *Vibrio harveyi* lux operon is regulated in *Vibrio cholerae*, then GacA would activate CsrB1 and CsrB2, repressing CsrA, which would lead to an indirect repression of LuxO (adapted from (Lenz *et al.* 2004; Lenz *et al.* 2005))

molecules produced by AinS or LuxS, LuxO represses LitR (Lupp and Ruby, 2004). LitR is a transcriptional activator of LuxR which, when bound to the C8-HSL from the AinS quorum sensing system or 3-oxo-C6HSL from LuxI, activates transcription of the *luxICDABEG* operon (Fidopiastis *et al.*, 2002; Lupp and Ruby, 2004; Lupp and Ruby, 2005). Recently, Kulkarni *et al.* designed a bioinformatic search program to find potential CsrBs designated CSRNA_FIND (Kulkarni *et al.*, 2006). This program searched for intergenic regions with A(R)GGA motifs that had a secondary structure of stem loops as identified by MFOLD. Their research indicates that there are GacA binding domains upstream of two CsrB-like genes in the *V. fischeri* genome (CsrB1 and CsrB2), both of which produce functional RNAs (Kulkarni *et al.*, 2006). It seems likely that in *V. fischeri* GacA also works through the CsrA/CsrB system, but we do not know if GacA exerts its influence on luminescence at LuxO as it does in *V. cholerae*.

Using luminescence as an indicator of GacA/CsrB1/CsrB2/CsrA regulation, we have elucidated the roles and circuitry of these proteins in *V. fischeri*. We confirmed that GacA regulates both CsrB1 and CsrB2, and these genes fully complemented all known defects in culture of the GacA mutation. Consistent with these findings, spontaneous CsrA mutations suppress GacA defects. Squid colonization studies supported the *in vitro* complementation data. Interestingly, the GacA/CsrA double mutants had a defect in initiating colonization that indicates the presence of CsrA and its proper regulation by GacA is important for efficient squid colonization. We further investigated how GacA, the CsrBs and CsrA interface with other luminescence regulators. The

inability of *litR*, *luxR* or even *luxICDABE* under the control of a GacA-independent promoter to complement the GacA-luminescence defect suggests GacA/CsrB/CsrA regulate light production not through quorum sensing activation, but through a post-transcriptional mechanism. We predict a novel binding site within the coding region of the *lux* mRNA that when deleted increases luminescence more than 70-fold. Thus, *V. fischeri* exhibits novel regulatory circuitry among the *Vibrios* studied so far.

Materials and Methods

Bacterial Strains, plasmids, media and growth conditions

Strains and plasmids are listed in table 1. All strains of *V. fischeri* used were derivatives of the sequenced squid symbiont strain ES114. *V. fischeri* was grown either in SWT (5g Tryptone, 3g Yeast Extract, 3 g glycerol per L in 70% instant ocean) (Boettcher and Ruby, 1990), LBS (10g Tryptone, 5g Yeast Extract, 20g NaCl, 50 ml 1 M Tris base pH 7.5 per L) (Graf *et al.*, 1994) or minimal media derived from CAS solid agar (0.58% K₂HPO₄, 4% NH₄Cl, and 50ml 1M Tris pH 7.5 supplemented with either 3 % glycerol, or 3% glycerol and 0.5% caseamino acids in artificial seawater (Graf and Ruby, 1998) at 28°C. Antibiotic concentrations for *V.fischeri* were erythromycin (Erm) 5µg/ml, chloramphenicol (Chl) 2.5µg/ml and kanamycin (Kan) 50µg/ml. All *E.coli* were grown in Luria-Bertani (LB) broth (Sambrook, Fritsch, and Maaniatis, 1989) or brain heart infusion (BHI) broth (Difco) at 37°C. Antibiotics used for *E.coli* were 150µg/ml Erm in BHI, 50µg/ml Kan in LB and 25µg/ml Chl in LB. For solid

Table 1.

Bacterial strains and plasmids used in this study		
Strain or plasmid	Description	Source or Reference
<i>E. coli</i> strains		
DH5 α	F- <i>RecA1 endA1 hsdRi7 supE44 thi-1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>) U169 Φ 80 <i>lacZ</i> Δ M15 λ -	Gibco-BRL, Inc.
DH5 α λ <i>pir</i>	F- <i>RecA1 endA1 hsdRi7 supE44 thi-1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>) U169 Φ 80 <i>lacZ</i> Δ M15 λ - with <i>lambda</i> <i>pir</i>	
CC118 λ <i>pir</i>	Δ (<i>arg-leu</i>) <i>araD</i> Δ <i>lacX74 galE galK phoA20 thi-1 rpsE rpoB argE</i> (Am) <i>recA1</i> , lysogenized with <i>lambda</i> <i>pir dam dcm</i>	Herrero, 1990
One Shot $\text{\textcircled{R}}$ Top10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139</i> Δ (<i>araleu</i>) 7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
<i>V. fischeri</i> strains		
ES114	Wild-type <i>E. scolopes</i> light organ isolate	Bottecher, 1990
VCW2F5	ES114 derivative with an internal in-frame deletion; Δ <i>gacA</i>	Whistler, 2003
VCW2H7	ES114 derivative with an frameshift mutation in <i>luxI</i> ; C6-HSL-	Lupp, 2003
ANS3	ES114 derivative with a complete deletion of <i>luxI</i> ; Δ <i>luxI</i>	This study
ANS8	Δ <i>luxI</i> derivative with the putative CsrA binding domain of <i>luxI</i> added; C6-HSL-	This study
LKS5	ES114 derivative with a <i>luxA</i> mutation	
VCW2D2	derivative of <i>gacA</i> mutant VCW2A1 (Whistler, 2003) with a frameshift; <i>csrA</i> Δ Δ 161(<i>GacA</i> ^{supϕ1})	This study
AEB3H8	derivative of <i>gacA</i> mutant VCW2A1 (Whistler, 2003) with base change; <i>csrA</i> T196G (<i>GacA</i> ^{supϕ1})	This study
Plasmids		
pVO8	pACYC, stable multicopy plasmid, <i>Chl</i> ^r , <i>Erm</i> ^r ,	Visick, 1997
pEVS79	pBCSK derivative, <i>Mob</i> ⁺ , <i>Chl</i> ^r	Stabb, 2002
pCR $\text{\textcircled{R}}$ 2.1TOPO $\text{\textcircled{R}}$	plasmid for cloning PCR products with adenine overhangs; <i>Km</i> ^r , <i>Amp</i> ^r	Invitrogen
pEVS104	R6K γ derivative of pRK2013; Δ <i>ColE1</i> , <i>oriT tra trb</i> Δ Tn903 <i>Km</i> ^r	Stabb, 2002
pVSV209	pES213 derivative, low copy number (7 per cell) promoterless <i>Chl</i> ^r and <i>gfp</i> , <i>rfp</i> , <i>Km</i> ^r	Dunn, 2005
pVCW3C3	pVO8 derivative with a 4kb fragment containing wild type <i>gacA</i> , <i>Chl</i> ^r	Whistler, 2003
pCL147	pVO8 derivative containing <i>luxI/CDABE</i> coding sequence in <i>P</i> _{lac} promoter orientation, <i>Chl</i> ^r	C. Lupp
pAEB1A1	pCR $\text{\textcircled{R}}$ 2.1 TOPO $\text{\textcircled{R}}$ derivative containing <i>csrB1</i> gene	This study
pAEB2H8	pVO8 derivative containing <i>csrB1</i> from pAEB1A1	This study
pAEB3F9	pCR $\text{\textcircled{R}}$ 2.1 TOPO $\text{\textcircled{R}}$ derivative containing <i>csrB2</i> gene	This study
pAEB1B4	pVO8 derivative containing <i>csrB2</i> from pAEB3F9	This study
pAEB1F3	pCR $\text{\textcircled{R}}$ 2.1 TOPO $\text{\textcircled{R}}$ derivative containing <i>litR</i> gene	This study
pAEB1F4	pVO8 derivative containing <i>litR</i> from pAEB1F3	This study
pAEB1E3	pCR $\text{\textcircled{R}}$ 2.1 TOPO $\text{\textcircled{R}}$ derivative containing <i>luxR</i> gene	This study
pAEB1E4	pVO8 derivative containing <i>luxR</i> from pAEB1E3	This study
pAEB1A3	pEVS79 derivative containing <i>csrA</i> deletion with 1.2kb upstream and 0.3kb downstream	This study
pAEB3A6	pEVS79 derivative containing <i>csrA</i> from pAEB1A3 with a <i>Kan</i> ^r gene inserted in upstream region	This study
pJCM24	pEVS79 derivative with a 1.5 kb region containing CsrA with a <i>EZ::TN<Kan-2></i> insertion	This study
pAEB3C6	pVSV209 derivative containing the promoter region of <i>csrB1</i>	This study
pAEB1D5	pCR $\text{\textcircled{R}}$ 2.1 TOPO $\text{\textcircled{R}}$ derivative containing promoter region of <i>csrB2</i>	This study
pAEB1A2	pVSV209 derivative containing the promoter region of <i>csrB2</i> from pAEB1D5	This study

media, 15g/L agar was added. For screening purposes, we added 40µg/ml 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-gal).

Molecular Techniques and Complementation

Standard molecular methods were used throughout (Ausubel et al) (, 1990; Sambrook, Fritsch, and Maaniatis, 1989). Cloning by polymerase chain reaction (PCR) was performed using either Fast Start Hifi (Roche) or Platinum Taq Hifi (Invitrogen) DNA polymerases using manufacturers protocols. The oligonucleotide primers (Integrated DNA Technologies) used for cloning are listed in Table 1. Primers were designed from the available genome sequence (Ruby, Urbanowski, Campbell, Dunn, Faini, Gunsalus, Lostroh, Lupp, McCann, Millikan, Schaefer, Stabb, Stevens, Visick, Whistler, and Greenberg, 2005) to regions flanking genes of interest.

For complementation studies with *csrB1* and *csrB2*, each gene was amplified using gene-specific primers. For *luxR* and *litR*, the genes were PCR-amplified using primers that had *SphI* and *SacI* or *PstI* and *SacI* sites respectively engineered into the 5' end of gene-specific primers. The PCR products were then cloned into pCR®2.1 TOPO® using the TOPO TA Cloning® Kit (Invitrogen) and transformed into chemically competent One Shot® Top10 *E. coli* using the manufacturers' protocols. Following plating on LB Kan containing X-gal, putative clones were identified among the white colonies and screened by PCR using the same gene-specific primers used for cloning. Positive clones containing *csrB1* (pAEB3F9) or *csrB2* (pAEB1B4) *luxR* (pAEB1E3) or *litR*

Table 2.

Primers designed for this study	
Primers	Sequence and description
CsrB1 primers	
CsrB1 F1	5' AGTCAAAGCGTAGTCTTATTGG3' forward primer for amplification and screening of <i>csrB1</i>
CsrB1 R2	5'CACTGAGGAGAAATGTAACCG3' reverse primer for amplification and screening of <i>csrB1</i>
CsrB1AvrII	5'ATGGACCCTAGGCAAGCGAAGAGCAAGCAGA3' for promoter region, contains AvrII restriction site
CsrB1Sal	5'TTCCAAGTCGACGAATGCTCCTTGAGTTATCTC3' for promoter region, contains Sal1 restriction site
CsrB2 primers	
CsrB2 F1	5'CTTACAAGCGAGTGAGATTTAGCG3' forward primer for amplification and screening of <i>csrB2</i>
CsrB2 R2	5'AGAGGAGGAACTATTGTGAGC3' reverse primer for amplification and screening of <i>csrB2</i>
CsrB2 FSal	5' GTCGACTAGACAAACAGGAAACCGCT3' for amplification of promoter region, contains Sal1 restriction site
CsrB2RStu	5'AGGCCTCCGACACCAATAAGATTACGC3' for amplification of promoter region, contains Stu1 restriction site
LitR primers	
LitR F1	5'CTGCAGTTGGCAAGGATATAAATATAATGG3' forward primer with Pst1 restriction site
LitF R2	5'GAGCTCTCCACGCTCACCCAACCTCTA3' reverse primer with Sac1 restriction site
LuxR primers	
LuxR F1	5'GCATGCTAGGTGAACAAATGGAACATTA3' forward primer with Sph1 restriction site
LuxR R2	5'GAGCTCATTAGAGCAACTTATTCGC3' reverse primer with Sac1 restriction site
CsrA primers	
CsrA F3	5'ATCACTAACCGCTGACCAG3' forward primer for amplification of <i>csrA</i>
CsrA R7	5'TCACCGTCTTCAGGAGGC3' reverse primer for amplification of <i>csrA</i>
CsrA F8	5'TGTCGGTTATCGTAAATCAAGC3' forward primer for sequencing <i>csrA</i>
CsrA R9	5'AACCGTCTATGAAACGACCA3' reverse primer for sequencing <i>csrA</i>

(pAEB1F3) exhibited the correct size PCR product by gel electrophoresis (0.7% agarose in 1X TAE). The *csrB1* and *csrB2* genes were then subcloned into the shuttle vector pV08 that is stably maintained in *V. fischeri* (Visick and Ruby, 1997) by digesting (pAEB3F9 and pAEB1B4) with *EcoRV* and *SacI* and digesting pV08 with *SmaI* and *SacI*, followed by heat inactivation the enzymes. The *luxR* and *litR* genes were subcloned into pV08 using the restriction enzyme whose sites engineered into the primers. Ligations were performed using T4 DNA ligase (Invitrogen) by temperature cycling (Lund *et al.*, 1996). The ligation products were then transformed into chemically competent *E. coli* DH5 α and plated onto LB Chl supplemented with X-gal. Plasmids containing the correct cloned products were identified among the white colonies by PCR screening with Master Taq® Kit (Eppendorf) using the same gene-specific primers used for cloning.

The plasmids were mobilized into wild-type ES114 and its derivatives by conjugation via triparental mating using a DH5 α helper strain containing the plasmid pEVS104 (Stabb and Ruby, 2002). Briefly, equal volumes of stationary phase cultures of the helper strain, *E. coli* donor and the recipient strain of *V. fischeri* were centrifuged individually, washed with LBS, resuspended together in LBS and spotted on LBS solid agar. This was incubated at 28°C overnight and the bacterial mixture was collected, diluted in sterile artificial seawater and plated onto LBS media containing antibiotic to select for the transconjugants. The plates were incubated at room temperature until visible transconjugant colonies

appeared, which were re-streaked for isolation on antibiotic containing media prior to use.

For *in trans* complementation with *csrB1*, *csrB2*, *gacA*, *litR*, *luxR*, and *luxICDABE* (in pCL147), cultures were grown at 28°C, shaking in SWT Chl media at 200rpm. Phenotypic complementation was tested as described in the phenotypic analysis of strains section.

Promoter-*gfp* fusions

Regions upstream of *csrB1* (~500bp) and *csrB2*(~250bp), predicted to contain the promoters (Kulkarni *et al.*, 2006), were amplified with a Platinum Taq Hifi (Invitrogen) using primers that contained restriction sites, *Sall* and *Stul* for *csrB2* and *AvrII* and *Sall* for *csrB1*. *csrB2* was cloned into pCR®2.1 TOPO® using the TOPO TA Cloning® Kit generating plasmid pAEB1D5 and then subcloned using the restriction enzymes *Sall* and *Stul*. The *csrB1* PCR product was digested directly with *AvrII* and *Sall* and each promoter was cloned into an available promoter fusion vector, pVSV209 a low copy number (n=7) plasmid (Dunn *et al.*, 2006) derived from a *V. fischeri* plasmid pES213 (Dunn *et al.*, 2006). The plasmid pVSV209 contains restriction sites allowing cloning of promoters upstream of promoterless *chl*^R and *gfp* which creates a transcriptional fusion that drives expression of the *gfp* reporter. The plasmid also contains constitutively expressed *kan*^R and *rfp* genes. Following ligation of promoters to the vector, plasmids were transformed into DH5α*λpir*. Transconjugants were selected on LB Kan, and the presence of the plasmid further confirmed by visual

inspection of RFP. Presence of the promoter within the plasmid was confirmed by PCR screening for the correct size product following electrophoresis. These promoter fusion plasmids along with the empty vector pVSV209 were mobilized by conjugation (Stabb and Ruby, 2002) into *V. fischeri* ES114 and the *gacA*Δ mutant. Putative transconjugants were selected on LBS Kan and the presence of RFP expression visualized.

To quantify promoter activity, strains were grown in 2ml SWT Kan with shaking for 9 h. Fluorescence was measured on 0.1 mL culture with 0.1mL media, as a control for background autofluorescence, using a plate reader (Tecan). RFP was measured using a 570nm excitation wavelength and read at 620nm emission wavelength. GFP was assessed using 480nm excitation wavelength and read at 515nm emission wavelength. Optical density was measured using a spectrophotometer (Eppendorf). Relative fluorescence was calculated as fluorescence intensity per unit of OD₆₀₀.

Phenotypic analysis of strains

All phenotypic analyses were based on those outlined previously (Whistler and Ruby, 2003). Briefly, luminescence and growth was determined from cultures grown at 28°C with shaking (200 RPM) in SWT or SWT Chl for strains with pV08 and its derivatives. Luminescence and density (OD₆₀₀) were measured from 1ml of culture at various points using a luminometer (Turner Designs) and a spectrophotometer (Eppendorf). LPS (Lipopolysacchride) was observed as difference in colony opacity on LBS plates. Growth on simple sugars

was assessed as cell density in minimal medium supplemented with glycerol or with glycerol and caseamino acids as a control. Growth yield OD_{600} was measured from cultures grown in SWT (with antibiotics if necessary) overnight (18hrs) at 28°C. Motility was assessed using motility media (1% Tryptone 70% Natural Seawater) with Bacto (Difco) agar at concentrations of 0.3 and 0.6%. For 0.3% agar, 3 μ l of mid-log cultures (0.4 OD_{600}) were spotted onto the plates and migration in mm was measured hourly for 9hrs to determine rate (mm/h). For 0.6% agar, individual colonies were picked using a toothpick and stabbed into the agar. The distance in mm of migration was measured after 24 hours. The experiments contained 3 replicates and were repeated at least 2 times. Data shown is from a representative experiment and error is expressed as SE.

Siderophore production was measured using a modified liquid chrome azurol S (CAS) liquid assay (Payne, 1994). Briefly, cultures were grown in minimal medium based on that used for CAS agar plates (Graf *et al.*, 1994) buffered with Tris (pH 7.4), and grown for eight hours with shaking. The density (OD_{600}) was measured and cells removed by pelleting. Equal volumes of the culture supernatant and a CAS assay solution were mixed along with 1% of shuttle solution (0.2M 5-Sulfosalicylic acid). After 20min the absorbance of these mixtures was measured using a plate reader (Tecan) at 630nm. Siderophore is reported percent siderophore (compared to the blank control) per OD_{600} .

Squid Colonization

Squid colonization was performed exactly as outlined previously (Whistler and Ruby, 2003). Briefly, exponentially growing bacteria (0.2-0.4 OD₆₀₀) were suspended in 50mL of filtered sea water (FSW) to an approximate final density of 3000 CFU/ml. Exact CFUs were determined by plating on LBS. Squid were added collectively to the sea water and after 3 hours, transferred to individual vials containing 4ml FSW. Each day the squid were transferred to new vials containing 4mls FSW. For strains containing plasmids, 1.25µg/ml ChI was added to the FSW.

Luminescence and colonization were assessed 12, 24 and 48 hours after hatching. To quantify the light organ bacterial populations, the squid were individually frozen at -80°C, which kills bacteria superficially associated with the squid, but does not kill bacteria within the light organ. Squid were then homogenized in FSW, the homogenate serially diluted and plated on LBS media. The plates were counted to determine the CFU per light organ.

CsrA mutant generation

Directed mutagenesis of *csrA* was attempted through homologous recombination as described previously (Stabb and Ruby, 2002). First, we generated a deletion construct suitable for mutagenesis by fusing 1.2kb of the upstream sequence to 0.3kb of the downstream sequence of *csrA* with PCR SOEing (Horton *et al.*, 1993). Although having only 300 bp of DNA flanking a mutation likely reduces efficiency of recombination, other mutants have been

successfully generated with less than 200 bp of flanking DNA (Whistler and Ruby, 2003). This strategy was necessary due to the presence of multiple tRNA genes immediately downstream of *csrA*. The presence of these sequences in an earlier construct facilitated recombination of the plasmid at a number of incorrect sites in the *V. fischeri* genome. Following successful deletion of *csrA* in the amplicon, the PCR product was cloned into a suicide vector, pEVS79 (Stabb and Ruby, 2002) generating plasmid pAEB1A3. To aid in selection of mutants an additional construct was also made with Kan^R from pMKm (Murillo *et al.*, 1994) inserted in a single *EcoRI* site upstream of *csrA* in pAEB1A3 generating plasmid pAEB3A6. The mutations were introduced into the genomes of wild-type *V. fischeri* and the *gacA* mutant by conjugation and derivatives with single crossovers were isolated by plating on LBS Chl grown for 24 h at room temperature, which enriches for *V. fischeri*. Individual colonies identified as *V. fischeri* based on morphology were streak purified onto LBS Chl for plasmid pAEB1A3, or LBS Kan for plasmid pAEB3A6, and the location of the crossover event on either side of the *csrA* gene determined by PCR. Derivatives with crossovers within the 300 bp (downstream) flanking DNA were then grown without selection. Derivatives of pAEB1A3 plated on LBS, and derivatives of pAEB3A6 plated on LBS Kan, were then screened for loss of Chl^R by replica plating indicating a second crossover event. Putative mutants were then screened by PCR. In over 5000 (3200 putative deletion mutants and 2000 putative Kan resistant mutants) colonies for each construct screened, no derivative harboring the deletion was ever identified. An additional attempt at

directed mutagenesis was made by introducing a transposon in the C-terminal region of *csrA* at nucleotide 148 as confirmed by sequencing using the EZ::TN™ <Kan-2> Insertion Kit following the manufacturers protocols (Epicentre) generating plasmid pJCM24. This mutation occurred 5 amino acids downstream of the mutation in *E. coli* (Romeo *et al.*, 1993) Conjugation and isolation of single and putative double crossover derivatives was done as described above. PCR screening of over 5000 putative mutants revealed no *csrA*::Kan^R mutants.

Spontaneous GacA suppressor mutants that mapped to *csrA* were isolated from cultures of the *gacA*::Kan^R mutant that were plated onto LBS or CAS agar and allowed to incubate for approximately 72 hours. Large opaque colonies were streak purified from the background of the translucent GacA mutants. The extent of *gacA* mutant suppression for these derivatives was determined. For five derivatives restored in LPS, luminescence, siderophore, growth yield, and growth on glycerol, the genomic DNA was extracted (Ausubel *et al.* (, 1990) and the *csrA* gene was amplified using HiFi polymerase using *csrA*-specific primers and the PCR product directly sequenced by the Hubbard Center for Genome Studies at the University of New Hampshire. The sequenced *csrA* genes from these *gacA* mutant suppressors was compared that of *csrA* from the published genome of *V. fischeri* (Ruby *et al.*, 2005) in order to identify the location of the mutation. Amplification and sequencing was performed multiple times to confirm that identified mutations were not the result of polymerase error.

Sequence analysis and comparison

The putative binding site for CsrA within *luxI* was found on observation of a series of adenines followed by a GGA motif, followed by five thymines. This was similar to some of the consensus data of other CsrA binding sites (Dubey *et al.*, 2005). This sequence was aligned with other *luxI* sequences in GenBank (*Vibrio salmonicida*, AF452135; *Vibrio fischeri* MJ1, AF170104; *Vibrio fischeri* ATCC 49387, AY261992; *Shewanella hanedai*, AB261992; *Vibrio anguillarum*, VAU69677; *Aeromonas salmonicida*, ASU65741; *Aeromonas hydrophila*, AY987586; *Pseudomonas aeruginosa* PAO1, AEO04091; *Pseudomonas fluorescens*, L48616; *Burkholderia vietnamiensis* G4, EF212890; *Burkholderia cepacia*, AJ422183). Sequence alignment was performed with the BLAST software package (Altschul *et al.*, 1990).

Results

Complementation of *in vitro* GacA mutant phenotypes with CsrB1 and CsrB2

Consistent with our working model whereby GacA represses CsrA via activation of the two sRNAs, we expected that either CsrB1 or CsrB2 would complement the GacA mutant. Using plasmids expressing *csrB1* or *csrB2* under the control of their own promoters, we examined their affect on known GacA defects including luminescence, siderophore production, motility, growth yield, growth on simple sugars and LPS (Table 3). Either construct restored all GacA-mutant phenotypes to wild-type.

Table 3. Complementation of *in vitro* GacA mutant phenotypes. Shown here are GacA mutants suppressed by a secondary mutation in *csrA* or complemented with *gacA*, *csrB1* or *csrB2* *in trans* with Wild type and the vector as controls. Each assay was replicated three times.

<i>V. fischeri</i> strain	Growth Yield OD	Value for Phenotype (SE)			Siderophore %siderophore units/OD	Growth in Simple Sugars	LPS
		Relative Luminescence Lum/OD	Motility 0.3% mm/Hour	Motility 0.6% mm/Hour			
Wild type	7.63 ± 0.14	3.18 ± 0.20	3.81 ± 0.06	0.20 ± 0.01	18.92 ± 0.87	+	OP
GacAΔ	2.11 ± 0.02	0 ± 0.00	4.19 ± 0.06	0.13 ± 0.01	0 ± 0.00	-	TR
<i>csrA</i> ΔA161	7.61 ± 0.57	2.67 ± 0.47	3.75 ± 0.14	0.20 ± 0.01	15.97 ± 0.87	+	OP
<i>csrA</i> T196G	7.92 ± 0.18	4.01 ± 0.37	2.88 ± 0.07	0.23 ± 0.00	14.85 ± 0.98	+	OP
Wild type + vector	7.53 ± 0.20	2.61 ± 0.08	3.31 ± 0.16	0.19 ± 0.00	16.32 ± 0.77	+	OP
GacAΔ + vector	2.79 ± 0.08	0 ± 0.00	3.94 ± 0.12	0.13 ± 0.01	0 ± 0.00	-	TR
GacAΔ + GacA	5.10 ± 0.33	5.80 ± 0.40	3.44 ± 0.06	0.19 ± 0.01	17.33 ± 0.42	+	OP
GacAΔ + CsrB1	6.24 ± 0.21	4.40 ± 0.59	2.94 ± 0.18	0.20 ± 0.02	16.33 ± 0.92	+	OP
GacAΔ + CsrB2	6.42 ± 0.05	3.47 ± 0.51	2.75 ± 0.10	0.18 ± 0.02	13.12 ± 1.25	+	OP

GacA suppressor mutations map to CsrA

We attempted to generate a *csrA* mutant and a *gacA-csrA* double mutant, a mutation in CsrA to test the model of CsrA regulation and would restore wild type phenotypes to the GacA mutant. Using homologous recombination, we attempted to replace the wild-type *csrA* with one of several mutations, including a complete *csrA* deletion and replacement of *csrA* with an erythromycin cassette, but these attempts were unsuccessful. Among the published *csrA* mutants, many have insertions near the C-terminus of the protein (Romeo *et al.*, 1993; Lenz *et al.*, 2005). We therefore attempted to replace the gene with several different extreme C-terminus insertion mutations, but again were unsuccessful. These data indicate that *csrA* may be an essential gene, or that the mutation is highly detrimental in *V. fischeri*.

Although our attempts to generate directed *csrA* mutants were unsuccessful, in culture the GacA mutant is very unstable and suppresses, meaning it acquires a second-site mutation that restores wild type phenotypes, quite readily. There are several classes of GacA mutant suppressors restored in phenotypes as would be expected for a *csrA/gacA* double mutant. We sequenced the *csrA* gene in five of these suppressors and found that two indeed carried mutations in the gene (Fig. 4). The first suppressor, *csrA*ΔA161 (GacA^{supS1}), has a deletion of an adenine at base 161 resulting in a frameshift and a change in the last 17% of the protein. The second, *csrA*T196G (GacA^{supKm12}), has a thymine to guanine transversion at position 196, that

	Domain 1	Domain 2
ES114	MLJLIRRVGETLMIGDEVTVTVLGVKGNQVRIGVGNAPKEYSVHREIYQRIQAEKTRAASQGNF	
csrAAA161	MLJLIRRVGETLMIGDEVTVTVLGVKGNQVRIGVGNAPKEYSVHREIYQRIQAEKVIHLINIKKVTFNIFV	
csrAT196G	MLJLIRRVGETLMIGDEVTVTVLGVKGNQVRIGVGNAPKEYSVHREIYQRIQAEKTRAASQGNFEEFNINIKGWIKQPKCLFLSFTIHI	

Fig. 4. GacA suppressor mutations in *csrA*. Amino acid alignment of CsrA in ES114 as well as the resultant amino acid changes in the suppressor mutants. The domains necessary for activity are underlined. The *csrAAA161* mutation results in a change in the last 13 amino acids of the protein. The mutation in *csrAT196G* does not change the amino acid sequence but an additional 20 amino acids are present at the C-terminus of the protein. The asterisk on the wild-type sequence indicates the location of the published *E. coli* transposon mutant (Romeo *et al.* 1993).

changes the stop to a glutamine and adds 20 amino acids on to the C-terminus of this 65 amino acid protein. We predict based on the difficulty in obtaining a null mutant, that neither of these suppressor mutations are null and probably maintain some functionality. We successfully replaced the *gacA::KanR* mutation with a wild-type *gacA* gene in the *csrAΔA161* (*GacA^{supS1}*) mutant, but the *csrA* mutation reverted, further thwarting our attempts to characterize a *csrA* mutation in an otherwise wild-type background. Still, these two suppressor mutants did allow us to determine the affect of a *csrA* mutation in a *gacA* mutant background (Table 3). All *in vitro* phenotypes of *csrAΔA161* and *csrAT196G* were the same as wild type with only one exception; *csrAT196G* had unusual motility in that it was more motile than the wild type on high percentage agar and less motile than the wild type on the lower percentage agar, a phenotype which is opposite of the effect of the *GacA* mutation.

Complementation of squid colonization phenotypes

Although addition of either *csrB1* or *csrB2* *in trans* as well as secondary mutations in *csrA* complemented *GacA* mutant phenotypes *in vitro*, some *GacA* defects differ from culture to the squid (i.e. luminescence). Therefore it is not clear whether squid-specific or *in vivo* traits would also be restored. *GacA* mutants have a severe initiation defect and once in the light organ, and achieve populations on average <2% that of wild type (Whistler and Ruby, 2003). As a result, they generally are not detectibly luminous. We examined the colonization attributes of our complemented and suppressor mutant strains at 12, 24 and 48

Table 4. Complementation of squid phenotypes. The ability for a bacterium to colonize a squid was measured as ability to colonize the squid (cfu/ light organ) and produce luminescence (specific luminescence). Percent luminous corresponds to the percentage of squid that are luminous (n=5) at 12, 24 and 48hrs. Specific luminescence is expressed as the mean of individual squid (luminescence/cfu x10⁴), (n=5).

<i>V. fischeri</i> strain	Wild type	GacAA	csrAAA161	csrAT196G	Wild type + pV08	GacAA+ vector	GacAA + GacA	GacAA + CsrB1	GacAA + CsrB2
Percent Luminous									
12hrs	0	0	0	0	0	0	0	0	0
24hrs	90	0	60	90	50	0	50	90	80
48hrs	100	0	90	100	80	0	80	100	100
CFU/Light Organ									
12hrs	(2.3±0.41)x10 ³	(0.6±0.66)x10 ³	(2.4±0.9)x 10 ³	(1.2±0.5)x 10 ³	(1.89±1.87)x10 ²	(8.05±0.42)x10 ¹	(2.34±1.31)x10 ²	(2.28±1.72)x10 ²	2.2x10 ²
24hrs	(1.39±0.65)x10 ⁶	(2.70±0.31)x10 ³	(5.53±1.37)x10 ⁴	(1.42±0.39)x10 ⁶	(8.68±6.25)x10 ⁴	(3.72±1.52)x10 ²	(1.89±0.84)x10 ⁴	(2.38±0.54)x10 ⁶	(6.57±2.22)x10 ⁴
48hrs	(2.17±0.63)x10 ⁶	(1.13±0.16)x10 ³	(2.05±0.11)x10 ⁶	(3.47±0.43)x10 ⁶	(1.08±0.07)x10 ⁶	(2.56±0.63)x10 ²	(4.58±1.76)x10 ⁴	(1.02±0.24)x10 ⁶	(5.61±1.40)x10 ⁴
Specific Luminescence									
24hrs	1.16±0.25	0	0.56±0.27	1.34±0.24	0.51±0.15	0	1.4±0.70	1.3±0.31	0.96±0.20
48hrs	1.15±0.04	0	1±0.20	1.18±0.11	1.24±0.19	0	1.86±0.30	1.09±0.30	1.25±0.29

hours for squid exposed to a standard inoculum (approximately 3000 CFU per ml seawater) for only three hours. We found that each of the CsrBs *in trans* fully complemented the GacA mutant and were indistinguishable from wild-type harboring the empty vector as a control (Table 4). For *csrA* Δ 161, the density of bacteria was low at 12 hours and at 24 hours its population density was still lower than wild-type. By 48 hours its population density was similar to that of wild type, indicating that *csrA* mutation impaired initiation, but did not substantially affect final growth yield.

Regulation of *csrB1* and *csrB2* by GacA

Although phenotypic complementation data supports our working model, we wished to examine the effect of GacA on *csrB1* and *csrB2* expression. To resolve whether or not GacA activates the expression of the *csrBs*, we made fusions of each of the *csrB* promoters to GFP and quantified expression in wild type and the *gacA* Δ mutant (Fig. 5). Compared to wild type, GFP was reduced by 31-fold for *csrB1-gfp* and 16- fold for *csrB2-gfp* in the *gacA* Δ background.

Examination of the mechanism of luminescence regulation

It is well established that GacA interfaces with quorum sensing in a number of bacterial species. For example, in several *Pseudomonas* spp. GacA influences AHL synthesis. GacA also regulates quorum sensing in *V.cholerae* and *V. harveyi*. Prior work on luminescence regulation by GacA in *V. fischeri* shows it has little effect on AHL production and that addition of exogenous AHL

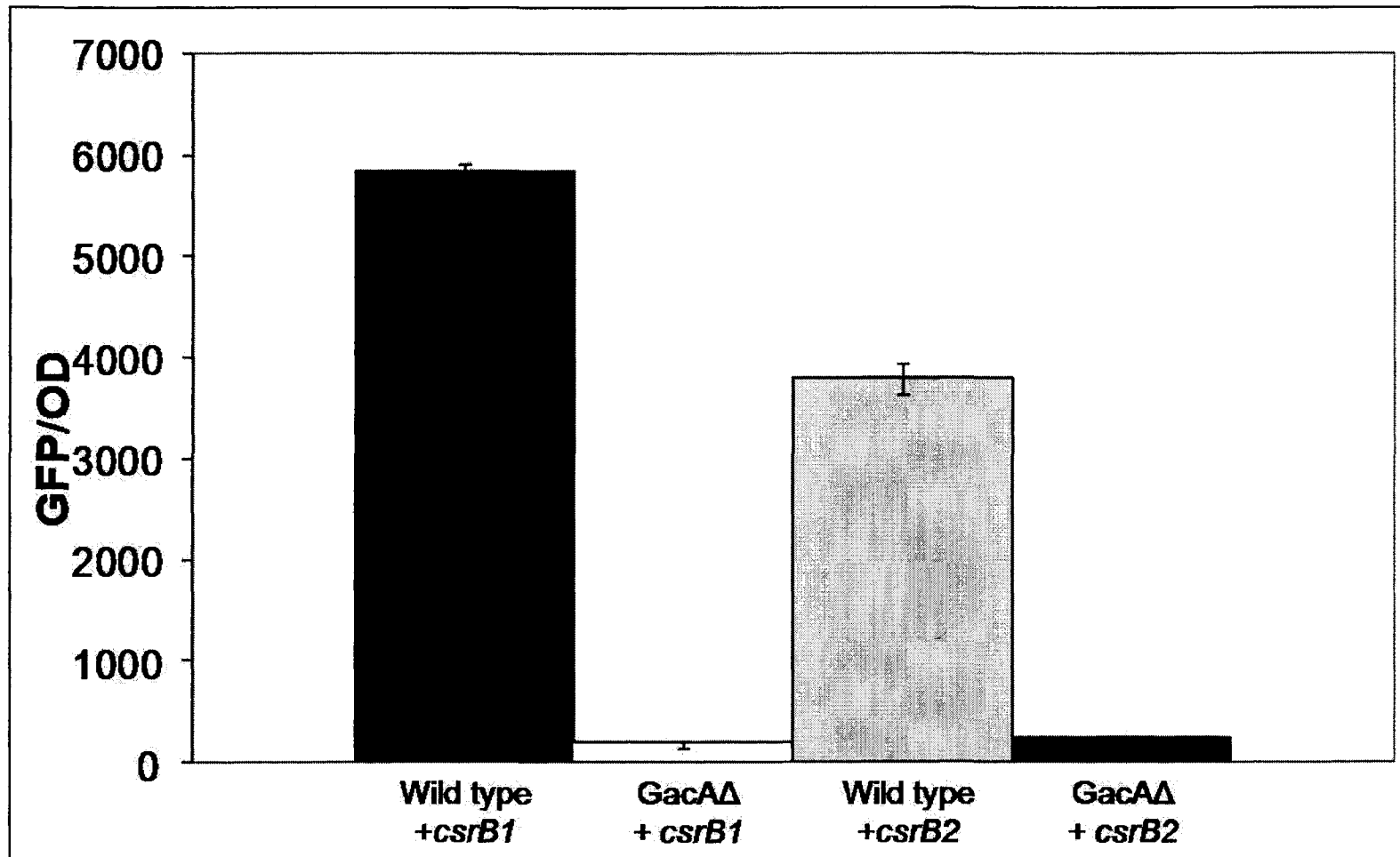


Fig. 5. *csrB1* and *csrB2* expression in wild type and GacAΔ. Gfp expression of promoter fusions relative to cell density (OD₆₀₀). Error bars indicate standard error (n=3).

does not restore luminescence suggesting differences in circuitry (Whistler and Ruby, 2003). If GacA/CsrB/CsrA regulation in *V. fischeri* and *V. cholerae* were conserved, GacA regulation would be funneled into the quorum sensing system via LuxO (Lenz *et al.*, 2005) and adding either *litR* or *luxR in trans* to the GacA mutant would bypass LuxO thus restore luminescence. However, neither *litR* nor *luxR* restored luminescence to the *gacAΔ* mutant (data not shown) indicating that GacA influences luminescence downstream of quorum-sensing activation.

Although GacA does not act through the known quorum sensing circuitry to activate *lux* transcription, this does not rule out an effect on transcription. To further elucidate whether GacA mutants are dark due to inhibition of transcription, we added *luxICDABE* expressed constitutively off of the P_{lac} promoter to the *gacAΔ* mutant, and as a control, to a *luxA* mutant and observed their luminescence over time (Fig.6). By eliminating the native P_{lux} promoter, we remove the requirement for activation. Although *luxICDABE* restored luminescence to a *luxA* mutant, it only had a small, positive effect on luminescence of the *gacAΔ* mutant. Similar luminescence levels in the *gacA* and *luxA* mutants harboring this construct would have indicated that GacA is required for transcription from the native P_{lux} promoter, whereas a lower level of luminescence would have indicated a post-transcriptional mechanism. These data strongly suggests that GacA regulates luminescence post-transcriptionally.

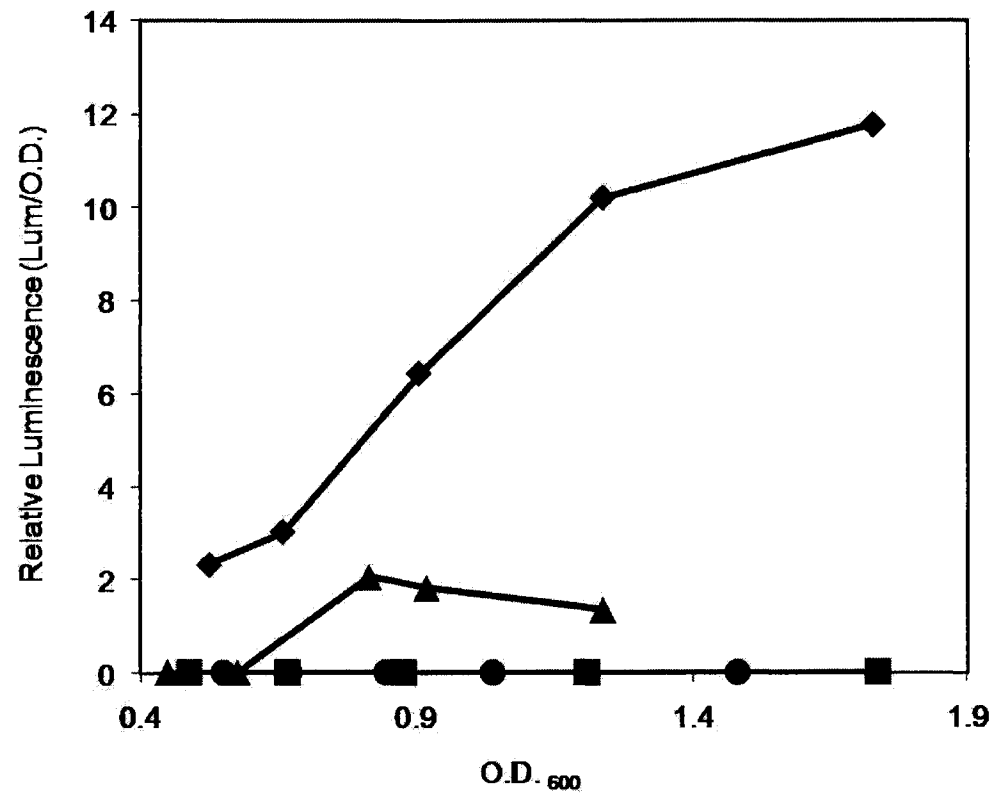


Fig. 6. Complementation of luminescence with *lux* expressed from P_{lac} promoter. Luminescence curve of *GacA*Δ complemented with *lux* operon *in trans* (▲) and the vector (●) and the *LuxA* mutant with *lux* (◆) and the vector (■) as a control. Note that *GacA*Δ is defective in growth yield.

Identification of a putative CsrA binding site within the *lux* coding sequence

CsrA represses translation of mRNAs by binding to stem-loops with (AR)GGA motifs located in the 5'UTR near the ribosomal binding site (Romeo, 1998). Several of these are often found in tandem, and facilitate cooperative and sequential binding by CsrA (Baker *et al.*, 2002). Upon observation of the deduced *luxI* mRNA sequence of *Vibrio fischeri* we noticed a possible stem-loop forming region containing a GGA motif (Fig. 7) similar to those previously reported as binding sites of CsrA. A second GGA that did not appear within a stem-loop forming region was also identified 16 bases downstream of this sequence. Alignments of the other sequenced *V. fischeri luxI* sequences, showed that this putative binding site, as well as the second GGA are conserved (Fig.7) but is absent in *ainS*, the other quorum sensing synthase. In all currently reported instances, the CsrA binding sites are located in the 5' UTR of regulated transcript; in contrast, this motif appears within the coding sequence of *luxI*. A survey of the LuxI homologs in related and unrelated species (including *Vibrio*, *Aeromonas*, *Pseudomonas*, *Shewanella* and *Burkholderia*) showed that the stem-loop is not conserved across species. However, a weak stem loop containing a GGA motif partially in the stem, believed not to be a significant impairment to binding (Dubey, Baker, Romeo, and Babitzke, 2005) was found in the coding sequence of several *Aeromonas* species *luxI* sequences. If the *luxI* region is truly a CsrA binding site then this is a novel location for CsrA-mRNA interaction.

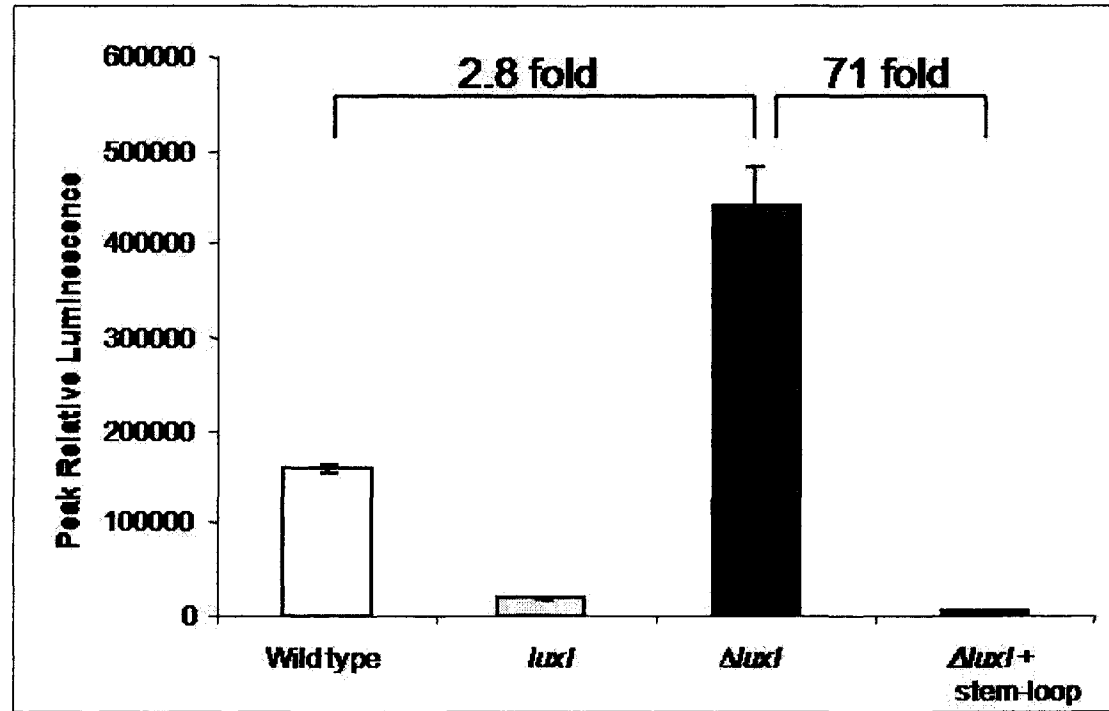


Fig . 8. Effects of putative CsrA binding site on luminescence. Luminescence expressed a maximum luminescence per OD₆₀₀ . Luminescence of wild type is higher than a luxI mutant with a mutation that does not eliminate the CsrA binding region. However, luminescence is greatly increased in the luxI deletion mutant. If just the putative CsrA binding site is added back to the luxI deletion mutant, it has a much lower luminescence.

To determine whether there is regulation at this potential CsrA target, we deleted the entire *luxI* gene, including both putative binding sites. We replaced the deletion with a short segment only containing the two GGA motifs. We observed the luminescence of these mutants (fig.8). Quite surprisingly, unlike the published *luxI* frame-shift mutant which is dim in culture (Lupp *et al.*, 2003) the *luxI* deletion mutant (which is unable to make one of the two AHL signals that activates *lux* transcription) was more luminous than wild type. Adding back the putative CsrA binding site resulted in a 71-fold reduction in luminescence. These data not only supported our hypothesis that this region is a CsrA binding site but it also suggested that under some conditions, post-transcriptional regulation of luminescence has a more significant effect than quorum-sensing.

Discussion

The GacA regulator has long been associated with both beneficial and pathogenic colonization. In recent years, several mechanistic studies have defined its interaction with other host-association regulons that include multiple classes of non-coding RNAs, the translational repressor CsrA/RsmA, as well as multiple quorum sensing pathways. Much significant work has been completed with only a few pathogenic species and these studies reveal some conservation of role and circuitry. But these studies by necessity have been largely removed from the host context. In this study, we begin to define the roles and interaction of the GacA, CsrB and CsrA regulators with quorum sensing in a bacterium that allows their study in a natural animal model. In keeping with the circuitry in other

bacterial species, in this study we demonstrate that, in *V. fischeri* GacA regulates both of the *csrBs* and GacA functions primarily to antagonize the repressor CsrA. We also determined that there are some striking differences in the regulatory circuitry. Specifically, the GacA/CsrB/CsrA regulators exert their control of luminescence predominantly by a post-transcriptional mechanism (Fig. 9), not by modulation of quorum sensing as has been found in other bacteria (Lenz *et al.*, 2004). We furthermore have identified a putative CsrA binding site located within the *luxI* coding sequence. Finally, animal colonization studies indicate that CsrA plays an important role in initiation of host colonization.

Regulation of luminescence and other factors important for host association by GacA, CsrA, CsrB1 and CsrB2

Though the relationship between GacA/CsrB/CsrA for *V. cholerae* and *V. fischeri* are the same, this study revealed curious differences in the placement of these regulators within their larger colonization cascades, perhaps as a reflection of the different lifestyles of these related bacteria. The VarA/CsrA interaction is important for virulence and quorum-sensing regulation in *V. cholerae* and further influences expression of the *V. harveyi lux* genes in this organism (Wong *et al.*, 1998; Lenz *et al.*, 2005). Similarly, the homologs in *V. fischeri* (GacA/CsrA) are important for symbiotic phenotypes and luminescence (Table 2) (Whistler and Ruby, 2003). Both VarA and GacA exert their influence by activating sRNAs (CsrB, C and D and CsrB1 and 2 respectively) which likely sequester the RNA binding protein CsrA. However it appears that this is where the similarity ends.

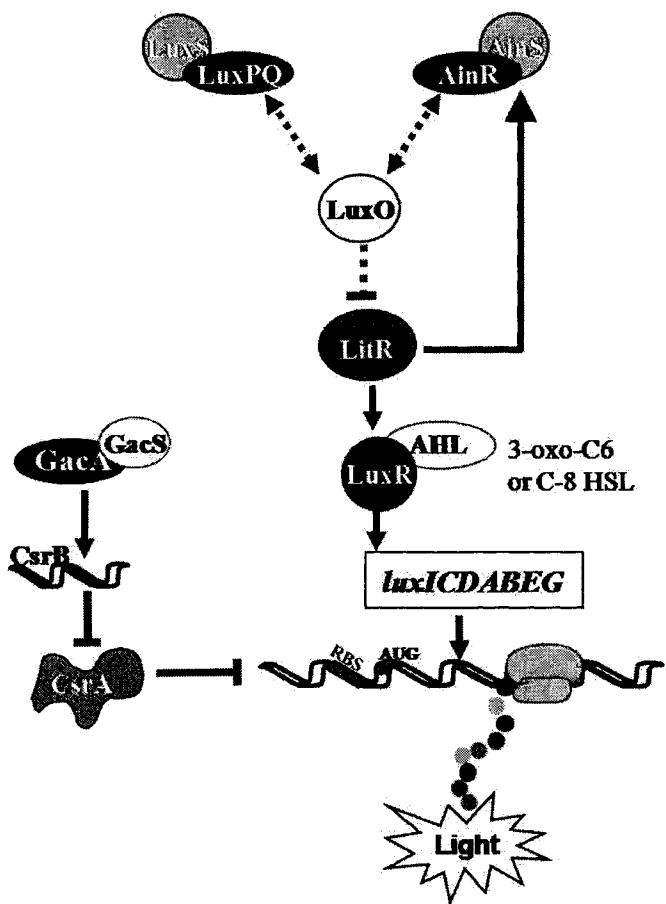


Fig. 9. New luminescence regulation model for *V. fischeri*. Based on data from this study, additions can be made to the current luminescence regulation model. It is now apparent that unlike the *V. cholerae* model, CsrA directly represses lux translation. Similar to *V. cholerae* and other organisms, GacA antagonizes CsrA via activation of CsrB1 and CsrB2 transcription.

In *V. cholerae*, this regulatory system functions in parallel with the quorum sensing systems to act on LuxO. In *V. fischeri*, GacA/CsrA mainly do not act upstream of LitR, but instead regulate luminescence and potentially other phenotypes post-transcriptionally. Relating to this theory that GacA/CsrA act on luminescence after transcription, the luminescence data showed that complete deletion of *luxI* has a positive effect on luminescence in culture. This seems contradictory to published data on a *luxI* mutant with a point mutation that inactivates the LuxI protein, dramatically diminishing luminescence (Visick *et al.*, 2000). The point mutation however, did not eliminate the putative CsrA binding site and therefore the transcript was likely under CsrA repression. By completely removing *luxI*, the putative CsrA binding site was also removed, eliminating repression. This putative binding domain has an even more dramatic effect than quorum sensing on luminescence *in vitro*, which demonstrates the importance of CsrA regulation. Having CsrA as a direct control mechanism may allow *V. fischeri* to further fine tune its luminescence expression by altering levels of CsrA and CsrB. Perhaps simultaneous quorum sensing activation of all virulence genes in *V. cholerae* is more useful in their host and so *V. cholerae* is better served by CsrA aiding in quorum sensing repression. However, it is also possible that important direct regulation of virulence transcripts by *V. cholerae* has been inadvertently overlooked due to the focused use of a heterologously expressed foreign transcript (*lux* from *V. harveyi*) as a measure of regulation, highlighting the usefulness of complimentary studies such as this for exploring the interface of translational and quorum sensing regulation.

The role of translational regulation during colonization

Although much work has been done to elucidate regulatory mechanisms of infection, with few notable exceptions, most conclusions about the relevance of transcriptional and translational hierarchies are inferred from *in vitro* data. For example, in *V. cholerae* CsrA regulation was elucidated using luminescence as a surrogate for virulence (Lenz *et al.*, 2005). In *E. coli* infectivity was inferred from motility, adherence and mucoidy (Romeo *et al.*, 1993). *Pseudomonas* virulence was assessed by production of extracellular products such as protease, HCN and pyocyanin (Pessi and Haas, 2001). Using a natural macrophage model, Swanson and colleagues have examined the roles of LetA (GacA homologue) and CsrA in the bacterial pathogen *Legionella pneumophilla* during macrophage infection (Molofsky and Swanson, 2003). In this system, CsrA mutants are highly infectious and those that overexpress CsrA are not. Analogous to CsrA over expression, the *V. fischeri* GacA mutant has a defect in colonization initiation. This shows that these two bacteria have a common need to repress CsrA in order to activate genes required for infectivity. However, once they are in the macrophage, CsrA mutants (LetA active) of *L. pneumophilla* are unable to multiply to a high density, which contrasts to the squid system where GacA mutants (CsrA active) are incapable of growing to a high density (Whistler and Ruby, 2003). Consistent with the LetA/CsrA regulation model, the infection defects of the *L. pneumophilla* CsrA mutant can be eliminated by additionally mutating LetA. In contrast, the GacA/CsrA suppressor mutants of *V. fischeri* were defective in initial colonization. This illustrates that in the *L. pneumophilla*

system CsrA/LetA are additional regulators of infection because without both them, infection occurs normally. However, in *V. fischeri*, the GacA/CsrA double mutants are defective at initiating infection, which means these regulators are important for normal colonization of the squid. Indeed, even though these regulatory relationships previously have been well characterized in a number of systems, we have laid the foundation with this work for elucidating their role in a natural animal system that compliments the findings from studying these regulators in other bacterial species.

CsrA binding within luxI coding sequence

In this study we discovered a putative CsrA binding site within the *lux* transcript in the coding region of *luxI* gene. When *luxI* was deleted from the *lux* operon, the mutant became brighter in culture (Fig. 8). This seems counterintuitive as this is a deletion of one of the quorum sensing synthases that induce luminescence. However, this highlights the significance of CsrA repression of luminescence in culture. When the putative CsrA binding domain was added back, the luminescence fell below that of wild type as a result of CsrA repression and loss of LuxI quorum sensing. It appears that quorum sensing and repression of CsrA are both important in luminescence induction. It also alludes to the fact that CsrA mutants may have an effect on 3-oxo-C6-HSL production. This is consistent with previous published data where the GacA mutant produced the same level of C8-HSL, but only produced half the amount of 3-oxo-C6-HSL compared to wild type- a difference that was not believed to be significant

enough to explain the luminescence defect, but which could be explained by CsrA repression of *lux*.

The placement of the putative CsrA binding site differs from those found in other organisms and raises many questions about CsrA regulation in *V. fischeri*. In the model organism *E. coli* the CsrA binding sites found were in the leader sequence and all had one site that overlapped the ribosomal binding site (Dubey *et al.*, 2005). The putative *luxI* binding site does not include the RBS and is within the coding sequence. The 5'UTR of the *lux* mRNA is very short, about 19 bases, and it may have been easier from an evolutionary standpoint to have a CsrA binding site within the coding sequence (Egland and Greenberg, 1999). CsrA is believed act by preventing the ribosome from binding thus destabilizing the mRNA (Romeo, 1998). Since the putative CsrA binding site located within the *lux* transcript of *V. fischeri* is downstream of the putative RBS, this may mean that when CsrA is bound, it does not block ribosome binding. Even if this is true CsrA would likely prevent translocation of the transcript through the ribosome. With the exception of the GGA motif, the *luxI* site does not fit with the consensus sequence for CsrA binding sites. It may be that *V. fischeri*'s CsrA recognizes a different consensus or that this operon is not as tightly regulated as others in the genome. Our findings suggest that future attempts to predict CsrA-regulated transcripts should include regions within the coding sequence of mRNAs.

Why would *V. fischeri* regulate luminescence post-transcriptionally whereas its relatives, *V. harveyi* and *V. cholerae* regulate luminescence at the transcriptional level (Lenz *et al.*, 2004)? An answer may be that *V. fischeri* needs

tighter control of luminescence because of the important biological role of luminescence for the success of its symbiosis with its natural hosts. Whereas luminescence is not required by *V. harveyi* during infections of marine life and the exact purpose of luminescence is still unknown, luminescence is a requirement of symbiosis by *V. fischeri* with its squid host (Visick *et al.*, 2000), and is also the basis of its symbioses with certain fishes. Post-transcriptional regulation allows bacteria to make rapid changes in expression (Romeo, 1998; Gottesman, 2002) and such rapid expression changes may be required of *V. fischeri* when it is associated with its host. Future work on the role of CsrA in this symbiosis may identify other CsrA-regulated symbiosis genes. This may allow us to further elucidate which genes are helpful or detrimental at key transitions in colonization. The colonization phenotypes of the GacA and GacA/CsrA mutants show that timing of expression is key in this symbiosis. It will be important to find the cues that mediate GacA/CsrA regulation to develop a better understanding of initiation of host infection by microbes.

CHAPTER 3

CONCLUSION

The significance of luminescence for *V. fischeri*'s symbiotic lifestyle is highlighted by its many points of control. At the transcriptional level, luminescence is derepressed by AinS/R and Lux S/PQ systems and induced by LuxI/AinS/LuxR (Lupp and Ruby, 2004). As we have discovered in *V. fischeri*, GacA, CsrA, CsrB1 and CsrB2 regulate luminescence post-transcriptionally. While in *V. cholerae* GacA and the quorum sensing systems work together regulate *lux* via HapR derepression, *V. fischeri* does not coordinate these two systems in the same way. Instead, GacA derepresses *lux* translation and the C8 and AI-2 quorum sensing-systems derepress *litR*, thereby influencing *lux* transcription. Why does this difference exist since these different regulatory approaches seem to be means to the same end? I would speculate that this difference in regulation has to do with the importance of HapR/LitR as an activator as well as the requirements imposed by the different lifestyles of the bacteria. In *V. cholerae* HapR is believed to regulate virulence, biofilm formation, protease as well as other genes, making it a very important regulator for this bacterium (Lenz *et al.*, 2005). LitR only has a small effect in *V. fischeri*. LitR activates LuxR, which already positively regulates itself, and has a slight positive effect on other symbiosis phenotypes (Lupp and Ruby, 2005). HapR is very important for the

pathogenic lifestyle of *V. cholerae* and so it would follow that this is the point of tightest control, whereas luminescence is very important for the symbiotic nature of *V. fischeri* and could be why GacA exerts its control at the post-transcriptional level.

CsrB1 and CsrB2 mutations

Although the main focus of this research was to elucidate the mechanism of GacA regulation of luminescence, as we found via CsrB1 and CsrB2 activation, GacA also regulates other symbiotic phenotypes through the same regulators. GacA mutants are defective in normal initiation and colonization of the squid (Whistler and Ruby, 2003). Both of these phenotypes as well as the *in vitro* phenotypes were all complemented by addition of *csrB1* or *csrB2 in trans*. Although untested, it would follow that knocking out these genes would mimic the GacA mutant phenotypically. Based on my promoter fusions and complementation which relied on the natural GacA-controlled promoters for *csrB1* and *csrB2*, there is some *csrB1* and *csrB2* expression in the absence of GacA which may mean that a CsrB1/CsrB2 double mutant may have a slightly more severe phenotype than a GacA mutant in that it may have greater growth yield and motility issues. I have generated a CsrB2 mutant by replacement of the gene with an erythromycin resistance cassette but this mutation has no discernable phenotype. It is likely CsrB1 and CsrB2 have redundant function as they can both complement GacA and similar redundancy of function has been found in *V. cholerae* with CsrB, C, and D (Lenz *et al.*, 2005). One could

speculate that this redundancy exists because of the importance of repressing CsrA which can be inferred from the deficiencies of the GacA mutant where CsrA is largely unchecked. However, it should be noted that CsrB1 *in trans* had a greater complementation effect than CsrB2 which fit with the differences in expression (Fig. 5) as the CsrBs *in trans* were expressed off their native promoters. This means that they are not fully redundant.

GacS Signal Identification

Since the discovery of GacS/GacA researchers have wondered what the signal is for this two-component pathway (Hrabak and Willis, 1992). GacA is important for the transition into stationary phase and this means that the GacS signal is likely to build up at this point in time (Reviewed in Heeb and Haas, 2001). The signal could be an internal metabolite that when it reaches a high enough concentration, it induces GacS autophosphorylation. In *Pseudomonas fluorescens* GacS appears to respond to “a solvent-extractable extracellular signal” (Kobayashi *et al.*, 2003) which does not narrow the possibilities much. In *L. pneumophila*, LetA/LetS (GacA/GacS) appear to respond to high levels of ppGpp (Hammer, Tateda, and Swanson, 2002). Work done by Craig Altiers group suggests that SirA(GacA homolog) and/or BarA (GacS homolog) respond to both unknown environmental signals as well as acetate (Lawhon *et al.*, 2002). From these attempts at isolating the ligand for GacS it is clear that there are a number of ideas that vary from species to species. While the majority of the GacS protein is fairly conserved between species, the periplasmic domain (the

region thought to bind the substrate) is highly divergent (Reviewed in Heeb and Haas, 2001). Based on this divergence as well as probable GacS regulators in different species I would venture that the signals for these proteins differs between groups or even species. While this belief is logical, it makes the discovery of the signal for GacS in *V. fischeri* and other species more difficult. To find it one may have to systematically add any number of potential extracellular products and internal metabolites to a culture of *V. fischeri*. A GacS mutant would serve as a good negative control and my *csrB1* and *csrB2* promoter fusions could serve as an indicator of GacA activity.

CsrA/CsrB regulation of GacA Mutant Phenotypes

My thesis work clearly shows that luminescence is regulated by GacA activation of CsrB1 and CsrB2, to repress CsrA, but how do they regulate the other GacA mutant phenotypes? In *E. Coli* CsrA has a direct positive effect on motility via FlhDC (a DNA binding protein that recognizes flagellar promoters) (Wei *et al.*, 2001). It is possible that motility in *V. fischeri* is modulated in the same way and that is why we see hyperflagellation and hypermotility of the GacA mutant on low concentration agars (Whistler and Ruby, 2003). The GacA mutant's lack of motility on high percentage agar (0.7%) cannot be explained by these means and may have to do with available energy. Microarray data indicates that GacA regulates siderophore transcriptionally. Sequences that matched the Fur box consensus were also located upstream of this operon as well as a number of other GacA-regulated genes indicating a likely role of Fur

repression (Boisvert, 2007). As for the other phenotypes including LPS and simple sugars utilization, there is no evidence of direct regulation of transcripts related to these phenotypes by CsrA in other bacteria. Therefore, it is not apparent whether or not CsrA directly binds these transcripts for these phenotypes. CsrA was originally named so because it regulated glycogen biosynthesis in *E.coli* via *glgCAP* repression (Romeo *et al.*, 1993). It follows that these GacA mutant phenotypes could arise secondarily from the disrupted metabolic state of the GacA mutant. It may be prudent to look for CsrA repression of the homologs of CsrA-regulated carbon storage genes of *E. coli* in *V. fischeri*, such as *glgCAP* and *cstA* (involved in nutrient scavenging under carbon starvation conditions) (Dubey *et al.*, 2003). CsrA has also been shown to regulate Hfq expression (a chaperone like protein involved in non-coding RNA stability), another global regulator (Baker *et al.*, 2007). Study of these genes will allow us to either include or eliminate them as CsrA targets and to identify if they are responsible for the indirect effects on LPS, and sugar utilization. Identification of the CsrA targets related to known phenotypes will give us a better understanding of CsrA regulation in *V. fischeri* as well as help to identify as yet unknown targets.

Identification of Other CsrA Binding Sites in *V. fischeri*

A significant outcome of this study is my identification of a novel location and structure for the putative binding of CsrA to a transcript. It will be interesting to see what other CsrA binding sites exist in *V. fischeri* because they may have a

different consensus from *E. coli*, changing the search approach in this, and possibly other organisms. By identifying the type and location of each binding site we may be able to determine what kind of influence CsrA exerts on the transcripts it regulates. In *E. coli* we have already seen a great diversity in CsrA binding regions; some with and without stem-loops and anywhere from one to six binding domains (Reviewed in Babitzke and Romeo, 2007). The *luxI* binding site is unusual in that the stem-loop is A-T rich and is beyond the ribosomal binding site (Dubey *et al.*, 2005). It may be that all the stem loops of CsrA binding sites in *V. fischeri* are A-T rich because of the nature of the genome (Ruby *et al.*, 2005). I would tend to believe that most CsrA binding sites in *V. fischeri* do overlap with the ribosomal binding site. However, the possibility of other sites in this location cannot be eliminated so it will still be important to search within coding sequences for CsrA binding sites. Researchers have found that CsrA can exert a positive influence by binding to the extreme 5' end of the transcript thereby preventing mRNA degradation (Babitzke, 2007). In addition to the possibility of this kind of regulation in *V. fischeri* it also raises questions of whether or not a binding site after the RBS also changes regulation by CsrA. Perhaps this gives the ribosome a chance to bind before CsrA, limiting the effect of CsrA. As other CsrA-regulated transcripts are found in *V. fischeri*, these questions will be answered.

Potential CsrA Mutation Techniques

Although my directed attempts at generating a mutant were unsuccessful,

a CsrA mutant of *V. fischeri* would be an excellent means of further elucidating CsrA regulation. A mutant would help to support data I have already found and aid in identifying additional, unknown, points of control by aiding in identifying changes in their expression in the mutant background. In addition, a CsrA mutant would be useful in identifying transcripts targeted by this protein by techniques like real-time PCR or for microarray experiments since, as we have shown with the *luxI* binding site, the targets do not always fit the mold, making bioinformatic techniques less useful. My previous attempts have failed because I had tried to completely inactivate the protein but this does not mean that future attempts using less severe mutations will. I believe that there are a few mutagenesis strategies that may be successful. Since the gene is highly pleiotropic and a complete deletion mutation seems to be lethal or to have a very significant impact on growth, complete inactivation in the wild type background may not be possible. However, spontaneous mutations that occurred in the C terminal region of the GacA/CsrA mutants were beyond the domains of significance and perhaps directed mutations in this end portion could be tolerated. By adding mutations specifically to that region, it may be possible to study targets of CsrA more effectively. This mutagenesis strategy would likely be successful for making a GacA/CsrA double mutant; however, we have never isolated a spontaneous CsrA mutant in the wild type background so this mutant may be less likely to occur. Other groups have made mutants that have an inducible CsrA so that the mutant is able to grow to a usable density for further experimentation (Molofsky and Swanson, 2003).

A Natural System for CsrA Study

Although the role of CsrA/CsrB in gene regulation has been studied and dissected in a number of pathogenic, and even some beneficial bacteria, due to the limitations of available host models, interpretations of the role of CsrA/CsrB in animal infection is highly speculative at this point. Work done in *E. coli* has been largely mechanistic and has clarified the structure and function of these antagonistic regulators (Romeo, 1998; Gutierrez *et al.*, 2006; Babitzke, 2007). Others have identified the binding capabilities with RNA (Baker *et al.*, 2002). Using plant models, groups have identified the importance of CsrA in more environmental conditions such as beneficial and pathogenic plant association of *Pseudomonas fluorescens* and *Erwinia carotovora* (Heeb *et al.*, 2002; Mukherjee *et al.*, 1996). These studies indicate a specific role of CsrA and CsrB in host-association but due to dramatic differences between plant and animal hosts (e.g. the nature of immunity) as well as the superficial nature of host-association of these bacterial species, there are again limitations as to how the data can be extrapolated to animal infections. Some work has been done studying the effect of CsrA on animal cells, for example *L. pneumophila* on macrophages and *Pseudomonas aeruginosa* on human lung cells, but these models are contrived and do not relate well with an intact host (Molofsky and Swanson, 2003; Mulcahy *et al.*, 2006). The natural infection model used to study *V. fischeri* is a clear advantage over these other systems and work on the role of CsrA and CsrB in infection should continue in this system.

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