Characterization of the novel auxiliary inhibitor, VieB, in the VieSAB three-component signal transduction system and insights into the regulation of *vieSAB* in *Vibrio cholerae*

A thesis

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ABSTRACT

Vibrio cholerae is a facultative, water-borne pathogen that causes a severe diarrheal disease known as cholera. Its ability to monitor environmental changes as it transitions between aquatic reservoirs and the human intestine is vital to its pathogenic lifestyle. One way *V. cholerae* alters its gene expression is by sensing changing external stimuli through the action of two-component signal transduction systems (TCS). One such system in *V. cholerae*, VieSAB, is comprised of the sensor kinase (VieS), response regulator (VieA), and an auxiliary protein (VieB). VieSAB has been shown to be important in the induction of virulence genes by controlling the concentration of the secondary messenger, cyclic-di-GMP (Tischler, Lee et al. 2002; Tischler and Camilli 2005; Tamayo, Schild et al. 2008). Even though VieSA behave similar to typical two-component systems (Martinez-Wilson, Tamayo et al. 2008), many questions relating to its function remain unanswered. Firstly, the role of VieB remains unclear, however preliminary data suggest that VieB may interact with VieSA to modulate its activity. Secondly, transcriptional regulation of *vieSAB* is poorly understood. Previous studies suggest that activated VieA is autoregulatory, however regulation of this operon has not been characterized.

In order to gain a better understanding of the VieSAB signal transduction system, the first goal of my thesis project was to identify and characterize the mechanism of VieB. Using biochemical techniques, I reveal the function and mechanism of action of VieB in the VieSA TCS. I provide evidence that VieB partially disrupts VieS autophosphorylation and completely interrupts phosphotransfer. Taken together, we

propose a working model whereby the inhibitory role of VieB in the VieSA phosphorelay provides negative feedback control over the signal output. Additionally, I describe attempts to characterize *vieB* genetically, however no phenotype has been observed thus far, suggesting that *vieB* may play only a minor role in controlling virulence gene expression at the end of infection.

In the second half of my thesis, I describe my work in characterizing the regulation of the *vieSAB* operon. While I provide evidence that *vieA* is not autoregulatory and the master virulence gene transcriptional regulator, *toxT*, does not control *vieSAB* expression under the conditions tested, I reveal the negative regulation of *vieSAB* by the cyclic-AMP receptor protein (CRP) during stationary phase. Interestingly, while CRP appears to up regulate *vieB* transcript, this does not correlate to VieB protein levels suggesting that there may be post-transcriptional regulation or differential expression within the *vieSAB* operon. Taken together, we hypothesize that incorporation of CRP into the regulation of *vieSAB* ties together carbon source availability with the modulation of virulence gene expression through cyclic-di-GMP.

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LIST OF ABBREVIATIONS

Amp	Ampicillin
Ara	Arabinose
BSA	Bovine Serum Albumin
CA	Catalytic, ATP-binding
CG	Composition Gradient
Cm	Chloramphenicol
CV	Column Volume
DGC	Diguanylate cyclase
DHp	Dimerization and histidine phosphotransfer domain
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GST	Glutathione S-transferase tag
HAMP	Histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and phosphatases
HK	Histidine kinase
His	poly Histidine (x6) tag
HPt	Histidine phosphotransfer domain
HTH	Helix-turn-helix domain
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
LB	Luria Broth

MALS	Multi-angle light scattering
MBP	Maltose-binding protein tag
mRNA	messenger RNA
PAS	Per-Arnt-Sim
PBP	Periplasmic binding protein
PBS	Phosphate buffered saline
PDE	Phosphodiesterase domain
REC	Receiver domain
RNA	Ribonucleic acid
RR	Response regulator
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SK	Sensor kinase
Sm	Streptomycin
sRNA	Small RNA
TBS	Tris buffered saline
TBS-T	Tris buffered saline, 0.1% Tween 20
TCS	Two-component system
TEV	Tobacco Etch Virus protease
TM	Transmembrane region
TPR	Tetratricopeptide repeat
UKF	Unknown function region

CHAPTER 1: INTRODUCTION

1.1 *CHOLERA* AND *VIBRIO CHOLERAE*

1.1.1 History of cholera

Cholera is a life-threatening disease, characterized by acute secretory diarrhea and resulting in severe dehydration. Even though cholera was first documented in India over 2,500 years ago, ancient Sanskrit texts dating back to the 5th century BC have been found that describe an illness that most believe to be cholera. Up until 1817, the disease was mostly confined to the subcontinent of India. However, that year marked the first recorded cholera pandemic, spreading out of the Bengal region of India and into Southeast Asia, China, Japan, the Middle East and even Russia (Colwell 2004). From 1817 to 1923 a total of six pandemics have plagued the world. During the 19-year long second pandemic, advances in global transportation allowed cholera to spread to Europe and eventually reach the United States in 1832 (Colwell 2004).

While it seemed that cholera was unstoppable, it was during the second pandemic in 1849 that a London physician, named John Snow, proposed that cholera was a communicable disease. In his published report "On the Mode of Communication of Cholera" in 1855, Dr. Snow presented his theory that the infectious agent of cholera existed in the stool samples of patients and it was this agent that contaminates the public's drinking water supplies, allowing transmission of the disease (Winkelstein 1995). While an Italian scientist, Filippo Pacini, in 1854 was first to observe comma-shaped forms in cholera patient stool samples, it was Robert Koch in 1884 that identified and isolated the causative agent of cholera, *Vibrio comma*, later renamed *V. cholerae* (Harris, LaRocque et al. 2012).

V. cholerae is a member of the gram-negative Vibrionaceae family and is a single, flagellated curved rod that naturally resides in brackish water and estuaries (Morris 2003; Sack, Sack et al. 2004). Within this species, *V. cholerae* can be further classified into over 200 serogroups based on the antigenic differences of the lipopolysaccharide O antigen. While all 200 serogroups inhabit the aquatic environment, only two are capable for causing epidemic cholera, O1 and O139 (Faruque, Siddique et al. 1999; Safa, Nair et al. 2010). While O1 causes the majority of disease, in 1992 the emergence of O139, a derivative of the O1 serogroup, and it's ability to contribute to disease, was recognized. The O1 serogroup can be subdivided into two biotypes, El Tor and classical (Safa, Nair et al. 2010), which can be grouped into two major serotypes, Ogawa and Inaba (Longini 2002). While previous pandemics were not well documented, it is believed that the first six pandemics were caused by the O1 classical biotype. Globally, we are in the seventh recorded pandemic of cholera, which began in 1961 and is dominated by the O1 El Tor biotype. Reports speculate that the recent switch from classical to El Tor is a result of the El Tor having outcompeted classical due to its ability survive better in the environment (Faruque, Albert et al. 1998; Reidl 2002; Faruque 2011). Indeed, classical has not been isolated from the environment since 1992 and is considered extinct but recent data, which will be discussed later in this chapter, suggests that classical may still be circulating.

1.1.2 Epidemiology and public health burden of cholera

Cholera affects millions worldwide and poses a global threat (Alam, Sultana et al. 2006). The number of reported cholera cases to the World Health Organization (WHO) in 2011, was 589,854, resulting in 7,816 deaths which represents a 3.5% increase from 2010 (Organization 2014). After 2011, the number of reported cholera cases has decreased

from 2012-2013. However this number is known to be a vast under-estimate, as some countries where cholera is endemic, such as Bangladesh, do not report cholera cases or outbreaks to the WHO. The current estimate of the cholera cases is 3 million per year worldwide resulting in 100,000 deaths (Alam, Sultana et al. 2006; Organization 2014).

Given that *V. cholerae* naturally resides in the aquatic environment, this pathogen can easily infiltrate and contaminate water sources in countries that are densely populated and do not have proper water infrastructure or sanitation procedures. Additionally, *V. cholerae* is subject to seasonal changes and climate variability, which can effect when cholera outbreaks occur. Increases in surface water temperatures and nutrients result in zooplankton blooms, which correlate with an increase in *V. cholerae* in these environments (Colwell 1996; Pascual, Rodo et al. 2000; Jutla, Akanda et al. 2011). Additionally, natural disasters such as floods, cyclones, or earthquakes also contribute to cholera outbreaks (Schwartz, Harris et al. 2006). Cholera is endemic in many parts of Asia, Africa and India and outbreaks coincide with the rainy seasons, occurring during or after heavy rain (Sack, Sack et al. 2004). In Haiti, a cholera epidemic began after a devastating earthquake hit the region in 2010, putting stress on an already weak water infrastructure system and contaminating a number of rivers, which many relied upon for water (Harris, Larocque et al. 2010).

Cholera is caused by the ingestion of *V. cholerae* via a contaminated water source and results in dehydration, vomiting and profuse watery diarrhea (known as rice water stool), where fluid loss can occur up to one liter per hour. Cholera that is left untreated can result in death within hours of the onset of symptoms (Harris, LaRocque et al. 2012). *V. cholerae* can infect all age groups and be highly fatal especially in naïve populations.

While the infectious dose is high, around 10^5-10^8 for a healthy individual, these numbers can drop dramatically for the very young and the immunocompromised individuals (Nelson, Nelson et al. 2011). The incubation period can range from 12 hours up to 5 days, however 75% of those infected do not display symptoms, even though bacteria are present in their stool 7-14 days post infection (Morris 2003; Weil, Khan et al. 2009; Organization 2014). This clinically asymptomatic state makes identifying and managing cholera outbreaks a challenge, as these bacteria are unknowingly being shed back into the water supply where they can infect others. Of those that are symptomatic, the nature of the disease creates a vicious cycle of fecal-oral spread, especially in areas where clean water is scarce. Within this shed rice water stool are tremendous numbers of infectious *V. cholerae* on the order of 10^8 CFU/mL. Furthermore, *V. cholerae* that are exiting the host are adept at subsequent infection, i.e., requiring a lower infectious dose (10 to 100 times lower than non-human passage *V. cholerae*) to cause disease. This phenomenon has been termed hyperinfectivity (Merrell, Butler et al. 2002; Alam, Larocque et al. 2005; Hartley, Morris et al. 2006). This hyperinfectious state can persist in the environment from 5 to 24 hours, adding to the burden of disease and to the difficulty of controlling outbreaks. Household transmission has also been documented, suggesting that person-to-person transmission can occur; *V. cholerae* in this setting is more infectious than *V. cholerae* that have adapted to the environment, further supporting a hypothesis of hyperinfectious state (Weil, Khan et al. 2009).

While cholera that is left untreated has a fatality rate of 70%, prompt treatment of cholera can reduce the fatality rate to below 1% (Lindenbaum, Greenough et al. 1967; Organization 2014). During outbreaks, the prevention of and controlling cholera focuses

on providing clean water, improving sanitation and rehydration therapy (Organization 2014). Since cholera causes dehydration and electrolyte imbalance, rehydration is key to combating this disease and could dramatically drop mortality rates, however there is still deaths associated with the disease (Sack, Sack et al. 2004; Mintz and Guerrant 2009; Walton and Ivers 2011; Organization 2014). In the 1960s, oral rehydration salts (ORS) were introduced that contained equimolar concentrations of sodium and glucose that helped to replace fluid loss during symptomatic cholera (Nalin, Cash et al. 1968; Guerrant, Carneiro-Filho et al. 2003). However, ORS is most effective when administered early during the disease and those that are severely dehydrated require intravenous rehydration therapy (Harris, LaRocque et al. 2012). In a case by case basis, antibiotics are sometimes given to those in the late stage of disease to aid in reducing *V. cholerae* concentrations and clinical symptoms, however this is not highly recommended due to concerns of breeding antibiotic resistance. Indeed, clinical isolates over the last decade have been shown to have resistances to commonly used antibiotics such as sulfamethoxazole-trimethoprim and streptomycin (Waldor, Tschape et al. 1996; Qadri, Chowdhury et al. 2007). More recently in Asia, isolates have been discovered to obtain resistances to tetracycline, erythromycin, and/or ciprofloxacin (Faruque, Islam et al. 2006; Kim, Wang et al. 2010).

In addition to ORS, vaccines are available but have limited protective efficacy against *V. cholerae*. Two orally administered whole cell killed vaccines have been widely tested and have been shown to be safe and provide short-term protection (Harris, LaRocque et al. 2012). While both vaccines contain multiple O1 biotypes and serotypes, only one is augmented with a segment of recombinant cholera toxin, which is thought to

boost efficacy. While a number of live attenuated vaccines have been developed none are currently used for treatment, as these vaccines were not effective or have not been tested in field studies (Levine, Kaper et al. 1988; Kotloff, Wasserman et al. 1992; Kenner, Coster et al. 1995; Sack, Sack et al. 1997; Richie, Punjabi et al. 2000; Cohen, Giannella et al. 2002; Ryan, Calderwood et al. 2006; Qadri, Chowdhury et al. 2007).

While attempts to develop vaccines and other preventative measures are on going, the ability of this organism to persist in the environment lends itself to continually cause disease and may never be eradicated. However, discussion of using phage-therapy for controlling cholera outbreaks is currently been revisited. Bacteriophage were discovered in the 20th century and research in their use for treating bacterial diseases, including *V. cholerae*, soon followed (Soothill 1992; Hanlon 2007). *V. cholerae* is a host for a number of virulent phages, which have been isolated from patient stool and the environment (Faruque, Islam et al. 2005; Jensen, Faruque et al. 2006; Seed, Bodi et al. 2011). Interestingly, a cycle between cholera outbreaks and bacteriophage densities has been observed, where as the outbreaks proceeds, bacteriophage populations increase concomitant with a decrease in cholera cases (Faruque, Naser et al. 2005). This cycle suggests that bacteriophage are able to kill *V. cholerae* both in the host and the environment, resulting in its ability to modulate outbreak duration (Seed, Yen et al. 2014). Since these bacteriophage are effective in killing *V. cholerae*, treatment of this disease by phage-therapy is currently under way in hopes of adding yet another tool to aid in the prevention and control of cholera.

1.2 Life cycle and Virulence Gene regulation of *V. cholerae*

1.2.1 Life aquatic: survival in the environment

As with other members of the *Vibrio* genus, *V. cholerae* is able to survive in marine or brackish water, where *V. cholerae* optimally lives in temperate water containing 0.5-3% salinity. Unlike the rest of the *Vibrio* genus, *V. cholerae* is unique in its ability to also survive in freshwater, where phosphate and nitrogen are limiting (Miller, Drasar et al. 1982; Schild, Tamayo et al. 2007; Nelson, Chowdhury et al. 2008). This unique trait is vital for *V. cholerae's* pathogenicity, as this freshwater reservoir provides access to its human host and ensures dissemination via fecal-oral spread (Faruque, Albert et al. 1998; Peterson 2002; Reidl 2002).

While living in this aquatic environment, *V. cholerae* survives by utilizing chitin, a modified un-branched long chain polysaccharide of N-acetylglucosamine (GlcNAc), as its sole carbon and nitrogen source. Utilization of chitin by *V. cholerae* requires two extracytoplasmic proteins, chitinases and chitin binding proteins (Mondal, Nag et al. 2014). These proteins bind and cleave chitin into soluble GlcNAc oligomers that can then be transported into the cell for growth. Therefore, *V. cholerae* is typically associated with zooplankton, copepods and shellfish, which use chitin in their exoskeletons (Colwell 1996).

Chitin is also important for the induction of natural competence in *V. cholerae* (Meibom, Blokesch et al. 2005). Competence in the aquatic environment allows for horizontal gene transfer between *V. cholerae* possibly with other closely related species. While competence promotes the acquisition of genes to improve the fitness in the aquatic environment, this also allows for exchange of virulence genes. Additionally, when naturally competent, *V. cholerae* may be more likely to integrate lysogenic phages into its genome, which are known to carry toxigenic and antibiotic resistance genes. Therefore,

evolution of *V. cholerae* due to its natural competence in the environment can result in highly pathogenic *V. cholerae* strains capable of large-scale outbreaks and epidemics.

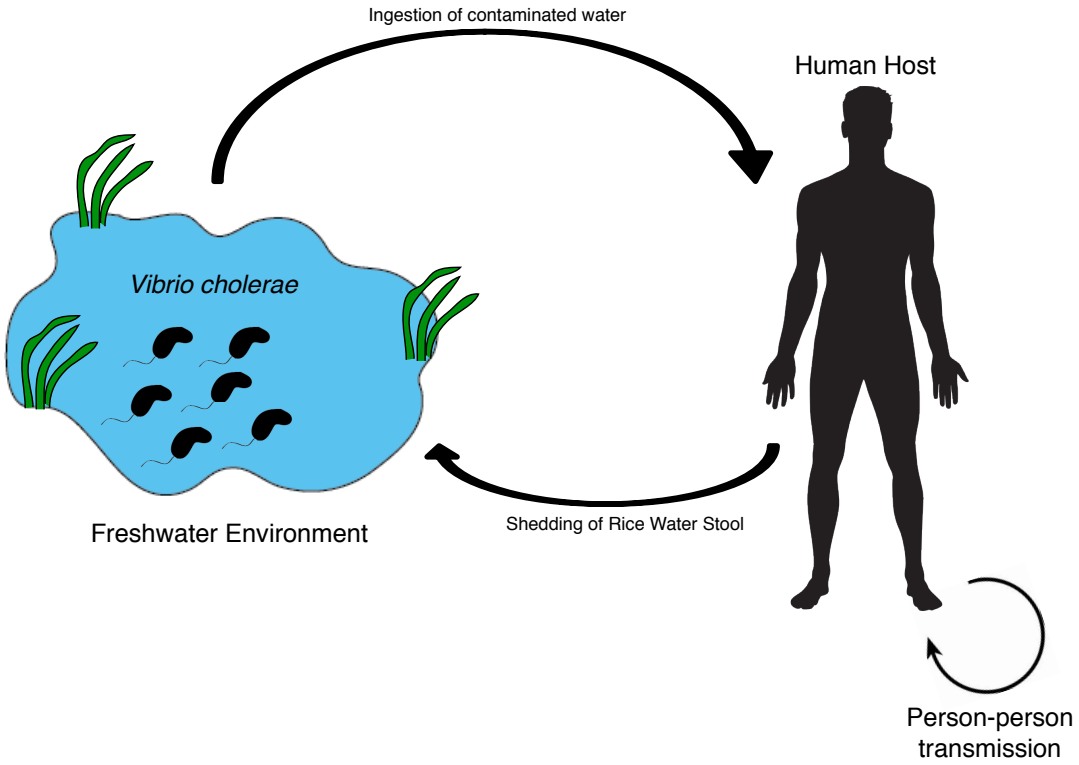
Biofilm formation also occurs during life in the aquatic environment, although *V. cholerae* can also exist in a planktonic state (Faruque, Biswas et al. 2006; Nelson, Chowdhury et al. 2007). Biofilms are usually formed on the chitinous surfaces of the zooplankton and copepods, which is ideal since *V. cholerae* uses these as a food source and promotes survival under the nutrient poor conditions. Biofilms are well known to provide bacteria with a defensive mechanism to protect themselves from a variety of stresses, such as antibiotics and UV radiation. Moreover, the ability of *V. cholerae* to form biofilms in freshwater also aids its transmission and survival in the host. Due to the high infectious dose required to successfully colonize and cause disease, formation of a biofilm is one way to ensure that a high number of bacteria are ingested. Additionally, once inside the host, biofilms are protective against host immune defenses and shields this pathogen from harsh stomach acids, ensuring a subset of live *V. cholerae* reach the small intestine (Zhu and Mekalanos 2003). Therefore, biofilms provide many advantages that increase the likelihood for successful colonization of the host resulting in disease.

1.2.2 There and back again: Life cycle of V. cholerae

In order for *V. cholerae* to successfully cause disease, it must overcome a number of hurdles; the first being ingestion by the human host. Once ingested, *V. cholerae* must survive killing by the low acidic environment of the stomach and pass through to the small intestines. In the small intestine, *V. cholerae* moves through the fluid filled lumen and intestinal mucus layer before it reaches the epithelial surface. Once at the cell surface *V. cholerae* begins to express important virulence factors, which will be discussed in

further detail below. These virulence factors are under the control of a highly complex regulatory network that ensures that infection proceeds in an organized fashion. The temporal and spatial expression allows this pathogen to bind, colonize, replicate and cause disease (Peterson 2002). As *V. cholerae* causes disease, production of rice water stool allows for dissemination out of the host. The end of infection is a critical moment in the life of *V. cholerae*, as it must prepare for survival in aquatic environment. As *V. cholerae* begins to exit the host, a subset of genes that are important for environmental survival, known as late genes, are up regulated thus preparing *V. cholerae* for its eventual release back into the environment (Figure 1-1)(Schild, Tamayo et al. 2007).

Figure 1-1. *V. cholerae* life cycle
V. cholerae is a natural inhabitant of marine ecosystems but can also contaminate and survive in freshwater. Upon ingestion of contaminated water by a human host, *V. cholerae* can travel through the gastrointestinal track, where in the small intestine this pathogen can colonize and replicate to high numbers. This, along with the expression of cholera toxin, results in the production of profuse watery diarrhea, known as rice water stool and subsequent severe dehydration. Rice water stool contains very high bacterial loads and it is through this route that *V. cholerae* is shed back into the aquatic environment. Here it can re-enter the cycle by ingestion by another human host. Direct person-to-person transmission has occurred in household settings and strikes rapidly, most likely due to the hyperinfectious state of *V. cholerae* as it exits the host.



1.2.3 Essential virulence genes for disease

As summarized above, *V. cholerae* must undergo a number of steps as it transitions from the aquatic environment to the host. Once *V. cholerae* finds itself in the small intestine, a number of virulence genes are expressed. In order to successfully bind epithelial cells, *V. cholerae* expresses a type IV bundle-forming pilus, called the toxin-coregulated pilus (TCP), which is essential for microcolony formation and intestinal colonization. It is thought that TCP-mediated microcolony formation is critical for resisting shear forces from peristalsis (Herrington, Hall et al. 1988; Lee, Hava et al. 1999; Childers and Klose 2007). TCP and the biosynthesis proteins need to assemble TCP are encoded on the *tcpA-H* operon, which is located on the *Vibrio* pathogenicity island-1 (VPI-1) (Childers and Klose 2007). In addition to TCP, the accessory colonization factor (ACF) is also necessary for efficient colonization of the small intestine as it is linked with TCP (Sharma, Jani et al. 2008). The divergently transcribed genes, *acfA* and *acfD* encode components that comprise the ACF virulence factor (Peterson and Mekalanos 1988), though the precise function of ACF remains to be discovered. Moreover, of particular interest for this work, VieA, a response regulator that controls virulence gene expression, is also up regulated immediately after binding to epithelial cells (Dey, Bhagat et al. 2013).

After successful colonization by TCP and ACF, another critical virulence factor, cholera toxin (CT), is expressed (Childers and Klose 2007). CT is encoded on the prophage, CTXΦ, which resides in the *V. cholerae* genome at a site distinct from the VPI-1 (Waldor and Mekalanos 1996; Waldor, Rubin et al. 1997; Childers and Klose

2007). CT is the prototype A-B toxin, encoded by *ctxAB*, and functions as an ADP-ribosylating toxin. CT is composed of two subunits, the A subunit which harbors the ADP-ribosylating catalytic activity and the B subunit which binds as a pentamer to GM_1 gangliosides, which are ubiquitous on the surface of intestinal epithelial cells. Through receptor mediated endocytosis and subsequent endosomal trafficking, the A subunit ultimately reaches the cytoplasmic side of the plasma membrane, where it ADP-ribosylates the $G_{s\alpha}$ regulatory subunit of the G-protein that regulates the adenylate cyclase enzyme activity (King and Van Heyningen 1973; Gill and King 1975; Gill and Rappaport 1979; Childers and Klose 2007). This results in increased cAMP level, which leads to Cl^- secretion and disruption of Na^+ import into intestinal cells. This osmotic imbalance is rectified by water transport into the lumen of the small intestine, which results in profuse secretory diarrhea and the hallmark symptom of cholera, rice water stool (Field 1965; Field 1979).

1.2.4 Regulation of virulence genes

1.2.4.i The Big Three: ToxT, ToxRS and TcpPH

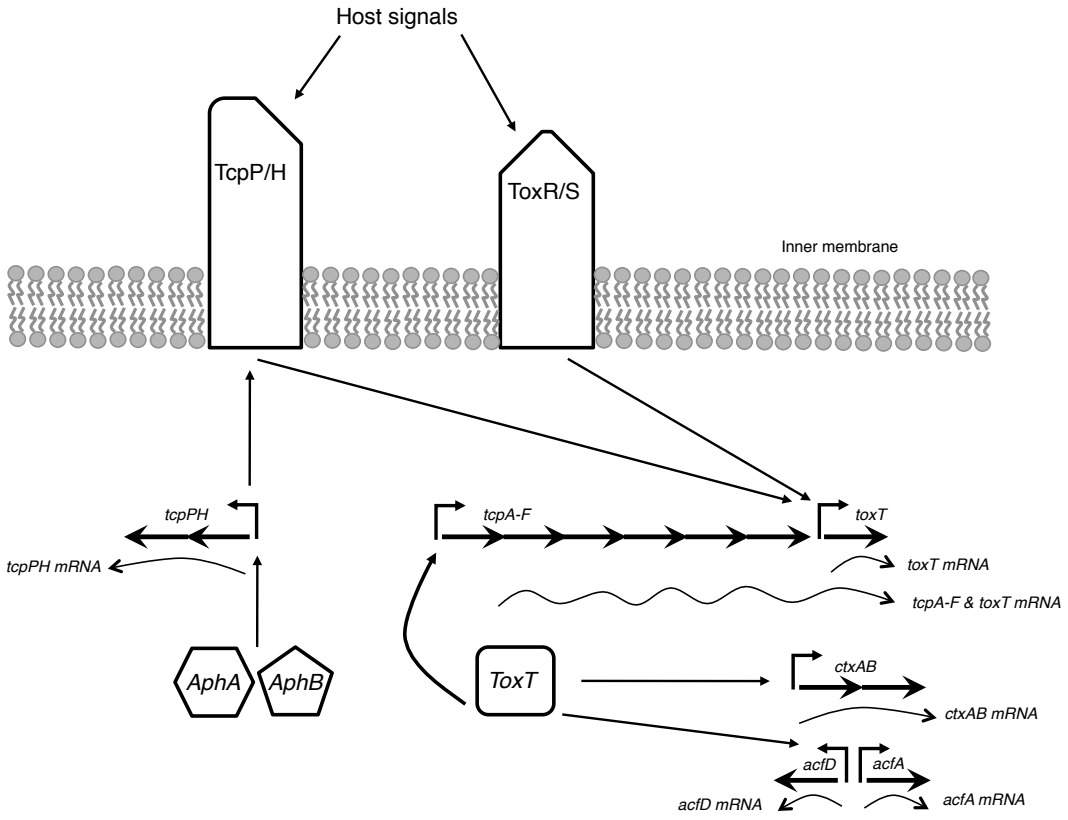
In order to cause disease, *V. cholerae* must express the essential virulence genes described above. A summary of the positive regulation of these genes is illustrated in Figure 1-2 and will be described below. Induction of the virulence genes, *tcpA-F*, *ctxAB* and *acfA/D* are under the control of the AraC-like transcription factor, ToxT (Higgins, Nazareno et al. 1992; Withey and DiRita 2005). ToxT contains a N-terminal cofactor binding and dimerization domain, and a C-terminal HTH DNA-binding domain involved in transcriptional activation (Prouty, Osorio et al. 2005; Weber and Klose 2011). ToxT is responsible for the expression of TCP, CT and ACF by directly binding their respective

promoters. The *toxT* gene itself, as well as several other virulence factors including *tcpA-F* and *acfA/D*, is located within the VPI-1 and is immediately downstream of *tcpA-F*. ToxT binds to an AT-rich sequence called a Toxbox, which is found within dozens of promoters scattered throughout the genome (Withey and DiRita 2006). In addition to ToxT regulating a number of virulence genes, it is also autoregulatory, as it can bind to a Toxbox within its own promoter and autoamplify (Yu and DiRita 1999). In addition to the ability of ToxT to positively regulate virulence genes, it has also been shown to be a repressor. ToxT can directly repress genes involved in biofilm formation, such as the type IV mannose-sensitive haemagglutinin (MSHA), further highlighting the importance of ToxT in *V. cholerae's* transition into the host (Hsiao, Toscano et al. 2008; Weber and Klose 2011).

The initial induction of *toxT* occurs via the ToxR/S and TcpP/H membrane-localized sensory proteins complexes, which also have DNA-binding transcriptional regulatory activity. Upon *in vivo* stimulation, ToxR/S and TcpP/H bind as a complex to the *toxT* distal promoter, which is located several genes upstream of *toxT* near one end of the VPI-1 (Childers and Klose 2007). While the precise signal(s) that ToxR/S and TcpP/H respond to are unknown, these regulators are activated *in vivo* suggesting that the stimuli may be a change in temperature, pH or the presence of bile salts (Childers and Klose 2007). In addition to inducing *toxT*, ToxR also can directly activate *ctxAB* to produce CT (Miller, Taylor et al. 1987). Presumably, the portion of the genome containing the promoters of *toxT* and *ctxAB* migrate to the inner-membrane where ToxR/S and TcpP/H are located, however the exact mechanism for how this regulation occurs is not clear.

In addition to ToxT, ToxR/S and TcpP/H, two additional positive regulators, AphAB have also been shown to play an important role in modulating *tcpPH*. The binding site for AphA lies directly upstream of the binding site for AphB, thereby allowing the AphAB complex to induce transcription, however the order in which AphA/B bind is essential. AphA is a winged helix DNA-binding protein that once bound to the *tcpPH* promoter, enhances the ability of the LysR-type transcriptional regulator, AphB, to bind and induce expression of the *tcpPH* operon (Murley, Carroll et al. 1999; Skorupski and Taylor 1999; Kovacikova, Lin et al. 2004; De Silva, Kovacikova et al. 2005). While AphB can bind in the absence of AphA, the binding is weaker and only occurs when AphB is over-expressed (Skorupski and Taylor 1999; Kovacikova and Skorupski 2001).

Figure 1-2. Positive regulation of *V. cholerae* virulence genes
As *V. cholerae* transitions into the host, these *in vivo* signals activate the membrane-bound regulators, TcpPH and ToxRS. Stimulated TcpPH and ToxRS activate *toxT* at its promoter, resulting in production of the transcription factor, ToxT. ToxT induces *tcpA-F* for the production of TCP and is also autoregulatory due to read-through from the *tcpA-F* promoter. ToxT also induces expression of *ctxAB, acfA* and *acfD*, resulting in cholera toxin and ACF expression. Additionally, AphAB is also produced early during infection and positively regulates *tcpPH*, however the regulation for *aphA* and *aphB* is currently unknown.



1.2.4.ii Repression by CRP

The cyclic-AMP receptor protein, CRP, has been well characterized in *E. coli* and is most commonly known for its crucial role in regulating carbon catabolite repression (CCR) by interacting with the α-subunit of RNA polymerase to promote transcription of genes involved in the metabolism of alternative carbon sources (Kolb, Busby et al. 1993; Busby 1999; Harman 2001; Krueger, Gregurick et al. 2003). CRP has also been shown to be a repressor or can behave as a co-activator or co-repressor, suggesting that the regulatory activity of CRP is global and includes a large number of genes (Botsford and Harman 1992; Kolb, Busby et al. 1993). Like most transcriptional activators, CRP acts as a homodimer and harbors a C' terminal HTH DNA-binding motif that is responsible for directly binding DNA within the target promoter to induce transcription (Aiba, Fujimoto et al. 1982; Busby 1999). A consensus sequence for CRP recognition and binding, TGTGA-N_6-TCACA, has been determined for *E. coli* but this sequence can vary between organisms (Hawley and McClure 1983). The N' terminus of CRP contains a ligand binding domain that is responsible for dimerization and the activation of CRP as a transcriptional regulator. In order for CRP to be active, it must bind the small molecule cyclic-AMP (cAMP) at this domain, forming a cAMP-CRP complex. cAMP is produced by the adenylate cyclase, *cyaA*. *cyaA* is involved with the glucose PTS system, where in the absence of glucose, phosphate is passed from the PTS to *cyaA*, resulting in *cyaA* activation and production of cAMP (Botsford and Harman 1992). However, CRP activity is bistable regarding cAMP concentrations as CRP has an additional cAMP-binding site located near the C' terminal HTH domain. When cAMP concentration is too low no CRP

will be bound to cAMP and CRP is inactive. As cAMP concentration increases, cAMP will reach a level that optimally binds CRP at the N' terminus, resulting in cAMP-CRP and its activation. However, if cAMP concentration becomes too high, CRP will bind this ligand at the second site and form a $cAMP_2$-CRP complex, resulting in a conformational change that renders this complex unable to bind DNA (Schultz, Shields et al. 1991). Therefore CRP activity is tightly controlled by cAMP concentration in the cell.

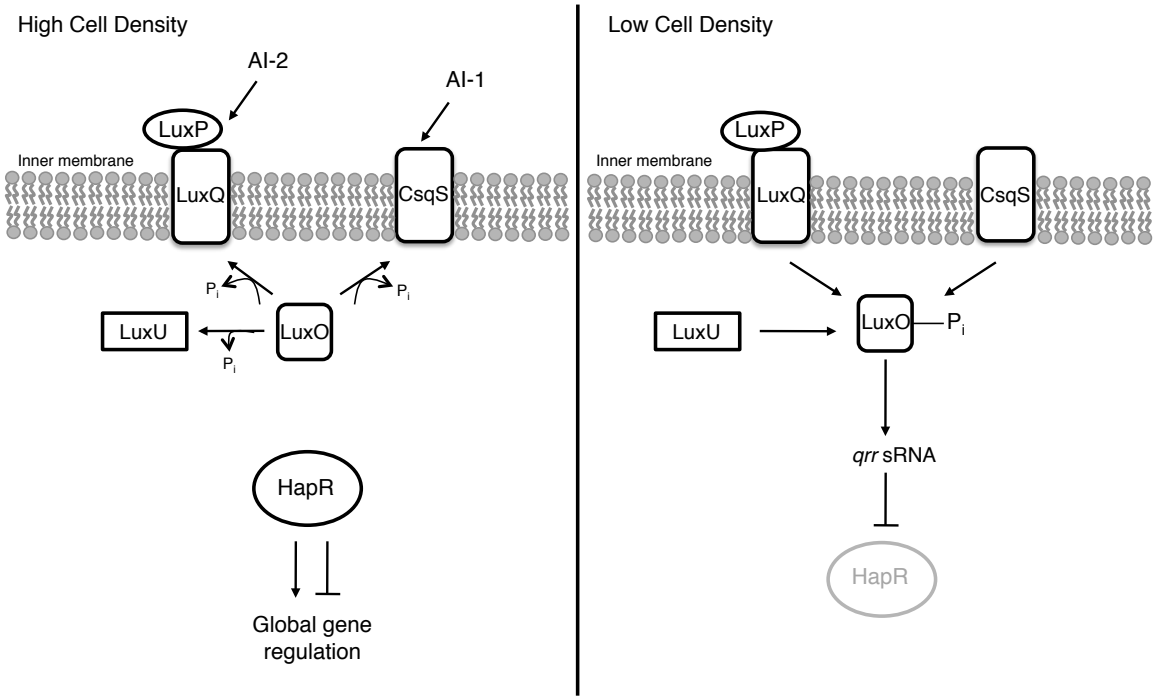
While CRP has been shown to regulate the *tcpPH* operon, in this case, CRP is a repressor (Kovacikova and Skorupski 2001). Interestingly, the binding site for CRP significantly overlaps with the binding site of AphA, which we know is important for AphB binding and activation at this promoter. *tcpPH* is therefore dual regulated though the activation by AphAB and repression by CRP. This is due to the direct competition for binding between AphA and CRP. Moreover, CRP has been shown to repress other virulence genes as well, such as the gene encoding CT and TcpA, however it is unclear if this repression is direct or indirect as these genes are in some way regulated by TcpPH (Skorupski and Taylor 1997). CRP has also been shown to play an important role in regulating virulence genes and pathogenesis in *Salmonella enterica* serovar Typhimurium (Curtiss and Kelly 1987; O'Byrne and Dorman 1994). Taken together, this highlights the importance of CRP in the regulation of virulence genes and reveals the integration of virulence and metabolism regulation to control pathogenesis.

1.2.4.iii Repression by quorum sensing

Building on this already complex regulatory system, quorum sensing (QS), or the measurement of bacterial population densities, also modulates virulence gene expression. QS is highly involved in the global regulation of genes throughout the life cycle of *V.*

cholerae and a summary of this network is illustrated in Figure 1-3. *V. cholerae* harbors two main QS networks, LuxPQ and CqsS which respond to their respective auto-inducers, AI-1 and AI-2 (Waters and Bassler 2005). When the cell population is low, the concentration of AI-1 and AI-2 are reduced. Under these conditions, the response regulator, LuxO, is phosphorylated by the sensor kinases, LuxQ, LuxU and CqsS. Phosphorylated LuxO leads to the production of the sRNA, *qrr*, which disrupts the translation of the central QS regulator, *hapR* (Lenz, Mok et al. 2004). Alternatively, when bacterial population densities are high, so is the concentration of auto-inducers. Under these conditions, LuxQ, LuxU and CqsS behave as phosphatases that remove phosphate from LuxO, resulting in low *qrr* and production of HapR, which can act as an activator or repressor for a number of genes.

Figure 1-3. Quorum sensing in *V. cholerae*
At low cell density (right), the concentration of autoinducers (AI) is low. Under this condition, the membrane bound sensor kinases LuxPQ, CsqS and the cytoplasmic sensor kinase, LuxU, phosphorylated the response regulator, LuxO. This phosphorylation leads to the expression of *qrr* sRNAs, which interfere with *hapR* mRNA translation and results in low levels of the transcription factor, HapR. Conversely, at high cell density (left), the concentration of AI is high and stimulates LuxQ, CsqS and LuxU phosphatase activity. This results in the dephosphorylation of LuxO, lowered *qrr* levels and production of HapR. HapR is able to activate or repress a variety of genes to induce changes in gene expression in order to survive.

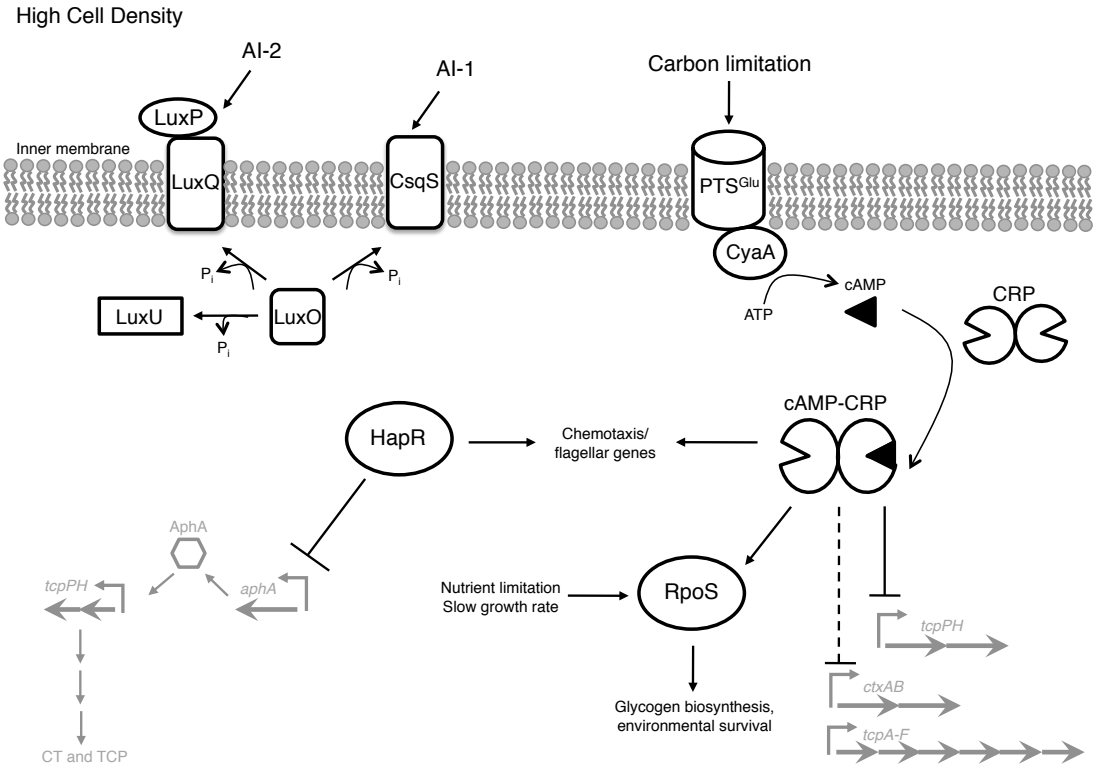


Overlay of the QS network on top of the *V. cholerae* life cycle provides a simplistic overview of virulence regulation by this system. Upon entry into the small intestine of the host, *V. cholerae* are at low cell density and are distance from each other, resulting in low concentrations of auto-inducers. Therefore, HapR is not expressed, allowing for expression of virulence genes required for colonization and infection. However, as *V. cholerae* beings to multiply and form microcolonies along the intestinal lining, bacterial populations and auto-inducers increase, especially late in infection. Under this high cell density, levels of HapR increase and lead to induction of chemotaxis and flagellar genes, allowing *V. cholerae* to detach and escape the intestinal lumen for exit out of the host (Nielsen, Dolganov et al. 2006). In addition, HapR represses *aphA*, thus repressing *tcpPH* and *toxT,* leading to the subsequent down regulation of virulence genes; this prepares *V. cholerae* for its exit into the environment (Kovacikova and Skorupski 2002; Miller, Skorupski et al. 2002; Zhu, Miller et al. 2002; Zhu and Mekalanos 2003; Nielsen, Dolganov et al. 2006).

Furthermore, since HapR is most active at high cell densities, this means that HapR is functional during stationary phase of growth; CRP is also activated in stationary phase due to the lack of preferred carbon sources and slowed growth rate. Interestingly, the cAMP-CRP complex promotes increased HapR expression (Hengge-Aronis 2002; Silva and Benitez 2004), revealing an important junction for virulence regulation between QS and metabolism. CRP activation leads to induction of chemotaxis and motility genes along with the production of the sigma-factor, RpoS, which plays a role in induction of genes necessary for environmental survival, such as glycogen biosynthesis (Schild,

Tamayo et al. 2007; Bourassa and Camilli 2009). Therefore, HapR and CRP appear to work in tandem to efficiently turn off the virulence cascade (Figure 1-4).

Figure 1-4. Repression of virulence by HapR and CRP
During stationary phase and high cell density, the Lux and CsqS QS systems are activated, resulting in the expression of HapR. HapR induces the expression of chemotaxis and flagellar genes but represses *aphA* expression, resulting in decreased expression of downstream virulence genes. Additionally, under these conditions, carbon limitation is sensed by the glucose-specific PTS transport system (PTS^{Glu}). This results in phosphorylation of adenylate cyclase (*cyaA*) and production of cAMP. cAMP binds to CRP, allowing CRP to up regulate chemotaxis and flagellar genes. cAMP-CRP also activates the sigma-factor, RpoS, which increases the production of glycogen biosynthesis genes, among other environmental survival genes. cAMP-CRP also directly represses *tcpPH* and indirectly represses the expression of *ctxAB* and *tcpA-F*. Together, HapR and CRP are able to shut down virulence when *V. cholerae* is exiting the host.



1.2.4.iv Role of cyclic-di-GMP

In addition to utilizing transcription factors, *V. cholerae* can also control the expression of virulence genes through the allosteric activator and cytoplasmic secondary messenger, cyclic diguanylate (cdGMP). cdGMP is common among Gram-negative and some Gram-positive bacteria, and is used as a global regulatory molecule to induce a wide range of physiological changes, such as biofilm formation, motility, adhesion, and the cell cycle (Krasteva, Giglio et al. 2012). Bacteria, including *V. cholerae*, vary cdGMP levels through the activities of diguanylate cyclases (DGCs) and cdGMP-specific phosphodiesterases (PDEs), which produce and degrade cdGMP respectively (Tischler and Camilli 2004; Tamayo, Schild et al. 2008; Krasteva, Giglio et al. 2012). DGCs harbor a conserved amino acid motif, GGDEF, which takes two GTP molecules and generates cdGMP. Breakdown is achieved by PDEs, which can be further sub-divided into two classes, those that contain a conserved EAL amino acid sequence and those that contain a HD-GYP sequence. Both result in degradation of cdGMP but the end products differ. cdGMP breakdown by EAL proteins result in linear di-GMP while cleavage by HD-GYP proteins produces two GMP molecules (Krasteva, Giglio et al. 2012). There are 31 PDEs and 40 DGCs annotated in the *V. cholerae* genome (Beyhan, Tischler et al. 2006). Opportunistic pathogens often have the highest number of cdGMP metabolizing enzymes, as they must adapt to different environments (Galperin, Nikolskaya et al. 2001; Romling, Gomelsky et al. 2005; Krasteva, Giglio et al. 2012). While *V. cholerae* is not an opportunistic pathogen, it does reside in two drastically different environments, thus requiring precision in sensing its environment and having tight control over intracellular

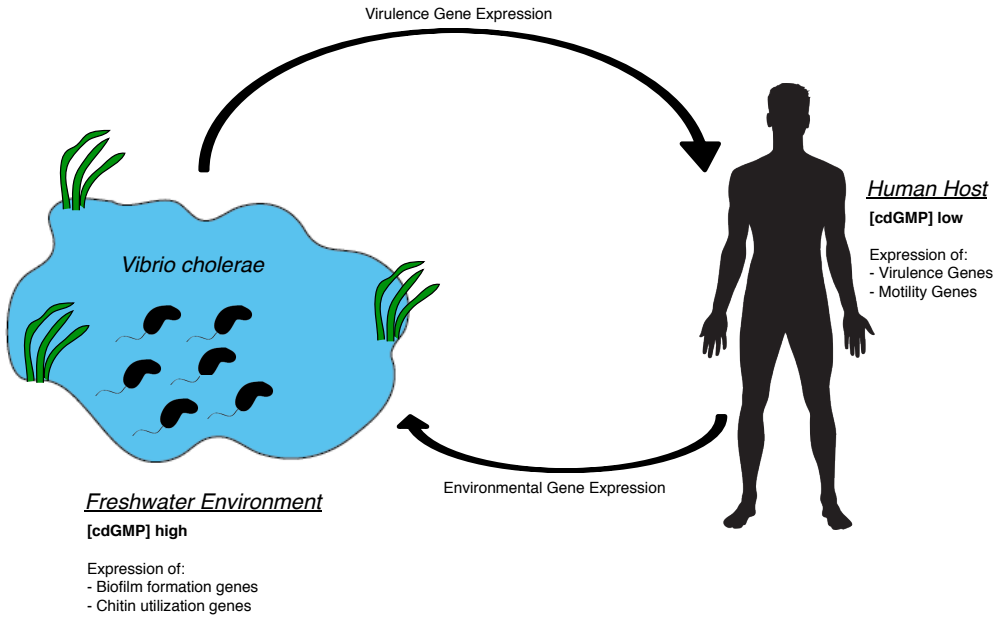
cdGMP levels to modulate gene expression. DGCs have been found on the STX/R391 family of mobile integrating conjugative elements (ICEs) in *V. cholerae* (Bordeleau, Brouillette et al. 2010). It is hypothesized that these DGCs may enhance the survival of *Vibrios* in the aquatic environment and could be a route of horizontal transfer of cdGMP metabolizing genes between different strains or even different species of bacteria.

Bacteria that utilize this secondary messenger encode numerous proteins that bind cdGMP, allowing the organism to sense intracellular levels. *V. cholerae* is able to monitor cdGMP concentration through protein sensors containing the PilZ domain (Benach, Swaminathan et al. 2007; Ko, Ryu et al. 2010). Typically, concentrations of intracellular cdGMP are modulated to either promote or prevent biofilm formation, where high concentrations of cdGMP promote biofilm formation and low concentrations result in biofilm dispersion. Interestingly, at low cdGMP level, virulence genes are expressed, which points to an important role of cdGMP in modulating virulence factor expression (O'Toole 2000; Tischler and Camilli 2005; Jenal and Malone 2006). Moreover, studies have shown that proteins containing PilZ domains are directly involved in biofilm and virulence gene regulation in *V. cholerae* (Pratt, Tamayo et al. 2007; Beyhan, Odell et al. 2008). In addition, cdGMP sensing riboswitches have been identified in *V. cholerae* and other bacteria, though their role in regulating virulence or biofilm formation genes has not been demonstrated (Smith and Strobel 2011).

In context of the life cycle of *V. cholerae*, as this pathogen enters the host, cdGMP concentrations must rapidly decrease presumably by *in vivo* signals that induce the expression of PDE-containing proteins. Therefore, as *V. cholerae* enters the small intestine, the low cdGMP level allows for the expression of virulence genes needed for

colonization and survival. Conversely, later in infection, the cdGMP concentration increases in order to prepare *V. cholerae* for its eventual exit back into the environment, where biofilm formation and chitin utilization genes will be necessary for survival (Figure 1-5). While this simple model of cdGMP modulation is supported by studies conducted by our lab (Tischler and Camilli 2005; Schild, Tamayo et al. 2007; Tamayo, Schild et al. 2008), other work suggests that cdGMP concentration appears to increase in response to bile salts, which suggests that cdGMP is high when *V. cholerae* is in the small intestine and regulation of this secondary messenger molecule is more complicated (Koestler and Waters 2014). However, the life cycle of this waterborne pathogen and the variety of signaling systems that *V. cholerae* utilizes to regulate virulence genes clearly underscores the apparent necessity for *V. cholerae* to incorporate a number of checkpoints and control mechanisms to properly regulate its transition between the host and environment.

Figure 1-5. Role of cdGMP in the *V. cholerae* life cycle
As *V. cholerae* moves from the aquatic environment into the host by ingestion, it must being to express virulence genes. Through the action of phosphodiesterases, cdGMP is cleaved, resulting in low intracellular cdGMP. This allows for the expression of virulence and motility genes and the repression of biofilm formation and other environmental genes. Alternatively, as *V. cholerae* transitions out of the host back into the aquatic environment, intracellular levels of cdGMP must be high. Diguanylate cyclases form cdGMP, resulting in the induction of environmental genes and repression of virulence genes.



1.2.4.v Differences in virulence among biotypes

El Tor and classical biotypes, while highly similar at the genetic level, do harbor a few important differences, especially in regards to the virulence genes. Among these is that there is diversity in the prophage between classical ($CTX\Phi^{Cla}$) and El Tor ($CTX\Phi^{El}$) and most interestingly, encodes distinctive CT genes. CT expression from $CTX\Phi^{Cla}$ causes more severe diarrhea than CT expression from $CTX\Phi^{El}$. However, recent data suggests that the El Tor biotype, and its CT, is evolving. It has been reported that variants have been isolated that contain combinations of El Tor and classical qualities, potentially leading to better survival in the aquatic environment, the host or both (Faruque, Asadulghani et al. 1998; Reidl 2002; Das 2012). Three variants have been identified so far: "Matlab", which mixes classical and El Tor traits; "Mozambique", which is an El Tor biotype with a tandem repeat of classical CTXΦ; and other El Tor variants that encode the El Tor CTXΦ but produce classical CT (Morita, Ohnishi et al. 2008; Das 2012). While classical strains are no longer found in the clinic, it is possible that classical still exists in the environment. If so, classical could play an important role in contributing to the evolution of the *V. cholerae* species and the disease.

In addition to differences in virulence genes, regulatory mechanisms also vary between classical and El Tor. Transcriptional profiling analysis revealed 524 genes are differentially regulated between the two biotypes during growth in virulence inducing conditions. Of importance for this work, the *vieSAB* system was expressed at a level 5-fold higher in the classical biotype (Beyhan, Tischler et al. 2006). Furthermore, the timing of virulence gene expression has also been shown to differ between El Tor and

classical. Under ToxR-inducing conditions in El Tor, expression of *tcpP* was lower at mid-log phase when compared to classical. However, later in growth, *tcpP* was expressed at a higher level than in classical, suggesting there are growth phase differences in TcpP expression between these biotypes (Murley, Behari et al. 2000). Perhaps the most striking difference in virulence gene regulation between the two biotypes is that virulence genes are maximally expressed *in vitro* in classical by growth in slightly acidified LB broth at 30°C with aeration or M9 minimal media supplemented with 0.5% glycerol and four amino acids (serine, asparagine, arginine, and glutamic acid [NRES]), whereas these conditions result in no virulence gene expression in El Tor. In contrast, El Tor maximally induces virulence genes *in vitro* during static growth in a bicarbonate containing medium (AKI) at 37°C (Iwanaga, Yamamoto et al. 1986). This difference in virulence gene regulation has been traced to differences in the *tcpPH* promoter, which alters the affinity for AphA binding. Therefore, differences in binding of AphA in classical and El Tor biotypes result in variable binding of AphB and differential expression of *tcpPH* and downstream virulence genes.

Despite the differences between biotypes, the ability of *V. cholerae* to recognize its environment and modify gene expression as it transitions between the aquatic environment and the host is an important aspect to its pathogenic life style. *V. cholerae* optimally lives in the aquatic environment, which is nutrient-poor (Miller, Drasar et al. 1982; Schild, Tamayo et al. 2007; Nelson, Chowdhury et al. 2008). However, once ingested, *V. cholerae* encounters a completely different environment. While nutrient dense, the small intestine has low levels of oxygen and antimicrobial host factors are present. Even within the small intestine, *V. cholerae* experiences different

microenvironments throughout the course of infection. Changes in nutrient availability and oxygen concentration, among other conditions, promote drastic changes in the gene expression profile in order for *V. cholerae* to adapt and survive (Merrell, Hava et al. 2002; Larocque, Harris et al. 2005; Nielsen, Dolganov et al. 2006; Schild, Tamayo et al. 2007; Nielsen, Dolganov et al. 2010). As a result of *V. cholerae's* ability to occupy a variety of ecological niches, it is a model organism for studying how bacteria sense and adapt to changing environments. One way *V. cholerae* senses its environment is through two-component systems (TCS), which will be discussed in detail below (Pratt, Ismail et al. 2010; Jang, Jung et al. 2011; Tsou, Liu et al. 2011).

1.3 TWO-COMPONENT SYSTEMS IN BACTERIA

1.3.1 Overview of two-component systems

Bacteria are remarkable for their ability to colonize practically every niche on the planet. Because virtually all environments are dynamic, efficient bacterial adaptation is key to fitness. Bacteria express genes required for optimal growth and suppress those genes that are unnecessary. To achieve this tight control, bacteria must detect the composition of the environment and convey this change to regulate specific enzymes and pathways needed to survive.

One universal mechanism by which bacteria sense their surroundings is through two-component systems (TCS). After stimulation of the TCS by its specific environmental stimulus, usually binding of a small molecule, a signal is then transmitted into the cytoplasm allowing the bacteria to modify gene expression and adapt. TCSs have been shown to recognize a wide array of environmental signals, such as nutrient and

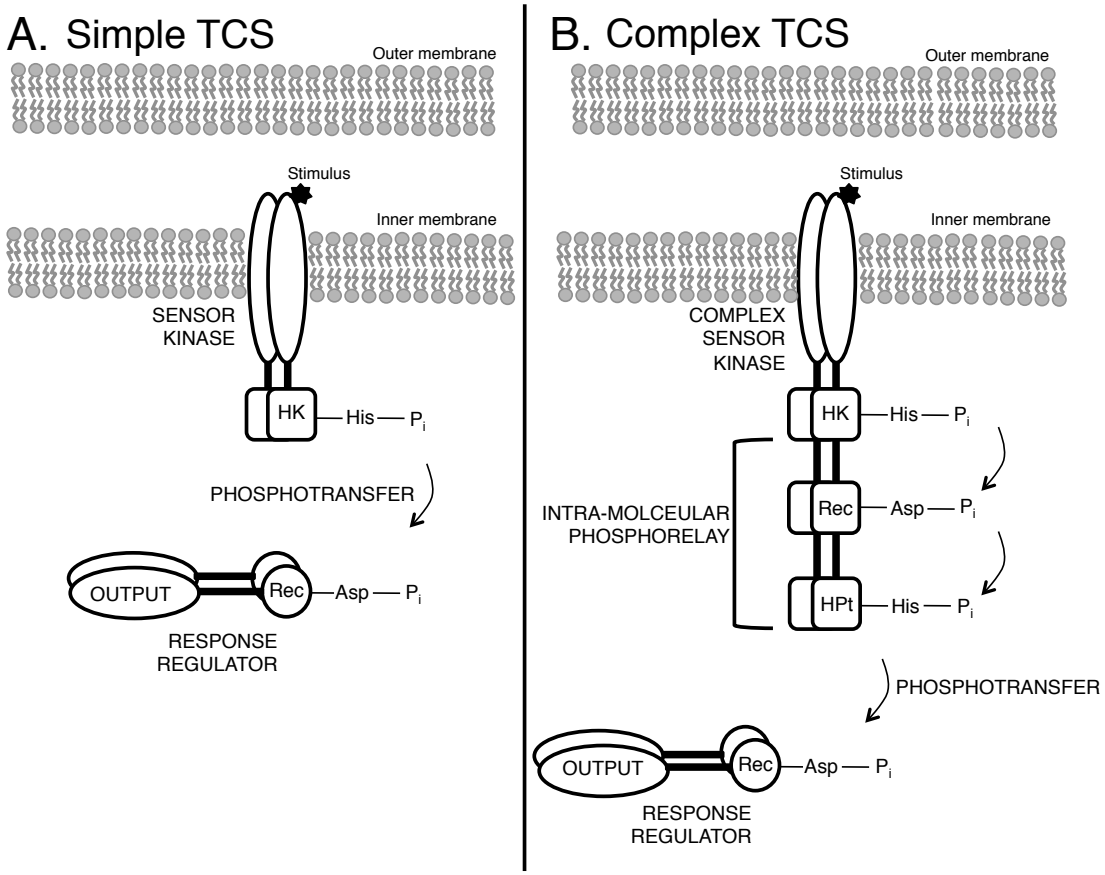
micronutrient concentrations, quorum signaling molecules, host derived hormones, changes in cellular redox state, pH and even antibiotics (Mitrophanov and Groisman 2008; Krell, Lacal et al. 2010). Given that some bacteria, like *V. cholerae*, endure dramatic environmental changes as they enter the human host, a number of TCSs have been implemented in bacterial pathogenesis (Gao and Stock 2009; Krell, Lacal et al. 2010; Tsou, Liu et al. 2011; Steele, O'Connor et al. 2012).

In the simplest form, TCSs are composed of an "orthodox" sensor kinase (SK), which is a dimer that contains a sensor domain and histidine kinase "transmitter" domain (HK), and a response regulator (RR). Figure 1-6 illustrates a membrane-bound periplasmic SK, however other types of SK exist and will be described in the next section. Upon binding and recognition of a stimulus by the periplasmic sensor domain, this results in autophosphorylation at the HK domain of the SK. This phosphoryl group is then passed from the HK domain to the receiver domain (Rec) on the RR, which results in its dimerization and activation; the phosphorylated RR is able to perform some output activity, which is typically changes in gene expression. In bacteria, the transmission of information from the SK to the RR is typically achieved through the transfer of phosphoryl group using His-P_i/Asp-P_i signaling cascades, however in *Myxococcus xanthus*, some Ser-P_i/Thr-P_i relays have been described (Lux and Shi 2005; Nariya and Inouye 2005). Typically, the SK harbors the phosphorylatable His residue and the RR harbors the phosphorylatable Asp residue with phosphotransfer flowing in the His→Asp direction (Figure 1-6 A).

Bacteria can also increase the complexity of the TCS. Inclusion of additional players in a phosphorelay allows for additional phosphotransfer events, which could be

used for additional sensory inputs, signal amplification, or provide opportunities for additional regulation, since the relay can be modified or disrupted at any transfer step (Hoch and Varughese 2001; Krell, Lacal et al. 2010). Phosphorelays can be incorporated through the addition of single domain proteins or through an "unorthodox" hybrid SK. "Unorthodox" hybrid SKs include supplementary domains in the cytoplasmic portion of the SK for additional phosphotransfer events, known as the intra-molecular phosphorelay. A common complex SK design is a HK-Rec-HPt (Histidine phosphotransfer) domain structure, which follows a phosphorelay pattern of His→Asp→His→Asp, with the last Asp residue being located on the RR (Figure 1-6 B). Of note, the same phosphorelay can be achieved using SK-independent single domain proteins that carry the Rec and HPt; examples of this type of TCS will be discussed later in this chapter.

Figure 1-6. Overview of TCS
(A) A simple TCS is comprised of a sensor kinase (SK) harboring a periplasmic sensor domain, a transmembrane region and a cytoplasmic histidine kinase domain (HK). Upon binding its stimulus, the SK undergoes a conformational change that allows for autophosphorylation at the His residue on the HK domain. This phosphate is then transferred onto the response regulator (RR) at the Asp residue on the receiver domain (Rec), resulting in dimerization, activation and performance of an output function. **(B)** A phosphorelay can be incorporated into the simple TCS through a hybrid SK, resulting in a complex TCS. The hybrid SK includes extra domains, like Rec and a histidine phosphotransfer domain (HPt) that provide additional phosphotransfer steps prior to the final phosphorylation of the RR.



1.3.2 Evolution and specificity of bacterial signaling

Since TCS are used to sense a multitude of signals in a variety of environments, it is not unexpected that most bacteria encode for a large number of these systems. Many TCSs are expressed constitutively, at least at low basal levels, in order to be prepared to respond to changing environments. Therefore a variety of TCSs are expressed at any given time; bacteria must ensure that activation or inactivation of a TCS does not occur nonspecifically, which can weaken the response when the appropriate stimulus is present. To avoid this issue, bacteria have evolved mechanisms to provide specificity between cognate SK-RR pairs to avoid cross-talk, i.e. the nonspecific phosphorylation of a RR by either the wrong SK or small molecular phosphodonors.

One such mechanism is co-localization of cognate SK and RR pairs (Angelastro, Sliusarenko et al. 2010), though this mechanism hasn't been explored for the vast majority of TCSs. Notable exceptions include cell cycle signaling in the dimorphic bacterium, *Caulobacter crescentus* and chemotactic signaling in *E. coli*. In *C. crescentus* cell cycle progression and cell division is controlled by the polar localization of the HK CckA. The location of CckA with either the phosphatase, PleC, at the flagellated pole or the kinase, DivJ, at the stalked pole, plays a critical role in determining activation of CckA kinase or phosphatase activity, which is controlled by a second polar-localized TCS, DivLK (Paul, Jaeger et al. 2008; Tsokos, Perchuk et al. 2011). By localizing the CckA to one pole, this not only modulates its activity but results in this signaling system being spatially protected from other TCS present elsewhere in the cell. Furthermore, the co-localization of chemotaxis proteins has been shown in *E. coli* to allow for the tight

regulation and rapid signal modification for chemotaxis movement towards or away from environmental signals (Maddock and Shapiro 1993; Hansen, Sourjik et al. 2010).

While spatial location is an efficient method for preventing cross-talk, some TCSs are needed throughout the cell. In this case, there are no longer protective compartments and two unrelated TCSs could be located very close to one another. Therefore, to limit cross-talk in this situation, bacteria have ensured cognate SKs and RRs pairs are biochemically specific for each other (Casino, Rubio et al. 2009; Capra 2010

). Studies suggest that RRs favor their cognate SKs even in the presence of nonspecific SKs or small molecular phospho-donors (such as acetyl-phosphate, [Ac-P]). For example, the phosphotransfer between a cognate SK-RR pair proceeds at a much higher rate than when the RR is incubated with a nonspecific SK (Lukat 1992; Mayover, Halkides et al. 1999). In further support of this mechanism, recent studies have isolated areas within the RR that are important for this specificity. Boll et al. have shown that in *Campylobacter jejuni,* the C' terminal domain of the FlgR RR, which also acts as the DNA-binding region, is important for limiting cross-talk from Ac-P (Boll and Hendrixson 2011). Furthermore, Skerker et al. have identified key amino acids in the SK that designate specificity to its RR (Skerker, Perchuk et al. 2008). By manipulating a few critical residues in the EnvZ SK of *E. coli*, the authors were able to change EnvZ specificity from its cognate RR, OmpR, to a non-cognate RR, RstB. Additionally, residues located on the RR have also been shown to provide specificity between SK/RR pairs (Capra 2010

). This and other data has built evidence to support the co-evolution of cognate SK/RR pairs for maintaining specificity (Skerker, Prasol et al. 2005; Kohanski and Collins 2008; Wuichet and Zhulin 2010; Reynolds, McLaughlin et al. 2011).

1.3.3 Sensor Kinase

The first component in the TCS that will be discussed in detail is the sensor kinase (SK). The SK is responsible for signal detection and transmitting information about the environment into the cell. This section will discuss the types of SK, what stimuli can be detected and the mechanisms for signal transduction.

1.3.3.i Sensing environments

There are three main categories for SKs with respect to how the SK senses its respective stimulus (Galperin 2006). SKs can either sense signals located in the cellular membrane, the cytoplasm or the periplasmic space, and it is through this array of sensors that bacteria can sample its environment and cellular state in order to adapt and survive.

Given the importance of the cellular membrane for a number of cellular processes such as growth, nutrient transport and energy conservation, it is not surprising that a number of TCSs are used to sense the status of the membrane and the activities that occur there. Generally, these SKs are located in the inner membrane and are anchored by their transmembrane helices. Cellular membrane SKs can be further subdivided into six main classes based on the number of transmembrane helices, which can range in number from 2 to 20, and the presence of a short or no extra-cytoplasmic regions (Mascher, Helmann et al. 2006). Additionally, these categories are loosely grouped by what the SKs sense, such as envelope stress, cell wall acting antibiotics, membrane fluidity and quorum sensing (Mascher, Helmann et al. 2006).

One example of a TCS that harbors a membrane SK is DesRK in *B. subtilis*. The SK is a part of the eponymous DesK-like subfamily of thermosensors, which sense membrane fluidity due to change in temperatures and consist of 4-6 transmembrane regions. DesRK is responsible for controlling the expression of *des*, encoding a fatty acid desaturase. When *B. subtilis* is growing in high temperatures (high membrane fluidity), DesK is not active as a kinase, thereby preventing *des* expression. However, upon a shift to lower temperatures (reduced membrane fluidity), DesK is stimulated, activating the RR, DesR, which results in strong expression of *des* and production of a cold shock response. While the mechanism of DesK sensing has yet to be completely solved, the ability of DesK to sense membrane fluidity due to temperature changes has been linked to four-five transmembrane regions located at the hydrophobic N' terminus (Aguilar, Cronan et al. 1998; Aguilar, Lopez et al. 1999; Aguilar, Hernandez-Arriaga et al. 2001; Cybulski, Albanesi et al. 2002; Hunger, Beckering et al. 2004). This is just one example of the type of TCSs that sense the cellular membrane in order to maintain membrane homeostasis.

The second group of SK that will be discussed are those that sense and respond to changes in the cytoplasm. The cytoplasm is home to many cellular processes that are vital to cell growth and survival. As such, cytoplasmic sensors detect a wide array of these processes, such as metabolism, DNA replication, protein synthesis, oxygen availability, and even chemotaxis and motility (Mascher, Helmann et al. 2006). These SKs are broadly grouped into three categories: (i) integral membrane proteins (ii) those associated with integral membrane proteins and (iii) SKs that reside only in the cytoplasm (soluble) and do not associate with the membrane or membrane proteins. Cytoplasmic SKs are

highly diverse with regards to the conserved domain structure, especially for the soluble cytoplasmic SK, making identification and classification of these SKs extremely difficult. Very little is known about most of the cytoplasmic SK, however a few well studied system have been characterized.

The KdpDE TCS in *E. coli* is responsible for adaptation to turgor pressure and osmolality changes by controlling the expression of the KdpFABC ATPase, which is the first response for *E. coli* when experiencing an osmotic upshift or K^+ limitation (Walderhaug, Polarek et al. 1992; Rothenbucher, Facey et al. 2006). KdpD reacts to osmotic shift by sensing K^+ and ATP concentrations along with ionic strength, all which are affected by osmolality changes. KdpD is a membrane cytoplasmic SK, with four transmembrane helices where both the N' and C' terminus are located in and responsible for cytoplasmic sensing. The N' terminus contains a Walker A and B motif, allowing for KdpD to bind ATP, while the C' terminus harbors the K^+ sensing domain (Heermann, Altendorf et al. 2003; Heermann, Fohrmann et al. 2003). Upon activation of KdpD by high osmolality and K^+ limitation, the cytoplasmic N' and C' termini interact, allowing for autophosphorylation and phosphotransfer to the RR, KdpE and production of the KdpFABC (Stallkamp, Altendorf et al. 2002).

While KdpD senses the cytoplasm from the membrane, NtrB is an example of a purely soluble cytoplasmic SK. Interestingly, less than one-fifth of the total number of identified SKs are soluble cytoplasmic SKs, which is quite surprising given the vast number of processes and potential signals that can occur in this cellular compartment (Mascher, Helmann et al. 2006). The NtrBC TCS in *E. coli* is important for regulating metabolism, specifically controlling genes required for the assimilation of ammonia and

alternative catabolism of nitrogen-containing compounds (Reitzer 1996). NtrB contains an N' terminal sensing domain, a domain containing the conserved His phosphorylation residue and a C' terminal transmitter domain. The internal domain containing the His residue is critical for the interaction between NtrB with another nitrogen sensor in the cytoplasm, PII. When nitrogen is in excess, PII interacts with NtrB, preventing its kinase activity. However, when nitrogen is limiting, PII no longer interacts with NtrB, allowing it to autophosphorylate and activate its RR, NtrC (Kamberov, Atkinson et al. 1995; Kramer and Weiss 1999; Pioszak, Jiang et al. 2000; Weiss, Kramer et al. 2002; Jiang 2003). These two systems are classic examples of two very different sub-groups within the cytoplasmic SK category.

The third and most important with respect to this work, is the membrane-bound periplasmic SK. Periplasmic SK are the largest and most well-studied group of SK, are present in both Gram-negative and Gram-positive bacteria and can sense a vast array of signals. Specifically, Gram-negative periplasmic SKs will be described. Most of these SK are located in the inner membrane spanning from the periplasm into the cytoplasm, which allows for the TCS to interact with and sense the extracytoplasmic environment (Hoch and Varughese 2001; Buelow and Raivio 2010). General diffusion porins in the outer membrane function to equilibrate the concentrations of small molecules outside the cell with the periplasm, and thus TCS sensing of conditions in the periplasm serves as a proxy for conditions outside the cell proper. Periplasmic SKs harbor an N' terminal periplasmic sensor domain, a transmembrane region typically consisting of two helices and a C' terminal cytoplasmic region that contains the site of autophosphorylation and phosphosignalling. Periplasmic SK can sense their respective signals in two main ways:

(i) by directly binding to small molecules or ligands or (ii) by binding to a periplasmic ligand-binding protein, thereby indirectly sensing the environment. However, mechanical, electrochemical, and concentration gradient changes have also been shown to be alternative mechanisms for signal detection (Mascher, Helmann et al. 2006). Unfortunately, the signals for most periplasmic SKs are unknown, making determining what and how these SK detect their signals difficult.

An example of the direct sensing model is CitA, the citrate sensor kinase in the CitAB TCS in *Klebsiella pneumoniae.* CitA is able to directly monitor citrate availability and is important for inducing genes necessary for anoxic metabolism of citrate (Sevvana, Vijayan et al. 2008). Conversely, the VirA SK, in the plant pathogen *Agrobacterium tumefaciens ,* can both directly and indirectly detect signals, though the method of detection depends on the signal. The VirAG TCS is responsible for sensing plant damage, where release of aldose monosaccharides and a drop in pH due to production of phenolic compounds are hallmarks of plant wounds. VirA can directly detect phenolic compounds at a cytoplasmic sensing region just downstream of the second transmembrane helix. Additionally, this region has also been shown to be involved in pH sensing (Gao and Lynn 2005). However, VirA can indirectly detect monosaccharides through the interaction with ChvE. ChvE is a periplasmic sugar-binding protein that interacts with the periplasmic VirA sensing domain when bound to sugars (Cangelosi, Ankenbauer et al. 1990; Shimoda, Toyoda-Yamamoto et al. 1990; Chang and Winans 1992; Heath 1995; Doty, Yu et al. 1996; Peng, Lee et al. 1998; Zhu, Oger et al. 2000). Therefore, VirA can utilize sensing domains on both sides on the membrane to accurately induce a response for adaptation. While VirAG can both directly and indirectly sense its stimuli, in the

LuxPQ quorum sensing system, LuxQ, is example of a SK that can only indirectly sense its signal though the periplasmic protein, LuxP. This system was described earlier (Figure 1-3) and will be highlighted again later in this chapter.

Since periplasmic SKs are sensing the extracytoplasmic environment, it has the challenging task of transmitting this information across the membrane to the cytoplasm in order to induce gene expression changes and a cellular response. How signal propagation is accomplished has been challenging to answer. However, some breakthroughs in understanding these mechanisms have been achieved and will be discussed next.

1.3.3.ii Signal Transduction

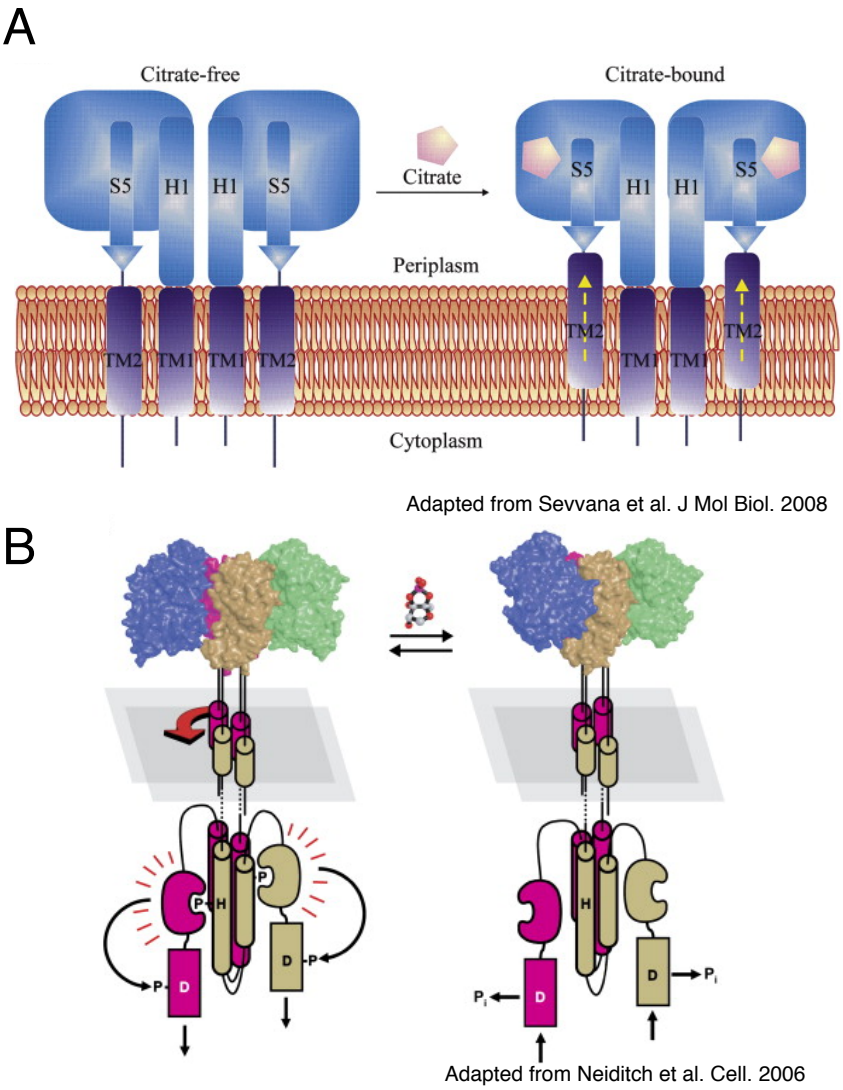
In order to relay information about the outside world to the bacterial cell, the SK must first recognize and detect its stimulus. Once the SK binds the signaling molecule, it must transmit that information through the membrane. A number of studies have provided compelling evidence that signal transmission is mediated through dramatic conformational changes in the sensing domain, which results in downstream structural changes in the transmembrane region and cytoplasmic portion of the protein (Ulrich, Kojetin et al. 2005; Neiditch, Federle et al. 2006; Casino, Rubio et al. 2009; Gao and Stock 2009; Casino, Rubio et al. 2010). These structural changes most often result in autophosphorylation of the SK at the HK domain. The structural changes throughout this process will be described below.

After ligand binding, CitA, an orthodox SK in the CitAB TCS that was described earlier, has been shown to undergo flexing in the central β-sheet within the dimerization region. This flexing results in a structural change that relocates the end of the β-sheet closer to the citrate-binding site. Though this conformation change, the second

transmembrane region of this periplasmic sensor is pulled away from the membrane in a piston-like fashion resulting in the transmission of this signal to the HK domain (Figure 1-7A) (Sevvana, Vijayan et al. 2008).

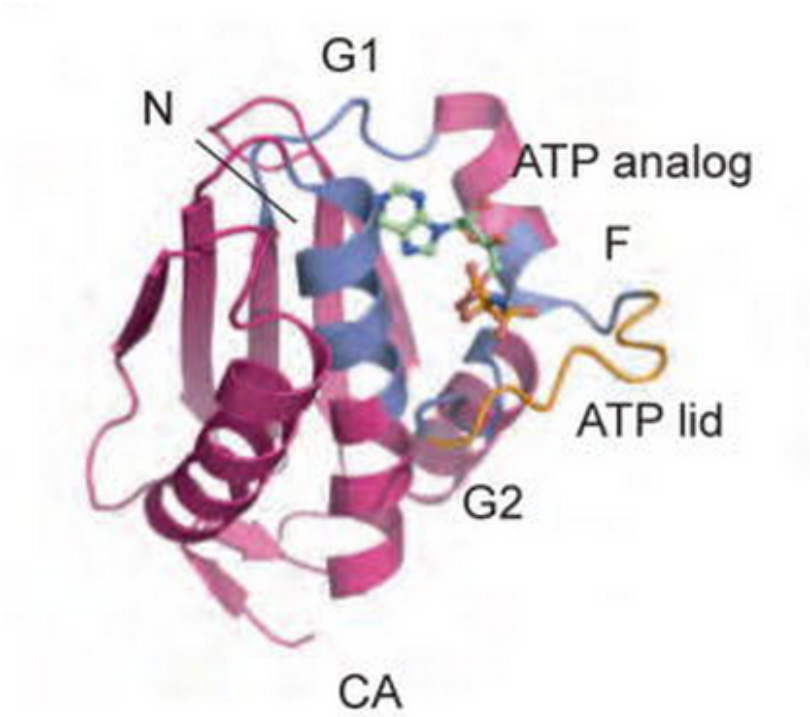
However, it appears that signal transduction can be achieved through different mechanisms. Much of our understanding about signal propagation is based on studies of the LuxPQ quorum sensing system in *Vibrio harveyi*. Similar to the LuxPQ system in *V. cholerae, V. harveyi's* LuxPQ system is located in the periplasm and uses the indirect sensing method, where LuxP is responsible for binding the autoinducer (AI-2) and subsequently binds LuxQ. The output of AI-2 bound-LuxPQ is dephosphorylation of LuxO. Through functional studies and protein crystallography, it was discovered that binding of AI-2, LuxP and LuxQ resulted in dimerization around AI-2 between two LuxPQ heterodimers. Unlike the piston action of CitA, signal transduction is thought to occur by a rotational movement. This rotation repositions the transmembrane α-helices and cytoplasmic domains of LuxQ, resulting in an asymmetrical dimer that prevents kinase activity and results in the subsequent dephosphorylation of LuxO (Figure 1-7B) (Neiditch, Federle et al. 2005). When LuxP is not bound to AI-2, LuxP binds a groove between two LuxQ domains, which prevents the formation of the quaternary structure (Neiditch, Federle et al. 2006). In this state, LuxQ behaves as a kinase (Neiditch, Federle et al. 2006). Interestingly, the association of LuxPQ in the absence of AI-2 blocks the AI-2 binding site; AI-2 can only bind LuxP when it reaches a critical threshold concentration, demonstrating a unique competitive inhibition at the sensor domain of the LuxQ.

Figure 1-7. Model for signal transmission of the CitA and LuxPQ SKs.
(A) CitA undergoes a conformational change after binding to citrate that causes a piston-like movement for the TM2 regions and transmission of the signal into the cytoplasm. Adapted from Sevvana et al., 2008, *J Mol Biol* 377(2):512-523 with permission. **(B)** LuxPQ, upon binding to AI-2, undergoes a rotational shift allowing for the formation of an asymmetrical periplasmic dimer that switches LuxPQ from kinase (left) to phosphatase activity (right). Adapted from Neiditch et al., 2006, *Cell* 126(6):1095-1108 with permission.



Once structural changes in the sensor domain have occurred, this causes a domino affect of downstream conformational changes that ultimately relay the signal. The sensor domain changes are thought to transmit this information through the membrane to a cytoplasmic coiled-coil region located just downstream of the last TM region (Gao and Stock 2009). This results in autophosphorylation at the HK. Within the HK domain there are two distinct sub-domains, a conserved long α-hairpin structure known as the dimerization and histidine phosphotransfer domain (DHp or HisKA) and the globular catalytic, ATP-binding domain (CA or ATPase). These sub-domains contain five highly conserved homology boxes, H (in the DHp domain) and N, G1, F, G2 (in the CA domain)(Gao and Stock 2009). The DHp domain is responsible for dimerization, which is required for HK activity, and also harbors the conserved His residue for phosphorylation in the H box. Unlike eukaryotic signaling mechanisms, in which the SK dimerizes upon signal binding, the bacterial SK appear to behave as constitutive dimers and signal activation only alters the dimerization interface to relay the signal to the HK (Gao and Stock 2009). The CA domain contains an ATP binding site created by the N, G1, F, and G2 boxes. Between the F and G2 boxes is a flexible region referred to as an "ATP lid" (Figure 1-8). The conformation of this lid is important not only for ATP binding but also for connecting this binding to the regulation of the SK through inducing interdomain conformational changes (Pearl and Prodromou 2006; Gao and Stock 2009).

Figure 1-8. Crystal structure of the PhoQ CA domain.
Structure of the prototypical PhoQ CA domain reveals the conserved N, G1, F, G2 boxes and the "ATP lid", which is important for ATP binding and catalysis. Adapted from Gao et al., 2009, *Annu Rev Microbiol* 63:133-154 with permission.



Adapted from Gao et al. Annu Rev Microbiol. 2009

The interaction between the DHp and CA regions is also important for SK regulation, where in the inactive state, the DHp and CA are connected by a large interface, preventing autophosphorylation and kinase activity. Disruption of this interaction through global conformational changes allows for autophosphorylation (Gao and Stock 2009). Autophosphorylation occurs when ATP binds the CA domain and the γ-phosphate is cleaved off the ATP molecule. This phosphoryl group is then transferred to the histidine residue in the DHp domain (Casino, Rubio et al. 2010; Gao and Stock 2010; Huynh and Stewart 2011). The nature of the His residue makes this conserved phosphorylation site an ideal phosphotransfer intermediate, as the bond between the His ring and the phosphate is highly unstable (Gao and Stock 2010). Autophosphorylation has been reported to occur in both *cis* and *trans* between the two SK peptides and this method of phosphorylation varies depending on the specific SK (Gao and Stock 2009).

For orthodox or canonical SK, autophosphorylation at the HK leads directly to the transfer of phosphate to its cognate RR. However, for unorthodox or hybrid SKs that incorporate an intramolecular phosphorelay, additional phosphotransfer steps are required to pass phosphate to the RR. As illustrated in Figure 1-6 B, the hybrid SK includes a receiver domain (Rec) and a histidine phosphotransfer domain (HPt), which uses a His→Asp→His relay to pass the phosphate to the RR. Phosphorylation that occurs at each residue induces a conformational change, allowing for the transfer of phosphate down the SK. The Rec domain contains a conserved Asp residue, serving as the first intermediate step in the phosphorelay. Just as for the His residue, Asp also has a high free energy of hydrolysis. The last step in the phosphorelay involves the kidney-shaped HPt

domain that contains a conserved all α-helical structure consisting of six anti-parallel helices, which generate a hydrophobic core. In the inner curve of the kidney shape lies the conserved His residue that is able to accept phosphate (Kato, Mizuno et al. 1997). The conserved His residue of the HPt domain is similar in instability to the His in the DHp, making the HPt domain an effective phosphotransfer intermediate (Xu, Carlton et al. 2009). However, unlike the HK domain it appears that the HPt domain itself does not harbor kinase activity and can therefore only assist in the transfer of phosphate to the RR (Gao and Stock 2009).

In addition to Rec and HPt domains, a hybrid SK can also harbor small domains, which are involved in signal recognition, protein-ligand, or protein-protein interactions. Examples of such domains are histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases (HAMP), and the Per-Arnt-Sim (PAS) domains, where 33% and 31% of all SK characterized have these domains, respectively (Gao and Stock 2009). Both PAS and HAMP domains, if present, are critical to the signaling mechanism but the specific role of these domains remains to be revealed for most TCSs (Gao and Stock 2009; Krell, Lacal et al. 2010). PAS and HAMP domains have high sequence and structural variability, allowing them to harbor a wide array of functions. Some PAS and HAMP domains have been shown to promote protein-protein interactions, be involved in signal detection or signal transduction (Taylor and Zhulin 1999; Anantharaman, Balaji et al. 2006; Gao and Stock 2009). A few PAS domains have been shown to be important for the kinase activity of the SK. However, PAS domains have also been shown to play a role in allowing some SKs to harbor phosphatase activity and reverse the flow of phosphate, i.e. from the RR back to the SK (Gao and Stock 2009).

1.3.3.iii SK phosphatase activity

For a SK to dephosphorylate the RR, it must harbor both phosphatase and kinase activity and is referred to as dual functioning SK (Gao and Stock 2013). While it seems that dual functioning SKs are a conundrum, the ability of the SK to both phosphorylate and dephosphorylate its cognate RR can be quite useful. Other mechanisms for dephosphorylating a RR, such as phosphatase activity by an additional protein or spontaneous hydrolysis (Figure 1-9), can be slow or requires incorporation of a number of other factors, making regulation complex. However the dual kinase/phosphatase activity of the SK is advantageous, as it ensures rapid inactivation of the RR. As with the forward flow of phosphate, the reverse flow is thought to be specific between a cognate SK/RR pair, allowing rapid shut down of an adaptive response, most often in the absence of signal. This mechanism also provides insulation from cross-talk, by preventing other SK or orphan phosphatases from dephosphorylating the RR in an inopportune moment.

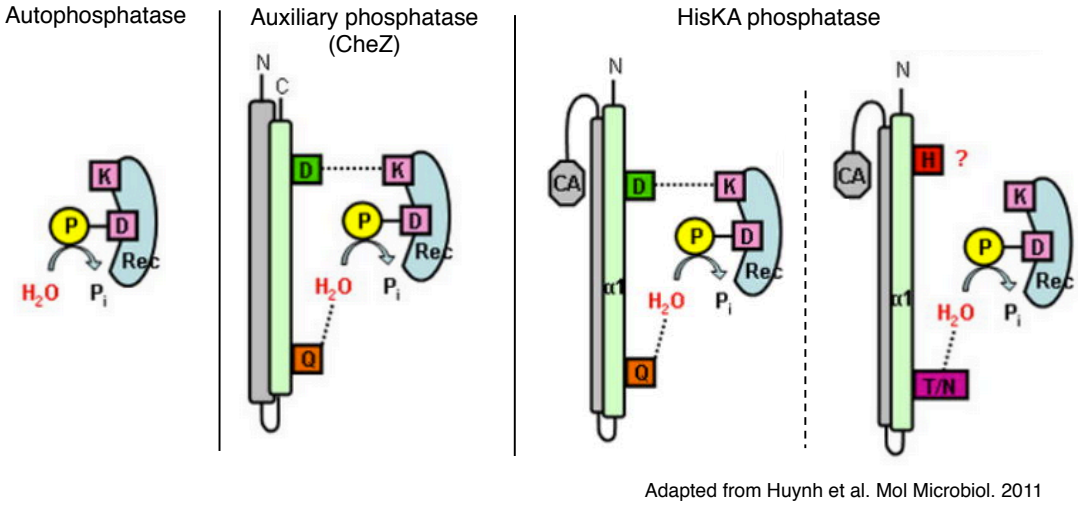
For orthodox SKs, the phosphatase activity is relatively simple. EnvZ is a dual functioning kinase and it is important for phosphorylating the RR, OmpR, which responds to osmolality changes in *E. coli*. Studies of EnvZ and its dephosphorylation of OmpR have shed light on how phosphatase activity is conducted. Biochemical and structural studies show that EnvZ harbors phosphatase activity by reversing transfer of phosphate from the RR to the SK (Hsing and Silhavy 1997). This activity has been localized to the conserved His residue located in the DHp domain, where the interaction between the DHp and CA domains mediates removal of the phosphate from the RR (Figure 1-10 A). While other residues seem to be important as well, the His243 is needed, but not required, for the hydrolysis reaction between phosphorylated OmpR and water

(Hsing and Silhavy 1997). Therefore, for some SKs, the residues that are important for kinase function are also important for phosphatase activity. Alternatively, it is common to find SKs that utilize independent residues for kinase and phosphatase functions, i.e., residues that are important for phosphatase activity are dispensable for kinase activity, and vice versa (Chen, Tsokos et al. 2009; Huynh, Noriega et al. 2010; Huynh and Stewart 2011). While the individual residues may vary, the locations of these residues are typically located in the HK domain, near the active site. However, some SKs do not require a HK domain to retain phosphatase activity. The $CheA_3$ SK in *Rhodobacter sphaeroides* lacks both the prototypical DHp and CA domain but retains phosphatase activity. This activity has been localized to an uncharacterized sub-region that appears to have no homology to a DHp or CA domain suggesting a novel phosphatase mechanism (Porter, Roberts et al. 2008).

With regards to hybrid SKs, it is still unclear exactly how phosphatase activity is achieved despite the many suggested mechanisms described. Depending on the domain structure of the hybrid SK, the route of dephosphorylation can vary. A common hybrid SK structure is HK-Rec-HPt. Based on studies of orthodox SKs, like EnvZ, it is possible that phosphatase activity could be associated with the HK DHp domain. However, the HPt domain, while important in forward flow of phosphotransfer, could also be involved in phosphatase activity (Huynh and Stewart 2011). During the forward flow of phosphate, the HPt domain, like the HK domain, transfers phosphate from its unstable phospho-His residue to the Asp residue of the RR Rec domain, so it is conceivable that this reaction may be reversible. In support of this possibility, structural data suggest that HPt domains may harbor chelated metal ions, which could play a role in phosphatase activity.

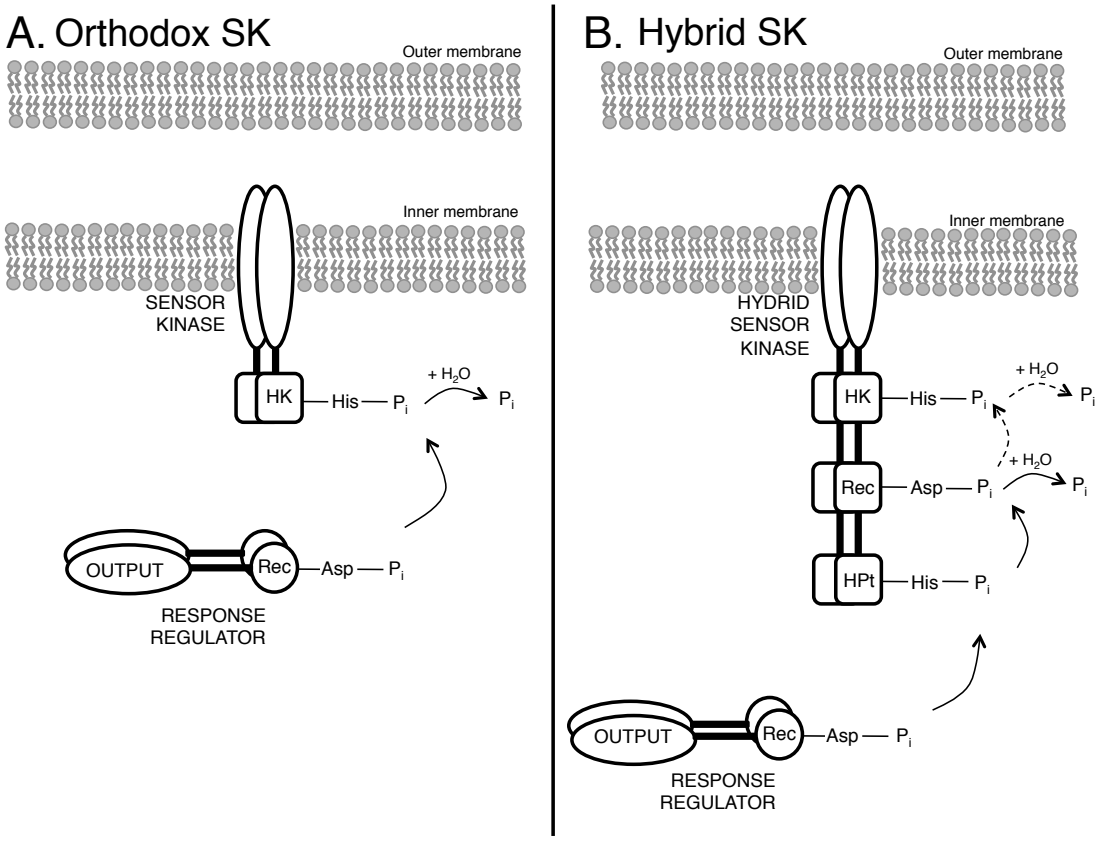
Furthermore, data on the anoxic redox control system, ArcBA, in *E. coli*, suggests that both the HPt and Rec domains of the ArcB hybrid SK are involved in phosphatase activity, while the entire HK domain is dispensable (Kato, Mizuno et al. 1997; Georgellis, Kwon et al. 1998). In this case, the Hpt is responsible for a partial "reversed phosphorelay", where the phosphate is transferred from the RR back to the HPt domain on the SK. The phosphate is then transferred to the Rec domain where hydrolysis and loss of the phosphate to the surrounding water occurs (Figure 1-10 B). Interestingly, the PAS domain also regulates the phosphatase activity of ArcB. In the presence of oxygen, formation of disulfide bridges in the PAS domain promotes ArcB homodimerization and phosphatase activity. While PAS domains have been described in other TCSs as being important for HK phosphatase activity, only recently has it been shown to be important for HPt activity (Yamada, Sugimoto et al. 2009; Gutu, Wayne et al. 2010; Bidart, Ruiz et al. 2012; Yeo, Zwir et al. 2012). Clearly, more in-depth studies of the phosphatase activity of both simple and hybrid SKs are needed to reveal the method for TCS shut-off by dual functioning kinases, though it seems that the mechanisms for phosphatase activity may be highly variable and SK specific.

Figure 1-9. Overview of the common mechanisms for phosphatase activity. Autophosphatase activity (left) of a Rec domain requires a Lys residue and nucleophilic attack at the Asp residue by water. Auxiliary phosphatases (middle, e.g. CheZ) assist in dephosphorylation in two ways: 1. By forming a salt bridge between its Asp residue and the Lys residue in the Rec domain. 2. Aiding in hydrolysis by positioning the water molecule near the phosphorylated Asp residue via its Gln residue. HisKA phosphatase activity (right) is proposed to follow a similar mechanism to auxiliary phosphatases. However, a variation on this (far right), utilizes a conserved Asn or Thr residue to coordinate hydrolysis; the importance of the conserved phosphorylatable His residue is unknown. Adapted from Huynh et al., 2011, *Mol Microbiol* 82(2):275-286 with permission.



Adapted from Huynh et al. Mol Microbiol. 2011

Figure 1-10. Overview of phosphatase activity of simple and hybrid SKs
(A) Phosphatase activity of orthodox SKs is thought to occur between the RR Rec domain and specifically the DHp domain of the SK. The conserved His residue is needed for the hydrolysis of phosphate to water. **(B)** Phosphatase activity of hybrid SKs is more complex and not clearly understood. Dephosphorylation of the RR could occur at the HK domain, similar to the orthodox SK. However, due to the presence of the HPt domain, it is also possible that the HPt and Rec domains are involved in dephosphorylation.

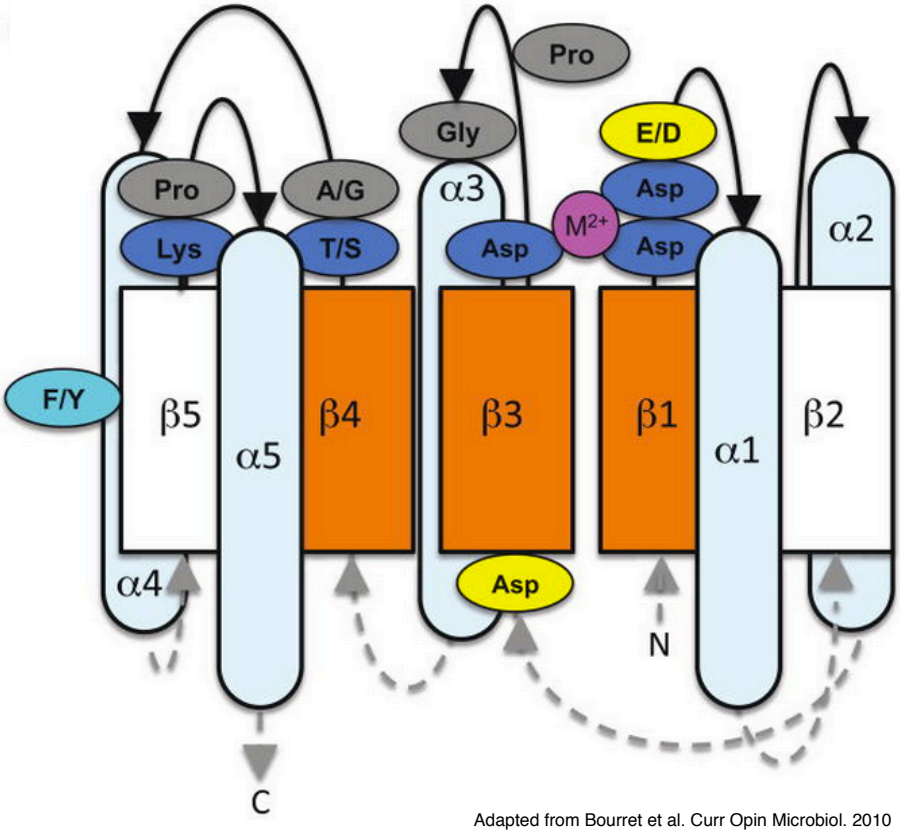


1.3.4 Response regulator

While the SK is involved in sensing and transmitting information into the cell, it is the RR that provides a means for cellular adaptation based on that information. The RR resides in the cytoplasm and is the output component of the TCS. Prior to activation, the RR loosely interacts with the SK via its Rec domain (Bourret 2010). Once the SK is activated and autophosphorylation occurs, the phosphate is passed by phosphotransfer from the HK to a conserved Asp in the Rec (Gao and Stock 2009; Gao and Stock 2010). While the SK was originally thought to perform most of the activity in the transfer of phosphate, in fact the Rec domain harbors a majority of the catalytic activity near the conserved Asp residue and is required for phosphotransfer; the phosphorylated SK acts mostly as phosphodonor. However, in fact, both the SK and RR are active in completing phosphotransfer. Studies suggest that the transfer of phosphate is through an associative mechanism that involves the formation of a pentacoordinate phosphorus intermediate (Mildvan 1979; Zhao, Copeland et al. 2008). For this mechanism to occur, the nucleophile (RR) and electrophile (SK) must be in close proximity. Therefore, during the transition state, conformational changes are observed for both the SK and RR, which alter the position the active sites, particularly the His and Asp residues, allowing for an optimal protein-protein interface for phosphotransfer (Zhao, Copeland et al. 2008). While it is thought that this mechanism is used for all SK-RR phosphotransfer reactions, it is possible that the mechanism could differ depending on the type of SK or the domain arrangement of these proteins.

While slight variations of the REC secondary structure have been described, generally, REC domains are characterized by a conserved $(\beta\alpha)_5$ structure that contain a run of hydrophobic residues in the three central β strands. At the C-terminal end of these central strands lies the active site containing four conserved Asp residues. One Asp is the site for phosphorylation while the other three are involved in chelating a divalent metal ion (Mg^{2+}, Zn^{2+}, or Mn^{2+}) that is necessary for function. Lys, Thr/Ser, and Phe/Tyr residues are located near the REC active site and are also critical for signal transduction (Figure 1-11) (Bourret 2010). Once the Asp has been phosphorylated the large net negative charge of the phosphate group causes a conformational change that activates the RR and further weakens the loose association between the RR and SK. This allows the RR to break away from the SK in order to perform its function.

Figure 1-11. Schematic of a RR Rec domain.
Rec domain structure consists of five highly conserved α helices, which surround five parallel β-sheets and are connected by loops. The most conserved β-sheets are indicated in orange. Black solid lines denote loops/residues present on the side of the protein that contains the active site, while grey dashed lines denote loops/residues on the opposite side. Highly conserved residues required for active site activity are shown in blue and less conserved residues in cyan. Grey residues represent conserved residues that are thought to be structurally important, where yellow residues represent residues that are commonly conserved but their function is unknown. Adapted from Bourret et al., 2010, *Curr Opin Microbiol* 13(2):142-149 with permission.



The conformational change that occurs for a phosphorylated RR also causes dimerization, which is required for most RR output activities. The most common RR output is changes in gene expression through DNA-binding. Indeed, most of the RRs that have been described throughout this chapter are transcription factors involved in a variety of cellular functions, such as motility, cell division, stress response, metabolism and virulence. RRs that induce changes in gene expression often have a helix-turn-helix (HTH) DNA-binding domain, which allows the active RR to bind specific promoter regions, modulating gene expression. While RRs that control gene expression are common, other outputs have been described (Buelow and Raivio 2010). Some RRs have been described to have only a Rec domain, where the role of this single domain RR is to participate in phosphotransfer from the SK to other output proteins. An example of a single domain RR is in the sporulation phosphorelay in *B. subtilis*, where the RR, Spo0F, harbors only a Rec domain. Spo0F is vital to the phosphorelay as it an intermediate step in the transfer of phosphate between the SK, KinA, and the final RR, Spo0A (Wang, Grau et al. 1997; Buelow and Raivio 2010). Other RRs have been reported to contain RNA-binding or enzymatic domains such as those involved in making or hydrolyzing c-di-GMP (Galperin, Nikolskaya et al. 2001; Galperin 2006). In *Pseudomonas*, the WspR RR in the Wsp phosphorelay harbors a GGDEF c-di-GMP synthesis motif and, when phosphorylated by its cognate SK, plays an important role in the production of c-di-GMP and in regulating biofilm formation and cytotoxicity (De, Navarro et al. 2009). Therefore, depending on the domains present in the RR, TCS can modulate bacterial adaption in a variety of ways.

1.3.5 Auxiliary proteins and inhibition of phosphosignalling

While most TCSs consist of a SK and RR, there are a number of signal transduction systems that have expanded on the standard two-component theme. Deviations on the TCS, such as three- or multi-component systems, incorporate additional regulatory proteins, or auxiliary proteins, to provide either positive or negative feedback to help fine-tune responses to environmental change (Buelow and Raivio 2010). Auxiliary proteins offer additional control to the signal transduction system using a variety of mechanisms and can reside in the cytoplasm, inner membrane, or periplasm.

In the sporulation phosphorelay in *B. subtilis*, numerous auxiliary proteins negatively control the progression of phosphotransfer (Wang, Grau et al. 1997; Perego 2001; Parashar, Mirouze et al. 2011). Protein inhibitors, like Sda and KipI, interact with the SK, KinA. Sda and KipI bind the DHp domain of KinA disrupting the transfer of the γ phosphate from ATP to the conserved His residue, thus preventing autophosphorylation. In addition to Sda and KipI, Rap proteins and Spo0E act as phosphatases at various stages in the phosphotransfer and dephosphorylate two important RRs, Spo0F and Spo0A. Not every auxiliary protein is involved in regulating the phosphorelay. SadARS, a three-component system involved in regulating biofilm maturation in *Pseudomonas aeruginosa*, contains the SK, SadA and two RRs, SadR and SadS (Kuchma, Connolly et al. 2005). While SadS appears to be a typical RR, harboring a REC and HTH domain, SadR, in addition to the Rec domain, contains an EAL PDE domain that hydrolyzes c-di-GMP. While the phosphorylation state of SadR has not been addressed, it is hypothesized that upon phosphorylation of SadR, this activates the EAL domain resulting in cleavage of c-di-GMP and biofilm dispersal. Clearly, the

incorporation of other RRs or auxiliary proteins can expand an already large number of processes that TCSs can regulate.

Just as bacteria need to activate TCSs to adapt to changing environments, they must also return the system to a resting, pre-activation state. As previously mentioned, TCSs can incorporate auxiliary phosphatases that dephosphorylate the RR, such as Rap proteins in *B. subtilis*. Additionally, simple mechanisms such as varying the stability of the phosphorylated RR are also a common technique. As described earlier, another way for down regulating TCS activity is to include inhibitors that halt the kinase activity of the SK or modulate the switch between kinase and phosphatase activity for dual SK (Chen, Tsokos et al. 2009). The NtrBC/PII TCS in *E. coli*, the KdpDE/LprF and LprJ TCS in *Mycobacterium tuberculosis*, and the LiaFSR TCS in *Streptococcus mutans* all incorporate auxiliary proteins that, through various binding mechanisms, modulate the kinase or phosphatase activity of their respective SKs (Steyn, Joseph et al. 2003; Ninfa and Jiang 2005; Suntharalingam, Senadheera et al. 2009).

One interesting example of SK stimulated phosphatase activity by auxiliary proteins is in the multi-component system in *Staphylococcus aureus*, SaeRSPQ (Jeong, Cho et al. 2012). SaeRS is a TCS that responds to neutrophil antimicrobial peptides and regulates production of α-haemolysis, coagulase and fibronectin-binding proteins, all which are important virulence factors. The SK, SaeS, naturally has a basal level of phosphatase activity on its RR, SaeR, but this activity is greatly enhanced by the binding of SaeS to a membrane protein complex, SaePQ. The expression of *saePQ* was shown to be under the control of SaeR, which suggests that SaePQ acts as a negative feedback mechanism over the SaeRS TCS, allowing the TCS to return to its inactivated state.

Clearly, signal transduction by TCSs is vital to the survival and adaptation of bacteria. Understanding how these TCS function and control behavioral changes can provide a greater understanding into how bacteria have evolved to cope with drastic environmental changes. For this work, the VieSAB three-component signal transduction system in *V. cholerae* will be explored to gain further insight into how this organism manages the transition between the aquatic environment and human host and how this system impacts pathogenesis.

1.4 VieSAB three-component system in *V. cholerae*

1.4.1 Role of vieSAB in V. cholerae pathogenesis

vieSAB was discovered using recombination-based *in vivo* expression technology (RIVET) in a screen to identify genes that are transcriptionally induced upon entry into the small intestine of infant mice (Lee, Hava et al. 1999); in this screen, *vieB* was the only gene hit out of the three genes. *vieSAB* is a putative operon on the large chromosome of *V. cholerae* between genes for a magnesium transporter and a secreted collagenase (Figure 1-12 A) and encodes for three respective proteins, VieSAB. This putative operon is conserved only in closely related *Vibrio* species. Arguing against co-expression of all three genes, *vieSA* are transcribed *in vitro* and *in vivo*, while *vieB* appears to only be induced during infection of the infant mouse small intestine and experimental infection of human volunteers (Camilli and Mekalanos 1995; Lee, Angelichio et al. 1998; Lee, Hava et al. 1999; Osorio, Crawford et al. 2005). There is no identifiable terminator or promoter between *vieA* and *vieB*, which if present, could explain the differential regulation of the latter gene. Moreover, the location of promoter(s) and mechanism(s) of transcriptional control of *vieSAB* remain uncharacterized.

vieSAB appears to be involved in pathogenesis, as a deletion of the entire operon in the classical biotype causes a substantial decrease in colonization of the mouse small intestine (Lee, Angelichio et al. 1998; Lee, Butler et al. 2001). Additionally, in the classical biotype, both *vieS* and *vieA* mutants exhibit increased biofilm formation compared to WT, and the *vieA* mutant has decreased motility (Martinez-Wilson, Tamayo et al. 2008). Conversely, in the El Tor biotype, *vieSAB* is expressed at a lower level and appears to be dispensable for virulence, however deletion of either *vieA* or the entire operon results in a reduction in CT and *toxT* in *in vitro* studies (Tischler, Lee et al. 2002; Tischler and Camilli 2005; Beyhan, Tischler et al. 2006; Tamayo, Schild et al. 2008). Between the classical and El Tor biotypes, the *vieSAB* locus and upstream promoter regions are 99.6% identical at the nucleotide level and the three proteins are nearly 100% identical at the amino acid level. Thus, it is unclear what lies behind the mutant phenotype discrepancy between the two biotypes. Presumably differences in regulatory systems outside the *vieSAB* locus or the presence of a redundant system in El Tor are responsible. However, taken together, these data suggest that *vieSAB* is involved in positive regulation of virulence genes and negative regulation of biofilm formation, playing a role in the ability of *V. cholerae* to transition from the environment to the host.

1.4.2 VieSA TCS

The VieSA system is homologous to bacterial TCS. VieS appears to be a complex SK most similar to the *Bordetella pertussis* hybrid SK, BvgS. VieS contains a transmembrane region and is thought to be localized to the inner membrane of *V. cholerae*. As a result, VieS's putative sensor domains reside in the periplasm and are able

to sense the extracytoplasmic environment. The nature of the signal responsible for activation of VieS remains unknown. However, the VieS sensor domain pockets are homologous to the periplasmic binding protein (PBPb) super-family and share sequence similarity to amino acid binding proteins (Figure 1-13). Certain L-amino acids, specifically serine (S), asparagine (N), arginine (R) and glutamic acid (E), are know to stimulate CT expression *in vitro* (Mey, Craig et al. 2012). Therefore, it is hypothesized that amino acids, specifically NRES, may be sensed by VieS. Of note, a VieS monomer contains two signal-binding pockets. The reason behind this is unclear but the presence of two binding sites may allow for cooperative binding or could permit varying levels of VieSAB activation depending on the number of sites bound. VieS is a hybrid SK, with a cytoplasmic domain structure of a putative PAS domain, then HK-Rec-HPt (Figure 1-12 B). VieS is capable of autophosphorylating at the HK and passing this phosphate down the intra-molecular phosphorelay, though it is not clear whether this phosphorylation is in *cis* or *trans*. Interestingly, VieS appears to utilize manganese instead of the more common magnesium, in its active site for autophosphorylation and phosphotransfer (Martinez-Wilson, Tamayo et al. 2008). Through phosphotransfer, VieS is able to specifically phosphorylate its cognate RR, VieA (Martinez-Wilson, Tamayo et al. 2008).

VieA shows homology to DNA-binding RRs and has a domain architecture of Rec-PDE-HTH (Lee, Angelichio et al. 1998) (Figure 1-12 B). The Rec domain is homologous to other RR Rec domains, such as CheY, and contains a conserved Asp residue (Asp52) for phosphorylation. The presence of the C'-terminal HTH domain points to VieA being able to bind DNA and regulate gene expression, however direct targets of activated VieA are unknown. Previous experiments suggest that *vieSAB* are

expressed at low levels in a VieA-dependent manner under certain *in vitro* conditions as well as during infection, pointing to VieA being autoregulatory (Lee, Angelichio et al. 1998; Tischler, Lee et al. 2002; Tischler and Camilli 2004) (Beyhan, Tischler et al. 2006). It is proposed that phosphorylated VieA binds a promoter upstream of *vieA*, allowing autoregulation of *vieA* or the entire *vieSAB* operon (Beyhan, Tischler et al. 2006; Martinez-Wilson, Tamayo et al. 2008). While binding of VieA to its own promoter has not been directly shown, autoregulation is common for most characterized RRs.

VieA also contains a PDE domain specifically belonging to the EAL family; previous studies show that VieA and the PDE activity of this protein contribute greatly to the regulation and production of CT through c-di-GMP cleavage (Tischler, Lee et al. 2002; Tischler and Camilli 2005). Interestingly, preliminary data indicate that regardless of the phosphorylation state of VieA the EAL domain remains functional, suggesting that phosphorylation of VieA only activates the HTH domain (Tamayo, Tischler et al. 2005). VieA is also responsible for the repression of the *Vibrio* exopolysaccharide synthesis genes involved in biofilm formation, though this function is independent of its DNA-binding activity and is most likely a direct result from c-di-GMP cleavage by the PDE domain (Tischler and Camilli 2004). Therefore, VieA appears to have dual functions. While phosphorylated VieA activates the HTH, allowing VieA to directly modulate expression of target genes, both unphosphorylated and phosphorylated VieA can continually cleave c-di-GMP resulting in global changes in gene expression via lowering the level of this secondary messenger molecule. If VieA is indeed autoregulatory, it is hypothesized that the phosphorylation of VieA by VieS would allow for up-regulation of

VieA production. This would result in a lowering of c-di-GMP concentration, resulting in suppression of environmental genes and expression of virulence genes, like CT.

1.4.3 VieB, an auxiliary protein

While VieSA behave similarly to a traditional TCS system, the inclusion of the auxiliary protein, VieB, classifies VieSAB as a three-component system. *vieB* is the last gene in the *vieSAB* operon and, as presented above, should be under the same regulatory control as *vieSA* based on sequence data alone. However, previous experimental data suggests that there may be differential regulation within the *vieSAB* operon and it is hypothesized that there may be additional promoters residing within the operon, though this has not been shown (Lee, Angelichio et al. 1998).

VieB consists of two conserved domains, Rec and a tetratricopeptide repeat (TPR), which resides at the N-terminal end of the protein (Figure 1-12 B). No recognizable DNA-binding domain appears to be present suggesting that VieB is not a typical RR. The latter half of the protein, referred to as the unknown function (UKF) domain, appears to be novel, having no homology to any protein or domain in the Genbank database. VieB's Rec domain, like VieA, has homology to the Rec domain of CheY and harbors the conserved Asp phosphorylation residue (Asp 62). The TPR domain is a conserved protein-protein interaction domain found in eukaryotes and bacteria. TPR domains are composed of tandem arrays of 34 degenerate amino acid sequences, which can range from 3-16 repeats. These repeats fold into two anti-parallel α-helical sub-domains, which results in a right-handed helical conformation that creates a channel or groove that allows for interactions with other proteins. While this secondary structure is

the most common, slight variations to the overall structure of TPR domains have also been described (Allan and Ratajczak 2011). In *Saccharomyces cerevisiae*, the TPR-containing protein, Tah1 was shown to interact with the heat shock protein, Hsp90 via a consensus binding sequence, MEEVD (Millson, Vaughan et al. 2008). However, depending on the Hsp90 isoform, this binding sequence varies and it remains unclear if a conserved binding sequence is present for TPR domains. Additionally, most TPR domain-containing proteins have been shown to bind proteins that do not contain this domain, suggesting that TPR domains do not necessarily interact with each other. However, structural data of the TPR-containing protein, YbgF from *E. coli*, has shown that monomers of the same protein can oligomerize via their TPR domains (Krachler, Sharma et al. 2010). Whether association can occur between two different proteins that harbor TPR domains remains ambiguous. TPR domains have been reported to take part in a variety of functions, such as transcriptional control, protein kinase inhibition, and protein folding (Allan and Ratajczak 2011). The presence of a TPR domain in VieB suggests that this protein may be involved in protein-protein interactions. Since previous data shows induction of *vieB* during colonization of the host and VieSA appears to be needed only during the early stages of infection, we hypothesized that VieB could be repressing the activity of VieSA (Lee, Hava et al. 1999). Indeed, preliminary data points to VieB acting as a negative feedback inhibitor of the VieSA phosphorelay, though this has not been clearly demonstrated (Martinez-Wilson 2008).

Attempts to gain insight into the importance of VieB through observable changes in CT expression, biofilm formation and motility have been inconclusive. A previous study looked for a VieB-dependent phenotype using a truncated allele of *vieB* in the El

Tor biotype. However, no changes in CT expression were observed, and biofilm and motility assays were not conducted (Tischler, Lee et al. 2002). While this truncated mutant no longer has the Rec and TPR domain, it retains a large majority of the UKF portion. This could result in the production of a small protein that may have function, which could explain the lack of observable phenotype. Therefore, a clean deletion of *vieB* is necessary for further investigation of a phenotype.

The VieSAB signal transduction system is clearly important in regulating c-di-GMP levels and modulating gene expression in *V. cholerae*. However, the biochemical and structural mechanisms of how this system interacts and carries out its function are not well characterized. Understanding these details will shed light on the importance of this system in virulence gene regulation and its contribution to modulating c-di-GMP levels as *V. cholerae* transitions between the environment and host. Additionally, study of this system could reveal a novel mechanism for phosphorelay modulation through the characterization of VieB, thus expanding the knowledge of how TCSs control their activity.

Figure 1-12. *vieSAB* and the VieSAB three-component system
(A) *vieSAB* operon orientation in the *V. cholerae* genome. *vieSAB* is located between *mgtE*, which encodes a magnesium transporter, and a secreted collagenase. Scale represents gene size in basepairs. **(B)** Domain architecture of VieS, VieA and VieB. Periplasmic binding protein, PBP; transmembrane region, TM; Per-Arnt-Sim, PAS; histidine kinase, HK; phospho-acceptor and dimerization DHp, HisKA; ATP-binding and catalytic CA, ATPase; receiver domain, Rec; histidine phosphotransfer, HPt; phosphodiesterase, PDE; helix-turn-helix DNA-binding, HTH; tetratricopeptide repeat, TPR; unknown region of function, UKF. Scale represents protein/domain size in amino acids.

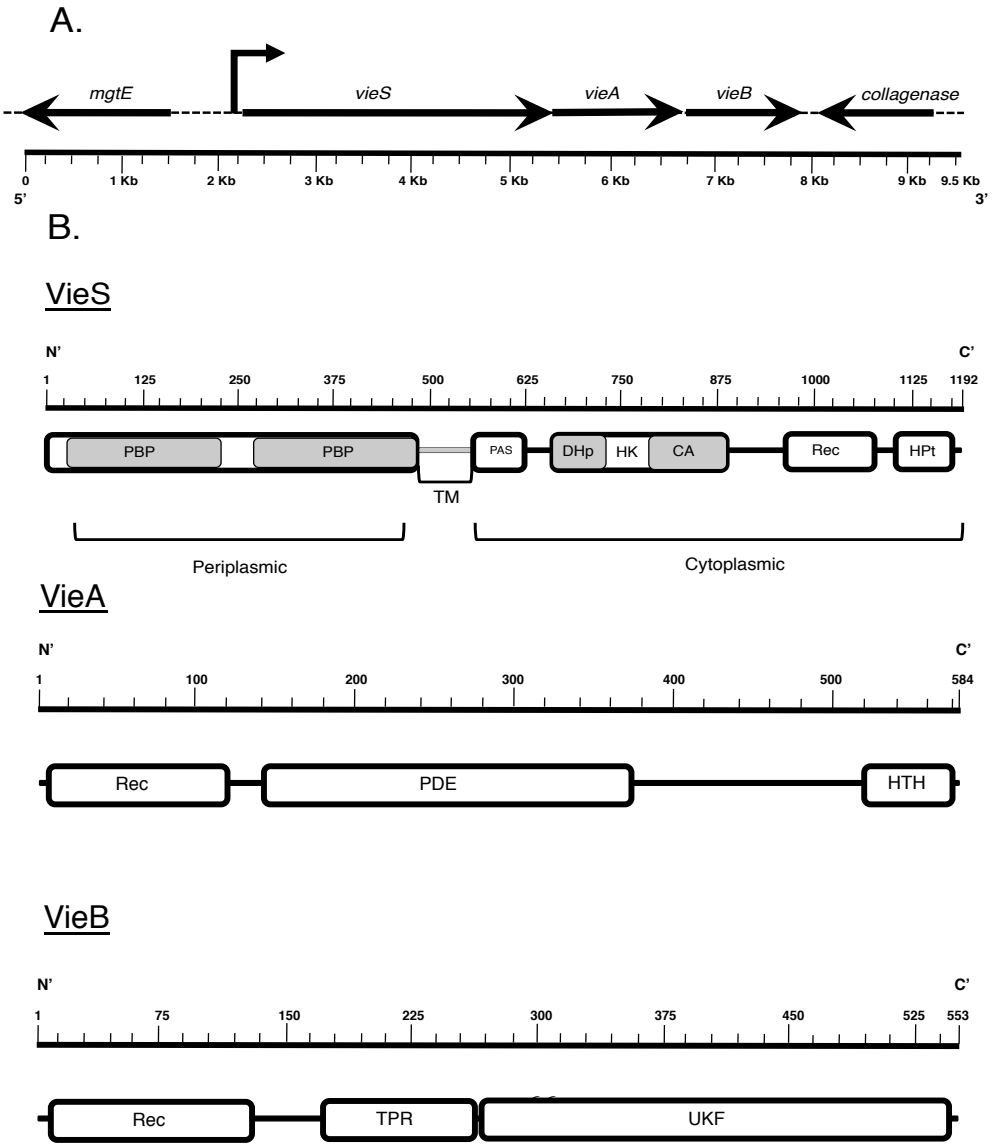
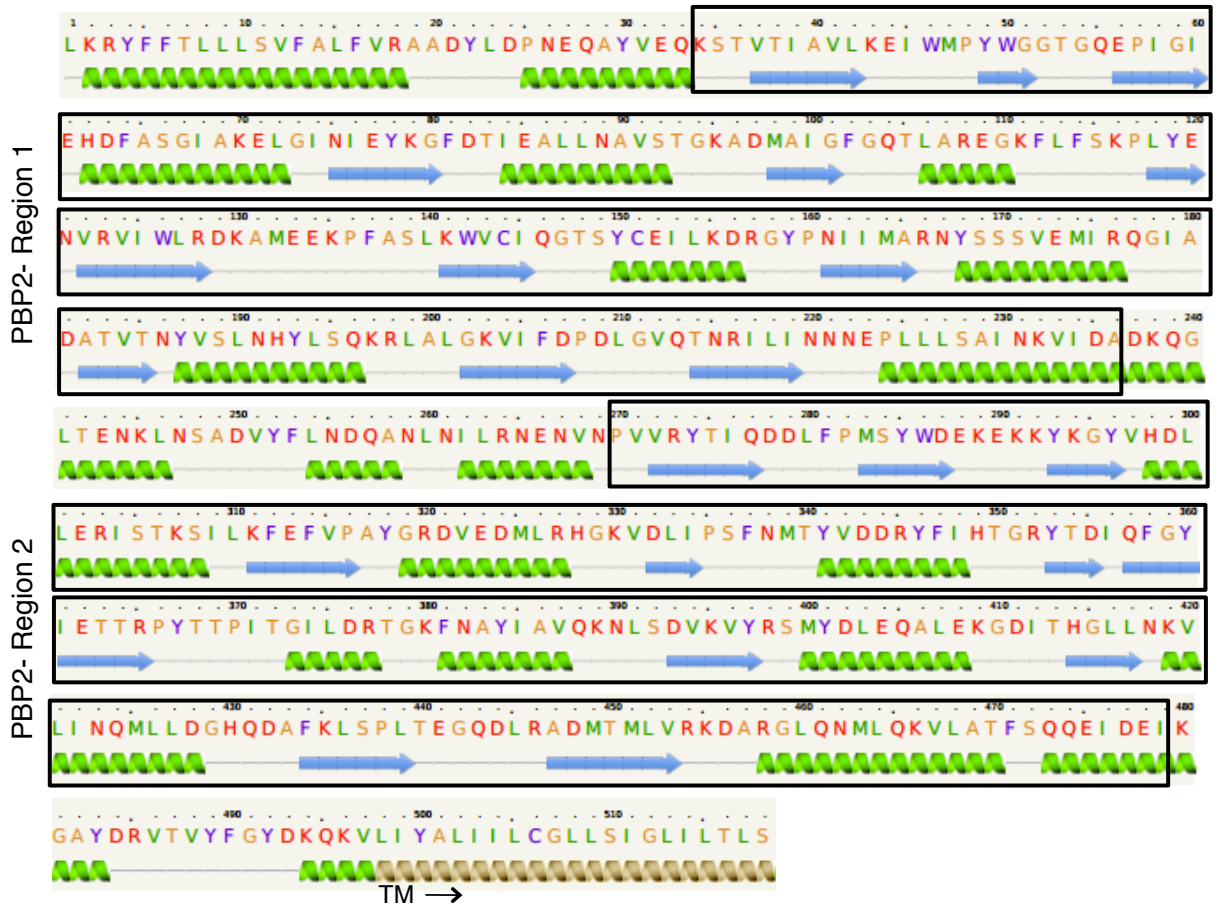


Figure 1-13. Predicted PBP binding regions within VieS.
Two putative PBP ligand-binding regions (boxed sequences) are predicted in the periplasmic region of each VieS monomer using the Phyre2 Protein Fold Recognition server. The predicted secondary structure for these regions is shown below the sequence. α-helices, green spirals; β-sheets, blue arrows; transmembrane region (TM), gold spiral.



CHAPTER 2: IDENTIFICATION AND CHARACTERIZATION OF THE ROLE OF VIEB ON THE VIESA TWO-COMPONENT SYSTEM

Some of the data presented in this chapter are included in the following manuscript, which has been recently accepted for publication.

Mitchell, S.L., Ismail, A.M., Kenrick, S.A. and Camilli, A. 2015 "The VieB Auxiliary Protein Negatively Regulates the VieSA Signal Transduction System in *Vibrio cholerae*." *BMC Microbiology*, Epub ahead of print

2.1 INTRODUCTION

A previous report identified the putative *vieSAB* operon using a genetic screen for positive regulators of virulence genes in *V. cholerae* (Lee, Angelichio et al. 1998). Much work has been done to understand the importance of the *vieSAB* operon and characterize the role of the VieSA TCS in regulating cdGMP levels (Tischler, Lee et al. 2002; Tischler and Camilli 2005; Tamayo, Schild et al. 2008). Additionally, a previous report has shown that *vieSA* encode a SK (VieS) and a RR (VieA), whereby VieS is able to autophosphorylate and transfer its phosphate to VieA (Martinez-Wilson, Tamayo et al. 2008). However, there has been little investigation of VieB, the putative third component of this signal transduction system.

Although sequence analysis of VieB revealed two conserved domains, an N-terminal Rec domain and a TPR domain, the C-terminal half of the protein has no known sequence homology. Interestingly, VieB lacks a C-terminal DNA binding domain suggesting that it may not behave as a typical RR. Although bioinformatic analysis gives some clues to VieB's role, the lack of sequence homology for the latter half of VieB offers little insight into the true function of this protein. However, since *vieB* is encoded in the *vieSA* operon, we hypothesized that VieB is involved with the VieSA TCS. Furthermore, the presence of the TPR domain points to VieB potentially being involved in protein-protein interactions, presumably with either VieS or VieA. A majority of well-studied auxiliary proteins have been described as inhibitors (Buelow and Raivio 2010). Therefore, we hypothesize that VieB may behave as an inhibitor of the VieSA TCS by interacting with one of these components.

In this chapter, we reveal the role and mechanism of action of VieB within the VieSAB phosphorelay.

2.2 RESULTS

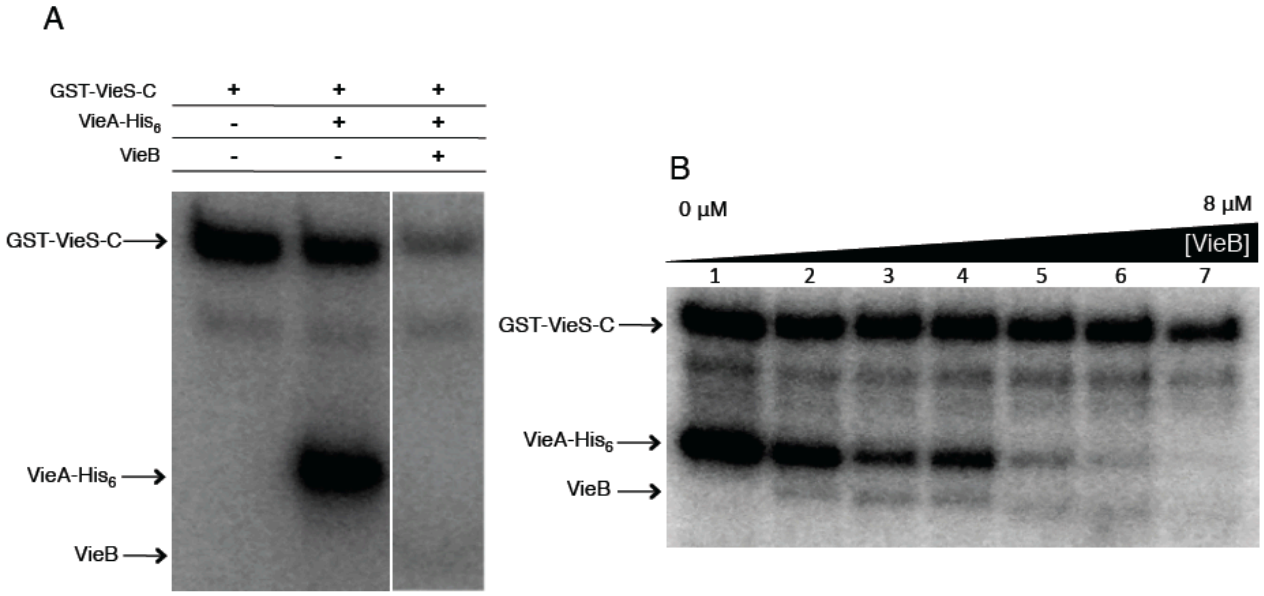
2.2.1 VieB is an inhibitor of VieA phosphorylation

To address if VieB affects the VieSA TCS, phosphotransfer assays were conducted to test if VieB is able to alter the transfer of phosphate between VieS and VieA. The cytoplasmic portion of VieS was purified using a N-terminal GST-tag (GST-VieS-C) and VieA was purified using a C-terminal His_6 tag (VieA-His_6). VieB was purified using an N-terminal His_6 tag, which was subsequently cleaved off for use in experiments (VieB). As expected, GST-VieS-C is able to autophosphorylate and transfer phosphate to VieA-His_6 (Figure 2-1A). However, when a five-fold molar excess of VieB was added to the reaction, there is a significant reduction in the amount of phosphorylated VieA-His_6 (Figure 2-1A). Interestingly, VieB does not appear to become readily phosphorylated during the reaction, providing preliminary evidence that VieB is not acting as a phosphate sink or is competing with VieA-His_6 for phosphate.

To further investigate the inhibitory role of VieB, its affect on phosphotransfer was tested over a range of VieB concentrations. As seen in Figure 2-1B, while GST-VieS-C can readily transfer phosphate to VieA-His_6 in the absence of VieB, as the concentration of VieB increases, there is a correlative decrease in the amount of phosphotransfer that occurs between GST-VieS-C and VieA-His_6. Furthermore, the amount of phosphorylated VieA-His_6 is reduced by approximately half when GST-VieS-C, VieA-His_6 and VieB are present in equal molar amounts (Figure 2-1B lane 4). Even though VieB appears to be most effective at disrupting phosphorylation of VieA-His_6

when in molar excess, VieB at a molar ratio of 0.25 is still able to inhibit phosphorylation of VieA-His_6 to some extent (Figure 2-1B lane 2). These data suggest that VieB is a dose-dependent inhibitor of VieA-His_6 phosphorylation and that VieB is not acting enzymatically.

Figure 2-1. VieB is a dose-dependent inhibitor of phosphotransfer.
(A) Purified GST-VieS-C was incubated with ^{32}P-ATP-γ either alone (lane 1) or in the presence of equimolar VieA-His$_6$ (lane 2) and 5 μM VieB (lane 3) for 30°C minutes at 30°C. **(B)** GST-VieS-C was incubated with ^{32}P-ATP-γ in the presence of equimolar VieA-His$_6$ and either 0, 0.25, 0.5, 1, 2, 4, or 8 μM VieB for 30 minutes at 30°C. Samples were stopped with the addition of 2X-denaturing sample buffer and separated using a 10% SDS-PAGE gel. Proteins labeled with ^{32}P were observed by radioautography. The radioautographs shown are a representative of three replicates. The band just below GST-VieS-C appears to be a VieS degradation band, which is present in all figures and can be ignored.



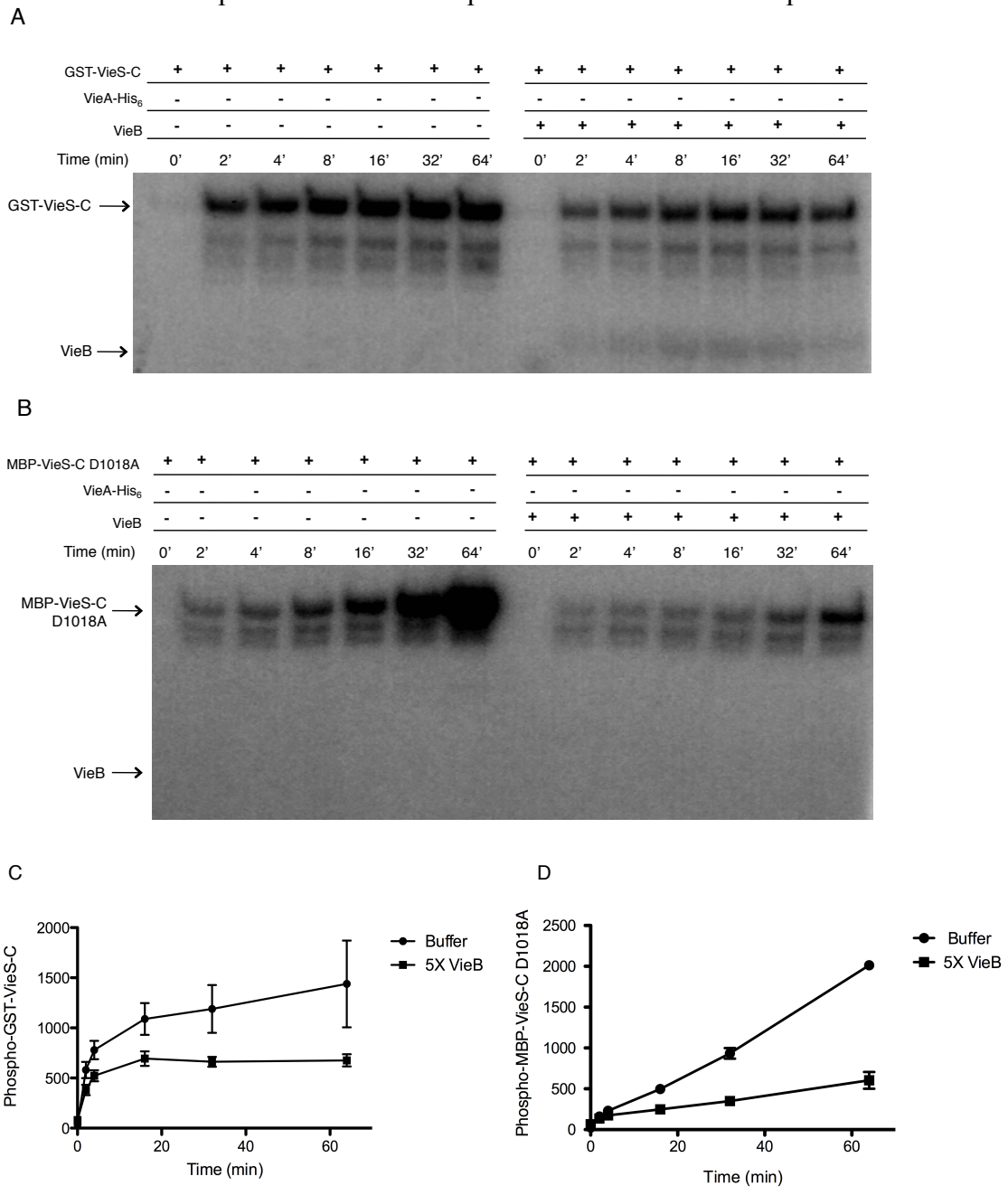
2.2.2 Effect of VieB on VieS phosphorylation

Interestingly, the amount of phosphorylated GST-VieS-C is relatively unaffected by increasing concentrations of VieB (Figure 2-1B). However, when VieB is in high amounts, there is a moderate but stable decrease in the amount of phosphorylated GST-VieS-C (Figure 2-1B, lanes 6-7). This suggests that VieB may be disrupting VieS phosphorylation. To test this, the accumulation of phosphate on GST-VieS-C was observed over time in the absence of VieA-His_6. While GST-VieS-C is able to become phosphorylated in the presence of five-fold molar excess VieB, the total amount of phosphate on GST-VieS-C is reduced when compared to in the absence of VieB (Figure 2-2 A,B). While these data suggest that VieB is having some affect on GST-VieS-C phosphorylation, it is not clear from this experiment if the effect is on VieS autophosphorylation, the intra-molecular relay within VieS or the stability of phosphate on VieS.

VieS is a complex SK, harboring a total of six phosphorylation sites in the homodimer, one site in the HK, the Rec and the HPt, per monomer. Due to the number of phosphorylation sites and phosphotransfer steps, it is possible that VieB could be altering one or more of these phosphorylation events. A previous report identified and characterized two auxiliary proteins that inhibited the autophosphorylation of another SK, KinA (Wang, Grau et al. 1997). Therefore, it is possible that VieB could affect the initial autophosphorylation at the HK domain. To test this hypothesis, we utilized an intra-molecular phosphorelay mutant, MBP-VieS-C D1018A, which has the conserved Asp residue in the Rec domain replaced with an Ala. This mutant is incapable of transferring

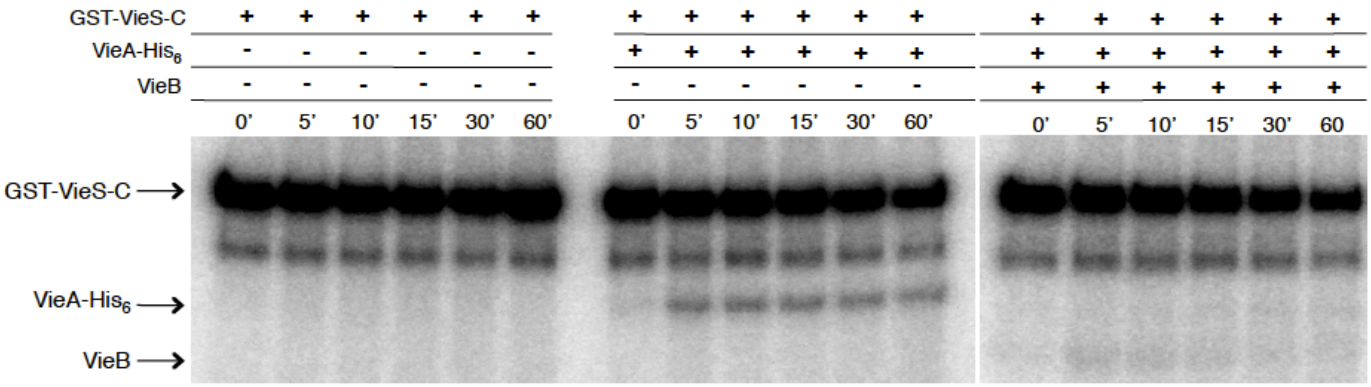
phosphate to VieA-His_6; MBP-VieS-C D1018A is only capable of HK autophosphorylation and it is unable to transfer this phosphate from the HK domain to the Rec or HPt domains. Thus this mutant allows the assessment of only the initial phosphorylation. In phosphotransfer assays, MBP-VieS-C D1018A is able to rapidly autophosphorylate to high levels over time in the absence of VieB (Figure 2-2C). When VieB is present in five-fold molar excess, MBP-VieS-C D1018A is still able to autophosphorylate, however the total amount of accumulated phosphate is lower than when VieB is absent (Figure 2-2D). Additionally, the rate of autophosphorylation is lessened in the presence of VieB, providing further evidence that VieB inhibits autophosphorylation of the VieS HK. However, the observation that this mutant is still able to acquire phosphate in the presence of VieB over time suggests that the inhibitory affect of VieB on the HK domain is not complete.

Figure 2-2. VieS moderately inhibits VieS-C autophosphorylation.
Purified GST-VieS-C or MBP-VieS-C D1018A was incubated with ^{32}P-ATP-γ in the presence or absence of 5 μM VieB over time at 30°C. Samples were stopped at indicated time points with the addition of 2X-denaturing sample buffer and separated using a 10% SDS-PAGE gel. VieS-C fusion proteins labeled with ^{32}P were observed by radioautography **(A,B)** and quantified in **(C,D)**.The radioautograph shown is a representative of three replicates. Error bars represent the SEM of three replicates.



While VieB appears to only partially inhibit autophosphorylation at the HK domain, it is able to completely prevent the phosphorylation of VieA. Therefore, we hypothesize that VieB must harbor an additional inhibitory function that effects either the VieS intra-molecular phosphorelay or the ability of the VieS HPt domain to transfer phosphate to VieA. To address the inhibitory effect of VieB on VieS's phosphotransfer, we assessed phosphotransfer between GST-VieS-C and VieA-His_6 in the absence of ATP. By allowing GST-VieS-C to become robustly phosphorylated then removing ATP and adding VieA-His_6, we prevent further autophosphorylation at the HK domain and can strictly address the intra-molecular and inter-molecular phosphotransfer steps. In the absence of ATP, phosphorylated GST-VieS-C is still able to transfer phosphate to VieA-His_6 (Figure 2-3). However, in the presence of VieB, the amount of phosphorylated VieA-His_6 is greatly reduced (Figure 2-3). These data suggest that VieB is indeed able to block the phosphotransfer, however these data are unable to distinguish between the intra- and inter-molecular phosphotransfer. Since VieB is able to partially affect autophosphorylation at the HK domain, implying that it can bind at or near the HK domain, we further hypothesize that VieB may be more likely to inhibit the intra-molecular phosphorelay between the HK and Rec domains rather than directly affecting the HPt domain located distally in the protein. Therefore, taken together, the additive effect of VieB's ability to partially inhibit autophosphorylation at the HK and block the transfer of any phosphate, presumably between the HK and Rec domains, allows VieB to completely inhibit phosphotransfer between VieS and VieA.

Figure 2-3. VieB inhibits VieS phosphotransfer to VieA-His_6.
Purified GST-VieS-C was incubated with ^{32}P-ATP-γ in the absence of VieA-His_6 and VieB for 30 minutes at 30°C. Excess ^{32}P-ATP-γ was removed from phosphorylated VieS-C constructs by gel filtration. P-GST-VieS-C was then incubated with additional $MnCl_2$ and either buffer, 1 μM VieA-His_6 or pre-mixed 1 μM VieA-His_6 and 5 µM VieB for 60 minutes. Samples were stopped at indicated time points with the addition of 2X-denaturing sample buffer and separated using a 10% SDS-PAGE gel. GST-VieS-C labeled with ^{32}P were observed by radioautography. The radioautograph shown is a representative of three replicates.



Interestingly, phosphorylated GST-VieS-C in the absence of ATP still appears to slightly decrease in the presence of VieB when compared to in the absence of VieB (Figure 2-3). Therefore, yet another possible property of VieB could be to affect the stability of phosphorylated VieS-C. To test this hypothesis, both wild type GST-VieS-C and the MBP-VieS-C D1018A mutant were allowed to autophosphorylate in the absence of VieB, after which the reaction was passed through a gel filtration column to remove excess ATP. The rate of loss of P-VieS-C was measured over time in the presence or absence of VieB. The MBP-VieS-C 1018A mutant was tested to specifically address the stability of phosphate at the HK domain since VieB partially affects autophosphorylation at this domain. As seen in Figure 2-4, the rate of loss is similar between both the wild type (Figure 2-4A) and mutant VieS-C (Figure 2-4B), regardless of the presence or absence of VieB. These data suggest that VieB does not alter the stability of phosphorylated VieS-C.

Another common mechanism of auxiliary proteins that behave as inhibitors of TCSs is harboring phosphatase activity (Wang, Grau et al. 1997; Perego 2001; Parashar, Mirouze et al. 2011). Since the addition of VieB appears to have its most drastic effect on the level of phosphorylated VieA-His_6, an alternative hypothesis is that VieB could itself be a phosphatase of VieA-His_6. To test this hypothesis, we conducted order of addition phosphotransfer experiments. When GST-VieS-C is first incubated with VieA-His_6 and VieB is added second, while reduced, there is still an observable amount of phosphorylated VieA-His_6 (Figure 2-5A). If VieB harbored phosphatase activity, it would be expected that this enzymatic activity would be relatively quick, especially when VieB

is in molar excess. However, an observable amount of phosphorylated VieA-His_6 remains in the presence of VieB suggesting that VieB is not an effective phosphatase of VieA-His_6. Alternatively, when GST-VieS-C is incubated with VieB first and then VieA-His_6 is added, there is a complete loss of phosphorylated VieA-His_6 and a total disruption of phosphotransfer. Taken together with VieB acting non-enzymatically, these data are supportive of VieB not being a phosphatase.

While it appears that VieB is not a phosphatase, it is possible that the decrease in phosphorylated GST-VieS-C could be due to the natural phosphatase activities of VieS. Indeed, it is common for SKs to harbor both kinase and phosphatase activity (Georgellis, Kwon et al. 1998; Bourret and Silversmith 2010; Huynh and Stewart 2011). Therefore we decided to test whether GST-VieS-C had phosphatase activity and if VieB stimulates that activity. To test this, we measured the rate of loss of phosphorylated VieA-His_6 in the presence of GST-VieS-C, GST-VieS-C and VieB, or in buffer alone. While there is an intrinsic slow rate of loss of phosphate when VieA-His_6 is in buffer alone this is common among RRs, though these rates can vary (Lukat, Stock et al. 1990; Wolanin, Webre et al. 2003). When in the presence of GST-VieS-C or GST-VieS-C plus VieB, the rate of loss is similar to that of the rate in buffer alone (Figure 2-5B). These data suggest that GST-VieS-C does not harbor phosphatase activity in the absence or presence of VieB. Of note, in some instances, phosphatase activity of the SK requires the full-length protein properly folded in a membrane. Therefore, since our construct only contains the cytoplasmic portion of the protein, our data cannot completely rule out that there may be phosphatase activity *in vivo*.

Figure 2-4. VieB does not affect the stability of phosphorylated GST-VieS-C. Purified GST-VieS-C or MBP-VieS-C D1018A was incubated with ^{32}P-ATP-γ in the absence of VieB for 30 minutes at 30°C. Excess ^{32}P-ATP-γ was removed from phosphorylated VieS-C constructs by gel filtration. P-VieS-C constructs were then incubated with additional $MnCl_2$ and buffer or with 5 μM VieB for 60 minutes. Samples were stopped at indicated time points with the addition of 2X-denaturing sample buffer and separated using a 10% SDS-PAGE gel. VieS-C constructs labeled with ^{32}P were observed by radioautography **(A,B)** and quantified in **(C)**.The radioautograph shown is a representative of three replicates. Error bars represent the SEM of three replicates.

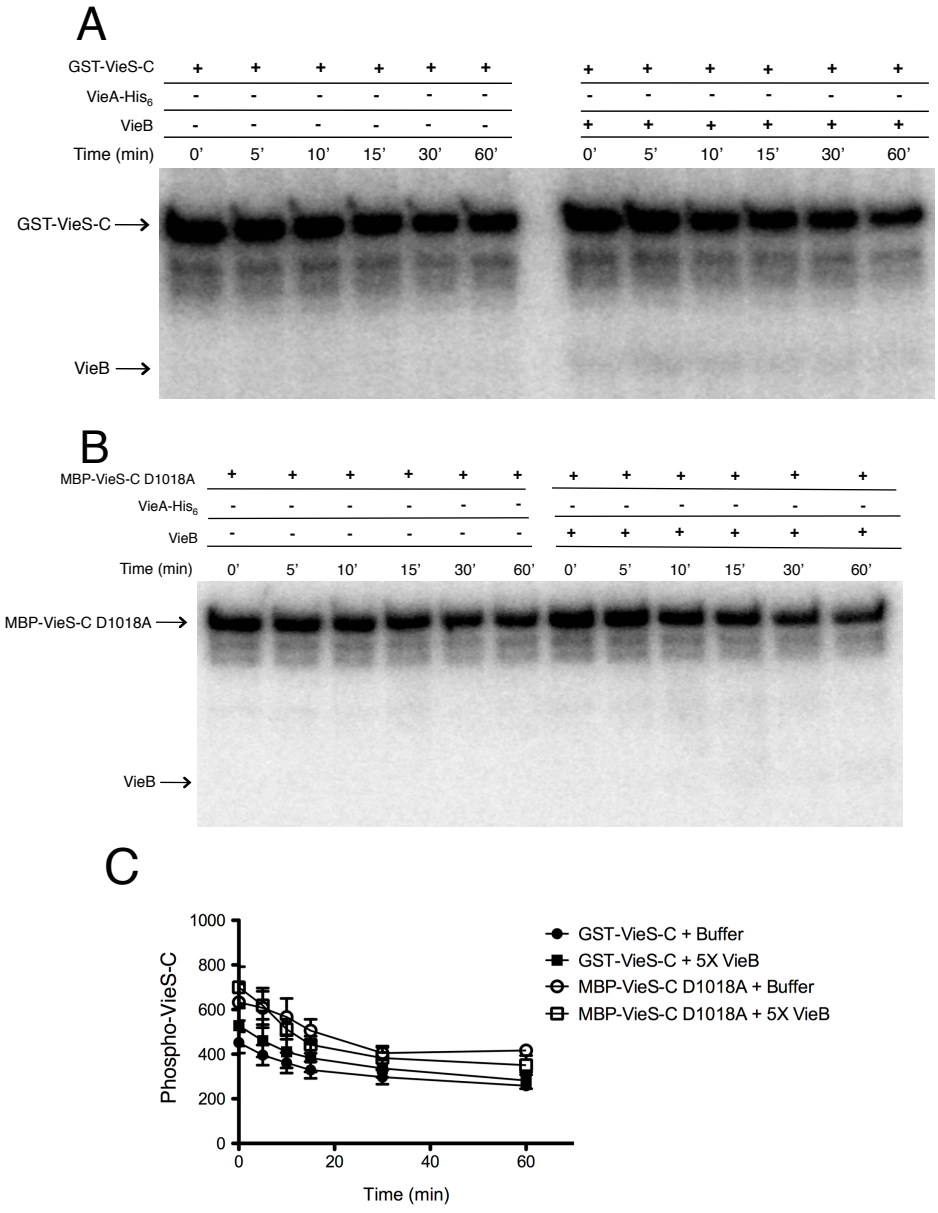
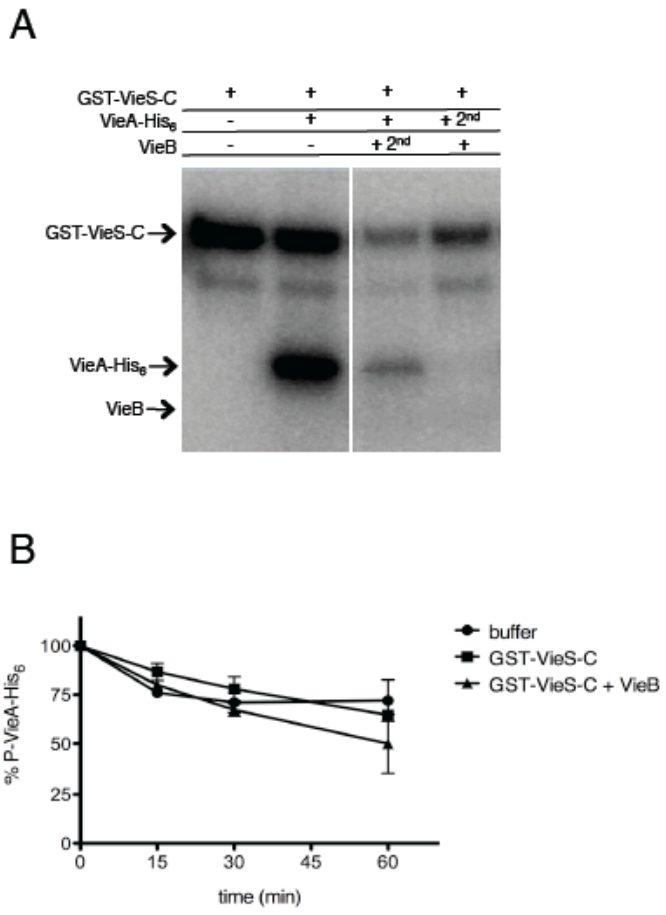


Figure 2-5. VieB is not a phosphatase and does not stimulate GST-VieS-C phosphatase activity.
(A) Previously described phosphotransfer assays were conducted at 30°Cin the presence of ^{32}P-ATP-γ with the following modifications: Lane 1- GST-VieS-C alone was incubated for 60 minutes, Lane 2- GST-VieS-C was incubated with 1 μM VieA-His_6 for 60 minutes, Lane 3- GST-VieS-C was incubated with 1 μM VieA-His_6 for 30 minutes then 5 μM wild type VieB was added for an additional 30 minutes, Lane 4- GST-VieS-C was incubated with 5 μM wild type VieB for 30 minutes then 1 μM VieA-His_6 was added for an additional 30 minutes. Proteins labeled with ^{32}P were observed by radioautography. The radioautographs shown are a representative of three replicates. **(B)** ^{32}P-VieA-His_6 was incubated with buffer, GST-VieS-C alone or GST-VieS-C and VieB for 60 minutes at 30°C. Samples were taken over time and quantified. 0 minute time point represents 100% ^{32}P-VieA-His_6. Error bars represent the SEM of three replicates.

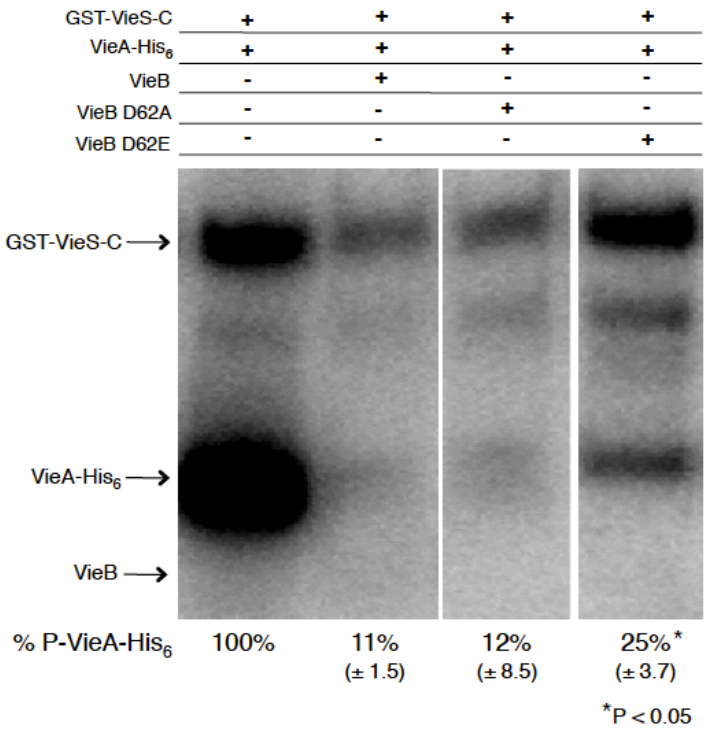


2.2.3 VieB mechanism of action

As described above, we have shown that VieB is an inhibitor of VieS-VieA phosphotransfer by the partial inhibition of VieS autophosphorylation and disruption of phosphotransfer. Interestingly, VieB harbors a Rec domain that contains a conserved phosphorylation site located at Asp62. In TCSs, the activity of the RR is regulated through phosphorylation at this conserved site, whereby phosphorylation usually results in activation of the RR. Since the Rec domain and the conserved Asp residue provides tight regulation of RR output activity, we hypothesized that phosphorylation of VieB could modulate its inhibitory activity. However, VieB is poorly phosphorylated by GST-VieS-C (Figure 2-1) and acetyl-phosphate, a small molecular phosphor-donor commonly used to phosphorylate RRs independently of the SK. Therefore to mimic the phosphorylated and unphosphorylated states of VieB, point mutations were made at the conserved Asp62 residue. By replacing D62 with an Ala (D62A), this mimics an unphosphorylated VieB, while replacing D62 with a Glu (D62E), we hypothesize, will mimic a phosphorylated state, based on the use of this mutation for artificially creating phospho-mimic RRs of the Ntr family. Using phosphotransfer assays, we assessed the ability of these mutants to inhibit the phosphorylation of VieA-His_6. Of note, while wild type VieB is not robustly phosphorylated by GST-VieS-C, there is a very low level of phosphorylation (Figure 2-1). Neither of these point mutants became phosphorylated during any of the phosphotransfer reactions, suggesting that the D62 residue is indeed the conserved phosphorylation site and no alternative phosphorylation residue exists (Figure 2-6). When VieB D62A is added to the reaction, this mutant was able to inhibit VieA-His_6 phosphorylation at wild type levels. However, the VieB D62E mutant is a

significantly weaker inhibitor of phosphotransfer, suggesting that the phosphorylation state of VieB affects its ability to act as an inhibitor (Figure 2-6). Interestingly, for VieB, the unphosphorylated state (D62A) is active while the "phosphorylated" state (D62E) is inactive, which is the opposite of how most RRs are regulated. While we expected the VieB D62E mutant to be completely inactive, there is still some level of phosphorylated VieA-His_6 present in the reaction (Figure 2-6). This may be due to the D62E mutation not completely mimicking the true phosphorylated state of VieB and/or simply due to electrostatic changes within the Rec domain for the D62E mutation.

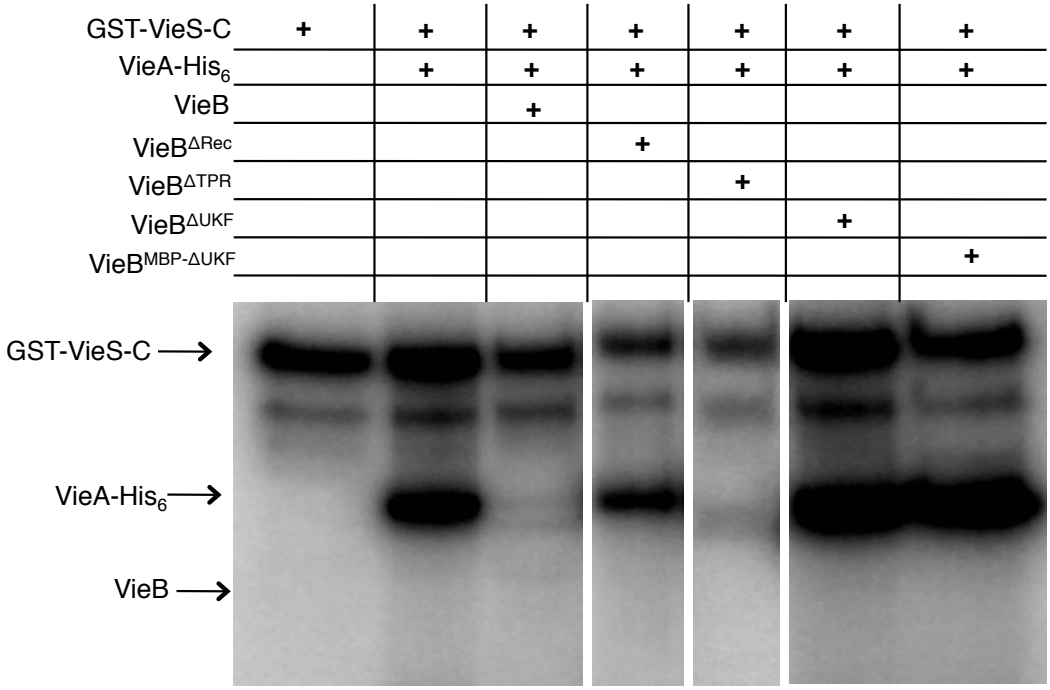
Figure 2-6. Mutation of the conserved Asp residue affects the inhibitory activity of VieB.
GST-VieS-C was incubated with ^{32}P-ATP-γ in the presence of equimolar VieA-His_6 (lane 1) and either 5 μM wild type VieB (lane 2), VieB D62A (lane 3), or VieB D62E (lane 4) for 30 minutes at 30°C. Samples were stopped with the addition of 2X-denaturing sample buffer and separated using a 10% SDS-PAGE gel. Proteins labeled with ^{32}P were observed by radioautography. The radioautographs shown are a representative of four replicates. Standard deviation is shown below. Stars denotes a significant P-value < 0.05 determine by a Mann-Whitney U test.



VieB harbors two conserved domains, the Rec and TPR, while the latter half of the protein is of unknown function (UKF). In order to understand what domains are important for VieB's inhibitory activity, we made domain truncations and tested these mutants using phosphotransfer assays. Due to the important regulatory role of the Rec domain and its significance in the recognition and interaction with the cognate SKs, we hypothesize that deletion of this domain would be necessary for its function. As expected, when this domain is deleted, this mutant is no longer able to inhibit phosphotransfer (Figure 2-7). Interestingly, when the TPR domain was replaced with a flexible linker, this mutant is still able to inhibit phosphotransfer, suggesting that this domain is not necessary for inhibition. This was quite surprising as we hypothesized that this domain would be important since TPR domains are known to mediate protein-protein interactions. When the UKF region was deleted, this mutant was no longer able to inhibit phosphotransfer (Figure 2-7). While this result suggests that the UKF region is needed for VieB inhibition, deletion of the UKF is approximately half of the protein. Therefore it is possible that this protein construct is misfolded, which could yield a similar result in phosphotransfer assays. In attempts to obtain a properly folded VieB$^{\Delta UKF}$, this construct was purified with an N-terminal MBP tag. This MBP-VieB$^{\Delta UKF}$ construct was still unable to inhibit phosphotransfer, providing no additional information as to if this region is required for VieB's inhibitor function (Figure 2-7). In attempts to determine if either of these constructs was properly folded, phosphotransfer assays were conducted in the presence of GST-VieS-C, as properly folded proteins should receive wild type levels of phosphorylation by GST-VieS-C. However, no detectable phosphorylation of either the

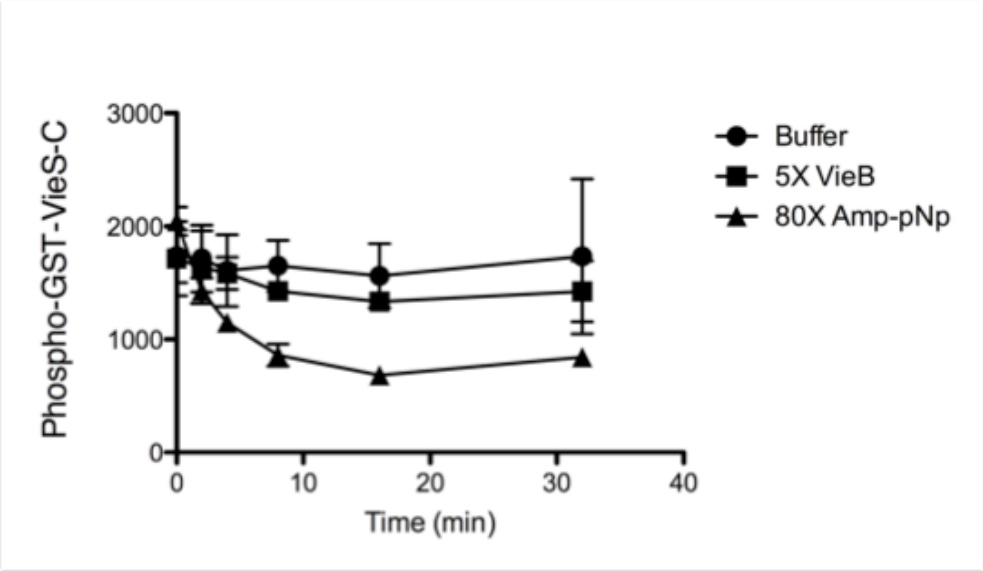
MBP-tagged or the untagged ΔUKF constructs were observed, suggesting that these constructs are not properly folded (data not shown). Taken together, this data suggests that the Rec domain, but not the TPR domain, is required for activity.

Figure 2-7. Domain truncation analysis of VieB
GST-VieS-C was incubated with ^{32}P-ATP-γ in the presence of equimolar VieA-His$_6$ and either 5 μM wild type VieB or various domain truncation mutants for 30 minutes at 30°C. Samples were stopped with the addition of 2X-denaturing sample buffer and separated using a 10% SDS-PAGE gel. Proteins labeled with ^{32}P were observed by radioautography.



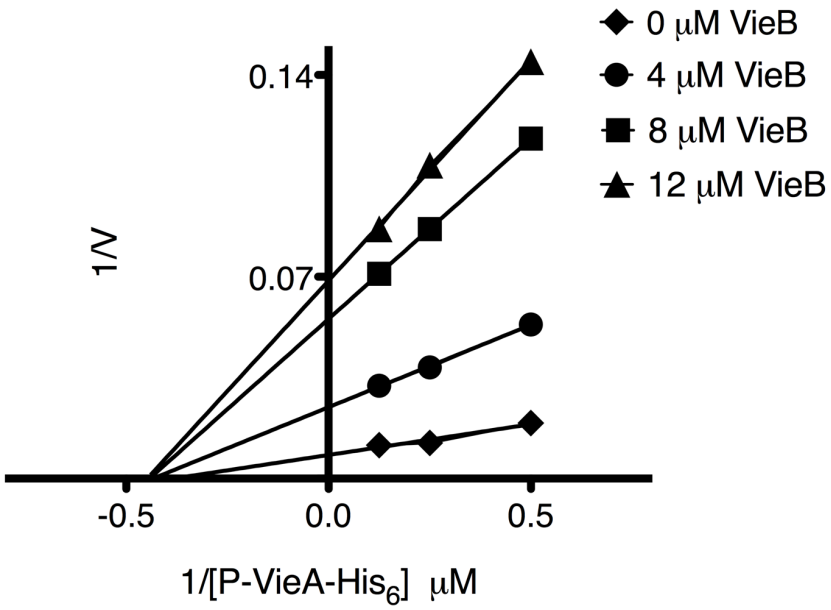
Since VieB is able to inhibit phosphorylation of VieA-His_6 by partially disrupting GST-VieS-C autophosphorylation and preventing phosphotransfer, we hypothesized that VieB may be acting as a competitive inhibitor. This model would suggest that VieB actively competes for the binding site at the HK domain and through this mechanism, is able to reduce autophosphorylation and block intra-molecular transfer. To first test this hypothesis, we compared the inhibition kinetics of VieB with that of a known competitive inhibitor of SKs, AMP-pNp. AMP-pNp is an analogue of ATP in which the third phosphate is bound by nitrogen, as opposed to oxygen, allowing this molecule to bind the ATP catalytic pocket but is unable to be cleaved to release P_i and ADP. Using phosphotransfer assays, we tracked the amount of phosphorylated GST-VieS-C in the presence of VieB and AMP-pNp. While both decrease the amount of phosphorylated GST-VieS-C, the total amount and the rate of decrease in the presence of AMP-pNp is much greater and quicker than that of VieB (Figure 2-8). These data suggest that VieB is not acting as a competitive inhibitor of VieS-C and may be acting noncompetitively.

Figure 2-8. VieB does not behave similar to the competitive inhibitor, AMP-pNp
Purified GST-VieS-C was incubated with ^{32}P-ATP-γ in the absence of buffer alone, wild type VieB or AMP-pNp at 30°C over time. Samples were stopped at indicated time points with the addition of 2X-denaturing sample buffer and separated using a 10% SDS-PAGE gel. GST-VieS-C constructs labeled with ^{32}P were observed by radioautography and quantified. The radioautograph shown is a representative of three replicates. Error bars represent the SEM of three replicates.



To further distinguish if VieB is behaving as a competitive or noncompetitive inhibitor, we generated a Lineweaver-Burk plot using phosphotransfer assays. A previous report described MBP-VieS-C kinase activity to operate under second order Michaelis-Menten kinetics (Martinez-Wilson, Tamayo et al. 2008). By varying the concentration of VieB over a range of VieA-His_6 concentrations, the rate (velocity) of phosphotransfer between GST-VieS-C and VieA-His_6 was calculated. As the concentration of VieB increased, the velocity at which GST-VieS-C was able to phosphorylate VieA-His_6 decreased. Extrapolation of the line for each VieB concentration reveals that all concentrations result in the same Michaelis K_m (*x*-intercept) but vary in the V_{max} (*y*-intercept), suggesting that VieB is a noncompetitive inhibitor of GST-VieS-C (Figure 2-9).

Figure 2-9. VieB acts as a noncompetitive inhibitor
A Lineweaver-Burk Plot was generated using the previously described phosphotransfer assay over a range of VieA-His_6 (2, 4, and 8 μM) and wild type VieB (0, 4, 8, and 12 μM) concentrations. VieA-His_6 phosphorylated with ^{32}P (P-VieA-His_6) was quantified by radioautography. These data represent the average velocity of three replicates (0, 4 and 12 μM VieB) and four replicates (8 μM VieB). V= velocity



Since VieB is a noncompetitive inhibitor of GST-VieS-C, we hypothesized that VieB should bind to VieS-C. To first address if VieB interacts with VieS, GST pull-down assays were conducted. Immobilized GST-VieS-C was able to pull down VieB and VieA-His_6 from whole cell lysate in which VieB and VieA-His_6 were over-expressed from an IPTG-inducible plasmid (Figure 2-10). To further characterize this interaction, size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) and composition-gradient MALS (CG-MALS) were used to quantify the self- and hetero-association affinities and stoichiometries for all VieSAB proteins and their combinations. Table 2-1 summarizes the results of these experiments. Both VieS-C and VieA-His_6 behave as putative homo-dimers while VieB remains as a monomer. As expected based on the characterization of other SK-RR pairs, the VieS-C dimer binds a single VieA-His_6 dimer with an equilibrium dissociation constant (K_D) of 1.38 μM. Interestingly, VieB binds as a single monomer to one VieS-C dimer, with a strong K_D of 0.467 μM. This affinity is approximately three-fold stronger than that of the Vie-C/VieA-His_6 interaction, which is consistent with VieB acting as an inhibitor. Of note, there were no other stoichiometries present for the VieB/VieS-C interaction, providing evidence that there is only one available VieB binding site per VieS-C dimer. Furthermore, VieB does not interact with VieA-His_6 as no detectable interactions between these two proteins were observed using MALS. We predict that the purified proteins in these experiments are unphosphorylated, as there is no ATP present during the MALS experiments. While the interaction kinetics may vary in the presence of ATP, taken together, these data further

support that VieB specifically interacts with VieS to inhibit phosphotransfer in its unphosphorylated state.

Since the VieB D62E mutant is a poor inhibitor of phosphotransfer, we hypothesized that this might be due to a weaker binding of this mutant to VieS-C. Therefore, we tested this mutant using SEC- and CG-MALS. Intriguingly, this mutant behaves as a monomer and is still able to bind a dimer of VieS-C with a surprisingly strong affinity, K_D=0.197 μM. This affinity is about two-fold stronger than that of the wild type VieB, which is unexpected given the inability of this mutant to inhibit phosphotransfer. While this provides little insight into how phosphorylation regulates the inhibitory activity of VieB, these data suggest that simply binding to VieS-C is not sufficient to inhibit and/or that the binding of the D62E is different from that of the wild type VieB.

Figure 2-10. VieB is pulled down by GST-VieS-C
GST-VieS-C can specifically pull down VieA-His_6 and VieB from whole cell lysates. Whole cell lysates were prepped from *E. coli* BL21 (D3E) strains over-expressing *vieA-His_6, His_6-VieB,* or the His tag empty vector. GST-VieS-C was immobilized and protein lysates were incubated for 30 minutes at 30°C. Reactions were washed with 150 µl of wash buffer three times and protein was eluted off the beads with 150 µl of elution buffer containing reduced glutathione. Samples were taken from the input lysate (Lys), flow through (FT), wash (W) and elution (E) fractions for analysis on 10% SDS-PAGE gel stained with Lumitein protein stain™. M = protein standard

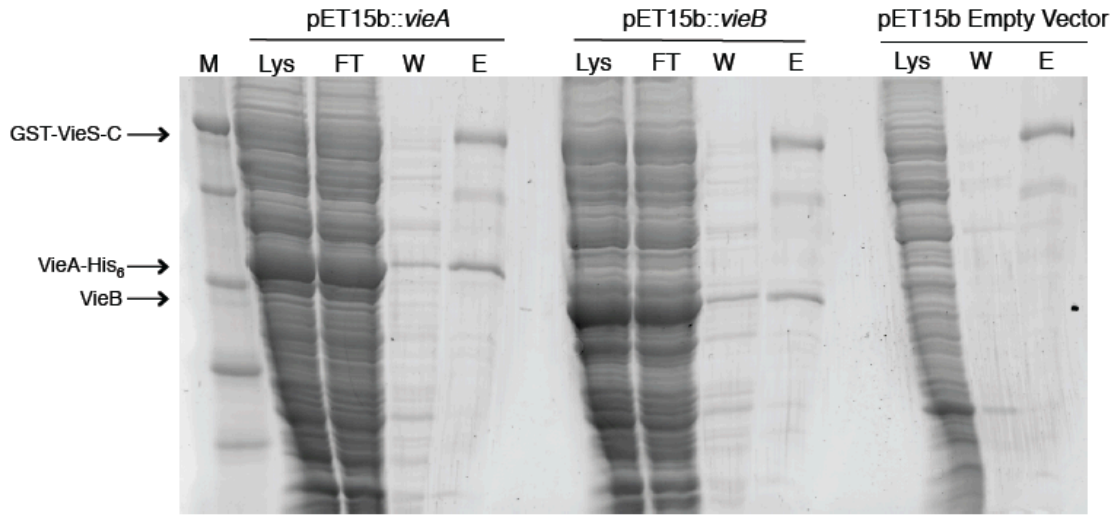


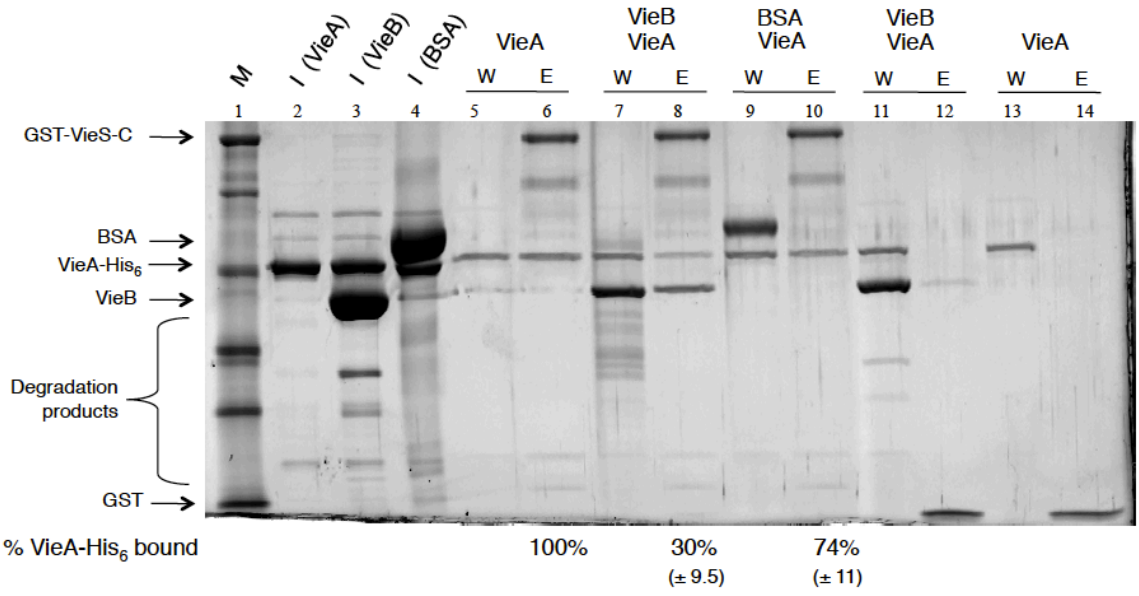
Table 2-1. VieB specifically interacts with VieS-C

Characterization of VieS-C, VieA-$His_{6,}$ wild type VieB and the VieB D62E point mutant (self-association) were determined by Size-Exclusion Chromatography Multi-angle Light Scattering. To determine the protein-protein interactions of various VieSAB protein combinations, hetero-association interaction kinetics were determined over a range of protein concentrations by Composition-gradient Multi-angle Light Scattering. Data for the hetero-association stoichiometry are represented in monomer units. These data represent the average and ±SD of three independently purified replicates.

Protein Combination	**Native Oligomeric State**		**Hetero-association stoichiometry**	***K_D* (μM)**
VieS-C + VieA-His_6	**VieS-C**	**VieA-His_6**	1 dimer : 1 dimer	1.38 ± 0.35
	Dimer (MW = 150 kDa)	Dimer (MW = 130 KDa)		
VieS-C + VieB	**VieS-C**	**VieB**	1 dimer : 1 monomer	0.467 ± 0.054
	Dimer (MW = 150 kDa)	Monomer (MW = 64 kDa)		
VieS-C + VieB D62E	**VieS-C**	**VieB D62E**	1 dimer : 1 monomer	0.197 ± 0.061
	Dimer (MW = 150 kDa)	Monomer (MW = 64 kDa)		
VieA-His_6 + VieB	**VieA-His_6**	**VieB**	N/A	N/A
	Dimer (MW = 130 kDa)	Monomer (MW = 64 kDa)		

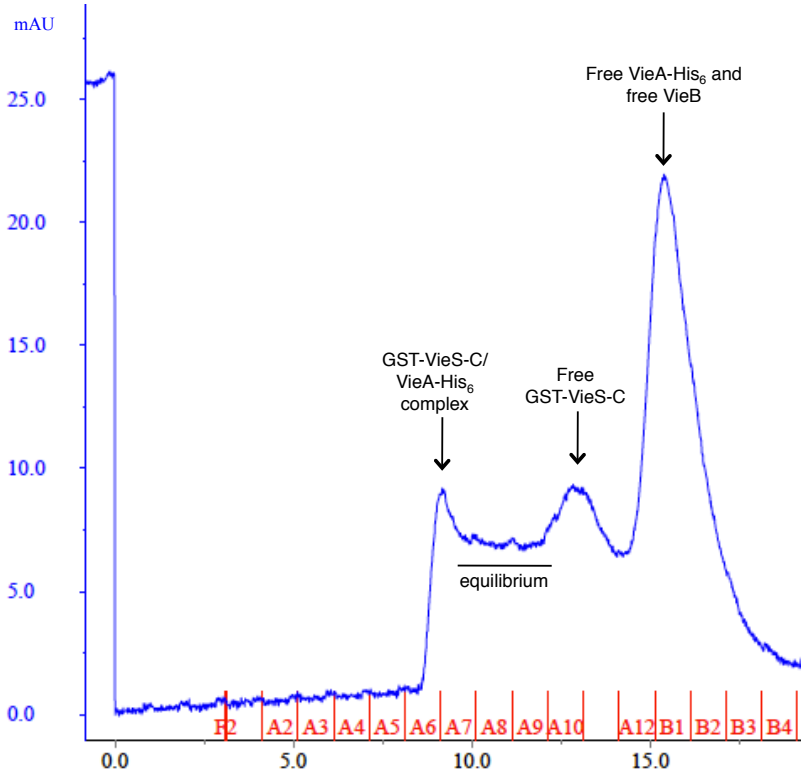
Given the noncompetitive model of VieB inhibition, it follows that the binding of VieB to VieS-C should have little or no effect on the VieS-C/VieA-His_6 interaction. To test this hypothesis, GST pull-down experiments were conducted with purified proteins. Since VieB inhibition is most effective at molar excess, we conducted these experiments with a five-fold molar excess of VieB over VieA-His_6. Additionally, to ensure that all sites on GST-VieS-C were occupied, both VieA-His_6 and VieB were in molar excess to GST-VieS-C. As expected, when VieA-His_6 is incubated with GST-VieS-C, some VieA-His_6 is present in the wash fraction since it is in molar excess of GST-VieS-C (Figure 2-11, lane 5). VieA-His_6 was pulled down with GST-VieS-C in the elution (Figure 2-11, lane 6) and this amount of VieA-His_6 was quantified and set to 100%. In the presence of VieB, the amount of VieA-His_6 pulled down in the elution was reduced by 70%, which is not consistent with VieB being a noncompetitive inhibitor (Figure 2-11, lane 8). However, as a negative control, BSA at a five-fold molar excess was used to control for nonspecific interactions between an unrelated protein and VieA-His_6. When BSA is present, there is a 26% reduction in the amount of VieA-His_6 that is bound to GST-VieS-C. Statistical analysis by one-way ANOVA and Dunn's Multiple Comparison reveals that this decrease is not significantly different from the decrease observed with VieB, which could suggest that the reduction seen in both reactions is nonspecific. As a negative control for VieA-His_6 and VieB nonspecific interactions with the GST tag, GST alone was bound to glutathione beads. Neither VieA-His_6 nor VieB was present in the elution fraction when only GST is bound, suggesting that proteins pulled down in the elution are specific to VieS-C (Figure 2-11, lane 11-14). Taken together, this supports the noncompetitive model of inhibition for VieB.

Figure 2-11. VieB does not disrupt the VieS/VieA interaction
GST-VieS-C (lane 4-9) or GST alone (lane 10-13) bound to glutathione beads were incubated with either, pre-mixed 5 µM VieA-His_6 and 25 µM VieB (VieB), 5 µM VieA-His_6 alone (VieA) or 5 µM VieA-His_6 and 25 µM BSA (BSA). Reactions were washed with 150 µl of wash buffer five times and protein was eluted off the beads with 150 µl of elution buffer containing reduced glutathione. Samples were taken from the input (I), wash (W) and elution (E) fractions for analysis on a 10% SDS-PAGE gel stained with Lumitein protein stain™. Lanes 1-3 indicates protein inputs, respectively. Percentage of VieA-His_6 bound is the average of four replicates with standard deviation shown below; gel shown is a representative of the replicates. Statistical significance was determined by one-way ANOVA and Dunn's Multiple Comparison. M = protein standard



Also in line with VieB being a noncompetitive inhibitor, we hypothesize that VieSAB should form a heterotrimeric complex. Therefore, we attempted to observe a trimeric complex by incubating GST-VieS-C, VieA-His_6 and VieB together and subsequently running the reaction over a gel filtration column. Figure 2-12 shows that GST-VieS-C/VieA-His_6 is able to form a stable complex. However, the GST-VieS-C/VieA-His_6/VieB complex is either not formed or is not stable over the column, as there is no defined peak corresponding to the heterotrimeric complex. Instead, gel filtration analysis shows a plateau that represents a state of equilibrium between binding and dissociation of VieB. Based on this analysis, it is unclear if VieSAB can form a heterotrimeric complex or if they can, how stable this complex is over time. However, collectively, the data presented above, particular the Lineweaver-Burk plot indicates that VieB's mechanism of action is through noncompetitive inhibition of VieS.

Figure 2-12. VieSAB do not form a heterotrimeric complex
Equal molar concentrations of GST-VieS-C, VieA-His_6 and VieB were mixed in Gel Filtration buffer and incubated for 30 minutes at 30°C. The protein mixture was then immediately run over a HiLoad Superdex200 gel filtration column that was equilibrated in Gel Filtration buffer. Fractions were monitored to observe peaks corresponding to various VieSAB protein complexes. Samples were taken from each peak fraction, added to 2X denaturing sample buffer and run on a 10% SDS-PAGE gel to observe which proteins were within each peak.



2.3 DISCUSSION

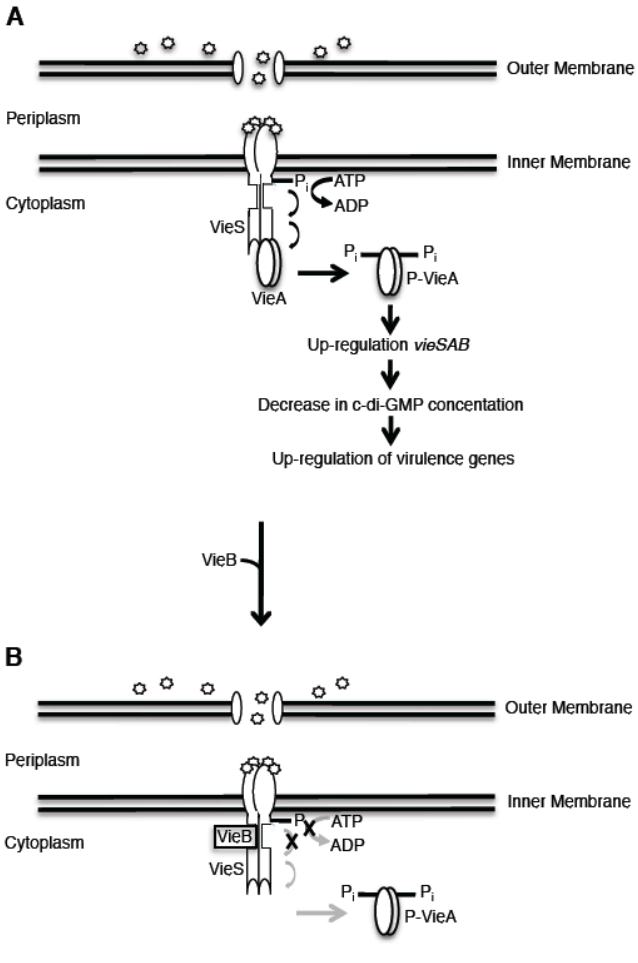
In these studies, we reveal the function of VieB, the previously uncharacterized protein in the VieSAB signal transduction system. Using phosphotransfer assays, we show that VieB is able to inhibit the phosphorylation of VieA by binding to VieS by partially disrupting autophosphorylation and interrupting the intra-molecular phosphorelay. Furthermore, we characterized the mechanism of action, where VieB appears to behave as a noncompetitive inhibitor of VieS. We propose that VieB inhibition of phosphotransfer between VieS and VieA provides a negative feedback mechanism to down-regulate the VieSA TCS.

vieSAB has been well-described to play a role in regulating virulence gene expression through the modulation of cdGMP by the PDE domain of VieA (Tischler, Lee et al. 2002; Tischler and Camilli 2005; Dey, Bhagat et al. 2013). Changes in cdGMP concentration are known to regulate gene expression in *V. cholerae*, where high intracellular cdGMP concentration induces environmental survival genes- such as biofilm formation and chitin utilization genes, and low intracellular cdGMP concentration induces virulence genes-such as CT, *tcpA* and *toxT* (Tamayo, Schild et al. 2008; Krasteva, Giglio et al. 2012). However, VieA, like many other RRs, also harbors a DNA-binding HTH motif suggesting that it may also regulate virulence gene expression directly through DNA binding. Unfortunately, direct binding of VieA to DNA has not been shown, although a previous microarray study provides some evidence that VieA may be autoregulatory (Beyhan, Tischler et al. 2006). Our data taken together with the findings of that study lets us present a working model that incorporates the inhibitory role of VieB into the VieSA TCS (Figure 2-13). When VieS becomes bound by an unknown

environmental stimulus, VieS autophosphorylates and transfers this phosphate down its intra-molecular relay to VieA. Based on VieA's predicted autoregulatory activity, we hypothesize that phosphorylated VieA results in its activation as a transcription factor and subsequent up-regulation of the *vieSAB* operon. This results in increased levels of VieA protein, rapid cleavage of cdGMP by the PDE enzymatic activity within VieA, and aids in triggering virulence gene expression needed for survival in the host (Figure 2-13A).

Over time, autoregulation of *vieSAB* leads to the accumulation of VieB. We hypothesize that VieB can then tightly bind to VieS noncompetitively, inhibit the autophosphorylation at the HK and disrupt further phosphotransfer between VieS's HK and Rec domains. This inhibition ultimately prevents phosphorylation of VieA and turns off its transcription factor activity. This reduction in phospho-VieA shuts off or at least decreases the expression of *vieSAB* and virulence genes due to the rise in cdGMP levels (Figure 2-13B). Since VieB is only a moderate inhibitor of autophosphorylation, it is possible that even though VieB is present, VieS could become phosphorylated. However, since VieB is also capable of blocking the intra-molecular phosphorelay, this autophosphorylation is not a problem and ensures VieA will remain unphosphorylated when VieB is present.

Figure 2-13. Proposed working model of VieB inhibition of VieSA TCS
Stimulation of the VieSA TCS by binding of external signal (stars) to VieS results in autophosphorylation and phosphotransfer to and activation of VieA. We propose that VieA activation results in the amplification of the *vieSAB* operon and up regulation of virulence genes. We hypothesize that this up regulation of *vieA*, leads to decreased intracellular levels of cdGMP and enhanced expression of virulence genes **(A)**. Over time, or at high levels of transcription of the *vieSAB* operon, VieB accumulates. This pool of VieB is able to tightly bind, noncompetitively, to VieS. We hypothesize that this binding partially disrupts autophosphorylation and interrupts the transfer of phosphate between the HK and REC domains of VieS, down-modulating the phosphorelay. This lack of phosphotransfer to VieA results in down regulation of *vieSAB* and virulence genes **(B)**. 'X' denotes the inhibitory action of VieB. Black arrows correspond to active phosphotransfer while grey arrows denote incompletion of transfer.



Additionally, we show that the conserved Asp provides a means to regulate the inhibitory activity of VieB. However, this regulation is reversed for from what is normally observed for Rec domain containing proteins, where the unphosphorylated (D62A) state is the active inhibitor and the "phosphorylated" (D62E) state is inactive. Given that inhibition requires VieB binding to VieS, this reversed regulation is an attractive model. We hypothesize that in its unphosphorylated state, VieB is able to efficiently bind VieS and prevent phosphotransfer to VieA. Therefore, phosphorylation of VieB can serve as a shut-off signal to allow VieS to regain function. While we originally hypothesized that phosphorylation would cause a conformation change in VieB that would completely abolish its ability to bind VieS thereby rendering it inactive, MALS data from VieB D62E mutant suggest that binding can still occur in the phosphorylated state. This suggests that the mere binding of VieB to VieS is not sufficient for inhibition but that the structure of VieB that is bound plays an important role. Nevertheless, our data cannot rule out the possibility that the D62E mutation does not adequately mimic a phosphorylated VieB conformation.

Interestingly, wild type VieB is not robustly phosphorylated by VieS, suggesting that VieS may not be its cognate SK and may not be responsible for regulating VieB. This observation is intriguing since *vieB* is located in the putative operon with *vieSA* and is clearly involved with the VieSA TCS. However, one explanation could be that another SK in the cell regulates VieB. While we have no evidence to support this hypothesis, it is not uncommon to have cross-regulation that connects two separate TCSs (Paul, Jaeger et al. 2008; Tsokos, Perchuk et al. 2011; Downey, Mashburn-Warren et al. 2014). If this

hypothesis is true, it could add even more complexity to the already intricate regulation of VieA, cdGMP levels and virulence gene expression.

Taken together, the studies presented here biochemically characterize VieB, the third component in the VieSAB signal transduction system. We present evidence that VieB provides a unique negative feedback mechanism over the VieSA TCS, allowing for tight regulation over VieA activity and cdGMP levels. This work provides a more comprehensive understanding of how VieSAB functions and further highlights the importance of this signal transduction system in regulating gene expression as *V. cholerae* transitions between the environment and human host. Future work is necessary for understanding which domains and residues are important for the complex interaction between VieB and VieS. Additionally, further investigation into the regulation of VieB by phosphorylation is required to fully understand how this auxiliary protein regulates the VieSA TCS.

CHAPTER 3: GENETIC CHARACTERIZATION OF VIEB

3.1 INTRODUCTION

Characterization of *vieB* in previous studies has offered little insight into its physiological function (Lee, Butler et al. 2001; Tischler, Lee et al. 2002). While *vieB* is highly expressed *in vivo* three hours post intragastric inoculation, it is not expressed under any *in vitro* condition tested thus far (Lee, Angelichio et al. 1998). Additionally, a partial deletion of *vieB* does not have a colonization defect in the infant mouse model of infection (Lee, Angelichio et al. 1998). This deletion also has no effect on the amount of CT expressed under virulence gene inducing conditions (Tischler, Lee et al. 2002). Therefore, to date, the physiological role of *vieB* remains unclear.

However, based on genetic analysis of the *vieSAB* operon, we can attempt to surmise under what conditions *vieB* may play a role in *V. cholerae* fitness. Firstly, a screen for positive regulators of virulence genes identified *vieB*, and subsequently the *vieSAB* operon (Lee, Butler et al. 2001). When the entire operon is deleted, this results in decreased expression of CT and the master virulence gene regulator, *toxT* (Tischler, Lee et al. 2002). Furthermore, a deletion of *vieS* and *vieA* results in increased biofilm formation and decreased motility and virulence gene expression, respectively (Tischler, Lee et al. 2002; Martinez-Wilson, Tamayo et al. 2008). It is well known that these phenomena are regulated by the intracellular concentration of cdGMP and VieA plays an important role in this modulation (Tischler and Camilli 2004; Tischler and Camilli 2005). In the previous chapter, we biochemically characterize VieB and reveal its mechanism of action as an inhibitor of the VieSA TCS. Therefore, based on these data and our proposed model of VieB (Figure 2-13), we hypothesize that a deletion of *vieB* should result in a

prolonged low concentration of cdGMP in the cytoplasm and may result in altered behaviors that are controlled by cdGMP, such as motility and biofilm formation.

In this chapter, we attempt to characterize *vieB* genetically, both in the classical and El Tor biotypes, specifically under conditions in which we know cdGMP plays an important role.

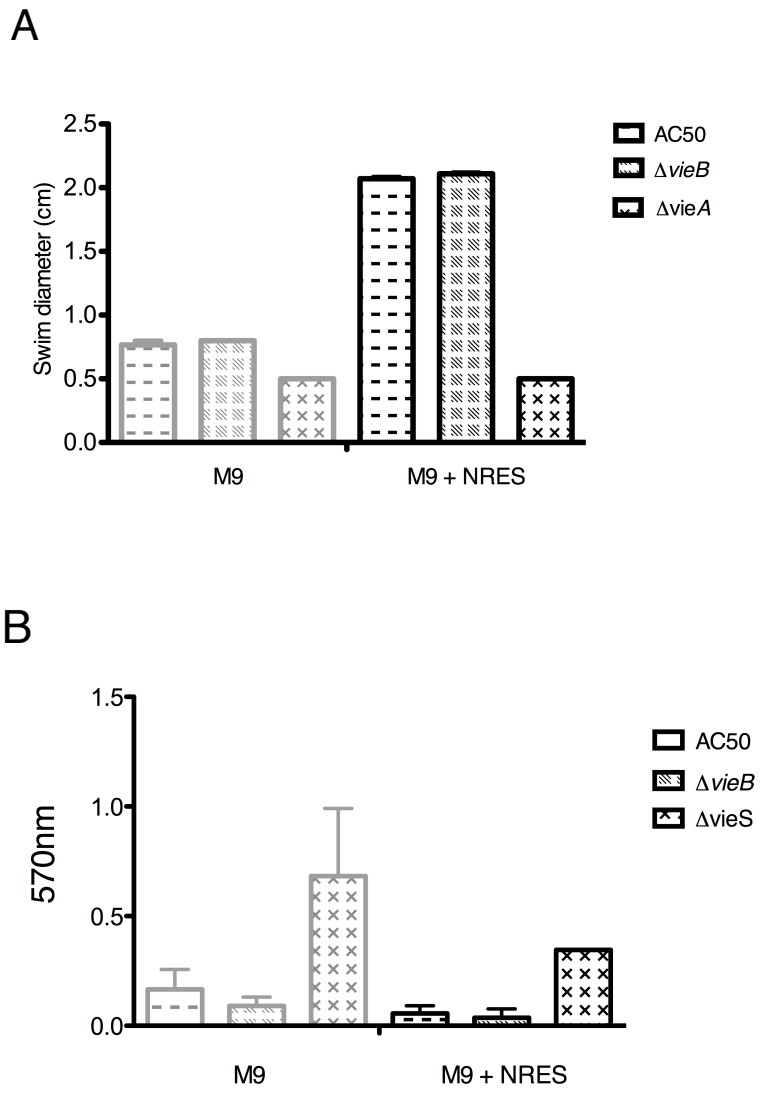
3.2 RESULTS

3.2.1 In vitro assays of Δ*vieB in the classical biotype*

Previous experiments utilized a partial deletion of *vieB*. This partial deletion lacked the Rec and TPR domains but retained part of the UKF sequence. While unlikely, it is possible that the truncated protein may maintain some function. Therefore, to ensure that there is no lingering effect of the partial deletion, we made a clean deletion of *vieB* in classical and El Tor biotype strains. Since Δ*vieSAB* appears to have a more significant role in the classical biotype (Beyhan, Tischler et al. 2006), we first tested the role of *vieB* in this background. Since VieSAB is needed for the expression of virulence genes in M9 NRES glycerol, it stands to reason that this system must be expressed and active under this condition. Therefore, we searched for additional phenotypes in M9 NRES glycerol that are known to be regulated by VieSAB, namely motility and biofilm formation. The Δ*vieB* mutant was tested in motility and single strain biofilm assays under non-inducing (M9 glycerol) and inducing (M9 NRES glycerol) conditions. Δ*vieA* was used as a control for motility assays, since a previous report demonstrates a deletion of *vieA* results in decreased motility (Martinez-Wilson, Tamayo et al. 2008). Δ*vieS* was used as a positive control for biofilm assays, as a deletion of *vieS* results in increased biofilm formation

(Martinez-Wilson, Tamayo et al. 2008). Based on our hypothesis that Δ*vieB* will have low cdGMP due to prolonged and/or higher VieA PDE activity, we would expect Δ*vieB* to have an advantage in motility but disadvantage in biofilm formation. However, Δ*vieB* does not have a defect in motility or biofilm formation (Figure 3-1).

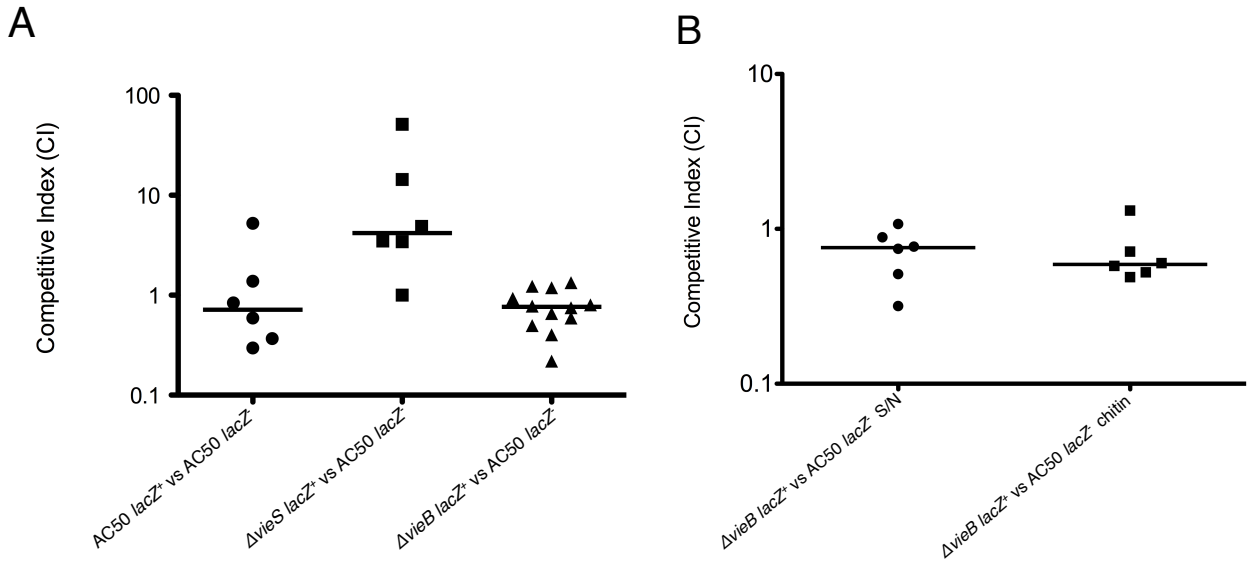
Figure 3-1. Δ*vieB* has no defect in motility or biofilm formation
(A) Wild type AC50, Δ*vieB* and Δ*vieA* strains were grown to stationary phase and the liquid culture was stabbed into M9 glycerol or M9 NRES glycerol minimal media plates containing 0.3% agar. Plates were incubated 30°C overnight and the swimming diameters were measured. **(B)** Wild type AC50, Δ*vieB* and Δ*vieS* overnight cultures were backed diluted 1:50 in M9 glycerol or M9 NRES glycerol media and allowed to undergo static growth at RT for 4 days. Biofilm formation was measured by staining with 0.5 mg/ml of crystal violent and absorbance was read at 570 nm wavelength.



It is possible that the loss of *vieB* results in a very small change in phenotype under these conditions, which is not detected in single strain assays. Therefore, in attempts to exacerbate possible phenotypes, we conducted biofilm and growth on chitin both as competition experiments between wild type and Δ*vieB*. Again, we predict that low cdGMP levels in Δ*vieB* will result in a decreased ability of this mutant to form biofilms compared to wild type. As expected, Δ*vieS* is able to outcompete wild type in biofilm competitions, however the Δ*vieB* has no defect in biofilm formation (Figure 3-2A). Since *V. cholerae* binds and utilizes chitin as a carbon and nitrogen source in the natural aquatic environment, we hypothesize that high cdGMP concentrations would be required for the expression of genes needed for chitin utilization. Therefore, in the chitin experiments, we hypothesize that Δ*vieB* will be somewhat defective at up regulating genes required for chitin utilization due to prolonged low cdGMP concentrations. However, there is no defect in the ability of Δ*vieB* to grow on chitin when compared to wild type (Figure 3-2B). These data suggest that *vieB* is not involved in controlling cdGMP levels under either of these biofilm formation or chitin utilization conditions tested.

Figure 3-2. Δ*vieB* is competes equally with wild type in biofilm and growth on chitin competition experiments

(A) A *lacZ*⁻ derivative of wild type AC50 and Δ*vieB lacZ*⁺ or Δ*vieS lacZ*⁺ were mixed 1:1 at an OD_{600}=0.1 in M9 NRES glycerol. Cultures underwent static growth at RT for 4 days. The biofilms were disrupted in PBS by vortexing with beads. Both inputs and outputs were plated on indicator plate containing X-gal, blue and while colonies were enumerated and a competitive index (CI) was calculated. **(B)** *lacZ*⁻ AC50 and Δ*vieB lacZ*⁺ strains were mixed 1:1 at an OD_{600}=0.1 and underwent static growth at 30°C for 4 days in the presence of chitin in 0.5X Instant Ocean. The supernatant was removed, vortexed and plated as above to determine a CI. The remaining chitin was vortexed and sonicated in 0.5X Instant Ocean to remove bound *V. cholerae* and was plated as above. CI was calculated for both the supernatant (S/N) and chitin-bound supernatant (chitin) samples.

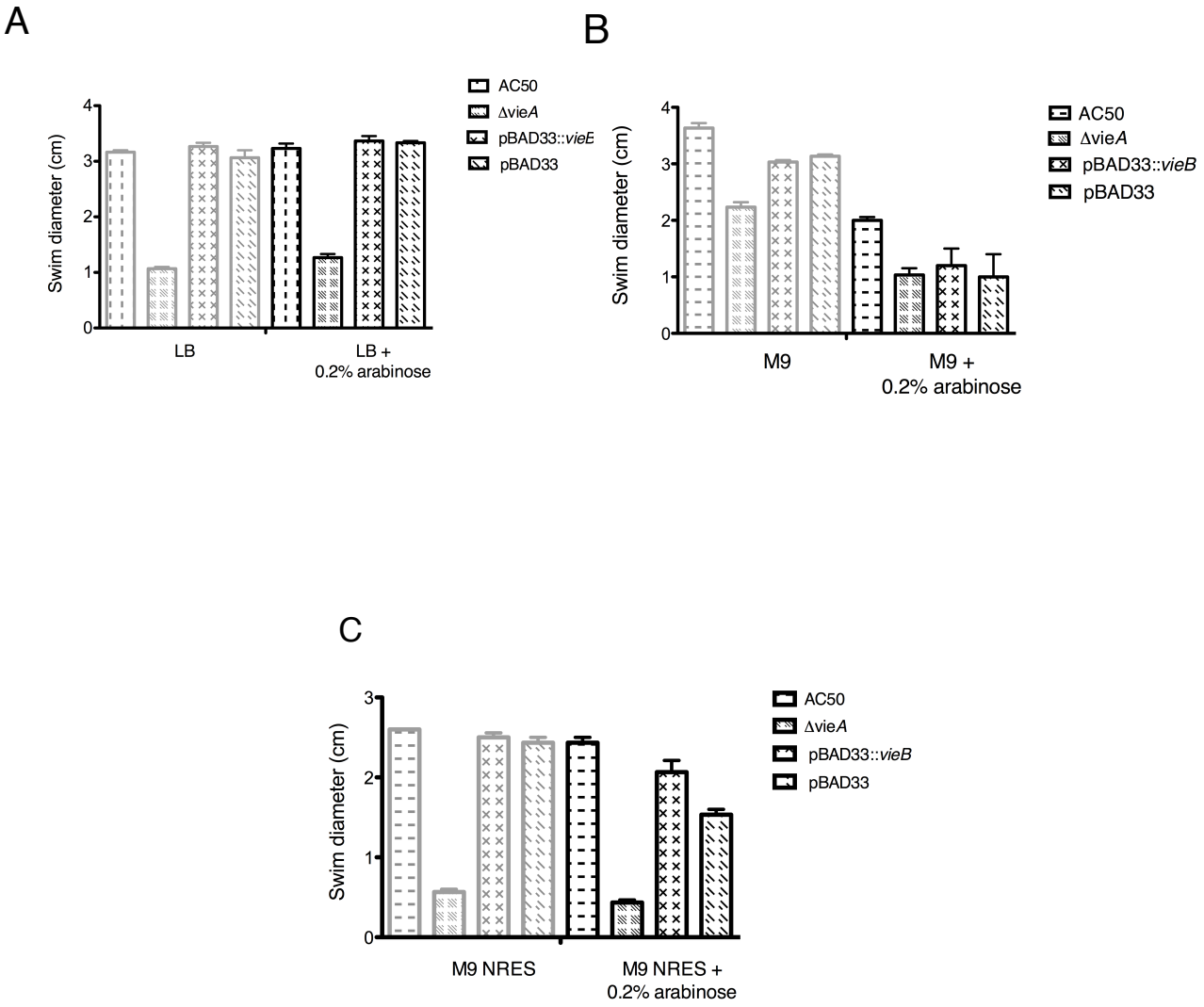


3.2.2 Over-expression of vieB in the classical biotype

Another possible explanation for why we did not detect a phenotype for the *vieB* deletion strain in motility, biofilm or growth on chitin experiments is that *vieB* may not be induced under these conditions. Therefore, we next decided to test if over-expressing *vieB* from an arabinose-inducible plasmid would affect motility in LB, M9 or M9 NRES glycerol. We hypothesized that over-expression of *vieB* would result in high cdGMP levels and result in decreased motility. As seen in Figure 3-3A, over-expression of *vieB* in LB does not result in decreased motility compared to both the wild type and empty vector control. This suggests that *vieB* has no effect on motility in LB, even when over-expressed. Of note, LB does not induce virulence genes or up-regulate *vieSA*; therefore this result may be due to lack of *vieSA* induction, which we hypothesize is necessary in order to see any effect of *vieB*. In M9 lacking arabinose, there is reduced motility of both the pBAD33::*vieB* and the empty vector strains (Figure 3-3B). While this reduction is in line with our hypothesis, the decrease is similar between both the pBAD33::*vieB* and the empty vector control. This suggests that the plasmid may be slightly toxic to the cells, which are already undergoing stress due to the poor growth medium. Additionally, this toxicity is amplified in the presence of arabinose as all strains, including wild type, took a total of 4 days to swim to a measurable swarm size (Figure 3-3B, black bars). Therefore in addition to plasmid toxicity there also appears to be toxicity due to arabinose under minimal media conditions. Interestingly, the plasmid toxicity is slightly reduced in M9 NRES, a slightly better growth medium, as all strains (with exception of the Δ*vieA* control) swim to similar diameters (Figure 3-3C). However, in the presence of arabinose

in M9 NRES, both strains harboring the pBAD33 plasmid exhibit a reduction in swimming diameter, with the empty vector having a larger decrease (Figure 3-3C). Therefore, it seems that arabinose is taxing to the cells when grown in minimal media and this stress is further exacerbated when driving expression off the plasmid. Though this toxicity makes the data hard to interpret, taken together, these data suggest that *vieB* is not drastically affecting cdGMP levels that manifest into a motility phenotype. However, it remains possible that a small effect of *vieB* over-expression may have been masked by the plasmid and arabinose toxicities.

Figure 3-3. *vieB* over-expression does not result in a motility phenotype
AC50, pBAD33::*vieB* and pBAD33 empty vector derivatives, and Δ*vieA* were grown to stationary phase and the liquid culture was stabbed into **(A)** LB, **(B)** M9 glycerol, or **(C)** M9 NRES glycerol, +/- 0.2% arabinose plates all containing 0.3% agar. Plates were incubated at 30°C for 17 hrs **(A)**, 4 days **(B)** or 1.5 days **(C)** and the swimming diameters were measured.



3.2.3 Transcription of vieSA between the wild-type and ΔvieB in the classical biotype

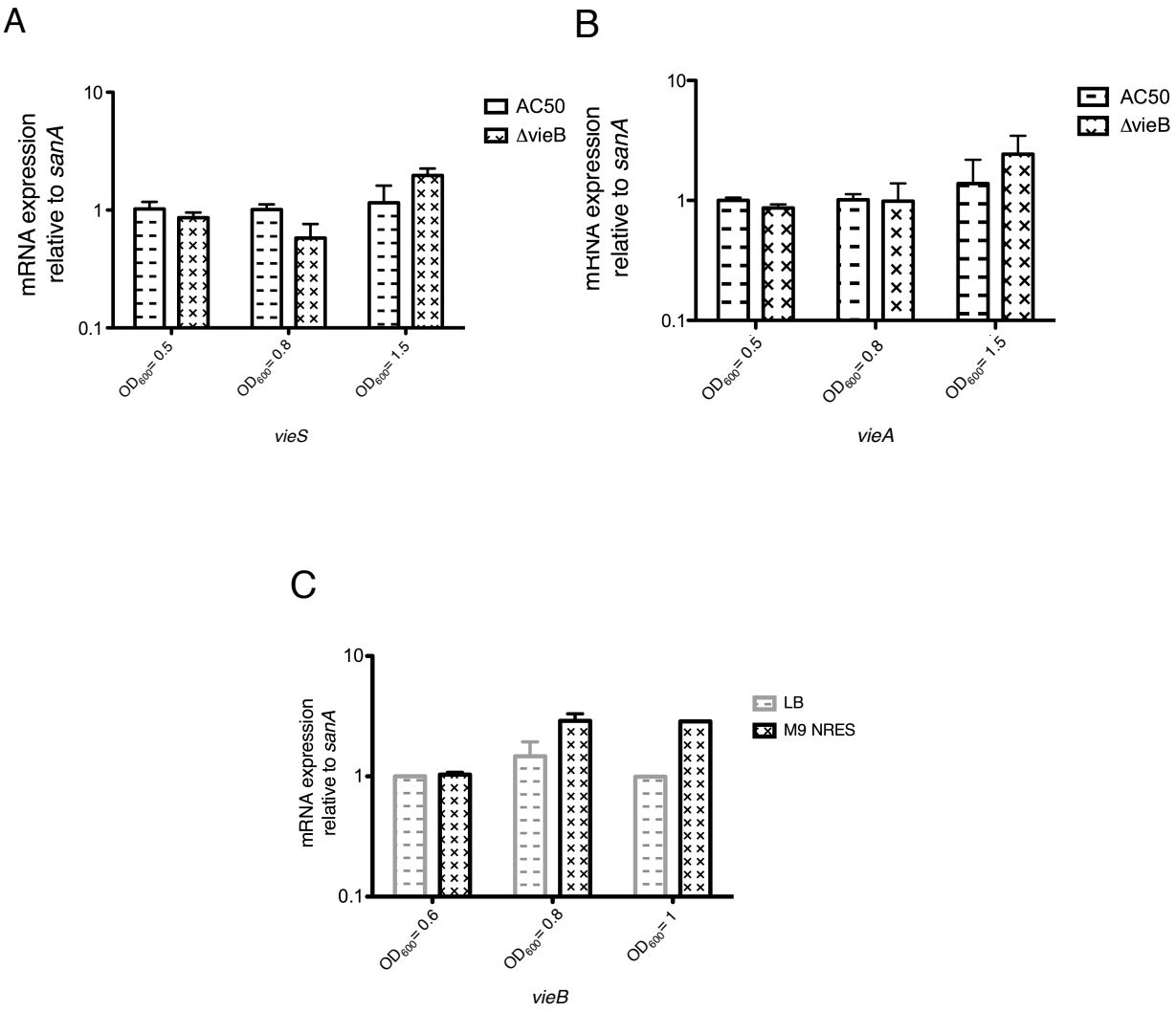
Our attempts to reveal a phenotype of *vieB* through cdGMP-controlled behavioral assays have been unsuccessful. Based on our model of VieB inhibition, we propose that VieB is involved in shutting off or decreasing *vieSAB* expression. Therefore, we aimed to address the role of *vieB* by measuring transcriptional changes in the Δ*vieB* mutant under virulence inducing conditions. We hypothesize that a deletion in *vieB* would result in extended and/or increased levels of *vieSA* transcripts. To test for increased *vieSA* transcripts, mRNA levels were compared between wild type and Δ*vieB* in M9 NRES glycerol throughout the growth phase of *V. cholerae*. Under these conditions, there is no significant increase in either *vieS* (Figure 3-4A) or *vieA* (Figure 3-4B) transcripts when compared to wild type, providing no evidence that *vieB* is having an impact on *vieSA* transcription.

A previous report provided evidence that only *vieSA* is expressed at low levels *in vitro* but *vieSAB* can be up regulated *in vivo* (Lee, Angelichio et al. 1998; Lee, Butler et al. 2001; Lombardo, Michalski et al. 2007). While M9 NRES glycerol is reported to mimic *in vivo* conditions regarding virulence gene induction (Callahan, Ryder et al. 1971), this *in vitro* condition may not correctly mimic this environment for *vieB* expression. Thus, after many attempts to observe a phenotype for *vieB*, we began to question whether M9 NRES glycerol was actually inducing expression of *vieB*. Therefore, we decided to test if *vieB* transcript is in fact up regulated in wild type when grown in M9 NRES glycerol when compared to the non-inducing condition, LB. As seen

in Figure 3-4C, *vieB* is not highly induced by M9 NRES glycerol at any point throughout growth. While there is a small increase at OD_{600}=0.8, this induction is just under 3-fold, which may not be enough expression to have any observable effect. Based on the data presented here, M9 NRES glycerol medium does not highly express *vieB*, which could explain the inability to detect a *vieB* phenotype.

Since *vieSAB* appears to be more important for the classical biotype, and because *vieB* is not induced in the El Tor biotype during growth in AKI medium (Lee, Angelichio et al. 1998), we hypothesized that the lack of a detectable phenotype in the classical biotype would also hold true for El Tor. Therefore, the *in vitro* experiments conducted in classical were not tested in El Tor.

Figure 3-4. *vieSAB* transcript levels are not elevated in Δ*vieB* or in M9 NRES
Overnight wild type AC50 or Δ*vieB* cultures were back diluted in M9 NRES glycerol or LB and grown at 30°C or 37°C, respectively. Samples were taken at the indicated OD_{600} for RNA isolation. Transcript levels were determined by qRT-PCR and are normalized to a house-keeping gene (*sanA*). For *vieS* **(A)** and *vieA* **(B)**, RNA samples were collected at OD_{600}=0.5, 0.8, 1.5 and are relative to AC50 at each respective OD_{600}. For *vieB* **(C)**, RNA samples were collected at OD_{600}=0.6, 0.8, 1 and are relative to LB at each respective OD_{600}.

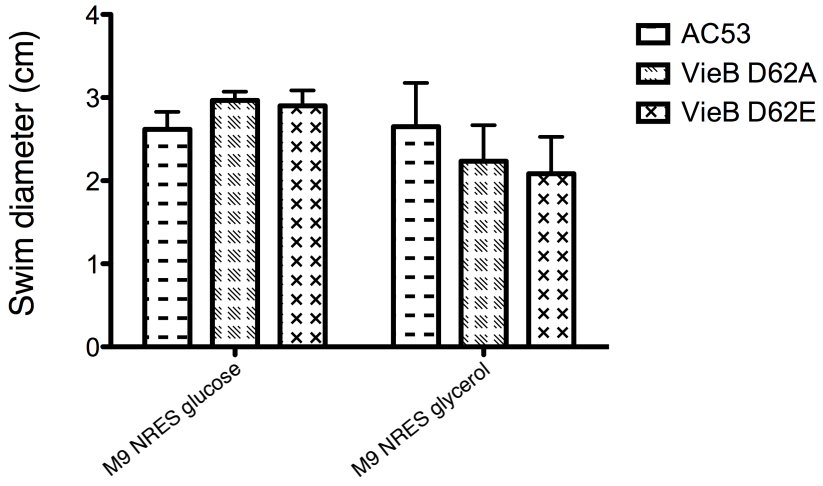


3.2.4 In vitro analysis of vieB D62 point mutants in the El Tor biotype

In Chapter 2, we showed indirect evidence that the inhibitory activity of VieB can be regulated by phosphorylation of the conserved Asp62 (D62) residue within the Rec domain. More specifically, we showed that, the D62A mutant inhibits the VieS/VieA phosphotransfer similar to wild type VieB, but the D62E mutant, which mimics the phosphorylated state, is a weaker inhibitor (Figure 2-7). Our working model proposes that the D62A will constantly block phosphotransfer, inhibiting the transcription factor activity of VieA and increasing cdGMP concentrations as a result of VieA no longer being able to up regulate its transcription. Conversely, the D62E mutant should behave similar to a *vieB* null, where D62E is unable to inhibit phosphotransfer, resulting in high VieA levels and low cdGMP. In further attempt to reveal a role of VieB, we decided to test these "regulatory-locked" point mutants for cdGMP-regulated phenotypes. These mutants were only tested in the El Tor biotype due to technical problems in trying to make them in the classical biotype.

We first assessed the *vieB* D62A and D62E point mutants in motility assays in M9 media. While there is some difference between wild type and the point mutants, the *vieB* D62A and D62E mutants behave similarly under both media conditions (Figure 3-5). Take together, these data suggest that neither point mutation has an effect on motility. However, as discussed above, this may be due to the lack of induction of *vieB* in this defined media.

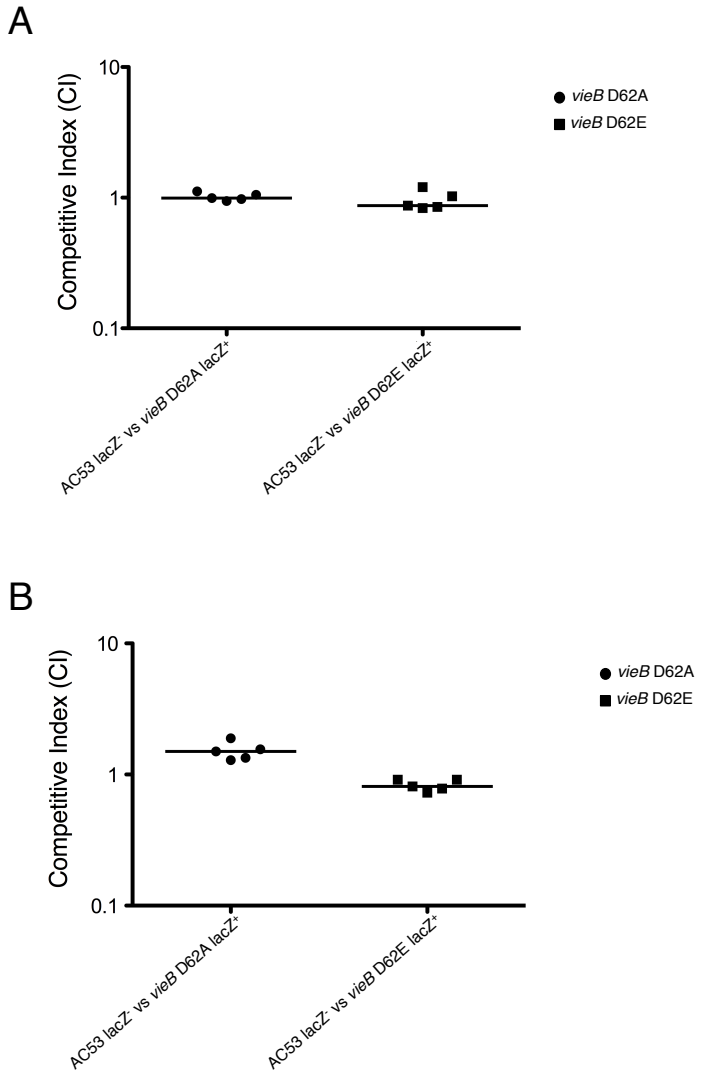
Figure 3-5. Neither *vieB* D62A nor D62E have a motility defect
Wild type AC53, *vieB* D62A and *vieB* D62E were grown to stationary phase and the liquid culture was stabbed into M9 NRES 0.5% glucose or 0.5% glycerol minimal media plates containing 0.3% agar. Plates were incubated 30°C overnight and the swimming diameters were measured.



Based on previous data, it was somewhat expected that the *vieB* point mutants would not reveal a phenotype in single strain motility assays. Therefore, we next decided to test these mutants in competition assays in hopes of amplifying any fitness difference of the *vieB* point mutants. To ensure that neither *vieB* D62A nor D62E has an *in vitro* growth defect, we tested their ability to grow in the presence of wild type in LB. As seen in Figure 3-6A, there is no *in vitro* growth defect of either mutant compared to wild type.

We next tested the ability of each mutant to survive in pond water in competition with wild type. This experiment was based on our hypothesis that VieB provides a negative feedback mechanism for the VieSA TCS and allows for expression of genes required for survival in the environment. As *V. cholerae* enters the nutrient-poor pond water environment, we hypothesized that rapid accumulation of cdGMP will be necessary for its survival. We predicted that the *vieB* D62A mutant might have an advantage over wild type given that this mutant will always be an active inhibitor of the VieSA TCS and should have a high cdGMP level. Alternatively, the *vieB* D62E mutant may have a disadvantage compared to wild type since this mutant will be unable to inhibit the VieSA TCS and will have a low level of cdGMP. While there is a slight increase in CI for the D62A mutant, this value is not statically different from a theoretical CI value of 1 (Figure 3-6B). These data show that both mutants compete equally with wild type after 24 hours of incubation in pond water, providing evidence that *vieB* has no effect on survival under these conditions.

Figure 3-6. *vieB* D62 point mutants compete equally with wild type for LB growth and pond survival.
A *lacZ*⁻ derivative of wild type AC53 and *vieB* D62A *lacZ*⁺ or *vieB* D62E *lacZ*⁺ overnight cultures were mixed 1:1 and back-diluted in LB to a final OD_{600} of 0.01. **(A)** 1 µl of the 1:1 mixture was added to 2 ml LB and cultures were grown at 37°C overnight. **(B)** 100 µl of the 1:1 mixture was added to 5 ml of autoclaved pond water and incubated statically at RT for 24 hours. Inputs and serial dilutions of the output were plated for CFU and a competitive index was calculated.



While the data thus far have not revealed a phenotype for either *vieB* point mutant, it is probable that *vieB* is not being expressed under these conditions. This explanation is most likely valid for the experiments conducted in M9 NRES glucose or glycerol and LB. While we hypothesize that VieB plays a role in increasing cdGMP levels, the pond water competition assay does not support this hypothesis. However, the inoculation that was added to the pond water was originally grown in LB, conditions where we know *vieSA*, and especially *vieB*, are not highly expressed. Since pond water is drastically different from the small intestine, it is thought that this environment does not induce expression of *vieB*. Based on the *in vivo* temporal and spatial expression of *vieB* (Lee, Angelichio et al. 1998), we hypothesize that *vieB* must be expressed just prior to *V. cholerae* exiting the host in order to aid in the transition to environment. However, because that study utilized a recombinase reporter system, which provides only a qualitative measure of gene expression, the magnitude of *vieB* expression late in infection remains unknown. Since LB does not induce *vieB*, one possibility for no observable phenotype could be due to a lack of *vieB* expression prior to entering the pond environment.

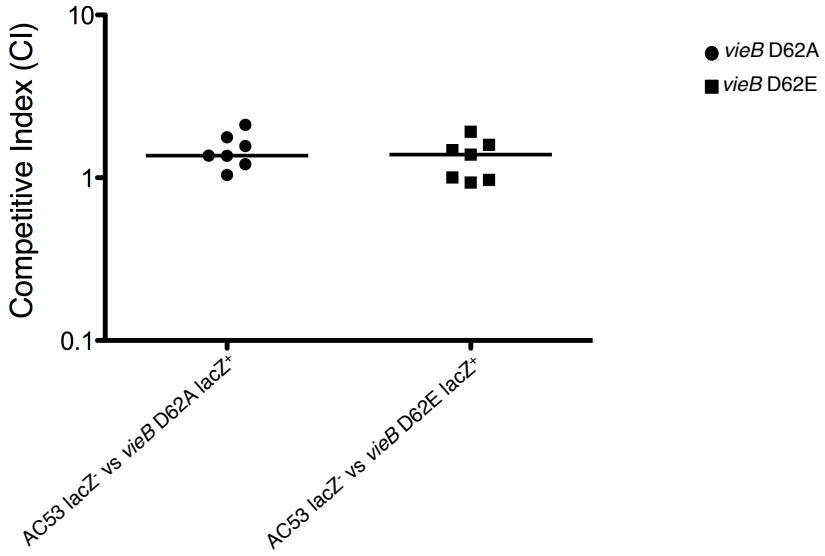
3.2.5 In vivo and transition assay analysis of vieB D62 point mutants in the El Tor biotype

To address the lack of *vieB* expression prior to entry into pond water, we needed a condition that would robustly induce *vieB* expression prior to the transition. Since there are currently no known *in vitro* conditions that can achieve this, we induced *vieB* by passing the point mutants through an infant mouse. To first ensure that there is no defect with either point mutant in the infant mouse model of infection, we tested the D62

mutants in competition with wild type. We hypothesized that both mutants will compete equally with wild type as we suspect that *vieB* is necessary during the transition period between the two environments. As expected, there is no statistical difference for either mutant in competition with wild type in the infant mouse model of infection (Figure 3-7). These data support our hypothesis that *vieB* is not needed for colonization and survival in the host.

Figure 3-7. *vieB* D62 point mutants compete equally with wild type in the infant mouse model of infection

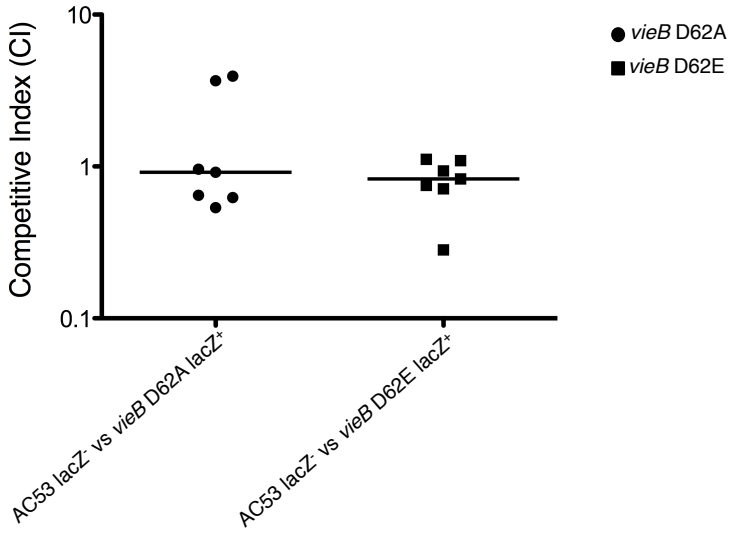
A *lacZ*⁻ derivative of the wild type strain AC53 and *vieB* D62A *lacZ*⁺ or *vieB* D62E *lacZ*⁺ overnight cultures were mixed 1:1 and back diluted in LB to a final OD_{600} of 0.01. 50 µl of the 1:1 mixture was inoculated into anesthetized infant mice by oral gavage. Inputs were plated for CFU. Mice small intestines were harvested at 24 hrs post inoculation, diluted and plated for CFU and a competitive index was calculated.



To test if *vieB* is required for the transition between the host and the environment, the outputs from each mouse were directly inoculated into pond water for 24 hours. We hypothesize that, as *V. cholerae* transitions into pond water, the expression of either constitutively active or inactive VieB should have an effect on the ability of each mutant to correctly modulate cdGMP level and ultimately affect its ability to survive. For the D62A mutant, we expect the cell to have high cdGMP, which would be beneficial for this mutant during the transition period. Alternatively, we predict that the D62E mutant to have low cdGMP and therefore be at a disadvantage during the transition. However, the *in vivo* to pond transition assay did not reveal any effect, where both mutants survived equally as well as wild type (Figure 3-8). These data suggest that *vieB* is not playing a significant role during the transition between the host and environment in our experimental design.

Figure 3-8. *vieB* D62 point mutants survive similar to wild type in an *in vivo* to pond transition assay

A *lacZ*⁻ derivative of the wild type AC53 and *vieB* D62A *lacZ*⁺ or *vieB* D62E *lacZ*⁺ overnight cultures were mixed 1:1 and back-diluted in LB to a final OD_{600} of 0.01. 50 µl of the 1:1 mixture was inoculated into anesthetized infant mice by oral gavage. Mice small intestines were harvested at 24 hrs post infection and plated for CFU for the input. Homogenates underwent a low-speed spin to pellet host cells and debris and 100 µl of supernatant was incubated in 5 ml of autoclaved pond water statically at RT for 24 hrs. 2 ml of cultures were concentrated 40-fold and outputs were serially diluted and plated for CFU. A competitive index was calculated.



3.3 DISCUSSION

In this chapter, we attempt to genetically characterize *vieB*. We hypothesized based on VieB's inhibitory action on the VieSA TCS that it should play a role in increasing cdGMP, therefore altering behaviors regulated by this secondary messenger molecule. Although we tested Δ*vieB*, over-expression of *vieB* and regulatory-locked point mutants in a range of conditions in both the classical and El Tor biotypes, no phenotype was observed. These data suggest that *vieB* is not important for the global regulation of cdGMP and gene expression changes between the host and environment. However, based on the biochemical analysis of VieB, we know that VieB can regulate the VieSA TCS, which is clearly involved in the modulation of cdGMP. Therefore, we suspect that *vieB* does have some significant physiological role but we were unable to find a condition where this could be observed.

There were a few notable issues that may have lead to our inability to detect the role of *vieB*. First, we were unable to find any *in vitro* conditions where *vieB* was highly expressed. A previous study aimed to find an *in vitro* condition where *vieB* is expressed but no condition tested was able to induce this gene (Lee, Angelichio et al. 1998). Taken together with our attempts to induce *vieB* in M9 NRES glycerol provides evidence that *vieB* may be regulated by additional factors that do not appear to regulate *vieSA*. Since VieB is an inhibitor, it would make sense that *V. cholerae* would impose tight regulation over its expression. This would ensure that *vieB* does not become expressed when it is not needed, since this could be detrimental to the cell when induced nonspecifically. It appears that the conditions when *V. cholerae* express *vieB* are strictly precise.

In order to address the lack of expression *in vitro* and in attempts to enhance any effect of *vieB* after induction, we assessed regulatory-locked *vieB* point mutants after passage through the infant mouse. As described above, there was no defect of either point mutant after 24 hours of incubation in pond water. However, it is possible that *vieB* may only be required within a narrow time frame during this transition period. Additionally, the term "transition period" is broad and encompasses a number of steps for exiting the host, such as detachment from epithelial cells, migration out of the small intestine and eventual exit into the environment. None of these three steps are specifically assayed in our infant mouse to pond transition model, since we physically remove and homogenize the small intestine to release the bacteria. Therefore, it is possible that *vieB* could be important at any one of these phases as *V. cholerae* exits the human host. Additionally, since we only sampled the pond water at 24 hours post inoculation it is possible that we may have missed the window where *vieB* plays a role. This could explain the lack of phenotype, as the *vieB* point mutants may initially have a defect during the first few hours of exiting the host but are able to recover to wild type levels over time. Indeed, while VieA is a main player in modulating cdGMP levels, there are many other active DGC- and PDE-containing proteins that could assist in cdGMP modulation in the absence of VieA (Beyhan, Odell et al. 2008). To address this concern, earlier time points during the pond incubation would be needed. Additionally, this transition assay would be worthwhile to test with the *vieB* point mutants and the Δ*vieB* in the classical biotype, as *vieSAB* is more important in this background.

Even though further experiments should be conducted to address the issues presented above, it is possible that *vieB* only plays a minor role in shutting down

virulence genes at the end of infection. Such a role may manifest as a minor increase in overall fitness for those bacteria that promptly turn off virulence genes. If true, observing a clear and significant role of *vieB* may be difficult.

CHAPTER 4: VIEA AUTOREGULATION OF VIESAB

4.1 Introduction

VieA plays an important role in the inverse regulation of virulence and environmental survival genes. A recent study showed that *vieA* transcription is up regulated immediately after binding to epithelial cells, presumably when virulence genes are needed in order to colonize and cause invasive disease (Dey, Bhagat et al. 2013). VieA has also been shown to be important for CT and ToxT expression (Tischler, Lee et al. 2002; Tischler and Camilli 2005). VieA's ability to regulate these genes is due to its ability to cleave cyclic-di-GMP (cdGMP). VieA cleaves cdGMP using an EAL style of PDE domain, which is capable of hydrolyzing cdGMP into its linear form. Therefore, VieA is thought to be responsible for modulating gene expression changes by lowering cdGMP concentrations to induce virulence genes and repress environmental genes. Indeed, expression of CT and *toxT* is dependent on the activity of the EAL domain of VieA (Tischler and Camilli 2005).

While the EAL domain of VieA has been well characterized, there has been little investigation into understanding the role of VieA's HTH DNA-binding motif. Since VieA harbors both a Rec and HTH domain, it suggests that VieA is behaving as a traditional RR. This further implies that VieA is capable of directly modulating gene expression changes in addition to its indirect regulation by cdGMP cleavage. A previous study addressed what genes composed the VieA regulon by performing a microarray under M9 NRES glycerol growth conditions (Beyhan, Tischler et al. 2006). By comparing wild type to a Δ*vieA*, they found a number of genes that were differentially regulated. Of note, a complete deletion of *vieA* removes both the HTH and PDE activity; therefore this study is unable to distinguish if VieA directly or indirectly regulates these

genes. Nevertheless, of the differentially regulated genes, there was a significant decrease in the expression of the entire *vieSAB* operon when *vieA* was absent. This suggests that VieA may be autoregulatory, however it is unclear whether this autoregulation is direct or indirect.

In this chapter, we attempt to address if VieA is autoregulatory and if so, is this regulation through direct DNA-binding or through the action of cdGMP cleavage by the PDE domain.

4.2 RESULTS

4.2.1 VieA autoregulation in the classical biotype

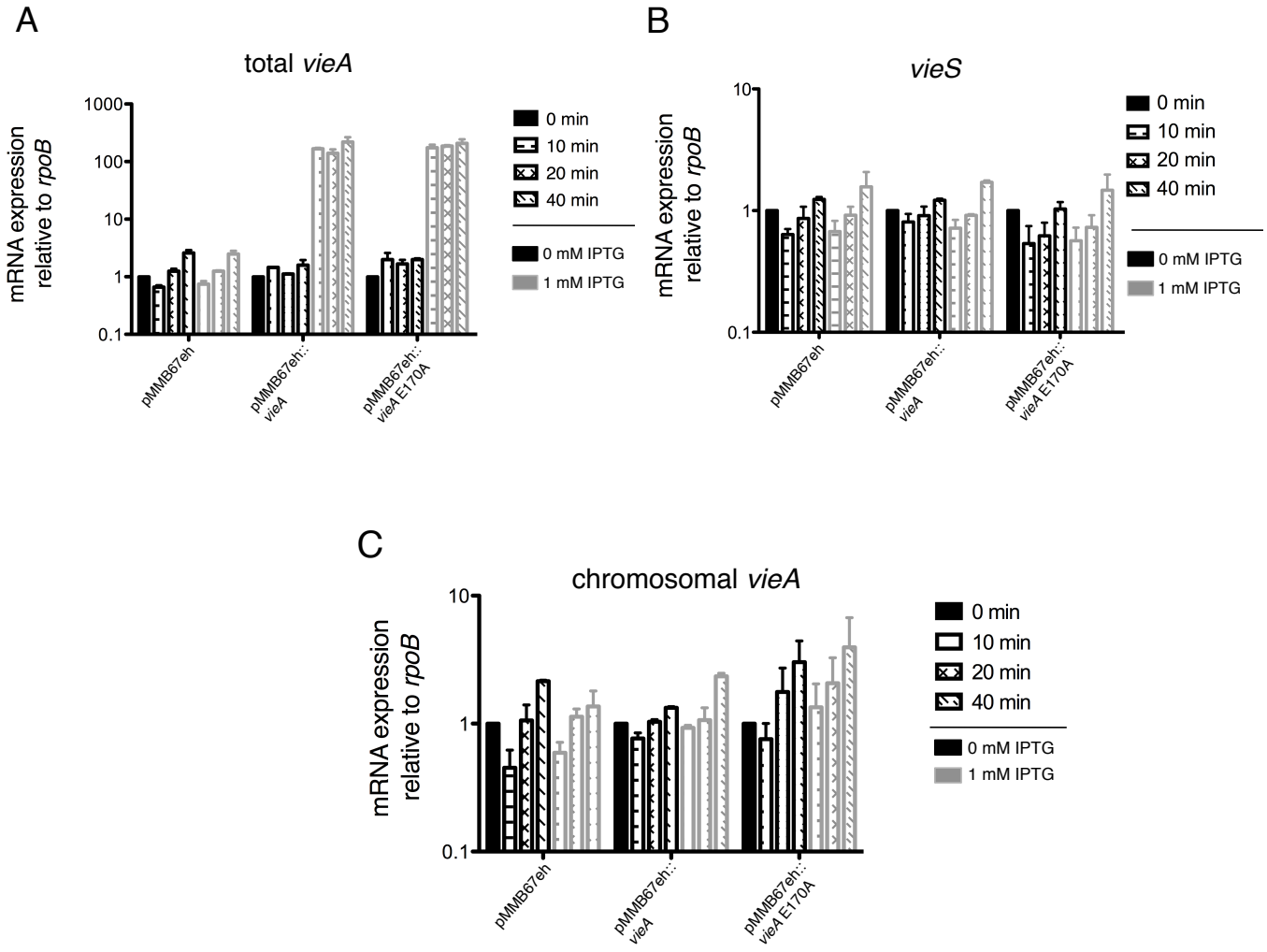
To address if VieA is autoregulatory, we choose to observe transcriptional differences of the *vieSAB* operon in the classical biotype. Due to concerns that *vieA* would not be highly expressed under M9 NRES glycerol growth conditions, we decided to artificially induce expression of *vieA* from an IPTG-inducible plasmid (pMMB67EH) in LB broth. Since we over-expressed *vieA* in the wild type background, to differentiate between the chromosomal and plasmid derived *vieA* transcripts, qRT-PCR primer sets were designed to distinguish total *vieA* (plasmid and chromosomal) and chromosomal *vieA*. Given that we hypothesize that *vieA* autoregulation is direct, we would expect that inactivation of the VieA PDE domain to have no effect. To test this hypothesis, a point mutation was made in the plasmid *vieA* gene. The mutation resulted in the Glu170 residue being replaced with an Ala, rending the PDE domain inactive (VieA E170A). Based on the hypothesis that *vieA* is autoregulatory, we predict that over-expression of *vieA* from the plasmid will lead to an increase in chromosomal *vieA* expression. If only *vieA* is increased, but not *vieS*, that would suggest that there is an internal promoter

within *vieS* that is specific for VieA autoregulation. However, if both *vieS* and *vieA* are increased, this result would suggest that VieA binds the main promoter upstream of the entire operon.

After induction with IPTG, the amount of total *vieA* transcript is increased significantly over time for both strains harboring plasmids containing *vieA* and *vieA* E170A but not the empty vector control (Figure 4-1A, grey bars). However, even though the total amount of *vieA* transcript is increased, there is no significant difference in chromosomal *vieA* transcript levels between the 0 minute time point and post-induction (Figure 4-1C, compared solid black to grey bars). This suggests that the large increase seen for total *vieA* is mostly comprised of the plasmid copy, not the chromosomal. This lack of induction also holds true for *vieS* transcript (Figure 4-1B, solid black compared to grey bars). While there appears to be an increase in *vieS* and chromosomal *vieA* at 40 minutes post-induction, this increase is only about 1.5-fold and 2.5-fold, respectively. If VieA were autoregulatory, we would expect this increase to be larger. Furthermore, these transcripts are increased to a similar level in the un-induced samples (Figure 4-1B-C, black bars). While it is possible that the promoter allows some leaky expression in the absence of IPTG, we would expect that full induction of plasmid *vieA* would increase these transcript levels higher than that of leaky expression. This suggests that the increase in the induced samples is not specific to VieA regulation. Taken together, these data suggest that VieA is not able to autoregulate through direct DNA-binding under the conditions tested.

Figure 4-1. Over-expression of plasmid *vieA* does not increase *vieS* or chromosomal *vieA* transcript levels

AC50 was transformed with either pMMB6EH empty vector, *vieA* or a *vieA* PDE mutant (E170A). Strains were scraped from a plate and back diluted in LB^{Amp50} to an OD=0.01. Strains were grown at 37°C until mid-exponential phase of growth, where the culture was split and 1mM IPTG was either added or not. Time points for RNA collection were taken at 0, 10, 20 and 40 minutes post-induction. qRT-PCR was used to determine changes in transcript levels of total *vieA* **(A)**, *vieS* **(B)** and chromosomal *vieA* **(C)**.



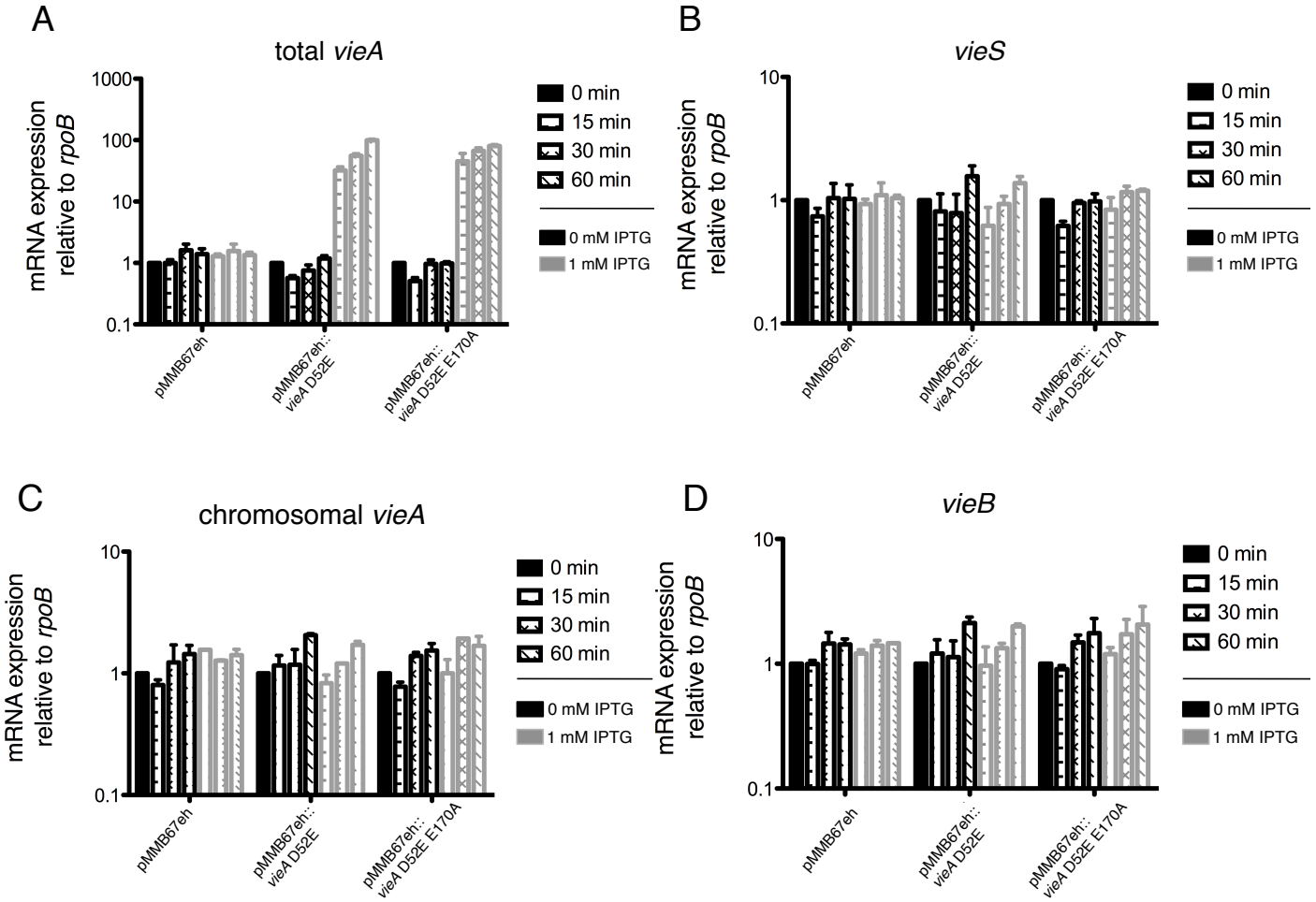
While these data suggest that VieA is not autoregulatory, it is possible that this lack of induction is due to VieA not be phosphorylated by VieS. We hypothesize that VieA is behaving as a traditional RR, where DNA-binding activity is stimulated by phosphorylation of the Rec domain. Indeed, since these experiments were conducted in LB, which is a non-activating condition for VieSAB, it is expected that VieS is not activated; VieA is not phosphorylated and therefore unable to bind DNA. Since we do not know the activation signal for VieS, to address this issue we made a point mutation in VieA that is commonly used for other RRs to mimic a constitutively active state. This VieA construct has the conserved phosphorylation residue, D52, in the Rec domain replaced with a Glu (D52E). Of note, for some RRs, this mutation does not result in constitutive activation so it is possible that VieA will not be activated by the D52E mutation. We repeated the above over-expression experiment using this active *vieA* D52E point mutant and the double mutant, *vieA* D52E E170A, which we hypothesize to be active for DNA-binding but is unable to cleave cdGMP. Again, induction by IPTG results in a significant increase in total *vieA* levels (Figure 4-2A, grey bars). However, despite having high expression of a constitutively active VieA, there is no significant increase of either *vieS*, *vieB* or chromosomal *vieA* transcript levels (Figure 4-2B-D, compare solid black and grey bars). Again, while a small increase is observed at the later time points post-induction, this increase is also observed for the non-induced samples, suggesting that this is nonspecific (Figure 4-2B-D, compare black and grey bars). The lack of observable increase in chromosomal *vieSAB* transcripts by VieA D52E provide further evidence that VieA is not directly regulating itself or this operon. However, it is possible

the D52E mutation does not result in VieA activation, which could also explain the observed results.

In both experiments, over-expression of the E170A mutation has no effect, regardless if the wild type or D52E *vieA* is being expressed. These results suggest that VieA PDE activity is not responsible for regulating *vieSAB,* which supports our hypothesis that VieA is not indirectly regulating *vieSAB*. Collectively, these data support the negative hypothesis that VieA does not regulate *vieSAB*, either directly or indirectly.

Figure 4-2. Over-expression of *vieA* D52E does not up-regulate chromosomal *vieSAB* expression

AC50 was transformed with either pMMB67EH empty vector, *vieA* D52E or a *vieA* D52E E170A (PDE mutant). Growth and IPTG induction was done as described in Figure 4-1. Time points for RNA collection were taken at 0, 15, 30 and 60 minutes post-induction. qRT-PCR was used to determine changes in transcript levels of total *vieA* **(A)**, *vieS* **(B)**, chromosomal *vieA* **(C)** and *vieB* **(D)**.



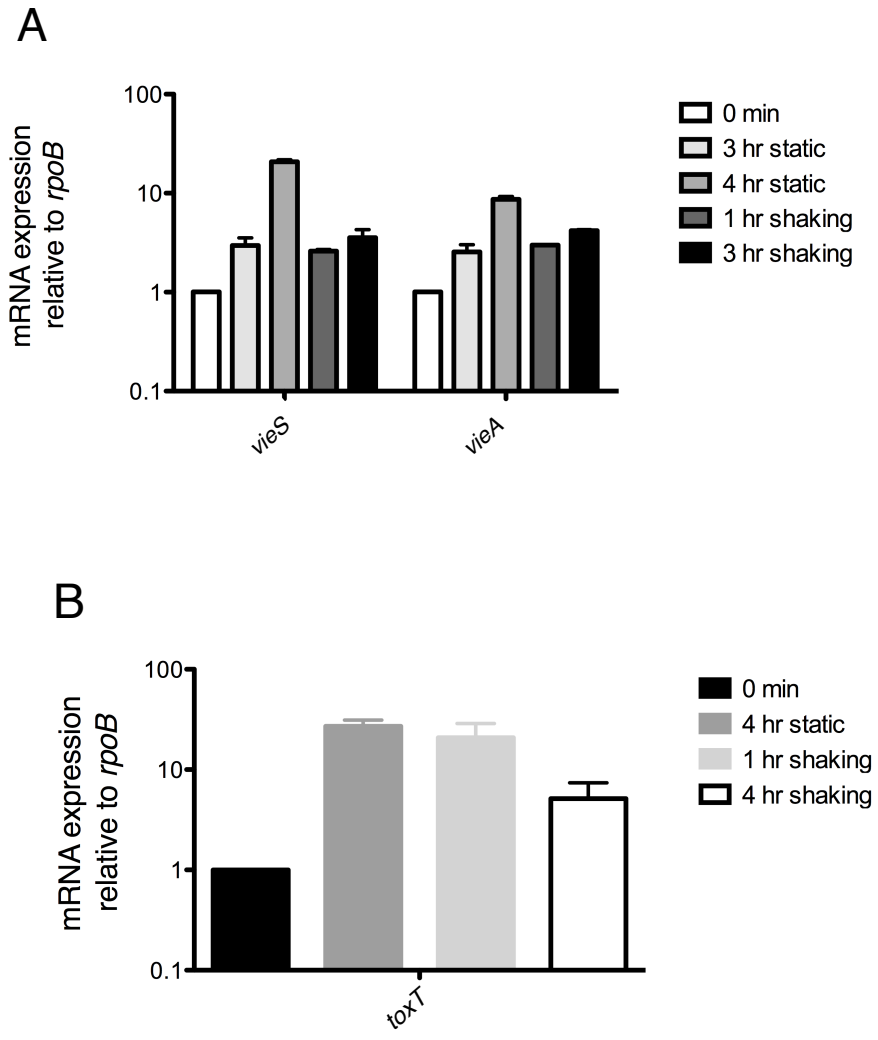
4.2.2 VieA and ToxT regulation of vieSAB in the El Tor biotype

The experiments in the classical biotype strain, described above, were conducted in LB broth. One possible reason that VieA regulation was not observed might be due to the lack of induction of the entire virulence cascade. Indeed, *vieA* appears to be highly expressed at the beginning of infection, around the same time a number of other virulence factors are also expressed. Since the *in vitro* condition that stimulates virulence gene expression in the El Tor biotype is growth in AKI medium, we decided to test for VieA regulation in the El Tor biotype in this condition. We hypothesize that if VieA autoregulation occurs only under virulence inducing conditions, we may be able to observe this regulation in AKI media.

Virulence gene induction in AKI requires growth for 4 hours in a filled culture tube (low surface area to volume ratio) static (without shaking), during which the culture becomes microaerophilic, followed by 3 hours of aerobic growth in a partially filled culture tube (high surface area to volume ratio) with vigorous shaking. To ensure growing *V. cholerae* in AKI induces that *vieSA*, we assessed mRNA levels of these genes in AKI over time. Indeed, *vieSA* are up regulated in this growth media (Figure 4-3A). This induction is at its highest when *V. cholerae* has undergone 4 hours of static growth and drops when cultures are transitioned to aerated growth. Furthermore, the master virulence gene transcriptional regulator, *toxT*, is also induced in AKI media. *toxT* induction begins at 4 hours of static growth and continues up to 1 hour of aerated growth (Figure 4-3B). While the induction kinetics for *vieSA* and *toxT* vary slightly, this would be expected, as these genes are most likely required at different times during virulence gene activation. Regardless, it is clear that AKI medium is able to induce the virulence

gene cascade and *vieSA*. Of note, *vieB* is not expressed in AKI, as previously reported (Lee, Angelichio et al. 1998). Therefore, *vieB* expression was not tested for any of the assays presented below.

Figure 4-3. *vieSA* and *toxT* are up-regulated in AKI medium
Wild type AC53 was grown statically in AKI media at 37°C for 4 hours and then grown for an additional 3 hours, aerobically (shaking). Time points for RNA collection were taken at 3 and 4 hours after static growth and 1 and 3 hours after aerobic growth. qRT-PCR was used to determine changes in transcript levels of *vieS* and *vieA* **(A)** and *toxT* **(B)**.



Since *V. cholerae* undergoes robust virulence gene induction in AKI medium, we investigated if VieA is autoregulatory under these conditions. We compared mRNA levels of *vieS* for wild type and a Δ*vieA* mutant. While this experiment is unable to distinguish if VieA regulation is direct or indirect, we decided to test VieA regulation using this mutant because the experiment could give more clear-cut results. Since *vieSA* and *toxT* is most highly expressed at 4 hours post static growth, we looked for differential regulation of *vieS* at this time point and also after 30 minutes of shaking. When grown in AKI, there is no significant change in *vieS* transcript levels in Δ*vieA* when compared to wild type at either the 4 hours or 30 minutes time point (Figure 4-4). These data suggest that VieA is not regulating *vieS* under AKI conditions. Of note, since *vieA* is deleted from the operon, this could disrupt any alternative promoter that exists at the end of *vieS* and beginning of *vieA*. Therefore, this experiment can only address the ability of VieA to bind the main promoter that lies upstream of the *vieSAB* operon.

Even though VieA appears to have no affect on *vieSAB* transcription in either biotype, we know that *vieSA* is up regulated in AKI. Therefore, we hypothesize that a regulator of this operon must be active under these conditions. Given that *toxT* is a master transcriptional regulator of virulence genes and is up regulated in AKI around a similar time as *vieSA*, we hypothesize that *toxT* might be regulating *vieSA* expression. Interestingly, *vieSA* is involved in the expression of *toxT* (Tischler and Camilli 2005). Therefore, it is possible that there is a regulatory loop that exists between VieA and ToxT. However, a deletion of *toxT* has no effect on *vieSA* expression when compared to

wild type in AKI (Figure 4-5), providing no evidence for *toxT* being the regulator of *vieSAB*.

Figure 4-4. VieA does not regulate *vieS* in El Tor under AKI growth conditions
Wild type AC53 and a Δ*vieA* mutant were grown statically in AKI media at 37°C. Time points for RNA collection were taken at 4 hours after static growth and 30 minutes after shaking growth. qRT-PCR was used to determine changes in transcript levels of *vieS*.

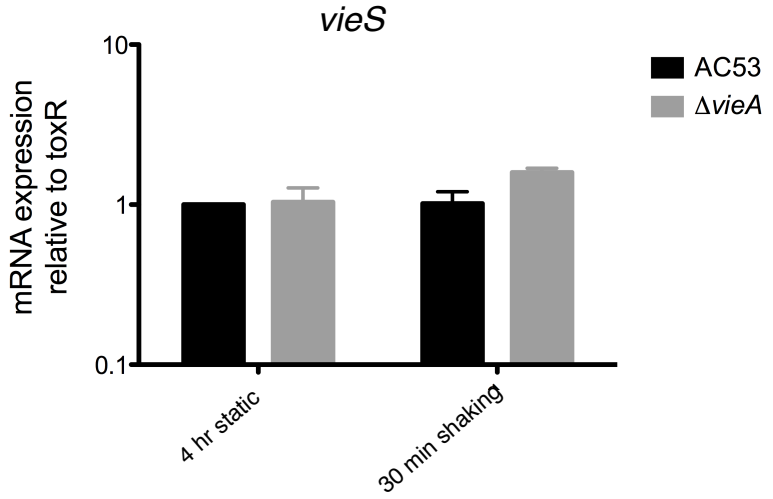
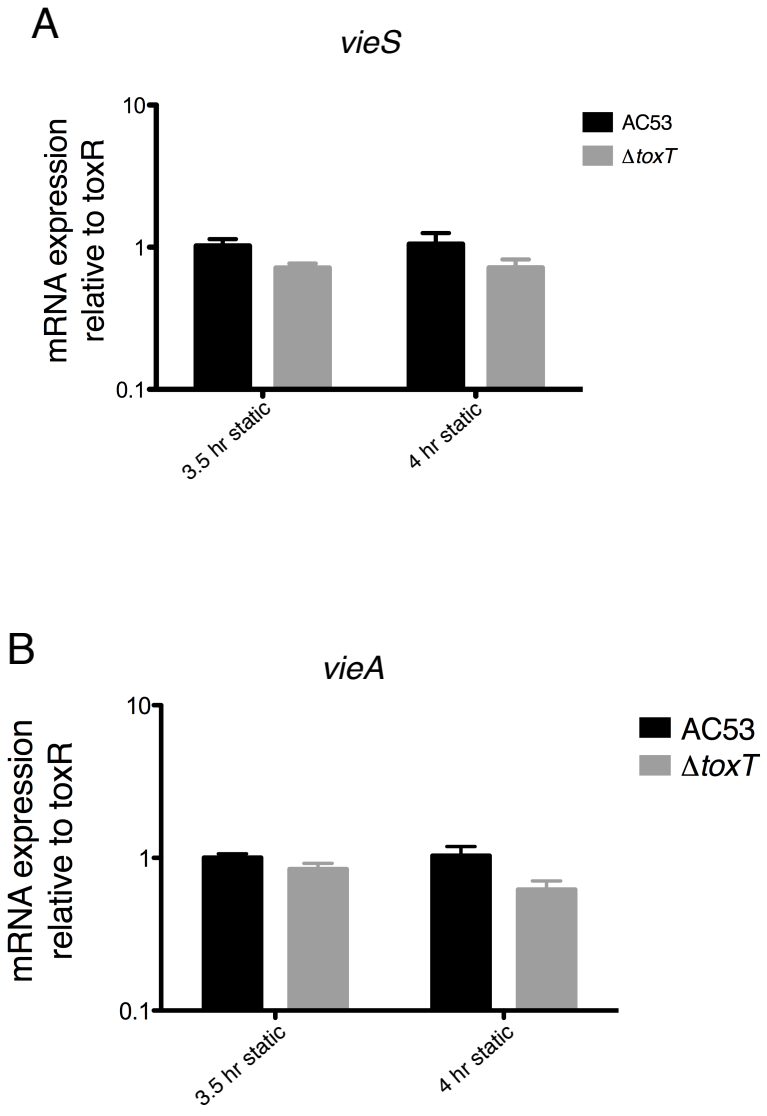


Figure 4-5. ToxT does not regulate *vieSA* in AKI medium
Wild type AC53 and a Δ*toxT* mutant were grown statically in AKI media at 37°C. Time points for RNA collection were taken at 3.5 hours and 4 hours after static growth. qRT-PCR was used to determine changes in transcript levels of *vieS* **(A)** and *vieA* **(B)**.



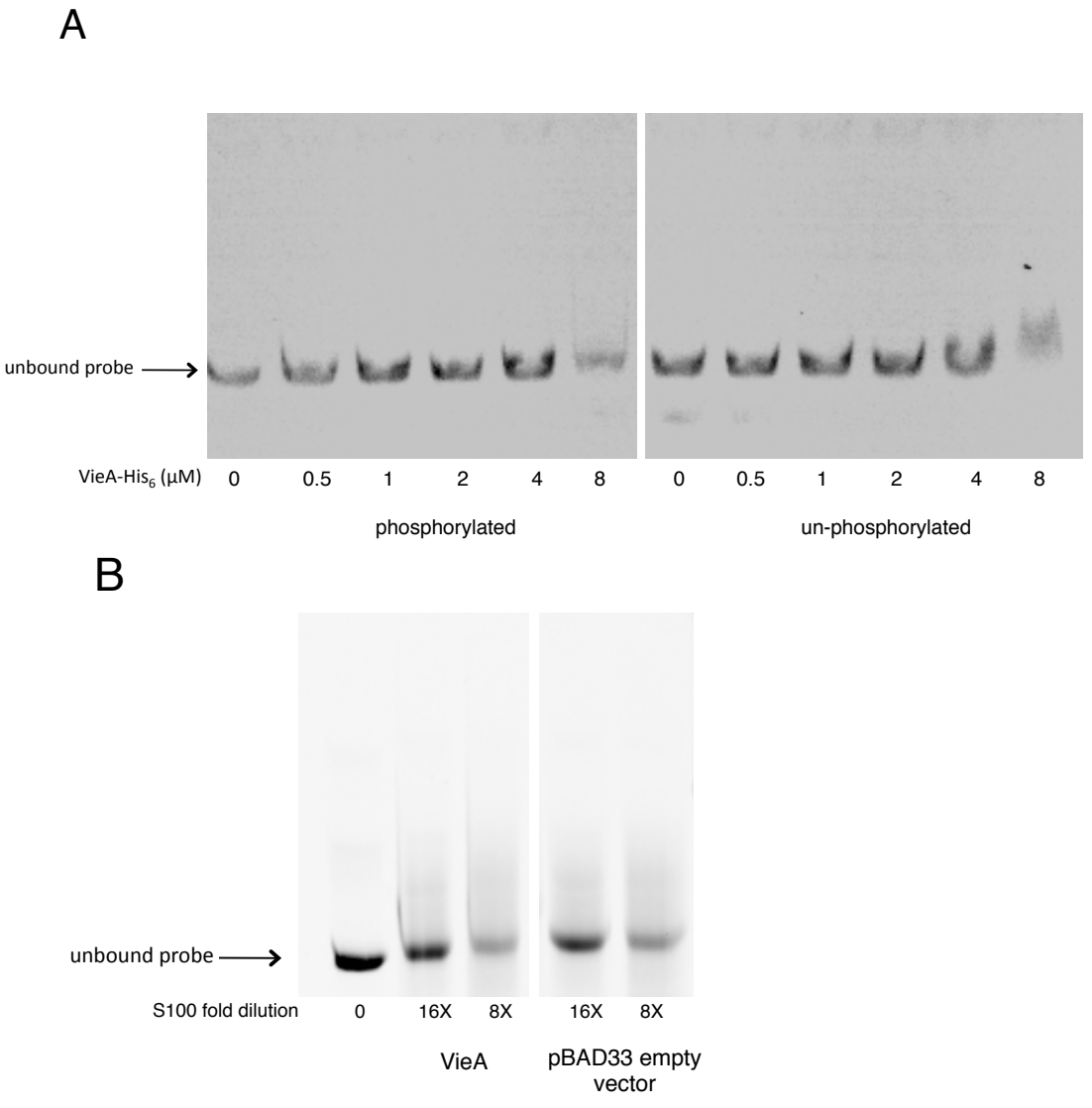
4.2.3 EMSA analysis of VieA to the promoter region of vieSAB

Since we were unable to observe regulation of the *vieSAB* operon by VieA *in vivo*, we decided to address VieA regulation of this operon *in vitro* using purified components. We hypothesize that VieA is able to regulate by directly binding the promoter region through its HTH motif. However, direct binding of VieA to DNA has not been shown. Therefore, in order to test if VieA can bind this promoter region, we conducted EMSA analysis using a Cy5 labeled probe consisting of the *vieSAB* promoter region. We first tested a purified VieA-His_6 construct in its ability to bind. However, VieA-His_6 is unable to bind the *vieSAB* promoter over a range of protein concentrations (Figure 4-6A, right panel). This is somewhat expected as this construct is not phosphorylated and we hypothesize that VieA must be phosphorylated in order activate the HTH motif. Therefore, we also addressed the ability of a phosphorylated VieA-His_6 to bind the *vieSAB* promoter. However, phosphorylated VieA-His_6 does not appear to bind the *vieSAB* probe either, even when 8 μM of this protein was used (Figure 4-6A, left panel). These data suggest that VieA-His_6 is unable to bind the promoter region of *vieSAB*.

Given that the purified VieA-His_6 construct is active in phosphotransfer assays, it is thought that it should also be active for DNA-binding. However, it is possible that this construct does not have a functional HTH motif. This could be due to the His_6 tag being present at the C'-terminus, which is also where the HTH domain is located. To address this issue, we over-expressed an un-tagged VieA construct in *E. coli*. Whole cell S100 fractioned protein supernatants were collected from both the empty vector and *vieA* over-expression strains. These protein fractions were subsequently tested in EMSA analysis.

We hypothesize that the lack of the His_6 tag should allow the HTH domain to be functional. However, the supernatant containing VieA is unable to bind the *vieSAB* probe (Figure 4-6B). While there is a slight reduction in the amount of unbound probe observed in the 8X dilution, no shift was observed. This decrease is most likely a result of probe degradation due to carry-over DNase that is present in the protein fraction. Taken together, this suggests that VieA is not able to regulate *vieSAB* through direct DNA binding.

Figure 4-6. VieA-His$_6$ and VieA protein extract is unable to bind *vieSAB* promoter
(A) VieA-His$_6$ was incubated with or without acetyl-phosphate (Ac-P) for 2 hrs at 30°C. After phosphorylation, the reaction was passed through an EdgeBio column to remove Ac-P. **(B)** DH5α containing pBAD33::*vieA* or pBAD33 empty vector were induced with 0.2% arabinose and grown at 37°C until mid-exponential phase. Cultures were harvested, lysed by bead beating and DNase treated. S100 fractions were collected by centrifugation at 100,000 x *g* for 1 hour. VieA constructs were incubated with the *vieSAB* promoter fragment probe for 30 minutes at 30°C and run on a 6% TBE native gel. The probe was observed using the Cy5 fluorescent channel.



4.2.4 Regulation of VieA PDE activity by phosphorylation

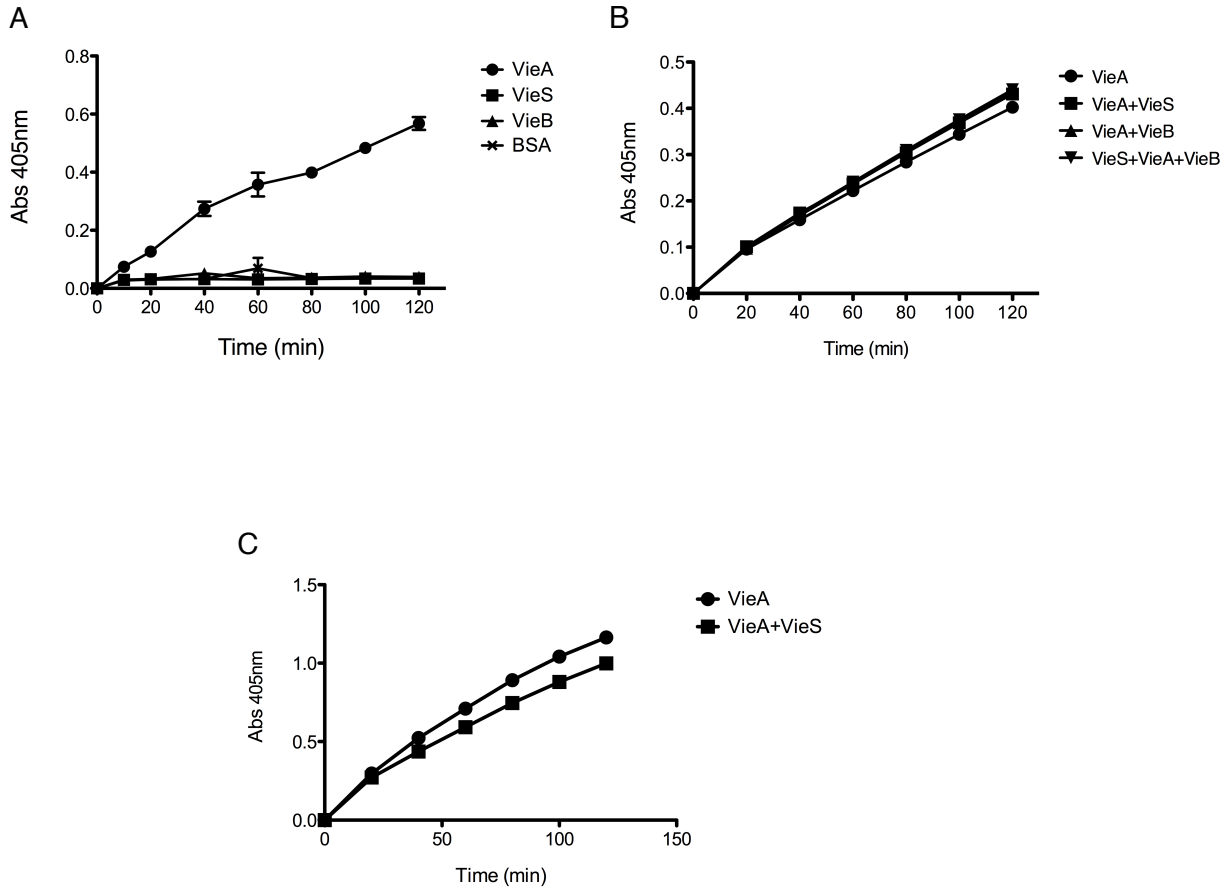
Given the strong homology of VieA's Rec and HTH domains to other RR proteins, we originally hypothesized that phosphorylation of its Rec domain results in its activation as a transcription factor. However, based on the studies presented in this chapter, it appears that VieA is not able to regulate the *vieSAB* operon or bind the *vieSAB* promoter region. While these experiments do not rule out VieA's ability to directly regulate other genes, an alternative hypothesis is that phosphorylation of VieA is does not activate its HTH domain. Instead, phosphorylation of VieA may regulate the PDE domain, thereby controlling when VieA can cleave cdGMP and induce virulence gene expression. Previous studies have provided some evidence to suggest that the PDE domain is active regardless of phosphorylation state (Tamayo, Tischler et al. 2005), but this has not been directly tested.

To test if phosphorylation of VieA regulates its PDE activity, we utilized bis (p-nitrophenyl) phosphate (bis-pNpp), a colorimetric substrate, which upon cleavage by EAL domain PDEs produces a yellow color that can be measured at an absorbance of 405 nm. To first test if the purified VieA-His_6 construct is active, we assayed bis-pNpp cleavage in standard assay buffer (Tris pH=8.5, 1 mM $MnCl_2$). When VieA-His_6 is incubated with this substrate, VieA-His_6 is able to rapidly cleave bis-pNpp over time (Figure 4-7A). As expected, when BSA, VieS or VieB are incubated with this substrate alone, there is no cleavage of bis-pNpp since none of these proteins harbor an EAL domain (Figure 4-7A). We next tested if the addition of VieS or VieB in the presence of ATP altered the ability of VieA-His_6 to cleave bis-pNpp. As seen in Figure 4-7B, VieA-His_6 is able to cleave bis-pNpp to similar levels regardless of whether ATP and VieS or

VieB is present in the reaction. Since this experiment was done in standard assay buffer, we predict that VieA-His_6 is mostly unphosphorylated, as there is little to no phosphotransfer occurring between VieS and VieA. These data suggest that ATP or the additional proteins alone do not affect PDE activity.

To address if phosphorylation of VieA modulates its PDE activity, we observed cleavage of bis-pNpp in phosphotransfer buffer (Tris pH=7.5, 100 mM KCl, 5% glycerol, 1 mM DTT, 1 mM $MnCl_2$, 25 μM ATP). Under these conditions we know that VieS is able to readily transfer phosphate to VieA. Therefore, we would expect that if phosphorylation has any affect on PDE activity that will be reflected in the ability of VieA-His_6 to cleave bis-pNpp when incubated with VieS. When VieA-His_6 is incubated with VieS, there is no significant difference in PDE activity when compared to VieA-His_6 alone (Figure 4-7C). While there is a slightly slower rate of cleavage in the presence of VieS, this may be due to the interaction between VieA and VieS, which could interfere with the ability of the PDE domain to bind bis-pNpp. Taken together, these data suggest that the PDE domain of VieA-His_6 is active regardless of its phosphorylation state.

Figure 4-7. VieA PDE activity is not regulated by its phosphorylation state
(A) Purified VieA-His_6 (circle), VieS-C (square), VieB (triangle) or BSA (x) was incubated in standard assay buffer (Tris pH-8.5, 1mM $MnCl_2$). **(B)** Purified VieA-His_6 was incubated either alone (red), with VieS-C (blue), VieB (orange) or VieS and VieB (green) in standard assay buffer (Tris pH-8.5, 1mM $MnCl_2$) in the presence of 25 μM ATP. **(C)** Purified VieA-His_6 was incubated either alone (red) or with VieS-C (blue) in phosphotransfer buffer (Tris pH-7.5, 100 mM KCl, 5% glycerol, 1 mM DTT, 1mM $MnCl_2$, 25 μM ATP). All reactions contained 5 mM bis-pNpp and were incubated at 30°C. Absorbance at 405 nm was measured over the 2-hour time course. BSA was used as a negative control.



4.3 DISCUSSION

In this chapter, we attempted to determine if VieA is autoregulatory and/or can regulate the *vieSAB* operon. Since VieA harbors a HTH motif, we hypothesized that VieA should be able to bind upstream or within the *vieSAB* operon to regulate *vieS* and/or *vieA* expression. Despite our efforts, no significant differences were observed in either a strain that over-expressed or was deleted for *vieA*, suggesting that VieA is not the transcriptional regulator of itself or its own operon. Due to the lack of data supporting VieA regulation, we addressed if phosphorylation, instead, regulates the PDE domain of VieA. However, we show that the PDE domain of VieA is active regardless of its phosphorylation state. Given the data presented in these studies, it is still unclear if VieA is behaving as a transcriptional regulator.

It has been reported in the literature that a D to E mutation at the conserved phosphorylation site within the Rec domain mimics RR phosphorylation and activation, however this mutation is most accurate for Ntr-like RRs. Even though VieA is CheY-like, we hypothesized that VieA D52E should be active for DNA-binding. However, over-expression of *vieA* D52E did not result in any transcriptional changes. While it is possible that the D52E mutation does not activate VieA, these data could also suggest that VieA is not able to regulate *vieSAB*. However, one alternative explanation for the lack of *vieSA* induction may be due to complications of having both a wild type and D52E mutant copy of *vieA* in the cell. Given that VieA behaves as a homodimer, it is possible that during over-expression, chimeras are formed between the wild type VieA and VieA D52E. If this occurs, these chimeric dimers may have altered conformations

that are unable to bind DNA. In order to address this issue, *vieA* D52E will need to be over-expressed in a background that either has the D52E mutation on the chromosome or has *vieA* deleted. Additionally, it would be worthwhile to conduct over-expression experiments in AKI or M9 NRES glycerol, as we hypothesize that the entire virulence gene cascade, especially VieSA, is needed for VieA regulation.

While the proposed experiments above would help in providing insight into VieA regulation, it is possible that deleting *vieA* may also disrupt any alternative promoter between *vieS* and *vieA*. If this is true, then VieA autoregulation will never be observed using a Δ*vieA* mutant. Unfortunately, we do not know of any genes that are directly regulated by VieA. Therefore, we are unable to rule out the possibility that either our over-expression system is not acting as expected or that the VieA HTH domain is non-functional. While a published microarray has identified genes that are regulated by VieA (Beyhan, Tischler et al. 2006), this approach was unable to determine whether regulation is occurring directly or indirectly. Since we know little about the VieA regulon, it would be very meaningful to identify genes that are directly regulated by VieA, outside of the *vieSAB* operon. Discovering genes that VieA directly regulate would be advantageous for two reasons: 1. It would provide evidence that VieA is a direct transcriptional regulator that harbors a functional HTH domain, and 2. It would allow for the incorporation of positive control genes into the experiments described above. This would provide assurance that over-expression of *vieA* is acting as we predict and allow more accurate interpretation of the results. Hopefully future experiments will be able to successfully uncover and characterize the transcriptional regulatory functions of this RR.

CHAPTER 5: CRP REGULATION OF THE VIESAB OPERON

5.1 INTRODUCTION

The cyclic-AMP receptor protein (CRP) is known to be a global regulator of carbon catabolite repression (CCR) in enteric bacteria. While CRP has mostly been described as a positive regulator of genes involved in the metabolism of alternative carbon sources, CRP has also been shown to regulate a wide range of other genes (Botsford and Harman 1992). CRP acts as a transcription factor by directly binding to DNA through its HTH motif, but to accomplish this, it must first bind to the small molecular ligand, cyclic-AMP (cAMP). Once the cAMP-CRP complex is formed, CRP binds a conserved binding site, TGTGA-N_6-TCACA, which is located either within or near the target promoter to promote or inhibit transcription.

In *V. cholerae*, CRP has been shown to play a role in virulence gene expression and pathogenesis (Skorupski and Taylor 1997; Kovacikova and Skorupski 2001; Liang, Pascual-Montano et al. 2007). Specifically, CRP has been described to negatively regulate two crucial virulence factors, the toxin co-regulated pilus, TcpA, and cholera toxin, CT (Skorupski and Taylor 1997). Furthermore, expression of *tcpPH*, which encodes for major virulence gene regulators, is under CRP regulation (Kovacikova and Skorupski 2001). Interestingly, *tcpPH* is dually regulated due to the overlapping binding sites of the transcriptional activator pair, AphAB, and the repressor CRP, suggesting that regulation of some virulence genes is highly complex. Taken together, these studies provide evidence that virulence gene expression in *V. cholerae* is intertwined with regulation of metabolism.

We found a number of putative CRP binding sites exists in the promoter region of *vieSAB*, suggesting that CRP may also negatively regulate this operon. In this chapter, we address the role of CRP in regulating the *vieSAB* operon and characterize this interaction.

5.2 RESULTS

5.2.1 Regulation of vieSAB by CRP in the El Tor biotype

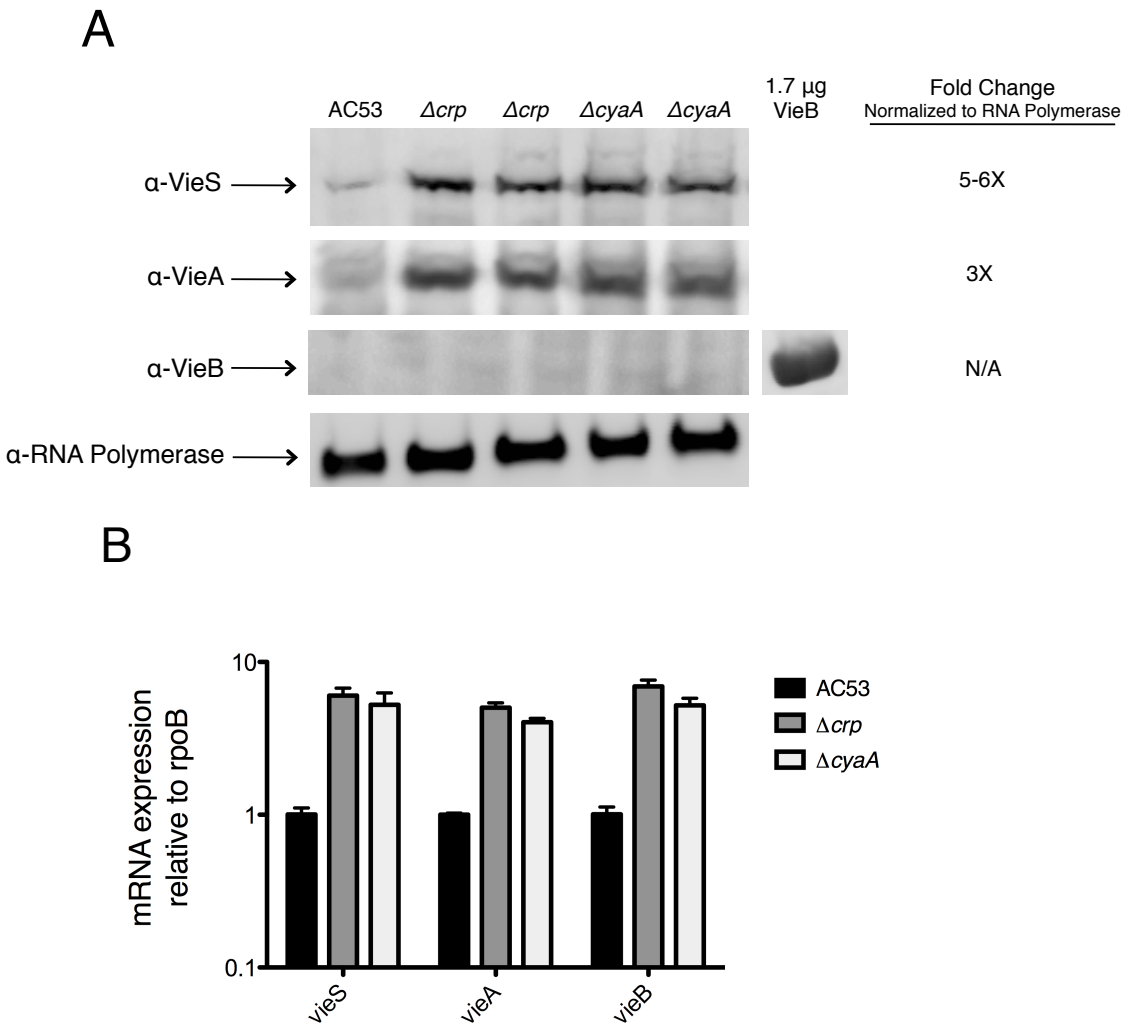
Given that *vieSAB* plays an important role in regulating the expression of virulence genes, we hypothesized that CRP may also negatively regulate this operon. As previously described, a Δ*crp* over-expresses two important virulence gene, CT and TcpA (Skorupski and Taylor 1997). This phenotype is only observed in the El Tor biotype as it enters stationary phase when grown in LB pH=6.5 at 30°C. Therefore, to test if CRP is a regulator of *vieSAB*, we looked for differential expression of the VieSAB proteins between wild type and Δ*crp* grown under these conditions. Since we know that low *vieSA* expression occurs *in vitro*, we would expect that there would be some level of protein present in the wild type. As seen in Figure 5-1A, wild type expressed a low level of VieS and VieA. When *crp* was deleted, there was a substantial increase in the amount of VieS and VieA protein that was expressed (Figure 5-1A). Given that *vieSA* are in an operon, we hypothesized that over-expression levels for VieS and VieA would be comparable for the Δ*crp* mutant. This was the case, as VieS was increased about 5- to 6-fold and VieA was increased about 3-fold over wild type. The approximately 2-fold difference in increased expression between VieS and VieA may be due to differential translation or stability of the proteins. Conversely, VieB is not expressed in any strain, suggesting that this condition does not induce VieB and furthermore, CRP does not control VieB expression.

In addition to Δ*crp*, we also tested VieSAB protein expression for the adenylate cyclase mutant, Δ*cyaA*. Adenylate cyclase is important for CRP regulation, as this enzyme is responsible for forming cAMP, which binds and stimulates the DNA activity of CRP. Therefore, without *cyaA*, there is little to no cAMP present in the cell, resulting in constitutively inactive CRP. Similar to Δ*crp*, Δ*cyaA* also over-expressed VieS and VieA about 5-6 fold and 3-fold, respectively, while VieB is not expressed (Figure 5-1A). These data further support that CRP negatively regulates the expression of VieSA proteins, but not VieB.

To address if CRP directly regulates *vieSAB* at the transcriptional level, we also tested for the over-expression of these transcripts for wild type, Δ*crp* and Δ*cyaA*. We hypothesized that transcript levels will correlate with the observed increase in protein levels. When either *crp* or *cyaA* is deleted, there was an increase in the expression of *vieS*, *vieA* and, surprisingly, *vieB* (Figure 5-1B). Transcripts for *vieS* and *vieA* both increased to approximately the same level (5-6 fold). This was not observed for VieS and VieA protein levels, where VieS was induced about 2-3 fold higher than VieA. There was also no correlation between *vieB* transcript and protein levels, as *vieB* transcription increased by 7-fold and 5-fold for Δ*crp* and Δ*cyaA*, but VieB protein was not detected. These data suggest that *vieSAB* may undergo post-transcriptional regulation that alters protein expression and this may especially hold true for *vieB*. Furthermore, there were slight variances in transcript expression between Δ*crp* and Δ*cyaA*, even though for protein, over-expression levels were the same. While Δ*crp* increased by 6-fold (*vieS*), 5-fold (*vieA*) and 7-fold (*vieB*), the Δ*cyaA* had slightly lower induction for *vieS* (5-fold), *vieA* (4-fold) and *vieB* (5-fold). Perhaps this difference in expression is due to residual

activity of CRP as a repressor in the Δ*cyaA* background. Taken together, these experiments show that CRP negatively regulates *vieSAB* expression during stationary phase.

Figure 5-1. CRP negatively regulates the expression of VieS and VieA, but not VieB
Wild type AC53, Δ*crp* or Δ*cyaA* strains were grow in LB pH=6.5 rolling at 30°C until stationary phase (OD=1.5-1.7). **(A)** 1 ml of culture was pelleted and resuspended in 2X denaturing sample buffer, boiled for 10 minutes and diluted 1:5 for VieSAB and 1:100 for RNA polymerase. 1.7 µg of purified VieB was included as a positive control for the VieB antibody. Diluted samples were run on a 4-12% gradient SDS-PAGE gel, transferred to nitrocellulose and probed for VieS, VieA, VieB and the α-subunit of RNA polymerase. Bands were quantified, values were normalized to RNA polymerase and the fold change compared to wild type was calculated. **(B)** Samples were taken for RNA collection. qRT-PCR was used to determine changes in transcript levels of *vieS*, *vieA*, and *vieB* relative to the house-keeping gene, *rpoB*.

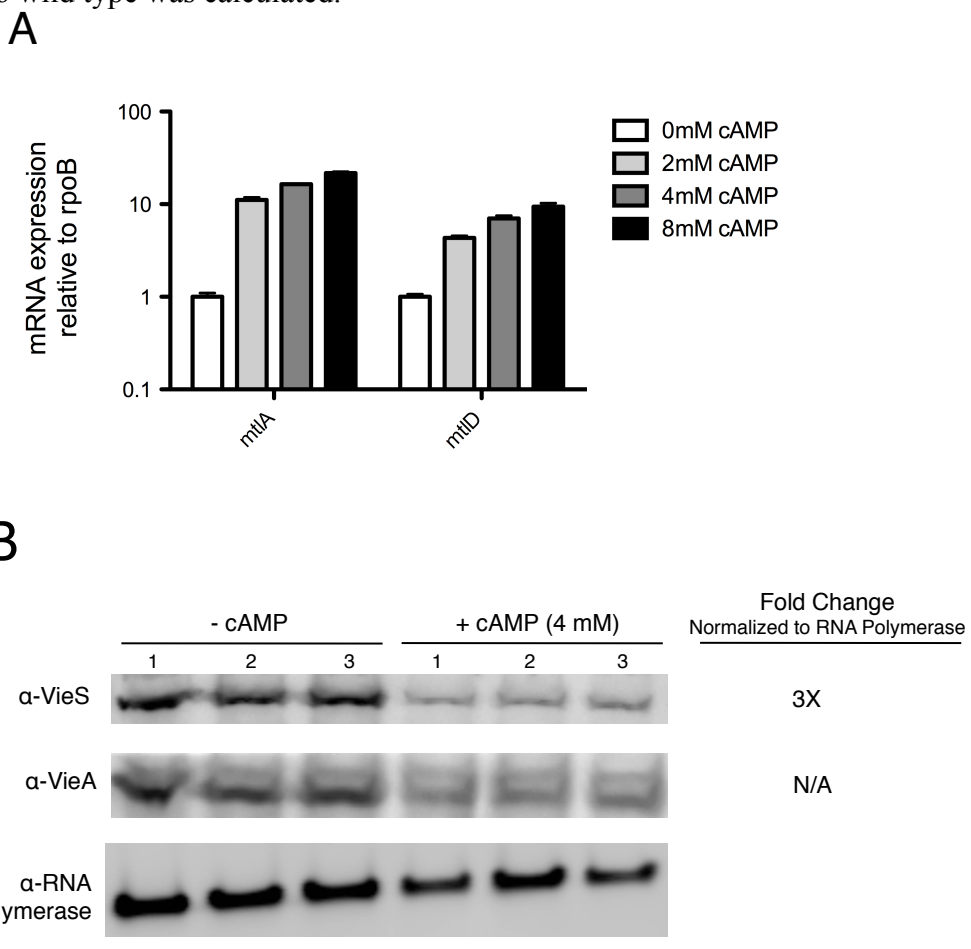


Since the cAMP level modulates the DNA-binding activity of CRP, we wanted to address if we could alter CRP regulation of *vieSAB* by controlling the amount of cAMP available in the cell. To find the optimal level of cAMP to induce CRP DNA-binding activity, we measured mRNA levels of *mtlA* and *mtlD* over a range of cAMP concentrations. *mtlA* and *mtlD* encode for the mannitol PTS EII enzyme and the mannitol dehydrogenase, respectively, and are known to be positively regulated by CRP (Zhou, Zhang et al. 2013). When Δ*cyaA* was grown in 2, 4, or 8 mM of exogenous cAMP, all concentrations were able to induce expression of both *mtlA* and *mtlD* to very similar levels (Figure 5-2A). While expression of *mtlA* was greater than that of *mtlD*, both genes are highly up regulated in the presence of cAMP.

To test if exogenous cAMP can effect VieSA protein expression, we grew Δ*cyaA* in the presence or absence of 4 mM cAMP. Since CRP negatively regulates VieSA expression, we hypothesized that in the absence of cAMP, CRP will be unable to bind DNA and VieSA should be over-expressed. Conversely, we'd expect that in the presence of cAMP, CRP will be active, and protein levels will be down regulated. VieS protein expression behaved as we expected, where the protein level increased in the absence of cAMP, although this 3-fold increase was less than what was observed for Δ*crp* (Figure 5-2B). Unexpectedly, no increase was observed for VieA protein in the absence of cAMP. While, visually, it appears that VieA protein was up regulated in the absence of cAMP, when normalized to the RNA-polymerase loading control, the values between the 0 and 4 mM cAMP samples are similar. In an attempt to address if the inability of 4 mM cAMP to induce VieA expression was valid, qRT-PCR was conducted on these samples.

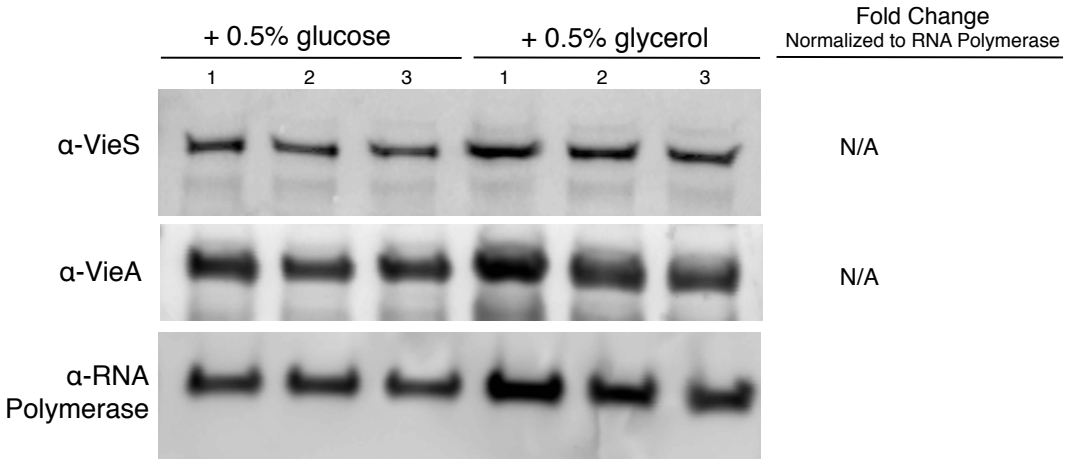
However, due to technical difficulties in analyzing the qRT-PCR data, we are unable to resolve this issue. In light of this, CRP repression at the *vieSAB* promoter should be tested using *lacZ* transcriptional fusions in the presence or absence of cAMP. Taken together, these data suggest that modulating CRP activity with cAMP is not sufficient to fully regulate *vieSAB*.

Figure 5-2. Altering cAMP levels does not replicate Δ*crp* VieSA expression
(A) Δ*cyaA* was grown at 30°C in LB containing various amounts of exogenous cAMP (0-8 mM). Samples were taken at stationary phase for RNA collection. qRT-PCR was used to determine changes in transcript levels of *mtlA* and *mtlD* relative to the house-keeping gene, *rpoB*. **(B)** Δ*cyaA* was grown at 30°C in LB pH=6.5 in the presence or absence of 4 mM exogenous cAMP. 1 ml of stationary phase culture was pelleted and resuspended in 2X denaturing sample buffer, boiled for 10 minutes and diluted 1:5 for VieSA and 1:100 for RNA polymerase. Diluted samples were run on a 4-12% gradient SDS-PAGE gel, transferred to nitrocellulose and probed for VieS, VieA and the α-subunit of RNA polymerase. Shown are three representative biological replicates of each strain. Bands were quantified, values were normalized to RNA polymerase and the fold change compared to wild type was calculated.



Since there was only a partial effect in VieSA expression in the exogenous cAMP experiment and there are slight differences in *vieSA* regulation between Δ*crp* and Δ*cyaA*, we decided to modulate cAMP levels by growing wild type in minimal media containing either glucose or glycerol as the sole carbon source. Given that CRP is involved in CCR, its DNA-binding activity can be modulated based on the carbon sources that are available. For example, glucose is a preferred carbon source. When cells are grown under this condition, cAMP levels will be low and CRP inactive. Conversely, when grown in glycerol (a non-preferred carbon source), cAMP levels will be high and CRP active for DNA-binding. Therefore, we hypothesized that VieSA protein expression will be up regulated in the presence of glucose and repressed in the presence of glycerol. However, no changes in VieS or VieA were observed between the cells grown in glucose versus glycerol (Figure 5-3). Since there was no detectable change for VieS or VieA protein levels, qRT-PCR was not conducted, however it is possible that there is differential expression at the transcript level, which should be addressed. Although it is possible that these two carbon sources did not yield the expected large difference in cAMP concentration and thus CRP repressor activity, it also may suggest that other mechanisms, in addition to CRP, are involved in regulating *vieSAB*.

Figure 5-3. Varying the carbon source does not regulate VieSA expression
Wild type AC53 was grown rolling at 30°C in M9 minimal media supplemented with NRES at pH=6.5 and either 0.5% glucose or 0.5% glycerol to stationary phase (OD=1.5-1.7). 1 ml of culture was harvested and the pellet resuspended in 2X denaturing sample buffer, boiled for 10 minutes and diluted 1:5 for VieSA and 1:100 for RNA polymerase. Diluted samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose and probed for VieS, VieA and the α-subunit of RNA Polymerase. Shown are three biological replicates. Bands were quantified, values were normalized to RNA polymerase and the fold change compared to wild type was calculated.



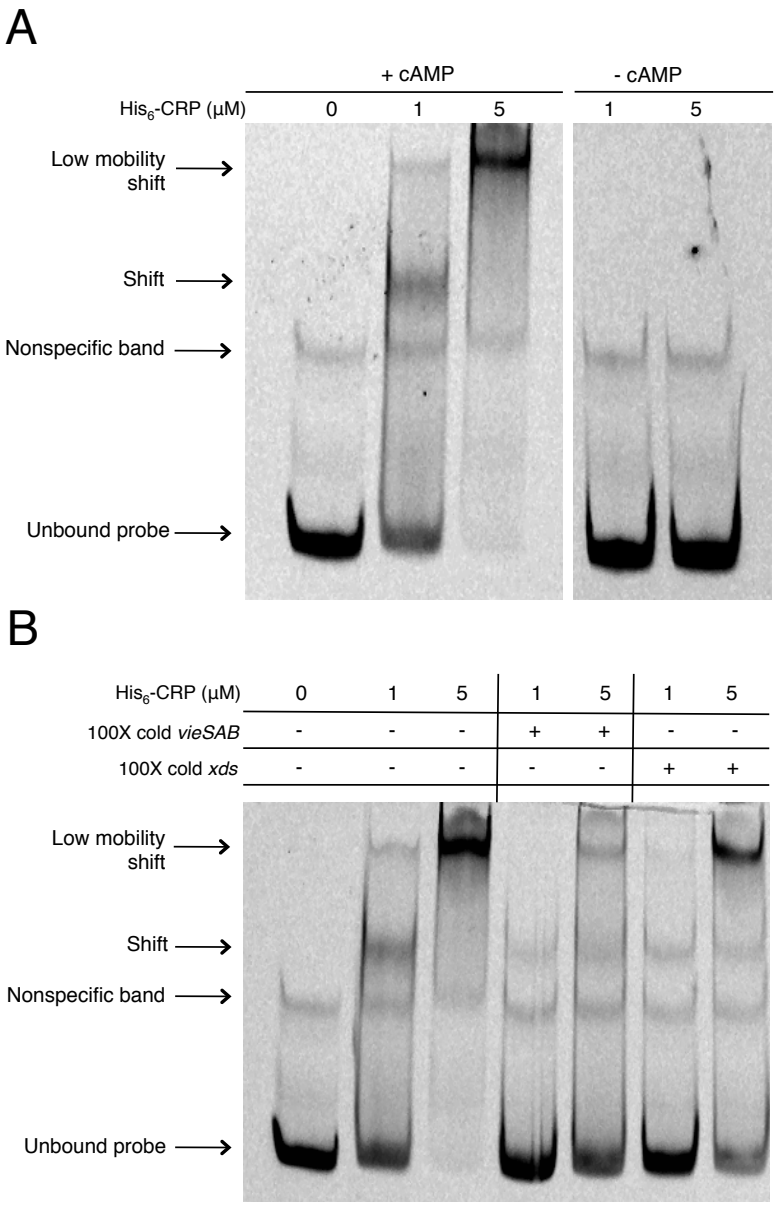
5.2.2. EMSA analysis of CRP and the vieSAB promoter

Due to both the ability of CRP to regulate VieSA expression *in vivo* and the presence of putative CRP binding sites in the promoter region of *vieSAB*, we hypothesized that CRP negatively regulates this operon by directly binding its promoter. To test this hypothesis, we conducted EMSA analysis using purified CRP protein and a labeled DNA probe containing the promoter region of *vieSAB*. As seen in Figure 5-4A (left panel), CRP at 1 μM was able to shift the *vieSAB* probe to a complex that migrated to approximately the middle of the gel, though the binding was not complete as a significant amount of unbound probe remains. However, when 5 μM CRP was incubated with the *vieSAB* probe, a complete shift of the probe was observed, resulting in a very slowly migrating complex near the tope of the gel (low mobility shift). This more slowly migrating complex compared to that observed with 1 μM CRP is consistent with their being two sites of occupancy for CRP that was predicted bioinformatically, where one is a lower affinity site. Given that the cAMP-CRP complex is required for CRP to bind DNA, we next addressed the ability of CRP to bind in the absence of cAMP. We hypothesized that without cAMP CRP will not be able to bind the promoter. Indeed, in the absence of cAMP, neither 1 nor 5 μM CRP could bind the *vieSAB* probe (Figure 5-4A, right panel). These data show that CRP is able to directly bind the promoter of *vieSAB* and cAMP is required for this binding.

Due to the high amounts of CRP protein that was needed to observe the complete shift of the promoter probe, we wanted to address the specificity of CRP binding to this promoter. We hypothesized that, if CRP binding were specific, the labeled *vieSAB* probe would be outcompeted only when in the presence of another specific probe that binds

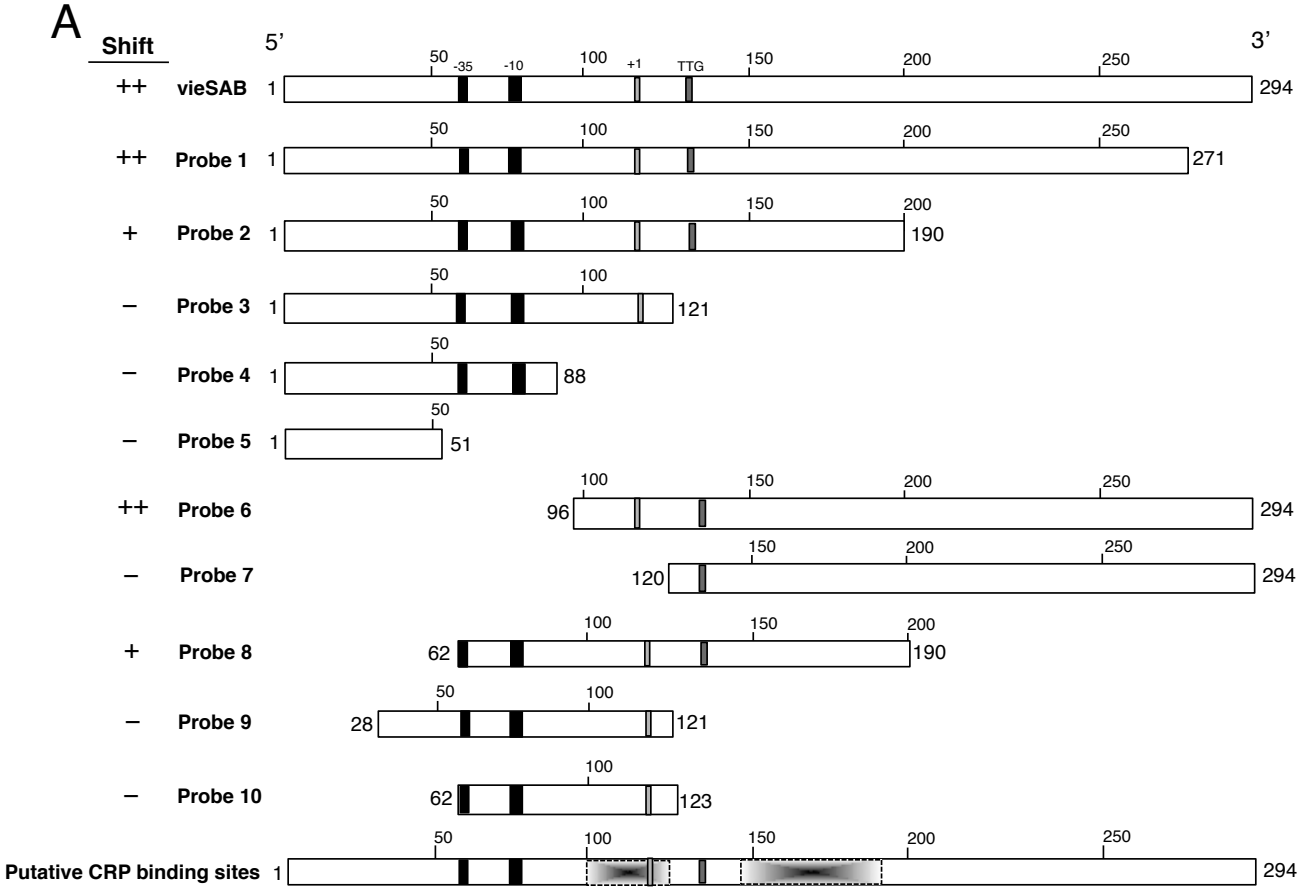
CRP. For this control, we incubated CRP and labeled *vieSAB* probe in the presence of either unlabelled (cold) *vieSAB* probe or cold nonspecific probe. Both cold probes were at a 100-fold excess over the labeled *vieSAB* probe. For the nonspecific probe, the promoter region of *xds*, which encodes for a secreted exonuclease, was used since CRP does not regulate this gene (McDonough, Lazinski et al. 2014). When CRP was incubated with labeled *vieSAB* probe and 100-fold cold *vieSAB* probe, the ability of CRP to shift the labeled probe was greatly reduced (Figure 5-4B). This was especially true for the slowest mobility complex (at 5 μM CRP), suggesting that the cold *vieSAB* probe is efficient at competing for CRP binding. However, when CRP and labeled *vieSAB* probe are incubated with 100-fold cold *xds* probe, CRP was able to bind and shift the labeled *vieSAB* probe to almost normal levels (Figure 5-4B). Even though there is some unbound labeled probe remaining when in the presence of 5 μM CRP, there is still a clear shift for the low mobility shift, suggesting that binding is not greatly affected by the excess of the nonspecific *xds* probe. Taken together, these data show that the cAMP-CRP complex is able to directly bind the *vieSAB* promoter and this interaction is specific.

Figure 5-4. The cAMP-CRP complex specifically binds the *vieSAB* promoter
(A) Purified His_6-CRP was incubated in the presence or absence of exogenous cAMP with a Cy5-labeled DNA probe comprised of the promoter region of *vieSAB*. **(B)** Purified His_6-CRP was incubated with Cy5-labeled *vieSAB* probe either alone, in the presence of 100X excess unlabelled *vieSAB* probe or in the presence of 100X excess unlabelled nonspecific probe (*xds*). For A and B, reactions were incubated for 15 minute at 30°C. Reactions were loaded on a 6% TBE native gel and run for 1 hr at 150 V at 4°C in the dark. Gels were scanned using the Cy5 channel to visualize labeled probe.

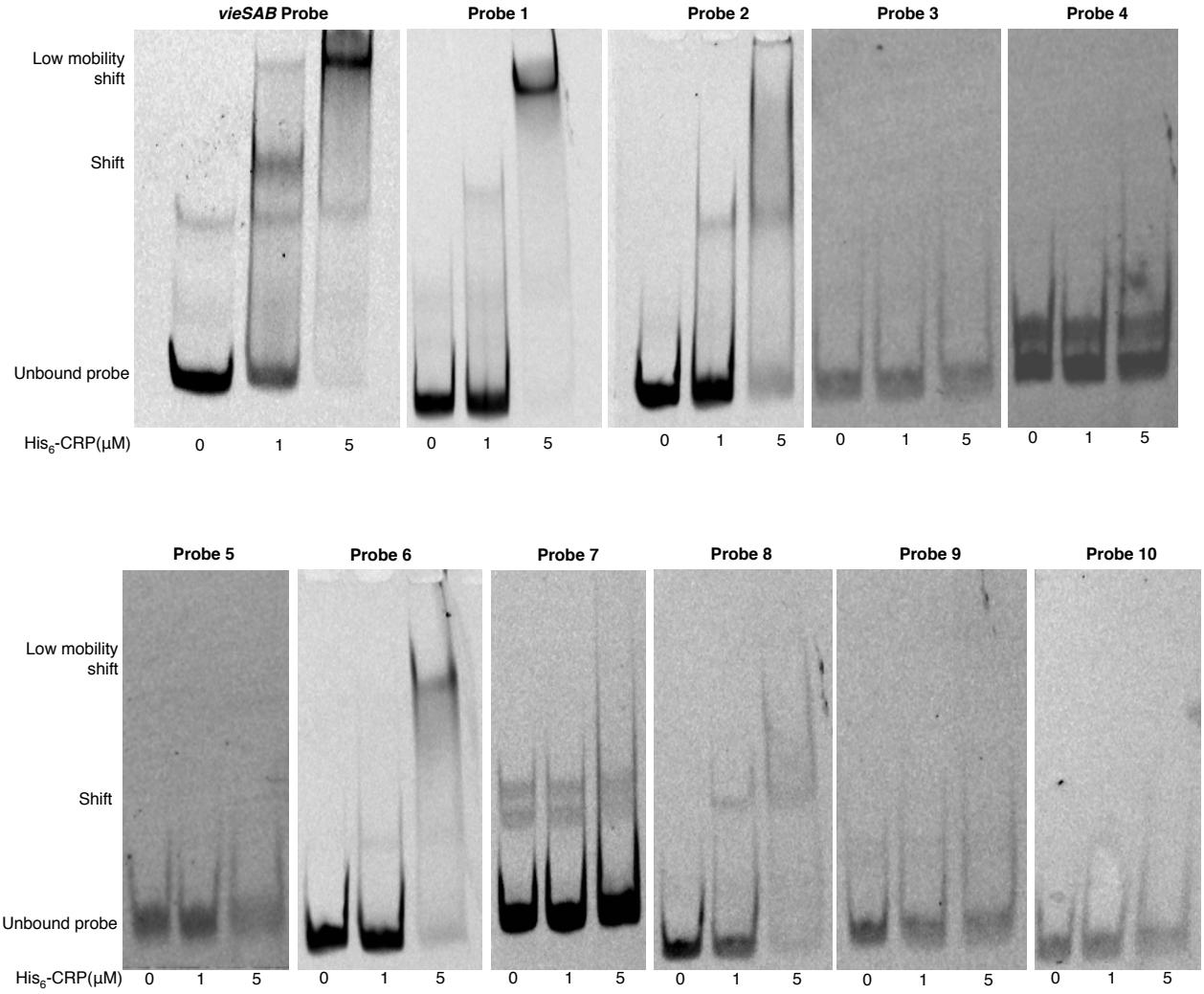


Based on the presence of two distinct shifts during the EMSA analysis, we hypothesized that the *vieSAB* promoter may have two or more CRP binding sites. In order to determine the location of the CRP binding sites within the *vieSAB* promoter, we constructed various truncations of the *vieSAB* probe from both the 3' and 5' ends. These truncated probes were then assayed using EMSA analysis with purified CRP. A schematic representation of the truncated probes is shown in Figure 5-5A, which depicts the location of the -10, -35, and the transcriptional and translational start sites. When the various probes were incubated with CRP, differences in the ability of CRP to bind were observed, specifically between probes 2 and 3 and probes 6 and 7 (Figure 5-5A-B). While a shift is detected for probe 2, there is no shift observed for probe 3, suggesting that the region removed between these probes is important for CRP binding. A similar observation was seen for probes 6 and 7, where there was a shift for probe 6 but not probe 7. Based on these EMSA data, two putative CRP bindings regions (hashed-lined boxes) were discovered in the *vieSAB* promoter and are illustrated in the last schematic in Figure 5-5A. Interestingly, probe 8 contains both putative CRP binding regions but is only able to weakly bind CRP resulting in a partial shift (Figure 5-5B). While we would expect this probe to bind similar to the full-length *vieSAB* probe, this suggests that either there is a third CRP binding site or the DNA conformation of this particular probe is not conducive for efficient CRP binding. Nevertheless, we have identified two putative CRP binding regions within the *vieSAB* promoter, providing further evidence that CRP regulates this operon through direct DNA binding.

Figure 5-5. Two putative CRP binding sites exists within the *vieSAB* promoter
(A) Schematic representation of 3' and 5' truncations of the *vieSAB* probe for EMSA analysis. Black regions represent the -10 and -35, while the light and dark grey regions represent the +1 transcriptional and translational start for *vieS*, respectively. Last schematic shows the location of two putative CRP binding sites (hashed-lined boxes) within the *vieSAB* promoter region. **(B)** Purified His_6-CRP was incubated with each of the truncated probes. Reactions were incubated for 15 minute at 30°C, loaded on a 6% TBE native gel and run for either 1 hr at 150 V (*vieSAB* and probes 1, 3-5, 7-10) or 1 hr 30 minute at 100 V (probes 2, 6) at 4°C in the dark. Gels were scanned using the Cy5 channel to visualize the labeled probes. '++' represents a complete shift; '+' represents a partial or weak shift; '–' represents no shift.



B


vieSAB Probe
Probe 1
Probe 2
Probe 3
Probe 4
Low mobility shift
Shift
Unbound probe
His6-CRP(μM)
0 1 5
Probe 5
Probe 6
Probe 7
Probe 8
Probe 9
Probe 10

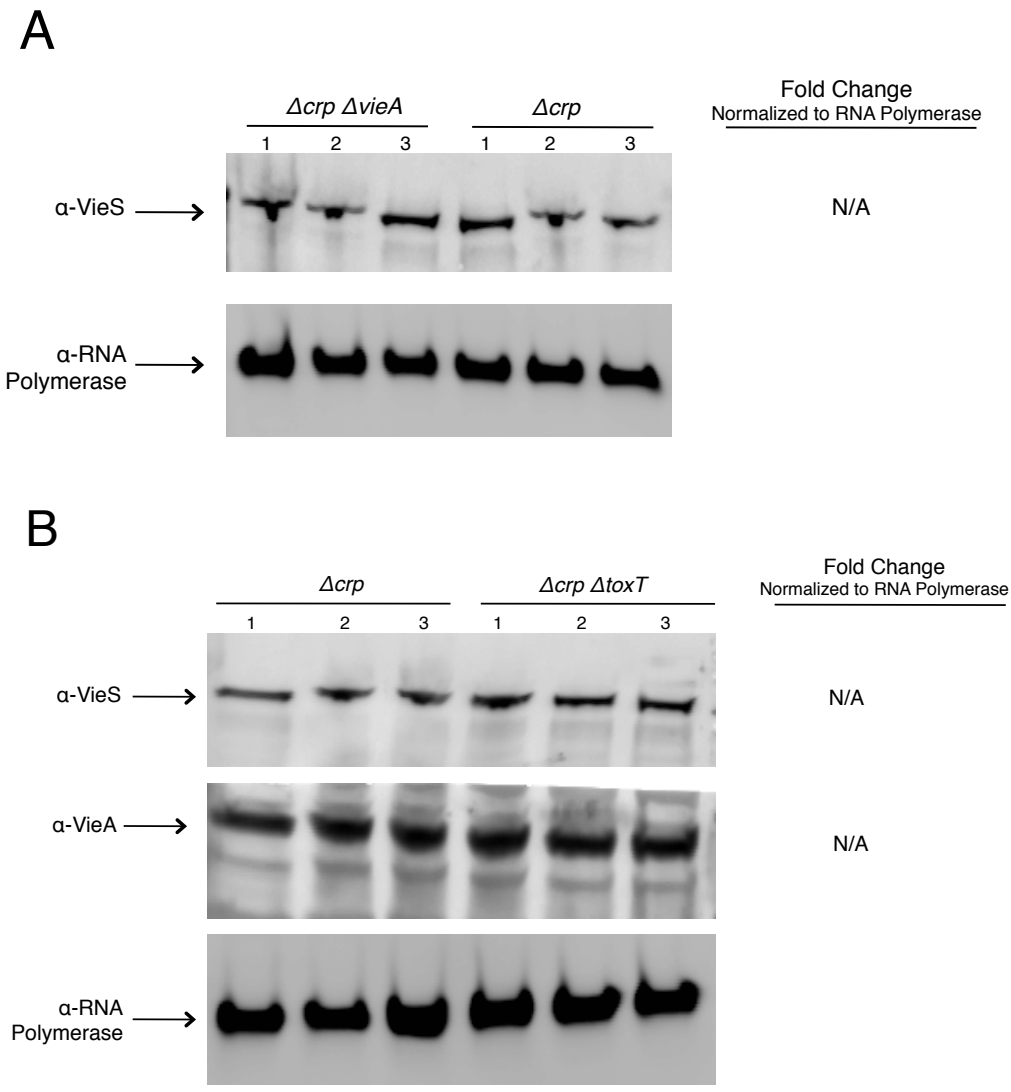
5.2.3. Positive regulation of vieSAB in Δcrp

Previously, we were unable to detect any regulation of the *vieSAB* operon by either VieA or ToxT. However, repression by CRP could explain the inability to observe VieA or ToxT regulation in previous experiments. Therefore, we tested for VieA or ToxT regulation in the absence of CRP. We hypothesized that in the Δ*crp* mutant, VieSA will be over-expressed, in part or in whole due to the positive regulation by VieA or ToxT and the lack of repression. However, in the absence of both regulators, VieSA levels would be down regulated due to the lack of positive regulation. We first addressed VieA regulation using the double mutant, Δ*vieA* Δ*crp* and comparing VieS protein expression to the single mutant, Δ*crp*. Given that there was no detectable expression of VieB protein in any strain, VieB was not tested. As expected based on previous data, when CRP was absent, we observed over-expression of VieS. However, when both CRP and VieA were absent, there was no change in VieS level (Figure 5-6A), suggesting that VieA is not regulating VieS. As noted earlier, since we deleted *vieA*, this experiment cannot rule out VieA autoregulation at an internal promoter and can only address VieA binding to the main promoter upstream of the *vieSAB* operon. Regardless, these data provide evidence that even in the absence of CRP, VieA is not able to regulate the *vieSAB* operon, at least not under the condition tested.

We next tested the ability of ToxT to regulate *vieSAB* in the absence of CRP using the Δ*toxT* Δ*crp* double mutant. Again, we hypothesize that in the absence of both the positive and negative regulation, VieSA levels will be down regulated when compared to the single Δ*crp* mutant. However, the levels of VieSA were unchanged between the double and single mutants (Figure 5-6B) and suggest that ToxT is also unable to regulate

the *vieSAB* operon. Taken together, these studies presented here and in Chapter 4 suggest that neither VieA nor ToxT are regulators of the *vieSAB* operon under the *in vitro* conditions tested.

Figure 5-6. Neither VieA nor ToxT are regulators of the *vieSAB* operon
Δ*crp*, Δ*crp* Δ*vieA* **(A)**, or Δ*crp* Δ*toxT* **(B)** were grown in LB pH=6.5 rolling at 30°C until stationary phase (OD=1.5-1.7). 1 ml of culture was pelleted and resuspended in 2X denaturing sample buffer, boiled for 10 minutes and diluted 1:5 for VieSA and 1:100 for RNA polymerase. Diluted samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose and probed for VieS and the α-subunit of RNA polymerase. Shown are three representative biological replicates of each strain. Bands were quantified, values were normalized to RNA polymerase and the fold change compared to wild type was calculated.



5.3 DISCUSSION

The studies presented in this chapter address the role of CRP in the negative regulation of the *vieSAB* operon. We show that in the absence of CRP, *vieSA* are up regulated at both the transcript and protein level. However for *vieB*, over-expression in the Δ*crp* mutant only occurred at the transcriptional level; in fact no VieB protein was detected for any strain tested. Additionally, we show the cAMP-CRP complex specifically binds to the promoter of the *vieSAB* operon at two or possibly more putative CRP binding sites. Taken together, these studies provide evidence that CRP is a negative regulator of *vieSAB*.

In light of these studies, we must reevaluate our hypothesis that there is positive regulation of the *vieSAB* operon by VieA and/or ToxT. Dual regulation between CRP and AphAB has been observed in *V. cholerae* for the virulence factors, *tcpPH*. Therefore, we hypothesized that a similar dual regulation may also hold true for the *vieSAB* operon, where CRP and a transcriptional activator compete for binding to the promoter region. However, deletion of VieA or ToxT in a Δ*crp* background did not alter VieS or VieA protein levels, suggesting that CRP may be the sole regulator of this operon. One caveat to this conclusion is that we only tested expression of vieSAB in a single *in vitro* growth condition, whereas the natural conditions under which this operon may be positively regulated could be different. Additionally or alternatively, it is possible that another virulence gene transcription factor is responsible for regulating *vieSAB*, such as ToxR or AphAB. Additional work is needed to resolve if there is a regulator that competes with CRP to control *vieSAB* expression.

Through EMSA analysis, we show that purified CRP can directly bind to the promoter of *vieSAB*. However, the concentration of CRP needed to bind is slightly higher than what is typically seen for transcriptional regulators, which are in the nanomolar range. While this suggests that CRP may be binding nonspecifically, we show that CRP cannot be outcompeted by a nonspecific probe (even when in 100-fold excess) and any probe truncation that does not harbor the putative CRP binding sites do not shift, suggesting that the observed binding with the *vieSAB* probe is bona fide. Therefore, the requirement for micro-molar concentrations of protein may be a result of the purified prep of His_6-CRP containing some inactive CRP protein. In this case, a higher concentration of protein would be needed to achieve observable binding. Indeed, purified CRP in the EMSA buffer appears to be slightly unstable when held at -20°C for more than 2 days and precipitates quickly after one freeze-thaw cycle or when at a concentration higher than approximately 30 μM. Therefore, altering the purification conditions and/or EMSA buffer may aid in achieving a more active prep that will allow for a lower concentration of CRP to be used to observe binding.

Based on the probe truncation analysis, we identified two potential regions that CRP binds within the *vieSAB* promoter. We hypothesize that one binding site is located in between the -10 and the translational start of *vieS*, overlapping the +1 transcriptional start site, and the other is located just downstream of the *vieS* translational start codon. Interestingly, probe 8 does not bind CRP very well even though this probes contains these two putative binding regions. While this could be due to probe 8 lacking a third cooperative binding site, based on the probe truncation experiments, we do not expect a third site to exist. An alternative explanation is that the DNA conformation of this shorter

probe is less optimal for CRP binding compared to longer probes. An additional probe that appends non-specific sequences onto both ends of probe 8 could be used to test this hypothesis.

Given that CRP represses the expression of *vieSAB* at the transcriptional level, the position of the CRP binding sites can provide insight into how repression is achieved. One mechanism for repression is through blocking the binding site of RNA polymerase, thereby preventing transcription. This mechanism has been described for CRP repression of *cyaA* in *E. coli* (Aiba, Fujimoto et al. 1982). Given that the two identified binding regions spans from the -10 into the open reading frame of *vieS*, this mechanism of repression is highly possible for CRP at this promoter. However, further investigation to uncover the exact locations and sequences of the CRP binding sites is necessary to reveal the mechanism of CRP repression of *vieSAB*. Perhaps the most informative approach would be to perform foot-printing to precisely map the locations of CRP binding. Generating site-specific mutations within the identified binding sequences to specifically disrupt CRP binding could follow foot-printing assays. Moreover, future work should also aim to address the differential regulation of VieS, VieA and VieB at the post-transcriptional level to fully understand how CRP regulates this operon.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Significance of This work

The overall goal of this work was to take a genetic and biochemical approach to gain a better understanding of the regulation and function of the VieSAB three-component system in *V. cholerae*. From this work, two notable achievements were accomplished. First, we reveal the role and mechanism of action of VieB, the previously uncharacterized auxiliary protein in the VieSAB three-component system. We hypothesize that VieB is a novel inhibitor that serves as a unique negative feedback mechanism to shut off or down regulate the VieSA TCS. Secondly, we uncover that *vieSAB* is directly regulated by the global transcription factor, CRP. While induction of *vieSAB* is still unclear, we show that CRP is involved in its repression during the stationary phase of growth, providing insight into the regulation of this operon.

In Chapter 2, we present biochemical evidence that the auxiliary protein, VieB, behaves as a noncompetitive inhibitor of the VieSA TCS. We show that VieB is able to achieve this inhibition by specifically binding VieS and partially disrupting VieS autophosphorylation and blocking the phosphotransfer to the VieA. Based on these data, we propose a working model for VieB inhibition of VieSA and its effect on virulence gene expression. Upon induction, VieS is able to autophosphorylate and pass this phosphate via the phosphorelay to VieA. Phosphorylation of VieA results in up regulation of *vieSAB* expression and increased VieA protein levels, which are able to enzymatically cleave cdGMP; this leads to the expression of virulence genes. However, over time, the up regulation of *vieSAB* leads to increased levels of the inhibitor, VieB. We hypothesize that VieB is then able to bind VieS and disrupt autophosphorylation and the intra-molecular phosphorelay. As a result of this inhibition, VieA is no longer

phosphorylated, resulting in down regulation of *vieSAB*; virulence genes are also down regulated due to the increasing cdGMP levels in the absence of VieA protein (Figure 2-13).

Unlike many characterized inhibitors of TCS, VieB is neither a phosphatase nor does it modulate the phosphatase activity of VieS. While VieB's function is similar to the of autophosphorylation inhibitor, KipI in *B. subtilis*, our data provide evidence that VieB has only minimal inhibitory activity on VieS autophosphorylation. More strikingly, and we hypothesize of greater importance, is its ability to block phosphotransfer VieA, either by inhibiting the intra-molecular phosphorelay within VieS or HPt phosphotransfer to VieA. Since VieB is able to incompletely inhibit VieS autophosphorylation, this led us to hypothesize that VieB is most likely interacting at the HK domain; expanding upon this, we would expect that VieB would therefore inhibit the intra-molecular phosphorelay between the VieS HK and Rec domains. Therefore, VieB may act similar to the Sda inhibitor of the orthodox SK, KinA, in *B. subtilis.* While originally thought to be an inhibitor of autophosphorylation (Rowland 2004), it was later shown that, Sda instead inhibits the transmission of the signal from the HK to RR (Whitten, Jacques et al. 2007). Conversely, based on our studies, VieB appears to affect both autophosphorylation and phosphotransfer. Additionally, VieB is unique since it appears to disrupt an intra-molecular phosphorelay of a hybrid SK. Taken together we believe that the dual function of VieB to partially block autophosphorylation and fully disrupt the intra-molecular phosphorelay provides a novel mechanism for the negative regulation of a TCS.

Additionally, we show that the inhibitory activity of VieB can be regulated by its phosphorylation state. While this regulation is used for the prototypical RR, to our

knowledge, this is a new mechanism by which inhibitors of TCS can be controlled. Using point mutants that we hypothesized to mimic a phosphorylated (D62E) or unphosphorylated (D62A) state, we show that phosphorylation leads to inactivation of the inhibitory function of VieB. While this is opposite to that of the typical RR, the reverse regulation by phosphorylation is an appealing model for an inhibitor like VieB: Given that VieB must be in its unphosphorylated state in order to inhibit VieS, phosphorylation of VieB provides a rapid shut-off signal, allowing VieS to regain function or return VieSA to its resting state. However, the D62E mutant retains strong binding to VieS, which was unexpected. This suggests that the mere binding of VieB to VieS is not sufficient for inhibition, and that the structure of VieB, which can be altered by phosphorylation, is important for its inhibitory activity.

Intriguingly, while VieB is regulated by its phosphorylation state, it is not readily phosphorylated by VieS or by the small molecule donor, acetyl-phosphate. This could suggest that VieB is simply inefficient in receiving phosphate. However, given the specificity of cognate SK-RR pairs and the kinetic preference for phosphotransfer between them, an alternative explanation is that another SK in the cell is responsible for phosphorylating VieB. Whether the phosphorylation occurs via an orphan SK or one that is coupled to a RR, cross-regulation is an interesting hypothesis, since this would result in the incorporation of another signaling system into the regulation of the VieSA TCS. While bacteria strive to prevent unwanted cross-talk, in some instances, the incorporation of two separate signals is useful for modulating a response, especially one that has a global effect in the cell. Indeed, signaling systems that engage in cross-regulation have been described (Gunn and Miller 1996; Kox, Wosten et al. 2000; Kato and Groisman

2004; Tsokos, Perchuk et al. 2011; Downey, Mashburn-Warren et al. 2014). A well-characterized example of cross-regulation is observed between the PmrAB and PhoPQ TCS in *Salmonella enterica*. While PhoPQ is responsible for sensing and responding to low Mg^{2+} concentration, the PmrAB TCS is important for the recognition of Fe^{3+}. Cross-regulation occurs via the PhoPQ induction of *pmrD.* PmrD is then able to stabilize the phosphorylated PmrA RR, preventing its dephosphorylation by the PmrB SK. Integrating the Mg^{2+} and Fe^{3+} signals between these TCSs allows for the precise control over a common response, virulence gene expression. Based on this model, the ability of VieSA to induce virulence gene expression could not only be regulated by VieS's signal but by an additional signal via another SK, which is connected to the VieSA TCS by VieB. Identifying and characterizing cross-regulation would provide further insight into the regulation of this TCS and how it modulates virulence gene expression.

In Chapter 5, we discovered that the global transcription factor, CRP, negatively regulates the *vieSAB* operon. We show that repression by CRP is direct and requires the cAMP-CRP complex. Additionally, we also found that repression of *vieSAB* occurs only in stationary phase, which is in agreement with previous studies that also observed CRP repression in stationary phase for other virulence genes (Skorupski and Taylor 1997; Kovacikova and Skorupski 2001; Liang, Pascual-Montano et al. 2007). Since VieSA is known to be an important regulator of *ctxAB* and *toxT*, one would expect that high expression of this system would be disadvantageous when *V. cholerae* is in the late stage of infection. Taken together with the known role of CPR repression of virulence genes late during infection, we hypothesize that CRP repression of *vieSAB* aids in shutting off virulence genes by increasing cdGMP concentrations through decreasing the VieA

protein levels. Therefore, uncovering the repression of *vieSAB* by CRP during the stationary phase sheds new light on when this system is required in the life cycle of *V. cholerae* and highlights the importance of rapidly shutting down virulence expression when *V. cholerae* is transitioning into the environment.

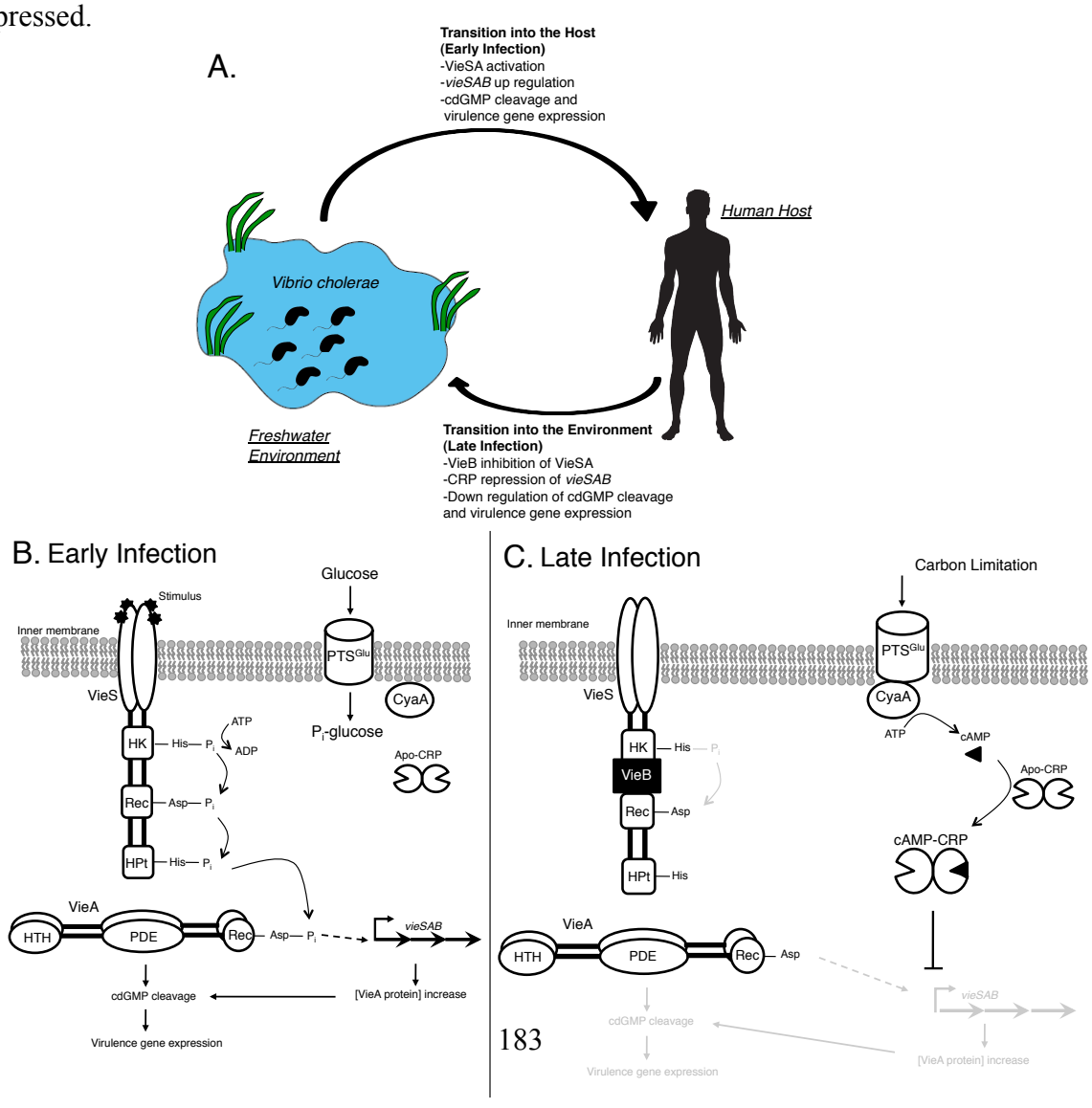
Taken together, we propose a working model for the role of both VieSAB and CRP in the regulation of virulence genes in context of the *V. cholerae* life cycle (Figure 6-1A). As *V. cholerae* colonizes the nutrient rich small intestine and is in the early stages of infection, glucose or other readily metabolizable carbohydrate is utilized as the preferred carbon source, resulting in low cAMP level and inactive CRP. Additionally, the VieSA TCS is activated by *in vivo* signals, allowing for induction of *vieSAB* and rapid cleavage of cdGMP due to high VieA levels. This *vieSAB* up regulation is hypothesized to be due to both CRP de-repression and the regulatory function of VieA, though this VieA regulation may be direct or indirect (Figure 6-1B). Together, this allows for expression of virulence genes once inside the host.

However, as *V. cholerae* multiplies to high numbers over the course of infection, this results in a microenvironment that is limited in preferred carbon source. Under this condition, cAMP level increases in the cell, which activates CRP and directly represses *vieSAB* expression at its promoter. Simultaneously, the accumulation of VieB over the course of infection allows this inhibitor to block VieA phosphorylation and activation by the partial inhibition of VieS autophosphorylation and interruption of the intra-molecular phosphorelay (Figure 1-6C). Therefore, CRP and VieB can efficiently down regulate virulence gene expression by increasing cdGMP level by concurrently repressing *vieSAB*

and shutting down the VieSA TCS, allowing for *V. cholerae* to prepare for its exit out of the host.

Taken together, the studies presented in this thesis have underscored the importance of *V. cholerae* to incorporate a variety of signals in order to accurately modify gene expression changes during its transition between the host and the environment. Through CRP and VieSAB, *V. cholerae* is able to tie together carbon availability and intracellular cdGMP levels through an *in vivo* induced TCSs to regulate the virulence cascade. Furthermore, these studies have expanded our knowledge of how auxiliary proteins control TCS, as we present a novel mechanism for the inhibitor, VieB. In sum, our work imparts a new complexity and better understanding of the intricate regulation of virulence genes in this pathogen.

Figure 6-1. Proposed model of CRP and VieSAB dual-regulation of virulence genes **(A)** Overview of CRP and VieSAB virulence gene regulation in *V. cholerae* life cycle. **(B)** Early during infection, CRP is inactive due to the presence of glucose and low cAMP level, resulting in de-repression of *vieSAB*. VieSA is activated by an *in vivo* stimulus. This results in VieA phosphorylation and up regulation of *vieSAB* leading to increased VieA protein, cdGMP cleavage and expression of virulence genes. **(C)** As *V. cholerae* replicates to high number within the small intestine, glucose becomes limiting. cAMP level rises, cAMP-CRP complex forms, and CRP represses of *vieSAB*. Additionally, VieB is able to inhibit VieS autophosphorylation and intra-molecular phosphotransfer, inactivating VieA. As a result, cdGMP level rises and virulence gene expression is repressed.



6.2 Future Directions

6.2.1 Structure and function analysis of VieB inhibition

The studies presented in this thesis have determined the mechanism of action of VieB, however the structural details of how this is achieved remain unclear. Firstly, we do not know which domains on VieS and VieB are important for their physical interaction. While domain truncation analysis of VieB revealed that the Rec domain, but not the TPR domain, was important for inhibition, the role of the UKF region is still unclear. Additionally, we were unable to decipher if VieB is disrupting the intra-molecular phosphorelay or phosphotransfer from the HPt domain to VieA. Due to partial inhibition of VieS autophosphorylation by VieB, we hypothesized that VieB is interacting with the HK domain and based on this location, is therefore blocking the VieS intra-molecular phosphorelay. While we favor this hypothesis, that VieB disrupts the VieS intra-molecular phosphorelay, our data do not rule out the possibility of VieB to block HPt phosphotransfer from VieS to VieA. Given the size of VieB (64 kDa) and the cytoplasmic portion of VieS (75 kDa), depending on the tertiary structure of VieB, it is possible that VieB could bind at both the HK and HPt domains. Determining which of these hypotheses are correct would not only further clarify the exact mechanism of VieB but it would also help to identify broad regions for its binding. In this light, future studies are necessary to distinguish the structural details of the VieS-VieB interaction.

To address the issues presented above structure/function analysis of the VieS-VieB interaction should be conducted in order to fully elucidate how VieB is able to

affect both autophosphorylation and phosphotransfer. Crystal structures for the cytoplasmic portion of VieS, VieB and their complex would be the most beneficial in order to identify sites of interactions. The VieS-VieB complex could clarify whether VieB is blocking the intra-molecular phosphorelay or HPt phosphotransfer based on where VieB interacts with VieS. In addition, structure-guided mutagenesis could then be used to identify the necessary residues that are critical for the VieS-VieB interaction and inhibition. Moreover, comparison of these proteins alone and in complex can aid in creating a detailed model for VieB inhibition by revealing the global and local conformational changes that occur. Overall, these structural studies should provide substantial, and very detailed, insight into the function of VieB inhibition.

6.2.2 Determine the role of VieB phosphorylation

An intriguing unknown in the proposed model of VieB inhibition is the regulation of its inhibitory activity by phosphorylation. Based on what is known about the structural and functional changes a classical RR undergoes upon phosphorylation, a straightforward hypothesis to explain the regulation of VieB would be that phosphorylation results in VieB dimerization and the inability to bind VieS. However, this does not appear to be the case: The VieB D62E phospho-mimic construct remains a monomer, which suggests that VieB does not undergo dimerization or form any quaternary structure after phosphorylation. Additionally, the D62E VieB point mutant was still able to tightly bind VieS, although it was a weaker inhibitor. This suggests that the binding of and inhibition by VieB are exclusive and that there may be specific residues or regions that are important for inhibition but not for binding and vice versa. Therefore, the phosphorylation of VieB may result in minor or local conformation

changes for those residues or regions that control the inhibitory activity of VieB. Again, structural studies could provide detailed information into how phosphorylation alters the structure of VieB. Comparing the crystal structures of the wild type VieB and D62E mutant could reveal the changes that modulate its inhibitory activity. Additionally, a complex between the VieB D62E and VieS could also be very informative as this could differentiate between regions or residues that are important for inhibition but not for binding and vice versa. However, it is possible that the D62E mutant does not accurately mimic a phosphorylated state. In this case, a "phosphorylated" VieB crystal structure can be achieved by incorporating a phosphate substitute, BeF_3, with wild type VieB, which is commonly used for obtaining phospho-mimic structures for RRs.

In addition to resolving structural differences between phosphorylated and unphosphorylated VieB, another interesting observation from these studies was the lack of VieB phosphorylation by VieS. Given the specificity of phosphotransfer between cognate SK and RR pairs, this lack of phosphorylation could suggest that another SK in the cell regulates VieB. Due to the lack of *vieB* expression under a number of *in vitro* growth conditions and the lack of a detectable phenotype for a Δ*vieB* strain, in order to probe for the second SK, a biochemical approach could be used. *In vitro* phosphotransfer assays that assess the ability of other SKs to phosphorylate VieB is a straightforward experiment that could be used for discovering this SK. Further characterization of this proposed SK and its ability to regulate VieB would be worthwhile, as it would provide a clearer understanding of how and when VieB regulates the VieSA TCS. If cross-regulation does occur, this would offer additional detail to the complex regulation of virulence gene expression through VieSAB.

6.2.3 Uncover phenotypes for vieB

Based on the biochemical studies presented in this work, VieB plays an important role in regulating the VieSA TCS. In spite of this, our attempts to detect a physiological role of *vieB in vitro* and *in vivo* have been unsuccessful. Since we hypothesize that VieB is needed late in infection to inhibit the VieSA TCS and increase cdGMP level, future investigation into the role of *vieB* should target the host to environment transition period. However, this transition period includes many stages and is extensive. While we conducted a mouse to pond transition assay, we only sampled the 24 hours time point of infection and then survival in the pond after 24 hours. Therefore, different time points both *in vivo* and in pond water should be taken to observe if there is any detrimental effect due to the loss of *vieB*. While additional time points could reveal a phenotype for *vieB*, it is still possible that no effect will be observed. This may be due to these studies being conducted in the El Tor background, where *vieSAB* appears to be less important. Therefore, the infant mouse colonization and mouse to pond transition experiments should also be conducted in the classical biotype. Finally, the infant mouse model may not replicate the conditions in the human host under which VieB is most important. For example, the infant mouse model does not result in secretory diarrhea, so perhaps it is during the exit from the human small intestine within secretory diarrhea that VieB is needed. Similar studies should therefore be carried out using the infant rabbit model, which does result in profuse secretory diarrhea.

6.2.4 Characterization of CRP repression and further investigation into the positive regulation of vieSAB

In these studies, we provide strong evidence that CRP is a repressor of the *vieSAB* operon, as a deletion of *crp* results in increased expression of *vieSAB* at both the transcript and protein levels. While we show that CRP can directly bind two broad regions within the *vieSAB* promoter, the exact locations and sequences that CRP is binding are unknown. In order to characterize CRP repression of *vieSAB* further, future studies should identify the precise binding sequences for both sites. Additionally, mutation analysis of these binding regions should be conducted to disrupt CRP binding, which could be assayed by EMSA and/or footprinting and by measuring transcript levels of *vieSAB*. These experiments would verify that the identified sequences are CRP binding sites and show that alteration of these sites results in the disruption of *vieSAB* regulation by CRP.

Attempts to show positive regulation of the *vieSAB* operon remain elusive, as we were unable to show regulation of *vieSAB* by either VieA or ToxT. However, a previous microarray showed that deletion of *vieA* resulted in decreased expression of the *vieSAB* operon, providing evidence for autoregulation. Due to the complexity of virulence regulation in *V. cholerae* it is likely that regulation of this operon involves the incorporation of a number of signals, specifically those that induce virulence but inactivate CRP DNA-binding. Additionally, given that CRP repression only occurs in stationary phase, the positive regulation of this operon may also be under strict temporal control. Therefore, testing for the positive regulation of *vieSAB* should be conducted throughout growth and under more stringent conditions that attempt to induce virulence and minimize carbon limitation.

While we hypothesize that the positive regulator is VieA, it is also possible that another transcription factor is responsible for *vieSAB* regulation but is controlled by VieA. Regardless, both require VieA activation by VieS. However, we do not know the nature of the signal that induces VieS; finding an *in vitro* condition that is capable of inducing VieSA, the virulence cascade and reduces carbon limitation may be difficult. In this case, it may be worthwhile to test for the regulation of *vieSAB* using an *in vivo* RIVET experiment. This experiment will allow for the proper induction and detection of temporal expression of *vieSAB* in either the presence or absence of a particular regulator, such as VieA. Since VieA is responsible for controlling cdGMP level, a PDE mutant should also be included to test for the effect of cdGMP concentration on *vieSAB* expression. Furthermore, given that we do not know if VieA is directly autoregulatory, other transcription factors that are involved in pathogenesis, such as ToxT, ToxR/S, TcpP/H, AphA, AphB and HapR, should also be tested for the direct regulation of this operon. By taking what was learned throughout these studies, hopefully a more targeted approach can be used to expose the regulation of this operon.

CHAPTER 7: MATERIALS AND METHODS

7.1 CLONING

All strains and primers used are listed in Tables 7-1 and 7-2, respectively. Bacteria were grown in Luria-Bertani (LB) broth with 100 µg/ml ampicillin (Amp) or streptomycin (Sm) at 37°C with aeration, unless otherwise stated. 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression when necessary. The cytoplasmic encoding portion of *vieS* (*vieS-C*) and full-length *vieB* (*vieB*) were constructed using PCR amplicons made from *V. cholerae* AC50 (classical) template DNA. For glutathione *S*-transferase (GST) tagged *vieS-C*, a silent mutation was created at the internal NdeI restriction endonuclease recognition site at position 2916. Overlap extension PCR (Horton 1989) was used to generate this mutant allele using primer pairs GST-VieS-C F/GST-VieS-C T2916C R and GST-VieS-C T2916C F/GST-VieS-C R. *vieB* was generated by PCR using the VieB F/R primers. The outer primers for all alleles introduce NdeI and BamHI restriction sites for subsequent cloning, unless otherwise stated. After amplification, PCR products were double-digested with NdeI and BamHI (New England Biolabs). Generation of the GST-*vieS-C* was ligated into a modified pGEX-TEV vector (Pratt, Ismail et al. 2010). *vieB* was fused to an N' terminal poly-Histidine (His_6) tag by ligation into a modified pET15b-TEV (Pratt, Ismail et al. 2010). The maltose binding protein (MBP) tagged *vieS-C* D1018A mutant was generated using overlap extension PCR using the primer pairs MBP-VieS-C F/MBP-VieS-C D1018A R and MBP-VieS-C D1018A F/MBP-VieS-C R. Specifically for this protein fusion the outer primers introduced BamHI and SalI restriction sites for subsequent cloning. Generation of the MBP-*vieS-C* D1018A was ligated into the pMALc2E vector. Ligations were transformed into *E. coli* DH5α by electroporation and plated on LB broth

supplemented with Amp. The insert in each plasmid was confirmed by DNA sequencing. The pMMB67EH::*vieA-His*$_6$ was purified from DH5α and transformed into *E. coli* BL21 (DE3) for protein expression (Tamayo, Tischler et al. 2005). The VieB D62 point mutants were constructed using the QuickChange site-directed mutagenesis method (Stratagene) using the pET15b::His$_6$-*vieB* vector described above. The plasmids were transformed into *E. coli* BL21 (DE3) after the mutations were confirmed by DNA sequencing.

For the MBP-VieB ΔRec and ΔUKF, constructs were generated using PCR using the primer pairs F NdeI 487VieB/R ctVieB st BamHI and F NdeI ntVieB/R 906VieB st BamHI, respectively. Specifically for the VieB ΔTPR construct, the PreScission protease sequence that was flanked on both sides by three repeats of the protein sequence, GGS, was used as a flexible linker to replace the TPR domain. This construct was created using overlap extension PCR using the primer pairs F NdeI ntVieB/R 486VieB (GGS)3 PreS and F PreS (GGS)3 907VieB/R ctVieB st BamHI. The final PCR products were cloned into the pMALc2E as described above for MBP-VieS-C D1018A. The inserts for the domain truncation mutants were confirmed by DNA sequencing.

Δ*vieB* mutant was constructed using PCR amplicons made from *V. cholerae* AC50 template DNA while the *vieB* D62 point mutants were constructed using PCR amplicons made from *V. cholerae* AC53 (El Tor) template DNA. For Δ*vieB*, an in-frame clean deletion was generated by overlap extension PCR using the primer pair Δ*vieB* F1/Δ*vieB* R1 and Δ*vieB* F2/Δ*vieB* R2. Δ*vieB* F1 and Δ*vieB* R2 contain the SphI restriction sites for subsequent cloning. The PCR product was digested and ligated into pCVD442*lac* that was digested with SphI. The ligation was electroporated into DH5α

λpir and plated on LB supplemented with Amp. The plasmid was prepped and transformed into SM10 λpir via electroporation, which was then mated with AC50. Allelic exchange was achieved by counter-selection on sucrose. Final deletion of the coding sequence was confirmed by PCR and sequencing. For the *vieB* D62 point mutants, the mutant alleles were generated by overlap extension PCR using the primer pairs VieB D62 3kb F/VieB D62A R or VieB D62E R and VieB D62A F or VieB D62E R/VieB D62 3kb R. Final PCR products were co-transformed with a drug marked selectable plasmid (pBAD harboring kanamycin resistance) into AC53 via congression by natural competence using the described protocol in Dalia et al. (Dalia, McDonough et al. 2014). Genomic DNA was prepped from a subset of transformants and were sequenced using the primers VieB D62 screen F/R for conformation of the mutations. For AC50 stains containing pBAD33::*vieB* and pBAD33, plasmids was prepped from AC965 and AC1173, respectively. AC50 was made electrocompetent by growing 500 ml cultures in LB plus Sm to an OD_{600}=0.5-0.7. Cultures were chilled for 10 minutes in an ice bath. Cells were pelleted and washed two times with cold 2 mM $CaCl_2$ by centrifuging at 5,000 x *g* for 15 minutes at 4°C. After the second wash, cells were resuspended in 15 ml of cold 10% glycerol and centrifuged at 4,000 x *g* for 40 minutes. After this wash, cells were resuspended in 1 ml of cold 10% glycerol; aliquots were made, snap-frozen in an ethanol bath and stored at -80°C. Purified plasmids were then transformed into electrocompetent AC50 by electroporation, were plated on LB agar containing 10 µg/ml chloramphenicol (Cm) and incubated at 37°C overnight.

pMMB67EH::*vieA* E170A was prepped from AC2388 and transformed into electrocompetent AC50 via electroporation as described above. *vieA* D52E and *vieA*

D52E E170A were generated using overlap extension PCR; D52E point mutations were made using pMMB67EH::*vieA* and pMMB67eh::*vieA* E170A as templates using the primer pairs F BamHI VieA/VieA D52E R and VieA D52E F/R VieA st SphI for both constructs. Outer primers incorporated the BamHI and SphI restriction sites for subsequent cloning. Double digested products were ligated into the pMMB67EH vector, ligations were transformed into *E. coli* DH5α via electroporation and plated on LB supplemented with Amp. Plasmids were prepped and sent for sequencing to confirm the D52E point mutations. Correct plasmids were then transformed into electrocompetent AC50. For AC53 Δ*vieA,* a clean in-frame deletion was generated by overlap extension PCR using the primer pairs dvieA 3kb F/dvieA deletion R and dvieA deletion F/dvieA 3kb R. Final PCR products were transformed by natural competence as described above (Dalia, McDonough et al. 2014). Transformants were screened using colony PCR with the primers dvieA screen F/dvieA screen R. Genomic DNA was prepped from transformants that gave the correct sized PCR band and were sequenced to validate the clean deletion.

Double mutants were generated by making the Δ*crp* in either the AC53 Δ*vieA* or Δ*toxT* background, as the Δ*crp* mutant is unable to become naturally competent. For Δ*crp* Δ*vieA* and Δ*crp* Δ*toxT*, a clean in-frame deletion of *crp* was generated by overlap extension PCR using the primer pairs dCRP 3kb F/dCRP deletion R and dCRP deletion F/dCRP 3kb R. Final PCR products were cleaned and transformed by congression into AC53 Δ*vieA* or AC53 Δ*toxT* (Dalia, McDonough et al. 2014). Transformants were screened using colony PCR with the primers dCRP screen F/dCRP screen R. Validation of the *crp* clean deletion was conducted as describe above. For His_6-CRP expression, a

crp amplicon was generated using PCR using the primers CRP NdeI F/CRP BamHI R. Primers incorporated the NdeI and BamHI restriction sites for subsequent cloning into the pET15b-TEV His tagged vector as described in above. The insert in each plasmid was confirmed by DNA sequencing prior to transformation into *E. coli* BL21 (DE3) for protein expression.

7.2 PROTEIN EXPRESSION AND PURIFICATION

E. coli BL21 (DE3) containing the protein expression vectors were grown in 1 L cultures to an OD_{600} = 0.5-0.8. Protein expression was induced by 1 mM IPTG and grown at 20°C for 16-17 hours. Cell cultures were harvested by centrifugation at 2, 990 x *g* for 20 minutes and resuspended in 25 ml of the following buffers: for GST-VieS-C, MBP-VieS-C D1018A and all MBP-tagged VieB domain truncation mutants, 20 mM Tris pH=8, 150 mM NaCl, 5 mM *beta* mercaptoethanol (βME), Complete EDTA-free protease inhibitor cocktail tablet (Roche); and for VieA-His_6, His_6-VieB, and His_6-VieB D62 mutants and His_6-CRP, 20 mM Tris pH=8, 150 mM NaCl, 25 mM Imidazole, 5 mM βME, Complete EDTA-free protease inhibitor cocktail tablet (Roche). The resuspended cells were lysed on ice with five, 30-second pulses (0.5 seconds on, 0.5 second off) of sonication at 50% amplitude with one-minute rest in between pulses. Cell debris was pelleted by centrifugation at 38,464 x *g* for 45-60 minutes at 4°C and the supernatant was collected for immediate protein purification.

For GST-VieS-C, supernatant was incubated on 7 ml of Glutathione Sepharose 4B beads (GE Healthcare) for 30 minutes at 4°C. The beads were washed with three column volumes (CV) of 20 mM Tris pH=8, 200 mM NaCl, 5 mM βME. Protein was eluted in 30 ml of 100 mM Tris pH=8, 20 mM reduced glutathione, 100 mM NaCl, 5 mM

βME. For VieS-C, the GST tag was removed by the addition of TEV protease overnight at 4°C. Both GST-VieS-C and VieS-C were diluted three-fold with Buffer A (20 mM Tris pH=8, 1 mM DTT) and applied directly to a 4 ml Source15Q anion exchange column (GE Healthcare) that has been equilibrated in Buffer A. Protein was eluted using a 0-50% (v/v) Buffer B (20 mM Tris pH=8, 1M NaCl, 1 mM DTT) gradient developed over 15 CV. The peak fractions were pooled and concentrated by a series of centrifugations at 2,514 x *g* at 4°C using the Amicon Ultra-15 10K centrifugal filters (Millipore). Protein was then applied to a Superose12 prep grade 16/70 gel filtration (GE Healthcare) column that was equilibrated in Gel Filtration Buffer (25 mM Tris pH=7.5, 100 mM KCl, 1 mM DTT, 5% glycerol (v/v)). For VieS-C, peak fractions were pooled and incubated a second time on fresh 2 ml of Glutathione Sepharose 4B beads equilibrated in Gel Filtration Buffer for 30 minutes at 4°C to remove any remaining GST tag or GST-VieS-C protein. Flow-through containing VieS-C was collected and concentrated as described above.

For MBP-VieS-C D1018A, MBP-VieB ΔRec, MBP-VieB ΔTPR, and MBP-VieB ΔUKF, supernatant was incubated on 5 ml of amylose high flow resin (New England Biolabs) for 30 minutes at 4°C. Beads were washed with five CV of 20 mM Tris pH=8, 150 mM NaCl, 5 mM βME and eluted in 30 ml of 20 mM Tris pH=8, 100 mM NaCl, 1 mM DTT and 30 mM maltose. For VieB ΔRec, VieB ΔTPR and VieB ΔUKF, the MBP tag was removed by the addition of TEV protease overnight at 4°C. Proteins were diluted three-fold with Buffer A and applied to a 4 ml Source15Q anion exchange column. All protein fusions were eluted using a 0-50% (v/v) Buffer B gradient developed over 15 CV.

The peak fractions were pooled, concentrated and applied to a Superose12 prep grade 16/70 gel filtration column equilibrated in Gel Filtration Buffer.

For VieA-His_6, His_6-VieB and His_6-VieB D62 point mutants, supernatant was incubated on 7 ml of His-Pur NiNTA beads (Thermo Scientific) for 30 minutes at 4°C. Beads were washed three times with three CV of Wash 1 (20 mM Tris8, 150 mM NaCl, 25 mM imidazole, 5 mM βME), three times with three CV of Wash 2 (same as Wash 1 except 50 mM imidazole), and eluted in 30 ml of Elution Buffer (same as Wash 1 except 300 mM imidazole). For VieB and the D62 point mutants, the His tag was removed by the addition of TEV protease overnight at 4°C. Proteins were diluted three-fold with Buffer A and applied to a 4 ml Source15Q anion exchange column. VieA-His_6 was eluted using a 0-40% (v/v) Buffer B gradient, while VieB and the D62 mutants were eluted using a 0-35% (v/v) Buffer B gradient, both developed over 15 CV. The peak fractions were pooled, concentrated, and applied to a Superose12 prep grade 16/70 gel filtration column equilibrated in Gel Filtration Buffer.

Purification of His_6-CRP was as follows: protein supernatants were incubated on 5 ml of His-Pur NiNTA beads (Thermo Scientific) for 30 minutes at 4°C. Beads were washed as described above for other His tagged protein fusions. Buffer exchange of the eluant containing His_6-CRP from the Elution buffer into EMSA buffer (10 mM Tris, pH=8, 50 mM KCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) was achieved by loading the eluant on the HiPrep 26/10 desalting column that was previously equilibrated in EMSA buffer. Fractions containing His_6-CRP were collected and pooled for subsequent use in EMSA reactions. These fractions were not concentrated due to the high likelihood of precipitation.

7.3 Biochemical Assays

7.3.1 Phosphotransfer assays

Phosphotransfer reactions were carried out as described by Martinez et al (Martinez-Wilson, Tamayo et al. 2008) with the following modifications. Phosphotransfer reactions were incubated in phosphotransfer buffer (Gel Filtration Buffer supplemented with 5 mM $MnCl_2$, 25 μM radiolabeled ^{32}P-ATP-γ [10 mCi/ml; Perkin Elmer, Boston, MA] and 1.25 μM cold ATP). GST-VieS-C, MBP-VieS-C D1018A and VieA-His_6 were at final concentrations of 1 μM while VieB, the VieB domain truncation mutants and the D62 mutants were at a final concentration of 5 μM, unless stated otherwise. Once reactions were stopped by the addition of 2X denaturing sample buffer (10 mM Tris pH=6.8, 4% (w/v) SDS, 20% (v/v) glycerol, bromophenol blue, βME), proteins were separated on 10% SDS-PAGE gel at 200 V for 45 minutes. Autoradiographs were recorded and quantified with FUJI FILM Image Reader FLA-9000 (Life Science) and FUJI FILM Multi-Gauge software (Life Science). Due to inherent experimental variation, an internal control of GST-VieS-C and VieA-His_6 phosphotransfer in the absence of VieB is included with every experiment.

7.3.2 Phosphorylated VieS stability and transfer assays

For phosphorylated VieS stability assays, 1 μM GST-VieS-C or MBP-VieS-C D1018A was incubated with 25 μM ^{32}P-ATP-γ for 30 minutes at 30°C. To remove excess ATP, phosphorylated VieS-C (P-VieS-C) constructs were passed through a Performa

DTR spin gel filtration column (EdgeBio) for two minutes at 750 x *g*. Additional $MnCl_2$ was added and either Gel Filtration Buffer or 5 μM VieB was added to P-VieS-C constructs and incubated at 30°C. The amount of P-VieS-C was measured over time. At indicated time points, reactions were stopped and analyzed as previously described for the phosphotransfer assays.

For phosphorylated VieS transfer assays, 1 μM GST-VieS-C was incubated with 25 μM ^{32}P-ATP-γ for 30 minutes at 30°C. To remove excess ATP, P-VieS-C constructs were passed through a Performa DTR spin gel filtration column (EdgeBio) as described above. Additional $MnCl_2$ was added and then either Gel Filtration Buffer, 1 μM VieA-His_6, 5 μM VieB or pre-mixed 1 μM VieA-His_6 and 5 μM VieB was added to P-VieS-C and incubated at 30°C. At indicated time points, reactions were stopped and analyzed as previously described for the phosphotransfer assays.

7.3.3 VieS phosphatase assay

GST-VieS-C was bound to Glutathione Sepharose 4B beads equilibrated in phosphotransfer buffer. Beads were washed and VieA-His_6 was added in the presence of 25 μM radiolabeled ^{32}P-ATP-γ and incubated for 30 minutes at 30°C. Beads were washed three times with 150 μl of 25 mM Tris pH=7.5, 150 mM KCl, 1 mM DTT by centrifugation for 30 seconds at 12, 000 x *g* using Pierce Spin Columns (Thermo Scientific) to collect phosphorylated VieA-His_6 (P-VieA-His_6). P-VieA-His_6 was incubated with new GST-VieS-C alone or pre-mixed GST-VieS-C and VieB in phosphotransfer buffer in the absence of [^{32}P-γ]. Samples were collected over time and reactions were analyzed and quantified as described above for the phosphotransfer assays.

7.3.4 Lineweaver-Burk Plot

Phosphotransfer assays were used to generate the Lineweaver-Burk plot except VieA-His_6 concentrations ranged from 2-8 μM and VieB concentrations ranged from 0-12 μM while GST-VieS-C remained constant (1 μM). Samples were taken at 0, 3.5, 7.5, and 15 minutes. The amount of phosphorylated VieA-His_6 was quantified over time in order to generate enzyme reaction velocities at each concentration combination. A double reciprocal plot was generated by plotting the inverse of the enzyme reaction velocity against the inverse of VieA-His_6 (substrate) concentration using GraphPad Prism5 (GraphPad). Extrapolation of each line to determine the intercept was determined by linear regression analysis.

7.3.5 Multi-Angle Light Scattering

Size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) was used to determine oligomeric state of each protein. SEC-MALS was conducted using the ÄKTA HPLC Explorer system (GE Healthcare) connected to a vacuum degasser (Alltech), a Superose12 10/300 GL gel filtration (GE Healthcare) column equilibrated in 0.1 μm filtered Gel Filtration Buffer that is directly connected to a DAWN HELEOS II light scattering detector (Wyatt Technology Corporation) and an Optilab T-rEX refractive index detector (Wyatt Technology Corporation). The column temperature was held at 4°C while light scattering detection was conducted at 25°C. A MALS baseline was established with Gel Filtration Buffer overnight. Light scattering and concentration data were acquired and analyzed with ASTRA software (version 6, Wyatt Technology Corporation) to determine molar mass in solution and the oligomeric sate of

each protein. All light scattering experiments were conducted using the untagged VieS-C protein, which is able to dimerize in the absence of the GST tag.

Composition gradient MALS (CG-MALS) was conducted to probe the hetero-association interactions between the VieSAB proteins (Attri 2005; Kameyama and Minton 2006; Halling, Kenrick et al. 2014). The Calypso II syringe pump system (Wyatt Technology Corporation) was used to inject various protein concentration mixtures into the DAWN HELEOS II light scattering detector that was attached to the UV detector of the ÄKTA HPLC Explorer system (GE Healthcare). Proteins were diluted into 0.1 μm filtered Gel Filtration Buffer at a predetermined stock concentration and were subsequently filtered through a 0.02 μm filter. After each injection, the flow was stopped for 30-180 seconds to allow protein interactions to reach equilibrium. Hetero-association stoichiometry and dissociation constants (K_D) were determined using the CALYPSO software (version 2.1.3, Wyatt Technology Corporation) based on the light scattering and UV signal acquired during the stopped flow for each concentration gradient. All CG-MALS experiments were conducted using the untagged VieS-C protein.

7.3.6 GST-pulldown assays

Unconjugated GST tag, used as a negative control for purified protein GST pull-downs, was purified as follows: TEV protease was added to previously purified GST-VieS-C overnight at 4°C. The sample was incubated on 500 μl of Glutathione Sepharose 4B beads that were equilibrated in Gel Filtration Buffer for 30 minutes at 4°C. Beads were washed and eluted as described above for GST-VieS-C. Protein was directly applied to a HiLoad 16/60 Superdex75 prep grade gel filtration (GE Healthcare) column to

separate GST alone from any un-cleaved GST-VieS-C protein. The peak fractions corresponding to GST tag alone were collected and used for subsequent experiments.

For GST pulldowns using whole cell lysates, 50 ml cultures of BL21 (D3E) strains that over-expressed pET15b-TEV::*vieA*-His_6, pET15b-TEV::His_6-*VieB,* or the pET15b-TEV::His tag empty vector were grown to OD_{600}=0.5-0.7 in LB plus Amp and induced with 1mM IPTG for 16 hours at 20°C. Whole cell lysates were collected by centrifugation for 10 minutes at 2, 990 x *g*. Cell pellets were resuspended in 4 ml 20 mM Tris pH=8, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EDTA and protease inhibitor cocktail (Roche). Resuspended cultures were disrupted via bead beating using 500 µl of 0.1 mm beads for 3 minutes and centrifuged for 2 minutes and 30 seconds at 16,000 *x g*. Supernatants were collected and 5 µl of Benzonase was added for 30 minutes at 37°C to remove DNA and RNA. Supernatants were spun at 9,000 x g for 15 minutes at 4°C to remove debris and were filtered through a 0.45 µm low protein-binding filter. GST-VieS-C was immobilized on 50 µl Glutathione Sepharose 4B beads for 30 minutes at 4°C using Pierce Spin Columns. Beads and bound GST-VieS-C were washed three times with 150 µl of 25 mM Tris pH=7.5, 150 mM KCl, 1 mM DTT by centrifugation for 30 seconds at 12, 000 x *g*. Whole cell protein lysates from each strain were incubated for 30 minutes at 30°C. Reactions were washed three times each with three CV of 25 µM Tris pH=7.5, 100 mM KCl, 1 mM DTT using the same centrifugation conditions. GST-VieS-C and bound proteins were eluted using 150 µl of the elution buffer described above for GST-VieS-C purification. Samples from the input lysate, flow through, wash and elution fractions were taken, 2X denaturing sample buffer was added, and were analyzed by 10% SDS-PAGE gel that was stained with Lumitein protein stain™ (Biotium). Gel images were

acquired using the FUJI FILM Image Reader FLA-9000 and protein bands were quantified using FUJI FILM Multi-Gauge software.

For purified protein pulldowns, 1 μM of either GST-VieS-C or GST tag was incubated on 50 μl Glutathione Sepharose 4B beads for 30 minutes at 4°C using Pierce Spin Columns. Bound protein was washed three times with 150 μl of 25 mM Tris pH=7.5, 150 mM KCl, 1 mM DTT by centrifugation described above. 5 μM VieA-His_6 that was premixed with either buffer, 25 μM VieB, or 25 μM BSA was incubated on the beads for 15 minutes at 30°C. Reactions were washed five times with three CV of 25 μM Tris pH=7.5, 100 mM KCl, 1 mM DTT using the same centrifugation conditions above. An additional dry spin was conducted to ensure all of the wash was removed from the column. GST-VieS-C or GST and all bound proteins were eluted using 150 μl of the elution buffer described above for GST-VieS-C purification. Samples were taken from the input, wash and elution fractions, analyzed and quantified as described above for whole cell lysate pulldown experiments.

7.3.7 VieSAB heterotrimeric complex assays

GST-VieS-C, VieA-His_6 and VieB were mixed at equal molar amounts in Gel Filtration Buffer and incubated for 30 minutes at 30°C. The protein mixture was immediately injected over Superdex 75 gel filtration column (GE Healthcare), which was previously equilibrated in Gel Filtration Buffer. Fractions were collected and samples were taken from each peak-containing fraction and mixed with 2X denaturing sample buffer. These samples were run on a 10% SDS-PAGE denaturing gel and proteins were observed by staining with Coomassie (10% Glacial Acetic Acid, 50% Methanol and 0.1%

Coomassie Brilliant Blue R250). The proteins present in each fraction were then assigned to each respective peak.

7.3.8 EMSA assays

For VieA EMSA experiments, VieA-His_6 was purified as described in Section 7.2. VieA-His_6 was incubated with or without 1M acetyl phosphate (Ac-P) for 2 hrs at 30°C in Gel Filtration Buffer supplemented with 0.1M $MgCl_2$. After phosphorylation, the reaction was passed through an EdgeBio column to remove excess Ac-P. For EMSA reactions using S100 protein fractions, DH5α containing pBAD33::*vieA* (AC964) or pBAD33 empty vector (AC1173) were grown in 50 ml cultures at 37°C until cultures reached an OD_{600} of 0.5-0.7. At this time, cultures were induced with 0.2% arabinose and grown at 20°C for 16 hours. Cultures were harvested by centrifugation for 10 minutes at 2,990 x *g*. Cell pellets were resuspended in 6 ml 20 mM Tris pH=8, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EDTA and protease inhibitor cocktail (Roche). Resuspended cultures were disrupted via bead beating using 500 µl of 0.1 mm beads for 2 minutes and 45 seconds and centrifuged for 4 minutes at 16,000 *x g*. Supernatants were collected and 5 µl of Benzonase was added for 30 minutes at 37°C. Supernatants were spun at 100,000 x *g* for 1 hour at 4°C to remove any remaining debris and the S100 supernatants were collected.

For the VieA EMSA reactions, the *vieSAB* promoter fragment was generated using AC50 template for PCR using the primer pair vieSAB PROBE F/vieSAB PROBE R. The PCR was labeled by the incorporation of Cy5-labeled dCTP (GE Healthcare) during the PCR reaction. VieA constructs (purified or protein fractions) were incubated over a range of concentrations with a final probe concentration of 5 nM, 0.1 mg/ml

sheared calf thymus DNA, 0.1 mg/ml BSA in Gel Filtration Buffer for 30 minutes at 30°C in the dark. Reactions were run on a 6% TBE native gel for 45 minutes at 150V in 1X TBE buffer.

For CRP EMSA and probe truncation experiments, the *vieSAB* probe truncations were generated using AC53 as template. PCR was used to generate each probe using the primer pairs listed in Table 7-4 (primer sequences are listed in Table 7-2). The PCR was labeled as described above. His_6-CRP was purified (Section 7-2) and incubated at either 0 μM, 1 μM, or 5 μM with a final probe concentration of 5 nM, 0.1 mg/ml sheared calf thymus DNA, 0.1 mg/ml BSA in EMSA buffer (10 mM Tris, pH=8, 50 mM KCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) in the presence or absence of 500 mM cAMP for 15 minutes at 30°C in the dark. Reactions were run on a 6% TBE native gel for run for either 1 hour at 150 V (*vieSAB* and probes 1, 3-5, 7-10) or 1 hour 30 minutes at 100 V (probes 2, 6) at 4°C in the dark in 0.5X TBE running buffer. For all EMSA experiments, the fluorescent probe was observed using the Cy5 fluorescent channel using the FUJI FILM Image Reader FLA-9000 (Life Science) and FUJI FILM Multi-Gauge software (Life Science).

7.3.9 VieA PDE activity assays

VieA-His_6, VieS-C and VieB were purified as previously described in Section 7.2. Reactions were incubated in the presence of 5 mM bis-pNpp (Sigma) in either standard assay buffer (Tris pH-8.5, 1mM $MnCl_2$ +/- 25 μM ATP) or in phosphotransfer buffer (Tris pH-7.5, 100 mM KCl, 5% glycerol, 1 mM DTT, 1mM $MnCl_2$, 25 μM ATP). Various combinations of proteins were incubated at 30°C over 2 hours, as indicated in the figure legend (Figure 4-7). BSA was used as a negative control. Absorbance at 405 nm

was measured over the 2-hour time course and used as a proxy for PDE cleavage. All values were normalized to the background absorbance of buffer alone. These values were plotted over time using GraphPad Prism5 (GraphPad) to observe the rate of the reaction for each combination of proteins.

7.4 Behavioral Assays

7.4.1 Motility assays

Motility assays for classical, the wild type AC50, Δ*vieB* and Δ*vieA*, strains were grown to stationary phase in M9 glycerol or M9 NRES glycerol at 30°C with aeration. For El Tor *vieB* D62 point mutants, wild type (AC53), *vieB* D62A and *vieB* D62E were grown to stationary phase in M9 NRES 0.5% glucose or 0.5% glycerol at 30°C with aeration. For both experiments, 1 µl of the liquid culture was stabbed into minimal media plates containing 0.3% agar and were incubated 30°C overnight and the swimming diameters were measured.

For over-expression of *vieB* in classical biotype, wild type AC50, Δ*vieA*, and AC50 derivatives containing either pBAD33::*vieB* or pBAD33 empty vector were grown to stationary phase in LB, M9 glycerol, or M9 NRES glycerol at 37°C (LB) or 30°C (M9 media) with aeration. 1 µl of the liquid culture was stabbed into each respective media that was either supplemented with or without 0.2% arabinose, which all contained 0.3% agar. Plates were incubated at 30°C for 17 hrs for LB, 4 days for M9 glycerol or 1.5 days for M9 NRES glycerol and the swimming diameters were measured.

7.4.2 Single strain biofilm assay

Wild type AC50, Δ*vieB* and Δ*vieS* were grown overnight in M9 glycerol or M9 NRES glycerol at 37°C with aeration. Cultures were backed diluted 1:50 in each respective media and allowed to undergo static growth at RT for 4 days in glass culture tubes. The liquid culture was removed and biofilm formation was measured by staining with 0.5 mg/ml of crystal violet for 30 minutes at RT. Excess stain was removed by washing with 1 ml of PBS three times. 1 ml of 100% ethanol was added and the absorbance was read at 570 nm.

7.4.3 In vitro competition assays

For biofilm formation competition assays, a wild type *lacZ*⁻ derivative of AC50 and Δ*vieB lacZ*⁺ or Δ*vieS lacZ*⁺ strains were grown overnight in M9 NRES glycerol. Cultures were matched based on OD_{600} and mixed 1:1 at a final OD_{600}=0.1 in M9 NRES glycerol. Inputs were plated on indicator LB plates supplemented with Sm and 10 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). 1:1 mixed cultures were back diluted into M9 NRES glycerol and cultures were underwent static growth at RT for 4 days in glass culture tubes. The liquid culture was removed and biofilms were washed once with 1 ml PBS. Biofilms were disrupted in 1 ml of PBS by vortexing with beads for 30 seconds. Outputs were plated on indictor plates and incubated at 30°C overnight. White and blue colonies were counted and a competitive index was calculated using this formula:

$$\text{C.I.} = \text{Output Ratio} / \text{Input Ratio}$$

Where the Output Ratio = (Output mutant CFU / Output wild type CFU) and the Input Ratio = (Input mutant CFU / Input wild type CFU).

For growth on chitin competition assays, a wild type *lacZ*⁻ derivative of AC50 and Δ*vieB lacZ*⁺ strains were grown overnight in M9 medium. Cultures were matched based on OD_{600} and mixed 1:1 to an OD_{600}=0.1 in M9. Inputs were plated for CFU on LB supplemented with Sm and 10 μg/ml X-gal. 1:1 mixed cultures were back diluted into 0.5X Instant Ocean and underwent static growth at 30°C for 4 days in the presence of chitin. The supernatant was removed, vortexed for 30 seconds and plated for CFU for planktonic bacteria. The remaining chitin was vortexed for 30 seconds and sonicated for 1 minute in 1ml of 0.5X Instant Ocean to remove bound *V. cholerae* and this supernatant was plated for output CFU on LB supplemented with Sm and X-gal as described above. A competitive index was calculated for both the supernatant and chitin-bound supernatant samples.

For *vieB* D62 mutants in the El Tor biotype, a wild type *lacZ*⁻ derivative of AC53 and either *vieB* D62A *lacZ*⁺ or *vieB* D62E *lacZ*⁺ strains were grown overnight in LB broth. Cultures were matched by OD_{600} and mixed 1:1 in LB to a final OD_{600} of 0.01. For LB in vitro growth, 1 μl of the 1:1 mixture was added to 2 ml LB and cultures were grown at 37°C overnight. For in vitro pond survival, 100 μl of the 1:1 mixture was added to 5 ml of autoclaved pond water and cultures were incubated statically at RT for 24 hours. The 1:1 mixtures were plated for input CFU, serial dilutions of the output were plated for CFU, and a competitive index was calculated as described above.

7.4.4 In vivo competition assay

A wild type *lacZ*⁻ derivative of AC53 and either *vieB* D62A *lacZ*⁺ or *vieB* D62E *lacZ*⁺ strains were grown overnight in LB plus Sm. Cultures were matched by OD_{600} and mixed 1:1 in LB to a final OD_{600} of 0.01. 50 μl of the 1:1 mixture was inoculated into

sedated infant mice, which were starved for 1 hour prior to inoculation, by oral gavage. Inputs from the 1:1 mixture were plated for CFU on LB plus Sm supplemented with X-gal plates. Mice small intestines were harvested at 24 hours post inoculation and homogenized in 1ml of PBS. Homogenates underwent a low-speed spin to remove cell debris for 2 minutes and were diluted and plated for CFU. A competitive index was calculated as previously described above.

7.4.5 In vivo mouse to pond transition assay

A wild type *lacZ*⁻ derivative of AC53 and either *vieB* D62A *lacZ*$^+$ or *vieB* D62E *lacZ*$^+$ strains were grown overnight in LB plus Sm. Cultures were matched by OD_{600} and mixed 1:1 in LB to a final OD_{600} of 0.01. 50 µl of the 1:1 mixture was inoculated into sedated infant mice, which were starved for 1 hour prior to inoculation, by oral gavage. Mice small intestines were harvested at 24 hours post inoculation and homogenized in 1ml of PBS. Homogenates underwent a low-speed spin for 2 minutes and diluted and plated for input CFU as described above. 100 µl of homogenate supernatant was directly inoculated into 5 ml of autoclaved pond water supplemented with Sm. Cultures were statically incubated at RT for 24 hours. 2 ml of cultures were concentrated 40-fold and outputs were serially diluted and plated for CFU on LB plus Sm supplemented with X-gal plates. A competitive index was calculated as described previously.

7.5 qRT-PCR ASSAYS

For Δ*vieB* transcriptional assays, overnight wild type AC50 or Δ*vieB* cultures were back diluted in M9 NRES glycerol or LB and grown with aeration at 30°C or 37°C, respectively. Samples were taken at the indicated OD_{600} and diluted to an OD_{600} of 0.3 in 500 µl of respective media.

For VieA autoregulation by *vieA* over-expression experiments, respective strains listed in the figure legend (Figure 4-1 and 4-2) were grown overnight at 37°C on LB plates supplemented with Amp. Colonies were scraped off the plate and back diluted to an OD_{600} of 0.01 in 3 ml of LB plus Amp. Cultures were grown with aeration at 37°C until an OD_{600} of 0.5-0.6 was reached. At this point, cultures were split and 1 mM IPTG was added or omitted. Samples were taken at the indicated times post IPTG induction and diluted to an OD_{600} of 0.3 in 500 µl of LB.

For VieA autoregulation experiments conducted in the AC53 background in AKI media, respective strains were grown at 37°C on LB plates supplemented with Sm. Colonies were scraped off the plate and were diluted into 1 ml of LB to an OD_{600} of 1. 100 µl of the diluted cultures were added to 10 ml of AKI media (AKI base with 3% $NaHCO_3$) in large glass culture tubes. Cultures were grown 4 hr statically and then the culture was divided in half into large culture tubes to increase the surface to volume ratio. These cultures were grown for 4 hours with aeration at 37°C. Samples were taken at the indicated time points and diluted to an OD_{600} of 0.3 in 500 µl of AKI media.

For CRP repression in acidic LB experiments (pH=6.5), strains were grown on LB plates supplemented with Sm overnight at 37°C. Single colonies were inoculated into 2 ml of LB pH=6.5 and grown at 30°C overnight, rolling. Overnight cultures were back diluted into fresh LB pH=6.5 to an OD_{600} of 0.01 and grown rolling at 30°C until cultures reached OD_{600} of 1.5-1.7. For *mtlA* and *mtlD* experiments, Δ*cyaA* was grown similarly to acidic LB experiments, except 0, 2, 4, or 8 mM exogenous cAMP was added to the fresh media upon back dilution. All samples were taken when cultures reached an OD_{600} of 1.5-1.7 and were diluted to an OD_{600} of 0.3 in 500 µl LB pH=6.5.

For all qRT-PCR experiments, 1 ml of RNA Protect (Qiagen) was added after sample collection, vortexed, and incubated at RT for 5 min. RNA samples were collected by centrifugation at 16,000 *x g*, the supernatant removed and the pellet stored at -80°C until ready for RNA extraction. RNA was extracted using the RNeasy kit (Qiagen), RNA treated to remove DNA using the DNase Turbo kit (Ambion) and cDNA was generated using the iScript Select SYBR Green RT-PCR kit (BioRad) per the manufacture's instructions. qRT-PCR was performed on the Stratagene Mx3000P instrument (Agilent) using IQ SYBR Green Supermix (BioRad) and primers listed in Table 7-3 to measure mRNA transcript levels for each respective gene.

7.6 Western Assays and antibody generation

7.6.1 Western analysis

For acidic LB (pH=6.5) experiments, strains were grown as described in Section 7-5 for CRP repression in acidic LB. For western blot experiments in M9 NRES containing 0.5% glucose or glycerol, strains were grown on M9 NRES plates containing 0.5% glycerol. Single colonies were inoculated into 2 ml of M9 NRES pH=6.5 supplemented with either 0.5% glucose or glycerol and allowed to grow overnight at 30°C. These cultures were back diluted into fresh media, respectively, and grown to stationary phase as described above. For exogenous cAMP experiments, Δ*cyaA* was grown as described above for acidic LB experiments expect 0 mM or 4 mM cAMP (Sigma) was added to the fresh media prior to back dilution.

For all western blotting experiments, 1 ml of culture was pelleted by centrifugation at 10,000 x *g* for 5 minutes at RT. Pellets were resuspended in 50 µl of 2X denaturing sample buffer and boiled for 10 minutes. Samples were diluted 1:5 for

VieSAB and 1:100 for RNA polymerase in sample buffer. Diluted samples were run on a NuPAGE 4-12% gradient SDS-PAGE gel in 1X MES SDS running buffer (Invitrogen) for 35 minutes at 200V and transferred to a nitrocellulose membrane in 1X NuPAGE transfer buffer (Invitrogen) for 90 minutes at 25V. The nitrocellulose was blocked for 1 hour, shaking, at RT with 2X NAP Blocker (G Biosciences). Blots were probed for VieS, VieA, VieB and RNA Polymerase for 1 hour at RT, shaking, using the following primary antibodies: mouse anti-VieS, rabbit anti-VieA (our laboratory) mouse anti-VieB and mouse anti-α RNA polymerase (Santa Cruz Biotechnology). Blots were then washed three times for five minutes each in 1X TBS containing 0.1% Tween 20 (Sigma). Cy5-fluorescently labeled secondary antibodies (anti-mouse or anti-rabbit) were added (Invitrogen) and incubated at RT, shaking, for 1 hour in the dark. The blots were washed in the dark as described above and bands were quantified using FUJI FILM Image Reader FLA-9000 (Life Science) and FUJI FILM Multi-Gauge software (Life Science). Values were normalized to RNA polymerase and the fold change compared to wild type was calculated.

7.6.2 Antibody generation

VieS and VieB antibodies were generated by intraperitoneal injection of mice with 10 μg of purified VieS-C or VieB proteins (for purification conditions see Section 7.1.2) mixed with Complete Freund's Adjuvant (MP Biomedicals) to increase antibody production. Mice were boosted 2 weeks later with 10 μg of purified VieS-C or VieB proteins mixed with INcomplete Freund's Adjuvant (MP Biomedicals). Two weeks after the boost, blood from the mice was harvested by terminal bleeds coupled with perfusion of the circulatory system with 10 ml of PBS. Red blood cells and debris were pelleted by

a centrifugation at 1,000 x *g* for 10 minutes at 4°C. Serum was collect and stored at -80°C.

Table 7-1. Strains and plasmids used in this study.
Laboratory collection; LC.

Strain	Description	Protein Expressed	Reference/Source
AC50	*V. cholerae* classical O395 biotype	N/A	(Taylor, Miller et al. 1987); Lab Collection (LC)
AC4713	*E. coli* BL21 (DE3) pGEX-TEV::*vieS-C*	GST-VieS-C	(Mitchell 2015)
AC4714	*E. coli* BL21 (DE3) pMMB67eh::*vieA-His$_6$*	VieA-His$_6$	(Tamayo, Tischler et al. 2005)
AC4715	*E. coli* BL21 (DE3) pET15b-TEV::*His$_6$-vieB*	His$_6$-VieB	(Mitchell 2015)
AC4716	*E. coli* BL21 (DE3) pET15b-TEV::*His$_6$-vieB D62A*	His$_6$-VieB D62A	(Mitchell 2015)
AC4717	*E. coli* BL21 (DE3) pET15b-TEV::*His$_6$-vieB D62E*	His$_6$-VieB D62E	(Mitchell 2015)
AC4002	*E. coli* BL21 (DE3) pMalc2E-TEV::*vieS-C*	MBP-VieS D1018A	(Martinez-Wilson, Tamayo et al. 2008)
AC5057	*E. coli* BL21 (DE3) pMalc2E-TEV::*vieB ΔRec*	MBP-VieB ΔRec	This study
AC5058	*E. coli* BL21 (DE3) pMalc2E-TEV::*vieB ΔTPR*	MBP-VieB ΔTPR	LC; Ayman Ismail
AC5059	*E. coli* BL21 (DE3) pMalc2E-TEV::*vieB*	MBP-VieB ΔUKF	This study

	Δ*UKF*		
AC1159	*V. cholerae* O395 pBAD33	N/A	LC; Anna Tischler/Anne Bishop
AC5060	*E. coli* BL21 (DE3) pET15b-TEV::His_6	His empty vector	This study
AC4282	*V. cholerae* O395 Δ*vieB lacZ*$^+$	N/A	LC; Andrew Camilli
AC53	*V. cholerae* E7964	N/A	LC
AC5061	AC53 *vieB D62A*	N/A	This study
AC5062	AC53 *vieB D62E*	N/A	This study
AC1383	AC50 Δ*vieA*	N/A	LC; Anna Tischler
AC1101	AC50 Δ*vieS*	N/A	(Martinez-Wilson, Tamayo et al. 2008)
AC3745	AC53 Δ*lacZ*	N/A	LC; Evan Bradley
AC61	AC50 *lacZ*$^-$	N/A	LC; Andrew Camilli
AC1173	*E. coli* DH5α pBAD33	Empty vector	LC; Anna Tischler
AC965	*E. coli* DH5α λpir pBAD33::*vieB*	VieB	LC; Anna Tischler
AC964	*E. coli* DH5α λpir pBAD33::*vieA*	VieA	LC; Anna Tischler
AC1109	AC50 0395 pMMB67eh	Empty vector	LC; Anna Tischler
AC1818	AC50 0395 pMMB67eh::*vieA*	VieA	LC; Anna Tischler
AC2388	N16961 pMMB67eh::*vieA* E170A	VieA E170A	LC; Rita Tamayo

AC5063	AC50 pMMB67eh::*vieA* E170A	VieA E170A	This study
AC5064	AC50 pMMB67eh::*vieA* D52E	VieA D52E	This study
AC5065	AC50 pMMB67eh::*vieA* D52E E170A	VieA D52E E170A	This study
AC3763	AC53 Δ*toxT*	N/A	LC; Evan Bradley
AC5066	AC53 Δ*vieA*	N/A	This study
AC5070	AC53 Δ*crp*	N/A	LC; Ankur Dalia
AC5071	AC53 Δ*cyaA*	N/A	LC; Ankur Dalia
AC5067	AC53 Δ*crp* Δ*vieA*	N/A	This study
AC5068	AC53 Δ*crp* Δ*toxT*	N/A	This study
AC5069	*E. coli* BL21 (DE3) pET15b-TEV::*His_6-crp*	CRP	This study

Table 7-2. Primers used in this study.
All primers are listed 5' to 3'. This table does not include qRT-PCR primers, which are listed below in Table 7-3. Underlined sequences represent restriction enzyme sites.

Primer Name	Primer Sequence 5'-3'
GST-VieS-C F	TCACTGTGCATATGACTGAGCAGCTACGTTGGTTGACG
GST-VieS-C R	GAGCGAGTCGGATCCTCAGAGATAACGACTGAGTACTTTGCGC
GST-VieS-C T2916C F	GTTGATTACTGACTGCCACATGCCACATCTTGATG
GST-VieS-C T2916C R	CATCAAGATGTGGCATGTGGCAGTCAGTAATCAAC
VieB F	TCACTGTGCATATGGCTGTACCTACTTTTGCTGAATTAAAAG
VieB R	GAGCGAGTCGGATCCTTACGCCTCAACTGATTCGCTTCGC
VieB D62A F	GATTTGATATTTTTATTTGCGCTTACAACTTCGGTAAGGGGTT
VieB D62A R	AACCCCTTACCGAAGTTGTAAGCGCAAATAAAAATATCAAATC
VieB D62E F	GATTTGATATTTTTATTTGCGAGTACAACTTCGGTAAGGGGTT
VieB D62E R	AACCCCTTACCGAAGTTGTACTCGCAAATAAAAATATCAAATC
MBP-VieS-C F	CGCGGATCCTTACGCAGCTCCGAACAAG
MBP-VieS-C R	ACGCGTCGACTTATTCGCTCTGATACTGATG
MBP-VieS-C D1018A F	TATGGCAATTGGTAATCAACAAATCATACTGCTCAGGATGTTGCGAGAGCTTTT
MBP-VieS-C D1018A R	CGAAAAGCTCTCGCAACATCCTGAGCAGTATGATTTGTTGATTACCAATTGCCA
Δ*vieB* F1	GCGCATGCAAGGTGTTTGTGGCGC
Δ*vieB* R1	GCGGTTACATAACTCTCGGTACTATTTTAATGTTACAAAACGC
Δ*vieB* F2	CCGAGAGTTATGTAACCGCCCTCTACAGAGGAC
Δ*vieB* R2	GCGCATGCAAATTACCTTCAAACCTGAGCAG
VieB D62 3kb F	CGAGTCGCTGTTTGTTCCTT
VieB D62 3kb R	TAGCTGCCGTTGAGATGTTG
VieB D62 screen F	GAGAGTTGACGGTTGCGAAA
VieB D62 screen R	AGACCTAATTCAGCATCGCCA
F NdeI 487VieB	TCACTGTGCATATGTACAACGATTACTATTTCGTGATC
R ctVieB st BamHI	GAGCGAGTCGGATCCTTACGCCTCAACTGATTCGCTTCGC
F NdeI ntVieB	TCACTGTGCATATGGCTGTACCTACTTTTGCTGAATTAAAAG
R 906VieB st BamHI	GAGCGAGTCGGATCCTCAAGTCTCTTTGTTAATTTCCACATAC
R 486VieB (GGS)3 PreS	GGGCCCCTGGAACAGAACTTCCAGTGATCCACCGGACCCT CCAGAGCCACCAAAAGGCTCCAGCTCGTCACACAAACTCAGAC
F PreS (GGS)3 907VieB	CTGGAAGTTCTGTTCCAGGGGCCCGGCGGGTCGGGTGGG TCTGGCGGTTCCTATCGTAACAATCATCAGATGCACC

F BamHI VieA	TCACTGGAGGATCCATGAAAATAATGATAGTAGAAGATGA
VieA D52E R	TCCATCTGCGGCATTCGAATCTCGCAAAACATCAAATCGATAC
VieA D52E F	GTATCGATTTGATGTTTTGCGAGATTCGAATGCCGCAGATGGA
R VieA st SphI	GCAGCTCAGCATGCCTATTTTAATGTTACAAAACGCACCA
dvieA 3kb F	GCGACGGTGACAAATTATGT
dvieA deletion R	TACAGCCATAACTCTCGGTACTACATAACGTTATTCGCTCTGATAC
dvieA deletion F	GTATCAGAGCGAATAACGTTATGTAGTACCGAGAGTTATGGCTGTA
dvieA 3kp R	GTTGAGGTGGTGGTGTTTGC
dvieA screen F	CCGGCTCCCTTCAACTACAG
dvieA screen R	AAACTTGCTTACCATTCAACCCC
vieSAB PROBE F	CTCTCAAAAAGATCACAACAACAGA
vieSAB PROBE R	GACCTGTCCCACCCCAGTAAG
dCRP 3kb F	GAGCACGCCAAACCAAGATTAA
dCRP deletion R	GAGACGGGTTATCGGGGCACTTACATAATAATCTCACTTCCTCTGC
dCRP deletion F	GCAGAGGAAGTGAGATTATTATGTAAGTGCCCCGATAACCCGTCTC
dCRP 3kb R	CGTTTTTTGATGATGGCGGAA
dCRP screen F	CCTACTTACTGGCGATGATTGA
dCRP screen R2	CGGTGGCTTTTATGAGTTTG
CRP NdeI F	TCACTGGACATATGGTTCTAGGTAAACCTCAAACCG
CRP BamHI R	GCAGCTCAGGATCCTTAGCGAGTGCCGTAAACCACGATG
vieS3 R	ATCCAGATTTCTTTTAATACTGC
CRP EMSA 6 R	GCAGCACGCACAAATAATG
CRP EMSA 1 R	CCCATGGCAGAGATCACAC
CRP EMSA 3 R	TAATGTAATAAAGATAAAATAGGC
CRP EMSA 2 R	GCTTTAGCGTAAATAAATCCTCC
CRP EMSA 7 F	CTATAAGTGTGATCTCTGCC
vieS2 F	GTCTGTGACGATTGAAACGATAT
CRP EMSA 9 F	CGTTGCCTATTTTATCTTTAT
CRP EMSA 2 F	GGAGGATTTATTTACGCTAAAGC

Table 7-3. qRT-PCR primers used in this study.

qRT-PCR Primer Name	Primer Sequence 5'-3'
vieS F	GCCATTCCGAGCATGATATT
vieS R	GCGCAAGAAATGAACACTCA
vieA F	GATATTCGAATGCCGCAGAT
vieA R	TCCACAGCGCTGAGTATCAC
vieA chrom F	CATCAGTATCAGAGCGAATAAC
vieA D52E F	GAGATTCGAATGCCGCAGAT
vieB F	CGCGAAGTCTACCTCGAAAC
vieB R	CGCCAGCAGTTCTTGTATCA
sanA F	TTGCTGTGGCTGACTATTGG
sanA R	CCAATACCACTGCAACCTGA
rpoB F	CTGTCTCAAGCCGGTTACAA
rpoB R	TTTCTACCAGTGCAGAGATGC
toxT F	TCTTGGTGATCTCATGATAAGGAA
toxT R	TTTCGAGAAGAACCCTGAAAAA
toxR F	CGACAAAGTCCCCACAATAC
toxR R	TGAGAGATGTCATGAGCAGC
mtlA F	CGTTAGCCTCTTTGGTTGGG
mtlA R	TACCGACAATCACGCCCATC
mtlD F	AAGTCAAAGTGGTCGGTAGC
mtlD R	GGCGATGGTTTTGGCAATGA

Table 7-4. Primer combination for EMSA probes.
Sequences of primers are listed above in Table 7-2.

EMSA Probe	Primer Pair
vieSAB	vieSAB PROBE F / vieSAB PROBE R
1	vieSAB PROBE F / vieS3 R
2	vieSAB PROBE F / CRP EMSA 6 R
3	vieSAB PROBE F / CRP EMSA 1 R
4	vieSAB PROBE F / CRP EMSA 3 R
5	vieSAB PROBE F / CRP EMSA 2 R
6	CRP EMSA 7 F / vieSAB PROBE R
7	vieS2 F / vieSAB PROBE R
8	CRP EMSA 9 F / CRP EMSA 6 R
9	CRP EMSA 2 F / CRP EMSA 1 R
10	CRP EMSA 9 F / CRP EMSA 1 R

CHAPTER 8: REFERENCES

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