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Characterization of the Novel Response Regulator Sype: A Dual Regulator of Biofilm Formation and Colonization in Vibrio Fischeri

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CHARACTERIZATION OF THE NOVEL RESPONSE REGULATOR SYPE: A DUAL REGULATOR OF BIOFILM FORMATION AND COLONIZATION IN VIBRIO FISCHERI

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

ANDREW ROBERT MORRIS

CHICAGO, IL

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ABSTRACT

The marine bacterium Vibrio fischeri establishes a specific, mutualistic association with its animal host, the Hawaiian bobtail squid Euprymna scolopes. The V. fischeri-E. scolopes symbiosis provides a natural model to investigate the mechanisms involved in host-microbe interactions. Colonization by V. fischeri is a dynamic process, in which the bacteria sense and respond to host-derived signals. To coordinate cellular behavior in response to environmental stimuli, V. fischeri employs a variety of regulatory devices, including two-component signaling (TCS) systems. Importantly, TCS regulators control one of the earliest stages of host colonization, the formation of a biofilm aggregate on the surface of the squid’s symbiotic light organ. Biofilm formation requires the sensor kinase RscS and the response regulator (RR) SypG, which regulate transcription of the symbiotic polysaccharide locus (syp). Preliminary studies suggested that the RR SypE may also exert control over biofilm formation. For my dissertation, I investigated the role of SypE in regulating biofilm formation and symbiotic colonization.

Bioinformatic analysis predicted that sypE encodes a novel, multi-domain RR consisting of a central receiver (REC) domain flanked by two terminal, effector domains of putative opposing function: an N-terminal RsbW-like serine kinase and a C-terminal PP2C-like serine phosphatase domain. Based on these preliminary studies, I
hypothesized that SypE could exert dual regulatory control over *syp* biofilm formation, depending on which of the terminal effector domains was active.

To understand the role of SypE in biofilm regulation, I performed a genetic analysis and assessed the contribution of the SypE domains to overall protein function. I found that the N- and C-terminal effector domains of SypE do indeed possess negative and positive regulatory activities, respectively. My studies further suggest that the central REC domain regulates these opposing activities. Finally, colonization studies indicate that regulation of SypE activity by the HK RscS is critical to permit symbiotic colonization of *E. scolopes*.

To further elucidate the role of SypE, I assessed the downstream targets of SypE’s regulatory activities. Bioinformatic analyses revealed that SypE’s effector domains exhibit similarity to proteins found in partner-switching systems. Co-immunoprecipitation studies revealed that SypE interacts with another putative partner-switching regulator, the STAS-domain protein, SypA. Using biochemical and genetic approaches, I determined that SypE regulates the phosphorylation state of SypA, and that phosphorylation inhibits SypA activity. Furthermore, I found that biofilm formation and symbiotic colonization require unphosphorylated SypA. This study demonstrates that regulation of SypA activity by the novel regulator SypE is a critical mechanism by which *V. fischeri* regulates biofilm development and colonization of its animal host. That regulation of SypA activity is critical for both biofilm formation *in vitro* and symbiotic colonization further demonstrates the utility of the *V. fischeri*-squid symbiosis as a model for the role of biofilm formation in host colonization.
CHAPTER ONE  
LITERATURE REVIEW  

INTRODUCTION  
Throughout life, animals continuously encounter and enter into associations with a diverse array of bacterial species. In regards to the animal host, these bacterial interactions can result in both beneficial and detrimental outcomes. A common public concern is the development of host diseases resulting from infection by pathogenic bacteria. The threat of evolving bacterial pathogens has prompted numerous investigations into the mechanisms by which pathogenic bacteria promote host infection. Similarly, the continued rise in antibiotic resistance among various disease-causing bacteria emphasizes the need for identifying new antimicrobial targets and treatments.

While historically, focus has been on understanding pathogenic interactions, growing evidence indicates that many, if not all, animals also establish long-lived, cooperative associations with specific bacterial consortia [1]. Importantly, these mutualistic associations are often essential for promoting and maintaining host homeostasis [2]. Studies using germ-free animals have revealed that interactions with symbiotic bacteria contribute to host development, nutrition, reproduction, and defense [3,4]. An example of such a cooperative association can be found in the mammalian gut, in which colonization by beneficial bacteria not only provides the host with nutritional benefits, but also promotes host immune system development [2].
Host colonization by both beneficial and pathogenic bacteria is a dynamic process, in which the bacteria continually sense and respond to the presence of their animal host. Importantly, many of the cellular and molecular mechanisms by which beneficial bacteria establish colonization of their animal hosts are similar to those described for bacterial pathogens. Like pathogens, beneficial symbionts have evolved mechanisms to sense and respond to host environments and to interact with host surfaces. Therefore, understanding the mechanisms by which bacteria establish and maintain beneficial associations with their animal hosts can also yield insight into the development of pathogenic interactions.

The ability of bacteria to detect and respond to specific conditions within the host is essential for establishing colonization. To coordinate cellular behavior in response to host signals, bacteria employ a variety of signaling and regulatory devices, including two-component signaling (TCS) systems and partner-switching networks. TCS pathways involve histidine-aspartate phosphotransfer between a histidine kinase and a response regulator, and represent the predominant form of signaling in bacteria. Partner switching systems are comprised of a network of proteins whose interactions are controlled through a reversible serine phosphorylation event. Partner switching systems consist of three primary conserved regulatory elements, a serine kinase/anti-sigma factor, a serine phosphatase, and an antagonist protein. Importantly, both TCS and partner switching systems have been shown to regulate key processes required for colonization by both pathogenic and beneficial bacteria [5].
Recent studies have identified a critical role for bacterial biofilms in promoting bacterial-host interactions. Biofilms, or multi-cellular, bacterial communities encapsulated in a matrix, represent a common mode of growth for many bacterial species. Importantly, both pathogenic and beneficial bacteria have been shown to produce biofilms to promote and/or maintain colonization of their animal hosts [6]. Production of a biofilm often contributes to multiple stages of colonization, including promoting association with host tissues and providing resistance to both host-derived defenses and antibiotics [7]. Given the importance of biofilms in mediating bacteria-host interactions, understanding the processes involved in biofilm formation is necessary for the development of approaches to control bacterial infections.

The mutualistic association between the marine bioluminescent bacterium *Vibrio fischeri* and its animal host, the Hawaiian squid *Euprymna scolopes*, provides an elegant, model system for investigating the mechanisms underlying bacteria-host interactions [8,9,10]. Research into this symbiosis has yielded novel insights into the bacterial and host processes required for colonization. Importantly, studies have demonstrated that *V. fischeri* possesses multiple TCS systems, several of which regulate symbiotic processes required for host colonization. This includes the regulation of one of the earliest events in colonization, the formation of a bacterial biofilm aggregate. Finally, work presented in this dissertation demonstrates that partner-switching regulators provide critical regulation over the production of this symbiotic biofilm and host colonization.
MUTUALISTIC ASSOCIATION OF VIBRIO FISCHERI AND EUPRYMNA SCOLOPES

Introduction

The marine bioluminescent bacterium *V. fischeri* establishes a specific, mutualistic association with its animal host, the Hawaiian bobtail squid *E. scolopes*. The *V. fischeri*-*E. scolopes* mutualism has been extensively studied for over 20 years [8,9,10]. In this symbiosis, *V. fischeri* exclusively colonizes the squid’s specialized symbiotic organ, termed the light organ. Upon colonization of the light organ, the bacteria grow to high cell density (approximately $10^{11}$ bacteria cells/ml) and produce bioluminescence, the end product of the symbiosis [8]. The squid host is proposed to utilize the bioluminescence produced by the bacteria as a form of camouflage, termed counterillumination [8,11], during the animal’s nocturnal activities.

The light organ of a juvenile *E. scolopes* is aposymbiotic upon hatching [8,12], and therefore the animal must acquire the *V. fischeri* cells from the surrounding seawater [12]. The light organ is centrally located within the squid’s mantle cavity and consists of two lobes. The surface of each lobe features a pair of ciliated appendages that extend into the mantle cavity [12]. The ciliated epithelial fields located on the surface of these appendages function in the recruitment of the symbiotic bacteria by directing the surrounding seawater toward pores that provide entry into the light organ (Fig. 1). Each lobe contains three pores, approximately 5-15 µM in diameter, which serve as entrances to one of three, distinct epithelium-lined crypts (Fig. 1). The pores located on the light organ surface open into the ciliated ducts, which lead to an antechamber space and,
ultimately, the deep crypt spaces (Fig. 1). It is within these epithelium-lined, deep crypts that the colonizing *V. fischeri* cells reside [12] [13].

The process of host colonization is highly dynamic and typically initiates within the first few hours post-hatching [12]. Upon establishing symbiotic association, the squid host is proposed to maintain a monospecific culture of *V. fischeri* for the duration of its lifetime [12]. The squid controls the levels of *V. fischeri* contained within its light organ by expelling the majority of bacteria (~90-95%) on a daily basis. Each morning, muscle tissue surrounding the light organ contract and expel the light organ contents, including *V. fischeri* symbionts, into the surrounding seawater [14,15]. The remaining bacterial symbionts repopulate the light organ over the course of the following day [14,15]. In this manner, the animal host plays an active role in maintaining colonization by controlling the bacterial load [14,15].

Most, if not all, animals are believed to establish long-term, specific associations with particular consortia of bacteria. The utility of the *V. fischeri*-*E. scolopes* mutualism as a model system is the ability to independently study both symbiotic partners, as both the animal host and bacterial symbiont can be naturally maintained and investigated separately. Furthermore, this symbiosis is highly specific, as only *V. fischeri* is capable colonization. This permits researchers to investigate the direct mechanisms by which a single bacterium and its animal host naturally establish and maintain a symbiotic association. Current evidence indicates that colonization is a tightly regulated process that involves specific communication between the squid and its bacterial symbiont. Indeed, studies have identified a variety of both bacterial and host processes, which
**Figure 1. Diagram of the squid light organ and colonization by *V. fischeri***. This figure was modified from a diagram by Emily Yip. Juvenile *E. scolopes* (shown on the left) contains a bilobed light organ, which is colonized by *V. fischeri*. The inset (shown on the right) depicts one of the light organ lobes and the general stages of colonization: (i) attachment and aggregation of *V. fischeri* cells in host mucus (tan) on the light organ surface. (ii) migration through the ciliated ducts and antechamber. (iii) colonization and persistence in the deep crypt spaces. Within the deep crypts, *V. fischeri* cells proliferate to high cell density and produce bioluminescence (shown in yellow).
contribute to particular stages of the colonization process. The process of symbiotic colonization proceeds in a series of progressive stages, including initiation, accommodation, and persistence [8]. During the initiation stage, recruited *V. fischeri* cells attach to the light organ surface and form compact, biofilm-like aggregates [16]. Following initiation, *V. fischeri* cells disperse from the aggregate and proceed to migrate into the light organ where they establish colonization within the deep crypts. During this accommodation stage, *V. fischeri* adapt to the light organ environment and proliferate to a high cell density. In the final persistence stage, *V. fischeri* and *E. scolopes* establish a daily pattern of symbiotic behaviors, which promote maintenance of the symbiosis for the lifetime of the animal host. In the following section, I will discuss in further detail the key host and bacterial responses involved in these various stages of colonization.

**Initiation of the *V. fischeri*-E. scolopes mutualism**

**(A) Overview**

Colonization of the squid light organ by *V. fischeri* occurs rapidly, generally within 12 hours after the juvenile squid hatch. Investigations into the *E. scolopes-V. fischeri* symbiosis indicate that colonization is not a passive process, but rather involves a number of adaptive responses on the part of both the squid host and bacterial symbiont. Newly hatched, juvenile squid are aposymbiotic, and therefore acquire their *V. fischeri* symbionts from the surrounding seawater. An early study found that *V. fischeri* cells constitute a small percentage (less than 0.1%) of the total bacteria within the ambient seawater [16,17]. Upon hatching, the juvenile squid ventilates seawater through its mantle cavity (approximately 1.3 μL every half second), which passes the bacteria-
containing seawater across the light organ surface [16]. This results in approximately one *V. fischeri* cell present per ventilation [16]. Despite its relatively low abundance, *V. fischeri* successfully gains access to the light organ by entering one of the six pores located on the light organ surface.

The initiation of colonization typically occurs within the first 3 hours after the juvenile *E. scolopes* and *V. fischeri* come into contact. One of the earliest events in colonization involves the formation of a bacterial aggregate on the surface of the symbiotic light organ [16,18]. The light organ possesses two features that appear to facilitate aggregate formation and colonization. First, the ciliated epithelial fields located on the surface appendages help direct seawater, containing both *V. fischeri* and other bacteria, towards the six pores that lead into the light organ [16]. Second, epithelial cells surrounding the pores secrete mucus, which promotes surface attachment by the bacterial cells present within the ventilated seawater [16]. Both *V. fischeri* and other Gram-negative bacteria are capable of aggregating within the squid-derived mucus layer, but *V. fischeri* quickly dominates in the secreted mucus [16,18,19]. The ability of *V. fischeri* to form aggregates in the squid-derived mucus depends upon the production of a specific extracellular polysaccharide [20]. The formation of this exopolysaccharide requires the symbiosis polysaccharide (*syp*) locus, whose gene products contribute to the production of a specific biofilm [21,22]. These host and bacterial responses serve to promote interaction between the symbiotic partners and initiate the colonization process.

(B) Host responses during colonization initiation
Accumulating evidence indicates that the juvenile squid actively recruit the *V. fischeri* symbionts from the surrounding seawater. This recruitment occurs early in the initiation stage, in which the ciliated appendages and mucus secretion concentrate the bacteria near the light organ pores [16]. Staining with fluorescently labeled lectins identified N-acetylneuraminic acid and N-acetylgalactosamine as two components present in the squid-derived mucus [16]. Mucus production was found to be induced upon exposure of the squid to seawater containing either Gram-negative or Gram-positive bacteria, but not to sterilized seawater alone [19]. This finding suggested that mucus shedding by the juvenile squid occurs in response to a bacterial product. Additional experiments identified that peptidoglycan, a common component of the bacterial cell envelope, is sufficient to induce this mucus shedding [19]. Peptidoglycan is categorized as a Microbe-Associated Molecular Pattern (MAMP), and is typically recognized through binding to specific MAMP receptors, including peptidoglycan recognition proteins (PGRPs) [23]. Analysis of an expressed sequence tag cDNA (EST-cDNA) library generated from the tissues of juvenile squid identified four putative PGRPs (EsPGRP1-4), which may contribute to the host response(s) to peptidoglycan [23]. Indeed, a recent study revealed that one of these PGRPs, EsPGRP2, localizes to the mucus secreting, surface epithelia and associates with the secreted mucus [24]. These studies suggest that binding of peptidoglycan to one or more of the EsPGRPs may mediate mucus shedding by the surface epithelia and promote bacterial aggregation.

*(C) Bacterial responses during colonization initiation*
A key process in the initiation of colonization involves the attachment of *V. fischeri* aggregates within the secreted mucus on the light organ surface [16]. Aggregation within the squid-secreted mucus is not limited to *V. fischeri*, as other Gram-negative, non-symbiotic species also aggregate [16]. However, a study revealed that *V. fischeri* rapidly dominates within the aggregates, accounting for approximately 99% of the aggregated cells [18]. This study further determined that the enhanced propensity of *V. fischeri* to aggregate could not be attributed to increased growth, but rather the accumulation of pre-existing *V. fischeri* cells [18]. These studies suggest that *V. fischeri* detects an-as-yet unidentified signal that specifically triggers the symbionts to form aggregates on the light organ surface.

Subsequent studies found that the *V. fischeri* aggregates represent a form of bacterial biofilm formation [25]. Investigation into this biofilm has led to the identification of a number of the genes involved in the formation and regulation of this biofilm. In particular, transposon insertional mutagenesis screens for mutants defective in colonization identified the regulatory gene *rscS* (regulator of symbiotic colonization, sensor), which encodes a histidine sensor kinase (HK) protein, and the *syp* biofilm locus, an 18-gene polysaccharide locus [21,26](Fig. 2). Both *rscS* and *syp* mutants exhibit severe defects in the initiation of colonization, which can be attributed to the inability of these mutants to aggregate on light organ surface [20,26]. The *syp* genes encode a number of structural proteins involved in the synthesis and export of a polysaccharide [21]. Mutational analyses determined that the majority of these structural *syp* genes are required for biofilm formation and efficient host colonization [21,22]. Current evidence
indicates that the *syp* locus and aggregate formation are under tight regulatory control [25]. In addition to the *syp* structural proteins, the *syp* locus encodes four regulatory proteins, including the HK SypF, the response regulators (RRs) SypG and SypE, and the putative anti-sigma factor antagonist SypA [21](Fig. 2). Genetic studies determined the HK RscS functions upstream of the RR SypG to control transcription of the *syp* locus [20,27] (Fig. 3). The overexpression of *rscS* activates the *syp* locus, resulting in increased aggregation on the light organ surface and enhanced colonization competency [20]. In laboratory culture, induction of the *syp* locus through *rscS* overexpression results in the production of biofilm phenotypes [20,21]. In addition to RscS, the HK SypF also appears to regulate *syp* locus activation, most likely through activation of SypG [28]. Current evidence suggests that the RR SypE also contributes to the regulation of *syp*-dependent biofilms [27]. The role of SypE in regulating biofilms and its relevance during host colonization are the subjects of this dissertation.

In addition to aggregation, early studies established that motility and chemotaxis are critical behaviors during the initiation [29,30,31]. *V. fischeri* motility is mediated by a polar tuft of flagella [32]. Analysis of motility mutants revealed that loss of motility does not dramatically impact aggregate formation, but does impair migration into the light organ [16]. These results indicate that migration is an active process, in which motile bacteria direct their movements toward the light organ. The directed migration of *V. fischeri* suggests that the cells respond to specific chemotactic signals. Indeed, several studies demonstrated that *V. fischeri* mutants defective in the chemotaxis regulators, CheY, CheA, or CheR, exhibit impaired host colonization [33,34,35].
Figure 2. The syp polysaccharide locus of *V. fischeri*. The syp locus consists of 18 genes arranged in four operons. The syp genes encode proteins with putative regulatory activities (red) or functions involved in polysaccharide synthesis and/or export (blue). The predicted or known functions of the individual syp genes are listed below.

- **sypA**: Anti-sigma factor antagonist
- **sypB**: Outer membrane protein
- **sypC**: Capsule biosynthesis export
- **sypD**: ATPase
- **sypE**: Response Regulator (RR)
- **sypF**: Sensor Kinase (HK)
- **sypG**: Response Regulator (RR)
- **sypH**: Glycosyltransferase
- **sypI**: Glycosyltransferase
- **sypJ**: Glycosyltransferase
- **sypK**: Oligosaccharide translocase
- **sypL**: Lipid A core-O-antigen ligase
- **sypM**: Acetyltransferase
- **sypN**: Glycosyltransferase
- **sypO**: Chain length regulator
- **sypP**: Glycosyltransferase
- **sypQ**: Glycosyltransferase
- **sypR**: Undecaprenyl galactosephosphotransferase
Figure 3. Transcriptional regulation of the syp locus. The HK RscS acts upstream of the syp-encoded RR SypG to control syp transcription. The current model predicts that RscS is activated upon sensing some as-yet-unidentified signal and serves as a phosphodonor for SypG. Phosphorylation is proposed to activate SypG, resulting in SypG-mediated activation of the individual syp operons. In addition to RscS, the syp-encoded HK SypF is also proposed to serve as a phosphodonor to SypG. Dashed arrow indicates potential direct phosphorylation of SypG by SypF.
V. *fischeri* have been shown to migrate towards a variety of chemotactants, including several squid-derived compounds [34,36]. Specifically, *V. fischeri* performs chemotaxis to both N-acetylneuraminic acid, a component of the squid-secreted mucus, and squid-derived chitin [34,36]. Chemotaxis toward chitin is critical for *V. fischeri* to migrate through the pores and ducts leading into the light organ [34]. These studies suggest that *V. fischeri* uses gradients of host-derived chemoattractants to efficiently colonize the light organ.

**Migration and accommodation of *V. fischeri* within the light organ**

**(A) Overview**

Following attachment and aggregation, *V. fischeri* cells disperse from the aggregate and migrate to one of six pores located on the light organ surface. The bacteria enter the pores, travel through the ciliated ducts, and migrate through an antechamber before reaching and colonizing the deep crypt spaces. Entrance to the deep crypts is restricted by a bottleneck region, which limits access to the crypt spaces. Within the deep crypt spaces, *V. fischeri* cells closely associate with the host epithelial, making direct contact with the microvilli located on the surface of the crypt epithelia [37].

During their migration, *V. fischeri* cells encounter a variety of host-derived antimicrobial defenses. These host defenses are presumed to function as specificity determinants, which restrict colonization by non-symbionts, while simultaneously enriching for *V. fischeri* cells [15,38,39]. After colonization, *V. fischeri* induces a series of morphological changes within the host light organ [13,37]. These symbiont-induced developmental changes occur within the tissues in direct contact with the bacteria (i.e. the
deep crypts) and in tissues distant from the site of infection. Studies in which antibiotics were used to cure *E. scolopes* of their *V. fischeri* symbionts revealed that some of these developmental changes are reversible, while others are permanent. Altogether, these host changes may serve to prevent further infection of the light organ by non-symbionts. In the following sections, I will discuss in further detail the host and bacterial behaviors that contribute to *V. fischeri* accommodation within the symbiotic light organ.

(B) Host responses during symbiont migration and accommodation

In addition to recruiting its bacterial symbionts, the squid host also possesses a variety of mechanisms that function in controlling and ensuring the specificity of colonization. These include the production of antimicrobial compounds that restrict entry and colonization by non-symbiotic bacteria. Soon after aggregating on the light organ surface, *V. fischeri* cells are exposed to low levels of nitric oxide (NO) produced by vesicles embedded in the host-derived mucus [40]. NO is a reactive nitrogen species that is commonly employed by eukaryotes as an antimicrobial agent [41]. Both NO and nitric oxide synthetase (NOS), the enzyme involved in NO production, are produced by epithelial tissues throughout the light-organ. During their migration through the ciliated ducts, *V. fischeri* cells are exposed to increased levels of NO [40]. The presence of NO during these early stages of colonization suggests that NO production may serve as an early specificity determinant. In support of this, the presence of NO-scavenging molecules results in an increase in the aggregation of both *V. fischeri* and non-symbiotic bacteria [40].
*E. scolopes* has also been shown to produce hypochlorous acid, a reactive oxygen species (ROS) that commonly functions as an antimicrobial agent [42,43]. An early study utilizing a cDNA library generated from the light organ tissues determined that a high proportion of the light organ transcripts encode putative ROS-generating peroxidases [42]. These putative peroxidases included a homolog of mammalian peroxidases, a halide peroxidase (HPO) involved in the production of hypochlorous acid from halide ions and hydrogen peroxide [42,43]. The HPO gene was found to be expressed at relatively higher levels in tissues that directly contact bacteria [43]. *V. fischeri* is sensitive to hypochlorous acid, suggesting that hypochlorous acid may serve to control the levels of colonizing bacteria. Together, the production of antimicrobial molecules, such as NO and hypochlorous acid, represents a means by which *E. scolopes* chemically responds to interaction with bacteria. These host responses may provide a chemical barrier that promotes colonization specificity, restricting colonization to its *V. fischeri* symbionts.

Similar to other cephalopods, *E. scolopes* possesses a single immune cell population, the macrophage-like hemocytes [15]. These host hemocytes transverse the epithelium of the light organ crypts and “sample” the crypt environment [15]. Similar to macrophages, the hemocytes function to engulf and kill bacterial cells. Indeed, within the light organ crypts of newly colonized juvenile *E. scolopes*, host hemocytes have been observed containing internalized *V. fischeri* [15]. Interestingly, hemocytes within the crypts of adult *E. scolopes* do not appear to engulf the bacteria, despite being surrounded by *V. fischeri* cells [15,44]. These studies suggest that the host hemocytes are “educated”
upon exposure to *V. fischeri*, eventually developing a tolerance for the bacterial symbionts. Indeed, hemocytes isolated from colonized *E. scolopes* bind and phagocytose *V. fischeri* to a lesser degree than related, non-symbiotic species [44]. This tolerance is not observed in hemocytes isolated from adult *E. scolopes* cured of their bacterial symbionts, suggesting that prior exposure to *V. fischeri* or a bacterial component is required for tolerance [44]. This same study determined that *V. fischeri* cells deleted for the outer membrane protein OmpU did not induce tolerance, and were engulfed by hemocytes at a higher frequency than wild-type cells, suggesting that OmpU recognition by host hemocytes contributes to *V. fischeri* tolerance and persistence [44]. The mechanism by which *V. fischeri* OmpU promotes the specific tolerance of *V. fischeri* cells currently remains unclear. Together, these studies reveal that the host immune system responds to the presence of the bacterial symbionts, resulting in the hemocytes developing an OmpU-dependent tolerance for the *V. fischeri* symbionts.

Colonization by *V. fischeri* induces a series of development changes in the host light organ, several of which are reversible upon removal of the bacterial symbionts. These symbiont-induced changes occur not only in areas in direct contact with the *V. fischeri* cells (i.e. the deep crypts), but also in areas distant from the infection site (i.e. the ciliated ducts and ciliated appendages) [45]. One of these symbiont-induced changes involves the constriction of the ciliated ducts. Soon after *V. fischeri* colonizes the deep crypts (approximately 6 hours post hatching), the tissues within the ducts exhibit an increase in actin synthesis, which corresponds to an increase in the deposition of filamentous actin at the apical surface of the duct epithelia, thus resulting in duct
constriction [46]. Duct constriction decreases the diameter of the light organ duct, which may serve to limit entry of additional bacteria after initial colonization [46]. Removal of the bacterial symbionts through antibiotic treatment results in the expansion of the ducts to their original state [19]. The bacterial ligands responsible for inducing these changes in the ducts remain to be determined.

Early in colonization, the epithelia of the ciliated appendages secrete mucus, which promotes bacterial aggregation and the initiation of infection. Following *V. fischeri* infection of the light organ, the presence of the symbiont induces the cessation of this mucus shedding [19]. Similar to duct constriction, mucus cessation is a reversible event, as removal of the bacterial symbionts restores mucus secretion by the ciliated epithelium [19]. Another dramatic change induced by *V. fischeri* is the regression of the ciliated appendages. Studies indicate that *V. fischeri* colonization delivers an irreversible signal that triggers the loss of the ciliated surface epithelium [45,47]. Around 12 hours after initial exposure, *V. fischeri* colonization signals the apoptosis of the ciliated epithelia and the gradual regression of the appendages. While the cessation of mucus shedding occurs relatively quickly after colonization (around 48 hours post initiation), the complete regression of the ciliated appendages occurs gradually over a 4-5 day period [13].

The observation that *V. fischeri* infection triggers host morphological changes has prompted investigations into the bacterial factors that signal these developmental processes. Several studies have focused on components of the bacterial cell envelope as possible bacteria-derived signals for host development. Similar to intact bacterial cells, the exposure of juvenile squid to cell surface fractions isolated from *V. fischeri* was found
to induce light organ development, including the apoptosis and regression of the ciliated appendages, supporting a role for bacterial cell envelope components [48]. Additional studies determined that two components of the cell envelope, lipopolysaccharide (LPS) and peptidoglycan (PG), are sufficient to induce light organ morphogenesis. Bacterial LPS was first demonstrated to signal the apoptosis of the ciliated epithelial fields, but did not induce regression of the ciliated appendages [49]. Further analysis determined that the induction of apoptosis is mediated by the lipid A moiety of LPS. The effect of LPS on the apoptosis of the epithelial fields is not restricted to *V. fischeri* lipid A, as LPS derived from non-symbiotic bacteria induced similar changes [49].

That LPS alone induced apoptosis, but not full regression of the ciliated appendages, suggested that additional bacterial molecules were required for inducing light organ morphogenesis. Subsequent studies determined that peptidoglycan represented the second signaling molecule [48]. Specifically, *V. fischeri* releases a tetrapeptide PG monomer, termed tracheal cytotoxin (TCT), that promotes light organ development [48]. Using a *V. fischeri* mutant defective in TCT release, it was determined that TCT is required for inducing regression of the ciliated appendages [50]. Although capable of triggering the regression of the ciliated appendages, TCT alone does not induce apoptosis in the epithelial field [48]. However, when TCT is provided in combination with LPS, the two morphogens have a synergistic effect on light organ morphogenesis, resulting in both apoptosis and ciliated appendage regression [48]. Together, TCT and LPS mimic the irreversible signal induced by intact *V. fischeri* cells,
indicating that the sensing of these bacterial molecules by *E. scolopes* is sufficient to signal light organ development.

Both LPS and PG are common bacterial components, produced by both *V. fischeri* and other non-symbiotic bacteria, and yet light organ development is specific to *V. fischeri* infection. It has been established that *V. fischeri* must enter the crypts in order to induce light organ morphogenesis, suggesting that engagement with host receptors likely occurs within the crypt spaces. Therefore, specificity in LPS and PG signaling is likely conferred through restricting host receptor activation to areas exclusively inhabited by *V. fischeri* cells.

(C) Bacterial symbiont responses during migration and accommodation

Following entry through the pores, *V. fischeri* cells migrate through the ciliated ducts leading to the light organ crypts. In addition to a role in colonization initiation [29,30], motility is also likely to be required for transit of the bacteria through the ciliated ducts and antechamber, as the ducts are lined with dense cilia that generate an outward directed current, thus generating an obstacle for the migrating bacterial cells [45]. The role of motility during duct migration is difficult to directly test, however, as nonmotile cells exhibit a severe initiation defect as a result of their failure to migrate to the pores [30].

During migration into the light organ, *V. fischeri* cells encounter a variety of host-derived antimicrobial defenses, including NO and ROS. Microarray analysis revealed that exposure of *V. fischeri* cells to NO results in the upregulation of an alternative respiratory oxidase (AOX) [38,51]. Importantly, AOX is relatively NO-resistant, which
allows aerobic respiration to proceed in the presence of NO when other cellular oxidases are inhibited [51]. A recent study determined that *V. fischeri* also possesses a homolog of the *Escherichia coli* flavohaemoglobin, Hmp, which is involved in the detoxification of NO by *E. coli* [39]. Transcriptome analysis indicates that, similar to AOX, Hmp expression is upregulated upon exposure of *V. fischeri* to NO. Furthermore, reporter studies revealed that *hmp* expression is activated at the initial aggregation stage of colonization, in which the cells first encounter squid-derived NO [39]. Importantly, Hmp is required for NO resistance in *V. fischeri*, as an *hmp* mutant fails to detoxify NO in culture and exhibits impaired host colonization, partially due to a defect in colonization initiation [39]. *V. fischeri* also possesses mechanisms to resist stress induced by the exposure to host-derived hypochlorous acid. *V. fischeri* encodes a periplasmic catalase, KatA, that converts hydrogen peroxide, a precursor to the production of hypochlorous acid, to water and oxygen [52]. This suggests that *V. fischeri* may prevent the host production of hypochlorous acid through the catalase activity of KatA. Indeed, a *katA* mutant exhibits a competitive colonization defect during mixed-culture infections, indicating that the catalase activity of KatA indeed contributes to colonization fitness [52]. These studies reveal that *V. fischeri* has evolved specific mechanisms to cope with the various host defenses it encounters during colonization.

**Persistence of *V. fischeri* within the light organ**

**(A) Overview**

Once *V. fischeri* reaches the deep crypt spaces, the bacteria proliferate in the nutrient-rich environment within the light organ [53]. Within 12 hours after initial
encounter, the bacteria achieve a high cell density and produce bioluminescence. Luminescence is regulated in a cell density-dependent manner, through a pheromone-mediated process termed quorum sensing. Quorum sensing describes a mechanism in which bacterial cells determine the population density through the release and detection of pheromones, or chemical signals termed autoinducers. The accumulation of these autoinducers at high-cell densities results in a change in gene regulation, including the induction of bioluminescence. Bioluminescence is required for successful colonization, as *V. fischeri* mutants deficient in light production initiate colonization, but fail to persist within the light organ [54,55]. These studies suggest that the squid host possesses mechanisms to ensure light production by its bacterial symbionts. Indeed, the *E. scolopes* light organ contains tissues capable of detecting and responding to light production [56].

Once association between *E. scolopes* and *V. fischeri* is established, both organisms develop a daily rhythm in their symbiotic behaviors. One of the most evident of these daily rhythms is the expulsion of the light organ contents, including *V. fischeri* cells and host hemocytes, into the surrounding seawater. Every dawn, *E. scolopes* expels approximately 95% of its bacterial symbionts within a thick mucus secretion [14,15]. Over the course of the following day, the remaining *V. fischeri* cells re-populate the light organ [14]. The process of daily expulsion and re-growth likely serves to control the population of bacterial symbionts. Another daily rhythm established during persistent colonization is the controlled variation in bacterial luminescence [14]. Luminescence varies greatly within the squid host, with levels typically reaching a peak in the hours before night [14]. A recent microarray study revealed that *V. fischeri* metabolism also
exhibits a daily rhythm [57]. *V. fischeri* appears to adjust its metabolic profile in response to the host’s daily rhythm of light organ expulsion [57].

(B) Host responses involved in *V. fischeri* persistence

Following the completion of colonization, *E. scolopes* initiates a daily cycle of symbiont expulsion and regrowth. A recent microarray analysis determined that, just prior to light organ expulsion, *E. scolopes* undergoes a dramatic change in gene expression, particularly in genes involved in cytoskeletal rearrangement [57]. Analysis of the light organ epithelium found that these changes in cytoskeletal gene expression coincide with the effacement of the epithelium and the release of epithelial tissue into the crypt spaces [15,57]. Early studies determined that this expulsion event is specifically triggered in response to light exposure, as animals maintained in the darkness fail to release their symbionts [14,58]. The relevance of expulsion to the light organ symbiosis currently remains unclear. It is likely that expulsion benefits the maintenance of symbiosis, possibly by controlling the levels of the symbiont population or by promoting luminescence. Additionally, expulsion may serve to increase the concentration of *V. fischeri* in the local environment, thus promoting transmission to newly hatched juveniles.

The production of bacterial luminescence represents a key contribution of *V. fischeri* to the light organ symbiosis. Light emission is proposed to aid *E. scolopes* during its nocturnal activities, serving as a form of camouflage. The squid host appears to exert control over the emission of bacterial luminescence. The ink sac and other tissues surrounding the light organ, including a layer of reflective tissues, serve to direct the light
emission downward, thus providing counter-illumination and masking the animal’s shadow [11]. In addition to directing the emission of light, the squid also appears to exert a level of control over the production of luminescence, occasionally suppressing light production [14]. Analysis of the specific luminescence, or the amount light produced per cell, found that \textit{V. fischeri} cells within the light organ exhibit a daily rhythm of increasing and decreasing levels of light production compared to released cells. Specifically, in periods of low luminescence (i.e. during the day), the specific luminescence of colonizing \textit{V. fischeri} cells is decreased relative to released cells [14].

In addition to controlling light emission, the squid also possesses mechanisms to ensure light production by the bacterial symbionts. \textit{V. fischeri} mutants deficient in luminescence are capable of initiating colonization, but fail to persist within the light organ [54,55]. The inability of non-luminescent strains to persist within the light organ may be due to a variety of reasons. One possibility is that \textit{E. scolopes} is capable of detecting bacterial light emission, and actively selects for luminescent symbionts.

Indeed, a recent transcriptome analysis of the light organ tissues revealed the expression of genes whose proteins exhibit similarity to those found in visual transduction systems, including photoreceptor proteins such as opsin [59]. The light organ is also surrounded by accessory tissues that are structurally similar to those found in the eye. These include an encapsulating layer of reflective tissue and a transparent, “lens”-like tissue layer [13]. A subsequent study confirmed that these accessory tissues indeed possess the ability to directly detect and respond to light stimulation [56]. Thus, the ability of the light organ tissues to directly sense light production may provide an additional mechanism by which
E. scolopes responds to its bacterial symbiont and, possibly, restricts colonization to luminescent bacteria.

(C) Bacterial symbiont responses involved in persistence

Once V. fischeri establishes residency within deep crypts, the bacteria undergo a change in cell morphology. Specifically, the bacterial cells become smaller and rounder than those grown in culture [32]. V. fischeri within the deep crypts also become nonflagellated, suggesting that motility at this stage of colonization is not required [32]. It currently remains unknown what signals or conditions within the light organ promote these morphological changes. Additionally, it is unclear what role the loss of flagella may play in colonization. It is has been proposed that the presence of flagella may be detrimental at this stage [8].

Within the light organ crypts, V. fischeri cells proliferate to a high cell density, reaching levels of approximately $10^{11}$ cells per mL of light organ fluid [60]. Growth of the bacteria is supported by the nutrients provided by the light organ environment, including host-derived amino acids [53]. Upon establishing colonization, V. fischeri cells adjust their metabolism in response to the light organ environment. V. fischeri metabolism exhibits a daily rhythm that coincides with the daily rhythm of the animal host. A recent transcriptome analysis characterized the gene profiles of both the squid host and bacterial symbionts at intervals throughout the daily cycle of light organ expulsion and re-population [57]. This study found that V. fischeri cells undergo various changes in gene expression (up to 17% of total genes) throughout the daily cycle of symbiosis [57]. The most dramatic changes in gene expression occurred around dawn, the
time period in which *E. scolopes* expels the contents of its light organ [57]. *V. fischeri* exhibited specific changes in the expression of genes involved in the metabolism of chitin and glycerol, both nutrient sources proposed to be provided by the squid host [57]. Genes involved in chitin metabolism were up-regulated in the period prior to the dawn expulsion, and subsequently down-regulated following the dawn expulsion and throughout the remainder of the day [57]. In contrast, genes involved in glycerol metabolism were significantly up-regulated in the hours following dawn and light organ expulsion [57]. The authors of this study proposed that these alterations in metabolism gene expression reflect the response of the symbionts to changes in the available host-supplied nutrients that accompany light organ expulsion [57]. In particular, effacement of the light organ epithelium during expulsion is thought to provide an increased source of glycerophospholipids, from which glycerol may be obtained and metabolized by *V. fischeri* [57]. Lipid analysis of *V. fischeri* cell membranes confirmed that the cells indeed incorporate host-derived fatty acids [57]. This suggests that the bacteria adjust their metabolism in response to the daily changes in the light organ environment and the availability of host-derived nutrients.

Upon achieving a high cell density, the bacteria initiate the production of luminescence via three quorum sensing systems. Each of these systems produce and detect a distinct autoinducer signal. The signal synthase protein LuxI produces the autoinducer N-3-oxohexanoryl-L-homoserine lactone (3-oxo-C6-HSL), which is secreted into the supernatant and, upon reaching an equilibrium, binds to the cellular receptor LuxR [61,62]. Together, LuxI-LuxR activate transcription of the *lux* genes, which encode
the proteins responsible for bioluminescence [63,64]. *V. fischeri* possesses two additional autoinducers: an N-octanoyl-homoserine lactone (C8-HSL) produced by the synthase AinS, and a proposed furanosyl borate diester, called autuinducer-2 (AI-2) produced by the synthase LuxS [65,66]. C8-HSL and AI-2 are predicted to signal through two independent receptors, the hybrid sensor kinases AinR and LuxP/Q, respectively [67,68]. Proliferation of *V. fischeri* to high cell densities within the deep crypts results in the accumulation of these autoinducers, which are subsequently detected by their cognate receptor proteins and induces expression of the *lux* luminescence genes [68]. Mutational studies of the *lux* genes and their regulators demonstrate that loss of bioluminescence impair symbiotic colonization [54,55].

**Conclusion**

The symbiotic association between *E. scolopes* and *V. fischeri* has been established as an elegant model system for investigating the processes involved host-microbe interactions. The study of this system over the last 20 years has yielded numerous insights into the mechanisms by which a bacterium and its animal host establish, develop, and maintain a symbiotic partnership. An important feature of this model system is the exclusive nature of the association, as only *V. fischeri* is capable of establishing successful colonization of the squid light organ. This exclusive partnership allows for direct investigation into the processes by which the bacterial symbiont and its animal host promote and ensure specificity over colonization. Both *V. fischeri* and *E. scolopes* actively participate in the colonization process, as each organism detects and adaptively responds to the presence of its symbiotic partner. *V. fischeri* has evolved a
variety of mechanisms that promote host interaction and allow the bacteria to adapt to conditions within the squid host. Similarly, *E. scolopes* contributes to colonization through the initiation of a number of physiological and developmental processes, many of which are triggered by interaction with its bacterial symbiont. Although two decades worth of investigation has resulted in the identification of many of the bacterial and host factors required for establishing colonization, many questions remain. In particular, the mechanisms by which *V. fischeri* regulates the expression of colonization factors, such as *syp* biofilm formation, remain areas of active study. This dissertation examines a particular mechanism by which *V. fischeri* regulates colonization of its animal host.

**TWO-COMPONENT REGULATORY SYSTEMS**

**Introduction**

The ability to sense and respond to changes within the environment is critical for the survival of any living cell. To modulate gene expression in response to environmental cues, cells employ a variety of signaling devices. A common mechanism by which bacteria coordinate cellular behavior in response to environmental cues is through the regulation of protein phosphorylation by protein kinases and phosphatases. Upon detection of an environmental stimulus a protein kinase autophosphorylates and then donates the phosphoryl group to a substrate protein(s). Kinases typically phosphorylate substrate proteins on specific amino acid residues, including serine (Ser), threonine (Thr), tyrosine (Tyr), histidine (His) and aspartate (Asp) residues [69,70]. As a result, phosphorylation can regulate a target protein’s activity, either by inducing specific conformational changes or by impacting protein-protein interactions [69].
In bacteria, a common form of signal transduction is through two-component signal (TCS) transduction systems involving His-Asp phosphotransfer [71]. In its simplest form, TCS systems consist of a histidine sensor kinase (HK) that autophosphorylates at a conserved histidine residue, and a downstream response regulator (RR) that catalyzes the transfer of the phosphoryl group from the HK to a conserved aspartate residue located within the RR [71](Fig. 4).

The typical HK protein consists of a variable N-terminal sensory domain connected to a highly conserved kinase catalytic domain [72]. Typically, sensing of an environmental stimulus by the HK’s sensory domain results in the activation of the protein’s ATP-dependent kinase domain, which subsequently autophosphorylates on a conserved His residue located in the kinase core. HKs are generally thought to function as homodimers and autophosphorylation occurs via an in trans mechanism, in which one HK subunit catalyzes the phosphorylation of the conserved His residue in the second subunit [72,73].

RRs catalyze phosphoryl transfer from the phosho-histidine of the HK to a conserved aspartate residue located within the RR’s N-terminal regulatory receiver (REC) domain [74](Fig. 4). Phosphorylation generally regulates the activity of an attached C-terminal effector domain [75,76]. The vast majority of RR proteins contain a DNA-binding effector domain and function as transcription factors [77,78]. As a result, RR phosphorylation commonly results in a change in DNA binding and, thus, altered gene expression [reviewed in [78]]. Alternatively, the RR REC domain may be attached to a large variety of other output domains, including those involved in protein-protein
Figure 4. Two-component signal transduction pathways. (A) In the typical two-component signaling (TCS) pathway, a HK protein autophosphorylates on a conserved histidine (His) and then donates the phosphoryl group to an aspartate-containing receiver (REC) domain on a cognate RR protein. REC Phosphorylation changes the activity of an output domain, resulting in a cellular response. Simple HK proteins consist of a variable sensory domain and a conserved kinase module, which includes a catalytic and ATPase (CA) domain responsible for ATP binding and catalyzing autophosphorylation at a His residue located in the dimerization and autophosphorylation (DHp) domain. (B) Phosphorelays represent a more complex (TCS) pathway, involving multiple phosphotransfer events. A hybrid HK protein transfers phosphoryl groups from the conserved His to an internal REC domain. A histidine phosphotransferase (Hpt) protein then shuttles the phosphoryl groups to the cognate RR. The Hpt module may exist either as an independent protein or as part of the hybrid HK.
interaction, ligand binding, or enzymatic activity [78,79].

The simple TCS system described above involves a single phosphotransfer event between a single HK and its cognate RR protein. However, many characterized TCS systems possess a more complex signaling circuitry involving multiple His-Asp phosphotransfer events [71,80](Fig. 4B). Multi-step phosphorelays represent a more elaborate form of the TCS system and include multiple phosphoprotein intermediates; upon autophosphorylation of the histidine kinase, the phosphoryl group first transfers to a conserved Asp residue located within a REC domain, followed by transfer to a second conserved His residue within a histidine-containing phosphotransfer (HPt) domain, and finally to a second conserved Asp located in the REC domain of a second RR [80](Fig. 4B). The intermediate His- and Asp-containing domains function as phosphotransfer elements and can be found either as independent signaling elements or “fused” in a single protein, as observed in hybrid HK proteins [71]. In the case of hybrid HKs, the proteins possess both the ATP-dependent, catalytic kinase domain (containing the phosphoacceptor His residue) and a REC domain (containing the intermediate Asp residue) (Fig. 4B). The HPt-domain, containing the intermediate phospho-accepting His residue, may also be found as a component within the hybrid HK or may exist as a separate protein (Fig. 4B). The HPt domains themselves do not exhibit kinase or phosphatase activity, in comparison to the catalytic, histidine kinase domain, but instead shuttle the phosphoryl group to the downstream RR. As a result of these multiple phospho-intermediates, the phosphorelay system provides an opportunity for additional regulatory checkpoints, as well as additional signaling inputs, to be incorporated into the signaling cascade [80].
In addition to phosphorelay systems, another means by which the simple TCS system can be modified to achieve greater complexity, and thus regulatory control, is through the incorporation of multiple HKs and/or RRs. In these more complex TCS systems, multiple HKs can mediate phosphorylation of a single, downstream RR protein (Fig. 5). Alternatively, a single HK may serve as a phosphoryl donor to multiple downstream RRs (Fig. 5). For example, the HK CheA, which regulates chemotaxis in *E. coli*, functions as a phosphoryl donor to two downstream RRs, CheY and CheB [81]. Similarly, the Lux quorum sensing system controlling bioluminescence in *Vibrio harveyi* provides an elegant example of multiple HK proteins (LuxQ, LuxN, and CqsS) that serve as phosphoryl donors to a single RR protein, LuxO [82,83]. These more complex variations on the TCS system, as with the phosphorelay scheme, provide greater control over cellular responses by integrating multiple signaling inputs [80].

The number of TCS systems within a particular bacterium varies greatly among different species, with the average bacterium utilizing between 10-50 TCS systems [71]. The observation that a single bacterium may possess multiple TCS components raises an important question as to how distinct TCS pathways achieve signal specificity or fidelity. One means by which cells may achieve specificity within TCS pathways is through regulating the relative levels of HK and RR pairs. Indeed, many cognate HKs and their RRs are often genetically linked, such that the expression of the HK and RR are co-regulated. Another mechanism by which bacteria prevent aberrant signaling between distinct TCS pathways is through controlling protein interactions between cognate HK and RR pairs [84]. Several studies have examined the structure of cognate HK/RR
Figure 5. Variations on the two-component signaling pathway. Depicted are two possible variations in signaling between HK proteins and downstream RR proteins. In the “One-to-Many” scenario, a single HK protein can function as a phosphodonor to multiple downstream RR proteins (RR1 and RR2). Alternatively, the “Many-to-One” scenario involves multiple HK proteins (HK1 and HK2) that both serve as phosphodonors to a common downstream RR. This figure was modified from Laub and Goulian (2007) [88].
complexes and utilized these structures to characterize the interacting interfaces and residues involved in HK/RR pairing [85,86]. By comparing the structures of the different HK/RR complexes reported in these studies, researchers have shown that binding between HK/RR pairs involves several conserved interacting interfaces between the RR’s regulatory REC domain and the histidine kinase domain of the cognate HK (reviewed in [84]). These initial studies have led to further computational analyses investigating the potential variable residues within these interaction interfaces that may confer binding specificity between cognate HK/RR pairs [87]. These studies indicate that specific residues within the interaction interfaces of cognate HK/RR pairs may function to control specificity in TCS pathways. Specificity between cognate HK/RR pairs may also be achieved through controlling RR dephosphorylation. Multiple HKs have been reported to possess bifunctional activity, capable of both phosphorylation and dephosphorylating their cognate RR [71,88]. Studies have investigated the role of HK phosphatase activity in controlling RR activation, and suggest that RR dephosphorylation by cognate HK may serve to prevent aberrant RR activation by non-cognate HKs [89,90]. Additionally, dedicated proteins phosphatases may serve to control RR phosphorylation and thus provide further control of gene expression. These studies suggest that TCS systems may possess multiple mechanisms to ensure proper signal transmission.

TCS systems have been implicated in the regulation of diverse bacterial processes, including the expression of factors involved in host colonization and pathogenesis [5]. Importantly, recent studies have shown that V. fischeri utilizes multiple TCS systems during colonization of E. scolopes [33,91]. These TCS systems contribute
to the regulation of numerous symbiotic behaviors, including bacterial aggregation, motility and bioluminescence [33,91,92]. In the following sections, I will summarize the key components of HKs and RRs and their roles in signal transduction. In addition, I will discuss the role of TCS systems during the *V. fischeri-E. scolopes* symbiosis, focusing on two-component regulators of *V. fischeri* aggregate formation and colonization initiation.

**Structure and function of histidine sensor (HK) kinases**

HK proteins typically possess a modular domain architecture and consist of at least three conserved domains that participate in distinct functional roles. The typical HK contains a variable sensory domain connected to conserved dimerization and catalytic kinase domains (Fig. 4). The key features of these HK domains and their roles in HK activity/function are discussed below.

(A) **Sensory domain**

The central function of a HK is to provide detection of specific environmental signals and to elicit the appropriate cellular response by modulating the activity of a downstream RR(s). The detection of specific environmental stimuli is mediated directly or indirectly by the HK’s N-terminal sensory domain. The sensory domains between individual HKs exhibit high sequence variability, suggesting that these domains have evolved to sense diverse environmental ligands [93]. In most cases, the specific ligands sensed by the sensory domain and the mechanism of detection remain unknown. The organization of the sensory domain within HKs can vary depending upon the detected stimulus. In the typical membrane-spanning HK, the sensory domain is formed by a folded, extracellular loop located between two transmembrane domains [94]. In other
membrane-spanning HKs, the sensor domain may be embedded within the membrane or located within the cytoplasm either N- or C-terminal to the protein’s transmembrane domain(s).

(B) The catalytic kinase core

In contrast to the variable sensory domain, the catalytic kinase core is highly conserved among HK proteins. The kinase core is composed of two key domains: a dimerization and histidine phosphotransfer (DHp) domain and a catalytic and ATP-binding (CA) domain [72]. The kinase core functions to bind an ATP molecule and autophosphorylate at a conserved His residue. The CA domain of histidine kinases belongs to the ATPase/Kinase GHKL (gyrase, Hsp90, histidine kinase, MutL) superfamily of proteins [95]. Members of the GHKL superfamily possess a structural kinase core that contains a conserved ATP-binding fold, termed the Bergerat fold [95]. The structure of the ATP-binding Bergerat fold consists of an α/β sandwich with a four-stranded antiparallel β sheet and three α helices [95]. The catalytic ATP-binding domain also includes several conserved amino acid motifs involved in ATP binding and hydrolysis. In the CA domain of HKs, these conserved amino acid motifs are termed the N, G1, G2, and G3 boxes. The N-box contains a conserved asparagine residue that coordinates the binding of a Mg\(^{2+}\) ion required for connecting the ATP phosphoryl groups to the nucleotide binding cleft [95]. The G-box motifs contain conserved glycine residues that contribute to the flexibility of the protein’s ATP-lid, which serves to cover the ATP binding pocket [72,95]. In contrast to other members of the GHKL superfamily, HKs contain an additional conserved motif, termed the F box, which furthers contributes
to the ATP-lid and the binding of ATP [72]. The DHp domain of HKs consists of a conserved H box motif, which contains the phospho-accepting His [72]. Phosphate transfer is proposed to occur through a nucleophilic attack on the \( \gamma \)-phosphate of the ATP molecule bound by the CA domain by the recipient His residue [96]. Autophosphorylation by several characterized HKs has been demonstrated to occur via an \textit{in trans} mechanism [73]. In this manner, the conserved His residue located in one subunit receives a phosphoryl group from the ATP bound by the CA domain in the other subunit [73,97]. Evidence suggests that HK autophosphorylation can also occur through an \textit{in cis} mechanism, in which the phosphoryl group is transferred from the CA domain to the conserved His residue within the DHp domain of the same subunit [85].

**Structure and function of response regulators (RRs)**

In TCS systems, the RR serves as the downstream component that mediates a cellular response upon receiving a phosphoryl signal from a cognate HK or a small phosphodonor (e.g. acetyl phosphate) [107,108]. The typical RR possesses a modular domain structure consisting of two key domains: a conserved regulatory receiver domain and an attached variable, output domain (Fig. 4). The role of the individual RR domains is described below.

(A) Regulatory receiver (REC) domain

The regulatory REC domain functions as a phosphorylation-mediated molecular switch, and contains a conserved aspartate (Asp) residue that serves as the phospho-acceptor site. The REC domain is mostly commonly found attached to other domains present in multi-domain RRs or hybrid HKs. The REC domain may also exist as a stand-
alone module, as in the case of the single-domain RR CheY, which regulates bacterial chemotaxis by interacting with a component of the flagellar apparatus [98]. The RR CheY serves as a model for understanding the structure and function of the REC domain. The REC domain generally adopts a double wound α/β fold topology consisting of a parallel, five-stranded β-sheet surrounded by five α-helices [99,100,101]. The active site of the REC domain consists of several highly conserved residues. In CheY, these include three Asp residues, Asp 12, Asp 13, and Asp 57, which participate in the binding of a Mg$^{2+}$ cation required for phosphoryl transfer [102,103]. One of these Asp residues, Asp 57 for CheY, was identified via mass spectrometry to be the phospho-acceptor site, receiving phosphoryl groups from the cognate HK CheA [104]. The REC active site contains two additional, highly conserved threonine (Thr 87) and lysine (Lys 109) residues, which are not required for REC phosphorylation, but are critical for signal transduction [102,105].

An early study found that RRs are capable of catalyzing their own autophosphorylation in the absence of their cognate HKs [106]. Using purified RR proteins CheY and CheB, Lukat et al. demonstrated that RRs can catalyze their own phosphorylation using small molecule phosphodonors, such as acetyl-phosphate [106]. In addition to this initial in vitro study, subsequent investigations have since demonstrated that autophosphorylation using relevant, small phosphodonors can also occur in vivo [107,108]. Together, these studies revealed that the REC domain plays an active role in promoting RR phosphorylation.
RR phosphorylation is proposed to occur through a similar mechanism regardless of whether the RR autophosphorylates using a small phosphodonor or through interaction with a cognate HK. REC phosphorylation requires the presence of bound divalent cation, typically a Mg$^{2+}$ metal ion, coordinated by the three conserved Asp residues [103]. Structural studies of Mg$^{2+}$-bound CheY, indicate that the carboxylate side chains of the Asp residues form a Mg$^{2+}$ binding cleft [109]. The bound Mg$^{2+}$ ion is thought to facilitate the coordination of the phosphoryl group’s oxygen atoms [109]. REC phosphorylation is then proposed to proceed through a nucleophilic attack on the phosphorous atom by the carboxylate side chain of the phospho-accepting Asp (Asp 57 in CheY) [109].

The additional residues conserved within REC domains are proposed to participate in the subsequent signal transduction events following phosphorylation. Structural studies indicate that phosphorylation of the RR REC domain induces subtle conformational changes within the protein [101,110]. A study examining the structures of CheY in either the unphosphorylated, Mg$^{2+}$-bound state or upon binding of beryllofluoride (BeF$_3$), which mimics the phosphorylated state [111], revealed that phosphorylation promotes subtle conformational changes across the surface of the REC domain [110]. Specifically, the largest phosphorylation-induced changes were observed in the α4/β5/α5 face of the REC domain, including a repositioning of the loop region between β sheet 4 and α helix 4 (β4/α4) and a modest change in the loop region between β sheet 5 and α helix 5 (β5/α5) [110]. The other highly conserved Thr 87 and Lys 109 residues within the REC domain are found within these structural regions [110]. These
residues likely contribute to REC domain signal transduction by mediating the conformational changes induced by phosphorylation. These studies indicate that the REC domain possesses both inactive and active conformational states, with phosphorylation generally favoring the active conformation. Indeed, recent studies have suggested that the REC domain may exist in an equilibrium state, in which the protein shifts between both the active and inactive conformations [112,113]. In this case, phosphorylation would serve to shift the balance toward stabilization of the active conformational state [112,113].

A critical step in any TCS system is the termination of the cellular response through the removal of the phosphoryl group from the REC domain of an activated RR. The dephosphorylation of an activated RR can occur through several possible mechanisms, including RR autodephosphorylation [114,115]. RR dephosphorylation can also be achieved through the phosphatase activity of the cognate HK(s) or a dedicated protein phosphatase(s). A number of HK proteins have been demonstrated, either genetically or biochemically, to possess phosphatase activity toward their phosphorylated, cognate RRs [71, 107, 116]. In this manner, HKs can tightly regulate the longevity of a cellular response by controlling both the phosphorylation and dephosphorylation of their cognate RR. Finally, bacteria may also utilize dedicated phosphatase proteins to control the RR phosphorylation. For example, the Rap phosphatases in *B. subtilis* function in the signaling cascade to control sporulation by promoting the dephosphorylation of the RR Spo0F, thus inhibiting sporulation [117].

**(B) RR effector domains**
The majority of bacterial RRs are multi-domain proteins, typically containing the regulator REC domain in combination with one or more effector or output domains. In general, phosphorylation of the REC domain elicits a cellular response through inducing the activation, or occasionally the inactivation, of an attached effector domain. Unlike the highly conserved REC domain, RR effector domains exhibit great diversity and, thus, participate in a wide range of cellular functions [78]. Additionally, a number of RRs lack an effector domain altogether, and exist as single-REC domain proteins [118]. The chemotaxis protein CheY and the sporulation regulatory protein Spo0F provide well-characterized examples of such single-domain RRs. The RR CheY exerts regulatory control over bacterial chemotaxis through a mechanism involving protein-protein interactions. Upon phosphorylation, CheY is activated to bind to the flagellar switch component FliM and induce a switch in flagellar rotation [98,119]. In contrast, the RR Spo0F regulates sporulation in Bacillus subtilis by participating in a phosphorelay, in which Spo0F shuttles phosphoryl groups from two cognate HKs (KinA/KinB) to a downstream HPr protein, Spo0B [120]. These studies demonstrate that the REC domain alone can exert regulatory activity. Although the REC domain can function as a stand-alone protein, most RRs contain the REC domain in combination with an effector domain, of which there are a variety. I will therefore provide a brief overview of the major classes of RRs containing characterized effector domains.

The majority of characterized RRs possess the REC domain attached to a DNA-binding effector domain, and therefore often function as transcriptional regulators [78]. Consequently, REC phosphorylation typically results in a change in DNA binding and
altered target gene expression. A comprehensive analysis of RRs with DNA-binding effector domains, indicated that most can RRs be classified into three major classes: the OmpR class, the FixJ/NarL class, and the NtrC class [78]. The OmpR class represents the most abundant family of RRs (30.1% of all RRs), and includes RRs containing a winged helix-turn-helix DNA-binding domain [78]. The FixJ/NarL class is the second most abundant class of RRs (10.1% of total RRs), and consists of RRs containing a helix-turn-helix DNA-binding domain [78]. Finally, the NtrC class of RRs (representing ~9.1% of all RRs) includes those that contain three distinct domains: a REC domain combined with both a $\sigma^{54}$-interacting-ATPase domain and a DNA-binding domain [78].

In addition to DNA-binding domains, the REC domain may also be found in combination with a variety of other effector domains, including those involved in protein-protein interactions, RNA-binding, or enzymatic activity [78]. Due to the large diversity of RR effector domains, I discuss here several representative examples of characterized RRs containing non-DNA binding effector domains. The RR PhyR, which regulates the general stress response in *Methylobacterium extorquens*, provides an interesting example of a RR that functions through the regulation of protein-protein interactions [121]. PhyR contains an N-terminal REC domain connected to a C-terminal effector domain that exhibits sequence similarity to the $\sigma^{E}$ subunit of RNA polymerase [122]. The $\sigma^{E}$-like effector domain of PhyR was found to regulate the general stress response by binding to and sequestering an anti-sigma factor, NepR, which allows the genuine sigma factor, $\sigma^{E_{cfG}}$, to associate with RNA polymerase and promote activation of stress response genes
Phosphorylation is proposed to activate PhyR’s $\sigma^E$-like effector domain and permit binding to NepR [123].

In recent years, the list of characterized RRs containing effector domains with proposed enzymatic activity has greatly expanded. These effector domains often possess enzymatic activities that further participate in signal transmission, such as enzymatic domains that function as diguanylate cyclases, phosphodiesterases, and serine/threonine protein kinases and phosphatases [78]. Among the best characterized of these RRs include CheB-like chemotaxis regulators, which combine the REC domain with a C-terminal methylesterase domain [124,125]. Another example of an extensively studied RR containing an enzymatic effector domain is the RR PleD, which regulates flagellar ejection and stalk biogenesis in *Caulobacter crescentus* [126]. PleD contains two REC domains (an active, N-terminal REC domain linked to a second, inactive REC domain), which are connected to a C-terminal diguanylate cyclase (GGDEF) domain involved in the synthesis of bis-(3’-5’)-cyclic diguanosine monophosphate (c-di-GMP), a bacterial second messenger [127]. In the case of PleD, REC phosphorylation is proposed to activate the attached GGDEF domain, resulting in the production of c-di-GMP, which ultimately serves to down-regulate motility and promote stalk production [128].

In addition to these characterized RRs, recent surveys have identified putative RRs containing the REC in association with a variety of effector domains with putative functions in signal transduction, including serine/threonine kinases, PP2C type serine phosphatases, c-di-GMP phosphodiesterases, and adenylate cyclases [79]. Together, these studies demonstrate the modular nature of the RR REC domain. The ability of the REC
domain to be connected to a variety of output domains provides great diversity in the functions of RR proteins.

**Two-component regulators of the *V. fischeri*-*E. scolopes* symbiosis**

(A) Overview

Bacteria typically employ a variety of colonization factors during infection of their respective eukaryotic hosts. Expression of these colonization factors is often under tight regulatory control, thus allowing the bacterium to coordinate its behavior in response to host-derived signals. TCS systems represent a common mechanism by which bacteria regulate the expression of colonization factors in response to environmental conditions. During the course of host colonization, *V. fischeri* cells encounter a variety of unique environmental conditions. These include exposure to host-derived antimicrobials in the ciliated ducts, association with the crypt epithelial cells, and interaction with host immune cells. Given the range of environments that the bacteria encounter, it is not surprising that *V. fischeri* employs multiple TCS systems to sense and adaptively respond to the various conditions and host signals. Indeed, *V. fischeri* contains 40 putative and known RR proteins, a subset of which has been shown to contribute to colonization fitness [33]. Of the TCS systems reported to impact host colonization, several appear to impact colonization fitness through global effects on cell physiology. The GacS/GacA regulatory system represents a well-studied example of a global TCS system. *V. fischeri* mutants lacking the RR *gacA* fail to efficiently colonize *E. scolopes* [129]. Further analyses determined that a *gacA* mutant exhibits defects in a number of symbiotic traits, including luminescence, motility, LPS production, and growth [91,129]. Due to the
pleiotropic effects of a gacA mutation, the specific role(s) of this global regulator during the colonization process remains an area of active study.

In contrast, other characterized TCS systems have been shown to regulate specific symbiotic traits and, therefore, contribute to particular stages of host colonization, such production of luminescence. Cell density-dependent light production in V. fischeri involves an intricate network of regulators, including multiple TCS regulatory proteins that control activation of the lux luminescence genes. The Lux phosphorelay consists of three hybrid HKs (LuxN, LuxP/Q, AinR) that donate phosphoryl groups to the HPt protein LuxU, which shuttles phosphoryl groups to the RR LuxO [130]. Mutations within several of these Lux regulatory genes result in the dysregulation of luminescence and defects in colonization [68,130,131]. In addition to luminescence, TCS regulators have also been shown to control one of the earliest stages of host colonization, the formation of a biofilm aggregate on the surface of the symbiotic light organ [15,16,25]. In the following section, I will discuss the role of TCS regulators in controlling syp aggregate formation and the initiation of host colonization, as knowledge of this regulatory network is pertinent to this dissertation work.

(B) Two-component regulation of symbiotic biofilm formation

An early event in the initiation of host colonization involves the formation of a biofilm-like aggregate by V. fischeri cells on the surface of the symbiotic light organ [16]. The production of this biofilm requires the symbiosis polysaccharide (syp) locus [21]. Current evidence indicates that activation of the syp locus and biofilm production is under tight regulatory control [25]. In particular, V. fischeri does not produce a syp
biofilm *in vitro* under normal, laboratory conditions, suggesting that *syp* expression and biofilm formation are restricted to conditions in which the bacteria encounter the host squid. Indeed, transcriptional reporter assays indicate that the *syp* locus is poorly expressed under normal culture conditions [20,27]. The regulation of *syp* biofilm formation involves multiple two-component regulator proteins, including the HK RscS [20,27](Fig. 3). Similar to *syp* mutants, an *rscS* mutant exhibits a defect in the ability of the cells to aggregate and initiate host colonization [20,26]. Overexpression of an *rscS* allele, *rscS1*, was found to activate *syp* transcription, resulting in a 100-fold increase relative to a vector control [20]. The *rscS1* allele contains a silent mutation at codon Leu25 and a mutation within the putative ribosome binding site; these changes are proposed to enhance ribosome binding and increase protein production [20,132]. *rscS1* overexpression also induces the production of *syp*-dependent biofilm phenotypes. *In vivo*, cells overexpressing *rscS1* form increased symbiotic aggregates on the light organ surface [20] (Fig. 6A). In laboratory culture, *rscS1* overexpression results in wrinkled colony formation on solid agar media and the formation of a biofilm pellicle at the air-liquid interface of static cultures [20] (Fig. 6B).

Mutational analysis of *V. fischeri* RRs determined that RscS activation of *syp* transcription and induction of biofilm phenotypes requires the *syp*-encoded RR SypG [27]. SypG belongs to the NtrC-like family of σ<sup>54</sup>-dependent transcriptional activators and contains an N-terminal REC domain linked to a central σ<sup>54</sup> interaction domain and a C-terminal DNA-binding domain. Similar to *rscS*, *sypG* overexpression results in the activation of the *syp* locus [27]. The promoter sequences upstream of the four putative
Figure 6. *syp*-dependent biofilm phenotypes induced by RscS. (A) Representative confocal microscopy images of GFP-labelled *V. fischeri* (green) aggregates on the surface of the symbiotic light organ of *E. scolopes* (counterstained in red). Images were taken from Yip *et al.*, 2006 [20]. (B) Representative images of *in vitro* biofilm phenotypes produced by wild-type cells carrying empty vector (pKV282) or pRscS plasmid (pARM7). *rscS*-overexpressing cells form a pellicle at the air-liquid interface of static cultures (Top panel) and exhibit wrinkled colony morphology (bottom panel) on solid agar media.
syp operons contain putative binding sites for σ^{54}-carrying RNA polymerase and SypG. The current model (Fig. 3) predicts that RscS, upon sensing an as-yet unidentified signal, initiates a phospho-relay and donates a phosphoryl group to SypG [27]. REC phosphorylation is proposed to activate SypG’s DNA-binding domain, resulting in the binding of SypG to the syp promoters and activation of syp transcription [27] (Fig. 3).

Another two-component regulator encoded by the syp locus is the hybrid HK SypF (Fig. 3). A recent study found that overexpression of an increased activity allele of sypF, sypF*, results in the production of robust biofilm phenotypes [28]. sypF* carries two mutations, resulting in a phenylalanine substitution at serine 247 (S247F) and isoleucine substitution at valine 439 (V439I) [28]. Genetic studies indicate that SypF acts upstream of the RR SypG to activate syp transcription and induce biofilm formation [28]. Additionally, SypF was reported to function upstream of a second RR, VpsR, to control activation of another biofilm locus, the bcs cellulose locus [28]. What role, if any, SypF may play during symbiotic colonization remains an area of active research.

In addition to SypG, the syp locus encodes a second RR SypE. SypE is a novel RR consisting of a central regulatory REC domain flanked by two effector domains that exhibit sequence similarity to partner-switching regulatory elements. SypE’s N-terminal effector domain exhibits sequence similarity to HPK (histidine protein kinase)-like serine kinases, including the serine kinase/anti-sigma RsbW of B. subtilis [133]. Additionally, SypE’s C-terminal effector domain exhibits sequence similarity to PP2C-like serine phosphatases, including the B. subtilis phosphatase RsbU [133]. The presence of a central REC domain flanked by two effector domains of apparent opposing function
distinguishes SypE as a unique RR protein with a novel domain architecture. Interestingly, SypE appears to exert opposing control over syp biofilm formation depending on whether rscS or sypG is overexpressed. Specifically, overexpression of RscS in a strain lacking sypE results in diminished biofilm phenotypes, indicated by a decrease in wrinkled colony formation relative to wild-type cells, suggesting a positive regulatory role for SypE [27]. In contrast, SypE strongly inhibits biofilms induced by SypG: overexpression of SypG in wild-type cells results in smooth colony formation and poor pellicle production, while its overexpression in a ΔsypE strain induces robust biofilms similar to that of an RscS-overexpressing wild-type strain [27]. The mechanism by which SypE exerts control over syp-dependent biofilm formation remains unknown. Furthermore, what role, if any, SypE’s opposing activities may play in regulating host colonization, particularly during symbiotic aggregation, has yet to be determined. This dissertation investigates the role of SypE in the regulation syp-dependent biofilm formation and symbiotic colonization.

**Conclusion**

TCS systems represent one of the most common mechanisms by which bacteria sense and adaptively respond to changes in their environment. The domains of TCS proteins possess a highly modular design, which permits different TCS pathways to sense diverse signals and elicit a multitude of cellular responses. HKs possess a variety of unique sensory domains, allowing the proteins to detect and elicit a signal cascade in response to diverse environmental stimuli. Similarly, RRs can possess the conserved REC domain in combination with a variety of different effector domains. As a result,
signal transmission to a RR can induce a specific cellular response dependent upon
the RR’s particular effector domain.

The modularity of TCS domains also allows these regulatory components to be
integrated into a variety of signaling systems with varying degrees of complexity. The
simplest TCS systems consist of a single phospho-transfer event between a HK and a
cognate RR. In contrast, more complex TCS systems, such as phosphorelays, incorporate
the HK, HPt, and RR domains in various combinations and involve multiple steps of
phospho-transfer. These multi-step, phosphorelay systems provide opportunities for
additional regulatory checkpoints or for the integration of various signaling inputs. In
HKs that possess both kinase and phosphatase activities, the regulation of these opposing
activities can control the levels of RR phosphorylation, and thus the duration of the
cellular response. It is also possible for RRs to receive phosphoryl signals from multiple
HKs, and therefore the net activities (either kinase or phosphatase) of these HKs can
provide even tighter control over RR phosphorylation levels.

V. fischeri utilizes multiple TCS systems to regulate symbiotic colonization of its
host E. scolopes [33,129,134]. Importantly, TCS regulators control one of the earliest
stages of host colonization, the formation of a biofilm aggregate on the surface of the
squid’s symbiotic light organ. The formation of this biofilm depends on the HK RscS and
the RR SypG, which regulate transcription of the syp biofilm locus [20,21,27].
Additionally, the syp-encoded RR SypE appears to contribute to syp biofilm regulation;
however, the role of SypE in the regulation of biofilms and host colonization has not been
fully investigated. The presence of two putative effector domains of opposing enzymatic
functions suggests that SypE may play regulatory dual roles. In this dissertation, I investigate the putative regulatory role of SypE in controlling syp biofilm formation and colonization initiation.

SERINE/THREONINE PHOSPHORYLATION IN BACTERIA

Introduction

Two-component signaling (TCS) systems involving phosphorylation of conserved histidine (His) and aspartate (Asp) residues represent a common form of signal transduction in bacteria [71]. While most bacterial signaling pathways utilize His-Asp phosphotransfer, the use of other phosphorylated amino acids to accomplish signal transduction has also been reported. These include signaling cascades that utilize the phosphorylation of conserved serine (Ser), threonine (Thr) or tyrosine (Tyr) residues. Ser/Thr phosphorylation cascades account for the majority of signaling pathways in eukaryotes, and initially were thought to be absent in bacteria [70]. However, several early studies indicated that Ser/Thr phosphorylation may also occur in bacteria [70,135]. Studies in the Gram-negative bacterium E. coli were the first to report the occurrence of Ser/Thr protein phosphorylation in a bacterial system [136]. These studies identified that the activity of the E. coli protein isocitrate dehydrogenase (IDH), an enzyme involved in the tricarboxylic acid cycle, was regulated through the phosphorylation and dephosphorylation at a conserved Ser residue [136]. A later study found that IDH phosphorylation was controlled through the activity of a cognate bifunctional kinase/phosphatase protein, AceK [137]. Subsequent research has since identified a
number of additional Ser/Thr kinases and phosphatases present in a variety of bacterial systems [70,135].

The typical Ser/Thr signaling system includes a Ser/Thr kinase (STK) protein and a downstream substrate protein(s). Ser/Thr phosphorylation pathways also generally include a cognate Ser/Thr phosphatase (STP) protein, which functions to dephosphorylate the substrate protein. The requirement for dedicated STPs within Ser/Thr signaling pathways is due to the stable nature of phosphorylated Ser and Thr residues. This feature distinguishes Ser/Thr signaling systems from the typical TCS system. Compared to the phospho-His and phospho-Asp residues present in TCS systems, which possess a high free energy and therefore undergo rapid hydrolysis [138], phospho-Ser and phospho-Thr residues are significantly less labile [138,139]. Therefore, the removal of the phosphoryl groups by a cognate STP is necessary for the controlled termination of the signaling response.

While the role of TCS systems in bacterial is well-established, the importance of signaling systems utilizing Ser/Thr phospho-transfer has only recently been appreciated. In recent years, a number of studies have demonstrated that, like TCS systems, Ser/Thr phospho-transfer systems are widespread among bacterial species and contribute to the regulation of diverse cellular responses [70]. In the following sections, I will discuss the key features of bacterial Ser/Thr kinases and phosphatases, focusing on the components relevant to this dissertation work.
Structure and function of bacterial serine kinases

Since the discovery that the *E. coli* protein IDH was regulated through Ser phosphorylation, the role of Ser/Thr phosphorylation in bacterial signaling systems has been heavily investigated. As a result, a large number of Ser/Thr kinases (STKs) have been identified and characterized in a variety of bacterial organisms. These STKs can generally be classified into two, broad groups based on the catalytic kinase domain: those whose catalytic domain shares structural homology to eukaryotic Ser/Thr kinases (eukaryote-like serine kinases, eSTKs) and those that are distinct from eukaryotic Ser/Thr kinases (non-eSTKs). In this section, I provide a brief overview of these two groups of bacterial STKs, but focus particularly on the structure/function of non-eSTKs, which is pertinent to this dissertation.

(A) Eukaryote-like serine kinases

The first eukaryote-like serine kinase (eSTK) identified in bacteria was the *Myxococcus xanthus* protein Pkn1, which contributes to *M. xanthus* development [140]. Pkn1 was demonstrated to autophosphorylate on conserved Ser and Thr residues, and analysis of the protein’s catalytic domain indicated that it shared sequence homology with eukaryotic, or Hanks type, STKs [140]. Bacterial genome analyses have since identified numerous eSTKs present in a wide range of bacterial systems. This includes the *Mycobacterium tuberculosis* protein kinase PknB, which represents the first bacterial eSTK to be crystallized and its structure determined [141]. Analysis of PknB, and subsequent bacterial eSTKs, indicated that, in addition to sequence homology, the catalytic domains of these proteins are structurally similar to eukaryotic STKs. As
observed in eukaryotic STKs, eSTKs possess a kinase catalytic domain that adopts a two-lobed structure in which ATP binding occurs in a catalytic cleft located between these two lobes [70]. In general, the N-terminal lobe participates in the binding and coordination of the ATP nucleotide, while the C-terminal lobe is involved in binding to the substrate protein and the initiation of phosphatase transfer [70]. These eSTKs are further characterized by the presence of 12 highly conserved residues that are located within the N- and C-terminal lobes of the catalytic domain. These conserved residues participate directly or indirectly in catalyzing phosphate transfer, either through coordination of the ATP molecule (phosphoryl donor) or substrate protein binding [70].

(B) Non-eukaryote-like serine kinases

Ser/Thr kinases that are homologous to those found in eukaryotes (eSTKs) represent the majority of STKs identified in bacterial systems. However, studies have also described a number of bacterial STKs that are distinct from eSTKs in both their overall protein sequence and structural features. These non-eSTK proteins include a class of Ser/Thr kinases that are more closely related to histidine sensor kinases (HKs) commonly found in TCS systems. These HPK (Histidine protein kinase)-like serine kinases exhibit both sequence and structural homology to HKs. The serine kinase SpoIIAB, which regulates the sporulation pathway in Bacillus subtilis and related Bacillus species, provides an example of a well-characterized HPK-like serine kinase [142](Fig. 7). Crystal structure analysis of SpoIIAB demonstrated that the HPK-like kinase domain contains the core elements of the ATP-binding Bergerat fold, conserved in the ATPase/Kinase superfamily of proteins [95,143]. Similar to HKs, the ATP-binding
Figure 7. Characteristics of SpoIIAB and non-eSTK proteins. (A) Schematic diagram of the catalytic kinase core domain of non-eSTK proteins. Shown are the locations of the N, G1, G2, and G3 boxes. (B) Schematic of B. subtilis non-eSTK proteins SpoIIAB and RsbW. Shown are key conserved residues within the individual motifs: invariant asparagine (N) of the N Box and the conserved aspartate (D) and glycine (G) residues of the G1 Box.
domain of HPK-like serine kinases consists of several conserved amino motifs termed the N, G1, and G2 boxes, which participate in Mg\(^{2+}\) ion coordination and ATP binding [95,142](Fig. 7). HPK-like serine kinases, however, lack the additional H-box motif containing the conserved His residue, which serves as the site of autophosphorylation in HKs [95]. As a result, HPK-like serine kinases phosphorylate their substrate proteins by a different mechanism than HKs. Phosphate transfer by HKs involves HK \textit{trans}-autophosphorylation at a conserved His residue, followed by phosphoryl transfer to an Asp located in the REC domain of a downstream RR. In contrast, HPK-like serine kinases do not autophosphorylate, and are proposed to directly transfer the phosphoryl group from ATP to a Ser residue within a cognate, substrate protein [142,144]. The mechanism of phosphate transfer by HPK-like serine kinases is proposed to occur through a direct nucleophilic attack on the γ-phosphate of the bound ATP by the Ser residue located on the substrate protein [142,144]. Therefore, although histidine kinases and HPK-like serine kinases share structural homology, they differ in their mechanisms of phosphate transfer and their amino acid targets.

**Structure and function of serine phosphatases**

The removal of phosphoryl groups from phosphorylated substrate proteins is critical for the termination of a signaling cascade and resetting of the system. In bacteria, the majority of characterized Ser/Thr phosphatases (STPs) exhibit homology to eukaryotic protein phosphatases [70]. These eukaryote-like Ser/Thr phosphatases (eSTPs) are categorized into two structurally distinct phosphatase families: the phosphoprotein phosphatases (PPPs) and the metal-dependent phosphatases (PPMs) [70].
Figure 8. Structural and domain characteristics of PP2C serine phosphatases. (A) Diagram depicting the catalytic core domain of the serine phosphatase PP2C belonging to the metal-dependent protein phosphatase (PPM) family. Indicated are the conserved aspartates (shown in red) and the neighboring residues involved in metal ion coordination. (B) The *B. subtilis* PP2C serine phosphatases SpoIIE and RsbU. Shown are the conserved aspartates required for catalytic activity.
Many eSTPs of the PPP family have been demonstrated to possess “dual specificity” and are capable of cleaving phosphoryl groups not only from Ser/Thr residues, but also from phospho-tyrosine and phospho-histidines [70]. I focus here on the structure and function of eSTPs belonging to the PPM family of phosphatases, as an understanding of these characteristics is relevant to the work presented in this dissertation. eSTPs of the PPM family are manganese/magnesium ion (Mg$^{2+}$/Mn$^{2+}$)-dependent phosphatases and share a conserved catalytic core domain with the human phosphatase PP2C [145,146](Fig. 8A). The PP2C catalytic core domain consists of a central β-sandwich composed of two β sheets each flanked by a pair of α helices [147]. Crystal structure analysis indicates that the central β-sandwich forms a metal-binding cleft, in which two metal cations (either Mg$^{2+}$ or Mn$^{2+}$) are located at the base of the cleft and hexa-coordinated with water and neighboring amino acids [147]. The proposed mechanism of dephosphorylation occurs through a nucleophilic attack of the target phosphorous atom by the metal-activated water molecule [147].

PP2C-like eSTPs have been identified in a variety of both Gram-positive and Gram-negative bacteria. The phosphatases PstP of *Mycobacterium tuberculosis* and PphA of *Thermosynechococcus elongatus* are among the best characterized of these PP2C-like eSTPs [147]. Studies indicate that PstP and PphA, and similar bacterial eSTPs, possess little overall sequence similarity to PP2C and other eukaryotic serine kinases of the PPM family [70,147]. Despite weak overall sequence homology, crystal structure analyses indicated that the catalytic domains of both PstP and PphA, and similar bacterial eSTPs, are structurally identical to those observed for the eukaryotic PP2C phosphatases.
Additionally, both PstP and PphA were found to possess several, highly conserved amino acid residues that form the metal-binding pocket of the active site of PP2C phosphatases [147]. These include several invariant Asp residues that have been demonstrated to be required for metal ion binding and \(\text{Mg}^{2+}/\text{Mn}^{2+}\)-dependent catalysis by both eukaryotic and bacterial PP2C-like phosphatases [145,146,148].

**Conclusion**

Protein phosphorylation on conserved Ser/Thr residues provides an additional mechanism by which a cell can facilitate signal transduction. The typical Ser/Thr phosphorylation cascade includes cognate Ser/Thr kinase and phosphatases, which together regulate the phosphorylation state of a downstream substrate protein(s). Signal transmission is initiated by the activation of a Ser/Thr kinase, which facilitates the phosphorylation of a downstream, substrate protein. Activation of a cognate Ser/Thr phosphatase protein(s) terminates the signaling cascade and resets the system.

Although bacteria can utilize Ser/Thr phosphorylation as a means of signal transduction, the majority of identified bacterial signaling systems utilize His-Asp phosphotransfer (e.g. TCS systems). This is in stark contrast to eukaryotic systems in which Ser/Thr phosphorylation represents the predominate form of signaling, and His-Asp phosphotransfer systems are relatively rare [71]. Currently, it remains unclear as to the reason(s) behind this different distribution of signaling mechanism among bacteria and eukaryotes. As previously discussed, a key difference between Ser-Thr and His-Asp phosphotransfer systems is the increased, inherent stability of phosphorylated Ser/Thr residues. While phosphorylated His/Asp residues typically undergo rapid hydrolysis,
phosphorylated Ser/Thr residues are considerably less labile. As a result, Ser/Thr phosphotransfer systems typically possess an additional, cognate Ser/Thr phosphatase necessary to remove the phosphoryl group and terminate the cellular response. It remains possible that bacterial cells may exploit these differences in the stability of the phosphorylated amino acid targets to achieve controlled transmission of cellular signals.

Interestingly, several studies have identified bacterial signaling pathways in which elements of both Ser/Thr and His/Asp phosphotransfer systems are combined. An interesting example can be found in the multi-domain response regulator (RR) protein PA3346, which functions in a two-component signaling pathway to regulate swarming motility in *Pseudomonas aeruginosa*. The RR PA3346 combines an N-terminal RR receiver domain coupled to a central PP2C-like serine phosphatase domain and a C-terminal HPK-like serine kinase domain [149]. In the case of the RR PA3346, Asp phosphorylation within the protein’s RR REC domain is proposed to regulate activity of the attached serine kinase and serine phosphatase domains [150]. It remains unclear as to the potential benefit(s) of integrating these distinct phosphotransfer pathways.

PARTNER SWITCHING REGULATORY SYSTEMS

Introduction

Many bacterial signaling systems utilize phosphate transfer as a means of signal transmission. An additional mechanism by which a cell can facilitate signal transmission is through the regulation of protein-protein interactions, such as partner-switching systems. First coined by Alper *et al.* (1994), the term “partner switch” describes a
network of interacting proteins whose interaction with cognate “partners” depends upon a reversible serine phosphorylation event [133]. Dependent upon which partner proteins interact, the outcome of this partner switching can either negatively or positively regulate a target protein, generally a transcription factor or enzyme. Specifically, the partner-switch mechanism involves several key regulatory elements, including a serine kinase/anti-sigma factor, a serine phosphatase, an antagonist protein/anti-anti sigma factor, and a target protein (often a sigma factor) [151].

The partner-switching mechanism has been best characterized in the Gram-positive bacterium *Bacillus subtilis*. In *B. subtilis* and other Gram-positive bacteria, partner-switching systems contribute to signaling networks that control the activity of downstream sigma factors, the subunit of RNA polymerase that provides promoter specificity. For example, *B. subtilis* possesses two distinct partner switching systems, which function in the regulation of the stress-responsive sigma factors sigma-F ($\sigma^F$) and sigma-B ($\sigma^B$) of the sporulation and general stress response pathways, respectively [152,153]. The *B. subtilis* RsbU-RsbV-RsbW signaling network, which regulates $\sigma^B$ of the general stress response, represents one of the most well-characterized examples of a partner-switching system (Fig. 9). In this regulatory pathway, an anti-sigma/serine kinase RsbW negatively regulates $\sigma^B$ activity by binding to $\sigma^B$ and preventing its association with the core RNA polymerase [154,155,156]. $\sigma^B$ is released by the action of an anti-sigma factor antagonist protein, RsbV, which binds RsbW and prevents its sequestration of $\sigma^B$ [153].
The ability of RsbV to function as an antagonist of RsbW depends on the phosphorylation state of a conserved serine residue (S56) located in the protein’s sulfate transport and anti-sigma factor antagonist (STAS) domain (Fig. 9). When phosphorylated by RsbW (functioning as a kinase), RsbV is rendered unable to bind and inhibit RsbW [153]. Dephosphorylation of RsbV is promoted by a set of serine phosphatases, RsbU and RsbP, which are activated by environmental and energy stress signals, respectively [157]. Thus, stress detection induces the dephosphorylation of RsbV, the inhibition of RsbW, and the release of \( \sigma^B \) and subsequent activation of the \( \sigma^B \) regulon [157]. In this regulatory network, RsbW functions as a regulatory switch, as it reversibly interacts with its cognate sigma factor (\( \sigma^B \)) and anti-sigma factor antagonist (RsbV).

Partner-switching systems have been identified in both Gram-positive and Gram-negative bacteria. In the following sections, I will briefly describe the key components of partner switching proteins relevant to this dissertation work, and discuss the characterization of partner-switching networks in other bacterial systems.

**Structure and function of partner-switching proteins**

(A) Anti-sigma factors/serine kinases

The canonical partner-switching system includes an RsbW-like regulatory protein that functions as both an anti-sigma factor and serine kinase [154,158]. As described above for the *B. subtilis* partner-switching paradigm, the RsbW regulator serves as a “molecular switch” by reversibly interacting with both a cognate sigma factor (\( \sigma^B \)) and anti-sigma factor antagonist (RsbV). RsbW and its homologs exhibit sequence similarity
Figure 9. Model of the *B. subtilis* partner-switching system controlling $\sigma^B$ activity. Detection of stress signals activates two serine phosphatases (RsbU/RsbP) that dephosphorylate the STAS-domain protein RsbV. Unphosphorylated RsbV binds to the anti-sigma factor RsbW, inducing the release of $\sigma^B$ and subsequent activation of $\sigma^B$-dependent genes. RsbW also functions as a serine kinase to phosphorylate RsbV, resulting in the release of RsbW by RsbV. Released RsbW subsequently binds and sequesters $\sigma^B$, inactivating the pathway.
to HPK (histidine protein kinase)-like serine kinases, which are structurally related to histidine sensor kinases of the bacterial two-component signal transduction systems [159]. Like histidine kinases, HPK-like serine kinases possess several conserved amino acid motifs, termed the N, G1, G2, and G3 boxes, involved in ATP-binding and kinase activity [95](Fig. 7). For example, crystal structure analysis of the RsbW homolog SpoIIAB from Bacillus stearothermophilus indicated that a conserved N box asparagine (Asn 50) is required for chelating a Mg$^{2+}$ ion that participates in ATP binding and kinase activity [143](Fig. 7). Additionally, conserved glycine residues located within the G box motifs contribute to the formation of an ATP-binding lid, a finding that is consistent with other HPK-like serine kinases [95,143]. These studies revealed that the key structural features of HPK-like serine kinases are conserved in SpoIIAB and its homologs, including RsbW (Fig. 7).

Another feature of many HPK-like serine kinases is their propensity to function as dimers [95]. Several studies have demonstrated that RsbW and SpoIIAB homodimerize through an interaction interface localized to the region between $\beta1$ and $\alpha1$ of each monomer [143]. Additionally, both RsbW and SpoIIAB exist as dimers when bound to their cognate sigma factors ($\sigma^B$ or $\sigma^F$, respectively) or antagonist proteins (RsbV or SpoIIAA, respectively), suggesting that dimerization is critical for mediating binding to their cognate partner switching proteins [143,160]. In characterized partner switching systems, an RsbW (or SpoIIAB) dimer typically forms a stable, long-lived complex with a single $\sigma^B$ (or $\sigma^F$ for SpoIIAB) molecule [143,160]. Disassociation of the RsbW- $\sigma^B$ complex predominantly occurs through the binding of the RsbV antagonist protein, in
which two RsbV monomers bind to the RsbW dimer and displace the bound $\sigma^F$ [161,162]. Analysis of *B. stearothermophilus* SpoIIAB bound to either its cognate sigma factor ($\sigma^F$) or antagonist protein (SpoIIAA) revealed that both partner proteins bind to similar, overlapping epitopes on SpoIIAB [162]. These binding sites are highly conserved in *B. subtilis* RsbW and SpoIIAB [162]. These findings support a model in which the release of the sequestered sigma factor ($\sigma^F$) is induced by the binding of the antagonist protein (SpoIIAA) to common docking sites on SpoIIAB [162].

**(B) Anti-sigma factor antagonists**

In the canonical partner-switching pathway, the activation of an anti-sigma factor antagonist protein (RsbV in *B. subtilis*) promotes its binding to a cognate anti-sigma factor (RsbW), which induces the release of the sequestered sigma factor ($\sigma^B$) [154]. The activity of the antagonist protein is regulated through the phosphorylation of a conserved Ser residue located in the protein’s STAS (sulfate transporter and anti-sigma factor antagonist) domain [142](Fig. 9). The structure and impact of phosphorylation has been best characterized in the antagonist protein SpoIIAA of *B. subtilis*, which regulates the sporulation pathway controlling $\sigma^F$ activity. Crystal structure analysis of SpoIIAA homologs from *B. subtilis* and related *Bacillus* species revealed that protein has a novel fold, consisting of a four-stranded $\beta$ sheet and 4 $\alpha$ helices with the site of phosphorylation (Serine-58 for SpoIIAA) located at the N-terminus of the $\alpha$-2 helix [163,164].
In characterized anti-sigma factor antagonists, phosphorylation regulates protein activity by reducing its affinity to bind its cognate anti-sigma factor [165,166]. Phosphorylation of a conserved Ser residue located in the protein’s STAS domain is proposed to regulate the interaction with a cognate anti-sigma factor through multiple mechanisms. The introduction of a negatively charged phosphate group on the antagonist protein has been proposed to alter its binding affinity for its cognate anti-sigma factor through electrostatic hindrance [167]. Additionally, nuclear magnetic resonance (NMR) spectroscopy revealed that phosphorylation of the *B. subtilis* antagonist SpoIIAA at Ser-58 induces subtle structural changes [167]. These phosphorylation-induced structural changes primarily occurred within the protein’s α3 helix, a highly conserved region that has been demonstrated to be involved in anti-sigma factor binding [167]. These studies indicate that phosphorylation likely alters the binding affinity of the antagonist protein for its cognate anti-sigma factor both through altering protein charge and by inducing subtle, structural changes in the protein’s interaction interface.

**Conservation of partner-switching systems in bacteria**

**(A) Partner-switching systems in Gram-positive bacteria**

Since its initial characterization in *B. subtilis*, the partner-switching mechanism has been identified as a signaling component in a wide range of Gram-positive bacteria. These include *Bacillus cereus* [168], *Bacillus anthracis* [169], *Mycobacterium tuberculosis* [170], *Staphylococcus aureus* [171], and *Listeria monocytogenes* [172,173]. In these systems, partner-switch modules are utilized in a manner similar to that observed in *B. subtilis*: primarily, the regulation of sigma factor activity. However, the output of
these modules varies among the individual bacteria. Partner-switching modules have been demonstrated to contribute to the regulation of the general stress response of *L. monocytogenes* and many other bacteria [172], biofilm formation in *S. epidermidis* [174], and the expression of virulence-associated genes in *M. tuberculosis* [170,175]. Additionally, bacteria may possess multiple partner-switching pathways regulating distinct sets of target proteins. For example, in addition to the RsbU/V/W module that regulates the general stress response, *B. subtilis* possesses a second set of partner-switching regulators, SpoIIAA/SpoIAB/SpoIIE. Similar to the σB regulatory pathway, these partner-switching proteins control the activity of σF, which regulates sporulation [176]. The partner-switching mechanism, while similar among diverse Gram-positives, has been adapted to regulate distinct cellular responses.

(B) Partner-switching systems in Gram-negative bacteria

Genome surveys suggest that partner-switching orthologs exist in a wide range of eubacteria [177,178]. Despite its predicted widespread distribution, partner switching has remained relatively uncharacterized within Gram-negative bacteria. Indeed, partner-switching systems have only been characterized in several Gram-negative bacteria, including *Bordetella bronchiseptica* and *Chlamydia trachomatis* [179,180]. The respiratory pathogen *B. bronchiseptica* utilizes a partner-switching module to control production of a type III secretion system (T3SS)[178]. The T3SS consists of a needle-like secretory apparatus that directly transports virulence proteins into the cytoplasm of host cells. In *B. bronchiseptica*, the T3SS contributes to colonization of the host trachea and the avoidance of the host immune response [178,181]. The production of the T3SS
requires transcription of a gene cluster, the *bsc* locus, which encodes multiple components of the secretory system [182]. Regulation of the T3SS depends upon a set of genes adjacent to the *bsc* cluster, the *btr* locus, which encode orthologs of the *B. subtilis* RsbU/V/W partner-switching proteins, BtrU/BtrV/BtrW. *In vitro* and *in vivo* analyses of the *B. bronchiseptica* proteins demonstrated that they constitute a regulatory network similar to their *B. subtilis* counterparts [179]. However, this partner-switching system deviates from that of the *B. subtilis* RsbU/V/W paradigm. First, disruption of any component of the BtrU/V/W partner-switching module results in the loss of Type III secretion [178], a result that is inconsistent with the *B. subtilis* model. Second, positive regulation of the T3SS requires both the formation of the BtrV/BtrW complex and its dissociation, via phosphorylation of BtrV by BtrW [179]. Finally, although the BtrU/V/W module regulates type III secretion, it does not control transcription of the *bsc* locus [179]. Instead, Kozak *et al.* (2005) suggest that these partner-switching proteins may regulate the T3SS at the posttranscriptional level possibly by interacting with yet unknown regulatory proteins or playing a structural role in the secretory pathway. Thus, although there is conservation of the partner-switching components, the regulatory mechanism appears to vary from that of the Gram-positive paradigm.

Genome analysis of the obligate intracellular pathogen *C. trachomatis* identified several components of a putative partner-switching module [180]. *In vitro* analysis of the candidate genes demonstrated that these proteins indeed interact. As with *B. bronchiseptica*, it appears that the *C. trachomatis* partner-switching system may vary from the *B. subtilis* paradigm, as *in vitro* binding assays failed to demonstrate an
interaction between the putative anti-sigma factor/serine kinase (RsbW) with any of the three sigma factors encoded in the C. trachomatis genome [180]. However, the lack of genetic tools and difficulty in culturing C. trachomatis have delayed analysis of this potential partner-switching module in vivo.

Together, these studies suggest that the partner-switching mechanism, previously observed only among the Gram-positives, also contributes to regulatory control in Gram-negative bacteria. It remains unknown how these partner-switching proteins regulate downstream targets. Furthermore, it remains unclear how widespread this regulatory mechanism is among Gram-negative bacteria.

**Conclusion**

Partner-switching systems represent an additional mechanism by which bacteria facilitate signal transmission. These signaling pathways utilize both protein phosphorylation and protein-protein interactions to achieve signal transduction and target gene regulation. The partner-switching paradigm has been best characterized in B. subtilis and other Gram-positive bacteria, in which partner-switching proteins typically regulate the activity of a target sigma factor. Although genome surveys indicate that partner-switching proteins are conserved in Eubacteria, the contribution of partner-switching systems to signaling in Gram-negative bacteria remains unclear.

A role for partner switching regulators in V. fischeri has yet to be investigated. Intriguingly, bioinformatic analysis suggests that partner-switching elements are present within the effector domains of the syp-encoded RR SypE. SypE’s N-terminal domain shares similarity to RsbW-like serine kinases, while the C-terminal domain of SypE
shares similarity to PP2C-like serine phosphatases. In this dissertation, I investigated the role of SypE in the regulation of biofilm formation and symbiotic colonization. In my characterization of SypE, I assessed the contribution of these putative partner-switching domains to the regulatory function of SypE.

BACTERIAL BIOFILM FORMATION

Introduction

Biofilms, or surface-associated community of cells encapsulated in a matrix, are ubiquitous and likely represent the preferred mode of growth for many bacterial species [7]. Throughout the environment, bacterial biofilms can be found attached to both abiotic (e.g. air-liquid interface) and biotic surfaces (e.g. plant or animal host tissues). Biofilms have been demonstrated to play multiple roles in the lifestyle(s) of diverse bacterial species. The formation of a biofilm often dramatically impacts bacterial physiology and can promote bacterial survival and growth by providing, among other things, antibiotic resistance and nutrient access.

Additionally, biofilms can assist in the colonization of host tissues by both pathogenic and non-pathogenic bacteria. Biofilms have been found to enhance various stages of bacterial infection, including attachment to host cells and transmission within the environment. Therefore, it is clear that understanding the processes involved in biofilm formation and identifying means of controlling these processes remain significant areas of research. In this dissertation, I investigate the regulatory mechanisms involved in V. fischeri biofilm formation, and assess the impact of biofilms on symbiotic
colonization of *E. scolopes*. In the following sections, I discuss several of the key cellular processes and factors that contribute to biofilm development.

**Stages of biofilm development**

Biofilm formation is a dynamic process in which bacterial cells form complex, multi-cellular communities in response to environmental conditions. Studies using model biofilm-forming organisms, such as *P. aeruginosa* and *E. coli*, have demonstrated that biofilm formation generally proceeds through a series of tightly regulated developmental stages. The initial stages of biofilm formation generally involve transient association of bacterial cells with a particular surface, in which the bacterial reversibly attach to the surface [183]. Initial surface contact is often mediated by a variety of bacterial structures, including flagella and pili. Upon contacting a surface, the bacterial cells undergo irreversible adhesion and proceed to establish clusters or microcolonies. In *Pseudomonas*, the formation of these bacterial clusters has been shown to involve twitching motility, in which the bacteria utilize type IV pili-mediated gliding motility to move across the attached surface [183]. The bacterial cells within the microcolonies proliferate and produce an extracellular polymeric matrix that encapsulates the biofilm cells. In the following stages, the microcolonies continue to mature and often develop complex, organized structures. Research has revealed that biofilm structures often exhibit intricate architecture, consisting of three-dimensional pillars and water channels that permit nutrient and waste exchange [184]. The final stage of biofilm formation is characterized by the dispersal of the biofilm-encased bacteria. The released bacterial cells return to the planktonic lifestyle, and may proceed to colonize and re-initiate biofilm
formation in a new niche. The dispersal from a biofilm may occur passively, as a result of external physical forces that disrupt the biofilms, such as increased water flow. Additionally, dispersal may involve regulated, active processes, such as the production of enzymes that degrade the extracellular matrix.

**Determinants of biofilm formation**

Given the variety of environmental conditions under which bacteria exist, it is not surprising that the mechanisms by which bacteria regulate and produce biofilms are also diverse. However, research has revealed several key bacterial processes and products that contribute to biofilm formation in many bacterial species. These include the use of particular bacterial structures to promote interaction with and attachment to a surface and the production of matrix components to encapsulate the attached cells. In the following sections, I will provide a brief overview of several key determinants of biofilm formation.

**Role of flagella and pili in biofilm formation**

The initial stages of biofilm formation involve the transient attachment of bacterial cells with a surface. Studies utilizing non-motile mutant strains either lacking flagella or possessing paralyzed flagella revealed that flagella contribute to initial surface attachment in a variety of bacteria [185,186,187]. It has been proposed that flagella-based motility may allow bacterial cells to overcome the repulsive forces generated by surface interaction [188]. Intriguingly, studies in other bacterial systems, such as *Vibrio cholerae*, have demonstrated that flagella not only contribute to initial attachment, but also play a role in later stages of biofilm formation. In particular, *V. cholerae* mot mutants, which produce a paralyzed flagellum, fail to form rugose colonies indicative of
biofilm formation. This failure to form biofilms can be attributed to the inability of these mutant cells to produce an exopolysaccharide required for matrix production [189]. The authors of this study proposed that the flagellar rotation may therefore function to both mediate motility and to signal the production of matrix components upon attachment to a surface. In addition to flagella, pili have also been demonstrated to promote surface attachment. Studies using mutants defective in pili production revealed a dramatic impairment in bacterial adhesion to a surface, and thus these cells fail to initiate biofilm formation [190]. Additionally, many bacteria have been shown to utilize retractable pili to facilitate motility across a surface. Studies in the bacterium *P. aeruginosa* demonstrated that type IV pili were required for twitching motility and the subsequent aggregation of the surface-associated bacteria [191,192]. Together, these studies indicate that bacteria utilize pili to promote multiple stages in biofilm formation.

Components of the biofilm extracellular matrix

The production of an extracellular matrix is a significant step in bacterial biofilm formation. Matrix production has been shown to promote both biofilm development and maintenance by contributing to cell-cell and/or cell-surface attachment.

Exopolysaccharides (EPS) represent a major component of most bacterial biofilm matrices. The EPS composition generally varies among diverse bacterial species. Furthermore, a bacterium may produce multiple types of EPS, which can contribute to formation of distinct biofilms. For example, *P. aeruginosa* possesses three EPS loci, which are involved in the production of three distinct types of EPS (termed alginate, Pel, and Psl polysaccharides) [193,194]. Production of alginate, a polymer of β-1-4-linked
mannuronic acid and guluronic acid, is involved in formation of a mucoid phenotype commonly associated with *P. aeruginosa* isolates from cystic fibrosis (CF) patients, and is proposed to contribute to persistence within the CF lung [195]. In contrast, lab strains of *P. aeruginosa* are devoid of alginate and instead utilize the Pel and Psl EPS to form biofilms. Analyses determined that the Pel polysaccharide contributes to the glucose-rich matrix component, while the Psl polysaccharide contributes the mannose- and galactose-rich matrix components [193]. Both the Pel and Psl EPS have been demonstrated to contribute to cell-cell and cell-surface attachment [196].

In addition to EPS, proteins are a common component of most biofilm matrices. Among the best-studied matrix proteins are curli fimbriae. Curli fimbriae are proteinaceous surface appendages that contribute to matrix production and biofilm in multiple organisms, including *E. coli* and *P. aeruginosa*. In *E. coli*, curli have been demonstrated to function in both cell-cell and cell-surface adhesion [197]. *P. aeruginosa* mutants defective in curli production exhibit impaired biofilm formation, suggesting that curli are likely a component of the biofilm matrix [193].

Another common component of the biofilm matrix is extracellular DNA (eDNA). Studies of *P. aeruginosa* biofilms revealed that eDNA is abundant within the biofilm matrix, and likely contributes to the overall integrity and structure of the biofilm matrix. In support of the role of eDNA in biofilm formation, studies demonstrated that DNase treatment of *P. aeruginosa* cultures both inhibited biofilm formation and disrupted previously formed biofilms [198]. The source of eDNA is generally chromosomal DNA, which is proposed to be released through either cell lysis or secretion. Investigation into
biofilm formation in *Staphylococcus epidermidis* found that eDNA was released into the biofilm matrix through the activity of an autolysin, AtlE, which functions in cell wall recycling and promotes cell lysis [199]. Together, these studies suggest that eDNA release is an essential mechanism by which numerous organisms promote biofilm formation.

**Biofilm dispersal strategies**

Dispersion of bacterial cells is a critical stage in the biofilm process, allowing the bacteria to colonize new niches. Biofilm dispersal has been proposed to occur through both passive mechanisms, as a result of environmental forces, and active, adaptive processes. Bacteria have been shown to utilize multiple mechanisms to actively facilitate biofilm dispersal. These include the secretion of enzymes that degrade the matrix components. For example, *P. aeruginosa* secretes a lyase that degrades alginate and promotes biofilm dispersal [200]. Recent studies of biofilms in the model organism *B. subtilis* have identified additional bacterial-produced factors that promote dispersal. In particular, *B. subtilis* produces both D-amino acids and norspermidine as molecular triggers that disrupt essential matrix components [201,202]. Additional analyses determined that D-amino acids induce the release of critical matrix proteins (amyloid fibers)[202], while norspermidine collapses the EPS component of the matrix [201]. Together, these studies indicate that bacteria actively promote the disassembly of and dispersal from biofilms.
Conclusion

Biofilms play a critical role in the survival and persistence of bacterial cells within the environment. The formation of a biofilm is a dynamic process, in which bacterial cells tightly control both the assembly and disassembly of complex, multicellular communities. Although studies have identified many of the bacterial factors and processes involved in biofilm formation, understanding the regulatory mechanisms controlling biofilm formation remains an area of active research. A significant area of research concerns understanding the roles biofilm formation plays during interaction of bacteria with their respective eukaryotic hosts. Both pathogenic and non-pathogenic bacteria have been shown to produce biofilms to facilitate colonization of host tissues and provide protection from host-derived defenses and/or antimicrobials. Importantly, *V. fischeri* has been demonstrated to produce a specific biofilm to initiate colonization of its animal host *E. scolopes*. The *V. fischeri-E. scolopes* symbiosis therefore provides an excellent model system to investigate the mechanisms by which bacteria control biofilm production and the impact of biofilm formation on host colonization.
CHAPTER TWO  
MATERIALS AND METHODS

Strains and Media. The bacterial strains used in this study are listed in Table 1. *V. fischeri* strain ES114, an isolate from *E. scolopes*, was used as the parental strain throughout this study [60]. *V. fischeri* derivatives were generated by conjugation, as previously described [26]. *E. coli* strains Tam1 λ *pir* (Active Motif, Carlsbad, CA), DH5α (Invitrogen, Carlsbad, CA), and GT115 (Invivogen, San Diego, CA) were used for cloning and conjugative purposes. *E. coli* strains were routinely cultured in Luria Bertani medium (LB) [203] or brain heart infusion medium (BHI) (Difco). For routine culturing, *V. fischeri* strains were grown in complex medium Luria-Bertani salt (LBS) [29]. For experimentation purposes, *V. fischeri* cells were occasionally grown in complex Sea Water Tryptone (SWT) medium [21] or in HEPES minimal medium [HMM [204]] containing 0.3% Casamino acids and 0.2% glucose [21]. The following antibiotics were added to *V. fischeri* media, where necessary, at the indicated concentrations: chloramphenicol (Cm) 2.5 μg mL⁻¹, erythromycin at 5 μg mL⁻¹, and tetracycline (Tc) at 5 μg mL⁻¹ in LBS and 30 μg ml⁻¹ in SWT. The following antibiotics were added to *E. coli* media, where necessary, at the indicated concentrations: Cm at 25 μg/ml⁻¹, kanamycin (Kan) at 50 μg ml⁻¹, Tc at 15 μg/ml⁻¹, or ampicillin (Ap) at 100 μg ml⁻¹. Agar was added to a final concentration of 1.5% for solid media.
Bioinformatics. Amino acid sequences of the *B. subtilis* 168 proteins (RsbW, RsbU, RsbV, SpoIAB, SpoIE, SpoIIA) and the *B. bronchiseptica* RB50 proteins (BtrU, BtrV, BtrW) were obtained from the National Center for Biotechnology Information (NCBI) database and the sequences of the *V. fischeri* ES114 SypA and SypE proteins were obtained from the ERGO light database from Integrated Genomics (http://www.ergo-light.com/ERGO). Amino acid sequence alignments of the SypA proteins with the indicated *B. subtilis* and *B. bronchiseptica* proteins were generated using the Clustal Omega multiple-sequence alignment program from EMBL-EBI (http://ebi.ac.uk/Tools/msa/clustalo) [205].

Molecular and genetic techniques. The *sypA* and *sypE* alleles used in this study were generated by PCR amplification using the primers listed in Table 3. PCR products were cloned into mobilizable plasmids pVSV105 or pKV282 using standard molecular techniques. For chromosomal insertion at the Tn7 site, the *syp* genes were subcloned into the mini-Tn7 delivery vector pEVS107 [206]. For *sypA* Tn7 insertions, the *sypA* genes were subcloned along with the upstream *P*_{sypA} promoter. For Tn7-insertion of *sypE*, the *sypE* genes were subcloned along with the upstream *P*_lacZ promoter. The alleles were inserted into the chromosomal Tn7 site of the indicated *V. fischeri* strains using a tetraparental mating as previously described [206]. To generate site-directed mutations in *sypA* and *sypE*, we utilized the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the primer Phos-*lacZ*-up-rev and select mutagenic primers (Table 3). Generation of the desired mutations was confirmed by sequence analysis using the Genomics Core Facility at the Center for Genetic Medicine at Northwestern University
(Chicago, IL) and ACGT, inc (Wheeling, IL). FLAG and Hemagglutinin (HA) epitope fusions to the C-terminus of SypE and SypA were generated by standard PCR using the primers listed in Table 3. Analysis of the epitope tagged-syp alleles indicated that the introduction of the epitopes impacted the activity of certain Syp proteins. In particular, I found that introduction of a C-terminal HA tag impaired SypE regulatory activity. I also observed that introduction of a C-terminal FLAG-epitope impaired SypA phosphorylation by SypE in vivo, but not in vitro.

To construct the ΔsypA deletion, I used PCR to amplify and clone sequences approximately 2 kb upstream and downstream of sypA into the pJET1.2 cloning vector and subsequently plasmid pEVS79 [207]. I then ligated these fragments to form a plasmid, pARM37, which was subsequently used to introduce the ΔsypA deletion into V. fischeri strains as previously described [27]. Colony PCR analysis was performed to confirm deletion of sypA.

To restore wild-type sypA or insert the mutant sypA allele at the native locus, I generated plasmids pARM135 and pARM160, which contained 750 bp upstream and 500 bp downstream of sypA and sypA<sup>S56A</sup>, respectively. The sypA genes and flanking sequences were confirmed by sequence analysis using ACGT, inc (Wheeling, IL). I introduced the plasmids into the recipient strain [KV4716] by conjugation and isolated stably Cm resistant (cm<sup>R</sup>) colonies, indicating integration of the sypA suicide construct into the chromosome. Stable cm<sup>R</sup> colonies were then cultured in LBS containing 0.2% arabinose to induce expression of the ccdB toxin gene, and plated onto LBS agar plates containing 0.2% arabinose. Arabinose induction selects for those cells that have
undergone a second recombination event, resulting in excision of the suicide plasmid and either restoration of the original ΔsypA allele or replacement with the wild-type or sypA<sup>S56A</sup> allele. Colony PCR was performed to identify those colonies containing the restored sypA allele.

**Wrinkled colony formation assay.** To observe wrinkled colony formation, the indicated *V. fischeri* strains were streaked onto LBS agar plates containing the necessary antibiotics. Single colonies were then cultured with shaking in LBS broth containing antibiotics overnight at either 24°C (for *rscS* overexpressing strains) or 28°C (for *sypG* overexpressing strains). The strains were then sub-cultured the following day to an OD<sub>600</sub> of 0.1 in 5 mL of fresh medium. Cells were spun down, washed twice in 70% artificial seawater (ASW) (280 mM MgSO<sub>4</sub>, 56 mM CaCl<sub>2</sub>, 1.68 M NaCl, 56 mM KCl), and re-suspended in 70% ASW and diluted to an OD of 0.1. 10 µL of re-suspended cultures were spotted onto LBS agar plates, containing necessary antibiotics, and grown for 48 h at either ~ 24°C (for *rscS* overexpressing strains) or 28°C (for *sypG* overexpressing strains). Images of the spotted cultures were acquired at the indicated time points using the Zeiss stemi 2000-C dissecting microscope. For RscS-induced wrinkling time course assays, spot development was followed over a course of 48 h and images taken at indicated time points [208].

**Pellicle formation assay.** The indicated *V. fischeri* strains were grown with shaking in either HMM or LBS containing necessary antibiotics. Cultures were grown at either 24°C (for *rscS* overexpressing strains) or 28°C (for *sypG* overexpressing strains) overnight, and then subcultured the next day to an OD of 0.1 in 1.5 mL of fresh medium in 24-well
microtiter dishes. Cultures were then grown statically at ~ 24°C (for rscS overexpressing strains) or 28°C (for sypG overexpressing strains) for 48 h. The strength of each pellicle was qualitatively evaluated by disrupting the air-liquid interface with a sterile pipette tip after 48 h of incubation. A pellicle is observed as a disruption at the culture surface. Cultures with no observable pellicle were scored as (-); cultures with an intermediate/weak pellicle were scored as (+); cultures with a strong pellicle that was able to be lifted from the culture intact were scored as (++).

**Confocal microscopy.** The indicated *V. fischeri* strains expressing green fluorescent protein (GFP) were grown statically in HMM containing Tc in 12 well microtiter plates with glass cover slips partially submerged into the culture medium. Cover slips were incubated with bacteria at room temperature for 24 h and removed for biofilm examination. A Zeiss LSM 510 confocal microscope (40X objective) was used to collect xy plane and z sections (xz and yz plane) images of the biofilms. Representative images were prepared using the Zeiss LSM Image Browser software.

**Symbiont aggregation assays.** Log-phase cells (OD$_{600}$ 0.3 to 0.6) were grown in 2 ml SWT medium at 22°C. Bacterial cells were then inoculated into filtered ASW at a concentration of 10$^6$ cells per ml. Juvenile squid were then placed into inoculated seawater and the two organisms were allowed to associate at room temperature for 3 h prior to dissection. The juvenile squid were removed to vials containing 2 ml filter-sterilized seawater and 5 µM of the counter-stain, CellMask (Invitrogen, Eugene, OR). The animals were then anesthetized in filtered artificial seawater containing 2% ethanol. Each squid was placed ventral side up on a depression well slide and dissected to remove
the mantle and funnel and expose the light organ. Fluorescently labeled light organs (blue) and GFP-labeled (green) bacteria were viewed using a Zeiss LSM 510 confocal microscope.

**Symbiotic colonization assays.** Colonization assays were performed using the indicated *V. fischeri* strains. To perform single-strain colonization assays, juvenile squid were placed into artificial seawater (ASW) (Instant Ocean; Aquarium Systems, Mentor, OH) containing roughly 1,000-1,500 *V. fischeri* cells per mL of seawater. Colonization assays were allowed to proceed for 16-18 h post inoculation, at which time the animals were washed twice in ASW and homogenized to release the light organ contents. Serial dilutions of the light organs were plated and colony-forming units (CFU) calculated. For competitive colonization assays, juvenile squid were placed into ASW containing approximately 1,000 *V. fischeri* cells per mL of seawater. Juvenile squid were inoculated with an approximate 1:1 ratio of mutant and wild-type cells, and colonization was allowed to proceed for 18 h. For these assays, one strain was marked with an erythromycin resistance (Em$^R$) cassette within the chromosome at the Tn7 site. Reciprocal experiments were also performed in which the other strain carried the Em$^R$ marker. The ratio of bacterial strains within the light organs of the animals was assessed through homogenization/plating assays as described previously [20]. The competitive colonization data are reported as the log-transformed relative competitive index (Log RCI). This index is generated by dividing the ratio of mutant to wild-type in the homogenate by the ratio present in the inoculum and calculating the log$_{10}$ value of that number.
**β-galactosidase assays.** The indicated *V. fischeri* strains that contained the PsypA-lacZ reporter fusion were grown (in triplicate) with shaking in LBS containing the necessary antibiotics at either 24°C (for *rscS* overexpressing strains) or 28°C (for *sypG* overexpressing strains). The following day, the strains were sub-cultured into fresh medium and grown with shaking for up to 24 h. Aliquots (1 mL) of cells were removed at 8 h and 24 h post-inoculation, concentrated, resuspended in Z-buffer, and lysed. The β–galactosidase activity [209] and total protein concentration [210] of each sample were assayed. β–galactosidase units are reported as units of activity per mg of protein.

**Co-Immunoprecipitation of SypE and SypA proteins.** The plasmids SypA-HA (pARM36) and SypE-FLAG (pARM80), SypE<sup>NTD</sup>-FLAG (pARM136), SypE<sup>ΔNTD</sup>–FLAG (pARM162), or the appropriate empty control vectors, were introduced by conjugation into ΔsypA ΔsypE *V. fischeri* cells. Bacterial strains were cultured in LBS containing Tc and Cm at 28 °C overnight with shaking and subsequently sub-cultured to an OD<sub>600</sub> of 0.5. Cells (~0.10 g) were harvested by centrifugation (13,000 x g for 10 min), and washed in 1 mL of phosphate-buffered saline (PBS). Samples were subsequently prepared and immunoprecipitated using the Dynabeads Co-immunoprecipitation Kit (Invitrogen, Oslo, Norway). Cell samples were re-suspended and lysed in 900 uL of Extraction Buffer (EB) (1X IP buffer, 100 mM NaCl, 1 mM DTT) (Invitrogen, Oslo, Norway). Rabbit anti-FLAG and anti-HA antibodies (25 µg, Sigma-Aldrich, St. Louis, MO) were coupled to magnetic Dynabeads (5 mg, Invitrogen) according the manufacturer’s protocol. As a negative control, dynabeads (1.5 mg) were coupled with
non-specific mouse anti-rabbit IgG antibody (5 µg, Promega). For the co-immunoprecipitation, antibody-coupled beads were incubated with 900 µL of whole cell extracts at 4°C with rocking for 1 h. Eluted samples were diluted with sample buffer and resolved using SDS-PAGE. Samples were then transferred to PVDF membrane and proteins were detected using rabbit anti-FLAG and anti-HA antibodies followed by a HRP-conjugated secondary antibody.

**Purification of SypA and SypE proteins.** Wild-type and mutant sypE and sypA-FLAG genes were PCR amplified using the primers listed in Table 3, and subsequently cloned into the GST-fusion vector pGEX-5X-1 (Amersham Biosciences) to generate N-terminal glutathione-S-transferase (GST) fusions (Table 2). The resulting plasmids were transformed into *E. coli* Tam1 cells. *E. coli* cultures were grown at 28°C to an OD600 of 0.5 and overexpression of the GST-fusion proteins was induced by the addition of 0.4 mM IPTG followed by further culturing overnight. Cells were harvested by centrifugation (10,000 x g) and resuspended and lysed in BugBuster protein extraction reagent (Novagen, EMD Chemicals Inc, San Diego, CA). The resuspended cells were incubated with rocking at room temperature for 20 mins and subsequently diluted in resuspension buffer (50 mM Na₂HPO₄, 1 M NaCl, 0.1% Tween-20, pH 8.0). Samples were centrifuged (12,000 x g) and the soluble supernatants applied to a Glutathione Sepharose 4B column (GE Healthcare, Piscataway, NJ), washed with cold 1X PBS, and the bound proteins were eluted by the addition of glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0). Eluted proteins were dialyzed using Slide-A-Lyzer dialysis cassettes in storage buffer (50% glycerol, 10 mM Tris-HCl, 100
mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol [DTT], 0.1 mM EDTA) (10 K MWCO, Thermo Scientific). Following dialysis, the concentration of purified protein was determined using a standard lowry assay.

To obtain purified phosphorylated SypA (SypA~P), E. coli GT115 cells were co-transformed with the GST-SypA-FLAG plasmid (pARM157) and either plasmid pCLD64 expressing only the N-terminal kinase domain of SypE or empty vector pVSV105. Overexpression and purification of the proteins was performed as described above.

**Western blot analysis of *V. fischeri* cell lysates**

The indicated *V. fischeri* strains were cultured in LBS containing the appropriate antibiotics overnight at either 24 °C (for *rscS*-expressing strains) or 28 °C (for *sypG*-expressing strains). 1 mL of cell cultures were spun down and cells lysed in 500 µL 2X sample (4% SDS, 40 mM Tris pH 6.3, 10% glycerol) buffer. Samples were resolved on 10-15% SDS-PAGE gels (10% 29:1 acrylamide: N, N’-methylene-bis-acrylamide, 375 mM Tris pH 8.6, 0.1% SDS), and transferred to PVDF membranes. The indicated Syp proteins were detected by western blot analysis using either a rabbit anti-FLAG antibody or rabbit anti-HA antibody (Sigma-Aldrich, St. Louis, MO) followed by a secondary, donkey anti-rabbit IgG antibody (Sigma-Aldrich, St. Louis, MO) conjugated to horseradish peroxidase (HRP), and visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific, Rockford, IL).

**in vitro kinase assay.** Purified GST-SypA containing a C-terminal FLAG epitope tag (either wild-type SypA or SypA<sub>S56A</sub>; 1 µg) was incubated at 28°C for 10 min in
phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 0.1 mM EDTA) in the presence or absence of 2 mM ATP (ATP Disodium, Trihydrate; Fischer Scientific, Fairlawn, NJ). Purified wild-type GST-SypE (2 µg) was added to the reactions and the samples incubated for an additional 30 min. Reactions were terminated by the addition of sample buffer and proteins were resolved on 10% SDS-PAGE gels containing 20-30 µM Phos-tag™ acrylamide (WAKO chemicals, Richmond, VA) and 40-60 µM MnCl2. Gels were fixed for 15 min in standard transfer buffer (20% MeOH, 50 mM Tris, 40 mM glycine) containing 1 mM EDTA to remove Mn2+ from the gel. Gels were incubated for an additional 20 min in transfer buffer without EDTA. Proteins were transferred to a PVDF membrane and detected by western blot analysis using an anti-FLAG antibody (Sigma-Aldrich).

**in vitro phosphatase assay.** Phosphorylated SypA protein (SypA~P) was purified from *E. coli* cells co-expressing the N-terminal kinase domain of SypE (SypE<sup>NTD</sup>). To confirm the phosphorylation state of the purified protein, samples were analyzed using Phos-Tag™ acrylamide SDS-PAGE coupled with anti-FLAG western blotting, as described earlier. For the phosphatase assay, purified SypA–P was preincubated alone in phosphatase buffer (50 mM Tris-HCl, 10 mM NaCl, 10 mM MgCl2, 1 mM DTT) at 28°C, followed by the addition of increasing concentrations of purified SypE<sup>CTD</sup> protein. Samples were incubated at 28°C for an additional 30 min and the reactions were terminated by the addition of 2X SDS sample buffer. To assess the phosphorylation state of SypA, samples were analyzed using Phos-Tag™ acrylamide SDS-PAGE coupled with anti-FLAG western blot analysis as described for the *in vitro* kinase assay.
Assessment of SypA phosphorylation \textit{in vivo}. The indicated \textit{V. fischeri} strains were cultured overnight with shaking in LBS containing the appropriate antibiotics at either 24°C (for \textit{rscS} overexpressing strains) or 28 °C (for \textit{sypG} overexpressing strains). Aliquots of cells (1 mL) were spun down, washed twice with 1X PBS, and standardized to the same amounts using \textit{OD}_{600} measurements. Samples were lysed in 2X SDS sample buffer and resolved on SDS-PAGE gels containing 25-30 µM Phos-tag\textsuperscript{TM} acrylamide (WAKO chemicals, Richmond, VA) and 50-60 µM MnCl\textsubscript{2}. Gels were fixed for 15 min in standard transfer buffer containing 1 mM EDTA, and then incubated an additional 20 min in transfer buffer without EDTA. Proteins were transferred to a PVDF membrane and the proteins were detected by western blot analysis using an anti-HA antibody (Sigma-Aldrich, St. Louis, MO).
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### Table 2. Plasmids used in this study

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CHAPTER THREE
EXPERIMENTAL RESULTS

SypE: A DUAL REGULATOR OF BIOFILMS AND SYMBIOTIC COLONIZATION

Introduction

Biofilm regulation in V. fischeri involves a number of putative and characterized regulatory proteins. Several of these regulators function to control the expression of the syp biofilm locus, predicted to be involved in polysaccharide synthesis and export (Fig. 1)[21]. The syp locus is required for both biofilm formation and efficient host colonization [21]. The syp locus is regulated at the transcriptional level by a two-component signaling (TCS) system consisting of the histidine sensor kinase (HK) RscS and the syp-encoded response regulator (RR) SypG (Fig. 3) [20,27].

In addition to SypG, the syp locus encodes a second putative RR, SypE. The SypE protein is unusual both in its structure and in its apparent function in biofilm regulation and symbiosis. Bioinformatic analysis predicts a novel, multi-domain RR with the regulatory REC domain in a central position flanked by two terminal effector domains of putative opposing function: an N-terminal RsbW-like serine kinase and a C-terminal PP2C-like serine phosphatase domain. The presence of a central REC domain flanked by two effector domains of apparent opposing functions distinguishes SypE as a unique RR with a novel domain architecture.
Previous studies identified SypE as a potential regulator of *V. fischeri* biofilms. Hussa *et al.* (2008) demonstrated that overexpression of *rscS* in a strain lacking *sypE* resulted in slightly diminished biofilm phenotypes, indicated by a decrease in wrinkled colony formation relative to wild-type cells overexpressing *rscS*. These results suggested a positive regulatory role for SypE. In contrast, SypE strongly inhibited biofilms induced by SypG: *sypG* overexpression in wild-type cells did not lead to wrinkled colony or pellicle formation, while its overexpression in a Δ*sypE* strain induced robust biofilms similar to that of an *rscS*-overexpressing wild-type strain [27]. These initial studies suggested that under distinct signaling conditions SypE may function to either negatively or positively impact *syp*-dependent biofilm production. However, the mechanism by which SypE exerts dual control over biofilm formation remained unclear. Additionally, the relevance of SypE during symbiotic colonization had yet to be investigated.

To further assess the mechanisms by which *V. fischeri* regulates symbiotic biofilm formation and host colonization, I chose to characterize the regulatory role of SypE in *syp*-dependent biofilm formation. I hypothesized that SypE plays a novel role as a dual regulator of biofilm formation, and that these opposing activities are mediated by the activity of the protein’s terminal effector domains. I further reasoned that SypE’s opposing regulatory activities must be controlled to permit biofilm formation and host colonization. To investigate the regulatory role of SypE, I performed a structure-function analysis of the SypE protein. Using a complementation approach, I assessed the potential contribution of the individual SypE domains to the regulation of *syp*-dependent biofilm
formation. Finally, I assessed the relevance of SypE’s regulatory activities during symbiotic colonization of *E. scolopes*.

**Impact of SypE on RscS induced biofilm phenotypes**

To better understand the putative role of SypE as a regulator of *syp* biofilms, I first examined the impact of *sypE* deletion on biofilm phenotypes induced by overexpression of *rscS*. Deletion of *sypE* was previously shown to reduce the ability of *rscS* overexpression to induce biofilm formation [27]. To better assess this subtle phenotype, I examined biofilm development over time by wild-type (WT) and Δ*sypE* mutant cells containing the *rscS*-expressing plasmid, pARM7 (pRscS) or the empty vector (pKV282). In addition, to verify that any phenotypes of the Δ*sypE* mutant were due to the loss of *sypE*, and were not the result of a polar effect on downstream genes, I complemented the *sypE* null strain with a wild-type *sypE* allele (*sypE<sup>+</sup>*) inserted in the chromosome at the Tn7 integration site. As a control, I inserted an empty Tn7 cassette (EC) at the same site.

First, I evaluated the formation of wrinkled colonies and found that *rscS*-expressing wild-type cultures consistently exhibited wrinkled colony morphology within 14 h of growth, while a Δ*sypE* mutant consistently exhibited a 4-6 h delay, initiating wrinkled colony formation at 18-20 h post-spotting (Fig. 10A). As expected, vector-carrying cells never formed wrinkled colonies (Fig. 10A). Complementation with wild-type *sypE* at the Tn7 site of a Δ*sypE* mutant fully restored the wild-type timing and pattern of RscS-induced wrinkled colony development (Fig. 10A). At a late (48 h) time point, all the strains exhibited a similar degree of wrinkled colony morphology, indicating
that deletion of \( sypE \) results in a delay in RscS-induced wrinkled colony development.

Second, I evaluated the production of pellicles over time. When grown statically in HEPES minimal medium (HMM), \( rscS \)-expressing wild-type cells produced a structured pellicle at the air-liquid interface within 24 h of incubation, while vector control cells failed to form any observable pellicle (Fig. 10B). The \( \Delta sypE \) strain formed a diminished pellicle at 24 h that was easily disrupted [27](Fig. 10B). To quantify this subtle decrease in pellicle formation, I assessed by confocal microscopy pellicle biofilms produced by wild-type and \( \Delta sypE \) strains overexpressing both \( rscS \) and GFP at 24 h post inoculation. \( rscS \)-expressing wild-type cells exhibited increased pellicle attachment relative to vector-containing cells, with an average biofilm thickness of 15 (+/- 2) and 4 (+/- 1) \( \mu m \), respectively (Fig. 10C). Compared to wild-type cells, the \( \Delta sypE \) mutant strain consistently exhibited a 50% reduction in RscS-induced pellicle attachment with an average biofilm thickness of 8 (+/- 1) \( \mu m \) (Fig. 10C). Complementation with \( sypE^+ \) restored the average biofilm thickness to 13 (+/- 1) \( \mu m \) (Fig. 10B and C). These results confirm that loss of \( sypE \) impairs the development of biofilm formation, and support the hypothesis that under RscS-overexpressing conditions SypE exerts positive regulatory activity.

**SypE domains and mutant library construction**

I hypothesized that the ability of SypE to exert dual regulatory control over biofilm formation may be mediated by the protein’s terminal, effector domains. To better understand the mechanism by which SypE exerts dual control over \( syp \) biofilm formation, I performed a BLAST bioinformatic analysis of the individual SypE domains. I
Figure 10. Impact of SypE on RscS-induced biofilms. (A) Time course of wrinkled colony formation. Cultures of the following strains were spotted onto LBS medium and colony morphology was assessed over a 48 h period: wild-type (WT) cells containing the empty Tn7 cassette (EC) [KV4389] and carrying RscS plasmid pARM7 or vector control (pKV282); ΔsypE cells carrying pARM7 and containing either EC [KV4390] or complemented with sypE⁺ [KV4819]. Black bar represents 2 mm. (B) Pellicle formation in static culture. The following strains were grown in HMM in 24-well plates for 48 h: WT cells containing EC [KV4389] and carrying the vector control pKV282 or pARM7; pARM7-carrying ΔsypE containing EC [KV4390] or complemented with sypE⁺ [KV4819]. A pipette tip was dragged over the surface of the air-liquid interface to visualize the pellicle, and relative pellicle strength was determined (C) Confocal microscopy of pellicles. Pellicle attachment to glass cover slips was visualized via confocal microscopy at 24h post inoculation. Representative views of the xy plane and z sections are shown for GFP-labeled derivatives of the strains described in panel B. White arrows indicate the air-liquid interface. Photographs are representative of at least 3 independent experiments.
Figure 11. SypE domain structure and sequence alignments. (A) SypE contains a central receiver (REC) domain flanked by a putative N-terminal RsbW-like serine kinase domain and a putative C-terminal PP2C-like serine phosphatase domain. (B) Sequence alignments of SypE’s N-terminal domain with the serine kinases RsbW and SpoIAB from *B. subtilis* and BtrW from *B. bronchiseptica*. (C) Sequence alignments of SypE’s C-terminal domain with the serine phosphatases RsbU and SpoIE from *B. subtilis* and BtrU from *B. bronchiseptica*. Conserved residues within the individual domains are indicated (see text for details).
Figure 12. SypE domain mutants. Representations of the various SypE domain mutants generated in this study are presented above. See text for a full description.
subsequently used the CLUSTAL OMEGA software to generate multiple sequence
alignments of the SypE terminal domains with the predicted orthologous proteins [205].
These alignments were subsequently used to identify conserved residues within SypE’s
domains to target for mutagenesis (Fig. 11).

Bioinformatic analyses indicated that the terminal effector domains of SypE share
sequence similarity with serine kinase and serine phosphatases commonly found in
partner switching regulatory systems. Specifically, the N-terminal domain of SypE
exhibits sequence similarity to HPK-like serine kinases of the Gyrase, Hsp90, histidine
kinase, MutL (GHKL) superfamily [133]. Further analysis revealed that SypE’s N-
terminal domain is most similar to a class of serine kinases/anti-sigmas, including RsbW
and SpoIIAB of Bacillus subtilis and BtrW of Bordetella bronchiseptica (Fig. 11B).
Sequence analysis revealed the presence of several conserved residues, including a highly
conserved asparagine (N52 in SypE) (Fig. 11B). In characterized HPK-like serine
kinases, this invariant asparagine is located within the N-box motif, and is required for
coordination of a Mg^{2+} ion involved in ATP binding and kinase activity [95].

In contrast, the C-terminal effector domain of SypE exhibits sequence similarity
to serine phosphatases of the PP2C family. These include the partner-switching
regulatory phosphatases RsbU and SpoIIE of Bacillus subtilis and BtrU of B.
bronchiseptica [133](Fig. 11C). Proteins of the PP2C family are Mg^{2+}/Mn^{2+}-dependent
phosphatases. Analysis of characterized PP2C phosphatases identified conserved amino
acid residues involved in phosphatase activity, including several invariant aspartate
residues required for coordination of divalent cation binding and catalytic activity
Sequence alignments revealed that these catalytic aspartates are conserved in SypE (Asp-443 and Asp-495) (Fig. 11C).

To investigate the putative regulatory activities of SypE’s terminal effector domains, I performed a structure-function analysis of SypE. With the help of a research technician, Cynthia Darnell, I generated a library of SypE domain mutants (Fig. 12). I then assessed the potential regulatory activity of the SypE mutants using a complementation approach.

**SypE mediated positive regulation of RscS-induced biofilms**

To determine the role(s) of the individual SypE domains in the positive regulation of RscS-induced *V. fischeri* biofilms, I introduced the mutant *sypE* alleles into the Tn7 site of a Δ*sypE* strain, and then assessed complementation by assaying for the restoration of RscS-induced biofilm phenotypes (i.e. wrinkled colony formation).

As previously observed, RscS-induced wild-type cells initiated wrinkled colony development within 16 h (Fig. 13A), while the Δ*sypE* strain exhibited a relative delay in RscS-induced wrinkling (Fig. 13C). Complementation with a wild-type copy of *sypE* restored wild-type biofilm development (Fig. 13D). Similar to WT *sypE*, I found that expression of a SypE N-terminal deletion mutant (SypE^ΔNTD) (Fig. 12) fully complemented the *sypE* deletion for wrinkled colony morphology (Fig. 13E). This mutant lacks the N-terminal 135 amino acids encoding the putative serine kinase domain but retains an intact C-terminal serine phosphatase domain and REC domain. These data indicate that the positive regulatory activity of SypE is retained in this mutant and thus does not require the N-terminal domain. I hypothesized that the positive activity retained
in the SypE\(^{\text{ANTD}}\) mutant protein may reside in the protein’s C-terminal, effector domain. Indeed, a deletion derivative that expresses only the C-terminal 245 amino acids (SypE\(^{\text{CTD}}\)) fully complemented the ΔsypE deletion, restoring wrinkled colony morphology to wild-type levels (Fig. 13F). These data thus demonstrate that the C-terminal effector domain of SypE alone is active and positively regulates RscS-induced biofilms.

The C-terminal domain exhibits sequence similarity to PP2C-like serine phosphatases and possesses several conserved residues, including conserved aspartate residues (D443 and D495) required for phosphatase activity (Fig. 11C). We reasoned that if SypE’s C-terminal domain functions as a phosphatase, then these conserved residues were likely required for the observed positive regulatory activity. We generated alanine substitutions in the context of both the C-terminal domain alone (SypE\(^{\text{CTD}, D443A}\) and SypE\(^{\text{CTD}, D495A}\)) and of the full-length protein (SypE\(^{D443A}\) and SypE\(^{D495A}\)). Mutation of either conserved aspartate in the context of the C-terminal domain alone (SypE\(^{\text{CTD}, D443A}\) and SypE\(^{\text{CTD}, D495A}\)) resulted in failure to complement the ΔsypE defect: these mutants exhibited delayed wrinkled colony morphology similar to the uncomplemented ΔsypE control (Fig. 13G and 13H, respectively). I concluded that the positive regulatory activity of the SypE C-terminal domain requires the conserved aspartate residues (D443 and D495), suggesting that SypE likely functions as a phosphatase to promote biofilms.

In the context of the full-length SypE, the SypE\(^{D443A}\) and SypE\(^{D495A}\) mutants again failed to complement the sypE deletion as observed at the early (16 h) time point (Fig. 13I and 13J, respectively), but unexpectedly also completely inhibited biofilm formation,
as indicated by smooth colony morphology even at 48 h (Fig. 14E and 14F). I hypothesized that loss of function in SypE’s C-terminus “locked” SypE into an inhibitory state. Consistent with that interpretation, a SypE^ACTD mutant, which contains the N-terminus and the central REC domain, but lacks the C-terminal domain, exhibited the same phenotype: this strain failed to form wrinkled colonies even at late time points (48 h post-incubation) (Fig. 14G). Taken together, these results demonstrate that an intact C-terminal domain is required for the positive regulatory activity of SypE and that loss of the C-terminal function results in a protein with constitutive inhibitory activity.

**Negative regulation of RscS-induced biofilms by SypE**

I hypothesized that the inhibitory activity observed upon disruption of SypE’s C-terminal domain likely resided in the protein’s N-terminal effector domain. To test this hypothesis, I evaluated biofilm formation in a ΔsypE strain that expressed only the 140 amino acids comprising the N-terminal domain (SypE^NTD). This strain failed to form biofilms, as indicated by the smooth colony morphology at 48 h (Fig. 14H). These results demonstrate that the isolated SypE N-terminal effector domain is active and sufficient to inhibit the development of RscS-induced biofilm phenotypes.

The N-terminal domain of SypE exhibits sequence similarity to HPK-like serine kinases, and possesses a conserved asparagine residue located within the N box motif predicted to be required for serine kinase activity (Fig. 11B). I asked whether the conserved asparagine N52 is required for the inhibitory activity of SypE by substituting alanine at that position. In the context of the inhibitory N-terminal domain alone (SypE^NTD,N52A), this substitution resulted in loss of inhibitory activity: expression of
Figure 13. Positive regulation of biofilm formation by SypE. Cultures of the following strains were spotted onto LBS medium at RT and wrinkled colony formation was assessed at an early time point (16 h) post-spotting: WT cells containing EC [KV4389] and carrying either RscS plasmid pARM7 (A) or vector control pKV282 (B), and ΔsypE mutants carrying pARM7 and containing EC (KV4390) (C) or complemented with WT sypE+ [KV4819] (D), sypE^ANTD [KV5124] (E), sypE^CTD [KV5204] (F), sypE^CTD, D443A [KV5314] (G), sypE^CTD, D495A [KV5345] (H), sypE^D443A [KV4886] (I), sypE^D495A [KV4887] (J), sypE^ACTD [KV5315] (K), or sypE^N52A, D192A [KV5205] (L). Photographs are representative of at least 3 independent experiments.
Figure 14. Negative regulation of biofilm formation by SypE. Cultures of the following strains were spotted onto LBS agar medium at RT and wrinkled colony formation was assessed at a late time point (48 h) post-spotting: WT cells containing EC [KV4389] and either RscS plasmid pARM7 [A] or vector control pKV282 (B); ΔsypE cells carrying pARM7 and containing EC [KV4390] (C) or complemented with WT sypE⁺ [KV4819] (D), sypE₄⁴₄₃A [KV4886] (E), sypE₄⁴₉₅A [KV4887] (F), sypEₜₐₜ [KV5315] (G), sypENₜₐₜ [KV5129] (H), sypENₜₐₜ, N52A [KV5143] (I), sypEN₅₂A [KV5142] (J), sypEN₅₂A, D₄₄₄₃A [KV5379] (K). Photographs are representative of at least 3 independent experiments.
SypE<sup>NTD, N52A</sup> in the ΔsypE strain resulted in wrinkled colony formation (Fig. 14I). Western blot analysis confirmed that the SypE<sup>NTD, N52A</sup> protein was expressed at levels similar to the SypE<sup>NTD</sup> control, indicating that the loss of inhibitory activity results from the N52A mutation (data not shown). As expected, the N52A mutation in the context of the full-length protein (SypE<sup>N52A</sup>) permitted complementation of the sypE deletion; the SypE<sup>N52A</sup> strain exhibited wrinkled colony morphology similar to the wild-type and sypE complemented strains (data not shown). These results indicate that the N52A mutation disrupts the inhibitory activity of the SypE protein, but does not impact its positive regulatory activity.

**Regulation of SypE’s opposing activities**

The above results demonstrated that SypE exerts both negative and positive control over biofilm formation through the opposing activities of its N- and C-terminal effector domains, respectively. In general, RR activity is regulated by the phosphorylation of a conserved aspartate residue located in the protein’s REC domain [71]. I therefore hypothesized that the phosphorylation state of the central REC domain likely controls the opposing activities of SypE’s terminal effector domains. To test this hypothesis, I evaluated biofilm formation by cells expressing a SypE mutant containing an alanine substitution at the conserved aspartate residue (D192), the predicted site of phosphorylation [27](Fig. 11). This substitution in other RRs results in a protein that cannot become phosphorylated, and whose activity therefore mimics that of the non-phosphorylated state (e.g., [82]). The SypE<sup>D192A</sup> mutant not only failed to complement the sypE deletion but also completely inhibited wrinkled colony as observed at the late
(48 h) time point (Fig. 15E). This inhibitory activity was not due to aberrant protein expression as western blot analysis indicated that the SypE^{D192A} protein was expressed at levels similar to wild-type SypE (data not shown). These results indicate that the conserved Asp residue (D192) in the REC domain is required for the positive regulatory activity of SypE. Thus, the phosphorylation state of this residue is likely important in controlling SypE regulatory activity.

I hypothesized that the D192A substitution “locks” SypE into a conformation that favors the activation of the inhibitory N-terminal domain. I reasoned that if the D192A mutation indeed “locks” SypE in a constitutive inhibitory state, then this activity should require an intact N-terminal domain and the invariant asparagine (N52). To test this, I combined the D192A and N52A mutations (SypE^{N52A, D192A}). In contrast to the inhibitory SypE^{D192A} mutant, cells carrying SypE^{N52A, D192A} exhibited biofilm formation indistinguishable from the uncomplemented (EC) control (Fig. 15F). This result could not be attributed to a defect in protein production of the double mutant (data not shown). These data demonstrate that the N52A mutation is epistatic to the D192A mutation, and that the constitutive inhibitory activity of the D192A mutant requires an intact N-terminal domain. While the SypE^{N52A, D192A} mutant lost inhibitory activity, the protein remained incapable of complementing the sypE deletion despite the fact that the positive-acting C-terminal domain remained intact (Fig 13L). These data therefore demonstrate that residue D192 is also required for promoting SypE’s positive regulatory
**Figure 15. Role of the REC domain in controlling SypE activity.** Cultures of the following strains were spotted onto LBS agar medium at RT and wrinkled colony formation was assessed at a late time point (48 h) post-spotting: WT cells containing EC [KV4389] and either RscS plasmid pARM7 (A) or vector control pKV282 (B); and ΔsypE mutants carrying pARM7 and containing EC [KV4390] (C) or complemented with WT sypE+[KV4819] (D), sypE^{D192A}[KV4885] (E), sypE^{N52A,D192A}[KV5205] (F). Photographs are representative of at least 3 independent experiments.
activity SypE, suggesting that phosphorylation may function to both activate the positive C-terminal domain and to inactivate the inhibitory N-terminal.

We noted that the constitutive inhibitory activity of the SypE^{D192A} mutant mimicked that observed previously in a C-terminal domain mutant, SypE^{D443A}. We predicted that the inhibitory activity of the SypE^{D443A} mutant would similarly depend upon residue N52, and tested this by generating a SypE^{N52A,D443A} double mutant. As expected, a SypE^{N52A,D443A} mutant failed to inhibit biofilm formation, and exhibited delayed wrinkled colony similar to uncomplemented ΔsypE control (Fig. 14K). Western blot analysis confirmed that the SypE^{N52A,D443A} protein was expressed at levels similar to wild-type SypE^{+} (data not shown)[212]. These data demonstrate that the N52A mutation is also epistatic to the D443A mutation, and suggest that in all cases SypE inhibitory activity requires an intact N-terminal domain.

**Role of SypE in regulating symbiotic colonization**

The ability to produce syp-dependent biofilms is essential for *V. fischeri* cells to efficiently colonize host squid [20,21]. Because SypE exerts both a subtle positive effect and a strong negative effect over biofilm formation, I asked if either or both of these activities were important to host colonization. I evaluated the role of SypE in colonization of juvenile *E. scolopes* using both single strain inoculations and mixed strain competitions. I found that deletion of *sypE* did not substantially impact the ability of *V. fischeri* to colonize squid, when in competition or presented alone (Fig. 16A and 16B, respectively), although there was a small advantage for wild-type cells to out-compete the ΔsypE mutant (mean Log RCI, -0.21 +/- 0.63). Similar results were also observed when
juvenile squid were exposed to a mixture of wild-type and ΔsypE cells both overexpressing rscS: the squid contained roughly equal numbers of both strains (data not shown). These results demonstrate that loss of sypE does not greatly impact colonization fitness, suggesting that the minor, positive activity SypE exerts over biofilm formation is not likely critical during colonization.

My in vitro studies suggested that, of the two roles of SypE, the inhibitory activity appears to play a more important role with respect to biofilm formation. I therefore asked whether the inhibitory activity of SypE impacts symbiotic colonization. To address this, I utilized the SypE<sup>D192A</sup> mutant, which exhibited constitutive inhibitory activity. I exposed squid to a mixture of wild-type and ΔsypE mutant cells complemented with either wild-type sypE<sup>+</sup> or the inhibitory sypE<sup>D192A</sup> allele. While the SypE<sup>+</sup> strain competed well for colonization with the wild-type strain (mean Log RCI, -0.07 +/- 0.44), the SypE<sup>D192A</sup> mutant exhibited a strong colonization defect (mean Log RCI, -1.59 +/- 0.52), with all squid colonized predominantly by the wild-type cells (Fig. 16C and 16D, respectively). These data indicate that the inhibitory activity of SypE is detrimental to colonization.

To explore this possibility further, I performed single-strain colonization experiments using strains that did not carry the rscS overexpression plasmid. Squid inoculated with wild-type cells contained levels of bacteria between 10<sup>4</sup> and 10<sup>5</sup> colony-forming units per animal, and those inoculated with the ΔsypE mutant complemented with wild-type sypE<sup>+</sup> were similarly proficient at colonization (Fig. 16E). However, the ΔsypE mutant complemented with sypE<sup>D192A</sup> exhibited a dramatic defect in host
colonization relative to wild-type cells: multiple squid remained completely uncolonized, while the others had a severe decrease in their level of colonization (2-3 log decrease) (Fig. 16E). These data demonstrate that residue D192 within the central REC domain, is necessary to promote efficient host colonization.

Finally, to confirm that the colonization disadvantage of the SypE<sup>D192A</sup> strain is due to the activity of the inhibitory domain, I assessed colonization by the SypE<sup>N52A, D192A</sup> expressing strain. Whereas the SypE<sup>D192A</sup> mutant inhibited host colonization, the SypE<sup>N52A, D192A</sup> double mutant achieved near wild-type levels of colonization (Fig. 16E). These data thus support our earlier observations that the inhibitory activity of a SypE<sup>D192A</sup> mutant resides in the N-terminal effector domain. These results are consistent with the biofilm phenotypes observed in culture, and suggest that the constitutive inhibition of colonization resulting from disruption of the predicted site of phosphorylation (D192) is mediated by the N-terminal effector domain. Together, these findings indicate that the inhibitory activity of SypE is relevant during host association, and the ability of the cell to turn off this activity is critical to permit symbiotic colonization.

**Impact of SypE on symbiotic aggregate formation**

*syp*-dependent biofilm formation is essential for the initial stages of host colonization, when *V. fischeri* cells aggregate in the squid-secreted mucus on the light organ surface. To determine if SypE impacts this early stage in symbiotic colonization, another graduate student, Valerie Ray, and I performed aggregation studies with the help of Elizabeth Heath-Heckman in the laboratory of Dr. Margaret McFall-Ngai at the
Figure 16. Impact of SypE on symbiotic colonization. (A, C, D) Competitive colonization with WT and sypE strains. Newly hatched squid were exposed to a mixed inoculum of WT (ES114) and sypE cells. The Log relative competitive index (Log RCI) is plotted on the x-axis. Position on the Y-axis is arbitrary. The black diamond and error bars indicate the average Log RCI and standard deviation for indicated data set. (B and E) Single-strain colonization assays. Newly hatched squid were exposed for 18 h to either WT cells or: (B) ΔsypE cells; (E) ΔsypE cells complemented with either sypE\textsuperscript{+}, sypE\textsuperscript{D192A}, or sypE\textsuperscript{N52A,D192A}. As a negative control, aposymbiotic (APO) juvenile squid were maintained in bacteria free water. Each circle represents the number of V. fischeri cells recovered from an individual animal. The dashed line indicates the limit of detection. The black bar indicates the average CFU and standard deviation for 15 animals. The data shown above are from one experiment and are representative of at least 3 independent experiments.
Figure 17. Inhibition of symbiotic aggregation by SypE. Newly hatched juvenile squid were inoculated with GFP-labeled derivatives of the following strains: (A) pARM7 (pRscS) in WT cells [KV4389] or ΔsypE cells complemented with WT sypE⁺ [KV4819] (B), sypE_{D192A} [KV4885] (C), or sypE_{N52A,D192A} [KV5205] (D). After 3 h, animals were stained with CellMask stain (blue color) and the light organs were examined by confocal microscopy. Representative images of *V. fischeri* cells aggregating on the light organ surface are shown. White arrows indicate the surface of the light organ appendages. The white, dashed arrow indicates single *V. fischeri* cells on the light organ surface.
Figure 18. Impact of SypE on host colonization in \( \Delta rscS \) mutant cells. Competitive colonization assay with \( \Delta rscS \) and \( \Delta rscS \Delta sypE \) mutant cells. Colonization assays were performed and Log RCI of the competing bacterial strains was calculated as described in Figure 7. Each circle represents a single animal. Closed circles represent animals in which no \( \Delta rscS \) cells were present in the homogenate. The position of the circles on the y-axis is merely for spacing. The black diamond and error bars indicate the average Log RCI and standard deviation for 15 animals. The data shown above are from one experiment and are representative of at least 3 independent experiments.
University of Wisconsin (Madison). Briefly, we assessed symbiotic aggregate formation by wild-type and \textit{sypE} mutant strains expressing GFP. To promote aggregate formation, these studies were performed using strains that also overexpressed \textit{rscS}. In agreement with previous data [20], wild-type cells exhibited large aggregates (green color) on the light organ surface (blue color) (Fig. 17A), as did the \textit{ΔsypE} strain complemented with wild-type \textit{sypE}+ (Fig. 17B). In contrast, the \textit{SypE}^{D192A} mutant strain formed no observable aggregates; only single cells were occasionally observed on the light organ surface (dashed arrow) (Fig. 17C). As expected, the aggregation defect of this mutant depended upon the inhibitory N-terminal domain, as a \textit{SypE}^{N52A, D192A} double mutant formed large aggregates similar to control (\textit{SypE}+) cells (Fig. 17D). These data suggested that the colonization defect of the \textit{SypE}^{D192A} mutant likely resulted from a loss of bacterial aggregation at the initiation stage. Thus, SypE appears to impact bacterial aggregation, a critical stage in the initiation of host colonization. Finally, these results support our earlier conclusion that the inhibitory activity of SypE must be deactivated to promote efficient aggregation and host colonization.

**Deletion of \textit{sypE} improves colonization by an \textit{rscS} mutant**

The previous experiments demonstrated that the conserved site of phosphorylation in the SypE REC domain is necessary to inactivate the inhibitory activity of the N-terminus and promote its positive activity. Furthermore, although SypE expressed from its native site in the chromosome inhibits biofilm formation induced by the overexpression of the RR SypG, it does not inhibit RscS-induced biofilm formation. These data indicate that upon RscS induction, this inhibitory activity of SypE must be
turned off. We therefore hypothesized that in a strain lacking \textit{rscS}, SypE is likely to exhibit constitutive inhibitory activity and negatively impact host colonization. We reasoned that this phenomenon could account, at least in part, for the severe colonization defect of an \textit{rscS} mutant [26]. To determine whether this is the case, I utilized a \textit{\Delta rscS} \textit{\Delta sypE} double mutant generated by another graduate student, Valerie Ray, and assessed the ability of this strain to compete with the \textit{\Delta rscS} parent to colonize juvenile squid. Indeed, the \textit{\Delta rscS} \textit{\Delta sypE} mutant cells dramatically outcompeted the \textit{\Delta rscS} single mutant (mean Log RCI, 1.76 +/- 0.66) with the majority of the animals exclusively colonized by the double mutant (open circles) (Fig. 18). These results indicate that SypE contributes to the colonization defect observed in an \textit{rscS} mutant, supporting our hypothesis and linking SypE-mediated regulation of colonization to the activity of RscS.

**Summary**

In this section, I identified a novel role for the RR SypE in controlling biofilm formation and host colonization by \textit{V. fischeri}. My initial interest in characterizing SypE was prompted both by its unusual domain architecture and by earlier studies that suggested that SypE contributes to biofilm regulation [27,28]. Bioinformatics indicated that SypE is a RR with a novel domain architecture: a central REC domain flanked by two effector domains with apparently opposing enzymatic activities. I hypothesized that SypE could exert dual regulatory control over \textit{syp} biofilm formation, depending on which of the terminal effector domains was active.

Using a genetic analysis of \textit{sypE} activity, I determined that SypE indeed exerts dual control over \textit{syp}-dependent biofilm formation. Specifically, the N-terminal putative
serine kinase domain inhibited RscS-induced biofilms, while the C-terminal, putative serine phosphatase domain positively impacted biofilm formation. Furthermore, the regulator activities of the N- and C-terminal effector domains required conserved residues consistent with these domains possessing serine kinase and serine phosphatase activity, respectively. These opposing activities appear to be controlled by the protein’s central REC domain, as mutation of the predicted site of phosphorylation (D192) resulted in loss of SypE positive activity. I interpret these data to indicate that REC phosphorylation shifts the equilibrium of the SypE conformational states such that the inhibitory N-terminus is inactivated, while the positively acting C-terminus is activated.

I also determined that the activity SypE is relevant during symbiotic colonization. While the minor positive role of SypE in promoting biofilm formation is relatively unimportant during host colonization, I showed that inhibition by SypE is a significant activity that must be overcome during the colonization process. Further studies established that SypE impacts host colonization during the initiation stage, in which *V. fischeri* forms aggregates in the squid-derive mucus on the light organ surface. SypE mutants that failed to produce biofilms exhibited severe defects in host colonization, which could be attributed to the inability of these cells to efficiently aggregate. Finally, I determined that the inhibitory activity of SypE contributes to the colonization defect observed in an *rscS* mutant. This work, published in Molecular Microbiology, establishes SypE as a key regulator of host colonization, restricting colonization to conditions in which RscS is overexpressed or activated [212].
SypE CONTROLS BIOFILMS AND SYMBIOTIC COLONIZATION THROUGH
REGULATION OF SypA ACTIVITY

Introduction

In the previous section, I established that SypE controls biofilms both negatively and positively through the opposing activities of its putative N-terminal serine kinase and C-terminal serine phosphatase domains. However, the downstream target of SypE’s regulatory activities and the mechanisms by which SypE restricts biofilms and colonization remained unknown. Given the results from my previous experiments, I reasoned that SypE likely regulates biofilm formation by controlling the phosphorylation state of a downstream regulatory protein, possibly another partner switching-like protein.

SypE is encoded downstream of another putative regulatory gene, sypA, which encodes a protein with sequence similarity to the B. subtilis antagonist protein RsbV [21,133](Fig. 2). Like RsbV, SypA contains a single STAS domain indicative of regulatory proteins that function as anti-sigma factor antagonists (Fig. 19)[133]. A role for SypA in regulating syp-dependent biofilm formation and/or symbiotic colonization had yet to be identified. However, given the similarity to characterized partner-switching regulators and genetic proximity to sypE, we speculated that SypA may represent the target of SypE regulatory activities.

In order to investigate the potential role of SypA as a regulatory target of SypE, I first assessed whether SypE interacts with SypA in vivo by performing co-immunoprecipitation assays. I then asked whether SypE’s N- and C-terminal effector domains indeed possess serine kinase and serine phosphatase activities, respectively, and
whether SypA represents a target of these opposing activities. To address these questions, I established both in vitro and in vivo assays to assess the impact of SypE activity on the phosphorylation state of SypA. Finally, I investigated whether SypE mediates control over biofilm formation and symbiotic colonization through regulation of SypA activity via epistasis analyses. The studies described in this section provide insight into the mechanism by which SypE restricts biofilm formation and symbiotic colonization.

**Analysis of SypE interaction with SypA in vivo**

To determine if SypE targets SypA to regulate biofilm formation, I first asked whether SypE interacts with SypA in vivo by performing a co-immunoprecipitation assay. Briefly, I generated FLAG- and HA-epitope tag fusions to the C-termini of SypE and SypA, respectively, and expressed the epitope-tagged alleles in a *V. fischeri* ΔsypA ΔsypE strain [KV4716]. I used anti-FLAG or anti-HA antibodies to immunoprecipitate either SypE or SypA, respectively, and then detected the immunoprecipitated proteins using western blotting analyses. Upon immunoprecipitation using anti-FLAG antibody, I detected not only immunoprecipitated SypE, but also SypA (Fig. 20A, left panel). In reciprocal experiments, I found that immunoprecipitation of SypA with anti-HA antibody resulted in the co-immunoprecipitation of SypE (Fig. 20A, right panel). These results indicate that SypA and SypE interact in vivo, a result that is consistent with the model that SypA serves as a target of SypE’s regulatory activity.

I next examined the domains of SypE required for mediating this binding to SypA. Based on similarity to *B. subtilis* orthologues, in which RsbW directly binds
Figure 19. SypA domain structure and sequence alignment. (A) SypA contains a sulfate transport and anti-sigma factor antagonist (STAS) domain indicative of regulatory proteins that function as anti-sigma factors antagonists. (B) Sequence alignments of SypA with antagonist proteins RsbV and SpoIIAA from *B. subtilis* and BtrV from *B. bronchiseptica*. The predicted phosphorylated serine residue (S56) of SypA is indicated.
RsbV [153], I hypothesized that binding to SypA would depend upon SypE’s N-terminal, RsbW-like domain. I first tested the ability of SypA to interact with an N-terminal deletion mutant of SypE (SypE^ANTD), a SypE derivative that lacked the first 135 amino acids (comprising the putative serine kinase domain) but retained positive regulatory activity mediated by the C-terminal domain. The FLAG-SypE^ANTD mutant failed to co-immunoprecipitate with SypA (Fig. 20B, lanes 1 and 3). This result suggested that the N-terminus of SypE is required for interaction with SypA. To explore this possibility further, I co-expressed SypA with a SypE mutant (SypE^NTD) that expressed only the first 140 amino acids of the N-terminus. I found that FLAG-SypE^NTD co-immunoprecipitated with SypA, indicating that the N-terminal domain alone is sufficient to mediate interaction with SypA (Fig. 20B, lanes 2 and 4). These data indicate that the N-terminal domain of SypE interacts with SypA in vivo.

**In vitro analysis of SypA phosphorylation by SypE**

The identification of SypA as a target protein capable of interacting with SypE provided a candidate substrate to test the activities of SypE’s terminal effector domains, e.g., kinase and phosphatase activities. To assess SypE’s kinase activity, I purified glutathione S-transferase (GST)-tagged versions of the proteins (SypE and SypA-FLAG). I then incubated purified SypA in kinase buffer either in the absence or presence of purified SypE. The samples were then resolved on SDS-PAGE gels containing Phos-tag™ acrylamide, which permits the separation of phosphorylated and non-phosphorylated forms of proteins by preferentially binding to and retarding the migration of phosphorylated proteins [215,216]. Following electrophoresis, SypA protein was
detected by western blotting using an anti-FLAG antibody. As shown in figure 21A, purified SypA incubated in kinase buffer alone (lane 2) migrated as a single species, corresponding to non-phosphorylated SypA. In contrast, upon co-incubation of SypA with SypE, two major bands were observed: both a lower, faster migrating SypA band (unphosphorylated SypA) and an upper, slower migrating band corresponding to phosphorylated SypA (SypA~P) (lane 3). This shifted SypA band was not observed upon resolving the reaction samples in parallel on gels lacking Phos-tag™ acrylamide, suggesting that the upper band observed in our Phos-tag™ gels represents phosphorylated SypA (data not shown). To confirm that the slower migrating, upper band observed indeed represented phosphorylated SypA, we co-incubated SypA with SypE in kinase buffer lacking ATP. As expected, only the lower (unphosphorylated) SypA band was present, indicating that the shifted SypA band represented phosphorylated SypA (Fig. 21A, lane 5). Together, these data demonstrate that SypE can phosphorylate SypA.

Phosphorylation of *B. subtilis* RsbV, and similar STAS-domain containing orthologs, occurs on a conserved serine residue [151], which in SypA corresponds to serine-56 (S56) [133](Fig. 19). To assess whether this conserved serine was required for SypA phosphorylation, I repeated the kinase reaction using a purified SypA mutant in which S56 was substituted with an alanine (SypA_{S56A}). The SypA_{S56A} sample migrated as a single, lower band, corresponding to unphosphorylated SypA (Fig. 21A, lane 4). These data suggest that phosphorylation of SypA by SypE depends on the predicted site of phosphorylation (S56) within SypA.
SypE’s serine kinase activity is proposed to reside in the protein’s N-terminal effector domain [212]. Unfortunately, attempts to assess the kinase activity of purified SypE N-terminal domain alone (SypE<sup>NTD</sup>) in vitro were unsuccessful, as the GST-SypE<sup>NTD</sup> protein failed to promote SypA phosphorylation in our in vitro assay (data not shown). As an alternative approach to assess whether the N-terminal domain of SypE possesses kinase activity, I asked whether expression of SypE’s N-terminal domain could promote SypA phosphorylation in <i>E. coli</i> cells. I introduced the GST-SypA plasmid into <i>E. coli</i> cells carrying either an empty vector (pVSV105) or a plasmid (pCLD64) expressing the untagged, N-terminal domain of <i>sypE</i> (SypE<sup>NTD</sup>), which we have previously shown is sufficient to inhibit biofilm formation (see Fig. 14). I then affinity-purified SypA from these SypE<sup>NTD</sup>-expressing cells and assessed the phosphorylation state of SypA. Whereas SypA purified from vector-containing cells appeared as a single, faster migrating band corresponding to non-phosphorylated SypA (Fig. 21B, lane 1), SypA purified from <i>sypE</i><sup>NTD</sup>-expressing cells predominately appeared as an upper, slower migrating band corresponding to phosphorylated SypA, although a small amount of unphosphorylated protein could be detected (Fig. 21B, lane 2). I conclude from these data that SypE, and specifically its N-terminal domain, indeed possesses serine kinase activity and can promote phosphorylation of SypA.

**In vitro analysis of SypA dephosphorylation by SypE**

The C-terminal domain of SypE exhibits sequence similarity to PP2C-like serine phosphatases, including the <i>B. subtilis</i> phosphatase RsbU. Given our finding that the N-terminal domain of SypE can phosphorylate SypA, I next questioned whether SypE’s C-
**Figure 20. Co-immunoprecipitation of SypE and SypA.** (A) Soluble lysates from ΔsypA ΔsypE cells [KV4716] carrying plasmids expressing FLAG-SypE (pARM80), HA-SypA (pARM36), or untagged SypE (pCLD48) and SypA (pARM13) control plasmids were used in immunoprecipitation assays with non-specific anti-rabbit IgG antibody (Lanes 1 and 5), anti-FLAG antibody (Lanes 2-4), or anti-HA antibody (Lanes 6-8). Samples were resolved using SDS-PAGE and subjected to western blot analysis with anti-FLAG (top panel) or anti-HA (bottom panel) antibodies. (+) indicates cells carrying the epitope tagged SypE (pARM80) and/or SypA (pARM36) plasmids. (-) indicates cells carrying control plasmids pCLD48 or pARM13. (B) Soluble lysates from ΔsypA ΔsypE cells [KV4716] carrying plasmids expressing HA-SypA (pARM36) and either FLAG-SypE<sup>NTD</sup> (pARM162) [Lanes 1 and 3] or FLAG-SypE<sup>NTD</sup> (pARM136) [Lanes 2 and 4] were used in immunoprecipitation assays with anti-FLAG or anti-HA antibody. Lanes 5 and 6, ΔsypA ΔsypE cells [KV4716] carrying both pARM36 and either pARM162 (lane 5) or pARM136 (lane 6) were immunoprecipitated with non-specific, anti-rabbit IgG. Samples were resolved using SDS-PAGE and subjected to western blot analysis with anti-FLAG (top panel) or anti-HA (bottom panel) antibodies.
Figure 21. *in vitro* phosphorylation of SypA by SypE. (A) *in vitro* phosphorylation of SypA by SypE. Purified SypA-FLAG (3 mg) and/or SypE proteins (2 mg) were incubated in kinase buffer in the presence or absence of ATP. Samples were resolved by SDS-PAGE on a 25 mM Phos-tag acrylamide gel and the proteins were detected via western blot analysis using an anti-FLAG antibody. Lane 1, SypE incubated in kinase buffer. Lanes 2 and 3, wild-type (WT) SypA-FLAG protein incubated in kinase buffer alone (Lane 2) or with SypE (Lane 3). Lane 4, SypA_{S56A}-FLAG protein incubated with SypE in kinase buffer. Lane 5, wild-type SypA-FLAG protein incubated with SypE in kinase buffer lacking ATP. (+) indicates reactions containing WT SypA-FLAG or SypE protein. (-) indicates reactions not containing purified protein. (S56A) indicates reactions containing SypA_{S56A} protein. (B) SypE-mediated phosphorylation of SypA in *E. coli* cells. SypA-FLAG protein was purified from *E. coli* cells carrying both pARM157 and either empty vector, pVSV105, (Lane 1) or plasmid pCLD64, which expresses the N-terminal, serine kinase domain of SypE (Lane 2). Samples were resolved using SDS-PAGE on a 25 mM Phos-tag™ acrylamide gel and proteins were detected by anti-FLAG western blot analysis. SypA~P denotes phosphorylated SypA.
Figure 22. SypE dephosphorylates SypA in vitro. Western blot analysis of in vitro SypA samples analyzed on Phos-Tag™ gels. Purified phosphorylated SypA protein (SypA~P; 2 mg) was incubated in Mg$^{2+}$-containing phosphatase buffer in the presence or absence of increasing concentrations of purified SypE C-terminal phosphatase domain (SypE$_{CTD}$; 2-10 mg). The reactions were terminated after 30' minutes and the samples resolved by SDS-PAGE on a 25 mM Phos-tag acrylamide gel. SypA proteins were detected by western blot analysis using an anti-FLAG antibody. Lane 1, SypE$_{CTD}$ incubated in buffer alone. Lane 2, non-phosphorylated SypA incubated in buffer alone. Lanes 3 and 7, phosphorylated SypA (SypA~P) incubated in buffer alone for 0' (Lane 3) and 30' (Lane 7) minutes. Lanes 4-6, phosphorylated SypA (SypA~P) incubated for 30' minutes in buffer containing 2 mg (Lane 4), 5 mg (Lane 5), or 10 mg (Lane 6) of purified SypE$_{CTD}$. (+) indicates reactions containing SypA-FLAG or SypE$_{CTD}$ protein. (-) indicates reactions not containing purified protein.
terminal domain possesses phosphatase activity and can dephosphorylate SypA~P. To address this question, I incubated phosphorylated SypA (SypA~P) protein in phosphatase buffer either in the presence or absence of the putative phosphatase domain of SypE (SypE<sup>CTD</sup>) and then assessed the phosphorylation state of SypA. Phosphorylated SypA incubated in phosphatase buffer alone (in the absence of SypE) remained largely phosphorylated as indicated by the presence of the upper (SypA~P) band (Fig. 22, Lanes 3 and 7), relative to the unphosphorylated SypA control (Fig. 22, Lane 2). Upon addition of increasing amounts of SypE<sup>CTD</sup>, I observed a decrease in the intensity of the upper (SypA~P) band and a corresponding increase in the lower, unphosphorylated SypA band (Fig. 22, Lanes 4-6). These data indicate that the C-terminal domain of SypE possesses phosphatase activity and is capable of dephosphorylating SypA <em>in vitro</em>. Together with the findings from the <em>in vitro</em> kinase assays, these results demonstrate that the N- and C-terminal domains of SypE indeed possess enzymatic activity and are sufficient to mediate SypA phosphorylation and dephosphorylation, respectively.

**Role of SypA in RscS-induced biofilm formation**

To investigate what role, if any, SypA may play in the regulation of <em>syp</em> biofilm formation, I first generated a <em>sypA</em> deletion strain and assessed biofilm formation upon introduction of the <em>rscS</em> expression plasmid (pARM7). In contrast to wild-type cells that displayed robust RscS-induced wrinkled colony morphology (Fig. 23B), the Δ<sup> </sup><em>sypA</em> mutant consistently failed to form biofilms, as indicated by the smooth colony morphology similar to vector-containing cells (Fig. 23C). To confirm that the loss of biofilm formation was indeed due to the absence of <em>sypA</em>, I introduced a wild-type copy
of \textit{sypA} into the chromosome at the Tn7 site. Single-copy expression of \textit{sypA} fully complemented the \textit{\Delta sypA} mutant (Fig. 23D). Together, these results identify SypA as an essential, positive regulator of RscS-induced biofilm formation.

As previously discussed, the activity of RsbV-like anti-sigma factor antagonists is regulated by the phosphorylation of a conserved serine residue, which inhibits protein activity [151,153]. Above, I determined that a conserved serine residue (S56) of SypA is required for SypA phosphorylation by SypE \textit{in vitro}. To address the role of S56 phosphorylation in regulating SypA activity, I generated \textit{sypA}\textsuperscript{S56D} and \textit{sypA}\textsuperscript{S56A} mutant alleles and assessed their ability to complement the \textit{\Delta sypA} mutant for biofilm formation. The aspartate substitution (SypA\textsuperscript{S56D}) was predicted to mimic the phosphorylated, inactive, state [151,217], while the alanine substitution (SypA\textsuperscript{S56A}) was predicted to “lock” the protein in the non-phosphorylated, active, state [151]. The \textit{sypA}\textsuperscript{S56D}–expressing strain was severely impaired in biofilm formation (Fig. 23E). This defect could not be attributed to poor expression, as a FLAG-tagged version of this mutant was present at steady-state levels similar to that of FLAG-tagged wild-type SypA protein and the SypA\textsuperscript{S56A} mutant (data not shown). In contrast to the aspartate mutant, the alanine substitution mutant (SypA\textsuperscript{S56A}) fully complemented the \textit{\Delta sypA} mutant, restoring wrinkled colony morphology (Fig. 23F). Together, these results indicate that S56 of SypA is critical for the regulation of SypA activity, and suggest that phosphorylation at this residue, possibly by SypE, inhibits SypA activity.
Epistasis analysis of SypA and SypE

Both SypA and SypE contribute to the regulation of syp biofilm formation. To further characterize the role of SypA in biofilm regulation, I next assessed whether SypA functions in the same regulatory pathway as SypE to control biofilm formation by performing an epistasis analysis. Briefly, I generated a sypA sypE double mutant and assessed the ability of this strain to form rscS-induced biofilms relative to that of the two single mutants. The ΔsypE strain exhibited wrinkled colony morphology similar to wild-type cells (Fig. 24), albeit with a slight delay in wrinkling (data not shown) as observed previously [212]. In contrast, the ΔsypA mutant failed to develop any observable biofilm phenotypes (Fig. 24). Similar to the sypA single mutant, the ΔsypA ΔsypE double mutant failed to produce biofilms (Fig. 24). Expression of sypA+ at the Tn7 site of the ΔsypA ΔsypE strain fully restored both wrinkled colony development to a level indistinguishable from the sypE single mutant (Fig. 24). These data demonstrate that SypA is epistatic to SypE, indicating that SypA functions downstream of SypE in the regulatory pathway controlling syp biofilm formation.

SypE inhibits biofilm formation by inactivating SypA.

The above results support the hypothesis that SypE functions upstream of SypA to regulate syp biofilm formation. In my genetic analysis of SypE, I demonstrated that expression of a sypED192A mutant constitutively inhibits biofilm formation in laboratory culture and impairs host colonization in vivo (Fig. 17 and Fig. 16, respectively). Additionally, the inhibitory activity of the SypED192A mutant required the protein’s N-terminal kinase domain, suggesting that SypED192A inhibits biofilm formation by
Figure 23. Role of SypA in biofilm formation. The RscS plasmid (pARM7) was introduced into wild-type (WT) or sypA strains. Cultures of the following strains were spotted onto LBS medium at 24°C and wrinkled colony formation was assessed at 48 h post-spotting: WT cells containing empty Tn7 cassette (EC) [KV4389] and carrying empty vector pKV282 (A) or pARM7 (B); pARM7-carrying ΔsypA cells containing EC [KV5079] (C), or complemented with WT sypA+ [KV5479] (D), sypA<sup>S56D</sup> [KV5480] (E), or sypA<sup>S56A</sup> [KV5481] (F). Images are representative of at least three independent experiments. Black bar represents 2 mm.
Figure 24. Epistasis analysis of SypE and SypA. Assessment of RscS-induced wrinkled colony formation. Cultures of the following strains were spotted onto LBS medium at 24°C and wrinkled colony formation was assessed at 48 h post-spotting: Wild-type (WT) cells containing empty Tn7 cassette (EC) [KV4389] and carrying empty vector pKV282 (A) or pRscS plasmid pARM7 (B); ΔsypE cells containing EC [KV4390] and carrying pARM7 (C); ΔsypA cells containing EC [KV5079] and carrying pARM7 (D); pARM7-carrying ΔsypA ΔsypE cells containing either EC [KV6392] (E) or WT sypA+ [KV6393] (F). Images are representative of at least three independent experiments. Black bar represents 2 mm.
constitutively activating the kinase domain to phosphorylate a downstream target protein (Fig. 15 and Fig. 16). Given the current findings, I hypothesized that SypE<sup>D192A</sup> inhibits biofilm formation by promoting the phosphorylation, and therefore the inactivation, of SypA. If so, then the “constitutively active”, non-phosphorylatable SypA<sup>S56A</sup> mutant, should be insensitive to SypE’s inhibitory activity; therefore, expression of sypA<sup>S56A</sup> should suppress the biofilm defect of the sypE<sup>D192A</sup> mutant.

To test this hypothesis, I generated <i>V. fischeri</i> strains expressing combinations of the <i>sypA</i> and <i>sypE</i> alleles: either the wild-type <i>sypA</i><sup>+</sup> or <i>sypA</i><sup>S56A</sup> allele (expressed from the native <i>sypA</i> locus) was combined with either the wild-type <i>sypE</i><sup>+</sup> or the constitutively inhibitory <i>sypE</i><sup>D192A</sup> allele (expressed at the Tn7 site of a <i>sypE</i> deletion mutant). As expected, SypE<sup>+</sup> strains expressing either <i>sypA</i> or <i>sypA</i><sup>S56A</sup> exhibited wrinkled colony morphology (Figs. 25A and B), while a strain that expressed the inhibitory <i>sypE</i><sup>D192A</sup> allele and wild-type <i>sypA</i> failed to produce biofilms (Figs. 25C). However, a strain expressing both the inhibitory <i>sypE</i><sup>D192A</sup> allele and the “constitutively active” <i>sypA</i><sup>S56A</sup> allele was fully competent to produce wrinkled colony morphology (Figs. 25D). These data indicate that expression of <i>sypA</i><sup>S56A</sup> suppresses the biofilm defect of the <i>sypE</i><sup>D192A</sup> mutant and further support my earlier epistasis experiments suggesting that SypA functions downstream of SypE to control biofilms.

**Analysis of SypA phosphorylation in vivo**

My genetic analyses suggested that the loss of biofilm formation in a SypE<sup>D192A</sup> mutant results from the inhibition of SypA activity, most likely mediated by the phosphorylation of SypA. I therefore asked whether SypA is indeed phosphorylated in <i>V. fischeri</i>. ...
fischeri (in vivo), and whether phosphorylation of SypA corresponds with loss of biofilm formation. To determine whether SypE<sup>D192A</sup> promotes SypA phosphorylation in vivo, I introduced plasmids expressing epitope-tagged SypA or SypA<sup>S56A</sup> into V. fischeri strains expressing either wild-type syphE or the inhibitory syphE<sup>D192A</sup> allele. I then assessed the in vivo phosphorylation state of the tagged SypA proteins using the Phos-tag™ SDS-PAGE assay coupled with western blot analysis. Cells expressing wild-type alleles of syphA and syphE consistently exhibited two bands: a predominant, lower band corresponding to unphosphorylated SypA and a faint, slower migrating band corresponding to phosphorylated SypA (SypA–P) (Fig. 26, Lane 2). In contrast, cells expressing syphA<sup>+</sup> and the inhibitory syphE<sup>D192A</sup> allele exhibited only the upper SypA–P band (Fig. 26, Lane 3), indicating that the majority of the SypA protein was phosphorylated under those conditions. As expected, cells co-expressing the syphA<sup>S56A</sup> mutant and either syphE<sup>+</sup> (Fig. 26, Lane 4) or syphE<sup>D192A</sup> (Fig. 26, Lane 5) exhibited only the lower band corresponding to non-phosphorylated SypA. These results corroborate my in vitro analyses indicating that residue S56 is required for SypA phosphorylation. Importantly, they demonstrate that SypA is indeed phosphorylated in vivo, and that SypA phosphorylation corresponds to inhibition of biofilm formation by SypE (e.g., in cells expressing inhibitory SyphE<sup>D192A</sup>).

**SypE inhibits symbiotic colonization by inactivating SypA.**

The above studies identified SypA as a critical regulator of biofilm formation in vitro, and previous work has shown a correlation between biofilm-formation and symbiotic colonization by *V. fischeri* [20,21]. Thus, I sought to verify the in vivo
Figure 25. A \textit{sypA}^{S56A} mutant suppresses the \textit{sypE}^{D192A} biofilm defect. Assessment of RscS-induced wrinkled colony formation. The pRscS plasmid pARM7 was introduced into Δ\textit{sypE} cells complemented with either wild-type \textit{sypE} or the inhibitory \textit{sypE}^{D192A} allele and expressing either wild-type \textit{sypA} or \textit{sypA}^{S56A}. Cultures of the following strains were spotted onto LBS medium at 24°C and wrinkled colony formation was assessed at 48 h post spotting: \textit{sypE}^{+} cells expressing wild-type \textit{sypA} [KV6213] (A) or \textit{sypA}^{S56A} [KV6215] (B); \textit{sypE}^{D192A} cells expressing either wild-type \textit{sypA} [KV6214] (C) or \textit{sypA}^{S56A} [KV6216] (D). Images are representative of at least three independent experiments. Black bar represents 2 mm.
Figure 26. Assessment of SypA phosphorylation in vivo. Western blot analysis of *V. fischeri* cell lysates analyzed on Phos-Tag™ gels. Soluble lysates from indicated *V. fischeri* strains were resolved by SDS-PAGE on 25 μM Phos-tag™ acrylamide gels and the proteins were detected by western blot analysis using anti-HA antibody. ΔsypA ΔsypE cells containing wild-type *sypE* [KV6424] and carrying pRscS plasmid (pCLD46) and plasmids expressing either untagged *sypA*⁺ (pARM13) (Lane 1), HA-tagged *sypA*⁺ (pARM36) (Lane 2), or HA-tagged *sypA*<sup>S56A</sup> (pARM78) (Lane 4); ΔsypA ΔsypE cells containing inhibitory *sypE*<sup>D192A</sup> [KV6425] and carrying pRscS plasmid (pCLD46) and plasmids expressing either HA-tagged *sypA*⁺ (pARM36) (Lane 3), or HA-tagged *sypA*<sup>S56A</sup> (pARM78) (Lane 5). (+) indicates cells expressing wild-type *sypA*-HA and/or *sypE*. (S56A) indicates cells expressing *sypA*<sup>S56A</sup>. (D192A) indicates cells expressing *sypE*<sup>D192A</sup>. (-) indicates cells expressing untagged *sypA*. Images are representative of at least three independent experiments.
relevance of sypA during host colonization using the various sypA mutant strains. First, I competed ΔsypA mutant cells against wild-type cells in mixed inoculation experiments, and found that the sypA mutant exhibited a dramatic competitive colonization defect (mean Log Relative Competitive Index (RCI), 1.38 +/- 0.51) (Fig. 27A). This defect in colonization could be abrogated by complementation with a wild-type allele of sypA+ (mean Log RCI, 0.15 +/- 0.47) (Fig. 27B). These data indicated that sypA is required for efficient symbiotic colonization of E. scolopes.

Next, I performed single-strain colonization assays. 18 h after exposure to V. fischeri, animals inoculated with wild-type cells contained bacterial levels of about 10^5 colony-forming units (CFU) per animal (mean= 1.2 x 10^5 cfu; Fig. 27C). In contrast, animals exposed to ΔsypA cells largely remained uncolonized, while those that were colonized contained significantly fewer (2-3 logs decreased) bacteria (mean= 3.9 x 10^2 CFU; Fig. 27C). As in the competitive colonization assays, we found that complementation with a wild-type allele of sypA+ restored colonization to levels similar to that of wild-type cells (mean= 8.1 x 10^4 cfu; Fig. 27C).

Our in vitro biofilm studies suggested that phosphorylation inhibits SypA activity. To explore the dependence of symbiotic colonization on unphosphorylated SypA, I assessed colonization by the ΔsypA mutant expressing the SypA_{S56D} protein, which mimics the phosphorylated state of SypA and failed to promote biofilms. As expected, the SypA_{S56D} strain exhibited a severe colonization defect, similar to that observed for the ΔsypA mutant (mean= 1.1 x 10^3 cfu; Fig. 27C). Together, these data suggest that
phosphorylation may serve to inhibit SypA activity, resulting in loss of biofilms and symbiotic colonization.

Finally, to test the hypothesis that SypE inhibits colonization through phosphorylation of SypA, I examined the ability of the “constitutively active” \( sypA^{S56A} \) allele to bypass the inhibition of colonization resulting from the \( sypE^{D192A} \) (constitutive kinase) allele. As previously observed, animals exposed to wild-type \( V. fischeri \) cells contained roughly \( 10^5 \) cfu/animal (mean= \( 6.8 \times 10^5 \) cfu; Fig. 28), while \( sypE^{D192A} (sypA^+) \) cells exhibited a severe defect in colonization: several animals remained un-colonized, while colonized animals exhibited a 2-3 log decrease in bacterial loads (mean = \( 5.66 \times 10^3 \) cfu; Fig. 28) [212]. In contrast, \( sypE^{D192A} \) cells expressing the \( sypA^{S56A} \) allele colonized to levels similar to wild-type \( V. fischeri \) (mean= \( 3.68 \times 10^5 \) cfu; Fig. 28).

These data demonstrate that the colonization defect of the \( sypE^{D192A} \) mutant can be suppressed by a constitutively active, SypE-insensitive derivative of SypA, indicating that the effect of SypE\(^{D192A}\) during colonization is to inhibit SypA activity. Furthermore, these results support my \textit{in vitro} biofilm assays, demonstrating that SypE functions through SypA to control both biofilms and colonization.

**Impact of SypA on syp locus activation**

To further investigate the positive role of SypA in promoting \( syp \)-dependent biofilm formation, I examined the effect of \( sypA \) expression on \( syp \) locus transcription. To determine if SypA regulates \( syp \) transcription, I utilized a \( sypA \) promoter-
\( lacZ \) fusion inserted in single copy in the chromosome of the wild-type and \( \Delta sypA \) strains, and then assessed reported activity upon introduction of the \( rscS \) plasmid (pCLD46).
Figure 27. Role of SypA in symbiotic colonization. (A and B) Competitive colonization with wild-type (WT) *V. fischeri* and *sypA* strains. Newly hatched squid were exposed to a mixed inoculum of WT and either Δ*sypA* [KV5079] cells (A) or Δ*sypA* cells complemented with *sypA*⁺ [KV5479] (B). The Log RCI is plotted on the x-axis. The position on the y-axis is arbitrary. Each circle represents a single animal. Closed circles indicate animals containing no *sypA* mutant cells. The black diamond and errors bars indicate the average Log RCI ± SD for indicated data set. Data shown are representative of at least three independent experiments. (C) Single-strain colonization by WT and *sypA* mutant strains. Newly hatched squid were exposed for 18 h to WT cells carrying empty vector (EC) [KV4389] or Δ*sypA* cells complemented with empty vector (EC) [KV5079], *sypA*⁺ [KV5479] or *sypA*₅₅₆D [KV5480]. As a negative control, aposymbiotic (APO) juvenile squid were maintained in bacteria free water. Each circle represents the number of *V. fischeri* cells recovered from an individual animal. The dashed line indicates the limit of detection (14 CFU/squid). The black bar indicates the average CFU for 10 animals. Data shown are from one experiment and are representative of at least three independent experiments.
**Figure 28. SypE inhibits colonization through SypA inactivation.** Single-strain colonization by wild-type (WT) *V. fischeri* and select mutant strains. Newly hatched squid were exposed for 18 h to either WT containing empty Tn7 cassette (EC) [KV4389] or *sypE<sup>D192A</sup>* mutant cells expressing wild-type *sypA* [KV6214] or *sypA<sup>S56A</sup>* [KV6216]. As a negative control, aposymbiotic (APO) juvenile squid were maintained in bacteria free water. Each circle represents the number of *V. fischeri* cells recovered from an individual animal. The dashed line indicates the limit of detection. The black bar indicates the average CFU for 10 animals. Data shown are from one experiment and are representative of at least three independent experiments.
Figure 29. Impact of SypA on syp locus activation. Transcription of the syp locus was monitored using a β-galactosidase activity assay. A transcriptional reporter construct consisting of the sypA promoter region fused upstream of a promoter-less lacZ gene was inserted at the chromosomal Tn7 site of wild-type [KV3246] or ΔsypA [KV4925] cells carrying pRscS plasmid (pCLD46) and either the pSypA plasmid (pARM13) or empty vector pKV282. Vector corresponds to pKV282 or, in the case of wild-type carrying two vectors, pVSV105 and pKV282. Cells were grown in LBS containing Tc and Cm for 24 h. Results shown are representative of at least 3 independent experiments. Error bars indicated the standard deviation.
Overexpression of \( rscS \) in either the WT or \( \Delta sypA \) reported strains resulted in similar levels of reporter activity, approximately 10-fold induction over vector control cells (Fig. 29), indicating that loss of \( sypA \) does not affect \( syp \) activation. Similarly, introduction of a \( sypA \) overexpression plasmid (pARM13) into the \( rscS \)-overexpressing strains had no impact on reporter activity in either a wild-type or \( \Delta sypA \) background (Fig. 29).

Together, these data demonstrate SypA does not contribute to the regulation of \( syp \) transcription, indicating that SypA, and thus SypE, regulates biofilm formation downstream of \( syp \) activation.

**Summary**

Based on sequence similarity partner switching regulatory proteins, I hypothesized that SypE controls biofilms and colonization through regulation of the activity of the \( syp \)-encoded regulator, SypA. Using a co-immunoprecipitation approach, I demonstrated that SypE interacts with SypA \textit{in vivo}, in a manner dependent upon its N-terminal, serine kinase domain. Furthermore, I established \textit{in vitro} and \textit{in vivo} assays to confirm that SypE’s N- and C-terminal effector domains indeed possess serine kinase and serine phosphatase activities, respectively, and that SypA serves as a target of these opposing activities. Mutational analyses determined that SypA plays an essential role in promoting biofilm and host colonization. Importantly, I determined that the ability of SypE to inhibit biofilm formation and host colonization is dependent upon its phosphorylation, and thus inactivation, of SypA. Together, these data indicate that regulation of SypA activity by SypE is an important mechanism by which \textit{V. fischeri} controls biofilm formation and symbiotic colonization. This study investigating the
interaction between SypE and SypA, and their impact on biofilm formation and colonization, was published in Molecular Microbiology [213].

THE ROLE OF SypE AS A NEGATIVE REGULATOR OF SypG-INDUCED BIOFILMS

Introduction

Previous studies established that biofilm formation in *V. fischeri* can be induced by the overexpression of either *rscS* or *sypG*. Although overexpression of *rscS* and *sypG* similarly induce activation of the *syp* locus in wild-type cells, SypG fails to promote the robust biofilm phenotypes (i.e. wrinkled colony or pellicle formation) as observed for *rscS*-expressing cells [27]. As previously discussed, the inability of *sypG* overexpression to induce biofilm formation is due to the inhibitory activity of SypE: overexpression of *sypG* in a Δ*sypE* mutant resulted in dramatic biofilm formation similar to that observed with *rscS*-overexpressing wild-type cells [27]. This suggests that *sypG* overexpression fails to mimic the effects of *rscS* in regards to the regulation of SypE activity.

In the previous chapters, I investigated the regulation of biofilms induced by overexpression of *rscS*, a condition in which SypE functions as a positive regulator. Therefore, assessment of SypE’s inhibitory activity required the use of mutant *sypE* alleles (e.g. a SypE<sup>D192A</sup> mutant). To better understand the mechanism by which SypE negatively regulates biofilm formation, I also examined SypE regulatory activity under *sypG* overexpression conditions, in which SypE naturally inhibits biofilm production. Using this approach, I asked whether SypE’s ability to naturally inhibit biofilms induced by *sypG* overexpression occurs through mechanisms similar to those observed with *rscS*
overexpression. In particular, I investigated whether the N-terminal, serine kinase domain of SypE was also required to mediate inhibition of SypG-induced biofilm formation. Additionally, I asked whether the inability of *sypG* overexpression to induce biofilms was due to the inhibition of SypA activity by SypE.

**Negative regulation of SypG-induced biofilms by SypE**

To better understand the mechanism by which SypE inhibits biofilms under *sypG*-overexpressing conditions, I utilized a complementation approach similar to that described previously (see part one of this chapter). Using the collection of *sypE* alleles that contained or lacked the individual SypE domains, I assessed the domains of SypE required for inhibition of SypG-induced biofilm phenotypes. I utilized a dual plasmid complementation approach, in which I co-expressed *sypG* and the mutant *sypE* alleles in a Δ*sypE* mutant and assessed complementation (i.e. restoration of biofilm inhibition). As previously reported, wild-type cells failed to produce biofilms, indicated by the smooth colony morphology on solid agar media (Fig. 30A), while Δ*sypE* cells exhibited dramatic SypG-induced biofilm phenotypes (Figs. 30B) [27]. Co-expression of wild-type *sypE* (pCLD48) fully complemented the *sypE* mutant and restored inhibition of biofilms, resulting in smooth colony morphology similar to wild-type cells (Fig. 30C).

I next examined the regulatory activity of a SypE^{ANTD} mutant (pARM3) that lacks the N-terminal kinase domain, and found that it failed to complement the *sypE* mutant, but instead permitted wrinkled colony formation similar to vector-containing cells (Figs. 30D). Similarly, expression of the C-terminal domain alone (pCLD67) failed to complement the *sypE* mutant and inhibit biofilm formation (Figs. 30E).
Figure 30. Regulation of SypG-induced biofilms by SypE. The SypG expression plasmid (pCLD56) was introduced into either wild-type cells [A] or ΔsypE mutant cells [KV3299] carrying either empty vector (pVSV105) [B] or the indicated SypE-complementing plasmids: full-length SypE (pCLD48)[C], SypE<sup>NTD</sup> (pARM3)[D], SypE<sup>CTD</sup> (pCLD67)[E], SypE<sup>NTD</sup> (pCLD64)[F], SypE<sup>N52A</sup> (pARM4)[G], SypE<sup>NTD, N52A</sup> (pCLD65)[H]. Cultures were spotted onto agar plates and wrinkled colony morphology was assessed at 48 h post-spotting. Images are representative of at least three independent experiments.
These results indicate that inhibition of SypG-induced biofilms requires the N-terminal domain of SypE. In my earlier analysis of SypE, I demonstrated that both the \(sypE^{\text{ANTD}}\) and \(sypE^{\text{CTD}}\) alleles were capable of promoting biofilms induced by \(rscS\) overexpression, indicating the resulting proteins were indeed produced and retained positive activity (see part one of this chapter). Although the \(sypE^{\text{ANTD}}\) and \(sypE^{\text{CTD}}\) mutants retained positive regulatory activity under \(rscS\)-overexpressing conditions, this activity was not apparent under our current (\(sypG\) overexpressing) conditions (i.e. expression of these alleles did not enhance or increase the rate of biofilm formation).

To further investigate the inhibitory role of the N-terminal domain, I assessed the inhibitory activity of a SypE\(^{\text{NTD}}\) mutant (pCLD64), which expresses only the N-terminal serine kinase domain and was shown to inhibit RscS-induced biofilms (Fig. 14). Similar to full-length SypE, SypE\(^{\text{NTD}}\) fully complemented the \(sypE\) mutant, restoring inhibition of wrinkled colony formation (Fig. 30F). Together, these data support the results from my \(rscS\)-overexpression studies, and demonstrate that the N-terminal domain of SypE is both necessary and sufficient to inhibit SypG-induced biofilm formation.

In my earlier analysis of SypE function, I found that the ability of SypE to inhibit RscS-induced biofilms required an intact N-terminal domain. In particular, a conserved asparagine (N52) was required for a mutant SypE variant to inhibit biofilms induced by RscS (Fig. 14). In characterized HPK-like serine kinases, this asparagine is required for catalytic kinase activity [151,179]. To ask whether the ability of SypE to inhibit SypG-induced biofilms also required this conserved asparagine, I assessed the inhibitory activity of a SypE\(^{\text{N52A}}\) mutant (pARM4), which contains an alanine substitution at this
site. As expected, expression of sypE<sup>N52A</sup> failed to complement the ΔsypE mutant: the colonies exhibited strong SypG-induced wrinkling (Figs. 30G). Similarly, this mutation in the context of the inhibitory N-terminal domain alone, SypE<sup>NTD, N52A</sup> (pCLD65), resulted in the loss of inhibitory activity: cells co-expressing sypE<sup>NTD, N52A</sup> and sypG exhibited robust wrinkled colony morphology (Figs. 30H). The failure to complement cannot be attributed to protein instability, as epitope-tagged versions of both the SypE<sup>N52A</sup> and SypE<sup>NTD, N52A</sup> proteins are stably expressed (data not shown)[212]. These results demonstrate that the inhibitory activity of SypE’s N-terminal, kinase domain requires conserved residue N52, consistent with that seen for other characterized HPK-like serine kinases. Together, these data support my earlier studies of SypE function under RscS activating conditions, and suggest that SypE functions as an inhibitory kinase to negatively regulate biofilm formation.

**Co-overexpression of sypG and sypA overcomes inhibition by SypE**

Our results indicate that SypE inhibits SypG-induced biofilms through the activity of its N-terminal, serine kinase domain. These findings suggest that SypE primarily functions as a kinase under SypG-inducing conditions, and likely inhibits biofilms by phosphorylating a downstream target protein. I hypothesized that SypE exerts negative control over SypG-induced biofilms by phosphorylating SypA [213]. I reasoned that if SypE prevents SypG-induced biofilms through phosphorylation of SypA, then co-overexpression of sypG and sypA may result in sufficiently high levels of SypA such that some of it could escape phosphorylation and thus inhibition by SypE. To test this, I co-overexpressed sypA and sypG from compatible plasmids in a wild-type (sypE<sup>+</sup>)
background and assessed biofilm formation. As controls, I first evaluated strains overexpressing either \( sypG \) or \( sypA \) alone, and found that biofilm formation was not induced (Fig. 31A and B). In contrast, cells co-overexpressing both \( sypG \) and \( sypA \) exhibited robust biofilm formation (Fig. 31C). These data indicate that providing excess SypA overcomes the inhibition of SypG-induced biofilms by SypE, and further suggest that SypA is the target for SypE’s inhibitory, kinase activity. In addition, the fact that biofilms were only induced upon co-overexpression of \( sypG \) and \( sypA \), but not when \( sypA \) was overexpressed alone, indicates that \( sypG \) is still required to induce \( syp \) transcription and the production of the Syp structural proteins.

**SypE inhibits SypG-induced biofilms through phosphorylation of SypA**

The previous results indicated that the inhibition of biofilm formation by SypE depends upon its ability to phosphorylate SypA. I reasoned that if this is correct, then expression of a SypA mutant (SypA^{S56A}) that cannot become phosphorylated should bypass the inhibition of SypG-induced biofilms by SypE. I thus assayed SypG-induced biofilm formation by cells that expressed from single copy in the chromosome the mutant protein SypA^{S56A}; this protein contains a substitution at a conserved serine required for phosphorylation, and fails to become phosphorylated either *in vitro* or *in vivo* when \( rscS \) is overexpressed (see part two of this chapter)[213]. I found that \( sypE^{+} \) cells expressing SypA^{S56A}, but not those that expressed wild-type \( sypA \) or contained the empty cassette, formed robust biofilms upon overexpression of \( sypG \) (Fig. 32). These data demonstrate that the non-phosphorylatable SypA^{S56A} mutant is insensitive to the inhibitory activity of
SypE, thus supporting the hypothesis that SypE inhibits the formation of SypG-induced biofilms via phosphorylation of SypA.

To assess directly whether SypA becomes phosphorylated under SypG-inducing conditions, I evaluated the *in vivo* phosphorylation state of SypA under both biofilm-inhibitory conditions (i.e. *sypG*-overexpressing wild-type cells) and biofilm-permissive conditions (i.e. *sypG*-overexpressing Δ*sypE* cells). Briefly, I introduced an epitope-tagged wild-type *sypA* allele (containing a C-terminal HA tag) in single copy in the chromosome (at the Tn7 site) of either Δ*sypA* cells or Δ*sypA* Δ*sypE* cells. I then overexpressed *sypG* and assessed the *in vivo* phosphorylation state of SypA by resolving cell lysates on SDS-PAGE gels containing Phos-tag™ acrylamide. SypA-HA proteins were detected by western blot analysis using an anti-HA antibody. As observed in figure 33, *sypG*-overexpressing wild-type cells (lane 2) exhibited a single, upper band corresponding to phosphorylated SypA (SypA~P). These data indicate that in wild-type cells overexpressing *sypG*, the majority of, if not all, SypA protein is in the phosphorylated state. In contrast, *sypG*-overexpressing Δ*sypE* cells (which are competent to produce biofilms) exhibited a single, lower band representing unphosphorylated SypA (Fig. 33, lane 3). Complementation with a wild-type allele of *sypE* (SypE⁺) restored SypA phosphorylation as indicated by the presence of the shifted SypA band (SypA~P) (Fig. 33, lane 4). Finally, I confirmed that the SypA<sup>S56A</sup> mutant also fails to become phosphorylated in *sypG*-overexpressing cells (Fig. 33, lane 5). These results demonstrate that SypE promotes SypA phosphorylation under SypG-inducing conditions, and
Figure 31. Co-overexpression of *sypG* and *sypA* induces biofilm formation. Biofilm formation by wild-type ES114 carrying either the *sypG* overexpression plasmid (pARM9) [A], the *sypA* overexpression plasmid (pARM13) [B], or both [C]. For A and B, the indicated vectors are pKV282 and pVSV105, respectively. The strains were cultured in LBS broth containing Tet and Cm. Cultures were spotted onto agar plates and wrinkled colony morphology was assessed at 48 h post-spotting. Images are representative of at least three independent experiments.
Figure 32. A *sypA* S56A mutant permits SypG-induced biofilm formation.
Assessment of SypG-induced wrinkled colony formation by *sypG*-overexpressing (pCLD56) wild-type cells (A), and *sypG*-overexpressing Δ*sypA* cells complemented with either wild-type *sypA*+ [KV5479] (B) or the *sypA*S56A allele [KV5481] (C). Cultures were spotted onto LBS medium containing Cm at 28°C and wrinkled colony formation was assessed at 48 h post spotting. Images are representative of at least three independent experiments.
Figure 33. Phosphorylation of SypA under SypG-inducing conditions. Western blot analysis of *V. fischeri* cell lysates resolved on Phos-tag™ gels. Soluble lysates from indicated strains were resolved by SDS-PAGE on 30 µM Phos-tag™ acrylamide gels and the proteins were detected by western blot analysis using anti-HA antibody. SypG-expressing (pCLD56) ΔsypA cells containing untagged *sypA* [KV5479] [lane 1] or HA-tagged wild-type *sypA* [KV6578] [lane 2]; ΔsypA ΔsypE cells expressing HA-tagged wild-type *sypA*⁺ [KV6580] and carrying pCLD56 [lane 3]; ΔsypA ΔsypE expressing HA-tagged wild-type *sypA*⁺ [KV6580] and carrying pCLD56 and pSypE plasmid (pCLD48)[lane 4]; ΔsypA cells expressing HA-tagged *sypA*⁺[KV6579] and carrying pCLD56 [lane 5]. (+) indicates cells expressing wild-type *sypA*-HA and/or *sypE*. (S56A) indicates cells expressing *sypA*⁺[S56A]. (-) indicates cells expressing untagged *sypA* or deleted for *sypE*. Images are representative of at least three independent experiments.
corroborate my biofilm assays indicating that, under SypG-inducing conditions, SypE functions as a kinase. Importantly, they verify that phosphorylation of SypA is critical for biofilm inhibition.

**Impact of SypE on syp locus activation**

Previous studies examining the regulation of the syp locus suggested that SypE may impact syp activation induced by sypG overexpression [27]. However, my results demonstrate that SypE regulates biofilm formation through control of SypA activity, which impacts biofilms downstream of syp activation. To address this apparent discrepancy and further characterize the regulatory role of SypE, I assessed the impact of the various sypE mutant alleles on SypG-induced syp transcription. I utilized a sypA promoter-lacZ fusion inserted in single copy in the chromosome of the wild-type and ΔsypE strains. I then assessed sypA promoter activity by measuring β-galactosidase activity upon co-expression of sypG and the sypE alleles.

As previously observed [27], wild-type cells overexpressing sypG exhibited high levels of syp transcription, as indicated by the significant increase in β-galactosidase activity relative to vector control cells (Fig. 34). Compared to the wild type, cells deleted for sypE exhibited a moderate (~1.5-fold), but consistent, increase in SypG-induced syp transcription (Fig. 34). Co-expression of wild-type sypE and sypG in the ΔsypE mutant restored syp transcription to near wild-type levels (Fig. 34). These results suggested that SypE does have a minor effect on SypG-induced syp transcription.

To further determine whether this effect on syp transcription could account for the observed biofilm phenotypes, I asked whether inhibition of transcription required the
SypE’s inhibitory N-terminal domain. I first tested whether a \textit{sypE} mutant lacking the inhibitory, N-terminal domain (SypE^{ANTD}), and thus unable to inhibit biofilm formation, impacted activation of \textit{syp} transcription by SypG. Surprisingly, I found that co-expression of SypE^{ANTD} and \textit{sypG} in Δ\textit{sypE} cells resulted in a decrease in \textit{syp} transcription similar to cells expressing wild-type \textit{sypE}, a result opposite to the observed biofilm phenotype (Fig. 34). These results suggested that while SypE may have a slight impact on \textit{syp} transcription, it is not sufficient to account for the observed effects of SypE on SypG-induced biofilms. In support of this, I found that expression of the N-terminal domain alone (SypE^{NTD}), which is sufficient to inhibit SypG-induced biofilm formation, had no observable impact on \textit{syp} transcription (Fig. 34). These data reveal that SypE indeed has a minor effect on \textit{syp} transcription, but that this effect is not sufficient to account for the dramatic effects on biofilm formation.

**Deletion of \textit{sypE} confers a competitive colonization advantage.**

The ability of \textit{V. fischeri} to produce \textit{syp}-dependent biofilms in laboratory culture directly correlates with the ability of the bacteria to efficiently colonize host juvenile squid [20,22,212]. We previously observed that the deletion of \textit{sypE} in otherwise wild-type cells did not significantly impact host colonization (Fig. 16)[33,212]. However, I questioned whether the presence of \textit{sypE} would impact colonization when \textit{sypG} is overexpressed. In other words, would the ability of the \textit{sypG}-overexpressing \textit{sypE} mutant cells to produce robust biofilms also promote colonization competence, or would the absence of \textit{sypE} have no impact? To address this question, I examined the ability of
**Figure 34. Impact of SypE on syp locus activation.** Transcription of the *syp* locus was monitored using a β-galactosidase activity assay. A transcriptional reporter construct consisting of the *sypA* promoter region fused upstream of a promoterless *lacZ* gene was inserted at the chromosomal Tn7 site of wild-type [KV3246] or Δ*sypE* [KV4926] cells carrying the pSypG plasmid (pCLD56) and indicated SypE expression plasmids: wild-type SypE (pCLD48), SypE<sup>ANTD</sup> (pARM3), and SypE<sup>NTD</sup> (pCLD64). Vector corresponds to pVSV105 or, in the case of wild-type carrying two vectors, pVSV105 and pKV282. Cells were grown in LBS containing Tc and Cm for 24 h. Results shown are representative of at least 3 independent experiments. Error bars indicated the standard deviation.
Figure 35. Deletion of sypE promotes host colonization. Competitive colonization assay with sypG-overexpressing wild-type (WT) and sypE mutant strains. Newly hatched squid were exposed to a mixed inoculum of WT carrying the pSypG plasmid (pCLD56) and either ΔsypE [KV4390] cells (A) or ΔsypE cells complemented with wild-type sypE+ [KV4819] (B) and carrying pCLD56. The Log RCI is plotted on the x-axis. The position of the circles on the y-axis is merely for spacing. Each circle represents a single animal. Open symbols indicate animals containing no WT cells. The black diamond and error bars indicate the average Log RCI and standard deviation for the indicated data set. Data shown are representative of at least three independent experiments.
sypG-overexpressing wild-type and ΔsypE strains to competitively colonize juvenile squid. I found that ΔsypE cells dramatically outcompeted wild-type cells for host colonization (Fig. 35A). These data suggest that upon overexpression of sypG, deletion of sypE confers a competitive colonization advantage. To confirm that the loss of sypE was responsible for the colonization advantage observed in the ΔsypE strain, I complemented the ΔsypE strain with a wild-type allele of sypE in trans in the chromosome. Indeed, the SypE-complemented (SypE+) cells failed to outcompete wild-type cells, when both were overexpressing sypG (Fig. 35B). These results reveal a critical role for SypE in inhibiting SypG-induced biofilm formation and host colonization.

Summary
In this portion of my dissertation, I continued my analysis of SypE function by examining the regulatory activity of SypE under sypG overexpression conditions, in which SypE naturally inhibits biofilm formation. Through complementation studies, I determined that SypE inhibits SypG-induced biofilms through the activity of its N-terminal, serine kinase domain. Further analysis determined that the inhibitory activity of the N-terminal domain required residue N52, a conserved asparagine that is necessary for kinase activity in other characterized serine kinases [95]. These results parallel those observed for the role of SypE in RscS-induced biofilm formation: expression of SypE^{NTD} alone also inhibited biofilm formation in a manner that depended on N52.

Next, I used both genetic and biochemical approaches to test the hypothesis that SypE inhibited SypG-induced biofilms by promoting the phosphorylation, and thus
inactivation, of SypA. I found that the inhibition of SypG-induced biofilms by SypE could be overcome by co-overexpressing sypA and sypG. This result suggested that providing high levels of SypA overcomes the inhibitory activity of SypE, presumably because some SypA escapes phosphorylation. In support of this idea, I demonstrated that expression from the chromosome of a sypA mutant that does not become phosphorylated (SypA^{S56A}) also permitted the formation of SypG-induced biofilms in wild-type (SypE^{+}) cells. Phos-tag\textsuperscript{TM} analysis confirmed that SypA indeed was predominantly phosphorylated under biofilm-inhibiting conditions (i.e., upon overexpression of sypG in wild-type cells), but unphosphorylated under biofilm-permissive conditions (i.e. upon overexpression of sypG in ΔsypE cells). Together, these studies demonstrate that under SypG-inducing conditions, SypE functions as an inhibitory kinase, and prevents biofilms by phosphorylating, and thus inactivating, SypA.

Finally, I observed that the enhanced ability of the ΔsypE strain to form biofilms upon overexpression of sypG provided these cells with a competitive colonization advantage, presumably due to enhanced aggregation outside of the symbiotic light organ. This work emphasizes the critical role of SypE in restricting syp biofilm formation, and subsequently symbiotic colonization.
CHAPTER FOUR
DISCUSSION

The goal of my dissertation was to characterize the mechanism by which the novel RR SypE regulates syp-dependent biofilm formation and, as a result, symbiotic colonization by *V. fischeri*. My interest in characterizing SypE was initially prompted both by its novel domain architecture and by earlier studies suggesting that SypE may both negatively and positively impact biofilm formation [27,28]. Bioinformatic analyses predicted that SypE was an unusual RR, with its three putative domains arranged in a unique configuration: a central REC domain flanked by two effector domains with apparently opposing activities. Based on these data, I hypothesized that SypE could exert dual regulatory control over syp biofilm formation, depending on which of the terminal effector domains was active. Given their similarity to regulatory proteins found in partner-switching systems, I speculated that SypE might control the activity of another syp-encoded regulator, SypA. Finally, I proposed that the mechanisms that I uncovered would provide insights into the process by which *V. fischeri* colonizes its symbiotic host. All of my hypotheses were supported by the work described in this dissertation. In addition, I made several unexpected and novel observations that I discuss in more detail below.
Characterization of SypE as a dual regulator of biofilm formation

To understand how SypE’s individual domains contributed to the protein’s apparent dual regulatory activities, I performed a genetic analysis by generating a variety of truncation and point mutant sypE alleles, expressing them in V. fischeri, and analyzing the resulting biofilm phenotypes. Using the truncation and deletion mutants, I found that SypE indeed possesses both negative and positive regulatory activities that are mediated by the N- and C-terminal effector domains, respectively. I also determined that these effector domains likely function as a serine kinase and serine phosphatase, respectively, using point mutants predicted to be disrupted for these activities.

Additionally, I investigated the putative roles of these domains through biochemical approaches. Experiments to test the putative enzymatic activities of SypE confirmed that SypE’s N-terminal serine kinase domain does, in fact, function as a kinase, while the C-terminal serine phosphatase domain exhibits phosphatase activity (Fig. 21 and 22, respectively). Although I was unable to observe kinase activity of the purified (GST-tagged) N-terminal domain of SypE in vitro, I did find that expression of SypE’s untagged N-terminal domain promoted phosphorylation of its target substrate in E. coli cells (Fig. 21B). Specifically, I determined that SypE controls the phosphorylation state of the syp-encoded protein, SypA. These findings corroborate my genetic analysis of SypE and allow us to assign kinase and phosphatase activity to SypE’s N- and C-terminal domains, respectively.

Although each domain was necessary and sufficient for the respective activities, my data suggested that one domain could influence the activity of another. In particular,
mutations within the C-terminal domain not only resulted in loss of SypE positive activity but, in the context of the full-length protein, shifted the protein’s activity to an inhibitory state. For example, expression of the \( \text{sypE}^{\text{D443A}} \) or \( \text{sypE}^{\text{D495A}} \) alleles, which were predicted to lack the positive activity and thus behave in a manner similar to vector control, completely inhibited biofilm formation (Figs. 14E and 14F). This inhibitory activity depended on an intact N-terminal domain, as a SypE\(^{\text{N52A,D443A}}\) double mutant exhibited a loss of inhibitory activity (Fig. 14K). I interpret these data to suggest that the SypE protein structure is sensitive to molecular perturbations. In support of this, I found that introduction of a HA-epitope tag to the C-terminus of the SypE protein also caused aberrant protein activity, such that the protein appeared “locked” in the constitutive inhibitory state (data not shown). I speculate that disruption of the C-terminal effector domain likely alters the protein conformation to favor activation of the inhibitory N-terminal domain, perhaps indirectly by impacting phosphorylation of the protein. This would not be unprecedented: mutations within the C-terminal DNA-binding effector domain of the RR OmpR not only affect DNA-binding, but also impact the phosphorylation state of the N-terminal REC domain [218]

In contrast to the C-terminus, disruption of the inhibitory, N-terminal domain of SypE did not appear to “switch” protein activity (i.e. constitutively activate the positive, C-terminal domain). It is possible that mutation of SypE’s N-terminal domain does indeed promote the activity of the positive, C-terminal domain, but that this effect is not apparent under the experimental conditions tested. Indeed, my data indicate that SypE functions as a positive regulator under RscS-inducing conditions, suggesting that the
inhibitory domain is typically inactivated under these conditions. Therefore, a mutation in the inhibitory domain of SypE is not likely to produce an observable effect on protein function. Together, these data indicate that the terminal effector domains of SypE are indeed active and, more importantly, exert opposing regulatory control over syp-dependent biofilm formation.

**Regulation of SypE activity by the central REC domain**

I next tested the hypothesis that SypE’s central REC domain regulates the opposing activities of the terminal domains. In general, RR activity is controlled through the phosphorylation of a conserved aspartate residue located in the REC domain [71]. REC domains are proposed to exist in an equilibrium of conformational states, which either activate or inactivate an attached effector domain [76]. Phosphorylation is proposed to shift this equilibrium and promote a particular conformational state, resulting in RR activation or inactivation [76]. My results indicate that an intact REC domain is required to promote the positive regulatory activity of SypE. I found that a SypE mutant that mimics the unphosphorylated state (SypE\textsuperscript{D192A}) exhibits constitutive inhibitory activity, thus preventing biofilm formation (Fig. 15E). I interpret these data to indicate that phosphorylation of SypE at residue D192 is required to overcome SypE’s default inhibitory activity (Fig. 36). In support of this hypothesis, the inhibitory activity of the D192A mutant indeed depended upon an intact N-terminal domain. Interestingly, the loss of inhibitory activity in the SypE\textsuperscript{N52A,D192A} mutant failed to permit the protein to exert its positive activity (Fig. 13L), suggesting that phosphorylation at residue D192 was still required for activation of the C-terminal domain and, thus, positive activity. I
similarly found that the conserved aspartate was required for positive regulatory activity displayed by a SypE mutant lacking the N-terminus (SypE$^{\text{ANTD}}$) (data not shown). Based on these data, I propose that REC phosphorylation shifts the equilibrium of the SypE conformational states such that the inhibitory N-terminus is inactivated, while the positively acting C-terminus is activated (Fig. 36). In my assessment of the REC domain, I attempted to identify mutations that would mimic the phosphorylated state of SypE, resulting in “constitutively active” SypE and dominant positive activity. However, I found that several mutations reported to mimic the phosphorylated state in select RRs, such as a SypE$^{\text{D192E}}$ [219] or a SypE$^{\text{D192E,D150K}}$ [220] mutant, failed to result in the constitutive activation of the positive C-terminal domain (data not shown). The inability of these mutations to result in a SypE mutant that mimics the phosphorylated state is not surprising, as few mutations have been found to universally activate all RRs [221]. Indeed, these common mutations have failed to activate several well-characterized RRs [74,222,223]. I hypothesize that, due to the complex domain structure of SypE, these mutations likely fail to mimic the conformational changes induced and/or stabilized by phosphorylation.

While my genetic evidence strongly suggests a role for phosphorylation in controlling SypE activity, confirmation that SypE indeed becomes phosphorylated awaits further investigation. In preliminary studies using V. fischeri strains expressing an epitope-tagged SypE, I attempted to assess the in vivo phosphorylation state of SypE under biofilm promoting or inhibiting conditions. However, the results from these
Figure 36. Model of *syp* biofilm regulation by SypE. (i) The RscS-SypG pathway induces transcription of the *syp* polysaccharide locus to promote biofilm formation and colonization. (ii) Under non-RscS activating conditions (i.e. upon induction with SypG), SypE functions as a serine kinase to phosphorylate SypA and inhibit biofilms and colonization. (iii) Under RscS activating conditions, phosphorylation of SypE is proposed to inactivate the inhibitory kinase domain, while promoting activation of the positive C-terminal phosphatase (PPase) domain. Phosphorylated SypE promotes the dephosphorylation, and thus activation, of SypA, resulting in biofilm formation and colonization. The dashed arrow indicates potential direct phosphorylation of SypE by RscS, resulting in inactivation of SypE inhibitory activity.
studies were inconclusive (data not shown). The difficulty in detecting SypE phosphorylation in vivo is not particularly surprising, given the labile nature of the phospho-Asp bond, which typically undergoes rapid hydrolysis [224]. Therefore, future studies of SypE phosphorylation may require the development of in vitro phosphotransfer assays using purified SypE and potential phosphodonors, such as acetyl phosphate or purified HK proteins (e.g. RscS).

**SypE as a negative regulator of symbiotic colonization**

In this work, I established a critical role for SypE in the regulation of symbiotic colonization of *E. scolopes.* Using the *sypE* mutant strains characterized in the in vitro biofilm assays, I assessed whether SypE’s positive and/or negative regulatory activities contributed to *V. fischeri* colonization fitness. My results indicate that the minor positive role of SypE in promoting biofilm formation is relatively unimportant, as deletion of *sypE* did not substantially affect host colonization (Fig. 16). It remains possible that the positive activity of SypE does indeed contribute to colonization fitness, but that this impact may not be readily evident given our current experimental conditions. Indeed, my in vitro biofilm studies indicate that loss of SypE’s positive activity only results in a delay in biofilm development (Fig. 10). This positive impact of SypE on RscS-induced biofilms is subtle, and not readily apparent at late time points (Fig. 10). Given these findings, it is possible that SypE may also have a subtle, positive impact on colonization, and therefore time course assays may also be necessary to assess this effect.

In contrast to the positive activity, I found that inhibition by SypE is a significant activity that must be overcome during the colonization process. *V. fischeri* cells
expressing SypE\textsuperscript{D192A}, a mutant disrupted at the predicted site of phosphorylation, exhibited a severe defect in host colonization (Fig. 16), which could be attributed to the inability of these cells to efficiently aggregate in the squid-derived mucus on the light organ surface, an essential stage in the initiation of host colonization (Fig. 17C). Thus, residue D192 within the central REC domain is absolutely required to inactivate the inhibitory activity of SypE. I propose that phosphorylation at residue D192 is necessary to shift the activity of SypE away from the inhibitory N-terminal domain, and permit activation of the positive C-terminal domain. Consistent with this hypothesis and my biofilm results, the inhibition of both host colonization and symbiotic aggregation by the SypE\textsuperscript{D192A} mutant depended upon an intact N-terminal domain, as a SypE\textsuperscript{N52A, D192A} double mutant exhibited near wild-type levels of colonization and aggregation (Fig. 16C and 17D). These results suggest that during normal colonization, the inhibitory activity of SypE must be “turned off”, to permit aggregate formation and efficient host colonization.

In support of the proposed negative role of SypE during colonization, I found that the enhanced ability of a ΔsypE strain to form biofilms upon overexpressing SypG provided these cells with a competitive colonization advantage, presumably due to enhanced aggregation outside of the symbiotic light organ (Fig. 35). The inhibitory role for wild-type SypE under sypG-overexpressing conditions is in direct contrast to my earlier analysis of a ΔsypE mutant under rscS-overexpressing conditions, in which I observed little impact of SypE on host colonization (Fig. 16). That SypE permits biofilms and colonization upon overexpression/activation of RscS, yet inhibits under
SypG-overexpressing conditions, suggests that a primary function of SypE is to prevent aberrant biofilm formation induced by the phosphorylation, and thus activation, of SypG in the absence of RscS signaling/activation. It is currently unclear under what conditions SypG may become activated without signaling through RscS. It remains possible that SypG may receive phosphoryl groups and become activated through other regulatory inputs in addition to RscS. Recently, Ray and Visick (2012) demonstrated that regulatory components within the Lux phosphorelay controlling bioluminescence also impact the regulation of syp biofilm formation. Importantly, this impact on syp biofilms appears to occur at the level of SypG and syp locus activation [225]. Thus, it is possible that, under conditions in which the Lux phosphorelay promotes SypG activation and syp induction, SypE functions as a negative regulator to prevent biofilm formation and restrict colonization. Alternatively, SypE may be important for preventing biofilm formation under conditions in which SypG might get phosphorylated due to inadvertent “cross-talk” from other sensor kinases that are activated. These possibilities remain to be investigated.

**Regulation of SypE activity by the HK RscS**

My genetic studies suggest that phosphorylation serves to regulate SypE activity. Although the identity of the cognate HK(s) that directly phosphorylates SypE remains to be verified, my current data does suggest that SypE activity is modulated, directly or indirectly, by RscS activation/overexpression. Our lab previously demonstrated that SypE inhibits biofilms induced by the RR SypG [27]. In contrast, I have shown here that upon rscS overexpression, SypE exhibits positive regulatory activity. These data
suggest that RscS expression/activation alters the activity of SypE (i.e. inactivates the protein’s inhibitory activity). The ability of RscS to inactivate SypE’s inhibitory activity depended upon the REC domain of SypE, as a SypE<sup>D192A</sup> mutant that cannot become phosphorylated failed to promote biofilm formation (Fig. 15E). I interpret these results to suggest that RscS modulates SypE activity through REC phosphorylation.

In addition to these genetic studies, I used an in vivo Phos-tag<sup>TM</sup> approach to evaluate the influence of RscS on the kinase activity of SypE. Specifically, I monitored the phosphorylation state of SypE’s downstream target protein, SypA. I found that SypA was predominantly phosphorylated under biofilm-inhibiting conditions (i.e., upon overexpression of <i>sypG</i> in wild-type cells), but unphosphorylated under biofilm-permissive conditions (i.e. upon overexpression of <i>rscS</i> in wild-type cells or overexpression of <i>sypG</i> in Δ<i>sypE</i> cells) (Fig. 26 and Fig. 33). These results support the genetic studies, confirming that SypE indeed functions as a serine kinase to phosphorylate SypA and inhibit <i>syp</i> biofilm formation. Importantly, these data also demonstrate that overexpression of <i>rscS</i> inactivates the inhibitory kinase activity of SypE, subsequently promoting SypA dephosphorylation and biofilm formation.

Consistent with the results of my biofilm assays, the regulation of SypE’s kinase activity by RscS required the intact REC domain of SypE: cells expressing the inhibitory SypE<sup>D192A</sup> mutant failed to produce RscS-induced biofilms, and this biofilm defect could be attributed to the constitutive phosphorylation of SypA by SypE (Fig. 26). These data thus suggest that RscS, directly or indirectly, promotes SypE phosphorylation at D192, resulting in the inactivation of SypE’s inhibitory kinase domain (Fig. 36).
Given the results—that (1) SypE inhibitory activity must be inactivated to permit biofilm formation and colonization, (2) inactivation of this inhibitory activity depends upon the REC domain and likely phosphorylation at D192, and (3) the HK RscS promotes this inactivation—we generated a model for the severe colonization defect observed in an \( rscS \) mutant [26](Fig. 36). In this model, RscS would function to both induce \( syp \) transcription and inactivate the inhibitory activity of SypE. We hypothesized that if this were true, then a \( \Delta rscS \Delta sypE \) double mutant should colonize better than an \( rscS \) single mutant. Indeed, I found that the \( \Delta rscS \Delta sypE \) mutant dramatically outcompeted the \( \Delta rscS \) mutant for colonization (Fig. 18). Although deletion of \( sypE \) did improve colonization fitness in an \( rscS \) mutant, it did not fully restore colonization competency to wild-type levels (data not shown). I predict that this is likely due to the additional role of RscS in activating SypG-dependent transcription of the \( syp \) locus and biofilm formation [27]. These experiments further support the model that the inactivation of SypE inhibitory activity is critical during symbiotic colonization. These data also provide insight into the previously established requirement for RscS in initiating colonization [26] by demonstrating that this regulator is necessary for the inactivation of SypE. Whether RscS directly phosphorylates SypE remains unknown. The possibility that RscS may serve as a phosphodonor to both SypG and SypE would not be unprecedented, as other studies have reported a single HK interacting with multiple RRs [e.g., [226]](Fig. 5).
Role for the HK SypF in regulating SypE activity?

It remains possible that RscS may exert indirect control over SypE by influencing the activity of another HK that can phosphorylate SypE. In many instances, RRs are encoded adjacent to their cognate HKs. Intriguingly, SypE is encoded upstream of the HK SypF, another known regulator of syp biofilm formation [28]. Given its genetic context, it is tempting to speculate that SypF may function with RscS to control SypE phosphorylation. Indeed, I have preliminary data that suggest that SypF, similar to RscS, can influence SypE’s activity. In my earlier SypE complementation studies, I originally utilized a dual plasmid complementation approach to assess SypE function. However, I found that overexpression of wild-type sypE from a multi-copy plasmid not only failed to complement a ΔsypE mutant under RscS-inducing conditions, but also completely inhibited biofilm formation (see appendix). Given my data indicating that phosphorylation is required to modulate SypE activity, I hypothesized that the inability of wild-type sypE to complement when overexpressed may be due to an excess of SypE protein relative to the levels of its cognate HK. As such, the majority of SypE protein could remain largely unphosphorylated and thus exhibit negative regulatory function. I reasoned that if this were the case, then co-expressing SypE with its cognate HK could restore this balance, and permit SypE to complement when overexpressed. Interestingly, I found that co-overexpressing sypE with sypF on a multi-copy plasmid permitted complementation of the ΔsypE mutant and restoration of RscS-induced biofilms (see appendix). Importantly, co-expressing sypF did not impact SypE’s inhibitory activity under SypG-inducing conditions, in which SypE naturally functions as a negative
regulator. These preliminary studies suggest that SypF may work in conjunction with RscS to modulate the activity of SypE. The role of SypF in biofilm regulation and its possible interaction with SypE and/or RscS is currently being investigated.

**SypE-SypA regulatory network and biofilm regulation**

As part of my dissertation, I established that SypE regulates biofilm formation and colonization through controlling the phosphorylation state of the syp-encoded regulator SypA. I initially focused on SypA as a target for SypE based on the similarity of these proteins to partner-switching regulators. Specifically, the N- and C-terminal effector domains of SypE are similar to the *B. subtilis* partner-switching proteins RsbW and RsbU, respectively, while SypA is similar to the STAS-domain protein RsbV [133]. In *B. subtilis*, RsbW and RsbU interact with and regulate the phosphorylation state of RsbV [151](Fig. 9). Using co-immunoprecipitation studies, I confirmed that SypE likewise interacts with SypA in *V. fischeri* (Fig. 20). Interaction with SypA appeared to be mediated by the N-terminus of SypE, as a derivative that included only the N-terminal domain co-immunoprecipitated with SypA, while an N-terminal deletion mutant failed to do so (Fig. 20). The observation that SypE’s RsbW-like domain mediates interaction with SypA is consistent with that reported for characterized *B. subtilis* orthologues, in which binding of RsbV to RsbW inhibits the anti-sigma activity of RsbW [153].

It currently remains unknown what role, if any, the binding between SypE’s N-terminus and SypA plays in biofilm regulation. One formal possibility is that SypA binding is necessary for SypE to efficiently phosphorylate, and thereby inactivate, SypA.
Another possibility is that binding by SypE may serve to sequester SypA, and in this manner control SypA activity. Mutational studies of RsbW homologs in other bacterial systems have demonstrated that these separate activities (i.e. binding to and phosphorylation of RsbV) can indeed be distinguished, and their regulatory roles examined [179,227]. In particular, the conserved aspartate and glycine residues located in the G-1 box motif of RsbW-like regulators are required for the binding of these proteins to their cognate RsbV antagonists [179,227]. Sequence analysis of SypE identified these conserved residues correspond to Asp-81 and Gly-83 (see Fig. 11B). What role, if any, these conserved residues may play in the inhibitory activity of SypE awaits further analysis.

In addition to binding SypA, SypE both phosphorylates and dephosphorylates it. Using Phos-tag™ analyses, I determined that SypE regulates the phosphorylation of SypA at conserved serine residue (S56). Furthermore, in vivo Phos-tag™ studies revealed that SypA phosphorylation correlated with the inhibition of biofilm formation by SypE (Fig. 26). To better understand the regulatory role of SypA, I also examined the impact of SypA mutants on syp biofilm formation and symbiotic colonization. Loss of SypA disrupted both RscS-induced biofilm formation and natural symbiotic colonization. In agreement with my biochemical studies, I also found that both biofilm formation and colonization fitness correlated with the phosphorylation state of SypA: (1) Cells expressing a phosphorylated mimic of sypA (sypA\textsuperscript{S56D}) failed to produce biofilms in response to rscS overexpression and were dramatically impaired in their ability to colonize E. scolopes (Fig. 23 and 27, respectively); and (2) Expression of a non-
phosphorylatable version of sypA (sypA<sup>S56A</sup>) suppressed the severe defect in biofilm formation and symbiotic colonization caused by the constitutive kinase allele of sypE (sypE<sup>D192A</sup>) (Fig. 25 and 28, respectively). Together, these experiments reveal a critical role for SypA in promoting biofilm formation and symbiotic colonization.

In my characterization of the SypE-SypA signaling network, I performed epistasis analyses to demonstrate that SypA functions downstream of SypE in a single regulatory pathway to control biofilm formation (Fig. 24). Interestingly, this result stands in stark contrast to the characterized partner switching networks described in <i>B. subtilis</i> and other Gram-positive systems, in which the serine kinase/anti-sigma (RsbW) functions as the downstream regulatory protein [151](Fig. 9). In the canonical partner switching system, the interaction of the switch protein (RsbW) with either of its “partner” proteins (RsbV or s<sup>B</sup>) dictates the output of the regulatory switch (<i>i.e. sigma factor activity</i>), while the upstream antagonist (RsbV) indirectly controls the output response (sigma factor activity) through antagonism of RsbW [151]. My studies, however, demonstrate that the ability of SypE to regulate biofilms is fully dependent upon SypA and the regulation of SypA activity. These results suggest that while SypE and SypA share similarity with partner switching proteins, the regulatory cascade in which these proteins participate likely deviates from the <i>B. subtilis</i> partner switching paradigm.

**Downstream target of the SypE-SypA regulatory pathway**

A remaining question regarding the SypE-SypA regulatory pathway is the mechanism by which SypA, as the downstream regulator, controls <i>syp</i> biofilm
formation. I confirmed using transcriptional reporter assays that *sypA* expression (either deletion or overexpression) does not affect RscS-induced activation of *syp* transcription (Fig. 29). These results indicate that the SypA contributes to *syp* biofilm regulation at some level downstream of SypG activation and *syp* transcription (Fig. 36). Interestingly, I observed that expression of *sypE* did affect induction of *syp* transcription by SypG. However, this effect on transcription was not sufficient to account for the observed biofilm phenotypes (Fig. 34). Given that RscS appears to work upstream of both SypG and SypE, I hypothesize that the REC domain of SypE may be responsible for the observed impact on *syp* transcription. If both SypE and SypG do indeed receive signals from the same upstream sensor kinase, then SypE’s REC domain may compete with SypG for phosphorylation, resulting in a decrease in SypG activation and *syp* transcription. This possibility awaits future study. In all, these studies indicate that the SypE-SypA signaling pathway regulates biofilm formation downstream of *syp* activation.

Based on the similarity of SypA to RsbV-like antagonist proteins, I originally hypothesized that SypA would interact with an additional RsbW-like anti-sigma factor, and thus indirectly control the activity of a downstream sigma factor involved in biofilm formation. However, analysis of the *V. fischeri* genome for RsbW-like orthologues (other than the N-terminal domain of SypE) failed to identify any potential candidate genes. Additionally, my preliminary studies have yet to identify a potential sigma factor whose activity might be regulated by SypA.
The possibility also remains that SypA may function in a manner distinct from that described in *B. subtilis* and the other Gram-positive systems, and thus may not regulate activity of a sigma factor. Partner-switching orthologues have been described in several other Gram-negative systems, including *Bordetella bronchiseptica* and *Chlamydia trachomatis*. Interestingly, these reports suggest that while these partner-switching regulators are conserved in these systems, their regulatory mechanisms also appear to deviate from the partner-switching paradigm. For example, the *btr* partner switching orthologues of *B. bronchiseptica* (*BtrU-BtrV-BtrW*) regulate type III secretion (TTS), but do not impact transcription of the known TTS genes [178,179]. The authors of this study proposed that the Btr partner switching proteins do not regulate a sigma factor, but instead may function post-translationally to control TTS, perhaps by interacting with hypothetical proteins involved in the secretory system [179]. Similarly, a study examining partner switching orthologues in *C. trachomatis* (*RsbU-RsbV1-RsbV2-RsbW*) failed to identify an interaction between these proteins and any of the three sigma factors encoded by the *C. trachomatis* genome [180]. These studies indicate that partner-switching regulators, although conserved in Gram-negative bacteria, may function in a manner distinct from their Gram-positive orthologues.

Additional studies are necessary to identify the mechanism by which SypA controls biofilm formation. Interestingly, my studies of SypA revealed that in addition to promoting biofilm formation, overexpression of *sypA* also had a broad impact on cell viability. In particular, a rotation student, Lindsay Callan, and I found that overexpression of *sypA* was deleterious to *V. fischeri* viability, resulting in a “cell
death” phenotype (see appendix). The “cell death” phenotype was observed as the formation of opaque, non-viable colonies containing small papillae of viable cells (see appendix). Initial characterization of the SypA-induced “cell death” phenotype, determined that this phenotype was only observed upon culturing the cells on solid agar media and at a higher temperature (28°C). We found that culturing the cells either in liquid media or at a lower, permissive temperature (24°C) completely suppressed the SypA-induced “cell death”, even if we subsequently shifted the temperature back up to 28°C. Through additional studies, we determined that this phenotype was enhanced under conditions in which we predict would increase SypA activity. For example, enhanced “cell death” was observed upon overexpression of sypA in a ΔsypE background or upon overexpression of the enhanced activity allele of sypA (sypA$^{S56A}$) (See appendix). The observation that the cell death phenotype was enhanced in ΔsypE mutant relative to wild-type (sypE+) cells further supports our earlier conclusion that a primary function of SypE is to inhibit SypA activity. It remains unclear whether the ability of SypE to suppress the SypA-induced cell death is due to the phosphorylation of SypA and/or the binding of SypA by SypE. These possibilities await future testing.

Finally, this cell death phenotype occurred independent of biofilm formation, as overexpression of sypA in a syp mutant background still caused cell death even in the absence of biofilm formation (data not shown). Together, these studies suggest that SypA may regulate a downstream target or cellular process that impacts cell viability, in addition to biofilm formation. I expect that further characterization of SypA will not only deepen our understanding of syp biofilm regulation in V. fischeri, but may also
provide insight into the function of partner-switching orthologues in other Gram-negative bacteria.

**Conservation of SypE and SypA regulators in other Vibrios**

Genome analyses indicate that the *syp* locus is relatively conserved among several *Vibrio* species, including both pathogenic and symbiotic bacteria [21]. Although biofilm formation has been investigated in diverse *Vibrio* species, the role of the *syp* cluster in species other than *V. fischeri* remains unclear. Several studies have demonstrated that the *syp* locus contributes to biofilms formed by the pathogenic bacterium *Vibrio vulnificus* [228,229]. In *V. vulnificus*, the *syp* cluster, termed the *rbd* locus, was demonstrated to contribute to the production of exopolysaccharides and the attachment of the cells to biotic and abiotic surfaces [228]. Given the conservation of the *syp* locus among *Vibrio* species, I performed a bioinformatic survey of *syp*-containing *Vibrio* genomes for genes encoding SypA and SypE homologues, specifically, or other potential partner-switching proteins (see Appendix Table 1). While SypA is well-conserved among the *syp*-containing *Vibrio* genomes, SypE appears to be absent in several species. For example, *V. vulnificus* possesses a SypA homologue (VV1633), but lacks any clear *sypE*-like genes. Intriguingly, the genome of *V. vulnificus* contains genes for additional RsbW- and RsbU-like proteins, VVA0582 and VVA1682, which encode a putative RsbW-like anti-sigma factor and an RsbU-like serine phosphatase, respectively. The genomes of *Vibrio parahaemolyticus* and *Aliivibrio salmonicida* similarly lack SypE, but encode RsbW and RsbU-like proteins elsewhere. Thus, in several *Vibrio* species lacking *sypE*, other putative partner-switching
components exist; whether or not they function as predicted or control the activity of SypA remains to be determined.

**Role for c-di-GMP in the regulation of syp biofilm formation**

In addition to characterizing the regulatory role of SypE, I performed preliminary studies to identify other factors involved in the regulation of syp biofilm formation, including the potential role of cyclic-di-GMP. Cyclic-di-GMP is a common bacterial second messenger, which in many bacterial systems contributes to the regulation of motility and biofilm formation. Specifically, increased cyclic-di-GMP levels often correspond to enhanced biofilm formation and decreased motility [230]. In support of a role for cyclic-di-GMP in regulating biofilm formation in *V. fischeri*, I found that overexpressing the putative phosphodiesterase genes (VF_0087 and VF_0091), which were predicted to reduce c-di-GMP levels, impaired wrinkled colony formation in a biofilm-producing strain (see Appendix). These preliminary data suggest that cyclic-di-GMP may promote *V. fischeri* biofilm formation. Additional studies are necessary to confirm that these genes indeed encode proteins with phosphodiesterase activity capable of degrading cyclic-di-GMP. Furthermore, the mechanism in which cyclic-di-GMP levels may impact biofilm formation remains to be determined.

**Significance**

This work provides further insight into the mechanisms by which *V. fischeri* regulates biofilm formation and, as a result, symbiotic colonization of *E. scolopes*. Importantly, these studies established a novel role for the unique RR SypE in restricting host colonization by *V. fischeri*. My results demonstrate that inactivation of SypE’s
inhibitory activity is critical to permit *V. fischeri* biofilm formation and the initiation 
host colonization. That inactivation of SypE’s inhibitory activity is necessary for both 
biofilm formation *in vitro* and host colonization *in vivo*, further emphasizes the utility of 
the *V. fischeri*-*E. scolopes* symbiosis as a model system for understanding the role of 
biofilms in host colonization. These studies not only deepen our understanding of 
SypE’s regulatory function, but also provide insights into the requirement for the HK 
RscS in initiating colonization by demonstrating that this regulator is necessary for the 
inactivation of SypE. Together, this dissertation work thus identifies SypE as a key 
regulator of symbiosis, and further contributes to our understanding of how *V. fischeri* 
coordinates biofilm formation during colonization of *E. scolopes*.

In addition to SypE, these studies also identify SypA as an essential regulator of 
biofilm formation and symbiotic colonization. Using both genetic and biochemical 
approaches, I have shown that the regulation of SypA activity by SypE is a critical 
mechanism by which *V. fischeri* controls the production of biofilms. My 
characterization of the SypE-SypA signaling pathway reveals yet another layer of 
regulatory control over symbiotic biofilm formation. Furthermore, the identification of 
SypA as a regulator downstream of *syp* activation provides an exciting avenue in which 
to identify new factors or processes involved in biofilm formation and host colonization.

The work described in this dissertation demonstrates that biofilm formation in *V. fischeri* is a tightly controlled process, involving both traditional two-component 
regulators and partner switching-like proteins. That regulatory elements from both 
signaling systems are integrated in SypE distinguishes this protein from the traditional
RR, and may represent a novel means by which a bacterium can control gene expression. Interestingly, SypE-like RRs appear to be present in other bacterial systems, including other Vibrios, such as *A. salmonicida*, and non-Vibrio species, including *Aeromonas veronii* and *Pseudomonas aeruginosa* [133,149]. What role these SypE-like proteins play in these other systems remains largely untested. However, a recent study in *P. aeruginosa* identified a RR, PA3346, that exhibits a similar domain architecture as SypE; PA3346 consists of an N-terminal REC domain, a central PP2C-like domain, and a C-terminal HATP (histidine kinase/ATPase) domain [149]. Importantly, the RR PA3346 was shown to regulate swarming motility through a partner-switching mechanism involving a SypA-like protein, PA3347 [150]. Therefore, SypE-like regulators may be utilized in other bacterial species as a means to integrate regulatory elements from distinct signaling systems to provide tighter control over gene expression.

Biofilm formation has been established as a common mechanism by which bacteria promote interaction with their eukaryotic hosts. In particular, the conservation of the *syp* biofilm locus in various *Vibrio* species exhibiting varied lifestyles (pathogens vs. mutualist symbionts) suggests that *syp*-dependent biofilms may play a role in diverse responses, such as the colonization of respective hosts. Therefore, understanding the cellular and molecular processes involved in regulating biofilms, and identifying approaches to manipulate these processes, remain critical areas of research. My characterization of the SypE-SypA regulatory pathway may provide novel insight into the mechanisms by which these related *Vibrios* and other bacteria restrict biofilm production.
I. Overexpression of *sypA* induces a cell death phenotype

I previously found that co-overexpression of *sypA* and *sypG* overcomes inhibition of biofilm formation by SypE and promotes biofilm formation in wild-type cells (Fig. 33). In my analysis of SypA, I observed that expression of *sypA*, in addition to controlling biofilms, also impacted cell viability under under particular culture conditions. Working with a rotation student, Lindsay Callan, we found that the co-expression of *sypA* and *sypG* in wild-type *V. fischeri* cells resulted in the formation of opaque, non-viable colonies which contained small papillae of viable cells (Appendix Fig. 1A). This cell death phenotype was primarily observed upon culturing the cells at a higher temperature (28°C), and was largely suppressed by culturing the cells at a lower, permissive temperature (24°C) (Appendix Fig. 1A). Furthermore, the cell death phenotype was only observed for strains cultured on solid agar media; culturing of the strains in liquid media appeared to suppress the cell death phenotype (data not shown).

Additional studies found that the cell death phenotype was enhanced in a Δ*sypE* strain co-expressing *sypA* and *sypG* (Appendix Fig. 1A), suggesting that the presence of SypE inhibits SypA activity and prevents cell death. To better understand this phenotype, we also examined the impact of overexpressing SypA<sup>S56A</sup>, which I have previously shown exhibits increased activity to promote biofilm formation. Compared to wild-type *sypA*, overexpression of *sypA<sup>S56A</sup>* , either alone or co-expressed with *sypG*, enhanced the cell death phenotype (Appendix Fig. 1B). Together, these preliminary studies suggest that overexpression of *sypA* is detrimental to cell viability. To identify unknown cellular targets downstream of SypA, we performed a preliminary transposon
mutagenesis screen using a mini-Tn5 transposon library generated in the ΔsypE mutant strain. We overexpressed the \(sypA^{S56A}\) allele in this mutant library and screened for suppressors of SypA-induced cell death. Using this approach, we identified several transposon mutants that failed to exhibit SypA-induced cell death. Subsequent analysis determined the location of the transposon insertions and identified 3 disrupted genes: VF0130 (MutM), VF0534 (MutS), and VFA0451 (HutU/Urocanate Hydratase). Initial analyses suggest that these genes encode proteins involved in DNA mismatch repair. Future studies are necessary to characterize these genes and to assess their potential roles in biofilm formation and SypA-induced cell death.
Appendix Figure 1. SypA-induced cell death phenotype. (A) Wild-type and ΔsypE cells carrying the pSypG plasmid (pARM9) and either empty vector (pKV282) or the pSypA plasmid (pARM13) were streaked onto LBS agar plates and grown for 24 h either RT (~24°C) or 28°C. (B) Wild-type and ΔsypE cells carrying the pSypA<sup>S56A</sup> plasmid (pARM9) were streaked onto LBS agar plates and grown for 24 h at either RT (~24°C) or 28°C.
II. Impact of SypF on SypE activity

To characterize the regulatory activity of SypE, I initially utilized a dual plasmid complementation approach to dissect the activities of the SypE domains. For this approach, I assessed the ability of the various *sypE* alleles to complement a Δ*sypE* mutant upon co-overexpression with *rscS*. I found that overexpression *sypE* not only failed to complement Δ*sypE* mutant for RscS-induced biofilms, but completely inhibited biofilm formation induced by RscS (Appendix Fig. 2A). Interestingly, overexpression of *sypE* did not inhibit RscS-induced biofilms in wild-type (*sypE*+) cells (Appendix Fig. 2A). I hypothesized that the inhibitory activity observed upon overexpression of *sypE* may result from an excess pool of SypE protein relative to the levels of its cognate HK protein. Thus, a portion of the SypE protein pool would remain unphosphorylated and function as an inhibitory kinase. I reasoned that if this were the case, then co-overexpression of *sypE* with its cognate HK could restore this balance, resulting in the inactivation of SypE’s inhibitory activity and the production of RscS-induced biofilms.

To test this hypothesis, I asked whether co-expression of *sypE* with the downstream HK *sypF* on a multi-copy plasmid (pARM68) could permit biofilm formation in an *rscS*-overexpressing Δ*sypE* mutant. Indeed, I found that Δ*sypE* cells carrying both the RscS and SypE-SypF plasmids produced biofilms similar to wild-type cells (Appendix Fig. 2B). These data indicate that co-overexpression of *sypF* with *sypE* inactivates the inhibitory activity of SypE and promotes RscS-induced biofilm formation. Additionally, I found that co-overexpression of *sypE* and *sypF* also permitted complementation of a Δ*sypE* mutant under SypG-inducing conditions: similar to *sypE*
alone, co-expression of *sypE*-*sypF*, restored wild-type inhibition of SypG-induced biofilms (Appendix Fig. 2B). Together, these data suggest that SypF may modulate SypE activity, promoting the inactivation of SypE activity under RscS-inducing conditions.

Interestingly, although *sypE* overexpression inhibited biofilms in Δ*sypE* cells, I found that its overexpression in wild-type cells permitted RscS-induced biofilms (Appendix Fig. 2A). Considering my data indicating that SypF may affect SypE activity, I speculate that this difference in SypE activity may be due to the inability of SypF to inactivate SypE. In particular, it is possible that the co-production and/or co-localization of SypE and SypF within the cell is necessary for SypF to regulate SypE activity. Additional studies are necessary to explore these possibilities.
Figure 2. Regulation of SypE activity by the HK SypF. The indicated *V. fischeri* strain were spotted onto LBS plates and wrinkled colony formation assessed at 48 h post-spotting. (A) Wild-type and ΔsypE cells carrying the pRscS plasmid (pARM7) and either empty vector (pVSV105), pSypE plasmid (pCLD48) or the pSypE-SypF plasmid (pARM68). (B) Wild-type and ΔsypE cells carrying the pSypG plasmid (pCLD56) and either empty vector (pVSV105), pCLD48, or pARM68.
Appendix Table 1. Putative partner switching genes among sequenced *syp*-containing *Vibrios*.

<table>
<thead>
<tr>
<th><em>Vibrio species</em></th>
<th>Predicted function (partner switching domain)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. fischeri</em> ES114</td>
<td></td>
</tr>
<tr>
<td>VFA1020 (<em>sypA</em>)</td>
<td>RsbV-like protein</td>
</tr>
</tbody>
</table>
| VFA1024 (*sypE*) | N-terminal RsbW-like kinase  
                          C-terminal RsbU-like phosphatase |
|  |  |
| *V. vulnificus* (YJ016) |  |
| VV1633 (*sypA*) | *sypA* (RsbV-like) |
| VVA1682 | RsbU-like phosphatase |
| VV0459 | RsbV-like protein |
| VVA1683 | RsbV-like protein |
| VVA0582 | RsbW-like kinase |
|  |  |
| *V. vulnificus* (CMCP6) |  |
| VV1_2658 (*sypA*) | *sypA* (RsbV-like) |
| VV1 0681 | RsbV-like protein |
| VV2 1159 | RsbV-like protein |
| VV2 0075 | RsbW-like kinase |
| VV2 1158 | RsbU-like phosphatase domain |
|  |  |
| *V. splendidus* (LGP32) |  |
| VSS 1519 | *sypA* (RsbV-like) |
| VSS 2746 | RsbV-like protein |
|  |  |
| *A. salmonicida* (LFI1238) |  |
| VFAL II0312 | *sypA* (RsbV-like) |
| VSAL II0328 | RsbV-like protein |
| VSAL II0329 | RsbU-like phosphatase domain |
|  |  |
| *V. parahaemolyticus* |  |
| VP1476 | *sypA* (RsbV-like) |
| VP2660 | RsbV-like protein |
| VPA 1049 | RsbU-like phosphatase domain |
|  |  |
| *V. harveyi* |  |
| VIBHAR 02233 | *sypA* (RsbV-like) |
| VIBHAR 03654 | RsbV-like protein |
| VIBHAR 05599 | *sypE* homology (RsbW and RsbU-like domains) |
In addition to the characterization of the SypE-SypA regulatory pathway, I investigated other potential determinants of syp biofilm formation. In particular, I examined the potential role of cyclic diguanylate (c-di-GMP) in regulating biofilm formation induced by rscS overexpression. C-di-GMP is a common second messenger in bacteria and has been demonstrated to play an important role in promoting biofilm formation in many bacteria [230]. The levels of c-di-GMP are controlled by the opposing activities of diguanylate cyclases, which synthesize c-di-GMP, and phosphodiesterases (PDEs), which degrade c-di-GMP.

To determine whether c-di-GMP may contribute to RscS-induced biofilms, I assessed the impact of PDE overexpression on RscS-induced wrinkled colony formation. I introduced plasmids pKV302 and pKV303, which overexpress the putative PDE genes VF0087 and VF0091, respectively, into the biofilm forming V. fischeri strain, KV4366. Compared to vector-containing cells which exhibited robust wrinkled colony formation, cells carrying either pKV302 or pKV303 exhibited delayed wrinkled colony formation (Appendix Fig. 3). Additionally, pKV302-carrying cells exhibited diminished wrinkled colony formation even at later time points (Appendix Fig. 3). These preliminary data suggest that a decrease in c-di-GMP levels upon overexpression of PDEs impairs biofilm formation in V. fischeri. Further studies are necessary to confirm that VF0087 and VF0091 indeed encode PDE proteins, and whether their overexpression decreases intracellular c-di-GMP levels.
Appendix Figure 3. Impact of phosphodiesterase overexpression on biofilm formation in *V. fischeri*. Time-course assay of wrinkled colony formation. Cultures of the following strains were spotted onto LBS medium at RT and wrinkled colony formation was assessed at various times up to 48 h post-spotting: Biofilm-forming *V. fischeri* strain [KV4366] carrying empty vector (pKV69) or plasmids overexpressing the putative phosphodiesterase genes VF0087 (pKV302) and VF0091 (pKV303).
IV. UV random mutagenesis screen.

To identify unknown cellular targets downstream of the biofilm regulator SypA, I performed a random UV mutagenesis screen. Briefly, a summer student, Colin Linke, and I utilized a random UV mutagenesis screen of a ΔsypA mutant in order to identify suppressor mutations that could restore biofilm formation. I previously demonstrated that deletion of sypA (either in a wild-type or a ΔsypE background) results in the loss of both RscS- and SypG-induced biofilm formation, which can be complemented by expressing sypA in trans. In this screen, we UV mutagenized the ΔsypA ΔsypE mutant strain carrying the sypG overexpression plasmid (pARM9). We then screened for colonies that exhibited wrinkled colony formation, indicative of biofilm formation. We screened ~40-50,000 colonies and identified 8 wrinkled colonies, that exhibited partial restoration of SypG-induced wrinkling. We selected one of the isolated colonies (that which exhibited the most apparent wrinkled colony phenotype) (Appendix Fig. 4), and sequenced the genomes of both the isolated UV mutant strain and the control (parental) strain. Sequence analysis indicates that the isolated UV mutant strain contains several unique nucleotide polymorphisms and chromosomal deletions relative to the parental control strain (Appendix Table 2). Future studies are necessary to determine which of the mutation(s), or combination of mutations, contribute to suppression of the ΔsypA biofilm defect.
Appendix Figure 4. Wrinkled colony formation by UV-mutant strain. Cultures of the following strains were spotted onto LBS medium at 28 °C and wrinkled colony formation was assessed at 24 h post-spotting: ΔsypA ΔsypE mutant (Parental) strain or UV-mutagenized ΔsypA ΔsypE strain carrying pSypG plasmid (pARM9).
### Appendix Table 2. Mutations identified in UV mutagenized strain

<table>
<thead>
<tr>
<th>Location (gene)</th>
<th>Predicted gene function</th>
<th>Amino Acid Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>VF_1697</td>
<td>Latent nuclear antigen</td>
<td>1035 (silent)</td>
</tr>
<tr>
<td>VF_1703</td>
<td>Endonuclease IV</td>
<td>86 (lose stop codon)</td>
</tr>
<tr>
<td>VF_1889</td>
<td>Chitoporin-</td>
<td>338 (silent)</td>
</tr>
<tr>
<td>VF_1889</td>
<td>Chitoporin</td>
<td>148 (Ala to Gly)</td>
</tr>
<tr>
<td>VF_1993</td>
<td>DnaJ- chaperone</td>
<td>146 (silent)</td>
</tr>
<tr>
<td>VFA_1022</td>
<td>Syp polysaccharide export protein</td>
<td>31 (Gly to Ser)</td>
</tr>
<tr>
<td>VF_2432</td>
<td>RNA-binding protein</td>
<td>113 (framshift at 3’ end)</td>
</tr>
<tr>
<td>VF_A1162</td>
<td>RtxA2- cytotoxin</td>
<td>Multiples</td>
</tr>
</tbody>
</table>

#### Point Mutations (intergenic regions)

<table>
<thead>
<tr>
<th>Location (gene)</th>
<th>Predicted gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upstream of VF_0318</td>
<td>Crp/Fnr transcriptional regulator</td>
</tr>
<tr>
<td>Upstream of VF_1582</td>
<td>Periplasmic protein of efflux system</td>
</tr>
<tr>
<td>Between VF_T0024-0025</td>
<td>tRNA-Arg genes</td>
</tr>
<tr>
<td>Upstream of VF_1757</td>
<td>Hypothetic (88 AA) protein</td>
</tr>
<tr>
<td>Upstream of VF_1812</td>
<td>Long-chain fatty acid transport protein</td>
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<tr>
<td>Upstream of VF_A0276</td>
<td>GGDEF protein</td>
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#### Chromosomal Deletions

<table>
<thead>
<tr>
<th>Location (gene)</th>
<th>Predicted gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 bp upstream of VF_A1047</td>
<td>MgtE (magnesium transporter)</td>
</tr>
<tr>
<td>50 bp upstream of VF_1583</td>
<td>Exonuclease III</td>
</tr>
<tr>
<td>41 bp deletion in VF_1757</td>
<td>Predicted membrane protein</td>
</tr>
</tbody>
</table>
REFERENCES


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VITA

The author, Andrew Robert Morris, was born in Winfield, Illinois, to Donna and Robert Morris. He earned his Bachelor of Arts degree in Biology from Loyola University Chicago in May 2006.

In August, 2007, Andrew entered the Integrated Program in Biomedical Sciences (IPBS) at Loyola University Chicago and later joined the Department of Microbiology and Immunology. He joined the laboratory of Dr. Karen Visick in 2008, where his researched focused on characterizing the role of the novel regulatory protein SypE in restricting biofilm formation and host colonization by Vibrio fischeri. Andrew was awarded a grant from the Conservation Medical Center of Chicago in 2010 and the Arthur J. Schmitt dissertation fellowship in 2012.

After completing his Ph.D., Andrew will begin a post-doctoral position in Dr. Alan Hauser’s laboratory at Northwestern University (Chicago, IL) where he will continue to study host-microbe interactions and in particular, the processes involved in Pseudomonas aeruginosa pathogenesis.