Elucidating Regulatory Connections between the Second Messenger Cyclic-Di-GMP with Biofilm and Motility Phenotypes of Vibrio fischeri

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LOYOLA UNIVERSITY CHICAGO

ELUCIDATING REGULATORY CONNECTIONS BETWEEN THE SECOND
MESSENGER CYCLIC-DI-GMP WITH BIOFILM AND MOTILITY PHENOTYPES OF

VIBRIO FISCHERI

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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BY
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Chapter 1

BACKGROUND

Review of Literature

*Vibrio fischeri* and its Symbiosis with *Euprymna Scolopes*

Host-microbe interactions are complex. Depending on the interplay between the microenvironment, microbial factors, and host factors, host-microbe interactions can result in many different outcomes. Among these interactions, symbiotic relationships are well studied and serve as models to investigate different bacterial processes in bacterial-host interactions. One such model is the symbiosis between the bacterium *Vibrio fischeri* and the Hawaiian bobtail squid *Euprymna scolopes*. These organisms have evolved a specific relationship that occurs in the context of the light organ of the squid host (reviewed in Stabb and Visick, 2013).

*V. fischeri* is a bioluminescent, Gram-negative bacterium that is found in marine environments (reviewed in Stabb & Visick, 2013). *V. fischeri* has the ability to grow within a myriad of different conditions compared to other bacterial species in the same genus (Ochman & Moran 2001). The ability to adjust to a variety of different conditions can be attributed to the complex genome of *V. fischeri*. *V. fischeri*’s genome includes two chromosomes, a larger chromosome needed for essential genes and a smaller secondary chromosome containing genes with specific niche functions, along with a plasmid known to encode a type IV secretion system. The versatility in the ability of *V.*
The ability of *V. fischeri* to adjust in different conditions primarily arises from the smaller chromosome, which encodes unique proteins needed for its specific lifestyle (Ruby et al. 2005).

Similar to the ability of *V. fischeri* to grow in a variety of different conditions, the bacterium can symbiotically associate with a variety of different animals. These symbioses primarily occur with the bacterium colonizing the light organ of different fishes or squids (reviewed in Ruby 1996). The ability to colonize a host is competitive, as some strains of *V. fischeri* can outcompete others for colonization within certain hosts but also have little ability to colonize a different host. These findings suggest that many *Vibrio* species have evolved to occupy specific niches (Nishiguchi 2002).

*V. fischeri'*s best studied symbiotic relationship is with *E. scolopes*, a species commonly referred to as the Hawaiian bobtail squid. This squid is found off the coast of Hawaii. These animals have a short life span; in fact, they have the quickest life cycle of any cephalopod. This acceleration in life span is due to the warm temperatures of their environment, the organism’s exponential growth rate and the small size the cephalopods reach as adults (Hanlon et al. 1997). Behaviorally, *E. scolopes* are primarily nocturnal, as they bury into the sand during the day and come out at night. They are also solitary in nature as, in confined environments, they are observed to scatter (Moynihan 1983). Due to their small size and solitary behavior, predation of *E. scolopes* is a threat. One way that animals like these small squid can be found by predators is through detection of the animal’s shadow (Anderson & Mather 1996). To of
fset this threat of detection from predators, *V. fischeri* camouflages *E. scolopes* by colonizing the light organ of the squid and luminescing. This luminescence masks the shadow of the squid and limits the threat of detection from predators (Jones & Nishiguchi 2004).

**Colonization by *Vibrio fischeri* of its Symbiotic Host**

While the symbiotic relationship is a clear advantage for the host *E. scolopes*, the bacteria are also aided by the environment of the cephalopod’s light organ (Ruby & Asato 1993). The light organ provides necessary nutrients for rapid growth of *V. fischeri*. A first phase of colonization is the exponential growth of the bacteria, leading to full coverage of the light organ (Ruby & Asato 1993). This is followed by a phase of restricted growth, potentially due to the depletion of available nutrients used during the phase of rapid growth. The rapid growth suggests that residing in the environment of the light organ is beneficial to *V. fischeri*. Further, *V. fischeri* is protected from competition or predation by other microbes due to the immune system of the squid, which eliminates non-*V. fischeri* bacteria (Davidson et al. 2004). Thus, this relationship is beneficial to both *E. scolopes* and *V. fischeri*.

While this mutualistic interaction is necessary for survival for both the bacteria and the host organism, the juveniles that arise from mating of the Hawaiian bobtail squid lack *V. fischeri* in their light organ. *E. scolopes* juveniles acquire *V. fischeri* following their hatching into seawater, if the bacteria are present in the external marine environment (Wei & Young 1998). To become colonized, the host must utilize a process to pull the bacteria into the body cavity that houses the light organ; this process is known as venting (Nyholm and McFall-Ngai, 2004). However, after the first contact
between the squid and marine bacteria, *E. scolopes* under native conditions will become and remain exclusively colonized by the marine bacterial symbiont *V. fischeri*.

This proficiency in outcompeting other bacteria is due to the coevolution between the marine bacterium and the Hawaiian Bobtail squid (Mandel et al. 2009). Evidence of this coevolution can be seen via the acquisition of the gene *rscS* by the *V. fischeri* strain ES114 (Mandel et al. 2009). This gene encodes a sensor kinase, RscS, that induces the production of exopolysaccharide Syp, which in turn leads to the initiation of biofilm formation on the surface of the light organ (Yip et al. 2005, 2006). The bacteria’s ability to form a biofilm is due to the increased production of the polysaccharide, leading to the formation of the extracellular matrix necessary for adhesion and defense (Donlan et al. 2001). The bacteria in the aggregate are thought to be protected from the environment through an extracellular matrix consisting of a combination of proteins, nucleic acids and polysaccharides (reviewed in Stabb & Visick 2013). The aggregate also appears to play an important role in permitting the bacteria to transition from the seawater environment to the host environment (Nyholm et al. 2000). Together, these data suggest that one advantage of coevolution between the squid-derived *V. fischeri* strains and the Hawaiian bobtail squid is enhanced biofilm formation, which promotes attachment and thus colonization.

**Pathway of *V. fischeri* to Reach the Light Organ for Colonization**

The light organ resides in the middle of the mantle cavity of *E. scolopes*. This organ has an array of ciliated epithelial cells that reside on the lateral surfaces. Anterior and posterior extensions project inside the mantle cavity and form a ciliated ring, giving the bacteria a higher probability of being directed to and into the light organ interior through
pores. The light organ contains six pores, with each pore opening into the inside of the light organ (McFall-Ngai & Ruby 1991). The light organ utilizes venting to create currents. The movements of long and short cilia result in the introduction of the bacteria within the mantle cavity into less turbulent zones (Nawroth et al. 2017): the smaller bacteria are brought closer to the entrance of the light organ and the larger particles pushed further away. The bacteria then aggregate near the pores in a biofilm-like form (Nyholm et al., 2000; Yip et al., 2006). After a period of time in the aggregate, a switch to dispersal occurs such that the bacteria can reach the interior of the light organ. This process of dispersal and motility is necessary for colonization as only the cells that can migrate through the mucus can reach the entrance and interior (Nyholm et al. 2000). Once inside the light organ, the bacteria must transition from the nutrient-limited seawater outside into an environment inside that is likely challenging due to an abundance of antimicrobials produced by the squid (Davidson et al. 2004). After traversing ducts and antechambers that are non-permissive for growth, the bacteria reach the suitable, nutrient-rich environment of the deep crypts in the light organ. Thus, early colonization of the deep crypt spaces in the light organ of *E. scolopes* by *Vibrio fischeri* requires a number of different factors, including motility.

**Syp Polysaccharide Production Promotes Symbiotic Biofilm Formation**

Within this pathway for colonization, an important area of investigation is the formation of a transient biofilm on the surface of the light organ and the subsequent switch to dispersal. The formation of the biofilm or aggregate occurs via a combination of factors, including the bacteria attaching to each other and interacting with the host-derived mucus secreted by the ciliated epithelium (Nyholm & McFall-Ngai 2003)
(Nyholm & McFall-Ngai 2004). It has also been shown that *V. fischeri* successfully outcompetes other bacterial species within the aggregates, with about 99% of the aggregated bacteria being *V. fischeri* (Nyholm & McFall-Ngai, 2003). Many factors likely contribute to the ability of *V. fischeri* to outcompete other bacterial strains during symbiotic aggregation, including resistance to host immune defenses and signaling between host and symbiont.

The ability of *V. fischeri* to aggregate on the surface of the light organ depends on the production of biofilm matrix molecules such as exopolysaccharides (Yip et al. 2006). A key exopolysaccharide important for aggregation is Syp, which produced by enzymes encoded within the symbiosis polysaccharide (syp) locus. As described above, this locus is upregulated by the signal transduction sensor kinase RscS. The modulation of the syp locus is advantageous for biofilm aggregation and colonization, as evidenced by the result that overexpression of the sensor kinase RscS led to a larger group of aggregating bacteria on the *E. scolopes* light organ surface and resulted in a colonization advantage (Yip et al. 2006). In contrast, an rscS null mutation conferred a defect in bacterial aggregation and colonization (Yip et al. 2006; Visick & Skoufos 2001). The extracellular signal(s) detected by the sensor kinase RscS to induce biofilm formation is as yet unknown. While one signal that promotes biofilm formation is calcium, this effect appears to be independent of RscS function (Tischler et al. 2018).

**Vibrio fischeri Leaves the Biofilm to Migrate into the Light Organ**

Dispersal from the transient biofilm on the surface of the light organ depends upon a switch between the previous sessile state of the bacteria to a planktonic state, leading to migration into the light organ (Nyholm et al. 2000). However, the coordination of this
switch to dispersal from the aggregation in *V. fischeri* is still unknown. While it is suggested that LapV, an adhesive extracellular protein that can adhere to other bacterial cells or the surface, contributes to this switch in bacterial states, the mechanism of this switch is unknown (Christensen et al. 2020).

This switch to dispersal is followed by motility and chemotaxis of the bacteria from the aggregate to the pore. Strains that are nonmotile fail to reach the pore and thus fail to colonize (Nyholm et al. 2000). Control over motility is necessary due to the substantial energy cost of the flagella-based motility that *V. fischeri* utilizes for movement. Migration from the pore to the interior is promoted through a chemotaxis gradient, with the bacteria migrating to chitin derivatives such as chitobiose (Mandel et al. 2012) (Kremer et al. 2013). Chitin synthases present around the pores produce chitin, which is degraded to chitin oligosaccharides, thus attracting the motile bacteria to migrate towards the pore (Kremer et al. 2013).

**The Switch to Dispersal is Influenced by C-di-GMP Through the LAP Pathway**

The subject of my thesis is the investigation of the switch in the bacterial state from aggregation to dispersal and the corresponding regulation of motility in *V. fischeri*. An influencer in the switch in the states between nonmotile sessile bacteria to motile planktonic bacteria is the intracellular second messenger cyclic-di-GMP (c-di-GMP) (Reviewed in Hengge, 2009). Higher levels of c-di-GMP lead to nonmotile bacteria that can form biofilms and low levels of c-di-GMP lead to motile bacteria (figure 1) (Chua et al. 2014). C-di-GMP is made and broken down through the use of two types of enzymatic proteins. These enzymatic proteins are diguanylate cyclases (DGCs) that form c-di-GMP from two GTP molecules through the enzymatic GGDEF domain and
phosphodiesterases (PDEs) that break down c-di-GMP down to GMP through the enzymatic EAL or HD-Gyp domain (figure 1) (Hisert et al. 2005; Kulasakara et al. 2006). *V. fischeri*’s genome encodes 50 putative c-di-GMP modulating enzymes: 28 DGCs, 12 PDEs, and 10 other proteins, including degenerate proteins without enzymatic function, or combinations with both DGC and PDE domains (Wolfe & Visick 2010). With a few exceptions that are known, these enzymes controlling *V. fischeri* physiology are unknown. A couple of diguanylate cyclases that are known to coordinate functions within *V. fischeri* are VF_0989 and VF_A0959. Strains lacking either of these two diguanylate cyclases exhibit increased motility under specific conditions due, at least in part, to an increase in flagellin synthesis; thus, when functional, these DGCs inhibit motility by inhibiting flagellin, and likely flagella, production (O’Shea et al. 2006).
Figure 1. The Bacterial Intracellular Second Messenger Cyclic-di-GMP Coordinates Cellular Response

C-di-GMP is an intracellular second messenger that coordinates cellular responses such as motility and biofilm formation. The levels of c-di-GMP are modulated by c-di-GMP modulating enzymes: Diguanylate Cyclases (DGCs), which produce c-di-GMP from 2 GTP molecules through their enzymatic GGDEF domains, and Phophodiesterases (PDEs), which degrade c-di-GMP to GMP through their enzymatic EAL or HD-GYP domains. The levels of c-di-GMP modulate bacterial states, with lower c-di-GMP levels promoting the switch to planktonic cells and higher levels of c-di-GMP leading to biofilm formation. C-di-GMP levels can also be influenced by signaling via attached sensory domains.

One pathway that responds to the levels of c-di-GMP to control the switch from biofilm aggregation to dispersal is the LAP pathway (Newell et al. 2009; Christensen et al. 2020). Because this pathway is best studied in *Pseudomonas*, it serves as a model for understanding the similar pathway that exists in *V. fischeri*. In *V. fischeri*, the extracellular adhesin, LapV, is anchored on the cell surface and contributes to biofilm formation by adhering to surfaces and/or other bacterial cells. The intracellular region of LapV includes a region that can be cleaved by the transmembrane protease, LapG. Cleavage by LapG relieves LapV’s connection to the cell surface, allowing cells to exit...
the biofilm. LapG’s activity is controlled by the regulator LapD, which sequesters LapG, resulting in a biofilm state as seen in figure 2. In turn, LapD’s activity is regulated by c-di-GMP (Newell et al. 2009). At low levels of c-di-GMP, LapD is inactive and cannot sequester LapG, but with increased levels of c-di-GMP, the secondary messenger can influence the active state of LapD (figure 2). C-di-GMP can bind to the inactive EAL domain of LapD, thus switching its conformation from inactive to active to sequester LapG.

The phosphodiesterase that controls this switch for LapD activation in *V. fischeri* is PdeV (Christensen et al. 2020). Deletion of pdeV alone results in biofilm formation under conditions that do not promote biofilm formation by the wild-type parent. This biofilm depends on the LapD sequestration protein and the LapV adhesin (Christensen et al. 2020). These data suggest that PdeV may degrade c-di-GMP in a localized manner. Levels of c-di-GMP can be adjusted locally through synthesis and degradation.

Preliminary data (Christensen and Visick, unpublished) suggested that a single putative DGC, *VF_A0343*, was responsible for producing the c-di-GMP needed for LapD function: deletion of *VF_A0343* in the ΔpdeV mutant background resulted in abrogation of biofilm formation. *VF_A0343* is encoded adjacent to *VF_A0344*, a putative PDE, and overlaps with the gene for *VF_A0342*, a putative DGC. The proximity of these genes suggests that they may function in the same pathway, and thus, these other enzymes may also regulate LapV-dependent biofilm formation. I addressed this question as part of my thesis work.
Figure 2. The Transition Between Biofilm Formation and Dispersal is Dependent on the Lap Pathway. The transition of the bacterial cell population from sessile to planktonic is dependent on the state of the extracellular proteins LapV. LapV is an extracellular protein that is thought to permit bacteria to adhere to other bacterial cells and/or to surfaces. Lap adhesins like LapV are transported to the outer membrane and extracellular space by a type 1 secretion system apparatus (T1SS) composed of subunits LapB, LapC and LapE. The extracellular protein LapV holds the bacteria in a biofilm, but once cleaved is lost from the surface, releasing the bacteria to permit dispersal. LapV is cleaved by the periplasmic protease LapG, which is available to cleave if it isn’t sequestered by the inner membrane spanning regulator protein, LapD. LapD sequesters LapG when activated by binding to the intracellular second messenger c-di-GMP. If c-di-GMP isn’t present at a sufficient concentration, then LapD is inactive and cannot sequester LapG, leading to dispersal. (Genes not drawn to scale) (Christensen et al. 2020).

Vibrio fischeri Utilizes Quorum Sensing to Control Bioluminescence

The mechanism of quorum sensing (QS) was discovered in V. fischeri (Nealson et al. 1970). This organism uses QS to control the onset of luminescence, resulting in the production of the enzyme luciferase when the bacteria reach the exponential phase of growth (Nealson et al. 1970). QS occurs during bacterial growth as the population of bacteria releases signaling molecules, known as autoinducers, that coordinate large scale processes such as bioluminescence and biofilm production (Waters & Bassler 2005). These autoinducers must reach a certain threshold concentration to coordinate the processes. Once the threshold is reached, autoinducers will indirectly change
expression of genes within the entire bacterial population to trigger cellular responses. The proteins responsible for the production of bioluminescence are encoded by the \textit{luxICDABEG} locus (Engebrecht et al. 1983). The reaction that produces bioluminescence is an oxidative reaction that releases light by the enzyme luciferase, which is encoded by the genes \textit{luxA} and \textit{luxB} (Boylan et al. 1989). The substrates for this reaction are made by LuxCDE and LuxG.

This control of luminescence is regulated directly by the LuxI-LuxR quorum sensing system. The direct regulator of bioluminescence is LuxR, which controls transcription of the \textit{luxICDABEG} operon (Hanzelka & Greenberg 1995). LuxR is activated by binding the autoinducer, \textit{N}-3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL), which is synthesized by the protein LuxI. Once a sufficient concentration of 3-oxo-C6-HSL is reached, where the concentration intracellularly and extracellularly are at equilibrium, the autoinducer can bind to the N-terminal domain of LuxR, activating it to promote transcription, increasing the levels of bioluminescence in the population (Stevens et al. 1994).

**Indirect Quorum Sensing Systems Use LitR to Coordinate Cellular Responses**

The other quorum sensing systems affecting bioluminescence in \textit{V. fischeri} indirectly regulate bioluminescence, while also controlling other processes such as flagellar-based motility and acetate metabolism (Lupp & Ruby 2005; Studer et al. 2008).
Figure 3. Quorum Sensing Systems Indirectly Regulate Bioluminescence Through Modulation of LitR Translation. The two quorum sensing systems, AinS-AinR and LuxS-LuxP/Q systems, work in parallel to modulate the translation of LitR through posttranscriptional regulation. At low cell density, the production of autoinducers from LuxS and AinS is low, allowing for kinase activity from LuxP/Q and AinR to promote autophosphorylation and subsequent transfer of the phosphoryl group to the response regulator LuxO through the phosphorelay LuxU. The response regulator LuxO activates the gene that produces the sRNA Qrr1, which sequesters litR and represses its translation. When at sufficient cell density, the autoinducers AI-2 and C8-HSL are produced and bind and activate phosphatase activity of AinR and LuxP/Q. This reverses the phosphorylation, dephosphorylating LuxO, which inactivates LuxO. Thus, there is no production of the Qrr1 sRNA, leading to unsequestered litR mRNA, which is then translated into LitR. LitR then activates luminescence production.

There are two parallel QS systems that can indirectly regulate bioluminescence production, the LuxS-LuxP-LuxQ and the AinS-AinR quorum sensing systems (Figure 3). These two systems are parallel to each other and control LuxR indirectly, and are predominant influencers of luminescence at lower densities. In particular, AinS, which produces the N-octanoyl-homoserine lactone (C8-HSL) that is sensed by the histidine kinase AinR, makes a substantial contribution to this regulatory control (Neiditch et al. 2006) (Figure 3). AinR controls the phosphorylation state of the protein LuxO, which when phosphorylated indirectly inhibits light production. The other quorum sensing system, the LuxS-P/Q system, similarly controls luminescence through synthesis of and response to autoinducer-2 (AI-2) (reviewed in Verma & Miyashiro 2013) (Figure 3). Both
quorum sensing systems work in parallel to control the phosphorylation of the phosphorelay protein LuxU, which in turn regulates the phosphorylation state of the response regulator protein LuxO. Low cell density leads to phosphorylation, while high cell density leads to dephosphorylation. Phosphorylated LuxO is activated to induce transcription of qrr1, which encodes the small RNA Qrr1 (Figure 3). Qrr1 post-transcriptionally sequesters the mRNA for the global regulator, LitR, which in turn activates transcription of luxR to promote luminescence production (Figure 4) (Miyashiro et al. 2010).

With the sensing of autoinducers by histidine kinases in separate quorum sensing systems, extra layers of regulation and complexity are present with autoinducer crosstalk and regulatory feedback loops. The AinS-AinR quorum sensing system doesn’t have sequence similarity to the LuxI-LuxR quorum sensing system and while both regulate bioluminescence production, LuxI/R is direct and AinS/R is indirect. However, the autoinducer synthesized by AinS, C8-HSL, can compete with the autoinducer 3-oxo-C6-HSL to bind to LuxR (Kuo et al. 1996). This competitive binding by C8-HSL to LuxR can lead to a decrease in luminescence at intermediate levels of bacterial population, but 3-oxo-C6-HSL overcomes the C8-HSL competitive binding at higher V. fischeri cell densities. Positive feedback loops allow the autoinducers, or the proteins further down the pathway that are activated by the autoinducers, to increase their own synthesis. The autoinducer 3-oxo-C6-HSL increases its own synthesis by upregulating the luxI gene, while the protein further down in the converging quorum sensing pathways, LitR, activates the synthesis of the autoinducer C8-HSL by activating ainS (reviewed in Verma & Miyashiro 2013).
Cell Density Modulates the Translation of LitR and Influences Cellular Responses

The modulation of LitR protein levels is dependent on cell density, with lower cell density leading to the sequestration of the \( \text{litR} \) mRNA and a decrease in production of LitR protein. With higher cell density, the \( \text{litR} \) mRNA is not sequestered and is translated into LitR protein. The production of LitR protein permits the function of LitR as a transcription factor, globally regulating cellular processes in *V. fischeri* through binding to promoters and either activating or inhibiting transcription of certain genes. Certain cell processes are regulated within the bacterium by the transcription factor LitR, such as the activation of bioluminescence production in *V. fischeri* and the inhibition of flagellar motility in *V. fischeri*. With the decrease in LitR protein resulting in less activity, bioluminescence production is not activated while the bacterial cells are in a low cell density planktonic, motile state.

Pleiotropic Regulator LitR Activates Luminescence and Inhibits Motility

LitR is a transcription factor that controls luminescence and other phenotypes. The LitR protein has homologs in other *Vibrio* species such as HapR from *V. cholerae* and LuxR from *V. harveyi*, and belongs to the TetR family of regulators (Fidopiastis et al. 2002). LitR, once activated, leads to an induction in bacterial luminescence (figure 4) and if the protein is absent or repressed, there is a delay in luminescence. LitR activates expression of \( \text{luxR} \), leading to an increase in the production of the LuxR-LuxI quorum sensing system and thus increased bioluminescence, along with controlling genes for other processes within the cell. In addition to controlling luminescence and metabolism, the LitR transcription factor also modulates the transcription of genes responsible for the flagellar-based motility of *V. fischeri* (Lupp & Ruby 2005). The motility genes that are
modulated by the AinS-R-Qrr1-LitR pathway are those encode proteins that assemble the flagellar apparatus. In the flagellar hierarchy, the early genes encode the regulatory proteins that control the motility regulon, the middle genes encode the structural proteins such as the type three secretion system and the hook and basal body proteins, while the late genes encode the chemotaxis, flagellar filament, and motor (force generator) proteins (Lupp & Ruby 2005). High levels of AinS-AinR (phosphatase) activity leads to inhibition of these genes, as seen by a microarray, and decreased motility is observed with activation of the transcription factor LitR.

**Hypothesis and Summary of Results**

My thesis project tackled two c-di-GMP-related problems. First, I sought to probe the function of LapD by identifying the source of c-di-GMP that promotes biofilm formation and prevents biofilm dispersal, and is degraded by PdeV. I used genetic approaches, including mutagenesis and complementation, paired with biofilm assays to investigate the relationship between the different diguanylate cyclases and phosphodiesterases within the operon encoding VF_A0343, with respect to PdeV activity. My work revealed that the VF_A0342-4 operon does not appear to be responsible for producing the c-di-GMP that is degraded by PdeV to control LapD function.

Second, I investigated the relationship between the pleiotropic regulator LitR and c-di-GMP. I hypothesized that LitR protein overproduction activates the expression of diguanylate cyclases that in turn increase the levels of c-di-GMP, causing motility to be inhibited. My investigation provides new insights into the complicated control over motility in *V. fischeri*. 
Chapter 2
MATERIALS AND METHODS

Strains and Media

All V. fischeri strains utilized in this investigation are listed in Table 1. V. fischeri strains were constructed by introducing DNA via transformations or conjugations as described below. Escherichia coli strain π3813 (Le Roux 2007) carrying plasmid pEVS104 (Stabb & Ruby 2002) was utilized as a helper strain for triparental conjugations. Plasmid pKV496 (Visick et al. 2018) encoding the FLP recombinase was used to remove antibiotic cassettes by resolving regions flanked by FRT sites. The plasmids pPMF5 (Cohen-Eichinger et al. 2021) and pVSV105 (Dunn et al. 2006) were introduced into the recipient V. fischeri strains to overproduce LitR protein and serve as the vector control, respectively. Other V. fischeri strains were generated through transformation (Pollack-Berti et al. 2010) using DNA products generated by PCR Splicing by Overlap Extension (SOE) (Visick et al. 2018) or using genomic DNA from already constructed strains. All V. fischeri strains were grown in LBS, which contains 1% tryptone, 0.5% yeast extract, 342 mM NaCl and 50 mM Tris pH 7.5 (Dunlap & Greenberg, 1988). Antibiotics were added to the LBS medium at the following final concentrations: Chloramphenicol (Cm) at 2.5 μg/ml, Erythromycin (Erm) at 5 μg/ml, and Spectinomycin (Spec) at 25 μg/ml. E. coli strains were grown in Lysogeny-broth (LB) (Bertani, 1951). Antibiotics were added to the LB medium at the following final
concentrations: Kanamycin (Kan) at 50 µg/ml and Chloramphenicol (Cm) at 12.5 µg/ml. Thymidine was added for tri-parental conjugations at a final concentration of 0.3 mM. Media utilized for phenotypic tests include Tris-buffered Tryptone Broth Salt (tTBS) (LBS media without the yeast extract); Motility plates (have lower levels of agar, about 17% of the agar in LBS agar medium: and lack yeast extract) (O'Shea et al. 2006); Sea Water Tryptone (SWT), which is composed of artificial seawater, yeast extract, tryptone, and salt (Boettcher & Ruby 1990); and SWTO, which is SWT with increased sodium chloride concentration (an additional 20 g per liter). In addition, Tris Minimal Medium (TMM) was used for transformations (Cohen-Eichinger 2021); it contains 100 mM Tris PH 7.5, 300 mM NaCl, 50 mM MgSO₄, 0.33 mM K₂HPO₄, 10 µM Ferrous Ammonium Sulfate, 0.1% Ammonium Chloride, 10 mM N-acetylglucosamine, 10 mM KCl, and 10 mM CaCl₂.

**Transformations**

Bacterial transformation was utilized to modify *V. fischeri* strains with constructed PCR DNA or genomic DNA. The recipient strain carrying a *tfoX*-overexpressing plasmid was grown in 5 mL of Tris Minimal Media (TMM) supplemented with Cm at 2.5 µg/ml overnight at 28°C for 16 hours at 225 rpm. The grown culture was sub-cultured 1:50 into a 125 mL flask with 20 mL TMM and Cm at 2.5 µg/ml and grown at 24°C at 225 rpm. The cultures were grown until they reached a suitable optical density (OD), an optical density at 600 nm (OD₆₀₀) of at least 0.3 for genomic DNA and an OD₆₀₀ of at least 0.5 for PCR DNA. Once reaching a suitable OD, 500 µL of cells were incubated with the DNA at varied volumes, 8-10 µL for genomic DNA or 18-22 µL for PCR DNA, at room temperature for 30 minutes. After the co-incubation of the DNA and cells, the cultures
were diluted with 500 µL LBS and grown for 90 minutes at 28°C at 225 rpm, then
inoculated onto plates with antibiotics to select for the cells that took up (and
recombined in) the introduced DNA. The cultures were plated either 90 minutes after
recovery or after overnight incubation and then left to grow at 28°C overnight for
colonies to form. The colonies were subsequently re-streaked for purity.

Conjugations

Tri-parental conjugations were utilized to introduce plasmid DNA into *V. fischeri*. The
three strains were the *V. fischeri* recipient (for example, wild-type strain ES114), the
donor *E. coli* carrying the plasmid of interest (such as pKV496, pVSV105, or pPMF5),
and *E. coli* carrying the helper plasmid pEVS104 (Stabb & Ruby, 2002). *E. coli* was
grown in 5 mL LB and antibiotics were added to the medium to maintain the plasmid
stability. p3813, which carries the helper plasmid pEVS104, is a thymidine auxotroph
and thus was grown in medium supplemented with 0.3 mM thymidine. All of these
strains were grown shaking at 225 rpm at 37°C overnight and, after about 16 hours, the
strains were sub-cultured 1:100 with the same conditions of growth and incubated for 3
hours. The recipient *V. fischeri* strains were incubated with 5 mL LBS overnight at 28°C
shaking at 225 rpm, then sub-cultured 1:100 with the same conditions and grown for 3
hours. After incubation, the donor *E. coli* strain (250 µl), the helper *E. coli* strain (250 µl)
and the recipient *V. fischeri* strain (1 ml) were combined together in a 1.5 mL Eppendorf
tube. A negative control (*V. fischeri* recipient alone) (1.5 mL) was also included. All of
the samples were then centrifuged at 13,000 rpm for 1.5 minutes. The supernatant was
discarded and the pellet was resuspended in the remaining liquid (about 10 µL). 10 µL
of the cellular suspension was spotted onto a LBS plate, which was incubated at 28°C
for 3 hours. Then, the triparental conjugation and negative control spots were streaked onto a plate with the appropriate antibiotic to select for the plasmid of interest. The plates were incubated at 28°C overnight. If no growth was observed for the negative control, the triparental conjugation streak was streaked for purity on a plate with the same antibiotic.

**PCR Reactions**

Primers utilized in this study are shown in Table 3. Promoter-lacZ fusion constructs, site-directed point mutants within the Promoter-lacZ fusions, and general gene deletions were generated using PCR SOEing reactions (Visick et al. 2018). 5 µL of 10X KOD polymerase buffer, 3 µL of 25 mM MgCl₂, 5 µL of 2 mM dNTPs, 1 µL each of 20 µM forward and reverse primers, 1 µL of DNA template, and 0.4 µL of the KOD HiFi polymerase were added to 33.6 µL of dH₂O. The reaction was processed using a T100 thermal cycler using the following PCR parameters: a denaturing step of 98°C for 15 seconds, an annealing step at 55°C for 5 seconds, and an extension step at 72°C for 40 seconds, with the cycles repeating thirty times. Each product was separated from primers and buffer using the Zymogen Clean and Concentrate kit (Irvine, Ca.). The resulting fragments were joined together through a PCR without primers; because of the overlapping regions, the strand of the overlap acted as a 3’ end used by the polymerase for the overextension. 5 µl of 10X KOD polymerase buffer, 3 µl of 25 mM MgCl₂, 5 µl of 2 mM dNTPs, about 100 ng of each of the fragments from the first PCR reaction, along with 0.4 µL of KOD HiFi polymerase, were combined with a variable amount of dH₂O to reach 50 µl as the final volume. The T100 thermal cycler was programed with the same protocol as above but with twenty cycles instead of thirty. The resulting “mega fragment”
was amplified using outside primers of the set used to make the original pieces for the gene deletions, or amplified with primers 2185 and 2876 for the Promoter-lacZ fusion constructs. This PCR was carried out similar to the first PCR with the same ingredients and parameters in the T100 thermal cycler. The final product was stored at 4°C if needed or utilized immediately for transformation.

**Bioinformatics**

Sequence alignments and analysis were performed using NCBI blast (Altschul et al. 1990). Predicting potential promoters for isolation was through the utilization of Bprom on softberry.com (Solovyev & Salamov 2011), and highlighting potential binding site and designing primers was done using SnapGene (Insightful Software).

**Shaking Biofilm Assay**

Shaking biofilm assays were utilized to measure the ability of genetically-modified *V. fischeri* strains to form a robust biofilm. Strains were inoculated from single colonies into a 5 mL culture of LBS and grown overnight with shaking at 28°C at 225 rpm. The cultures were sub-cultured into smaller “blue-capped” tubes (13 x 100 mm) containing 2 mL of LBS supplemented with 10 mM calcium chloride. The OD$_{600}$ of the cultures were measured and normalized to sub-culture into the blue capped tubes at a starting OD$_{600}$ of 0.2. The tubes were shaken for 24 hours at 24°C at 225 rpm. The tubes were gently removed from shaking, and the phenotype was documented with photography and measurements of the cell density (OD$_{600}$), using a one to ten dilution.

**Wrinkled Colony Assay**

The wrinkled colony assay measures biofilm phenotypes on agar medium, with colony architecture and cohesiveness of the colony (evaluated by disruption with a
toothpick) used to estimate the formation of biofilm extracellular matrix. To perform this assay, genetically-modified *V. fischeri* strains were inoculated into 5 mL of either LBS or tTBS and grown overnight at 28°C with shaking at 225 rpm. After overnight growth, 50 μL of these cultures were then sub-cultured into 5 mL of fresh medium and grown at 28°C with shaking at 225 rpm for one to two hours. The sub-cultures were normalized to an OD<sub>600</sub> of 0.2 with the media used for the overnight and sub-culture. 10 μL of 0.2 normalized cell density culture were then spotted onto either LBS or tTBS plates. These plates were made with 25 mL of agar medium and were either not supplemented, supplemented with either 10 mM calcium chloride or 9.7 mM pABA (p-Aminobenzoic Acid), or supplemented with both (Dial and Visick, unpublished). The spots were allowed to dry, then the plates were inverted and incubated at 24°C for 24 hours, 48 hours, 72 hours and 96 hours. Images of the colonies were taken using the Zeiss 2000-C dissecting microscope and then the colonies were disrupted with a toothpick, and pictures were retaken.

**Motility Assay**

Motility assays are measurement of the bacteria’s ability to migrate through “soft agar” media using flagella-driven motility. This assay allowed for the identification of diguanylate cyclases that impact LitR-mediated inhibition of bacterial motility. Motility plates were made a day before the experiment. 5 mL of tTBS with Cm was inoculated with the bacteria and grown overnight at 28°C with shaking at 225 rpm. 50 μL of the grown culture was sub-cultured into 5 mL of fresh TBS Tris medium with Cm and grown at 28°C with shaking at 225 rpm for two to three hours. The cell density was measured by spectrophotometer and was normalized to an OD<sub>600</sub> of 0.2 with TBS. 10 μL of the
normalized culture was spotted onto the motility agar either in the center of the plate or equidistant from other spots. These plates were incubated at 28°C for 4-6 hours and pictures were taken. The diameter of the motility of the bacteria was also measured with a ruler. Plates were also supplemented with other additives such as 10 mM calcium chloride or 35 mM magnesium sulfate (O’Shea et al. 2005).

Miller Assay

Miller assays (Miller, 1972) are utilized to look at transcriptional expression of certain genes and permit elucidation of what type of regulation is occurring. Strains were streaked onto LBS agar plates with Cm (to select for maintenance of the litR plasmid or vector control) two days before the experiment, and the day before a colony was inoculated into 5 mL of LBS with 2.5 μg/mL Cm and grown overnight at 24°C shaking with 225 rpm. These cultures were started in duplicate or triplicate for consistent findings. After overnight growth, the strains were sub-cultured into 20 mL of LBS with 2.5 ug/mL of Cm in a 125 mL baffled flask and grown with at 24°C with shaking at 225 rpm. Samples were taken at four, ten, and/or twenty-two hour timepoints. At the indicated times, 1 mL samples were taken from each culture, and another 100 μL was used to take OD₆₀₀ measurements of the cultures cell density using a 1:10 dilution of the culture. The 1 mL samples were centrifuged at 13,000 rpm for 1.5-2 minutes, then the supernatants were decanted and discarded. The cell pellet was resuspended with 1 mL of Z buffer (6 mM disodium phosphate, 3 mM sodium phosphate dibasic dihydrate, 1 mM potassium chloride, 3.85 mM β-mercaptoethanol at PH 7.0), and then, to lyse the cells, 100 μL of chloroform was added and the mixture was vortexed. The lysate from the cultures was diluted with Z buffer, typically a 1:1 ratio (0.5 mL of the supernatant
and 0.5 mL of Z buffer), although it was adjusted as needed for the addition of more supernatant or more Z buffer. The solution for the β-galactosidase substrate, o-Nitrophenyl-β-galactoside (ONPG), was made by dissolving 0.004 grams of ONPG in 1 mL of Z buffer. The reaction was started when 0.2 mL of the ONPG solution was added to the diluted supernatant and the mixture was vortexed. Time was recorded at the start of the reaction. After the diluted supernatant and ONPG solution turned yellow, sodium carbonate was added to stop the reaction, followed by vortexing. The end time was recorded to determine the duration of the entire reaction. 200 µL of the solution was added to the 96 well plate and the absorbances at 420 nM and 550 nM were measured using the BioTek Synergy microplate reader.

**Luminescence Assay**

The luminescence assay measures the production of bioluminescence by *V. fischeri* and modified *V. fischeri* strains. The bacterial strains were inoculated into 5 mL of SWT and shaken overnight at 28°C at 225 rpm. The culture was sub-cultured into 30 mL of fresh SWTO with Cm. The sub-culture was normalized to an OD$_{600}$ of 0.05. After sub-culturing, the luminescence was measured with the GLOMAX 20/20 luminometer at a 6 second frequency and the OD$_{600}$ was measured using a spectrophotometer. These measurements were taken every hour until the luminescence started increasing exponentially, at which time measurements were taken every thirty minutes. These measurements continued until luminescence started to decrease or for a total of eight hours.
### Table 1. Strains Used in this Study

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<tr>
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<td>AR114</td>
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<td>AR116</td>
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<td>AR119</td>
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<td>AR121</td>
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Table 2. Promoter-lacZ Reporters

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<th>V. fischeri Strain</th>
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<tr>
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<td>-283 to +29</td>
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<td>AR76</td>
<td>-70 to +243</td>
<td>PVF_A0692-lacZ</td>
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<td>AR78</td>
<td>-158 to +19</td>
<td>PVF_0989-lacZ</td>
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<td>AR79</td>
<td>-150 to +72</td>
<td>PVF_A0381(-VF_1180)-lacZ</td>
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<td>AR80</td>
<td>-64 to +85</td>
<td>PVF_1200(-VF_2706)-lacZ</td>
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<td>AR101</td>
<td>-274 to +119</td>
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Note: These promoter regions were identified relative to the -10 site upstream of the translational start site.

Note: All promoters were located between yeiR and glmS in the chromosome.

Note: When in parenthesis the promoter region was located in a putative small gene proximal to the target gene.
### Table 3. Plasmids used in this Study

<table>
<thead>
<tr>
<th>Plasmid(s)</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>pEVS104</td>
<td>Plasmid used for conjugation purposes</td>
<td>Stabb and Ruby, 2002</td>
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<tr>
<td>pVSV105</td>
<td>Empty plasmid used as vector control</td>
<td>Dunn et al. 2006</td>
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<tr>
<td>pKV496</td>
<td>Plasmid containing enzyme flippase for FRT site recombination</td>
<td>Visick et al. 2018</td>
</tr>
<tr>
<td>pPMF5</td>
<td>pVSV105 containing <em>litR</em></td>
<td>Cohen-Eichinger et al. 2021</td>
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<tr>
<td>pJJC4</td>
<td>Competence plasmid</td>
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</tr>
<tr>
<td>pFY4535</td>
<td>c-di-GMP biosensor</td>
<td>(Zamorano-Sanchez et al., 2019)</td>
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### Table 4. Primers used in this Study

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Note: Lower-case letters represent 'tails' that are not complementary to the template sequence.
Chapter 3

EXPERIMENTAL RESULTS

*Vibrio fischeri*’s ability to symbiotically associate with the Hawaiian Bobtail squid is dependent on the bacteria reaching the deep crypt space of the light organ to colonize (reviewed in Stabb and Visick 2013). The pathway for *V. fischeri* from the marine environment into the crypts of the light organ begins with venting of the seawater into the mantle of the squid where the bacteria form a transient biofilm on the surface of the light organ (Nyholm et al. 2000). This is followed by dispersal from the biofilm and migration into the pore of the light organ (Mandel et al. 2012). Upon reaching the deep crypt spaces, colonization and symbiosis occur. Bacterial aggregation on the light organ surface, and subsequent dispersal from this biofilm towards the crypts, are essential steps during colonization initiation. Because little is known about the switch from the nonmotile sessile biofilm state to the planktonic motile state, dispersal is the focus of my investigation in this thesis (reviewed in Stabb and Visick 2013).

Recent research revealed a mechanism that dictates switching between biofilm and dispersal states through the Large Adhesive Protein (LAP) pathway, first and best characterized in *Pseudomonas* (Newell et al. 2009). In *V. fischeri*, the pathway appears to function similarly. The extracellular adhesion protein, LapV, is anchored on the cell surface and aids in forming biofilms. LapV can be cleaved by the periplasmic protease LapG, which switches the bacteria to dispersal. The regulatory protein LapD controls
LapG’s proteolytic activity by sequestering it, resulting in a biofilm state. The activity of LapD can also be modulated, through sufficient levels of the second messenger c-di-GMP (Newell et al. 2009). The protein that locally breaks down c-di-GMP influencing the LAP system was identified as the phosphodiesterase (PDE) PdeV (Christensen et al. 2020). Deletion of pdeV itself results in biofilm formation not observed for the wild type strain. While the phosphodiesterase that locally decreases c-di-GMP levels is identified, the diguanylate cyclase (DGC) that produces the c-di-GMP broken down by PdeV is unknown. Through phenotypic experiments, such as shaking biofilm assays and wrinkled colony assays, I intend to identify which DGC works in tandem with PdeV to modulate the levels of c-di-GMP and control the activity of LapD. Strains carrying deletions of DGC genes in combination with ΔpdeV can be screened to identify which mutations revert phenotypes back to dispersal.

Utilization of Phenotypic Assays to Elucidate the DGC relationship with PdeV

Another member in the lab evaluated biofilm formation by pdeV DGC double mutants. Of the 28 DGCs encoded in V. fischeri’s genome, only the DGC VF_A0343, when deleted in combination with ΔpdeV, led to a reversion from biofilm formation to dispersal. I replicated these results by constructing a double ΔVF_A0343 ΔpdeV mutant and assessing it with a shaking biofilm assay (figure 5). My result also suggested that the source of the c-di-GMP broken down by PdeV was solely from the DGC VF_A0343, and that the enzymes responsible for the modulation of the levels of c-di-GMP that control the regulatory protein LapD were identified. However, this conclusion could be complicated by VF_A0343’s location in the genome. It is in an operon with two other c-di-GMP modulating enzymes: VF_A0342 and VF_A0344. VF_A0342 is another DGC
while VF_A0344 is a PDE. When both VF_A0343 and VF_A0344 are deleted in combination with \( \Delta pdeV \), the resulting strain exhibits a biofilm phenotype indistinguishable from that of the \( \Delta pdeV \) single mutant (figure 5). A strain carrying a deletion of the entire operon along with \( \Delta pdeV \) also forms a biofilm. Finally, a strain lacking VF_A0342, VF_A0343 and PdeV exhibits a dispersal phenotype. These results complicate the originally proposed conclusion, requiring a new proposed mechanism for modulating the c-di-GMP levels that encompasses two PDEs, PdeV and VF_A0344, that may degrade the c-di-GMP produced by the DGC VF_A0343.

These complicated phenotypes were observed not just in liquid culture, but also on solid agar plates as well. In the agar assay, ten microliter spots of culture are spotted onto solid agar TBS plates, and are observed every 24 hours up until 72 hours, with disruption at 72 hours. The colony spots of the mutant strains will either produce a biofilm phenotype, consisting of wrinkled architecture and/or adhesiveness when disrupted, or a "planktonic" phenotype, fully smooth colonies that lack adhesion when disrupted. The phenotypes for the various strains on the solid TBS Ca agar at 72 hours were similar to the phenotypes found in shaking liquid culture (figure 6).
Figure 5. Shaking Biofilm Phenotypes of Mutants Carrying Deletions of the VF_A0342-0344 Operon in Conjunction with ΔpdeV Mutant strains carrying various deletions in the VF_A0342-344 operon in combination with ΔpdeV were grown in 2 mL of LBS supplemented with 10 mM calcium for 24 hours. Pictures were taken at 40 hours after inoculation. Strains used: KV8513, KV8969, KV9284, AR3, AR4, AR7, AR9, AR8.

All of the strains had a biofilm phenotype except ES114, the ΔVF_A0343 ΔpdeV mutant and the ΔVF_A0342 ΔVF_A0343 ΔpdeV mutant (figure 6). The lack of architecture and adhesiveness in these two mutant strains and wild type suggested that the mutants behave similarly in states of biofilm formation and dispersal independent of liquid or solid media.
Figure 6. Some Mutants with Operon Deletions in Combination with \( \Delta pdeV \) Exhibit the Biofilm Phenotypes. Mutant cultures were spotted onto TBS agar medium supplemented with 10 mM calcium. Pictures were taken with Zeiss 2000-C dissecting microscope 72 hours after spotting before and after disruption with a toothpick. Strains used: KV8513, KV8969, KV9284, AR3, AR4, AR7, AR9, AR8.

Removal of an Antibiotic Cassette Reverts Dispersal Phenotypes

Deletion of \( \Delta VF_A0343 \) in the \( \Delta pdeV \) mutant switched the phenotype of the single \( \Delta pdeV \) mutant from biofilm to dispersal. To determine whether the deletion of
ΔVF_A0343 was the reason for this reversion or if it was an artifact due to polar effects on the downstream PDE VF_A0344, the antibiotic cassettes utilized to generate the deletion were removed using Flp recombinase. I confirmed the loss of the antibiotic cassette by screening for erythromycin-sensitive colonies. I then re-evaluated the phenotypes of the modified mutants through shaking biofilm assays. Mutant strains ΔVF_A0343 ΔpdeV and ΔVF_A0342 ΔVF_A0343 ΔpdeV, which had previously produced turbid cultures, now formed biofilms (figure 7A). With the disappearance of the original phenotypes, I cannot conclude that the DGC VF_A0343 produces the c-di-GMP locally for the LAP pathway. The more probable reason for the phenotype switching from biofilm formation to dispersal when VF_A0343 was deleted from the ΔpdeV mutant is due to the promoter from the erythromycin cassette; this promoter likely promotes overexpression of downstream gene VF_A0344, which encodes a PDE. In this scenario, the ΔVF_A0343::Erm mutation would lead to increased PDE activity of VF_A0344, which could break down the c-di-GMP locally produced for the LAP pathway. To assess this hypothesis, a copy of VF_A0344 was inserted into a ΔpdeV mutant that contained a deletion of the entire ΔVF_A0342-4 operon (figure 7B). When tested with a shaking biofilm assay, the VF_A0344 complemented strain restored the dispersal phenotype (figure 7B). I conclude that the original striking results, which were inferred to be due to identification of the sole DGC modulating the concentration of c-di-GMP in conjunction with PdeV, were due to a polar effect. I further conclude that multiple (unknown) DGCs likely generate the c-di-GMP that is removed by PdeV.
Figure 7. Biofilm Formation and Dispersal are Impacted by Presence/Absence of an Erythromycin Resistance Cassette due to Altered Activity of VF_A0344. A. \(\Delta pdeV\) with VF_A0342-4 operon mutations containing or lacking an Erm\(^R\) cassette were grown in LBS + calcium and evaluated using the shaking biofilm assay and observed 24 hours later. B. The original conclusion for the observed phenotypes, that VF_A0343 synthesizes the c-di-GMP degraded by PdeV, was overturned by the phenotypes of the strains that lacked the Erm\(^R\) cassette. A new hypothesis, that the Erm\(^R\) promoter caused overexpression of the PDE VF_A0344, leading to increased PDE activity and degradation of c-di-GMP, was developed. The introduction of a copy of VF_A0344 into the \(\Delta pdeV\) strain lacking the VF_A0342-344 operon restored turbidity, as assessed in the shaking biofilm assay 24 hours after inoculation, supporting the second hypothesis. Strains used: KV8513, KV8969, AR10, AR11, AR12, AR3, AR4, AR7, AR13, AR26.
**Luminescence is Activated by the Transcription Factor LitR, While LitR Inhibits Motility**

A hallmark in the symbiotic relationship between *V. fischeri* and *E. scolopes* is the production of bioluminescence by the bacterium after colonizing the deep crypts of the host’s light organ. An important protein that the symbiotic relationship depends on is the regulatory protein LitR. LitR activation results in induction of bioluminescence. In the absence of LitR protein, a delay of luminescence is observed (Fidopiastis et al. 2002).

The transcription factor LitR is a global regulator that works to regulate transcription of genes by binding to the promoter regions and either repressing or activating genes (Fidopiastis et al. 2002). The availability of LitR is dependent on quorum sensing, with posttranscriptional repression of LitR occurring through sequestration of the *litR* mRNA by the sRNA Qrr1 and chaperone protein Hfq at low cell density (Miyashiro et al. 2010). During high cell density, Qrr1 is not produced, allowing for *litR* translation. Along with activating luminescence, many other processes are regulated by LitR, including acetate metabolism and motility (Lupp & Ruby 2005). The control by LitR of luminescence is well-known, but the mechanism for the inhibition of motility has not yet been elucidated. High levels of the second messenger c-di-GMP coordinate biofilm formation by the bacteria while low levels of c-di-GMP coordinate more motile planktonic states in bacteria (Chua et al. 2014). We propose that the transcription factor LitR could work through diguanylate cyclases to inhibit motility, perhaps by activating their transcription. Using motility assays, Miller assays, and a c-di-GMP biosensor, I attempted to determine if LitR works through DGCs to inhibit motility.
Overproduction of the LitR Protein Leads to an Arrest in Motility

To measure the impact of LitR on motility in *V. fischeri*, LitR was overproduced through plasmid-based overexpression in either wild type or *litR* mutant strains. Motility of these strains was compared with the same strains that contained an empty plasmid (vector control). The strains were spotted on semisolid agar and, after six hours, the diameter of migration was measured. For strains in which LitR protein was overproduced, motility was arrested with a diameter of 6 mm. In contrast, the vector control strains exhibited robust migration, with wild type at 21 mm and ΔlitR at 25 mm (figure 8). With this experiment, I conclude that increased production of LitR protein correlates to less motility while loss of LitR results in increased motility. These results are consistent with previous findings that LitR inhibits motility (Lupp & Ruby 2005).

Figure 8. Motility was Arrested When Upon LitR Overproduction, while it is Increased Upon Disruption of *litR* The comparison of motility by strains that either overexpress *litR* by plasmid, or produce normal levels of LitR production with the empty plasmid used as a control. The strains compared were the wild type strain and ΔlitR strains with either a vector control plasmid or a LitR overproduction plasmid. These strains were inoculated and spotted onto TBS media with Cm at 2.5 µg/mL. These strains that were spotted onto TBS Cm agar plates for six hours and the motility was observed by measuring the diameter of the spots and photographing the resulting migrating cells. Strains used: JC23, JC24, JC25, JC26.
Arrest in Motility by Overproduction of LitR can be Overcome by Deletion of Certain DGC Genes

We hypothesized that LitR-mediated inhibition of motility depends on activation by LitR of certain genes that inhibit motility (Lupp et al. 2005). Specifically, we proposed that LitR could activate the genes for diguanylate cyclases (DGCs), resulting in increased levels of c-di-GMP, which in turn inhibit motility. I assessed this possibility by generating and screening the motility of LitR-overproducing strains that carry DGC gene deletions. If LitR works through a particular DGC, deleting the corresponding gene would disrupt the inhibitory effect of LitR overexpression on motility. I spotted each strain onto semisolid motility agar and measured migration relative to the LitR-overproducing wild type strain at four hours. I found 6 DGC mutants with increased motility compared to wild type: ΔVF_1200, ΔVF_A0381, ΔVF_0989, ΔVF_A0959, ΔVF_A0692 and ΔVF_A0567 (figure 9).
Figure 9. The Deletion of Certain DGCs Disrupts the LitR-mediated Arrest of Motility

LitR-overproducing DGC mutant strains were spotted onto semisolid motility agar that is supplemented with 35 mM magnesium to promote motility and 2.5 ug/mL Cm to maintain plasmid stability. Migration was documented by photography after 4 hours. Strains used: JC25, JC26, AR38, AR39, AR53, AR54, AR55, AR56, AR69, AR70, AR65, AR66.

Because single DGC deletions increased motility of the LitR-overproducing strain only modestly, we hypothesized that a greater effect might be observed with strains that carried two or more DGC mutations. Thus, I generated multiply mutant stains that overproduced LitR and used the same approach as above to assess the impact of multiple mutations on motility. Unfortunately, the multiple DGC mutant strains \( \Delta VF_{1200} \Delta VF_{A0959} \Delta VF_{A0989}, \Delta VF_{1200} \Delta VF_{0989} \), and \( \Delta VF_{1200} \Delta VF_{A0989} \).
phenocopied the migration of ΔVF_1200 on motility plates (figure 10). Overall, these results suggest that the DGCs VF_1200, VF_A0381, VF_A0959, VF_0989, VF_A0567, and VF_A0692 do not function together but rather individually produce c-di-GMP that impacts motility in some as-yet unknown way.

Figure 10. Multiple DGC Deletions Don’t Have Synergistic Effects but Rather Phenocopies a Single DGC Deletion LitR-overproducing strains with mutations in multiple DGC genes were spotted onto semisolid motility agar supplemented with 35 mM magnesium to promote motility and 2.5 ug/mL Cm to maintain plasmid stability. The migration of these strains was observed after 4 hours on the motility agar and documented with photography. Strains used: JC25, JC26, AR83, AR81, AR84, AR82, AR85.

The Transcription Factor LitR may modulate Transcription of Diguanylate Cyclase Genes

The overproduction of LitR protein led to an arrest in motility, with this arrest being partially disrupted by the inactivation of genes for six diguanylate cyclases: VF_1200, VF_A0381, VF_A0959, VF_0989, VF_A0567 and VF_A0692. These results provide some support for our hypothesis that LitR could inhibit motility by activating diguanylate cyclases. To determine if LitR exerts control over these diguanylate cyclase genes, I made promoter-lacZ reporter constructs to measure transcription of the DGC genes using a Miller assay. LitR is a known transcription factor, and thus modulates
transcriptional expression through binding in the promoter region of the genes it controls. Thus, to determine if LitR can control these DGCs, and to see if this control is direct or indirect, I evaluated reporter activities of the six DGC constructs under different LitR conditions. Specifically, these reporter strains were modified to produce increased LitR production via plasmid based overexpression, wild type levels of LitR production with an empty plasmid as a vector control or with plasmid-less wild type, or no LitR protein (the litR gene was deleted).

The reporter activities of the six DGC genes were first assessed with plasmid-based overexpression of LitR. I expected that, if LitR overproduction inhibits motility, then LitR must activate the DGCs, resulting in increased transcription. This hypothesis appeared to be correct for the diguanylate cyclase VF_A0381 as the pVF_A0381 exhibited increased β-galactosidase activity upon LitR overproduction compared to the vector control (figure 11B). This result suggested that LitR activates transcription of VF_A0381, increasing DGC activity to produce more c-di-GMP and inhibit motility. While the hypothesis was accurate for VF_A0381, the opposite was observed for the DGCs VF_A0959 and VF_1200. The transcription was slightly decreased for pVF_A0959 when LitR protein was overproduced compared to vector control (figure 11C) and expression was significantly decreased for pVF_1200 with LitR overproduction compared to the vector control (figure 11A). These data suggest that LitR may inhibit the activity of the DGCs VF_1200 and VF_A0959. Promoters for the other three DGCs, VF_0989, VF_A0567, and VF_A0692, exhibited no significant change in expression between LitR overproduction and normal production of LitR protein.
Using as a guide the measurements revealing that overproduction of LitR either activated or inhibited specific DGCs, I subsequently measured transcription of the three DGCs that had exhibited a difference, this time comparing levels in a \textit{litR} deletion strain versus the Wild Type. I reasoned that, if increased LitR production led to an inhibition of transcription of \textit{pVF\_1200} and \textit{pVF\_A0959} and activation of \textit{pVF\_A0381}, then the lack of LitR production would cause the opposite effect. However, the deletion of \textit{litR} caused a decrease in transcription for \textit{VF\_1200} and \textit{VF\_A0959} (figure 12A, B), while not impacting transcription of \textit{VF\_A0381} (figure 12C). Potentially, a certain minimal threshold of LitR protein may be needed to activate but an increased amount of LitR protein leads to a repression of transcription.

Overall, the biggest effects occurred when LitR was overproduced, resulting in inhibition of \textit{VF\_1200} and activation of \textit{VF\_A0381}. However, whether the regulation was direct or indirect was unknown. To begin to determine the type of regulation LitR has on these promoters, I decided to mutate putative LitR binding sites near the \textit{VF\_1200} and \textit{VF\_A0381} promoters (figure 13).
Figure 11. Overproduction of the Transcription Factor LitR Activates VF_A0381 While Inhibiting VF_1200 and VF_A0959 
The transcription from the promoters of three DGCs under LitR overproduction conditions were measured using Miller assays. These samples were collected in duplicate following growth in LBS. A. pVF_1200 transcription was measured at multiple time points between 4 hours and 22 hours. The left bar graph directly compares LitR overexpression and vector control at the indicated time points while the right graph shows the levels over time. B. pVF_A0381 transcription was measured following growth of the reporter strain at 4 hours in LBS C. pVF_A0959 transcription was measured following growth of the reporter strain for 4 hours in LBS. Strains used: AR94, AR87, AR100, AR93, AR112, AR113.
Figure 12. Loss of LitR Diminished Transcription of VF_1200 and VF_A0959 but Caused no Significant Change in VF_A0381 Activity

The impact of deletion of litR on transcription of VF_1200, VF_A0959, and VF_A0381 was measured via Miller assay following growth of reporter strains in duplicate in LBS and SWTO for 4 hours and 22 hours. A. VF_1200  B. VF_A0381  C. VF_A0959. Strains used: AR80, AR104, AR79, AR102, AR101, AR118.
Utilizing SnapGene (Insightful Software) to view promoter regions, BPROM (Solovyev & Salamov 2011) to predict the promoters, and a putative LitR binding site reported by the Miyashiro lab in a seminar, I identified possible LitR binding sites in the promoter regions forVF_1200 (figure 13A) andVF_A0381 (figure 13B). I then made mutations in the context of the promoter-lacZ reporter fusions. Specifically, I mutated two nucleotides in a putative LitR binding site forVF_1200 since it was positioned between the -35 and -10 hexamer sequences of the promoter (figure 13A) and, forVF_A0381, I deleted the LitR binding site since it was positioned upstream and separated from the promoter (figure 13B). With the proposed LitR binding site inactivated or removed, LitR should lose ability to directly regulate the identified DGCs. I introduced thelitRoverexpression plasmid (or vector control) into the reporter strains carrying the mutated promoter regions and evaluated transcription through Miller assays. ForVF_1200, the mutated bases resulted in increased transcription overall, regardless of whether LitR was overproduced or not (figure 14A). Thus, the original bases within theVF_1200promoter appear to be generally inhibitory for transcription. ForVF_A0381, deletion of the putative LitR binding site led to no significant change when LitR is overexpressed. Surprisingly, however, this deletion caused, in the vector control strain, increased transcription to near the level observed with LitR overexpression (figure 14B). These results suggest both that the deleted sequences are inhibitory and LitR overproduction suppresses or by-passes this inhibition. Overall, while this work suggests that the transcription factor LitR influences transcription of DGC genesVF_1200, VF_A0381 andVF_A0959, its impact on regulatory control is not straightforward and not fully consistent with its impact on motility; we had expected that
LitR would activate these DGCs. While LitR overproduction increased expression of VF_A0381, it inhibited the other DGC genes. Thus, we conclude that inhibition of motility by LitR overproduction cannot be attributed to its regulation of c-di-GMP production.

Figure 13. The Proposed LitR Binding Sites were Identified and Either Mutated or Removed

The proposed LitR binding sites were predicted through identification of the conserved sequences (unpublished data). To ensure that transcription wasn’t lost from mutating the promoter region, BProm was utilized (Solovyev & Salamov 2011) to located the -10 and -35 regions of the promoter as indicated on the SnapGene figures (Insightful Software). The LitR binding sites were then either removed for VF_A0381 (removed -62 to -83), or changed nucleotides through site-directed mutagenesis (TT(-21,-22) → GG).
Figure 14. Transcription of VF_A0381 and VF_1200 was increased upon removal of the putative LitR binding site. Putative LitR binding sites were mutated (VF_1200) or removed (VF_A0381) in the context of the respective promoter-lacZ reporters. Miller assays were used to measure the resulting transcription of strains carrying these altered reporters and either the litR overexpression plasmid or the vector control. The strains were grown in triplicate for 4 hours in LBS. A. VF_1200 with and without the putative LitR binding site. B. VF_A0381 with and without the LitR binding site. Strains used: AR119, AR120, AR121, AR122.

**Global Levels of C-di-GMP are Inversely Correlated to Protein Levels of LitR**

It was hypothesized that global c-di-GMP levels would increase in conjunction with increased production of LitR protein, due to the fact that LitR overproduction arrests motility which coincides with increased c-di-GMP levels. Additionally, deletion of certain DGC genes increased motility when LitR is overproduced. However, the transcription of those DGC genes was more complex than originally hypothesized. Thus, an mCherry c-di-GMP riboswitch biosensor was utilized to measure global c-di-GMP levels in litR deletion and overexpression strains. Strains carrying the biosensor were spotted onto
solid agar, and c-di-GMP levels estimated from the resulting red/pink color of the spots. LitR overproduction decreased global c-di-GMP levels as there was less red fluorescence by the litR overexpression strain compared to vector control on both TBS and LBS agar. With the addition of calcium, both LitR overexpression and vector control strains exhibited increased c-di-GMP levels. In contrast, the addition of magnesium led to decreased levels of c-di-GMP for both strains (figure 15). These data indicate that the impact of these cations was independent of LitR.

Consistent with the results of LitR overproduction, deletion of litR resulted in increased red fluorescence compared to wild type, suggesting that LitR inhibits global c-di-GMP levels (Figure 15). Also consistent with the overproduction results, litR was not needed for the impact of calcium and magnesium addition, which led to increased and decreased global levels of c-di-GMP, respectively (figure 15).
Figure 15. LitR Protein Levels are Inversely Related with Global C-di-GMP levels

C-di-GMP levels were estimated using an c-di-GMP mCherry riboswitch biosensor that was introduced into wild-type, litR mutant, litR overexpression, and vector control strains. These strains were spotted on TBS or LBS agar plates containing 2.5 μg/mL Cm with different additives, including 10 mM calcium chloride and 35 mM magnesium sulfate. Strains used: AR57, AR58, KV8762, KV9549.

Luminescence was increased upon overproduction of a DGC.

LitR is a global regulator that coordinates the cell’s physiological responses, and is a link between the outside environment and the cell response. A well-known response that is regulated through the LitR protein is the production of bioluminescence, as LitR can bind to the luxR promoter region and activate transcription to increase luminescence (Fidiopastis et al. 2002). We proposed that LitR may increase bioluminescence production not only by activating luxR transcription but also by increasing the concentration of a second messenger that also coordinate cell
processes, c-di-GMP. To elucidate the relationship between c-di-GMP and luminescence, we overproduced a non-native DGC and PDE in *V. fischeri* and measured luminescence. The DGC overproduction strain had an increase in luminescence relative to the vector control and to a strain that carried the DGC variant that lacked an intact active site (figure 16). However, increased PDE activity resulting from PDE overproduction yielded no significant difference in luminescence production. Overall, these data suggest that increased c-di-GMP production can lead to an increase in luminescence. We speculate that, if LitR can activate specific DGCs, then those enzymes might contribute to an increase in the production of bioluminescence.

![Graph showing luminescence/OD over optical density for PDE and DGC Q/E](image)

**Figure 16. Increased Expression of a Non-native DGC Led to an Increase in Bioluminescence Production** Bioluminescence and growth were measured over time from strains that overproduced a non-native DGC, its catalytically-inactive variant, a non-native PDE, or the vector control. The data are shown as luminescence (RLU) divided by optical density (OD$_{600}$) plotted against optical density (OD$_{600}$). Strains used: KV8154, KV8155, KV8156, KV8157.

Overall, my investigation into LitR’s relationship with motility determined that LitR overproduction arrested motility of *V. fischeri*. This arrest in motility could be partially disrupted when certain DGC genes were deleted, suggesting that the identified DGCs, VF_1200, VF_A0381, VF_A0959, VF_0989, VF_A0567 and VF_A0692, contribute to
motility inhibition. We then hypothesized that LitR overproduction activates transcription of those identified genes. However, upon LitR overproduction, transcription was inhibited for a majority of those DGC genes. We conclude that the relationship between LitR, those DGCs, and motility may be complex. However, consistent with the general inhibition of DGC genes mediated by LitR overproduction, global levels of c-di-GMP were lower when higher levels of LitR protein were produced and the opposite occurred when litR was deleted. Certain additives such as Mg lowered global c-di-GMP levels while Ca increased global c-di-GMP levels, but these effects were independent of LitR. Finally, LitR-controlled luminescence was increased with an increase in c-di-GMP levels. Whether LitR controls transcription of a DGC gene(s) that contributes to the increase in light production remains to be determined. Overall, this work provides insight into the second messenger c-di-GMP’s complex contribution to cellular responses within the bacteria, whether it controls the switch from biofilm formation to dispersal in its collaboration with the transcription factor LitR in inhibition of motility.
Chapter 4

Discussion

The pathway for *V. fischeri* to colonize its host depends on a switch from biofilm formation to dispersal and the bacteria’s subsequent migration into the symbiotic light organ. To date, the only known influencer of this switch in *V. fischeri* is the large adhesive protein (LAP) system. The Lap-mediated switch depends on proteolytic cleavage of LapV by LapG (Christensen et al. 2020) and sequestration of LapG by LapD (Newell et al. 2009). The LapD regulatory protein remains active to sequester when sensing sufficient levels of the second messenger c-di-GMP, but releases LapG to promote dispersal when levels are low (Newell et al. 2009). Work completed in this investigation probed the identification of diguanylate cyclases (DGCs) that produce the c-di-GMP sensed by the regulatory protein LapD.

A second possible mediator of the biofilm-dispersal switch is the transcription factor LitR. This protein influences the regulation of machinery that coordinates the cellular responses of motility and luminescence (Lupp & Ruby 2005; Fidopiastis et al. 2002). LitR inhibits motility by regulating genes that assemble flagellar biosynthesis. Here, I proposed LitR also could alter the levels of c-di-GMP to inhibit motility. My work has shown that, while its precise transcriptional control over DGCs remains unclear, LitR does indeed exert influence over the production of c-di-GMP.
Investigations of the Switch Between Biofilm Formation and Dispersal

Through a combination of shaking biofilm assays and wrinkled colony assays, I disproved the hypothesis that the DGC VF_A0343 produced the c-di-GMP that was locally degraded by PdeV. Instead, my work revealed that the gene replacement mutation ($\Delta$VF_A0343::Erm) caused polar effects arising from overexpression of VF_A0344 by the upstream erythromycin cassette promoter. This was true both in liquid as well as on solid media. These findings, in combination with previous work generating and testing $\Delta$pdeV $\Delta$DGC double mutants (Christensen and Visick unpublished), suggested that multiple DGCs, rather than a single one as was originally proposed, produce the c-di-GMP needed to activate LapD (figure 17).

For further determination on the similarities between the LAP system in P. fluorescens and the LAP system in V. fischeri, I would generate mutations inactivating different enzymatic regions of the system to confirm that the system works as we propose. The regulatory protein LapD has a degenerate EAL domain, which contains the enzymatically inactive KVF amino acids in place of the EAL motif and is used to sense c-di-GMP rather than degrade it (Newell et al. 2009). It also contains a degenerate GGDEF domain, with the enzymatically inactive RGGEF amino acids in place of the GGDEF motif; in Pseudomonas, this domain is used to switch the protein from inactive to active (Navarro et al. 2011). One mutation that could be made would be to inactivate the c-di-GMP sensing domain of LapD by generating a K575A substitution in the LapD c-di-GMP sensing site KVF, the degenerate EAL motif (Newell et al. 2009). Another interesting mutation would be one that prevents LapD from changing into the
active state; this could be achieved by generating an R248A substitution in the
degenerate GGDEF domain RGGEF, as it was done in the LapD homolog in *P.
fluorescens* (Navarro et al. 2011). These mutations, preventing c-di-GMP binding or the
switch to an active state, would both prevent LapD from sequestering LapG and thus
would keep the bacteria in a dispersed state. Other mutations would be to inactivate the
EAL domain of PdeV, thus disrupting phosphodiesterase activity and promoting biofilm
formation. Finally, it would be important to verify that LapD binds to LapG when c-di-
GMP levels are increased using an assay such as a pull-down.

To identify which DGCs together produce the c-di-GMP degraded by PdeV, I would
measure partial phenotypes at earlier time points in shaking biofilm of a ΔpdeV mutant,
looking for small increases in OD$_{600}$ reflecting reduced biofilm formation. I would also
perform similar experiments on plates, looking for differences in colony architecture.
Then, based on those partial phenotypes, I would generate strains that carried multiple
deletions of DGCs to determine if I could achieve a switch from biofilm formation to
dispersal. Depending on the DGCs identified, we could inactivate enzymatic GGDEF
domains or sensory domains such as a CSS domain, for example.

Beyond evaluating biofilm formation or dispersal with the indicated mutants, the
impact of mutations in the pathway on transcription of the lapV gene or the
对应ing protein production could be measured. This would allow us to expand
our understanding of the pathway by probing the impact of various mutations on the
most downstream component of the pathway. Overall, the switch between biofilm
formation and dispersal is dependent on the phosphodiesterase PdeV along with
multiple, as-yet unknown, DGCs.
**Figure 17. Two or More DGCs Produce the C-di-GMP that is Degraded Locally by PdeV to Modulate LapD Activity**

To engage the switch from dispersal to biofilm formation, sufficient c-di-GMP is needed to activate the regulatory protein LapD. While the PDE PdeV locally degrades c-di-GMP and switches biofilm formation to dispersal, two or more DGCs are needed to produce the c-di-GMP needed for biofilm formation from dispersal.

**LitR Inhibits Motility and Overproduction Leads to an Arrest of Motility**

Microarray data revealed that LitR can directly or indirectly regulate genes known to assemble flagellar machinery (Lupp & Ruby 2005), consistent with its ability to inhibit motility. What was not known and what we uncovered through this investigation is the second messenger c-di-GMP’s ability to contribute to this LitR-mediated inhibition of motility. We had good reason to hypothesize that c-di-GMP could play a role due to its ability to coordinate cell states such as the switch to biofilm formation from planktonic cells (Chua et al. 2014) as well as previous work identifying MifA (VF_0989) and MifB (VF_A0959) as DGCs that influence *V. fischeri* motility (O’Shea et al. 2006). Through observing bacterial movement of a LitR-overproducing strain, we determined that, after 6 hours of growth, the bacterial spot appeared to be arrested in migration. However,
after 20 hours, I saw that the bacteria had migrated through the soft agar, although the amount of migration remained substantially reduced compared to wild type (unpublished data). I hypothesize that LitR, produced at a high level due to plasmid-based overexpression, directly regulates motility early on but at later stages the production of LitR is insufficient to fully inhibit the motility.

I further hypothesized that motility isn’t inhibited by direct LitR regulation but indirectly by controlling c-di-GMP to coordinate the nonmotile state. This c-di-GMP-mediated influence on motility could be through controlling a regulatory protein similar to the *V. cholerae* homolog FlrA; c-di-GMP binding to this regulatory protein inactivates its ability to bind DNA and thus prevents activation of flagellar biosynthesis genes (Srivastava et al. 2013). *V. fischeri* also uses a FlrA homolog to control flagella gene transcription (Millikan & Ruby 2003, 2004), but its ability to bind c-di-GMP has yet to be assessed. The concentration of c-di-GMP coordinates the ability of bacteria to be motile or nonmotile and thus the modulation of this second messenger by DGCs and PDEs would be important in the inhibition of motility. My work revealed that the deletion of certain DGC genes partially overcame the arrest of motility caused by LitR overproduction, reinforcing the idea that an increase in c-di-GMP levels will inhibit bacterial motility and suggesting that LitR also needs c-di-GMP to inhibit motility. These data could also justify that while LitR can independently regulate motility, c-di-GMP can also independently regulate motility through its concentration, as higher levels of c-di-GMP dictate the bacterial state to be sessile as opposed to planktonic (Chua et al. 2014). What is unknown is if LitR influences the c-di-GMP modulating enzymes and if that regulation is direct or indirect.
LitR Influences the Transcription of the Identified DGCs

My work identified a set of six DGCs that appeared to contribute to LitR-mediated inhibition of motility. Thus, I wondered if LitR regulates the transcription of these genes. I measured the transcription of the promoters upstream of these genes comparing variable levels of LitR. I found that, while LitR does indeed regulate the transcription of a subset of the identified genes, the regulation was more complex than expected. There was not a clear relationship between the DGC’s activity and increased levels of LitR protein. For the DGC VF_1200, wild-type levels of LitR were needed for activation of DGC transcription but increased levels of LitR protein led to inhibition of expression. Many transcription factors activate at low levels and inhibit at higher levels (reviewed in Browning & Bugsby 2004), and this could be the case for LitR-mediated control over VF_1200. The DGC VF_A0381 was also activated by LitR overproduction, but exhibited no difference in expression in the litR mutant relative to wild-type cells. We concluded that LitR overproduction is necessary to activate VF_A0381 transcription under the conditions used for these experiments.

Because the promoters for VF_1200 and VF_A0381 exhibited the greatest response to LitR overproduction, I sought to identify and disrupt possible LitR binding sites, to begin to determine if the effect of LitR could be direct. Indeed, I was able to identify sequences that were inhibitory to expression for both promoter regions. However, despite this identification, LitR still exerted an effect on transcription, making it difficult to make any conclusions about the nature of LitR’s influence over transcription of these genes. A possibility for the altered transcription of VF_A0381 could also be through polar effects of the erythromycin antibiotic cassette. Thus, the increase in expression in
pVF_A0381 when the LitR binding site is removed could be due to the promoter being moved closer to the erm cassette. By adding a terminator to the end of the erm cassette, we could remove these polar effects and retest the expression to determine if LitR is inhibitory. It is also possible that LitR acts both directly and indirectly. One way we could fully determine an interaction between LitR and the genes for these c-di-GMP producing enzymes would be to utilize an electrophoretic mobility shift assay (EMSA). This approach involves the incubation of DNA target molecules, tagged with a fluorescent marker such as fluorescein, with increasing concentrations of LitR protein, followed by electrophoresis through a polyacrylamide gel. If LitR can bind to these promoter regions, we would observe a shift in band size due to the increased size of the DNA molecules when complexed with the LitR protein.

An important caveat for the comparisons in transcription being made in this thesis is that all the Miller assay experiments were carried out during growth in liquid, while the phenotypes were observed during growth on semi-solid motility plates. Thus, due to the difference in media type, the samples could exhibit different levels of regulation by LitR. To determine if this difference in growth conditions actually leads to a difference in regulation, I conducted a Miller assay by collecting samples from motility plates. This was carried out with motility plates after six hours of growth. In this experiment, I found that LitR overproduction still inhibited transcription from promoters of the DGCs VF_1200 and VF_A0959 and still activated the promoter for VF_A0381 (unpublished data). With observation of this regulation by LitR, we can confidently conclude that the regulation by LitR is not dependent on growth on motility agar. Thus, overall, LitR appears to exert control over this certain subset of DGCs, but mostly negative
regulation, a direction that does not coincide with its inhibition of motility. A possible explanation for the identified DGCs having opposite regulation with different levels of LitR protein could be that LitR overproduction actually “resets” the system: low levels exert one effect, while higher levels, such as would be achieved with increased cell density due to quorum sensing, exert the opposite effect. This phenomenon is seen with luminescence, as it increases until a peak occurs and then diminishes. Whether this is due to LitR activity or another effect such as decreased oxygen at higher cell densities remains unknown. But, potentially, bacterial messengers like c-di-GMP could contribute to this decline. It would be of interest to determine if LitR overexpression can diminish light production by *V. fischeri*. Additional work will be necessary to fully understand if and how LitR influences motility (and luminescence) via its control over these (or other) DGC genes.

**Global Levels of C-di-GMP Decrease with Increasing LitR Protein Levels**

My observations indicated that LitR influences transcription of certain identified DGCs, but via an as-yet undefined mechanism. To understand this phenomenon further, I asked if the global levels of c-di-GMP in *V. fischeri* were regulated by LitR. Using a c-di-GMP riboswitch reporter plasmid, I found an inverse relationship, with increased LitR protein levels resulting in lower global levels of c-di-GMP. Thus, LitR appears to inhibit global c-di-GMP levels. While certain DGCs were activated by LitR, a significant number of other DGCs (or else the most active DGCs) must be inhibited by LitR. Alternatively, LitR could activate PDEs. This relationship was observed under different media conditions, including LBS and TBS. When supplements such as Ca$^{2+}$ and Mg$^{2+}$ were provided, global c-di-GMP levels were significantly altered. With Ca$^{2+}$
added into the medium, the c-di-GMP levels were increased significantly regardless of whether litR was deleted or overexpressed, indicating that the Ca^{2+}-inducing signal is epistatic to LitR regulation. With the addition of Mg^{2+}, global c-di-GMP levels were decreased significantly, also regardless of the levels of LitR. While Ca^{2+} is known to increase c-di-GMP production (Tischler and Visick, unpublished data), it was previously unknown that Mg^{2+} causes a decrease in global c-di-GMP. This effect, however, is consistent with the known ability of Mg^{2+} to promote motility of V. fischeri (O'Shea et al. 2006). To further elucidate how Mg^{2+} inhibits global c-di-GMP production, the activity of the c-di-GMP riboswitch biosensor in mutants defective for phosphodiesterases and diguanylate cyclases grown in the presence and absence of Mg^{2+} could be measured through flow cytometry. Overall, these data thus revealed (1) increased LitR protein decreases c-di-GMP levels, presumably by inhibiting c-di-GMP production or activating c-di-GMP degradation and (2) Mg^{2+} is a major inhibitor of c-di-GMP production in V. fischeri.

**An Increase of C-di-GMP Concentration Leads to an Autoinduction of Luminescence**

LitR is known to inhibit motility, but the most researched function of LitR is its ability to activate genes needed for luminescence production. To date, no connection between c-di-GMP and luminescence has been reported. Here, I found that increased c-di-GMP leads to an increase of bioluminescence production. To evaluate the impact of c-di-GMP on luminescence, I compared vector control, an overexpressed non-native DGC and an overexpressed non-native PDE. The strain with the overexpressed DGC had increased bioluminescence production compared to the rest of the strains. While an
increase in c-di-GMP concentration leads to increased luminescence, LitR activates luminescence but decreases c-di-GMP. As described above, an experiment measuring luminescence of a LitR-overproducing strain will be telling with respect c-di-GMP’s contribution to control bioluminescent production. While additional work is needed, my findings provide exciting new directions for the luminescence field.

**Figure 18. LitR controls DGCs to Either Inhibit Motility or Activate Luminescence**
The transcription factor LitR is produced when bacteria are at a high cell density and will be able to inhibit motility or activate luminescence. LitR inhibits transcription of VF_1200 and VF_A0959 while activating VF_A0381, but the regulation remains poorly understood. LitR decreases the global c-di-GMP levels, however an increase in c-di-GMP leads to an increase in luminescence. Overall, complex layers of regulation are in place between LitR and c-di-GMP producing enzymes and LitR has another function which at this time is unknown.

**Overall Conclusions**

The regulation LitR exerts on certain cell processes such as motility and luminescence can occur through direct control of motility and luminescence genes. However, other conditions within the cell can also influence these cellular processes. The mechanism of how LitR influences these systems appear to be complex, with direct (or near-direct) regulation of structural genes as well as potentially indirect regulation by controlling transcription of DGC or PDE genes; these effects would modulate levels of the second messenger c-di-GMP, which can in turn change protein activity. I propose
that LitR inhibits transcription of the genes for DGCs VF_A0959 and VF_1200 while activating that of VF_A0381, leading to motility inhibition (figure 18). However, LitR may also contribute indirectly to regulate these genes and thus motility (figure 18). I also propose that LitR decreases global c-di-GMP levels by inhibiting the Diguanylate Cyclases with layers of complex regulation, even though both LitR and increased c-di-GMP levels activate luminescence (figure 18). LitR connects the cell’s sensor pathway with the cellular response, and what I have discovered is that, along with LitR’s direct regulation of luminescence, it may also modulate luminescence and motility through the second messenger c-di-GMP, perhaps ultimately to downregulate these processes. Along with LitR’s ability to regulate genes to coordinate cell responses, LitR activity could also be modified by alternative pathways from posttranscriptional modification such as ligand binding within its putative sensory domain. Potential identification of this domain through alignment and BLAST analysis and mutation could lead to more insight into LitR with multiple phenotypic assays. Further investigation is necessary to elucidate the connection between LitR and the identified DGCs, as transcriptional regulation is more complex than expected. With more in-depth experiments, the indirect regulation LitR has on the DGCs can be elucidated along with a deeper understanding of the inverse connections between LitR, c-di-GMP levels, and luminescence.
REFERENCES


VITA

The author, Ali Razvi, was born in Orlando, Florida on December 31st, 1996 to Batool and Mehdi Razvi. He attended the Ohio State University in Columbus, Ohio where he earned a Bachelor of Science in Microbiology.

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