TO STICK OR SWIM: CYCLIC-DI-GMP MEDIATED INVERSE REGULATION OF BIOFILMS AND MOTILITY IN *VIBRIO CHOLERAE*

By

Disha Srivastava

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Biochemistry and Molecular Biology-Doctor of Philosophy

2014

ABSTRACT

TO STICK OR SWIM: CYCLIC-DI-GMP MEDIATED INVERSE REGULATION OF BIOFILMS AND MOTILITY IN *VIBRIO CHOLERAE*

By

Disha Srivastava

Bacteria sense and respond to environmental cues to control important developmental processes. Decoding the language of chemical signaling in bacteria and the mechanisms by which these signals control coordinated behavior impacts our understanding of the role of bacteria in human health, the environment, and industrial processes. Bacteria exist in communities and often perform coordinated activities such as production and secretion of extracellular enzymes, luminescence, biofilm formation, and virulence. *Vibrio cholerae*, the causative agent of the diarrheal disease Cholera provides an excellent model system to study the effect of environmental signals on bacterial phenotypes.

In *Vibrio cholerae*, c-di-GMP affects transcriptome changes regulating many important phenotypes, such as biofilms and motility. I have identified two c-di-GMP binding transcription factors, VpsR and FlrA, involved in biofilm and motility, respectively.

Currently, ten c-di-GMP binding transcription factors are known in bacteria, three of which are from *V. cholerae*. Three c-di-GMP binding transcription factors; FleQ, FlrA and VpsR belong to the NtrC-like enhancer binding protein family (EBPs). EBPs consist of an N-terminal receiver domain, central AAA+ domain (ATPase associated with diverse activities), and a C-terminal helix-turn-helix DNA

binding domain. AAA+ domains are involved in ATP hydrolysis which drives open complex formation initiating transcription. The AAA+ domains are widespread in bacteria and are found in both transcription factors and other cellular machinery. The transcription factor VpsR binds c-di-GMP to induce biofilm gene expression. Alternatively, binding of c-di-GMP to FlrA, the master regulator of flagellar biosynthesis in *V. cholerae*, abrogates its ability to initiate downstream flagellar gene expression leading to a repression in motility. VpsR and FlrA exhibit the most homology in the AAA+ domain. I have discovered that the AAA+ domain of FlrA is important for interacting with c-di-GMP and demonstrated that two arginine residues are important for this binding. I have also isolated constitutively active mutants of VpsR which can be utilized to study the mechanism how c-di-GMP controls VpsR activity. Lastly, I have shown that other c-di-GMP regulated genes in *V. cholerae* are not regulated by known c-di-GMP binding transcription factors, suggesting that other unidentified machinery are involved in c-di-GMP signaling.

To my Grandmother, Sheela Srivastava,
Who has and will always be my pillar of strength…

ACKNOWLEDGMENTS

I would like to first and foremost thank Dr. Christopher M. Waters for his guidance and mentorship without which this work would not have been possible. His infectious enthusiasm for science and research has been and will always be a great source of inspiration for me. I would also like to acknowledge my Guidance committee members Dr. Rob Britton, Dr. Zach Burton, Dr. Bill Henry, Dr. Lee Kroos for their constant advice and support.

I would like to sincerely thank all members of the Waters lab for their assistance and support. I would also like to thank Lauren Priniski for construction of pLLP15, Benjamin R. Pursley for construction of pBRP1, pBRP2, BP27 and BP55, and Amanda Ngouajio for construction of pALN17 and pALN23.

Last, but not the least I, would like to thank my parents (Ram Srivastava and Madhu Srivastava), my husband (Angad Garg) and my dear friends (especially Deepika Sharma and Ankit Malik), who have been extremely supportive and encouraging through this whole process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AAA+ .....ATPases Associated with diverse cellular Activities
AI ..... Quorum sensing autoinducer
c-di-GMP .....Cyclic dimeric guanosine 3',5'-monophosphate
CV .....Crystal violet
CVEC .....conditionally viable environmental cells
CT ..... Cholera enterotoxin
DGC .....Diguanylate cyclase enzyme
EBP..... Enhancer binding proteins
GTP..... Guanosine-5' triphosphate
IPTG ..... Isopropyl ß-D1-thiogalactopyranoside
LB .....Luria-Bertani media
LC-MS/MS.....Liquid chromatography coupled with tandem mass spectrometry
MMS .....Methyl Methane Sulfonate
MS .....motor switch
OD..... Optical density
ORF ..... Open reading frame
PBS..... Phosphate buffered saline
PCR.....Polymerase chain reaction

PDE .....Phosphodiesterase enzyme
QS ..... Quorum sensing
REC .....Reciever domain
R.L.U .....Relative luminescence units
RNAP..... RNA polymerase
SDS .....Sodium dodecyl sulfate cells
TCP..... Toxin co-regulated pilus
Tris 2-Amino-2-hydroxymethyl-propane-1,3-diol

VPS ....Vibrio polysaccharide

CHAPTER 1

INTRODUCTION

1.1 Cyclic-di-GMP signaling in bacteria

C-di-GMP was first discovered as an allosteric activator of cellulose synthesis in *Gluconoacetobacter xylinus* in the late 1980's (Hengge, 2009, Weinhouse *et al.*, 1997, Ross *et al.*, 1987). C-di-GMP, predicted to be present in 85% of all bacteria, controls the switch between biofilm formation and motility (Galperin, 2004). It is synthesized from two GTP molecules by diguanylate cyclase (DGC) enzymes

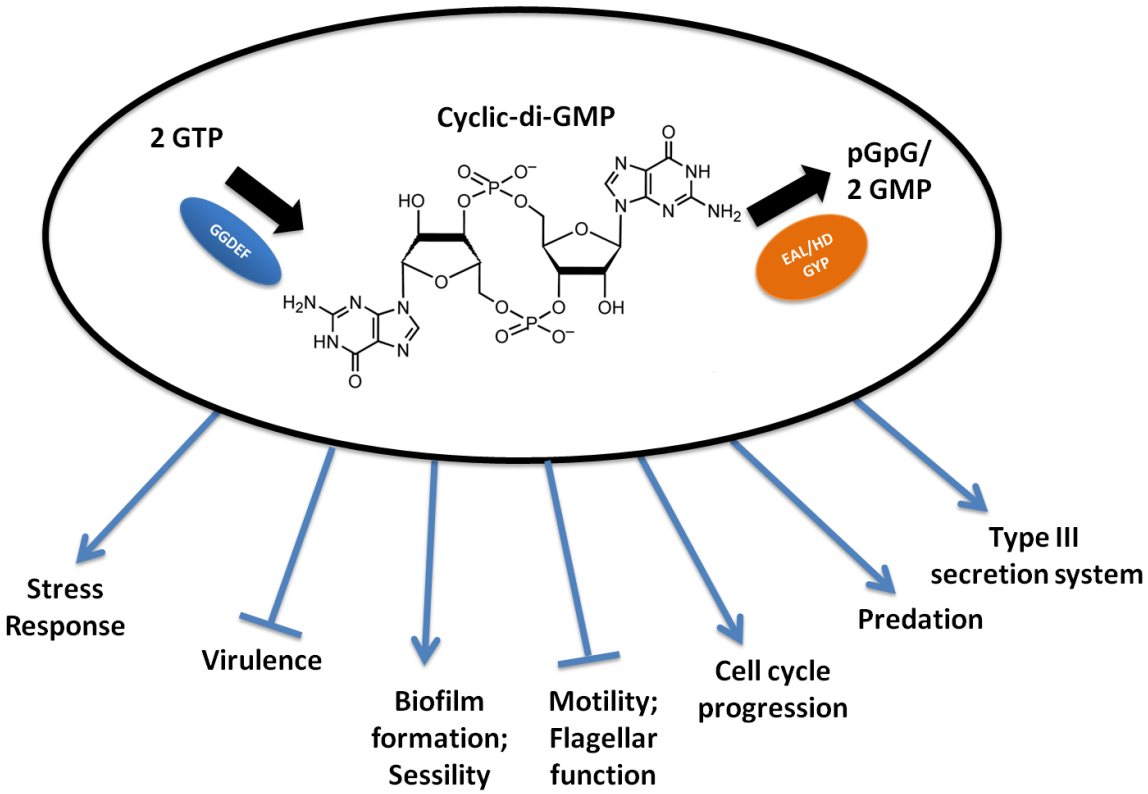


Figure 1: Cyclic-di-GMP signaling in bacteria. Bis-(3'-5')- cyclic dimeric guanosine monophosphate (c-di-GMP) is synthesized in the cell by the action of diguanylate cyclases and degraded by the action of phosphodiesterases as shown. It regulates many important phenotypes in the bacterial cell, some of which are listed in the figure.

containing GGDEF domains consisting of approximately 170 amino acids (Ryjenkov *et al.*, 2005). Conversely, c-di-GMP is degraded by phosphodiesterase (PDE) enzymes containing EAL or HD-GYP domains that are approximately 250 amino acids in length (Fig. 1) (Schmidt *et al.*, 2005). These enzymes form nearly ubiquitous families in multiple bacterial genomes and their modular architectures hint at their probable signaling functions.

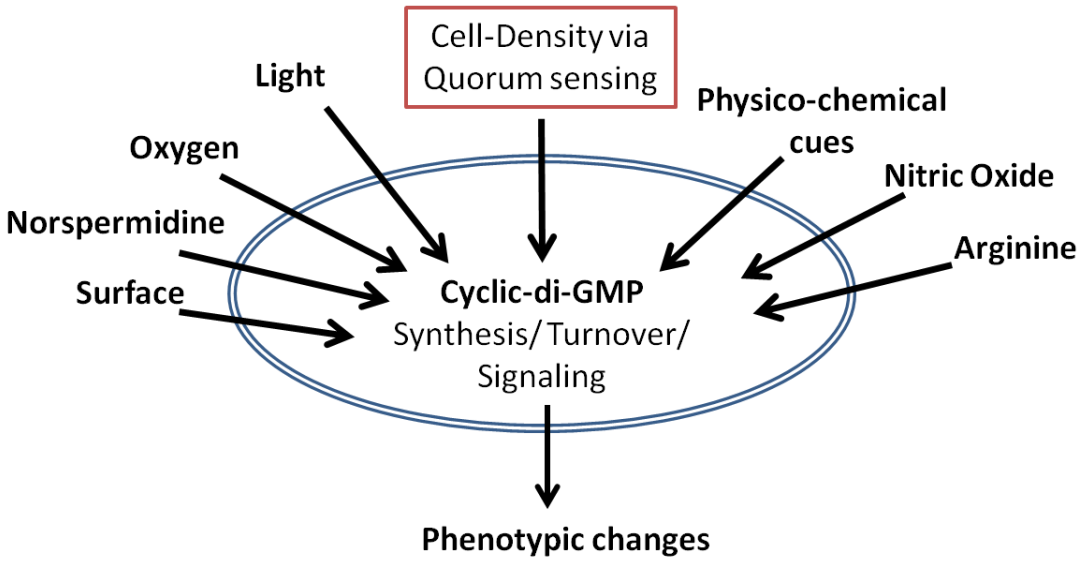


Figure 2: C-di-GMP signaling integrates environment information into phenotypic output. The synthesis and degradation of c-di-GMP is controlled by multiple environmental signals to modulate downstream phenotypic changes. As shown, information regarding local cell density via QS pathways, chemical cues such as oxygen, NO and others is relayed into the c-di-GMP signaling network composed of multiple signaling pathways (not depicted here) to allow bacteria to appropriately adapt and respond to different environments (Srivastava & Waters, 2012).

Proteins containing GGDEFs and EAL or HD-GYPs are typically modular in nature with the enzymatic domain linked to various amino terminal sensory domains (Galperin, 2004, Römling *et al.*, 2005, Römling *et al.*, 2013). These sensory domains respond to environmental or host derived cues to control the downstream enzymatic activity (Fig. 2). Thus far, only a handful of specific environmental signals have been identified including norspermidine, oxygen, light, nitric oxide, and arginine (Fig. 2) (Tuckerman *et al.*, 2009, Karatan *et al.*, 2005, Carlson *et al.*, 2010, Bernier *et al.*, 2011, Merritt *et al.*, 2007, Kanazawa *et al.*, 2010).

C-di-GMP signaling employs multiple pathways. A striking feature of c-di-GMP signaling is that many bacteria encode a wide array of c-di-GMP synthesis and degradation proteins. For example, *Escherichia coli* K12 encodes 12 GGDEF-containing proteins, 10 EAL-containing proteins, and 7 proteins that have both a GGDEF and EAL domain (Galperin, 2004). Although the enzymatic domains are conserved, each of these proteins has a unique N-terminal sensory domain that is predicted to respond to a specific cue. The changes in levels of c-di-GMP are sensed by c-di-GMP receptor proteins or riboswitch RNAs which regulate downstream phenotypes (Hengge, 2009). Interestingly, regulation of downstream phenotypes occurs at many levels including transcriptional regulation, post-transcriptional modulation, or direct control of an enzymatic response or protein activity (Krasteva *et al.*, 2010, Tao *et al.*, 2010, Ross et al., 1987, Paul *et al.*, 2010).

The role of c-di-GMP in controlling the transition from a motile to sedentary state has been observed in many bacteria including, but not limited to, *Pseudomonas aeruginosa, Salmonella enterica* serovar Typhimurium, and *V. cholerae* (Hengge, 2009, Römling et al., 2013). However, it is clear that c-di-GMP impacts a wide array of other fundamental bacterial behaviors including cell cycle propagation, development, fimbriae synthesis, type three secretion system, RNA modulation, stress response, bacterial predation, and virulence (Fig. 1) (Duerig *et al.*, 2009, Jenal & Malone, 2006, Kuchma *et al.*, 2005, Weber *et al.*, 2006, Hobley *et al.*, 2012, Tamayo *et al.*, 2007, He & Zhang, 2008). It is likely that this list will continue to grow.

1.2 Cholera and its causative agent, *Vibrio cholerae*

Vibrio cholerae is a gram-negative bacterium belonging to the Vibrionaceae family commonly found in the marine environment. It was first described by Robert Koch in 1883 as the comma shaped bacterium that causes the disease Cholera (Reidl & Klose, 2002, Barua, 1992). The history of cholera-like diseases dates back to the time of Hippocratus, although, the modern history of the disease began in the early 19th century (Barua, 1992). Up to 200 serogroups of *V. cholerae* exist in the environment but only O1 and O139 serogroups are infectious (Reidl & Klose, 2002). The O1 serogroup is found in two biotypes called Classical and El Tor. There have been numerous known pandemics of cholera, the fifth and the sixth pandemic were attributed to the Classical strain of *V. cholerae,* this biotype was replaced by the O1

El Tor biotype in the seventh pandemic and has since then emerged to be the major disease-causing agent (Barua, 1992, Calia *et al.*, 1994, Barrett & Blake, 1981). It is believed that the El Tor biotype is better able to adapt and survive, and thus was able to replace the Classical biotype (Pradhan *et al.*, 2010). Another serogroup conversion event led to the emergence of the O139 biotype, which is sometimes referred to as the eighth pandemic (Albert, 1994, Barua, 1992, Preston *et al.*, 1993). These events highlight the ability of *V. cholerae* to evolve in environmental niches mostly through exchange of genetic material (Meibom *et al.*, 2005, Blokesch &

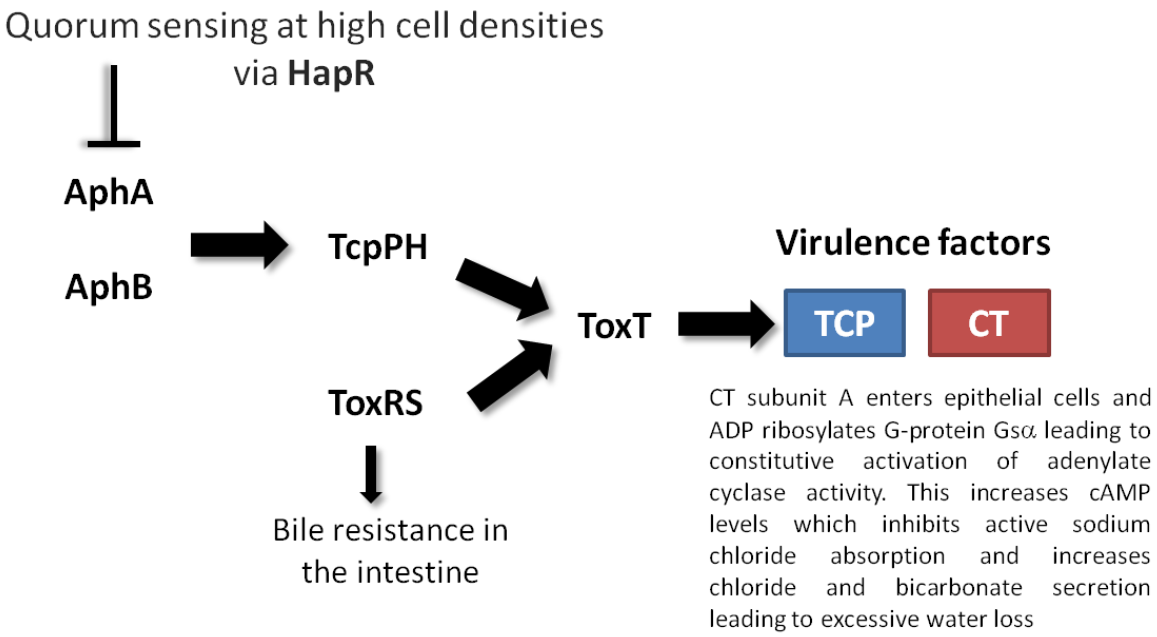


Figure 3: Gene expression cascade regulating virulence of *V. cholerae* in the human host. Only a few key players are shown in the pathway. As shown, QS regulates the virulence cascade in high cell density state by repressing the levels of AphA. TcpH, ToxR and ToxT are important transcriptional activators of virulence gene expression. TCP and CT are virulence factors expressed in the human host. Modified from (Zhu *et al.*, 2002).

Schoolnik, 2007).

In the environment, *V. cholerae* exists primarily in a sessile state as biofilms on chitinous surfaces of marine organisms but also some bacteria exist in a free living state. This sessile association with surfaces in a biofilm state provides protection from the harsh conditions in the marine environment. It also helps with the persistence of the virulent strains between epidemics (Reidl & Klose, 2002).

- **Virulence factors and disease**

Virulent strains of *V. cholerae* contain two genetic elements that make them infectious, the CTX phage and the Vibrio Pathogenicity Island (VPI) (Reidl & Klose, 2002). The CTX phage codes for the Cholera toxin subunits that causes severe diarrhea. The VPI codes for the toxin co-regulated pilus that functions as a colonization factor and a CTX phage receptor (Reidl & Klose, 2002). Other proteins expressed from derived genetic elements and from the *V. cholerae* genome, function as regulatory, virulence and colonization factors in the virulence cascade to cause disease in the human host (Fig. 3).

V. cholerae infection is acquired with ingestion of contaminated food or water. The infectious dose of *V. cholerae* is very high ranging between 10^4-10^9 cells since the bacteria are susceptible to the harsh acidic conditions of the stomach. It is hypothesized that biofilms serve to shield the bacteria from the acidic barrier of the stomach (Reidl & Klose, 2002, Merrell *et al.*, 2002). Disease symptoms occur by a process involving colonization of bacteria, expression of virulence factors and action of toxins on small intestine epithelial cells (Reidl & Klose, 2002). Secretion of

Cholera toxin leads to acute watery diarrhea due to ADP ribosylation activity of the CT-A subunit (Fig.3) (Herrington *et al.*, 1988). The mortality rate due to excessive water loss in severe cases can be as high as 20% (Reidl & Klose, 2002, Barua, 1992).

Transition from the aquatic environment to the human host exposes *V. cholerae* to a series of changes in the environmental conditions such as temperature, pH and osmolarity. To adapt to these changes *V. cholerae* has developed the ability to perform phenotypic adaptations and modulate these responses. This makes *V. cholerae* an excellent model system to study phenotypic changes in response to environmental conditions.

- **Biofilm development and its significance**

Biofilm formation in *V. cholerae* is important for survival of the bacterium in aquatic reservoirs and disease transmission (Zhu & Mekalanos, 2003). Biofilm formation by *V. cholerae* occurs on biotic surfaces of chitinous animals such as copepods and zooplankton (Huq *et al.*, 1983). Biofilms are critical for the development of conditionally viable but non-culturable *V. cholerae* cells (CVEC) because mutants in biofilm formation are unable to enter the CVEC state (Faruque *et al.*, 2006, Kamruzzaman *et al.*, 2010). CVECs are thought to be important for spread of disease because this state increases the ability of *V. cholerae* to survive stress and starvation outside of the host (Faruque et al., 2006).

As mentioned, biofilm formation also increases resistance of *V. cholerae* to the acidic environment in the stomach which is essential for the passage of bacteria to

the small intestine (Zhu & Mekalanos, 2003). Although mutants defective in biofilm formation are not compromised for colonization in a murine or rabbit disease model, stool from *V. cholerae* patients contains a mixture of infectious biofilm-like aggregates along with planktonic cells, suggesting biofilm formation occurs *in vivo* . Furthermore, *in vitro* biofilm formation increases the infectivity of *V. cholerae* (Tamayo *et al.*, 2008).

Development of biofilms in *V. cholerae* relies on the expression of two linked operons termed *vps*I and *vps*II that encode proteins essential for exopolysaccharide (EPS) production (Fig.4). Expression of these operons is increased at high levels of c-di-GMP (Yildiz & Schoolnik, 1999, Lim *et al.*, 2006). At least two transcriptional activators, VpsT, a member of the LuxR family of transcription regulators, and VpsR, a NtrC-like transcriptional regulator, positively control biofilm development in *V. cholerae* by activating expression of the *vps* operons (Yildiz *et al.*, 2001, Casper-Lindley & Yildiz, 2004). It has been shown that the expression of *vpsT* is induced under conditions of high levels of c-di-GMP and repressed at high cell density by the QS regulator HapR (Hammer & Bassler, 2003, Waters *et al.*, 2008). VpsT was shown to bind c-di-GMP and this binding was shown to activate expression of *vps* and repress expression of flagellar assembly genes (Krasteva et al., 2010). The mechanism by which *vpsT* expression itself is induced by c-di-GMP was unknown; however, it was hypothesized that VpsT bound to c-di-GMP induces its own expression via a positive feedback loop (Krasteva et al., 2010). We have

shown that VpsR binds c-di-GMP to induce *vpsT* expression leading to increased VPS synthesis (Chapter 2).

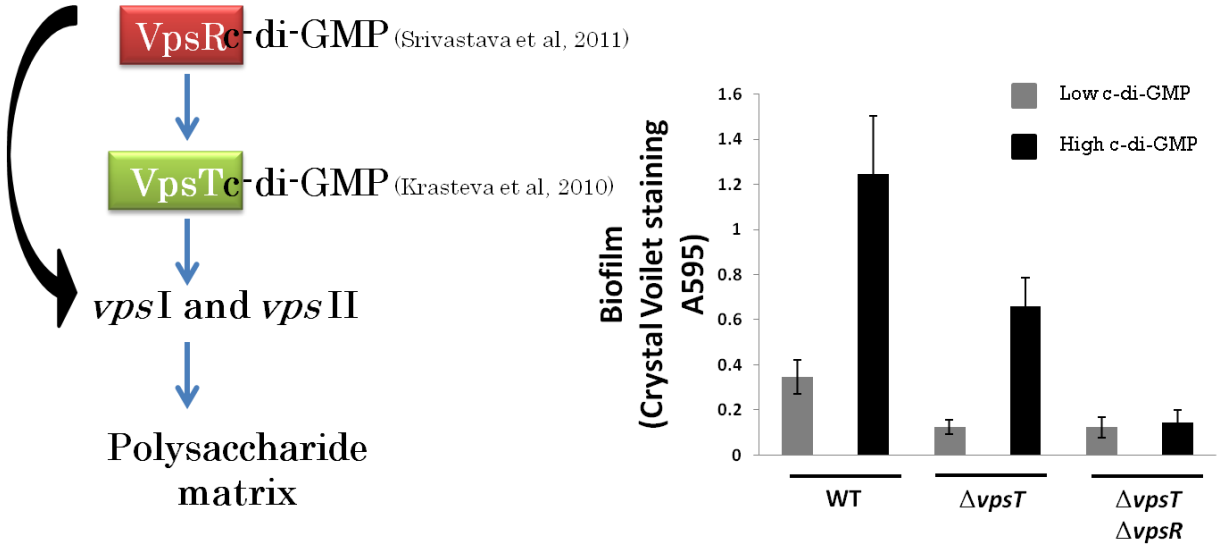


Figure 4: Biofilm formation is induced at high levels of c-di-GMP. The left panel shows the cascade of gene expression involved in biofilm development in *V. cholerae*. Two transcriptional activators VpsR and VpsT induce downstream *vps* gene expression. VpsT was shown to be a c-di-GMP binding transcription factor. In Chapter 2, we have shown VpsR binds to c-di-GMP. The right panel shows biofilm formation in the WT strain versus Δ*vpsT* and Δ*vpsT* Δ*vpsR* strains. It is evident that c-di-GMP mediated induction of biofilms is dependent on both VpsT and VpsR. In a Δ*vpsT* strain some induction of biofilms is still observed whereas, VpsR is essential for any observable biofilm formation.

- **Motility and flagellar structure**

V. cholerae is a motile bacterium with a single polar flagellum. Motility has been shown to be important for virulence in the rabbit ileal loop model of infection;

however, non-motile mutants do not exhibit defective colonization in the infant mouse model (Krukonis & DiRita, 2003). The relationship between motility and virulence is unclear. It is believed that motility is required in the small intestine before the step of colonization to move through the mucosal layer towards the epithelial cells (Krukonis & DiRita, 2003, Reidl & Klose, 2002). Genetic evidence suggests that motility and virulence genes are inversely regulated (Krasteva et al., 2010, Syed *et al.*, 2009). Motility is also believed to play a role in biofilm formation in some infectious strains (Watnick *et al.*, 2001, Watnick & Kolter, 1999). *V. cholerae* primarily exists in the sessile state in the environment but it can also exist in a free living state. In the O1 Classical biotype, initial attachment of bacteria to the surface requires the presence of the flagellum (Watnick & Kolter, 1999, Watnick et al., 2001). However, in the O139 strains, it has been shown that loss of the flagellum leads to increased production of VPS and biofilm formation (Watnick et al., 2001). Genes for biofilms and motility are also inversely regulated in *V. cholerae* in the O1 strains (Reidl & Klose, 2002, Beyhan *et al.*, 2006).

The flagellar genes are found in multiple separated clusters in the *V. cholerae* chromosome (Prouty et al., 2001). The expression of flagellar biosynthesis genes in *V. cholerae* occurs by a cascade of gene expression involving four classes of genes expressed in a sequential manner (Fig. 5) (Prouty et al., 2001). *flrA*, flagellar regulatory protein A, is the only gene in class I. It codes for a σ 54-dependent transcription factor that activates expression of Class II genes. Class II genes consist of structural components of the MS ring, switch and export apparatus. Class

II genes also consist of regulatory genes; *fliA*, that codes for alternative sigma factor σ28 and *flrBC*, that codes for two component system consisting of FlrB, a histidine kinase and FlrC, a σ54-dependent transcription factor. Expression of Class II genes then leads to expression of Class III genes coding for basal body, hook and filament proteins. Class IV genes are mostly σ28 dependent and code for additional filament genes and motor proteins. Expression of flagellar genes and formation of flagella is a coordinated process involving regulation by alternative sigma factors σ54 and σ28

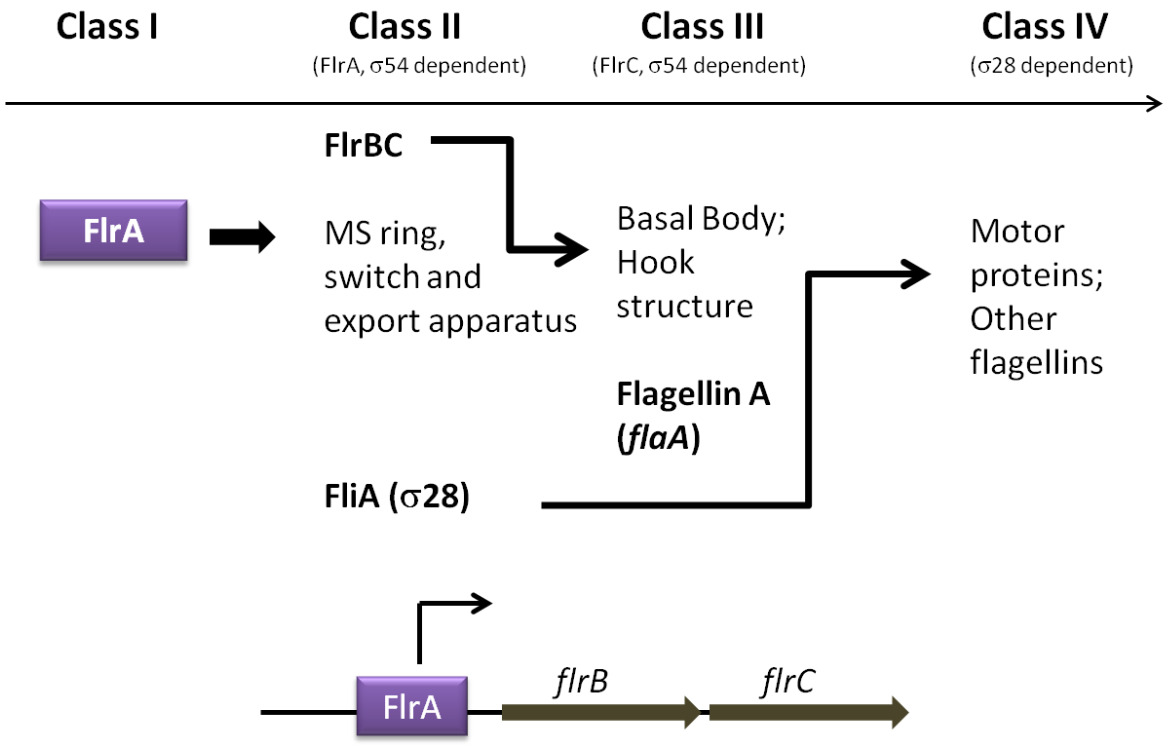


Figure 5: Flagellar regulatory cascade in *V. cholerae*. Flagellar gene regulatory network in *V. cholerae* is organized into four classes of gene to ensure temporal and coordinated expression (modified from (Prouty *et al.*, 2001). Bottom panel represents the regulation of ClassII genes *flrBC* by FlrA, a class I transcriptional activator. It is believed that FlrA directly regulates *flrBC* expression however, no direct evidence has been shown (discussed in detail in Chapter 3).

and controlled assembly of the flagellar structure (Prouty et al., 2001).

C-di-GMP has been shown to inhibit expression of components of the flagellar biosynthesis regulon (Beyhan et al., 2006, Krasteva et al., 2010). Transcriptome profiling studies have previously reported decreases in the expression of the Class III and IV flagellar biosynthesis genes in response to high levels of c-di-GMP (Beyhan et al., 2006). Moreover, a subset of these Class III and IV genes were shown to be negatively regulated by VpsT, suggesting this transcription factor might link c-di-GMP and flagellar biosynthesis; however, it is unknown if this regulation is direct (Krasteva et al., 2010). Overall, the regulation of flagellar gene expression by c-di-GMP is poorly understood.

1.3 Cyclic-di-GMP and its role in *V. cholerae* lifestyle

C-di-GMP signaling in *V. cholerae* has been extensively studied (Hammer & Bassler, 2009, Lim et al., 2006, Liu *et al.*, 2010, Lim *et al.*, 2007, Beyhan et al., 2006, Srivastava *et al.*, 2011). *V. cholerae* contains 61 predicted c-di-GMP synthesis/degradation enzymes, although a subset of these might be enzymatically inactive (Galperin, 2004). In *V. cholerae,* c-di-GMP positively regulates biofilm development (Beyhan et al., 2006, Tischler & Camilli, 2004) and negatively controls motility and virulence (Krasteva et al., 2010, Tamayo et al., 2007, Tischler & Camilli, 2005). Biofilm expression is highly induced by c-di-GMP through induction of the *vps* (vibrio polysaccharide) genes while c-di-GMP represses expression of the flagellar biosynthetic genes (Beyhan et al., 2006, Krasteva et al., 2010, Liu et al.,

2010). Transcription of virulence factor genes is also repressed by c-di-GMP although the molecular mechanism for how this occurs is unknown (Cotter & Stibitz, 2007). These results and other observations have led to a model wherein c-di-GMP levels are high in *V. cholerae* during environmental persistence where the organism exists primarily as a biofilm and repressed upon infection of humans (Tamayo et al., 2007).

V. cholerae transitions between aquatic environmental reservoirs and human

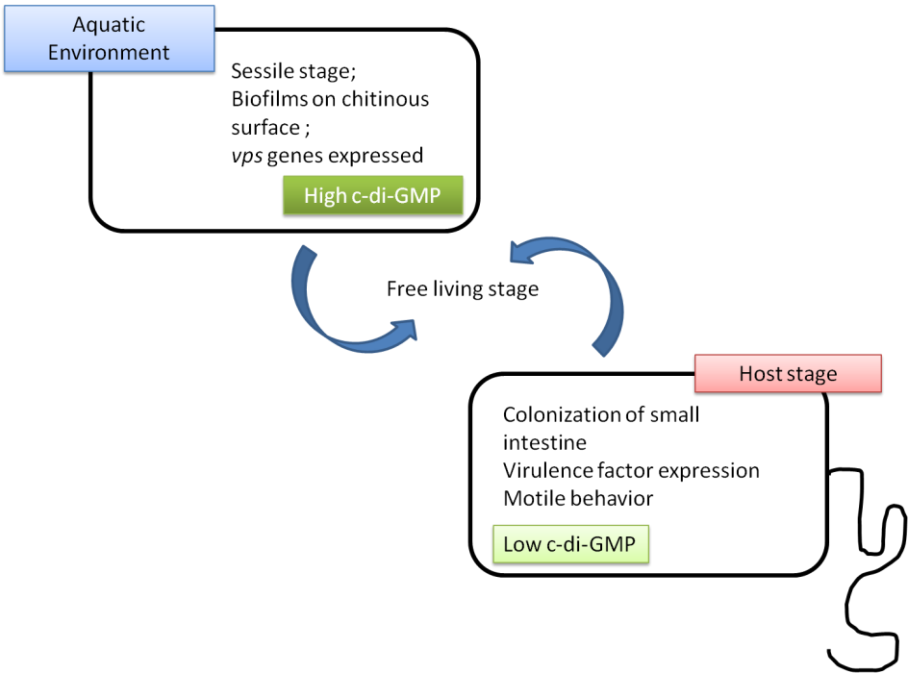


Figure 6: Life cycle of human pathogen *V. cholerae* illustrating two distinct phases of its lifestyle. *V. cholerae* exists in a sedentary phase in the aquatic environment where it forms biofilms and in an infectious phase in the human host where it is motile and activates its virulence cascade. The levels of c-di-GMP are important in this transition. As depicted, the levels are hypothesized to be high in the aquatic compartment and low in the human host.

host, this transition requires fine-tuning of signaling responses (Fig. 6). C-di-GMP in conjunction with QS controls the transition of *V. cholerae* between motile (low c-di-GMP) and sessile (high c-di-GMP) developmental states. C-di-GMP levels are hypothesized to be high in aquatic reservoirs due to controlled activity of DGCs/GGDEFs where biofilm development is promoted and lower upon intestinal colonization in the host due to activation of PDE/EALs where the bacteria are in a motile state and virulence cascade is initiated (Fig. 6) (Tischler & Camilli, 2004, Tischler & Camilli, 2005, Tamayo et al., 2007). Thus, c-di-GMP is believed to control biofilms and virulence (also motility) inversely.

C-di-GMP regulates many phenotypes in *V. cholerae* at the level of gene expression. According to microarray data, 4.3% of the *V. cholerae* genome is differentially expressed in response to high levels of c-di-GMP (Beyhan et al., 2006). Responses include increases in the expression of biofilm/vibriopolysaccharide development genes (*vps*), *eps* genes involved in extracellular protein secretion, and *msh* genes required for mannose sensitive hemagglutinin and a decrease in expression of *fla*, flagellar assembly genes. Currently it is known that VpsT is a c-di-GMP binding transcription factor that positively regulates *vps* gene expression (Krasteva et al., 2010). In this dissertation work, I have discovered and described two other c-di-GMP dependent transcription factors (VpsR and FlrA) involved in regulation of some of these gene regulatory networks in *V. cholerae* (Srivastava et al., 2011, Srivastava *et al.*, 2013).

- **Regulatory connections between quorum sensing and c-di-GMP signaling in *V. cholerae***

The quorum sensing (QS) system of *V. cholerae* has been well characterized, although not every *V. cholerae* strain encodes a complete, functional QS system (Joelsson *et al.*, 2006). This QS system possesses two parallel sensory circuits that respond to two specific AIs, a furanosyl borate diester named AI-2 and a hydroxylated alkyl ketone named CAI-1 (Higgins *et al.*, 2007) (Fig. 7). Signal perception of AI-2 in the periplasm by LuxPQ and CAI-1 by CqsS modulates a phosphorelay cascade that ultimately results in phosphorylation or dephosphorylation of LuxO, the central response regulator of the pathway (Ng & Bassler, 2009, Chen *et al.*, 2002). At low cell density, the AI receptors function as kinases and LuxO is phosphorylated. Phospho-LuxO activates expression of four *qrr* sRNAs, which then repress expression of the master high-cell density transcription factor HapR by destabilizing the *hapR* mRNA (Lenz *et al.*, 2004, Tu & Bassler, 2007). HapR is the master high-cell-density QS transcriptional regulator in *V. cholerae,* and it both activates and represses high-cell-density QS target genes (Miller *et al.*, 2002). Recently, the transcription factor AphA was shown to be the master low-cell-density QS regulator of *V. cholerae* (Rutherford *et al.*, 2011). At high cell density, interaction of the receptors with AIs switches their activity to phosphatases, leading to dephosphorylation of LuxO. The *qrr* sRNAs are no longer expressed and HapR is produced (Tu & Bassler, 2007). In *V. cholerae*, QS controls many developmental phenotypes such as biofilms, virulence factor expression,

extracellular protease production, and competence (Hammer & Bassler, 2003, Kovacikova & Skorupski, 2002, Vance *et al.*, 2003, Meibom et al., 2005). Biofilms and virulence are induced at low cell densities whereas protease production and competence induction occur at high-cell-densities (Ng & Bassler, 2009).

QS modulation of c-di-GMP occurs at multiple levels throughout the *V. cholerae* QS signal cascade (Waters et al., 2008) (Fig. 7). The *qrr* sRNAs were shown to directly stimulate translation of the mRNA encoding the GGDEF protein VCA0939 independently of master regulator HapR (Hammer & Bassler, 2007). The induction of VCA0939 by *qrr* RNAs is consistent with a high intracellular concentration of c-di-GMP in the low-cell density state, although a deletion of VCA0939 did not significantly affect biofilm formation (Hammer & Bassler, 2007, Bardill *et al.*, 2011). However, this result is not surprising as single mutations in complex c-di-GMP signaling networks often do not exhibit strong phenotypes due to redundancy and the activity of VCA0939 remains to be tested.

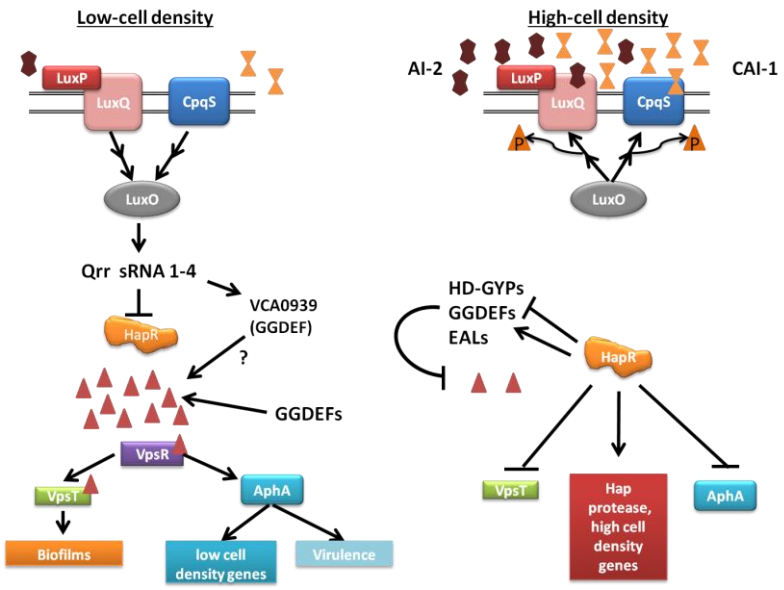


Figure 7: Control of c-di-GMP by the QS system of *V. cholerae*. QS mediated control of c-di-GMP in *V. cholerae* occurs at multiple levels. Left panel of this figure shows the signaling cascade at low-cell density, the AIs AI-2 (brown double pentagon) and CAI-1 (orange double triangle) are low, causing the histidine kinase receptors to phosphorylate the response regulator LuxO Phosphorylated LuxO activates the expression of *qrr* sRNAs which repress HapR expression by destabilization of *hapR* mRNA. VpsR and VpsT, two transcriptional activators that directly bind to c-di-GMP, positively regulate biofilm genes. Also, expression of AphA, the master QS low-cell density regulator is induced by VpsR and c-di-GMP to activate low-cell density expressed genes. Virulence factor expression is also induced by AphA but thought to be repressed by c-di-GMP, and this contradiction is not currently understood. The right panel shows the cascade at high-cell densities, the increase in AI-2 and CAI-1 reverses the flow of phosphate in the QS cascade leading to decreased *qrr* sRNA expression. HapR represses c-di-GMP levels in cells and also directly represses *vpsT* and *aphA* expression, decreasing biofilm formation, virulence factor expression, and low cell density gene expression.

QS control of c-di-GMP levels in *V. cholerae* also occurs downstream of the *qrr* sRNAs. Expression of HapR at high-cell density controls the transcription of 14 different GGDEFs and EALs, ultimately resulting in decreased c-di-GMP levels and lower *vps* expression (Waters et al., 2008). Likewise, a mutation in *hapR* that caused a smooth to rugose transition in colony morphology led to higher levels of c-

di-GMP (Beyhan *et al.*, 2007, Waters et al., 2008). Similarly, a screen to identify QS regulated genes showed that HapR regulated the transcription of four HD-GYP domain containing proteins that may degrade c-di-GMP (Hammer & Bassler, 2009). Increased production of the VCA0681 HD-GYP protein reduced c-di-GMP levels, decreasing *vps* gene expression and biofilm formation (Hammer & Bassler, 2009).

1.4 Molecular mechanisms of c-di-GMP mediated gene regulation in bacteria

As mentioned above, regulation of intracellular c-di-GMP levels occurs by sensing of environmental factors by c-di-GMP synthesis and degradation enzymes (Fig.1 and 2). The c-di-GMP effector systems present in bacterial cells sense the changing levels of the second messenger and in turn modulate the downstream phenotypic responses. C-di-GMP effectors are c-di-GMP binding proteins or riboswitch RNA, the activities of which are modulated by binding to c-di-GMP. C-di-GMP was first shown to be an allosteric activator for the enzyme cellulose synthase in *Gluconoacetobacter xylinus* (Ross *et al.*, 1986). Since the discovery of this first effector, many proteins have been shown to bind and respond to c-di-GMP (Boyd & O'Toole, 2012, Römling et al., 2013). The first domain described to bind c-di-GMP was the PilZ domain (Amikam & Galperin, 2006); these domains are widely present in many bacteria in proteins such as Alg44 in *P. aeruginosa* and *YcgR* in *E. coli* (Amikam & Galperin, 2006, Ryjenkov *et al.*, 2006). Not all bacteria that have conserved GGDEF and EAL domain proteins contain PilZ domains suggesting that domains other than PilZ can bind c-di-GMP. Other proteins not containing the PilZ

domain have been demonstrated to bind c-di-GMP such as the EAL domain of FimX (Kazmierczak *et al.*, 2006, Huang *et al.*, 2003), PelD and FleQ from *P. aeruginosa* (Whitney et al., 2012*, Hick*man & Harwood, 2008), the RXXD domain of GGDEFs (Christen *et al.*, 2006), VpsT and CpsQ from *V. cholerae* and *Vibrio parahemolyticus* respectively (Ferreira et al., 2012, Krasteva et al., 2010) and also RNA riboswitches (GEMM motif of class I riboswitch) from *V. cholerae* (Sudarsan et al., 2008). The activities regulated by these effectors range from control of transcription initiation and modulating enzyme function to altering activity of cellular molecular machinery. It is clear that c-di-GMP can modulate outputs through varied mechanisms. Since so many c-di-GMP regulatory enzymes and effectors exist in bacteria, it is believed that discrete phenotypic outputs are regulated by spatial and temporal regulation of c-di-GMP metabolizing enzyme activities and effectors (Massie *et al.*, 2012, Römling et al., 2013).

A key mechanism by which c-di-GMP regulates bacterial behaviors is through the control of transcription initiation. Transcriptional regulation by effectors such as VpsT in *V. cholerae* (Krasteva et al., 2010) and FleQ in *P. aeruginosa* (Hickman & Harwood, 2008) have been demonstrated to be regulated by direct binding of c-di-GMP to these proteins. The transcription factors that bind to c-di-GMP to induce or repress gene expression belong to five families of proteins 1) CRP (e.g., Clp and Bcam1349 (Leduc & Roberts, 2009, Fazli *et al.*, 2011)), 2) TetR (e.g., LtmA (Li & He, 2012)), 3) FixJ/LuxR (e.g., VpsT and CpsQ (Krasteva et al., 2010, Ferreira *et al.*, 2011)), 4) the NtrC family enhancer binding proteins (EBPs) (e.g., FleQ, VpsR, and

Transcription factor	Organism	K_d	Functions controlled	Reference	Domains present
FleQ	*Pseudomonas aeruginosa*	20μM	Motility and Biofilms	Hickmann *et al*, 2008 Baraquet *et al*, 2013	AAA+; sigma 54 Walker B motif
Clp	*Xanthomonas campestris*	0.2μM	Virulence	Tao *et al*, 2009 Chin *et al*, 2010	CRP like; D70, R154, R166, and D170 residues are involved in binding
VpsT	*Vibrio cholerae*	3.2μM	Biofilms	Krasteva *et al*, 2010	LuxR like; Interface of dimer
Bcam1349	*Burkholderia cenocepacia*	----	Biofilm formation and virulence	Fazli *et al*, 2011	CRP/FNR family proteins; binding site unknown (18% homology to XcClp)
MrkH	*Klebsiella pnuemoniae*	----	Fimbriae expression	Wilksch *et al*, 2011	PilZ domain
VpsR	*Vibrio cholerae*	1.6μM	Biofilms	Srivastava *et al*, 2011	AAA+; unknown
CpsQ	*Vibrio parahemolyticus*	----	Quorum sensing regulation	Ferreira *et al* , 2011	Same as VpsT
LtmA	*Mycobacterium stegmatis*	0.83μM	Mycobacterium cell wall metabolism	Li et al, 2012	TetR type HTH domain; Binding site unknown
FlrA	*Vibrio cholerae*	0.378μM	Motility	Srivastava *et al*, 2013	AAA+, sigma 54; Between REC and AAA+ domain
BrlR	*Pseudomonas aeruginosa*	2.2μM	Drug resistance in biofilms	Chambers *et al*, 2014	MerR family; binding site unknown

Table 1: List of known c-di-GMP binding transcription factors in bacteria. It is evident from this table that c-di-GMP binding transcription factors belong to many classes of transcriptional regulators. These factors regulate a multitude of phenotypes in bacteria. For many of these factors, c-di-GMP binding motifs remain uncharacterized.

FlrA (Hickman & Harwood, 2008, Srivastava et al., 2011) and 5) the MerR family protein, BrlR (Chambers *et al.*, 2014).

All known c-di-GMP binding transcription factors to date are listed in Table 1.

These factors are involved in regulation of diverse phenotypes such as biofilms, virulence and drug resistance. The mechanism of action to some extent has been characterized for three transcription factors Clp, VpsT and FleQ. In the case of VpsT from *V. cholerae*, c-di-GMP binds to a dimer interface to binding motif W[F/L/M][T/S]R (Krasteva et al., 2010). It was shown using structural characterization that binding of c-di-GMP to VpsT leads to c-di-GMP dependent dimerization of VpsT which activates downstream *vps* gene expression (Krasteva et al., 2010). For the Clp protein from *Xanthomonas campestris*, c-di-GMP binds to the cNMP binding domain (residue E 99) which induces a conformational change in the protein preventing it from binding promoter DNA of *engXCA* genes that code for endoglucanase proteins that act as essential virulence factors during infection (Tao et al., 2010). Further structural characterization of XcClp has identified other residues important for c-di-GMP binding (Table 1) (Chin *et al.*, 2010). Another Clp (CRP like) protein Bcam1439 was identified in *Burkholderia cenocepacia* as a c-di-GMP binding transcription factor. Bcam1439 has only 18% homology to XcClp and residues important for c-di-GMP binding to XcClp are not conserved in Bcam1439 suggesting that its c-di-GMP binding site is distinct (Fazli et al., 2011). For FleQ, it was recently demonstrated that c-di-GMP interacts with the Walker B motif of the AAA+ domain and is able to repress ATPase activity of FleQ (Baraquet & Harwood, 2013). Another protein FleN is required for this response(Hickman & Harwood, 2008). FleQ was shown to activate the expression of *pel* gene in response to high levels c-di-GMP but repress it at low levels of c-di-GMP (Hickman & Harwood,

2008, Baraquet *et al.*, 2012, Baraquet & Harwood, 2013). Structural characterization of FleQ, FlrA, MrkH, LtmA, VpsR and BrlR is pending. The various domains that interact with c-di-GMP and the diverse mechanisms elucidated for some of these factors suggest that no one common theme for c-di-GMP mediated transcriptional regulation and much remains unknown about these mechanisms.

CHAPTER 2

Integration of Cyclic di-GMP and Quorum Sensing in the Control of *vpsT* and *aphA* in *Vibrio cholerae*

PREFACE

Vibrio cholerae transitions between aquatic, environmental reservoirs and infection in the gastrointestinal tract of human hosts. The second messenger molecule, cyclic di-GMP (c-di-GMP), and quorum sensing (QS) are important signaling systems that enable *V. cholerae* to alternate between these distinct environments by controlling biofilm formation and virulence factor expression. Here, a conserved regulatory mechanism in *V. cholerae* is identified that integrates c-di-GMP and QS to control the expression of two transcriptional regulators: *aphA*, an activator of virulence gene expression and an important regulator of the quorum sensing pathway, and *vpsT*, a transcriptional activator that induces biofilm formation. Surprisingly, expression of *aphA* was induced by c-di-GMP. C-di-GMP activation of both *aphA* and *vpsT* requires the transcriptional activator VpsR, which binds to c-di-GMP. The VpsR binding site at each of these promoters overlaps with the binding site of HapR, the master QS regulator at high-cell density. Our results suggest that *V. cholerae* combines information conveyed by QS and c-di-GMP to appropriately respond and adapt to divergent environments by modulating the expression of key transcriptional regulators.

2.1 Introduction

Bacteria use multiple signaling pathways to monitor and appropriately respond to changing surroundings. Small molecule chemical signals convey information about the presence, nature, number, and characteristics of the surrounding bacterial species as well as the composition of the environment. Properly responding to changing environments is vital to the survival of bacteria. *Vibrio cholerae*, the causative agent of cholera, alternates between a motile, virulent state within the host and a sessile, biofilm state in aquatic environmental reservoirs (Faruque et al., 2006). Quorum sensing (QS) and cyclic-di-GMP (c-di-GMP) signaling are two chemical signaling systems that control this transition (Hengge, 2009).

QS allows bacteria to sense the population density and species composition of the surrounding bacterial consortium through the secretion and detection of chemical signals called autoinducers to collectively control behaviors (Waters & Bassler, 2005). In *V. cholerae*, in the high-cell density QS state, both biofilm formation and virulence factor expression are repressed (Miller et al., 2002). C–di-GMP is a nearly ubiquitous bacterial second messenger that induces biofilm formation and represses motility (Hengge, 2009). In contrast to QS, c-di-GMP activates expression of genes necessary for biofilm formation in *V. cholerae* (Beyhan et al., 2006). However, similar to QS, c-di-GMP is thought to repress expression of virulence factors (Tischler & Camilli, 2005, Tamayo et al., 2007).

The QS regulatory pathways that control biofilm formation and virulence factor expression have been largely elucidated. HapR, the master high-cell density regulator of the QS signaling cascade, represses biofilm formation by directly binding to and inhibiting transcription of the biofilm activator, *vpsT* (Waters et al., 2008). Additionally, HapR production reduces the intracellular levels of c-di-GMP (Waters et al., 2008). Inhibition of virulence factor expression by QS is mediated by HapR repression of the virulence gene activating protein, *aphA* (Kovacikova & Skorupski, 2002). Interestingly, *aphA* is also the master regulator of the QS low-cell density state in *V. cholerae* and *Vibrio harveyi* (Rutherford et al., 2011). Although much is known about QS control of biofilms and virulence factor expression, the molecular mechanism by which c-di-GMP controls biofilm formation and virulence factor expression is less understood.

Other transcriptional activators that have been shown to bind and regulate gene expression in response to c-di-GMP are FleQ and Clp from *Pseudomonas aeruginosa* and *Xanthomonas campestris,* respectively (Hickman & Harwood, 2008, Chin et al., 2010). The regulation of gene expression by c-di-GMP has been shown to occur in many bacterial species; however, the molecular mechanisms responsible for this regulation have been elucidated for only a subset of genes (Hengge, 2009). Further characterization of transcriptional effectors that bind c-di-GMP in *V. cholerae* and other bacteria will shed light on how c-di-GMP controls important phenotypes like biofilm formation and virulence factor expression.

Here, we report that *aphA* expression is induced by c-di-GMP. We determine that this induction occurs via a common regulatory mechanism encoded in both the *aphA* and *vpsT* promoters that integrates QS and c-di-GMP signaling. Induction of *vpsT* and *aphA* is independent of VpsT but requires VpsR, a transcriptional activator that directly binds the promoters of these genes. VpsR binds c-di-GMP with a dissociation constant (K_d) of 1.6 μM. Both *vpsT* and *aphA* are directly repressed by HapR at a binding site that overlaps with the VpsR binding site (Lin *et al.*, 2007, Waters et al., 2008). Finally, we identify additional promoters activated by c-di-GMP that are not dependent on VpsR, suggesting there are multiple signal transduction pathways linking c-di-GMP to regulation of gene expression in *V. cholerae*. Furthermore, we have also isolated constitutive mutants of VpsR that can activate *vpsT* expression in the absence of c-di-GMP. Characterization of these mutants will help in understanding the mechanism of action of VpsR.

2.2 Materials and Methods

- **Bacterial strains and culture conditions**

All strains, primers, and plasmids used in this study are listed in the appendix section (Table 3 and 4). *V. cholerae* strains used in the study were derived from El Tor biotype strain C6706str2 and contain a mutation in *vpsL* (Thelin & Taylor, 1996). This mutation renders *V. cholerae* unable to make biofilms. Therefore, even under conditions of high levels of c-di-GMP, the cells do not flocculate enabling

accurate readings of reporter gene expression. Strains carrying mutations in *lrp*, *vpsT* and *vpsR* were constructed using the pKAS32 suicide vector as described (Skorupski & Taylor, 1996). This procedure generated unmarked deletions of the entire coding sequences of *vpsT* and *vpsR*. To generate the Δ*lrp* mutant, a gene encoding resistance to tetracycline, *tetA*, was amplified from pBR322 and inserted between the DNA encoding the upstream (550bp) and downstream (500bp) *lrp* flanking regions in pKAS32. Selection for crossover events was performed in the presence of a plasmid expressing wild type *lrp* (pDS15). These steps were performed because we were unable to generate a deletion of *lrp* using the standard protocol described by Skorupski (Skorupski & Taylor, 1996). pDS15 was cured prior to further analysis, and the deletion mutation was stably maintained in the absence of pDS15. Δ*alsS* was constructed by natural transformation and homologous recombination of a PCR product encoding the *cat* open reading frame from pKD3 flanked by FRT sites fused between 500bp upstream and downstream of *alsS* coding sequence (Datsenko & Wanner, 2000). This method created a chloramphenicol resistant *alsS* mutant. The unmarked deletion mutant was generated by flipping out the *cat* gene using the plasmid pTL17 which overexpresses the flippase enzyme (Long *et al.*, 2009). pTL17 was cured before performing any assays, and pCMW75 was introduced through biparental mating. All strains of *V. cholerae* were grown in Luria-Bertani medium (LB). Antibiotics were obtained from Sigma and used at the following concentrations (μg/ml): ampicillin 100, kanamycin 100, chloramphenicol 10, and tetracycline 10 unless stated otherwise. BW29427 and S17 *Escherichia coli*

strains were used as the donors in biparental conjugation to mobilize plasmids into *V. cholerae* (Reddy, September 2007).

- **Molecular methods**

DNA manipulation was performed using standard procedures (Sambrook *et al.*, 1989). T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (NEB) and Stratagene. PCR reactions were performed with iProof DNA Polymerase (NEB) and Phire DNA polymerase (Finnzymes). Promoter deletion constructs were cloned into the SpeI and BamHI restriction sites of pBBRlux (Waters & Bassler, 2006) (refer to Table 4 for primer sequences). pKAS32 cloning of *lrp*, *vpsT*, *and vpsR* was accomplished using primers listed in Table 4. Overexpression constructs for protein purification were engineered into pTXB1 for HapR, Lrp and YcgR, and pET28b for VpsR as per the manufacturer's instructions (IMPACT, NEB and Novagene). VpsR over-expression constructs were engineered into pAR3 (arabinose inducible promoter) vector using EcoRI and HindIII sites on the vector.

- **Screen to identify *V. cholerae* c-di-GMP responsive promoter*s* and measurement of gene expression**

A promoter library of *V. cholerae* was previously constructed by inserting SauIIIA restricted genomic DNA fragments into the BamHI restriction site of pBBRlux (Hammer & Bassler, 2007). From approximately 150,000 clones, 5,000 inserts that encoded promoters as determined by expression of the luciferase operon

were rearrayed to generate a *V. cholerae* promoter enriched library (Hammer & Bassler, 2007). These clones were pooled, the plasmids were isolated, retransformed into a donor *E. coli* strain, and ultimately conjugated into *V. cholerae* strain CW2034 containing the plasmid pCMW75 (Waters et al., 2008). pCMW75 overexpresses *qrgB*, a *V. harveyi* GGDEF protein under the control of the Ptac promoter (Waters et al., 2008). Induction of QrgB with IPTG produces high levels of c-di-GMP in *V. cholerae* (Waters et al., 2008). Bioluminescence of 960 isolates from the promoter-enriched library containing pCMW75 were measured on a plate reader (Spectra Max B5, Molecular devices) in the presence and absence of 0.1mM IPTG in LB medium after 7 hours and adjusted for growth by concurrent measurement of OD_{600}. Clones showing significant changes in bioluminescence in response to IPTG were re-assayed in triplicate. The fold-change was calculated by dividing the induced values by the uninduced values (Table 2). The inserted genomic DNA was sequenced using primers homologous to sequences upstream and downstream of the SpeI and BamHI restriction sites of pBBRlux (Waters & Bassler, 2006). Gene expression studies were performed similarly by assessing the expression of promoter-*lux* reporter fusions in pBBRlux for *aphA*, *vpsT* and their promoter deletion constructs) and virulence genes in the presence of the plasmids pCMW75 and pCMW98 at the uninduced versus induced (0.1mM IPTG) state. pCMW98 encodes a mutant allele of QrgB in which the active site has been mutated to the amino acid sequence AAEEF. This mutant is unable to synthesize c-di-GMP (Waters et al., 2008).

- **5'RACE for identification of *vpsT* start site**

RNA was prepared from *V. cholerae* WT cultures using the RNeasy RNA extraction kit (Qiagen). The transcription start site of *vpsT* was determined using 5'-RACE (Invitrogen) as per the manufacturer's instructions. The primers used are *vpsT*-GSP1 and *vpsT*-GSP2 (Table 4).

- **Purification of proteins and electrophoretic mobility shift assays**

HapR, Lrp, and YcgR were purified with the IMPACT protein purification system using the pTXB1 plasmid as described by the manufacturer's instructions (NEB). Purified proteins were stored in 20 mM Tris (pH 7.5), 1 mM EDTA, and 10 mM NaCl, in 20% glycerol. VpsR was purified using the pET28B plasmid as per the manufacturer's instructions (Novagene) as a C-terminal 6XHistidine tagged protein fusion and dialyzed in storage buffer (20 mM Tris pH 7.5, 1 mM EDTA, 1 M NaCl, 0.1 mM DTT). DNA probes for Electrophoretic mobility shift assays (EMSA) were prepared using PCR amplification with primers CMW234 and CMW235 tagged with 5' FAM (5-Carboxyflourescein) which are complementary to upstream and downstream sequences in pBBRlux that lie adjacent to the SpeI and BamHI restriction sites. The promoter fragment constructs harboring *vpsT* shown in Fig. 10B were used as the templates to generate the probes. 10 nM probe was incubated at 30^0C and 4^0C for 30 minutes with the proteins (HapR (42 nM to 800 nM) and VpsR (25 nM to 650 nM respectively, 1 μL dIdC (1 mg/mL stock) in a final 20 μL volume in respective protein buffers (Waters et al., 2008). EMSAs were performed

on 5% polyacrylamide TBE gels and visualized using *Typhoon* FLA 9000 scanner (GE healthcare Life Sciences).

- **Acetoin production assay**

V. cholerae cultures (with and without 0.1mM IPTG) were grown in MR-VP media (Benjaminson *et al.*, 1964) from overnight cultures for 24hrs shaking at 37^0C. The Voges Proskauer test was performed with 100 µL of culture by addition of 30 µL Solution A (5% Naphthol) followed by addition of 10 µL Solution B (40% KOH) (BD life sciences). After five minutes, the color development was quantified in the Spectra Max 96 well plate reader at 550 nm (Van Houdt *et al.*, 2007). Absorbance values at 600 nm were also recorded and data was analyzed by ratio of OD_{550} nm to OD_{600} nm. The experiment was repeated in triplicate.

- **Cyclic di-GMP binding assay**

VpsR and YcgR proteins were purified using 6X Histidine tagged affinity purification and IMPACT protein purification kit from NEB, respectively, as per the manufacturer's instructions. [^{32}P]-labeled c-di-GMP was generated using the purified cytoplasmic portion of the GGDEF VC2370 that does not contain the first 142 amino acid residues (VC2370-142) (De *et al.*, 2009). Reactions consisted of a total volume of 100 µL containing 10 µM VC2370 in buffer (75 mM Tris-Cl, pH 7.8, 250 mM NaCl, 25 mM KCl, 10 mM MgCl2), 12.5µM [α-^{32}P]-GTP (800 Ci/mmol,Perkin Elmer) or 12.5 µM unlabeled GTP were incubated at room

temperature for 30 minutes (Hickman & Harwood, 2008). Antarctic phosphatase (NEB) was added and reactions were incubated an additional 30 minutes to remove any residual [α-^{32}P]-GTP. Reactions were then heated at 100°C for 5 minutes, subsequently cooled on ice, and spun at15000 *g* for 10 minutes to remove denatured protein and collect the supernatants (Hickman & Harwood, 2008). The amount of c-di-GMP synthesized was assessed by running the unlabeled control reaction on UPLC-MS-MS against known c-di-GMP standards (Bobrov *et al.*, 2011). For binding reactions, 400nM protein in binding buffer (10mM $MgCl_2$, 20mM Tris pH 7.8 and 50mM NaCl) was incubated with varying amounts of [^{32}P]-labeled c-di-GMP (0.125 μM-1.14 μM) in a 20 μL volume for 30 minutes at room temperature. The binding was assessed using a filter binding technique (Hickman & Harwood, 2008). Preparations were loaded on nitrocellulose membrane (0.2μm, Whatmann) through a vacuum slot blot (Hybri Dot Manifold 1050MM, BRL). Sample wells were washed with 3 mL binding buffer to wash away unbound [^{32}P]-labeled c-di-GMP. The membrane was removed, dried, and individual wells were quantified by scintillation counting (cpm/min) (Hickman & Harwood, 2008). For competition experiments with unlabeled c-di-GMP and GTP, 3 μM unlabeled nucleotides were incubated with the preparations after 15 minutes of incubation with 1 μM [^{32}P]-labeled c-di-GMP in 20 μl volume and processed similarly

- **Screen for constitutive mutants of VpsR**

Random mutagenesis of *vpsR* was carried out using error prone PCR reaction with Taq polymerase (Invitrogen) in buffer containing 10mM Tris-Cl pH8.3, 50mM

KCl, 7mM $MgCl_2$ and 0.5mM $MnCl_2$ using forward and reverse primers containing BamHI and HindIII. This mutagenesis reaction created 3-7 nucleotide changes per amplicon. The mutants were cloned in arabinose inducible overexpression vector (pAR3). The mutants were mated into S17λpir strain containing pDL1711 and screened for high *vpsT* expression in the presence of 0.002% arabinose 7 hours post inoculation. A strain containing a GGDEF encoding vector (*qrgB*; pBRP2) under IPTG control with WT *vpsR* overexpression vector (pDS130) was used a positive control. The mutants that showed high *vpsT* expression were selected. These selected mutants were then tested for *vpsT* induction at low versus high levels of c-di-GMP. Mutants that had high *vpsT* expression and remained uninducible by c-di-GMP were selected for further analysis. Receiver domain truncation mutants of VpsR were overexpressed from plasmids pDS126 and pDS127 and tested in a S17 *E. coli* strain containing pBRP2 for *vpsT-lux* expression in the presence of 0.002% arabinose. The cultures were grown for 5 hours before addition of 0.1mM IPTG to induce c-di-GMP synthesis, luminescence recordings were done 7 hours post inoculation.

- **_vpsT_ promoter deletion and insertion constructs to test promoter looping**

Deletions and insertions of 5 bp increments were introduced in the *vpsT* promoter by PCR (Fig. 24) between the VpsR binding site and the -35 promoter element using the primers listed in table 4. These promoters were cloned in the pBBRlux vector (plasmids listed in Table 4). These constructs were tested in *V. cholerae* in the presence of 0.1mM IPTG with a mutant form of *qrgB*, *qrgB**

(AADEF; pBRP1 vector) and WT *qrgB* (GGDEF, pBRP2 vector) in the presence of 0.1mM IPTG. pDL1711 was used as a positive control for *vpsT* expression.

2.3 Results

- Identification of *Vibrio cholerae* c-di-GMP responsive promoters

To determine the molecular mechanism by which changes in the levels of c-di-GMP are coupled to the regulation of gene expression in *V. cholerae*, we performed a genetic screen to isolate promoters regulated by c-di-GMP. A library of random *V. cholerae* genomic fragments driving expression of a promoterless *luxCDABE* operon in the plasmid pBBRlux was used (Hammer & Bassler, 2007). C-di-GMP levels in the cell were modulated by introducing a plasmid encoding *qrgB*, a *V. harveyi* GGDEF protein, under the control of an IPTG inducible Ptac promoter. Strains containing a Δ*vpsL* mutation were used in this study because this mutation eliminates biofilm formation. This strategy is essential for accurate reading of reporter gene expression under conditions of high levels of c-di-GMP. Without the ability to form biofilms, the cells do not aggregate but, rather, remain as a well dispersed planktonic culture. Induction of QrgB by IPTG leads to increased levels of c-di-GMP in *V. cholerae* (Waters et al., 2008). Therefore, promoters induced by c-di-GMP show increased luciferase expression following IPTG addition while promoters repressed by c-di-GMP show decreased luciferase expression following IPTG addition.

Changes in bioluminescence readings with and without IPTG were measured for 960 independent clones. From these, we identified 7 unique c-di-GMP responsive promoters (Table 2). Expression of all of the promoters isolated in this screen increased in the presence of c-di-GMP. Four of these promoters drive genes encoding hypothetical proteins; one promoter is located in the ORF of VC1673, a putative transporter protein, one promoter drives expression of VC2108,

Table 2 : Cyclic-di-GMP regulated promoters identified in screen

FRAGMENT	DESCRIPTION	FOLD CHANGE*
1:B8	VCA0213-hypothetical	2.8
1:F6	VC2647-*aphA*, central regulator of virulence gene expression and Quorum sensing	3.9
2:G12	VC2610-hypothetical	1.3
4:H4	VC2108-erythronate-4-phosphate dehydrogenase	1.8
5:A6	VC1899-hypothetical	2.9
6:C9	VC1673-transporter family	3.9
9:C11	VCA0055,-conserved hypothetical	4.8

* Fold change= luminescence value at high levels of c-di-GMP/ luminescence at low levels of c-di-GMP

erythronate-4-phosphate dehydrogenase, and one promoter maps upstream of the virulence and QS regulator, *aphA* (VC2647). Our screen was not carried to

saturation and did not identify promoters that have been previously shown to be regulated by c-di-GMP (Krasteva et al., 2010, Beyhan et al., 2006).

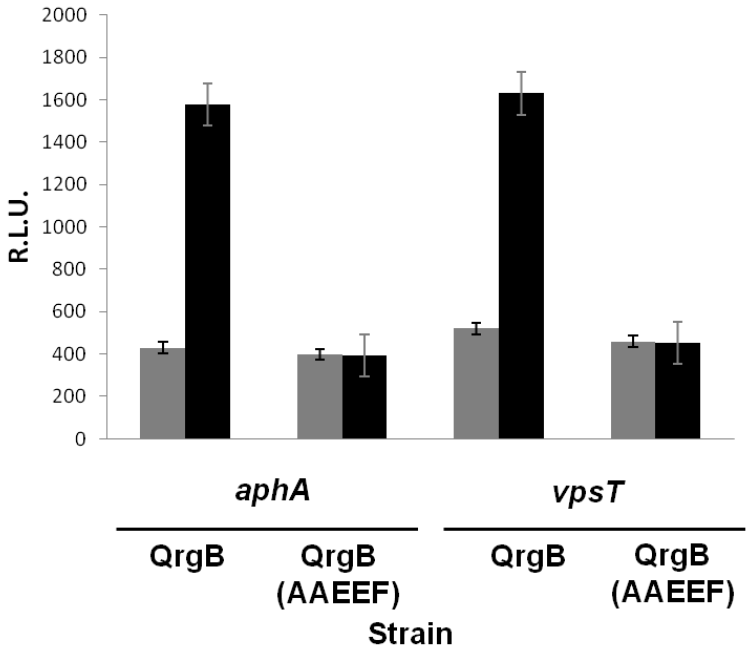


Figure 8:*aphA* and *vpsT* are induced at high levels of c-di-GMP. Luciferase production from *aphA-lux* and *vpsT-lux* following overexpression of a GGEEF enzyme (QrgB) and the corresponding QrgB AAEEF active site mutant was determined in the Δ*vpsL* strain. Gray bars indicate non-induced cultures. Black bars indicate addition of 0.1 mM IPTG. Error bars indicate the standard deviation. Relative light units (R.L.U.) are calculated by dividing the raw bioluminescence by the optical density of the culture at 600 nm.

The transcription activator AphA, along with its coactivator, AphB, positively regulates virulence in *V. cholerae* by increasing the expression of *tcpPH* (Kovacikova *et al.*, 2004). TcpP, along with TcpH, induces *toxT*, which activates expression of genes encoding the two major virulence factors of *V. cholerae*, cholera toxin (CT) and the toxin co-regulated pilus (TCP) (Häse & Mekalanos, 1998).

Earlier studies of c-di-GMP in *V. cholerae* have reported that c-di-GMP negatively regulates virulence (Tamayo et al., 2007), although the influence of c-di-GMP on *aphA* expression has not been examined. Thus, our observation that c-di-GMP activates *aphA* expression was surprising. However, consistent with our results, *aphA* functions as the master QS regulator at low cell density in *V. cholerae* (Rutherford et al., 2011), a state in which the intracellular concentration of c-di-GMP is relatively high (Waters et al., 2008).

To confirm that c-di-GMP induces the transcription of *aphA*, we reconstructed a transcriptional fusion of the *aphA* promoter in pBBRlux and examined its expression upon *qrgB* overexpression. As a control, we also examined expression following overproduction of a *qrgB* allele encoding a non-functional active site (GGEEF→AAEEF). This mutant protein has previously been shown to be incapable of c-di-GMP synthesis (Waters et al., 2008). Identical to the original *aphA* clone identified in the screen, expression of *aphA* was induced by c-di-GMP (Fig. 8). Furthermore, no induction of *aphA* occurred upon overexpression of the *qrgB* active site mutant, confirming that induction occurs through synthesis of c-di-GMP (Fig. 8). Similarly, a transcriptional fusion of the *vpsT* promoter to luciferase in pBBRlux was induced by c-di-GMP upon overexpression of wild type QrgB but not the QrgB active site mutant derivative (Fig. 8), confirming our previous observation (Waters et al., 2008) .

To examine the consequence of c-di-GMP induction of *aphA* on the expression of genes encoding virulence factors, we constructed *lux* transcriptional fusions of the virulence genes *tcpA, ctxA, tcpP*, and *toxT*. Expression of these genes was 1.2-1.5 fold higher at high concentrations of c-di-GMP when the cells were grown in the virulence inducing AKI media (data not shown). Induction of *aphA* expression by c-di-GMP in AKI was similar to the induction observed in LB. Therefore, we conclude that while c-di-GMP increases transcription of the *aphA* promoter, induction of downstream virulence genes is modest under the conditions tested here (data not shown).

- **C-di-GMP reduces acetoin production through induction of *aphA***

In addition to controlling virulence factor expression, AphA regulates numerous other genes (Kovacikova *et al.*, 2005, Rutherford et al., 2011). AphA reduces acetoin synthesis through repression of the VC1588-VC1593 operon harboring biosynthetic genes for acetoin and 2,3-butanediol synthesis (Kovacikova et al., 2005). To test if the induction of *aphA* by c-di-GMP impacts additional *aphA* controlled phenotypes, we measured the acetoin produced at low versus high levels of c-di-GMP using a Voges Proskauer test (Benjaminson et al., 1964), which assesses the amount of acetoin produced by bacteria in the medium. Bacteria were grown in MR-VP medium (Difco), a medium that contains a mixture of glucose and buffered peptone. This environment induces *V. cholerae* to make acetoin to combat acidic effects of metabolic end products (Kovacikova et al., 2005). Induction of QrgB, which

generates high levels of c-di-GMP, reduced acetoin production in the Δ*vpsL* mutant (Fig. 9). We hypothesized that this reduction was due to increased expression of *aphA.* Indeed, induction of c-di-GMP synthesis in an *aphA* mutant strain did not significantly reduce acetoin production, showing that the impact of c-di-GMP on acetoin requires *aphA.* Overexpression of *aphA* significantly reduced acetoin production, even when the Ptac promoter driving its expression was not induced

with IPTG. We interpret this result to mean that low levels of *aphA* expression from the uninduced Ptac promoter on a multicopy plasmid are sufficient to fully repress acetoin production. As expected, acetoin production was also abolished in the *alsS* deletion mutant encoding the enzyme alpha-acetolactate essential for this biosynthetic pathway (Kovacikova et al., 2005). These results indicate that c-di-GMP induction of *aphA* alters expression of a subset of the genes controlled by *aphA.* As *aphA* is the major low-cell-density regulator of the QS pathway in *V. cholerae* (Rutherford et al., 2011), induction of *aphA* by c-di-GMP would be expected to alter the expression of numerous genes and phenotypes although this remains to be formally examined.

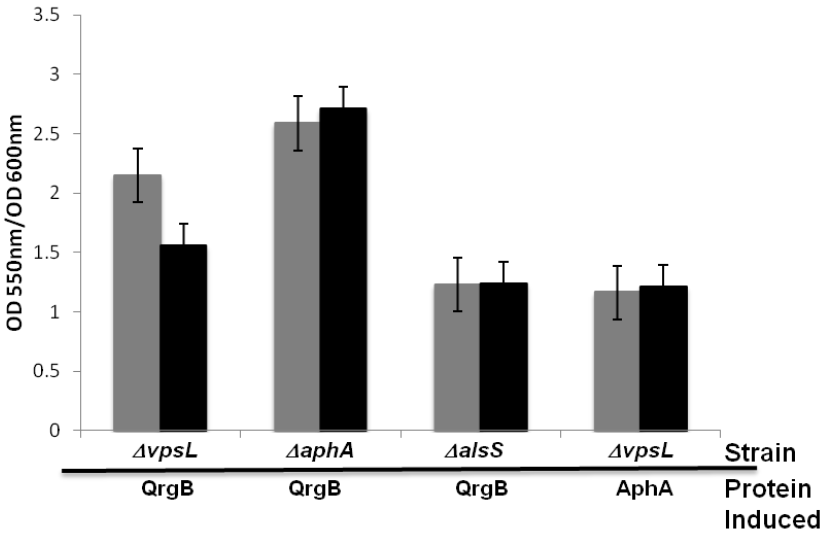


Figure 9: C-di-GMP represses acetoin production through induction of *aphA*. Acetoin production was assessed by the MR-VP test. The first three strains examined contain the pTac-*qrgB* overexpression vector while the fourth has a pTac-*aphA* overexpression vector. Each strain was tested under non-inducing conditions (gray) and following addition of 0.1mM IPTG (black). Error bars indicate the standard deviation.

- ***aphA* and *vpsT* encode similar promoter architectures**

C-di-GMP induces the transcription of *vpsT,* leading to increased biofilm formation in *V. cholerae* (Waters et al., 2008, Beyhan et al., 2006). VpsT is a transcriptional activator of the LuxR, CsgD, and FixJ family (Casper-Lindley & Yildiz, 2004). The mechanism of c-di-GMP induction of *vpsT* expression is not known; however, comparison of the promoter architectures of *vpsT* to that of *aphA* indicates they could share a similar regulatory control mechanism. Expression of *aphA* is controlled by three global regulatory proteins; HapR, Lrp, and VpsR (Lin et

al., 2007). Lrp, a transcriptional regulator that responds to changes in the cell's metabolic state (Brinkman *et al.*, 2003), induces expression of *aphA* by binding to a site between bases -138 to -123 (Fig. 10A). VpsR, a transcriptional activator of biofilm formation (Yildiz et al., 2001), activates *aphA* expression by binding in the -88 to -70 region (Fig. 10A). HapR, the master high-cell density transcription regulator of the quorum sensing system in *V. cholerae*, represses *aphA* expression by binding to the nucleotides located -85 to -57 from the transcription start site (Fig. 10A) (Lin et al., 2007). HapR represses *aphA* expression by excluding VpsR binding (Lin et al., 2007).

A binding site for VpsR was predicted to exist in the *vpsT* promoter (Lin et al., 2007). Genetic evidence also suggests that VpsR is essential for *vpsT* expression, although a direct interaction between VpsR and the *vpsT* promoter has not been shown (Casper-Lindley & Yildiz, 2004). We also identified a predicted binding site for Lrp on the *vpsT* promoter located upstream of the predicted VpsR binding site. Lrp has not been reported to be a regulator of *vpsT*. A HapR binding site is also predicted in the *vpsT* promoter, and we have previously shown that HapR directly binds to and represses *vpsT* expression, although the exact binding site has not yet been identified (Waters et al., 2008). Importantly, similar to their organization at the *aphA* promoter, the predicted binding sites for VpsR and HapR also overlap in the *vpsT* promoter (Fig. 10B). Therefore, we wondered if *aphA* and *vpsT* could share a common regulatory mechanism that is activated by c-di-GMP.

To examine this possibility, we first determined that the *vpsT* transcriptional start site is 20 base pairs upstream of the translational start site at a T nucleotide using 5'-RACE. This base will now be referred to as +1 (Fig. 10B). Using this information, we can now define the predicted binding sites of Lrp, HapR, and VpsR relative to the transcription start site to be -163 to -148, -144 to -123, and -136 to -118, respectively (Fig. 10B). While the locations of these predicted binding sites relative to one another in *vpsT* indeed parallels their corresponding sites in the *aphA* promoter, the spacing between these binding sites and the transcription start site is greater in the *vpsT* promoter (118 bases) than the *aphA* promoter (57 bases) (Fig. 10). The implications, if any, of this difference in spacing are currently unknown.

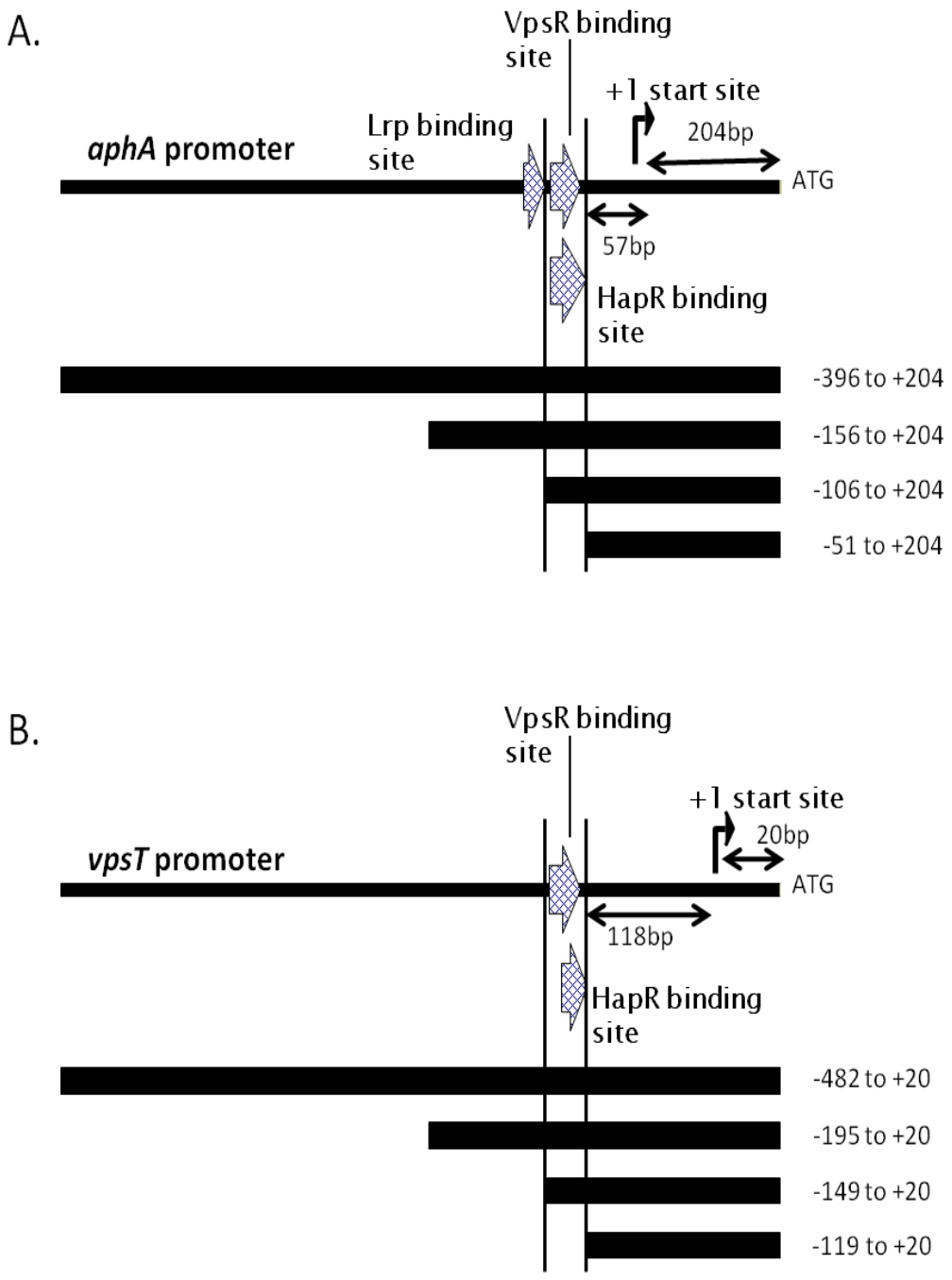


Figure 10 : Architectures of the *aphA* (A) and *vpsT* (B) promoters. The VpsR, HapR, and Lrp binding sites, transcription start sites, and translation start sites for the *aphA* and *vpsT* promoters are shown. The black bars underneath each promoter indicate the promoter deletion constructs that were made in this work.

- **C-di-GMP activation of the *aphA* and *vpsT* promoters requires VpsR**

To further examine the roles of HapR, VpsR, and Lrp in c-di-GMP regulation of *aphA* and *vpsT*, we constructed transcriptional fusions of *aphA* encoding the base pair at -396 to the translation start site, and of *vpsT* encoding the base pair from -482 to the translation start site in a *lux* reporter plasmid (see Fig. 10). The expression of these two fusions was measured in response to increased c-di-GMP levels in Δ*vpsL,* Δ*vpsL*Δ*lrp*::*tetA*, Δ*vpsL*Δ*hapR*, and Δ*vpsL*Δ*vpsR* mutant strains. C-di-GMP induction of transcription from these promoters is maintained in the Δ*lrp*::*tetA* and Δ*hapR* mutants for both *aphA* and *vpsT* (Fig. 11). Expression of *aphA* was lower in a Δ*lrp*::*tetA* mutant than in the wild type strain because Lrp activates *aphA* expression (Higgins et al., 2007). No difference in *vpsT* expression was observed in the Δ*lrp*::tetA mutant suggesting that Lrp does not regulate *vpsT* expression in the conditions examined here. Expression of both *aphA* and *vpsT* was increased in a Δ*hapR* strain, confirming previous findings that QS regulates these promoters through HapR repression (Waters et al., 2008, Kovacikova & Skorupski, 2002). The fold induction of *aphA* expression in the Δ*hapR* mutant was reduced as basal expression in the low c-di-GMP condition was greatly increased; suggesting that HapR repression of *aphA* predominates.

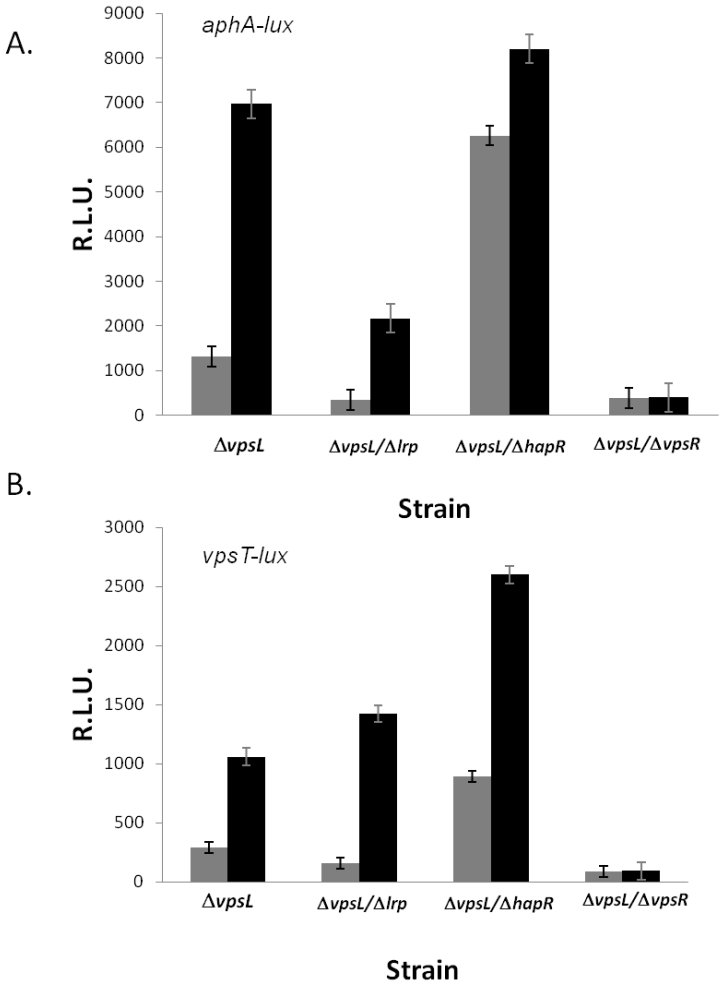


Figure11:VpsR is required for c-di-GMP mediated induction of *aphA* and *vpsT*. Expression of *aphA-lux* **(A)** and *vpsT-lux* **(B)** constructs was analyzed in Δ*vpsL*, Δ*vpsL*Δ*lrp*::*tetA*, Δ*vpsL*Δ*hapR* and Δ*vpsL*Δ*vpsR* mutants containing the Ptac-*qrgB* overexpression vector under non-inducing conditions (gray) and following addition of 0.1 mM IPTG (black). Error bars and Relative light units (R.L.U.) are as in Figure 8.

The fold induction of expression of *vpsT* by c-di-GMP in the Δ*hapR* mutant, however, was greater than that observed for *aphA*. Therefore, c-di-GMP induction may play a larger role relative to HapR repression in the regulation of *vpsT* than *aphA.* But, c-di-GMP induction was consistently observed in the Δ*hapR* mutant for

both genes. Importantly, neither *aphA* nor *vpsT* was induced in the Δ*vpsR* mutant strain. These results suggest that VpsR, but not HapR or Lrp, is essential for the c-di-GMP mediated induction of *aphA* and *vpsT*. Furthermore, Lrp does not regulate *vpsT* expression in these conditions.

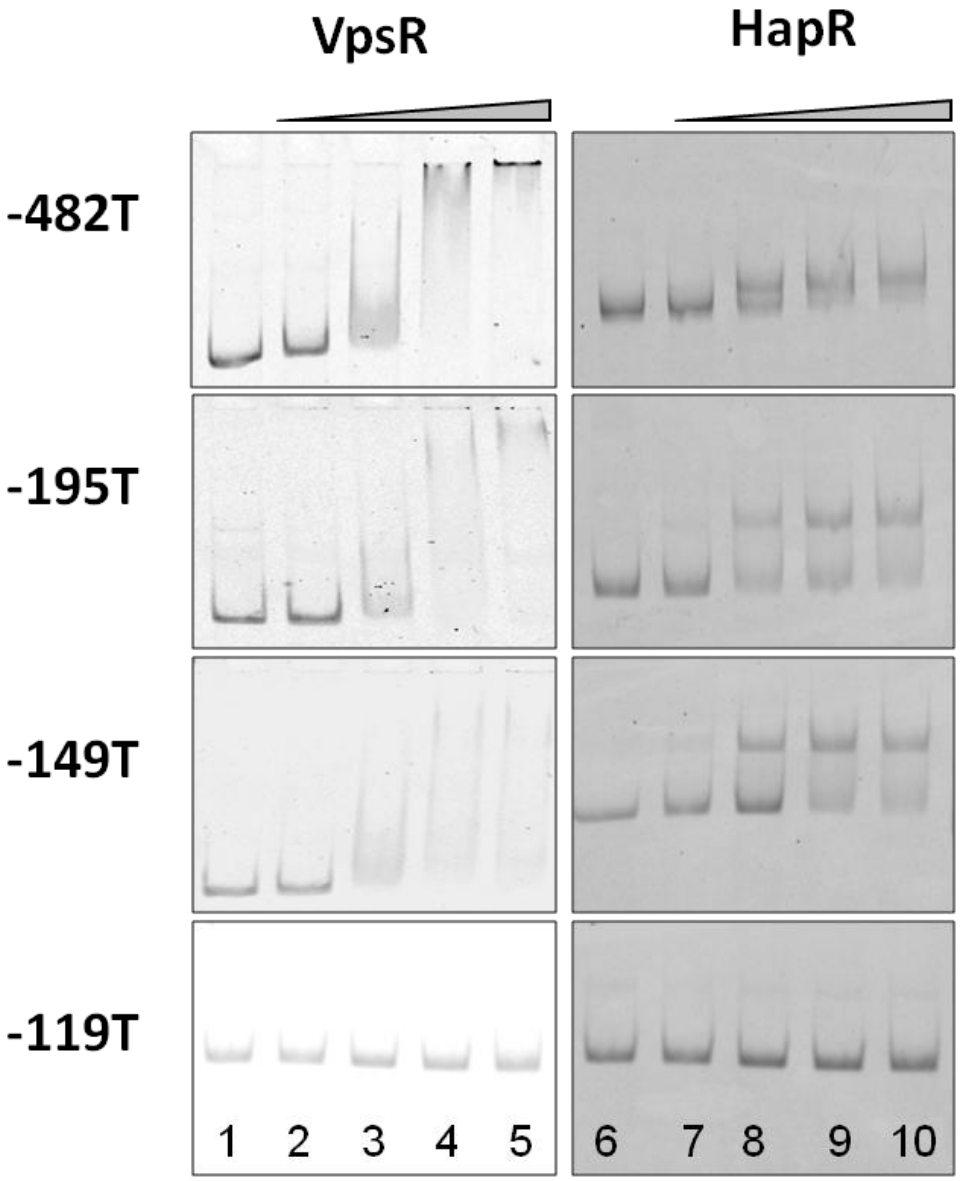


Figure 12: VpsR and HapR bind to the *vpsT* promoter at the predicted binding sites. The *vpsT* promoter deletion constructs shown in Figure 3 were used to generate fluorescent probes for EMSAs with purified VpsR and HapR. 10nM probe was used in all lanes. Lanes 1 and 6 contain no protein. Lanes 2-5 contain 25, 120, 360, and 650 nM VpsR, respectively. Lanes 7-10 contain 42, 230, 620, and 800 nM HapR, respectively.

- **VpsR directly binds to the *vpsT* promoter**

HapR, Lrp, and VpsR are known to bind the *aphA* promoter while only HapR has been shown to bind the *vpsT* promoter (Waters et al., 2008). To determine if VpsR and HapR also bind to the *vpsT* promoter at the predicted binding site (Fig. 10B), these proteins were purified and electrophorectic mobility shift assays (EMSA) were performed with each protein and a fluorescently labeled *vpsT* DNA probe. This probe contained the sequence from -482 to +20 of the *vpsT* promoter (Fig. 10B). As we have previously observed, HapR binds to the *vpsT* promoter (Waters et al., 2008). Furthermore, VpsR also binds to the *vpsT* promoter (Fig. 12, top panels).

To determine if the predicted binding sites for HapR and VpsR are correct, we constructed 5'-truncated transcriptional fusions of the *vpsT* promoter starting at -482, -195, -149, and -119 to the +20 translation start site in pBBRlux. These constructs will be referred to -482T, -195T, -149T and -119T respectively (Fig. 10B). Fluorescent probes of each of these derivatives were generated, and EMSAs were performed with purified HapR and VpsR (Fig. 12). The results indicate that VpsR and HapR bind to the *vpsT* promoter fragments -482T, -195T and -149T, but do not bind to the -119T fragment. This finding suggests that, indeed, the HapR and VpsR binding sites at the *vpsT* promoter lie between -149 to -119, as predicted. In addition, the binding of these proteins is specific as neither protein bound to the smallest fragment. Importantly, this result suggests that the binding sites for HapR

and VpsR overlap, similar to their relative locations in the *aphA* promoter (Fig. 10B).

- **The VpsR and HapR binding sites are required for c-di-GMP induction of *aphA* and *vpsT***

Expression of both *aphA* and *vpsT* is induced by c-di-GMP, and they share binding sites for both HapR and VpsR. Our mutation analysis showed that VpsR is critical for the c-di-GMP induction of the *vpsT* and *aphA* promoters (Fig. 11). To test if the VpsR binding sites encoded in the *aphA* and *vpsT* promoters are important for this regulation, we analyzed c-di-GMP induction of the -428T, -195T, -149T, and -119T *vpsT* transcriptional fusions in pBBRlux. Similarly, we engineered corresponding transcriptional fusions of the *aphA* promoter in pBBRlux referred to as -396A, -156A, -106A and -51A (see Fig. 10A). These *aphA* constructs contain binding sites for Lrp, HapR, and VpsR (-396A and -156A), only HapR and VpsR (-106A), or neither of these regulators (-51A) (Higgins et al., 2007). The expression levels of these *aphA-lux* and *vpsT-lux* promoter fusions were determined at varying concentrations of intracellular c-di-GMP by inducing expression of QrgB via increasing amounts of IPTG. This strategy increases the intracellular c-di-GMP concentrations in a dose-dependent manner from 1 to 10 μM (unpublished observation). In the *aphA* and *vpsT* promoter derivatives, promoter fragments encoding the overlapping binding sites for HapR and VpsR were induced by c-di-GMP in an IPTG-dependent manner, while the shortest fragments that lack the HapR and VpsR binding sites, -51A and -119T, were not significantly induced (Fig.

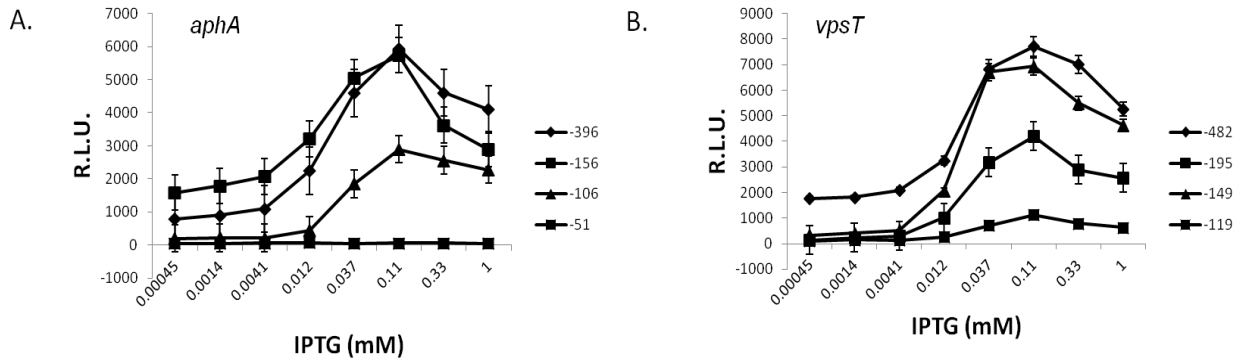


Figure13: Transcriptional response of *aphA* and *vpsT* to increasing levels of c-di-GMP. The *aphA* **(A)** and *vpsT* **(B)** promoter deletion constructs shown in Figure 10 were constructed as transcriptional fusions to the luciferase operon and introduced into a Δ*vpsL V. cholerae* mutant containing the Ptac-*qrgB* overexpression plasmid. C-di-GMP levels were increased by adding IPTG at concentrations from 0.45 μM to 1 mM. Error bars and Relative light units (R.L.U.) are as in Figure 8.

13). This result suggests that the VpsR binding site is critical for c-di-GMP induction of *aphA* or *vpsT.*

- **VpsT is not required for c-di-GMP induction of *vpsT* or *aphA***

Binding of c-di-GMP by VpsT induces a change in its oligomeric state leading to a form of the protein capable of promoting transcription (Krasteva et al., 2010). Genetic evidence has suggested that VpsT auto-activates its own expression (Beyhan et al., 2007), and it was hypothesized that induction of *vpsT* by c-di-GMP could occur through increased auto-activation by VpsT bound to c-di-GMP (Krasteva et al., 2010). To examine if VpsT mediates c-di-GMP induction of *aphA* and its own expression, we constructed a Δ*vpsT* mutant and analyzed c-di-GMP induction of the -396A (*aphA-lux*) and -482T (*vpsT-lux*) promoter constructs in the Δ*vpsL* and

Δ*vpsL*/Δ*vpsT* mutants (Fig. 14). The expression of *aphA* and induction by c-di-GMP was unaffected by deletion of *vpsT*. This result shows that *vpsT* does not regulate *aphA in* the conditions examined here. Alternatively, although the overall expression of *vpsT* was reduced in the Δ*vpsT* mutant, confirming that *vpsT* auto activates its own expression, the *vpsT* promoter remained inducible by c-di-GMP, similar to the induction of *vpsT* by c-di-GMP observed in the WT strain. Therefore, we conclude that VpsT is not required for c-di-GMP-mediated induction of either *aphA* or *vpsT*.

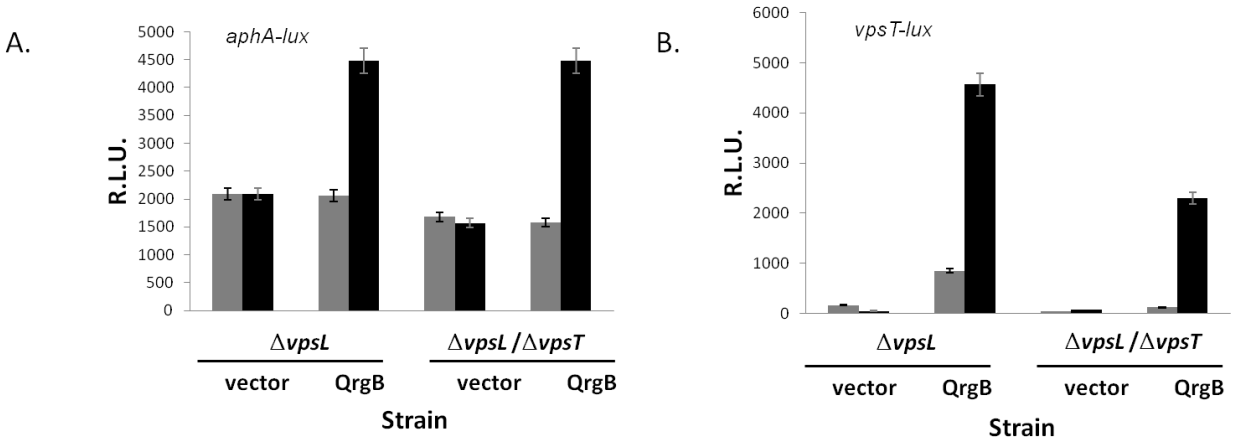


Figure 14:C-di-GMP activation of *aphA* and *vpsT* is independent of VpsT. Expression of *aphA-lux* **(A)** and *vpsT-lux* **(B)** in the Δ*vpsL* strain and the Δ*vpsL* /Δ*vpsT* mutant containing a vector control or the Ptac-*qrgB* overexpression vector was measured under non-inducing conditions (gray) or following induction with 0.1 mM IPTG (black). Error bars and Relative light units (R.L.U.) are as in Figure 8.

- **VpsR binds to c-di-GMP**

VpsR belongs to the NtrC family of transcriptional regulators that harbor a phosphorylation site in the amino terminus (Yildiz et al., 2001). The cognate kinase for VpsR phosphorylation has not yet been identified. Like other NtrC regulators, VpsR encodes consensus sequences for interaction with sigma 54 (Yildiz et al., 2001). However, expression of the *vpsT* and *aphA* promoters is not affected by a deletion of *rpoN,* the gene encoding sigma 54 (Syed et al., 2009). VpsR harbors predicted ATP binding and helix turn helix DNA binding domains like other members of the NtrC family (Buck *et al.*, 2000, Yildiz et al., 2001). FleQ, another NtrC-like regulator of *Pseudomonas aeruginosa*, directly binds c-di-GMP to regulate *pel* gene expression (Hickman & Harwood, 2008). To determine if VpsR also binds to c-di-GMP, a filter-binding assay was performed. In this experiment, purified protein incubated with [^{32}P]-labeled c-di-GMP was bound to a nitrocellulose membrane using a slot blot apparatus, extensively washed, and bound radioactivity was quantified using scintillation counting. We observed a dose dependent increase of binding when 400 nM of purified VpsR was incubated with varying amounts of [^{32}P]-labeled c-di-GMP (Fig. 15A). In the absence of protein, minimal binding was observed.

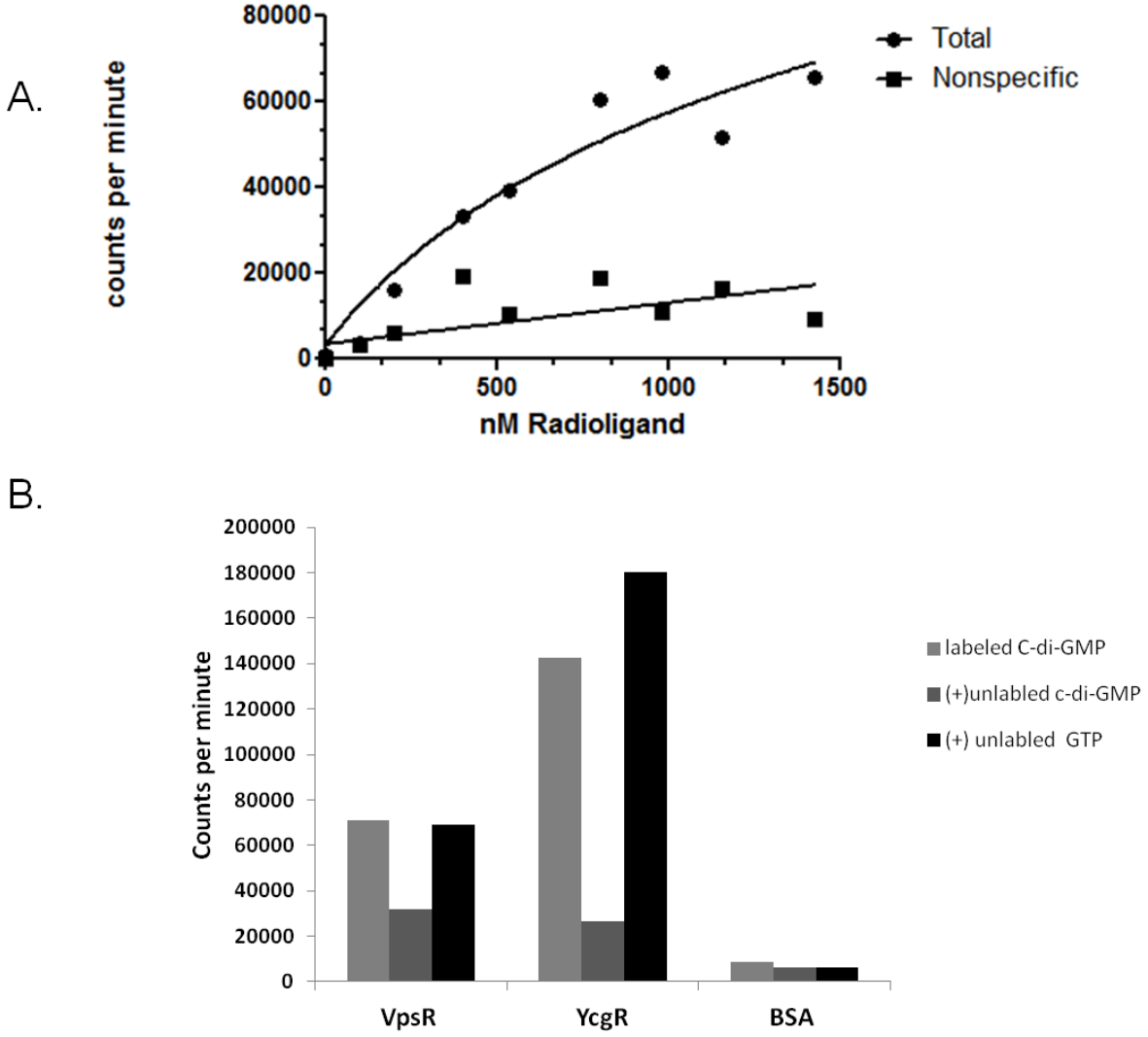


Figure 15: VpsR binds c-di-GMP. **(A)** Purified VpsR (400nM, circles) and buffer control (square) reactions were incubated with varying concentrations of [^{32}P]-labeled c-di-GMP to generate a saturation binding curve. The data was analyzed with Graph pad Prism 5.0 using non-linear regression analysis. This experiment was repeated four times and a representative curve is shown. **(B)** Purified proteins (400 nM) indicated on the X axis were incubated with 1 μM [^{32}P]-labeled c-di-GMP with and without 3 μM unlabeled c-di-GMP and GTP. This experiment was repeated twice with similar results.

Saturation binding of VpsR to c-di-GMP was examined four times independently to determine the binding affinity of VpsR to c-di-GMP. Averaging the data from these four experiments yielding a disassociation constant (K_d) of this interaction to be 1.6 μM with a standard deviation of 0.66. The data for saturation binding was analyzed using GraphPad software using the specific binding equation (Prism).

To determine if binding of c-di-GMP to VpsR is specific, we performed a similar filter binding assay examining binding of one concentration of [^{32}P]-labeled c-di-GMP to VpsR, BSA, and the protein YcgR in the presence of excess unlabeled c-di-GMP or GTP. YcgR is a PilZ encoding protein that directly binds to c-di-GMP to control motility (Ryjenkov et al., 2006, Fang & Gomelsky, 2010). YcgR bound to the most [^{32}P]-labeled c-di-GMP while BSA did not retain any radioactivity. VpsR retained two fold less [^{32}P]-labeled-c-di-GMP than YcgR (Fig. 15 B). Addition of a 3-fold excess of unlabeled c-di-GMP reduced binding of both VpsR and YcgR while 3-fold excess of unlabeled GTP had no effect (Fig. 15B). This experiment was repeated three times with similar results. Thus, we conclude that the observed binding of c-di-GMP to VpsR is specific.

Addition of c-di-GMP does not change the *in vitro* DNA binding profile of VpsR to the *vpsT* or *aphA* promoter in the conditions we tested (data not shown). This result differs from the other described c-di-GMP binding transcriptional regulators, FleQ, Clp, and VpsT, all of which differentially bind DNA *in vitro* in the presence and absence of c-di-GMP (Hickman & Harwood, 2008, Krasteva et al., 2010, Chin et

al., 2010). Therefore, we conclude that VpsR binds c-di-GMP; however, it remains to be determined how this binding affects VpsR function at these promoters.

- **VpsR is not required for c-di-GMP-mediated induction of additional *V. cholerae* promoters**

In addition to the promoter controlling *aphA*, our screen identified six other promoters that are activated by c-di-GMP (Table 1). To determine if VpsR is required for the c-di-GMP-mediated regulation of these promoters, they were introduced (as promoter-*lux* fusions) into the Δ*vpsL* and Δ*vpsL*Δ*vpsR* mutants containing inducible *qrgB*. Here, we present results for two isolates (1:B8 and 5:A6, regulating genes VCA0213 and VC1899, respectively) as representatives (Fig. 16). The other four promoters behaved similarly to these two (data not shown). In contrast to what we found for *aphA* and *vpsT*, none of the other six promoters required VpsR for c-di-GMP induction, indicating that their c-di-GMP-regulation occurs via a VpsR-independent mechanism. Because VpsT is not expressed in a Δ*vpsR* mutant (Casper-Lindley & Yildiz, 2004), we also infer that VpsT is not required for induction of these six promoters. Taken together, our results indicate that multiple signal transduction pathways in *V. cholerae* link c-di-GMP to transcription regulation.

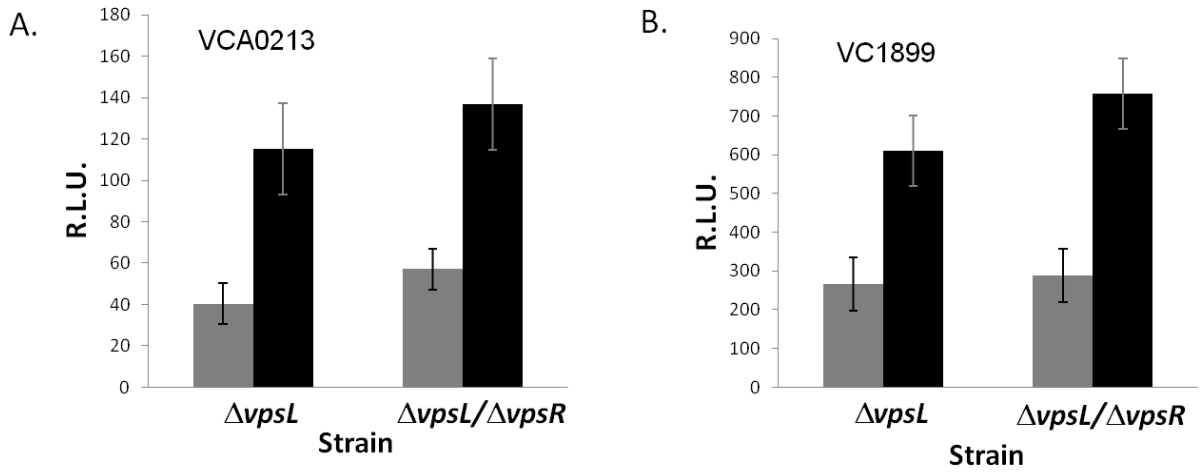


Figure 16: VpsR is not required for c-di-GMP-mediated activation of other *V. cholerae* promoters. The expression of two promoter constructs VCA0213 **(A)** and VC1899 **(B)** (from Table 2) was analyzed in the Δ*vpsL* and the Δ*vpsL*Δ*vpsR* mutants containing the Ptac-*qrgB* overexpression vector under non-inducing conditions (gray) and following addition of 0.1 mM IPTG (black). Error bars and Relative light units (R.L.U.) are as in Figure 8.

- **Insight into VpsR mechanism of action**

VpsR binds to c-di-GMP to activate *vpsT* gene expression (Fig. 15). We do not clearly understand the mechanism of this activation. VpsR is a member of the enhancer binding protein family (EBP), but it is non-canonical in both its sequence and function. Firstly, VpsR unlike most other EBPs, functions in combination with σ70 instead of σ54 to regulate *vpsT* expression (data not shown). Also, it lacks the GAFTGA motif in the AAA+ domain essential for σ 54 interaction (Francke *et al.*, 2011). Secondly, VpsR has sequence identity to the EBP family, but it is mutated in the key conserved residues of the Walker B motif in the AAA+ domain (DE to ND mutation) that makes it unlikely to perform ATPase function (Fig. 17). Lastly, we

do not see a change in the DNA binding profile of VpsR in the presence of c-di-GMP suggesting that either the change in conformation is too small to observe or non-existent. These observations lead to many questions regarding how c-di-GMP impacts the mechanism of action of VpsR to induce *vpsT* expression.

There are other known examples of proteins that have some sequence similarity to VpsR that also function with σ70. One well known example is TyrR (Yang *et al.*, 2002, Yang *et al.*, 2004). TyrR is a σ54-independent EBP that regulates gene expression in response to aromatic amino acids (Pittard *et al.*, 2005). TyrR binds to

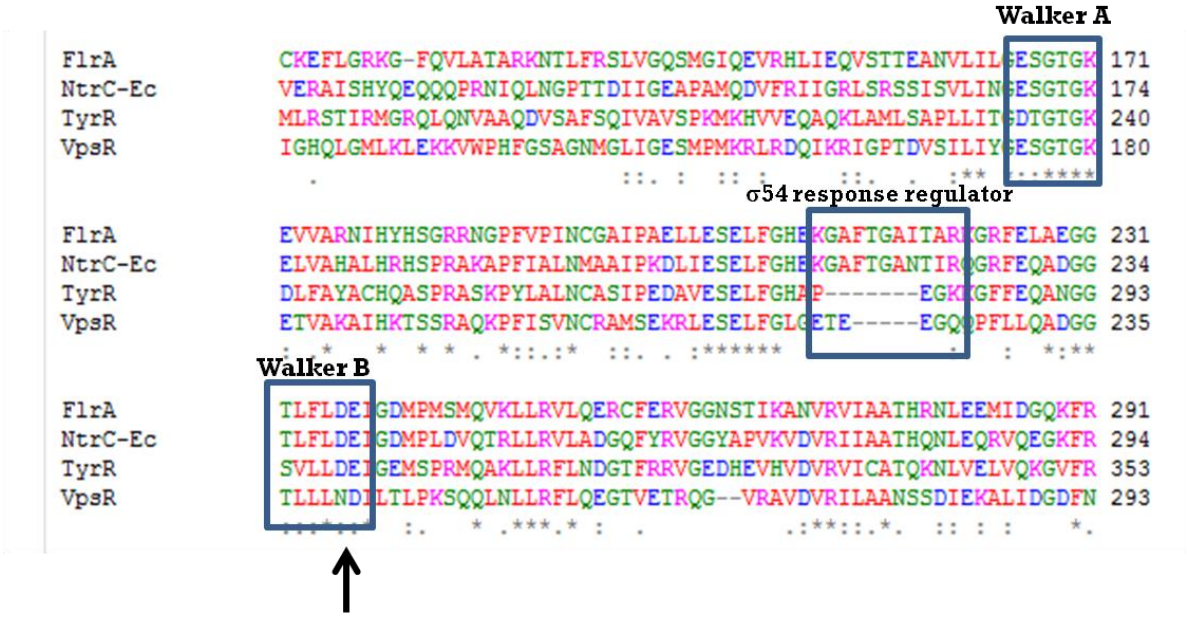


Figure17: VpsR is a non canonical EBP. Clustal W alignment of amino acid sequences of NtrC (*E.coli*), FlrA (*V. cholerae*), TyrR (*E.coli*) and VpsR (*V. cholerae*) highlighting the important regions in the AAA+ domain. The arrow at the bottom points out to the mutation in VpsR Walker B domain. σ 54 interaction region is also highlighted to show the missing GAFTGA (interaction) domain in TyrR and VpsR.

phenylalanine or tyrosine via its N-terminal receiver domain to regulate downstream gene expression (Yang et al., 2004, Pittard et al., 2005). TyrR however interacts with ATP through its AAA+ domain and unlike VpsR does not contain a mutation in its Walker B motif. Other σ54-independent EBP examples include HupR and RcNtrC from *E.coli* and *Rhodobacter capsulatus* respectively (Dischert *et al.*, 1999, Richard *et al.*, 2003). HupR is negatively regulated by its cognate sensor kinase by phosphorylation whereas RcNtrC is positively regulated by phosphorylation (Cullen *et al.*, 1996, Richard et al., 2003, Dischert et al., 1999). These examples show that the non-cognate EBPs can be functionally regulated by varied mechanisms. If we can define the mechanism of VpsR and characterize the interaction motif for c-di-GMP on VpsR, we can use this information to identify other VpsR-like EBPs in bacteria.

- **REC domain of VpsR is required for VpsR function**

VpsR contains a REC domain at its N terminus which has a conserved target aspartate for phosphorylation (D59). It has been observed that a D59E mutant of VpsR has increased activity suggesting a role for phosphorylation in VpsR regulation. No kinases that phosphorylate VpsR have been identified in *V. cholerae*. We have previously shown that VpsR (D59E) and VpsR (D59A) are able to induce *vpsT* expression in response to c-di-GMP (Christopher M. Waters, unpublished data). Thus, c-di-GMP does not appear to affect *vpsT* expression by regulating VpsR phosphorylation, although *vpsT* expression is low in response to VpsR (D59A)

suggesting the importance of this phosphorylation (data not shown). We have shown that VpsR can induce *vpsT* expression in the heterologous organism, *E. coli* suggesting that if phosphorylation of VpsR is important, it does not occur via a *V. cholerae* specific kinase (Fig. 18). This experiment was performed in *E. coli*, where we introduced the *vpsT*-lux vector pDL1711, arabinose inducible *vpsR* overexpression vector (pDS125) and IPTG inducible *qrgB*, a GGDEF protein that

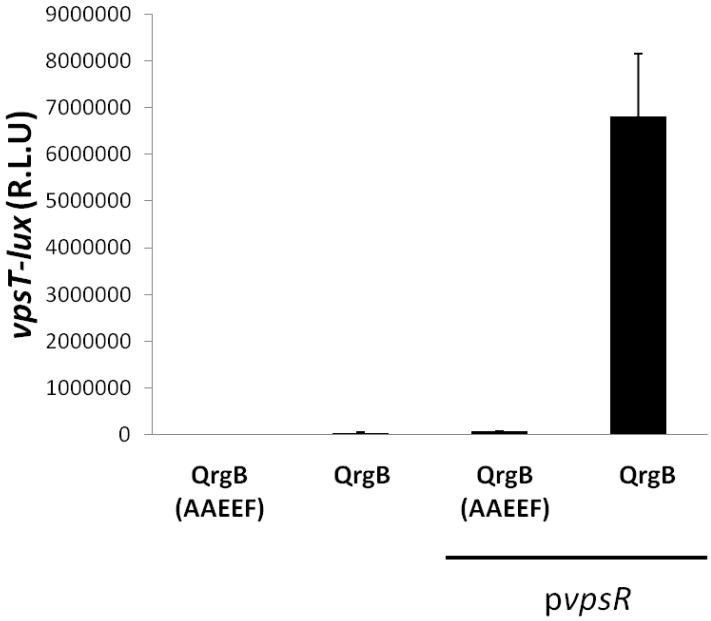


Figure 18: *vpsT* induces in *E.coli* in the presence of VpsR and c-di-GMP. *vpsT-lux* expression in the presence of *vpsR* overexpressed from arabinose inducible promoter along with a GGDEF (*qrgB*) overexpressed from an IPTG inducible promoter in *E. coli* S17λpir. Expression of *vpsT* is highly induced in the presence of both VpsR and c-di-GMP suggesting that VpsR can lead to c-di-GMP mediated induction of *vpsT* in an heterologous organism. The cultures were grown for 5 hours with 0.002% arabinose (for p*vpsR* expression) before addition of 0.1mM IPTG to induce c-di-GMP. and the luminescence and O.D 600nm readings were take 2-3 hours after addition of IPTG. Gray bars indicate non-induced cultures. Black bars indicate addition of 0.1 mM IPTG Error bars represent standard deviation.

synthesizes c-di-GMP (pBRP2).

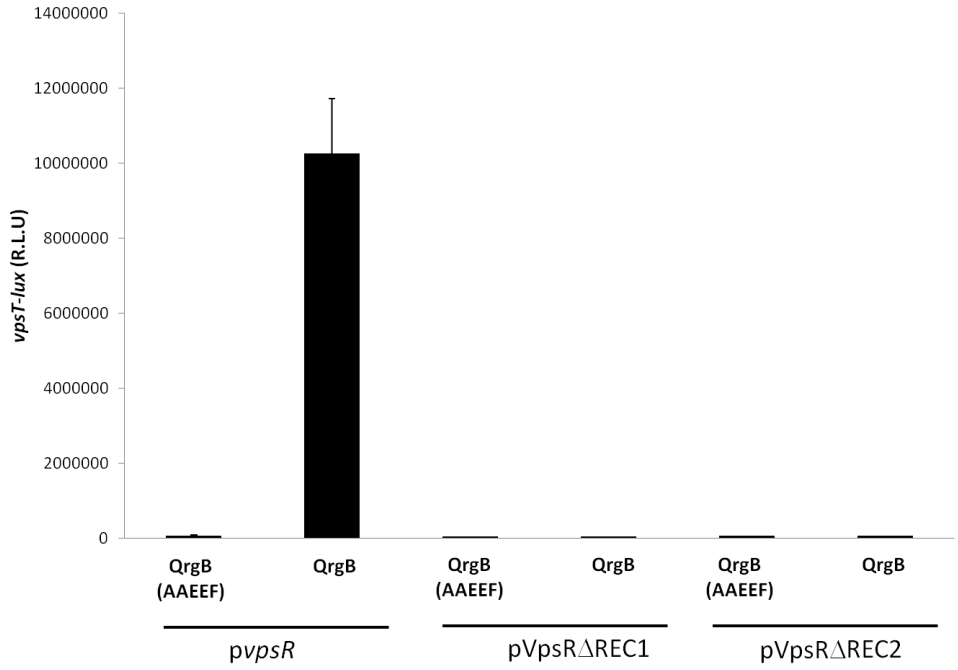


Figure 19: The REC domain of VpsR is important for function. Truncation mutants in the receiver domain of VpsR were tested in an *E. coli* S17λpir assay as described previously. ΔREC1 is a deletion of residue 1 to 126 and REC2 is a deletion of residue 1-142. Both mutants are inactive in initiating transcription suggesting the importance of REC domain in VpsR activity. The cultures were grown for 5 hours with 0.002% arabinose (for p*vpsR* expression) before addition of 0.1mM IPTG to induce c-di-GMP and the luminescence and O.D 600nm readings were take 3 hours after addition of IPTG. Error bars represent standard deviation.

The N-terminal domains of EBPs can regulate the function of these proteins in either a positive or a negative manner (Rappas *et al.*, 2005, Schumacher *et al.*, 2006). In positive regulation, phosphorylation leads to activation of protein by promoting conformational changes that lead to oligomerization. In negative

regulation, phosphorylation leads to inactivation of protein mostly due to formation of a closed dimer (Schumacher et al., 2006). In order to test what role REC domain of VpsR plays in its activity, we made truncation of VpsR in its REC domain VpsRΔREC1 (127-443aa) and VpsRΔREC2 (143-443aa), deleting the REC domain completely in both cases but leaving the complete linker region between AAA+ and REC domain in ΔREC1 and deleting half of the linker domain in ΔREC2 respectively. We tested the expression of *vpsT* in response to these mutants versus WT VpsR. Both VpsR truncations were unable to activate and induce *vpsT* expression suggesting that the REC domain of VpsR functions in a positive manner (Fig. 19). These truncations were being overexpressed in *E. coli* (data not shown) suggesting that the truncation does not have an effect on the stability and expression. We have not tested c-di-GMP binding to these truncations, and it is possible that the REC domain is important for interaction with c-di-GMP.

- **The AAA+ domain is important for VpsR function**

In order to characterize the molecular mechanism by which c-di-GMP activates VpsR function, we wanted to also identify the important residues that c-di-GMP interacts with. VpsR contains an AAA+ domain but the relevance of this domain in VpsR function is unknown. As mentioned previously, the Walker B motif of VpsR contains a mutation that possibly renders VpsR inactive for ATPase function (Fig. 17). It is possible that VpsR can still function by binding ATP as the Walker A motif is fully conserved. The AAA+ domain is a positively charged region which is able to

interact with negatively charged ATP. We hypothesized that the AAA+ domain region of VpsR interacts with c-di-GMP. To test our hypothesis, we performed site directed mutagenesis of the AAA+ domain (changing conserved residues to alanine, one or two at a time) and tested the effect of these mutations on *vpsT* expression in the presence and absence of c-di-GMP. We mutated important functional residues (from CLUSTAL analysis with other known EBPs) in the Walker A and Walker B domain; VpsR-G179A/K180A (Walker A) and VpsR-T236A/L237A (Walker B). Mutation of both Walker A and Walker B domains failed to activate *vpsT* expression in presence of c-di-GMP suggesting the importance of the AAA+ domain in VpsR function (Fig. 20). We know that both mutants are expressed as full length protein in *E. coli* (data not shown). This suggests that AAA+ domain is required for VpsR activity and conserved residues are essential for this function.

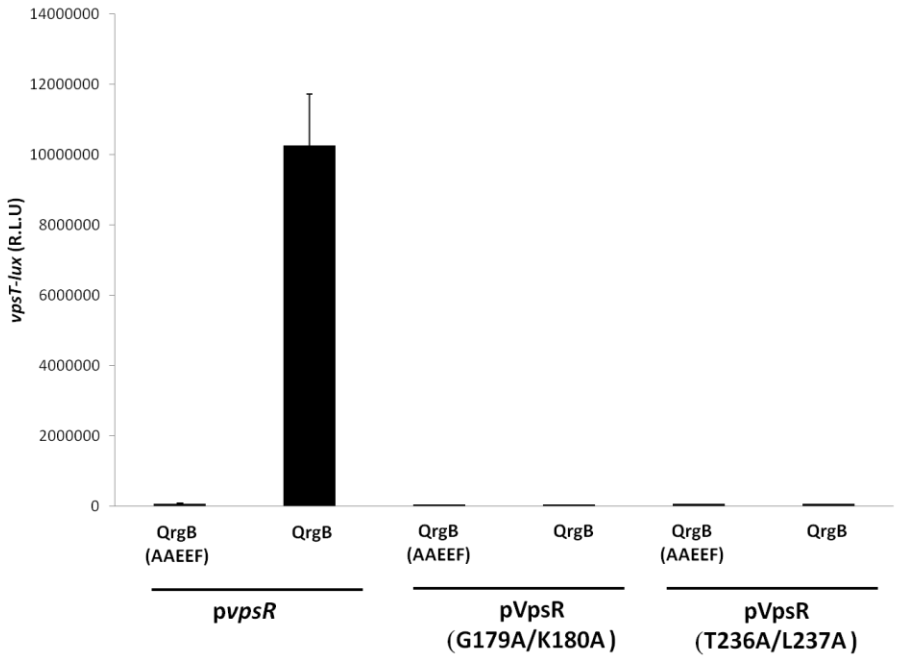


Figure 20: AAA+ domain of VpsR is important for function. Site directed mutants in the AAA+ domain motifs Walker A and Walker B were constructed and tested in *E. coli* S17λpir assay as described previously. The cultures were grown for 5 hours with 0.002% arabinose (for p*vpsR* expression) before addition of 0.1mM IPTG to induce c-di-GMP and the luminescence and O.D 600nm readings were take 3 hours after addition of IPTG. Error bars represent standard deviation.

- **Screen for constitutive mutants of VpsR**

We performed a genetic screen to isolate VpsR mutants that exhibit constitutive activity using the *E. coli* system described above (Fig.17). Random mutants of VpsR-6XHIS were generated using $MnCl_2$ based error-prone PCR and screened for high *vpsT-lux* expression in the absence of c-di-GMP. This screen is more powerful

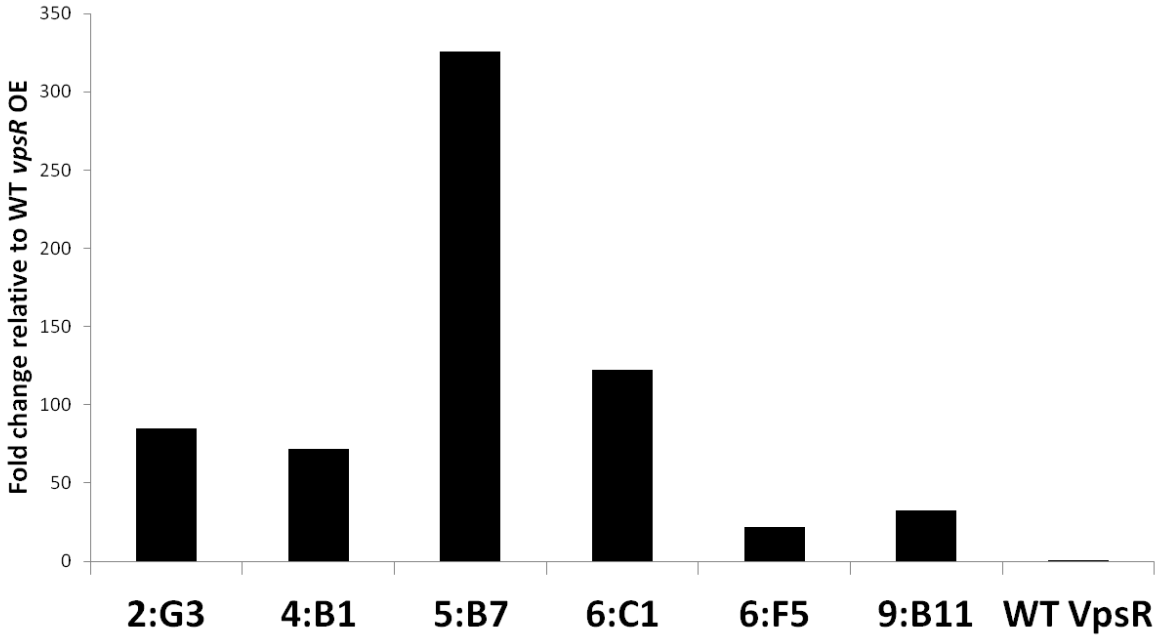


Figure 21: Screen for constitutive mutants of VpsR. Random mutagenesis was performed for *vps*R using $MnCl_2$ in a Taq polymerase PCR reaction. These random mutants were then tested for rescuing *vps*T-lux expression in S17λpir *E.coli* strain in the absence of GGDEF over expression vector. An *E. coli* strain containing WT VpsR overexpression vector was used as a control. Fold change in expression is calculated by dividing the R.L.U of the individual wells by the R.L.U of WT VpsR overexpression vector. The cultures were grown for 5 hours with 0.002% arabinose (for p*vpsR* expression) and the luminescence and O.D 600nm readings were taken.

than the approach identifying VpsR mutants that do not activate transcription in the presence of c-di-GMP because it is hard to distinguish that class of mutants

from mutations that inactivate VpsR function. We created a random collection of *vpsR* mutants and cloned them in our arabinose inducible vector, pAR3. These mutants were then tested in the *E. coli* S17λpir strain containing pDL1711 (*vpsT-lux*) for *vpsT* expression. The expression of these mutants was compared to an *E. coli* strain containing WT VpsR. We identified 6 isolates that induced *vpsT* expression 30-300 fold as compared to WT VpsR (Fig. 21). These mutant alleles were sequenced and all isolates had upto 3-6 nucleotide changes (data not shown).

To test if the increase in *vpsT* expression in the presence of these mutants was indeed due to their c-di-GMP blind nature, we tested these mutants in the presence of c-di-GMP (Fig. 21 shows a select few mutants). Most of the mutants we isolated lead to higher *vpsT* expression but were still inducible by c-di-GMP suggesting that they are not blind to c-di-GMP. This could be due to conformational changes in VpsR that lead to its over-activation or increased sensitivity to c-di-GMP binding (low levels of c-di-GMP are present in *E.coli*). One of the mutants we isolated (V27E/Q37H/V49D) was c-di-GMP blind. This mutant has three amino acid changes and all the residues mutated are in the REC domain (Fig. 22). This is interesting since we saw that a deletion in REC domain made VpsR inactive (Fig. 19).

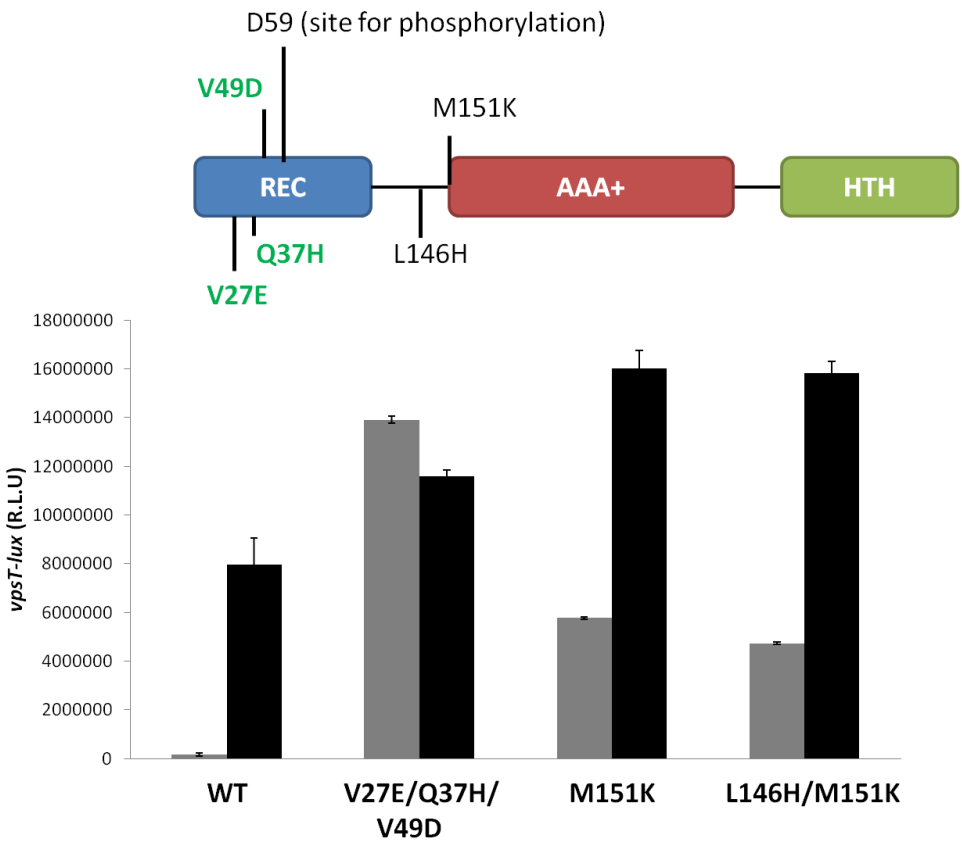


Figure 22: Screen for c-di-GMP blind VpsR mutants. Random mutants isolates of VpsR were tested for rescuing *vps*T-lux expression in S17 *E.coli* strain in the presence and absence of GGDEF over expression vector. An *E.coli* strain containing WT VpsR overexpression vector was used as a control. The cultures were grown for 5 hours with 0.002% arabinose (for p*vpsR* expression) before the addition of IPTG. Gray bars indicate non-induced cultures. Black bars indicate addition of 0.1 mM IPTG. Luminescence and O.D 600nm readings were taken 3 hours post IPTG addition. Error bars indicate standard deviation.

We analyzed this mutant further by making single mutants in each residue (Fig. 23). A mutation in V27E leads to inactivation of the protein. Mutation in V49D did not affect VpsR activity. We have not been able to obtain the Q37H mutant as yet. Further characterization of this c-di-GMP blind mutant can help us understand

VpsR function in response to c-di-GMP. We will be testing these mutants for c-di-GMP binding, binding to *vpsT* promoter using EMSA and DNAseI footprinting and also oligomerization using gel filtration chromatography.

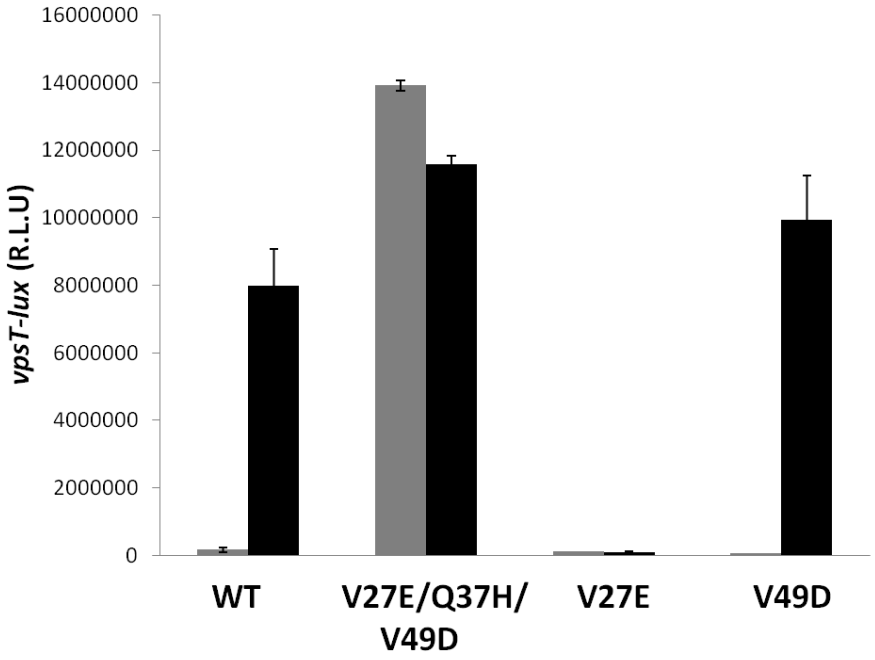


Figure 23: Further analysis of constitutive VpsR mutants. Single mutants in V27E and V49D were tested for rescuing *vpsT-lux* expression in S17 *E. coli* strain in the presence and absence of GGDEF over expression vector. An *E. coli* strain containing WT VpsR overexpression vector was used as a control. The cultures were grown for 5 hours with 0.002% arabinose (for p*vpsR* expression) before the addition of IPTG. Gray bars indicate non-induced cultures. Black bars indicate addition of 0.1 mM IPTG. Luminescence and O.D 600nm readings were taken 3 hours post IPTG addition. Error bars indicate standard deviation.

- **VpsR does not appear to function by DNA looping**

The binding site for VpsR in the *vpsT* promoter region is unique since it is 118 bp upstream of the +1 transcription start site (Fig. 10B). Since VpsR is a non-canonical EBP (absence of σ54 interaction sequence and mutation in WalkerB site as shown in Fig.17 and it is a σ70 dependent activator) the location of this binding site is intriguing. However, classical EBPs are known to bind >100 bp upstream of +1 and have the ability to loop the DNA to contact the RNAP polymerase σ54 complex (Francke et al., 2011). We hypothesized that VpsR is able to loop DNA to activate transcription of the *vpsT* promoter. To test this, we created insertions (+5,+10,+15) and deletions (-5, -10, and -15, and -53 (i.e., the entire region between VpsR binding site and the -35 element) constructs of the *vpsT* promoter. As one turn of the DNA helix is approximately 10 bp, if the placement of binding site is important for activation due to looping we would expect insertions and deletions in increments of 5 will significantly affect activation; however, changes in increments of 10 will not. We also created a deletion construct where we deleted the entire region between -35 and the binding site to test the significance of this region (Fig. 24). Addition of 5, 10, and 15 bases all significantly decreased *vpsT* expression, but the promoter remained inducible by c-di-GMP. Deletion of 5 and 15 bp did reduce overall expression, although the promoters remained inducible by c-di-GMP while deletion of 10 bp did not have an impact on *vpsT* expression. Interestingly, deletion of the region between the VpsR binding site and the -35 site reduced expression

significantly, but this promoter was still inducible by c-di-GMP suggesting VpsR is able to function from a short distance to activate transcription (Fig. 24). We see an effect on *vps*T promoter expression by both addition and deletion of bases between VpsR binding site and -35 promoter element for all constructs tested except deletion of 10bp. The most drastic effect was seen on addition of 15 bp, and all constructs with changes in increments of 5 have lower expression. Overall our results do not support DNA looping as the mechanism of VpsR action.

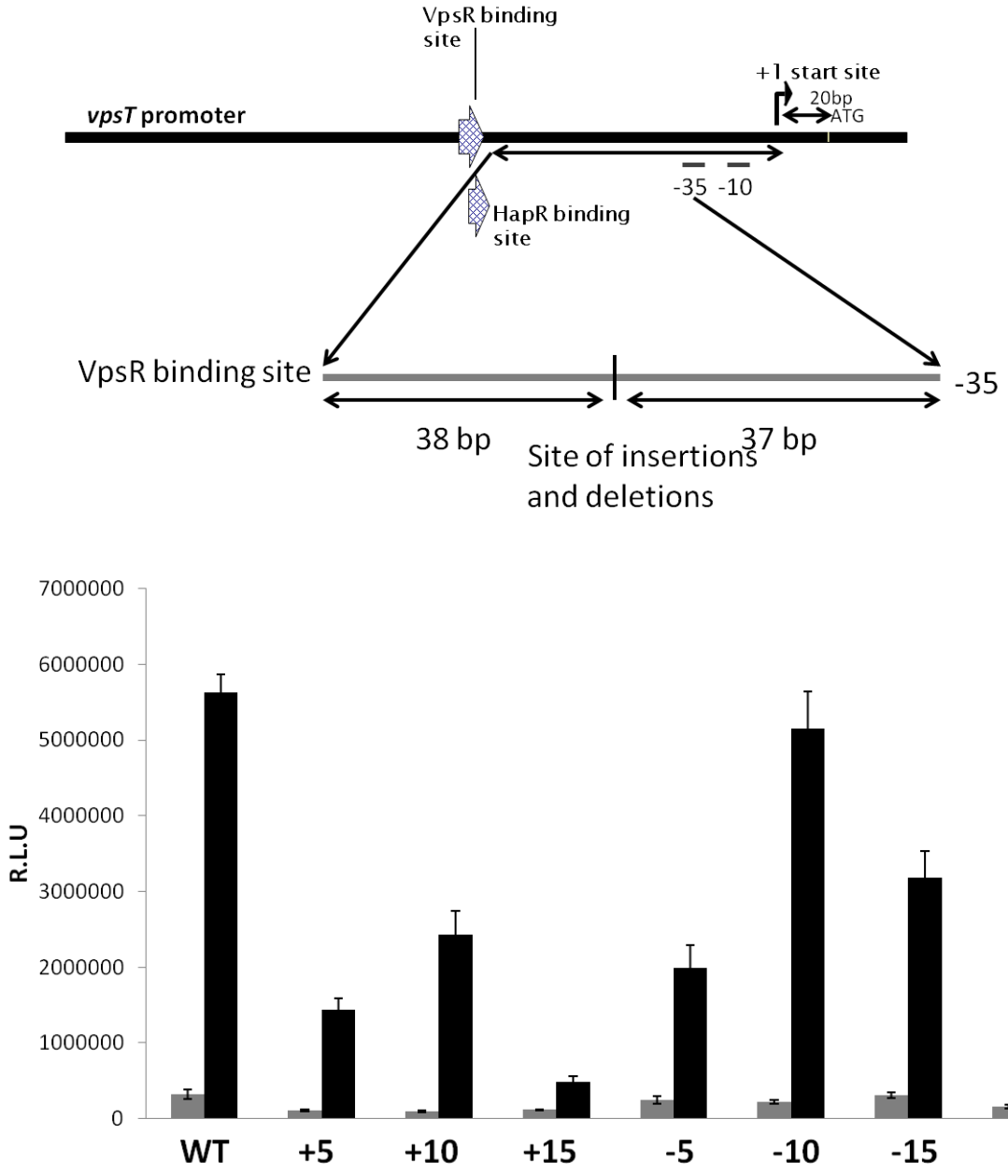


Figure 24: *vpsT* DNA looping constructs. A) Representation of the *vps*T promoter showing the location of the insertions and deletions tested. B) *vpsT* promoters tested in Δ*vpsL* strain of *V. cholerae* at low versus high c-di-GMP. The data is represented as Relative luminescence units and the error bars represent standard deviation. Gray bars indicate non-induced cultures. Black bars indicate addition of 0.1 mM IPTG. Error bars indicate standard deviation.

2.4 Discussion

Here, we show that the integration of QS and c-di-GMP in the control of *aphA* and *vpsT* occurs through a common mechanism. These two transcriptional regulators function as checkpoints at the apices of cascades regulating entry into the low-cell density QS state, virulence cascade, and biofilm developmental pathways. Our results lead to four important conclusions. First, the expression of *aphA*, a central regulator of the virulence cascade and the master low-cell density regulator of the QS pathway, is induced by c-di-GMP. This finding was unexpected as c-di-GMP is thought to play a negative role in virulence gene expression (Tamayo et al., 2007); however, it is consistent with *aphA* functioning at low-cell density as c-di-GMP is high in this state (Waters et al., 2008). Second, a shared regulatory mechanism controls c-di-GMP-mediated induction of *aphA* and *vpsT*. Although both promoters interact with HapR and VpsR, only VpsR is required for c-di-GMP induction of their transcription. Importantly, c-di-GMP induction was not dependent on VpsT. Third, VpsR directly binds c-di-GMP. Fourth, we have identified six additional promoters whose transcription is induced by high levels of c-di-GMP independently of VpsR. We are examining the regulation of these genes to identify additional c-di-GMP-dependent transcriptional regulators of *V. cholerae*.

Activation of *aphA* and *vpsT* by c-di-GMP is mediated through a mechanism involving VpsR, demonstrating that they share a common regulatory pathway. Furthermore, the expression of both genes is repressed by the QS regulator HapR

(Kovacikova & Skorupski, 2002, Waters et al., 2008). Therefore, joint regulation of HapR and VpsR represents a central control module integrating QS and c-di-GMP mediated regulation. Binding of HapR at the *aphA* promoter excludes VpsR binding (Lin et al., 2007). Further experimentation is required to determine if HapR similarly excludes VpsR binding at the *vpsT* promoter. This control module appears to combine important information about the surrounding bacterial community and the local environment. We propose that integrating the information from these two major sensory pathways at the apices of the biofilm, low-cell density QS state, and virulence cascades functions akin to a regulatory checkpoint. We are currently identifying other genes/promoters with similarly arranged HapR and VpsR binding sites to determine the extent of c-di-GMP and QS cross-wiring in other *V. cholerae* developmental pathways.

We examined whether VpsR might function by binding to c-di-GMP to further activate the expression of *aphA* and *vpsT*. We found that VpsR binds c-di-GMP with a K_d of 1.6 μM and we hypothesize that this binding is important for the c-di-GMP mediated induction of *vpsT* and *aphA* promoters. Interestingly, VpsT and VpsR share a similar binding affinity (Krasteva et al., 2010). The intracellular concentration of c-di-GMP in *V. cholerae* ranges from 10 μM at low-cell density to 1 μM at high-cell density (Massie et al., 2012). Thus, the binding affinities of VpsR and VpsT fall within the normal physiological concentrations of c-di-GMP in the cell, although we expect the *in vitro* binding affinity of VpsR determined here is less than the true *in vivo* value due to an incomplete replication of the *in vivo*

environment in our filter binding assay. C-di-GMP binding to both of the major transcriptional activators of biofilm formation in *V. cholerae*, VpsT and VpsR, increases positive induction of biofilm development genes and possibly amplifies the response of this second messenger. We have recently isolated some constitutive mutants of VpsR that are able to induce *vps*T expression in the absence of c-di-GMP (Fig. 22). Studying these mutants in more detail will provide insights into the mechanism of action of VpsR.

C-di-GMP has been previously reported to exert a negative effect on the virulence of *V. cholerae* (Tamayo et al., 2007, Tischler & Camilli, 2005). In the Classical biotype of *V. cholerae*, the phosphodiesterase (PDE) VieA functions to reduce the c-di-GMP levels *in vivo* (Tischler & Camilli, 2005). The PDE activity of VieA is required for full expression of *toxT* and production of CT. Mutation of *vieA* causes a 10-fold decrease in *V. cholerae* colonization in an infant murine mouse model (Tamayo *et al.*, 2005). Regulation of virulence and colonization by c-di-GMP in the El Tor biotype (the biotype studied here) is not as well understood because VieA does not play a role in controlling c-di-GMP levels. Rather, CdpA, a PDE in *V. cholerae* El Tor C6706, is suggested to modulate c-di-GMP levels during infection by repressing biofilm formation and positively increasing CT production (Tamayo et al., 2008). However, mutation of *cdpA* has no effect on colonization in the murine model. Consistent with a lack of a major negative role for c-di-GMP in El Tor infection, overexpression of VdcA, a *V. cholerae* GGDEF protein, only reduced colonization levels 3-fold versus the wild type strain (Tamayo et al., 2008). The

effect of VdcA overexpression *in vivo* on ToxT expression was determined using the RIVET system. These experiments showed a subpopulation of *V. cholerae* expressed virulence at both high- and low- levels of c-di-GMP. Interestingly, both wild type El Tor and the VdcA overexpression strain exhibited heterogenous *toxT* expression, suggesting that *V. cholerae* may exist in multiple development stages *in vivo* (Tamayo et al., 2008).

As these previous results have proposed that c-di-GMP inhibits virulence in early stages of infection, our discovery that *aphA* is activated by c-di-GMP was unexpected. AphA was recently shown to be an important low cell density master regulator in *V. cholerae* and *V. harveyi* controlling the expression of genes involved in motility, Type 3 secretion, acetoin production, and multiple hypothetical genes (Rutherford et al., 2011, Kovacikova et al., 2005). Our results showing the regulation of *aphA* by c-di-GMP leading to altered production of acetoin suggests that other genes may be jointly controlled by c-di-GMP and QS through induction of *aphA* expression.

AphA controls the expression of the *tcpPH* operon, which leads to activation of *toxT* expression. However, under laboratory conditions, we did not observe a significant induction of the transcription of virulence genes known to lie downstream of AphA (such as *toxT*) at high c-di-GMP levels. One possibility is that c-di-GMP induction of *aphA* occurs under many conditions while AphA activation of the remainder of the virulence cascade may be context dependent and only occurs in specific environments such as in the host. Alternatively, c-di-GMP could negatively

control virulence gene expression independent of *aphA* at additional points in the virulence cascade.

Interestingly, AphA itself induces *vpsT* expression and biofilm formation, further linking these two central regulators (Rutherford et al., 2011, Yang *et al.*, 2010). The AphA binding site at the *vpsT* promoter is located 240 bases upstream of the translation start site of *vpsT.* Our observation that truncated promoter fragments that do not contain this sequence, specifically -195T and -149T (Fig. 10B), maintain c-di-GMP induction suggests that AphA is not required for this process. However, we would predict that c-di-GMP induction of AphA would further amplify expression of *vpsT.* Further work on the connections between biofilms and virulence in *V. cholerae* is required to characterize the interplay between the biofilm and virulence signal transduction pathways and the potential *in vivo* role for c-di-GMP in these processes.

In *V. cholerae*, biofilm formation and virulence have been reported to be inversely regulated, and c-di-GMP has been suggested to inhibit *in vivo* disease development. Our results showing that expression of the virulence regulator *aphA* is activated by c-di-GMP hint that c-di-GMP may, in some cases, have a positive function during *in vivo* infection. Furthermore, as *aphA* is the master low-cell density regulator of the QS pathway, induction of *aphA* by c-di-GMP has a significant impact on the low-cell density state in *V. cholerae*. Clearly, the QS and c-di-GMP signaling pathways in *V. cholerae* controlling biofilm formation, virulence factor expression, and numerous other phenotypes is intricately intertwined at

many levels. We hypothesize these connections allow *V. cholerae* to sense and combine information about the extracellular community and surrounding environment to optimally adapt to ever changing conditions.

We know that c-di-GMP can activate *vpsT* and *aphA* expression by binding to VpsR but we do not understand the mechanism of this activation. As discussed previously, c-di-GMP does not affect the phosphorylation of VpsR or VpsR binding to *vpsT* promoter. Not much is known about VpsR-like non-canonical EBPs and thus it is important to understand the molecular mechanism of c-di-GMP mediated activation of VpsR. We have attempted to characterize the mechanism of action of VpsR by using a few approaches described above. First, we looked at the relevance of the REC domain in VpsR function. We have shown that REC domain of VpsR is involved in positive regulation of VpsR function (Fig.19) and further work on the effect of phosphorylation of the REC domains on VpsR structure or oligomerization will provide more insight into the significance of this domain. Interestingly, we also discovered important residues in the REC domain that when mutated make VpsR constitutively active in the absence of c-di-GMP (Fig.22). This suggests that the REC domain is involved in VpsR activation by c-di-GMP either directly or via a conformation change. Secondly, we characterized the importance of AAA+ domain for VpsR activity. We have shown that the conserved residues in the AAA+ domain (ATP binding site) are important for VpsR function (Fig.20). Mutation of these residues led to inactivation of VpsR suggesting that the AAA+ domain plays an important part in VpsR activity. Further characterization of the AAA+ domain by

ATP binding studies and ATPase assays will aid in our understanding of the function of this domain. Lastly, we focused on the relevance of the long distance (118bp) between VpsR binding site and -35 promoter motif on VpsR function which suggests that some mechanism is involved in interaction of the VpsR c-di-GMP complex with the promoter region. From our results, we were not able to conclusively show if DNA looping is required in the activation of *vpsT* expression by c-di-GMP and VpsR (Fig. 24). Extension of the studies performed here and further characterization of the mechanism of action of VpsR using other approaches will aid in understanding of VpsR-like c-di-GMP binding EBPs in bacteria.

CHAPTER 3

Cyclic di-GMP inhibits *Vibrio cholerae* motility by repressing induction of transcription and inducing extracellular polysaccharide production

PREFACE

Cyclic di-GMP (c-di-GMP) controls the transition between sessility and motility in many bacterial species. This regulation is achieved by a variety of mechanisms including alteration of transcription initiation and inhibition of flagellar function. How c-di-GMP inhibits the motility of *V. cholerae* has not been determined. FlrA, a homolog of the c-di-GMP binding *P. aeruginosa* motility regulator FleQ, is the master regulator of the *V. cholerae* flagellar biosynthesis regulon. Here we show that binding of c-di-GMP to FlrA abrogates binding of FlrA to the promoter of the *flrBC* operon, deactivating expression of the flagellar biosynthesis regulon. FlrA does not regulate expression of extracellular *Vibrio* polysaccharide (*VPS*) synthesis genes. Mutation of the FlrA amino acids R135 and R176 to histidine abrogates binding of c-di-GMP to FlrA, rendering FlrA active in the presence of high levels of c-di-GMP. Surprisingly, c-di-GMP still inhibited the motility of *V. cholerae* only expressing the c-di-GMP blind FlrA(R176H) mutant. We determined that this flagellar transcription-independent inhibition is due to activation of *VPS* production by c-di-GMP. Therefore, c-di-GMP prevents motility of *V. cholerae* by two distinct but functionally redundant mechanisms.

3.1 Introduction

Cyclic di-GMP (c-di-GMP) is a nearly ubiquitous second messenger in bacteria that regulates a wide array of molecular processes in the cell such as biofilm formation, motility, cell signaling and differentiation (Hengge, 2009, Römling et al., 2005, Römling et al., 2013, Srivastava & Waters, 2012). C-di-GMP regulates these processes via a number of mechanisms including the control of transcription initiation. Recently, a number of transcription factors responding to c-di-GMP have been identified; however, these transcription factors have diverse functional domains indicating that c-di-GMP utilizes a range of mechanisms to control gene expression (Chin et al., 2010, Wilksch *et al.*, 2011, Hickman & Harwood, 2008, Srivastava et al., 2011, Krasteva et al., 2010, Fazli et al., 2011). VpsT and VpsR are two c-di-GMP binding transcription factors that have been identified in *Vibrio cholerae* (Krasteva et al., 2010, Srivastava et al., 2011). However, we have identified a number of genes whose expression is induced by c-di-GMP independent of VpsT and VpsR (Srivastava et al., 2011). This finding suggests that *V. cholerae* encodes additional regulatory proteins that alter transcription initiation in response to c-di-GMP.

C-di-GMP represses *V. cholerae* flagellar-based motility in a low-agar motility assay (Liu et al., 2010, Beyhan et al., 2006), but the molecular mechanism by which this occurs has not been fully elucidated. C-di-GMP controls bacterial motility via regulation of transcriptional induction and posttranscriptional

mechanisms. For example, c-di-GMP inhibits motility in *Salmonella enterica* by binding to the flagellar associated regulatory protein YcgR and through induction of cellulose synthesis by the *bcs* genes (Ryjenkov et al., 2006, Zorraquino *et al.*, 2013). In *Vibrio parahaemolyticus,* c-di-GMP inhibits expression of the *laf* gene cluster through the Scr regulatory system preventing swarming motility (Ferreira *et al.*, 2008, Trimble & McCarter, 2011).

The expression of flagellar biosynthesis genes in *V. cholerae* occurs by a cascade of gene expression involving four classes of genes expressed in a sequential manner (Prouty et al., 2001), and c-di-GMP has been shown to negatively inhibit expression of components of the flagellar biosynthesis regulon. Transcriptome profiling studies have previously reported decreases in the expression of the Class III and IV flagellar biosynthesis genes in response to high levels of c-di-GMP (Beyhan et al., 2006). Moreover, a subset of these Class III and IV genes were negatively regulated by VpsT, suggesting this transcription factor might link c-di-GMP and flagellar biosynthesis; however, it is unknown if this regulation is direct (Krasteva et al., 2010).

The master regulator of flagellar biosynthesis in *V. cholerae* is the transcription factor FlrA (Prouty et al., 2001, Klose & Mekalanos, 2002). FlrA is a σ 54-dependent enhancer binding protein (EBP) that contains an N-terminal receiver domain, central **A**TPase **A**ssociated with diverse cellular **A**ctivities (AAA+) domain, and a C-terminal DNA binding domain. σ54-dependent EBPs typically bind

100-1,000 bp upstream of the -12/-24 σ54 promoter (Rappas *et al.*, 2007). Upon activation by phosphorylation or dephosphorylation of the REC domain, these proteins oligomerize into ring shaped structures which loop the DNA and activate transcription. This activation requires the hydrolysis of ATP by the AAA+ domain (Chen *et al.*, 2008). EBPs are centrally important regulators of bacterial function as they control motility, biofilm formation, quorum sensing, and virulence factor expression (Bush & Dixon, 2012, Francke et al., 2011). EBPs are widespread in bacteria — a recent analysis of sequenced genomes identified 4,850 EBPs, occupying nearly every branch of the bacterial phylogenetic tree (Francke et al., 2011).

FleQ, the FlrA ortholog encoded by *P. aeruginosa* was the first transcription factor demonstrated to directly bind to c-di-GMP(Hickman & Harwood, 2008). c-di-GMP does not significantly regulate expression of the flagellar biosynthesis gene cluster in *P. aeruginosa* (Hickman & Harwood, 2008). In the absence of c-di-GMP, FleQ directly represses expression of the *pel* operon that encodes the machinery necessary for extracellular polysaccharide production (Hickman & Harwood, 2008). Upon increased levels of c-di-GMP, FleQ binds to this second messenger and, in conjunction with the accessory protein FleN, alters its binding arrangement on the *pel* promoter to activate *pel* gene expression in a σ70-dependent manner (Baraquet et al., 2012, Hickman & Harwood, 2008). C-di-GMP is thus able to convert FleQ from a repressor to an activator(Baraquet et al., 2012).

Both FlrA and FleQ lack the conserved Asp residue required for phosphorylation of their REC domain, and what role, if any, this domain plays in FleQ or FlrA function is unknown (Prouty et al., 2001, Jyot *et al.*, 2002, Dasgupta & Ramphal, 2001).

In this study, we determined how c-di-GMP inhibits motility in *V. cholerae.* Based on the homology between FlrA and FleQ, we hypothesized that FlrA is a c-di-GMP binding transcription factor in *V. cholerae*. Although homologs to FleQ are widespread in bacteria, c-di-GMP binding to these proteins has not been examined. We found that FlrA binds to c-di-GMP resulting in inhibition of FlrA binding to a Class II flagellar promoter, *flrBC*. Unlike FleQ, FlrA does not regulate the expression of extracellular polysaccharide biosynthetic genes in *V. cholerae*. We identified two missense mutations in FlrA, R135H and R176H that abrogate binding to c-di-GMP, leading to constitutive activity of these FlrA mutants even at high levels of c-di-GMP. Structural modeling suggests these arginine residues form a c-di-GMP binding site located at the junction of the REC and AAA+ domain. We show that REC domain of FlrA is important for its activity and has a negative effect on c-di-GMP binding. Surprisingly, c-di-GMP inhibits the motility of *V. cholerae* cells expressing only the FlrA(R176H) c-di-GMP-blind mutant, leading us to determine that c-di-GMP induction of *Vibrio* polysaccharide (*VPS*) negatively inhibits motility independent of FlrA control of gene expression. Thus, c-di-GMP inhibits motility of *V. cholerae* through both transcriptional control of flagellar genes and non-flagellar posttranscriptional input.

3.2 Material and Methods

- **Culture conditions, molecular methods, and expression analysis**

All strains used in this study are listed in Table 4. All plasmids and primers used are listed in Table 4. *V. cholerae* C6706str2 El Tor biotype strain was used in the study. All strains used for luminescence assays have a deletion of the *vpsL* gene rendering them unable to make biofilms. This mutation aids in accurate luminescence readings by preventing flocculation at high c-di-GMP levels. The *flrA* mutation was created in the Δ*vpsL* and WT strains using the plasmid pDS54 via the pKAS32 suicide plasmid (Skorupski & Taylor, 1996). All plasmids were introduced into *V. cholerae* using biparental matings with *E. coli* S17-λpir. All *V. cholerae* and *E. coli* strains were grown in Luria-Bertani (LB) medium. Antibiotics were obtained from Sigma Aldrich and were used at the following concentrations (μg ml^{-1}) unless stated otherwise: ampicillin, 100; kanamycin, 100; and chloramphenicol, 10. Standard molecular procedures were used for DNA manipulations (Sambrook et al.). All molecular enzymes were purchased from New England BioLabs (NEB), Roche and Stratagene. PCR utilized Phusion Polymerase for cloning and preparation of probes for EMSA. Gene expression studies were performed using luminescence reporter constructs of promoters inserted into the pBBRlux expression vector as previously described (Srivastava et al., 2011). The plasmids used for gene expression studies are compatible in *V. cholerae* and *E. coli* and were maintained using respective antibiotics in the medium. To measure gene expression of transcriptional fusions, both *V. cholerae* and *E. coli* were diluted 1/150 in 150 μL

LB with the appropriate antibiotics in microtiter plates and grown with shaking at 220 rpm. Gene expression was measured at five and seven hours for *V. cholerae* and *E. coli,* respectively, using a SpectraMax M5 microplate spectrophotometer system (Molecular Devices Sunnyvale, CA). All plasmids constructed are listed in Table 4.

- **Protein purification and c-di-GMP binding assays**

YcgR, FlrA, FlrA(R135H), and FlrA(R176H) were purified from the Bl21(DE3) strain by overexpression from the respective pTXB1 vectors as per manufacturer's details in 20 mM Tris, 1 mM EDTA and 250 mM NaCl buffer. YcgR was used a positive control for c-di-GMP binding and purified as previously described (Srivastava et al., 2011). [^{32}P]-labeled c-di-GMP was generated using the purified WspR(R242A) protein from *P. aeruginosa* as previously described (Srivastava et al., 2011). For binding reactions, 250 nM protein in binding buffer (20 mM Tris [pH 7.8], and 150 mM NaCl) was incubated with varying amounts of [^{32}P]-labeled c-di-GMP (0.125 μM to 1.5 μM) in a 20μL volume for 30 min at room temperature. Binding was assessed using a filter-binding technique as previously described (Srivastava et al., 2011). For competition experiments, 950 nM [^{32}P]-labeled c-di-GMP was used and equal amounts of unlabeled c-di-GMP and GTP were added to reactions. For c-di-GMP binding assays with FlrA truncations, proteins were provided by our collaborators at Rutgers (Matt Neiditch and Atul Khataokar).

- **Electrophoretic mobility shift assays (EMSA)**

The *flrBC* promoter probe was amplified from the pDS49 vector using 3' and 5' FAM labeled primers. Similarly a *vpsL* probe was also amplified from pBH629 (Waters et al., 2008). EMSA assays were performed as previously described (Waters et al., 2008) . Binding of 10 nM to 200 nM FlrA protein to 10 nM of the *flrBC* promoter was determined. 100 nM FlrA was tested for binding to 10 nM of the *vpsL* promoter. For EMSA reaction with c-di-GMP, ATP and GTP added, 60 nM protein was incubated with 10 nM probe and 40 μM and 80 μM nucleotides and then analyzed on 5% TAE acrylamide gels.

- **DNaseI Footprinting**

The *flrBC* promoter probe was amplified from pDS49 vector using a 5' FAM-labeled CMW324 primer and 3' unlabeled-primer CMW1834. 22.2 nM of the *flrBC* promoter, FlrA protein ranging from 133 to 1,067 nM, and 1 μg of poly(dI-dC) were incubated for thirty minutes at room temperature in the presence of 17.33 mM Tris pH 8.2, 216.67 mM NaCl, 0.867 mM EDTA pH 8.0, and 17.33% glycerol. For reactions containing c-di-GMP, 44.4 μM of c-di-GMP was added to the reaction with *flrBC* promoter, FlrA, and poly(dI-dC). To initiate the footprinting assay, 0.15U of DnaseI enzyme (Roche) was added. The reaction was then allowed to proceed for ten minutes at room temperature, quenched with 0.25 mM EDTA pH 8.0, and purified using phenol extraction and ethanol precipitation. After resuspension of digested

DNA fragments in 25 μL ddH2O, 5 μL of digested fragments were mixed with 4.9 μL HiDi formamide and 0.1 μL 500 LIZ size standards prior to submission using the ABI Prism 3130xl Genetic analyzer (MSU Research Technology Support Facility). Chromatograms were aligned using PeakScanner Software v1.0.

To obtain sequencing reactions, unlabeled template DNA, pDS49, and unlabeled 5' primer were used with Thermo Sequenase Dye Primer Manual Sequencing Kit (USB) according to manufacturer's instructions. After PCR reaction, 1 μL of each sequencing reaction was diluted in 4 μL ddH2O and submitted for sequencing analysis on the ABI Prism 3130xl Genetic analyzer as described previously.

- **Comparative Modeling**

The FlrA-RC comparative model (residues 1-378) was generated using ModWeb. The *Aquifex aeolicus* NtrC1-RC X-ray crystal structure (Protein Data Bank code 1NY5) was used as the modeling template. The FlrA-RC dimer was generated by structurally aligning FlrA-RC monomers with the dimeric NtrC1-RC structure (Eswar *et al.*, 2003). Molecular graphics were produced with PyMOL (DeLano, 2002).

- **Motility assays**

Motility of *V. cholerae* strains were tested by stabbing an overnight culture into 0.375% LB agar plates (containing appropriate antibiotics +/- IPTG) using a 200 μl tip as previously described (Edmunds *et al.*, 2013) . High levels of c-di-GMP were

obtained by introduction of QrgB overexpression (pBRP2). Low levels of c-di-GMP correspond to overexpression of QrgB* (pBRP1).

3.3 Results

- **FlrA directly binds to c-di-GMP**

FlrA was purified and examined for binding to radiolabeled c-di-GMP using a previously described filter binding assay (Srivastava et al., 2011). We observed a dose-dependent increase in FlrA binding to radiolabeled c-di-GMP (Fig. 25A). This experiment was performed two times with similar results and the dissociation coefficient (K_d) was determined to be 0.378 μM c-di-GMP with a standard deviation of 0.043 μM. This K_d is lower than that of VpsT and VpsR, but is within the range of c-di-GMP levels in *V. cholerae* that are typically low μM (Koestler & Waters, 2013). This K_d value is significantly lower than what was observed for FleQ (15-25 μM) (Hickman & Harwood, 2008), although we have observed *P. aeruginosa* on average has lower levels of c-di-GMP than *V. cholerae* consistent with other published results (Simm *et al.*, 2009).

To determine if c-di-GMP binding to FlrA is specific, we incubated radiolabeled c-di-GMP with FlrA in the presence of equal amounts of unlabeled c-di-GMP (Axxora) or GTP. Purified YcgR from *Escherichia coli*, a known c-di-GMP binding protein that controls flagellar function, was used as a positive control (Ryjenkov et al., 2006), and bovine serum albumin (BSA) was used as a negative

control to determine non-specific binding. Binding of c-di-GMP to FlrA was competed by unlabeled c-di-GMP but not unlabeled GTP, indicating binding specificity (Fig. 25B). Identical results were observed for YcgR. This experiment was performed three times with similar results, and a representative experiment is shown.

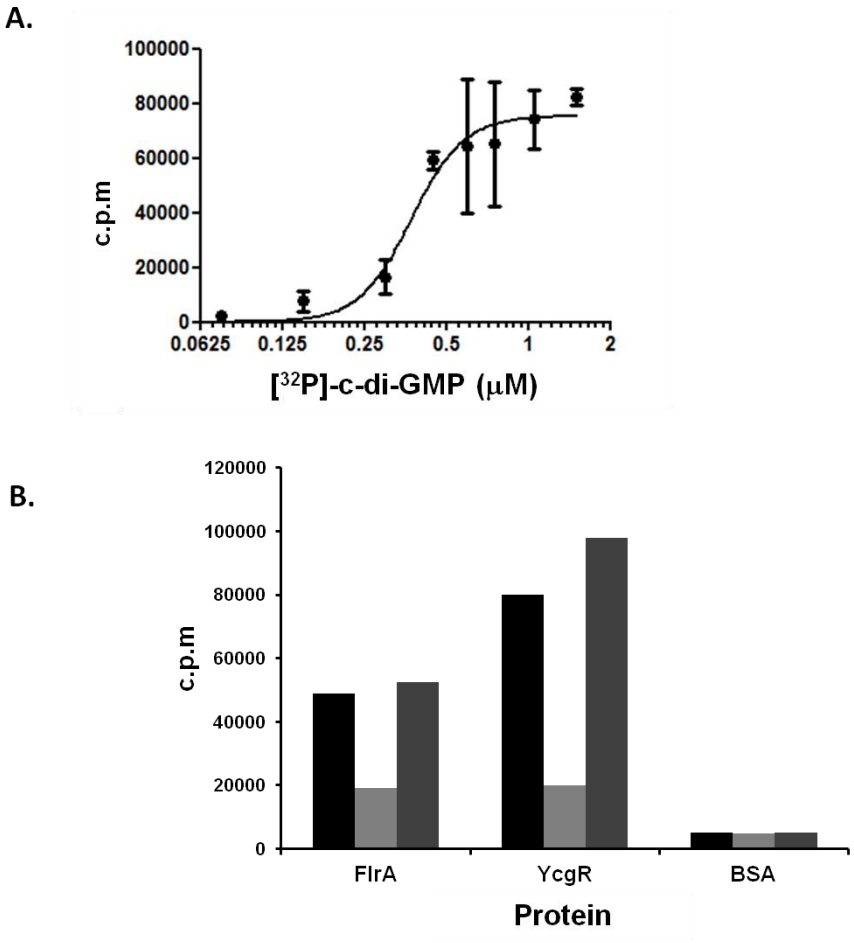


Figure 25: FlrA binds to c-di-GMP. (A) Purified FlrA (300 nM) was incubated with increasing concentrations of [^{32}P]-labeled c-di-GMP and binding was measured using a filter binding assay. The data are expressed as counts per minute (C.P.M.) and analyzed with GraphPad Prism 5.0 using non-linear regression analysis. Error bars indicate standard deviation (n=2). **(B)** Purified FlrA, YcgR or BSA (300 nM) was incubated with [^{32}P]-labeled c-di-GMP (950 nM) with addition of buffer alone (black bars), 950 nM unlabeled c-di-GMP (light gray), or 950 nM unlabeled GTP (dark gray). This experiment was repeated thrice with similar results, and one representative experiment is shown.

- **FlrA-regulated flagellar biosynthesis genes are repressed by c-di-GMP**

Based on our observation that FlrA binds to c-di-GMP and high levels of c-di-GMP inhibit motility, we hypothesized that the flagellar biosynthesis regulon would be repressed by c-di-GMP in an FlrA-dependent manner. To test this prediction, we constructed transcriptional fusions of the Class II *flrBC* promoter, Class III *flgB* and *flaA* promoters, and Class IV *flgM* promoter with luciferase (*lux*) and examined expression of these reporters at low and high levels of c-di-GMP (Prouty et al., 2001). The levels of c-di-GMP were altered by overexpressing the active diguanylate cyclase (DGC) *qrgB* (Waters et al., 2008). As a negative control, an inactive allele of *qrgB* (*qrgB** GGEEF→AAEEF) was similarly induced (Waters et al., 2008). Consistent with previous transcriptomic findings (Beyhan et al., 2006), expression of the Class III genes *flgB* and *flaA* and the Class IV gene *flgM* were significantly repressed by increased c-di-GMP (Fig. 26A). Additionally, we found that expression of the Class II genes *flrBC*, which are directly regulated by FlrA, was also repressed by c-di-GMP (Fig. 26A). The *flrBC* genes encode the FlrB histidine kinase and cognate FlrC response regulator that are essential for initiating Class III and Class IV gene expression (Correa *et al.*, 2000).

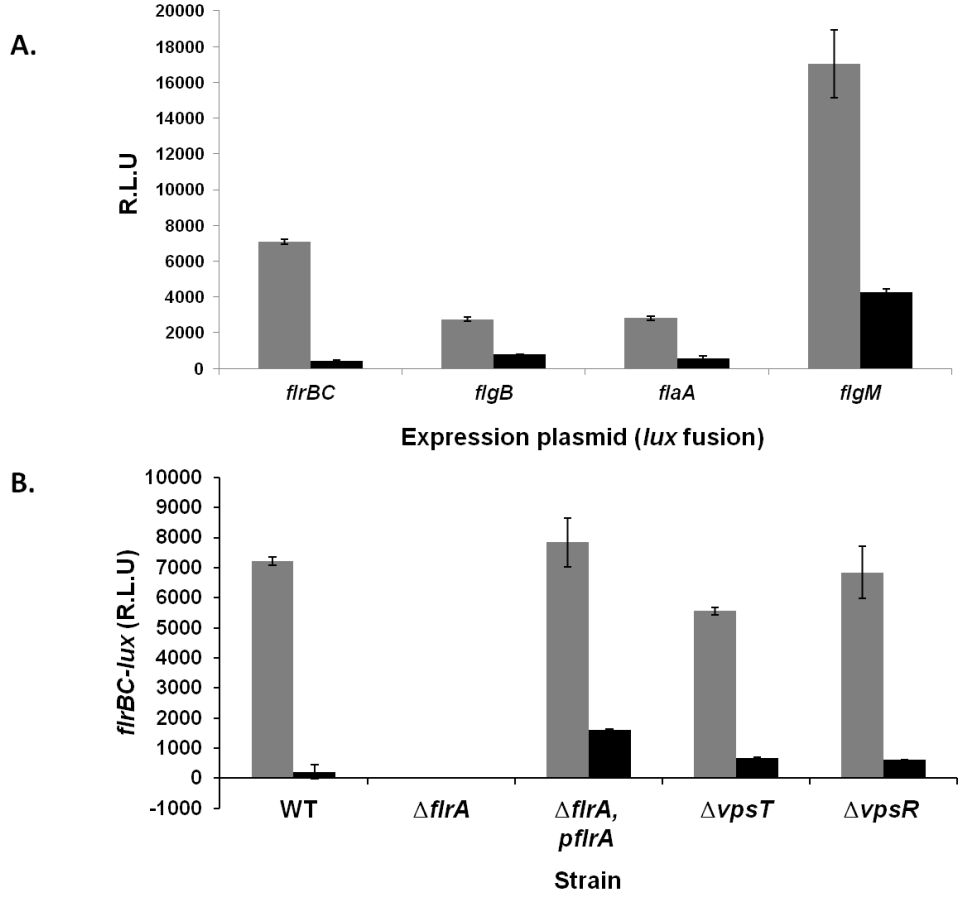


Figure 26: Flagellar gene regulation is repressed by c-di-GMP in an FlrA-dependent manner. (A) Luciferase production in WT *V. cholerae* from *flrBC-lux, flgB-lux, flaA-lux*, and *flgM-lux* and **(B)** *flrBC-lux* expression analyzed in WT, Δ*flrA*, Δ*flrA* complemented with WT FlrA (*pflrA*), Δ*vpsT* and Δ*vpsR V. cholerae* strains. High (black bars) and low (gray bars) intracellular levels of c-di-GMP were generated by overexpression of the DGC QrgB or its active site variant, QrgB*, respectively. Relative light units (R.L.U.) represented on the Y-axis are calculated by dividing the raw bioluminescence by the optical density of the culture at 600 nm. Error bars indicate standard deviation (n=3). The differences in expression between low and high levels of c-di-GMP for all promoter constructs were statistically significant (One tailed T-test, P value<0.05).

To examine the role of FlrA in this c-di-GMP mediated repression, we constructed a Δ*flrA* deletion mutant and assessed *flrBC-lux* expression at low versus high levels of c-di-GMP. As previously observed, expression of *flrBC* was dependent on FlrA and we observed no expression of *flrBC* at both low and high levels of c-di-GMP in the Δ*flrA* mutant (Fig. 26B) (Klose & Mekalanos, 2002). Complementation of the Δ*flrA* mutation was achieved by expressing *flrA* from a multicopy plasmid, and the repression of *flrBC* by c-di-GMP was restored (Fig. 26B). Both VpsT and VpsR are c-di-GMP binding transcription factors in *V. cholerae*, and VpsT has been implicated in the c-di-GMP repression of motility genes (Krasteva et al., 2010). Therefore, we examined if c-di-GMP could inhibit *flrBC* expression in Δ*vpsT* and Δ*vpsR* mutants. In both of these mutants, *flrBC* expression was repressed by c-di-GMP similar to the WT strain, showing that VpsT and VpsR are not required for the c-di-GMP mediated regulation of *flrBC* (Fig. 26B). The expression of *flgB*, *flgM* and *flaA* were similarly repressed by c-di-GMP in the absence of *vpsT* or *vpsR* (data not shown).

- **FlrA binding to *flrBC* promoter is abrogated in the presence of c-di-GMP**

The above genetic experiments suggest that FlrA could link changes in the intracellular concentration of c-di-GMP to the control of the flagellar biosynthesis gene expression. However, an alternative hypothesis is that an additional regulatory factor or factors are necessary for c-di-GMP control of *flrBC*, but these

factors are not evident in the Δ*flrA* mutant as the *flrBC* genes are not expressed. To distinguish between these hypotheses, we examined if c-di-GMP impacted the *in*

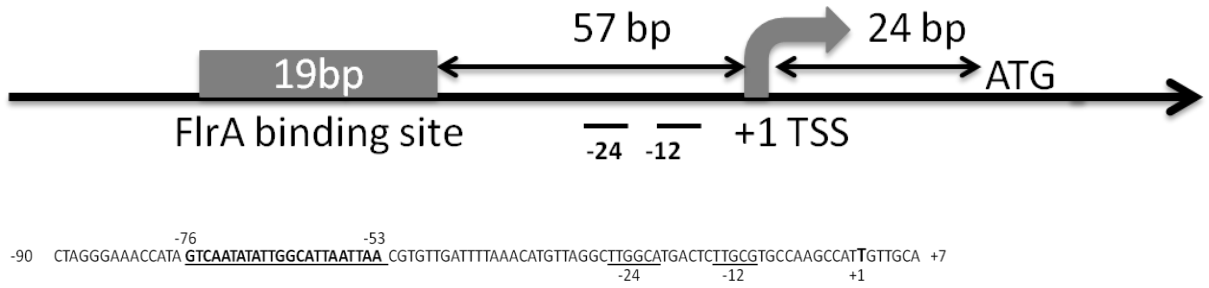


Figure 27: Architecture of *flrBC* promoter. *flrBC* promoter schematic showing the +1 transcription start site and FlrA binding site as deciphered from primer extension and DNaseI footprinting assays, respectively. The sequence depicted below shows the *flrBC* promoter. The underlined region is the binding site determined by DNAseI footprinting experiments. σ54 promoter is shown by the -24 and -12 elements.

vitro binding of FlrA to the *flrBC* promoter region. First, the transcription start site for *flrBC* was characterized with primer extension analysis using the *flrBC-lux* plasmid as a template in the WT and Δ*flrA* mutant (data not shown). We determined that the transcription start site (denoted +1TSS in Fig. 27) is 24bp upstream of the translation start site of *flrBC* operon.

We further characterized the binding of FlrA to *flrBC* using an electrophoretic mobility shift assay (EMSA) and a DNase I footprinting assay. EMSA analysis indicates that FlrA binds to the *flrBC* probe in a concentration dependent manner, confirming that FlrA directly binds to the *flrBC* promoter (Fig. 28). FlrA did not bind the *vpsL* promoter indicating that binding of FlrA at *flrBC* is specific (Fig. 28). We next examined binding of FlrA to the *flrBC* promoter region

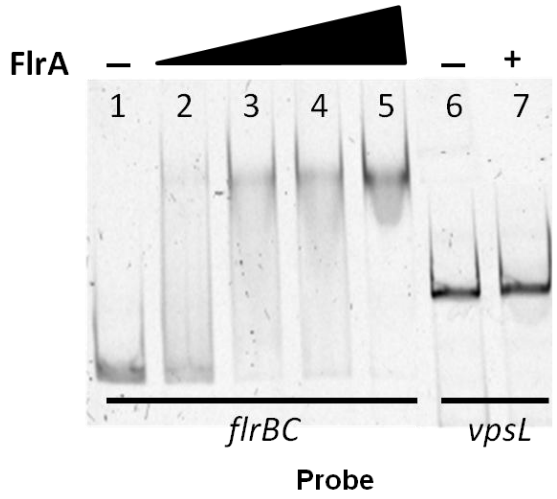
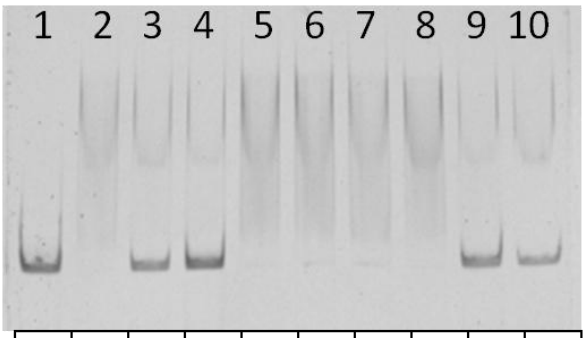


Figure 28: FlrA binds *flrBC* promoter (A) The *flrBC* promoter (10 nM) was incubated with no protein (lane 1) or 10, 30, 80, and 200 nM FlrA (lane 2-5, respectively). Lanes 6 and 7 consist of a DNA fragment encoding the *vpsL* promoter (10 nM), and lane 7 contains 100 nM FlrA.

using a DNAse I footprinting assay and observed protection of a specific region of the promoter from -76 to -54 bp at increasing FlrA concentrations (data not shown, depicted in Fig.26). This binding site consisted of an inverted repeat sequence of ATTG(A/G)C (underlined in Fig. 27).

We hypothesized that decreased expression of *flrBC* by c-di-GMP might occur through c-di-GMP inhibition of FlrA binding at the *flrBC* promoter. We tested this hypothesis by performing an EMSA of FlrA bound to the *flrBC* promoter in the presence and absence of c-di-GMP. Indeed, increasing amounts of c-di-GMP clearly inhibited FlrA binding to the *flrBC* promoter (Fig. 29, lanes 3 and 4). This inhibition is specific to c-di-GMP as we observe no change in FlrA binding to the *flrBC* promoter in the presence of ATP or GTP (Fig. 29, lanes 5-8). FlrA is a σ54-

dependent member of the NtrC-like EBP transcription factor family (Klose & Mekalanos, 2002). Binding and hydrolysis of ATP by the central AAA+ of EBPs is often necessary for transcription induction (Bose *et al.*, 2008, Bush & Dixon, 2012). We therefore examined if ATP abrogated the c-di-GMP inhibition of DNA binding by FlrA (Fig. 29, lanes 9 and 10). However, addition of equivalent amounts of ATP did not inhibit the ability of c-di-GMP to antagonize FlrA, suggesting the c-di-GMP independently interacts with FlrA or, alternatively, c-di-GMP binding to FlrA supersedes FlrA binding to ATP. We performed a similar experiment using excess ATP levels more analogous to the concentrations found *in vivo* but similarly observed no impact of ATP on c-di-GMP-inhibition of FlrA DNA binding (data not shown).



	1	2	3	4	5	6	7	8	9	10
FlrA	--	+	+	+	+	+	+	+	+	+
C-di-GMP	--	--	+	++	--	--	--	--	+	++
ATP	--	--	--	--	+	++	--	--	+	++
GTP	--	--	--	--	--	--	+	++	--	--

Figure 29:FlrA binding to the *flrBC* promoter is inhibited by c-di-GMP. (A) The *flrBC* promoter (10 nM) was incubated with no protein (lane 1) or 60 nM FlrA (lanes 2-10). 40 μM (+) and 80μM (++) of c-di-GMP, ATP, or GTP were added as indicated.

- **FlrA does not regulate the *vps* genes in *V. cholerae***

FleQ represses the expression of the *pel* promoter in the absence of c-di-GMP but functions as an activator of *pel* expression when bound to c-di-GMP (Hickman & Harwood, 2008, Baraquet et al., 2012). The *pel* operon encodes gene products which synthesize a glucose rich extracellular polysaccharide (EPS) that is a component of the biofilm matrix (Friedman & Kolter, 2004). We tested if FlrA similarly regulates

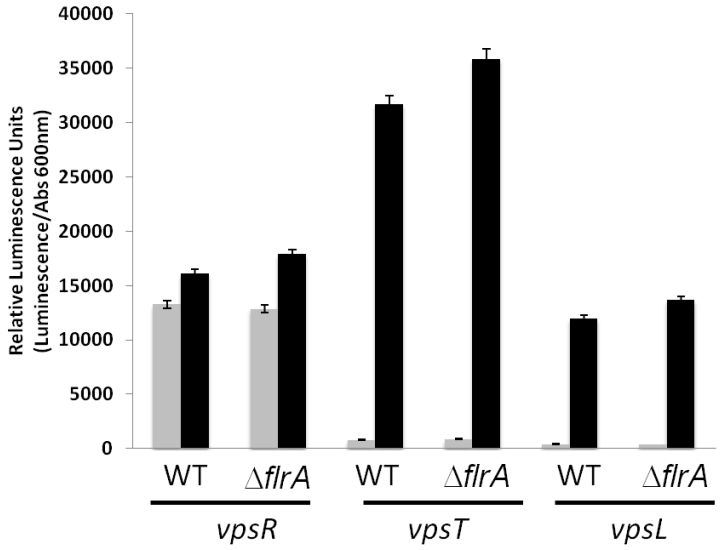


Figure 30: FlrA does not regulate *vps* gene expression. The expression of *vpsR*, *vpsT* and *vpsL*. Luciferase production from *vpsR-lux, vpsT-lux, vpsL-lux* in WT and *ΔflrA V. cholerae* strains. High (black bars) and low (gray bars) intracellular levels of c-di-GMP were generated by overexpression of the DGC QrgB or its active site variant, QrgB*, respectively.

production of VPS in *V. cholerae*. First, we measured biofilm formation of the Δ*flrA* mutant at low and high c-di-GMP using a standard crystal violet assay and observed similar biofilm formation to the WT strain (data not shown). The Δ*flrA* mutant lacks flagella (Klose & Mekalanos, 2002), and flagella have previously been implicated as important for surface attachment (Watnick & Kolter, 1999). However, we analyzed mature biofilm after 12 hours of growth, and our results indicate that the absence of flagella has no effect at this time point in our assay.

We next examined the impact of FlrA on the expression of the *Vibrio* polysaccharide (*vps*) genes. Biofilm formation in *V. cholerae* occurs through expressi

on of the *vpsI* and *vpsII* operons, which are regulated by VpsR and VpsT (Casper-Lindley & Yildiz, 2004, Beyhan et al., 2007, Krasteva et al., 2010, Srivastava et al., 2011). To test if FlrA regulates *vps* gene expression, we assessed induction of *vpsL*, *vpsT* and *vpsR* by c-di-GMP in the Δ*flrA* mutant. Each of the *vps* genes showed identical induction by c-di-GMP in the WT and the Δ*flrA* mutant, showing that FlrA does not regulate these genes (Fig. 30). These results suggest that although FlrA and FleQ are both c-di-GMP binding transcription factors, their c-di-GMP-dependent regulons are distinct.

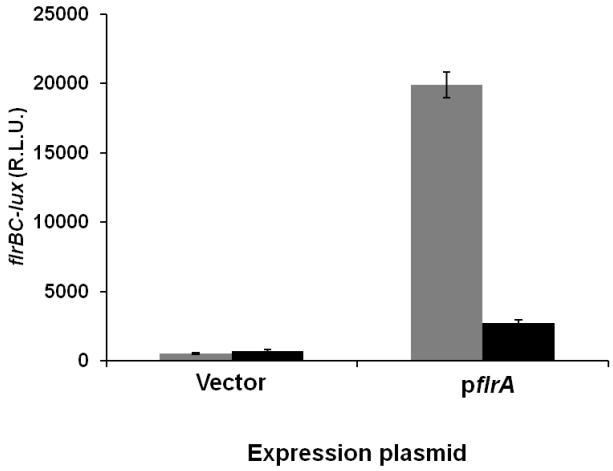


Figure 31: FlrA activates *flrBC-lux* expression in *E. coli* and is inhibited by c-di-GMP. *flrBC-lux* expression was measured in *E. coli* containing a vector control (vector) or expressing FlrA (p*flrA*). C-di-GMP levels were modified by overexpression of QrgB which produces high c-di-GMP levels (black) or the corresponding active site mutant that generates low c-di-GMP (gray). Error bars indicate the standard deviation (n=3).

- **FlrA does not require accessory proteins to respond to c-di-GMP**

FleN, a putative ATPase which binds to ATP, is required for FleQ to respond to c-di-GMP and activate *pel* transcription (Hickman & Harwood, 2008, Dasgupta & Ramphal, 2001, Baraquet et al., 2012). FleN directly interacts with FleQ but not the promoter DNA (Hickman & Harwood, 2008, Baraquet et al., 2012). The inhibition of FlrA binding to the *flrBC* promoter by c-di-GMP in the EMSA and DNase I footprinting experiments does not require any additional proteins (Fig. 28 and 29). Therefore, we hypothesized that FlrA does not function with a protein analogous to FleN to regulate *flrBC* in response to c-di-GMP. To further test this idea, we examined if *flrBC* could be regulated by c-di-GMP in an FlrA-dependent manner in the heterologous host *E. coli*. For these experiments, the *flrBC-lux* transcriptional reporter was co-introduced into *E. coli* along with *flrA* expressed from a compatible vector. We observed robust expression of *flrBC-lux* only in the presence of FlrA (Fig. 31). Increasing the intracellular concentration of c-di-GMP by overexpression of QrgB led to significant repression of *flrBC* in *E. coli* (Fig. 31). As the flagellar biosynthesis regulatory cascades of *V. cholerae* and *E. coli* are inherently different (Chilcott & Hughes, 2000), and *E. coli* does not encode any obvious FleN homologs, we conclude that FlrA does not function, at least at the *flrBC* promoter, in conjunction with a FleN-like protein.

- **Identification of c-di-GMP blind mutants of FlrA**

Of the eight known transcription factors that have been identified to directly bind and respond to c-di-GMP, three of them (VpsR, FlrA, and FleQ) belong to the EBP family (Hickman & Harwood, 2008, Srivastava et al., 2011). Each of these three transcription factors has a conserved central AAA+ domain. The AAA+ domain typically binds to and hydrolyzes ATP to drive closed to open complex formation at σ54-dependent promoters (Chen et al., 2008). As the AAA+ domain has evolved to bind purine nucleotides, we hypothesized that FlrA interacts with c-di-GMP via its AAA+ domain. In support of this hypothesis, deletion of the N-terminal receiver domain of FleQ did not abrogate c-di-GMP binding (Hickman & Harwood, 2008).

The AAA+ domain of EBPs contains a number of conserved motifs: Walker A, Walker B, second region of homology (SRH), sensor I (SI), and sensor II (SII) (Schumacher et al., 2006). The Walker A domain is a conserved stretch of amino acids (GxxGxGK[S/T]) that functions with the Walker B domain to bind and hydrolyze ATP (Schumacher et al., 2006, Bose et al., 2008, Bush & Dixon, 2012). The FlrA Sensor II motif is followed by 30 amino acids that are conserved amongst the AAA+ domains of FleQ homologues (Baraquet et al., 2012, Francke et al., 2011). Moreover, deletion of 20 amino acids from this conserved region in FleQ was shown to abolish c-di-GMP binding (Baraquet et al., 2012). However, no single amino acid mutations that inhibited binding of c-di-GMP to FleQ have been identified

(Baraquet et al., 2012). Therefore, to identify c-di-GMP binding mutants of FlrA, we targeted the Walker A and Sensor II domains with alanine scanning mutagenesis. To expedite the analysis, two to three conserved residues in the Walker A and Sensor II sites were concurrently mutated to alanine. These mutants were overexpressed in *E. coli* in the presence of the *flrBC-lux* reporter at both high and low levels of c-di-GMP to identify mutants that are insensitive to c-di-GMP-mediated inhibition.

The phenotypes of all the mutants we generated are indicated in Fig. 32. Mutations in the Sensor II region (i.e. amino acids 349-356) were indistinguishable

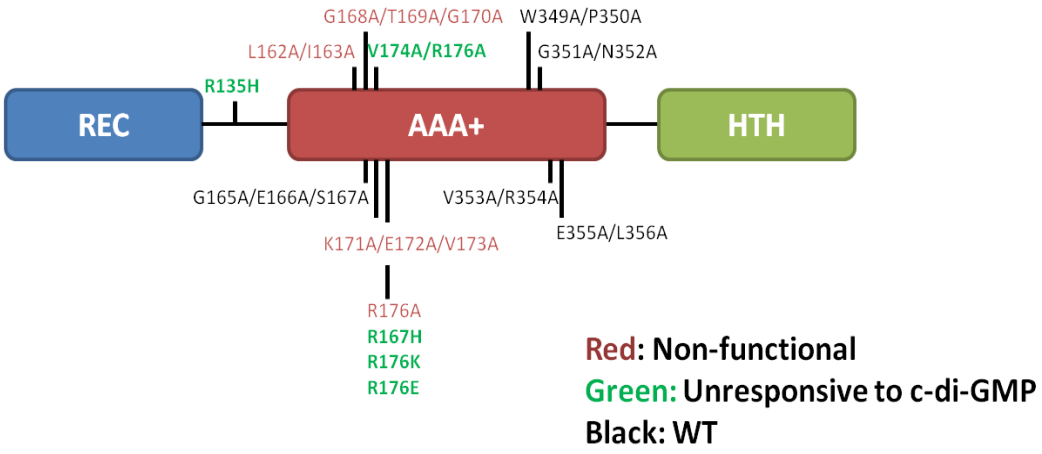


Figure 32: Schematic representation of site directed mutants of FlrA. The site directed mutants constructed here are shown on the FlrA schematic highlighting their location on the protein. Mutations colored in red were non-functional showing no expression of *flrBC* in the luminescence assay. Residues listed in bold black showed activity similar to WT with decreased *flrBC-lux* expression at high levels of c-di-GMP. Residues listed in green were unresponsive to c-di-GMP leading to full *flrBC-lux* expression even at high levels of c-di-GMP.

from WT FlrA. However, disruption of residues in the Walker A exhibited more

dramatic phenotypes. Many of these mutations produced nonfunctional FlrA variants that are not able to induce *flrBC* in any condition, suggesting the Walker A box is necessary for FlrA to activate gene expression. However, we also isolated one double mutant, V174A/R176A, which was unresponsive to c-di-GMP and showed constitutive expression of *flrBC* in *E. coli* even at high levels of c-di-GMP (Fig. 33). The V174 and R176 residues lie 2 amino acids downstream of the Walker A box and are partially conserved amongst EBPs.

To determine if the c-di-GMP blind phenotype was attributed to mutation of V174, R176, or both we individually mutated each residue to alanine. The V174A mutant was similar to WT FlrA. Alternatively, the R176A was nonfunctional and could not significantly induce *flrBC* expression even at low levels of c-di-GMP (Fig. 33). Therefore, we generated more conservative mutations of R176, R176H and R176K, and found that both of these mutations were insensitive to c-di-GMP, promoting induction of *flrBC* expression even in the presence of a high intracellular c-di-GMP concentration (Fig. 33). We also generated a R176E mutation, which reverses the charge at this residue. Interestingly, *flrBC* expression was induced in the presence of high c-di-GMP with the R176E FlrA mutant although to much lower levels than WT FlrA (Fig. 33). This mutant analysis suggests that R176 is an important residue for c-di-GMP mediated repression of FlrA activity.

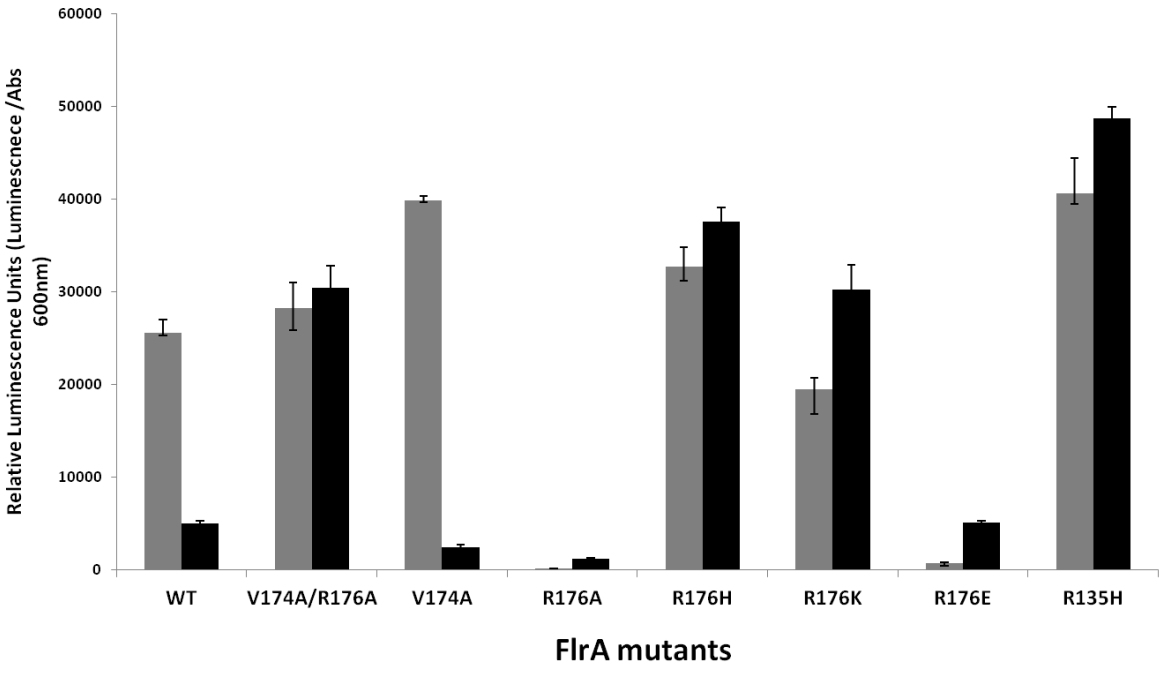


Figure 33: The R176 and R135 residues of FlrA are important for responding to c-di-GMP *in vivo*. The ability of the FlrA mutants listed on the x-axis to drive *flrBC-lux* expression in *E. coli* at high levels of c-di-GMP (black bars) or low levels of c-di-GMP (gray bars) produced by expression of QrgB or QrgB*, respectively, was determined. Error bars indicate the standard deviation (n=3).

As arginines have been shown to mediate binding of proteins to c-di-GMP (Benach *et al.*, 2007, Steiner *et al.*, 2012, Habazettl *et al.*, 2011), we hypothesized that residue R176 formed part of a c-di-GMP binding pocket in FlrA. To further explore this hypothesis, we created a homology model of an FlrA dimer from amino acids 1-378 containing the REC and AAA+ domain based on the crystal structure of NtrC (Fig. 34). Interestingly, we observed a pocket located in the interface of two FlrA monomers that was flanked by R176. Similarly, the residue R135 also flanks

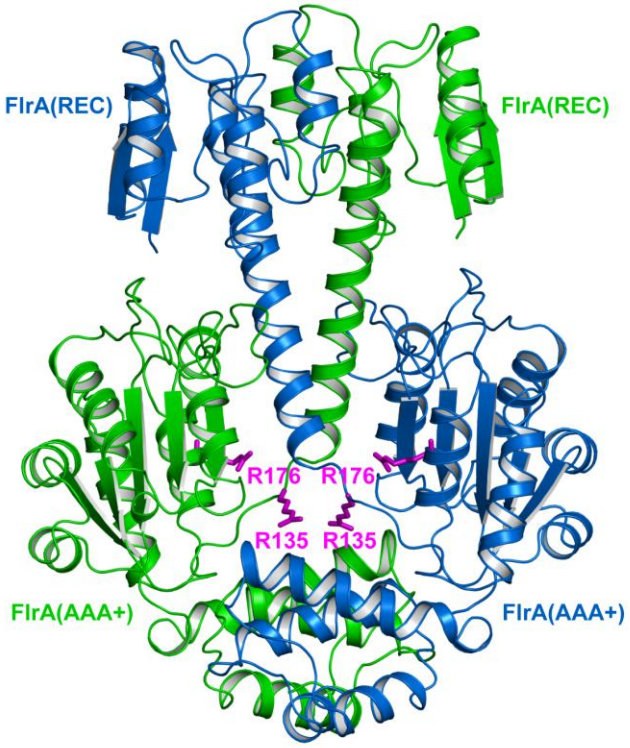


Figure 34: FlrA-RC comparative model. FlrA-RC monomers (green and blue cartoons) are shown in the inactive, dimeric conformation observed in the NtrC1-RC crystal structure (1NY5). Mutations in residues R135 or R176 (magenta sticks) abolished the ability of FlrA to bind and respond to c-di-GMP. The dimensions of this pocket are consistent with fitting a dimer of c-di-GMP molecules base stacked via ends.

this pocket and we hypothesized it could be important for binding to c-di-GMP. To test this hypothesis, we created a R135H mutant of FlrA, and found that it induced *flrBC–lux* expression at high levels of c-di-GMP similar to FlrA R176H (Fig. 33). These data suggest that R135 and R176 are functionally important for FlrA inhibition by c-di-GMP.

- **FlrA(R135H) and FlrA (R176H) do not bind or respond to c-di-GMP *in vitro***

The c-di-GMP blind phenotype of FlrA(R135H) and FlrA(R176H) could be due to disrupted c-di-GMP binding or other changes in protein conformation. To distinguish these possibilities, we measured binding of these FlrA mutants to c-di-GMP *in vitro*. Indeed, both the FlrA(R135H) and FlrA(R176H) mutants were unable to bind to c-di-GMP, exhibiting binding levels equivalent to the BSA non-specific control (Fig. 35A, 35C). We conclude that R135 and R176 are important for binding of FlrA to c-di-GMP. We further examined the R135H and R176H mutant proteins by determining if c-di-GMP could inhibit their interaction with the *flrBC* promoter in an EMSA. Indeed, FlrA(R135H) and FlrA(R176H) bound to the *flrBC* promoter in the same manner as WT FlrA; however, addition of c-di-GMP did not inhibit this binding (Fig. 35B, 35D).

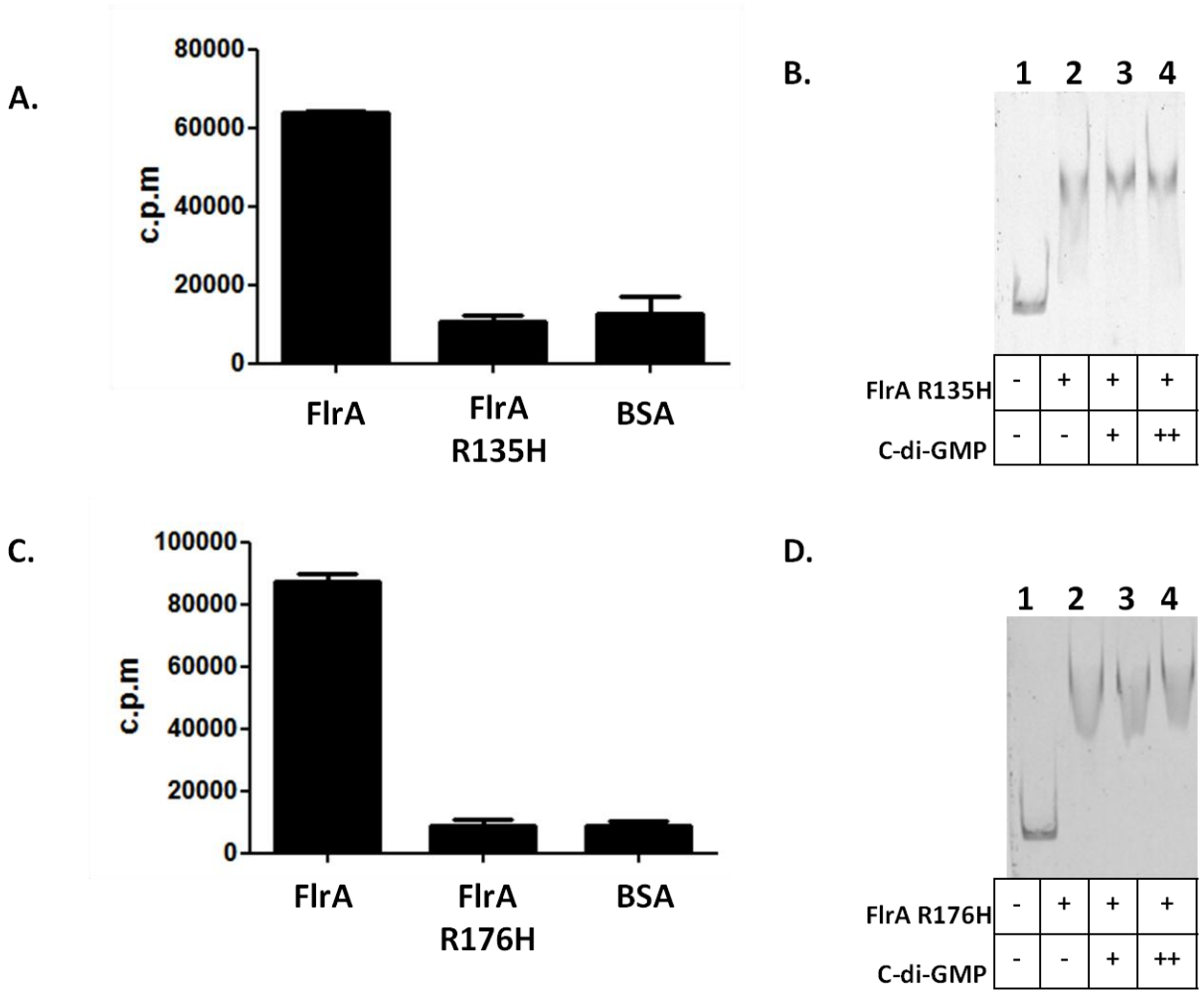


Figure 35: The FlrA(R135H) and R(176H) mutants do not bind and respond to c-di-GMP. (A and C) Binding of the purified proteins (250 nM) indicated on the x-axis to [^{32}P]-labeled c-di-GMP (950 nM) was determined using a filter binding assay. Error bars indicate standard deviation. **(B and D)** EMSA analysis was performed to measure the *in vitro* response of purified FlrA(R135H) and FlrA (R176H) to c-di-GMP respectively. The *flrBC* probe (10 nM) was incubated with no protein (lane 1) or 60 nM FlrA(R135H) and FlrA (R176H) (lanes 2-4). Lane 3 and 4 contain 40 μM (+) and 80 μM (++) of c-di-GMP, respectively.

- **REC domain of FlrA is required for activity**

Enhancer binding proteins are primarily regulated by phosphorylation of the REC domain by a cognate kinase (Rappas et al., 2005). Phosphorylation can either affect protein function in a positive or a negative manner. In positive regulation, phosphorylation leads to activation of protein function by enhancing oligomerization essential for contact with RNAP σ54 complex whereas in negative regulation

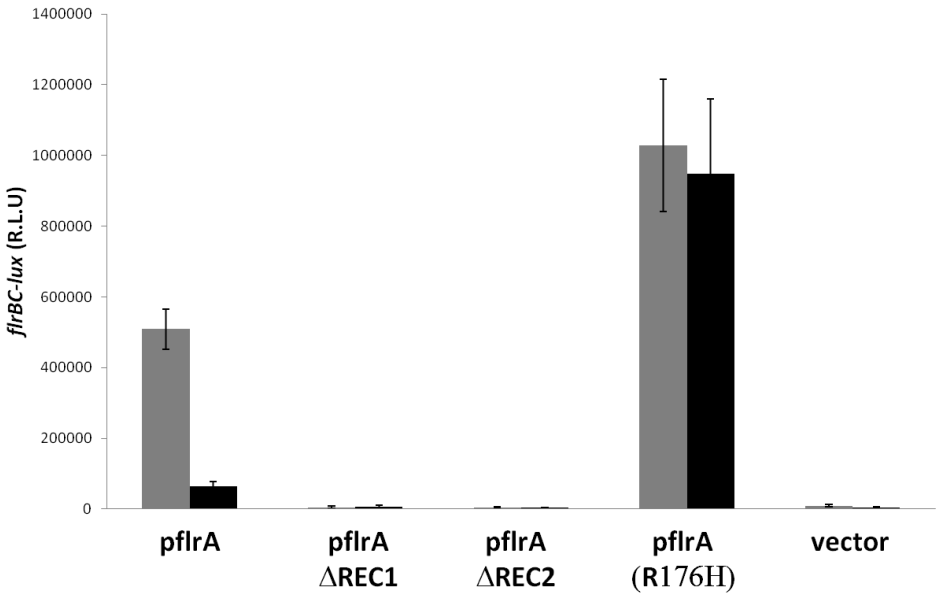


Figure 36: REC domain of FlrA is important for function. Truncation mutants in the receiver domain of FlrA were tested in E.coli S17 assay as described previously. ΔREC1 is a deletion of residue 1 to 129 and ΔREC2 is a deletion of residue 1-138. Both mutants are inactive in initiating transcription suggesting the importance of REC domain in FlrA activity. R176H c-di-GMP blind mutant of FlrA is used as a control. The cultures were grown for 5 hours with 0.002% arabinose (for p*flrA*expression) before addition of 0.1mM IPTG to induce c-di-GMP and the luminescence and O.D 600nm readings were take 3 hours after addition of IPTG. High levels of c-di-GMP (black bars) or low levels of c-di-GMP (gray bars) produced by expression of QrgB or QrgB* under control of IPTG. Error bars indicate the standard deviation (n=3).

phosphorylation inactivates the protein function (Rappas et al., 2005, Schumacher et al., 2006). Both FlrA and FleQ lack the conserved Asp residue required for phosphorylation of REC domain (Prouty et al., 2001, Jyot et al., 2002, Dasgupta & Ramphal, 2001), and the role of this domain in the regulation of these proteins is not clear. To test if REC domain regulates FlrA function, we made truncations in the REC domains (residues ΔREC1 (1-129) and ΔREC2 (1-138) respectively). We tested these truncations in our previously described *E. coli* assay for activating *flrBC-lux* expression. Both REC domain truncations were inactive in our assays suggesting the importance of the REC domain in FlrA function (Fig. 36). We know that these proteins were expressing in *E. coli* by analysis of overexpression extracts on SDS PAGE gels (data not shown). This could also mean that the REC domain is important for the correct conformation of FlrA.

- **REC domain of FlrA limits c-di-GMP binding**

Our collaborators at Rutgers University (Mathew Neiditch and Atul Khataokar) are working on solving the crystal structure of FlrA. They have developed several truncated constructs of FlrA to facilitate structure development. We tested the truncated versions of FlrA in our c-di-GMP binding assay to assess the importance of each domain in binding c-di-GMP. As the c-di-GMP blind mutants we discovered were clustered in between REC domain and AAA+ domain, we hypothesized that the FlrA-RC (amino acids 1-384) truncation would exhibit the greatest binding to c-di-GMP. Interestingly we see that FlrA-RC binds c-di-GMP to the same level as

FlrA WT, but FlrA-CD (amino acids 123 to 488) binds significantly higher amounts of c-di-GMP than both WT and RC (Fig. 37). This suggests that REC domain is somehow limiting the amount of c-di-GMP FlrA can interact with. This could define a new regulatory function for the REC domain wherein it prevents binding of c-di-GMP to FlrA to prevent inactivation of protein function. Further experiments need to be performed to analyze this in detail. Structural analysis of FlrA bound to c-di-GMP will be instrumental in uncovering the mechanism of action of REC domain in FlrA.

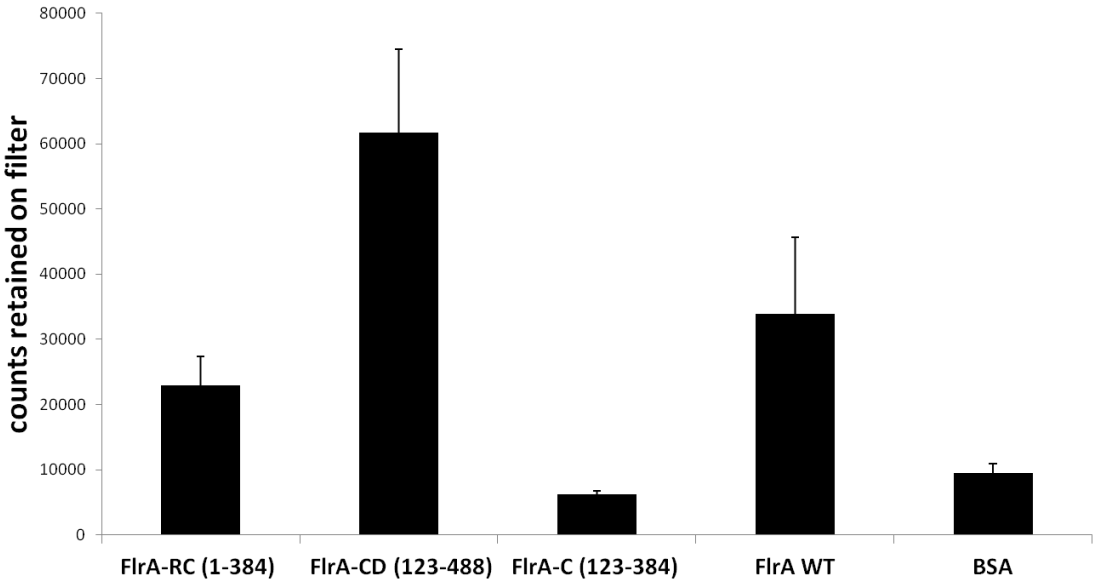


Figure 37: REC domain inhibits c-di-GMP binding. Truncations of FlrA were tested for c-di-GMP binding using the filter binding assay described previously. 1μM P^{32} labeled c-di-GMP was incubated with 400nM protein and counts retained on filter after washing were measured using scintillation counting. Error bars are standard deviations (n=2).

- **c-di-GMP inhibits motility by inactivating FlrA and inducing VPS synthesis**

To assess if inhibition of *V. cholerae* flagellar based motility by c-di-GMP is due solely to inactivation of FlrA, we complemented the non-motile Δ*flrA* mutant with WT FlrA or the c-di-GMP blind FlrA(R176H) mutant and determined motility at both low and high levels of c-di-GMP (Fig. 38). As previously observed, c-di-GMP strongly inhibits motility of *V. cholerae* when expressing WT FlrA. Surprisingly, a similar inhibition was observed when *V. cholerae* only expressed the c-di-GMP blind mutant FlrA(R176H). This result suggested that c-di-GMP inhibits motility in *V. cholerae* via an alternative mechanism(s) in addition to but independent of transcriptional control of the flagellar biosynthesis gene regulon. In *E. coli* and *Salmonella,* c-di-GMP directly inhibits motility by binding to the protein YcgR to influence flagellar function (Paul et al., 2010, Boehm *et al.*, 2010, Fang & Gomelsky, 2010). C-di-GMP was also recently shown to inhibit motility of *Salmonella* partially through induction of extracellular cellulose synthesis (Zorraquino et al., 2013). As *V. cholerae* does not encode an obvious homolog of YcgR, we hypothesized that induction of *VPS* synthesis by c-di-GMP inhibits motility. To test this hypothesis, we complemented a Δ*flrA*Δ*vpsL* double mutant with WT FlrA and FlrA(R176H). Deletion of *vpsL* prevents formation of the *VPS* extracellular polysaccharide. In this mutant, c-di-GMP repression of motility was maintained upon complementation with WT FlrA, presumably through repression of the flagellar biosynthesis genes (Fig. 38). However, complementation of the Δ*flrA*Δ*vpsL* mutant with FlrA(R176H) led to enhanced motility that was no longer significantly inhibited by c-di-GMP.

These results show that c-di-GMP inhibits motility of *V. cholerae* by two distinct but functionally redundant mechanisms: repression of flagellar biosynthesis gene expression through inhibition of FlrA and induction of *VPS* biosynthesis.

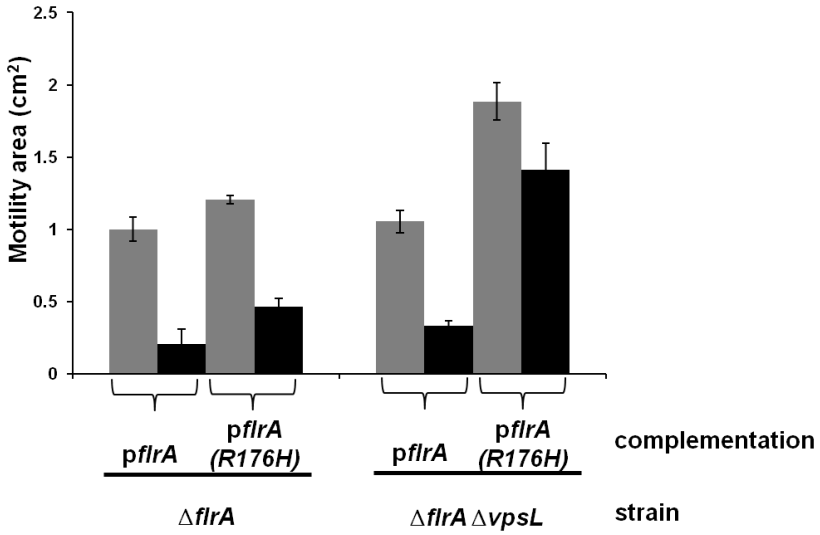


Figure 38: Cyclic-di-GMP mediated inhibition of *V. cholerae* motility is dependent on repression of flagellar gene expression and induction of *VPS*. Motility of Δ*flrA* and Δ*flrA*Δ*vpsL V. cholerae* mutants through low percentage agar plates was assessed after introduction of WT FlrA and FlrA(R176H) complementation plasmids. High levels of c-di-GMP were generated by induction of QrgB (black bars) while low levels of c-di-GMP were produced by induction of QrgB* (gray bars). Error bars indicate standard deviation (n=3).

- ***V. cholerae*** **encodes unidentified c-di-GMP-dependent transcriptional machinery**

The identification of FlrA brings the total number of c-di-GMP dependent transcription factors in *V. cholerae* to three. We have previously identified seven c-di-GMP dependent promoter-*lux* fusions that are induced by c-di-GMP in *V. cholerae*, only one of which shows dependence on VpsT or VpsR (Srivastava et al., 2011). All seven of these promoters were fully induced by c-di-GMP in the Δ*flrA*

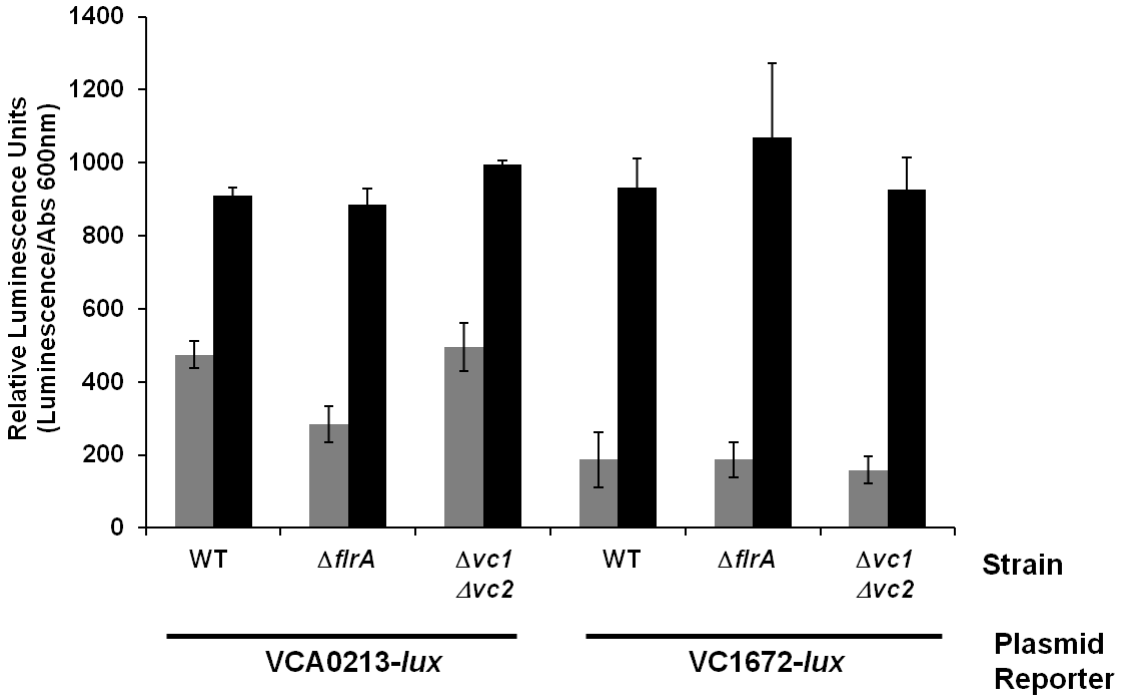


Figure 39: A subset of c-di-GMP inducible promoters are regulated independently of FlrA and the c-di-GMP binding riboswitches. Induction of the VCA0213-*lux* and VC1673-*lux* transcriptional fusions by c-di-GMP was measured in WT *V. cholerae* and the Δ*flrA* and riboswitch (*vc1* and *vc2*) mutants. High levels of c-di-GMP were generated by induction of QrgB (black bars) while low levels of c-di-GMP were produced by induction of QrgB* (gray bars). Error bars indicate the standard deviation (n=3).

mutant, indicating that none of these promoter fusions required FlrA for their induction. Two representative promoters, VCA0213-*lux* and VC1763-*lux*, are shown although all seven promoters behaved similarly (Fig. 39). *V. cholerae* also encodes two c-di-GMP binding riboswitches called Vc1 and Vc2 located near the genes VCA0811 (*gbpA*) and VC1722 (*tfoY*), respectively, that are predicted to alter gene expression in response to c-di-GMP (Sudarsan et al., 2008). All seven promoters maintained induction by c-di-GMP in a mutant deleted for both of these riboswitches, showing that they too are dispensable for the c-di-GMP mediated regulation of these genes (Fig. 39). Therefore, only one of the seven promoter-*lux* fusions that we previously identified to be induced by c-di-GMP requires VpsR, VpsT, FlrA, or the c-di-GMP riboswitches for c-di-GMP mediated regulation, suggesting that additional c-di-GMP dependent transcriptional machinery remains to be identified.

3.4 Discussion

The study of c-di-GMP mediated regulation of transcription has led to the identification of ten c-di-GMP binding transcription factors including FlrA (Table 1; Chapter 1). It is clear that c-di-GMP does not regulate transcription through one conserved mechanism but involves many types of transcription factors (discussed in chapter 1).

EBPs possess a conserved domain architecture consisting of an N-terminal receiver domain, a central AAA+ domain, and a C-terminal DNA binding domain

(Bush & Dixon, 2012, Francke et al., 2011). These regulators typically bind far upstream of σ54 promoters and oligomerize upon phosphorylation of the receiver domain. This oligimerization promotes looping of the DNA stimulating contact of the EBP with σ54-RNA polymerase complex at the promoter (Bush & Dixon, 2012). Hydrolysis of ATP by the AAA+ domain drives closed to open complex formation, initiating transcription (Chen et al., 2008). Interestingly, our analysis of the FlrA dependent *flrBC* promoter identified a putative transcription start site located downstream of a σ54 consensus promoter sequence; however, the binding site of FlrA is only 73-53 bp from the transcription start site. This location is much closer than traditional σ54-dependent EBPs and future studies are needed to determine how FlrA functions as a transcriptional activator. The binding site for FleQ and FlrA are similar in sequence /ATTG/(A/G)C/ but not in arrangement. Whereas we identified two FlrA binding sites in *flrBC* located in close proximity as an inverted repeat (Fig.26), the two distinct regions protected by FleQ in a DNAse I protection assay each contained one binding site (Baraquet et al., 2012).

Our determination that mutation of R135 and R176 abolished the ability of FlrA to bind and respond to c-di-GMP suggests that c-di-GMP interacts with the REC and AAA+ domain interface. This finding combined with molecular modeling hint at a c-di-GMP binding pocket in FlrA flanked by arginine residues. Arginine residues have been shown in other proteins to mediate an interaction with the c-di-GMP phosphate moieties (Steiner et al., 2012, Benach et al., 2007, Habazettl et al., 2011), consistent with our identification of two important arginines in FlrA that

mediates binding to c-di-GMP. It is generally thought that the positive charge of arginines interacts with the negatively charged phosphates of c-di-GMP. *V. cholerae* contains 12 EBPs including FlrA and VpsR, some of which have conservation of either R135 or R176 but not both together. These residues are conserved in the FlrA homolog, FleQ but not in VpsR. VpsR is a non-canonical EBP since it functions with σ70 and has a mutation in the Walker B motif of the AAA+ domain which hints at it being unable to hydrolyze ATP (Francke et al., 2011). Interestingly, it was recently demonstrated that FleQ interacts with c-di-GMP through its Walker B motif in the AAA+ domain (Baraquet & Harwood, 2013). It was shown that c-di-GMP competes with ATP to bind to WalkerB site and decreases FleQ ATPase activity(Baraquet & Harwood, 2013). This effect of c-di-GMP on FleQ is potentiated by the presence of another protein FleN which also inhibits FleQ ATPase activity and also aids in FleQ binding to DNA. It was suggested in this work that FleQ ATPase activity is needed in the regulation of flagellar gene expression and decrease in this activity may lead to small decreases seen at high c-di-GMP levels on flagellar gene expression(Baraquet & Harwood, 2013).

Although FlrA and FleQ are orthologs, their functional response to c-di-GMP is markedly different. First, in *V. cholerae*, c-di-GMP strongly inhibits the expression of *flrBC* and other members of the flagellar biosynthesis gene cascade. In contrast, c-di-GMP does not significantly repress flagellar gene synthesis in *P. aeruginosa,* even though FleQ is absolutely required for the expression of flagellar genes (Hickman & Harwood, 2008). Also, FleQ requires FleN for its effect on gene

expression (Hickman & Harwood, 2008, Baraquet & Harwood, 2013). Second, we determined that FlrA does not regulate the *V. cholerae vps* operon, which encodes the genes necessary for production of VPS polysaccharide. In contrast, FleQ functions as both a repressor and activator of the *P. aeruginosa pel* genes (Hickman & Harwood, 2008, Baraquet et al., 2012). Thus, although both FleQ and FlrA bind c-di-GMP, the downstream networks controlled by these regulators are dissimilar. Third, full-length FlrA binds robustly to *flrBC* promoter DNA *in vitro* at only 6-fold excess protein (10 nM probe with 60 nM protein); however, full-length FleQ requires a much higher excess of protein to bind target promoters *in vitro* (Hickman & Harwood, 2008). Lastly, FleN, an accessory protein encoding a putative ATPase, is required for FleQ to fully respond to c-di-GMP (Hickman & Harwood, 2008, Dasgupta & Ramphal, 2001). Our results suggest that FlrA alone is sufficient to respond to c-di-GMP at the *flrBC* promoter, and an accessory protein like FleN is not required. FlhG, the FleN homolog in *V. cholerae,* has been shown to negatively regulate FlrA expression, however, no physical interaction between these proteins has been demonstrated (Correa *et al.*, 2005). However, it is possible that c-di-GMP-mediated control of FlrA at other target promoters could require FlhG or other accessory factors.

Regulation of motility by c-di-GMP occurs at multiple levels such as arresting flagellar rotation via a backstop brake mechanism (Paul et al., 2010, Boehm et al., 2010, Fang & Gomelsky, 2010), repression of flagellar genes (Krasteva et al., 2010, Beyhan et al., 2006, Ferreira et al., 2008), and impediment of flagellar rotation by

accumulation of extracellular polysaccharide around cells (Zorraquino et al., 2013). With the isolation of the FlrA(R135H) and FlrA(R176H) c-di-GMP blind mutants, we were able to address the impact of c-di-GMP-mediated transcription repression of the flagellar biosynthesis regulon on cell motility. Interestingly, we still observed a c-di-GMP-mediated decrease in motility of an Δ*flrA* mutant complemented with FlrA(R176H) analogous to the WT strain. This result suggested that c-di-GMP represses motility by an alternative mechanism independent of flagellar gene regulation. Indeed, a strain that cannot synthesize VPS complemented with FlrA(R176H) shows virtually no inhibition of motility by c-di-GMP. These results indicate that extracellular synthesis of VPS can inhibit motility similar to the results recently demonstrated in *Salmonella enterica* (Zorraquino et al., 2013). These two mechanisms that we have identified function redundantly to inhibit motility in *V. cholerae*; either process is sufficient for motility inhibition by c-di-GMP. Only when both processes are removed (i.e. expression of FlrA(R176H) in the Δ*flrA*Δ*vpsL* mutant) is the motility of *V. cholerae* is largely insensitive to c-di-GMP.

We speculate that these two mechanisms of repression operate at different time scales. Whereas production of VPS by c-di-GMP should occur relatively quickly, transcriptional repression of flagellar biosynthesis will not impact preexisting flagella but would only exert its effects on newly formed cells. Our observations are consistent with an emerging theme that flagella function is controlled at short time scales by direct modulation of activity and at longer time scales by transcriptional regulation (Guttenplan & Kearns, 2013).

EBPs are a widespread family of transcription factors of critical importance in the regulation of biofilm formation, motility, virulence, and quorum sensing (Visick, 2009, Wolfe *et al.*, 2004, Jovanovic *et al.*, 2011, Saldías *et al.*, 2008, Dasgupta & Ramphal, 2001, Francke et al., 2011). We propose that the interaction of c-di-GMP with EBPs is an important mechanism by which c-di-GMP controls gene expression. In some cases, such as VpsR and FleQ, binding of c-di-GMP leads to an induction of gene expression (Srivastava et al., 2011, Baraquet et al., 2012), whereas here we show that in the case of FlrA, c-di-GMP binding inhibits the activity of the transcription factor. Understanding the molecular mechanisms by which c-di-GMP controls EBPs will allow systematic identification of additional c-di-GMP regulated EBPs in a wide variety of bacterial species.

CHAPTER 4

C-di-GMP activates base excision repair pathway in *V. cholerae*

PREFACE

C-di-GMP regulates multiple processes in *V. cholerae* and other bacteria. It is evident form the number of c-di-GMP regulatory domains (61 EAL and GGDEF domains) present in *V. cholerae* that this second messenger is an important part of its life cycle (Galperin, 2004). Work from our lab and others have shown that levels of c-di-GMP are highly variable in bacterial cells and are regulated by varied environmental inputs (Massie et al., 2012, Römling et al., 2013). Multiple c-di-GMP regulatory pathways exist in cells; these pathways must contain other unknown c-di-GMP effector proteins such as enzymes, cytoplasmic proteins and transcription factors. We know from our previous work that other genes are regulated by c-di-GMP independent of the known c-di-GMP binding transcription factors and riboswitches in *V. cholerae,* suggesting the existence of other c-di-GMP binding proteins such as novel transcription factors (Srivastava et al., 2011, Srivastava et al., 2013). We have previously isolated 6 c-di-GMP inducible promoters from *V. cholerae* and virtually nothing is known about the regulation of these promoters by c-di-GMP (Srivastava et al., 2011, Srivastava et al., 2013). Here, I describe our efforts to determine the regulatory machinery that controls the 6:C9 promoter isolate in response to c-di-GMP.

4.1 Introduction

In order to search for unknown c-di-GMP effectors, we focused on 6:C9, a c-di-GMP regulated promoter that was identified in the above mentioned screen (refer to Table 1) (Srivastava et al., 2011). The 6:C9 promoter was interesting since it was located in the middle of an ORF (VC1673) in the direction of the coding sequence, which was unique amongst the promoters discovered in the screen. VC1673 codes for an ABC transporter protein called VexK involved in efflux of detergent specific substrates (Bina *et al.*, 2008). These proteins are called resistance nodulation deficient or RND efflux pump proteins. There are 14 proteins of this family in *V. cholerae*; VexA-M and VexR. These proteins have been shown to be required for antimicrobial resistance, efficient colonization and virulence factor production in infant mouse model of *V. cholerae* infection (Bina et al., 2008). VexK is believed to be important for growth of *V. cholerae* in specific environmental conditions such as high bile acid concentrations in the small intestine (Bina et al., 2008).

The location of 6:C9 promoter suggested to us that it is possibly not regulating the expression of *vexK* (VC1673). We hypothesized that 6:C9 instead regulated c-di-GMP dependent expression of upstream gene VC1672 (Fig. 42). VC1672 codes DNA-3-methyladenine glycosidase I, also known as *tag,* a protein involved in the base excision repair pathway (Krokan *et al.*, 1997).

The base excision repair pathway is a DNA repair mechanism in the cells which protects cells against damaged bases. In general, this pathway recognizes a

damaged base that can occur due to many reasons such as spontaneous deamination of bases, radiation, oxidative stress, alkylating agents or replication errors (Krokan et al., 1997, Lindahl, 1979). Several DNA glycosylases are involved in the base excision repair pathways, some of which have narrow substrate specificities (Krokan et al., 1997). DNA glycosylases cleave the glycosylic bond between the base and the sugar to remove the defective base leaving an apurinic/apyrimidinic site also known as AP-site (Krokan et al., 1997). On cleavage of the damaged base, the AP-site is recognized by the downstream components of the pathway to repair the lesion (as shown in Fig. 40). This pathway has been widely studied in *E. coli* (Friedberg, 1995) and it has been shown that the glycosylases involved in the first step of the BER pathway are specific to the damage caused. For alkylation induced damage, two DNA glycosylases have been widely studied in *E.coli*; *alkA* and *tag* (Friedberg, 1995). AlkA is the more widely studied enzyme and has wide substrate specificity. Tag on the other hand is able to efficiently remove only 3-meA bases. Exposure to alkylating agents confers resistance to further damage in *E. coli* by induction of *alkA, alkB* and *ada* genes in a process known as the adaptive response (Clarke *et al.*, 1984, Friedberg, 1995, Lindahl *et al.*, 1988). Tag is believed to be constitutively expressed (Friedberg, 1995). In the adaptive response, low levels of alkylating agents such as N-methyl-N' -nitro-N-nitrosoguanidine (MNNG) that are toxic to cells can induce resistance to higher levels of alkylating agents (Lindahl et al., 1988). This response is distinct from the SOS response wherein the products of *recA* and *lexA* genes are induced.

The SOS response is induced by damaging effects of multiple DNA-damaging agents including UV damage.

AlkA and Tag proteins have no sequence similarity but are still able to perform the same function of removal of alkylated bases. This suggests that these proteins evolved independently to cleave base-sugar bonds in double-stranded alkylated DNA (Lindahl et al., 1988). It has been shown that *alkA* expression is induced by the adaptive response by the action of Ada protein, which is a transcriptional activator activated by methylation (Teo *et al.*, 1986). Overexpression of *tag* in a Δ*alkA* mutant strain is able to protect against alkylation induced damage (Sakumi *et al.*, 1986). Also overexpression of *alkA* leads to a defect in growth of *E. coli* whereas *tag* overexpression does not cause any growth defects (Steinum & Seeberg, 1986, Lindahl, 1979, Lindahl et al., 1988).

In the work presented here, we study the 6:C9 promoter isolate from the screen described in Chapter 1 (Table 1) in order to better understand the transcriptional regulation by c-di-GMP. The 6:C9 promoter was most highly induced by c-di-GMP (Srivastava et al., 2011) and was located in the middle of an ORF (VC1673) (Fig. 42). Using promoter analysis we were able to demonstrate that c-di-GMP induces expression of VC1672 or *tag*, which codes for DNA-3-methyladenine glycosidase I, an enzyme involved in the BER pathway. We also show that high levels of c-di-GMP provide resistance to the alkylating agent methyl methane sulfonate (MMS). Similar resistance to MMS was observed on overexpression of VC1672. Finally, a genetic screen was developed to discover the regulator of VC1672 expression and we

have isolated three mutants that lose the c-di-GMP dependent induction of 6:C9 and VC1672 expression. This is the first demonstration in our knowledge of the role of c-di-GMP in a mutagenesis repair pathway in bacteria.

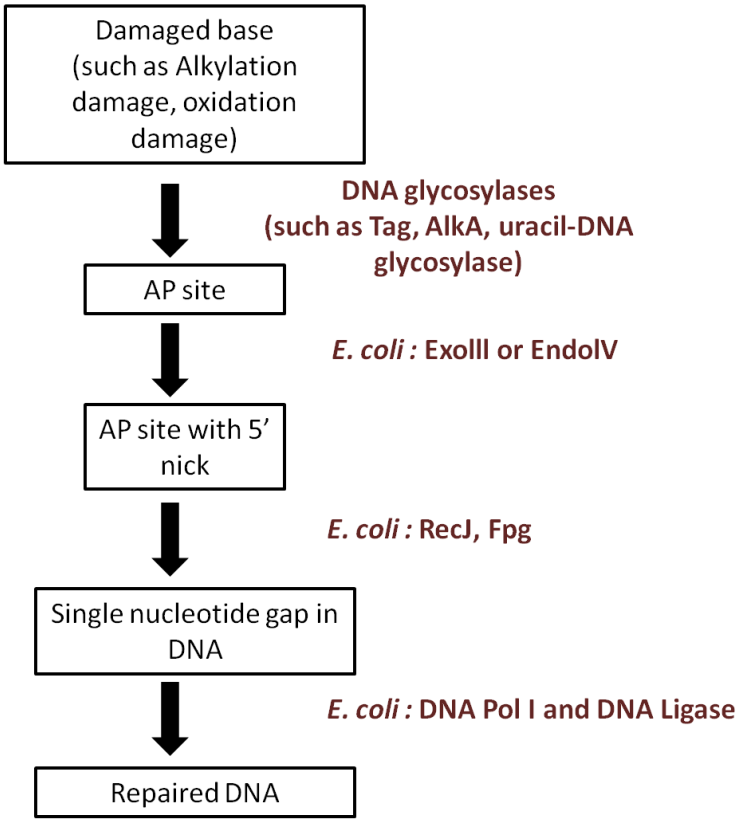


Figure 40: Base excision repair pathway. This figure represents the steps involved in the base excision repair pathway in bacteria and lists some gene products involved in the pathway.

4.2 Material and methods

- **Bacterial strains and culture conditions**

All strains, primers, and plasmids used in this study are listed in Table 3 and 4. Δ*VC1672* was constructed using the pKAS32 vector(Skorupski & Taylor, 1996). All strains of *V. cholerae* were grown in Luria-Bertani medium (LB). Antibiotics were obtained from Sigma and used at the following concentrations (μg/ml): ampicillin 100, kanamycin 100 and chloramphenicol 10unless stated otherwise. S17λpir *Escherichia coli* strains was used as the donor in biparental conjugation to mobilize plasmids into *V. cholerae* (Reddy, September 2007).

- **Molecular methods**

DNA manipulation was performed using standard procedures (Sambrook et al., 1989). T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (NEB) and Stratagene. PCR reactions were performed with iProof DNA Polymerase (NEB) and Phire DNA polymerase (Finnzymes). Promoter deletion and extension constructs were cloned into the SpeI and BamHI restriction sites of pBBRlux (Waters & Bassler, 2006) (refer to Table 4 for primer sequences). pKAS32 cloning of *VC1672* constructs was accomplished using primers listed in Table 4. Overexpression constructs for VC1672 was engineered into pEVS143 vector using primers listed in Table 4.

- **Screen to identify 6:C9 and VC1672 regulator**

A mutant library of *V. cholerae* was constructed by mating in pRL27c plasmid with Δ*vpsL* and BP55 strain respectively. In the Δ*vpsL* mutant library, pBRP2 (GGDEF overexpressing vector) and 6:C9-lux were subsequently mated in using S17λpir strain. Kan, cam and amp positive colonies were then picked for the screen in 96 well Costar plates. Similarly, in the BP55 mutant library created using the same Tn5 plasmid, pDS129 (6:C9-3-lux) was mated in using S17λpir strain. Kan and cam positive colonies were picked for the screen. 1500 and 1000 colonies from the Δ*vpsL* and BP55 mutants were picked respectively. These were grown overnight in a 96 well plates at 37°C. Plates were diluted using a 96 well plate replicator in white clear bottom Costar plates. Luminescence was recorded in the presence and absence of 0.1mM IPTG in LB medium after 7 hours and adjusted for growth by concurrent measurement of OD_{600}. Clones that showed no significant changes in bioluminescence in response to IPTG were re-assayed in triplicate.

- **Growth assays for MMS sensitivity**

Overnight cultures for the strains tested were diluted 1/1000 in clear Costar 96 well plates. All low c-di-GMP strains contained pBRP1 plasmid (*qrgB**: AADEF, mutant GGDEF) and all high c-di-GMP strains contained pBRP2 plasmid (*qrgB*: active GGDEF). Strains were grown in presence of 0.1mM IPTG and varying amounts of MMS 100μM, 200μM and 300μM. The growth assays were performed in

Perkin Elmer Multimode plate reader EnVision at 37°C, 200rpm, with readings at 10 min. intervals. The data was analyzed using the EnVision manager software.

4.3 RESULTS

- **6:C9 induction by c-di-GMP is not dependent on any known c-di-GMP effectors**

We hypothesize that c-di-GMP mediated 6:C9 regulation occurs via an unknown c-di-GMP binding protein. In order to test this, we examined the expression of the 6:C9-lux reporter in response to c-di-GMP in the absence of all known c-di-GMP binding transcription factors in *V. cholerae*. We show that 6:C9 promoter is not regulated by any c-di-GMP binding transcriptional machinery in *V. cholerae* namely; VpsT, VpsR, FlrA and *vc1* and *vc2* riboswitches (Fig. 41). This strongly suggests that the pathway for 6:C9 promoter regulation requires other c-di-GMP binding machinery.

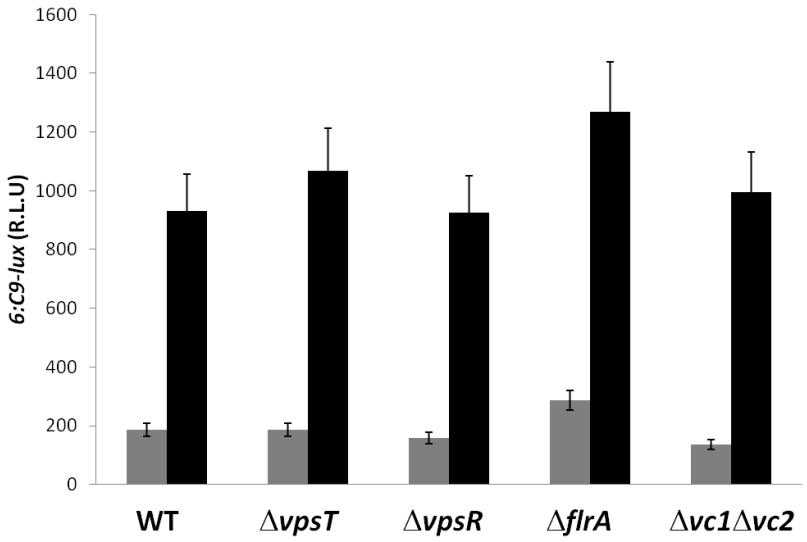


Figure 41: Regulation of 6:C9 promoter by c-di-GMP does not require known c-di-GMP effectors. The 6:C9 promoter is not regulated by any known c-di-GMP effectors in *V. cholerae*. We examined 6:C9-lux expression in WT, Δ*vpsT*, Δ*vpsR*, Δ*flrA*, and Δ*vc1*Δ*vc2*. The expression was measured at 6 hours post inoculation. Gray bars represent low c-di-GMP levels and black bars represent high c-di-GMP. c-di-GMP was induced from a GGDEF (*qrgB*) overexpression by addition of 0.1mM IPTG to the media. Error bars represent standard deviation.

- *Promoter analysis of 6:C9*

Sequence analysis of the 6:C9 promoter region indicated that it is comprised of a long stretch of DNA (1462bp) (Fig. 42). To further characterize this promoter region, we made promoter deletion constructs to define a small fragment required for c-di-

GMP induction. Fig. 42 shows the fragments constructed and fused to *lux* operon in our pBBRlux reporter vector. We tested the expression of these constructs *in V. cholerae* strain containing inducible GGDEF (*qrgB*) vector pCMW75. We see that the c-di-GMP inducible region exists at the 3' end of the original promoter, in the region denoted by red dotted lines (Fig. 42). Deletion of the 3' end of original 6:C9 promoter leads to inactivation of this promoter (6:C9-2). Also, deletion of the 5' end of the original 6:C9 promoter leads to increased expression suggesting the role of a c-di-GMP independent repressor at the 5' end (6:C9-1). We hypothesized that the 6:C9 promoter regulates the expression of the upstream gene VC1672 as it is oriented in its direction. To test this, we extended the promoter construct to the region between VC1673 and VC1672. This fragment was still inducible by c-di-GMP suggesting that the expression of VC1672 is regulated by c-di-GMP (6:C9-3). The intergenic region between VC1673 and VC1672 by itself is not inducible by c-di-GMP (6:C9-4). This analysis shows that the c-di-GMP inducible region lies within the 6:C9-2 and 6:C9-3 promoters (Dotted lines represent the fragment being referred to in Fig. 42).

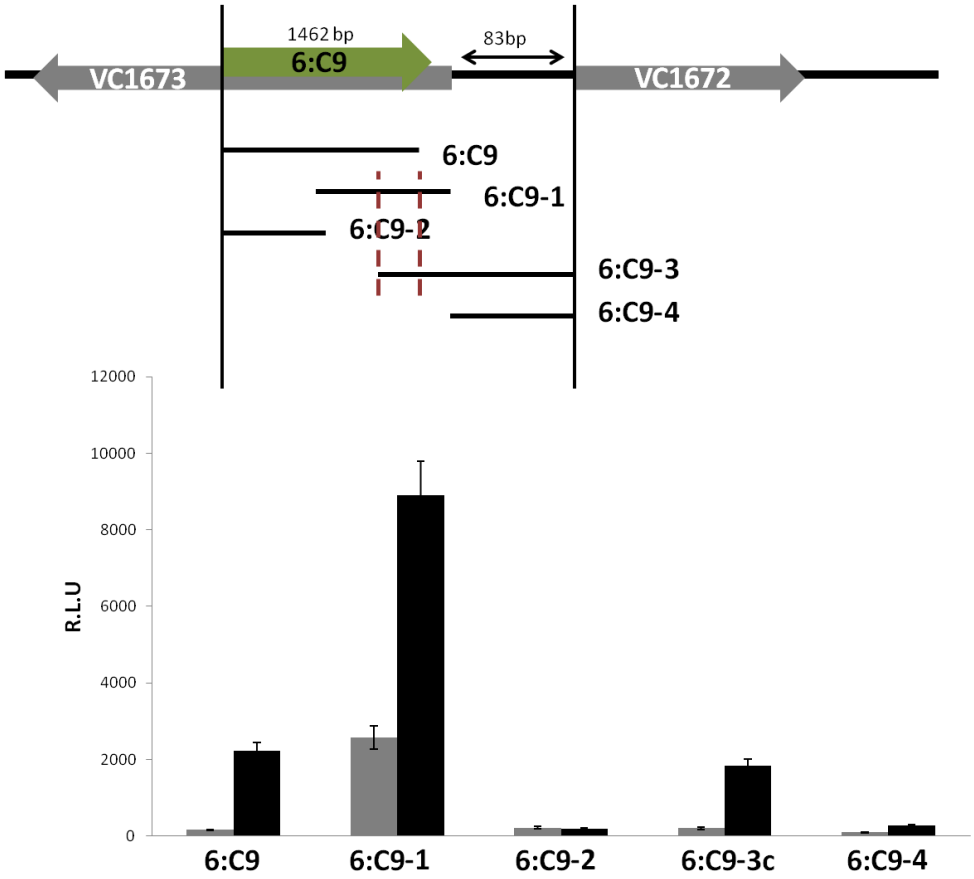


Figure 42: Promoter analysis of 6:C9. We constructed promoter deletion and extension constructs for 6:C9 promoter to understand its regulation shown in a schematic representation (upper panel). Black lines denotes the start and end positions of the constructs. These constructs were fused to *lux* operon in reporter vector pBBRlux. The expression was assessed at low versus high levels of c-di-GMP (lower panel). Gray bars represent low c-di-GMP and black bars represent high c-di-GMP. Error bars represent standard deviation.

- **C-di-GMP mediated induction of VC1672 protects against alkylation damage**

VC1672 also known as *tag* codes for DNA-3-methyladenine glycosidase I, this enzyme is involved in the base excision repair pathway as mentioned above. The expression of this gene is known to be constitutive in *E. coli* (Friedberg, 1995). We

observed an induction of this gene in response to c-di-GMP, which led us to hypothesize that c-di-GMP induces VC1672 to protect against alkylation induced damage. To test this, we grew *V. cholerae* strains at low and high c-di-GMP levels in the presence of alkylating agent Methyl Methane Sulfonate (MMS). MMS is an alkylating agent that methylates DNA on N7-deoxyguanosine and N3-deoxyadenosine (Lundin *et al.*, 2005). Damage caused by MMS causes double stranded breaks, stalling of replication forks and increase in mutation rates (Lundin et al., 2005). Exposure of bacterial cells to MMS significantly slows their growth; however, cells can repair the damage caused by MMS through the base excision repair pathway. MMS has previously been used to study the effects of alkylation damage and quantify effects of the base excision repair pathway in *E. coli* (Ljungquist et al., 1976). Our low c-di-GMP strain has a vector over-expressing a mutant GGDEF (AADEF, *qrgB**) and our high c-di-GMP strain is overexpressing an active GGDEF (*qrgB*); under the control of an IPTG inducible promoter. We performed growth curves for all strains at 0.1mM IPTG. The results are depicted as O.D 600nm on the Y axis and time on the X-axis (Fig. 43).

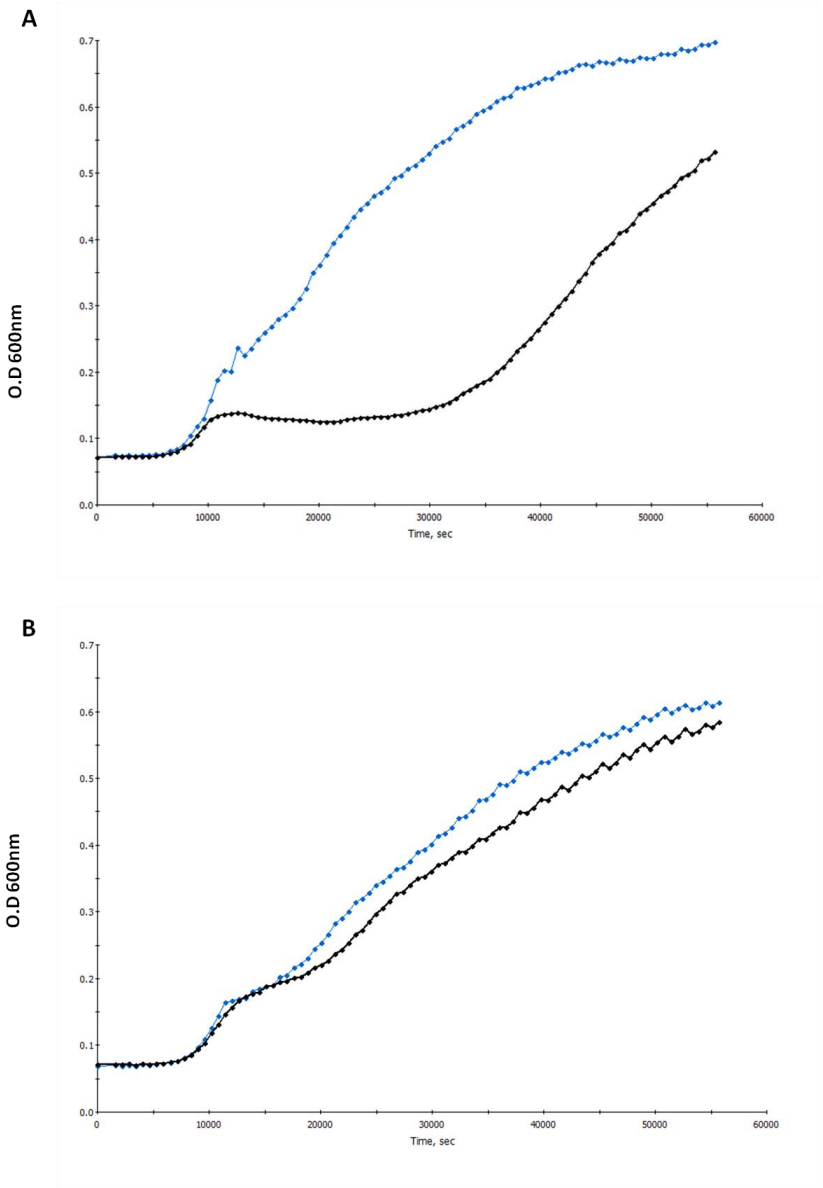


Figure 43: Alkylating agent methyl methane sulfonate (MMS) affects growth of *V. cholerae* and this effect is modulated by c-di-GMP. All strains were growth with 0.1mM IPTG from a 1/1000 inoculation in a 96 well plate and growth was monitored every 10 minutes using the Perkin Elmer (EnVision) 96 well plate reader. **(A)** Low c-di-GMP strain containing a mutated GGDEF (*qrgB*: AADEF) inducible from an IPTG regulated promoter grown in the presence of no MMS (blue) and 300μM MMS (black). **(B)** High c-di-GMP strain containing an active GGDEF (*qrgB*: GGDEF) inducible from an IPTG regulated promoter grown in presence of no MMS (blue) and 300μM MMS (black). High c-di-GMP strain grows slow as compared to the low c-di-GMP strain.

We have previously seen that high levels of c-di-GMP have a negative effect on the growth of *V. cholerae*, and we observe this slow growth in our experiment (data not shown). Addition of MMS to a low c-di-GMP strain significantly reduces its growth rate (Fig. 43A). Although we do see a negative effect of increasing MMS in our high c-di-GMP strain, it is much less in comparison to the low c-di-GMP strain (Fig. 43B). There is a gradual decrease in growth as MMS is increased for both strains (data not shown). This suggests that the high c-di-GMP strain is better protected against the effect of MMS damage (Fig. 43B).

- **Overexpression of VC1672 protects against MMS induced damage**

As mentioned previously the expression of *tag* is known to be constitutive in *E. coli* (Lindahl, 1979, Lindahl et al., 1988). We see an increase in expression of *tag* at high levels of c-di-GMP. Also, we observe that high levels of c-di-GMP in *V. cholerae* lead to a protection against the MMS induced damage or cell death. We hypothesized that overexpression of VC1672 in both low and high c-di-GMP strains will lead to a protection against MMS damage. We used VC1672 overexpression vector under the control of an IPTG inducible promoter in our low and high c-di-GMP strains described earlier and performed an overnight growth curve assay at 0.1mM IPTG and 300μM MMS. Addition of MMS to both low and high c-di-GMP strains overexpressing VC1672 did not have an effect on their growth suggesting that increase in VC1672 expression leads to protection against MMS damage (Fig.

44). These results support our earlier hypothesis that c-di-GMP induces VC1672 expression which leads to an increase in the base excision repair of alkylated bases.

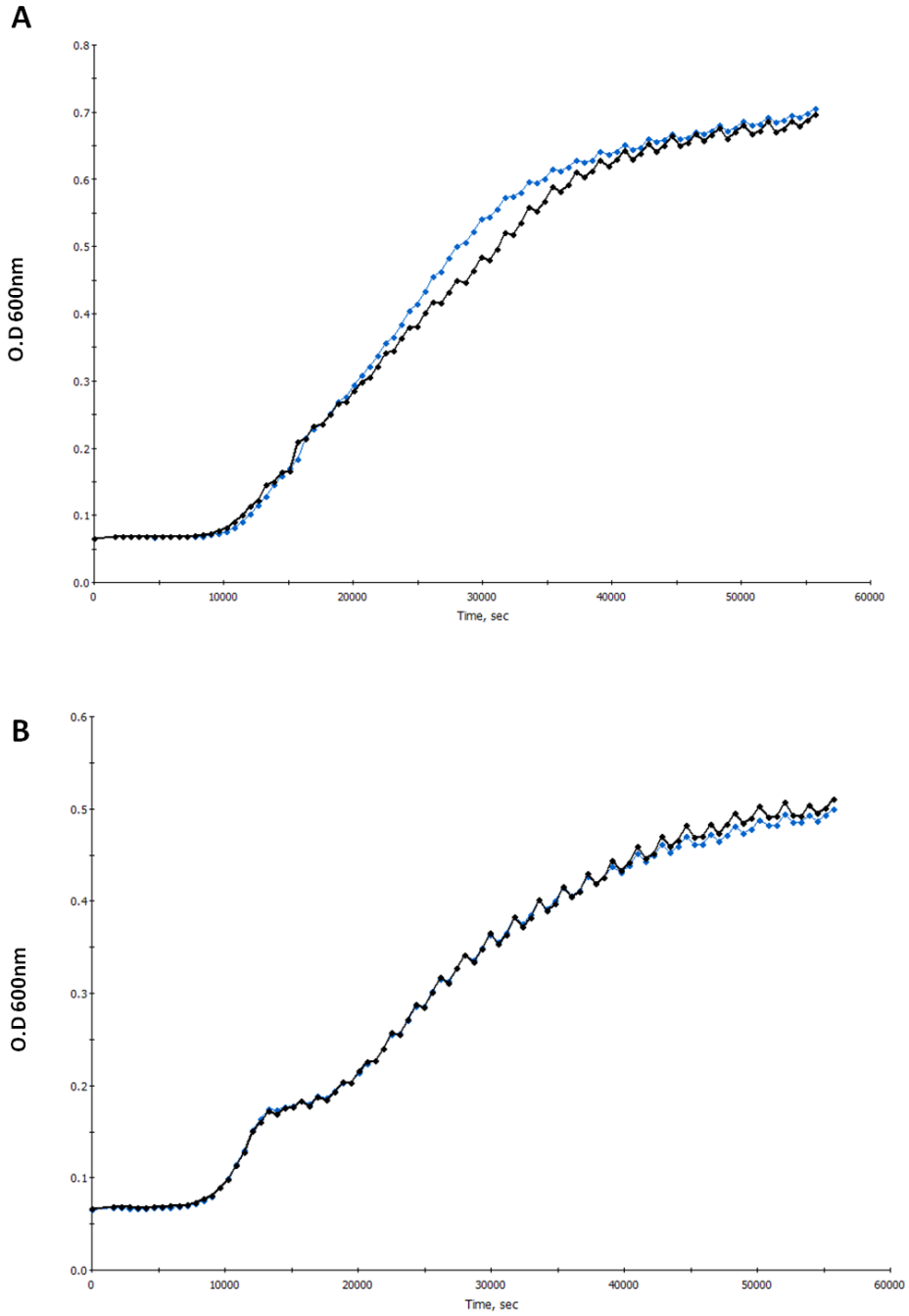


Figure 44: Overexpression of VC1672 (*tag*) alleviates effects of MMS on growth. VC1672 was overexpressed from an IPTG inducible plasmid in low **(A)** and high **(B)** c-di-GMP strains with (black) and without (blue) 300 μM MMS.

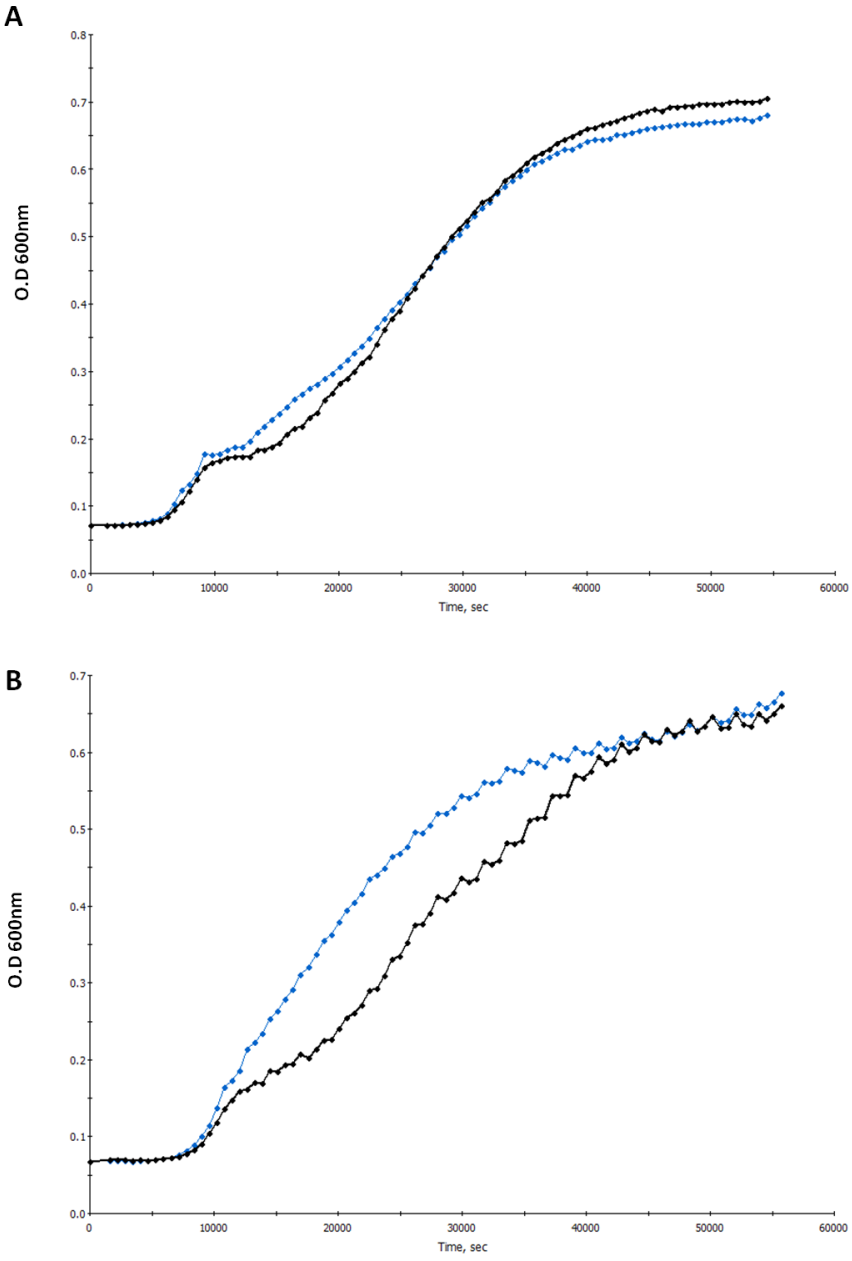


Figure 45: Effect of VC1672 deletion on MMS sensitivity. Deletion of VC1672 leads to loss of sensitivity to MMS. ΔVC1672 strain at low **(A)** and high **(B)** c-di-GMP background with (black) and without (blue) 300 μM MMS.

- **Deletion of VC1672 also protects against MMS induced damage**

To test if the effect of c-di-GMP on base excision pathway occurs solely due to VC1672 induction, we constructed a VC1672 deletion strain. We hypothesized that we would not observe protection against MMS at high c-di-GMP levels in this strain. We performed similar growth curve experiments described above at low and high levels of c-di-GMP in the ΔVC1672 strain without and with 300μM MMS. Surprisingly, we observed that our deletion strain was resistant to MMS irrespective of the c-di-GMP levels (Fig. 45). It is possible that a deletion in *tag* gene leads to induction of the other DNA glycosylase *alkA*, which is able to protect against MMS damage. We do not fully understand the expression patterns and importance of DNA repair genes in *V. cholerae* and more work needs to be done to address this result. Also, we can test the effect of c-di-GMP on growth in a double knockout strain Δ*alkA*Δ*tag* to understand this response further.

- **Screen for proteins important for VC1672 induction**

As previously mentioned, we have shown that 6:C9 and VC1672 expression is not regulated by any known c-di-GMP binding effectors (Fig. 41). To identify the factors involved in regulation of the 6:C9 promoter, we designed a genetic screen using a Tn5 transposon mutant library of *V. cholerae* using the pRL27c plasmid (De Lorenzo & Timmis, 1994). The expression of 6:C9-*lux* was measured in the mutant library at low versus high levels of c-di-GMP. We screened 1500 colonies containing independent Tn5 insertions, the 6:C9-lux vector, and pBRP2 (a GGDEF

overexpression vector) in the absence and presence of 0.1mM IPTG (giving low and high levels of c-di-GMP). From this screen, we obtained two isolates that were unresponsive to high levels of c-di-GMP. The expression of 6:C9 –*lux* in both isolates was high even at low levels of c-di-GMP and did not significantly change at high levels of c-di-GMP, this suggests the loss of a repressor that functions in a c-di-GMP dependent manner (Fig. 46). We hypothesize that in case of these mutants a repressor has been inactivated which can bind the promoter at low levels of c-di-GMP and inhibit expression but at high levels of c-di-GMP this repressor is inactivated leading to increased expression from promoter. These mutants are being characterized and analyzed further.

We performed another genetic screen using the 6:C9-3-*lux* construct. We chose this construct as it appears to regulate the VC1672 gene directly. The *V.*

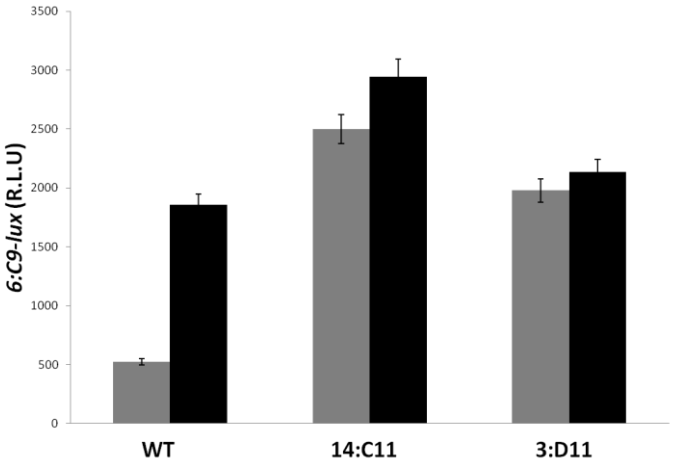


Figure 46: Screen for the 6:C9 regulator. Two mutants were isolated from the mutant screen. The expression of both isolates was high in comparison to WT and we did not observe a further increase in expression upon c-di-GMP induction. Gray bars represent low c-di-GMP and black bars represent high c-di-GMP. Error bars represent standard deviation.

cholerae strain used for this screen has a chromosomal insertion of the IPTG-inducible GGDEF overexpression cassette (BP55) (Benjamin R. Pursley, unpublished work). We created a Tn5 mutant library of this strain using plasmid pRL27c and tested the expression of 6:C9-3-*lux* in the presence and absence of 0.1mM IPTG. On screening 1000 colonies, we isolated one mutant that did not show induction in 6:C9-3 –*lux* expression (Fig. 47). Since this mutant shows low expression of 6:C9-3 –*lux* at both low and high levels of c-di-GMP, we hypothesize that this occurs due to a loss of an activator which can activate expression in response to c-di-GMP. We are currently analyzing this mutant further.

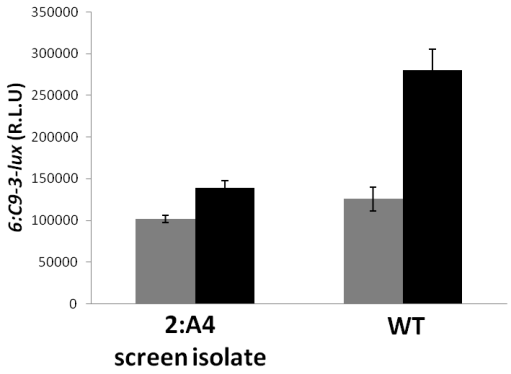


Figure 47: Screen for 6:C9-3 regulator. We isolated one mutant in which the expression of 6:C9-3 promoter was not inducible by c-di-GMP. Gray bars represent low c-di-GMP and black bars represent high c-di-GMP. Error bars represent standard deviation.

4.4 Discussion

C-di-GMP is known to regulate biofilms and motility; however, this second messenger is involved in regulation of a myriad of other bacterial processes (Fig. 1,

Chapter 1). Here we show that c-di-GMP can induce the expression of gene VC1672 also known as *tag*, which is involved in the base excision repair pathway. It is likely that c-di-GMP can mount an adaptive response through the induction of the *tag* gene in the presence of an alkylating agent. This is the first report of the role of c-di-GMP in DNA repair.

Methylating or alkylating agents can trigger the adaptive responses in *E. coli* and other bacteria by generating an intracellular signal. This signal is a DNA methylation product, a stereoisomer of a methyl phosphotriester (Teo et al., 1986). The Ada protein transfers this methyl group to one of its cysteine residues in a self-methylation event that converts the Ada protein from a weak to a strong transcriptional activator, thereby inducing *alkA, alkB* and its own expression (Teo et al., 1986).

We propose that c-di-GMP levels are modulated by sensing of the methylating agent by c-di-GMP regulatory enzymes, either a GGDEF or an EAL protein. The modulation of c-di-GMP levels is sensed by a specific effector protein which then induces *tag* expression to mount resistance to the alkylating agent. As mentioned previously, *tag* overexpression is able to suppress effects of an *alkA* mutation suggesting that Tag is able to perform efficiently without AlkA. We do not currently understand the role of AlkA and Ada proteins in base excision repair in *V. cholerae* and further analysis of these mutants similar to the ones performed with the *tag* gene will help us understand this pathway better. We see that deletion of

VC1672 (*tag*) does not eliminate protection against MMS induced damage. It is possible that deletion of one component of the base excision repair pathway is able to induce the adaptive response which leads to cells being resistant to MMS. Further work with a double knockout strain Δ*tag*Δ*alkA* will help us understand this response better.

We are currently trying to identify the effector employed by c-di-GMP to induce VC1672 expression using the screens described above. We have found three mutant isolates in the screens described above, two of these mutants suggest a deletion of a c-di-GMP dependent repressor (Fig. 46) and one suggests deletion of a c-di-GMP dependent activator (Fig.47). Analysis of the screen isolates by sequencing and further analyses of identified proteins by c-di-GMP binding assays will help us identify a novel c-di-GMP effector protein. We will also test the c-di-GMP mediated induction of other screen isolates (Table1; Chapter 1) in this mutant to assess if this effector is involved in regulation of other c-di-GMP regulated genes.

CHAPTER 5

Concluding remarks

5.1 Conclusions and Significance of this dissertation

Bacteria have the ability to exist and thrive in many environmental conditions. This ability is exemplified by the model organism *V. cholerae* studied in our lab. As mentioned previously, *V. cholerae* can transition from its environmental niche, the marine environment to its human host by making drastic alterations in its behavior. It exists in primarily a sessile biofilm-like state in the marine environment whereas it is required to be motile and free-living during infection of the human host. C-di-GMP, the second messenger studied in our lab is crucial for this transition. This work has characterized the role of c-di-GMP in the inverse regulation of biofilms and motility.

We have shown that transcriptional regulation is important for this sessile to motile transition (Fig. 48). Specifically, we have shown that c-di-GMP regulates the activity of two EBP family transcription factors, VpsR and FlrA, to regulate biofilm and motility genes respectively. This work is the first to show differential regulation of two transcription factors by c-di-GMP in the same organism. C-di-GMP acts as an activator for VpsR inducing the downstream genes in response to binding to VpsR. But it acts as an anti-activator for FlrA preventing it from activating downstream flagellar genes upon binding to FlrA. We were also able characterize the mechanism of action of FlrA by showing that c-di-GMP binding requires two arginine residues R135 and R176 in the REC and AAA+ domain. We show that the inverse regulation of biofilms and motility by c-di-GMP also involves the effect of increased

polysaccharide on motility directly. This could be due to a direct effect of VPS on movement of flagella as has been shown for *Salmonella* and *E. coli*.

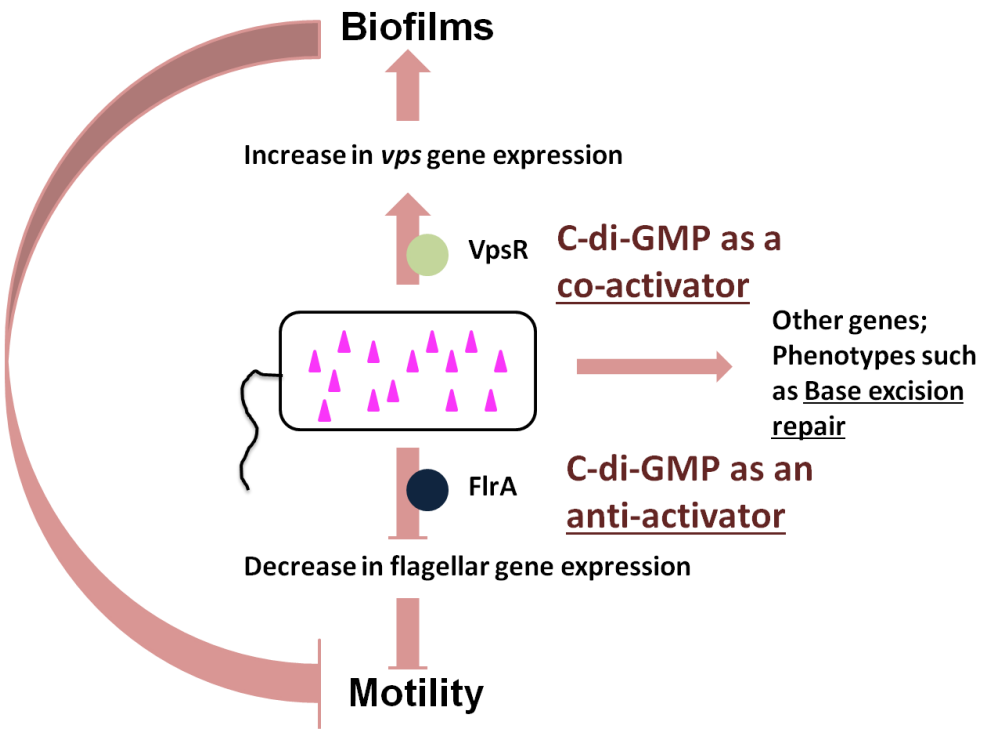


Figure 48: Model for c-di-GMP mediated inverse regulation of biofilms and motility.

C-di-GMP mediated regulation of phenotypes in bacteria is far from being understood. As mentioned previously, c-di-GMP regulates many phenotypes in bacteria, a list that is likely to grow in the near future (Fig.1). We have shown that c-di-GMP regulates the base excision repair pathway in *V. cholerae* (Chapter 4). Much work needs to be done to understand this response in greater detail. What regulators are involved in this process, how c-di-GMP is involved in sensing the

alkylation damage, and whether this occurs in other bacteria are only some of the questions that arise from this observation.

In this work, we have begun to elucidate the details of how biofilms and motility are regulated by c-di-GMP, but virtually nothing is known about other pathways of c-di-GMP transcriptional regulation. From our work, we have isolated 6 promoters that do not require any known c-di-GMP binding effectors in *V. cholerae*. This strongly suggests that c-di-GMP regulation employs unidentified machinery in *V. cholerae* to control these genes (Fig. 48).

Studying the currently known c-di-GMP binding proteins (such as transcription factors) will improve our understanding of how c-di-GMP controls protein activity. Here we have studied two c-di-GMP binding transcription factors VpsR and FlrA. Both VpsR and FlrA belong to the enhancer binding protein family. Identification of an EBP-specific c-di-GMP binding motif using these proteins will help us reveal other c-di-GMP binding EBP's in bacteria. We used the two arginine residues (R135 and R176) identified as being important for FlrA c-di-GMP binding to scan EBPs in *V. cholerae*. There are 12 EBP homologues in *V. cholerae* other than VpsR and FlrA. On performing Clustal W analysis, we see that R135 was conserved in two of those proteins and R176 was conserved in 4, however, we did not find another EBP in *V. cholerae* in which both residues were conserved (data not shown). VpsR contains Lysine in place of R176 and a methionine in place of R135. We changed the analogous lysine residue to alanine in VpsR but did not

observe a change in the c-di-GMP mediated effect on *vpsT* expression (data not shown). This suggests that c-di-GMP regulation of VpsR differs markedly from FlrA. We predict that a large scale bioinformatics search involving all EBPs (~5000 in bacteria) can help us uncover other FlrA like EBPs (Francke et al., 2011).

During our study of *vps* gene expression, we observed that the regulation of *vps* genes by c-di-GMP possibly involves signal amplification. Work from our lab and others has shown *vps* genes are highly induced by high levels of c-di-GMP by the activity of two c-di-GMP binding transcription factors VpsR and VpsT (Krasteva et al., 2010, Srivastava et al., 2011). We have shown that VpsR activates *vpsT* expression which then in combination with VpsR activates downstream *vps* genes to maximize the expression of VPS. We see an increase in expression of *vpsR* at high levels of c-di-GMP as well; however, it is much less in comparison to the induction we observe in *vpsT*, *vpsL* and VC0917 expression at high c-di-GMP levels (Fig. 49). *vpsL* and VC0917 promoters represent genes from *vpsI* and *vpsII* regions respectively. The baseline expression of the most downstream *vpsI* and *vpsII* genes is very low at low levels of c-di-GMP. As c-di-GMP levels increase, the expression of *vpsI* and *vpsII* increases by more than 40-fold. The expression of *vpsR* and *vpsT* is induced by 2-4 and 8-10 fold respectively. This observation makes it likely that the c-di-GMP signal is amplified by transcription factors VpsR and VpsT. This is also reflected by the dissociation constants of VpsR and VpsT. The levels of c-di-GMP vary from low μM amounts to up to 10 μM amounts (Massie et al., 2012). The dissociation constant for VpsR is around 1.6 μM, almost half that of VpsT (3.2 μM)

(Srivastava et al., 2011, Krasteva et al., 2010). Although these constants were calculated using different methods, they may reflect the real differences in c-di-GMP sensing. We believe that as c-di-GMP levels increase in *V. cholerae,* these gradual increases in c-di-GMP are sensed by the biofilm gene cascade. The base line expression level of *vpsR* is higher than other *vps* genes, including *vpsT,* and possibly low levels of c-di-GMP are able to induce *vpsR* expression. We do not yet understand the regulation of *vpsR* expression by c-di-GMP. The modest increases in c-di-GMP are sensed by VpsR which in turn induces expression of *vpsT*. VpsT then senses a further increase in c-di-GMP. We observe a very high induction in *vps* genes due to this additive effect of VpsR and VpsT bound to c-di-GMP. This observation gives us insight into the regulation by c-di-GMP; as levels of c-di-GMP increase, the cells want to maximize the expression of VPS and build a biofilm. The amplification of signal gives the cells an advantage to achieve this biofilm state at a faster rate.

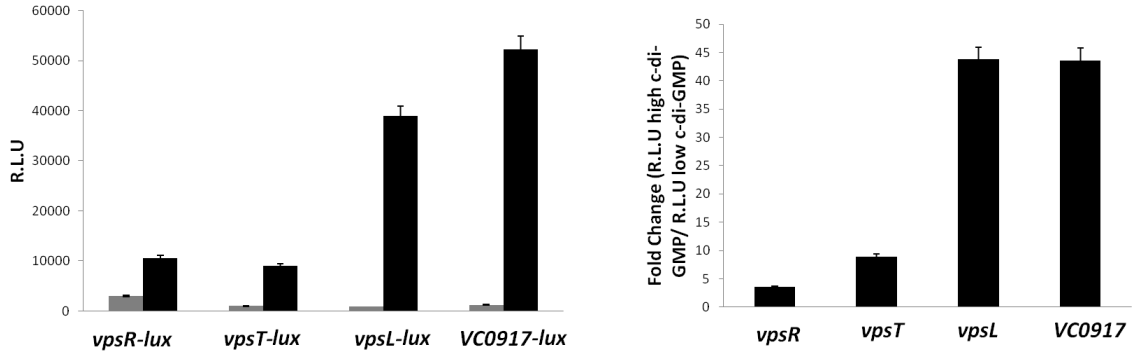


Figure 49: *vps* gene regulation by c-di-GMP involves signal amplification. Expression of *vpsR*, *vpsT* and *vpsI* and *vpsII* genes in response to low and high levels of c-di-GMP (right panel). Gray bars represent low c-di-GMP and black bars represent high c-di-GMP. Error bars represent standard deviation. Left panel displays the same data in form of fold change in gene expression at high versus low levels of c-di-GMP of *vps* genes. Error bars represent standard deviation.

Bacterial signaling is a composed of complex network of enzymes and other factors that sense and respond to the environment, and c-di-GMP signaling is no exception. The levels of c-di-GMP are regulated by environmental factors which in turn effect phenotypic changes. How these multiple networks integrate to produce the desired changes in responses is a fundamental question. Understanding regulation of c-di-GMP regulatory enzymes, identification of new c-di-GMP effectors, and characterizing the mechanism of action of these effectors will help us piece this critical puzzle of c-di-GMP signaling together in the future.

5.2 Future perspectives

Studies discussed in this dissertation shed light on the role of c-di-GMP in transcriptional regulation of biofilm and flagellar genes for regulation of sessile and motile lifestyles respectively. Greater insights into c-di-GMP regulation can be achieved by the following studies described below.

1. Mechanism of action of VpsR

To further understand how VpsR functions in response to c-di-GMP and activates *vpsT* and downstream *vps* gene expression, it is essential to characterize the mechanism of action of VpsR.

- We have shown that VpsR binds to *vpsT* and *aphA* promoters and is required for c-di-GMP mediated induction of these genes. We know from Electrophoretic mobility shift assay and DNAse I footprinting experiments that c-di-GMP does not affect the binding of VpsR to promoter DNA. How c-di-GMP affects VpsR structure is crucial in understanding the activation mechanism of VpsR. **Structural determination** of VpsR with and without c-di-GMP will aid in our understanding the functionality of this class of EBPs. Currently no structure exists for any EBP that contains all three conserved domains. Most structures are truncations of either the REC or the HTH DNA-binding domain. Thus, structural analysis of full length VpsR will be instrumental in understanding the function of VpsR and other VpsR-like EBPs.
- We will also focus on studying the effect of c-di-GMP on oligomerization of VpsR using **Gel Filtration analysis**. We know from DNAseI footprinting and EMSA analysis that VpsR binds to a specific DNA binding site depicted in Fig. 10B. Addition of c-di-GMP to these experiments does not generate any more or less DNAse I sensitive areas, suggesting the VpsR binding site is unchanged

(unpublished data, Meng-Lun Hsieh). However, it is possible that the oligomerization of VpsR occurs without affecting DNA binding.

- VpsR contains a REC domain which has a conserved Aspartate (D59) for phosphorylation. The significance of this phosphorylation is poorly understood. It is not currently known which kinase phosphorylates VpsR. We know that a mutant that mimics constitutive phosphorylation (D59E) of VpsR has higher expression of *vpsT*, but is still inducible by c-di-GMP (unpublished work, Christopher M. Waters). Also, the VpsR D59A mutant has lower expression of *vpsT* but the induction by c-di-GMP is maintained (unpublished work, Christopher M. Waters). It can be concluded that regulation via phosphorylation is independent of c-di-GMP. However, further insight into what proteins are involved in phosphorylation of VpsR will be essential in understanding this regulation. I have shown in this work that a truncation in the REC domain leads to inactivation of VpsR (Fig. 19). Also, in our constitutive mutant screen, most of the residues we found mutated were in the REC domain (Fig. 21). It will be very interesting to study the **c-di-GMP binding ability** of these REC truncated mutants and constitutive mutants using our previously described c-di-GMP binding assay. Furthermore, we can study VpsR function by characterizing the REC truncated mutants and constitutive mutants using EMSA and DNAseI footprinting analysis. This will give us insight into how c-di-GMP affects VpsR conformation on the *vpsT* promoter.

- We can further this analysis by using WT VpsR and constitutive VpsR mutants in **KMnO4 footprinting analysis** using RNAP and promoter complex. This will give us insight into open complex formation by VpsR and RNAP. Furthermore, we can assess the ability of WT VpsR and the constitutive mutants using ***in-vitro* transcription analysis**. Here, we can test the ability of VpsR to initiate transcription in the presence of minimal transcriptional machinery. It will clarify whether other proteins are not needed for this activation, and we can test the effect c-di-GMP on VpsR directly.

- VpsR binds far upstream of the start site of *vpsT* promoter. The relevance of the region between promoter and VpsR binding site in not fully understood. We have shown that deletions and insertions in this region (in 5 and 10 bp increments) affect expression of *vpsT* but the c-di-GMP induction is maintained. Even the deletion of 53 base pairs (entire region between the VpsR binding site and -35 site) does not affect c-di-GMP mediated induction. To further understand VpsR mediated *vpsT* activation, we can use these promoter constructs (described in Fig. 10B) in the **in-vitro transcription experiments.** Also, we will be studying *vpsT* induction in *E. coli* strains containing mutations in nucleoid proteins (IHF, HU, HNS) that are utilized by some transcription factors that require DNA looping as a part of their mechanism of transcription initiation.

- The role of the AAA+ domain in VpsR function is currently unknown. As mentioned previously, VpsR is a non-canonical EBP as it has a mutation in the Walker B motif (DE to ND) that should make its ATPase activity deficient. To understand what role the AAA+ domain plays in VpsR function, we made mutations in the critical residues of the Walker A and Walker B motifs. Mutations in both the Walker A and Walker B domain inactivated VpsR. We have shown that these proteins bind c-di-GMP and also bind the *vpsT* promoter (unpublished data; Disha Srivastava and Meng-Lun Hsieh). It is important to analyze what role the AAA+ domain plays in VpsR activity. We can test this by performing **ATP binding assays** and **ATPase assays** on VpsR. It is possible that VpsR requires ATP binding for function or is able to hydrolyze ATP in spite of having a mutation in its Walker B motif. Furthermore, it has been demonstrated for some EBPs that they require ATP binding to oligomerize but are unable to hydrolyze it.

2. **Further insight into the mechanism of action of FlrA**

We have shown that FlrA binds to c-di-GMP and this binding abrogates FlrA interaction with DNA. We have characterized that the interaction of c-di-GMP with FlrA requires two arginine residues (R135 and R176).

- Further studies such as **structural characterization** of FlrA in c-di-GMP bound and unbound forms will be instrumental in understanding how c-di-GMP changes FlrA conformation upon binding. As mentioned previously, no full

length structure for EBP has been characterized previously, and thus this structure will be the first of its kind to help better understand EBP function.

- FlrA binds to the *flrBC* promoter at an inverted repeat sequence ATTG(A/G)C. Most EBPs interact with DNA as dimers. We can deduce from our active site that this is likely the case for FlrA. However, we do not know if FlrA oligomerizes to activate *flrBC* expression and if c-di-GMP affects this process. Gel filtration analysis of FlrA with and without c-di-GMP will help understand the role of c-di-GMP in oligomerization of FlrA.
- Receiver domains of FlrA like EBPs do not contain a conserved residue for phosphorylation. How these proteins are regulated and if REC domains are involved in this regulation are not clearly understood. We have seen that the REC domain of FlrA is important for its function by analyzing REC truncations of FlrA. Also, we see that the REC domain of FlrA has a negative effect on c-di-GMP binding. In order to fully understand the mechanism of FlrA action, we need to explore the function of the REC domains in more detail. This can be achieved by studying the REC truncation mutants by gel filtration and DNAse I footprinting.

3. Regulation of base excision repair by c-di-GMP

We have shown that high levels of c-di-GMP protect against the damaging effects of alkylating agent MMS. This effect occurs by the induction of VC1672 expression, a DNA glycosylase involved in base excision repair. Overexpresison of

VC1672 also protects against MMS induced damage and cell death. This effect can be studied further by characterizing the pathway of VC1672 induction by c-di-GMP.

- From our genetic screen to identify VC1672 regulator, we have isolated three mutants that show no induction of VC1672 expression at high levels of c-di-GMP. These mutants will be studied further to characterize the factors involved. The factor once identified will be tested for promoter binding using EMSA and c-di-GMP binding. This work will potentially help us in identification of another c-di-GMP binding transcription factor.
- We can also test if c-di-GMP plays a role in regulation of other mutagenesis pathways such as deamination induced damage repair, oxygen damage repair, and UV-photoreactivation repair pathways.
- Expression analysis of other base excision repair genes by c-di-GMP will be done to test if the pathway is controlled at multiple levels by c-di-GMP. Also, MMS-induced damage will be analyzed in the Δ*alkA*Δ*tag* strain to test the mechanism of MMS resistance we observe in Δ*tag* strain.
- Furthermore, it will be interesting to test if this control of base excision repair pathway by c-di-GMP is conserved in other bacteria such as *E.coli* where the adaptive response is well-characterized.

4. Differential expression of genes in a population

Biofilms and motility are two very distinct states for *V. cholerae*. We and others have shown that these phenotypes are inversely regulated at the level of gene expression. However, it is possible that within a biofilm state, differential populations of *V. cholerae* cells exist wherein most cells are capable of forming *vps* but some have the ability to be motile. This idea comes from the observation that most bacterial cells show differential gene expression in a population (Elowitz *et al.*, 2002, Rosenfeld *et al.*, 2005). Also, the levels of c-di-GMP can vary within a population. Cells that have low c-di-GMP and high flagellar gene expression are poised to undergo dispersion from a biofilm which is essential for the bacteria to find new food sources and reach the human host.

- To test this idea, a dual reporter vector harboring *vpsT-gfp* and *flrBC-mcherry* (an *rfp* variant) can be constructed and tested at varying levels of c-di-GMP by microscopy and FACS. This will aid in dissecting the expression pattern at multiple levels. One, it will assess what c-di-GMP level is essential for regulation of these genes at the basal level, a possible mechanism by which these genes are regulated in response to c-di-GMP. Second, it will address the issue of how biofilm and motility expression control might be segregated. If the expression of reporters is heterogeneous, this can hint at possible division of labor between cells that undergo biofilm pathway and motility pathway. Thirdly, this reporter vector can be analyzed in the biofilm-like lifestyle followed by confocal

microscopy to visualize cells that express one or the other promoter. The construct can also be used to dissect control of expression at the levels of what specific GGDEFs are important for biofilm and motility control by analyzing the expression of *vpsT*-*gfp* and *flrBC*-m-cherry simultaneously in various GGDEF overexpression strains of *V. cholerae* El Tor C6706.

- Both microscopy and FACS (Fluorescence assisted cell sorting) can be used to identify and sort these differential populations. Microscopy is a more sensitive technique and will give a clear picture of expression differences between fluorescent reporters that can be quantified using software such as CellC, which can enumerate the cells based on intensity of fluorescence. With FACS, the differential populations can be sorted and analyzed further by transcriptome analysis and proteomics.

APPENDIX

Table 3: List of bacterial strains used in the work		
V. cholerae strains		
Strain	**Description**	**Reference**
C6706	ElTorSm^R	(Waters et al., 2008)
CW2034	Δ*vpsL*	(Waters et al., 2008)
CW2036	Δ*vpsL*, Δ*hapR*	(Waters et al., 2008)
WN310	Δ*vpsL*, Δ*vpsR*	this work
DS01	Δ*vpsL*, Δ*lrp*	this work
1195	Δ*vpsL*, Δ*vpsT*	this work
DS08	Δ*vpsL*, Δ*alsS*	this work
DS09	Δ*vpsL*, Δ*aphA*	this work
DS12	Δ*flrA* Δ*vpsL*	this work
DS13	Δ*flrA*	this work
JP1195	Δ*vpsT*Δ*vpsL*	
WN310	Δ*vpsR*Δ*vpsL*	
BP27	Δ*vc1*Δ*vc2* Δ*vpsL*	Benjamin R. Pursley (unpublished)
DS14	Δ*VC1672*Δ*vpsL*	this work
BP55	Chromosomal insertion of *qrgB* (GGDEF) under control of an IPTG inducible promoter in Δ*vpsL*	Benjamin R. Pursley (unpublished)

Table 3 (Cont'd)

***E. coli* strains**		
DH10B		Invitrogen
S17λpir-		(De Lorenzo & Timmis, 1994)
BW29427	pir+, requires DAP 300μg/ml	(Reddy, September 2007)
ER2566	Strain for protein purification	NEB (IMPACT)

Table 4: List of plasmids used in the work

Plasmid name	Description	Forward primer	Reverse primer	Reference
pCMW75	qrgB in overexpression vector under pTac control			(Waters et al., 2008)
pEVS141	Vector backbone for pCMW75 and pCMW98			(Hammer & Bassler, 2003)
pCMW98	Active site mutant of qrgB in pEVS141			(Waters et al., 2008)
pDL1711	-431 to '+68 of *vpsT* promoter cloned in pBBRlux	ATAactagtCGCTTGATTAAACGTTTGTC	ATAggatccTTCACCCCTCCTAACACATCA	(Miller et al., 2002)
pCMW110	-147 to '+68 of *vpsT* promoter cloned in pBBRlux	ATAactagtCTTTTTATTAAGCAACTTGG '-364	ATAggatccTTCACCCCTCCTAACACATCA	this work
pCMW128	-101 to '+68 of *vpsT* promoter cloned in pBBRlux	ATAactagtCAGCTATTGATATTCTTAAT '-410	ATAggatccTTCACCCCTCCTAACACATCA	this work
pCMW129	-71 to '+68 of *vpsT* promoter cloned in pBBRlux	ATA act agt GCT CAA TTA CAG CAA GAC	ATAggatccTTCACCCCTCCTAACACATCA	this work

Table 4 (cont'd)				
pDS5	-396 to '+204 of *aphA* promoter cloned in pBBRlux	ATA act agt GCT CAA TTA CAG CAA GAC	ATA gga tcc GAC ATG TCT TCA ATC CA	this work
pDS6	-156 to '+204 of *aphA* promoter cloned in pBBRlux	GGT act agt AAC AAA TCG CTA AAT GTC	ATA gga tcc GAC ATG TCT TCA ATC CA	this work
pDS7	-106 to '+204 of *aphA* promoter cloned in pBBRlux	GGT act agt CAA CTT TGT GGC CTT TTG	ATA gga tcc GAC ATG TCT TCA ATC CA	this work
pDS8	-51 to '+204 of *aphA* promoter cloned in pBBRlux	ATA act agt CTA ATC AGC ATA TTT GTA	ATA gga tcc GAC ATG TCT TCA ATC CA	this work
pRH1	*tcpA* promoter in pBBRlux	ATAactagtGTGACTGA AAGTCATCTCTTC	ATAggatccCGTGT TCTTCTTTTACAA AC	this work
pRH2	*ctxA* promoter in pBBRlux	ATAactagtCTCCTGCG TCTTTTGGTTTT	ATAggatccCATAT AATGCTCCCTTT GTTT	this work
pRH3	*tcpP* promoter in pBBRlux	ATAactagtAAGGCAGT AAAAGCCAACGTAAT GA	ATAggatccCCCCA TTACTTTACATTT TCTT	this work
pRH4	*toxT* promoter in pBBRlux	ATAactagtAGTAAGCA CGGGTATACCAA	ATAggatccCGTTC TACTCTGAAGAT ATATA	this work
1:B8	fragment cloned in pBBRlux identified in screen			this work
1:F6	fragment cloned in pBBRlux identified in screen			this work

Table 4 (cont'd)				
2:G12	fragment cloned in pBBRlux identified in screen			this work
4:H4	fragment cloned in pBBRlux identified in screen			this work
5:A6	fragment cloned in pBBRlux identified in screen			this work
6:C9	fragment cloned in pBBRlux identified in screen			this work
9:C11	fragment cloned in pBBRlux identified in screen			this work
pDS22	YcgR-C terminal intein tagged in pTXB1	ATAcatatgAGTCATTA CCATGAG	ATAgctcttcGTCGC GCACTTTGTC	this work
pZD46	*aphA* KO construct in pKAS32			
pDS33	Overexpression construct of *aphA* pEVS143	GGT gaa ttc ATG TCA TTA CCA CAC G	GGT gga tcc TTA TGC CAT CGC GTT CA	this work
pCMW159	*tetA* cloned in between lrp upstream and downstream sequences in pKAS32	AACTCAACCCTCATA TTGAG	TGCGAGATCTCG CTCACTAG	this work
pDS15	Overexpression construct of lrp in pEVS143	GGTgaattcATGGTGGA TAGTTAT	GGTggatccGTGAT TAAAACTCGC	this work

Table 4 (cont'd)				
pEVS143	overexpression vector with pTac promoter			(Hammer & Bassler, 2003)
pKAS32	Suicide vector for mutant construction			(Skorupski & Taylor, 1996)
pBBRlux	*luxABCDE* containing promoter less plasmid luxABCDE containing promoter less plasmid			(Hammer & Bassler, 2003)
pCMW135	VpsR C- terminal intein tag	GGTcatatgAGCACTCAATTCCGT	GGTgctcttcGTTTTCATCGGTGAT	this work
EMSA primers		CMW234	CMW235	(Waters et al., 2008)
		/56-FAM/ATTTTGCGGCCGCAACTAGA	/56-FAM/CCGCGGTGGCGGCCGCTCTA	
Δ*alsS* primers		CMW621 alsS upstream FP, CMW623 *alsS* ds FP, 422 pKD3 cat FP	CMW622alsS upstream RP, CMW624 *alsS* ds RP, 423 pKD3 cat RP	this work
		ATGGCACCTTTCCGAGTG, TAAGGAGGATATTCATATGGAGGTTTAACCCATAAAG, GTGTAGGCTGGAGCTGCTTC	GAAGCAGCTCCAGCCTACACAAGTGATCCTTAGTTACCT, CAGTGCTAGAGCGCGAGT, CATATGAATATCCTCCTTA	
pAR3	Arabinose inducible overexpression vector			Bagdasarian M.
pDS125	VpsR overexpression cloned in pAR3	GGTccatggAGGAGCTAAGGAAGCTAAAATGAGCACTCAATTCCGT	GGTaagcttTTAGAAGTTTTCATCGGT	this work

Table 4 (cont'd)

pDS130	VpsR-6XHIS cloned in pAR3	GGTccatggAGGAGCTAAGGAAGCTAAAATGAGCACTCAATTCCGT	AAG CTT TTA GTG GTG GTG GTG GTG GTG GAA GTT TTC ATC GGT	this work
pBRP1	*qrgB** mutant overexpression in IPTG inducible vector; Ampicillin selection			Benjamin R. Pursley
pBRP2	*qrgB* overexpression in IPTG inducible vector; Ampicillin selection			Benjamin R. Pursley
pDS126	VpsRΔREC (126-443) in pAR3	GGTccatggAGGAGCTAAGGAAGCTAAAATGCTGAAGTTGGAA	GGTaagcttTTAGAAGTTTTCATCGGT	this work
pDS127	VpsRΔREC (143-443) in pAR3	GGTccatggAGGAGCTAAGGAAGCTAAAATGGGCTTGATTGG	GGTaagcttTTAGAAGTTTTCATCGGT	this work
pDS134	VpsR (G179A/K180A; Walker A) in pAR3	GGTccatggAGGAGCTAAGGAAGCTAAAATGAGCACTCAATTCCGT	GGTaagcttTTAGAAGTTTTCATCGGT	this work
pDS135	VpsR (T236A/L237A; Walker B) in pAR3	GGTccatggAGGAGCTAAGGAAGCTAAAATGAGCACTCAATTCCGT	GGTaagcttTTAGAAGTTTTCATCGGT	this work
2:G3	VpsR mutant isolate of pDS125			this work
4:B1	VpsR mutant isolate of pDS125			this work
5:B7	VpsR mutant isolate of pDS125			this work
6:C1	VpsR mutant isolate of pDS125			this work
6:F5	VpsR mutant isolate of pDS125			this work

Table 4 (cont'd)				
9:B11	VpsR mutant isolate of pDS125			this work
pDS146	VpsR (M151K) in pAR3	Mutagenesis primer: CCGGCAACATGGGCTTGATTGGGGAATCTAAGCCTATGAAGCGTTTGCGCGATCAGATCAA		this work
pDS160	VpsR (V27E) in pAR3	Mutagenesis primer: TGGGAGGTACCTATGAACCCTGGCTGGCTGAGTTGGAAAAAGTGGGTTGGCGCTGTACT		this work
pDS161	VpsR (V49D) in pAR3	Mutagenesis primer: CAGATTTGCGCAAAGCCGATGCGTTATTTGACGAGACTGGGCCATGTATTGGTATTGTGG		this work
pDS137	10bp insertion in *vpsT* promoter in pBBRlux	FP BamHI: ATAactagtCGCTTGATTAAACGTTTGTC FP 10bp insertion: ACTGATACATATTCCGCTTGATTAA	RP SpeI: ATAggatccTTCACCCCTCCTAACACATCA RP 10bp insertion: ATGTATCAGTCTGAAATTTAATCTG	this work
pDS153	5bp insertion in *vpsT* promoter in pBBRlux	FP BamHI: ATAactagtCGCTTGATTAAACGTTTGTC FP 5bp insertion: ATTTCAGACTGAATTCCGCTTGATT	RP SpeI: ATAggatccTTCACCCCTCCTAACACATCA RP 5 bp insertion: TCAGTCTGAAATTTAATCTGTACAA	this work

Table 4 (cont'd)

pDS154	15bp insertion in *vpsT* promoter in pBBRlux	FP BamHI: ATAactagtCGCTTGATTAAACGTTTGTC FP 15bp insertion: GACAGATTCCGCTTGATT	RP SpeI: ATAggatccTTCACCCCTCCTAACACATCA RP 15bp insertion: CTGTCATGTATCAGTCTG	this work
pDS162	5bp deletion in *vpsT* promoter in pBBRlux	FP BamHI: ATAactagtCGCTTGATTAAACGTTTGTC FP 5bp deletion: TTGTACAGATTAAATATTCCGCTTGATTAA	RP SpeI: ATAggatccTTCACCCCTCCTAACACATCA RP 5bp deletion: TTAATCAAGCGGAATATTTAATCTGTACAA	this work
pDS163	10bp deletion in *vpsT* promoter in pBBRlux	FP BamHI: ATAactagtCGCTTG ATTAAACGTTTGTC FP 10bp deletion: AATTGTACAGATTATCCGCTTGATTAAA	RP SpeI: ATAggatccTTCACCCCTCCTAACACATCA RP 10bp deletion: TTTAATCAAGCGGATAATCTGTACAATT	this work
pDS164	15bp deletion in *vpsT* promoter in pBBRlux	FP BamHI: ATAactagtCGCTTGATTAAACGTTTGTC FP 15bp deletion: TGAATTGTACAGATGCTTGATTAAACGT	RP SpeI: ATAggatccTTCACCCCTCCTAACACATCA RP 15bp deletion: ACGTTTAATCAAGCATCTGTACAATTCA	this work

Table 4 (cont'd)

pDS165	53bp deletion in *vpsT* promoter in pBBRlux	FP BamHI: ATAactagtCGCTTGATTAAACGTTTGTC FP 53bp deletion: AGTCAATGTTCAGAAAAAGGTTAAGAAACCTTC	RP SpeI: ATAggatccTTCACCCCTCCTAACACATCA RP 53bp deletion: GAAGGTTTCTTAACCTTTTTCTGAACATTGACT	this work
pBH625	*vpsL* promoter driving *lux* operon in pBBRlux vector			(Hammer & Bassler, 2009)
pDL1723	*vpsR* promoter driving *lux* operon in pBBRlux vector			
pKAS32	Suicide plasmid for generation of mutants			
pBBRlux	*lux* operon containing reporter plasmid			(Hammer & Bassler, 2007)
pBRP1	*qrgB** (inactive GGDEF mutant) IPTG inducible overexpression vector			Benjamin R. Pursley(unpublished)
pBRP2	*qrgB* (active GGDEF) IPTG inducible overexpression vector			Benjamin R. Pursley(unpublished)
pDS1	FlrA overexpression construct in pTXB1	ATA cat atg CAG AGT TTT AGC GAA ACT	gcg ttg CAT GTT GTA TTT GC	NEB IMPACT system ™ this work

Table 4 (cont'd)

pDS54	pKAS32 derivative for *flrA* mutant construction	FP up: ATA gca tgc CAG TTA AAA ACG GCG GCG AT FP ds: ATA tct aga TAG GGA AAC CAT AGT CAA TA	RP up: ATA tct aga AGG TGA GAT TAT TTG CCT TT RP ds: ATA gtc gac TCG TGC AGT CGG TCA ACC AA	this work
pLLP15	*flrA* overexpression cloned in pEVS143	GGTaggcctAGGAGCTA AGGAAGCTAAAATGC AGAGTTTAGCGAAAC	GGTggatccGCGTT GCATGTTGTATTT GCG	Lauren Priniski
pDS49	*flrBC* promoter in pBBRlux	GGtactagTCGCAAATA CAAC	GGTggatccGAATG CTGCTCTT	this work
EMSA and DNaseI footprinting primers		**CMW234** /56-FAM/ATTTTGCGGCCG **CMW1834** CCGCGGTGGCGGCCG	**CMW235** GGCAACTGA FAM/CCGCGGTGGC CCGCTCTA CTCTA	(Srivastava et al., 2011)
pDS72	*flaA* promoter in pBBRlux	GGTactagtAATGGGTG TCAA	GGTggatccACACG TTGGTAT	this work

Table 4 (cont'd)

pDS73	*flgB* promoter in pBBRlux	GGTactagtGTTGTAACCCTG	GGTggatccAGGGCTCTGTCA	this work
pDS74	*flgM* promoter in pBBRlux	GGTactagtCAAGTCAGTGGT	GGTggatccTTAACGACTGTC	this work
pDS82	G168A/T169A/G170A mutation on pLLP15	Mutagenesis primer: GGAAGCCAACGTGCTGATCCTCGGTGAGTCGGCCGCGGCTAAAGAAGTGGTTGCGCGTAACATTCACT		this work
pDS83	K171A/E172A/V173A mutation on pLLP15	Mutagenesis primer: GTGCTGATCCTCGGTGAGTCGGGCACGGGTGCAGCAGCGGTTGCGCGTAACATTCACTACCATTCAGG		this work
pDS84	V174A/R176A mutation on pLLP15	Mutagenesis primer: TCGGTGAGTCGGGCACGGGTAAAGAAGTGGCTGCGGCTAACATTCACTACCATTCAGGACGCCGTAA		this work
pDS88	G351A/N352A mutation on pLLP15	Mutagenesis primer: TCAATTCGATGATGGAGCATGACTGGCCGGCTGCTGTGCGTGAACTTGCCAACTTGGTTGAGCG		this work

Table 4 (cont'd)

pDS89	V353A/R354A mutation on pLLP15	Mutagenesis primer: CGATGATGGAGCATG ACTGGCCGGGTAATG CGGCTGAACTTGCCA ACTTGGTTGAGCGTA TGGT		this work
pDS90	G165A/E166A/S167A mutation on pLLP15	Mutagenesis primer: TACGCGCAACCACTT CTTTACCCGTGCCCG CCGCAGCGAGGATCA GCACGTTGGCTTCCG TGGTCGAG		this work
pDS91	L162A/I163A mutation on pLLP15	Mutagenesis primer: GAGCAAGTCTCGACC ACGGAAGCCAACGTG GCGGCCCTCGGTGA GTCGGGCACGGGTA AAGAAGT		this work
pDS92	W349A/P350A mutation on pLLP15	Mutagenesis primer: CGCGTGCGATCAATT CGATGATGGAGCATG CCTGGGCGGGTAATG TGCGTGAACTTGCCA ACTTGG		this work
pDS136	E355A/L356A	Mutagenesis primer: TGGAGCATGACTGGC CGGGTAATGTGCGTG GCCGCGCCAACTTGG TTGAGCGTATGGTCA TCCTG		this work

Table 4 (cont'd)

pDS93	R176A mutation on pLLP15	Mutagenesis primer: GAGTCGGGCACGGG TAAAGAAGTGGTTGC GGCTAACATTCACTA CCATTCAGGACGCCG TAAT		this work
pDS101	V174A mutation on pLLP15	Mutagenesis primer: CTCGGTGAGTCGGGC ACGGGTAAAGAAGTG GCTGCGCGTAACATT CACTACCATTCAGGA CGC		this work
pDS103	R176H mutation on pLLP15	Mutagenesis primer: GAGTCGGGCACGGG TAAAGAAGTGGTTGC GCATAACATTCACTA CCATTCAGGACGCCG TAA		this work
pDS104	R176K mutation on pLLP15	Mutagenesis primer: GAGTCGGGCACGGG TAAAGAAGTGGTTGC GAAAAACATTCACTA CCATTCAGGACGCCG TAAT		this work
pDS105	R176E mutation on pLLP15	Mutagenesis primer: GAGTCGGGCACGGG TAAAGAAGTGGTTGC GGAGAACATTCACTA CCATTCAGGACGCCG TAAT		this work

Table 4 (cont'd)

pDS110	R176H mutation in pDS1	Mutagenesis primer: GAGTCGGGCACGGG TAAAGAAGTGGTTGC GCATAACATTCACTA CCATTCAGGACGCCG TAA		this work
pDS123	R135H mutation in pLLP15	Mutagenesis primer: TTGCGACCGCACGCA AAAACACCCTGTTTC ATAGCCTTGTTGGGC AAAGCATGGGG		this work
pDS102	FlrA overexpression in pAR3	GGTccatggAGGAGCTA AGGAAGCTAAAATGC AGAGTTTAGCGAAAC	GGTaagcttGCGTT GCATGTTGTATTT GCG	this work
pDS116	FlrA (R176K) in pAR3	Mutagenesis primer: GAGTCGGGCACGGG TAAAGAAGTGGTTGC GAAAAACATTCACTA CCATTCAGGACGCCG TAAT		this work
pDS117	FlrAΔREC1 in pAR3	GGTccatggAGGAGCTA AGGAAGCTAAAATGC TGAAGTTGGAA	GGTaagcttGCGTT GCATGTTGTATTT GCG	this work
pDS118	FlrAΔREC2 in pAR3	GGTccatggAGGAGCTA AGGAAGCTAAAATGG GCTTGATTGG	GGTaagcttGCGTT GCATGTTGTATTT GCG	this work
pDS129	6:C9-3 promoter in pBBRlux	GGTactagtCTTTGTTT AAAACCATGC	GGTggatccGCTTA TCCTTCTTCATTC	this work
pALN17	6:C9-1 promoter in pBBRLux	GGTactagtCGGTGCCT ACAACATCCG	GGTggatccGGATC ATACCGAGTA	Amanda Ngouajio
pALN23	6:C9-2 promoter in pBBRLux	GGTactagtGATCTGTT CTTCCGT	GGTggatccGGGTC AGCGTACATCAC ATC	Amanda Ngouajio
pDS132	6:C9-4 promoter in pBBRlux	GGTactagtCATTGGGT TAT	GGTggatccGCTTA TCCTTCTTCATTC	this work

Table 4 (cont'd)

pDS138	pKAS32 VC1672 KO construct	VC1672upFPSacI: GGTgagctcTCGGTGAAGGAGTCGC VC1672dsFPcompup: ATGAAGAAGGATAAGCTGATTCACCAAGAGCT	VC1672upRPcompds: AGCTCTTGGTGAATCAGCTTATCCTTCTTCAT VC1672dsRPKpnI : ACCggtaccGCCAGCATGAACAAAA	this work
pDS139	VC1672 overexpression in pEVS143	GGTgaattcAGGAGCTAAGGAAGCTAAAATGATGAATGCGGAACA	ACCggatccTCAGAGCTTGTCTGC	this work

REFERENCES

REFERENCES

The PyMOL Molecular Graphics System. In. Schrödinger, LLC, pp.

Albert, M.J., (1994) Vibrio cholerae O139 Bengal. *J Clin Microbiol* **32**: 2345.

Amikam, D. & M.Y. Galperin, (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* **22**: 3-6.

Baraquet, C. & C.S. Harwood, (2013) Cyclic diguanosine monophosphate represses bacterial flagella synthesis by interacting with the Walker A motif of the enhancer-binding protein FleQ. *Proc Natl Acad Sci USA* **110**: 18478-18483.

Baraquet, C., K. Murakami, M.R. Parsek & C.S. Harwood, (2012) The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the *pel* operon promoter in response to c-di-GMP. *Nucleic Acids Res* **40**: 7207-7281.

Bardill, J.P., X. Zhao & B.K. Hammer, (2011) The *Vibrio cholerae* quorum sensing response is mediated by Hfq-dependent sRNA/mRNA base pairing interactions. *Mol Microbiol* **80**: 1381-1394.

Barrett, T.J. & P.A. Blake, (1981) Epidemiological usefulness of changes in hemolytic activity of *Vibrio cholerae* biotype El Tor during the seventh pandemic. *J Clin Microbiol* **13**: 126-129.

Barua, D., (1992) History of cholera. In: Cholera. Springer, pp. 1-36.

Benach, J., S.S. Swaminathan, R. Tamayo, S.K. Handelman, E. Folta-Stogniew, J.E. Ramos, F. Forouhar, H. Neely, J. Seetharaman & A. Camilli, (2007) The structural basis of cyclic diguanylate signal transduction by PilZ domains. *EMBO J* **26**: 5153-5166.

Benjaminson, M.A., A.J. Weil & B.C. Deguzman, (1964) Voges-Proskauer Test - Expeditious Techniques for Routine Use. *J Bacteriol* **87**: 234-&.

Bernier, S.P., D.G. Ha, W. Khan, J.H. Merritt & G.A. O'Toole, (2011) Modulation of *Pseudomonas aeruginosa* surface-associated group behavior by individual amino acids through c-di-GMP signaling. *Res. Microbiol.* **162**: 680-688.

Beyhan, S., K. Bilecen, S.R. Salama, C. Casper-Lindley & F.H. Yildiz, (2007) Regulation of rugosity and biofilm formation in *Vibrio cholerae*: comparison of VpsT and VpsR regulons and epistasis analysis of *vpsT*, *vpsR*, and *hapR*. *J Bacteriol* **189**: 388-402.

Beyhan, S., A.D. Tischler, A. Camilli & F.H. Yildiz, (2006) Transcriptome and phenotypic responses of *Vibrio cholerae* to increased cyclic di-GMP level. *J Bacteriol* **188**: 3600-3613.

Bina, X.R., D. Provenzano, N. Nguyen & J.E. Bina, (2008) *Vibrio cholerae* RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. *Infect. Immun.* **76**: 3595-3605.

Blokesch, M. & G.K. Schoolnik, (2007) Serogroup Conversion of *Vibrio cholerae* in Aquatic Reservoirs. *PLoS Pathog* **3**: e81.

Bobrov, A.G., O. Kirillina, D.A. Ryjenkov, C.M. Waters, P.A. Price, J.D. Fetherston, D. Mack, W.E. Goldman, M. Gomelsky & R.D. Perry, (2011) Systematic analysis of cyclic di-GMP signalling enzymes and their role in biofilm formation and virulence in *Yersinia pestis*. *Mol Microbiol* **79**: 533-551.

Boehm, A., M. Kaiser, H. Li, C. Spangler, C.A. Kasper, M. Ackermann, V. Kaever, V. Sourjik, V. Roth & U. Jenal, (2010) Second Messenger-Mediated Adjustment of Bacterial Swimming Velocity. *Cell* **141**: 107-116.

Bose, D., N. Joly, T. Pape, M. Rappas, J. Schumacher, M. Buck & X. Zhang, (2008) Dissecting the ATP hydrolysis pathway of bacterial enhancer-binding proteins. *Biochem Soc Trans* **36**: 83-88.

Boyd, C.D. & G.A. O'Toole, (2012) Second messenger regulation of biofilm formation: breakthroughs in understanding c-di-GMP effector systems. *Ann Rev Cell Dev Biol* **28**: 439-462.

Brinkman, A., T. Ettema, W. de Vos & J. van der Oost, (2003) The Lrp family of transcriptional regulators. *Mol Microbiol* **48**: 287-294.

Buck, M., M.T. Gallegos, D.J. Studholme, Y. Guo & J.D. Gralla, (2000) The bacterial enhancer-dependent sigma 54 (sigma N) transcription factor. *J Bacteriol* **182**: 4129.

Bush, M. & R. Dixon, (2012) The Role of Bacterial Enhancer Binding Proteins as Specialized Activators of σ54-Dependent Transcription. *Microbiol. Mol. Biol. Rev.* **76**: 497-529.

Calia, K.E., M. Murtagh, M.J. Ferraro & S.B. Calderwood, (1994) Comparison of *Vibrio cholerae* O139 with *V. cholerae* O1 classical and El Tor biotypes. *Infect. Immun.* **62**: 1504-1506.

Carlson, H.K., R.E. Vance & M.A. Marletta, (2010) H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Mol. Microbiol.* **77**: 930-942.

Casper-Lindley, C. & F.H. Yildiz, (2004) VpsT is a transcriptional regulator required for expression of *vps* biosynthesis genes and the development of rugose colonial morphology in *Vibrio cholerae* O1 El Tor. *J Bacteriol* **186**: 1574-1578.

Chambers, J.R., J. Liao, M.J. Schurr & K. Sauer, (2014) BrlR from *Pseudomonas aeruginosa* is a c-di-GMP-responsive transcription factor. *Mol Microbiol* **195**: 4678-4688.

Chen, B., T.A. Sysoeva, S. Chowdhury & B.T. Nixon, (2008) Regulation and action of the bacterial enhancer-binding protein AAA+ domains. *Biochem Soc Trans* **36**: 89.

Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B.L. Bassler & F.M. Hughson, (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549.

Chilcott, G.S. & K.T. Hughes, (2000) Coupling of Flagellar Gene Expression to Flagellar Assembly in *Salmonella enterica* Serovar Typhimurium and *Escherichia coli*. *Microbiol. Mol. Biol. Rev* **64**: 694-708.

Chin, K.H., Y.C. Lee, Z.L. Tu, C.H. Chen, Y.H. Tseng, J.M. Yang, R.P. Ryan, Y. McCarthy, J.M. Dow, A.H. Wang & S.H. Chou, (2010) The cAMP receptor-like protein CLP is a novel c-di-GMP receptor linking cell-cell signaling to virulence gene expression in *Xanthomonas campestris*. *J. Mol. Biol* **396**: 646-662.

Christen, B., M. Christen, R. Paul, F. Schmid, M. Folcher, P. Jenoe, M. Meuwly & U. Jenal, (2006) Allosteric control of cyclic di-GMP signaling. *J. Biol. Chem.* **281**: 32015-32024.

Clarke, N.D., M. Kvaal & E. Seeberg, (1984) Cloning of *Escherichia coli* genes encoding 3-methyladenine DNA glycosylases I and II. *Mol Gen Genet* **197**: 368-372.

Correa, N.E., C.M. Lauriano, R. McGee & K.E. Klose, (2000) Phosphorylation of the flagellar regulatory protein FlrC is necessary for *Vibrio cholerae* motility and enhanced colonization. *Mol Microbiol* **35**: 743-755.

Correa, N.E., F. Peng & K.E. Klose, (2005) Roles of the regulatory proteins FlhF and FlhG in the *Vibrio cholerae* flagellar transcription hierarchy. *J Bacteriol* **187**: 6324-6332.

Cotter, P.A. & S. Stibitz, (2007) c-di-GMP-mediated regulation of virulence and biofilm formation. *Curr. Opin. Microbiol.* **10**: 17-23.

Cullen, P.J., W.C. Bowman & R.G. Kranz, (1996) In vitro reconstitution and characterization of the *Rhodobacter capsulatus* NtrB and NtrC two-component system. *J. Biol. Chem.* **271**: 6530-6536.

Dasgupta, N. & R. Ramphal, (2001) Interaction of the Antiactivator FleN with the Transcriptional Activator FleQ Regulates Flagellar Number in *Pseudomonas aeruginosa*. *J Bacteriol* **183**: 6636-6644.

Datsenko, K.A. & B.L. Wanner, (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad Sci U S A* **97**: 6640.

De Lorenzo, V. & K. Timmis, (1994) Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5-and TnlO-derived minitransposons. *Methods Enzymol* **235**: 386-405.

De, N., M.V.A.S. Navarro, R.V. Raghavan & H. Sondermann, (2009) Determinants for the activation and autoinhibition of the diguanylate cyclase response regulator WspR. *J. Mol. Biol* **393**: 619-633.

Dischert, W., P.M. Vignais & A. Colbeau, (1999) The synthesis of *Rhodobacter capsulatus* HupSL hydrogenase is regulated by the two-component HupT/HupR system. *Mol Microbiol* **34**: 995-1006.

Duerig, A., S. Abel, M. Folcher, M. Nicollier, T. Schwede, N. Amiot, B. Giese & U. Jenal, (2009) Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. *Genes Dev.* **23**: 93-104.

Edmunds, A.C., L.F. Castiblanco, G.W. Sundin & C.M. Waters, (2013) Cyclic di-GMP Modulates the Disease Progression of *Erwinia amylovora*. *J Bacteriol* **195**: 2155-2165.

Elowitz, M.B., A.J. Levine, E.D. Siggia & P.S. Swain, (2002) Stochastic gene expression in a single cell. *Science* **297**: 1183-1186.

Eswar, N., B. John, N. Mirkovic, A. Fiser, V.A. Ilyin, U. Pieper, A.C. Stuart, M.A. Marti-Renom, M.S. Madhusudhan, B. Yerkovich & A. Sali, (2003) Tools for comparative protein structure modeling and analysis. *Nucleic Acids Res* **31**: 3375-3380.

Fang, X. & M. Gomelsky, (2010) A post translational c-di-GMP dependent mechanism regulating flagellar motility. *Mol Microbiol* **76**: 1295-1305.

Faruque, S.M., K. Biswas, S.M.N. Udden, Q.S. Ahmad, D.A. Sack, G.B. Nair & J.J. Mekalanos, (2006) Transmissibility of cholera: In vivo-formed biofilms and their relationship to infectivity and persistence in the environment. *Proc. Natl. Acad. Sci.* **103**: 6350-6355.

Fazli, M., A. O'Connell, M. Nilsson, K. Niehaus, J.M. Dow, M. Givskov, R.P. Ryan & T. Tolker-Nielsen, (2011) The CRP/FNR family protein Bcam1349 is a c-di-GMP effector that regulates biofilm formation in the respiratory pathogen *Burkholderia cenocepacia*. *Mol Microbiol* **82**: 327-341.

Ferreira, R.B., D.M. Chodur, L.C. Antunes, M.J. Trimble & L.L. McCarter, (2011) The Scr network in *Vibrio parahaemolyticus*: output targets and transcriptional regulation by a c-di-GMP responsive circuit. *J Bacteriol*.

Ferreira, R.B.R., L.C.M. Antunes, E.P. Greenberg & L.L. McCarter, (2008) *Vibrio parahaemolyticus* ScrC modulates cyclic dimeric GMP regulation of gene expression relevant to growth on surfaces. *J Bacteriol* **190**: 851-860.

Ferreira, R.B.R., D.M. Chodur, L.C.M. Antunes, M.J. Trimble & L.L. McCarter, (2012) Output targets and transcriptional regulation by a cyclic dimeric GMP-responsive circuit in the *Vibrio parahaemolyticus* Scr network. *J Bacteriol* **194**: 914-924.

Francke, C., T. Groot Kormelink, Y. Hagemeijer, L. Overmars, V. Sluijter, R. Moezelaar & R.J. Siezen, (2011) Comparative analyses imply that the enigmatic Sigma factor 54 is a central controller of the bacterial exterior. *BMC Genomics* **12**: 385.

Friedberg, E.C., Walker, G. C. and Suede, W. , (1995) *DNA Repair and Mutagenesis*. ASM Press, Washington DC.

Friedman, L. & R. Kolter, (2004) Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J Bacteriol* **186**: 4457-4465.

Galperin, M.Y., (2004) Bacterial signal transduction network in a genomic perspective. *Environ. Microbiol* **6**: 552-567.

Guttenplan, S.B. & D.B. Kearns, (2013) Regulation of flagellar motility during biofilm formation. *FEMS Microbiol Rev* **37**: 849-871.

Habazettl, J., M.G. Allan, U. Jenal & S. Grzesiek, (2011) Solution structure of the PilZ domain protein PA4608 complex with cyclic di-GMP identifies charge clustering as molecular readout. *J. Biol. Chem* **286**: 14304-14314.

Hammer, B.K. & B.L. Bassler, (2003) Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol. Microbiol.* **50**: 101-104.

Hammer, B.K. & B.L. Bassler, (2007) Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci USA* **104**: 11145-11149.

Hammer, B.K. & B.L. Bassler, (2009) Distinct sensory pathways in *Vibrio cholerae* El Tor and classical biotypes modulate cyclic dimeric GMP levels to control biofilm formation. *J Bacteriol* **191**: 169-177.

Häse, C. & J. Mekalanos, (1998) TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc. Natl Acad Sci U S A* **95**: 730.

He, Y.W. & L.H. Zhang, (2008) Quorum sensing and virulence regulation in *Xanthomonas campestris*. *FEMS Microbiol. Rev* **32**: 842-857.

Hengge, R., (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**: 263-273.

Herrington, D.A., R.H. Hall, G. Losonsky, J.J. Mekalanos, R. Taylor & M.M. Levine, (1988) Toxin, toxin-coregulated pili, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J Experimen Med* **168**: 1487-1492.

Hickman, J.W. & C.S. Harwood, (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* **69**: 376-389.

Higgins, D.A., M.E. Pomianek, C.M. Kraml, R.K. Taylor, M.F. Semmelhack & B.L. Bassler, (2007) The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* **450**: 883-886.

Hobley, L., R.K.Y. Fung, C. Lambert, M.A.T.S. Harris, J.M. Dabhi, S.S. King, S.M. Basford, K. Uchida, R. Till & R. Ahmad, (2012) Discrete Cyclic di-GMP-Dependent Control of Bacterial Predation versus Axenic Growth in *Bdellovibrio bacteriovorus*. *PLoS Pathog.* **8**: e1002493.

Huang, B., C.B. Whitchurch & J.S. Mattick, (2003) FimX, a multidomain protein connecting environmental signals to twitching motility in *Pseudomonas aeruginosa*. *J Bacteriol* **185**: 7068-7076.

Huq, A., E. Small, P. West, M. Huq, R. Rahman & R. Colwell, (1983) Ecological relationships between Vibrio cholerae and planktonic crustacean copepods. *App Environ Microbiol* **45**: 275.

Jenal, U. & J. Malone, (2006) Mechanisms of Cyclic-di-GMP Signaling in Bacteria. *Annu. Rev. Genet.* **40**: 385-407.

Joelsson, A., Z. Liu & J. Zhu, (2006) Genetic and phenotypic diversity of quorum-sensing systems in clinical and environmental isolates of *Vibrio cholerae*. *Infect. Immun.* **74**: 1141-1147.

Jovanovic, M., E.H. James, P.C. Burrows, F.G. Rego, M. Buck & J. Schumacher, (2011) Regulation of the co-evolved HrpR and HrpS AAA+ proteins required for *Pseudomonas syringae* pathogenicity. *Nat Commun* **2**: 177.

Jyot, J., N. Dasgupta & R. Ramphal, (2002) FleQ, the major flagellar gene regulator in *Pseudomonas aeruginosa*, binds to enhancer sites located either upstream or atypically downstream of the RpoN binding site. *J Bacteriol* **184**: 5251-5260.

Kamruzzaman, M., S. Udden, D.E. Cameron, S.B. Calderwood, G.B. Nair, J.J. Mekalanos & S.M. Faruque, (2010) Quorum-regulated biofilms enhance the development of conditionally viable, environmental *Vibrio cholerae*. *Proc. Natl. Acad Sci U S A* **107**: 1588.

Kanazawa, T., S. Ren, M. Maekawa, K. Hasegawa, F. Arisaka, M. Hyodo, Y. Hayakawa, H. Ohta & S. Masuda, (2010) Biochemical and physiological characterization of a BLUF protein/EAL protein complex involved in blue light-dependent degradation of cyclic diguanylate in the purple bacterium *Rhodopseudomonas palustris*. *Biochem.* **49**: 10647–10655.

Karatan, E., T.R. Duncan & P.I. Watnick, (2005) NspS, a predicted polyamine sensor, mediates activation of *Vibrio cholerae* biofilm formation by norspermidine. *J Bacteriol* **187**: 7434-7443.

Kazmierczak, B.I., M.B. Lebron & T.S. Murray, (2006) Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. *Mol Microbiol* **60**: 1026-1043.

Klose, K.E. & J.J. Mekalanos, (2002) Distinct roles of an alternative sigma factor during both free swimming and colonizing phases of the *Vibrio cholerae* pathogenic cycle. *Mol Microbiol* **28**: 501-520.

Koestler, B.J. & C.M. Waters, (2013) Exploring environmental control of cyclic di-GMP signaling in *Vibrio cholerae* by using the ex vivo lysate cyclic di-GMP assay (TELCA). *Appl. Environ. Microbiol* **79**: 5233-5241.

Kovacikova, G., W. Lin & K. Skorupski, (2004) *Vibrio cholerae* AphA uses a novel mechanism for virulence gene activation that involves interaction with the LysR-type regulator AphB at the tcpPH promoter. *Mol Microbiol* **53**: 129-142.

Kovacikova, G., W. Lin & K. Skorupski, (2005) Dual regulation of genes involved in acetoin biosynthesis and motility/biofilm formation by the virulence activator AphA and the acetate-responsive LysR-type regulator AlsR in *Vibrio cholerae*. *Mol Microbiol* **57**: 420-433.

Kovacikova, G. & K. Skorupski, (2002) Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. *Mol Microbiol* **46**: 1135-1147.

Krasteva, P.V., J.C. Fong, N.J. Shikuma, S. Beyhan, M.V. Navarro, F.H. Yildiz & H. Sondermann, (2010) *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. *Science* **327**: 866-868.

Krokan, H., R. Standal & G. Slupphaug, (1997) DNA glycosylases in the base excision repair of DNA. *Biochem J* **325**: 1-16.

Krukonis, E.S. & V.J. DiRita, (2003) From motility to virulence: Sensing and responding to environmental signals in *Vibrio cholerae*. *Curr Opin Microbiol* **6**: 186-190.

Kuchma, S.L., J.P. Connolly & G.A. O'Toole, (2005) A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **187**: 1441-1454.

Leduc, J.L. & G.P. Roberts, (2009) Cyclic di-GMP allosterically inhibits the CRP-like protein (Clp) of *Xanthomonas axonopodis* pv. citri. *J Bacteriol* **191**: 7121–7122.

Lenz, D.H., K.C. Mok, B.N. Lilley, R.V. Kulkarni, N.S. Wingreen & B.L. Bassler, (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**: 69-82.

Li, W. & Z.G. He, (2012) LtmA, a novel cyclic di-GMP-responsive activator, broadly regulates the expression of lipid transport and metabolism genes in *Mycobacterium smegmatis*. *Nucleic Acids Res* **40**: 11292-11307.

Lim, B., S. Beyhan, J. Meir & F.H. Yildiz, (2006) Cyclic-diGMP signal transduction systems in *Vibrio cholerae*: modulation of rugosity and biofilm formation. *Mol Microbiol* **60**: 331-348.

Lim, B., S. Beyhan & F.H. Yildiz, (2007) Regulation of Vibrio polysaccharide synthesis and virulence factor production by CdgC, a GGDEF-EAL domain protein, in *Vibrio cholerae*. *J Bacteriol* **189**: 717.

Lin, W., G. Kovacikova & K. Skorupski, (2007) The quorum sensing regulator HapR downregulates the expression of the virulence gene transcription factor AphA in *Vibrio cholerae* by antagonizing Lrp- and VpsR-mediated activation. *Mol Microbiol* **64**: 953-967.

Lindahl, T., (1979) DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. *Prog Nucleic Acid Res Mol Biol.* **22**: 135.

Lindahl, T., B. Sedgwick, M. Sekiguchi & Y. Nakabeppu, (1988) Regulation and expression of the adaptive response to alkylating agents. *Ann Rev Biochem* **57**: 133-157.

Liu, X., S. Beyhan, B. Lim, R.G. Linington & F.H. Yildiz, (2010) Identification and characterization of a phosphodiesterase that inversely regulates motility and biofilm formation in *Vibrio cholerae*. *J Bacteriol* **192**: 4541-4552.

Ljungquist, S., T. Lindahl & P. Howard-Flanders, (1976) Methyl methane sulfonate-sensitive mutant of *Escherichia coli* deficient in an endonuclease specific for apurinic sites in deoxyribonucleic acid. *J Bacteriol* **126**: 646-653.

Long, T., K.C. Tu, Y. Wang, P. Mehta, N. Ong, B.L. Bassler & N.S. Wingreen, (2009) Quantifying the integration of quorum-sensing signals with single-cell resolution. *PLoS biology* **7**: e1000068.

Lundin, C., M. North, K. Erixon, K. Walters, D. Jenssen, A.S. Goldman & T. Helleday, (2005) Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. *Nucleic Acids Res* **33**: 3799-3811.

Massie, J.P., E.L. Reynolds, B.J. Koestler, J.-P. Cong, M. Agostoni & C.M. Waters, (2012) Quantification of high-specificity cyclic diguanylate signaling. *Proc. Natl. Acad Sci U S A* **109**: 12746-12751.

Meibom, K.L., M. Blokesch, N.A. Dolganov, C.Y. Wu & G.K. Schoolnik, (2005) Chitin induces natural competence in *Vibrio cholerae*. *Science* **310**: 1824-1827.

Merrell, D.S., S.M. Butler, F. Qadri, N.A. Dolganov, A. Alam, M.B. Cohen, S.B. Calderwood, G.K. Schoolnik & A. Camilli, (2002) Host-induced epidemic spread of the cholera bacterium. *Nature* **417**: 642-645.

Merritt, J.H., K.M. Brothers, S.L. Kuchma & G.A. O'Toole, (2007) SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J Bacteriol* **189**: 8154-8164.

Miller, M., K. Skorupski, D. Lenz, R. Taylor & B. Bassler, (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* **110**: 303-314.

Ng, W.L. & B.L. Bassler, (2009) Bacterial quorum-sensing network architectures. *Annu Rev Genet* **43**: 197-222.

Paul, K., V. Nieto, W.C. Carlquist, D.F. Blair & R.M. Harshey, (2010) The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a "backstop brake" mechanism. *Molecular cell* **38**: 128-139.

Pittard, J., H. Camakaris & J. Yang, (2005) The TyrR regulon. *Mol Microbiol* **55**: 16-26.

Pradhan, S., A.K. Baidya, A. Ghosh, K. Paul & R. Chowdhury, (2010) The El Tor biotype of *Vibrio cholerae* exhibits a growth advantage in the stationary phase in mixed cultures with the classical biotype. *J Bacteriol* **192**: 955-963.

Preston, N., T. Iida, J. Shrestha, K. Yamamoto, T. Honda, M.J. Albert, M. Rivas, C. Toma, E. Miliwebsky & M. Caffer, (1993) Cholera isolates in relation to the" eighth pandemic". *The Lancet* **342**: 925-927.

Prism, G., 5.0. 2007. GraphPad Software. *Inc. San Diego, California. USA*.

Prouty, M.G., N.E. Correa & K.E. Klose, (2001) The novel sigma54- and sigma28-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. *Mol Microbiol* **39**: 1595-1609.

Rappas, M., D. Bose & X. Zhang, (2007) Bacterial enhancer-binding proteins: unlocking σ54-dependent gene transcription. *Curr Opin Struct Biol* **17**: 110-116.

Rappas, M., J. Schumacher, F. Beuron, H. Niwa, P. Bordes, S. Wigneshweraraj, C.A. Keetch, C.V. Robinson, M. Buck & X. Zhang, (2005) Structural Insights into the Activity of Enhancer-Binding Proteins. *Science* **307**: 1972-1975.

Reddy, C.A., (September 2007) Methods for General and Molecular Microbiology. *ASM press* **Edition 3**.

Reidl, J. & K.E. Klose, (2002) Vibrio cholerae and cholera: out of the water and into the host. *FEMS microbiology reviews* **26**: 125-139.

Richard, C.L., A. Tandon, N.R. Sloan & R.G. Kranz, (2003) RNA polymerase subunit requirements for activation by the enhancer-binding protein *Rhodobacter capsulatus* NtrC. *J. Biol Chem* **278**: 31701-31708.

Römling, U., M.Y. Galperin & M. Gomelsky, (2013) Cyclic di-GMP: the First 25 Years of a Universal Bacterial Second Messenger. *Microbiol. Mol. Biol. Rev* **77**: 1-52.

Römling, U., M. Gomelsky & M.Y. Galperin, (2005) C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* **57**: 629-639.

Rosenfeld, N., J.W. Young, U. Alon, P.S. Swain & M.B. Elowitz, (2005) Gene regulation at the single-cell level. *Science* **307**: 1962-1965.

Ross, P., Y. Aloni, H. Weinhouse, D. Michaeli, P. Weinberger-Ohana, R. Mayer & M. Benziman, (1986) Control of Cellulose Synthesis in *Acetobacter xylinum*. A Unique Guanyl Oligonucleotide is the Immediate Activator of the Cellulose Synthase. *Carbohydrate Res* **149**: 101-117.

Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, E. De Vroom, G. Van der Marel & J. Van Boom, (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* **325**: 275-281.

Rutherford, S.T., J.C. van Kessel, Y. Shao & B.L. Bassler, (2011) AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. *Genes Dev* **25**: 397-408.

Ryjenkov, D.A., R. Simm, U. Römling & M. Gomelsky, (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. *J. Biol. Chem.* **281**: 30310-30314.

Ryjenkov, D.A., M. Tarutina, O.V. Moskvin & M. Gomelsky, (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J Bacteriol* **187**: 1792-1798.

Sakumi, K., Y. Nakabeppu, Y. Yamamoto, S.-i. Kawabata, S. Iwanaga & M. Sekiguchi, (1986) Purification and structure of 3-methyladenine-DNA glycosylase I of *Escherichia coli*. *J Biol Chem* **261**: 15761-15766.

Saldías, M.S., J. Lamothe, R. Wu & M.A. Valvano, (2008) *Burkholderia cenocepacia* requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. *Infect. Immun* **76**: 1059-1067.

Sambrook, J., E. Fritsch & T. Maniatis, (1989) *Molecular cloning*. Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY.

Schmidt, A.J., D.A. Ryjenkov & M. Gomelsky, (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol* **187**: 4774-4781.

Schumacher, J., N. Joly, M. Rappas, X. Zhang & M. Buck, (2006) Structures and organisation of AAA+ enhancer binding proteins in transcriptional activation. *J Struct Biol* **156**: 190-199.

Simm, R., M. Morr, U. Remminghorst, M. Andersson & U. Romling, (2009) Quantitative determination of cyclic diguanosine monophosphate concentrations in nucleotide extracts of bacteria by matrix-assisted laser desorption/ionization,time-of-flight mass spectrometry. *Anal Biochem* **386**: 53-58.

Skorupski, K. & R. Taylor, (1996) Positive selection vectors for allelic exchange. *Gene* **169**: 47-52.

Srivastava, D., R.C. Harris & C.M. Waters, (2011) Integration of cyclic di-GMP and quorum sensing in the control of *vpsT* and *aphA* in *Vibrio cholerae*. *J Bacteriol* **193**: 6331-6341.

Srivastava, D., M.-L. Hsieh, A. Khataokar, M.B. Neiditch & C.M. Waters, (2013) Cyclic di-GMP inhibits Vibrio cholerae motility by repressing induction of transcription and inducing extracellular polysaccharide production. *Mol Microbiol* **90**: 1262-1276.

Srivastava, D. & C.M. Waters, (2012) A Tangled Web: Regulatory Connections between Quorum Sensing and Cyclic di-GMP. *J Bacteriol* **194**: 4485-4493.

Steiner, S., C. Lori, A. Boehm & U. Jenal, (2012) Allosteric activation of exopolysaccharide synthesis through cyclic di-GMP-stimulated protein–protein interaction. *EMBO J* **32**: 354-368.

Steinum, A.-L. & E. Seeberg, (1986) Nucleotide sequence of the tag gene from *Escherichia coli*. *Nucleic Acids Res* **14**: 3763-3772.

Sudarsan, N., E. Lee, Z. Weinberg, R. Moy, J. Kim, K. Link & R. Breaker, (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* **321**: 411.

Syed, K.A., S. Beyhan, N. Correa, J. Queen, J. Liu, F. Peng, K.J. Satchell, F. Yildiz & K.E. Klose, (2009) The *Vibrio cholerae* flagellar regulatory hierarchy controls expression of virulence factors. *J Bacteriol* **191**: 6555-6570.

Tamayo, R., J.T. Pratt & A. Camilli, (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu. Rev. Microbiol.* **61**: 131-148.

Tamayo, R., S. Schild, J.T. Pratt & A. Camilli, (2008) Role of cyclic di-GMP during El Tor biotype *Vibrio cholerae* infection: characterization of the in vivo-induced cyclic di-GMP phosphodiesterase CdpA. *Infect. Immun.* **76**: 1617-1627.

Tamayo, R., A. Tischler & A. Camilli, (2005) The EAL domain protein VieA is a cyclic diguanylate phosphodiesterase. *J. Biol. Chem* **280**: 33324.

Tao, F., Y.W. He, D.H. Wu, S. Swarup & L.H. Zhang, (2010) The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. *J Bacteriol* **192**: 1020-1029.

Teo, I., B. Sedgwick, M.W. Kilpatrick, T.V. McCarthy & T. Lindahl, (1986) The intracellular signal for induction of resistance to alkylating agents in *E. coli*. *Cell* **45**: 315-324.

Thelin, K.H. & R.K. Taylor, (1996) Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. *Infect. Immun.* **64**: 2853-2856.

Tischler, A.D. & A. Camilli, (2004) Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Mol Microbiol* **53**: 857-869.

Tischler, A.D. & A. Camilli, (2005) Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression. *Infect. Immun.* **73**: 5873-5882.

Trimble, M.J. & L.L. McCarter, (2011) Bis-(3'-5')-cyclic dimeric GMP-linked quorum sensing controls swarming in *Vibrio parahaemolyticus*. *Proc Natl Acad Sci USA* **108**: 18079-18084.

Tu, K.C. & B.L. Bassler, (2007) Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev.* **21**: 221-233.

Tuckerman, J.R., G. Gonzalez, E.H.S. Sousa, X. Wan, J.A. Saito, M. Alam & M.A. Gilles-Gonzalez, (2009) An oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochem.* **48**: 9764-9774.

Van Houdt, R., A. Aertsen & C.W. Michiels, (2007) Quorum-sensing-dependent switch to butanediol fermentation prevents lethal medium acidification in *Aeromonas hydrophila* AH-1N. *Res. Microbiol* **158**: 379-385.

Vance, R.E., J. Zhu & J.J. Mekalanos, (2003) A constitutively active variant of the quorum-sensing regulator LuxO affects protease production and biofilm formation in *Vibrio cholerae*. *Infect. Immun.* **71**: 2571.

Visick, K.L., (2009) An intricate network of regulators controls biofilm formation and colonization by *Vibrio fischeri*. *Mol Microbiol* **74**: 782-789.

Waters, C.M. & B.L. Bassler, (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* **21**: 319-346.

Waters, C.M. & B.L. Bassler, (2006) The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* **20**: 2754-2767.

Waters, C.M., W. Lu, J.D. Rabinowitz & B.L. Bassler, (2008) Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of *vpsT*. *J Bacteriol* **190**: 2527-2536.

Watnick, P.I. & R. Kolter, (1999) Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* **34**: 586-595.

Watnick, P.I., C.M. Lauriano, K.E. Klose, L. Croal & R. Kolter, (2001) The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholera*e O139. *Mol Microbiol* **39**: 223-235.

Weber, H., C. Pesavento, A. Possling, G. Tischendorf & R. Hengge, (2006) Cyclic-di-GMP mediated signalling within the σS network of *Escherichia coli*. *Mol Microbiol* **62**: 1014-1034.

Weinhouse, H., S. Sapir, D. Amikam, Y. Shilo, G. Volman, P. Ohana & M. Benziman, (1997) c-di-GMP-binding protein, a new factor regulating cellulose synthesis in *Acetobacter xylinum*. *FEBS letters* **416**: 207-211.

Whitney, J.C., K.M. Colvin, L.S. Marmont, H. Robinson, M.R. Parsek & P.L. Howell, (2012) Structure of the cytoplasmic region of PelD, a degenerate diguanylate cyclase receptor that regulates exopolysaccharide production in *Pseudomonas aeruginosa*. *J Biol Chem* **287**: 23582-23593.

Wilksch, J.J., J. Yang, A. Clements, J.L. Gabbe, K.R. Short, H.W. Cao, R. Cavaliere, C.E. James, C.B. Whitchurch, M.A. Schembri, M.L.C. Chuah, Z.X. Liang, O.L. Wijburg, A.W. Jenney, T. Lithgow & R.A. Strugnell, (2011) MrkH, a

Novel c-di-GMP-Dependent Transcriptional Activator, Controls *Klebsiella pneumoniae* Biofilm Formation by Regulating Type 3 Fimbriae Expression. *PLoS Pathog.* **7 (8)**: e1002204.

Wolfe, A.J., D.S. Millikan, J.M. Campbell & K.L. Visick, (2004) *Vibrio fischeri* σ54 controls motility, biofilm formation, luminescence, and colonization. *Appl. Environ. Microbiol.* **70**: 2520-2524.

Yang, J., H. Camakaris & J. Pittard, (2002) Molecular analysis of tyrosine-and phenylalanine-mediated repression of the tyrB promoter by the TyrR protein of *Escherichia coli*. *Mol Microbiol* **45**: 1407-1419.

Yang, J., J.S. Hwang, H. Camakaris, W. Irawaty, A. Ishihama & J. Pittard, (2004) Mode of action of the TyrR protein: repression and activation of the tyrP promoter of *Escherichia coli*. *Mol Microbiol* **52**: 243-256.

Yang, M., E.M. Frey, Z. Liu, R. Bishar & J. Zhu, (2010) The virulence transcriptional activator AphA enhances biofilm formation by *Vibrio cholerae* by activating expression of the biofilm regulator VpsT. *Infect. Immun.* **78**: 697-703.

Yildiz, F.H., N.A. Dolganov & G.K. Schoolnik, (2001) VpsR, a Member of the Response Regulators of the Two-Component Regulatory Systems, Is Required for Expression of *vps* Biosynthesis Genes and EPS(ETr)-Associated Phenotypes in *Vibrio cholerae* O1 El Tor. *J Bacteriol* **183**: 1716-1726.

Yildiz, F.H. & G.K. Schoolnik, (1999) *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad Sci U S A* **96**: 4028.

Zhu, J. & J.J. Mekalanos, (2003) Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Dev. Cell* **5**: 647-656.

Zhu, J., M.B. Miller, R.E. Vance, M. Dziejman, B.L. Bassler & J.J. Mekalanos, (2002) Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **99**: 3129-3134.

Zorraquino, V., B. García, C. Latasa, M. Echeverz, A. Toledo-Arana, J. Valle, I. Lasa & C. Solano, (2013) Coordinated cyclic-di-GMP repression of Salmonella motility through YcgR and cellulose. *J Bacteriol* **195**: 417-428.