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Identifying Novel Factors Involved in Biofilm Formation by Vibrio Fischeri

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IDENTIFYING NOVEL FACTORS INVOLVED IN BIOFILM FORMATION BY

VIBRIO FISCHERI

A DISSERTATION SUBMITTED TO
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BY

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For my grandfather, William H. Ray, Ph.D.
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ABSTRACT

Biofilm formation by bacteria provides them with a means by which to survive harsh environmental conditions and/or promote colonization of a particular surface, either biotic or abiotic. Due to the resistance properties of biofilms, biofilm-forming bacteria are difficult to treat in the context of host infections. Thus, investigating the processes that control biofilm formation will promote the development of novel therapeutics. To study biofilm formation in the context of a host, I utilized as a model system the marine bacterium *Vibrio fischeri*, which must be competent to form a biofilm to efficiently colonize its squid host *Euprymna scolopes*. Biofilm formation by *V. fischeri* requires the *syp* polysaccharide locus, as well the sensor kinase (SK) RscS and the response regulators (RRs) SypG, required for *syp* transcription, and SypE, a negative regulator of biofilm formation. However, I predicted that other factors were also involved in biofilm formation. Thus, my dissertation work focused on identifying novel factors involved in this process.

I first performed random transposon mutagenesis on a biofilm-competent strain of *V. fischeri* and screened for colonies that exhibited defects in wrinkled colony formation, an established biofilm phenotype. This analysis permitted the identification of members of the Lux luminescence pathway as regulators of biofilm formation, specifically, the SK LuxQ and the histidine phosphotransferase LuxU. Overall, my data support a model in
which the Lux pathway bifurcates at LuxU to regulate biofilm formation at or above the level of the RR SypG.

Next, I shifted my focus to better understand SypG and its regulon. Specifically, I sought to determine whether this regulator was the direct transcriptional activator of the syp locus. I found that, indeed, SypG recognizes and appears to bind to a predicted enhancer sequence, the syp enhancer (SE), to promote transcription of the syp locus. Additionally, I identified three new putative members of the SypG regulon. I predicted that, since these genes (called bam and bal) appeared to be SypG-regulated, they would also be involved in biofilm formation. Through deletion of these genes and analysis of the corresponding mutant strains, I determined that the bam genes are necessary for biofilm maturation, while the bal genes appear to regulate bioluminescence.

Thus, the work from my dissertation identified novel factors involved in biofilm formation and filled in critical gaps in our understanding of how the syp locus is regulated. It has also provided new information regarding the control of cellular bioluminescence. Overall, my work provides further insight into how complex pathways intersect to promote a particular function.
CHAPTER ONE
LITERATURE REVIEW

I. INTRODUCTION

In recent years, we have learned that bacteria interact with their hosts in ways other than causing disease. For example, bacteria play an integral role in the development of our immune system and maintaining the health of our gastrointestinal tract [reviewed in (Kelly & Mulder, 2012)]. Such bacteria-host interactions require specific sets of events to occur (in both the bacteria and host), yet the regulation of many of these events remains poorly understood. One tightly regulated process by which bacteria interact with their hosts is the formation of a biofilm, which is a community of bacteria encased in a self-produced matrix. Bacteria within a biofilm are often more resistant to standard antibiotic and chemical treatments, making them difficult to treat. However, biofilm formation is not limited to pathogenic bacteria and plays a critical role in the beneficial association of some bacteria with their hosts. Therefore, it is necessary to understand how bacteria regulate biofilm formation to better understand how bacteria interact with their hosts.

To study biofilm formation, I utilized a model system comprised of the marine bioluminescence bacterium Vibrio fischeri and its symbiotic host, the Hawaiian bobtail squid Euprymna scolopes. During this symbiotic association, V. fischeri must be competent to form a biofilm to effectively colonize its squid host. My dissertation work
focused specifically on understanding how biofilm formation is controlled by *V. fischeri*. Thus, in this introductory Chapter of my dissertation, I provide a detailed review of the literature necessary to understand and appreciate the findings presented herein.

I will first discuss the process of biofilm formation, starting with a detailed analysis of the biofilm developmental process, followed by specific examples of biofilm formation and its regulation in model biofilm-forming organisms. Next, I will provide a brief outline of the *V. fischeri*-*E. scolopes* symbiosis, with a focus on the initial stages of colonization, which requires biofilm formation by *V. fischeri*. Ultimately, this section will led into one that provides a detailed review of the known factors necessary for the regulation and formation of the *V. fischeri* biofilm.

Lastly, I will describe the Lux pathway, which regulates the production of cellular bioluminescence in a cell-density dependent manner. This section provides background information necessary to appreciate my finding that specific regulators of this pathway are also involved in controlling biofilm formation. Taken together, this information provides a comprehensive overview of the current state of the literature relevant to the work presented in this dissertation.

**II. BIOFILMS**

**Introduction**

Bacteria can be found as planktonic (e.g., motile) cells or as sessile communities called biofilms, which are composed of bacterial cells encased in an extracellular matrix. This switch from a planktonic to a sessile lifestyle is trigger by a variety of environmental
factors, depending on the bacteria, and ultimately leads to the protection of the biofilm inhabitants from such things as predation (Matz et al., 2005), desiccation (Chang et al., 2007), host defenses (Donlan & Costerton, 2002), and antibiotic assaults (Mah, 2012). Biofilm-forming bacteria are often involved in human disease, such as otitis media, urinary tract infections from indwelling catheters, and infection of the cystic fibrotic lung [reviewed in (Donlan & Costerton, 2002)]. Because of the protection provided to the bacteria by the biofilm matrix, these infections are extremely difficult to treat using standard means, underscoring the need to understand how bacteria regulate and form biofilms so that new treatments and interventions can be generated. Thus, this section will cover the process of biofilm development, including the stages of biofilm formation and the idea of heterogeneous populations within a biofilm, and will then focus on biofilm formation by the model biofilm organisms Pseudomonas aeruginosa and Vibrio cholerae.

**Biofilm development**

The process of bacterial biofilm formation begins when bacteria sense some change in their environment. Karatan and Watnick (2009) review a variety of triggers that promote biofilm formation, which include, but are not limited to: 1) a decrease (or increase) in a particular nutrient source, 2) a change in osmolarity, temperature, pH, or the concentration of inorganic molecules, such as iron and phosphate, 3) host-derived signals, 4) mechanical signals, such as an approaching surface, 5) the presence of antimicrobials, 6) quorum sensing signals, or 7) a change in the concentration of second
Figure 1. Model for biofilm development. The biofilm lifestyle is often initiated via an environmental cue, which results in one or more cells attaching to a surface, first reversibly, then irreversibly (A). The cells divide and begin to produce an extracellular matrix (green). The biofilm then matures, often taking the form of mushroom-like structures with a stalk and cap (B). At this stage, the components of the matrix (e.g., eDNA, proteins, and polysaccharides) that are released from the cells of the biofilm promote cell-cell, cell-matrix, and cell-surface interactions and provide structural integrity to the biofilm (inset for B). Certain cells of the biofilm are eventually released in a process known as dispersal (C). These dispersed cells can then seed biofilm formation in new locations.
messenger molecules (e.g., c-di-GMP). Regardless of the specific trigger that initiates biofilm formation, it is followed by a well-established developmental process (Fig. 1) (Stoodley et al., 2002), which will be reviewed in this section. Biofilm formation starts with (A) reversible, and then irreversible, attachment to a surface (or between cells), followed by (B) maturation of the biofilm through production of an extracellular matrix, and finally (C) dispersal of cells from the biofilm to seed a new niche. Furthermore, during the process of biofilm formation, cells within the biofilm experience different conditions that can lead to (D) heterogeneity within the population.

(A) Initial and irreversible attachment

For a bacterium to make contact with a surface, it must first overcome the repulsive forces between itself and that surface. To overcome these repulsive forces, bacteria utilize a variety of mechanisms, from random (Brownian) motion to fluid flow or even directed motility [reviewed in (Dunne, 2002, Donlan, 2002, Palmer et al., 2007)]. For example, flagellar motility is necessary for initial biofilm attachment by Escherichia coli (Pratt & Kolter, 1998), P. aeruginosa (O'Toole & Kolter, 1998, Klausen et al., 2003a, Klausen et al., 2003b), V. cholerae (Watnick & Kolter, 1999), and Listeria monocytogenes (Lemon et al., 2007). Once a cell has contacted a surface, it then uses appendages such as pili and flagella to remain attached to that surface. Furthermore, since many of these appendages are also utilized for motility, they can be used to expand the surface area covered by the biofilm (Pratt & Kolter, 1998, Shrout et al., 2006). At this stage, despite these physical interactions between the cell and a surface, the cell retains the ability to leave the surface (Fig. 1A). After contacting a surface, and sometimes in
response to specific cues from the environment, bacteria then initiate a change in their transcriptional profile, leading to the production of more adhesins and components of the extracellular matrix that promote a more robust and ‘irreversible’ attachment to the surface (Fig. 1) (Stoodley et al., 2002).

(B) Maturation and the extracellular matrix

The next stage of biofilm formation is maturation (Fig. 1B), in which the cells continue to secrete an extracellular matrix, which will ultimately encase the cells and allow for the growth, structuring, redistribution, and protection of the cells by these matrix components. Biofilm maturation is often described as the stage in which cells form capped, mushroom-like towers and pillars, between which lie channels and pores that permit the flow of fluids containing oxygen, nutrients and other small molecules; these 3-dimensional (3D) structures are called multi-layer biofilms. However, biofilms can also be flat, single layer structures that cover considerable surface area; such biofilms are called monolayer biofilms (Karatan & Watnick, 2009). Monolayer biofilms are thought to form when cell-surface interactions are favored over cell-cell interactions (Karatan & Watnick, 2009). However, the form that a biofilm takes varies between different bacterial species and can even vary for the same organism depending on the environmental conditions. For example, *P. aeruginosa* forms multilayer biofilms when grown with glucose, but monolayer biofilms when grown with succinate due to an increase in the population of cells that exhibit swarming motility (Shrout et al., 2006).

One of the most important steps during biofilm maturation is the production and ordering of the extracellular matrix, which is mainly composed of polysaccharides,
proteins, and eDNA (Fig. 1B inset), though other molecules, such as surfactants (rhamnolipid for \textit{P. aeruginosa}; \textit{Bergstrom et al.}, 1946b, \textit{Bergstrom et al.}, 1946a)) and outer membrane vesicles (\textit{Schooling & Beveridge}, 2006, \textit{Shibata & Visick}, 2012), also comprise the biofilm matrix. These molecules make up the “House of Biofilm Cells” (\textit{Flemming et al.}, 2007) and are thought to account for up to 90\% of the dry mass of the biofilm (\textit{Flemming & Wingender}, 2010). Furthermore, the matrix helps protect the cells against UV irradiation (\textit{Elasri & Miller}, 1999), predation (\textit{Matz et al.}, 2005), pH gradients (\textit{Davey & O'Toole G}, 2000), desiccation (\textit{Chang et al.}, 2007), antimicrobials (\textit{Stewart & Costerton}, 2001), and host defenses (\textit{Donlan & Costerton}, 2002). Thus, the matrix gives substance and structure to the biofilm. The major components of the matrix (polysaccharides, eDNA, and proteins) will be covered in detail here; for reviews that describe the biofilm matrix, see (\textit{Sutherland}, 2001b, \textit{Branda et al.}, 2005, \textit{Karatan & Watnick}, 2009, \textit{Flemming & Wingender}, 2010).

\textit{(i) Polysaccharides}

Polysaccharides often make up a large part of the extracellular matrix, though the type of polysaccharide produced depends on the organism. It is even possible for one organism to produce more than one type of polysaccharide (not necessarily at the same time). For example, \textit{P. aeruginosa} and \textit{E. coli} can produce at least three different polysaccharides: Psl (\textit{Friedman & Kolter}, 2004b, \textit{Jackson et al.}, 2004), Pel (\textit{Friedman & Kolter}, 2004b, \textit{Friedman & Kolter}, 2004a), and alginate (\textit{Carlson & Matthews}, 1966) for \textit{P. aeruginosa}, and PNAG (\textit{Wang et al.}, 2004), cellulose (\textit{Zogaj et al.}, 2001), and colonic acid (\textit{Prigent-Combaret et al.}, 1999) for \textit{E. coli}. However, determining the exact
composition of a particular polysaccharide is difficult, since these molecules are associated with the cell and other materials, often leading to contamination of the sample (Flemming & Wingender, 2010). In some cases, the composition of the polysaccharide is known, such as for alginate and Psl from *P. aeruginosa* (Schürks *et al.*, 2002, Byrd *et al.*, 2009) or PNAG, which is produced by diverse bacteria, including *E. coli* (Karatan & Watnick, 2009).

Polysaccharides, when present, can be critical in promoting biofilm maturation, as these molecules promote cell-cell interactions within the biofilm: deletion of genes necessary for polysaccharide production leads to cells that can still attach to a surface, but are unable to promote biofilm maturation (Watnick & Kolter, 1999, Danese *et al.*, 2000, Ma *et al.*, 2009). However, some polysaccharides are necessary for cell-surface attachment, as is the case for PNAG produced by *E. coli* (Wang *et al.*, 2004).

Polysaccharides may also serve other, non-structural functions. For example, it is speculated that they may trap and bind cations as a means to provide essential nutrients to the cells within the biofilm (Sutherland, 2001a). Additionally, polysaccharides may serve as signaling molecules; a recent report suggests that extracellular Psl from *P. aeruginosa* can stimulate the production of Psl from planktonic cells (Irie *et al.*, 2012). Overall, polysaccharides are an essential part of the biofilm matrix, serving structural and, apparently, non-structural roles to aid in the maturation of the biofilm and ultimately provide protection of cells within the biofilm.

*(ii) eDNA*
Another important, and more recently appreciated, part of the biofilm matrix is extracellular DNA or eDNA. Similar to polysaccharides, the main role of eDNA is likely to provide structure and integrity to the biofilm. For example, eDNA forms a grid-like pattern at the substratum of the *P. aeruginosa* biofilm (Allesen-Holm et al., 2006), while it forms thick strands that span water channels between biofilm structures in nontypable *Haemophilus influenzae* biofilms (Jurcisek & Bakaletz, 2007). Although eDNA can be derived from genomic DNA, as is the case for *P. aeruginosa* and *Pseudomonas putida* (Steinberger & Holden, 2005), it may also be distinct from genomic DNA, as is the case for the gammaproteobacteria strain F8 (Bockelmann et al., 2006).

How is genomic eDNA released into the biofilm matrix? At least in some cases, the most likely answer is through cell death, though it is possible that mechanisms exist for the active excretion of DNA. If it occurs through cell death, this event need not indicate that the cell population is unhealthy or dying. Rather, this cell death event can be ‘natural’ or mediated by subsets of cells within the biofilm. Specifically, there are examples in which autolysins/toxins are secreted and target a susceptible sub-population within the biofilm, as is the case for *Bacillus subtilis* (Gonzalez-Pastor et al., 2003), *Enterococcus faecalis* (Thomas et al., 2008), *Staphylococcus epidermidis* (Qin et al., 2007), and *Staphylococcus aureus* (Rice et al., 2007). An added benefit of specific, directed cell lysis is that the contents of those cells are released, providing a source of nutrients to the remaining cells within the biofilm. eDNA is also thought to serve as a source of DNA for horizontal gene transfer to expand the diversity of the cells within the biofilm (Flemming & Wingender, 2010). Given the more recent identification of eDNA
as a critical structural component in biofilm maturation, this molecule may play many unidentified roles during biofilm formation.

(iii) Proteins

Proteins are another well-established component of the biofilm matrix. These molecules can be anchored to or associated with the cell surface or released into the extracellular space. Proteins that play a structural role often promote cell-matrix, cell-cell, and cell-surface interactions. However, there are only a few examples of known matrix proteins from a select set of organisms. Furthermore, even though some of the proteins are known to be associated with the extracellular matrix and are important for biofilm formation, the exact role for many of these proteins is unknown.

In *P. aeruginosa*, the cell-associated (and secreted) protein CdrA promotes cell-matrix interactions by binding to the Psl polysaccharide (Borlee *et al.*, 2010), while the lectins (carbohydrate-binding proteins) LecA, specific for D-galactose (Gilboa-Garber *et al.*, 1972), and LecB, specific for L-fucose (Garber *et al.*, 1987), are thought to promote cell-cell interactions (Tielker *et al.*, 2005). In *B. subtilis*, one of the major components of the matrix is the TasA protein (Branda *et al.*, 2006), which forms amyloid fibers that promote biofilm integrity and cell-cell interactions (Romero *et al.*, 2010). TasA is typically cell associated and anchored in the membrane by the TapA protein (Romero *et al.*, 2011). A more recently identified protein of the *B. subtilis* biofilm matrix is BslA (Ostrowski *et al.*, 2011, Hobley *et al.*, 2013). This protein is a hydrophobin, which is a type of protein found in fungi that provide spores with water resistant properties (Hobley...
et al., 2013). During *B. subtilis* biofilm formation, BslA appears to self-assemble and coat the surface of the biofilm, making it hydrophobic (Kobayashi & Iwano, 2012).

Certain *Staphylococcus* spp. actually encode a number of surface-associated biofilm matrix proteins. These proteins include the accumulation-associated protein Aap from *S. epidermidis* (Rohde et al., 2005) and the related protein SasG from *S. aureus* (Corrigan et al., 2007), the extracellular matrix binding protein (Embp) (Christner et al., 2010) from *S. epidermidis*, and the *S. aureus* surface protein C (SasC) (Schroeder et al., 2009) and the fibronectin-binding proteins FnbpA and FnbpB from *S. aureus* (O'Neill et al., 2008), which all appear to be involved in mediating attachment and cell aggregation/intercellular adhesion.

Another set of proteins important for biofilm formation are the Bap family of proteins, which are found in a variety of organisms, including *S. aureus* (Cucarella et al., 2001), *E. faecalis* (Toledo-Arana et al., 2001), *P. fluorescens* (Hinsa et al., 2003), and *Salmonella enterica* serovar Enteritidis (Latasa et al., 2005). These are large proteins (usually >1,800 amino acids) with multiple domains containing tandem repeats [reviewed in (Lasa & Penades, 2006)]. Proteins in the Bap family are either secreted or anchored to/associated with the cell surface, though their exact function remains unknown. However, it is suggested that, given the tandem repeats found within these proteins, they likely promote cell-cell interactions by binding to each other.

Some bacteria (e.g., *E. coli*, *Salmonella* spp., and *P. aeruginosa*) utilize proteinaceous fibers, such as flagella, pili, and curli (a type of fimbriae) to mediate cell-cell and cell-surface interactions (Karatan & Watnick, 2009). These fibers can also be
used to structure the biofilm and its components during maturation. For example, *P. aeruginosa* uses type IV pili and twitching motility to promote biofilm maturation; this type of motility allows cells to climb the stalks of the mushroom-like structures to promote cap formation (Klausen et al., 2003a, Barken *et al.*, 2008). Hence, proteins (and proteinaceous fibers) play a major role in biofilm formation by promoting cell-surface, cell-cell, and cell-matrix interactions to help build and maintain the 3D architecture of the mature biofilm.

The matrix as a whole is designed to protect cells from the outside environment, though it isn’t just a chaotic sea of molecules. The matrix is a structured environment that leads to the establishment of gradients of such things as nutrients, oxygen, waste/toxic compounds, and signaling molecules that either promote cells to maintain or break the biofilm lifestyle. Since gradients exist within the biofilm, not all cells experience the same conditions and, as such, will exhibit different characteristics, leading to heterogeneity of the biofilm community (see section D below). Overall, the biofilm matrix is indeed the “House of Biofilm Cells” (Flemming *et al.*, 2007) and promotes efficient maturation of the biofilm. Future studies should be aimed at providing a better understanding of the composition of the biofilm matrix, including structure-function relationships between known matrix components, as well as the identification of new matrix components.

**Dispersal**

The last stage of biofilm development is dispersal, which is the release of cells or portions of the biofilm from the mature structure to seed a new niche(s) (Fig. 1C).
Individual cells that leave or are released from the biofilm are phenotypically different than those within the biofilm (Purevdorj-Gage et al., 2005), suggesting that some cue has promoted the differentiation of these cells. Given that gradients exist within the biofilm, it is likely that cues from within the biofilm promote cellular differentiation [reviewed in (McDougald et al., 2012)]; these cues are often opposite to those that promote biofilm formation. For example, changes in the level of the small intracellular signaling molecule c-di-GMP have been linked to dispersal: high levels of c-di-GMP promote biofilm formation by *P. aeruginosa*, while low levels promote dispersal (Petrova & Sauer, 2012).

Dispersal is sometimes characterized by the hollowing of the central cavity of a microcolony due to cell lysis (Fig. 1C); this has been well established for *P. aeruginosa* (Webb et al., 2003). Such an event would be advantageous not only to free up space for new growth within the biofilm cavity, but it would release the contents of the lysed cell into the extracellular space, such as enzymes, proteases, and other molecules that could facilitate dispersal. For example, *B. subtilis* produces norspermidine, which appears to interact directly with the exopolysaccharide to promote dispersal (Kolodkin-Gal et al., 2012). *B. subtilis* also releases D-amino acids (specifically D-methionine, D-tryptophan, D-leucine, and D-tyrosine) at later stages of biofilm development. Initially, it was found that these D-amino acids were incorporated into the peptidoglycan and altered the association of an amyloid-like fiber (composed of the TasA protein) with its accessory protein TapA, ultimately promoting fiber release (Kolodkin-Gal et al., 2010); TapA anchors the fiber to the cell wall and also helps in fiber assembly (Branda et al., 2006, Romero et al., 2011). However, a new report has demonstrated that these D-amino acids
are growth inhibitory and cause a decrease in \( eps \) and \( tapA \) expression (Leiman et al., 2013). These data suggest that it is likely the decrease in \( tapA \) expression that promotes amyloid fiber release, though further studies are necessary to fully understand the role of D-amino acids in \( B. subtilis \) biofilm disassembly. Overall, dispersal mechanisms will vary depending on the organism and the environmental and biofilm conditions. As a result, a thorough understanding of this process will depend upon the study of additional organisms and of model organisms exposed to different conditions.

(D) Heterogeneity within the biofilm community

The various stages of biofilm formation, attachment, maturation, and dispersal, come with changes in the transcriptional profiles of the cells within the biofilm. It is well established that, while bacterial cells within a biofilm may be clonal, they exhibit heterogeneous characteristics. A prime example of this heterogeneity is found during biofilm formation by the Gram-positive organism \( B. subtilis \) [reviewed in (Lopez & Kolter, 2009)]. In \( B. subtilis \), an ‘undifferentiated’ cell becomes motile and then, due to the levels of various signaling molecules and regulators within that individual cell, can differentiate into a variety of different cell types, such as toxin-producing cells that lyse other cells to release their contents, cells that secrete proteases to degrade extracellular proteins into a nutrient source, or cells that produce the extracellular matrix that holds the community together. Ultimately, the cell either terminally differentiates into a spore or is lysed to release nutrients for consumption and/or DNA that is taken up by competent cells; competence is thought to help ‘diversify’ the community.
P. aeruginosa also appears to promote genetic diversity during biofilm formation: plating biofilm bacteria results in distinct colony types (i.e., large, small, and wrinkled) (Boles et al., 2004). Additionally, these distinct colony types retained their respective phenotype through repeated passaging, suggesting that a genetic change is responsible for the altered colony morphology. These changes are likely mediated by RecA-dependent recombination (though other mechanisms likely exist) (Boles et al., 2004). The result, distinct subsets of cells (e.g., altered motility or polysaccharide production), may allow for increased resistance to stress (from both outside and inside the biofilm) on a community level. This idea is known as the insurance hypothesis, which predicts that functionally diverse populations are able to thrive because certain subpopulations are better able to cope with changing conditions, benefiting the community as a whole (Yachi & Loreau, 1999, Boles et al., 2004). Thus, biofilm formation and regulation varies between different bacterial species and leads to varying transcription profiles, not only between biofilm and planktonic cells, but also between cells within the biofilm. Taken together, it is very evident that bacteria are far more complex than previously believed.

**Biofilm formation by Pseudomonas aeruginosa**

Most of what we know about biofilm formation has come from studying a select few model organisms, one of which is the opportunistic pathogen P. aeruginosa. This organism was one of the first used to describe the developmental stages of biofilm formation (Sauer et al., 2002) and was used as the model for the developmental stages in the well-known Stoodly et al. review on biofilm formation (Stoodley et al., 2002). A major reason P. aeruginosa is a model biofilm organism is because of its involvement in
infecting the lungs of cystic fibrosis (CF) patients (Lam et al., 1980). *P. aeruginosa* initially infects the CF lung as a non-mucoid biofilm-forming strain that subsequently undergoes conversion to a mucoid biofilm-forming strain (Govan & Deretic, 1996, Ramsey & Wozniak, 2005), which promotes evasion of the immune system and persistent infection (Ramphal & Vishwanath, 1987, Pier et al., 2001, Leid et al., 2005). Since the matrix components play a critical role in the ability of *P. aeruginosa* to form a biofilm, they will be the main focus of this next section, and include: (A) the alginate, Psl, and Pel polysaccharides, (B) eDNA, (C) proteins and proteinaceous fibers, and (D) the biosurfactant rhamnolipid.

(A) The alginate, Psl, and Pel polysaccharides

*P. aeruginosa* is known to produce at least three different polysaccharides. One of the most well characterized polysaccharides is alginate, which was originally called ‘slime’ (Carlson & Matthews, 1966). This polysaccharide is an acetylated polymer consisting of non-repeating monomers of β1,4 linked L-guluronic and D-mannuronic acids (Evans & Linker, 1973). Overproduction of alginate results in a mucoid colony morphology, a phenotype exhibited by most *P. aeruginosa* strains isolated from the lungs of CF patients (Lam et al., 1980). However, colonization of the CF lung is initiated via non-mucoid strains, which then undergo a conversion to the mucoid phenotype (Govan & Deretic, 1996, Ramsey & Wozniak, 2005). The most common cause for this conversion is a mutation in *mucA*, which encodes an anti-sigma factor that normally sequesters the sigma factor responsible for alginate expression, AlgT/U (Govan & Deretic, 1996). Without the anti-sigma factor, AlgT/U promotes constitutive expression of the alginate
biosynthetic gene cluster, resulting in the overproduction of alginate and the mucoid phenotype. However, alginate does not appear to be necessary for biofilm formation by non-mucoid strains (Wozniak et al., 2003), which require the Psl and, to a lesser extent, Pel polysaccharides.

The Psl polysaccharide is produced by the polysaccharide synthesis locus (psl), which is comprised of 15 genes (Friedman & Kolter, 2004b, Jackson et al., 2004, Matsukawa & Greenberg, 2004); only 11 of these genes are required for Psl polysaccharide production (Byrd et al., 2009). This polysaccharide is composed of a pentasaccharide containing D-mannose, D-glucose, and L-rhamnose and is generated using GDP-D-mannose, UDP-D-glucose, and dTDP-L-rhamnose (Byrd et al., 2009). Psl can be found as a high molecular weight form associated with the cell surface (in a helical pattern) or a smaller soluble form found in the supernatant (Byrd et al., 2009). It is speculated that the smaller form is due to cleavage or breakdown of the cell-associated product (Mann & Wozniak, 2012), though this has not been verified.

Psl plays a role in both non-mucoid and mucoid biofilm formation by P. aeruginosa. During non-mucoid biofilm formation, this polysaccharide promotes cell-cell and cell-surface interactions during the initial stages of biofilm formation (Ma et al., 2006), coats the outside of the biofilm during maturation (Ma et al., 2009), and is also organized (via twitching motility mediated by type IV pili) into fiber-like structures within the mature biofilm (Wang et al., 2013). Psl was also recently shown to be a critical structural component used to connect bacteria in biofilms formed by mucoid strains of P. aeruginosa (Ma et al., 2012). However, Psl is not only involved in structuring the
biofilm. It also appears to serve as an extracellular signal to increase the levels of c-di-GMP in the cell (via the diguanylate cyclases SiaD and SadC), which in turn increases Psl production, as well as production of the matrix protein CdrA (discussed below in Proteins and proteinaceous fibers) (Irie et al., 2012). Thus, Psl not only serves as a scaffold, but a signal. It seems possible that this unique signaling role of Psl could be a common theme for polysaccharides during biofilm formation that has yet to be identified.

The third polysaccharide produced by *P. aeruginosa* is the Pel polysaccharide, which was identified in a screen for mutants that were unable to form a pellicle (Friedman & Kolter, 2004a). The *pel* locus is a seven-gene operon, though it does not appear to encode all the proteins necessary for polysaccharide biosynthesis (Franklin et al., 2011). A study by Ghafoor *et al.* found that Pel production increased when Psl was absent, and suggested that cross-talk exists in regulation of the two polysaccharide loci or that competition may exist for factors and precursors necessary for polysaccharide biosynthesis (Ghafoor *et al.*, 2011); the latter possibility could explain why certain genes are lacking in the *pel* locus. However, unlike alginate and Psl, the exact structure of the Pel polysaccharide has not been elucidated; Pel is likely a glucose-rich polysaccharide that is unrelated to cellulose (Friedman & Kolter, 2004b, Friedman & Kolter, 2004a).

During the initiation of biofilm formation, the Pel polysaccharide can compensate for the loss of type IV pili during attachment (Vasseur *et al.*, 2005), while it serves a more structural role later in biofilm formation to promote cell-cell interactions. Additionally, expression of the Pel polysaccharide has been shown to promote increased tolerance to aminoglycosides (Colvin *et al.*, 2011).
Regulation of these polysaccharide loci is complex and involves the second messenger c-di-GMP for *alg*, *psl*, and *pel* regulation, a number of 2CST systems for *psl* and *pel* regulation, and the transcriptional regulator AmrZ, for *alg* and *psl* regulation. In *P. aeruginosa*, high levels of the second messenger c-di-GMP promote biofilm formation (Hickman *et al.*, 2005, Merritt *et al.*, 2007). c-di-GMP is generated via diguanylate cyclases (GGDEF proteins) from two GTP molecules and degraded by phosphodiesterases (EAL or HY-GPY proteins) into pGpG (Simm *et al.*, 2004). *P. aeruginosa* encodes 41 proteins involved in the synthesis and degradation of c-di-GMP (17 GGDEF proteins, 5 EAL proteins, 16 GGDEF/EAL proteins, and 3 HD-GYP proteins) (Kulasakara *et al.*, 2006, Ryan *et al.*, 2009). Of these c-di-GMP synthesizing and degrading proteins, a number are known to be involved in biofilm formation (Kulasakara *et al.*, 2006), such as the diguanylate cyclases WspR (Hickman *et al.*, 2005), RoeA (Merritt *et al.*, 2010), SadC (Merritt *et al.*, 2007), and SiaD (Klebensberger *et al.*, 2009), and the phosphodiesterases Arr (Hoffman *et al.*, 2005), BifA (Kuchma *et al.*, 2007), PvrR (Drenkard & Ausubel, 2002), and RocR (Rao *et al.*, 2008). Along with proteins that make and break c-di-GMP, there are c-di-GMP receptor proteins. In *P. aeruginosa*, two of these proteins, Alg44 (Remminghorst & Rehm, 2006) and PelD (Lee *et al.*, 2007b), regulate transcription of the *alg* and *pel* loci, respectively; it is currently unclear how *psl* is regulated via c-di-GMP.

Since there are a variety of proteins that make and break c-di-GMP, do they all exert the same impact on biofilm formation? The answer appears to be no, in some cases. For example, the diguanylate cyclases RoeA and SadC, which would ultimately increase
c-di-GMP levels, impact biofilm formation at different levels: RoeA is involved in controlling polysaccharide production, while SadC regulates flagellar motility (Merritt et al., 2010). Furthermore, these proteins are differentially localized, and these authors suggest that, instead of one large pool of c-di-GMP, there may be smaller localized pools. Therefore, the role of c-di-GMP and individual c-di-GMP regulatory proteins in biofilm formation requires further work.

The pel and psl operons are also regulated via a complex network of proteins including the GacS/A two-component signal transduction (2CST) system, the small regulatory RNAs (sRNAs) RsmY and RsmZ, and the translational repressor RsmA (Parkins et al., 2001, Brencic et al., 2009, Irie et al., 2010). Briefly, when GacS is activated, it initiates a phosphorelay involving its cognate response regulator (RR) GacA, which promotes transcription of the sRNAs rsmY and rsmZ. These sRNAs then bind to RsmA to inhibit its function; RsmA binds to mRNA transcripts to either inhibit translation (for psl and pel) or promote translation (for motility genes). Additional levels of regulation also impact this system. One level of regulation consists of controlling GacS activity: the SK RetS inhibits GacS function, while the SK LadS indirectly promotes GacS function (Goodman et al., 2004, Ventre et al., 2006). Another level consists of control of the sRNAs: the histidine phosphotransferase protein HptB indirectly inhibits rsmY transcription (Bordi et al., 2010), while the cytoplasmic SK BifS and its cognate RR BifR inhibit rsmZ transcription (Petrova & Sauer, 2010). Ultimately, this complex regulatory cascade is involved in the switch between the planktonic and sessile lifestyle.
Psl and alginate are also regulated by the transcriptional regulator AmrZ. This transcription factor inversely regulates the alg (Tart et al., 2006) and psl (Jones et al., 2013) loci by promoting and inhibiting expression of these genes, respectively. Since it is known that non-mucoid strains undergo a conversion to mucoid strains during CF lung infections (Govan & Deretic, 1996, Ramsey & Wozniak, 2005), understanding the role of AmrZ during biofilm formation may provide better insight into the non-mucoid-to-mucoid conversion (Ramphal & Vishwanath, 1987, Pier et al., 2001, Leid et al., 2005). However, since Psl is also necessary for biofilm formation by mucoid strains of P. aeruginosa, there are likely additional levels of regulation involved in controlling psl (and alg) expression.

Overall, P. aeruginosa uses a variety of mechanisms to regulate the production of its three polysaccharides, each of which play a unique and important role in the various stages of biofilm formation by this organism. Although P. aeruginosa biofilm formation is known to be involved in chronic wound (Kirketerp-Moller et al., 2008), contact lens (Robertson et al., 2011), and urinary tract infections (Mittal et al., 2009), it is probably best known for its role in colonizing the lungs of CF patients. Thus, with the knowledge gained from studying biofilm formation by P. aeruginosa, novel therapeutics may be identified/generated that will target specific stages of biofilm formation to prevent and treat infections caused by this organism.

(B) The role of eDNA in P. aeruginosa biofilm formation

eDNA is an important component of P. aeruginosa biofilm formation at early and late stages of maturation. eDNA is abundantly found in early biofilms (Allesen-Holm et
al., 2006) and appears to maintain the structure and integrity of the growing biofilm: DNAse I treatment of young biofilms (12, 36, and 60 h old) dissolved the biofilm, while older biofilms (84 h old) were mostly unaffected (Whitchurch et al., 2002). After 2 days of growth, eDNA is present throughout the biofilm, while at day 5 it is primarily localized to the interior of the stalk of mushroom-like structures, and by day 6 it is centered in the cap of these structures (Allesen-Holm et al., 2006); eDNA localization to the stalk is likely mediated by Psl (Yang et al., 2007). It is hypothesized that the eDNA within the forming stalk permits bacteria to migrate to the top, using type IV pili and twitching motility, where they form the cap of the mushroom-like structures (Klausen et al., 2003a, Barken et al., 2008); type IV pili were previously shown to bind DNA (van Schaik et al., 2005).

eDNA can also be used as a nutrient source during biofilm formation. This molecule can be broken down into carbon, nitrogen, and phosphate by the EddB (extracellular DNA degradation) protein (Mulcahy et al., 2010), though the role of this protein during biofilm formation has not been explored. Additionally, a more recent study demonstrated that eDNA protects cells from aminoglycosides, likely by serving as a ‘shield’ (Chiang et al., 2013). Taken together, there appears to be a variety of roles for eDNA during biofilm formation by *P. aeruginosa*.

The eDNA present in *P. aeruginosa* biofilms appears to be genomic in origin (Allesen-Holm et al., 2006), although it is unclear how DNA release is mediated during biofilm formation. There is some evidence that quorum sensing may be involved in this process. Specifically, the PQS system is implicated in controlling the release of DNA:
loss of this system resulted in decreased amounts of DNA release, while overproduction of PQS increased the amount of DNA release (Allesen-Holm et al., 2006, D'Argenio et al., 2002). Since prophage induction is also under the control of quorum sensing, it has been suggested that DNA release may be due to induction of a prophage (Spoering & Gilmore, 2006); however, this has yet to be experimentally determined. DNA release also appears to be controlled by the iron concentration, as DNA release and pqs expression decreases with increasing amounts of iron (Yang et al., 2007). Overall, it appears that these mechanisms of DNA release are linked, though it is likely not the only means to release DNA into the biofilm matrix.

(C) Proteins and proteinaceous fibers

Another important part of the P. aeruginosa biofilm matrix is proteins and proteinaceous fibers. Only a few proteins have been identified as members of the extracellular matrix of the P. aeruginosa biofilm, which include the LecA and LecB lectins and CdrA. LecA is specific for D-galactose (Gilboa-Garber et al., 1972) and has recently been shown to contain a second sugar-binding site for glucose (Blanchard et al., 2013). This protein appears to be involved in maintaining the biofilm architecture, as loss of LecA resulted in a decrease in the height and surface coverage of the biofilm (Diggle et al., 2006). However, the exact role of this protein during biofilm formation is unclear. The other lectin, LecB, is specific for D-galactose (Gilboa-Garber et al., 1972) and is localized to the outer membrane (Tielker et al., 2005), where it appears to bind to the major outer membrane porin OprF to impact biofilm formation (Funken et al., 2012); LecB and OprF are predicted to be involved in cell-cell interactions, though this has not
been verified. LecB is also known to impact the synthesis of type IV pili, which are involved in twitching motility and biofilm formation (Sonawane et al., 2006). Whether this role of LecB in biofilm formation is related to its interaction with OprF is unknown.

The third matrix protein is CdrA, which is a large adhesin found in the extracellular matrix, likely transported outside of the cell by its partner transporter, CdrB (Borlee et al., 2010). These proteins are encoded within the cdrAB (cyclic diguanylate-regulated TPS partner A and B) locus, aptly named because this locus is up-regulated when the concentration of c-di-GMP is high and encodes proteins with sequence similarity to two-partner secretion (TPS) systems (Borlee et al., 2010). CdrA can bind to both the small and large forms of the Psl polysaccharide (Borlee et al., 2010), suggesting that this protein could potentially mediate both cell-matrix and cell-cell interactions, respectively. Further studies are necessary to fully define the role of CdrA in biofilm formation.

Biofilm formation also depends on proteinaceous fibers, including flagella, type IV pili, and Cup fimbriae. Both flagella and type IV pili are necessary for efficient surface attachment at the initial stages of biofilm formation (O'Toole & Kolter, 1998). Additionally, flagella and type IV pili are necessary for cap formation during biofilm maturation (Barken et al., 2008). Type IV pili mediate twitching motility, likely along eDNA in the stalk (Klausen et al., 2003a, Barken et al., 2008), while it is predicted, but not confirmed, that flagella promote cap formation via swarming motility (as opposed to swimming motility). An additional proteinaceous adhesive structure used for biofilm formation is the chaperone usher pilus or Cup fimbriae (Vallet et al., 2001), which are
necessary for microcolony formation and cell-cell interactions (Kulasekara et al., 2005, Ruer et al., 2007).

Together, the proteins and proteinaceous fibers of the *P. aeruginosa* biofilm play a critical role in the initial development and maturation of the biofilm. Whereas some of the functions of the proteins and proteinaceous fibers are known, there are probably other, as yet unknown roles for them during biofilm formation. Though relatively little is known about the role of LecA in biofilm formation, current studies to inhibit *P. aeruginosa* biofilm formation are targeting LecA (Reymond et al., 2013, Grishin et al., 2013). Future studies should be aimed at understanding the role of this protein in biofilm formation, as well as uncovering other matrix components necessary for this process.

(D) The biosurfactant rhamnolipid

Another molecule necessary for efficient biofilm formation is the biosurfactant rhamnolipid, which is produced by a variety of bacteria (Abdel-Mawgoud et al., 2010). Rhamnolipids were first identified from *P. aeruginosa* (formally *Pseudomonas pyocyanea*) (Bergstrom et al., 1946b, Bergstrom et al., 1946a). This molecule was originally described as an oily glycolipid (pyolipic acid) consisting of L-rhamnose and β-hydroxydecanoic acid (Jarvis, 1949, Hauser & Karnovsky, 1954). In *P. aeruginosa*, rhamnolipid synthesis requires the *rhlAB* operon, which encodes the enzyme rhamnosyltransferase (Ochsner et al., 1994). This operon is under the control of the quorum sensing system RhlR-RhlI (Ochsner & Reiser, 1995, Medina et al., 2003a, Medina et al., 2003b, Medina et al., 2003c), one of the two LuxR-LuxI-like systems in *P. aeruginosa*; RhlI produces a signaling molecule known as an autoinducer (AI) and RhlR
is a transcriptional regulator that, when bound to the AI, promotes transcription of the 
*rhlAB* operon (Ochsner et al., 1994, Ochsner & Reiser, 1995).

Rhamnolipids are necessary for the structuring of the biofilm, as well as its dispersal. Expression of *rhlA* is first detected in 2-day old biofilms (Davey *et al.*, 2003). In older biofilms, rhamnolipid is found in the stalks and is critical for maturation into the mushroom-like capped structures (Lequette & Greenberg, 2005, Pamp & Tolker-Nielsen, 2007), possibly by facilitating twitching and swarming motility. Rhamnolipid is also necessary to maintain the channels that separate the mushroom-like towers during biofilm formation: whereas channels were initially observed for both an *rhlA* mutant and the wild-type strain in young biofilms, these channels were not maintained in the *rhlA* mutant at later time points (Davey *et al.*, 2003). An additional role of rhamnolipid is in dispersal, which likely involves the disruption of cell-cell, cell-matrix, and cell-surface interactions (Boles *et al.*, 2005); however, this has yet to be determined.

Overall, *P. aeruginosa* biofilms are some of the most well-studied and serve as an important model for biofilm formation by bacteria. However, bacterial biofilm formation and its regulation vary, even between bacteria from the same genus and closely related species. For example, *P. syringae* uses the levan polysaccharide during biofilm formation (Osman *et al.*, 1986), while *P. fluorescens* and *P. putida* utilizes the Lap matrix proteins (Hinsa *et al.*, 2003), which are part of the Bap family of proteins, none of which are produced by *P. aeruginosa*. This variation in biofilm formation likely speaks to the environmental conditions experienced by the bacteria and gives a continuing reason to study biofilm formation by both model organisms and diverse species.
Biofilm formation by *Vibrio cholerae*

Another model organism used to study biofilm formation is *V. cholerae*, which is found in aquatic ecosystems (Faruque *et al.*, 1998). *V. cholerae* is best known as the causative agent of the human diarrheal disease cholera. During infection, both planktonic and biofilm-associated bacteria (as aggregates), which are more infectious, can be recovered from stool samples isolated from infected individuals (Faruque *et al.*, 2006), suggesting that biofilm formation is important for host colonization. Like other biofilm-forming bacteria, *V. cholerae* uses a variety of mechanisms to promote biofilm formation. Due to the multitude of serotypes, slight differences in biofilm formation between these serotypes exist (i.e., altered polysaccharide composition or changes in the relative contribution of matrix associated proteins). In general, interaction with and attachment to a surface is mediated by flagella and at least three different pili (TCP, MSHA, and ChiRP), while maturation is mediated by the *Vibrio polysaccharide* (VPS) or the O-antigen polysaccharide and the matrix proteins Bap1, RbmA, and RbmC. Although little is known about dispersal, some studies have shown that the quorum sensing signal AI-2, shown to inhibit VPS production (Hammer & Bassler, 2003), and nitric oxide (NO), through an unknown mechanism (Barraud *et al.*, 2009), may be involved in this process. This particular section will discuss: (A) flagella and pili mediated attachment, (B) the *V. cholerae* matrix, and (C) regulation of biofilm formation.

(A) Flagella- and pili-mediated attachment

*V. cholerae* can use a variety of mechanisms to promote attachment to a surface, including flagella- and pili-mediated motility and adhesion. The role of flagella in biofilm
formation is often viewed as a means to propel the cell towards a surface. However, flagella serve additional roles during biofilm formation. First, flagella are used as adhesins to promote attachment to a surface (Watnick & Kolter, 1999). Second, loss of the flagella leads to an increase in VPS production (Watnick et al., 2001), which appears to be linked to the flagellar motor (Lauriano et al., 2004). It is suggested that, when a cell encounters a surface, a mechanical signal is transduced via the flagellar motor that promotes biofilm formation by increasing VPS production (Lauriano et al., 2004). The idea of the flagellar motor transducing a mechanical signal to promote biofilm formation does not appear to be unique to *V. cholerae*. Cairns et al. have demonstrated that, in *B. subtilis*, a decrease or loss of flagellar rotation promotes biofilm formation by inducing expression of the biofilm protein BslA (Cairns et al., 2013). These studies have identified a unique role for flagella, specifically the flagellar motor, in biofilm formation, which may be a common mechanism used by a variety of biofilm-forming bacteria.

Pili are also necessary for the initiation of biofilm formation by *V. cholerae*. However, each of the three pili utilized by this organism are important under different conditions. For example, the toxin co-regulated pilus (TCP) promotes attachment to chitin in the environment (Reguera & Kolter, 2005), as well as to host tissues during colonization (Herrington et al., 1988), while the mannose-sensitive haemagglutinin type IV pili (MSHA) appears to be involved in accelerating attachment to abiotic surfaces (Watnick et al., 1999, Watnick & Kolter, 1999). Additionally, TCP and MSHA are inversely regulated during infection: induction of the transcription factor ToxT promotes expression of the TCP and represses *msh* expression, while the pre-pilin peptidase TcpJ...
degrades unprocessed MshA protein (Hsiao et al., 2008). The last pilus is the chitin regulated pilus, or ChiRP. Little is known about the role of ChiRP in mediating biofilm formation, except that it may be involved in attaching to chitin surfaces, such as crab shells (Meibom et al., 2004). Overall, both flagella and pili are necessary for efficient biofilm formation by *V. cholerae*.

(B) The *V. cholerae* matrix

Biofilm formation by *V. cholerae* is also dependent on components of the biofilm matrix, which, like other biofilms includes polysaccharides, proteins, and eDNA. *V. cholerae* is capable of producing two different polysaccharides, depending on the environmental conditions (e.g., freshwater vs. saltwater). The first is the *Vibrio* polysaccharide, or VPS, which appears to be induced mainly in freshwater-based medium (Kierek & Watnick, 2003a), suggesting that this is the polysaccharide used for biofilm formation in freshwater environments. The second polysaccharide is the O-antigen polysaccharide, which appears to be induced in saltwater-based medium and depends on Ca²⁺ (Kierek & Watnick, 2003b), suggesting that this is the polysaccharide used for biofilm formation in saltwater environments.

The VPS polysaccharide is the best studied polysaccharide produced by *V. cholerae*. Synthesis and export of this polysaccharide requires the *vps* genes, which reside in two loci, *vps*-1 (*vpsU, vpsA-K*) and *vps*-2 (*vps-L-Q*) (Yildiz & Schoolnik, 1999, Fong et al., 2010). These two loci are separated by the *rbm* gene locus, which encodes RbmA and RbmC, proteins that are also involved in biofilm formation (discussed below) (Fong et al., 2006, Fong & Yildiz, 2007). The polysaccharide is likely synthesized from
nucleotide sugar precursors produced by VpsA, a predicted UDP-N-acetylglucosamine 2-epimerase and VpsB, a predicted UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase (Fong et al., 2010), as well as from monosaccharides from the environment (Kierek & Watnick, 2003a). Initiation and elongation of the polysaccharide appears to involve the glycosyltransferases VpsD (class 4), VpsI (class 4), VpsK (class 26), and VpsL (which contains a bacterial sugar transferase domain) (Fong et al., 2010). Polymerization and export of the polysaccharide appears to be mediated by VpsE, a predicted polysaccharide export protein, VpsH, a predicted polysaccharide polymerase, VpsN, a predicted polysaccharide export protein, and VpsO, a predicted chain length determinant protein. VpsC and VpsG are predicted to be acetyltransferases that would modify the polysaccharides, and VpsU, is a predicted phosphotyrosine-protein phosphatase, which plays an unknown role in polysaccharide production. Lastly, the remainder of the Vps proteins, VpsF, VpsJ, VpsM, VpsP, and VpsQ are proteins of hypothetical function. The relative importance of each of the vps genes to VPS synthesis has been determined, and the majority of these genes are necessary for biofilm formation by V. cholerae (Fong et al., 2010). Regulation of the vps loci is discussed further below.

Many studies have sought to determine the composition of VPS. However, these studies have yielded somewhat conflicting results due to the use of different serotypes of V. cholerae. For example, VPS from V. cholerae O139 is composed of N-acetylglucosamine, glucose, galactose, and mannose (Kierek & Watnick, 2003b), while VPS from the El Tor strain 92A1552 mainly consists of glucose and galactose, with lesser amounts of N-acetylglucosamine, mannose, and xylose (Yildiz & Schoolnik,
Additionally, VPS from the El Tor strain TSI-4 consists mainly of mannose and N-acetylglucosamine, with lesser amounts of galactose and 6-deoxy-galactose (Wai et al., 1998). Thus, it is unclear whether these differences are artifactual, due to contamination by other extracellular products during VPS purification, or real and due to the specific activities of the VPS biosynthetic proteins in different serotypes (Karatan & Watnick, 2009).

The second polysaccharide is the O-antigen polysaccharide, which is involved in $vps$-independent biofilm formation. This polysaccharide is induced when $V. cholerae$ cells are grown in seawater-based medium, a condition that is unable to promote $vps$ gene expression (Kierek & Watnick, 2003a). $vps$-independent biofilm formation appears to require flagella and the MSHA pili for attachment, as well as genes for the O-antigen and capsule (Kierek & Watnick, 2003b). Additionally, $Ca^{2+}$ is an integral part of the biofilm matrix of $vps$-independent biofilms, and is proposed to mediate salt-bridge formation between O-antigen moieties to promote cell-cell interactions (Kierek & Watnick, 2003b). The removal of $Ca^{2+}$ from the growth medium promotes dissolution of $vps$-independent biofilms, which could occur when biofilms are transported from a seawater environment to a freshwater environment, possibly facilitating the spread of cholera. However, little else is known about $vps$-independent biofilm formation.

Another critical component of the biofilm matrix is proteins. Biofilm formation by $V. cholerae$ is known to require the genes encoding the matrix proteins RbmA, RbmC, and Bap1, which are co-regulated with the $vps$ loci (Yildiz et al., 2004, Moorthy & Watnick, 2005, Fong et al., 2006, Fong & Yildiz, 2007); $rbmA$ and $rbmC$ lie between the
two vps loci, while bap1 is encoded elsewhere on the genome (Fong & Yildiz, 2007). RbmA is a secreted protein that is critical for biofilm structure and fitness (Fong et al., 2006) and is predicted to bind to carbohydrates via tandem fibronectin II folds to facilitate cell-cell and cell-matrix interactions (Giglio et al., 2013). RbmC and Bap1 are large, secreted proteins that are 47% similar to each other and contain multiple tandem repeats (Moorthy & Watnick, 2005, Karatan & Watnick, 2009); these proteins are unrelated, but structurally similar to proteins of the Bap family. Furthermore, their tandem repeats resemble those found in integrins (FG-GAP domains), which are receptors involved in cell-cell and cell-matrix interactions between eukaryotic cells (Hynes, 1987, Moorthy & Watnick, 2005). Although it is unclear how Bap1 is secreted, RbmC appears to be a type II secretion system substrate (Sikora et al., 2011). With regards to Bap1 and RbmC function, there appears to be some level of redundancy in the function in the V. cholerae El Tor A1552 strain, as only deletion of both genes significantly reduced biofilm formation (Fong & Yildiz, 2007). However, in the V. cholerae O137 strain, deletion of bap1 by itself negatively impacted biofilm formation (Moorthy & Watnick, 2005), highlighting again that different serotypes of V. cholerae exhibit differences in the control of biofilm formation.

More recently, a study by Berk et al. used epitope-tagged Bap1, RbmA, and RbmC and direct staining for VPS to observe biofilm formation initiated by a single cell over time, specifically assessing the expression and location of individual matrix components (Berk et al., 2012). Initial attachment of a cell was followed by expression of RbmA and then Bap1, which mediate cell-cell and cell-surface interactions, respectively.
RbmC was expressed next, and, together with Bap1 and VPS, encased the aggregate of cells. VPS accumulated at discrete sites on the cell surface, as soon as 15 minutes after cell attachment, with the number of spots increasing over time. However, in the absence of VPS, the matrix proteins did not accumulate on the cell surface, though they were still synthesized. Not only is VPS necessary for matrix protein retention, but VPS retention depended on the matrix protein RbmC: in the absence of RbmC, little VPS was associated with the cell. Though other studies have examined the expression of genes and proteins during biofilm formation (Whiteley et al., 2001, Davey et al., 2003, Moorthy & Watnick, 2005), this is the first to provide such a detailed look into the localization, timing of appearance, and interaction of individual molecules due to the major advances in technology. Given these advances in technology, the ability to examine biofilm formation over time, starting at a single cell, and further examining expression of individual molecules will likely become a common practice for biofilm researchers and will greatly increase our understanding of this process.

There also appears to be a role for eDNA in biofilm formation by *V. cholerae*. eDNA appears critical for both initial attachment and structural maturation of the biofilm, along with the extracellular nucleases Dns and Xns (Seper et al., 2011). Though it is unclear how eDNA is released (likely through autolysis), it is found close to the cell surface and is likely processed into small fragments via the nucleases. These nucleases exhibit distinct activities: Dns is an endonuclease that degrades both circular and linear DNA, while Xns is an exonuclease that only degrades linear DNA. Additionally, under phosphate-limiting conditions, these nucleases appear to degrade the eDNA, which in
turn serves as a source of phosphate for the cells. This study thus defines a critical role for eDNA and the nucleases Dns and Xns in biofilm formation by *V. cholerae*.

Overall, the composition of the *V. cholerae* matrix not only influences biofilm formation, but the location of biofilm formation. For example, *vps*-dependent biofilms are likely formed in freshwater environments, while *vps*-independent biofilms are likely formed in saltwater environments. Since most studies focus on understanding *vps*-dependent biofilm formation, little is known about *vps*-independent biofilm formation. Do the matrix proteins play a role in *vps*-independent biofilm formation? Is there a role for eDNA in *vps*-independent biofilm formation? Thus, it would be of interest to understand more about *vps*-independent biofilm formation, as the ability of *V. cholerae* to form a biofilm in different environments likely facilitates the spread of cholera.

(C) Regulation of biofilm formation

Biofilm formation by *V. cholerae* is under a considerable amount of regulatory control. Multiple mechanisms have been identified that contribute to this regulation, such as modulation of c-di-GMP levels, quorum sensing, and the phosphoenoylpyruvate phosphotransferase system (PTS). However, these regulatory mechanisms are not mutually exclusive, as c-di-GMP levels impact quorum sensing regulators, which are also under metabolic control, partially mediated by PTS. Quorum sensing also regulates c-di-GMP, making it necessary to identify the levels at which these pathways intersect and where they diverge to better understand how biofilms formation is regulated by *V. cholerae*. 
One mechanism used to regulate biofilm formation is modulation of the levels of the ubiquitous second messenger c-di-GMP. *V. cholerae* is predicted to contain at least 31 GGDEF, 12 EAL, 10 GGDEF/EAL, and 9 HD-GYP domain containing proteins (Galperin *et al.*, 2001). *V. cholerae* also has a number of c-di-GMP receptor/binding proteins, including five PilZ domain proteins, two of which (PlzB and PlzC) impact biofilm formation in an unknown manner (Pratt *et al.*, 2007) and the c-di-GMP receptor VpsT, a transcriptional regulator of VPS expression (Krasteva *et al.*, 2010). c-di-GMP positively regulates vps gene expression, and thus biofilm formation, while decreasing expression of genes involved in virulence, such as ctxAB (for cholera toxin) and toxT, a transcriptional activator of ctxAB (Tischler & Camilli, 2005). Additionally, the phosphodiesterase MbaA (Bomchil *et al.*, 2003), CdgC (Lim *et al.*, 2007), and VieA (Tischler & Camilli, 2004) control biofilm formation by decreasing the level of c-di-GMP in the cell; the effect of MbaA can be relieved by norspermidine, which binds to the periplasmic sensor NspS to modulate MbaA activity (Karatan *et al.*, 2005). Finally, c-di-GMP also feeds into the regulation of the quorum sensing system (discussed below), another regulator of biofilm formation, thus resulting in a complex network of regulators.

Quorum sensing-mediated regulation of biofilm formation by *V. cholerae* involves the master regulator of the quorum sensing system, HapR. This regulator represses expression of the vps genes, as well as the genes for their transcriptional activators, VpsR (Yildiz *et al.*, 2001) and VpsT (Casper-Lindley & Yildiz, 2004). HapR also represses transcription of genes encoding diguanylate cyclases, such as cgdA (Beyhan *et al.*, 2007, Waters *et al.*, 2008), leading to decreased c-di-GMP levels and
decreased biofilm formation. Finally, because HapR also activates expression of a gene encoding a hemagglutinin/protease (*hapA*), HapR has been implicated in promoting dispersal of the *V. cholerae* biofilm (Finkelstein *et al.*, 1992, Zhu & Mekalanos, 2003)

Since mature biofilms likely experience high cell density conditions, due to the entrapment of quorum sensing signaling molecules within the matrix, it makes sense that HapR, which is mainly active under conditions of high cell density, would be linked to dispersal.

Another mechanism used to regulate biofilm formation by *V. cholerae* is the phosphoenolpyruvate phosphotransferase system (PTS) (Houot & Watnick, 2008). This system is involved in transporting specific sugars (e.g., glucose) into the cell and is likely involved in sensing favorable carbon sources in the environment. Furthermore, since the PTS is a phosphotransfer cascade, it is also thought to serve as a measure of the cell’s stores of high-energy phosphate reserves (Karatan & Watnick, 2009). It appears that there are three independent pathways from the PTS that are involved in regulating *vps* gene expression (Houot *et al.*, 2010). Pathway one involves the phosphorylated form of the EI subunit and leads to repression of *vps* expression. Pathways two and three promote *vps* expression and involve the EIIA<sup>Gluc</sup> subunit (and Mlc, a transcriptional regulator of PTS components) and the EIIA<sup>Ntr1</sup> subunit (a member of the nitrogen-related PTS). Thus, the nutritional status of the cell is a key factor in promoting and repressing biofilm formation.

Overall, *V. cholerae* uses a variety of mechanisms to promote and repress biofilm formation, which link together to form a complex regulatory network. Even though biofilm formation is important during infection of the human host, it is likely that biofilm
formation is also necessary in the natural environment, possibly as a reservoir for *V. cholerae*. Thus, understanding how *V. cholerae* promotes biofilm formation not only expands our knowledge of biofilm formation in general, but could also lead to better treatments and prevention of the disease caused by this organism.

**Conclusions**

Biofilm formation by bacteria is a complex process that involves multiple regulatory mechanisms, which vary from bacteria to bacteria and even strain to strain. However, the general principles of biofilm formation, such as the developmental process and components of the matrix, appear to be universal (Fig. 1). Regardless of whether a bacterium forms a monolayer or multilayer biofilm, the cells must still attach to the surface (or each other) and produce the extracellular matrix composed of polysaccharides, proteins, and eDNA. The uses and localization of these components will ultimately vary, not only between bacteria, but due to the environmental and cellular conditions.

Given that biofilm formation appears to be a universal process by bacteria, it is critical to continue to understand how it is regulated. In addition, infections caused by biofilm-forming organisms are difficult to treat, making it necessary to identify ways to better understand biofilm formation in the context of a host to provide more appropriate treatments. One way to accomplish this is through the use of model systems, which is a currently underdeveloped field. Since we have an increased understanding of biofilm formation on abiotic surfaces, it is time to determine whether this information translates
to biofilm formation on biotic surfaces so that we can better appreciate the complexity and utility of this process.

III. THE VIBRIO FISCHERI-EUPRYMNA SCOLOPES SYMBIOSIS

Introduction

To fully understand how a particular bacterial process, such as biofilm formation, impacts host colonization, it is necessary to utilize a model system. One such system is the symbiotic relationship between the bioluminescent marine bacterium *Vibrio fischeri* and its host the Hawaiian bobtail squid *Euprymna scolopes*. *V. fischeri* is the sole colonizer of a specific squid organ, the light organ (Fig. 2A and B); this colonization results in a life-long symbiosis. Since juvenile squid hatch without their symbiont, *V. fischeri* must either be acquired from the surrounding seawater (in nature) or provided to the squid (in the laboratory setting) to promote this symbiosis. In addition, *V. fischeri* must be competent to form a biofilm at one of the earliest stages of colonization (Fig. 2C), making this an ideal study to examine natural biofilm formation during colonization of a host by its bacterial partner. *V. fischeri* is also genetically tractable and the bacterium and the host can be studied separately, making it possible to study biofilm formation in the laboratory setting before utilizing the animal model.

Studies of the *Vibrio*-squid symbiosis have provided a wealth of information about the processes involved in colonization, the environments experienced by the bacteria during colonization, and the host responses to its symbiotic partner [reviewed in (Nyholm & McFall-Ngai, 2004, Ruby, 1996, Stabb, 2006, Stabb & Visick, 2013)]. These
Figure 2. The light organ of juvenile *E. scolopes*. (A) Juvenile *E. scolopes*. The light organ can be seen as a dark mass in the center of the body (mantle cavity) between the dotted lines. (B) A cartoon depiction of the light organ. This organ is bi-lobed, with a set of three pores located on each side at the base of two ciliated epithelial appendages. Each pore connects to a deep crypt space via a duct and antechamber (only one is represented for simplicity). *V. fischeri* cells are represented as white ovals with a curved line representing flagella. The bacteria aggregate outside of the light organ in the squid-secreted mucus (lines extending from the light organ surface). After aggregation, these cells migrate to the pores, through the ducts and the antechambers to the deep crypt spaces where they grow to high cell densities and bioluminesce. (C) A confocal microscopy image from (Yip *et al.*, 2006), showing one side of the squid light organ with both appendages on the right side of picture (top and bottom). The three pores can be seen in the middle of the image, with an aggregate of *V. fischeri* (green) located above one of the pores (indicated by the white triangle).
studies have identified three main stages of colonization: initiation, accommodation, and persistence [reviewed in (Ruby, 1996)]. Each stage requires that the bacteria and host communicate to promote processes that will ultimately lead to efficient colonization and a productive symbiosis. I will first describe the anatomy of the light organ to orient the reader for further discussions of the colonization process. Then, since my dissertation is focused on biofilm formation by *V. fischeri*, but does not rely heavily on the use of this model system, I will focus mainly on the initiation stage, which requires biofilm formation by *V. fischeri*. I will then briefly describe the accommodation and persistence stages, as well as the host developmental responses to its bacterial partner so that the reader can gain an overall view of the utility of this system.

**Anatomy of the light organ**

The squid light organ is a bi-lobed organ with two appendages (one long and one short) on either side (Fig. 2B) that are covered in cilia (McFall-Ngai & Ruby, 1991). The cells on the surface of the appendages are capable of secreting mucus (Nyholm *et al.*, 2000, Nyholm *et al.*, 2002), which appears to be utilized by the bacteria to remain close to the organ during colonization (Fig. 2B and C). *V. fischeri* enters the light organ via three pores located at the base of the appendages (total of six pores) (Fig. 2B). Each pore leads to a duct, followed by a large antechamber, which bottlenecks before opening into a deep crypt space (Fig. 2B) (McFall-Ngai & Ruby, 1991, Montgomery & McFall-Ngai, 1993). These deep crypt spaces are where *V. fischeri* is ultimately housed during its symbiotic association with *E. scolopes*. 
Initiation

During the initiation stage of colonization, newly hatched juvenile squid (Fig. 2A) obtain their symbiotic partner from the surrounding environment. Seawater containing *V. fischeri* and other bacteria is continuously flushed through the body cavity (mantle) and, as a result, across the light organ surface. The presence of bacteria and bacterial products (i.e., peptidoglycan) induces the squid to secrete mucus to the light organ surface (Fig. 2B) (Nyholm et al., 2002). Within about 3 hours after exposure of squid to *V. fischeri*, the bacteria attach to the light organ surface (Altura et al., 2013).

The initiation of the symbiosis actually appears to be a two-step process, starting with attachment of *V. fischeri* cells to the ciliated field of the light organ (bacteria-host interaction), followed by aggregation of the bacteria (bacteria-bacteria interaction) (Fig. 2C) (Nyholm et al., 2000, Yip et al., 2006, Altura et al., 2013). When juvenile squid are exposed to *V. fischeri* at an environmentally relevant concentration (i.e., $5 \times 10^3$ CFU/ml; (Jones et al., 2007)), only a few *V. fischeri* cells (approx. 5) are found associated with the cilia (Altura et al., 2013). However, attachment to the cilia, *per se*, is not specific to *V. fischeri*, as a non-squid symbiont (MJ11) and *V. parahaemolyticus* KNH1 are also able to attach to the cilia at this stage (Altura et al., 2013); however, of these strains, only the *V. fischeri* squid symbiont (ES114) is able to colonize the light organ (Nyholm & McFall-Ngai, 2003, Mandel et al., 2009). In addition, this initial attachment does not appear to require that *V. fischeri* be competent to form a biofilm (i.e., aggregate).

Biofilm formation is, however, critical for the next stage of initiation, which is aggregation (Fig. 2C). *V. fischeri* cells unable to promote biofilm formation are unable to
aggregate and fail to efficiently colonize the squid host (Yip et al., 2005, Yip et al., 2006, Morris et al., 2011, Morris & Visick, 2013b, Morris & Visick, 2013a, Shibata et al., 2012). It also appears that an aminopeptidase, PepN, is involved in aggregate formation: juvenile squid exposed to a pepN mutant are unable to form defined aggregates, possibly leading to the observed delay in colonization by this mutant (Fidopiastis et al., 2012); the reason behind this phenotype is poorly understood. Overall, the ability to aggregate likely allows the bacteria to stay closely associated so that they are not swept away during the constant ventilation of seawater through the squid mantle cavity.

The bacteria only remain in the aggregate for a few hours before transitioning to a planktonic state to migrate towards the light organ pores (Nyholm et al., 2000). While bacterial motility is not necessary for aggregation, it is necessary for *V. fischeri* to enter into the light organ (Nyholm et al., 2000). This migration is likely due to some chemotactic gradient associated with the pores. *V. fischeri* is able to sense and swim towards chitin, which is found lining the ducts of the light organ (Mandel et al., 2012); *V. fischeri* cells unable to sense chitin could not efficiently colonize the squid. Furthermore, polymeric chitin in the squid mucus appears to be hydrolyzed into chitobiose by a host-derived endochitinase, which may produce a chemotactic gradient to promote entry into the light organ (Kremer et al., 2013). Together, these data suggest that chitin and/or chitobiose may be signals to draw *V. fischeri* towards the pores and into the light organ.

*V. fischeri* can also sense and swim towards N-acetylneuraminic acid, a component of the mucus, and nucleosides (DeLoney-Marino et al., 2003). However, it is unclear whether these signals promote migration from the aggregate towards the pores. *V.*
*fischeri* encodes at least 43 proteins predicted to be involved in chemotaxis (Ruby *et al.*, 2005, Mandel *et al.*, 2008, Brennan *et al.*, 2013), so it is highly possible that more than one of these proteins are involved in chemotaxis towards the pores. Overall, initiation involves multiple processes that ensure that *V. fischeri* is in the right location to promote its entry into the light organ.

**Accommodation**

Once *V. fischeri* cells enter the light organ, they migrate through ducts and antechambers, both of which are non-permissive to colonization, to the deep crypt spaces (Fig. 2B) where colonization and growth occur (Montgomery & McFall-Ngai, 1993, Graf & Ruby, 1998, Davidson *et al.*, 2004, Sycuro *et al.*, 2006). The ducts and antechambers contain high concentrations of nitric oxide (NO) and other antimicrobial compounds, to which *V. fischeri* is able to sense and respond, accordingly (Davidson *et al.*, 2004, Dunn *et al.*, Wang *et al.*, 2010a, Wang *et al.*, 2010b). Furthermore, the light organ is patrolled by macrophage-like immune cells, known as hemocytes (Nyholm & McFall-Ngai, 1998), yet *V. fischeri* is able to reside within the deep crypts. One study suggests that these hemocytes become ‘educated’ to the presence of *V. fischeri* within the light organ, and this apparent ‘tolerance’, mediated by both host and bacterial factors, prevents removal of the symbiont (Nyholm *et al.*, 2009).

Once in the deep crypt spaces, the squid supplies the bacteria with nutrients, including amino acids in the form of small peptides, which permit rapid bacterial growth (Graf & Ruby, 1998). At high cell densities, the bacteria initiate production of bioluminescence, which the squid uses to avoid detection by predators: the light, which is
directed downward, disrupts the shadow that would otherwise result from the down-welling moonlight shining on the animal as it forages for food in shallow bays at night (Jones & Nishiguchi, 2004). Motility, which is essential for entry, appears unnecessary at this stage of colonization, as most bacteria lack flagella in the deep crypts (Ruby & Asato, 1993).

**Persistence**

The last stage of colonization is termed persistence. At dawn every morning, the squid expels approximately 95% of the bacteria into the seawater (Boettcher et al., 1996, Lee & Ruby, 1994). The remaining *V. fischeri* cells then re-populate the light organ within a few hours. Thus, this partnership is dynamic, yet it is maintained for the life of the squid.

A clearer picture of the interaction between *V. fischeri* and *E. scolopes* during this life-long symbiosis is beginning to develop due to transcriptomic and proteomic studies. Wier et al. used transcriptomics to examine the changes experienced by the bacterium and host at 6-h intervals over the day-night cycle (Wier et al., 2010). The greatest changes in transcript levels for both partners occurred around dawn, which coincides with the time at which the squid expels 90-95% of its symbiotic partner. At this time, *E. scolopes* up-regulates cytoskeleton-related genes, coincident with ultrastructural changes in the epithelium, which normally consists of polarized cells with microvilli; these cells are subsequently restructured, resulting in microvilli blebbing. These membrane blebs then appear to serve as a nutrient source for the remaining *V. fischeri* population, as *V. fischeri* up-regulated genes involved in anaerobic respiration of glycerol and was found to
incorporate fatty acids from these blebs into its lipid membrane. After the *V. fischeri* population has re-grown during the day, the cells then seem to switch from anaerobic respiration to chitin fermentation until dawn the next day. Proteomic studies are also being utilized to analyze the protein profiles of both the host and symbiont (Schleicher & Nyholm, 2011). Overall, these studies are beginning to provide a glimpse into the dynamic relationship that occurs each day between the squid and its symbiotic partner.

**Host development**

During symbiotic colonization, *V. fischeri* promotes a series of morphogenic changes in the squid light organ. For example, mucus secretion, which facilitates aggregate formation on the surface of the light organ, ceases in animals colonized by *V. fischeri* (Nyholm et al., 2002). In addition, the epithelial cells within the appendages on the surface of the light organ undergo apoptosis (Foster & McFall-Ngai, 1998). Subsequently, the appendages undergo regression and are lost over a 4-day period (Doino & McFall-Ngai, 1995, Montgomery & McFall-Ngai, 1994). These changes are a normal part of light organ maturation and considered hallmarks of successful colonization; likely, most if not all of these changes contribute to the specificity of the association and prevent subsequent “superinfection” by additional *V. fischeri* cells. These developmental events are triggered by bacterial cell wall components released from colonizing bacteria, in particular LPS and a component of peptidoglycan (Foster et al., 2000, Koropatnick et al., 2004). Although these signals are known, much remains to be learned about the bacterial factors controlling host development.
Conclusions

It is clear from this brief description of the *Vibrio*-squid symbiosis that the bacteria experience numerous different environments as they transition from seawater to symbiosis. Specifically, *V. fischeri* must adhere to the surface of the light organ and form a biofilm-like aggregate (Fig. 2). The cells then leave this aggregate and enter the pores to travel through the ducts and antechambers to colonize the deep crypts, where *V. fischeri* grows rapidly on host-supplied nutrients and induces bioluminescence. Thus, it seems likely that *V. fischeri* recognizes and responds to specific signals in different environments to enter into a productive relationship with its squid host. One critical stage, initiation, requires the formation of a biofilm-like aggregate, as well as genes known to be involved in biofilm formation. Since the main focus of my dissertation is on biofilm formation by *V. fischeri*, I will provide an in-depth review of the regulators involved in this process in the next section.

IV. BIOFILM FORMATION BY *VIBRIO FISCHERI*

Introduction

Biofilm formation is an important survival and colonization strategy used by bacteria. To date, few models have been described that allow for the study of natural biofilm formation during host colonization; the *V. fischeri*-squid symbiosis is one of these. During the initiation stage of colonization, *V. fischeri* cells form a biofilm-like aggregate in mucus on the surface of the squid light organ (Fig. 2C), and subsequently disperse from the aggregate to enter the organ (Nyholm et al., 2000). For *V. fischeri*, it
seems likely that this aggregate is a form of biofilm, as its formation depends upon genes with known roles in biofilm formation in culture (Yip et al., 2006, Morris et al., 2011). Whether or not the bacteria exist in a biofilm at a subsequent stage of colonization remains to be determined.

Surprisingly, under traditional growth conditions in laboratory culture, *V. fischeri* fails to form any substantial amount of biofilm, although some adherence to the test tube surface can be detected (Hussa et al., 2008, Yip et al., 2005). Thus, it is possible that *V. fischeri* recognizes some host-specific signal(s) in its environment to promote aggregation on the surface of the light organ. However, in the absence of the signal(s), biofilm formation can be induced via multi-copy expression of key two-component signal transduction (2CST) regulators (i.e., the SK RscS or the RR SypG). Furthermore, there is a correlation between a strain’s ability to promote biofilm formation and its ability to colonize the host: strains unable to promote biofilm formation exhibit reduced or defective host colonization (Yip et al., 2005, Yip et al., 2006, Morris et al., 2011, Morris & Visick, 2013b, Morris & Visick, 2013a, Shibata et al., 2012). This correlation between biofilm formation and natural host colonization makes *V. fischeri* an ideal organism for the study of biofilm formation. This section thus focuses on what is known about biofilm formation by *V. fischeri*. First, because many of the *V. fischeri* biofilm regulators are 2CST regulators, I will briefly describe 2CST systems. Then, I will describe the regulation of biofilm formation by *V. fischeri*, the known components of the biofilm matrix, and the conservation of the *syp* locus in other *Vibrio* spp.
**Two-component signal transduction systems**

Bacteria use two-component signal transduction systems to adapt to changes in their environment. A ‘simple’ 2CST system is typically composed of a membrane-bound histidine sensor kinase (SK), which senses some environmental or cellular signal (e.g., membrane stress), and a response regulator (RR), which elicits the appropriate cellular response (e.g., transcriptional regulation of a gene or set of genes) (Fig. 3A). More specifically, when the SK senses a signal, it autophosphorylates on a conserved histidine residue, located within its transmitter domain (HisKA), and serves as a phospho-donor to its partner RR (Stock et al., 2000, West & Stock, 2001). The RR then catalyzes the transfer of the phosphoryl group to a conserved aspartate residue, located within its receiver (REC) domain (Bourret et al., 1990). RR phosphorylation is thought to stabilize the protein in a particular conformation, typically activating an attached effector domain (e.g., DNA binding domain), which promotes the necessary response (Stock et al., 2000).

A more complex type of 2CST system is known as a phosphorelay. A typical phosphorelay relies on multiple phosphotransfer events that occur between two or more proteins (Fig. 3B). One common type of phosphorelay involves a hybrid SK, which contains multiple residues involved in phospho-transfer, and a partner RR (West & Stock, 2001). The hybrid SK usually contains a conserved histidine residue (H1) located within its transmitter domain (HisKA), a conserved aspartate residue (D2) located within a REC domain, and a second conserved histidine (H2) located within a histidine phosphotransferase (Hpt) domain; alternatively, the Hpt domain may be contained within a separate protein. The RR contains the second conserved aspartate residue (D2), located
Figure 3. Two-component system regulators. (A) A simple TCS system composed of a sensor kinase (SK) and a response regulator (RR). A SK typically contains: 1) a periplasmic signal sensing loop, with at least two transmembrane spanning regions (depicted by the black boxes), and 2) HisKA and HATPase-c domains (light grey boxes with black lettering); the latter domain binds ATP and promotes phosphorylation of a conserved histidine residue located in the HisKA domain (depicted as an H above the HisKA domain) upon signal receipt. SKs can also contain cytoplasmically-located signaling domains, such as PAS or HAMP domains (not shown). A RR typically contains: 1) a receiver (REC) domain (depicted as a darker grey box with white lettering), which catalyzes the transfer of the phosphoryl group from the SK to a conserved aspartate residue (indicated by a D above the REC domain) within this domain, and 2) an attached effector domain, depicted here as a helix-turn-helix (HTH) DNA binding domain (very light grey box with black lettering); effector domains are not always DNA binding domains. (B) A more complex TCS system is known as a phosphorelay, and typically contains multiple proteins for a total of four domains with residues that become phosphorylated. These domains, known as HisKA, REC, Hpt (histidine phosphotransferase), and REC, contain H1, D1, H2 and D2, respectively. Two representative phosphorelays are depicted. At the top, a hybrid sensor kinase is depicted that contains three of the four domains involved in phosphotransfer to a RR, while at the bottom, a hybrid sensor kinase with two of the four domains is shown along with a
separate phosphotransferase protein containing an Hpt domain that serves as the phosphodonor to the RR. As with the simple TCS system, a signal stimulates the hybrid SK to autophosphorylate on H1. The phosphoryl group is transferred intramolecularly to D1 and, in either the same protein or a second protein, to H2. The phosphorelay is completed by donation of the phosphoryl group to D2 on the RR.
within its REC domain. Upon sensing a signal, the hybrid SK autophosphorylates on H1 and subsequently transfers the phosphoryl group intra-molecularly to D1 and then H2 (West & Stock, 2001). The RR then catalyzes the transfer of the phosphoryl group from H2 to D2, which promotes RR activity (West & Stock, 2001).

**Regulation of biofilm formation**

Regulation of biofilm formation by *V. fischeri* requires multiple regulatory proteins (Fig. 4 and 5). Many of these proteins are 2CST system regulators, with the exception of SypA, whose function is unknown. Thus, this next section will provide detailed information about regulation of *V. fischeri* biofilm formation by: (A) the orphan hybrid SK RscS, the *syp* encoded RRs (B) SypG and (C) SypE, (D) the small STAS domain protein SypA, and (E) the hybrid SK SypF and the RR VpsR.

(A) RscS

The gene *rscS* (*regulator of symbiotic colonization – sensor*) encodes a hybrid SK that was first identified in a screen for mutants unable to colonize juvenile *E. scolopes* (Visick & Skoufos, 2001). The mutant defective for *rscS* exhibited a severe defect in initiating colonization: when exposed to the *rscS* mutant, most squid remained uncolonized. It was subsequently determined that this colonization defect resulted from the inability of the *rscS* mutant to aggregate on the surface of the light organ (Yip et al., 2006). These experiments revealed for the first time a bacterial determinant necessary for aggregate formation, thus supporting the identification of this process as a stage critical to colonization. They also demonstrated that, rather than being passively taken up by the squid, *V. fischeri* plays an active role in promoting colonization. More recently, the
Figure 4. Model of *syp*-dependent biofilm formation. The hybrid SK RscS is predicted to activate SypG via phosphorylation, thus promoting transcription of each of four operons in the *syp* locus (from promoters indicated by the bent arrows). The *syp* genes encode proteins involved in the production and transport of a polysaccharide involved in biofilm formation. SypE is predicted to work downstream of *syp* transcription and inhibit SypA via phosphorylation on a conserved serine residue. When RscS becomes active, the inhibitory activity of SypE is inactivated, promoting biofilm formation, as well as upstream of the RR VpsR, which is necessary for the production of cellulose, another component of biofilm formation. Biofilm formation in culture correlates with aggregation of *V. fischeri* within the squid secreted mucus, which promotes subsequent colonization events. The signals sensed by RscS and SypF are currently unknown. Furthermore, it is unknown whether cellulose promotes colonization.
Figure 5. Domain structures of the proteins that regulate the syp locus and cellulose.

(A) Each SK contains a cytoplasmically-located signal domain (either PAS or HAMP; very light grey box with black lettering) and the HisKA and HATPase-c domains (light grey boxes with black lettering), with the predicted, conserved histidine numbered and shown above the HisKA domain. RscS and SypF are hybrid SKs and contain a REC (dark grey box with white lettering) and Hpt (very dark grey box with white lettering) domain; the predicted, conserved aspartate and histidine residues are numbered and shown above these domains, respectively. The hybrid SKs each have two predicted transmembrane spanning regions (black boxes) flanking a periplasmic loop likely involved in the detection of an external signal. (B) All of the RRs contain a REC domain (grey boxes with white lettering) with the predicted, conserved aspartate residue numbered and shown above this domain. All of these RRs, with the exception of SypE, contain a HTH DNA binding domain (light grey boxes with black lettering). Two of these RRs (SypG and VpsR) contain an AAA+ domain (dark grey box with white lettering), which is involved in interactions with RNA polymerase carrying the alternative sigma factor σ54. SypE contains an N-terminal putative RsbW-like serine kinase domain and a C-terminal PP2C-like serine phosphatase domain, and CheY lacks these other domains. (C) SypA contains a single STAS (sulphate transporter and anti-sigma factor antagonist) domain, with the conserved serine (S56), which becomes phosphorylated by SypE, shown above the protein.
importance of RscS was further emphasized when it was discovered that the gene that encodes this hybrid SK was missing in the fish symbiont MJ11, a strain of *V. fischeri* incapable of colonizing squid (Mandel et al., 2009). When *rscS* was introduced into MJ11, the resulting strain became competent to colonize squid (Mandel et al., 2009). Thus, this work determined that *rscS* — a single regulatory gene — was able to specify the interaction between a bacterium and its host. Together, these data demonstrated that RscS is a critical factor necessary for colonization, due to its ability to promote aggregation.

RscS is now known to regulate the *symbiosis polysaccharide* (*syp*) locus (Fig. 4); this locus is comprised of 18 genes necessary for the regulation, production and transport of a polysaccharide critical for biofilm formation (discussed below in Biofilm matrix components)(Yip et al., 2005). Overexpression of *rscS* from either of two increased activity alleles, termed *rscS1* and *rscS2*, caused a marked increase in *syp* transcription (Yip et al., 2006). Overexpression of these alleles also led to the production of distinctive *syp*-dependent phenotypes associated with biofilm formation. These phenotypes included wrinkled colony formation on solid media, pellicle formation at the air-liquid interface of static cultures, cell aggregation in liquid cultures grown with shaking, increased attachment to glass surfaces (Fig. 6), and increased hydrophobicity (not depicted).

Furthermore, examination of the wrinkled colonies formed by *rscS1*-containing cells using scanning and transmission electron microscopy revealed the presence of an extracellular matrix between the cells and at the colony surface.
Figure 6. *V. fischeri* biofilm phenotypes. The left image in each section depicts a non-biofilm forming *V. fischeri* strain, while the right image depicts a biofilm-forming strain. (A) Wrinkled colony formation of spotted cultures on solid agar media. (B) Pellicle formation at the air-liquid interface of a static culture. The pellicle can be seen as a ‘bunched’ cell mass, generated to promote visualization by dragging a sterile pipette tip over the surface. (C) Bacterial aggregation of shaking cultures. ‘Stringy’ material, observed for the biofilm forming strain, consists of clumps of adherent cells. (D) Glass attachment of either statically-grown or shaking cultures, visualized following staining with crystal violet. Cells in static cultures attach at the air-liquid interface of the tube, while cells in shaking cultures can attach throughout the tube. These images are compiled from a variety of strains that produced or lacked biofilms.
These dramatic biofilm phenotypes begged the question, are they relevant to symbiosis? The answer was yes, as an \( rscS1 \) overexpression strain showed a dramatic increase in the size of the symbiotic aggregate relative to the control (Yip et al., 2006). Furthermore, aggregate formation depended on \( syp \): loss of \( sypN \) abolished the increase in aggregate formation induced by RscS overexpression. Finally, cells overexpressing \( rscS1 \) substantially out-competed the vector-containing control strain during mixed inoculation experiments, indicating that the increased ability to aggregate conferred an advantage to the cells during colonization (Yip et al., 2006). Thus, these studies revealed a clear correlation between biofilm formation in culture and both symbiotic aggregation (i.e., biofilm formation) and colonization in an animal model.

What are \( rscS1 \) and \( rscS2 \), and why do they have increased activity? These alleles were generated in a study that sought to determine the function of RscS. It was predicted that the signal sensed by RscS might exist only in the context of the symbiosis, and thus, a signal-insensitive mutant might be necessary to evaluate the role of RscS in culture. It was expected that the increased-activity alleles isolated, \( rscS1 \) and \( rscS2 \), would contain changes to the coding sequence that made the RscS protein independent of the inducing signal. Instead, however, each increased activity allele contained a mutation in or near the putative ribosome binding site (RBS), while the \( rscS1 \) allele also contained a silent mutation at codon Leu\(_{25}\) (Geszvain & Visick, 2008a, Yip et al., 2006). Consistent with their locations, these mutations caused an increase in the level of the RscS protein, but did not impact the \( rscS \) transcript level (Geszvain & Visick, 2008a). Importantly, no RscS protein was detected when the wild-type allele was expressed from the chromosome or
overexpressed from a plasmid lacking the mutations (Geszvain & Visick, 2008a),
suggesting that this protein is normally expressed poorly or not at all under standard
culturing conditions. Surprisingly, when the effects of the mutations in \( rscSI \) were
separately assessed, the silent mutation at codon Leu\(_{25}\) appeared to exert a greater impact
on RscS activity than the RBS-linked mutation. The silent mutation substitutes a rare Leu
codon (used in 4% of Leu codons in \( V. fischeri \)) for a more common Leu codon (used in
\( \sim 20\% \) of Leu codons). This Leu codon occurs within a region (\( L_{23}ML_{25}TRN_{28} \)) that
contains, with one exception, all rare codons (Geszvain & Visick, 2008a). Thus, there
may be multiple levels at which the production of RscS protein is controlled. These
studies concluded that the increase in protein production likely accounts for the increase
in RscS activity and subsequent biofilm phenotypes when \( rscSI \) and \( rscS2 \) are
overexpressed. It will be of interest to determine the extent of regulatory control over
RscS translation in \( V. fischeri \). Furthermore, future studies should address whether simple
overexpression is sufficient to overcome a need for a specific, potentially host-associated
signal, or if the signal naturally exists in laboratory culture but depends upon sufficient
protein production.

The identification of phenotypes for both RscS and the \( syp \) locus paved the way
for understanding the specific roles of RscS and other biofilm regulators. RscS is an
orphan hybrid SK (\( rscS \) is physically unlinked to a RR gene), and thus its partner(s) was
initially unknown. However, the \( syp \) locus encodes two RRs, SypE and SypG, and since
RscS regulates the \( syp \) locus, it was possible that one of these RRs could serve as a
partner for RscS. Indeed, it is now known that RscS functions upstream of both of these
RRs (Fig. 4)(Hussa et al., 2008, Morris & Visick, 2013b). The current knowledge of the roles of SypE and SypG in biofilm formation and colonization will be described further below.

Structurally, RscS resembles the hybrid SKs ArcB and BvgS (Visick & Skoufos, 2001). These proteins contain three conserved domains (HisKA, REC, and Hpt) with residues predicted to be involved in phosphotransfer (H412, D709, and H867 in RscS) (Fig. 5A). Investigation of the roles of the conserved residues in RscS supported the function of RscS as a hybrid SK: mutations of H1 (H412Q), D1 (D709A), and H2 (H867Q) abolished (H1 and D1) or diminished (H2) induction of RscS-dependent biofilm phenotypes (Geszvain & Visick, 2008b). Since H2 was not essential for RscS-induced phenotypes, the signal transduction pathway may be complicated. For example, it is possible that the phosphorelay is branched, with RscS donating its phosphoryl group to another Hpt domain-containing protein, or that phosphotransfer to the RR could occur directly from H1 to D2. Such events are not unprecedented (e.g., ArcA/B (Georgellis et al., 1997)), but further studies are needed to fully dissect the dispensability of the Hpt domain for RscS function.

RscS also contains a large periplasmic (PP) loop and a PAS domain (Fig. 5A), both of which are implicated in signal recognition. The PP loop is ~200 amino acids and flanked on either side by 2 transmembrane (TM) regions (Fig. 5A). The PAS domain is located in the cytoplasm after the second TM region. In other SKs, the PP loop is responsible for sensing an environmental signal, and transmitting that information through TM regions to the cytoplasmic signaling portion of the protein [reviewed in
This appears to be the case for RscS, as disruption of the PP loop or the first transmembrane domain promoted RscS activity (Geszvain & Visick, 2008b), indicating that these regions may serve to negatively regulate RscS function. In contrast, disruption of the cytoplasmically localized PAS domain abolished RscS function (Geszvain & Visick, 2008b), indicating that this domain may positively regulate RscS function. PAS domains can sense signals such as oxygen, redox potential, light, and small molecules such as ATP, and frequently require binding of a cofactor for signal transduction (Taylor & Zhulin, 1999). RscS may sense an FAD cofactor, since its PAS domain shares homology with the PAS domain of NifL from *Azotobacter vinelandii*, which senses an FAD cofactor (Key et al., 2007). In support of this idea, conserved residues required for FAD binding are essential for RscS activity (Geszvain & Visick, 2008b). Taken together, it seems likely that RscS senses multiple signals using the PP loop and PAS domain, which regulate its function accordingly. Thus, determining what these signals are and understanding how RscS responds to them will provide a better understanding of how biofilm formation is regulated, especially in the context of the squid host.

(B) SypG

SypG is a predicted RR encoded within the *syp* locus and, like RscS, is also necessary for biofilm formation and host colonization (Fig. 4) (Yip et al., 2005, Hussa et al., 2007). SypG is a member of the NtrC family of RRs and contains 3 predicted domains: an N-terminal REC domain, a C-terminal DNA binding domain, and between those two domains, a $\sigma^{54}$ interaction domain (Fig. 5B). This latter domain is predicted to
provide the ATPase activity necessary for transcription: RNA polymerase containing σ^{54} binds to DNA and forms a closed complex, but can not form the transcriptionally active open complex without the help of an activator protein to provide the energy [reviewed in (Buck et al., 2000, Wigneshweraraj et al., 2008)]. Thus, SypG is predicted to recognize and bind to a particular sequence, called an enhancer sequence, to help initiate transcription of a gene or set of genes; this sequence is typically located 80-150 bp upstream of a promoter.

Intriguingly, the *syp* locus contains at least four promoters (associated with *sypA*, *sypI*, *sypM*, and *sypP*) (Fig. 4) with σ^{54} recognition sequences (Barrios et al., 1999, Yip et al., 2005); three of the four promoters were confirmed by primer extension analyses (Yip et al., 2005). In addition, each promoter contains a conserved enhancer sequence that could serve as a binding site for a σ^{54}-dependent activator. This sequence exhibits dyad symmetry (i.e., an inverted repeat [IR]) and consists of two, 6-bp half-sites separated by a 3-bp intervening sequence (TTCTCANNTGAGAA). The predicted enhancer sequence located upstream of *sypI*, *sypM*, and *sypP* is a perfect IR, while that located upstream of *sypA* diverges in the 3’ half-site (TTCTCANNTGCAAA). Since SypG is predicted to bind to such a sequence, it seems reasonable to predict that SypG is the direct transcriptional activator of this locus.

Whether SypG is the direct transcriptional activator of this locus is unclear, though SypG is known to control *syp* transcription: overexpression of *sypG* from a multi-copy plasmid caused a 37- to 70-fold increase in *syp* transcription over that of the vector control strain (Yip et al., 2005). Furthermore, this SypG-induced transcription depended
upon the presence of $\sigma^{54}$, as disruption of the $rpoN$ gene, which encodes $\sigma^{54}$, abolished transcriptional activation of $syp$ (Yip et al., 2005). Coincident with induction of $syp$ transcription, overexpression of $sypG$ induced biofilm formation. Specifically, $sypG$ overexpression induced a 3.5-fold increase in glass attachment when cells were grown statically and a $>30$-fold increase in glass attachment when cells were grown with shaking, relative to the vector control strain. In addition, biofilms formed under either condition (static or shaking) depended upon a functional copy of $rpoN$ (Yip et al., 2005). Taken together, these data demonstrated that SypG activates $syp$ transcription, and thus biofilm formation (Fig. 4), in a $\sigma^{54}$-dependent manner; data that implicates SypG as the direct transcriptional activator of the $syp$ locus will be presented in Chapter Three, Section II.

Because both RscS and SypG induce $syp$ transcription and biofilm formation, and the ability of RscS to induce $syp$ transcription depends upon the presence of an intact SypG (Hussa et al., 2008), RscS and SypG appear to represent a two-component pair (Fig. 4). However, the biofilm phenotypes displayed by the two overexpression strains were dissimilar: overexpression of $rscS$ resulted in the formation of wrinkled colonies and a strong pellicle (Yip et al., 2006), while overexpression of $sypG$ only led to weak pellicle formation (Hussa et al., 2008). Two possibilities could account for these differences: 1) when overexpressed in the absence of its SK, SypG is not sufficiently activated to promote transcription of the genes required for strong biofilm phenotypes or 2) RscS signals through a second RR to either inactivate a negative regulator or activate a positive regulator to promote biofilm formation. To assess the first possibility, a
constitutively active version of SypG (SypG*) was generated, in which the conserved
aspartate residue (D53) within the REC domain (Fig. 5B) was changed to a glutamate
(D53E); these types of mutations have been shown to promote RR activity in the absence
of phosphorylation in other RRs (e.g., CheY, NtrC, and LuxO (Freeman & Bassler,
1999a, Sanders et al., 1989, Sanders et al., 1992)). Overexpression of sypG* led to an
increase in syp transcription, but could not induce wrinkled colony formation or enhance
pellicle formation (Hussa et al., 2008). Support for the second possibility came from an
unexpected result: when sypG was overexpressed in a mutant lacking SypE, the second
predicted RR encoded within the syp locus, the cells formed wrinkled colonies and
pellicles that were indistinguishable from those induced by RscS (Hussa et al., 2008).
Subsequent analyses, described in the SypE section below, supported the idea that RscS
promotes inactivation of an inhibitory activity of SypE (Morris et al., 2011). These results
thus support the hypothesis that RscS and SypG function together to promote syp-
dependent biofilm formation, but suggest that control over biofilm formation is complex
and extends beyond transcriptional activation of the syp locus. This regulatory control
may serve to restrict biofilm formation to those times when V. fischeri encounters its
host.

(C) SypE

The SypE RR is unusual. It contains a centrally located REC domain that is
flanked by effector domains with opposing enzymatic functions (Fig. 5B) (Morris &
Visick, 2010). The N-terminal domain of SypE exhibits sequence similarity to SK-like
serine kinases (i.e., RsbW) (Fig. 5B) found in the GHKL (Gyrase, Hsp90, HK, MutL)
superfamily, which contain a conserved asparagine residue [D52 in SypE (Morris & Visick, 2010)] necessary for ATP binding (Dutta & Inouye, 2000). The C-terminal domain exhibits sequence similarity to the PP2C family of serine phosphatases (Fig. 5B), which contain invariant aspartate residues [D443 and D495 in SypE (Morris & Visick, 2010)] involved in catalytic activity (Adler et al., 1997, Jackson et al., 2003). To date, no RR protein with this unique domain structure has been characterized.

Consistent with the presence of two domains of opposing activity, SypE exerts both positive and negative control over biofilm formation. First, as described above, SypE antagonizes SypG-induced biofilm formation: \( sypG \) overexpression induced robust biofilms only when \( sypE \) had been disrupted (Hussa et al., 2008), indicating that SypE functions as a negative regulator of biofilm formation. Second, SypE enhances biofilms produced by RscS overexpression: overexpression of \( rscS \) in a strain deleted for \( sypE \) resulted in wrinkled colony formation that was delayed relative to the \( sypE^+ \) control (Hussa et al., 2008, Morris et al., 2011). Taken together, these data suggest that SypE functions to both promote and inhibit biofilm formation.

How is this dual regulatory activity controlled? The answer to this question came from an elegant study that examined the function and interplay between the three domains of SypE and their impact on biofilm formation and host colonization. This study assessed the ability of a variety of SypE mutants to control biofilm formation induced by RscS (Morris et al., 2011). It found that the C-terminal putative serine phosphatase domain was responsible for the positive regulatory activity of SypE (i.e., promoting biofilm formation), while the N-terminal putative serine kinase domain was responsible
for the negative regulatory activity of SypE (i.e., inhibiting biofilm formation) (Fig. 7).

The negative and positive functions of the two domains depended upon conserved residues predicted to be necessary for serine kinase and serine phosphatase activities, respectively. Finally, mutation of the conserved aspartate (D192A), predicted to be the site of phosphorylation in the REC domain (Fig. 5B), ‘locked’ SypE into an inhibitory state. Thus, it appears that D192 and, presumably, its phosphorylation is necessary for switching the activity of SypE from negative to positive (Fig. 7).

Which, if any, of these activities is important for colonization? A deletion of sypE exerted little effect on colonization, suggesting that the positive activity is not critical. However, expression of sypE<sup>D192A</sup>, the REC domain mutant that is ‘locked’ into the inhibitory state, severely impaired colonization (Morris et al., 2011). For example, in single strain colonization experiments, most squid remained uncolonized, while those that became colonized contained up to 1000-fold fewer bacteria than wild-type colonized squid. This colonization defect could be attributed to a defect at the aggregation stage: whereas RscS-overexpressing wild-type cells formed large aggregates above the light organ pores, RscS-overexpressing sypE<sup>D192A</sup> cells were unable to aggregate (Morris et al., 2011). These data, along with previous studies of RscS, demonstrate the importance of symbiotic aggregation in promoting colonization, as well as indicating that SypE naturally becomes inactivated during this stage of host colonization.

To date, no studies have confirmed phosphorylation of SypE, but it seems reasonable to expect that D192 becomes phosphorylated in response to some as-yet-unknown signal. The next question is, what SK works upstream of SypE? Morris et al.
Figure 7. Biofilm regulation mediated by SypE and SypA. SypE contains an N-terminal serine kinase domain (RsbW), a centrally located REC domain with the conserved site of phosphorylation D192, and a C-terminal serine phosphatase domain (PP2C). SypA contains a single STAS domain with the conserved site of phosphorylation S56. (A) In its unphosphorylated form, SypE functions as a serine kinase to phosphorylate SypA on S56, which inhibits biofilm formation. (B) When SypE becomes phosphorylated on D192, it switches from serine kinase to serine phosphatase activity, which promotes the removal of phosphoryl groups of SypA, leading to subsequent biofilm formation.
(2011) reasoned that RscS functions upstream of SypE, because overexpression of RscS but not SypG seemed to turn off the inhibitory activity of SypE (Fig. 4). Thus, RscS would have two activities: 1) activating SypG and 2) inactivating the negative regulatory activity of SypE. As a result of the latter activity, SypE would function as a negative regulator of colonization in an \textit{rscS} mutant. If so, then deletion of \textit{sypE} should suppress at least part of the colonization defect of an \textit{rscS} mutant. Indeed, an \textit{rscS sypE} double mutant colonized better than the single \textit{rscS} mutant (Morris et al., 2011), supporting the hypothesis that SypE functions as a negative regulator in the absence of RscS. Thus, during symbiotic colonization, SypE may serve to restrict colonization until RscS is activated. Whether RscS directly or indirectly influences the phosphorylation state of SypE remains to be determined.

One remaining question is the identity of the target of SypE’s activity. Morris \textit{et al.} recently demonstrated that this target is the \textit{syp}-encoded protein SypA (Fig. 4), a small protein predicted to contain a single STAS (sulphate transporter and anti-sigma factor antagonist) domain (Morris & Visick, 2013b). This study demonstrated that SypE and SypA interact and that both of SypE’s enzymatic activities target SypA: the N-terminal serine kinase domain phosphorylates SypA, while the C-terminal serine phosphatase domain dephosphorylates SypA (Fig. 7). In its unphosphorylated form, SypA promotes biofilm formation and host colonization (discussed in the SypA section below), though the role of this protein in biofilm formation is currently unknown. However, both SypE and SypA appear to function below \textit{syp} transcription (Fig. 4) (Morris & Visick, 2013a). In summary, SypE normally inactivates SypA via phosphorylation, inhibiting biofilm
formation (Fig. 7). However, when SypE becomes phosphorylated (presumably on D192), it switches activities and dephosphorylates SypA, leading to biofilm formation and subsequent host colonization (Fig. 7).

(D) SypA

SypA, which is encoded by the first gene in the syp locus, is an additional regulator of biofilm formation (Fig. 4). SypA is a small protein that contains a single STAS (sulphate transporter and anti-sigma factor antagonist) domain (Yip et al., 2005). SypA is critical for biofilm formation and host colonization, as deletion of the sypA gene results in a complete loss of RscS-induced biofilm formation, as well as host colonization (Morris & Visick, 2013b). STAS domain proteins typically contain a conserved serine residue, S56 for SypA, which serves as a site of phosphorylation (Fig. 5C); phosphorylation controls the activity of these types of proteins. This appears to be the case for SypA: in a sypA mutant, expression of SypA$^{S56A}$ (which mimics the unphosphorylated state of this protein) promotes biofilm formation, while expression of SypA$^{S56D}$ (which mimics the phosphorylated state of this protein) is unable to promote biofilm formation (Morris & Visick, 2013b). These data indicate that S56 on SypA is critical for SypA’s activity and suggest that phosphorylation may regulate the activity of this protein (Fig. 7). Furthermore, when squid were exposed to a sypA mutant carrying the sypA$^{S56D}$ allele, they exhibited a colonization defect, again highlighting the importance of SypA to biofilm formation and host colonization by V. fischeri.

Proteins like SypA are known to be involved in a regulatory mechanism called partner switching, which is employed by a variety of bacteria, such as B. subtilis. In B.
In *V. fischeri*, SypE (discussed above) and SypA exhibit similarities to proteins in partner switching systems: SypE contains a serine kinase and serine phosphatase domain (Fig. 5B and 7), while SypA could be an anti-sigma factor antagonist due to its STAS domain (Fig. 5C and 7) (Morris et al., 2011, Morris & Visick, 2013b). In addition, SypE and SypA physically interact and SypE phosphorylates SypA via its N-terminal serine kinase domain and dephosphorylates SypA using its C-terminal serine phosphatase domain (Fig. 7) (Morris & Visick, 2013b). Furthermore, SypA is phosphorylated on a conserved serine, S56 (Fig. 5C and 7), which is critical for biofilm formation and host
colonization. However, SypE and SypA do not appear to follow the standard model of partner switching.

In other systems, the serine kinase and serine phosphatase domains are contained within separate proteins. In contrast, SypE contains both of these domains in its N- and C-termini, respectively (Fig. (Morris & Visick, 2010). Furthermore, in these other systems, the serine kinase functions downstream of the anti-sigma factor antagonist (Morris & Visick, 2010), which does not appear to be the case for SypE. Instead, the phenotype of a sypA mutant is epistatic to that of a sypE mutant: a single sypE mutant exhibits a slight delay in biofilm formation, while both the single sypA mutant and the double sypA sypE mutant were unable to promote biofilm formation (Morris & Visick, 2013b). A similar result was obtained when constitutively active sypA and sypE mutant alleles were examined: while SypE^{D192A} inhibits biofilm formation and host colonization in the presence of wild-type SypA, it is unable to do so in the presence of SypA^{S56A}, which cannot be phosphorylated (Morris & Visick, 2013b). Thus, SypA plays a critical role in controlling biofilm formation by functioning downstream of SypE (Fig. 4 and Fig. 7), though whether SypA functions as an anti-sigma factor antagonist or promotes another function is currently unknown. Whatever activity SypA promotes appears to occur downstream of syp transcription (Morris & Visick, 2013a).

(E) SypF and VpsR

The syp locus contains another regulatory gene, located between the two RR genes (sypE and sypG), that encodes a putative hybrid SK, SypF (Fig. 4). SypF contains three conserved domains (HisKA, REC, and Hpt) and residues predicted to be involved
in phosphotransfer (H250, D549, and H705, respectively) (Fig. 5A). Similar to RscS, SypF contains 2 additional putative signaling regions, a PP loop and a cytoplasmic domain (Fig. 5A). Whereas RscS contains a PAS domain, SypF contains a HAMP domain (Fig. 5A). Similar to PAS domains, HAMP domains are often involved in signal transduction. HAMP domains contain a highly conserved helix-turn-helix fold, a motif also common in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases; the fold but not the amino acid sequence is conserved, making it difficult to identify these domains (Kishii et al., 2007). While the HAMP domain and periplasmic loop of SypF are likely involved in signal transduction, the signal(s) sensed by SypF are unknown.

Like RscS, SypG, and SypE, SypF also appears to serve as a regulator of biofilm formation in V. fischeri (Fig. 4). Overexpression of a signal-independent allele of sypF, sypF*, resulted in a 10-fold increase in syp transcription and four distinct biofilm phenotypes: 1) cell aggregation (the clumping of cells in liquid culture under shaking growth conditions, not to be confused with symbiotic aggregation), 2) wrinkled colony formation, 3) pellicle formation, and 4) increased glass attachment (3 fold increase vs. the wild-type strain) (Darnell et al., 2008). Sequence analysis of the sypF* allele showed that this allele contains two amino acid substitutions, one at residue 247 (S247F) and the other at residue 439 (V439I). Of these two mutations, the S247F substitution, located in the HisKA domain three residues N-terminal to the conserved histidine (H250), appeared to be more important for the increased activity of sypF* (Darnell et al., 2008). Due to the
proximity of this residue to the conserved histidine, it is likely that this mutation impacts the predicted kinase activity of SypF.

Because SypF is a hybrid SK, it is predicted to exert its effect by acting through a downstream RR. In the chromosome sypF is adjacent to sypG (Fig. 4), thus, it seemed reasonable to expect that SypF worked by activating SypG. Indeed, disruption of sypG eliminated the 10-fold induction of syp transcription caused by overexpression of sypF* (Darnell et al., 2008). Furthermore, disruption of sypG also diminished SypF*-induced cell aggregation, a known syp-dependent biofilm phenotype. Surprisingly, however, the SypF*-induced wrinkled colony and pellicle formation phenotypes were only diminished, not eliminated, by loss of sypG. In addition, loss of sypG did not impact SypF*-induced glass attachment. These results suggested that some of the SypF*-induced phenotypes are SypG-dependent (syp transcription and cell aggregation), while other phenotypes are only partially dependent upon SypG (wrinkled colony and pellicle formation), and yet others were SypG-independent (glass attachment). Overall, while SypF appears to work upstream of SypG to regulate the syp locus (Fig. 4), it also appears to work through another regulator(s) to control biofilm formation.

What is the identity of the other regulator(s)? Besides SypG, V. fischeri is predicted to encode 39 other RRs (Hussa et al., 2007). Of these RRs, a prime candidate was VpsR, an NtrC-like $\sigma^{54}$-dependent activator (Fig. 4 and 5B). In a related Vibrio, V. cholerae, VpsR controls biofilm formation by activating expression of the vps polysaccharide locus (Yildiz et al., 2001). In V. fischeri, a vpsR mutant produces colonies that are mucoid and opaque, distinct from the smooth and yellow-ish colonies formed by
the wild-type strain (Darnell et al., 2008), suggesting that VpsR might play a role in biofilm formation. Indeed, overexpression of sypF* in a vpsR mutant led to diminished wrinkled colony and pellicle formation, similar to when sypG was disrupted, as well as a total loss of glass attachment (which was unaffected by loss of sypG). However, disruption of vpsR did not impact cell aggregation (which was diminished by loss of sypG). Thus, wrinkled colony and pellicle formation appear to depend on both syp and vpsR, while syp transcription and cell aggregation (i.e., cell-cell interactions) are sypG-dependent phenotypes, and glass attachment (i.e., cell-surface interactions) is a vpsR-dependent phenotype. When sypF* was overexpressed in a double sypG vpsR mutant, all SypF*-induced biofilm phenotypes were lost (Darnell et al., 2008), indicating that syp and vpsR together account for all of these phenotypes. Thus, SypF* (and likely wild-type SypF) appears to modulate biofilm formation through regulation of the syp locus and an unknown, vpsR-dependent pathway (Fig. 4).

To begin to elucidate the role of VpsR in biofilm formation, a vpsR overexpression construct was generated. Overexpression of vpsR increased glass attachment under static growth conditions and allowed for weak pellicle formation, but it did not induce wrinkled colony formation (Darnell et al., 2008), suggesting that VpsR plays a role in biofilm formation that is distinct from syp. However, despite the role of the V. cholerae homolog in controlling the vps polysaccharide locus, VpsR-induced biofilm formation did not require the vps-like locus present in V. fischeri (Darnell et al., 2008, Grau et al., 2008, Yildiz & Visick, 2009). Instead, disruption of another polysaccharide locus, responsible for the production of cellulose, eliminated glass attachment and
diminished pellicle formation. It also eliminated another phenotype associated with VpsR and SypF*: overexpression of either regulator caused an increase in the binding of the dye Congo Red, a phenotype that is associated with cellulose production (Teather & Wood, 1982). Together, these data suggest that SypF works upstream of VpsR to regulate cellulose biosynthesis (Fig. 4) and implicate cellulose as a player in V. fischeri biofilm formation and, specifically, cell-surface interactions.

Of these regulators (SypF and VpsR), only VpsR has been investigated for a role in host colonization. When juvenile squid were exposed to a mixture of the vpsR mutant and wild-type V. fischeri, the resulting symbiotic animals contained a higher percentage of the wild-type strain (Hussa et al., 2007), suggesting that the vpsR mutant exhibited a slight colonization defect. However, since this mutant exhibited multiple phenotypes (motility, colony morphology, cellulose biosynthesis) (Darnell et al., 2008, Hussa et al., 2007), it is unclear whether the slight colonization defect resulted from a lack of one of these processes, from the loss of a combination of them, or from an as-yet-unknown factor. Further studies are needed to better assess the roles of SypF and VpsR in host colonization.

In conclusion, these studies: 1) further demonstrate the complexity of biofilm formation in V. fischeri; 2) indicate that vpsR is conserved, but serves a distinct role in biofilm formation in V. fischeri relative to V. cholerae; 3) suggest that cell-surface interactions (glass attachment) depend upon cellulose, while cell-cell interaction (pellicles and cell aggregation) depend upon syp; and 4) suggest that the cellulose biosynthetic gene cluster is regulated by multiple proteins. Many unanswered questions
remain, including the identity of the environmental signal(s) that stimulate the formation of syp- and cellulose-dependent biofilm formation and the role, if any, of cellulose in promoting host colonization.

**Biofilm matrix components**

As mentioned in previous sections, wild-type *V. fischeri* does not exhibit biofilm formation unless a regulatory gene (*rscS* or *sypG*) is overexpressed (Yip et al., 2006; Hussa et al., 2008). Induction of biofilm formation leads to discernable phenotypes, such as wrinkled colony formation, the production of a pellicle at the air-liquid interface in static cultures, and glass attachment (Fig. 6). These phenotypes are likely due to the production of biofilm components, such as polysaccharides, proteins, and eDNA. While little is known about the role of eDNA and proteins, there is some information regarding the role of the Syp polysaccharide and, to a lesser extent, outer membrane vesicles (OMVs) in biofilm formation by *V. fischeri*.

*V. fischeri* appears to produce at least two different types of polysaccharides, the Syp polysaccharide and cellulose. Production of the Syp polysaccharide requires genes encoded within the *symbiosis polysaccharide* (*syp*) locus (Fig. 4), which was identified via a transposon mutagenesis screen for novel genes involved in the symbiotic association between *V. fischeri* and its squid host (Yip et al., 2005); transposon insertions within this locus resulted in strains that were defective in host colonization. The *syp* locus contains 18 genes, divided into at least four operons (*sypA-H, sypI-L, sypM-O, and sypP-Q*) (Fig. 4). Four of these genes encode regulatory proteins (SypA, SypE, SypF and SypG) (Fig. 4). The remainder of this locus encodes proteins that appear to be involved in
polysaccharide synthesis and transport, and are divided into three classes: glycosyltransferases, polysaccharide exporters, and ‘other’ (Fig. 4) (Yip et al., 2005, Shibata et al., 2012).

The glycosyltransferases, which would catalyze the transfer of a sugar to the growing polysaccharide chain, are SypH, SypI, SypJ, SypN, SypP, and SypQ. These proteins are predicted to be glycosyltransferase 1 family proteins, with the exception of SypQ, which is predicted to be a glycosyltransferase 2 family protein. The predicted polysaccharide exporters are SypC, a Wza-like polysaccharide exporter, and SypK, an RfbX/Wzx O-antigen unit translocase or flippase. Lastly, the ‘other’ proteins are SypB, an OmpA-like regulator of cellulose production, SypD, an EspG/Wzc-like chain length determinant, SypL, an RfaL/WaaL-like lipid A core-O-antigen ligase, SypM, a sugar O-acetyltransferase, SypO, a Wzz-like chain length determinant protein, and SypR, a WbaP-like undecaprenyl-phosphate galactose phosphotransferase.

To understand the role and importance of the syp structural genes during biofilm formation, Shibata et al. constructed in-frame deletions of each of these genes and examined the ability of these mutants to colonize juvenile squid and promote wrinkled colony and pellicle formation, as well as glass attachment (Shibata et al., 2012). With the exception of sypB and sypI, the syp structural genes are required for host colonization and wrinkled colony and pellicle formation. However, these genes are not necessary for glass attachment (Shibata et al., 2012), suggesting that Syp is not involved in this phenotype. These data are consistent with similar data obtained from Darnell et al. (Darnell et al., 2008). These authors also analyzed Syp-dependent polysaccharide production by these
mutants via western blot analysis with an antibody that presumably recognizes the Syp polysaccharide. Compared to the control strain, the glycosyltransferase mutants (with the exception of the \textit{sypI} mutant) and the \textit{sypK} mutant did not stain with the antibody, while \textit{syp} mutants from the ‘other’ category exhibited altered banding patterns (with the exception of the \textit{sypB} and \textit{sypO} mutants). These data fit nicely with the predicted roles of the \textit{syp} structural genes and indicate that the majority of these genes are necessary for biofilm formation and host colonization by \textit{V. fischeri}.

It is unclear whether the Syp polysaccharide is associated with the cell and/or released into the extracellular matrix. Evidence suggests that at least some of the Syp polysaccharide may be cell associated, as nonproteinaceous molecules that depend upon an intact \textit{syp} locus can be obtained via LPS extraction (Shibata & Visick, 2012, Shibata et al., 2012). In addition, little is known about the composition of this polysaccharide. One study found that the lectin Concanavalin A bound to the polysaccharide (Yip et al., 2006), suggesting that this molecule contains glucose and/or \(\alpha\)-linked mannose residues. Thus, an exciting future direction would be to determine the composition of this polysaccharide, the identity of which could aid in our understanding of the role of this molecule during biofilm formation and host colonization.

Although little is known about the role of eDNA and proteins in the biofilm matrix, Shibata \textit{et al.} identified another possible component of the matrix: outer membrane vesicles or OMVs (Shibata & Visick, 2012). OMVs are normally produced by wild-type \textit{V. fischeri}, though OMV production is increased in strains of \textit{V. fischeri} induced for biofilm formation (i.e., \textit{rscS} overexpression). Additionally, the OMVs
produced by biofilm-induced strains are antigenically distinct from those produced by wild-type cells: biofilm-specific antibodies only recognize molecules on OMVs produced by biofilm-induced cells. Surprisingly, the increase in OMV production and the differences in the surface of these OMVs depends upon an intact syp locus: deletion of sypK results in wild-type levels of OMV production and OMVs that are not recognized by the biofilm specific antibody. Thus, biofilm-induced OMV production appears to be syp-dependent, and it seems likely that these OMVs may be decorated with the Syp polysaccharide. However, the role of OMVs in biofilm formation is unclear, as is the pathway that ultimately leads to OMV production; some evidence suggests that DegP, a predicted periplasmic serine protease-chaperone, may be involved in this process (Shibata & Visick, 2012). Determining the role of OMVs in biofilm formation and host colonization would be an interesting area of study.

Overall, some of the components of the biofilm matrix have been identified. The Syp polysaccharide seems to make up a large part of the matrix and mainly appears to promote cell-cell interactions. In addition, OMVs also seem to be a part of the biofilm matrix, though their exact role is unclear. To date, no studies have identified any matrix proteins or a role for eDNA in biofilm formation. However, given the importance of these components to biofilm formation by other bacteria, it seems likely that they would also be involved in biofilm formation by V. fischeri. Thus, future studies should focus on understanding the composition of the biofilm matrix and the mechanisms by which these components impact natural biofilm formation both inside and outside the host.
Conservation of the syp locus in other *Vibrio* spp.

*V. fischeri* is not the only Vibrio that contains the *syp* locus. As of 2005, all sequenced *Vibrios*, with the exception of *V. cholerae*, contained a *syp* or *syp*-like locus (Yip et al., 2005). More recently, it appears that the *syp* locus is also conserved in *Aeromonas* spp. (K. Visick, personal communication). Though some sequence divergence is evident, the majority of the genes within this locus are conserved, with the exception of *sypE*, which is absent from the *syp* locus of most *Vibrio* species, and *sypF*, which is poorly conserved. However, the role of the *syp* and *syp*-like locus from these other bacteria is poorly understood. Given that this locus is involved in biofilm formation and host colonization by *V. fischeri*, it seems likely that it also may play a role in promoting biofilm formation and host colonization in these other bacteria.

Only two studies have examined the role of the *syp* locus outside of *V. fischeri*; both of these studies have examined this locus in *V. vulnificus*. The first study identified a *syp*-like locus in *V. vulnificus* that was regulated by an NtrC-like protein and involved in production of an exopolysaccharide (Kim et al., 2009); this polysaccharide is likely involved in promoting cell-cell interactions during biofilm formation. The second study further assessed the role of this *syp*-like locus, now termed the *rbd* locus, in biofilm formation by *Vibrio vulnificus* (Guo & Rowe-Magnus, 2011). The *rbd* locus contains 18 genes that are divided into four operons (*rbdA*-H, *rbdI*-L, *rbdM*-O, and *rbdP*-R); it appears that only the *sypE*-like gene is absent from this locus and is replaced by a gene that encodes a predicted phosphonate transport system substrate-binding protein. Each operon contains a promoter with a predicted $\sigma^{54}$ binding site, as well as an enhancer.
binding site that is almost identical to that found in *V. fischeri*. Similar to regulation of the *syp* locus, the *rbd* locus encodes a SypG-like protein, RbdG, which is likely the direct transcriptional activator of this locus. Expression of the *rbd* locus enhances *V. vulnificus* biofilm formation, which likely plays a role in the ecology of this organism. Together, these data support the hypothesis that *syp*-like loci in other bacteria function in biofilm formation. In addition, this work validates the use of *V. fischeri* as a model organism to study biofilm formation, specifically *spy*-dependent biofilm formation in the context of host colonization, as the *syp* locus is likely important for this process in a variety of other, more pathogenic *Vibrio* spp.

**Conclusions**

*V. fischeri* is an ideal organism for the study of biofilm formation, as it is genetically tractable and its ability to form a biofilm can be studied in the context of the natural host. Biofilm formation by *V. fischeri* is mediated by a complex network of regulators, though the exact function of some of these regulators, such as SypF and VpsR, has yet to be determined. In addition, the majority of these regulators are necessary for host colonization, indicating the importance of biofilm formation for this process. Of note, the biofilm forming capacity of different *V. fischeri* isolates varies depending on the relative salinity and temperature of the seawater (Chavez-Dozal & Nishiguchi, 2011), suggesting that the environmental conditions experienced by *V. fischeri* will impact its ability to form a biofilm. Though there is still much to learn about biofilm formation and its regulation by *V. fischeri*, the information that is available
extends our understanding about biofilm formation by bacteria, as well as the role of biofilm formation during bacteria-host interactions.

V. QUORUM SENSING MEDIATED REGULATION OF BIOLUMINESCENCE BY VIBRIO FISCHERI

Introduction

Bacteria utilize a variety of mechanisms to respond to their environment. One such mechanism is quorum sensing (QS), which relies on cell density and the production of signaling molecules called autoinducers (AIs). At a threshold concentration (e.g., high cell density), these signals can be then taken up into the cell to promote a specific response. The response can be mediated via AI signaling to 2CST regulators or by the direct binding of the AI to a transcription factor to promote the expression of a gene or set of genes. In V. fischeri, both of these QS mechanisms are utilized to regulate the production of cellular bioluminescence. The pathway involved in this regulation is the Lux pathway, which consists of the Lux phosphorelay and the LuxR/LuxI circuit. This pathway is critical for V. fischeri to enter into a productive symbiosis with its squid host: the squid utilize the bacterially-produced bioluminescence to avoid predation while they forage in shallow bays at night (Jones & Nishiguchi, 2004). In addition, this pathway contributes to biofilm regulation by V. fischeri, which will be discussed further in Section I of Chapter Three.

Control of bioluminescence by V. fischeri

V. fischeri utilizes a complex network of regulators to control light production in a cell density-dependent manner: bioluminescence is repressed at low cell densities and
induced at high cell densities. Specifically, there are two separate AI-based regulatory
circuits that control bioluminescence that I will discuss in this section. The first is (A) the
LuxR/LuxI circuit, in which the transcription factor LuxR binds to the AI produced by
LuxI to promote transcription of the genes necessary for the production of
bioluminescence (i.e., the lux genes) (Fig. 8). The second regulatory circuit is (B) the Lux
phosphorelay, which consists of the hybrid SKs LuxQ and AinR, whose activities rely on
AI signaling, the histidine phosphotransferase (Hpt) LuxU, and the RR LuxO (Fig. 8).
The Lux phosphorelay is ultimately linked to the LuxR/LuxI circuit via (C) LuxO, the
sRNA Qrr1, and LitR, the direct transcriptional activator of luxR (Fig. 8). I will also
provide additional information on (D) the AI synthases AinS and LuxS and (E) the hybrid
SK AinR; though the exact role of (F) LuxQ and LuxU are unknown, I will provide
information regarding the predicted functions of these proteins, as they will be the topic
of discussion in Section I of Chapter Three.
(A) The LuxR/LuxI circuit

The LuxR/LuxI circuit from V. fischeri was one of the first AI-based regulatory
circuits described in bacteria (Nealson & Markovitz, 1970, Eberhard, 1972, Engebrecht et
al., 1983, Kaplan & Greenberg, 1985, Fuqua et al., 1994). LuxR is a transcription factor
that becomes competent to activate transcription when bound to the AI N-3-oxo-hexanoyl
homoserine lactone (3-O-C₆-HSL), synthesized by LuxI (Fig. 8) [reviewed in (Sitnikov et
al., 1995)]. During growth in culture, luminescence is initially low, as is the
concentration of 3-O-C₆-HSL, but as the cell density increases, so does the concentration
of this AI. This increased concentration of AI favors its interaction with LuxR, which
Figure 8. Models of bioluminescence regulation by Lux in V. harveyi and V. fischeri.

(A) In V. harveyi, at low cell densities the AI concentration is low and the hybrid SKs (LuxN, CqsS, and LuxQ) act as kinases, serving as phospho-donors to the Hpt protein LuxU, which serves as the phospho-donor to the RR LuxO. LuxO becomes active when phosphorylated and promotes the transcription of five sRNAs (qrr1-5), which, in conjunction with Hfq, bind to and destabilize the mRNA transcript of the transcriptional activator LuxR\textsubscript{Vh}. Without LuxR\textsubscript{Vh}, the lux operon is not transcribed and luminescence is not induced. (B) In V. harveyi, at high cell densities the AI concentration increases (represented by the cross, sun, and star), and thus these molecules can interact with their respective hybrid SK and induce a conformational change that promotes phosphatase over kinase activity (in the case of LuxQ, AI-2 binds to LuxP, a periplasmic associated protein, which promotes the conformational change in LuxQ). As phosphatases, the hybrid SKs promote the removal of phosphoryl groups from LuxO, through LuxU. Unphosphorylated LuxO is no longer able to promote transcription of qrr1-5, in which case LuxR\textsubscript{Vh} is produced. LuxR\textsubscript{Vh} binds to the promoter region of the lux operon and promotes transcription, thus inducing luminescence. (C) In V. fischeri, Lux regulation at low cell densities and high cell densities is predicted to be similar to that of V. harveyi, except: 1) there is only one qrr sRNA, which, in conjunction with Hfq, binds to and destabilizes the mRNA transcript for the transcriptional activator protein LitR, which is similar to LuxR\textsubscript{Vh}; 2) at high cell densities, LitR is produced and activates transcription of luxR in V. fischeri, which, when bound by its AI 3-O-C6-HSL promotes transcription of...
the *lux* operon in *V. fischeri*. *V. harveyi* does not contain this LuxR/I circuit, while *V. fischeri* does not contain CqsS or CqsA.
becomes competent to induce transcription of the \textit{luxICDABEG} operon, which encodes the proteins necessary for light production (Fig. 8) (Engebrecht & Silverman, 1984, Meighen, 1991, Stevens \textit{et al.}, 1994). The \textit{luxA} and \textit{luxB} genes encode the two subunits of the enzyme luciferase, which utilizes long-chain aliphatic aldehyde, reduced flavomononucleotide (FMNH$_2$), and oxygen to produce light, aliphatic acid, oxidized flavomononucleotide (FMN) and water (Ziegler & Baldwin, 1981). The other \textit{lux} genes are thought to encode an aliphatic acid reductase complex (LuxC, LuxD, and LuxE), involved in recycling aliphatic acid to aldehyde (Boylan \textit{et al.}, 1989), and a protein that reduces FMN to FMNH$_2$ using NADH (LuxG) (Zenno & Saigo, 1994). These proteins together are sufficient to permit a non-bioluminescent organism, such as \textit{E. coli}, to produce light (Engebrecht \textit{et al.}, 1983, Engebrecht & Silverman, 1984).

As a result of this AI-based regulatory mechanism, \textit{luxR} and \textit{luxI} are not only required for luminescence in culture, but luminescence during host colonization. While \textit{luxR} and \textit{luxI} mutants only exhibit a slight decrease in luminescence in culture, during host colonization these mutants produce no detectable light at any time during colonization (Visick \textit{et al.}, 2000), indicating that these regulators are critical for luminescence induction. Furthermore, the \textit{luxR} and \textit{luxI} mutants exhibited a persistence defect: they were unable to sustain the high colonization levels of the wild-type strain (Visick \textit{et al.}, 2000). These data suggest that these regulators are necessary for \textit{V. fischeri} to persist within the light organ. Similarly, mutants defective for the luciferase gene \textit{luxA} (Visick \textit{et al.}, 2000) or the entire \textit{lux} operon (Bose \textit{et al.}, 2008) exhibited a defect in persistence, suggesting that the colonization defects of \textit{luxR} and \textit{luxI} mutants can be
attributed to a lack of bioluminescence. Thus, bioluminescence regulation, at least the LuxR/LuxI circuit, and bioluminescence itself are critical determinants of host colonization. However, this circuit, and ultimately luminescence, is regulated by another AI-based system, the Lux phosphorelay.

(B) The Lux phosphorelay

The second AI-based regulatory circuit in *V. fischeri* is the Lux phosphorelay, which also reports on cell density but does not rely on the AI produced by LuxI. The Lux phosphorelay is similar to the well-studied pathway in *V. harveyi* (Fig. 8) (Ng & Bassler, 2009), which consists of three AI synthase proteins (LuxS, LuxM, and CqsA), which produce the signals detected by three hybrid SKs (LuxQ, in association with LuxP, LuxN, and CqsS, respectively), the Hpt protein LuxU, and the RR LuxO. Briefly, at low cell densities when the AI concentrations are low, the hybrid SKs exhibit kinase activity, donating phosphoryl groups to LuxU, which serves as a phospho-donor to LuxO. Phosphorylated LuxO directs the transcription of 5 small RNAs (sRNAs) (*qrr1*-5), which bind to and destabilize the mRNA transcript of a transcription factor known as LuxR and which we will designate as LuxR<sub>Vh</sub>; the *V. harveyi* protein is not homologous to LuxR from *V. fischeri*. LuxR<sub>Vh</sub> serves as the direct transcriptional activator of the *luxCDABEGH* operon. Thus, at low cell densities, little LuxR<sub>Vh</sub> is produced due to transcript instability, and luminescence is not induced. As the cell density increases, AI signaling induces the hybrid SKs to switch from kinase activity to phosphatase activity, promoting the removal of phosphoryl groups from LuxO, through LuxU.
Unphosphorylated LuxO is no longer active, leading to increased LuxR\textsubscript{Vh} and subsequent induction of luminescence.

The Lux phosphorelay in \textit{V. fischeri} is similar to that of \textit{V. harveyi}, and is predicted to function in largely the same way (Fig. 8). \textit{V. fischeri} contains homologs of LuxP, LuxQ, LuxU, LuxO, and LuxS, as well as LuxN and LuxM (called AinR and AinS, respectively). However, there are a few distinct differences between the Lux phosphorelays in \textit{V. harveyi} and \textit{V. fischeri}: 1) LuxM and AinS do not produce the same AI (Cao & Meighen, 1989, Kuo \textit{et al.}, 1994), 2) \textit{V. fischeri} does not encode CqsS or CqsA, 3) \textit{V. harveyi} does not contain the LuxR/LuxI circuit, and 4) LuxR\textsubscript{Vh} is homologous to LitR from \textit{V. fischeri}, which is the transcriptional regulator for \textit{luxR} from \textit{V. fischeri}. Similar to \textit{luxR}\textsubscript{Vh}, \textit{litR} is subject to LuxO-mediated regulation, which will be discussed further below. The following sections will describe the role of these regulators in bioluminescence control.

(C) The RR LuxO, the sRNA Qrr1, and the transcriptional regulator LitR

(i) \textit{LuxO}

LuxO is a RR that links the Lux phosphorelay to the LuxR/LuxI circuit in \textit{V. fischeri} (Fig. 8). LuxO was initially identified as a regulator of bioluminescence in \textit{V. harveyi} (Bassler \textit{et al.}, 1994b). LuxO homologs were subsequently identified in two \textit{V. fischeri} strains: ES114, a squid symbiont, and MJ1, an organism that does not colonize squid, but in which luminescence has been extensively studied (Miyamoto \textit{et al.}, 2000). In both strains, LuxO exerts negative control over bioluminescence: \textit{V. fischeri luxO} mutants exhibited an increase in luminescence over the respective wild-type strains.
(Lupp et al., 2003, Miyamoto et al., 2000). These results are similar to what was observed in *V. harveyi*, and indicate that LuxO serves as an inhibitor of luminescence in both species.

The LuxO protein from MJ1 has been characterized in greater detail than that of ES114. The MJ1 protein shares 72% identity with LuxO from *V. harveyi*, and contains the conserved REC domain and putative site of phosphorylation, D47, in its N-terminus (Miyamoto et al., 2000). Similar to SypG, LuxO also contains a centrally located putative σ^54^-interaction domain, with a predicted ATP-binding site, and a C-terminal putative DNA binding domain. These features are conserved in the ES114 protein, as the two *V. fischeri* proteins differ at only two residues (V/G 114 and G/C 177). Thus, like the *V. harveyi* protein, *V. fischeri* LuxO likely activates transcription at a σ^54^-dependent promoter to control expression of genes involved in luminescence. In support of that idea, Wolfe et al. demonstrated that an *rpoN* mutant exhibited increased luminescence compared to the control strain (Wolfe et al., 2004); *rpoN* is the gene for σ^54^. In addition, Miyashiro et al. demonstrated that LuxO promotes transcription of an sRNA, designated *qrr1* (Miyashiro et al., 2010), which, in other *Vibrios*, is regulated in a σ^54^-dependent manner. In *V. fischeri*, this sRNA represses production of LitR, the transcriptional activator of *luxR*; Qrr1 and LitR will be discussed in more detail below. Together, these data indicate that, indeed, LuxO from *V. fischeri* likely functions as predicted.

During squid colonization, a luxO mutant exhibits an initiation defect: when used to inoculate juvenile *E. scolopes*, the luxO mutant only reaches 37% of the colonization level of wild-type *V. fischeri* at 12 hours post-inoculation (Lupp & Ruby, 2005). This is
an early time point, before the animals become fully colonized; thus, these data indicate that the luxO mutant has a defect in initiating colonization. However, at 72 hours post-inoculation, the colonization levels of the wild-type strain and the luxO mutant are comparable (Lupp & Ruby, 2005), suggesting that the luxO mutant is only defective at initiation, not persistence. This is perhaps not surprising, as at this late time point, luminescence is fully induced and LuxO should be inactivated. The luxO mutant is also defective in colonization when the wild-type strain is present: when juvenile squid were inoculated with a mixture of wild-type and luxO mutant strains, the luxO mutant exhibited a colonization disadvantage relative to the wild-type strain at 24-48 hours post-inoculation (Hussa et al., 2007, Lupp & Ruby, 2005, Miyashiro et al., 2010). Whether the competition defect can be attributed to the initiation defect of the luxO mutant, or to the loss of control over a persistence factor not required during single strain colonization, remains to be determined.

What could account for the initiation defect of the luxO mutant? As mentioned above, bioluminescence is only required at later stages of colonization (i.e., persistence) (Lupp & Ruby, 2005, Visick et al., 2000) and, thus, light per se cannot account for the initiation defect of the luxO mutant. Array experiments revealed that mutations in luxO impacted expression of motility genes and, in support of this, luxO mutants exhibited decreased migration in motility assays (Hussa et al., 2007, Lupp & Ruby, 2005). Previous studies have demonstrated that motility is essential for the initiation of colonization (e.g., (Graf et al., 1994)) and, thus, the motility defect could be responsible for the initiation defect of the luxO mutant. Alternatively, other targets of LuxO activity identified in the
array analysis, including regulatory proteins and genes involved in nutrient uptake and metabolism, could account for the initiation defect of the luxO mutant. Thus, the precise role of LuxO in initiation remains unclear. Taken together, LuxO is a critical regulator of bioluminescence, though further studies of LuxO and LuxO-mediated activities are necessary to fully understand how LuxO impacts luminescence, other cellular processes, and ultimately host colonization.

(ii) Qrr1

As mentioned above, the Lux phosphorelay is linked to the LuxR/LuxI circuit by LuxO. However, this link is indirect. According to the model generated from V. harveyi, LuxO would regulate the transcription of an sRNA that, in conjunction with Hfq, would bind to and destabilize the litR mRNA transcript in V. fischeri, ultimately resulting in little or no luxR transcription and decreased luminescence (Fig. 8). This sRNA is called qrr. Unlike V. harveyi, which contains 5 qrr genes, and V. cholerae, which contains 4 qrr genes (Lenz et al., 2004), V. fischeri contains only one qrr gene, qrr1 (Miyashiro et al., 2010). This gene is transcribed divergently from luxO and, as the model predicts, is regulated in a cell density-dependent manner via LuxO: qrr1 levels are increased at low cell density, and decreased at high cell density. Furthermore, in a luxO mutant, qrr1 expression is low regardless of cell density (Miyashiro et al., 2010).

In accordance with the model, Qrr1 regulates litR mRNA levels and luminescence: a qrr1 mutant exhibited increased litR transcript levels and luminescence similar to a luxO mutant (Miyamoto et al., 2003, Miyashiro et al., 2010), suggesting that Qrr1 negatively regulates luminescence through controlling litR transcript levels.
Furthermore, Qrr1 itself is sufficient to regulate litR mRNA transcript levels: overexpression of qrr1 in a luxO qrr1 double mutant resulted in a decrease in litR transcript levels (Miyashiro et al., 2010). Thus, LuxO’s control of qrr1 ultimately links the Lux phosphorelay to the LuxR/LuxI circuit.

The role of qrr1 during squid colonization is unclear, as a qrr1 mutant does not exhibit a colonization defect unless mixed with the wild-type strain (Miyashiro et al., 2010). These results are not surprising, as Qrr1 is a repressor of luminescence, which is required for efficient host colonization. However, this study only examined the 48 h time point post-inoculation. It would thus be of interest to determine whether qrr1 is necessary at earlier stages of colonization, such as initiation, as other regulators of luminescence (but not luminescence itself) are known to be important during this stage of colonization.

(iii) LitR

The gene for litR was identified in a search for LuxR_Vh homologues in V. fischeri (Fidopiastis et al., 2002). Since V. fischeri appeared to encode a LuxR_Vh-like regulator, LitR was predicted to impact luminescence regulation. This was indeed the case, as loss of LitR from V. fischeri resulted in decreased luminescence as compared to the control strain. Unlike LuxR_Vh, LitR is not the direct transcriptional activator of the lux operon. However, LitR is the direct transcriptional activator of lux_RV (Fig. 8); Fidopiastis et al. demonstrated that LitR binds to a sequence located in the lux_RV promoter region (Fidopiastis et al., 2002).

In addition to regulating luminescence, LitR has also been shown to regulate motility (Lupp & Ruby, 2005), acetate metabolism (through AinS) (Studer et al., 2008),
and the alternative sigma factor RpoQ, which appears to negatively regulate motility and bioluminescence in an unknown manner (Cao et al., 2012). Thus, LitR appears to feed into multiple pathways to regulate more than just luminescence, though the implications of this regulation have yet to be determined.

During symbiotic colonization, litR mutants do not exhibit a colonization or persistence defect (Fidopiastis et al., 2002). Surprisingly, however, during a mixed inoculum experiment using the wild-type strain and the litR mutant, the litR mutant outcompeted the wild-type strain for colonization at 48 h post-inoculation. These data suggest that LitR is not necessary for colonization, though its presence likely promotes certain functions that are slightly inhibitory to colonization. They also indicate that the LuxR/LuxI circuit must dominate over the Lux phosphorelay in symbiosis, as lux mutants have a competitive disadvantage at 48 h in symbiosis (Visick et al., 2000). Taken together, LitR is a critical regulator of luminescence, at least under laboratory culture conditions and, like LuxO, links the Lux phosphorelay to the LuxR/LuxI circuit.

(D) The autoinducer synthases AinS and LuxS

In many 2CST systems, the SK(s) have been well-characterized, while the signal(s) sensed by these components are unknown or less well characterized. However, in *V. fischeri*, there have been limited studies of the hybrid SKs (AinR and LuxQ), while multiple studies have examined the importance of the signal synthase proteins AinS and LuxS, which produce the AI signal molecules C8-HSL and AI-2, respectively (Fig. 8). Thus, this next section will focus on the role of AinS and LuxS in regulating luminescence.
(i) *AinS*

*AinS* synthesizes the AI N-octanoyl-homoserine lactone (C₈-HSL) that is proposed to signal through the hybrid SK AinR (Gilson *et al.*, 1995, Kimbrough & Stabb, 2013). C₈-HSL also appears to serve as a second AI that activates LuxR-mediated transcription of the *lux* operon (Kuo *et al.*, 1994, Kuo *et al.*, 1996). *AinS* was initially identified and characterized as a luminescence regulator in the *V. fischeri* strain MJ1 (Kuo *et al.*, 1994, Kuo *et al.*, 1996, Gilson *et al.*, 1995). However, recent studies have focused on *AinS* from the squid symbiont ES114, which will be the focus of discussion here.

In *V. harveyi*, LuxM (a homolog of *AinS*) synthesizes an AI sensed by the hybrid SK LuxN, which functions through LuxU to control the activities of LuxO [reviewed in (Ng & Bassler, 2009)]. Thus, although the *AinS*-produced signal is distinct from that produced by LuxM, it likely functions in a similar manner, signaling to the hybrid SK AinR to regulate LuxO activity in *V. fischeri*. Indeed, an *ainS* mutant of ES114 produced no detectable light in culture (Lupp *et al.*, 2003). Surprisingly, this mutant also exhibited a growth yield defect, reaching only 75% of the wild-type level (Lupp *et al.*, 2003). This growth yield defect could be suppressed by the addition of exogenous C₈-HSL, indicating that the loss of the signal molecule was indeed responsible for the phenotype. Thus, it appears that, like LuxO, the *AinS*-produced signal regulates more than just luminescence. In support of this idea, an array analysis that compared transcripts from wild-type and *ainS* mutant cells revealed differences in the control of genes involved in metabolism and nutrient uptake (Lupp & Ruby, 2005). Subsequently, Studer *et al.* demonstrated that the
AinS mutant was unable to utilize the acetate that is normally secreted and assimilated by *V. fischeri* cells (Studer et al., 2008). As a result, the mutant cultures became acidified, which caused the loss of cell viability. Thus, AinS activity appears to regulate multiple cellular functions, including luminescence and acetate utilization.

The *V. harveyi* paradigm predicts that the AinS-produced signal, C₈-HSL, should function to control LuxO activity. If this were true, then the phenotypes of the *ainS* mutant should depend on LuxO function. Indeed, this was the case: a double *ainS luxO* mutant exhibited luminescence and growth yield phenotypes indistinguishable from the *luxO* single mutant (i.e., high luminescence and normal growth) (Lupp et al., 2003). These results are consistent with the idea that AinS or, more specifically, C₈-HSL acts upstream of LuxO to control both luminescence and acetate utilization.

To understand how *ainS* itself was regulated, Lupp and Ruby evaluated levels of C₈-HSL and *ainS* transcription during growth of ES114 (Lupp & Ruby, 2004). They found that over time the concentration of C₈-HSL increased over 2,000-fold, concurrent with the increase in luminescence. Furthermore, *ainS* transcription was initially low, but increased exponentially until cells reached stationary phase, consistent with the increase in the amount of C₈-HSL produced during growth. These data suggested that *ainS* autoregulates. The same pattern of *ainS* transcription occurred in *luxR* mutants, indicating that *ainS* autoregulates independently of the LuxR/LuxI circuit. However, autoregulation depended upon the Lux phosphorelay, as *ainS* transcription was decreased in an *ainS* mutant, but could be restored to normal levels by the disruption of *luxO* (Lupp & Ruby,
Thus, the C₈-HSL signal not only controls luminescence and metabolism, but also its own production, in a manner that depends on LuxO.

Although the ainS mutant produced no light in culture (Lupp et al., 2003), similar to a luxA mutant (Visick et al., 2000), during colonization it produced detectable levels of light (between 10-40% of that observed for the wild-type strain) (Lupp et al., 2003). These data indicate that other factors contribute to luminescence control during symbiosis. This mutant also exhibited an initiation defect: at an early time (12 hours) following inoculation, the ainS mutant reached only 45% of the wild-type colonization level (Lupp & Ruby, 2005). This defect likely occurs after the aggregation stage, as this mutant did not appear to be defective in the timing of aggregate production or the size of the aggregate. Finally, the ainS luxO double mutant exhibited the same initiation defect as the luxO single mutant (37% and 36% colonization, respectively), which was not much different than the single ainS mutant (45% colonization). These results support the idea that AinS and LuxO function in the same pathway, and suggest that the initiation defect of the ainS mutant is likely due to some LuxO-regulated process.

In addition to its initiation defect, the ainS mutant exhibited a persistence defect: at 24 hours post-inoculation, the levels of colonization by the mutant were 75% that of wild-type, and further decreased to 20% at 72 hours post-inoculation (Lupp & Ruby, 2004). However, this persistence defect was not due to the defect in acetate utilization: an acs mutant, which is similarly defective in acetate utilization, achieved the same level of colonization as the wild-type strain at 48 hours post-inoculation (Studer et al., 2008). Finally, disruption of luxO in the ainS mutant background restored colonization levels to
that achieved by both the wild-type and single luxO mutant strains, indicating that the
defect of the ainS mutant depends upon LuxO function (Lupp & Ruby, 2005).

In summary, the AinS-produced signal (C₈-HSL) controls multiple phenotypes via
its control over LuxO activity, and plays roles in symbiosis during at least two stages of
colonization. Given the differences in the environments at these stages, it seems
reasonable to expect that different C₈-HSL-controlled processes are important in
initiation vs. persistence. However, further studies of AinS are necessary to determine
which genes under C₈-HSL control participate at these different times, and what other
regulators might contribute to dictate the appropriate cellular response.

(ii) LuxS

The second AI produced by V. fischeri is synthesized by LuxS. In V. harveyi, LuxS produces the AI AI-2, a furanosyl borate diester (Chen et al., 2002). V. fischeri also
encodes a LuxS protein, and it appears to produce a similar autoinducer molecule (Fig.
8): supernatants from wild-type V. fischeri, but not a luxS mutant, contained AI-2
activity, as measured by the ability to activate luminescence of a V. harveyi luxS mutant
(Lupp & Ruby, 2004). Furthermore, AI-2 from V. fischeri appears to signal through
LuxP/Q as it does in V. harveyi (Fig. 8): wild-type supernatant from V. fischeri was
unable to induce luminescence in a V. harveyi luxQ mutant. To date, there have been no
studies of LuxP in V. fischeri, nor confirmation of the structure of the autoinducer
molecule produced by LuxS; work investigating LuxQ will be discussed in Section I of
Chapter Three.
In *V. fischeri*, a *luxS* mutant reaches about 70% of the luminescence achieved by the wild-type strain (Lupp & Ruby, 2004), suggesting that AI-2 contributes to luminescence regulation in *V. fischeri*, although not to the same extent as C8-HSL. Consistent with its mild effect on luminescence, *luxS* transcription was low and unchanged throughout growth of the cells in culture, and AI-2 levels varied only 4-fold. These data indicate that *luxS* is constitutively expressed, rather than autoregulated like *ainS*. Finally, as predicted from the *V. harveyi* model, AI-2 from *V. fischeri* functions upstream of LuxO: the luminescence output of a double *luxS luxO* mutant resembled the *luxO* single mutant (i.e., increased luminescence) (Lupp & Ruby, 2004). Together, these data suggest that LuxS plays a role, albeit a minor one, in controlling LuxO activity and thus luminescence regulation in culture.

Consistent with this relatively small role in luminescence regulation in culture, the *luxS* single mutant had no observable impact on either symbiotic bioluminescence or squid colonization (Lupp & Ruby, 2004). However, combining the *luxS* and *ainS* mutations resulted in a synergistic defect in colonization: the double mutant reached colonization levels that were 50-75% that of the *ainS* single mutant at both 24 and 48 hours post-inoculation (Lupp & Ruby, 2004). In contrast, this synergistic effect did not appear to occur during colonization initiation: at 12 hours post-inoculation, the *luxS ainS* mutant was no different than that of an *ainS* single mutant (both were approximately 45% that of wild-type), indicating that either AinS plays distinct roles during initiation and persistence, or that AI-2 (produced by LuxS) plays a stronger role during persistence than in initiation. In either case, LuxS and AinS appear to function in the same pathway to
regulate luminescence and LuxO activity, since an *ainS luxS luxO* triple mutant did not exhibit a persistence defect, but resembled the *luxO* single mutant. Thus, LuxS is involved in regulating luminescence, though to a lesser extent than AinS. However, the role of LuxS during squid colonization is unclear and merits further investigation.

(E) AinR

Until recently, little was known about the role of the hybrid SKs LuxQ and AinR in controlling bioluminescence; this section will focus on what is currently known about the role of AinR in regulating bioluminescence. The function of AinR is predicted to be similar to that of LuxN from *V. harveyi* (i.e., serve as a kinase at low cell densities and a phosphatase at high cell densities to modulate LuxO phosphorylation) (Fig. 8). This appears to be the case, as loss of AinR decreased expression of a *qrr1-lacZ* reporter fusion when C$_8$-HSL was present (Kimbrough & Stabb, 2013), indicating that AinR likely signals through LuxO to regulate Qrr1 levels. In addition, expression of *ainR* in a *V. harveyi luxN* mutant (in the presence of its predicted AI C$_8$-HSL) increased luminescence, suggesting that AinR can functionally complement a *luxN* mutant of *V. harveyi*. Taken together, these data demonstrate that AinR appears to function as predicted and, for the first time, suggest that AinR likely responds to C$_8$-HSL. However, AinR also appears to respond to a variety of AIs: HSLs with unsubstituted or substituted acyl chains of six to ten carbons appear to promote AinR phosphatase activity, while acyl chains of four or more than twelve carbons were unable to promote this activity (as measured via the *qrr1-lacZ* reporter) (Kimbrough & Stabb, 2013); the implications of this finding have yet to be determined. This study also found that deletion of *ainR* destabilizes
the ainSR transcript, leading to decreased levels of the C₈-HSL in an ainR mutant. Since this is one of the first studies to examine the role of AinR in the control of bioluminescence by V. fischeri, many question remain, such as, why does AinR sense multiple AIs and does this regulator impact squid colonization? Given the multiple roles of the AinS-produced AI, a deeper understanding of AinR function will help determine how AinS impacts luminescence regulation overall.

(F) LuxQ and LuxU

While the role of the hybrid SK LuxQ and the Hpt LuxU will be discussed in greater detail in Section I of Chapter Three, I will provide a brief description of their predicted roles in luminescence regulation based on the model generated from V. harveyi (Fig. 8). Similar to AinR, LuxQ is predicted to function at the top of the Lux phosphorelay. However, in V. harveyi, LuxQ is associated with the periplasmic protein LuxP (Neiditch et al., 2005), which senses AI-2 (Bassler et al., 1994a); similar to other SKs, the LuxP/Q complex is part of a dimer (Neiditch et al., 2006). In the absence of or at low concentrations of AI-2 (or in the absence of LuxP; (Neiditch et al., 2006)), LuxQ exhibits kinase activity (Freeman & Bassler, 1999b). As the AI-2 concentration increases, this molecule binds to LuxP and promotes an asymmetric rotation of the LuxP/Q dimer (Neiditch et al., 2006), which then promotes LuxQ phosphatase activity.

Ultimately, the activities of LuxQ funnel into LuxU, which is a predicted Hpt protein. However, the only known role of LuxU is to shuttle phosphoryl groups between the SKs and the RR LuxO. In other Vibrios, such as V. anguillarum and V. alginolyticus, some evidence suggests that LuxU may regulate the activity of other RRs (Croxatto et al.,...
However, this activity ultimately feeds back into the Lux pathway. Overall, LuxQ and LuxU serve to regulate the phosphorylation state of LuxO and thus the production of cellular bioluminescence.

**Conclusions**

Both the control of bioluminescence and its production by *V. fischeri* are important processes for this organism to promote an efficient symbiosis with its squid host. However, this regulation is complex and involves two linked AI-based regulatory circuits. The Lux phosphorelay dictates the phosphorylation state of the RR LuxO, which controls the levels of the Qrr1 sRNA, and ultimately the levels of LitR, which controls luxR transcription. Furthermore, in *V. harveyi*, there are multiple levels of feedback within the Lux pathway, providing extra regulatory control over this system. These feedback loops likely exist within the Lux pathway of *V. fischeri*, but have not yet been investigated in any detail, and thus represent an important area of future research.

Overall, the Lux pathway is necessary for luminescence, which is critical for persistence of *V. fischeri* within the squid light organ. In addition, regulators within this pathway, but not luminescence, *per se*, appear to control a number of other cellular functions (e.g., acetate metabolism and motility) that are important at the initiation of colonization (Lupp & Ruby, 2005, Visick et al., 2000). Thus, to better understand how *V. fischeri* functions both inside and outside of its host, future studies should be aimed at determining how the Lux pathway controls these other processes, as well as uncovering new levels of regulation by this pathway.
CHAPTER TWO
MATERIALS AND METHODS

**Bacterial strains and media.** *V. fischeri* strains utilized in this study are listed in Table 1. All strains used in this study were derived from strain ES114, a bacterial isolate from *Euprymna scolopes* (Boettcher & Ruby, 1990). *V. fischeri* strains were grown in one of the complex media LBS (Graf et al., 1994), Sea Water Tryptone (SWT) (Yip et al., 2005), or SWTO (Bose et al., 2007), or in the minimal medium HMM (Ruby & Nealson, 1977). All derivatives of *V. fischeri* were generated via conjugation, as previously described (DeLoney et al., 2002). *E. coli* strains GT115 (Invivogen, San Diego, CA, USA), TAM1 (Active Motif, Carlsbad, CA, USA), TAM1 pir (Active Motif, Carlsbad, CA, USA), CC118 pir (Herrero et al., 1990), β3914 (Le Roux et al., 2007), and π3813 (Le Roux et al., 2007), were used for the purposes of cloning, plasmid maintenance, and conjugation. *E. coli* strains were grown in LB (Davis et al., 1980) or BHI (Difco). Solid media were made using agar to a final concentration of 1.5%. The following antibiotics were added to growth media as necessary, at the indicated final concentrations: chloramphenicol (Cm) at 2.5 µg/ml (*V. fischeri*) or 12.5, 20, or 25 µg/ml (*E. coli*); erythromycin (Em) at 5 µg/ml (*V. fischeri*) or 150 µg/ml (*E. coli*); Tetracycline (Tc) at 5 µg/ml in LBS or 30 µg/ml in HMM, SWT, and SWTO (*V. fischeri*) or 15 µg/ml (*E. coli*); ampicillin (Ap) at 100 µg/ml (*E. coli*). Along with any necessary antibiotics, diaminopimelate (DAP) was added to a
Table 1. *V. fischeri* strains used in this study.

<table>
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<th>Strains</th>
<th>Relevant Genotype</th>
<th>Reference</th>
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<tr>
<td>ES114</td>
<td>wild-type</td>
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<td>(Hussa et al., 2007)</td>
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KV5472  ∆luxO ∆luxU ∆sypE  (Ray & Visick, 2012)
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KV6716  ∆SE-A::SE-A  (Ray et al., 2013)
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KV7079  ∆bamA ∆bamC  This study
KV7080  ∆balA ∆balB  This study
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KV7128  ∆balA ∆balB ∆balC  This study
KV7216  attTn7::P_{bamA}−lacZ ErmR  This study
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KV7274  ∆bamA ∆bamB ∆bamC attTn7::bamA-FLAG  This study

This study
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</table>
final concentration of 0.3 mM for *E. coli* strain β3914 and thymidine was added to a final concentration of 0.3 mM for *E. coli* strain π3813.

**Molecular techniques.** All plasmids were constructed using standard molecular biology techniques, with restriction and modification enzymes obtained from New England Biolabs (Beverly, MA, USA) or Thermofisher (Pittsburgh, PA, USA). Plasmids and primers used in this study are listed in Table 2 and Table 3, respectively. In some cases where PCR was used to generate DNA fragments, PCR cloning vector pJET1.2 (Fisher Scientific, Pittsburgh, PA, USA) or pCR1.2-TOPO (Life Technologies, Grand Island, NY) was used as an intermediate vector prior to cloning into the final vector. Gibson Assembly® (New England Biolabs, Beverly, MA, USA) was also used, in some instances, to construct plasmids. Unmarked deletions in *V. fischeri* were generated as previously described (Le Roux et al., 2007, Shibata & Visick, 2012). Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For complementation in single copy from the chromosome, the appropriate DNA fragment was cloned with its native promoter or upstream of a P<sub>lacZ</sub> promoter in the mini-Tn7 delivery vector pEVS107. Insertion at the Tn7 site of the chromosome was performed via Tetraparental mating (McCann *et al.*, 2003) with wild-type *V. fischeri, E. coli* carrying pEVS104 (Stabb & Ruby, 2002), *E. coli* carrying the pEVS107 derivatives, and *E. coli* carrying the Tn7 transposase plasmid pUX-BF13 (Bao *et al.*, 1991). To identify the site of insertion of the 3 non-syp Tn mutants, we cloned the Tn, with flanking DNA, as previously described (Lyell *et al.*, 2008). All plasmids constructed in this study were sequenced at the Genomics Core Facility at the Center for
### Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Relevant Prim</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pARM9</td>
<td>pVSV105 + sypG</td>
<td>N/A</td>
<td>(Morris &amp; Visick, 2013a)</td>
</tr>
<tr>
<td>pARM36</td>
<td>pKV282 + sypA-HA</td>
<td>N/A</td>
<td>(Morris &amp; Visick, 2013b)</td>
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<tr>
<td>pEAH73</td>
<td>pKV69 carrying wild-type sypG; Cm(^{\prime}) Te(^{\prime})</td>
<td>N/A</td>
<td>(Hussa et al., 2008)</td>
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<tr>
<td>pEAH90</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (FL + SE-A)</td>
<td>N/A</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pEAH120</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (No SE-I)</td>
<td>783, 786</td>
<td>This study</td>
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<tr>
<td>pEAH121</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (FL + SE-I)</td>
<td>784, 786</td>
<td>This study</td>
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<tr>
<td>pEAH123</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (No SE-M)</td>
<td>787, 718</td>
<td>This study</td>
</tr>
<tr>
<td>pEAH124</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (FL + SE-M)</td>
<td>788, 718</td>
<td>This study</td>
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<tr>
<td>pEAH128</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (No SE-A)</td>
<td>675, 713</td>
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<tr>
<td>pEVS104</td>
<td>Conjugal helper plasmid (tra trb); Kn(^{R})</td>
<td>N/A</td>
<td>(Stabb &amp; Ruby, 2002)</td>
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<td>pEVS107</td>
<td>Mini-Tn7 delivery plasmid; oriR6K, mob; Kn(^{R}), Em(^{R})</td>
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<td>(McCann et al., 2003)</td>
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<td>pKJW4</td>
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<td>1547, 1548</td>
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<td>pKJW5</td>
<td>pKV300 ΔCm(^{R}) + SE-A - 14TA</td>
<td>1549, 1550</td>
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<td>pKV276</td>
<td>pEAH73 with D53E mutation in sypG; Cm(^{\prime}) Te(^{\prime})</td>
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<td>pKV69</td>
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<td>pKV300</td>
<td>pKV69 + P(_{sypA}) sypA (No SE-A); Cm(^{R}), Tc(^{R})</td>
<td>675, 807</td>
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<tr>
<td>pKV301</td>
<td>pKV69 + P(_{sypA}) sypA (SE-A); Cm(^{R}), Tc(^{R})</td>
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<td>pKV311</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (IG + SE-P)</td>
<td>719, 720</td>
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<td>937, 786</td>
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<td>pKV313</td>
<td>pEVS107 + P(_{sypA}) - lacZ Em(^{R}) (FL + SE-P)</td>
<td>791, 720</td>
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<td>pKV314</td>
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<td>pKV363</td>
<td>Cm(^{R}), oriT, oriR6K, ccdB</td>
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<td>(Shibata et al., 2012)</td>
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<td>pKV437</td>
<td>pKV300 ΔCm(^{R}) + SE-I-down</td>
<td>1384, 1385</td>
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<td>pKV438</td>
<td>pKV300 ΔCm(^{R}) + SE-I-up</td>
<td>1386, 1387</td>
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<td>pKV439</td>
<td>pKV300 ΔCm(^{R}) + SE-M</td>
<td>1388, 1389</td>
<td>This study</td>
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<td>pKV440</td>
<td>pKV300 ΔCm(^{R}) + SE-P</td>
<td>1390, 1391</td>
<td>This study</td>
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<tr>
<td>pKV444</td>
<td>pKV300 ΔCm(^{R}) + SE-A - 15TC</td>
<td>1398, 1399</td>
<td>This study</td>
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<td>pKV445</td>
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<td>1400, 1401</td>
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<td>pKV446</td>
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<td>1402, 1403</td>
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<tr>
<td>pKV447</td>
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<td>1404, 1405</td>
<td>This study</td>
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<td>pKV448</td>
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<td>1406, 1407</td>
<td>This study</td>
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<tr>
<td>pKV449</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A - 10AG</td>
<td>This study</td>
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<td>pKV450</td>
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<td>pKV451</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A - 5AG</td>
<td>This study</td>
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<tr>
<td>pKV452</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A - 4CT</td>
<td>This study</td>
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<td>pKV453</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A - 3AG</td>
<td>This study</td>
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<td>pKV454</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A - 2AG</td>
<td>This study</td>
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<tr>
<td>pKV455</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A - 1AG</td>
<td>This study</td>
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<tr>
<td>pKV461</td>
<td>pMSM25 with SE-A inserted at non-native ApaI site; restores SE-A</td>
<td>This study</td>
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<tr>
<td>pKV465</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; (No SE-A)</td>
<td>N/A</td>
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<tr>
<td>pSLN4</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A Δ4 bp</td>
<td>This study</td>
<td></td>
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<tr>
<td>pSLN5</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A Δ7 bp</td>
<td>This study</td>
<td></td>
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<tr>
<td>pSLN7</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A Δ10 bp</td>
<td>This study</td>
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<tr>
<td>pSW7848</td>
<td>Mobilizable suicide vector; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>N/A</td>
<td>Marie-Eve Val</td>
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<tr>
<td>pVAR17</td>
<td>pSW7848 containing 2 kb sequence flanking sypE derived from pCLD19</td>
<td></td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR18</td>
<td>pSW7848 containing 3.3 kb sequence flanking luxU</td>
<td>995, 996, 1017, 1018</td>
<td>(Ray &amp; Visick, 2012)</td>
</tr>
<tr>
<td>pVAR29</td>
<td>pKV363 containing ~500 bp flanking SE-A on either site (deletes SE-A)</td>
<td>935, 936, 675, 807</td>
<td>This study</td>
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<tr>
<td>pVAR30</td>
<td>pKV363 containing 850 bp sequencing flanking luxQ</td>
<td>1286, 1287, 1288, 1304</td>
<td>(Ray &amp; Visick, 2012)</td>
</tr>
<tr>
<td>pVAR31</td>
<td>pKV363 containing 1.1 kb sequence flanking luxP</td>
<td>1282, 1283, 1284, 1303</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR36</td>
<td>pKV363 containing 1.1 kb sequence flanking luxO</td>
<td>1319, 1320, 1344, 1345</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR37</td>
<td>pKV363 containing 1.1 kb sequence flanking luxO and luxU</td>
<td>1319, 1321, 1344, 1346</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR45</td>
<td>pSV105 + sypG-FLAG</td>
<td>1249, 1438</td>
<td>This study</td>
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<tr>
<td>pVAR52</td>
<td>pEVS107 with P&lt;sub&gt;lac&lt;/sub&gt;Z containing 2.3 kb luxQ-FLAG allele</td>
<td>1314, 1437</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR53</td>
<td>pEVS107 with P&lt;sub&gt;lac&lt;/sub&gt;Z containing 2.3 kb luxQ-&lt;i&gt;A216P&lt;/i&gt;-FLAG allele</td>
<td>849, 1425</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR54</td>
<td>pEVS107 with P&lt;sub&gt;lac&lt;/sub&gt;Z containing 2.3 kb luxQ-&lt;i&gt;H378A&lt;/i&gt;-FLAG allele</td>
<td>849, 1426</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR55</td>
<td>pEVS107 with P&lt;sub&gt;lac&lt;/sub&gt;Z containing 400 bp luxU-FLAG allele</td>
<td>1312, 1422</td>
<td>(Ray &amp; Visick, 2012)</td>
</tr>
<tr>
<td>pVAR56</td>
<td>pEVS107 with P&lt;sub&gt;lac&lt;/sub&gt;Z containing 400 bp luxU-&lt;i&gt;H61A&lt;/i&gt;-FLAG allele</td>
<td>849, 1427</td>
<td>(Ray &amp; Visick, 2012)</td>
</tr>
<tr>
<td>pVAR57</td>
<td>pKV301 ΔCm&lt;sup&gt;8&lt;/sup&gt; (+ SE-A)</td>
<td>N/A</td>
<td>This study</td>
</tr>
<tr>
<td>pVAR58</td>
<td>pKV300 ΔCm&lt;sup&gt;8&lt;/sup&gt; + SE-A</td>
<td>1036, 1037</td>
<td>This study</td>
</tr>
<tr>
<td>pVAR62</td>
<td>pKV363 containing 1.1 kb sequence flanking ainR</td>
<td>1323, pr-NL35</td>
<td>(Ray &amp; Visick, 2012)</td>
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<tr>
<td>pVAR77</td>
<td>pKV363 containing 1.2 kb sequencing flanking bamA</td>
<td>1532, 1533, 1675, 1535</td>
<td>This study</td>
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<tr>
<td>pVAR78</td>
<td>pKV363 containing 850 bp sequencing flanking balA</td>
<td>1659, 1676, 1661, 1662</td>
<td>This study</td>
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<tr>
<td>pVAR80</td>
<td>pKV363 containing 1.2 kb sequencing flanking balA</td>
<td>1665, 1666, 1667, 1668</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Primers</td>
<td>Notes</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
<td>--------------------------</td>
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<tr>
<td><strong>pVAR81</strong></td>
<td>pKV363 containing 1.2 kb sequencing flanking balC</td>
<td>1667, 1668</td>
<td>This study</td>
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<tr>
<td><strong>pVAR83</strong></td>
<td>pKV300 ΔCmR + SE-A – 2AC</td>
<td>1628, 1629</td>
<td>This study</td>
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<tr>
<td><strong>pVAR84</strong></td>
<td>pKV300 ΔCmR + SE-A – 2AT</td>
<td>1630, 1631</td>
<td>This study</td>
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<tr>
<td><strong>pVAR88</strong></td>
<td>pEVS107 containing ~2.8 kb fragment with the native bamA promoter and bamA and balA</td>
<td>1754, 1755</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pVAR90</strong></td>
<td>pEVS107 containing ~2.7 kb fragment with the native BamC promoter and BamC and balC</td>
<td>1758, 1759</td>
<td>This study</td>
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<tr>
<td><strong>pVAR91</strong></td>
<td>pKV300 ΔCmR + sypA-HA (+SE-A)</td>
<td>1798, 1800</td>
<td>This study</td>
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<tr>
<td><strong>pVAR92</strong></td>
<td>pKV300 ΔCmR + sypA-HA (ΔSE-A)</td>
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<td>This study</td>
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<td><strong>pVAR93</strong></td>
<td>pEVS107 containing ~2.7 kb fragment with the native bamB promoter and bamB and balB</td>
<td>1756, 1757</td>
<td>This study</td>
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<tr>
<td><strong>pVAR94</strong></td>
<td>pEVS107 containing ~2.5 kb fragment with bamA with a C-terminal FLAG-tag</td>
<td>1754, 1644, 1830</td>
<td>This study</td>
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<td><strong>pVAR95</strong></td>
<td>pEVS107 containing ~2.5 kb fragment with bamA</td>
<td>1754, 1833</td>
<td>This study</td>
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<td><strong>pVAR96</strong></td>
<td>pEVS107 containing ~3.3 kb fragment with the native bamA promoter fused to lacZ</td>
<td>1754, 1821, 1824, 1827</td>
<td>This study</td>
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<tr>
<td><strong>pVAR97</strong></td>
<td>pEVS107 containing ~3.3 kb fragment with the native BamC promoter fused to lacZ</td>
<td>1758, 1823, 1826, 1827</td>
<td>This study</td>
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<td><strong>pVS105</strong></td>
<td>Mobilizable vector, CmR</td>
<td>N/A</td>
<td>(Dunn et al., 2006)</td>
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</table>

1. Relevant primers for plasmids generated in this study; N/A, not applicable.
2. pVAR57 and pVAR58 differ in the presence of 5 bp non-native (“scar”) sequence on the 3’ side of SE-A present in pVAR58 but not pVAR57.
Table 3. Primers used in this study.

<table>
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<th>Primer</th>
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<td>559</td>
<td>ggtaccGGTACCTCATTCCGATTTCTCATAG</td>
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Genetic Medicine at Northwestern University (Chicago, IL) or ACGT (Wheeling, IL) to ensure that the insertion contained the desired sequence or mutation.

Plasmids that contained epitope (HA) tagged *sypA* that either contained or lacked the SE-A sequence upstream of the *sypA* gene were generated via PCR using pARM37 (Morris & Visick, 2013b) as a template and the indicated primers (Table 2 and 3) using Gibson Assembly (Gibson *et al.*, 2009) (master mix obtained from New England Biolabs, Beverly, MA, USA). The resulting plasmids were then introduced into *V. fischeri*.

**Transposon mutagenesis and identification of mutants with wrinkling defects.**

Transposon mutants were generated as described previously (Lyell *et al.*, 2008). Briefly, plasmid pEVS170, containing the mini-Tn5 transposon, was introduced into *V. fischeri* strain KV3299 via conjugation. Ex-conjugates were then pooled and the *sypG* overexpression plasmid pEAH73 was introduced via conjugation. The resultant ex-conjugates were then screened for their ability to form wrinkled colonies. Any mutants found to be defective for wrinkled colony formation after 2 days were then cured of their *sypG* overexpression plasmid and the plasmid was re-introduced. Any mutant that still exhibited a defect was considered to be a biofilm-defective mutant.

**Southern blot analysis.** Southern blot analysis was performed as described previously (Visick & Skoufos, 2001, Yip *et al.*, 2005), except chromosomal DNA was digested with KpnI and probed for the *syp* locus or with PstI and probed for Tn sequences. All Tn mutants exhibited a pattern consistent with only one Tn insertion.

**Luminescence assays.** *V. fischri* cultures were grown in fresh SWT overnight at 24°C with shaking, then diluted to an optical density at 600 nm (OD$_{600}$) of ~0.01 in 30 ml of
SWTO and incubated at 24°C with vigorous shaking. Samples were taken every 30-60 minutes. At each time point, bioluminescence (using a Turner Designs TD-20/20 luminometer at the factory settings and a large, clear scintillation vial) and OD\textsubscript{600} (using a cuvette) were measured for each sample. Maximum luminescence was observed at OD\textsubscript{600} measurements between 1.5 and 2 for all strains. Specific luminescence was calculated as relative luminescence (the relative light units of 1 ml of culture integrated over a 6-second count) divided by the OD\textsubscript{600}. For cultures supplemented with exogenous C8-HSL, we used 120 nM purified C8-HSL (Sigma Aldrich, St. Louis, MO) dissolved in a carrier (ethyl acetate and 0.01% (v/v) glacial acetic acid), which was spotted onto the bottom of the flask and allowed to dry before SWTO and cells were added. Addition of the carrier alone had no effect on growth or luminescence.

**Wrinkled colony assay.** *V. fischeri* strains were cultured overnight at 28°C with shaking in LBS containing Tc, then sub-cultured 1:100 into fresh LBS containing Tc and grown under the same conditions for 3 to 4 h the next day. Sub-cultures were standardized to an OD\textsubscript{600} of 0.2 and 10 µl aliquots were spotted onto LBS agar plates containing Tc and incubated at 28°C (for *sypG*-overexpressing strains) or 24°C (for *rscS*-overexpressing strains). Spotted cultures were then monitored from the time the start of wrinkled colony formation became apparent to the point at which wrinkled colony development ceased or the appropriate data set was collected. Each set of strains for a particular experiment was spotted onto the same plate to account for any minor plate-to-plate variations. Each assay was performed at least 2-3 times. To ensure that cultures spotted at an OD\textsubscript{600} of 0.2 resulted in the same number of cells inoculated per spot, we evaluated the correlation
between cell number and OD. Specifically, we determined the cell number of the pEAH73-containing strains \( \Delta sypE, \Delta sypE \Delta luxU, \Delta sypE \Delta luxQ, \) and \( \Delta sypE \Delta ainR \) using cultures normalized to an OD\(_{600}\) of 0.2, and found no significant difference in the number of colony-forming units obtained from dilutions of the normalized cultures of these strains.

For experiments designed to evaluate the SE, wrinkled colony assays were performed as described above, with the exception that the strains were cultured in and grown on LBS media containing Tc and Cm. Wrinkled colony development was monitored over time, and representative images are shown at the indicated time points. Although the specific end point of the assay on the third day varied between 68 and 72 h, there were no significant differences observed in wrinkled colony development within that time frame. We classified strains that carried \( sypA \) constructs with mutant SE-A sequences into three groups: similar to the positive control, similar to the negative control, or intermediate, based on the timing and extent of wrinkled colony development

**Pellicle assay.** *V. fischeri* strains were cultured overnight at 28°C with shaking in LBS containing Tc, then sub-cultured 1:100 into fresh LBS containing Tc and grown under the same conditions for 3 to 4 h the next day. Sub-cultures were standardized to an OD\(_{600}\) of 0.2 in 2 ml in a 24 welled-plate. Plates were incubated statically at 24°C for up to 72 h.

**Stickiness assay.** *V. fischeri* strains were grown as indicated above for the wrinkled colony assay. At the indicated time point, the spot was disturbed with a toothpick. A spot was considered sticky if it exhibited biofilm-like properties (i.e., the spot remained ‘bunched’ after disruption).
Exogenous complementation assay. *V. fischeri* strains were grown as indicated above for the wrinkled colony assay (LBS containing Tc). For the mixing experiments, 10 µl of each sub-culture standardized to OD₆₀₀ 0.2 was mixed, and then 10 µl of that mixture was spotted. For the spot-touching experiments, the standardized cultures were spotted close to each other and monitored over time as they grew close to or into each other.

Construction of *syp*- and *bam*-promoter lacZ fusions and β-galactosidase assays. To evaluate promoter activity the promoter region of *sypA*, *sypI*, *sypM*, and *sypP* (containing or lacking SE sequences) or *bamA* and *bamC* (containing the SE sequence) were fused upstream of a promoterless lacZ gene. The reporter fusions were introduced into the Tn⁷ delivery plasmid pEVS107 (McCann et al., 2003) and subsequently into the chromosome of wild-type *V. fischeri* (ES114), as described above; the *sypA* reporter fusion plasmid pEAH90 (Table 2) was also introduced into the Δ*sypE* and Δ*luxU* Δ*sypE* mutants. This approach permitted us to assay the transcription from the *syp* or *bam* promoters present in single copy in the chromosome, in the presence of either the vector control (VC - pKV69) or *sypG* (pEAH73) plasmids.

To assay β-galactosidase activity from the reporter fusions (Table 1), the strains containing either the VC or *sypG* plasmids were grown in HMM (for *syp* reporter fusions) or LBS (for *bam* reporter fusions) containing Tc. Samples (50 µl) were collected at 12 and/or 24 hours and 50 µl of Pierce β-galactosidase Assay Reagent (Pierce Biotechnology, Rockford, IL) were added to each sample. Measurements were taken in a microtiter dish using an ELx800 Absorbance Microplate Reader (BioTek, Winooski, VT)
with the appropriate settings. β-galactosidase activity was determined as previously described (Miller, 1972). P-values were calculated using the student’s t-test.

**Development of a biofilm-based assay of SypG activity.** As an indirect measure of sypA promoter activity, we developed an assay based on the observation that a strain that contained two plasmids, one that expressed sypG (from the lac promoter) and one that expressed sypA, but not one or the other plasmid, could produce a biofilm (Morris & Visick, 2013a). When cultures were spotted onto LBS agar plates (containing Cm and Tc), the resulting colony developed a wrinkled morphology (indicative of biofilm formation) or remained smooth (indicative of the lack of biofilm formation). In the published work, sypA was under the control of both its native promoter and the vector-based lac promoter. Here, to understand the role of the SE sequences in promoting sypA transcription, we cloned the sypA gene under the control of its native promoter region, either lacking or containing the syp enhancer sequence upstream of sypA (SE-A), in an orientation opposite to that of the lac promoter contained within pKV69 (Visick & Skoufos, 2001) to generate pKV300 and pKV301, respectively. Derivatives of pKV300, which lacks SE-A but instead contains a non-native ApaI restriction site (gggcc^c), were generated as follows: pKV300 was digested with ApaI and NcoI, for which a site is located within the Cm^R gene of the cloning vector. Sets of complementary primers were generated that contained SE sequences as described in the text and Table 3 and, on the ends, non-native sequences complementary to the ApaI and NcoI restriction sites (Fig. 9). These sets of primers were annealed and ligated into the ApaI/NcoI-digested pKV300. Each resulting plasmid contained SE sequences and a 5 bp non-native “scar” (ggccc) as a
Figure 9. Construction of pVAR58 and other derivatives of pKV300. The genomic context of SE-A (red bold letters) is shown and compared to pKV300, which contains only the sequence downstream of SE-A (bold black letters). pKV300 was cut with NcoI and Apal as indicated and the annealed primer set 1036/1037 was ligated into the linearized vector. The resulting plasmid, pVAR58, contains an insertion of the SE-A sequence flanked on both sides by 5 non-native nucleotides. These additional nucleotides had no impact on the ability of SypG to recognize the SE-A sequence in our biofilm-based assay (see Figure 29). Other pKV300 derivatives were generated in a similar manner.
result of ligation to the Apal site (Fig. 9). All derivatives were compared to pVAR58, which was similarly derived from pKV300 by the insertion of the wild-type SE-A at Apal/NcoI, and also contains the non-native “scar” (Fig. 9). The pKV300 derivatives all lost Cm\(^R\), but retained Tc\(^R\). These Tc\(^R\) plasmids were fully compatible with pARM9 (Morris & Visick, 2013a), the pVSV105-based Cm\(^R\) plasmid that carries the sypG gene. Both pARM9 and the pKV300 derivatives were introduced into wild-type strain ES114 via conjugation and selection with Cm and Tc. As a negative control, pKV300 was digested with Apal and NcoI, the overhangs were filled-in, and the plasmid was self-ligated to obtain pKV465. To assess the consequence of the insertion of the non-native “scar” sequences, pVAR57 was generated from pKV301 by deletion of the sequences between Apal and NcoI; the Apal site in pKV301 is on the 5’ end of SE-A, and thus there is no “scar” between SE-A and the remainder of the promoter region in front of sypA. Additional constructs were generated in the same way, with the insertion of specific SE sequences into the Apal/NcoI sites of pKV300.

**Colonization assay.** Experiments involving *E. scolopes* animals were carried out using approaches described in an Animal Component of Research Protocol (ACORP) approved by Loyola University’s Institutional Animal Care and Use Committee (IACUC) (LU #107314, 201297). To perform single strain colonization assays, juvenile *E. scolopes* squid were placed in artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) and incubated with 700-1500 *V. fischeri* cells per ml of seawater for 3-4 hours. The squid were then washed in artificial seawater and placed into fresh (*V. fischeri*-free) artificial seawater and colonization was allowed to proceed for 13-16 hours post-wash (total
experiment time 16-20 hours). The squid were then homogenized to release the contents of their light organs and the homogenates were diluted and plated onto SWT to determine the colony forming units (CFU) per squid.

**Western blot analysis.** To evaluate the levels of protein produced by the luxU-H61A mutant, we expressed epitope (FLAG)-tagged versions of this allele and the wild-type control from plasmids (pVAR44 and pVAR49, respectively) in the ΔluxU mutant (KV4830). Strains were grown overnight at 28°C with shaking in LBS containing Tc. Samples were collected (1 ml) and pelleted, then resuspended in 500µl 2X SDS loading buffer (4% SDS, 10% 2-mercaptoethanol, 0.005% bromophenol blue, 20% glycerol, 0.1M Tris pH 7), boiled for 5 min, and then loaded onto a 15% SDS polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane and probed with anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO). Protein bands were visualized using a horseradish peroxidase-conjugated secondary antibody and ECL reagents (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL).

Western blot analysis (as described above, with the exception that samples were loaded onto a 10% SDS polyacrylamide gel and an anti-HA antibody was used to probe for SypA) was also used to evaluate the levels of SypA protein produced when either pVAR91 (+SE-A) or pVAR92 (ΔSE-A) were expressed with either vector control (VC – pVSV105) or a sypG plasmid (pARM9) in the wild-type (ES114) strain.

**Sample preparation for transmission electron microscopy.** Strains ES114, KV6897 (ΔbamABC), and KV5069 (ΔsypL), all overexpressing rscS, were grown as described
above in the wrinkled colony assay and spotted individually. KV6897 and KV5069 were also grown and spotted for a spot-touching experiment. Samples were harvested after 72 h as indicated below. For the individual spots, a sample from the edge of the spot was removed using a clean razor blade and placed into a 1.5 ml Eppendorf tube. For the touching spots, a sample at the interface of the touching spots was removed using a clean razor blade and placed into a 1.5 ml Eppendorf tube; note that since KV6897 overexpressing \( rscS \) is sticky, the sample mostly contains this strain.

For staining \textbf{without} ruthenium red, samples were mixed with 1 ml of a 1\% gluteraldehyde solution (840 \( \mu l \) 10 mM phosphate buffer, pH 7 and 160 \( \mu l \) 8\% gluteraldehyde) and incubated overnight at 4\(^\circ\)C. The liquid was then removed from the samples, which were then washed with 1.4 ml of 0.5 M NH\(_4\)Cl three times. Next, 400 \( \mu l \) of a 1\% osmium Tetroxide solution (300 \( \mu l \) 10 mM phosphate buffer, pH 7 and 100 \( \mu l \) 4\% osmium Tetroxide) was added to each sample and incubated overnight at 4\(^\circ\)C. The liquid was then removed from the samples, which were washed with 1.4 ml of 0.5 M NH\(_4\)Cl twice. Next, 200 \( \mu l \) of ddH\(_2\)O was added to each sample. The samples were then taken to the agarose enrobement step below.

For staining \textbf{with} ruthenium red, 1 ml of 2.4\% gluteraldehyde, 0.1 M sodium cacodylate, and 0.1\% ruthenium red mixture (310 \( \mu l \) 8\% gluteraldehyde, 0.01 g ruthenium red, and 690 \( \mu l \) 0.1 M sodium cacodylate) was added to the samples and incubated for 1 h at 37\(^\circ\)C. The liquid was then removed and the samples were washed with 1.4 ml of 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), and 1.8 mM KH\(_2\)PO\(_4\), at pH 7.4). Next, 1 ml of a 2\% osmium Tetroxide, 0.1 M sodium cacodylate,
0.1% ruthenium red solution (500 µl 4% osmium Tetroxide, 0.001 g ruthenium red, and 500 µl 0.1 M sodium cacodylate) was added to each sample and incubated for 3 h at room temperature. The liquid was then removed and the samples were washed with 1.4 ml of 1X PBS twice. Next, 200 µl of ddH₂O was added to each sample. The samples were then subjected to agarose enrolement.

Samples were then dehydrated in ethanol and then encased in resin. Briefly, 4 ml of a 30% ethanol was added to each sample and incubate for 1 h at room temperature with agitation (using a platform rocker). The liquid was then removed and a 50% ethanol solution was added and incubated for 1 h at room temperature with agitation. This process was repeated for a 70% and 100% ethanol solution. Next, resin was prepared by weight (i.e., 25 g) using vinylcyclohexane dioxide (VCD or ERL-4221 – 5.9 g), D.E.R. (3.5 g), nonenyl succinic anhydride (NSA – 15.4 g), and 2-dimethylaminoethanol (DMAE – 0.24 g). Next, 4 ml of a 1:1 ratio of 100% ethanol and resin was added to each sample and incubated for 1 h at room temperature with agitation. The ethanol resin mix was removed and 4 ml of 100% resin was added to each sample and agitated overnight. Lastly, the samples were removed to a BEEM® embedding capsule, size 00 tube (Electron Microscopy Science, Hatfield, PA), filled with resin, and hardened at 65°C overnight in an oven. Samples were then submitted to the Core Imaging Facility (Loyola University Medical Center, Maywood, IL) for sectioning. Dr. Adam Driks (LUMC) performed TEM analysis of the samples.

**Bioinformatics.** Dr. Beth Hussa used the IR for the *syp* enhancer sequence (TTCTCANNNTGMDWN) to search for additional potential SypG binding sites within
the 500 bp regions upstream of promoter-proximal ORFs in the *V. fischeri* genome using the genome-scale DNA pattern search in Regulatory Sequence Analysis Tools (RSAT) [http://rsat.ulb.ac.be; (van Helden, 2003, Thomas-Chollier *et al.*, 2008, Thomas-Chollier *et al.*, 2011)].
CHAPTER THREE
EXPERIMENTAL RESULTS

I. LuxU CONNECTS QUORUM SENSING TO BIOFILM FORMATION IN *Vibrio fischeri*

Introduction

*V. fischeri* promotes biofilm formation through the *symbiotic polysaccharide (syp)* locus (Fig. 4), which encodes genes involved in the regulation, production, and transport of a polysaccharide necessary for biofilm formation (Yip et al., 2005, Yip et al., 2006, Shibata & Visick, 2012). This locus is transcriptionally controlled by the RR SypG, encoded within the *syp* locus, and σ^54 (Yip et al., 2005). Another regulator of the *syp* locus is the SK RscS, which functions upstream of SypG to promote *syp* transcription (Yip et al., 2006, Hussa et al., 2008). Under standard laboratory conditions, *V. fischeri* does not form robust biofilms (i.e., wrinkled colonies and pellicles). However, robust biofilms that are dependent on the *syp* locus can be induced by overexpression of either *rscS* (Yip et al., 2006) or *sypG*; for SypG to induce biofilm formation, the biofilm inhibitor protein SypE must be absent or inactivated (Hussa et al., 2008, Morris et al., 2011). Although much is known about the role of the *syp* locus and its regulation during biofilm formation, little is known about other factors that influence biofilm formation in this organism. Thus, in this study, I sought to identify other (non-*syp*) components involved in this process.

Transposon mutagenesis reveals a regulatory connection between *syp* and *lux*
To better understand the requirements for biofilm formation by *V. fischeri*, I performed a random transposon mutagenesis of KV3299, a strain that lacks the *syp* biofilm inhibitor protein SypE. I then induced biofilm formation by introducing the *sypG* overexpression plasmid pEAH73; under these biofilm-inducing conditions, *V. fischeri* forms wrinkled colonies (Hussa et al., 2008). I screened approximately 5000 mutants for those exhibiting a defect in wrinkled colony formation and found 27 that appeared to form smooth colonies. To verify the phenotypes of these mutants, I cured them of their *sypG* overexpression plasmid and then re-introduced it. All of the mutants remained defective in wrinkled colony formation and fell into two classes. Class 1 mutants (24 total) exhibited smooth colony morphology (compare Fig. 10A and B), while class 2 mutants (3 total) exhibited a substantial delay (approximately 6 h) in the start of wrinkled colony formation, but appeared similar to that of the parent strain at later time point (compare Fig. 10A and C; asterisks indicate the time at which wrinkled colony formation is apparent, typically identified by ridge formation around the outer edge of the spot).

To determine whether the transposon had inserted within the *syp* locus, a location predicted to disrupt wrinkling, I performed Southern blot analysis on each mutant (data not shown). I found that the class 1 (smooth) mutants mapped within this locus, while the class 2 (delayed) mutants were unaltered in their *syp* regions. These results confirm the assumption that wrinkled colony formation induced by *sypG* overexpression depends on the *syp* locus. Because my goal was to identify novel (non-*syp*) factors involved in biofilm formation, I pursued characterization of the class 2 mutants. Upon cloning and sequencing the DNA flanking the site of the Tn insertion in each class 2 mutant, I found
Figure 10. Transposon mutagenesis reveals other regulators of biofilm formation in *V. fischeri*. (A-C). Time-course assays of wrinkled colony formation induced by *sypG* overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45.5 h post-spotting for the following strains: Δ*sypE* control (pEAH73/KV3299) (A), a representative class 1 mutant (pEAH73/KV5872; *syp::Tn5 ΔsypE*) (B), and a representative class 2 mutant (pEAH73/KV4431; *luxQ::Tn5 ΔsypE*) (C). An * indicates the time at which wrinkled colony formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments. (D). A graphical depiction of the predicted *luxPQ* genes (block arrows) and approximate Tn insertion sites (black triangles). There are 2 bp between the predicted translational stop site of *luxP* and the predicted translational start site of *luxQ*. 
that one insertion mapped near the end of VF_0707, while the other two were within VF_0708 (Fig. 10D). These genes are predicted to encode LuxP and LuxQ, respectively, two proteins proposed to be involved in controlling bioluminescence in V. fischeri (Fig. 8).

**Loss of LuxQ affects bioluminescence and biofilm formation**

In V. fischeri, LuxP and LuxQ are predicted to regulate bioluminescence due to their sequence similarity to the well-characterized proteins of V. harveyi (58% and 44% identical, 72% and 67% similar, respectively) and to the functional conservation of other members of the Lux regulatory pathway between V. harveyi and V. fischeri (reviewed in (Stabb et al., 2008)); however, the functions (bioluminescence or otherwise) of these two proteins in V. fischeri have not yet been assessed through mutagenesis studies. Thus, to understand the functions of these putative regulators, I first asked whether the luxP and luxQ genes were indeed involved in controlling bioluminescence. Since the Tn insertions were in a ΔsypE background, it was necessary to ask whether loss of SypE impacted luminescence (Fig. 11A); I found that it did not substantially impact luminescence, regardless of whether I used OD\textsubscript{600} (Fig. 11B) to estimate cell number or determined the number of colony forming units (CFU; Fig. 11C) to calculate the specific luminescence.

Next, I assessed the impact of the Tn mutations on luminescence. The model (Fig. 8), generated from work in V. harveyi (Bassler et al., 1994a, Neiditch et al., 2005), predicts that a luxP mutant should fail to transmit the AI signal to LuxQ, causing LuxQ to remain a kinase; as a result, the levels of phosphorylated LuxO should be higher and luminescence should be lower. The model also predicts that the luxQ mutant should
Figure 11. Luminescence of ES114, ΔsypE, and ΔluxU ΔsypE in culture. Cultures were grown in SWTO and incubated at 24°C with vigorous shaking. Luminescence and OD$_{600}$ were measured over time until maximum luminescence was achieved for the following strains: ES114 (black circles), ΔsypE (black squares; KV3299), and ΔluxU ΔsypE (black triangles; KV4830). (A) Data are plotted as specific luminescence (Sp. lum.; relative luminescence divided by OD$_{600}$) versus OD$_{600}$. The inset depicts a close-up of the luminescence levels of ES114 and ΔsypE to show the error bars. (B) Data are plotted as luminescence divided by OD at one time point around maximal luminescence (OD ~1.5). (C) Data are plotted as luminescence divided by CFU at one time point around maximal luminescence (OD ~1.5), in which samples were taken and the CFUs were calculated. Samples were taken in triplicate for each strain, with the average and standard deviation (error bars) represented. These data are representative of 2 independent experiments.
exhibit a decrease in phosphorylated LuxO, leading to increased luminescence. I found that all three mutants exhibited an increase in luminescence relative to their parent (ΔsypE) (Fig. 12A).

Since the luxP Tn mutant did not exhibit the predicted luminescence phenotype, I hypothesized that the Tn insertion, which was located at the end of luxP, was polar on luxQ. To test this prediction, I constructed in-frame deletions of both luxP (ΔluxP) and luxQ (ΔluxQ) in both the ΔsypE and wild-type backgrounds. Neither mutation impacted growth of V. fischeri (data not shown). Regardless of the background, loss of LuxP decreased bioluminescence, while loss of LuxQ increased bioluminescence, as predicted (Fig. 12B and data not shown). The luminescence of the ΔluxQ mutant could be restored to the level of the luxQ+ control by expression of an epitope-tagged version of luxQ (luxQ-FLAG) in single copy from the chromosome (Fig. 12C). Together, these data indicate that: 1) LuxP and LuxQ are involved in controlling bioluminescence, as predicted, 2) the Tn insertion within luxP was polar on luxQ, and 3) luxP and luxQ likely comprise an operon (Fig. 10D).

Since the Tn insertion in luxP was polar on luxQ, I predicted that the 6 h delay in biofilm formation initially observed from the Tn mutants was likely due to loss or disruption of luxQ. To test this prediction, I examined wrinkled colony formation by the ΔluxQ (ΔsypE) mutant that overexpressed sypG. Like the Tn mutants, this mutant also exhibited a delay in wrinkled colony formation relative to the control (compare Fig. 13A & B). This delay in biofilm formation could be complemented by expression of luxQ-
Figure 12. Luminescence of lux mutants in culture. Cultures were grown in SWTO and incubated at 24°C with vigorous shaking. Luminescence and OD\textsubscript{600} were measured over time until maximum luminescence was achieved (between OD\textsubscript{600} 1.5 and 2). All data are plotted as specific luminescence (Sp. lum.; relative luminescence divided by OD\textsubscript{600}) versus OD\textsubscript{600} and are representative of at least 3 independent experiments.

A. ΔsypE control (black squares; KV3299), luxP::Tn\textsubscript{5} ΔsypE (white circles; KV4430), luxQ::Tn\textsubscript{5} ΔsypE (grey diamonds; KV4431), luxQ::Tn\textsubscript{5} ΔsypE (black triangles; KV4432)

B. ΔsypE control (black squares; KV3299), ΔluxP ΔsypE (white circles; KV5347), ΔluxQ ΔsypE (black circles; KV5394), ΔluxU ΔsypE (grey triangles; KV4830), ΔluxQ ΔluxU ΔsypE (black diamonds; KV6008)

C. ΔsypE attTn\textsubscript{7}::erm control (black squares; KV4390), ΔluxQ ΔsypE attTn\textsubscript{7}::erm (black circles; KV5973), ΔluxQ ΔsypE attTn\textsubscript{7}::luxQ-FLAG (white circles; KV5902), ΔluxQ ΔsypE attTn\textsubscript{7}::luxQ-A216P-FLAG (grey triangles; KV5904), ΔluxQ ΔsypE attTn\textsubscript{7}::luxQ-H378A-FLAG (black diamonds; KV5903)

D. ΔsypE control (black squares; KV3299), ΔluxO ΔsypE (white circles; KV5468), ΔluxU ΔsypE (black triangles; KV4830), ΔluxO ΔluxU ΔsypE (grey diamonds; KV5472)

E. ΔsypE attTn\textsubscript{7}::erm control (black squares; KV4390), ΔluxU ΔsypE attTn\textsubscript{7}::erm (black triangles; KV5974), ΔluxU ΔsypE attTn\textsubscript{7}::luxU-FLAG (white triangles; KV5905), ΔluxU ΔsypE attTn\textsubscript{7}::luxU-H61A-FLAG (white circles; KV5906)

F. ΔsypE control (black squares; KV3299), ΔainR ΔsypE (grey circles; KV6169), ΔluxU ΔsypE (black triangles; KV4830), ΔainR ΔluxU ΔsypE (black diamonds; KV6259)
The data for KV3299 in panel B are the same as that shown in panel D. The data for KV4390 in panel C are the same as that shown in panel E.
Figure 13. The effect of luxQ point mutations on biofilm formation. Time-course assays of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28˚C. Wrinkled colony formation was monitored up to 45 h post-spotting for the following strains: ΔsypE attTn7::erm control (pEAH73/KV4390) (A), ΔluxQ ΔsypE attTn7::erm (pEAH73/KV5973) (B), ΔluxQ ΔsypE attTn7::luxQ-FLAG (pEAH73/KV5902) (C), ΔluxQ ΔsypE attTn7::luxQ-A216P-FLAG (pEAH73/KV5904) (D), and ΔluxQ ΔsypE attTn7::luxQ-H378A-FLAG (pEAH73/KV5903) (E). An * indicates the time at which wrinkled colony formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments.
FLAG in single copy from the chromosome of the ∆luxQ mutant (Fig. 13A-C). Thus, LuxQ appears to control both bioluminescence and biofilm formation in *V. fischeri*. In contrast, the luxP mutation exerted relatively little effect on biofilm formation (Fig. 14); thus, my subsequent studies focused on LuxQ and other Lux regulators.

**LuxU exerts a more substantial impact on biofilm formation than LuxO**

In *V. harveyi*, LuxQ functions through the histidine phosphotransferase LuxU to control the phosphorylation state of the RR LuxO (Fig. 8) (Freeman & Bassler, 1999b, Freeman & Bassler, 1999a). Since LuxQ is involved in controlling biofilm formation by *V. fischeri*, I asked whether LuxU and LuxO were also involved. Thus, I generated deletions of both luxU (∆luxU) and luxO (∆luxO) in the ∆sypE background. However, it was necessary to first confirm that the mutants exhibited the predicted pattern of luminescence [i.e., increased bioluminescence; for luxO mutants, this has been previously reported (Lupp et al., 2003, Hussa et al., 2007)] (Fig. 8). As expected, both mutants exhibited an increase in bioluminescence relative to their parent (Fig. 12D). Neither mutant exhibited a growth defect (data not shown). Finally, a ∆luxU ∆luxO (∆sypE) mutant exhibited a luminescence phenotype similar to that of the individual mutants (Fig. 12D). Overall, these data confirm that LuxU functions to control bioluminescence in *V. fischeri*, as predicted.

Next, I introduced the sypG plasmid into the ∆luxU and ∆luxO mutants and assessed wrinkled colony formation. Similar to the loss of LuxQ, loss of LuxU resulted in a delay (about 7 h) in wrinkled colony formation (compare Fig. 15A & B). However, loss of LuxO resulted in only a slight, but reproducible delay (1.5 h) in wrinkled colony
Figure 14. Wrinkled colony formation by ΔluxP. Time-course assays of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28˚C. Wrinkled colony formation was monitored up to 40 h post-spotting for the following strains: ΔsypE control (pEAH73/KV3299) and ΔluxP ΔsypE (pEAH73/KV5347). An * indicates the time at which wrinkled colony formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments.
Figure 15. Wrinkled colony formation by luxU and luxO mutants. Time-course assays of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45 h post-spotting for the following strains: ΔsypE control (pEAH73/KV3299) (A), ΔluxU ΔsypE (pEAH73/KV4830) (B), ΔluxO ΔsypE (pEAH73/KV5468) (C), and ΔluxO ΔluxU ΔsypE (pEAH73/KV5472) (D). An * indicates the time at which wrinkled colony formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments.
formation (compare Fig. 15A and C); I also observed the same slight delay for a luxO::kan mutant (data not shown), confirming the results of the ΔluxO mutant. These data suggest that, under our conditions, LuxU plays a more critical role than LuxO in controlling biofilm formation. These results also suggest that LuxU may function independently of LuxO to control biofilm formation. To investigate this possibility further, I evaluated biofilm formation by the ΔluxO ΔluxU (ΔsypE) mutant. I predicted that if LuxU functions through LuxO to regulate biofilm formation, then loss of both LuxU and LuxO would result in a phenotype similar to loss of LuxO alone (i.e., a 1.5 h delay). This was not the case: loss of both regulators resulted in an 8 h delay in wrinkled colony formation (compare Fig. 15A and D). This delay supports the hypothesis that the two regulators function independently to impact biofilm formation. Furthermore, these data contrast with the luminescence results, in which the phenotypes of the luxU, luxO, and luxU luxO mutants were similar (Fig. 12D), and which suggest that LuxU likely functions through LuxO to control bioluminescence. Together, these data suggest that the Lux pathway bifurcates at LuxU to control both bioluminescence and biofilm formation (Fig. 8). Since loss of LuxU resulted in a more severe biofilm phenotype than loss of LuxO, I chose to pursue the role of LuxU (and its inputs) in the current study.

**LuxQ kinase activity promotes biofilm formation**

My current data suggest that LuxQ functions as a positive regulator of biofilm formation. Because this SK is predicted to function as both a kinase and a phosphatase (Freeman & Bassler, 1999a, Neiditch et al., 2006), I asked whether the ability of LuxQ to positively regulate biofilm formation depended upon its kinase and/or phosphatase
activity. Previous work from *V. harveyi* had demonstrated that certain point mutations cause the loss of one activity but not the other (i.e., kinase activity is lost, while phosphatase activity is retained and vice versa) (Neiditch et al., 2006). Thus, I generated point mutations in the *V. fischeri* luxQ gene that are predicted to cause either loss of phosphatase activity (luxQ-A216P; kin+/phos-) or loss of kinase activity (luxQ-H378A; kin-/phos+), while retaining the other activity, respectively. I then expressed these luxQ alleles in single copy from the chromosome of the ΔluxQ mutant. To confirm that these LuxQ derivatives were functional, I examined their ability to control light production. According to the model (Fig. 8) and work from *V. harveyi* (Neiditch et al., 2006), a phosphatase mutant (LuxQ-A216P, kin+/phos-) should exhibit a decrease in bioluminescence (due to an increase in phosphorylated LuxO), while a kinase mutant (LuxQ-H378A, kin-/phos+) should exhibit an increase in bioluminescence (due to a decrease in phosphorylated LuxO). Indeed, each mutant exhibited the expected pattern of luminescence (Fig. 12C), indicating that the proteins produced were functional and behaved as predicted.

I next assessed the ability of these alleles to complement the ΔluxQ mutant with respect to the timing of wrinkled colony formation. I found that the phosphatase mutant, LuxQ-A216P (kin+/phos-), could complement the luxQ mutant, restoring the timing of wrinkled colony formation to approximately that of the control strain (luxQ+) and the wild-type-complemented ΔluxQ mutant (compare Fig. 13A, C, and D). In contrast, the kinase mutant, LuxQ-H378A (kin-/phos+), failed to complement the luxQ mutant; this strain exhibited wrinkled colony formation that was indistinguishable from the ΔluxQ
parent (compare Fig. 13A, B, and E). These data suggest that the kinase activity of LuxQ, but not its phosphatase activity, is necessary to regulate biofilm formation.

**The impact of LuxQ on biofilm formation depends on LuxU**

My data indicate that LuxQ (specifically its kinase activity) and LuxU are necessary to regulate biofilm formation. According to the model (Fig. 8), LuxQ is predicted to function through LuxU. To test this hypothesis, I first asked whether LuxQ functioned through LuxU to regulate bioluminescence. If this were the case, I would expect that a \textit{luxQ luxU} mutant would phenocopy a \textit{luxU} mutant, and indeed it did (Fig. 12B). To further evaluate this regulatory connection, I expressed the \textit{luxQ-A216P} (kin+/phos-) allele in the \textit{luxQ luxU} mutant. Whereas, in the context of the \textit{luxQ} (luxU\textsuperscript{+}) background this allele decreased luminescence, it failed to do so when \textit{luxU} was also disrupted: the levels of luminescence produced by the \textit{luxQ luxU} mutant expressing \textit{luxQ-A216P} (kin+/phos-) were indistinguishable from that of the \textit{luxU} mutant (Fig. 16A). These data suggest that LuxQ functions through LuxU to regulate bioluminescence.

Next, I asked whether LuxQ functioned through LuxU to control biofilm formation. I first evaluated biofilm formation by the \textit{luxQ luxU} (sypE) mutant. I found that the double mutant exhibited a delay in wrinkled colony formation similar to that seen with the individual \textit{luxQ} and \textit{luxU} mutants (Fig. 17), rather than an additive delay. Thus, these results suggest that LuxQ and LuxU function in the same pathway to regulate biofilm formation. To probe this relationship further, I utilized the \textit{luxQ-A216P} allele, which permits complementation of the \textit{luxQ} mutation (Fig. 13). Specifically, I expressed this allele in the \textit{luxQ luxU} mutant with the expectation that if LuxU were necessary for
Figure 16. Luminescence and wrinkled colony formation by luxQ and luxU mutants. 
(A) Luminescence of lux mutants in culture. Cultures were grown in SWT and incubated at 24˚C with vigorous shaking. Luminescence and OD_{600} were measured over time until maximum luminescence was achieved (between OD_{600} 1.5 and 2) for the following strains: ΔluxQ attTn7::erm control (black triangles; KV5973), ΔluxU ΔsypE attTn7::erm (black diamonds; KV5974), ΔluxQ ΔsypE attTn7::luxQ-A216P-FLAG (white circles; KV5904), and ΔluxQ ΔluxU ΔsypE attTn7::luxQ-A216P-FLAG (black circles; KV6054). All data are plotted as specific luminescence (Sp. lum.; relative luminescence divided by OD_{600}) versus OD_{600} and are representative of at least 3 independent experiments.

(B) Time-course assays of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28˚C. Wrinkled colony formation was monitored up to 42 h post-spotting for the following strains: ΔsypE attTn7::erm control (pEAH73/KV4390), ΔluxQ ΔsypE attTn7::luxQ-A216P-FLAG (pEAH73/KV5904), and ΔluxQ ΔluxU ΔsypE attTn7::luxQ-A216P-FLAG (pEAH73/KV6054). An * indicates the time at which wrinkled colony
formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments.
Figure 17. Wrinkled colony formation of luxQ and luxU mutants. Time-course assays of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45.5 h post-spotting for the following strains: ΔsypE control (pEAH73/KV3299) (A), ΔluxQ ΔsypE (pEAH73/KV5394) (B), ΔluxU ΔsypE (pEAH73/KV4830) (C), and ΔluxQ ΔluxU ΔsypE (pEAH73/KV6008) (D). An * indicates the time at which wrinkled colony formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments.
LuxQ to regulate biofilm formation, then the ability of LuxQ-A216P to promote biofilm formation should not be apparent in the absence of LuxU. Indeed, this was the case (Fig. 16B). These data suggest that the ability of LuxQ to positively regulate biofilm formation depends upon LuxU.

Biofilm formation depends on the conserved site of phosphorylation in LuxU

In _V. harveyi_, LuxU serves as a phosphotransferase, shuttling phosphoryl groups between the SKs and the RR LuxO (Fig. 8). This role depends upon the conserved site of phosphorylation, His58 (Freeman & Bassler, 1999b), which is homologous to H61 on LuxU in _V. fischeri_. To determine whether the _V. fischeri_ homolog functions in a similar manner, I first constructed an epitope-tagged version of _luxU_ (_luxU-FLAG_). Expression of this allele in single copy from the chromosome of the Δ_luxU_ mutant restored luminescence to that of the control (Fig. 12E), as well as the normal timing of wrinkled colony formation (compare Fig. 18A-C). Next, I substituted the predicted, conserved histidine for an alanine (H61A) in the _luxU-FLAG_ construct and introduced this allele into the chromosome of the Δ_luxU_ mutant. The Δ_luxU_ mutant expressing the _luxU-H61A_ allele failed to restore luminescence to the level of the parent (Fig. 12E) and exhibited the same 6 h delay in wrinkled colony formation as the uncomplemented Δ_luxU_ mutant (compare Fig. 18B and D). To ensure that the lack of complementation was not due to a reduction or loss of the protein, I performed western blot analysis and found that protein was expressed from both wild-type and _luxU-H61A_ alleles (Fig. 19). Together, these data suggest that the conserved site of phosphorylation in LuxU is necessary to regulate
Figure 18. Wrinkled colony formation by complemented $\Delta luxU$ mutants. Time-course assays of wrinkled colony formation induced by $sypG$ overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45 h post-spotting for the following strains: $\Delta sypE\ att\mathrm{Tn}7::\erm$ control (pEAH73/KV4390) (A), $\Delta luxU\Delta sypE\ att\mathrm{Tn}7::\erm$ (pEAH73/KV5974) (B), $\Delta luxU\Delta sypE\ att\mathrm{Tn}7::\luxU\text{-FLAG}$ (pEAH73/KV5905) (C), and $\Delta luxU\Delta sypE\ att\mathrm{Tn}7::\luxU\text{-H61A-FLAG}$ (pEAH73/KV5906) (D). An * indicates the time at which wrinkled colony formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments.
Figure 19. Western blot analysis of LuxU-FLAG and LuxU-H61A-FLAG. Cells extracts from ΔluxU (KV4830) expressing either luxU-FLAG from pVAR44 (lane 1) or luxU-H61A-FLAG from pVAR49 (lane 2) were probed with an anti-FLAG antibody. LuxU is predicted to be 13 kD. This blot is representative of three independent experiments.
biofilm formation. Thus, it appears that key residues predicted to be involved in phosphotransfer are required for regulation of biofilm formation.

**AinR does not function as predicted to regulate bioluminescence and exerts no impact on biofilm formation**

It has been predicted that, like the *V. harveyi* Lux pathway, multiple SKs feed into LuxU to control bioluminescence in *V. fischeri* (Visick, 2005, Stabb et al., 2008, Miyashiro & Ruby, 2012). In particular, the SK AinR is proposed to function at the same level as LuxQ (Fig. 8) (Gilson et al., 1995, Stabb et al., 2008). To determine whether AinR was involved in controlling bioluminescence and biofilm formation, I generated a \( \Delta \text{ainR} (\Delta \text{sypE}) \) mutant and first assessed its luminescence phenotype; at the time of this work, no study of AinR had assessed its role in controlling bioluminescence in liquid culture. The model (Fig. 8) predicts that, similar to loss of LuxQ, loss of AinR would result in an increase in luminescence. However, this was not the case: loss of AinR resulted in a consistent but very slight decrease in luminescence as compared to the control (Fig. 12F and Fig. 20). To determine whether AinR functioned through the known phosphorelay pathway (i.e., through LuxU), I generated a \( \Delta \text{ainR} \Delta \text{luxU} (\Delta \text{sypE}) \) mutant and assessed its luminescence phenotype. I expected that the double mutant would exhibit the luminescence phenotype of the luxU single mutant. Surprisingly, this mutant consistently exhibited an intermediate luminescence phenotype: the \( \Delta \text{ainR} \Delta \text{luxU} \) mutant was brighter than the \( \Delta \text{ainR} \) mutant, but not as bright as the \( \Delta \text{luxU} \) mutant (Fig. 12F). These data suggested that, under these conditions, AinR plays only a minor role in controlling bioluminescence.
Figure 20. Luminescence of ainR mutants in the presence and absence of C8-HSL. Cultures were grown in SWTO and incubated at 24°C with vigorous shaking. Luminescence and OD$_{600}$ were measured over time until maximum luminescence was achieved for the following strains: ΔsypE control (squares; KV3299), ΔainR ΔsypE (circles; KV6169), ΔluxU ΔsypE (triangles; KV4830), ΔainR ΔluxU ΔsypE (diamonds; KV6259). (A) Luminescence in the absence of C8-HSL (black symbols). (B) Luminescence in the presence of 120 nM C8-HSL (white symbols). The inset for panel A depicts a close-up of the luminescence levels between the ΔsypE control and ΔainR ΔsypE mutant. The data for ΔsypE in panel A (black squares) is the same as those in panel B (black squares). Data are plotted as specific luminescence (Sp. Lum.; relative luminescence divided by OD$_{600}$) versus OD$_{600}$ and are representative of at least 3 independent experiments.
As a putative SK, AinR is predicted to recognize and respond to the autoinducer (AI) N-octanoyl-homoserine lactone (C8-HSL) (Gilson et al., 1995). Thus, the diminished luminescence phenotypes of the ΔainR and ΔainR ΔluxU mutants could result from a failure of this mutant to respond to C8-HSL. Alternatively, deletion of ainR could impact expression of the upstream gene ainS, which encodes the C8-HSL synthase protein. An impact on AinS synthesis could lead to decreased amounts of C8-HSL and decreased light production, potentially via direct control of the lux operon, as previously demonstrated (Kuo et al., 1996, Egland & Greenberg, 2000). To distinguish between these possibilities, I added exogenous C8-HSL to the ainR mutants and controls. I reasoned that, if the ainR mutants were defective in their response to C8-HSL, then they would still exhibit diminished luminescence relative to their controls. This appeared not to be the case, however, as addition of C8-HSL to the ΔainR and ΔainR ΔluxU mutants increased their luminescence levels to those of the control strain and the ΔluxU mutant, respectively (Fig. 20B). These data suggest that the decrease in luminescence by both the ΔainR and ΔainR ΔluxU mutants is likely due to decreased levels of C8-HSL, whose activity in promoting luminescence is largely or fully independent of the function of AinR, at least under our conditions. Thus, the role of AinR in controlling luminescence remains unclear.

Although AinR did not function as predicted in controlling bioluminescence, I wondered whether loss of AinR would impact biofilm formation. This was not the case: the ΔainR mutant exhibited no defect in biofilm formation (in the absence or presence of C8-HSL) (compare Fig. 21A and B and data not shown), while the ΔainR ΔluxU mutant
Figure 21. Wrinkled colony formation by ainR mutants. Time-course assay of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 49 h post-spotting for the following strains: ΔsypE control (pEAH73/KV3299), ΔainR ΔsypE (pEAH73/KV6196), ΔluxU ΔsypE (pEAH73/KV4830), and ΔainR ΔluxU ΔsypE (pEAH73/KV6259). Data are representative of at least three independent experiments.
exhibited the defect of the luxU mutant (compare Fig. 21C and D) and could be complemented when the wild-type allele of luxU-FLAG was expressed in single copy from the chromosome (data not shown). Thus, AinR has no impact on biofilm formation, and its role in controlling bioluminescence remains unclear. Further work will be necessary to determine what role AinR plays, if any, in controlling luminescence in V. fischeri.

**LuxU, but not RscS, is necessary to regulate syp-dependent biofilm formation under SypG-inducing conditions**

Since the only known role of LuxU is to serve as a phosphoryl-donor (Freeman & Bassler, 1999b, Shikuma et al., 2009) and LuxU impacts syp-dependent biofilm formation, I hypothesized that it could function upstream of SypG, a RR known to be required for transcription of the syp locus (Yip et al., 2005). Since previous studies had demonstrated that the SK RscS functions upstream of the RR SypG to control syp-dependent biofilm formation (Fig. 4) (Yip et al., 2006, Hussa et al., 2008), I questioned the relative importance of these two potential inputs, RscS and LuxU, on SypG-induced biofilm formation. I thus generated sypE mutants with deletions in luxU, rscS, or both and evaluated SypG-induced biofilm formation. Surprisingly, only loss of LuxU exerted an impact: whereas the luxU mutant exhibited a delay in wrinkled colony formation (compare Fig. 22A and C), the rscS mutant showed no significant defect in biofilm formation under these conditions (compare Fig. 22A and B). Even when the rscS and luxU mutations were combined, this mutant exhibited the same delay as the luxU mutant alone and could be complemented when the wild-type allele of luxU-FLAG was
Figure 22. Wrinkled colony formation by luxU and rscS mutants. Time-course assays of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 43 h post-spotting for the following strains: ΔsypE control (pEAH73/KV3299) (A), ΔrscS ΔsypE (pEAH73/KV6268) (B), ΔluxU ΔsypE (pEAH73/KV4830) (C), and ΔrscS ΔluxU ΔsypE (pEAH73/KV6269) (D). An * indicates the time at which initiation of wrinkled colony formation was apparent, typically identified by the presence of ridges around the outer edge of the spot. Data are representative of at least three independent experiments.
expressed in single copy from the chromosome (compare Fig. 22C and D and data not shown). Overall, these data indicate that LuxU plays a more important role than RscS in controlling biofilm formation when sypG is overexpressed.

**LuxU functions at or above SypG to impact syp transcription**

Since LuxU is necessary to promote syp-dependent biofilm formation, I sought to determine whether LuxU functioned upstream of SypG to control its activation (phosphorylation). If so, then I would expect that a phosphorylation-independent allele of SypG would be “blind” to the presence or absence of LuxU. I thus overexpressed a version of SypG in which the conserved site of phosphorylation, D53, was substituted for a glutamate (D53E). This substitution in RRs has previously been shown to promote the active state of the RR (Sanders et al., 1989, Sanders et al., 1992, Freeman & Bassler, 1999a). Indeed, this substitution in SypG caused an increase in SypG activity, as measured by syp transcription (Hussa et al., 2008). Consistent with this increased activity, when overexpressed in the ΔsypE mutant, sypG-D53E induced wrinkling sooner than when the wild-type allele of sypG was overexpressed (9-10 h vs. 13-15 h, respectively). When sypG-D53E was overexpressed in the ΔluxU ΔsypE mutant, the timing of wrinkled colony formation was not delayed, but rather was similar to that of the luxU+ control (Fig. 23A). These data are consistent with a model in which LuxU functions at or above the level of SypG.

It remains formally possible that the accelerated wrinkling effects of the sypG-D53E allele, combined with the delayed wrinkling caused by the loss of LuxU, results in a strain with a net timing of biofilm formation similar to the wild-type strain. Thus, to
Figure 23. The role of LuxU in syp activation. (A) Time-course assays of wrinkled colony formation induced by sypG-D53E overexpression using plasmid pKV276. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 24 h post-spotting for the following strains: ΔsypE control (pKV276/KV3299) and ΔluxU ΔsypE (pKV276/KV4830). Data are representative of at least three independent experiments.

(B) SypG-induced syp transcription from P_{sypA}-lacZ reporter strains. Cultures of sypG overexpressing strains ΔsypE attTn7::P_{sypA}-lacZ (white bars; pEAH73/KV4926) and ΔluxU ΔsypE attTn7::P_{sypA}-lacZ (grey bars; pEAH73/KV5516) were inoculated in LBS containing Tc and grown at 28°C with shaking. Samples were collected at 12 and 24 h and assessed for β-galactosidase activity (in Miller units) as a measure of promoter activity. All experiments were performed in triplicate. Data are a combination of two independent experiments with error bars representing the standard error. The P-value refers to the variation between the two samples as indicated by the brackets.
further probe the level at which LuxU exerts its impact on biofilm formation, I asked whether loss of LuxU affected transcription of the SypG-controlled sypA gene using a lacZ reporter fusion. I assayed β-galactosidase activity from the reporter expressed from the chromosomes of the ΔluxU and the luxU+ strains that overexpressed the wild-type allele of sypG. Loss of LuxU resulted in a decrease in syp transcription at two time points tested (12 and 24 h) (Fig. 23B). Thus, these data suggest that LuxU functions at or above the level of syp transcription, potentially due to an impact on SypG activation (Fig. 24).

Summary

In this study, I identified a novel connection between the Lux pathway and biofilm formation in *V. fischeri*. Specifically, I found that disruption of either the gene encoding the SK LuxQ or the gene encoding the histidine phosphotransferase LuxU caused a delay in SypG-induced biofilm formation. Surprisingly, this effect was independent of LuxO, which exerted only a minor impact on biofilm formation. However, LuxU does seem to function through LuxO to regulate bioluminescence. Thus, the Lux pathway appears to bifurcate at LuxU to regulate bioluminescence through LuxO and biofilm formation via a SypG-dependent pathway.

My data permit me to propose a model in which LuxQ functions through LuxU to regulate syp-dependent biofilm formation via activation of the RR SypG (Fig. 24). Support for the idea that LuxQ and LuxU serve as phosphoryl-donors to a downstream regulator of biofilm formation is as follows: 1) the kinase activity of LuxQ is necessary to promote biofilm formation, 2) the predicted, conserved site of phosphorylation in LuxU is necessary to regulate biofilm formation, 3) the only known role of LuxU in the
Figure 24. Model for regulation by the Lux and Syp pathway. Bioluminescence is regulated by the Lux phosphorelay, composed of the sensor kinases (SKs) LuxQ (which interacts with the periplasmic protein LuxP), and possibly AinR, the phosphotransferase LuxU, and the response regulator (RR) LuxO. Phosphoryl-transfer (dashed, double-sided arrows) is predicted to occur between the SKs, LuxU, and LuxO. Under low cell density conditions, LuxO is phosphorylated by the kinase activity of the SKs and activates transcription of the qrr sRNA, which binds to the transcript of litR and prevents its translation. LitR controls expression of LuxR, which promotes transcription of the lux operon (when bound to the AI produced by LuxI) (not depicted), leading to subsequent light production (bioluminescence). Regulators shaded in gray indicate those found in this study to be involved in biofilm formation. RscS is an SK known to control biofilm formation. Phosphorylation of the RR SypG is predicted to activate transcription of the syp locus, which encodes proteins thought to regulate, produce, and transport a polysaccharide necessary for biofilm formation. The specific activity of LuxU in activating biofilm formation is unknown, but it appears from the current study to work at or above the level of syp transcription, likely at the level of SypG activation (indicated by a question mark).
literature is as a histidine phosphotransferase (Freeman & Bassler, 1999b, Shikuma et al., 2009), 4) LuxQ depends on LuxU to regulate biofilm formation, and 5) the downstream RR of the Lux pathway, LuxO, is not required for the effect of LuxU on biofilm formation. Together, these data suggest that phosphotransfer is necessary for LuxQ and LuxU to regulate biofilm formation via a regulator distinct from LuxO. In support of the idea that LuxU serves as an input to regulate the activity of the RR SypG, I found that: 1) a “constitutively active” allele of SypG overcomes the requirement for LuxU, and 2) LuxU functions at or above the level of syp transcription. Overall, these data suggest that LuxU functions at or above the level of SypG, potentially at the level of SypG phosphorylation. This possibility is further supported by the fact that SypG and LuxO have similar domain structures (both are σ^54-dependent RRs) and exhibit 50% identity to each other. However, proof of such a possibility awaits additional biochemical experimentation; to date, attempts to examine the phosphorylation state of SypG have been unsuccessful. Thus, while my data support the hypothesis that LuxU could serve as a phosphoryl-donor to SypG, the regulation is clearly complex and may include currently unknown regulators.
Introduction

Previous studies have demonstrated that SypG and σ^{54} are necessary to promote syp transcription (Yip et al., 2005). SypG is a multi-domain protein that contains an N-terminal receiver (REC) domain with the predicted site of phosphorylation (D53), a centrally located AAA+ domain (for ATP hydrolysis), and a C-terminal DNA binding domain (Yip et al., 2005). Based on the presence of these domains and the overall similarity of SypG to proteins such as NtrC and LuxO (Lilley & Bassler, 2000), SypG is predicted to function as a σ^{54}-dependent transcriptional activator. Transcription by the σ^{54}-containing holoenzyme differs from other holoenzymes in that RNA polymerase recognizes sequences at -12 and -24 (rather than -10 and -35) and fails to initiate transcription without the help of a σ^{54}-dependent transcriptional activator [reviewed in (Buck et al., 2000, Wigneshweraraj et al., 2008, Bush & Dixon)]. These activator proteins typically bind to an enhancer sequence exhibiting dyad symmetry (i.e., an inverted repeat or IR) located 80-150 bp upstream of the promoter sequence. Upon activation (e.g., phosphorylation), the activator protein oligomerizes (typically into a hexamer), interacts with σ^{54} via DNA bending, and provides the energy (via ATP hydrolysis) necessary for RNA polymerase to promote open complex formation, leading to subsequent transcription of a particular gene(s).
The *syp* locus contains four promoters (upstream of *sypA*, *sypI*, *sypM*, and *sypP*), each with a predicted $\sigma^{54}$ recognition sequence (Fig. 4); of these, the promoters for *sypA*, *sypI*, and *sypM* have been confirmed via primer extension (Yip et al., 2005). In addition, multicopy expression of *sypG* has been shown to induce expression of two *syp::lacZ* reporters (*sypD::Tn10lacZ* and *sypN::Tn10lacZ*) in a $\sigma^{54}$-dependent manner. Finally, SypG is required for the RscS-induced transcription of *sypA* (Yip et al., 2005). From these studies, we concluded that SypG likely regulates transcription from at least two of the four putative $\sigma^{54}$-dependent *syp* promoters. Additionally, bioinformatic analyses of the *syp* promoters revealed a conserved 22 bp element present 50-90 bp upstream of each predicted $\sigma^{54}$ recognition sequence (Yip et al., 2005). This conserved region includes an IR consisting of two 6 bp half-sites separated by a 3 bp intervening sequence. Three of these sequences associated with the *syp* locus (*sypI*, *sypM*, and *sypP*) contain perfect IRs, while that associated with *sypA* diverges in the 3' half-site (Fig. 25). The position and composition of this conserved element is consistent with those of other enhancer-binding sequences bound by $\sigma^{54}$-dependent activators such as NtrC (Bush & Dixon, 2012). We hypothesized that this 22 bp sequence, designated the *syp* enhancer (SE) sequence, serves as a SypG-binding site to facilitate transcriptional activation at each of the *syp* promoters (Yip et al., 2005). Thus, in this work, I assessed the role of the SE sequence in *syp* transcription, biofilm formation, and host colonization, in collaboration with fellow graduate student Justin Eddy and building on the results of previous lab members Elizabeth Hussa and Michael Misale.
Figure 25. The conserved *syp* enhancer (SE) sequences. (A) The nucleotide sequence of each *syp* enhancer (SE) is shown with the inverted repeat underlined and in bold. Note that *sypI* contains two tandem SE sequences (SE-I-up and SE-I-down). (B) Logo sequence analysis [weblogo.berkely.edu (Schneider & Stephens, 1990, Crooks *et al.*, 2004)] comparing SE-A, SE-I-up, SE-I-down, SE-M, and SE-P.
SypG-mediated induction of syp promoters requires a conserved 22 bp sequence

Evidence gathered to date suggested that SypG, a putative $\sigma^{\text{54}}$-dependent transcriptional activator, serves as the direct transcriptional activator of the syp locus (Fig. 4) (Yip et al., 2005, Hussa et al., 2008, Darnell et al., 2008). However, whether SypG activates all four syp promoters ($\text{sypA}$, $\text{sypI}$, $\text{sypM}$, and $\text{sypP}$) and whether its activity depends on the conserved syp enhancer (SE) sequences located upstream of these promoters remained unknown. To answer these questions, we generated $\text{lacZ}$ transcriptional reporter fusions to sequences upstream of the sypA, sypI, sypM, and sypP genes. These constructs included the entire intergenic (IG) sequence and, in some cases, sequences in the adjacent upstream gene (Fig. 26). The fusions were placed in single copy in the chromosome of wild-type (ES114) V. fischeri at a benign site distal to the syp locus (the Tn7 site). We then introduced either a multi-copy SypG-expression vector ($\text{p} \text{sypG}$) or the vector control (VC). SypG expressed from $\text{p} \text{sypG}$ induced a substantial increase in $\beta$-galactosidase activity of each of the reporters (Fig. 27, IG set). These data thus establish an important role for SypG in inducing syp transcription at each of the syp promoters. They also indicate that a SypG-responsive promoter exists upstream of sypP, which had been previously unconfirmed.

To begin to delimit the regulatory region important for SypG-mediated syp induction, we made additional reporter derivatives with 5’ truncations. Specifically, we generated a set of constructs, designated FL (full-length), that began at the previously documented SE sequence (Yip et al., 2005), and a second set, designed TR (truncation) that began immediately downstream of that sequence (Fig. 26). For strains containing the
Figure 26. Representation of the SE regions of the *syp* locus and regions used for *lacZ*-fusion derivatives. The promoter regions for (A) *sypA*, (B) *sypI*, (C) *sypM*, and (D) *sypP* are shown. The *syp* genes are indicated with block arrows, with the relevant *syp* gene shaded in gray. The predicted $\sigma^{54}$ and SE sequences are indicated with boxes. Numbers and brackets represent the distance within the indicated region. The promoter derivative used in the *lacZ*-fusion construct is indicated beneath each promoter region. Intergenic (IG) and full-length (FL) constructs each contains the associated SE sequence, with the exception of FL *sypI*, which lacks SE-I-up. The truncation (TR) constructs lack SE sequences.
Figure 27. Activity of sydA, sydI, sydM, and sydP reporter fusions. pEAH73 (psypG)-containing derivatives of the indicated strains were grown in HMM containing Tc for 24 h and then harvested for a β-galactosidase assay to measure promoter activity (Miller units). IG, FL, and TR refer to intergenic, full-length, and truncated constructs as defined in the text and Fig. S3. (A) PsypA-lacZ – IG (KV4522), FL (KV3246), TR (KV3636), (B) PsypI-lacZ – IG (KV4523), FL (KV3629), TR (KV3628), (C) PsypM-lacZ – IG (KV4524), FL (KV3632), TR (KV3631), and (D) PsypP-lacZ – IG (KV4525), FL (KV4526), TR (KV4527). Error bars represent the standard deviation. These graphs are representative of at least two independent experiments.
FL constructs, with one exception, we saw similar levels of β-galactosidase activity, indicating that the sequences included in these shorter constructs were sufficient for the observed SypG-mediated induction (Fig. 27, FL set). The exception was *sypI*, and upon closer inspection we noticed that the *sypI* promoter region contained two sets of the conserved SE sequence (Fig. 25); the upstream SE sequence was truncated in the *sypI* FL construct (Fig. 26). These data suggest either that both sets of the sequence are required for optimal transcriptional activation from the *sypI* promoter, or that the upstream sequence is the more important of the two. For strains containing the TR constructs, we found that the ability of SypG to induce transcription was severely diminished or abolished (Fig. 27, TR sets). Thus, SypG requires the SE sequences to induce *syp* transcription.

**Development of a biofilm assay for *sypA* expression**

The β-galactosidase reporter experiments confirmed our prediction that the SE sequences located upstream of the four *syp* promoters were necessary for SypG-mediated transcription, but did not address the requirement for specific nucleotides. To identify nucleotides that are critical for *syp* activation, we developed a biofilm-based assay of SypG activity. We used this assay instead of the β-galactosidase assay due to technical difficulties associated with generating numerous strains with single copy point mutant derivatives of the *lacZ* fusion constructs. The biofilm assay provides an indirect measure of SypG activity based on its ability to promote biofilm formation. Previous studies demonstrated that expression of SypG from a multi-copy plasmid fails to promote biofilm formation, even though it induces *syp* transcription (Hussa et al., 2008). This effect is due
to the inhibitory activity of SypE, which phosphorylates and inactivates SypA, a protein necessary for biofilm formation (Fig. 7) (Morris & Visick, 2013b). However, biofilm formation is induced when the sypG plasmid is introduced along with a second plasmid that contains the SypG-controlled gene sypA (along with its upstream regulatory region) (Fig. 28) (Morris & Visick, 2013a). We hypothesized that, under these conditions, SypG induces expression of high levels of SypA such that some SypA escapes the inhibitory phosphorylation mediated by SypE, thereby permitting biofilm formation to proceed.

Here, we tested one part of this hypothesis, that biofilms form due to the ability of SypG to activate the sypA promoter present on the multi-copy plasmid. First, we asked whether SypG expressed from a multi-copy plasmid increased the levels of SypA protein, and found that it did (Fig. 29). As predicted from the β-galactosidase results, this increase in SypA protein levels depended on the presence of an intact sypA enhancer (SE-A) sequence (Fig. 29).

Next, we asked whether biofilm formation induced upon introduction of the sypG and sypA plasmids similarly depended on the presence of the SE-A sequence. To evaluate biofilm formation, we spotted a culture of cells onto a plate and monitored the development of the spot from a smooth to wrinkled morphology over time. We anticipated that the decreased amount of SypA due to the loss of SE-A would result in diminished or delayed wrinkled colony development. As previously demonstrated (Hussa et al., 2008, Morris & Visick, 2013a), cells that contained either the sypG plasmid or the sypA plasmid alone formed smooth colonies (Fig. 30A and data not shown), while cells that contained both plasmids formed wrinkled colonies (Fig. 30B). In contrast, cells that
Figure 28. Model for the *sypA*-based biofilm assay. Overexpression of *sypG* alone (from a plasmid, grey block arrow in open circle) activates transcription of the *syp* locus, but is unable to promote biofilm formation due to the inhibitory activity of SypE (grey hexagon) on SypA (black oval). However, co-overexpression of *sypG* and *sypA* [black block arrow in open circle with promoter (P) and *syp* enhancer (SE) sequence (white box)] promotes biofilm formation, likely by SypG-mediated *sypA* expression, leading to excess SypA, which can escape the inhibitory activity of SypE and promote biofilm formation.
Figure 29. Assessment of SypA protein levels in strains with \textit{sypG} and \textit{sypA} plasmids. Western blot analysis was performed on the following strains to determine the level of SypA in strains that contained vector control (VC – pVSV105) or the \textit{sypG} plasmid (pARM9) and a plasmid containing an HA-tagged allele of \textit{sypA} with or without the SE-A sequence [pVAR91 (+SE-A) and pVAR92 (-SE-A), respectively]: VC and pVAR91 (lane 1), pARM9 and pVAR91 (lane 2), VC and pVAR92 (lane 3), and pARM9 and pVAR92 (lane 4).
Figure 30. Biofilm formation induced by co-overexpression of sypG and sypA. Images show the wrinkled colony morphologies of spotted cultures at 68 h post-spotting for ES114 containing the sypG overexpression plasmid pARM9 and either vector control (VC) or a sypA plasmid derivative that contains or lacks the sypA enhancer (SE-A) as indicated: (A) VC – pKV282, (B) WT SE-A (pVAR57), (C) ΔSE-A (pKV465), and (D) SE-A\textsuperscript{R} (pVAR58). Data are representative of at least three independent experiments.
contained the sypG plasmid along with a sypA plasmid lacking SE-A (ΔSE-A) were unable to form wrinkled colonies within the same time frame as the positive control (compare Fig. 30B and C). Although colonies formed by this strain did eventually wrinkle, the extent of wrinkling was minimal even at the late time point of this data set (Fig. 30). This strain thus served as the negative control throughout the remainder of these experiments.

To verify that wrinkled colony formation depended on SE-A, we restored SE-A to the ΔSE-A construct at an engineered Apal restriction site (SE-A-restored or SE-A^R). Cells containing this modified plasmid formed wrinkled colonies with the same timing and intensity as the native SE-A-containing control strain (Fig. 30B and D and data not shown). Although restoration of SE-A resulted in the additional insertion of 5 bp of non-native sequences between the enhancer and the promoter, we could detect no consistent differences in biofilm formation by cells carrying the restored or native SE-A constructs. Because the SE-A^R construct most closely matched other constructs we subsequently generated, we used it as a positive control throughout the remainder of these experiments.

Together, our data indicate that SE-A plays an important role in biofilm formation. Because SE-A was also necessary for sypA-lacZ reporter expression and induction of SypA protein production, we conclude that biofilm formation in this assay depends on the ability of SypG to induce sypA transcription and, as a result, promote SypA protein production. This assay thus provides a means to perform an in-depth analysis of the sequences necessary for SypG-mediated syp transcription and biofilm formation.
Identification of nucleotides necessary for SypG-mediated biofilm induction

We next constructed *sypA* plasmids with modifications to the SE-A sequence. First, we generated nested deletion derivatives (Fig. 31A). Loss of the four 5'-most nucleotides (-4 bp) did not affect the timing of wrinkled colony formation, suggesting that these sequences are not important for *sypA* induction (compare Fig. 31C & D). However, loss of the first seven nucleotides (-7 bp) caused a minor delay in biofilm formation, resulting in a slightly smaller wrinkled colony at later time points, indicating that *sypA* induction is likely diminished (compare Fig. 31C and E). When SE-A was truncated even further to remove a portion of 5' half-site of the IR (-10 bp), biofilm formation was reduced to that of the negative control (compare Fig. 31B and F). Thus, the presence of an intact IR is sufficient to promote biofilm formation, but the presence of sequences upstream of the IR permits the best induction of biofilm formation.

To further understand the requirements of the SE-A sequence for biofilm formation, we generated transition mutations (exchanging A for G and T for C and vice versa) at each of the nucleotides within the 5' and 3' half-sites of the IR region (Fig. 31A, underlined regions). Only two substitutions resulted in wrinkled colony formation that was comparable to or better than that of the control strain, 3AG and 1AG (compare Fig. 32A and C). In particular, the strain carrying the 3AG substitution in SE-A consistently exhibited wrinkled colony formation that was faster than the positive control (data not shown). Of note, 3AG is a change that makes SE-A more closely match a perfect IR sequence; in contrast to the other SE sequences (SE-I-up, SE-M, and SE-P), which
**Figure 3. The effect of truncating SE-A in the sypA-dependent biofilm assay.** (A) The full-length and 5’ truncation derivatives of SE-A generated in this study are indicated. (B-E) To assess the importance of the SE-A sequence, wrinkled colony formation was monitored up to 68 h post-spotting for ES114 containing the sypG overexpression plasmid pARM9 and a sypA plasmid derivative containing the indicated full-length or truncated SE-A sequence: (B) ΔSE-A (pKV465), (C) SE-A^R^ (pVAR58), (D) SE-A -4 bp (pSLN4), (E) SE-A -7 bp (pSLN5), and (F) SE-A -10 bp (pSLN7). Data are representative of at least three independent experiments.
Figure 32. The effect of SE-A transition mutations in the sypA-dependent biofilm assay. The impact of transition mutations within the SE-A sequence was assessed using the sypA-dependent biofilm assay. Images show the wrinkled colony morphologies of spotted cultures at 71.5 h post-spotting for ES114 containing the sypG overexpression plasmid pARM9 and a sypA plasmid derivative containing the indicated point mutation: (A) controls: ΔSE-A (pKV465) and SE-A$^R$ (pVAR58), (B) 5' half-site: 15TC (pKV444), 14TC (pKV445), 13CT (pKV446), 12TC (pKV447), 11CT (pKV448), and 10AG (pKV449), and (C) 3' half-site: 6TC (pKV450), 5GA (pKV451), 4CT (pKV452), 3AG (pKV453), 2AG (pKV454), and 1AG (pKV455). Data are representative of at least three independent experiments. Refer to Fig. 32A for the numbering scheme for the individual nucleotides within SE-A.
contain perfect IR sequences (TTCTCA-N3-TG\text{AGAA}), SE-A contains an imperfect IR (TTCTCA-N3-TG\text{CAA}) (Fig. 25).

We categorized the biofilm phenotypes of the remaining strains into two classes: (i) those with diminished biofilm formation relative to the positive control (intermediate phenotype) and (ii) those that were biofilm-defective (indistinguishable from the negative control) (Fig. 32). The former category included those with substitutions within the highly conserved 5' half-site of SE-A (15TC, 13CT, 12TC, and 11CT). The latter category included strains with substitutions within both the 3' half-site (6TC, 5GA, 4CT, and 2AG) and the 5' half-site (14TC and 10AG). We interpret the reduced and defective biofilm formation of these strains to mean that SypG has reduced or defective binding to the SE sequences and thus fails to promote the same levels of sypA transcription as the positive control. Thus, we conclude that 10 of the 12 nucleotides of the IR play important roles in promoting SypG-mediated biofilm formation in this assay.

Because our conclusions were based on a single base change, we expanded our screen for a subset of these nucleotides (positions 2, 4, 5, and 14) by generating the other two possible base changes (transversions). For position 2A, a change to T was permissive to promote biofilm formation, but a change to C was not (Fig. 33B). Similarly, for 4C, a change to A, which brings the 3' half-site closer to perfect, was permissive, but a change to G was not (Fig. 33C). In contrast, for 5G and 14T, all substitutions resulted in biofilm formation that was indistinguishable from the negative control (compare Fig. 33A and D-E), indicating that the original bases at these positions are indeed critical determinants for sypA expression. These data thus indicate that while some changes are more permissive
Figure 33. The effect of transversion point mutations in the sypA-dependent biofilm assay. The impact of SE-A transversion mutations was assessed using the sypA-dependent biofilm assay. Images show the wrinkled colony morphologies of spotted cultures at 72 h post-spotting for ES114 containing the sypG overexpression plasmid pARM9 and a sypA plasmid derivative containing the indicated point mutation: (A) controls: ΔSE-A (pKV465) and SE-A^R (pVAR58), (B) 2AG (pKV454), 2AT (pVAR84), and 2AC (pVAR83), (C) 4CT (pKV452), 4CA (pKJW11), and 4CG (pKJW10), (D) 5GA (pKV451), 5GT (pKJW8), and 5GC (pKJW9), and (E) 14TC (pKJW4), 14TG (pKJW5), and 14TA (pKV452). The original transition mutation constructs are included for reference. Data are representative of at least three independent experiments. Refer to Fig. 32A for the numbering scheme for the individual nucleotides within SE-A.
than others, most of the nucleotides within the IR play key roles in SypG-induced biofilm formation.

**Evaluation of other syp enhancers**

We next wondered whether replacing the SE-A sequence with other SE sequences (i.e., those associated with *sypI*, *sypM*, and *sypP*) would similarly promote biofilm formation in our assay. Indeed, insertion of either SE-M or SE-P promoted biofilm formation in the context of this assay (compare Fig. 34B, E, and F). Interestingly, when we evaluated the two *sypI* enhancers (SE-I-up and SE-I-down; see Fig. 25), we found that SE-I-down promoted biofilm formation, whereas SE-I-up did not (compare Fig. 34B, C and D). Thus, in the context of the *sypA* promoter region, the upstream *sypI* enhancer (SE-I-up), which is further removed from the IR consensus, is insufficient to promote biofilm formation. These results stand somewhat in contrast to what we observed for transcription from the *sypI* promoter, in which loss of the upstream, less-conserved SE sequence led to a loss of transcription; I will discuss this apparent contradiction in the Discussion section. Overall, the SE sequences associated with the other *syp* promoters are able to promote biofilm formation in our assay, indicating that SypG is able to recognize these sequences. In addition, chromatin immunoprecipitation (ChIP) assays performed by former graduate student Justin Eddy confirmed that SypG does indeed bind to the *syp* promoter regions.

**VF_A1019, VF_A0120, and VF_A0550 contain syp enhancer sequences**

Equipped with the information about the specific nucleotides critical for SypG-mediated activation, we next examined the *V. fischeri* genome for potential SypG binding
Figure 34. The impact of substitution of SE-I-up, SE-I-down, SE-M, and SE-P for SE-A in the *sypA*-dependent biofilm assay. The ability of SypG to recognize the other SE sequences was assessed using the *sypA*-dependent biofilm assay. Images show the wrinkled colony morphologies of spotted cultures at 72 h post-spotting for ES114 containing the *sypG* overexpression plasmid pARM9 and a *sypA* plasmid derivative with the indicated SE sequence in place of SE-A: (A) ΔSE-A (pKV465), (B) SE-A<sup>R</sup> (pVAR58), (C) SE-I-up (pKV437), (D) SE-I-down (pKV438), (E) SE-M (pKV439), and (F) SE-P (pKV440). Data are representative of at least three independent experiments.
sites. Using the Regulatory Sequence Analysis Tools (RSAT) program (van Helden, 2003, Thomas-Chollier et al., 2008, Thomas-Chollier et al., 2011), we identified a number of potential binding sites (Table 4); this bioinformatic analysis was performed by former graduate student Beth Hussa. We further predicted that a member of the SypG regulon would also require a $\sigma^{54}$ binding site, and found that the promoter regions of only three genes (other than $syp$) contained both a putative SypG binding site and a putative downstream $\sigma^{54}$ binding site. One potential SypG-binding sequence was located immediately adjacent to the $syp$ locus in front of the divergently transcribed gene, $VF_A1019$ (Fig. 35A). The other two were located upstream of $VF_A0120$ and $VF_A0550$ (Fig. 35A). Upon closer inspection of the predicted SE sequences associated with these genes, we noted that the 5' half-site was an exact match to the $syp$ SE sequences, but the 3' half-site deviated from the IR sequence (Fig. 35A and B). Compared to the 3' half-site of SE-A, the 3' half-site of SE-1019 (the SE sequence associated with $VF_A1019$) differs at a single at position 1 (A to T) (Fig. 35A), a position that does not appear important for SypG’s recognition of SE-A (Fig. 32C). Similarly, the 3' half-site associated with $VF_A0120$ (SE-0120) differs at nucleotide 4 (C to A), a change that was permissive in the context of SE-A (Fig. 33C). Finally, the 3' half-site associated with $VF_A0550$ (SE-0550) has nucleotide changes at positions 1-4; some substitutions at these positions were permissive for biofilm formation in the context of SE-A (Fig. 32C and 33B and C). Therefore, we asked whether SypG could recognize these SE sequences by replacing SE-A upstream of $sypA$ with the SE sequences from $VF_A1019$ (SE-1019), $VF_A0120$ (SE-0120), or $VF_A0550$ (SE-0550) in our plasmid-based biofilm assay. We
Table 4. Bioinformatics search for the SE sequence in *V. fischeri*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>σ⁵⁴</th>
<th>Name</th>
<th>Predicted protein</th>
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<tr>
<td>VF_A1020</td>
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<td>sypA</td>
<td>Sulphate transporter, anti-sigma factor antagonist</td>
</tr>
<tr>
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<td>sypI</td>
<td>Group 1 glycosyltransferase</td>
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<tr>
<td>VF_A1083</td>
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<td>Methyl-accepting chemotaxis protein</td>
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¹It is unclear which gene is associated with the predicted SE sequence.
Figure 35. The effect of SE-1019, SE-0120, and SE-0550 in the sypA-dependent biofilm assay. (A) The SE sequences (shaded) and predicted σ^{54} binding sites of sypA (SE-A), VF_A1019, VF_A0120, and VF_A0550 are shown. (B) Logo sequence analysis [weblogo.berkely.edu (Schneider & Stephens, 1990, Crooks et al., 2004)] comparing SE-A, SE-1019, SE-0120, and SE-0550. (C) The ability of SypG to recognize the putative SE sequences associated with VF_A1019, VF_A0120, and VF_A0550 was assessed using the sypA-dependent biofilm assay. Images show the wrinkled colony morphologies of spotted cultures at 68 h post-spotting for ES114 containing the sypG overexpression plasmid pARM9 and s sypA plasmid derivative containing the indicated SE sequence in place of SE-A: ΔSE-A (pKV465), SE-A^R (pVAR58), SE-1019 (pKV463), SE-0120 (pKV462), and SE-0550 (pKV464). Data are representative of at least three independent experiments.
found that introducing any of these SE sequences in place of SE-A induced biofilm formation to a level above that of the negative control (Fig. 35C), and indeed, to a degree indistinguishable from the positive control (Fig. 35C), suggesting that SypG can recognize these SE sequences despite their differences. In further support of this conclusion, ChIP work performed by Justin Eddy indicated that SypG binds to the VF_A0120 promoter region (the other promoters were not tested). Taken together, these data suggest that VF_A1019, VF_A0120, and VF_A0550 may comprise part of the SypG regulon.

**The sypA enhancer is important for biofilm formation and colonization**

To further investigate the importance of the SE-A sequence, we deleted it (SE-A + 5 bp upstream) from its native location in the chromosome and evaluated wrinkled colony formation. Because this strain expresses the SypE biofilm inhibitor, we induced syp using the sensor kinase RscS, as this regulator functions both to activate syp transcription via SypG (Hussa et al., 2008) and to inactivate SypE (Fig. 4) (Morris et al., 2011). Whereas the wild-type strain (SE-A⁺) formed wrinkled colonies by about 21 h, biofilm formation by the ΔSE-A strain was both delayed and diminished (Fig. 36A-C). To verify that the defect of the ΔSE-A strain was due to this deletion, we restored the SE-A sequence to its native location in the chromosome (ΔSE-A::SE-A). This strain exhibited wrinkled colony formation similar to that of the positive control (compare Fig. 36B and D). Thus, these data indicate that loss of SE-A from its native location in the chromosome indeed disrupts biofilm formation.
Figure 36. Wrinkled colony development by ΔSE-A and restored strains. The impact of deleting SE-A from its native position in the chromosome was assessed by monitoring wrinkled colony formation for 72 h post-spotting for the following strains: (A) WT (ES114) carrying pKV69 (vector control), (B) WT (ES114) carrying the \textit{rscS} overexpression vector pKG11, (C) ΔSE-A (KV4970) carrying pKG11, and (D) ΔSE-A::SE-A (KV6716) carrying pKG11. Data are representative of at least three independent experiments.
We next asked whether SE-A is similarly important for host colonization by *V. fischeri*. Previous studies have revealed a clear correlation between the ability to form a biofilm and colonization competence: strains unable to promote biofilm formation *in vitro* are also unable to proficiently colonize the host *in vivo* (Yip et al., 2006, Shibata et al., 2012, Morris & Visick, 2013b, Morris & Visick, 2013a). Furthermore, these experiments do not depend on overexpression of any regulatory protein such as RscS or SypG. We thus inoculated juvenile *E. scolopes* squid with wild-type *V. fischeri*, the ∆SE-A mutant, or the ∆SE-A::SE-A strain, and evaluated colonization at an early time point. At this time point, wild-type-inoculated animals contained on average 4x10^4 bacteria, while squid inoculated with the ∆SE-A strain were uncolonized or contained, on average, fewer than 150 bacteria (Fig. 37). Finally, squid inoculated with the SE-A-restored strain (∆SE-A::SE-A) contained wild-type levels of bacteria (Fig. 37). These data indicate that the SE-A sequence is indeed necessary for efficient initiation of host colonization, and underscore the importance of this enhancer sequence to the lifestyle of *V. fischeri*.

**Summary**

In this study, we sought to determine whether a conserved sequence within the *syp* locus, the *syp* enhancer or SE sequence, was necessary for the σ^54-dependent transcriptional activator SypG to promote *syp* transcription, biofilm formation, and host colonization by *V. fischeri*. We have: 1) demonstrated that the SE sequence is necessary for SypG-mediated *syp* transcription from the four *syp* promoters (*sypA, sypI, sypM*, and *sypP*), 2) identified critical nucleotides within the 5' and 3' half-sites of the *sypA* enhancer (SE-A) necessary for SypG-mediated activation of biofilm formation, 3) demonstrated
Figure 37. Colonization by WT, ΔSE-A, and restored strains. The role of SE-A in colonization was assessed by inoculating newly hatched juvenile E. scolopes squid with WT (ES114 - squares), the ΔSE-A mutant (KV4970 - triangles), or the SE-A-restored strain (ΔSE-A::SE-A - KV6716 - circles) for 3 hours, followed by transfer to fresh artificial seawater and incubation for another 13-16 h. The number of CFU per squid was then assessed. Each marker represents an individual squid. The dashed line represents the limit of detection (14 CFU). The data shown are combined from two individual experiments (black and white symbols).
that SypG is able to recognize the SE sequences associated with the *syp* locus, as well as those associated with genes outside of the *syp* locus, 4) determined that SypG binds to the *syp* promoter regions (worked performed by Justin Eddy), and 5) found that loss of SE-A leads to defective biofilm formation and host colonization. Overall, this work filled in a critical gap in the Syp regulatory circuit by demonstrating that SypG binds to *syp* promoter regions in a manner that depends on SE sequences and allowed identification of three new potential SypG targets.
III. IDENTIFICATION OF NOVEL MATRIX PROTEINS THAT CONTROL BIOFILM FORMATION IN *Vibrio fischeri*

**Introduction**

When bacteria sense a particular signal that promotes biofilm formation, the cells typically adhere to a surface and initiate the production of an extracellular matrix. This matrix is comprised of such components as polysaccharides, eDNA, and proteins. Many studies have focused on the role of polysaccharide and eDNA during biofilm formation. Thus, much less is known about the role of extracellular proteins during this process, or even the identity of such proteins, making this an underdeveloped area of research.

Biofilm matrix proteins have been, perhaps, best studied in *V. cholerae*. Biofilm formation by *V. cholerae* requires the genes encoding the matrix proteins RbmA, RbmC, and Bap1, which are co-regulated with the *vps* polysaccharide loci (Yildiz et al., 2004, Moorthy & Watnick, 2005, Fong et al., 2006, Fong & Yildiz, 2007); in some *V. cholerae* serotypes, there appears to be some level of redundancy in the function of RbmC and Bap1 (Fong & Yildiz, 2007). A recent study examined the expression, localization, and interaction of the matrix proteins with the VPS polysaccharide, starting from attachment of a single cell, providing one of the first comprehensive views for the role of matrix proteins during biofilm formation (Berk et al., 2012).

In contrast to *V. cholerae*, however, little is known about the role of matrix proteins in biofilm formation by other bacteria. Furthermore, for many good bacterial biofilm models, including *V. fischeri*, biofilm matrix proteins have yet to be identified. However, we recently identified three new putative members of the SypG regulon in *V.
that I predicted might be involved in biofilm formation. Indeed, my findings, as described in this section, indicate that these genes are necessary for biofilm maturation and one of them is a secreted product. Thus, I hypothesize that the gene products may represent the first identified biofilm matrix proteins in *V. fischeri*.

*bam* and *bal* constitute part of the SypG regulon

We previously identified three unlinked two-gene sets (pairs of genes) as putative members of the SypG regulon, namely *VF_A1019-VF_A1018, VF_A0120-VF_A0121,* and *VF_A0550-VF_A0549* (Ray et al., 2013), herein called *bamA-balA, bamB-balB,* and *bamC-balC.* These gene sets were identified due to the presence of an SE sequence (the SypG binding site) upstream of a putative σ^{54} binding site. In addition, we demonstrated that (i) these SE sequences could be recognized by SypG when substituted in place of the native SE sequence upstream of the *sypA* promoter and (ii) SypG could bind to the *bamB* promoter region in a ChIP assay (Ray et al., 2013). To confirm that SypG could also regulate *bamA* and *bamC,* I generated transcriptional reporter constructs that fuse the promoter regions of these genes to *lacZ,* and introduced the fusions, in single copy, in the chromosome of wild-type *V. fischeri* at a benign site (the Tn7 site). Next, I introduced either a multi-copy SypG-expression plasmid or the vector control (VC) into these strains. Finally, I measured the β-galactosidase activity of each reporter to determine relative promoter activities. Expression of SypG in these strains led to a dramatic increase in β-galactosidase activity relative to the VC-containing strains (Fig. 38), indicating that *bamA* and *bamC* are indeed regulated by SypG and thus comprise part of the SypG regulon.
Figure 38. SypG induces transcription from \textit{bam} promoters. The ability of SypG to induce transcription from the \textit{bamA} and \textit{bamC} promoters was assessed using reporter fusions to a promoterless \textit{lacZ}. \(\beta\)-galactosidase activity was assessed, as a measure of promoter activity, from \textit{sypG}-overexpressing (psypG; pEAH73) and vector-control (VC; pKV69) derivatives of KV7216 (P\textsubscript{bamA}-lacZ) and KV7220 (P\textsubscript{bamC}-lacZ) grown as described in Materials and Methods. The \(\beta\)-galactosidase activity is shown as Miller units. Error bars represent the standard deviation and the \(P\)-value refers to the variation between the two samples indicated by the line. These data are representative of at least two independent experiments.
BamA-C are conserved proteins, while BalA-C are conserved lipoproteins

Bioinformatic analyses of the bam and bal gene sets indicated that bamA and balA, which are located immediately adjacent to the syp locus, clearly form an operon, as the two genes overlap by 38 nucleotides, while bamB-balB and bamC-balC likely do so, as the genes are separated by only 11 and 12 nucleotides, respectively. Importantly, the predicted V. fischeri proteins are similar to each other: the three Bam proteins are 37% identical and 53% similar to each other, while the three Bal proteins are 26% identical and 50% similar to each other (Fig. 39) (Altschul et al., 1990, Altschul et al., 1997). The Bam proteins contain no hypothetical domains of known or unknown function, but they each contain a putative Sec-dependent signal sequence (Fig. 40)(Petersen et al., 2011), suggesting that these proteins are exported to the periplasmic space or out of the cell. In contrast, the Bal proteins appear to be lipoproteins: in addition to a predicted lipoprotein signal sequence, the Bal proteins contain a lipobox sequence (Babu et al., 2006) that includes an invariant cysteine predicted to become acylated following transport across the inner membrane (Nakayama et al., 2012) (Fig. 40). The apparent absence of inner membrane retention signals suggests that the Bal proteins may be sorted to the outer membrane (Tokuda & Matsuyama, 2004). Finally, while the syp locus is primarily conserved only in Vibrio spp., the bam-bal gene sets are present in numerous Vibrio spp. and other marine bacteria (Fig. 41). This conservation suggests that the bam and bal genes could play similar roles in the physiology and/or ecology of various marine microbes.
Figure 39. Alignment of the Bam and Bal proteins, respectively. Protein alignment of (A) BamA, BamB, and BamC or (B) BalA, BalB, and BalC. Residues in red are conserved between all three proteins, while those in blue are conserved between two of the three proteins.
Figure 40. Putative signal sequences of the Bam and Bal proteins. (A) Signal sequence prediction program SignalP (Petersen et al., 2011) predicts the presence of a signal sequence at the N-termini of BamA, BamB, and BamC. The inverted triangle indicates the predicted site of cleavage. (B) The Database of Bacterial Lipoproteins (DOLOP) (Babu et al., 2006) predicts that the Bal proteins are indeed lipoproteins based on the presence of the following sequences: a charged residue (blue), followed by a hydrophobic stretch of amino acids (green), and a lipobox sequence ending in an cysteine residue (red); bold black sequences after the invariant cysteine do not appear to contain an inner membrane retention signal (Tokuda & Matsuyama, 2004).
Figure 41. Conservation of Bam and Bal in other bacteria. Conservation of the (A) Bam protein and (B) the Bal protein in other bacteria. Sequences of BamA and BalA, respectively, were submitted to KEGG (Kanehisa & Goto, 2000, Kanehisa et al., 2014) for comparison with other proteins. The indicated trees were generated with a subset of the conserved proteins with the *V. fischeri* protein boxed for reference in each tree.
**Disruption of the bal genes impacts bioluminescence**

To probe function of Bam and Bal, I generated in-frame deletion mutants defective for each *bam* or *bal* gene. Because the similarity of the proteins to each other suggested they could have overlapping or redundant functions, I also generated double and triple *bam* and *bal* mutants. I then evaluated the impact of these mutations on motility, luminescence and growth. None of the mutations substantially impacted motility or growth (data not shown). The *bam* mutants all exhibited wild-type luminescence, as did the *balA* mutant (Fig. 42 and 43). Surprisingly, however, various *balB* and *balC* mutants exhibited alterations in luminescence. Relative to the wild-type strain, the single *balB* mutant exhibited a slight, yet reproducible decrease in luminescence, while any mutant that contained the *balC* deletion exhibited increased luminescence (Fig. 43). Together, these data suggest that BalC is a negative regulator of luminescence and that BalB could positively regulate luminescence. Of note, the luminescence experiments were carried out in the absence of SypG overexpression, suggesting that the basal level of transcription is sufficient to impact luminescence under these conditions. The roles of these proteins in luminescence and the likelihood that they are lipoproteins prompted us to name them *bal*, for bioluminescence-associated lipoprotein gene (*balA* (*VF_A1018*), *balB* (*VF_A0121*), and *balC* (*VF_A0549*), respectively). How the *bal* genes impact luminescence will be the subject of future work.

**The bam genes contribute to wrinkled colony formation**

Due to their coordinate regulation with the *syp* locus, which is critical for biofilm formation by *V. fischeri* (Yip et al., 2005, Shibata et al., 2012, Morris & Visick, 2013b), I
Figure 42. Mutation of *bam* does not impact bioluminescence. The impact of *bam* mutations on bioluminescence was assessed by growing single, double, and triple *bam* mutants and the wild-type control in SWTO and monitoring luminescence over time and calculating specific luminescence as described in Materials and Methods. The strains assessed are as follows: wild-type (ES114), Δ*bamA* (KV6886), Δ*bamB* (KV6638), Δ*bamC* (KV6787), Δ*bamAB* (KV7078), Δ*bamAC* (KV7079), Δ*bamBC* (KV6712), and Δ*bamABC* (KV6897). These data are representative of at least two independent experiments.
Figure 43. Mutation of bal impacts bioluminescence. The impact of bal mutations on bioluminescence was assessed by growing single, double, and triple bal mutants and the wild-type control in SWTO and monitoring luminescence over time and calculating specific luminescence as described in Materials and Methods. The strains assessed are as follows: wild-type (ES114), ΔbalA (KV6890), ΔbalB (KV6924), ΔbalC (KV6923), ΔbalAB (KV7080), ΔbalAC (KV7081), ΔbalBC (KV7369), and ΔbalABC (KV7128). These data are representative of at least two independent experiments.
hypothesized that the *bam* and/or *bal* genes might also play a role in biofilm formation. To evaluate biofilm formation, I used strains in which the sensor kinase gene *rscS* is overexpressed. In contrast to their importance in bioluminescence, the *bal* genes played little to no role in biofilm formation: a strain deleted for all three *bal* genes exhibited wrinkled colony formation similar to that of the control (Fig. 44). The biggest impact was exerted by *balB*, which when deleted alone or in the context of a *balA* deletion caused a minor (~5 h) delay in wrinkled colony formation (Fig. 44). However, at a later time point (70 h), there was no difference in wrinkled colony formation for the *balB* or *balAB* mutants compared to the control (Fig. 44). Taken together, these data suggest that the *bal* genes play a relatively minor role in biofilm formation, with *balB* exerting the biggest impact.

Unlike the triple *bal* mutant, the triple *bam* mutant exhibited a severe defect in wrinkled colony formation, largely failing to wrinkle (Fig. 45). This defect could not be attributed to a single *bam* gene, as deletion of individual *bam* genes had no substantial impact on wrinkled colony formation relative to the control (Fig. 45). However, deletions of combinations of two *bam* genes revealed that *bamA* and *bamB* are the more important genes: only the *bamAB* double mutant was severely defective for wrinkled colony formation, though this mutant did exhibit some wrinkling at later time points (Fig. 45). These data indicate that the presence of either *bamA* or *bamB* is sufficient to promote wrinkled colony formation. Furthermore, they suggest that there is likely some overlap in function of the Bam proteins. Indeed, complementation of the triple mutant with the *bamA-bala* operon alone permitted robust wrinkled colony formation similar to the
Figure 44. Impact of \textit{bal} mutations on biofilm formation. To assess the impact of \textit{bal} on biofilm formation, I overexpressed \textit{rscS} (pKG11) in wild-type (control) and \textit{bal} mutants and assessed the ability of these strains to form wrinkled colonies over time. In the experiment shown, cultures were spotted onto LBS medium containing Tc and incubated at room temperature for 70 h, with images collected at the indicated times. The strains assessed were pKG11-containing derivatives of the following strains: wild-type control (ES114), \textit{ΔbalA} (KV6890), \textit{ΔbalB} (KV6924), \textit{ΔbalC} (KV6923), \textit{ΔbalAB} (KV7080), \textit{ΔbalAC} (KV7081), \textit{ΔbalBC} (KV7369), and \textit{ΔbalABC} (KV7128). The images are representative of at least two independent experiments.
Figure 45. Impact of bam mutations on biofilm formation. To assess the impact of bam on biofilm formation, I overexpressed rscS (pKG11) in wild-type (control) and bam mutants and assessed the ability of these strains to form wrinkled colonies over time. In the experiment shown, cultures were spotted onto LBS medium containing Tc and incubated at room temperature for 70 h, with images collected at the indicated times. The strains assessed were pKG11-containing derivatives of the following strains: wild-type control (ES114), ΔbamA (KV6886), ΔbamB (KV6638), ΔbamC (KV6787), ΔbamAB (KV7078), ΔbamAC (KV7079), ΔbamBC (KV6712), and ΔbamABC (KV6897). The images are representative of at least two independent experiments.
control (Fig. 46). Due to these results and our further characterization of the biofilm defect described below, I named these genes bam, for biofilm architecture maturation (bamA (VF_A1019), bamB (VF_A0120), and bamC (VF_A0550), respectively). Because the triple bam mutant exhibited the most severe defect in wrinkled colony formation, the remainder of our work focused on the phenotypes associated with this triple mutant, hereafter termed, for simplicity, the bam mutant.

**The bam mutant retains the ability to form a pellicle**

I next examined the ability of the bam mutant to produce a pellicle, a biofilm that forms at the air/liquid interface of a static liquid culture. This phenotype, like wrinkled colonies, is induced by rscS overexpression and depends upon the syp locus (Yip et al., 2006, Hussa et al., 2008). Although we have previously observed a strong correlation between the formation of wrinkled colonies and pellicles (Yip et al., 2006, Hussa et al., 2008, Shibata et al., 2012, Morris & Visick, 2013b, Morris & Visick, 2013a), this was not the case for the bam mutant: despite being unable to form a wrinkled colony, the bam mutant was competent to form a pellicle at the air/liquid interface of a static liquid culture (Fig. 47). I noted, however, that while pellicles by the control exhibited a wrinkled phenotype, those formed by the bam mutant consistently exhibited little to no wrinkling (Fig. 47). These data suggested that, while the bam genes are not required for pellicle formation per se, they may be involved in the maturation of the *V. fischeri* biofilm by building and/or maintaining the 3D architecture of the biofilm. Additionally, because the syp locus is necessary for pellicle formation
Figure 46. The biofilm defect of the bam mutant can be complemented with BamA. The ability of the bamA-balA operon to complement the triple bam mutant was assessed via wrinkled colony formation. In the experiment shown, cultures were spotted onto LBS medium containing Tc and incubated at room temperature for 22 h. The strains assessed were pKG11-containing derivatives of: wild-type (control; ES114), ΔbamABC (KV6897), ΔbamBC (KV6712), and ΔbamABC attTn7 bamA-balA (KV7062). The images are representative of at least two independent experiments.
Figure 47. Pellicle formation by the bam mutant. To evaluate the role of bam in pellicle formation, I grew rscS (pKG11)-containing wild-type (control; ES114) and bam mutant (ΔbamABC; KV6897) strains statically in LBS containing Tc for 72 h. Pellicle production can be observed due to the 3D architecture visible on the surface of the static culture. The images are representative of at least two independent experiments.
(Hussa et al., 2008, Shibata et al., 2012, Morris & Visick, 2013b), these data suggest that the \textit{bam} mutant may still produce the Syp polysaccharide, a possibility that I address below.

The \textit{bam} mutant retains the ability to produce the Syp polysaccharide

Because the \textit{bam} mutant exhibited somewhat contradictory phenotypes (unable to form a wrinkled colony but competent to form a pellicle), I explored the colony phenotype further. Specifically, I wondered whether the lack of wrinkled colony formation was caused by a defect in the ability to build/maintain 3D architecture, similar to what I observed for pellicle formation. I therefore compared the colony morphology of the \textit{bam} mutant with those of the biofilm-competent positive control and a representative \textit{syp} mutant, \textit{ΔsypL}; the \textit{sypL} mutant is defective for the formation of both wrinkled colonies and pellicles (Shibata et al., 2012). At the indicated time point, the positive control exhibited a wrinkled phenotype with “sticky” properties: when perturbed with a toothpick, the whole colony was readily pulled away intact from the agar surface (Fig. 48A). In contrast, the toothpick slid through the \textit{sypL} mutant colony, resulting in a clear path with the rest of the colony remaining unperturbed (Fig. 48B). When I assessed the morphology of the \textit{bam} mutant, I found that this mutant exhibited “sticky” properties not unlike the positive control: despite the lack of wrinkling, the colony was pulled away intact from the agar surface by the toothpick (Fig. 48C). These data suggested that, similar to what I observed for pellicle formation, the \textit{bam} mutant is capable of forming a biofilm, but unable to promote biofilm maturation. Thus, these data further support my hypothesis that the \textit{bam} genes are involved in building and/or maintaining the 3D
Figure 48. The *bam* mutant colony is sticky. To assess the “stickiness” of various strains, I spotted cultures onto LBS medium containing Tc and incubated them at room temperature for 48 h. All strains overexpressed *rscS*. Images were collected for the spot before (left) and after (right) disruption (with a toothpick) for the following strains: (A) wild-type ES114, (B) ΔsypL (KV5069), (C) ΔbamABC (KV6897), and (D) ΔsypL ΔbamABC (KV7060). When a sticky colony is perturbed with a toothpick, the whole colony is readily pulled away intact from the agar surface (regions of the colony distal to the toothpick are dislodged). In contrast, perturbation of non-sticky colonies only dislodges cells within the path of the toothpick.
architecture of the mature *V. fischeri* biofilm.

Because the *bam* mutant exhibited “sticky” properties lacking in the *sypL* mutant, I next questioned whether the stickiness of the *bam* mutant depended upon an intact *syp* locus. I thus generated a *bam sypL* mutant and expressed *rscS* to examine the resulting colony morphology. Similar to the *sypL* single mutant (Fig. 48B), the *bam sypL* mutant was not “sticky” (Fig. 48D), indicating that an intact *syp* locus is necessary for the “stickiness” of the *bam* mutant. Together, these data suggest that the *bam* mutant retains the ability to produce the Syp polysaccharide. Furthermore, these results suggest that, while the *bam* genes are coordinately regulated with the *syp* locus, the *bam* gene products function in a pathway distinct from Syp polysaccharide production.

**The wrinkled colony defect of the *bam* mutant can be exogenously complemented**

Because *bam* and *syp* appear to function in distinct pathways to control biofilm formation, I wondered whether a mixture of the *rscS*-overexpressing *bam* and *sypL* mutants could produce wrinkled colonies (i.e., could one mutant exogenously complement the other?). Indeed, whereas neither mutant alone could produce wrinkled colonies, a mixture of the two strains resulted in wrinkled colony formation (Fig. 49A). This result was not limited to mixtures of the *bam* and *sypL* mutants, as mixtures of the *bam* mutant with any of the *syp* structural mutants behaved in the same manner (Fig. 50). Moreover, neither a mixture of the *bam* and *bam sypL* mutants nor a mixture of the *sypL* and *bam sypL* mutants resulted in exogenous complementation (data not shown). These data suggest that both *bam* and *syp* are necessary for exogenous complementation and, ultimately, wrinkled colony formation. Finally, in contrast to the *syp* structural mutants,
Figure 49. A mixture of biofilm-defective strains permits wrinkled colony formation. (A) I assessed the ability of the biofilm-defective *syp* and *bam* mutants to complement each other for wrinkled colony formation by spotting a mixture of the two strains (pKG11-containing Δ*syp*L (KV5069) and pKG11-containing Δ*bam*ABC (KV6897)) onto LBS medium containing Tc. As controls, I spotted the two strains separately as well as the biofilm-proficient pKG11-containing wild-type strain (ES114). In the experiment shown, cultures were spotted onto plates and incubated at room temperature for 70 h. (B) To assess the requirement for *sypG* in exogenous complementation of the *bam* mutant, I spotted a mixture of the pKG11-containing Δ*bam*ABC mutant with the pKG11-containing Δ*sypG* mutant (KV1787). As controls, I spotted the two strains separately as well as the biofilm-proficient pKG11-containing wild-type strain (ES114). In the experiment shown, cultures were spotted onto plates and incubated at room temperature for 53 h.
Figure 50. A mixture of biofilm-defective *bam* and *syp* strains permits wrinkled colony formation. I assessed the ability of each of the biofilm-defective *syp* mutants to complement the *bam* mutant for wrinkled colony formation by spotting, onto LBS medium containing Tc, a mixture of the pKG11-containing Δ*bamABC* mutant with each of the following pKG11-containing *syp* mutant strains: Δ*sypB* (KV5145), Δ*sypC* (KV5192), Δ*sypD* (KV5067), Δ*sypH* (KV5193), Δ*sypI* (KV5068), Δ*sypJ* (KV5664), Δ*sypK* (KV5097), Δ*sypL* (KV5069), Δ*sypM* (KV5194), Δ*sypN* (KV5098), Δ*sypO* (KV5146), Δ*sypP* (KV5044), Δ*sypQ* (KV5099), and Δ*sypR* (KV5195). As controls, I spotted each strain separately as well as the biofilm-proficient pKG11-containing wild-type strain ES114. In the experiment shown, cultures were spotted onto plates and incubated at room temperature for 48 h.
mixing the *bam* mutant with the *sypG* mutant, which cannot activate expression of *syp* or *bam*, did not result in wrinkled colony formation (Fig. 49B). Thus, not surprisingly, exogenous complementation requires a SypG-dependent product(s). Whether the *bam* mutant provides the Syp polysaccharide to the *syp* mutants, or alternatively, the *syp* mutant provides Bam or a Bam-dependent product to the *bam* mutant, remains unknown. However, it is clear that both Bam and Syp are required for wrinkled colony (and wrinkled pellicle) formation, further supporting the idea that Bam and Syp comprise separate, but necessary pathways leading to the production of a mature biofilm.

Next, because mixtures of the *bam* and *syp* mutants resulted in wrinkled colony formation, I wondered whether exogenous complementation was due to a diffusible factor. If this were true, then spotting the *bam* and *sypL* mutants close to each other, but not touching, should promote wrinkled colony formation for one or both strains. This was not the case, however (Fig. 51A). Thus, whatever factor is necessary for exogenous complementation may not be highly diffusible. When, instead, the two strains (*bam* and Δ*sypl*) were spotted very close and allowed to grow into each other, wrinkling occurred at the interface of the two spots (Fig. 51B). Upon further examination, the wrinkling only appeared on the *bam* mutant side of the interface: while the *bam* mutant sometimes exhibited weak wrinkling, this wrinkling never occurred at the edges of the colony unless it was in contact with the *sypL* mutant. From these data, I predict that the *bam* mutant may receive some factor from the *syp* mutant, either Bam itself or a molecule whose synthesis or secretion is Bam-dependent.
Figure 51. The *syp* mutant complements the *bam* mutant. As an initial test of the nature and direction of the complementation that occurs between the *syp* and *bam* mutants, I spotted the mutants separately, both adjacent but not touching (A) and touching (B). In the experiment shown, pKG11-containing Δ*sypL* (KV5069) and Δ*bamABC* (KV6897) strains were used. Cultures were spotted onto plates and incubated at room temperature for 92 h (A) or 66 h (B). Wrinkling can be observed on the *bam* mutant side of the interface of the touching colonies.
**BamA is a secreted product**

My cell mixing experiments suggested that Bam or a Bam-dependent product present in the cell matrix promotes biofilm maturation. Because the Bam proteins contain a putative signal sequence, I hypothesized that these proteins may themselves be secreted out into the matrix. To assess this possibility, I selected BamA as a representative Bam protein, and generated a version with an epitope tag (BamA-FLAG). Expression of either the BamA-FLAG protein or the untagged BamA control complemented the bam mutant for wrinkled colony formation (Fig. 52A). I then examined the presence of BamA-FLAG in cell-free supernatants of the bam mutant via western blot analysis with an anti-FLAG antibody. I observed a band at ~100 kDa, which is slightly bigger than the predicted size of BamA (~75 kDa) (Fig. 52B). This band was absent in the strain that expressed untagged BamA, suggesting that, despite the apparent molecular weight difference, the antibody is detecting BamA in the supernatant fraction. BamA was also present in the cell pellet (Fig. 52B); the faint band at ~37 kDa appears to be non-specific, as it is also present in the strain expressing untagged BamA. Together, these data support the identification of BamA (and likely BamB and BamC) as *V. fischeri* biofilm matrix proteins.

**TEM analysis reveals differences in the extracellular matrix of the wild-type, bam, and sypL mutants**

Since neither the bam nor sypL mutants could promote wrinkled colony formation separately, but could when mixed or spotted so that they would grow into each other, I
Figure 52. BamA is a secreted protein. (A) To determine whether bamA-FLAG could complement the bam mutant, I overexpressed rscS (pKG11) in wild-type (control; ES114), the bamABC mutant (KV6897), the bamBC mutant (KV6712), the bamABC mutant containing an untagged allele of bamA (KV7276), and the bamABC mutant containing a tagged allele of bamA (bamA-FLAG; KV7274) and assessed the ability of these strains to form wrinkled colonies over time. In the experiment shown, cultures were spotted onto LBS medium containing Tc and incubated at room temperature for 22 h, at which point, images were collected. (B) To determine whether BamA is secreted from V. fischeri cells, I collected the cell pellet and cell supernatant (supe.) from the rscS-overexpressing ΔbamABC mutant that contains either untagged bamA or bamA-FLAG. These samples were resolved on a 10% SDS-PAGE, and ultimately the presence of BamA-FLAG was assessed by western immunoblotting with an anti-FLAG antibody. Sizes of the marker proteins are as indicated on the left.
asked whether I could observe differences in the extracellular matrix of these mutants by microscopy. Therefore, with the help of Dr. Adam Driks, I used transmission electron microscopy (TEM) to analyze ultra-thin sections of biofilm colonies (obtained from the spot-touching experiments), produced by rscS-overexpressing strains, including the biofilm-competent wild-type control, the triple *bam* mutant, the *sypL* mutant, and the interface of the *bam* and *sypL* mutants. I used the stain Ruthenium red to enhance staining of polysaccharide. As expected, cells in close association were present in all samples (Fig. 53). An electron-dense thread-like material was readily detected in the extracellular matrix of the control, *bam* mutant, and interface samples, but not in the *sypL* mutant sample (Fig. 53). The thread-like material was significantly more abundant in the control strain colonies than in the *bam* mutant colonies (Fig. 53A and B). The interface sample possessed the thread-like structures, but at a density that was intermediate between that of the control and *bam* mutant (Fig. 53D). I speculate that the thread-like material could be the Syp polysaccharide, since it was absent in the *sypL* mutant (Fig. 53C). I also observed numerous outer membrane vesicles in all the samples. Intriguingly, those seen in the *sypL* sample appeared largely cell-associated, while those observed in the other samples were present mostly in the extracellular space. Thus, SypL specifically or Syp polysaccharide in general may promote release of outer membrane vesicles. Of note, some cells of the *sypL* mutant exhibited a “swollen-cell” phenotype, which was previously observed for other *syp* mutants (Shibata et al., 2012); in addition, the cells for this mutant didn’t appear to be as tightly packed as cells within the samples of the other strains. Taken together, these data suggest that there are distinct differences between the control, *bam*, and *sypL*
Figure 53. TEM analysis of biofilm mutants. I used TEM to visualize wrinkled and smooth colonies formed by spotting the following pKG11-containing strains on LBS medium containing Tc: (A) wild-type (ES114), (B) *bamABC* (KV6897) and (C) *sypL* (KV5069). I also collected (D) samples from the interface of touching cultures of the pKG11-containing *bamABC* and *sypL* mutant strains. Thin arrows indicate the thread-like material (likely a polymer or polysaccharide), arrowheads indicate outer membrane vesicles, and a star indicates the “swollen-cell” phenotype (only present in the *sypL* mutant strain).
mutants. While these experiments do not reveal the exact function of the Bam proteins, they do suggest that Bam is likely an important part of the biofilm matrix. Thus, this work reveals the first V. fischeri matrix protein, a novel protein unlike other characterized matrix proteins, and uncovers a role for it in biofilm maturation.

**Summary**

Until recently, SypG was only known to regulate the *syp* locus. Now, SypG is known to also regulate the *bam* genes. These genes are coordinately regulated with the *syp* locus, and also involved in biofilm formation. However, it is only when all three *bam* genes were deleted that I observed a severe defect in wrinkled colony formation. These data suggest that the Bam proteins may promote similar functions during biofilm formation. Surprisingly, the *bam* mutant could form a pellicle, and upon further examination, the *bam* mutant exhibited other biofilm properties (i.e., it formed a smooth but “sticky” colony). Together, these data suggest that the *bam* genes are involved in building and/or maintaining the 3D architecture of the mature *V. fischeri* biofilm.

I also demonstrated that, while *bam* and *syp* may be coordinately regulated, these loci appear to comprise separate pathways leading to biofilm formation. In support of this idea: (1) the *bam* mutant still produces the Syp polysaccharide and (2) mixing the *bam* and *syp* mutant cells (or spotting them close so that they touch) restored wrinkled colony formation (i.e., exogenous complementation). However, exogenous complementation only occurred on the *bam* mutant side of the spots, which suggests that the *syp* mutant may provide Bam or a Bam-dependent product to the *bam* mutant. Indeed, this is likely
the case, as BamA can be found in cell-free supernatant. Furthermore, TEM analysis suggests that Bam may be involved in organizing the matrix.

Lastly, each *bam* gene appears to comprise an operon with the gene directly downstream, *bal*. However, the *bal* genes only play a minor role in biofilm formation, although they control bioluminescence in a currently unknown manner. Overall, since SypG regulates the *bam* genes, and likely the *bal* genes, I have thus uncovered a further connection between the regulation of biofilm formation and bioluminescence. In addition, I have identified the first bifilm matrix proteins in *V. fischeri*, proteins that are likely novel, as they are not similar to any characterized matrix proteins in other bacteria.
The overall goal of my dissertation was to identify and characterize novel genes involved in biofilm formation by *V. fischeri*. This work was driven by the fact that, in other bacteria, biofilm formation is a complex process involving multiple regulators and effectors. However, in *V. fischeri* only the *syp* locus and its regulators have been well characterized (Yip et al., 2005, Hussa et al., 2008, Shibata et al., 2012, Morris & Visick, 2013b). Thus, to search for other components involved in biofilm formation, I performed a random transposon mutagenesis. I found that transposon insertions within the gene encoding the hybrid SK LuxQ of the Lux luminescence pathway impacted biofilm formation. Since the Lux pathway is known to regulate biofilm formation in other *Vibrios*, and this regulation occurs below the level of the RR LuxO (Croxatto et al., 2002, Hammer & Bassler, 2003, Enos-Berlage et al., 2005, Lee et al., 2007a, Zhang et al., 2012), I predicted that the same would be true for *V. fischeri*. This was not the case, as only LuxQ and the histidine phosphotransferase (Hpt) LuxU, but not LuxO, were involved in regulating biofilm formation under my conditions. I subsequently went on to determine the mechanisms by which LuxQ and LuxU regulated biofilm formation. I hypothesize that these regulators function to activate SypG to promote *syp* transcription, and thus biofilm formation.
Because SypG is a critical regulator of biofilm formation, I shifted my focus to understanding SypG and the SypG regulon. Previous studies have identified SypG as a predicted $\sigma^{54}$-dependent regulator, and demonstrated that SypG-mediated $syp$ transcription depends upon $rpoN$, the gene encoding $\sigma^{54}$ (Yip et al., 2005). However, the sequences necessary for SypG to promote $syp$ transcription remained unknown. Thus, I next tested and confirmed the prediction that the $syp$ enhancer (SE) sequence, located upstream of the four $syp$ promoters, was required for SypG-mediated $syp$ transcription and biofilm formation, as well as host colonization.

During this study, I also sought to determine whether SypG regulated genes outside of the $syp$ locus. I predicted that, besides an SE sequence, these genes would also require a putative $\sigma^{54}$ recognition sequence. We identified three genes ($bamA$, $bamB$, and $bamC$) that met these requirements. We also found that SypG could recognize the SE sequences associated with these genes. Given these data, I predicted that the $bam$ genes would be coordinately regulated with the $syp$ locus and also involved in biofilm formation. My current data suggest that this is the case. Furthermore, while $bam$ and $syp$ are coordinately regulated, these genes comprise separate pathways leading to biofilm formation and appear to be secreted proteins in the biofilm matrix. I hypothesized that the $bam$ gene products were secreted components of the biofilm matrix that are involved in building and/or maintaining the 3D architecture of the mature $V. fischeri$ biofilm. Indeed, this appears to be the case. Overall, the majority of my hypotheses have been supported by the work described in my dissertation. In addition, I have made several novel observations, which I discuss below.
LuxQ and LuxU regulate syp-dependent biofilm formation

In this study, I found that regulators of the Lux luminescence pathway (LuxQ and LuxU) were involved in biofilm formation (Fig. 54). Specifically, the Lux pathway bifurcates at LuxU to regulate bioluminescence through the RR LuxO and biofilm formation at or above the level of the RR SypG. Furthermore, regulation of biofilm formation requires the kinase activity of LuxQ and the predicted conserved site of phosphorylation on LuxU. Taken together, these data suggest that phosphotransfer within the Lux pathway is necessary to regulate biofilm formation. Therefore, I predict that LuxU serves as a phosphoryl donor to activate SypG, which would lead to syp transcription and, ultimately, biofilm formation.

The idea that LuxU can function independently of LuxO is not novel. It has been previously proposed that, in two other Vibrio spp., LuxU functions independently of LuxO to control the activity of downstream targets of the Lux pathway. The first example is from the fish pathogen Vibrio anguillarum. In this organism, VanU (a LuxU homolog) functions through VanO (a LuxO homolog) to positively regulate the expression of the sRNAs qrr1-4 (Croxatto et al., 2004). However, VanU also appears to inhibit the expression of these sRNAs in a VanO-independent manner (Croxatto et al., 2004, Weber et al., 2011). Weber et al. hypothesize that VanU function through another RR to repress expression of qrr1-4 (Weber et al., 2011). Similarly, in Vibrio alginolyticus, Liu et al. propose that LuxU functions, at least in part, independently of LuxO to control expression of a downstream regulator, LuxT, likely through a different RR (Liu et al., 2011). However, in neither case has a downstream RR been identified. What ultimately
Figure 54. Model for regulation of biofilm formation and bioluminescence by V. fischeri. Biofilm formation is regulated via the SK RscS (light blue box) and the RR SypG (green oval), which is the direct transcriptional activator of the syp locus (lettered block arrows). This locus encodes genes necessary for the production and transport of a polysaccharide required for biofilm formation. Biofilm formation is inhibited by the RR SypE (orange box), which inhibits SypA (light green oval). SypA is only active when SypE is inactivated via phosphorylation, presumably via RscS. The function of SypA in promoting biofilm formation is currently unknown. Biofilm formation is also regulated by two regulators of the Lux luminescence pathway, the SK LuxQ and the Hpt LuxU, which appear to function to activate SypG, thus promoting syp transcription. SypG also activates transcription of three, two-gene sets called bam (purple block arrows) and bal (teal block arrows). The bam genes encode proteins involved in maturation of biofilm formation, while the bal genes encode predicted lipoproteins involved in controlling bioluminescence. Both bioluminescence and biofilm formation are necessary for host colonization.
sets my work apart from these studies is that LuxU functions independently of LuxO to regulate processes outside of the Lux pathway. Furthermore, I have strong evidence as to the identity of the RR whose activity LuxU regulates.

An intriguing finding from my work is that LuxU likely regulates the activity of multiple RRs, namely LuxO and SypG. There are only a few examples in the literature in which a single domain Hpt protein, such as LuxU, interacts with more than one target RR. *Caulobacter crescentus* ChpT is one such example. ChpT phosphorylates the RRs CtrA and CpdR with equal affinity *in vitro* (Biondi *et al.*, 2006); these phosphorylation events are critical during cell cycle progression. Phosphorylation of CtrA activates this protein, permitting it to bind DNA and control, among other things, DNA replication (Domian *et al.*, 1997, Quon *et al.*, 1998, Jacobs *et al.*, 2003). In contrast, it is the unphosphorylated form of CpdR that is active; in this state, CpdR indirectly promotes degradation of (unphosphorylated) CtrA (Iniesta *et al.*, 2006), permitting the cell to replicate its DNA. Thus, the same phosphorelay controls two separate RRs to exert opposite effects on protein activity.

An additional, well-studied, example of an Hpt protein interacting with two RRs occurs in the yeast *Saccharomyces cerevisiae*. In this organism, the Hpt protein YPD1 serves as a phosphoryl-donor to the RRs SSK1 and SKN7 under hypo-osmotic conditions (Li *et al.*, 1998). However, YPD1 interacts differently with each RR. For example, YPD1 stabilizes the phosphorylated state of the RR SSK1 via protein-protein interactions, but does not form stable complexes with the RR SNK7 (Janiak-Spens *et al.*, 2000). Phosphorylation of SSK1 inactivates this regulator until the cell experiences hyper-
osmotic conditions, in which case SSK1 is rapidly dephosphorylated and activates a
downstream pathway involved in controlling osmotic stress genes (Posas et al., 1996,
Posas & Saito, 1998). In contrast, phosphorylation of SNK7 promotes activation of a
downstream pathway involved in controlling genes for the cell wall and cell cycle
(Morgan et al., 1995, Li et al., 1998, Bouquin et al., 1999). These activities of YPD1
allow for the coordinated regulation of multiple pathways in S. cerevisiae. It is possible
that LuxU similarly provides a mechanism for coordination of two distinct pathways in V.
fischeri.

One question that remains is why only the kinase activity of LuxQ, but not its
phosphatase activity, is important for biofilm formation. Making LuxQ a “constitutive”
kinase through three predicted routes (LuxQ-A216P mutation, deletion of luxP, or
deletion of luxS) did not (reproducibly, in the case of LuxQ-A216P) promote accelerated
biofilm formation by V. fischeri (Fig. 13D, Fig. 14, and Appendix Fig. 1); it is possible
that biofilm formation cannot be accelerated under my experimental conditions.
Potentially, similar to the yeast system described above, LuxU could interact differently
with LuxO and SypG, serving as a phosphoryl-donor to both, but only removing the
phosphoryl groups from LuxO. To address this possibility, future work could assess
phosphorylation by purified proteins to determine whether LuxQ can serve as a kinase
and a phosphatase to one or both regulators. These experiments would provide more
information regarding interactions within the Lux pathway, as well as those occurring
outside of this pathway.
My work not only provided insight into the control of biofilm formation by *V. fischeri*, but also examined the model of bioluminescence regulation in *V. fischeri*. While LuxP, LuxQ, LuxU, and LuxO appeared to function to regulate bioluminescence as predicted (or as previously shown for LuxO (Miyamoto et al., 2000)) (Fig. 12B & D and 24), the SK AinR did not. Loss of AinR led to a slight decrease in luminescence compared to the control strain, while loss of both AinR and LuxU resulted in an intermediate level of luminescence relative to either single mutant (Fig. 12F and 20).

However, the decreased luminescence of the *ainR* and *ainR luxU* mutants could be overcome by the addition of exogenous C₈-HSL, which is normally produced by the AI synthase AinS. From these data, I concluded that deletion of *ainR* likely impacted expression of the gene encoding *ainS* (located directly upstream of *ainR*); these results were recently confirmed by Kimbrough and Stabb (Kimbrough & Stabb, 2013). Thus, it appears that whatever role C₈-HSL plays in controlling luminescence is independent or mostly independent of AinR, under my experimental conditions. Therefore, the question of whether AinR controls luminescence remains open.

There is recent evidence that AinR is indeed involved in regulating luminescence. In particular, AinR was shown to functionally replace LuxN in *V. harveyi* (Kimbrough & Stabb, 2013); luminescence was restored to control levels when C₈-HSL was added. Furthermore, in *V. fischeri*, loss of AinR led to decreased *qrr1* transcription (Kimbrough & Stabb, 2013), suggesting that AinR could function to control LuxO, which is known to regulate *qrr1* expression (Miyashiro et al., 2010). However, these data do not account for the fact that loss of AinR in *V. fischeri* has little impact on luminescence. Maybe there is
a difference in the signal input strengths between LuxQ and AinR; this has been demonstrated for LuxQ and LuxN in *V. harveyi* (Freeman & Bassler, 1999a, Freeman *et al.*, 2000). It is also possible that LuxQ and AinR do not interact with LuxU to the same extent. For example, if AinR does not interact well with LuxU, AinR might not exert a significant impact on luminescence regulation through the phosphorelay. It also seems plausible that AinR does not function through the known phosphorelay (i.e., AinR could bypass LuxU and donate phosphoryl groups directly to LuxO) or that AinR does not function through the phosphorelay at all (i.e., since AinR appears to regulate *qrr1* expression, it may function through a regulator outside of the Lux pathway to control *qrr1* transcription). However, in either case, I would have expected to observe a change in luminescence, though this effect may be over-shadowed by the fact that loss of AinR impacts *ainS* expression, leading to decreased levels of C₈-HSL. Perhaps these studies will also have the added benefit of providing an explanation for why *V. fischeri* encodes AinR instead of LuxN (which is common in other Vibrios (Milton, 2006)); while these regulators are considered homologues, they are only 35% identical, suggesting that there may be differences in the way they ultimately function. Overall, the role of AinR in controlling bioluminescence is unclear and requires further investigation.

If AinR does not function through the known phosphorelay to regulate bioluminescence, does LuxQ serve as the only input to LuxU? My data suggest that this is not the case: loss of LuxU resulted in a greater increase in luminescence than loss of LuxQ (Fig. 12B). In both *V. cholerae* and *V. harveyi*, three SKs feed into LuxU (LuxQ, CqsS, and VpsS, and LuxQ, LuxN, and CqsS, respectively) (Ng & Bassler, 2009). In *V.
fischeri, no gene for CqsS exists, but two putative VpsS homologs (VF_1296 and VF_A0360) are present ((Shikuma et al., 2009) and Fitnat Yildiz, personal communication). I have preliminary data that indicate neither of these genes is involved in regulating bioluminescence (data not shown). Yet another possibility stems from a recent study that demonstrated that the cytoplasmic SK HqsK serves as an input to LuxU in V. harveyi (Henares et al., 2012). This kinase senses NO through an interaction with H-NOX, a NO/oxygen-binding protein. Hence, it is possible that a similar kinase regulates luminescence in V. fischeri.

Intriguingly, V. fischeri contains a gene (VF_A0072) encoding a predicted SK that is 41% identical and 62% similar to HqsK from V. harveyi (Altschul et al., 1990, Altschul et al., 1997). Furthermore, VF_A0072 is located directly downstream of the gene (hnoX) that encodes H-NOX in V. fischeri (Ruby et al., 2005); it is possible that these genes comprise an operon. A recent study demonstrated that, while an hnoX mutant did not exhibit a luminescence defect in culture, squid colonized by this mutant became luminescent faster than those colonized by the wild-type strain (Wang et al., 2010a). These experiments suggest that hnoX may be involved in controlling luminescence, though whether this is through an interaction with the SK encoded by VF_A0072 has yet to be determined. It remains curious that the hnoX mutant did not exhibit a luminescence phenotype in culture. Perhaps an explanation for this result is that the luminescence experiments from this study were carried out using MS media containing N-acetyl-glucosamine, which may not be an effective media to utilize when trying to detect subtle differences in luminescence. Therefore, it would be of interest to examine luminescence
of the hnoX mutant, as well as a VF_A0072 mutant using SWTO medium, which I have used to examine subtle differences in luminescence for various lux mutants (e.g., see Fig. 12). Overall, VF_A0072 is a promising candidate that may regulate luminescence through LuxU, similar to V. harveyi.

One inconsistency from my data is the fact that loss of LuxQ resulted in the same biofilm defect as loss of LuxU, but not the same luminescence defect: loss of LuxU increased luminescence to a greater extent than did loss of LuxQ. As stated above, from the luminescence data, it appears that there is an additional input into LuxU to regulate luminescence. If there really are multiple inputs into LuxU, then the biofilm defects of the luxQ and luxU mutants should have been distinguishable. Furthermore, since it is likely that a second input to luminescence regulation exists, it is possible that this regulator may function differently under biofilm-inducing conditions than under luminescence-inducing conditions. Thus, it is currently unclear why loss of LuxQ causes different luminescence and biofilm phenotypes.

My work also examined the relative importance of established Syp biofilm regulators. For example, this was the first study to examine the role of RscS in biofilm formation under SypG-inducing conditions (overexpression of sypG in a ΔsypE background). Previous studies had already demonstrated that: 1) RscS functions upstream of SypG to induce syp transcription in a manner that depends upon sypG (Hussa et al., 2008) and 2) RscS is critical in symbiotic biofilm (aggregate) formation and colonization (Visick & Skoufos, 2001, Yip et al., 2006). However, these previous studies only explored how overexpression of rscS impacts biofilm formation in culture or how its loss
or overexpression impacts symbiotic aggregation and colonization, whereas my work examines the role of RscS in activating SypG, and subsequently, *syp* transcription. Specifically, I found that under my experimental conditions (*sypG* overexpression in the absence of *sypE*), loss of RscS had little impact on biofilm formation. This is not necessarily surprising, as Morris *et al.* recently demonstrated that one of the major roles of RscS appears to be inactivating the biofilm inhibitor protein SypE (Morris *et al.*, 2011). Thus, my work provides further evidence that the role of RscS in inactivating SypE is critical for biofilm formation, while the role of RscS in activating SypG may be secondary.

The above observations raise the question of how RscS itself is regulated. Two specific questions come to mind: 1) what is the signal that promotes RscS function and 2) how is *rscS* expression regulated. Previous evidence from our lab suggests that RscS may require an FAD cofactor to promote biofilm formation (Geszvain & Visick, 2008b). Since FAD would likely be derived from a metabolic process by *V. fischeri*, and wild-type *V. fischeri* does not form robust biofilms *in vitro* (i.e., under standard laboratory conditions), it is possible that a squid or squid-associated factor promotes an increase in the cellular FAD concentration, leading to subsequent biofilm formation *in vivo* (i.e., during squid colonization). However, it is also plausible that the signal sensed by RscS is present both *in vivo* and *in vitro*, and the issue lies in expression of *rscS*. Support for this idea comes from previous studies that demonstrate that, via primer extension analysis, *rscS* appears to be transcribed under standard laboratory conditions (the actual level of transcription was not evaluated) (Visick & Skoufos, 2001), though little RscS protein is
produced by either wild-type cells or cells that overexpress the wild-type allele of rscS (Geszvain & Visick, 2008a). It was only when a mutation was acquired within rscS that changed a rare codon to a more common codon that overexpression of rscS led to biofilm formation and detectable levels of the protein product (Geszvain & Visick, 2008a). Thus, I predict that a limiting concentration of tRNAs for rare codons, or the lack of machinery necessary for their incorporation, leads to inadequate translation of the rscS mRNA transcript and, subsequently, little RscS protein. Furthermore, I hypothesize that, in vivo, a signal is received to increase production of these missing factors, which then promotes rscS expression and subsequent biofilm formation during host colonization. If these predictions are true, then the actual signal for RscS function is likely present under both in vitro and in vivo conditions. Overall, a more thorough understanding of how specific biofilm regulators, such as RscS and SypG, are controlled is necessary to increase our understanding of their role(s) in biofilm formation.

While RscS is known to be necessary for host colonization, my preliminary data for the impact of a luxU mutation on the ability of V. fischeri to colonize squid—when no regulators were overexpressed—revealed, at most, a mild defect due to loss of LuxU (V.A.R and K.L.V., unpublished data). These data suggest, perhaps not surprisingly, that our biofilm-inducing conditions do not fully reflect the dynamics in nature (during colonization). Alternatively, it is distinctly possible that our in vitro biofilm assay is more sensitive than what we can observe in vivo. Thus, I would need to examine aggregation by the luxU mutant to determine whether loss of LuxU contributes to this in vivo process.
It is also possible that the minor delay in biofilm formation observed from the *luxU* mutant *in vitro* does not translate into a significant delay in biofilm formation during host colonization. Since we do not know the signals or all the players necessary to promote biofilm formation *in vivo*, it is difficult to know whether loss of LuxU would cause a noticeable defect during host colonization. However, I expected to observe lower levels of colonization from the *luxU* mutant in my experiments, as *luxO* and *luxOD43E* (which encodes a ‘constitutively’ active LuxO) mutants exhibit decreased colonization at 12 h post-inoculation (Lupp et al., 2003, Lupp & Ruby, 2005). One caveat to my experiments is that I only examined host colonization by the *luxU* mutant 18-24 h post-inoculation. Furthermore, there is no documentation in the literature of any colonization defect for a *luxO* mutant at these time points during single strain inoculation experiments. Thus, it is possible that I did not choose the right time point to observe a defect in host colonization for the *luxU* mutant. Further experiments are necessary to determine the role of LuxU in host colonization.

One additional possibility to consider about the roles of LuxQ and LuxU in biofilm formation is that this regulation is purely an artifact of *sypG* overexpression. While *rscS* appears to be transcribed to some level under standard laboratory conditions (Visick & Skoufos, 2001), it does not appear to be efficiently translated (Geszvain & Visick, 2008a). Therefore, under my *sypG* overexpression conditions, it seems plausible that SypG lacks a natural phosphodonor; this lack of RscS protein would explain why loss of RscS had no impact on biofilm formation under my conditions (Fig. 22). Since SypG and LuxO are 50% identical and 66% similar, and LuxU likely serves as a
phosphodonor to LuxO in *V. fischeri*, it is possible that I forced an interaction between LuxU and SypG that does not normally exist. This possibility would explain why loss of LuxU did not greatly impact squid colonization: rscS should be expressed during colonization, possibly negating any impact of LuxU on biofilm formation. It is of interest to note, however, that not all symbiosis-competent strains of *V. fischeri* encode a functional RscS protein (Mandel et al., 2009, Gyllborg *et al.*, 2012). Therefore, it is not unreasonable to imagine that an additional pathway, such as Lux, could contribute to syp induction and biofilm formation during colonization. Furthermore, since strains of *V. fischeri* that colonize the squid are typically ‘dim’ (Schuster *et al.*, 2010), it is possible that *V. fischeri* has modified the Lux phosphorelay to favor kinase activity over phosphatase activity, which could potentially support my hypothesis that LuxU phosphorylates SypG. Overall, while it is possible that the regulation I uncovered in our strain of *V. fischeri* (ES114) is an artifact, this regulation may be relevant in other strains of *V. fischeri* and merits further investigation.

In support of a genuine link between Lux and Syp at the level of syp transcription, there is yet another connection between these pathways: overexpression of sypK, which encodes a putative flippase necessary for biofilm formation (Shibata *et al.*, 2012), leads to increased transcription of the sRNA *qrr1* (Miyashiro *et al.*, 2013). Consistent with this observation, SypK overexpression also decreases luminescence and increases motility in a *qrr1*-dependent manner. Current evidence suggests that SypK may function through SK LuxQ to mediate this regulation (Miyashiro *et al.*, 2013). Continuing to investigate how the Lux pathway is regulated will lead to a better understanding of this pathway in *V.*
*fischeri*, and should also serve to deepen our understanding of this pathway in other *Vibrio* spp., especially those that contain a *syp* or a *syp*-like locus.

One intriguing question from the above work is whether the role that SypK plays in controlling *qrr1* expression is distinct from its role in biofilm formation. If SypK plays distinct roles in these two processes, then it should be possible to isolate SypK mutants that promote biofilm formation but not *qrr1* expression and vice versa. Isolation of these mutants would allow for the study of SypK’s activities separately, as well as together. Furthermore, these mutants could be used to determine the relevance of SypK’s regulation on both bioluminescence and biofilm formation in the context of the squid host. Overall, this link between the Syp and Lux pathways is another example of how two complex pathways interact to promote a specific cellular function.

Lastly, looking more broadly at bioluminescence and biofilm regulation, I propose that these two pathways may also be connected via the sugar phosphotransferase system (PTS). In *V. cholerae*, the PTS system regulates biofilm formation, specifically *vps* expression (Houot et al., 2010): the EIIA\textsubscript{Gluc} subunit positively regulates *vps* expression, while the EIIA\textsubscript{Ntr} subunit negatively regulates *vps* expression. In *V. fischeri*, the EIIA\textsubscript{Gluc} subunit has been implicated in negatively controlling bioluminescence (Visick *et al.*, 2007); however, the level at which this regulation occurs within the Lux pathway is unclear. While it is highly unlikely that the PTS controls biofilm formation through its control of bioluminescence, it is possible that the PTS system controls both phenotypes separately in *V. fischeri*, especially given the connection between the EIIA\textsubscript{Gluc} subunit and biofilm formation in *V. cholerae*. Thus, determining how the Syp and Lux
pathways are linked to other, more global regulatory pathways, such as the PTS, will allow us to more fully understand how a particular process truly impacts the physiology and ecology of a particular organism.

**The SE sequence is necessary for SypG-mediated syp transcription, biofilm formation, and host colonization**

SypG is a critical regulator of biofilm formation that appears to integrate signals from RscS and Lux. However, when I began my work, gaps in our knowledge of this important regulator remained. Specifically, it was unknown whether SypG, could bind to conserved sequences, termed syp enhancer (SE), within the syp locus to serve as the direct transcriptional activator of this locus. Therefore, I sought to determine the requirements of the SE sequences for syp transcription, biofilm formation, and host colonization. I also questioned whether this sequence was associated with genes outside of the syp locus and, if so, whether SypG could recognize those SE sequences.

Through truncation analyses and lacZ-reporter fusions, I demonstrated that syp reporter fusions that contain the SE sequence were activated in a SypG-dependent manner, while reporter fusions that lack the SE sequence were not (Fig. 26 and 27). The only exception was the sypI promoter, which contains tandem SE sequences (Fig. 25A). I found that truncation of the upstream SE sequence (SE-I-up) resulted in a loss of syp transcription (Fig. 27B), suggesting that either the upstream sequence or the presence of both sequences is necessary for syp transcription. Surprisingly, in our biofilm-based assay, I found that only the downstream SE-I sequence (SE-I-down) was sufficient to promote biofilm formation when used to control sypA expression (Fig. 36C and D).
Although the IR of SE-I-up deviates from consensus, the individual changes are permissive with respect to SE-A. One simple explanation is that the transcription analyses were performed in the context of the native sypI promoter, while the biofilm-based experiments were carried out in the context of the sypA promoter; perhaps some combination of nucleotide substitutions prevents recognition by SypG in the context of the biofilm assay.

Of the other Vibrios that contain a syp-like locus (e.g., *V. parahaemolyticus* and *V. vulnificus*) (Yip et al., 2005, Yildiz & Visick, 2009, Guo & Rowe-Magnus, 2011), the SE sequence is present and well conserved, but the promoter regions upstream of the sypI equivalent only contain one SE sequence [(Guo & Rowe-Magnus, 2011) and unpublished data]. Thus, the presence of tandem SE sequences at the sypI promoter of *V. fischeri* may suggest that extra regulation is necessary at the sypI operon. The question then becomes why would extra regulation be necessary at this particular promoter. One explanation is that the SE sequences at this promoter are immediately adjacent to the sypH gene, such that transcription through the sypH gene could hinder transcription from the sypI promoter. Thus, it may be necessary to recruit more SypG via an additional SE sequence to promote effective transcription of this operon. Another explanation, though not mutually exclusive from the first, is that SE-I-down may not be positioned correctly to promote transcription in a σ^{54}-dependent manner; this SE sequence is only 58 bp from the predicted σ^{54}-binding site, while most enhancer binding sequences are located 80-150 bp from the σ^{54}-binding site. However, SE-I-down could serve to direct SypG binding to SE-I-up, which would explain why loss of SE-I-up in the transcription assay only led to basal
levels of transcription. This prediction could also support the results I observed in the biofilm assay, as, individually, SE-I-down would have been positioned correctly to promote biofilm formation and SE-I-up would not have an adjacent SE sequence to facilitate SypG binding. Overall, the presence of tandem SE sequences at the *sypI* promoter likely promotes cooperative binding of SypG, which may be necessary for efficient transcription. Thus, further investigation of the *sypI* promoter and the tandem SE sequences may provide insights about the regulation of this operon, as well as regulation of the *syp* locus as a whole.

The *sypI* operon is not the only place where SE sequences are adjacent to one another: the SE sequences associated with *sypA* and *VF_A1019* are located four bp apart on opposite strands of the DNA. This positioning raises the question of whether these SE sequences serve to regulate transcription of both genes or just the individual genes. My transcriptional data for the *sypA* promoter suggest that, while SE-1019 would remain intact when SE-A is truncated, SE-1019 alone is not sufficient to promote efficient transcription of the *sypA* promoter (Fig. 27A). While I have demonstrated that SypG recognizes (Fig. 35C) and regulates transcription of *VF_A1019* (Fig. 38), I have not performed a truncation analysis of its promoter region. Thus, it is currently unclear whether SE-A could contribute to the regulation of *VF_A1019* transcription.

A question that arises from the above observation is whether SypG has more affinity for SE-A or SE-1019, and if so, how this difference in affinity for these SE sequences would impact transcription of *sypA* and *VF_A1019*. Because transcription from σ54-dependent promoters typically requires DNA bending, and the DNA would bend
towards RNA polymerase, it seems likely that SypG binding to one site would prevent transcription from the other site. It also seems possible that the presence of two SE sequences promote cooperative binding, which is a situation similar to what I predicted for the need for tandem SE sequences at the \textit{sypI} promoter (discussed above). Overall, there are a multitude of possibilities to explore regarding the unique arrangement of the SE sequences between \textit{sypA} and \textit{VF}_{\textit{A1019}}, with future studies hopefully providing a better understanding about how these elements (and others necessary for transcription of these genes) regulate expression of \textit{sypA} and its operon, as well as \textit{VF}_{\textit{A1019}}.

Besides the presence of two SE sequences between \textit{sypA} and \textit{VF}_{\textit{A1019}}, there is a large amount of space (241 bp) between SE-A and \textit{VF}_{\textit{1019}}. This observation raises the question of whether additional factors, such as DNA binding sites for other proteins or sRNAs, are present in this region, and how these factors might impact transcription of \textit{sypA}. I would predict that there would be a number of other factors necessary to regulate the \textit{sypA} operon, since this operon encodes critical regulators of biofilm formation (i.e., SypA and SypE, and possibly SypF and SypG). Furthermore, the same regulatory factors and components could impact transcription of \textit{VF}_{\textit{A1019}}, which is involved in biofilm maturation (see Section III of Chapter Three). Thus, determining the other factors and elements present in the region between \textit{sypA} and \textit{VF}_{\textit{A1019}} is critical to our overall understanding of the regulation of biofilm formation.

An interesting finding from my transcriptional analysis was that I only detected transcriptional activation for the \textit{sypM} and \textit{sypP} reporter fusion strains carrying the \textit{sypG} overexpression plasmid when grown in HMM, not LBS (data not shown), while I
detected activation for the *sypA* and *sypI* reporter fusion strains when grown in either medium. It is possible that, since the *sypA* and *sypI* promoters contain tandem SE sequences, more SypG is recruited to these promoter regions through cooperative binding. An additional possibility is that the *sypM* and *sypP* promoters have different environmental or regulatory requirements for activation than do the *sypA* and *sypI* promoters. It could also simply be that the affinity of SypG for the SE sequences differs between the various promoter regions. In either case, a further examination of the *syp* promoter regions is necessary to fully understand regulation of the *syp* locus.

In this study, I also performed an in-depth analysis of the specific nucleotides necessary for SypG to recognize the SE sequence. This work was facilitated by the development of a novel biofilm-based assay that depended on the ability of SypG to activate transcription of *sypA*; increased SypA levels can overcome SypE-mediated inhibition, thus promoting biofilm formation (Fig. 28-30) (Morris & Visick, 2013a). I categorized the phenotypes of our different strains into three simple categories—similar to the positive control, similar to the negative control, or an intermediate phenotype. Although there was a range of intermediate phenotypes, the assay was not robust enough to permit categorization of the intermediate phenotypes into sub-categories. Nevertheless, we submit that the strains with diminished or disrupted biofilm formation represent those in which SypG-mediated transcription of *sypA* is diminished or defective. This conclusion is supported by results from both β-galactosidase and ChIP experiments. Using this biofilm-based assay, I found that at least one and potentially up to three bases immediately upstream of the IR sequence contribute to SypG-mediated activation (Fig.
Further truncation experiments are necessary to determine which of these bases are needed for efficient transcription.

Perhaps not surprisingly, the IR sequences themselves were the critical determinants in this assay. Our ability to replace the SE-A sequence with specific point mutant derivatives permitted us to understand which positions were necessary for SypG recognition and which sequences were ‘flexible’. Indeed, some flexibility was already suggested by the fact that the SE-A sequence differs from the other SE sequences in that it contains an imperfect IR, whereas SE-I-down, SE-M, and SE-P each contain a perfect IR (Fig. 25). I determined that mutations at position 1 or 3 of the SE-A IR did not cause a defect or delay in biofilm formation (Fig. 32C). This result explains why SypG recognizes the SE sequence associated with *sypA*, as SE-A has an A at position 3, while the other *syp* SE sequences have a G (Fig. 25 and Fig. 32C). These data also explain why SypG is able to recognize SE sequence associated with genes outside of the *syp* locus (i.e., SE-1019 and SE-0550) that have changes at position 1 and/or 3 (Fig. 35A).

Most of the other bases were required for SypG-mediated activation, as changes at nucleotides other than positions 1 and 3 resulted in reduced or no biofilm formation (Fig. 32). However, some specific changes in a required nucleotide were permissive. For example, at positions 2 and 4, an A to T change and a C to A change, respectively, were permissive, but not other changes (Fig. 33B and C). Consistent with the A to T change at position 2 being permissive, this sequence is naturally present in SE-0550, which can also promote biofilm formation. As for the C to A change at position 4, this specific change brings SE-A closer to the perfect IR consensus present in the other SE sequences.
Combining our knowledge of the critical SE sequences necessary for SypG-mediated activation with bioinformatic analyses, we identified a number of genes that have a predicted SE sequence, some of which also have a putative $\sigma^{54}$-recognition sequence associated with them (Table 4). Of the non-syp SE sequences, I chose to characterize three, SE-1019, SE-0120, and SE-A0550, since these sequences also appeared to be associated with a putative downstream $\sigma^{54}$-binding site. Of note, the three genes ($VF_A1019$, $VF_A0120$, and $VF_A0550$) encode proteins that are similar to each other [37% identical and 53% similar; (Altschul et al., 1990, Altschul et al., 1997)]. I predict that, since SypG recognizes the SE sequences associated with them, these genes constitute part of the SypG regulon and might also be involved in biofilm formation. I assessed this prediction in Section III of Chapter Three and provide discussion on this topic below.

The $V.\ fischeri$ SE sequence, specifically that associated with sypA, is necessary not only for syp transcription and biofilm formation, but also for host colonization. It is striking that deletion of only 27 bp from the $V.\ fischeri$ genome ($\Delta$SE-A) resulted in the inability to efficiently colonize the squid host (Fig. 37). This effect can be attributed to the severe decrease in transcription of the sypA operon (Fig. 27A), as sypA is known to be a critical colonization determinant (Morris & Visick, 2013b), while the divergently transcribed $VF_A1019$ does not appear to be required for colonization (Yip et al., 2005). Furthermore, re-introduction of SE-A into its native location on the chromosome restored colonization competence (Fig. 37), suggesting that the colonization defect was indeed due to the loss of this small sequence. Because the colonization experiments did not rely
on the overexpression of regulatory proteins, this work implicates the SE-A sequence (and likely, SE-I, SE-M, and SE-P) as a critical regulatory sequence in *V. fischeri* and gives us a better understanding of the biology of this organism.

Importantly, the work performed here to understand the role of a conserved sequence in biofilm formation and colonization has the potential for impacting more than our understanding of the marine bacterium *V. fischeri*. Numerous *Vibrio* species contain a similar locus, including the human pathogens *V. vulnificus* and *V. parahaemolyticus* (Yip et al., 2005, Yildiz & Visick, 2009). The SE sequence we identified in *V. fischeri* (Yip et al., 2005) is nearly identical to a conserved sequence found in *V. vulnificus* within the *rbd* locus, a *syp*-like locus similarly involved in biofilm formation (Guo & Rowe-Magnus, 2011). Similar to SypG, the SypG-like regulator, RbdG, is capable of inducing expression of the *rbd* genes, although the role of the conserved IR sequences in transcriptional control has yet to be investigated (Guo & Rowe-Magnus, 2011). Of note, the SE-like sequence associated with *rbdA*, *rbdM*, and *rbdP* contain perfect IR sequences, while the SE-like sequence associated with *rbdI* is not a perfect IR. The similarities between these two studied loci are striking, but the differences will be informative. Undoubtedly, work on one locus will provide insights into the other as well as into numerous other *Vibrio* species that contain this locus.

**The bam genes are involved in biofilm maturation, while the bal genes impact cellular bioluminescence**

The work described here identifies the first *V. fischeri* matrix protein, BamA, a novel protein unlike other characterized matrix proteins, and uncovers a role for it in
biofilm maturation. BamA represents the first member of a family of similar but uncharacterized proteins that includes *V. fischeri* BamB and BamC as well as proteins encoded by a variety of other bacteria. The genes for *bamA*, *bamB*, and *bamC* were previously identified as putative members of the SypG regulon (Ray et al., 2013), and I demonstrated here via reporter assays that SypG indeed regulates transcription of the *bam* genes. The coordinate regulation of *bam* with the *syp* polysaccharide locus provided the first indication that *bam* might function in biofilm formation.

Given the similarity of the Bam proteins, it is perhaps not surprising that they appear to have overlapping functions with respect to biofilm formation: deletion of one or a combination of two of the *bam* genes did not significantly impact wrinkled colony formation, while deletion of all three genes caused a severe defect. The exception to this was the *bamAB* double mutant, which exhibited a significant defect in this phenotype. Interestingly, BamA and BamB are more similar to each other than they are to BamC, suggesting that their function may be more conserved. Furthermore, while deletion of *bamA* alone did not substantially impact biofilm formation, complementation of the triple *bam* mutant with just *bamA* permitted near normal biofilm formation. These results are analogous to those found in *V. cholerae* for matrix proteins Bap1 and RbmC, which have sequence similarity and can partially complement each other (Fong & Yildiz, 2007, Berk et al., 2012). The Bam proteins are not similar to the *V. cholerae* matrix proteins, nor to other known matrix proteins. Future work using comparative studies should identify regions of the *V. fischeri* Bam proteins critical for their function.
The *bam* genes are located upstream of lipoprotein genes, *bal*, that I similarly predicted would be involved in biofilm formation, and thus, the finding that the *bal* genes impact cellular bioluminescence, but not biofilm formation, was somewhat surprising. However, I previously established a link between bioluminescence and biofilm formation by determining that specific Lux regulators (*LuxQ* and *LuxU*) were involved in controlling biofilm formation, likely through activation of SypG (Chapter Three, Section I). An additional connection between these pathways has recently been made through the finding that overexpression of *sypK* impacts *lux* regulation (Miyashiro et al., 2013). My findings thus indicate a third point of intersection between these two pathways.

I observed the biggest impact of *bal* on bioluminescence upon deletion of *balC* (singly or in combination with the other *bal* genes) (Fig. 3). Intriguingly, this increase in luminescence was observed in the absence of *sypG* (or *rscS*) overexpression, suggesting that either the level of basal transcription from the SypG-dependent *bam* promoter is sufficient for *bal* expression, or else there is a second promoter that drives *bal* expression. Since I did not observe a defect in luminescence from the triple *bam* mutant, it seems unlikely that there is a promoter within the *bam* genes to drive *bal* expression.

The *bal* genes encode predicted lipoproteins (Babu et al., 2006) that contain a predicted signal sequence (Petersen et al., 2011) but lack an inner membrane retention signal (Tokuda & Matsuyama, 2004). These data suggest that the Bal proteins are localized to the outer membrane, possibly in the inner leaflet, facing the periplasm. In this position, the Bal proteins could impact the signaling activity of the *lux* regulators that are located partially (*e.g.*, the SKs *LuxQ* and *AinR*) or completely (*e.g.*, *LuxP*) in the
periplasm. BalC could, for example, promote LuxQ kinase activity. Alternatively, the
connection between Bal and bioluminescence could be indirect. Additional work is
necessary to understand the contribution of the Bal proteins to the production of cellular
bioluminescence. Overall, this is the first time that lipoproteins have been implicated in
controlling bioluminescence in the *Vibrios* and thus the *bal* genes represent novel factors
involved in the control of cellular bioluminescence.

My work investigating the contribution of Bam to biofilm formation has
permitted a deeper understanding of the role played by the Syp polysaccharide in biofilm
formation. Because our studies of the *syp* genes had revealed a tight correlation between
wrinkled colony formation and pellicle formation (Shibata et al., 2012), I was initially
surprised to find that the triple *bam* mutant, which formed smooth colonies, retained the
ability to form a pellicle. This observation prompted a further examination of the smooth,
“biofilm-defective” colony produced by the *bam* mutant, which revealed that it retained
biofilm properties (e.g., it was “sticky”); the “stickiness” of the *bam* mutant was lost in a
*bam syp* mutant. Taken together, these data suggest that Syp is responsible for the
“stickiness” phenotype, while Bam promotes the development of the 3D architecture
observed for both colonies and pellicles. The subsequent cell-mixing experiments
indicated that these two distinct SypG-dependent processes function together: mixtures of
the *bam* and *syp* mutants, which individually fail to form wrinkled colonies, together
produced a wrinkled colony. Furthermore, the *bam-syp* spot-touching experiments
suggest that the Syp polysaccharide may not be a communal (i.e., shared) product, as I
did not observe wrinkling within the *syp* mutant spot. Thus, Syp may be cell-associated
or poorly diffusible. In this regard, the *V. fischeri* biofilm is similar to the VPS-dependent biofilm of *Vibrio cholerae*, as the VPS polysaccharide also does not appear to be a communal product (Absalon *et al.*, 2011). These experiments have thus provided a new phenotype (“stickiness”) and a new approach (mutant mixing) to probe the pathways that lead to biofilm formation, which we will utilize in the future to characterize the communal nature of matrix components, and to categorize additional biofilm-defective mutants.

Because the spot-touching experiments suggested that the *bam* mutant was supplied with a missing factor from the *syp* mutant, and because bioinformatic analyses of the Bam proteins revealed the presence of a Sec-dependent signal sequence at their N-termini (Petersen *et al.*, 2011), I predicted that Bam might be a secreted factor. Indeed, I could find BamA in cell-free supernatants (Fig. 52B). Given the presence of a putative Sec-dependent signal sequence, it is likely that the Sec pathway is responsible for secretion of BamA (and presumably the other Bam proteins) to the periplasm. However, it is unclear how BamA crosses the barrier of the outer membrane. It is unlikely to be dependent for export on the Syp proteins, as all of the structural *syp* mutants retained the ability to complement the *bam* mutant in mixing experiments. In *V. cholerae*, Type II secretion appears to be involved in secretion of at least one matrix protein (Sikora *et al.*, 2011). Future work will probe the requirement for this pathway in secretion of Bam and potentially other matrix proteins.

Due to the conservation of the Bam proteins in other *Vibrio* spp. and marine bacteria, their function may be relevant to marine environments. However, my
preliminary experiments did not reveal a role for \textit{bam} in initiation of symbiotic colonization (unpublished results). This is perhaps not surprising, given that the biofilm formed by \textit{V. fischeri} during symbiotic colonization is transient in nature, a process that may be independent of the development of biofilms with substantial 3D architecture. Because \textit{syp} mutants exhibit severe colonization defects, it is likely that the “stickiness” contributed by the Syp polysaccharide is a key event in proficient colonization. It will be of interest to determine if/when during symbiotic colonization the Bam protein can be found outside \textit{V. fischeri} cells.

How do the Bam proteins function to promote biofilm maturation? The answer to this question remains unclear. TEM analysis revealed the presence of a thread-like material between cells in the wild-type (Fig. 53A) and, to a lesser extent, the \textit{bam} mutant (Fig. 53B). Since the thread-like material was absent in the \textit{sypL} mutant colonies (Fig. 53C), I predict that this substance may be the Syp polysaccharide. Whether Bam directly interacts with the Syp polysaccharide, and whether it helps to organize the Syp polysaccharide, retain it on the cell surface or an abiotic surface, or plays some other role, remains to be determined.

The TEM analyses also revealed a new role for \textit{sypL} and/or the Syp polysaccharide in the release of outer membrane vesicles. However, the function of these vesicles, if any, in biofilm formation remains to be determined. An interesting possible vesicle function is delivery of periplasmic Bam to the biofilm matrix. If this were the case, then the deficiency of the \textit{sypL} mutant in vesicle release could account for the limited diffusion of Bam in the spot-touching experiments.
In summary, this work has revealed a new factor involved in biofilm maturation by *V. fischeri*. Because the Bam proteins are unrelated to other known biofilm matrix proteins (Altschul et al., 1990, Altschul et al., 1997), they represent a novel class of matrix proteins. Thus, understanding the exact function of the Bam proteins in promoting 3D architecture of biofilm formation, and how their function compares to other characterized matrix proteins, are important areas of future study.

**Significance**

Biofilm formation by bacteria is becoming very problematic for the medical community. Since bacteria in biofilms are more resistant to chemical and antibiotic assaults, it is particularly difficult to treat these types of infections. Thus, it is necessary to continue to understand how biofilm formation is regulated. In my dissertation work, I utilized the marine bioluminescent bacterium *V. fischeri*, a good biofilm model due to its requirement for this trait during colonization of its symbiotic host. The work I presented here has greatly improved our understanding of the factors necessary for biofilm formation by *V. fischeri*. My work has also provided a better understanding of the pathway involved in the production of cellular bioluminescence, which is also critical for host colonization.

Specifically, I demonstrated that the Lux luminescence pathway bifurcates at LuxU to regulate bioluminescence through the RR LuxO and biofilm formation at or above the level of the RR SypG; this activity requires the kinase activity of the SK LuxQ. While the idea of bifurcation within the Lux pathway has been previously suggested (Croxatto et al., 2004, Liu et al., 2011, Weber et al., 2011), the finding that this
bifurcation leads to regulation of biofilm formation is novel. In addition to this link between Lux and Syp, I have helped characterize a link between Syp and Lux, in which the predicted flippase SypK impacts transcription of the sRNA qrr1; this regulation likely impacts the activity of LuxQ.

My work has also filled in a critical gap in our understanding of the Syp pathway, namely that SypG is indeed the direct transcriptional activator of this locus. This study also permitted the identification of other members of the SypG regulon, bam and bal, which encode proteins involved in biofilm maturation and bioluminescence control, respectively; these genes appear genetically linked and likely comprise an operon. Of particular interest, the bal genes encode predicted lipoproteins, and no previous work (in other Vibrios) has documented lipoprotein involvement in luminescence regulation. Thus, understanding how these genes regulate this process is an exciting area of future study.

Overall, I have identified/characterized three previously undocumented links between biofilm formation and bioluminescence, and through my analysis of the syp promoters, uncovered novel genes involved in both processes. Together, my findings suggest that the control of bioluminescence and biofilm formation is a tightly regulated process that depends on regulators of both pathways. In addition, my work may have implications for the regulation of biofilm formation and bioluminescence (or other quorum sensing systems) by other Vibrios and marine bacteria, as the bam and bal genes are conserved in these species.
Ecological Relevance

*V. fischeri* is an excellent model biofilm-forming organism to study, not only because of its genetic tractability, but because biofilm formation can be studied in the natural context of host colonization. Specifically, it is at during the initial stages of colonization that *V. fischeri* cells form a *syp*-dependent, biofilm-like aggregate on the surface of the squid’s symbiotic organ prior to migrating inside where they grow to high cell density. While it is unclear whether *V. fischeri* cells reside within a biofilm in the deep crypt spaces, there is evidence that the cells are embedded in a matrix (likely of both bacterial and host origins). Biofilm formation is critical at the initiation stage of this symbiosis, but it remains unclear whether biofilm formation by *V. fischeri* is necessary for subsequent stages of host colonization.

I speculate that the Lux regulators LuxQ and LuxU, which I have shown to contribute to the control over biofilm formation *in vitro*, may function during the initiation stage of colonization during the formation of the *syp*-dependent aggregate. At the low cell density conditions within the early aggregate, LuxQ should function as a kinase (due to low signal concentration) to donate phosphoryl groups to LuxU, which would then both promote SypG activation and inhibit bioluminescence. Indeed, it is known that light production is not necessary at this stage of colonization (REF). For strain ES114, the strain that I used for my studies, it is unlikely that Lux is the primary input to activate SypG *in vivo*. Instead, it seems likely that RscS is the primary input, since RscS is required for aggregate formation. How, then, would Lux and RscS work to regulate biofilm formation? I hypothesize that the Lux input serves to ‘prime’ *syp*
transcription until enough RscS is produced and activated by its (unknown) signal; at this point, the Lux input may serve as a positive feedback loop. If this were the case, then syp transcription could be turned on more quickly and to a greater level, which might explain why aggregate formation occurs within such a short time frame. For other, non-ES114 strains of *V. fischeri*, it is possible that the Lux input may play a more important role in activating the syp pathway. For example, strain SR5 lacks the gene for *rscS*, yet still colonizes the squid in a syp-dependent manner (REF).

I further speculate that biofilm maturation is not a key stage during the initial stages of colonization. My work has shown that, in the absence of Bam, cells competent to produce the Syp polysaccharide are proficient at forming both pellicles and “sticky” colonies, and thus the Syp polysaccharide is likely to be sufficient for the formation of the biofilm-like aggregate on the surface of the light organ. Furthermore, the biofilm formed by *V. fischeri* is transient; although careful studies have not been performed, the current dogma suggests that the cells spend at most 2-3 hours within the aggregate before migrating into the light organ. In this time frame, there may not be sufficient time to accumulate enough Bam (or other matrix components, besides the Syp polysaccharide) to promote biofilm maturation.

Even if Bam is not critical for the initial aggregate formation, this result would not rule out a role for Bam at subsequent stages of colonization, such as within the deep crypt spaces. In that location, *V. fischeri* resides within a matrix of unknown origin, and may, in fact, exist as a biofilm. If so, then I propose that it is likely existing as a ‘mature’ biofilm, complete with the Bam matrix component. Furthermore, I propose that Bam, and
the mature biofilm architecture, may be important for the ability of *V. fischeri* to (1) resist host-imposed stresses and/or (2) adhere tightly to the host epithelial cells. The position of *V. fischeri* cells within the light organ appears to be important for their retention, as each day at dawn, the squid expel 95% of their symbionts, leaving the remaining 5% to repopulate the organ during the day. It will be of interest to determine if Bam is present in the matrix of cells within the deep crypt spaces, and if so, whether there is a particular pattern to its localization.

It is also possible that neither Lux nor Bam play critical roles with respect to biofilm formation in the context of the squid host. If no evidence can be found to support a role for these proteins, then it is likely that these processes are necessary for *V. fischeri* to be maintained in a distinct location outside of its squid host. Since biofilms can form on a variety of surfaces, *V. fischeri* could use biofilm formation is attach to such surfaces as seaweed or mollusk shells. These environmental reservoirs are also subjected to the ocean currents (i.e., some level of sheer stress), making the ability to form a biofilm an advantage to *V. fischeri*. Indeed, a mature biofilm would better withstand this force (and others), especially if one function of Bam is to impart structural integrity to the biofilm. Thus, although my work didn’t reveal the ecological importance of Lux and Bam, many possibilities exist. My work has uncovered novel factors involved in the control and development of biofilms by *V. fischeri*, and has laid the groundwork for asking more pointed question about the overall lifecycle and ecology of this compelling microbe.
APPENDIX

ADDITIONAL STUDIES
I. The role of other Lux regulators in biofilm formation by *V. fischeri*

Biofilm formation by bacteria is regulated by a variety of processes. In *V. fischeri*, biofilm formation is regulated by the Syp pathway and, as my work has demonstrated, by LuxQ and LuxU, two members of the Lux luminescence pathway. However, the Lux pathway is comprised of multiple regulators, some of which have known functions outside of luminescence regulation (e.g., LuxO and LitR are involved in controlling motility (Hussa et al., 2007, Lupp & Ruby, 2005)). Thus, to determine whether these other regulators were involved in regulating biofilm formation by *V. fischeri*, I tested various lux mutants for their role in this process; strains used in the appendix are listed in Appendix Table 1.

First, I examined the role of LuxS in biofilm formation by *V. fischeri*. LuxS produces AI-2, which is predicted to be sensed by LuxP/Q in *V. fischeri* (Lupp & Ruby, 2004). According to the model generated from *V. harveyi*, a luxS mutation should result in increased LuxQ kinase activity, leading to decreased luminescence (Bassler et al., 1994a). This appears to be the case in *V. fischeri*, as a luxS mutant exhibits a decrease in luminescence relative to the wild-type strain (Lupp & Ruby, 2004). Since a luxS mutant exhibits increased LuxQ kinase activity, and this activity is necessary for biofilm formation, I predicted that this mutant might exhibit precarious biofilm formation. My preliminary data suggest that this is not the case, as this mutant exhibits wrinkled colony development along the same timeframe as the control strain (Appendix Fig. 1); I could not detect any increased or precocious biofilm formation in my preliminary experiments. Together with the luminescence data, my biofilm results lend further support to my
### Appendix Table 1. Strains used in the appendix.

<table>
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<tr>
<th>Strains</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
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<tr>
<td>KV4427</td>
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<td>(Lupp &amp; Ruby, 2005)</td>
</tr>
<tr>
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</tr>
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<tr>
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<td>$luxA$::erm $\Delta$sypE</td>
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Appendix Figure 1. Wrinkled colony formation by a *luxS* mutant. Time-course assays of wrinkled colony formation induced by *sypG* overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45 h post-spotting for the following strains: Δ*sypE* (KV3299) and Δ*sypE luxS::kan* (KV4827). Data are representative of at least two independent experiments.
conclusion (Chapter Three Section I) that the kinase activity of LuxQ is necessary to regulate biofilm formation. However, it is possible that I might not be able to observe a weak effect on biofilm formation when *luxS* is deleted. Thus, I would need to perform an epistasis analysis to determine whether a *luxQ luxS* mutant exhibits the bioluminescence and biofilm phenotypes of the *luxQ* mutant (i.e., brighter than wild-type and delayed, respectively), the *luxS* mutant (i.e., expected to be dimmer than wild-type and not delayed, respectively), or a different set of phenotypes. Thus, these experiments could allow me to determine whether LuxS has any function outside of its predicted role to function through LuxP/Q.

Next, I further examined the role of LuxO in biofilm formation. LuxO is the RR at the bottom of the phosphorelay (Fig. 8). My studies (Chapter Three Section I) revealed that loss of LuxO resulted in a minor, yet reproducible 1.5 h delay in biofilm formation (compare Fig. 15A and C) and a *luxU luxO* mutant exhibited an additive delay in biofilm formation as compared to either single mutant (Fig. 15). These data indicated that LuxU and LuxO function independently to regulate biofilm formation. As part of that work, I investigated the impact of expression of a ‘constitutively’ active *luxO* allele, *luxOD47E* on biofilm formation. My preliminary data suggest that, under *sypG*-overexpression conditions, the *luxOD47E* mutant exhibits a slight delay in wrinkled colony formation (Appendix Fig. 2). However, since previous studies have demonstrated that his mutant exhibits a slight growth yield defect (Lupp & Ruby, 2005), and I have not examined growth yield of this mutant under my conditions, it is possible that the delay in biofilm formation is due to the growth yield defect of this mutant. Thus, it is difficult to make any
Appendix Figure 2. Wrinkled colony formation by *luxO* and *litR* mutants. Time-course assays of wrinkled colony formation induced by *sypG* overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45 h post-spotting for the following strains: Δ*sypE* (KV3299), Δ*sypE luxO::kan* (KV4828), Δ*sypE luxO-D47E* (KV4736), and Δ*sypE litR::kan* (KV4837). Data are representative of at least two independent experiments.
conclusions about the role of a LuxO phospho-mimic in SypG-induced biofilm formation. Surprisingly, however, I found that, in the absence of rscS or sypG overexpression, cells that expressed luxOD47E exhibited weak pellicle formation and aggregation in static LBS cultures (Appendix Fig. 3); this was not observed for wild-type cells or other lux mutants. Thus, it is clear that the phosphorylation state of LuxO could play a role in its ability to promote biofilm formation, but whether it depends on the syp locus remains to be determined.

I hypothesize that LuxO may exert its effect on biofilm formation by functioning as a transcriptional activator of the syp locus. Obviously, because a luxO mutant exhibits a very minor defect in wrinkled colony formation, the impact of LuxO on syp transcription would be small. However, in support of this possibility, LuxO and SypG are both predicted σ54-dependent regulators (Miyamoto et al., 2000, Yip et al., 2005), and the sequences that they recognize are similar (LuxO – TTGCA-NNN-TGCA vs. SypG – TTCTCA-NNN-TGCAAA) (Lenz et al., 2004, Yip et al., 2005). Data from a former technician (Michael Misale) and rotation student (Krystal White) suggest that SypG can recognize the LuxO-binding sequence: changing SE-A to more resemble the LuxO-binding site does not hinder SypG’s ability to recognize this sequence in the context of our biofilm-based assay. Thus, since SypG can recognize the LuxO-binding site, it is possible that LuxO could recognize the SE sequence. I predict LuxO could be used to initiate syp transcription so that when the signal for biofilm formation is received, the system is primed and ready to go.
Appendix Figure 3. Pellicle formation by a luxO-D47E mutant. Cultures of the control (ES114) or the luxO-D47E mutant (KV4736) were grown statically in LBS medium and incubated at 24°C for 72 h. Data are representative of at least two independent experiments.
Regulators below LuxO also appear to play some role in regulating biofilm formation. LuxO regulates the sRNA Qrr1, which is predicted to destabilize the mRNA transcript for LitR (Miyashiro et al., 2010), the direct transcriptional activator of luxR (Fidopiastis et al., 2002). While I have not assessed the role of qrr1 in biofilm formation, I found that disruption of litR led to a 3 h delay, as well as a slight overall defect in wrinkled colony formation (Appendix Fig. 2). In support of a role for LitR in biofilm formation, Fidopiastis et al. observed a change in the colony opacity of the litR mutant (Fidopiastis et al., 2002); in other Vibrios, colony opacity has been correlated with biofilm formation (e.g., (Yoshida et al., 1985, Enos-Berlage et al., 2005)). However, since previous studies have demonstrated that LitR regulates more than just luminescence (i.e., motility (Lupp & Ruby, 2005) and acetate metabolism (through AinS) (Studer et al., 2008)), the impact of LitR on biofilm formation may be indirect. Furthermore, the litR mutant exhibits a growth yield defect (Lupp & Ruby, 2005), which could also account for at least part of the defect in biofilm formation by this mutant. If the defect in biofilm formation is not due to the growth yield defect, then these data would suggest that LitR plays an important role in biofilm formation. Since LitR is a transcriptional regulator, I would predict that it might regulate transcription of genes necessary for efficient biofilm formation by V. fischeri. Additionally, it is possible that LitR regulates transcription of an unidentified gene (or set of genes) involved in biofilm formation. Thus, identifying the role of LitR in biofilm formation may elucidate non-syp/bam gene involved in this process and should be an area of further investigation.
I also investigated the roles of LuxR and LuxI in biofilm formation. LuxR is the direct transcription factor for the *lux* operon, and is activated by binding to the AI produced by LuxI (Engebrecht & Silverman, 1984, Meighen, 1991, Stevens et al., 1994). I found that disruption of *luxR* led to a minor delay in wrinkled colony formation (Appendix Fig. 4), suggesting that this regulator plays a minor role in this phenotype. Because the AI produced by LuxI functions with LuxR, I expected that the *luxI* mutant would exhibit the same biofilm phenotype. Surprisingly, I found that disruption of *luxI* led to a severe defect in wrinkled colony formation (Appendix Fig. 4). One possible explanation to account for these results is that LuxI or the AI that it produces (3-O-C₆-HSL) is involved in regulating more than just luminescence (i.e., 3-O-C₆-HSL could be sensed via another receptor to promote some, non-luminescence function). However, there is no precedence for this in the literature.

An additional possibility is that the *luxI* mutant acquired a secondary mutation. To address this possibility, I would re-make this strain and assess biofilm formation. If the original *luxI* mutant acquired a secondary mutation, then I would expect to see a difference in biofilm formation as compared to the new *luxI* mutant. However, if I obtain the same results as before, these data would suggest that LuxI (or the AI it produces) is necessary for biofilm formation. In this case, I would then determine whether the defect in biofilm formation was due to the absence of 3-O-C₆-HSL or the loss of LuxI itself by assessing biofilm formation by the *luxI* mutant in the presence and absence of 3-O-C₆-HSL. If the addition of 3-O-C₆-HSL complements biofilm formation, then 3-O-C₆-HSL is necessary for this process and thus, potentially, involved in signaling to another regulator.
Appendix Figure 4. Wrinkled colony formation by a *luxO-D47E* mutant. Time-course assays of wrinkled colony formation induced by *sypG* overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45 h post-spotting for the following strains: Δ*ypE* (KV3299), Δ*ypE luxR::erm* (KV4835), and Δ*ypE luxI* (frameshift; KV4838). Data are representative of at least two independent experiments.
of biofilm formation. However, if biofilm formation is not complemented by the addition of 3-O-C₆-HSL, then I would conclude that LuxI has another, previously unidentified function; I would pursue this area further via mutational analysis of LuxI. Overall, the role of LuxR and LuxI in biofilm formation is unclear and requires further study.

Lastly, I examined a luxA mutant for wrinkled colony formation; LuxA and LuxB together form the luciferase enzyme involved in light production (Ziegler & Baldwin, 1981). Surprisingly, I found that this mutant exhibited a slight delay in biofilm formation (Appendix Fig. 5). It is possible that this delay could result from the build-up of a factor(s) involved in the reaction for light production (i.e., long-chain aliphatic aldehyde, FMNH₂, and oxygen). Since the luxA mutant does not exhibit a growth defect (Visick et al., 2000), this is likely not the case. However, since we don’t know the specific cellular conditions necessary to promote biofilm formation, examining whether a build-up of factors necessary for the luminescence reaction impacts biofilm formation could provide information on factors necessary for this process.

Taken together, my data indicate that other Lux regulators, besides LuxQ and LuxU, are also involved in controlling biofilm formation by V. fischeri. While the majority of these effects are relatively small, and likely indirect, following up on these observations could provide necessary information about the Lux pathway, as well as the physiology of V. fischeri in general. Furthermore, previous studies have demonstrated that Lux regulators, but not light per se, are involved in initiating colonization of the squid host (i.e., (Lupp & Ruby, 2005, Visick et al., 2000)). Thus, my observations,
Appendix Figure 5. Wrinkled colony formation by a luxA mutant. Time-course assays of wrinkled colony formation induced by sypG overexpression using plasmid pEAH73. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. Wrinkled colony formation was monitored up to 45 h post-spotting for the following strains: ΔsypE (KV3299) and ΔsypE luxA::erm (KV5191).
combined with these previous studies, could led to a better understanding of the impact of Lux regulators and biofilm formation on the initiation of host colonization by *V. fischeri*.

**II. Role of LuxU in RscS-induced biofilm formation**

In my studies, I examined the role of LuxU on biofilm formation under SypG-inducing conditions. However, biofilm formation can also be induced via overexpression of *rscS* (Yip et al., 2006). Thus, I sought to determine the role of LuxU in biofilm formation under RscS-inducing conditions. My preliminary studies indicated that, under these conditions, loss of LuxU (in an otherwise wild-type background) led to biofilm formation that was no different from the control strain (Appendix Fig. 6). These results are in contrast to what I observed under SypG-inducing conditions: loss of LuxU resulted in delayed biofilm formation. One possibility for this difference is that RscS may regulate other, currently unidentified, components necessary for biofilm formation that can compensate for the loss of LuxU. Thus, under SypG-inducing conditions, *rscS* (and the components it regulates) may not be readily expressed, making it easier to discern the role of LuxU in biofilm formation. Another possibility I addressed in my Discussion is that the role of LuxU in biofilm formation is simply an artifact of *sypG* overexpression. Regardless, the differences observed between RscS- and SypG-inducing conditions can be used to our advantage to study components with a relatively minor influence on biofilm formation, leading to better understand how this process is regulated.
Appendix Figure 6. Wrinkled colony formation by a luxU mutants overexpressing rscS. Cultures were spotted onto LBS medium containing Tc and incubated at 28°C. All strains overexpress rscS (pKG11). Wrinkled colony formation is represented at 17.6 h post-spotting for the following strains: wild-type (ES114) and \( \Delta \text{luxU} \) (KV4829).
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VITA

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After completing her Ph.D., Valerie will begin a post-doctoral position in Dr. Daniel Wozniak’s laboratory at the Ohio State University (Columbus, OH) where she will continue to study biofilm formation using the model biofilm organism *Pseudomonas aeruginosa*. 