NUCLEOTIDE SECOND MESSENGERS SIGNAL THROUGH TRANSCRIPTIONAL AND
POSTTRANSCRIPTIONAL REGULATORS TO CONTROL THE PRODUCTION OF A
COLONIZATION FACTOR IN *VIBRIO CHOLERAE*

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ABSTRACT

Ankunda Therese Kariisa: Nucleotide Second Messengers Signal Through
Transcriptional and Posttranscriptional Regulators to Control the Production of a
Colonization Factor in *Vibrio cholerae*
(Under the direction of Rita Tamayo)

The diarrheal human pathogen *Vibrio cholerae* causes millions of cases of
severe cholera disease every year, resulting in substantial morbidity and mortality.
Although *V. cholerae* is primarily an aquatic organism, it is also adept at colonizing and
flourishing in the human small intestine. To persist and transition between the aquatic
environment and the host, *V. cholerae* controls the production of colonization factors
and virulence determinants. *V. cholerae* alters this through the production of intracellular
second messengers, such as cyclic diguanylate (c-di-GMP) and cyclic adenosine
monophosphate (cAMP), that relay information about the extracellular environment to
intracellular effectors. Within the cell, c-di-GMP interacts with a variety of effector
molecules, such as RNA riboswitches and proteins, to achieve transcriptional, post-
transcriptional and post-translational control of regulated processes. cAMP signals
primarily through its receptor, the cAMP Receptor Protein (CRP), and regulates
processes at the level of transcription. In this study, we examine the regulation of *gbpA*
by c-di-GMP and cAMP; GbpA is a colonization factor that contributes to attachment of
V. cholerae to the host small intestine and to chitinous surfaces. A c-di-GMP riboswitch,
Vc1, was recently identified in the 5' untranslated region of *gbpA*. Our results show that

Vc1 can bind c-di-GMP *in vitro* and that mutations that reduce binding abrogate GbpA production. Thus, c-di-GMP positively regulates *gbpA* expression via Vc1. In addition, we defined an additional mechanism of regulation of *gbpA* in which c-di-GMP negatively impacts activation *gbpA* transcription initiation by acting through cAMP-CRP. These studies identify two distinct signals, c-di-GMP and cAMP, that contribute to the regulation of *gbpA.* Our findings highlight the complex contribution of nucleotide second messengers in the adaptation of *V. cholerae* to host and aquatic environments.

ACKNOWLEDGMENTS

DEDICATIONS

This dissertation is dedicated to my late father Dr. G.M.B Kariisa. He lived a

remarkable life, and his legacy will forever inspire me in every way.

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LIST OF ABBREVIATIONS AND SYMBOLS

V.   *Vibrio*

E.   *Escherichia*

CT   Cholera Toxin

TCP   Toxin co-regulated pilus

CTXΦ   Lysogenic bacteriophage

VPI   Vibrio pathogenicity island

LPS   Lipopolysaccharide

cAMP   Cyclic adenosine 3',5'-monophosphate

c-di-GMP   Cyclic dimeric guanosine 3',5'-monophosphate

CRP   cAMP receptor protein

PTS   phosphoenolpyruvate carbohydrate phosphotransferase transport system

DGC   Diguanylate cyclase

PDE   Phosphodiesterase

CpdA   cAMP phosphodiesterase

AC   Adenylate cyclase

UTR   5' untranslated region

SHAPE   Selective 2'-hydroxyl acylation analyzed by primer extension

Δ   Deletion

CHAPTER 1: INTRODUCTION

CHOLERA DISEASE

Cholera is a gastrointestinal disease characterized by profuse watery "rice water" diarrhea (1-3). The voluminous stool can reach volumes of up to 1 L per hour, and if left untreated, cholera can result in death due to dehydration within hours of onset of symptoms (4). The World Health Organization (WHO) estimates that approximately 1.4 billion people worldwide are at risk of contracting cholera, predominantly people living in developing countries (5). The highest burden of cholera is observed in areas where it is endemic, about 2.8 million cases and 91,000 deaths occur annually (5). Within these at-risk populations in cholera-endemic areas, roughly 50% of disease and death occurs in children under the age of 5 (5), likely because young children are more susceptible to cholera due to a naïve immune system and poor protective immunity (5). In contrast, in areas where cholera is not endemic, the number of cholera cases and deaths annually is 87,000 and 2,500, respectively (5). Furthermore, in non-endemic areas, children and adults are equally susceptible to cholera due to lack of previous exposure (5,6). Currently, over 99% of the reported incidence and burden of cholera occurs in Bangladesh, India and Sub-Saharan Africa (5,7). In the developed world, cholera has been completely eradicated, and isolated outbreaks are rapidly contained and resolved.

As mentioned above, the incidence and burden of cholera is predominantly restricted to the developing world. Reasons for this include poor water sanitation, poor

health care infrastructure, high population density and environmental factors. Environmental factors can include increasing rainfall, rising water temperatures and natural disasters, such as earthquakes and floods. The contribution of these factors to cholera transmission was most recently observed in Haiti during the outbreak that started in October 2010. Following the earthquake on January 12th 2010, Haiti's already frail infrastructure incurred significant damages that had detrimental effects on the health care and sanitation sectors. To aid with relief efforts, seemingly healthy UN Nepalese health care workers, among others, were recruited to Haiti. One or more of the workers were asymptomatically infected with pathogenic *Vibrio cholerae*, a bacterium indigenous to marine and brackish waters and the causative agent of cholera. Using genomic approaches, it was determined from independent stool samples that the outbreak was caused by a single pathogenic strain of *V. cholerae* (8). Furthermore, comparative genomics revealed that the Haitian strain was nearly identical to contemporary Nepalese *V. cholerae* strains. Thus, it was concluded that the first strain of pathogenic *V. cholerae* in Haiti was introduced into water sources by Nepalese UN workers (9). The aquatic environment, ambient temperature and stagnant fresh water promoted *V. cholerae* outgrowth and further contamination of water sources. Due to the large number of people displaced from their homes, many were obliged to live in very dense and often overcrowded camps. Favorable *V. cholerae* growth conditions and high population densities, coupled with the lack of access to clean water, led to the rapid transmission of cholera within a week of the arrival of the UN Nepalese health care workers. The resulting outbreak devastated the lives of many in Haiti. As of January 7th

2014, the Haitian officials reported 697,256 cases and 8,534 deaths due to cholera (10). Notably, this occurred in a region in which cholera had not previously been reported.

CHOLERA HISTORY

Cholera has been endemic in the Ganges delta region of the Indian subcontinent for many centuries. However, the first reports of cholera were not until 1817, when it crossed into Russia. The transmission event was likely due to the rise in trade and travel. In addition to spreading to Russia, the outbreak extended to parts of China and the Middle East. This outbreak resulted in the first cholera pandemic, the Asiatic cholera pandemic, which lasted until 1824. In the second pandemic, from 1829-1851, cholera reached the Americas and Europe for the first time. The third and fourth pandemics, which spanned 1852-1875, introduced cholera into parts of South America and North Africa and Sub-Saharan Africa, where it remains a significant health concern. During the fifth and sixth pandemics, from 1881-1923, cholera outbreaks were the most widespread in history affecting parts of Africa, the Middle East, South East Asia and Europe. However, the deaths from cholera were lower during the later pandemics due to greater understanding of cholera transmission and increased surveillance. Collectively, over the last 200 years, there have been seven cholera pandemics. The current and ongoing seventh pandemic, which includes the outbreak in Haiti, began in 1961 in Indonesia. In the last 50 years, the seventh has spread rapidly to other countries in Asia, Europe, Africa and Latin America (3).

Before transmission of cholera was understood, the miasma theory, which suggested that cholera was transmitted through bad air, was widely perpetrated. It was not until 1854, when physician John Snow investigated the pattern of disease

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transmission during a cholera outbreak in London, that the oral-fecal route of

transmission gained support. Snow traced the source of the outbreak to the broad street

pump in London and urged authorities to remove the handle of the pump to curb the

outbreak. As a result of Snow's investigation, monitoring of cholera transmission

founded the field of epidemiology and cholera became the first reportable disease (11).

Vibrio cholerae AND PATHOGENESIS

The causative agent of the disease cholera is the Gram-negative, curved rod,

facultative bacterium called *Vibrio cholerae* (12). *V. cholerae* has two circular

chromosomes composed of four million base pairs that encode roughly 4000 genes. *V.

cholerae* was first isolated in 1854 by an Italian anatomist named Filippo Pacini (13).

Pacini went on to publish extensively on *V. cholerae*, describing its pathogenesis and

proposing methods for cholera treatment. In 1883, Robert Koch independently identified

V. cholerae as the causative agent of cholera disease, following research in Egypt and

Calcutta (12).

V. cholerae is primarily an aquatic bacterium, commonly found in the fresh water

reservoirs, where it can grow planktonically or associate in a biofilm with various

organisms such as zooplankton and phytoplankton (14,15). In the environment, *V.

cholerae* is often associated with crustaceans, such as copepods, where it survives by

feeding on their chitinous exoskeleton, which provides a carbon and nitrogen source

(16-18). *V. cholerae* species can also survive on larger crustaceans such as arthropods,

on chironomid egg masses and within amoebae (19-21). Environmental factors that

affect water levels and water temperatures, such as floods and climate change, can

promote the bloom and distribution of zooplankton and increase the spread of cholera in susceptible areas (22). As a species, *V. cholerae* exhibits genetic diversity, and in aquatic reservoirs over 200 serogroups have been identified (1). Serogroups are differentiated by O antigen variations in the lipopolysaccharide (LPS). Serogroups can be subdivided into biotypes, and biotypes can be subdivided into serotypes. Only serogroups O1 and O139 have been associated with cholera disease, suggesting that *V. cholerae* has primarily evolved to survive in the aquatic environment (3,23). Pathogenic O1 and O139 serogroups are distinguished from non-pathogenic strains by the presence of the Vibrio pathogenicity island (VPI) genomic island and the lysogenic bacteriophage CTXΦ. CTXΦ and VPI encode two key players in *V. cholerae* pathogenesis, cholera toxin (CT) and toxin co-regulated pili (TCP), respectively (24, 25). The O1 serogroup is composed of two biotypes, El Tor and classical, and each biotype contains the Inaba and Ogawa serotypes (23). The classical and El Tor biotypes differ in the type of CTXΦ and their number of VPIs; the classical biotypes encode one (VPI-1) and the El Tor biotypes encode two (VPI-1 and VPI-2) (26). The classical biotype was responsible for the first six pandemics but it has been largely replaced by the El Tor biotype, which was responsible for the seventh pandemic and most of the ongoing outbreaks (23). The O139 serogroup was first identified in 1992 and has since caused severe epidemics in South Asia (23, 27, 28). In addition to its role in pathogenesis, TCP acts as the receptor for CTXΦ (29). Therefore, evolution of pathogenic *V. cholerae* relies on the lateral transfer of VPI, which contains the gene encoding TCP, followed by CTXΦ. This suggests that *V. cholerae* strains in the environment became accidental human pathogens after acquiring the ability to colonize the mammalian host and cause

disease through horizontal gene transfer. This finding, along with the recent emergence of the serogroup O139, draws concerns that new strains of pathogenic *V. cholerae* may continue arise (30). In addition, many O1 and O139 variants isolated in the last few decades possess the SXT genetic element, which confers resistance streptomycin and co-trimoxazole (31).

V. cholerae is normally transmitted to a human host through contaminated food or water. Upon ingestion, pathogenic *V. cholerae* can colonize the small intestine (2, 3). Colonization can be asymptomatic and result in shedding for only a few days, or symptomatic and result in diarrhea and shedding for up to 2 weeks (32, 33). Symptomatic patients can experience either mild or severe diarrhea, and if left untreated, severe diarrhea is fatal in over 50% of cases due to rapid dehydration (34). Human infections may play an important role in the *V. cholerae* life cycle as bacterial shedding can lead to the introduction or reintroduction of large numbers of *V. cholerae* into aquatic reservoirs. For instance, human carriers coming from regions where cholera is endemic can seed new outbreaks of cholera in naïve areas (see Haiti outbreak above). In addition, pathogenic strains shed by a host are hyperinfectious for up to 5 hours post-infection. Hyperinfectious bacteria, which have an infectious dose $1/10^{th}$ of environmentally derived strains, may accelerate the transmission of cholera during outbreaks (35). Indeed, mathematical models that incorporate hyperinfectivity into their calculations can reproduce the explosive rates of transmission often observed during outbreaks (36).

The main driver of cholera symptoms is the enterotoxin cholera toxin (CT). CT is a multimeric protein composed of five B subunits (B_5) and one A subunit (A_1) (37).

Production and secretion of A_1B_5 by *V. cholerae* during infection leads to the engagement of B_5 with the GM1 ganglioside receptor on intestinal epithelial cells and the translocation of A_1 into the host cell cytosol (38). Once cytoplasmic, A_1 becomes enzymatically active and ADP-ribosylates the $Gs\alpha$ subunit of host adenylate cyclase, locking it into a constitutively active GDP bound conformation. This irreversibly activates the adenylate cyclase leading to uncontrolled synthesis of cAMP and activation of the Cystic Fibrosis transmembrane receptor (CFTR) (39, 40). The consequence of activating this signaling cascade is the massive efflux of ions and water into the intestinal lumen and the hallmark profuse and watery diarrhea. Massive fluid losses can reach volumes of 1 L per hour and result in death due to dehydration within hours of onset of disease (4).

In response to severe diarrheal symptoms from cholera disease, aggressive rehydration therapy can reduce mortality from over 50% to 0.2% (3). The composition of this therapy is critical to restoring normal electrolyte homeostasis, and depending on the severity of the patient's condition it is administered via oral or intravenous routes (41, 42). Additionally, in response to diarrhea, antibiotic treatment can be administered to help reduce symptoms (in about 50% of cases) and shorten the duration of disease (34, 43). In rare incidences, tetracycline, erythromycin and ciprofloxacin resistant strains have been isolated (44, 45). Therefore, the emergence of antibiotic resistant strains may make the treatment of cholera more challenging and costly. Nevertheless, antibiotics are secondary treatments to rehydration therapy since dehydration is the primary cause of morbidity and mortality. In an effort to prevent cholera outbreaks several vaccines have been developed, although none have led to the implementation

of a major vaccine program. One major obstacle that remain is limited long-term efficacy; only 50% of patients retain protective immunity when challenged 2-3 years following vaccination (46, 47). Although long-term protection is poor, vaccinating a proportion of a population at-risk can reduce the overall incidence of cholera transmission even in unprotected individuals (48). This phenomenon, termed "herd immunity", may be an important consideration when implementing future vaccine programs to combat cholera.

COLONIZATION FACTORS IN *V. cholerae*

To cause disease in a mammalian host and persist in the aquatic environment, *V. cholerae* must produce colonization factors that mediate attachment to the various surfaces it encounters (49). One of the best-studied colonization factors is TCP, which is encoded within VPI in epidemic and pandemic strains (25, 29). Along with CT, the second essential virulence factor is TCP, which plays an important role in *V. cholerae* colonization of the small intestine and is coregulated with CT (50, 51). Infections models using mice and humans have confirmed that TCP is absolutely required for colonization and subsequent disease (50, 51). TCP is a type IV pilus composed of TcpA subunits that make up length of the pilus structure, and help mediate microcolony formation through pilus-pilus interactions (52). Modifications to the pili that abrogate these interactions, but preserve pilus structure, reveal that microcolony formation is required for colonization of the mammalian intestine and infection by CTXΦ (52). Furthermore, imaging of *V. cholerae* TCP *in vivo* shows that they play a protective role against the antimicrobial effects of bile by forming bundles around the bacteria and serving as a

physical barrier (53). In recent studies, TCP was shown to directly play a role in attachment to Caco-2 human intestinal epithelial cells (53).

      V. cholerae in the aquatic environment, including those recently shed from an infected human, must establish interactions with biotic and abiotic surfaces in the water (14, 15). In the environment, *V. cholerae* will typically engage these surfaces as a biofilm, to resist environmental stresses (54). When biofilm formation on the chitin shell of the squid pen was measured, TCP was identified as an important factor in late stage maturation biofilm, but not initial attachment to chitin (55). Interestingly, using a test-tube to measure biofilm production, TCP was shown to be dispensable during all stages of biofilm formation (56). Therefore, although TCP does not function as an adhesin for chitin, it is important for biofilm maturation and potentially bacterial fitness. Overall, TCP appears to play a role in colonization in both the mammalian host and in the environment.

      Two other pili have been implicated in colonization of chitin and biofilm production: mannose-sensitive hemagglutinin-like pili (MSHA) and chitin regulated pili (ChiRP). MSHA was shown to participate in biofilm formation on abiotic surfaces, but not on chitin (56). However, despite the negligible contribution of MSHA to biofilm production on chitin, MSHA appears to be important for the initial attachment to the chitinous exoskeletons of zooplankton such as copepods (57, 58). ChiRP is another chitin binding pilus that is produced during growth with chitin and/or chitin oligosaccharides. Studies to identify factors required for chitin utilization, found that a strain deficient in *pilA*, which encodes a predicted ChiRP subunit, exhibited a fitness defect during surface growth on chitin (17).

Two nonpilus adhesins have been found to play a dual role in colonizing the host and environment: FrhA and GbpA. In a transcriptome analysis of flagellar regulatory mutants, *frhA* was identified as a positively regulated target of the flagellar regulatory hierarchy (59). FrhA is a hemagglutinin involved in binding to and agglutination of erythrocytes and attachment to human epithelial cells (59). Consistent with these roles, *frhA* mutants showed a colonization defect in the infant mouse model of infection. Interestingly, FrhA was also shown to be important for the early stages of biofilm production on abiotic surfaces and chitin beads (59). Collectively, these studies suggest that FrhA plays a role in the host and environmental phases of the life cycle of pathogenic *V. cholerae*.

The second nonpilus adhesin, GbpA, is a N-acetylglucosamine (GlcNAc)-binding protein that is encoded by locus VCA0811 (60). GbpA is secreted by the type two secretion system onto the bacterial cell surface, where it recognizes GlcNAc modifications found on some proteins, and GlcNAc derived carbohydrates such as chitin (60). GbpA is composed of four domains (1-4), and it exists as a monomer in solution. Domains 1 and 4 bind chitin oligosaccharides and GlcNAc modified glycans, whereas domains 3 and 4 are dispensable for chtin binding but are important for GbpA binding to the bacterial cell surface (61). GbpA production is positively regulated by its substrate, GlcNAc (17). When grown on chitin, *V. cholerae* secretes chitinases that release chitin oligosaccharides and GlcNAc monomers. GlcNAc is transported into *V. cholerae* and phosphorylated by specific phosphoenolpyruvate transport system (PTS) components, and serves as a carbon and nitrogen source (62). In addition, phospho-GlcNAc can bind the transcriptional repressor NagC, leading to the derepression of genes important for

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GlcNAc sensing and metabolism, including *gbpA* (62). Interactions of *V. cholerae* with mucin, which also contains GlcNAc moieties, can also result in increased *gbpA* expression through NagC depression, suggesting that during *V. cholerae* colonization of the small intestine GbpA production might be elevated (63). GbpA levels also are affected post-translationally through the activities of the quorum-sensing regulated proteases, HapA and PrtV (64). HapA and PrtV are produced at high cell density and are believed to aid in detachment of *V. cholerae* from surfaces so that the bacterium can transition to a new surface, e.g., from the host back into the aquatic environment or between surfaces in the environment (64).

V. cholerae* strains deficient in *gbpA* are significantly attenuated in binding mucin and intestinal epithelial cells, due to failed interactions with GlcNAc containing glycoprotein modifications (60, 63). Consistent with these results, GbpA is important for colonization and disease in the infant mouse (60). In addition, GbpA is important for colonizing the chitinous exoskeleton of the crustacean *Daphna magna*, suggesting that it also plays a role in *V. cholerae* attachment to similar environmental organisms (58, 60). Therefore, along with TCP and FrhA, GbpA adds to the repertoire of *V. cholerae* colonization factors that possess dual functions in host pathogenesis and environmental persistence (49).

NUCLEOTIDE SECOND MESSENGERS

The production of a particular colonization factor under the right environmental condition can be critical to bacterial survival. As bacteria transition between different environments, various stimuli influence bacterial behaviors in a myriad of ways. For example, signals sensed at the cell surface can regulate intracellular levels of small

molecule second messengers, which relay information about the extracellular milieu to intracellular effectors. In this section we will focus on nucleotide second messengers implicated in bacterial signaling. Production of these intracellular nucleotides, which include c-di-GMP, c-di-AMP, c-GMP-AMP, cGMP, cAMP, and (p)ppGpp, has been shown to regulate biofilm formation, motility, virulence, nutrient acquisition, stress responses and sporulation (reviewed in 65-68) (Figure 1.1). Accordingly, dysregulation of these signals likely impairs the ability of bacteria to transition between disparate environments.

Cyclic adenosine 3',5'-monophosphate (cAMP) was first identified as a second messenger in liver cells in 1957 by Earl Sutherland, and as a signaling molecule regulating carbon metabolism in *Escherichia coli* in 1969 (69, 70). In bacteria, intracellular cAMP levels are heavily influenced by the availability of extracellular nutrient sources, and cAMP along with cAMP Receptor Protein (CRP) govern the utilization of nutrient sources. Cyclic guanosine 3',5'-monophosphate (cGMP) was first identified in 1963 in rat urine and levels correlated with the hormonal state in the animal (71). A role for cGMP is largely restricted to eukaryotic cells, where it is associated with transmembrane signal transduction, protein kinase activity and many other important processes (72). In bacteria, production of cGMP was observed as early as 1974, but only recently has a function for cGMP in intracellular signaling been ascribed (73). In studies using *Rhodospirillum centenum*, a cGMP specific synthase was indentified and cGMP production was linked to cyst formation (74). The linear nucleotides guanosine 3',5'-bispyrophosphate (ppGpp) and guanosine 3'-diphosphate, 5'-triphosphate (pppGpp) are also intracellular bacterial second messengers. (p)ppGpp production in *E.*

coli was first indentified in 1970 and later classified as an "alarmone" produced in response to nutrient starvation and other stresses (75, 76).

It was not until several decades following the discovery of cAMP, cGMP and (p)ppGpp that cyclic dinucleotides were identified. Perhaps the most broadly studied is cyclic dimeric guanosine 3',5'-monophosphate (c-di-GMP), identified in 1989 as an allosteric regulator of bacterial cellulose synthase in *Gluconacetobacter xylinum* (77). The increased interest in c-di-GMP as a ubiquitous bacterial second messenger in the last decade, led to the identification of its role in virulence, motility, and biofilm formation in a vast number of organisms. About 20 years later, in 2008, cyclic dimeric adenosine 3',5'- monophosphate (c-di-AMP) was identified as second messenger in Gram-positive bacteria (78). Since the discovery of c-di-AMP, studies have shown that it regulates bacterial cell growth, sporulation, stress responses, antimicrobial resistance and virulence (79). Lastly, cyclic guanosine monophosphate adenosine monophospate (c-GMP-AMP) was identified in 2012. A function for c-GMP-AMP has been ascribed to few organisms; in *V. cholerae* c-GMP-AMP production during infection promotes chemotaxis and in numerous Deltaproteobacteria, including *Geobacter* species, it is predicted to function in extracellular electron transfer (80-82).

c-di-GMP SIGNALING

The second messenger c-di-GMP is widely recognized to play an important role in bacterial adaptation to changing environmental conditions (66, 83). Numerous studies have demonstrated that c-di-GMP modulates biological processes including motility, virulence, production of adhesins and exopolysaccharide matrix, cell cycle progression, and responses to environmental stresses (Figure 1.2). Genetic studies suggest that *V.*

cholerae modulates intracellular c-di-GMP levels as it transitions from its native aquatic environment into the host intestine. Reduction of c-di-GMP is required to promote bacterial motility and increase expression of virulence factors, and there is evidence suggesting that *V. cholerae* may downregulate c-di-GMP production during early stages of infection (84-88). Specifically, the production of VieSA, a two-component system composed of the response regulator c-di-GMP phophodiesterase VieA, is required for virulence in the mouse and CT production *in vitro*. Conversely, production of c-di-GMP promotes biofilm formation, which may enhance *V. cholerae* survival on chitin and other aquatic surfaces (89-91). Accordingly, inactivation *vieA* results in increased biofilm production, and similarly overexpression of a diguanylate cyclase, VC0956, increases biofilm production (91, 92). The molecular basis of c-di-GMP-dependent regulation in *V. cholerae* involves impacts on transcriptional, posttranscriptional and posttranslational mechanisms.

In response to extracellular cues, which are largely unknown, bacterial cells adjust the intracellular level of c-di-GMP through the opposing activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which synthesize and hydrolyze c-di-GMP, respectively. The enzymatic activity of DGCs is supplied by a GGDEF domain, named for conserved residues in the catalytic site (93-96) Two distinct domains, EAL and HD-GYP, also named for conserved residues, confer c-di-GMP hydrolytic activity to PDEs (97-99). The PDE and DGC enzymatic domains are often present in proteins in conjunction with sensory and regulatory domains such as PAS, HAMP, blue light sensing (BLUF), haemerythrin, GAF, CHASE, MASE and/or receiver domains linked to two-component signal transduction systems (83). The linkage of DGC and PDE

enzymatic domains with these additional modules provides a post-translational means for a variety of stimuli to impact DGC and PDE activity and thus the level of c-di-GMP in the cell. Transcriptional and post-transcriptional regulation of DGC and PDE gene expression also influences intracellular c-di-GMP levels.

c-di-GMP SIGNALING VIA PROTEIN AND RNA BASED RECEPTORS

To date there are several known intracellular c-di-GMP receptors, which include protein and riboswitch sensors. Protein sensors include transcription factors that directly sense c-di-GMP to modulate gene expression, or proteins that contain PilZ domains, degenerate EAL domains, I-site domains or GIL domains (GGDEF I-site like domains) (100). The specific interactions of these receptors with c-di-GMP mediate the physiological changes observed in response to changes in the level of the second messenger. In the following section, we discuss the various types of c-di-GMP effectors, how the effectors recognize c-di-GMP, and how c-di-GMP sensing contributes to the regulation of biological processes. For comprehensive reviews of c-di-GMP signaling and effectors, we direct readers to other excellent reviews, details to follow will focus on receptors found in *V. cholerae* (101, 102).

PILZ DOMAIN EFFECTORS

In an early study of the regulation of the cellulose synthase in *G. xylinus*, Benziman and co-workers observed that c-di-GMP stimulates the activity of the cellulose synthase BcsAB (77). The PilZ domain present in the α-subunit of BcsAB was later shown to directly bind c-di-GMP (111). Sequence analysis of the PilZ protein led to

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the discovery of this domain in a variety of bacteria and suggested that the PilZ domain may play an important regulatory role. Genes encoding a PilZ domain are broadly distributed in bacterial genomes (103). As with GGDEF, EAL and HD-GYP domains, bacterial genomes often encode multiple proteins containing PilZ domains in combination with other domains, suggesting broad regulatory roles for PilZ proteins. Numerous PilZ domain c-di-GMP receptors with diverse signaling functions and precise modes of action have been identified.

As in many bacterial species, in *V. cholerae* c-di-GMP inhibits swimming motility and favors biofilm formation; in addition, c-di-GMP negatively regulates virulence gene expression (87, 91, 92, 104, 105). *V. cholerae* encodes 5 proteins containing PilZ domains, PlzA, PlzB, PlzC, PlzD, and PlzE. *In vitro*, PlzC and PlzD bind c-di-GMP via their PilZ domains (106); it is unclear whether PlzA, PlzB and PlzE bind c-di-GMP under conditions not tested. *In vivo*, mutation of *plzB* or *plzC* results in decreased motility, mutation of *plzB* or plzD results in decreased biofilm formation, and mutation of *plzB* or both *plzC* and *plzD* results in reduced colonization of the infant mouse small intestine through unknown mechanisms (106). PlzD contains the RxxxR motif that is conserved among PilZ domains (and among all 5 PilZ domain proteins of *V. cholerae*). The arginines in this motif are essential for binding of c-di-GMP in PlzD and PilZ domains in other species (106, 111). Structural studies show that PlzD (VCA0042) exists as a dimer in both apo- and ligand-bound forms (107) (PBD ID 1YLN; R Zhang, M Zhou, S Moy, F Collart, and A Joachimiak, 2005). The PilZ and the other domain in the protein, the YcgR-N domain, are connected by a short seven-residue loop that contains the RxxxR motif. This loop is referred to as the "c-di-GMP switch", as it directly contacts the

c-di-GMP ligand and functions in the conformational change induced by c-di-GMP binding (107). In the *apo* structure, the C-terminal PilZ domain is located far from the two-fold axis and it makes no contact with the YcgR-N domain in the same monomer or in another monomer. In contrast, in the c-di-GMP bound structure of PlzD, the PilZ and YcgR-N domains in a single monomer are found in close proximity, with one c-di-GMP molecule packed tightly in their mutual interface (107). Thus, c-di-GMP binding changes the extended *apo*-PlzD structure into a more compact ligand-bound structure in which the PilZ domains make new contacts across the dimer interface.

To date, PilZ domain proteins have been identified in a variety of organisms and associated with functions that include flagellar motor activity, type IV pilus assembly, alginate and cellulose production and virulence (106, 108-111). The mechanisms of action of PilZ domain containing proteins are not always apparent from sequence and structural information; even PilZ domain proteins with very similar domain architectures can display distinct binding stoichiometries and mechanistic properties. It is likely that there are additional modes of action employed by this class of proteins, and that this diversity contributes to generating the broad regulatory effects observed.

c-di-GMP SENSING TRANSCRIPTION FACTORS

Transcription factors that sense c-di-GMP have been reported in several bacterial species. These regulators interact with c-di-GMP via various motifs, and the DNA binding region of the transcription factor lends specificity, allowing targeted regulation of gene expression. Thus, c-di-GMP can be broadly employed to control a wide variety of pathways. In *V. cholerae*, three transcription factors that sense c-di-GMP

have been identified to date, each of which was previously characterized for their roles in motility and/or biofilm development.

The master regulator of flagellar biosynthesis in *V. cholerae* is FlrA. FlrA is a σ54-dependent enhancer binding protein containing AAA+ ATPase and DNA-binding domains. FlrA binds c-di-GMP, which prevents FlrA from interacting with the *flrBC* promoter and activating flagellar gene expression (112). Regulation of FlrA activity by c-di-GMP defines the mechanism for the previously observed phenomenon that flagellar motility is inhibited by c-di-GMP in *V. cholerae*. In addition, transcriptome analysis revealed that over 300 genes are predicted to be regulated by FlrA, only 50 of which fall within the flagellar/chemotaxis operon (59).

The LuxR family transcription factor VpsT promotes *V. cholerae* biofilm formation by activating the expression of *vps* genes required for exopolysaccharide production (113). VpsT consists of an N-terminal REC domain and a C-terminal HTH domain involved in DNA binding. VpsT does not appear to be a response regulator, as no cognate sensor kinase has been identified and residues for phosphor-transfer in the REC domain are poorly conserved. Instead, VpsT binds c-di-GMP (K_D ~ 3.2 µM), an interaction that promotes VpsT binding to target promoters (89). Structural analysis of VpsT with c-di-GMP revealed that two intercalated c-di-GMP molecules interact with VpsT and stabilize the REC dimerization interface. c-di-GMP interacts with VpsT via a four residue W[F/L/M][T/S]R sequence within the REC domain (89). Importantly, mutation of residues in this motif abolish binding of c-di-GMP and target promoters *in vitro*, which abolishes regulation of *vps* genes and biofilm production *in vivo* (89).

The transcriptional regulator VpsR is an NtrC-family σ54-dependent enhancer binding protein that activates biofilm genes in *V. cholerae* (114, 115). VpsR contains an AAA+ ATP binding domain and a HTH DNA binding domain, and VpsR binds c-di-GMP *in vitro* (K_D ~ 1.6 μM), possibly via its Walker A box (116). However, binding of c-di-GMP does not affect the ability of VpsR to interact with the target promoters tested *in vitro* (116). It is possible that binding of c-di-GMP does not affect the structure of VpsR and thus does not impact VpsR promoter-binding function. Alternatively, c-di-GMP binding to VpsR may affect transcription of only a subset of VpsR-regulated genes, or may affect the subcellular localization or stability of VpsR.

CLASS I AND CLASS II c-di-GMP SENSING RIBOSWITCHES

In addition to the many types of protein sensors of c-di-GMP, RNA sensors of c-di-GMP have also been identified and are widely distributed in bacterial genomes. Riboswitches, *cis*-acting regulatory elements found in the 5' untranslated region (UTR) of some mRNA, consist of an aptamer that binds a specific ligand and an expression platform that regulates downstream gene expression. Regulation typically occurs as a result of conformational changes in the RNA structure in response to ligand binding. The two most common modes of gene regulation by riboswitches involve control of transcription read-through or translation initiation. The aptamer and expression platforms are modular in that a given aptamer can work in conjunction with different expression platforms depending on the specific riboswitch (117-120).

Two classes of c-di-GMP sensing riboswitches have been identified, class I and class II (121-123). To date, there are approximately 500 class I c-di-GMP riboswitches predicted in a wide range of pathogenic and non-pathogenic bacteria. The class I c-di-

GMP riboswitches contain a GEMM motif, so named because they often reside in the 5'

UTR of genes with functions predicted to relate to the environment, membrane or

motility (124). Several studies indicate that the GEMM motif represents the aptamer of

the c-di-GMP riboswitch, however the regions anticipated to correspond to the

expression platform appear to be highly divergent among putative class I riboswitches.

The class II c-di-GMP riboswitch shares no structure or sequence homology with the

class I riboswitch. class II riboswitches are less abundant than class I riboswitches, with

45 putative class II riboswitches identified to date (125).

Due to the lack of sequence and structural similarity, the two riboswitch classes

interact with c-di-GMP via distinct mechanisms. In the class I riboswitch Vc2 from *V.*

cholerae, c-di-GMP binds at the junction of three helices, P1, P2, and P3 (123, 126).

Vc2 makes contacts with c-di-GMP via three nucleotides, G20, C92, and A47. One

guanine base from c-di-GMP forms a Hoogsteen pair with G20, while the other forms a

Watson–Crick pair with C92. A47 bridges the P2 and P3 helices of Vc2 and also

contacts c-di-GMP by stacking between the guanine bases, producing extensive base

stacking interactions between the RNA and ligand. P2 and P3, as well as c-di-GMP

contact residues, are highly conserved among the class I riboswitches, suggesting that

these features are critical for c-di-GMP recognition by these riboswitches (123, 126). In

the class II riboswitch, no canonical base pairings are made with c-di-GMP, but stacking

interactions are observed. Three conserved adenosines, A13, A70, and A61, intercalate

below, between, and above the two guanine bases of c-di-GMP, respectively. In

addition, one guanine base of c-di-GMP is recognized as part of a base triple with A69

and U37 (base triples are clusters of three RNA nucleobases that interact edge-to-edge

by hydrogen bonding). The other guanine base of c-di-GMP forms a single hydrogen bond with G73 and contacts the 2′-OH of A70 and a fully hydrated magnesium ion (122, 125). Furthermore, several of the nucleotides directly involved in recognizing c-di-GMP, A69, A70 and G73, are conserved in at least 90% of class II sequences identified to date (122).

While predictions can be made as to how sensing of c-di-GMP by a riboswitch impacts the biology of an organism based on the identity of the downstream gene, little has been demonstrated experimentally. The class I riboswitch Vc2 in *V. cholerae*, for instance, has been well characterized *in vitro* and has been shown to regulate a reporter gene in response to c-di-GMP *in vivo* using the heterologous bacterial host *E. coli* (124). The exact mechanism of action for Vc2 remains unknown; however, c-di-GMP has no effect on transcript length, therefore the mechanism of action is likely post-transcriptional. Vc2 lies upstream of VC1722, which is predicted to encode a protein with homology to TfoX, a factor involved in uptake of exogenous DNA (17, 124, 127). However, the consequence of c-di-GMP binding to Vc2 and consequent regulation of VC1722 in *V. cholerae* is unknown. The second class I riboswitch in *V. cholerae* is called Vc1 and lies upstream of *gbpA*, which encodes the GlcNAc binding protein involved in the interaction of *V. cholerae* with environmental chitin and with the host intestine. No studies of the Vc1 riboswitch or its relevance to *V. cholerae* biology have been reported. The *in vivo* role of Vc1 will be discussed in Chapter 2.

cAMP AND THE PHOSPHOENOLPYRUVATE TRANSPORT SYSTEM

cAMP signaling is widespread and highly conserved among eukaryotic and prokaryotic organisms. In bacteria, intracellular cAMP levels are heavily influenced by

the availability of extracellular nutrient sources (128). In brief, for Gram-negative

bacteria, uptake of a preferred carbon source by the phosphoenolpyruvate (PEP)-

carbohydrate phosphotransferase transport system (PTS) is dependent on key

components of the pathway and the intracellular ratio of PEP to pyruvate. When PEP to

pyruvate levels are high, a phosphoryl group from PEP is transferred to the PTS

component enzyme I (EI), which initiates a phosphorylation cascade important for

utilization of PTS dependent sugars. EI transfers the phosphoryl group to histidine

protein (HPr), HPr transfers the phosphoryl group to EIIA, EIIA transfers the phosphoryl

group to EIIBC and EIIBC transfers the phosphoryl group to the incoming sugar. PTS

dependent sugars are transported into the cell by their cognate EIIC specific

transporter. The EII components of PTS are usually membrane bound and form a

complex to facilitate uptake and phosphorylation of the incoming sugar. Once

phosphorylated, the sugar is metabolized through glycolysis, which in turn influences

the intracellular pool of PEP and the rate of EI phosphorylation. Whereas some PTS

components are specific for a particular sugar, for instance EIIBC, some PTS

components can act on multiple different incoming sugars, for instance EIIA, HPr and

EI. Accordingly, *V. cholerae* deficient in EIIA is unable to grow on the PTS sugars

glucose, GlcNAc, sucrose or tetrahalose (129). During growth on non-PTS carbon

sources, the phosphoryl group on EIIA is not transferred to the incoming nutrients.

Phosphorylated EIIA can interact with the enzyme adenylate cyclase (AC) to stimulate

its activity, leading to increased intracellular cAMP production. Therefore, the

phosphorylation state of PTS components, namely EIIA, and the levels of intracellular

cAMP can serve as a measure of PTS and non-PTS nutrient availability. In addition, the

rate of glycolysis and PEP to pyruvate ratios, which are heavily influenced by the type of carbon source available, will also influence the PTS pathway and cAMP production.

V. cholerae has 25 genes that encode proteins with known or predicted roles as PTS components; these include two EI homologs, three HPr homologs and nine EIIA homologs (130). In *V. cholerae*, sugars dependent on PTS for uptake include sucrose, mannitol and fructose (129). Interestingly, unlike in *E. coli* in which glucose uptake is strictly PTS-dependent, *V. cholerae* can also utilize glucose in a PTS independent manner; genetic deletion of several PTS components still allows for glucose utilization. Similarly, a *V. cholerae* strain deficient for the EIIB^{GlcNAc} transporter, can still grow in media with GlcNAc as the sole carbon source, suggesting non-PTS utilization of GlcNAc may occur (62).

The role of PTS systems in *V. cholerae* has proven to be very complicated and extends beyond utilization of carbon sources. For instance, several components of PTS, such as EI, HPr, EIIA and EIIBC have been shown to influence biofilm production and colonization of the mammalian small intestine (129-134).

cAMP SIGNALING VIA THE cAMP RECEPTOR PROTEIN

The phosphorylation state of PTS components heavily influences intracellular cAMP production. To date, the only known sensor of cAMP is the cAMP receptor protein (CRP) (128). cAMP-CRP act as a regulatory complex that promotes the expression of genes required for growth on non-PTS carbon sources. In numerous organisms, including *V. cholerae*, the cAMP-CRP complex can also regulate the expression of genes associated with quorum sensing, motility, biofilm production, competence and virulence (Figure 1.3) (16, 135-140). As such, the bacterium

incorporates information about extracellular nutrient availability to accordingly regulate factors involved in these processes.

Early studies looking at the role of cAMP-CRP in *V. cholerae* showed that strains deficient in *cya*, the gene encoding AC, have increased biofilm production, and increased *vps* expression (136). The same was observed for a *crp* mutant, implicating the cAMP-CRP complex in inhibiting biofilm production. cAMP-CRP was also shown to repress the expression of *vpsT*, one of the master regulators of biofilm production in *V. cholerae* (137). Interestingly, cAMP-CRP controls the expression of ten genes predicted to encoded enzymes with DGC and PDE activity, suggesting that cAMP-CRP signaling can influence intracellular c-di-GMP turnover (136). Deletion of one of the predicted DGCs, *cdgA,* eliminated the elevated biofilm production observed in a *crp* mutant, suggesting cAMP-CRP controls biofilm production by influencing c-di-GMP production (136).

A role for cAMP-CRP in inducing competence was also observed. *V. cholerae* can become competent for natural transformation in the presence of chitin (127). Interestingly, cAMP-CRP can enhance the chitin induced competence pathway at several junctures (16). cAMP-CRP promotes attachment to chitin, promotes utilization of chitin as a carbon source, and enhances the expression of competence genes *pilA*, *pilM* and *comEA* (16, 141). These data suggest that cAMP-CRP promotes DNA uptake by activating important genes in the competence pathway.

Colonization and virulence factors are also regulated by cAMP-CRP. *V. cholerae* deficient in *cya* and/or *crp* produces elevated levels of CT and TCP, suggesting that cAMP-CRP negatively regulates their production (140). Interestingly, these strains are

attenuated for colonization in a mouse model despite elevated levels of CT and TCP, suggesting the cAMP-CRP pathway promotes the expression of other genes required for colonization and growth in a host (137, 140). Indeed, cAMP-CRP is a positive regulator of the outer membrane proteins OmpT, OmpU and OmpW, as well as genes involved in chemotaxis and metabolism (136, 137). cAMP-CRP also positively regulates motility, however, the importance of motility in *V. cholerae* pathogenesis remains unclear (137). Quorum sensing is another process controlled by the cAMP-CRP pathway in *V. cholerae*. HapR, the master regulator of quorum sensing is produced at high-cell density when levels of the autoinducer, CAI-1, are high. Strains deficient in *csqA*, the gene that encodes the CAI-1 synthase, have reduced *hapR* expression and increased production of CT and TCP (142). cAMP-CRP acts as a positive regulator of *cqsA* expression, therefore, the effect of cAMP-CRP on CT and TCP may be linked to its regulation of *csqA* (138).

Although phenotypic assays have established an important role for cAMP-CRP in numerous processes, the cAMP-CRP regulon is likely much broader. In a screen to identify the transcriptome profile of *cya* and *crp* mutants, up to 20% of the *V. cholerae* genome were differentially regulated compared to wild type (136). Regulation by cAMP-CRP can occur by direct interactions with a target promoter, or indirectly through its effect on other regulators. cAMP-CRP can also act as a transcriptional regulator in a co-regulatory or antagonistic fashion. For instance, cAMP-CRP can inhibit TcpH, an activator of virulence gene expression, by competing with AphAB for binding at the *tcpPH* promoter (143). In an analogous but opposite fashion, ToxR can inhibit cAMP-

CRP-dependent activation of the *ompT* , encoding an outer membrane protein, by competing with the cAMP-CRP for binding at the *ompT* promoter (144).

REGULATION OF *gbpA* BY c-di-GMP AND cAMP

c-di-GMP has pleiotropic effects on bacterial physiology and broadly impacts gene expression. With the exception of a handful of transcription factors that have been shown to regulate gene expression in response to c-di-GMP, the molecular basis of gene regulation by c-di-GMP is poorly understood. The identification of c-di-GMP specific riboswitches distributed widely among bacterial genomes positions these regulatory RNA domains to serve as important c-di-GMP effectors modulating downstream gene expression. Elegant studies have biochemically characterized a representative GEMM riboswitch, Vc2 from *V. cholerae*, but the functionality of c-di-GMP riboswitches in their native genetic contexts has been largely unexplored (123, 126, 145). In this thesis, we examine Vc1, a putative c-di-GMP riboswitch in *V. cholerae.* Vc1 is located in the 5' untranslated region of *gbpA*, which encodes a colonization factor. We hypothesize that c-di-GMP positively regulates *gbpA* through Vc1.

Another major signaling network common to many bacteria is the nutrient-responsive transcriptional regulator CRP. In *V. cholerae*, a recent transcriptome analysis revealed that CRP represses the production c-di-GMP by repressing the expression of a DGC, *cdgA* (136). These findings suggest that the c-di-GMP and CRP regulatory pathways interact, and that CRP signaling can inhibit c-di-GMP signaling. Herein, we describe the transcriptional regulation of *gbpA*, by CRP and c-di-GMP. We show that c-di-GMP inhibits transcriptional activation of *gbpA* by CRP, which directly

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binds the gbpA promoter in a cAMP-dependent manner. These findings suggest that c-di-GMP directly interferes with the interaction of cAMP-CRP and the *gbpA* promoter via an unidentified regulator. The use of two distinct second messenger signaling mechanisms to regulate *gbpA* transcription may allow *V. cholerae* finely modulate GbpA production, and therefore colonization of aquatic and host surfaces, in response to discrete environmental stimuli.
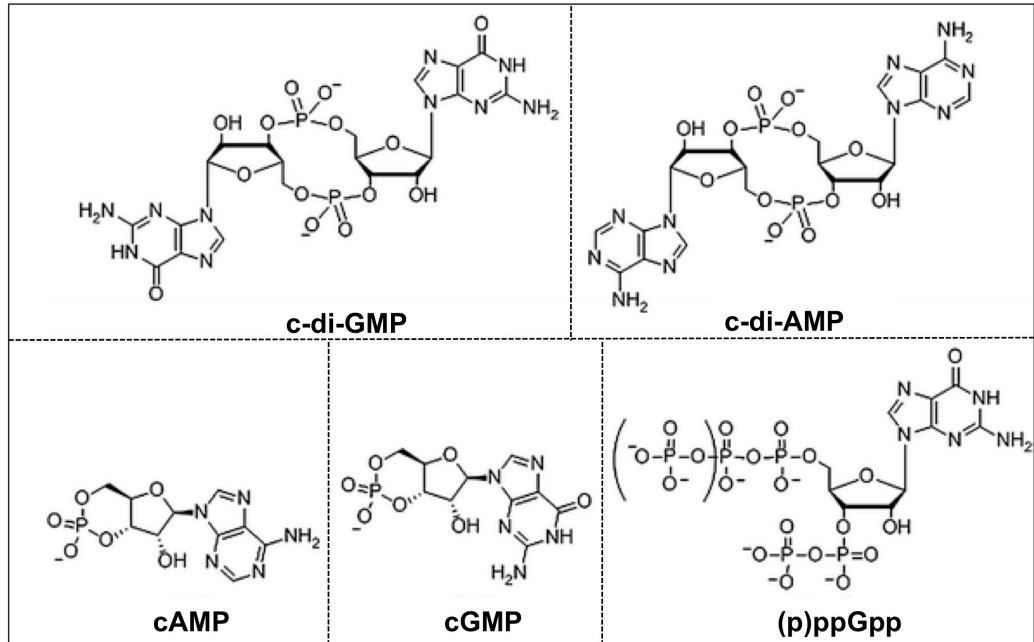
Figure 1.1. Structures of nucleotide second messengers. *Adapted from* (67)
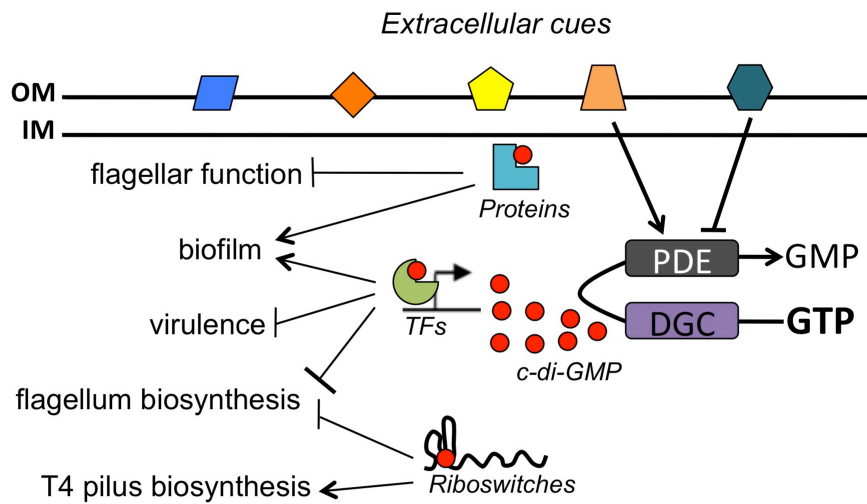
Figure 1.2. c-di-GMP regulated processes in bacteria. In response to extracellular cues, intracellular levels of c-di-GMP (red circles) are modulated by the activity of DGC and PDE enzymes. Intracellular c-di-GMP receptors include protein effectors, transcription factors and riboswitches. Modulation of c-di-GMP levels controls the transition between sessile to motile lifestyles.
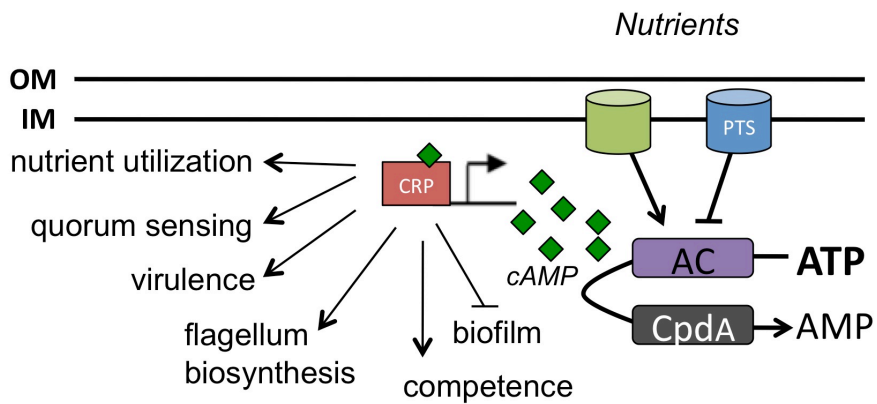
Figure 1.3. cAMP regulated processes in bacteria. In response to nutrient availability, intracellular levels of cAMP (green diamonds) are modulated by the activity of AC and CpdA enzymes. CRP is the receptor for cAMP and together they regulate the expression of genes required for nutrient utilization and other important bacterial processes.

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CHAPTER 2: THE RNA DOMAIN VC1 REGULATES DOWNSTREAM GENE EXPRESSION IN RESPONSE TO CYCLIC DIGUANYLATE IN *VIBRIO CHOLERAE*

Summary

In many bacterial species, including the aquatic bacterium and human pathogen *Vibrio cholerae*, the second messenger cyclic diguanylate (c-di-GMP) modulates processes such as biofilm formation, motility and virulence factor production. By interacting with various effectors, c-di-GMP regulates gene expression or protein activity. One class of c-di-GMP receptors is the GEMM riboswitch, representatives of which have been shown to bind c-di-GMP *in vitro*. Herein, we examined the *in vitro* and *in vivo* function of a putative GEMM riboswitch in *Vibrio cholerae* called Vc1, which lies upstream of the gene encoding GbpA, a colonization factor that contributes to attachment of *V. cholerae* to environmental and host surfaces containing N-acetylglucosamine moieties. We provide evidence that Vc1 RNA interacts directly with c-di-GMP *in vitro*, but with possibly via residues different from those in a canonical GEMM riboswitch. By isolating the regulatory function of Vc1, we show that the Vc1 element positively regulates downstream gene expression in response to c-di-GMP and that the mutation of a conserved residue in Vc1 reduces the interaction *in vitro* and *in vivo*. The same mutation on the *V. cholerae* chromosome significantly reduced the expression of *gbpA* and production of the GbpA protein. Together these data suggest

that the Vc1 element responds to c-di-GMP *in vivo*, but may not function as a high affinity riboswitch. Positive regulation of *gbpA* expression by c-di-GMP via Vc1 may influence the ability of *V. cholerae* to associate with chitin in the aquatic environment and the host intestinal environment.

Importance

Cyclic diguanylate (c-di-GMP) has pleiotropic effects on bacterial physiology and broadly impacts gene expression. With the exception of a handful of transcription factors that have been shown to regulate gene expression in response to c-di-GMP, the molecular basis of gene regulation by c-di-GMP is poorly understood. The identification of c-di-GMP specific riboswitches distributed widely among bacterial genomes positions these regulatory RNA domains to serve as important c-di-GMP effectors modulating downstream gene expression. Elegant studies have biochemically characterized a representative GEMM riboswitch, Vc2 from *Vibrio cholerae*, but the functionality of c-di-GMP riboswitches in their native genetic contexts has been largely unexplored. This study investigates whether the putative c-di-GMP riboswitch Vc1 from *V. cholerae* is functional *in vitro* and *in vivo*, and reveals important differences from the canonical riboswitch Vc2.

Introduction

Cyclic diguanylate (c-di-GMP) is a ubiquitous second messenger important for bacterial adaptation to environmental conditions. In response to largely undefined signals at the cell surface, changes in intracellular c-di-GMP concentration can relay information to target effectors, regulating processes such as biofilm formation, motility and virulence gene expression (reviewed in (1-3). The intracellular level of c-di-GMP is

controlled by the activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), enzymes responsible for the synthesis and degradation of c-di-GMP, respectively (4-10). Bacterial genomes often contain multiple genes encoding putative DGC and PDE enzymes, potentially reflecting a requirement for tight regulation of c-di-GMP levels and the need to modulate c-di-GMP in response to diverse extracellular cues.

Various classes of intracellular c-di-GMP receptors have been identified and can control c-di-GMP regulated processes via transcriptional, post-transcriptional or post-translational mechanisms. The best characterized are protein-based sensors of c-di-GMP, such as transcriptional regulators and proteins containing the PilZ domain. In the human diarrheal pathogen, *Vibrio cholerae*, transcriptional regulation can occur through the transcription factors VpsT and VpsR, which directly sense c-di-GMP and modulate the expression of target genes involved in biofilm development (11-14). Recently, the master regulator of motility genes FlrA was found to sense c-di-GMP (15). Binding of c-di-GMP to FlrA reduced the interaction between the transcription factor and a target flagellar gene promoter, thus decreasing flagellar gene expression and, in turn, bacterial motility. Analysis of the five PilZ domain proteins encoded by *V. cholerae* indicate that at least two of the PilZ proteins can bind c-di-GMP, and that the PilZ proteins regulate motility, biofilm formation and virulence of *V. cholerae*, likely through post-transcriptional mechanisms (16).

In addition to protein sensors of c-di-GMP, post-transcriptional regulation by c-di-GMP can occur through riboswitches, *cis* acting regulatory elements found in the 5' untranslated region (UTR) of some mRNA (17-19). Riboswitches consist of an aptamer

domain that binds a specific ligand and an adjacent expression platform that regulates downstream gene expression in response to ligand binding. Regulation typically occurs as a result of ligand-induced conformational changes in the RNA structure (20, 21). Most commonly, riboswitches regulate gene expression through control of either premature transcriptional termination or translational initiation. Two types of c-di-GMP sensing riboswitches have been identified, class I and class II (17, 22). The two classes share no structure or sequence homology. The class I c-di-GMP riboswitches contain a GEMM motif, which is widespread in bacteria and so named because it often resides in the 5' UTR of genes with functions predicted to relate to the environment, membrane or motility (17, 23). Based on the co-crystal structure of c-di-GMP with the *V. cholerae* aptamer Vc2, GEMM motifs are predicted to contain two adjacent stem-loops, termed P2 and P3, a tetraloop-tetraloop receptor motif that stabilizes the interaction between P2 and P3, and a P1 stem that forms through base pairing between the flanking 5' nucleotides of P2 and the flanking 3' nucleotides of P3 (17, 19, 23). The nucleotides of Vc2 that contact c-di-GMP lie at the junction of P1, P2, and P3.

Although the aptamer of the GEMM riboswitch has been defined and some specific nucleotides required for efficient gene expression identified (17), few studies have directly addressed the functionality of a c-di-GMP riboswitch *in vivo*, in its native genetic context, and the impact of c-di-GMP sensing on the physiology of a bacterium. *Clostridium difficile* encodes numerous putative c-di-GMP riboswitches (both class I and class II), some of which lie upstream of genes encoding surface proteins and organelles such as flagella and Type IV pili (17, 22). Artificial elevation of c-di-GMP in *C. difficile* represses flagellar gene expression and swimming motility (24-26). These findings are

consistent with prior work showing that the GEMM riboswitch Cd1 upstream of a flagellar operon functions as an "off switch" in response to c-di-GMP; mutations in Cd1 that impair its interaction with c-di-GMP result in increased reporter gene expression in a heterologous bacterial host (17). Conversely, the class II riboswitch upstream of a pilin gene functions as an "on switch" in response to c-di-GMP in *C. difficile* (27). C-di-GMP positively regulates type IV pilin gene expression through direct interaction with the riboswitch, promoting cell aggregation.

The biochemistry and function of the second predicted GEMM riboswitch in *V. cholerae,* called Vc1, has not been examined. The predicted GEMM motif of Vc1 has 75% overall identity with the well-characterized Vc2 GEMM motif, with 85% identity in the region that encodes the first stem-loop, P2 (See Figure 1A). The residues that contact the c-di-GMP ligand in Vc2 (G20, A47 and C92) are conserved in Vc1 (G12, A39, and C104, respectively). The nucleotides that form the interhelical Watson-Crick base pair, universally conserved among GEMM riboswitches, are also present in Vc1 (C36 and G90 in P2 and P3 of Vc1, respectively). While critical features of the GEMM riboswitch are conserved in Vc1, the differences between Vc2 and Vc1 may help define broadly the mechanisms by which c-di-GMP acts through GEMM riboswitches.

Vc1 lies upstream of *gbpA*, which encodes a well-characterized *V. cholerae* colonization factor. GbpA is a secreted protein that aids in colonization of surfaces in aquatic environments (the natural habitat of *V. cholerae*) and the small intestine (the tissue colonized by pathogenic *V. cholerae*) (28). GbpA recognizes chitin, a polymer of N-acetylglucosamine (GlcNAc) found in the exoskeletons of zooplankton and crustaceans colonized by *V. cholerae* in aquatic reservoirs (28). GbpA also plays a role

in colonization of the small intestine by interacting with GlcNAc present in mucin and on the surface of intestinal epithelial cells (28, 29). Accordingly, *V. cholerae* mutants lacking *gbpA* are attenuated both in an animal model of infection and attachment to chitinous surfaces. Studying Vc1 may thus provide insight about the role of c-di-GMP and Vc1 in modulating attachment of *V. cholerae* to environmental and host surfaces.

In this study, we combine biochemical and genetic approaches to test the hypothesis that the Vc1 sequence upstream of the *gbpA* open reading frame functions as a riboswitch to control *gbpA* expression in response to c-di-GMP. We provide evidence that Vc1 directly interacts with c-di-GMP in vitro, and interfering with Vc1 sensing of c-di-GMP impairs downstream gene expression in vivo. Vc1 has properties that distinguish it from the GEMM c-di-GMP riboswitch characterized to date, but is nonetheless a c-di-GMP responsive RNA element. These data suggest that c-di-GMP signaling through Vc1 promotes *gbpA* expression and GbpA-dependent adherence to host and environmental surfaces.

Materials and Methods

Growth conditions and media. *V. cholerae* C6706 and isogenic mutant strains (Table S1) were cultured at 37°C in Luria-Bertani (LB) broth containing 100 µg/ml streptomycin (Sm), 10µg/ml chloramphenicol (Cm), and/or 50 µg/ml ampicillin (Amp), as appropriate.

Artificial manipulation of intracellular c-di-GMP. The reduction of the intracellular level of c-di-GMP in *V. cholerae* was achieved as described previously (30,

31). Briefly, overnight cultures of *V. cholerae* harboring pBAD33 ("vector"),

pBAD33::*vieA* ("pPDE"), pBAD33::*vieA-E170A* ("pPDE^{mut}") or pBAD33::VCA0956

("pDGC") were back-diluted 1:100 in LB-Cm broth and grown at 37°C with shaking.

Induced cultures contained 0.2% L-arabinose unless otherwise specified. Samples were

collected for western blotting, β-galactosidase assays and/or qRT-PCR analysis as

described below.

5' Rapid Amplification of cDNA Ends (RACE). RNA was collected using

TRIsure (Bioline) and the RNeasy kit (Qiagen) as described previously (32). A *gbpA*

specific primer, gbpAsp1, was used to make cDNA from RNA in a reverse transcription

reaction with the Tetro cDNA Synthesis Kit (Bioline), using the manufacturer's protocol.

Next, Terminal transferase, TdT (NEB), was added to the reaction to introduce a

homopolymeric A sequence to the 5' end of the cDNA molecules, using the

manufacturer's protocol. PCR using nested primers specific to the 5' homopolymeric tail

(Race1g, Race1c, Race1a) and *gbpA* (gbpAsp2) were used to amplify the cDNA

products. Sequencing of amplified cDNA products identified the +1 site of transcription.

Genetic manipulations. Strains and plasmids used in this study are listed in

Table S1. All oligonucleotide primers used for cloning are listed in Table S2. Details

regarding the generation of strains used in this study are described in the Supplemental

Methods.

GbpA antibody production. Anti-GbpA antiserum was produced by Yenzym 192 Antibodies, LLC, South San Francisco, CA. Antiserum was raised in rabbits to a synthetic peptide (CSNATQYQPGTGSHWEMAWDKR) that corresponds to GbpA from *V. cholerae*. The animal facilities were NIH/OLAW/PHS assured, USDA certified, and IACUC regulated.

Western blot analysis. Overnight cultures were diluted 1:100 and grown in LB broth at 37°C with aeration until mid-exponential phase (OD_{600} ~ 0.6-0.8). Equal volumes of supernatant, normalized to OD_{600}, were collected, and proteins were precipitated using 10% trichloroacetic acid (TCA). TCA-precipitated samples were separated by electrophoresis, transferred onto nitrocellulose membranes, and probed with rabbit anti-GbpA antibodies. Goat α-rabbit IgG conjugated with IR800 dye (Thermo Scientific) was used as the secondary antibody. Membranes were imaged using an Odyssey imaging system (LI-COR). At least three independent experiments were performed, and a representative image is presented. Densitometry analyses were carried out using Odyssey software by normalizing the intensities of the bands corresponding to GbpA to those of a cross reactive band that did not change intensity in any of the strains or conditions tested (indicated by asterisks in relevant images).

***In vitro* transcription.** For SHAPE analysis, *V. cholerae* C6706 genomic DNA was used as the template in PCR with primers T7linkF + T7R, yielding a product consisting of the T7 promoter and the -15 to +665 portion of the *gbpA* transcript. The PCR product was used as template for *in vitro* transcription of the RNA using the

Ambion MEGAscript® T7 Kit, according to the manufacturer's instructions. The RNA was precipitated with ethanol and suspended in water. RNAs were resolved on a denaturing 6% polyacrylamide gel (1X TB, 7M urea, 6% acrylamide). The desired RNA bands were excised from the gel and placed in RNase-free water overnight at 4°C to elute the RNA. The eluted material was ethanol precipitated to recover RNA, and the RNA was suspended in TE buffer.

For equilibrium dialysis, $Vc1^{WT}$ and $Vc1^{G12T}$ templates used for the transcription reactions were amplified from C6706 and C6706 $Vc1^{G12T}$ genomic DNA, respectively, using T7Vc1F + Vc1R3. $Vc2^{WT}$ and $Vc2^{G20T}$ were amplified from C6706 genomic DNA and pCVD442::$Vc2^{G20T}$, respectively, using T7Vc2F + T7Vc2R. RNA was transcribed from the resulting DNA as described above, yielding products with the T7 promoter and the -15 to +140 region of the *gbpA* 5' UTR (Vc1 and $Vc1^{G12U}$; numbering according to 5' RACE results) or the -2 to +209 region of the VC1722 transcript (Vc2 and $Vc2^{G20U}$; numbering according to 3IRW entry in PDB).

SHAPE analysis of the *gbpA* mRNA. Vc1 RNA (10 µl, 7 µM) was denatured by heating at 95 $^{\circ}$C for 2 minutes and snap-cooled on ice for 2 minutes. RNA (2 µl) was combined with 6 µl of 3.3× folding buffer (33 mM HEPES, 333 mM $MgCl_2$, 333 mM NaCl, pH 8), 2 µl of water and either 10 µl of water or 10 µl of 1 mM c-di-GMP(33). The RNA was folded at 37 $^{\circ}$C for 30 min. Folded RNA (9 µl) was added to 1 µl of 30 mM 1M7 (in DMSO) or to 1 µl DMSO only, immediately mixed thoroughly, and incubated at 37°C for 3 min. RNA was precipitated with ethanol and suspended in TE buffer. SHAPE adducts were detected by primer extension as described (33). 1M7 and DMSO treated

RNA, as well as untreated RNA (later used for sequencing), were mixed with fluorescently labeled DNA oligonucleotides that anneal to *gbpA* (Vc1SHAPE_R). Primer extension was initiated by addition of Superscript III reverse transcriptase (Invitrogen). Sequence information was generated using unmodified RNA by performing primer extension in the presence of dideoxynucleotide triphosphates (ddNTPs). Primer extension products were ethanol precipitated and suspended in formamide. Products were resolved on an ABI 3500 capillary electrophoresis instrument and analyzed using QuShape (34). Each experiment was performed at least three times, and statistical significance was determined using Student's t-test.

Equilibrium dialysis. *In vitro* transcribed Vc1, Vc1^{G12U}, Vc2 and Vc2^{G20U} RNA were denatured by heating at 95°C for 2 minutes and snap-cooled on ice for 2 minutes. RNA was then combined with 6 µl of 5X folding buffer (50 mM HEPES, 500 mM MgCl$_2$, 500 mM NaCl, pH 8) and added in a 30 µl total volume to chamber A of an equilibrium dialysis device, Dispo Equilibrium Dialyzer (Harvard Apparatus). c-di-GMP^{32} was diluted 1:50, combined with 6 µl of 5× folding buffer, and added in a 30 µl total volume to chamber B of the equilibrium dialysis device. The final concentrations of RNA and ligand in the binding reactions were: 15 nM c-di-GMP^{32}, 20 mM Vc1 and Vc1^{G12U}, and 10 mM Vc2 and Vc2^{G20U}. Samples were allowed to equilibrate for 10 hours, and then 2 µl were removed for scintillation counting. For competition experiments, following 10 hours equilibration, the contents of chamber B were removed and replaced with 100 µM GTP or 100 µM c-di-GMP diluted in 5X folding buffer. Samples were allowed to equilibrate for an additional 10 hours, then 2 µl were removed for scintillation counting.

Binding was calculated as the percentage of the total radioactivity present in the chamber (A) containing RNA. Each experiment was repeated independently at least three times.

RNA isolation and quantitative real-time PCR. Transcriptional analyses by quantitative reverse transcriptase PCR (qRT-PCR) were done essentially as previously described, using mid-exponential phase (OD_{600} ~ 0.5-0.7) cultures (32). For cDNA synthesis, 200 ng of RNA was reverse transcribed using the Tetro cDNA Synthesis Kit with random hexamers as primers (Bioline). As negative controls, reactions were run without reverse transcriptase. cDNA samples were combined with 2× SYBR/fluorescein mix (SensiMix; Bioline), 7.5 µM of each primer (RPB2F + RPB2R for *rpoB*, gbpAqF2 + gbpAqR2 for the *gbpA* ORF) (Table S2). The reactions were run using a MyiQ thermocycler (Bio-Rad) and the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. Melt curves were performed to verify the amplification of single products, and all values were adjusted for primer binding efficiency, as calculated from a standard curve of C6706 genomic DNA. Data were analyzed using the ΔΔCt method, with values normalized to the specified reference strain/condition, and transcript levels normalized to those of the housekeeping gene *rpoB* in each sample (32, 35). For each strain a minimum of three biological samples was tested. Statistical significance was determined by unpaired t-test.

Beta-galactosidase assays. Dilutions of overnight cultures were assayed for β-galactosidase activity using ortho-nitrophenyl-β-D-galactoside as a substrate as

described previously (36). At least three independent experiments were done and the data were combined. Statistical analyses were done using unpaired t-tests.

Supplemental Methods

Genetic manipulations and strain construction. To make deletions by allelic exchange, using genomic DNA from *V. cholerae* C6706, ~800 bp fragments upstream and downstream of the sequences to be deleted were amplified by PCR using primers named according to the pattern geneF1 + geneR1 for the upstream region of homology and geneF2 + geneR2 for the downstream region of homology. The primers introduced restriction sites (underlined sequences in Table S2) that allowed ligation of the two PCR products to each other and into the suicide vector pCVD442. The ligations were transformed into DH5αλ*pir* by electroporation and transformed colonies were identified on LB-Amp agar. The desired clones that contain the upstream and downstream fragments were identified by PCR using primers geneF1 + geneR2 and/or pCVDseqF + pCVDseqR, which flank the multiple cloning site of pCVD442. For deletion of *gbpA*, the resulting pCVD442::Δ*gbpA* plasmid was transformed into Sm10λ*pir* by electroporation and grown on LB-Amp agar. The resulting strain was mated with *V. cholerae* C6706 on LB agar for ~8hrs, then transconjugants were selected on LB-Sm-Amp agar. Sm-Amp-resistant transconjugants were grown in LB broth in the absence of selection for 8 hours, then dilutions of the cultures were plated on LB-Sm agar containing 10% sucrose. Sucrose-resistant, Amp-sensitive colonies were screened for the relevant deletion by PCR using the corresponding geneF0 + geneR2 primers. To create a *V. cholerae* strain with a deletion of Vc1 (ΔVc1), fragments upstream and downstream to

Vc1 were amplified using Vc1F + placP2R and placP2F + Vc1R primers, respectively,

using genomic DNA from *V. cholerae* C6706 as the template. The upstream and

downstream fragments were digested with the relevant enzymes and ligated into

pCVD442. The ligation was transformed into DH5αλ*pir* by electroporation and

transformed colonies were identified on LB-Amp agar. The desired clone was identified

by PCR using primers pCVDseqF + pCVDseqR. The resulting pCVD442::ΔVc1 plasmid

was transformed into Sm10λ*pir* by electroporation, then mated with *V. cholerae* C6706

on LB agar. Mutants containing the Vc1 deletion were made and identified as described

above.

Point mutations in Vc1 and Vc2 were generated by incorporating the desired

nucleotide changes into self-complementary primers. These primers were used in

separate PCRs to amplify two, overlapping fragments which were then cloned into the

allelic exchange vector pCVD442. Genomic DNA from *V. cholerae* C6706 was used in

all cases. Specifically, to generate the "P1" mutation consisting of CACAC to GTGTG

mutations in the putative P1 stem of Vc1, the upstream fragment was amplified using

Vc1F + VC1BR and the downstream fragment with primers Vc1BF + Vc1R. A similar

technique was used to create point mutations in Vc1. For the Vc1^{G12T} mutant, primer

combinations Vc1F + Vc1gTR and Vc1gTF + Vc1R were used to amplify the two

overlapping fragments; for the Vc1^{A39T} mutant, Vc1F + Vc1aTR and Vc1aTF + Vc1R

were used; for the Vc1^{C104G} mutant, Vc1F + Vc1cGR and Vc1cGF + Vc1R were used;

and for Vc2^{G20T}, Vc2G20TF1 + Vc2G20TR1 and Vc2G20TF2 + Vc2G20TR2 were used.

For all four Vc1 mutants, the resulting pairs of fragments were combined and used as

the template in PCR reactions with Vc1F and Vc1R as primers, to yield a spliced

product containing the desired nucleotide changes. For the Vc2 mutation, Vc2G20TF1 +

Vc2G20TR2 were used to obtain the spliced product. The spliced PCR products were

digested with the appropriate restriction enzymes, for which restriction sites were

introduced with the outer primers, then cloned into similarly digested pCVD442.

Plasmids containing insert were identified by PCR, and point mutations were confirmed

by sequencing. The confirmed plasmids were introduced into *V. cholerae* C6706 via

SM10λ*pir*, and the desired *V. cholerae* mutants were identified, as described above. All

mutations to the *V. cholerae* chromosome were confirmed by sequencing.

The plasmid-borne *lacZ* reporter constructs were made by amplifying the 5' UTR of

gbpA from genomic DNA of *V. cholerae* C6706 "Vc1^{WT}", Vc1^{P1}, Vc1^{G12T}, Vc1^{A39T}, or

Vc1^{C104G} using primers gbpArbF and gbpAPrR and digested with *Eco*RI and *Sal*I. The

Vc1 fragments were ligated into similarly-digested pP*lacthiM#2-lacZ*, then ligations were

transformed into DH5α. Amp-resistant clones were screened by PCR using primers

pLacSeq + gbpAPrR. The resulting plasmids have Vc1 (wild type or mutant) as a

translational fusion to *lacZ*, with the *lac* promoter driving transcription. Likewise, the

pP*gbpA*-Vc1-*lacZ* and pP*gbpA*-ΔVc1-*lacZ* plasmids were constructed by amplifying

P*gbpA*-ΔVc1-Vc1 from *V. cholerae* C6706 and DVc1, respectively, by PCR using

gbpAP2F + gbpAR2. The PCR products were digested with *Eco*RI and *Sal*I and ligated

into pP*lacthiM#2-lacZ* digested with the same enzymes. The ligation reaction was

transformed into DH5α cells by electroporation, and resulting Amp-resistant colonies

were screened with primers pLacSeq + gbpAR2.

To make the chromosomal Vc1 reporter strains, the pP*lac*-Vc1-*lacZ* and pP*lac*-

Vc1^{G12T}-*lacZ* vector backbones were amplified to exclude the P*lac* promoter gbpArbF +

PlacZR3, which introduced *Eco*RI and *Sph*I sites, respectively. The PlacUV5 promoter

was amplified from p2UY35A using PlacUV5F + PlacUV5R, which also introduced *Sph*I

and *Eco*RI sites, respectively (37). The PlacUV5 promoter and Vc1-*lacZ* vector (now

lacking Plac) were digested with *Sph*I and *Eco*RI, ligated together, and cloned in *E. coli*

DH5α, yielding plasmid pPlacUV5-Vc1UTR-*lacZ* and pPlacUV5-Vc1UTR^{G12T}-*lacZ*.

To make the chromosomal Vc2 reporter strain, the pP*lac*-Vc1-*lacZ* vector

backbone was amplified to exclude the P*lac* promoter and the Vc1 riboswitch using

placZF + PlacZR3, which introduced *Sal*I and *Sph*I sites, respectively. The Vc2

riboswitch, found in the 5' UTR of VC1722, was amplified from genomic DNA of *V.*

cholerae C6706 using Vc2rsF and Vc2Rrs2, which introduce *Eco*RI and *Sal*I sites,

respectively. The PlacUV5 promoter was amplified from p2UY35A using PlacUV5F +

PlacUV5R, which also introduced *Sph*I and *Eco*RI sites, respectively (37). The PlacUV5

promoter, Vc2 riboswitch and the *lacZ* vector (now lacking Plac and Vc1) were digested

with *Sph*I and *Eco*RI, *Sal*I and *Eco*RI, and *Sph*I and *Sal*I, respectively. The three

products were ligated together, and cloned in *E. coli* DH5α, yielding plasmid pPlacUV5-

Vc2UTR-*lacZ*.

The resulting plasmids, pPlacUV5-UTR-*lacZ*, pPlacUV5-UTR^{Vc1G12T}-*lacZ,* and

pPlacUV5-Vc2UTR-*lacZ*, were used as the template for amplification of the fusions with

primers PlacF5 + PlacR5, which introduced *Stu*I and *Not*I sites, respectively. The PCR

products were digested with *Stu*I and *Not*I and cloned into similarly digested pJL1, a

suicide vector which allows targeting of DNA into the *lacZ* gene of *V. cholerae* (38). The

pJL1 constructs were introduced into *V. cholerae* C6706 by conjugation via SM10λ*pir E.*

coli. Incorporation of the fusions in the *V. cholerae lacZ* gene was achieved by standard

allelic exchange methods and confirmed by PCR with lacZsF +lacZsR and by sequencing. The resulting chromosomal reporter strains were transformed with vector, pPDE and pPDE^{mut} plasmids by electroporation to allow manipulation of c-di-GMP (9, 31).

Results

The predicted structure and c-di-GMP binding sites of Vc1. We first defined the 5' UTR of *gbpA* by identifying the transcriptional start site using 5' Rapid Amplification of cDNA Ends (5' RACE). To rule out potential differences in the length of the *gbpA* 5' UTR in response to c-di-GMP, 5' RACE was performed with RNA collected from strains with both wild type and low c-di-GMP levels. The intracellular level of c-di-GMP in *V. cholerae* was reduced by ectopic expression of a c-di-GMP PDE. Briefly, *V. cholerae* harboring a plasmid that allows inducible expression of the well-characterized c-di-GMP PDE, VieA, was used to test the effect of depleting intracellular c-di-GMP (9, 31). *V. cholerae* containing vector only, treated identically to *V. cholerae* with pPDE, serves as an unperturbed "wild-type" c-di-GMP control. 5' RACE showed that the transcriptional start site (+1) remains unchanged between *V. cholerae* with native and low c-di-GMP levels and is 225 nucleotides upstream of the annotated translational start site of *gbpA* (Figure 2.1A). The predicted GEMM motif of Vc1 is thus encoded within the first 111 nucleotides of the *gbpA* 5' UTR. Using this information and the GEMM consensus sequence and structure, the RNA structure of the first 120 bases of the *gbpA* 5'UTR, which encompass the GEMM motif of Vc1, was modeled and is consistent with the accepted secondary structure of Vc2 (Figure 2.1A and 2.1B).

Mutations in predicted c-di-GMP contact residues of Vc1 affect downstream gene expression. To examine the role of Vc1 in regulating gene expression in response to c-di-GMP, we constructed a translational reporter consisting of the *gbpA* 5' UTR fused to *lacZ* from *Escherichia coli* (Figure 2.7). The heterologous promoter P_{lac} drove expression, allowing constitutive, P_{gbpA} independent transcription initiation during growth in rich medium. We generated four derivatives of the reporter plasmid, each with a mutation in Vc1 predicted to interfere with sensing c-di-GMP. The "Vc1^{P1}" derivative contains a stretch of five nucleotide changes in Vc1: C4G, A5T, C6G, A7T, C8G. The other three mutant reporters each contain a single point mutation in a nucleotide predicted to interact directly with the c-di-GMP ligand: G12T, A39T and C104G (noted in Figures 2.1A and 2.1B) (19). The plasmids were introduced into *V. cholerae*, and reporter activity was measured using a β-galactosidase assay. Relative to the wild type reporter, the Vc1^{P1}, G12T and A39T mutations significantly reduced, but did not eliminate, β-galactosidase activity (Figure 2.2A). These results suggest that G12 and A39 may be important for sensing c-di-GMP and for full expression of the downstream gene. Conversely, the C104G mutation caused a modest increase in β-galactosidase activity, suggesting it is not required for sensing c-di-GMP. To confirm this result, an alternate mutation, C104A, was made, but this mutation also did not have an effect on β-galactosidase activity (data not shown).

We next assessed the functionality of Vc1 in its native genetic context. The four mutations described above, P1, G12T, A39T and C104G, were individually introduced into the chromosomal Vc1 in *V. cholerae* C6706 via allelic exchange, and GbpA

production was measured by western blot. A strain with an in-frame deletion of *gbpA* served as a control. Consistent with the β-galactosidase reporter assays, the level of GbpA decreased significantly in the P1 and G12T mutants (Figure 2.2B and 2.2C). GbpA protein abundance was not significantly altered in the A39T and C014G mutants. Thus, unlike in Vc2, in which each of the equivalent nucleotides plays critical roles in c-di-GMP sensing, the G12 mutation in Vc1 is the only one to significantly impact downstream gene expression.

Evidence that Vc1 interacts directly with c-di-GMP *in vitro*. To test whether Vc1 interacts with c-di-GMP, we used several approaches. First, we used selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) chemistry to probe the potential interaction between Vc1 and c-di-GMP (39). SHAPE uses acylating agents, such as 1-methyl-7-nitroisatoic anhydride (1M7), which selectively form adducts at the 2'-hydroxyl group of an RNA nucleotide. The ability of 1M7 to react with a nucleotide depends on the local flexibility of that nucleotide within an RNA structure, and flexibility correlates with the constraint of the nucleotide within the structure (40). SHAPE has been used successfully to assess the interactions between several riboswitches and their target ligands, including the Vc2 aptamer and c-di-GMP (33, 41). A comparison of structures for a GEMM riboswitch in the presence and absence of c-di-GMP has not been reported to date, and we used SHAPE chemistry to assess the differences between *apo*-Vc1 and Vc1 with c-di-GMP.

An *in vitro* transcript of the *gbpA* mRNA, encompassing the entire 5' UTR of *gbpA* and 440 bases of the open reading frame (ORF), was folded in the presence or

absence of c-di-GMP, subjected to 1M7 modification and analyzed by SHAPE. An RNA sequence that extends downstream of the Vc1 aptamer was used because of the possibility that neighboring RNA sequence impacts the structure of the riboswitch. Statistically significant differences in reactivity were observed in three distinct regions of the GEMM motif in the presence and absence of c-di-GMP (Figure 2.3, asterisks). Nucleotides U9, U10, and U11, located adjacent to the first predicted c-di-GMP contact residue G12, showed a significant increase in reactivity in the presence of c-di-GMP. A40 showed a decrease in reactivity in the presence of c-di-GMP and is adjacent to the second predicted contact residue for c-di-GMP, A39. An increase in reactivity in the presence of c-di-GMP was also observed in the "P1" region immediately 5' of Vc1. No changes were observed near or around C104. As a control, we performed SHAPE with Vc1 with and without another guanosine nucleotide, GTP. No statistically significant differences in Vc1 reactivity were observed in the presence of GTP relative to *apo*-Vc1, indicating that GTP and Vc1 do not interact (Figure 2.8). Together these results suggest that c-di-GMP specifically interacts with Vc1 via the G12 and A39 regions, but not the C104 region, though indirect effects of c-di-GMP on the reactivity of the G12 and A39 regions are also possible.

We next used equilibrium dialysis to assess the interaction between Vc1 RNA and c-di-GMP. These experiments were conducted using a two-chamber device (separated by a 5000 Da MWCO membrane) in which RNA was added to one chamber and ^{32}P-labeled c-di-GMP (c-di-GMP^{32}) was added to the second. After 10 hours incubation to allow equilibration of c-di-GMP^{32} between chambers, samples from each chamber were analyzed by scintillation counting. Binding is calculated as the

percentage of the total radioactivity present in the chamber containing RNA. If no

binding of c-di-GMP^{32} occurs, c-di-GMP^{32} will distribute equally between the two

chambers; if RNA capable of binding c-di-GMP^{32} is present, c-di-GMP^{32} will accumulate

in that chamber. As expected, when no RNA was added, c-di-GMP^{32} was detected

equally in both chambers (Figure 2.4A). Vc2 RNA, which served as a positive control,

sequestered c-di-GMP^{32}, indicating that Vc2 binds c-di-GMP as expected (17-19, 42).

Vc1 RNA also sequestered c-di-GMP^{32}, but less so than Vc2 RNA (77% versus 66% of

c-di-GMP in the RNA chamber, respectively), suggesting that Vc1 RNA binds c-di-GMP

but perhaps with a lower affinity than Vc2 (Figure 2.4A). Analysis of the denatured and

refolded RNAs by native gel electrophoresis revealed multiple folded species for Vc1

and Vc2, both in the presence and absence of c-di-GMP (data not shown), which may

also have contributed to differences in c-di-GMP sequestration.

We showed above that the G12T mutation in Vc1, but not the A39T or C104G

mutations, reduces downstream gene expression, as measured both in reporter assays

and in chromosomal mutants (Figure 2.2). The binding of the mutant Vc1 RNAs to c-di-

GMP was assessed using equilibrium dialysis to determine whether the mutants have

similar profiles *in vitro*. The G12U mutation in the Vc1 RNA significantly diminished

sequestration of c-di-GMP, but did not fully eliminate it, unlike the equivalent mutation in

Vc2 (Figure 2.3B). That the Vc1^{G12U} mutant RNA retained some ability to sequester c-di-

GMP^{32} indicates that this residue is not absolutely required for c-di-GMP binding.

We examined the specificity of Vc1 for c-di-GMP using a two-step equilibrium

dialysis binding experiment. Vc1, as well as the Vc2 positive control, were allowed to

interact with radiolabeled c-di-GMP for 10 hours as described above, and the proportion

of c-di-GMP sequestered in the RNA-containing chamber was measured (Figure 2.4B, black bars). For the second step, the non-sequestered radiolabeled c-di-GMP was removed, and excess non-radiolabeled ("cold") c-di-GMP or GTP was added. After an additional 10 hours to allow binding, the remaining radioactivity associated with the RNA was assessed (Figure 2.4B, grey bars). The interaction between c-di-GMP^{32} and Vc1 is specific, because addition of unlabeled c-di-GMP, but not unlabeled GTP, shifted the distribution of c-di-GMP^{32}; cold c-di-GMP, but not GTP, competed with radiolabeled c-di-GMP for binding with Vc1 (Figure 2.4B). Interestingly, addition of unlabeled c-di-GMP did not compete with c-di-GMP^{32} for Vc2 binding over the timescale of the dialysis assay, consistent with previous work showing that c-di-GMP dissociates very slowly from the Vc2 RNA (42). Conversely, Vc1 may have a faster off rate than Vc2, as c-di-GMP^{32} could be displaced by the added unlabeled c-di-GMP over the 10 hour time course of the assay.

Decreasing intracellular c-di-GMP reduces Vc1-dependent gene expression. The data presented above suggest that c-di-GMP signaling through Vc1 promotes downstream gene expression. We therefore determined the effect of altering the intracellular c-di-GMP concentration on GbpA production, using plasmid-borne, inducible diguanylate cyclase (pDGC) and phosphodiesterase (pPDE) enzymes to artificially increase or decrease intracellular c-di-GMP levels in *V. cholerae*, respectively (9, 43). For this study, the *gbpA* 5'UTR containing Vc1 was fused to a *lacZ* reporter gene and placed under the control of the constitutive P$_{lacUV5}$ promoter (37). This translational reporter was integrated into the *V. cholerae* chromosome within the native

lacZ gene, generating the "P*lacUV5*-Vc1UTR-*lacZ*" strain. Thus, the response of the Vc1

element to c-di-GMP could be assessed in isolation from the native promoter and

downstream gene, in single copy. A promoterless *gbpA* 5' Vc1UTR-*lacZ* fusion strain

served as a control to ensure that sequences within the 5' UTR do not initiate

transcription. pDGC, pPDE, pPDE^{mut} and control vector were introduced into the

reporter strains to allow manipulation of c-di-GMP as described (9, 43). As expected,

the strains with the promoterless fusion had baseline β-galactosidase activity,

regardless of c-di-GMP level (Figure 2.9). The *V. cholerae* P*lacUV5*-Vc1UTR-*lacZ* strain

with reduced intracellular c-di-GMP (pPDE, + L-arabinose) showed a greater than 50%

decrease in β-galactosidase activity compared to *V. cholerae* with wild-type levels of c-

di-GMP (vector, + L-arabinose) (Figure 2.5). Conversely, increasing intracellular c-di-

GMP through the ectopic production of a diguanylate cyclase (VCA0956) significantly

increased β-galactosidase activity (Figure 2.9). In addition, expression from pPDE^{mut}, in

which a glutamic acid residue essential for PDE activity has been mutated to an alanine

(VieA-E170A) (9), resulted in no change in β-galactosidase activity, indicating that

altered expression is specifically due to decreased c-di-GMP (Figure 2.9).

To further examine the importance of the Vc1- c-di-GMP interaction in promoting

downstream gene expression, we examined *V. cholerae* containing a derivative of

P*lacUV5*-Vc1UTR-*lacZ* in which the Vc1 sequence contains the G12T mutation (P*lacUV5*-

Vc1UTR^{G12T}-*lacZ*). In this strain, reporter activity is reduced compared to that of *V.*

cholerae with the wild type P*lacUV5*-Vc1UTR-*lacZ* fusion, and when compared to wild

type c-di-GMP levels, reducing c-di-GMP does not reduce reporter activity (Figure 2.5).

These results suggest decreasing c-di-GMP availability in the cell has a similar effect as

the G12 mutation, supporting a role for Vc1 in *gbpA* expression in response to c-di-GMP.

We further examined whether Vc1 can regulate gene expression in response to a range of c-di-GMP concentrations by growing the P*lacUV5*-Vc1UTR-*lacZ* strain with pPDE in medium with a range of arabinose concentrations, which induces a range of PDE production and c-di-GMP levels. The resulting β-galactosidase activity decreased in a dose-dependent fashion, with a gradual reduction in activity as PDE production increased and c-di-GMP level decreased (Figure 2.6). The P*lacUV5*-Vc1UTR-*lacZ* strain with vector served as a negative control, and the addition of arabinose did not affect β-galactosidase activity in this strain.

Discussion

The second messenger c-di-GMP plays a central role in bacterial adaptation to extracellular conditions, often mediating the switch between motile and sessile lifestyles. Numerous studies have revealed that, while c-di-GMP impacts common biological processes, it does so through diverse regulatory mechanisms. Recent work in *C. difficile* has implicated riboswitches in sensing c-di-GMP to control the production of bacterial surface organelles, such as flagella and Type IV pili, through post-transcriptional mechanisms involving the control of premature transcription termination (17, 22, 24-27). In this study, we investigated the *in vivo* role of the putative c-di-GMP riboswitch Vc1 in regulating gene expression *in cis* in *V. cholerae*.

Several lines of evidence support a role for Vc1 in sensing c-di-GMP in *V. cholerae*. Of key importance is the finding that the Vc1 sequence itself influences gene

expression *in cis*, in response to changes in c-di-GMP. Specifically, isolating the Vc1 sequence by fusing it to a reporter gene (*lacZ*), placing under the transcriptional control of a heterologous, constitutive promoter, and integrating in the *V. cholerae* chromosome rendered reporter activity regulatable by c-di-GMP. Depletion of c-di-GMP through the ectopic expression of a c-di-GMP phosphodiesterase gene reduced downstream gene expression, while expression of a diguanylate cyclase gene augmented expression. These regulatory effects are attributable specifically to altered c-di-GMP, as expression of a mutant PDE gene incapable of c-di-GMP hydrolysis did not affect expression. Moreover, mutating a single residue in the Vc1 sequence, the G12 nucleotide predicted to be involved in c-di-GMP binding, eliminated regulation of downstream expression by c-di-GMP. These findings are bolstered by experiments showing that the same mutation at the native *gbpA* locus similarly inhibited downstream gene expression. SHAPE and equilibrium dialysis studies using Vc1 RNA corroborate a direct interaction between Vc1 and c-di-GMP. SHAPE analysis indicated that the reactivity of Vc1 changed in the presence of c-di-GMP, specifically in regions predicted to be contact points between c-di-GMP and GEMM aptamers. Moreover, the G12 mutation that reduced downstream gene expression similarly diminished the Vc1—c-di-GMP interaction. Together these results support a role for the Vc1 element in regulation of *gbpA* expression in response to c-di-GMP, and point to Vc1 functioning as an "on" switch in response to c-di-GMP.

However, some of our results are in conflict with attribution of GEMM riboswitch function to Vc1. For example, despite conservation of key residues in Vc1, the interaction of c-di-GMP with Vc1 RNA appears to be less than that with Vc2 RNA. These results may reflect a lower binding affinity of Vc1 for c-di-GMP compared to the

affinity of Vc2, which is extremely high (10 pM) (42). The assay we used lacks the

sensitivity necessary to determine the K_D of Vc1 and c-di-GMP, though a future

comparison of binding affinities of Vc1, Vc2 and other c-di-GMP riboswitches would be

needed to know the range of sensitivities of GEMM riboswitches for c-di-GMP. In

addition, the equilibrium dialysis data suggest that the rate of dissociation of c-di-GMP

from Vc1 and Vc2 differs. The addition of excess, non-radiolabelled competitor c-di-

GMP to the equilibrium chambers led to displacement of labeled c-di-GMP from Vc1,

but not from Vc2. These results suggest that binding of c-di-GMP by Vc1 is reversible,

which may allow for more rapid adaptation and modulation of GbpA production.

Furthermore, in Vc2, G20, A47 and C92 are contact points for c-di-GMP and are critical

for c-di-GMP binding. Previous analyses of Vc2 showed that mutating G20 (equivalent

to G12 in Vc1) reduced binding of c-di-GMP by 30,000-fold (42). SHAPE analysis of

Vc1 showed that the regions adjacent to G12 and A39 had significantly altered reactivity

in the presence of c-di-GMP, suggesting that those residues participate in c-di-GMP

sensing. However, the equivalent residues in Vc1, G12, A39 and C104, are not

essential for c-di-GMP binding. The Vc1 G12 mutation reduces, but does not eliminate,

the interaction with c-di-GMP *in vitro* or downstream gene expression in vivo. Thus,

while several lines of evidence indicate that c-di-GMP signals through Vc1, this element

differs from the canonical GEMM riboswitch Vc2. Non-conserved sequences in Vc1 may

alter its interaction with Vc1, or, despite high conservation between Vc1 and Vc2, it is

possible that c-di-GMP makes alternate contacts with Vc1. Attempts to identify alternate

binding residues using error prone PCR to generate mutant derivatives of the PlacUV5-

Vc1UTR-lacZ reporter failed to identify single point mutations that eliminate reporter

activity, so additional experimentation is necessary to determine how the interaction of c-di-GMP with Vc1 differs from that with Vc2. Thus, Vc1 may be a poor riboswitch, or not a riboswitch in the strictest definition. Nonetheless, the Vc1 sequence is capable of regulating downstream gene expression in response to c-di-GMP. Other putative c-di-GMP riboswitches may also vary in their interactions with and responses to c-di-GMP.

Induction of a c-di-GMP phosphodiesterase using a broad range of inducer concentrations to achieve a spectrum of intracellular c-di-GMP levels showed that Vc1 promotes downstream gene expression in a dose-dependent manner. The ability of Vc1 to respond to a range of c-di-GMP levels is consistent with work done previously using a reporter controlled by an engineered c-di-GMP riboswitch, which also suggested that reporter activity varied depending on intracellular c-di-GMP levels (44).

Unlike the c-di-GMP riboswitches described to date, the sequence immediately 3' of the predicted Vc1 aptamer does not indicate a likely mechanism of gene regulation. There are no obvious Rho-independent transcription terminator or anti-Shine Delgarno sequences. The same is true for the region 3' of the Vc1 aptamer. Quantitative reverse transcriptase PCR analysis of the entire *gbpA* transcript, which involved probing for the aptamer as well as four additional regions along the length of the mRNA, showed that the G12 mutation in Vc1 results in significantly reduced transcript abundance uniformly across the *gbpA* mRNA (data not shown). Additional work is needed to determine whether this occurs as a result of Vc1 controlling transcriptional read-through or transcript stability, or as an indirect effect on transcript stability in the absence of translation elongation. The mechanism by which the canonical GEMM riboswitch Vc2 regulates VC1722 expression is similarly unknown.

Several lines of evidence suggest that c-di-GMP levels change in *V. cholerae* during transitions between its native aquatic environment and the host intestine. For example, *V. cholerae* forms biofilms on surfaces in the environment, such as the chitin, and numerous studies have shown that biofilm formation is positively regulated by c-di-GMP (13, 31, 45). Furthermore, increased intracellular c-di-GMP enhances the binding of *V. cholerae* to chitin *in vitro* in a process dependent on the hemagglutinin FrhA (46). Conversely, reduction of c-di-GMP is required to promote bacterial motility and increase expression of virulence factors in the intestinal tract (13, 31, 45). Genetic evidence suggests that c-di-GMP levels again increase during later stages of infection (47). In the context of *V. cholerae* biology, the effect of Vc1 sensing of c-di-GMP to augment GbpA production may contribute to colonization of environmental (chitin) and host surfaces. Determining the role of c-di-GMP in controlling c-di-GMP—regulated processes of *V. cholerae* is complicated by the finding that the *gbpA* promoter is negatively regulated by c-di-GMP (Chapter 2). To determine the role of Vc1 in controlling chitin binding or host colonization would thus require testing Vc1 mutants for altered phenotypes. Unfortunately, despite using the same *V. cholerae* strain and numerous approaches, we were unable to reproduce the effects of an in-frame deletion of *gbpA* on *V. cholerae* binding to chitin or host colonization, so we could not assess the role of Vc1 specifically in these processes. Future work will define the roles of Vc1 and Vc2 in modulating c-di-GMP-regulated processes in response to extracellular cues in *V. cholerae*.

Figures

A

```
         1              12
         |              |
Vc1  GGTCACACUUUGGCAAACCCUUUGAAAAAAUGGGACGC
Vc2  GTCACGCACAGGGCAAACCAUUCGAAAGAGUGGGACGC
     |          ********* ** ****  * ****** **
     9                              P2

Vc1  AAAGCUUCCGGUCUGUCGGUCAAGAAAGCUCAUAA--A
Vc2  AAAGCCUCCGGCCUAAACCA---------GAAGACA-
     ** *** ***** **                   *   *
                       P3
                                104
                                |
Vc1  GCCCUAUUACACCUAGGAUAGCGGGGUUACCAAUUCGG
Vc2  ---------UGGUAGG-UAGCGGGGUUACCGAUGGCA
              ****  ********** *** **
                       P3
```
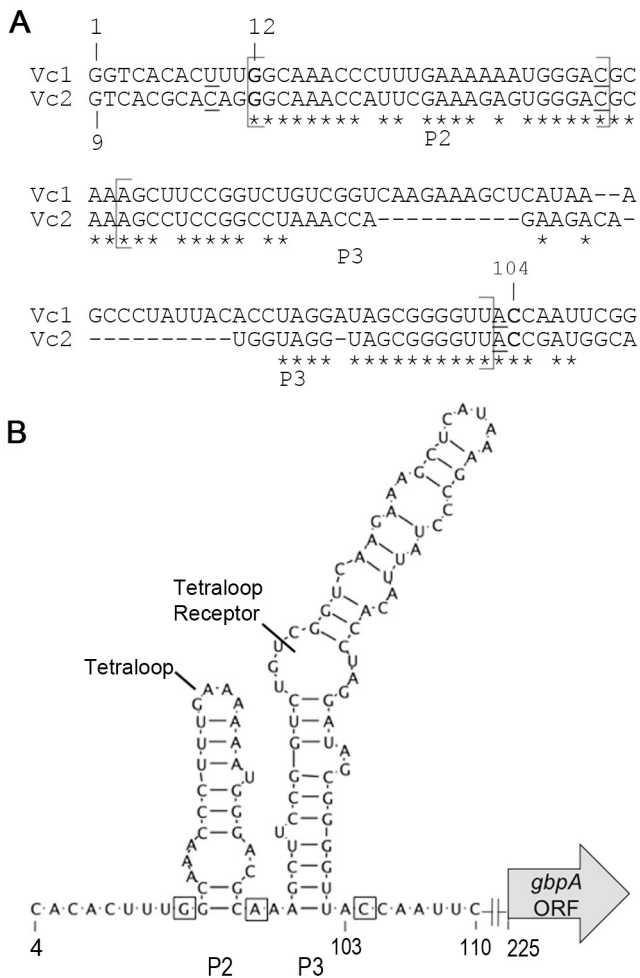
B

Figure 2.1. Vc1 secondary structure and putative contact residues for c-di-GMP. (A) Identification of the +1 site of transcription, which is 225 base pairs upstream of the annotated translational start site, using 5' RACE. Vc2 and Vc1 were aligned using ClustalW2. Asterisks represent nucleotides that are conserved between Vc1 and Vc2. Nucleotides bolded and underlined are contact residues for c-di-GMP in Vc2 and are predicted contact residues for c-di-GMP in Vc1. The regions predicted to encode P2 and P3 are labeled. (B) The predicted structure of Vc1 based on an alignment with Vc2 and the consensus GEMM aptamer structure. The predicted tetraloop and tetraloop receptor are noted.
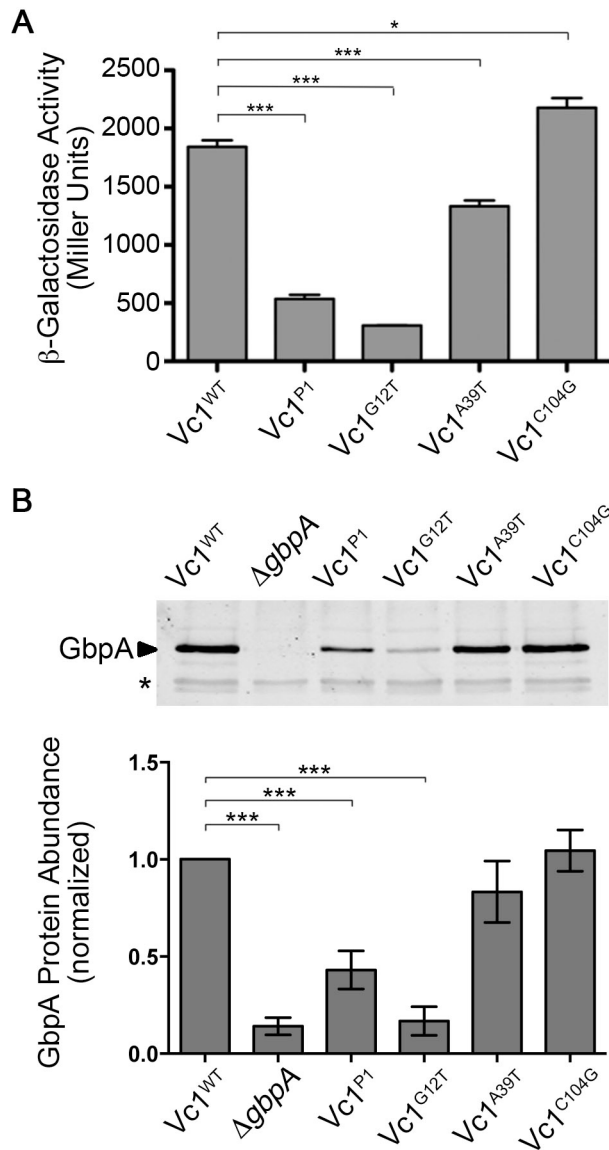
71

Figure 2.2. Vc1 influences downstream gene expression. (A) β-galactosidase activity of *V. cholerae* strains with plasmid-borne *lacZ* translational fusions to wild type Vc1 or mutant derivatives. Transcription initiation is controlled by the *lac* promoter. Three independent experiments were done, and the means and standard deviations are shown. *** $P < 0.001$, * $P < 0.05$ by unpaired t-test. (B) Western blot analysis of the GbpA levels in the supernatants of cultures of wild type *V. cholerae*, an isogenic Δ*gbpA* strain, or strains with mutations in Vc1 (P1, G12T, A39T, or C104G). The image shown is representative of three independent experiments. (C) Densitometry was done by comparing the intensity of the GbpA band to that of a cross-reactive band in the same lane (indicated by an asterisk), then normalizing to the wild type values. Shown are the means and standard deviations for three independent experiments. *** $P < 0.001$ by unpaired t-test.
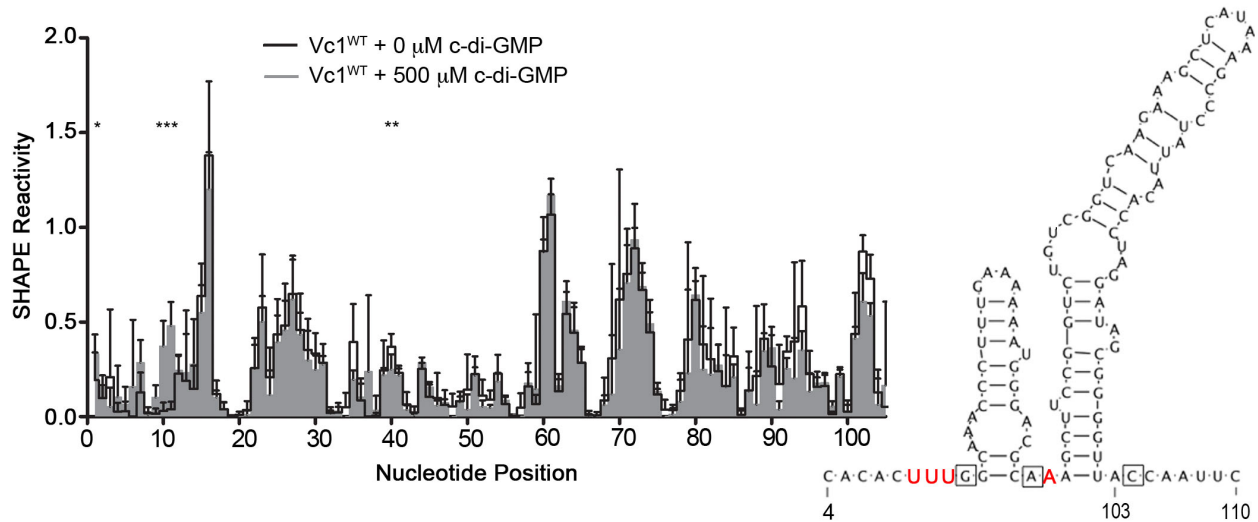
Figure 2.3. c-di-GMP impacts the reactivity of specific regions in Vc1 RNA. *In vitro* transcribed Vc1 RNA was incubated with or without 500 µM c-di-GMP and analyzed by SHAPE. SHAPE reactivity values for each nucleotide position were obtained by averaging values from five independent experiments. The inset shows in red text the location of the nucleotides with significantly altered reactivity, mapped on the predicted structure of Vc1. The boxes denote the G12, A39 and C104 residues. * $P < 0.05$ by unpaired t-test comparing SHAPE reactivity values for each nucleotide position, with or without c-di-GMP, indicating significant changes in reactivity in response to c-di-GMP.
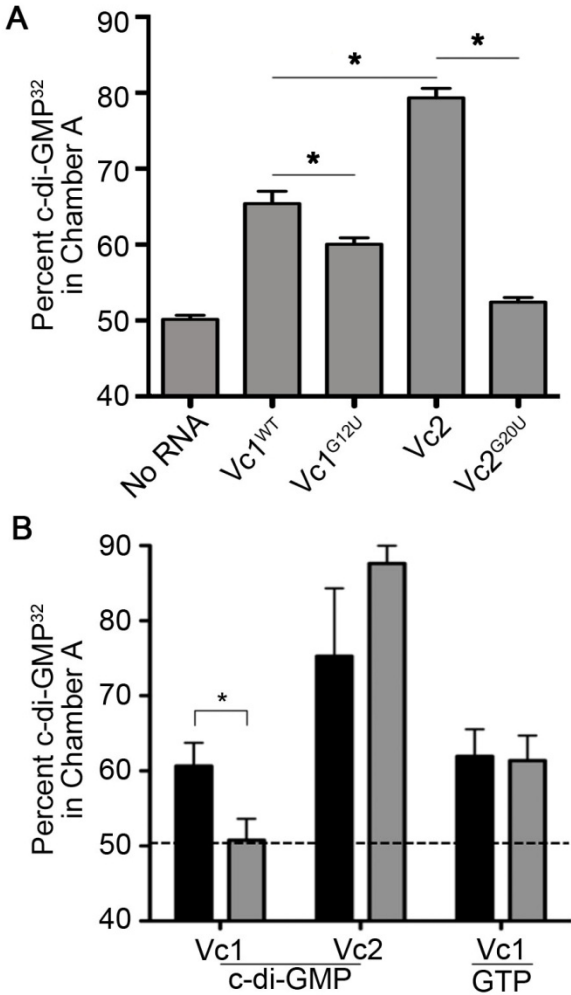
Figure 2.4. Vc1 directly and specifically interacts with c-di-GMP. (A) Binding of c-di-GMP[32] to *in vitro* transcribed Vc1 RNA and mutant derivatives was measured by equilibrium dialysis as described in the Materials and Methods. Vc2 and Vc2[G20U] RNA were included as controls, and no-RNA samples were used as a negative control. (B) The specificity of binding to c-di-GMP to Vc1 and Vc2 RNA was assessed by equilibrium dialysis, allowing c-di-GMP[32] to bind and reach equilibrium as the first measurement (black bars), then competing with unlabeled, excess c-di-GMP or GTP and determining the percentage of c-di-GMP retained in the RNA chambers (grey bars). * *P* < 0.05 by unpaired t-test.
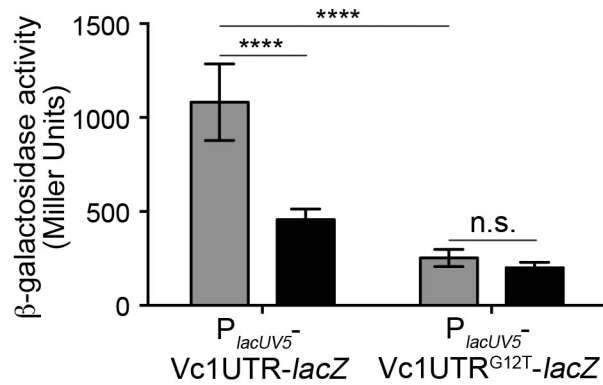
Figure 2.5. Lowering intracellular c-di-GMP reduces Vc1-dependent gene expression. β-galactosidase activity of *V. cholerae* strains with chromosomal, translational fusions of *E. coli lacZ* to the *gbpA* 5' UTR with wild type Vc1 or Vc1^{G12T}, with transcription initiation under the control of the constitutive P$_{lacUV5}$ promoter(37). Each reporter strain carried either vector (wild-type c-di-GMP level; grey bars) or pPDE (reduced c-di-GMP; black bars). Strains were grown in rich medium with 0.2% L-arabinose to induce VieA PDE production and c-di-GMP depletion. Three independent experiments were done, and the means and standard deviations are shown. **** $P < 0.0001$, n.s. = not significant by unpaired t-test.
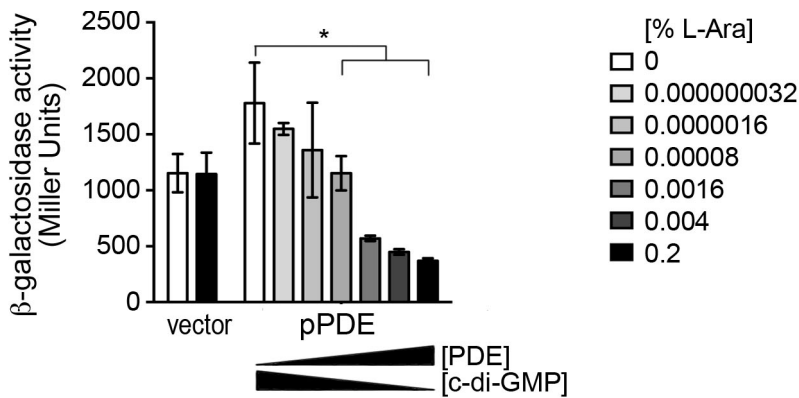
Figure 2.6. Vc1 can respond to dose dependent changes in intracellular c-di-GMP levels. Dose response analysis using the P_{lacUV5}-Vc1UTR-*lacZ* (A) and P_{lacUV5}-Vc2UTR-*lacZ* (B) reporter strains, each with vector or pPDE, grown in rich medium with a range of arabinose concentrations. Increasing PDE production corresponds with decreasing intracellular c-di-GMP. Measurements of β-galactosidase activity were done with three biological replicates, and the means and standard deviations are shown. * $P < 0.05$ by unpaired t-test.
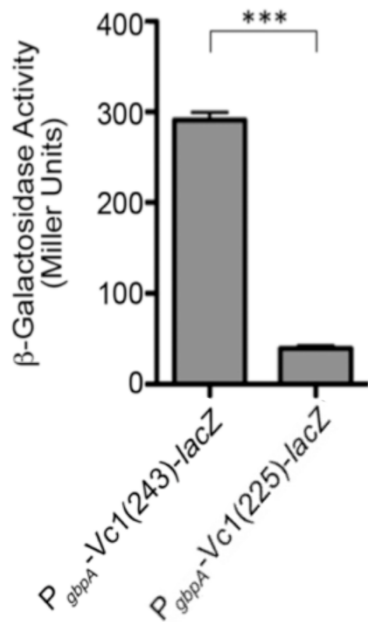
Figure 2.7. The translation start site of *gbpA* is at position +243. To create a translational fusion reporter plasmid, we first identified the methionine start codon in the *gbpA* ORF. The *E. coli lacZ* gene was substituted for the *gbpA* ORF beginning at either the methionine codon at position +225 (the annotated translational start site), or the methionine codon at position +243, in each case using the native translational initiation sequence. The β-galactosidase activity of *V. cholerae* strains with *lacZ* translational fusions to P$_{gbpA}$-Vc1(225) or P$_{gbpA}$-Vc1(243) that encompass the *gbpA* promoter and the 225 bases or 243 bases of the 5' UTR, respectively, was measured using a Miller assay. β-galactosidase activity was approximately 7-fold higher when the second methionine was included in the fusion, suggesting that the annotated translational start site is incorrect and the 5'UTR of *gbpA* is 243 bp long. *** $P < 0.001$ by unpaired t-test.
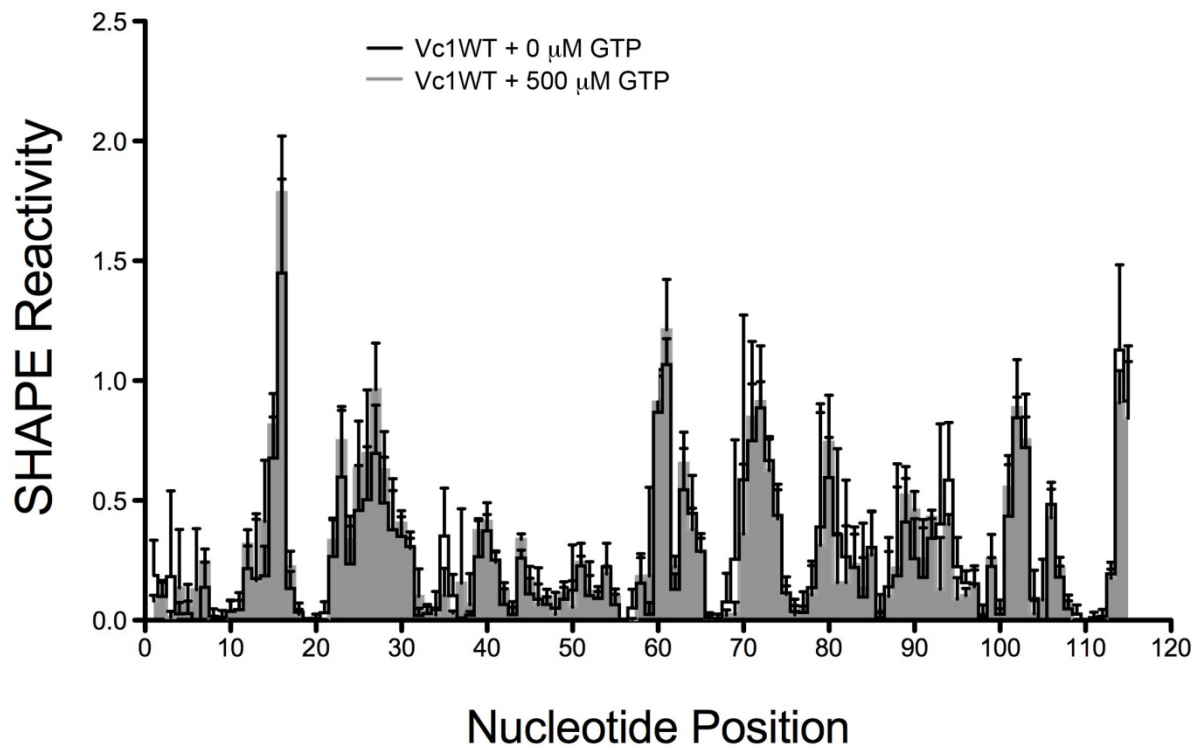
Figure 2.8. GTP does not induce reactivity changes in Vc1 RNA. *In vitro* transcribed Vc1 RNA was incubated with or without 500 µM GTP and analyzed by SHAPE. SHAPE reactivity values were obtained by averaging three independent experiments. The data were analyzed by unpaired t-test comparing SHAPE reactivity values for each nucleotide position; no significant differences were identified.
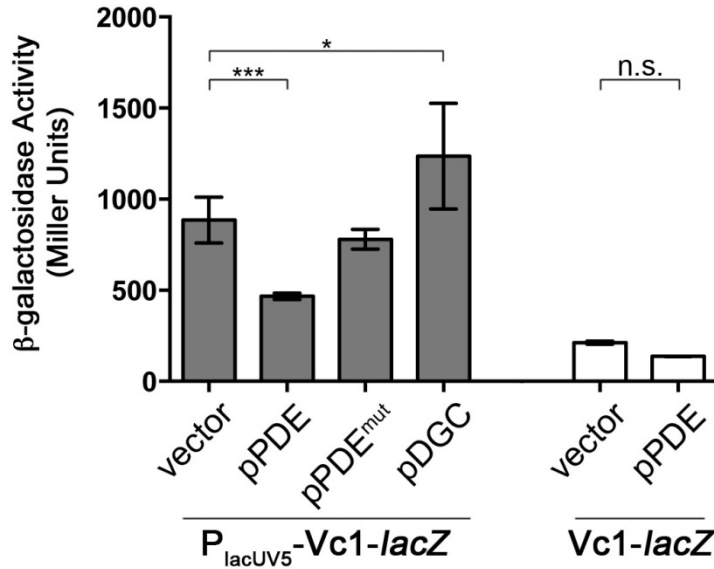
Figure 2.9. The effect of PDE (VieA) and DGC (VCA0956) gene expression on P_{lacUV5}-UTR-*lacZ* reporter activity is specifically due to reduction of c-di-GMP. The β-galactosidase activity was measured for *V. cholerae* strains with chromosomal translational fusions of *E. coli lacZ* to the wild type *gbpA* 5' UTR, with either vector (wild-type c-di-GMP level), pPDE (reduced c-di-GMP), pPDE^{mut} (inactive PDE, c-di-GMP) or pDGC (DGC VCA0956, increased c-di-GMP). Transcription initiation was controlled by the constitutive P_{lacUV5} promoter. The β-galactosidase activity of control strains containing a promoterless UTR-*lacZ* fusion inserted in the chromosome, with either vector (wild type c-di-GMP) or pPDE (reduced c-di-GMP) confirm that no element in the UTR drive transcription. All strains were grown in rich medium with 0.2% L-arabinose to induce gene expression. Three independent experiments were done, and the means and standard deviations are shown. * $P < 0.05$, *** $P < 0.001$ by two-way ANOVA and Tukey's multiple comparisons test.

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Table 2.1. Strains and plasmids used in this study.

Strain	Strain Description	Reference
Escherichia coli		
DH5α	F⁻ φ80*lacZ* ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (rK-, mK+) *phoA supE44 thi-1 gvrA96 relA1* λ- *tonA*	Invitrogen; (48)
DH5αλpir	F⁻ Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gvrA96 relA1* λpir	48)
SM10λpir	*thi thr leu tonA lacY supE recA*::RPA-2-Te::Mu λpirR6K, Km^r	(49)
Vibrio cholerae		
	C6706 (O1 El Tor)	(50)
	C6706 pBAD33	This study
	C6706 pBAD33::*vieA* (pPDE)	This study
	C6706 pBAD33::*vieA*E170A (pPDE^{mut})	This study
	C6706 Δ*lacZ*	(51)
	C6706 Δ*lacZ* pP*lac*-Vc1-*lacZ*	This study
	C6706 Δ*lacZ* pP*lac*-Vc1^{P1}-*lacZ*	This study
	C6706 Δ*lacZ* pP*lac*-Vc1^{A39T}-*lacZ*	This study
	C6706 Δ*lacZ* pP*lac*-Vc1^{G12T} -*lacZ*	This study
	C6706 Δ*lacZ* pP*lac*-Vc1^{C104G}-*lacZ*	This study
	C6706 Δ*gbpA* (VCA0811)	This study
	C6706 Vc1^{P1}	This study
	C6706 Vc1^{G12T}	This study
	C6706 Vc1^{A39T}	This study
	C6706 Vc1^{C104G}	This study
	C6706 Vc1^{G12T} pBAD33	This study
	C6706 Vc1^{G12T} pBAD33::*vieA* (pPDE)	This study
	C6706 PlacUV5-Vc1UTR-*lacZ*	This study

	C6706 PlacUV5-Vc1UTR-*lacZ* pBAD33	This study
	C6706 PlacUV5-Vc1UTR-*lacZ* pBAD33::*vieA* (pPDE)	This study
	C6706 PlacUV5-Vc1UTR-*lacZ* pBAD33::*vieA*E170A (pPDE^mut)	This study
	C6706 PlacUV5-Vc1UTR^Vc1G12T-*lacZ*	This study
	C6706 PlacUV5-Vc1UTR^Vc1G12T-*lacZ* pBAD33	This study
	C6706 PlacUV5-Vc1UTR^Vc1G12T-*lacZ* pBAD33::*vieA* (pPDE)	This study
	C6706 PlacUV5-Vc1UTR^Vc1G12T-*lacZ* pBAD33::*vieA*E170A (pPDE^mut)	This study

Plasmids		
pP*lac*thiM#2-*lacZ*	The TPP riboswitch encoded in the 5' UTR of *E. coli* *thiM* was mutated to function as an ON switch and cloned into pP*lac-lacZ*. Origin of replication *colEI*. Amp^R	(52)
pP*lac*-Vc1-*lacZ*	Vc1 allele from C6706 cloned into plasmid pP*lac*thiM#2-*lacZ*	This study
pP*lac*-Vc1^P1-*lacZ*	Vc1^P1 allele cloned into plasmid pP*lac*thiM#2-*lacZ*	This study
pP*lac*-Vc1^A39T-*lacZ*	Vc1^A39T allele cloned into plasmid pP*lac*-Vc1-*lacZ*	This study
pP*lac*-Vc1^G12T-*lacZ*	Vc1^G12T allele cloned into plasmid pP*lac*-Vc1-*lacZ*	This study
pP*lac*-Vc1^C104G-*lacZ*	Vc1^C104G allele cloned into plasmid pP*lac*-Vc1-*lacZ*	This study
pBAD33	Expression vector, P*ara* promoter, Cm^R	(53)
pBAD33::*vieA*	*vieA* in pBAD33 ("pPDE")	(31)
pBAD33::*vieA*E170A	*vieA*E170A (allele encoding enzymatically inactive VieA) ("pPDE^mut")	(31)
pCVD442	*oriR6K* plasmid with a polylinker, *mobRP4*, *bla*, and *sacB*	(54)
pCVD442::Δ*gbpA*	Allelic exchange vector for in-frame deletion of *gbpA*	This study
pCVD442::Vc1^P1	Allelic exchange vector for mutation of P1 stem	This study
pCVD442::Vc1^G12T	Allelic exchange vector for mutation of nucleotide G12 in Vc1	This study

	Allelic exchange vector for mutation of nucleotide A39 in Vc1	This study
pCVD442::Vc1^{A39T}	Allelic exchange vector for mutation of nucleotide A39 in Vc1	This study
pCVD442::Vc1^{C104G}	Allelic exchange vector for mutation of nucleotide C104 in Vc1	This study
pCVD442::Vc2^{G20T}	Allelic exchange vector for mutation of nucleotide G20 in Vc2	This study
pJL1	Allelic exchange vector for integration into *V. cholerae lacZ* gene	(38)
p2UY35A	Template used for amplification of PlacUV5 promoter	(37)
pJL1:: PlacUV5-Vc1UTR-*lacZ*	Allelic exchange vector for integration of PlacUV5-Vc1UTR-*lacZ* translational fusion into *V. cholerae lacZ* gene (WT 5' UTR of *gbpA* containing Vc1)	This study
pJL1:: PlacUV5-Vc1UTR^{G12T}-*lacZ*	Allelic exchange vector for integration of PlacUV5-Vc1UTR^{G12T}-*lacZ* translational fusion into *V. cholerae lacZ* gene (mutant 5' UTR of *gbpA* containing Vc1)	This study

Table 2.2. Primers used in this study.

Primer Name	Oligonucleotide sequence (5' to 3') *	Reference
gbpAF1	TTGCATGCTACTCGTCAGGTCTTTGG	This study
gbpAR1	TTGGTACCCATCACAGACTCTTCTTTG	This study
gbpAF2	TTGGTACCTAAGTTATCCTCCCTCTTAC	This study
gbpAR2	TTGAGCTCTTTCTCTGGATGGGAGTC	This study
gbpAF0	GCAAACGGTAGCAAGAAG	This study
pCVDseqF	CTGTTGCATGGGCATAAAGTTGCC	This study
pCVDseqR	ACACAGGAACACTTAACGGCTGAC	This study
gbpAPrR	CGATGTCGACCATCACAGACTCTTCTTTGTTAGC	This study
gbpAP2F	CCGAATTCCCGAGTAAAGCATCAACCTTTCATATTG	This study
gbpArbF	CCGAATTCAGTAAATTTGCTCTCGGTCACAC	This study
Race1a	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTA	This study
Race1g	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTG	This study
Race1c	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTC	This study
Race2	GACTCGAGTCGACATCG	This study
gbpAsp1	GTGAATCTGAACTTCAGCACC	This study
gbpAsp2	GCACCTTCAGCAACGCATAAG	This study
gbpAsp3	TTGCCAAAGTGTGACCGAGAG	This study
gbpAsp4	ACCAAGAGAGAAAGCGAAGTC	This study
placP2R	TTGCATGCTTGCCAAAGTGTGACCGAGAG	This study
placP2F	TTGCATGCCAGCTAACAAAGAAGAGTCTGTG	This study
Vc1F1	CCTCTAGATACTCGTCAGGTCTTTGG	This study
Vc1R2	AAGAGCTCCGAGTGTACACGGTATCG	This study
Vc1R3	AGCAACGCATAAGACAAACG	This study
Vc1gTF	CACACTTT**T**GCAAACCCTTTGAAAAAATGG	This study

Vc1gTR	AGGGTTTGC**A**AAAGTGTGACCGAGAG	This study
Vc1aTF	AATGGGACGC**T**AAGCTTCCGGTCTG	This study
Vc1aTR	CGGAAGC**A**TTGCGTCCCATTTTTTC	This study
Vc1cGF	GGTTA**G**CAATTCGGTTTATACCG	This study
Vc1cGR	ATTG**C**TAACCCCGCTATCC	This study
Vc2G20TF1	CC<u>GAGCTC</u>TCAGAGATGCCTTAATAGCTC	This study
Vc2G20TR1	CAG**T**GCAAACCATTCGAAAG	This study
Vc2G20TF2	GTTTGC**A**CTGTGCGTGA	This study
Vc2G20TR2	GG<u>CCCGGG</u>GCGGCGGATAAAATCATTGC	This study
T7linkF	TAATACGACTCACTATAGGGCCTTCGGGCCAACGGTCACACTTTGGCAAACCC	This study
T7R	CATCACGCGACAATGGCTGGT	This study
gbpAR2	CGAT<u>GTCGAC</u>TTTAGGTTGTTTTTTCATCACAG	This study
T7Vc1F	TAATACGACTCACTATAGGGAGAAGTAAATTTGCTCTCGGTCACAC	This study
T7Vc2F	TAATACGACTCACTATAGGGAGAGGAAAAATGTCACGCACAGG	This study
T7Vc2R	CATGCTGTTAGTCTCGGAGTATTG	This study
PlacZR3	GCGCATGCTCACATTAATTGCGTTGCGCTCTCG	This study
PlacUV5F	GCGCATGCAAACCCTATGCTAC	This study
PlacUV5R	GCGAATTCACACACTATACGAGCCG	This study
PlacF5	CCAGGCCTAATAGGCGTATCACGAGG	This study
PlacR5	CCGCGGCCGCGTCTTTCGACTGAGCCTTTC	This study
PlacZsF	AGCACACTAACTACCAGC	This study
PlacZsR	GTTTGTATGTGGTGGATGAG	This study
RPB2F	CTGTCTCAAGCCGGTTACAA	(35)
RPB2R	TTTCTACCAGTGCAGAGATGC	(35)
gbpAqF2	CTCTGGTATCAGTGGATTAGCG	This study
gbpAqR2	GTATTGAATCGCGCCACAGT	This study

Vc1BF	GT**AAATTT**GCTCTCGGTGTGTGTTTGGCAAACCCTTTG	This study
Vc1BR	CAAAGGGTTTGCCAAACACACACCGAGAGC**AAATTT**AC	This study
pLacSeq	ATTGTCTCATGAGCGGATAC	This study
placZF	ATGCAA**GTCGAC**CTGCTGGATC	This study

* The underlined sequences represent restriction enzyme recognition sites, and the bold and underline sequences represent point mutations. Italicized sequences encode a 6-histidine tag.

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CHAPTER 3: TWO NUCLEOTIDE SECOND MESSENGERS REGULATE THE PRODUCTION OF THE *VIBRIO CHOLERAE* COLONIZATION FACTOR GBPA

Summary

Background: The nucleotide second messengers cAMP and c-di-GMP allow many bacteria, including the human intestinal pathogen *Vibrio cholerae*, to respond to environmental stimuli with appropriate physiological adaptations. In response to limitation of specific carbohydrates, cAMP and its receptor CRP control the transcription of genes important for nutrient acquisition and utilization; c-di-GMP controls the transition between motile and sessile lifestyles often, but not exclusively, through transcriptional mechanisms. In this study, we investigated the convergence of cAMP and c-di-GMP signaling pathways in regulating the expression of *gbpA*. GbpA is a colonization factor that participates in the attachment of *V. cholerae* to N-acetylglucosamine-containing surfaces in its native aquatic environment and the host intestinal tract.

Results: We show that c-di-GMP inhibits *gbpA* activation in a fashion independent of the known transcription factors that directly sense c-di-GMP. Interestingly, inhibition of *gbpA* activation by c-di-GMP only occurs during growth on non-PTS dependent nutrient sources. Consistent with this result, we show that CRP binds to the *gbpA* promoter in a cAMP-dependent manner *in vitro* and drives

transcription of *gbpA in vivo*. The interplay between cAMP and c-di-GMP does not broadly impact the CRP-cAMP regulon, but occurs more specifically at the *gbpA* promoter.

Conclusions: These findings suggest that c-di-GMP directly interferes with the interaction of CRP-cAMP and the *gbpA* promoter via an unidentified regulator. The use of two distinct second messenger signaling mechanisms to regulate *gbpA* transcription may allow *V. cholerae* to finely modulate GbpA production, and therefore colonization of aquatic and host surfaces, in response to discrete environmental stimuli.

Introduction

Nucleotide second messengers in bacteria play a key role in relaying information from the extracellular environment to intracellular effectors, resulting in an adaptive physiological response. Production of these intracellular nucleotides, which include c-di-GMP, c-di-AMP, cGMP, cAMP, and (p)ppGpp, regulates fundamental processes including biofilm formation, motility, nutrient acquisition, stress responses, sporulation and, in some pathogens, virulence factor production (reviewed in [1-4]).

c-di-GMP, utilized by many Gram-negative and Gram-positive bacteria, usually regulates transitions between motile and non-motile lifestyles. Specifically, c-di-GMP negatively regulates flagellar motility by inhibiting flagellum biosynthesis or function, and positively regulates adherence and biofilm development in many species [5]. The intracellular levels of c-di-GMP are regulated by the opposing activities of diguanylate cyclase (DGC) biosynthetic enzymes and phosphodiesterase (PDE) hydrolytic enzymes [6-12]. Many organisms contain numerous genes that encode proteins with known or

predicted functions as DGC and/or PDE enzymes. To date there are several known intracellular c-di-GMP receptors, including protein and RNA-based sensors. Proteins that directly sense c-di-GMP include transcription factors, proteins containing the well-characterized PilZ domain, and others such as catalytically inactive PDEs that retain the capacity to bind c-di-GMP [13]. In addition, two distinct classes of c-di-GMP sensing riboswitch, broadly present in bacterial genomes, have been identified [14-17]. The presence of numerous c-di-GMP metabolic enzymes and an array of intracellular c-di-GMP receptors points to complex c-di-GMP signaling networks that modulates a wide array of cellular functions.

In the human diarrheal pathogen *Vibrio cholerae*, c-di-GMP promotes biofilm formation and inhibits flagellar motility [18-20]; in addition, c-di-GMP inhibits the expression of virulence genes including those encoding the major virulence factor, cholera toxin [21]. To date, three transcription factors that directly sense c-di-GMP have been identified in *V. cholerae*: VpsT, VpsR and FlrA [22-24]. Each of these was previously characterized as a regulator of biofilm formation and/or motility [20, 25-28]. Five proteins containing a PilZ domain have been described in *V. cholerae*, some of which have roles in biofilm, motility and/or virulence [29]. The *V. cholerae* genome also encodes two c-di-GMP riboswitches, Vc1 and Vc2, which lie upstream of *gbpA* and VC1722, respectively [14].

cAMP signaling is widespread in bacteria, and intracellular cAMP levels are heavily influenced by the availability of extracellular nutrient sources [30]. In Gram-negative bacteria, uptake of a preferred sugar by the phosphoenolpyruvate-carbohydrate phosphotransferase transport system (PTS) relies on a sugar-specific

transporter, EIIA. Following uptake of a PTS sugar, EIIA and other PTS components participate in a phosphorylation cascade that culminates phospho-transfer from EIIA to the incoming sugar. The phosphorylation state of EIIA serves as a measure of PTS sugar availability. Under conditions of PTS sugar limitation (and absence of a PTS sugar substrate), EIIA remains phosphorylated and stimulates the adenylate cyclase (AC), triggering cAMP biosynthesis. To date, the only known sensor of cAMP in bacteria is the cAMP receptor protein (CRP) [30]. Together, the cAMP-CRP complex promotes the expression of genes involved in the uptake and utilization of non-PTS nutrient sources.

V. cholerae uses the cAMP-CRP system to respond to the absence of PTS-dependent sugars such as *N*-acetylglucosamine, sucrose, mannitol and fructose [31]. Unlike *E. coli*, in which glucose utilization is PTS-dependent, glucose can be utilized via PTS and non-PTS mechanisms in *V. cholerae* [31]. *V. cholerae* has also adapted the cAMP-CRP pathway to promote motility and inhibit biofilm formation [32, 33]. Transcriptome analyses have shown that *cya* and *crp* deletion mutants (which lack cAMP biosynthesis and sensing, respectively) have increased expression of genes involved in extracellular matrix production, reduced expression of genes involved in flagellum biosynthesis and chemotaxis [32, 33]. In addition, a *crp* deletion mutant has reduced expression of genes important for colonization and is attenuated in a mouse model of infection [32]. Interestingly, the cAMP-CRP signaling pathway impinges upon the c-di-GMP signaling network; a *crp* mutant has altered expression of numerous genes involved in c-di-GMP metabolism [33].

V. cholerae naturally inhabits aquatic reservoirs, where it associates with chitinous surfaces such the exoskeletons of zooplankton and crustaceans [34]. *V. cholerae* can be ingested through consumption of contaminated food or water and subsequently colonize the small intestine and cause diarrheal disease in humans. The colonization factor GbpA aids in the attachment of *V. cholerae* to surfaces in its native aquatic environment and in the host intestine [35, 36]. GbpA recognizes N-acetylglucosamine (GlcNAc), a component of mucin and a modification of glycoprotein and lipids found on the surface of the intestinal epithelium, thus mediating interactions with the host intestinal epithelium [35, 36]. GlcNAc also comprises the polymer chitin, a major component of the exoskeletons of crustaceans and zooplankton, thus serving as a substratum for *V. cholerae* colonization in aquatic reservoirs [35]. In addition to serving as a ligand for GbpA, GlcNAc also regulates *gbpA* expression via the transcriptional regulator NagC [37, 38]. In *V. cholerae* and some other bacterial species, the transcription factor NagC regulates gene expression in response to GlcNAc and typically controls genes involved in GlcNAc uptake and metabolism [38-42]. Transcriptional profiling of a *V. cholerae nagC* mutant indicated that NagC represses *gbpA* transcription [38], suggesting that *gbpA* expression is down-regulated in the presence of mucin- and chitin-derived GlcNAc in the small intestine and in the aquatic environment, respectively [36-38].

In this study, we investigated the combined roles of the second messengers cAMP and c-di-GMP in regulating *gbpA* transcription. We report that c-di-GMP inhibits *gbpA* transcription independently of the previously described c-di-GMP riboswitch. Expression of *gbpA* is also influenced by the availability of PTS-dependent carbohydrates, and is

accordingly regulated by cAMP-CRP through direct binding of this complex to the *gbpA* promoter. Together, our results indicate that *gbpA* transcription is regulated by both c-di-GMP and cAMP, in opposing fashion. The c-di-GMP and cAMP second messenger signaling pathways may thus function together to modulate the production of GbpA in response to discrete extracellular stimuli, likely impacting the ability of *V. cholerae* to colonize GlcNAc-containing surfaces in the aquatic and host environments.

Materials and Methods

Growth conditions and media. *Escherichia coli*, *V. cholerae* C6706 and mutant derivatives were cultured at 37 °C with aeration in Luria-Bertani (LB) broth containing 100 µg/ml streptomycin (Sm), 10µg/ml chloramphenicol (Cm), and/or 50 µg/ml ampicillin (Amp), as appropriate. Where specified, *V. cholerae* was grown in M9 minimal medium (Fisher Scientific) supplemented with trace metals (1 ml l^{-1} of 5% MgSO$_4$, 0.5% MnCl$_2$4H$_2$O, 0.5% FeCl$_3$, 0.4% nitrilotriacetic acid) [43] and 0.5% (w/v) of the indicated carbon source.

Strain construction. Strains and plasmids included in this study are listed in Table S1. Primer information is contained in Table S2. The *gbpA*, *nagC*, *crp*, *cpdA, cya, vpsT, vpsR and flrA* genes were mutated by standard allelic exchange methods. A deletion in the Vc1 sequence upstream of *gbpA* was deleted in a similar manner. Using genomic DNA from *V. cholerae* C6706 as template, ~800 bp fragments upstream and downstream of the sequences to be deleted were amplified by PCR using primers named according to the pattern geneF1 + geneR1 for the upstream region of homology

and geneF2 + geneR2 for the downstream region of homology. The primers introduced restriction sites (underlined sequences in Table 2) allowing ligation of the resulting fragments to each other and into the pCVD442 suicide vector. The exception is the *vpsR* mutation, for which the two fragments were joined by splicing by overlap extension, and then ligated into pCVD442. The ligations were transformed into DH5αλ*pir* by electroporation and transformed colonies were identified on LB-Amp agar. The desired clones containing the upstream and downstream fragments were identified by PCR using primers geneF1 + geneR2 and/or pCVDseqF + pCVDseqR, which flank the multiple cloning site of pCVD442. The allelic exchange steps were done as described previously [44]. Colonies were screened for the desired deletion by PCR using the corresponding geneF0 + geneR2 primers.

The pP*gbpA*-Vc1-*lacZ* and pP*gbpA*-ΔVc1-*lacZ* plasmids were constructed by amplifying the *gbpA* promoter and 5' UTR from *V. cholerae* C6706 and DVc1 genomic DNA, respectively, by PCR using gbpAP2F + gbpAR2. The PCR products were digested with *Eco*RI and *Sal*I and ligated into pP*lac*thiM#2-*lacZ* [45] digested with the same enzymes. The ligation reaction was transformed into DH5α cells by electroporation, and Amp-resistant colonies obtained were screened with primers pLacSeq + gbpAR2. The resulting plasmids have P*gbpA*-UTR (wild type or ΔVc1) as a translational fusion to *lacZ*, with the *lac* promoter driving transcription. The plasmids were introduced by electroporation into *V. cholerae* C6706 in which the endogenous *lacZ* gene was inactivated [46]. The pBAD33 and pBAD33::*vieA* plasmids were introduced into the indicated strains by electroporation.

Manipulation of the intracellular c-di-GMP level in *V. cholerae*. The intracellular c-di-GMP level was depleted through the ectopic expression of a *V. cholerae* c-di-GMP phosphodiesterase gene, *vieA*, as described previously [47, 48]. Briefly, *V. cholerae* bearing pBAD33 ("vector") or pBAD33::*vieA* ("pPDE") were grown in LB-Cm broth at 37°C with aeration. At early exponential phase ($OD_{600} \sim 0.2$), 0.2% L-arabinose was added to the cultures to induce *vieA* transcription from the P_{BAD} promoter. Samples were collected at mid-exponential phase ($OD_{600} \sim 0.5$-0.7) for western blotting or qRT-PCR analysis as described below.

Reporter activity assays. The β-galactosidase activity of stationary phase samples of strains containing *lacZ* reporter fusions was measured using a Miller assay [49]. Strains were grown in LB broth overnight (14-16h), diluted 1:10 in LB broth, and 100 µl were assayed for hydrolysis of ortho-nitrophenyl-β-D-galactoside. At least three independent experiments were done, and the data were combined. Statistical analyses were done using unpaired t-tests.

GbpA antibody production. Anti-GbpA antiserum was produced by Yenzym 192 Antibodies, LLC, South San Francisco, CA. Antiserum was raised in rabbits to a synthetic peptide (CSNATQYQPGTGSHWEMAWDKR) that corresponds to GbpA from *V. cholerae*. The animal facilities were NIH/OLAW/PHS assured, USDA certified, and IACUC regulated.

GbpA and CRP detection by western blot. Equal-volume samples of mid-exponential phase cultures, normalized to OD_{600}, were collected. For detection of GbpA, the samples were centrifuged to remove the bacteria. Supernatant proteins were TCA precipitated, separated by electrophoresis, and subjected to western blotting with rabbit anti-GbpA antibodies. For detection of CRP, cells grown as above were collected by centrifugation. Whole lysates were electrophoresed and transferred to nitrocellulose membranes. CRP ($\sim$23.6 kDa) was detected using anti-CRP monoclonal antibodies (Neoclone). For cell lysates, RNA Polymerase β subunit ($\sim$150 kDa) served as a loading control and was detected with monoclonal antibodies (AbCam). In all western blots, goat α-rabbit IgG conjugated with IR800 dye (Thermo Scientific) was used as the secondary antibody. The blots were imaged using an Odyssey imaging system (LI-COR). At least three independent experiments were done, and a representative image is shown. Densitometry analyses were done using the Odyssey software. The intensities of the bands corresponding to GbpA in supernatants were normalized to those of a cross reactive band (indicated by asterisks in relevant images). For CRP quantification, the intensities of the bands of CRP in lysates were normalized to those of RNAP.

RNA purification and analysis using quantitative real-time PCR.
Transcriptional analyses using quantitative reverse-transcriptase PCR (qRT-PCR) were done as previously described [50]. Briefly, RNA was purified from mid-exponential phase ($OD_{600} \sim 0.5$-0.7) cultures. Genomic DNA was removed using the TURBO DNA-free kit (Ambion). For cDNA synthesis, RNA (200 ng) was reverse transcribed using the

Tetro cDNA Synthesis Kit (Bioline). We included control reactions without reverse transcriptase for every cDNA sample. For the real time PCR reaction, cDNA and control samples were combined with 2× SYBR/fluorescein mix (SensiMix; Bioline) and 7.5 µM of each primer (named according to the scheme gene-qF and gene-qR for forward and reverse primers, respectively, Table S2). We used the following program to amplify target cDNA: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. Melt curves were included to verify amplification of single products. The data were analyzed using the $\Delta\Delta$Ct method, with Ct values normalized to the specified reference strain/condition, and to the Ct values of the reference gene *rpoB* and/or *gyrA* in each sample [50-52]. For each strain/condition, a minimum of three independent samples was tested. Statistical significance was determined by unpaired t-test.

Cloning and expression of *crp* and purification of the recombinant protein.
The CRP gene was amplified from *V. cholerae* 6706 genomic DNA using primers CRPeF + CRPeH6R, which incorporate *Eco*RI and *Sal*I restriction sites into the product, respectively. The CRPeH6R primer introduces 6 histidine codons at the 3' end of the gene. The PCR product was digested with *Eco*RI and *Sal*I and ligated into similarly digested pMMB67EH, a low-copy vector allowing IPTG-inducible gene expression [53]. Ligations were transformed into *E. coli* DH5α cells. Ampicillin-resistant colonies containing pMMB67EH with *crp-his6* insert were identified by PCR, yielding pMMB::*crp*-his6.

E. coli DH5α containing pMMB::*crp*-his6 was grown in LB broth at 37°C with aeration to early exponential phase (OD$_{600}$ ~ 0.2), at which point IPTG was added for a 0.5 mM final concentration. The culture was grown at 37°C with aeration to mid-exponential phase (OD 600 nm ~ 0.7), then cells were collected by centrifugation. Cells were suspended in His6 lysis buffer consisting of 10 mm Tris, pH 8, 300 mm NaCl, 50 mm NaH$_2$PO$_4$, 10% glycerol, 1 mm phenylmethylsulfonyl fluoride, and 5 mM imidazole [10]. The cells were lysed by sonication, and CRP was purified by affinity chromatography with Ni-NTA resin (ThermoFisher) using the general methods described previously [10, 48]. The eluates were analyzed by SDS-PAGE and coomassie staining. CRP-containing fractions were dialyzed against 5 mM Tris 8.0, 10 mM MgCl2, 5 mM KCl, 5 mM CaCl2, 10% glycerol using 10,000 MWCO Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific). The CRP preparations were estimated to be > 95% pure. Glycerol was added to a final concentration of 20%, and the protein concentration was determined using the BCA Protein Assay Kit (Pierce). Aliquots of the protein were stored at -20°C.

Electrophoretic mobility shift assays (EMSAs). A 293 base pair *gbpA* promoter fragment was amplified from *V. cholerae* 6706 genomic DNA using primers gbpAP2F + Vc1R2. A 133 base pair non-specific control DNA fragment internal to the *gbpA* ORF was amplified with gbpAqF2 + gbpAqR2. EMSAs were done as previously described [54]. Binding reactions were done in 10 mM Tris pH 2.5, 1 mM EDTA, 100 mM KCl, 0.1 mM DTT, 5% glycerol (v/v) and 0.01 mg/ml BSA (final concentrations). The *gbpA* promoter fragment, CRP, and cAMP (Sigma-Aldrich) were used at final

concentrations of 2.5 ng/ml, 11 ng/ml, and 33 mM, respectively. C-di-GMP (Biolog) was used at 33 mM, or 333 mM when in competition with cAMP. The non-specific DNA fragment (75 ng) was added to every mixture. After 1 hour incubation at room temperature (~25°C), the samples were electrophoresed on a 6% TAE polyacrylamide gel. The gel was stained with GelRed (Biotium, Inc.) in TAE for 10 minutes, and then visualized under UV light.

Results

Low c-di-GMP induces *gbpA* transcription in a Vc1-independent manner.

A putative c-di-GMP riboswitch, named Vc1, was previously predicted upstream of the *gbpA* open reading frame. Using a heterologous reporter, we have found that depleting intracellular c-di-GMP or mutating a conserved residue in Vc1 impairs downstream gene expression (Chapter 2). Thus, c-di-GMP appears to promote *gbpA* expression via Vc1. Based on these data, we predicted that depleting intracellular c-di-GMP and preventing its stimulation of Vc1 would result in decreased *gbpA* expression. To test this, we used a previously described strategy for ectopic expression of a c-di-GMP phosphodiesterase (PDE), *vieA*, to manipulate intracellular c-di-GMP levels in *V. cholerae*. The abundance of the *gbpA* transcript and GbpA protein in *V. cholerae* bearing pPDE, which allows arabinose-induced expression of the PDE, was compared to those in *V. cholerae* with vector. Contrary to our expectation, western blot analysis of GbpA production showed that lowering c-di-GMP levels through PDE production resulted in a 2.8-fold increase in GbpA (Figure 3.1A). Regulation by c-di-GMP occurred at the level of gene expression, as quantitative real time PCR analysis indicated that

gbpA transcript levels were 11.1-fold higher in *V. cholerae* with depleted c-di-GMP (Figure 3.1B).

We next tested whether c-di-GMP induction of GbpA production is dependent on the putative riboswitch Vc1. To do this, we assessed the effect of depleting c-di-GMP in *V. cholerae* lacking the Vc1 sequence. This strain, ΔVc1, lacks nucleotides 17-202 of the transcript, which encompass most of the 5'UTR including the c-di-GMP riboswitch, but retains the native *gbpA* promoter and the native ribosomal binding site. The pPDE and control plasmids were introduced into the ΔVc1 mutant, and the strains were assessed for changes in *gbpA* expression. In the ΔVc1 strain with vector, *gbpA* transcript (Figure 3.1B) and GbpA protein (Figure 3.1A) were diminished by 75% and 83%, respectively, compared to the wild type with vector, suggesting that removal of nucleotides 17-202 reduces expression. Yet depletion of c-di-GMP in the ΔVc1 strain (pPDE) increased GbpA production 3.4-fold (Figure 3.1B) and *gbpA* transcription 15.4-fold (Figure 3.1A), an effect equivalent to that seen in the wild type background.

In addition, we used *lacZ* fusions to either the wild type *gbpA* promoter and 5'UTR (P_{gbpA}-Vc1-*lacZ*) or the promoter and 5'UTR lacking Vc1 (P_{gbpA}-ΔVc1-*lacZ*) as reporters of *gbpA* expression. We measured β-galactosidase activity in *V. cholerae* with these plasmid-borne reporters, each with either vector or pPDE. In both the P_{gbpA}-Vc1-*lacZ* and P_{gbpA}-ΔVc1-*lacZ* reporter strains, reduction of c-di-GMP (pPDE) resulted in a statistically significant ~2-fold increase in β-galactosidase activity (Figure 3.1C). Thus, c-di-GMP inhibition of *gbpA* expression is independent of Vc1, indicating that low intracellular c-di-GMP concentrations promote *gbpA* transcription initiation via the *gbpA* promoter.

The c-di-GMP responsive regulators VpsT, VpsR and FlrA are not involved in the regulation of *gbpA* transcription by c-di-GMP.

We next aimed to determine the mechanism by which c-di-GMP inhibits *gbpA* transcription. We hypothesized that a c-di-GMP sensing transcription factor interacts with the *gbpA* promoter, either inhibiting expression in response to c-di-GMP, or alleviating activation in response to c-di-GMP. We first focused on three previously identified c-di-GMP binding transcription factors, VpsT, FlrA and VpsR, as potential mediators of c-di-GMP regulation of *gbpA* expression [22-24]. Transcriptional profiling studies analyzing *V. cholerae vpsT*, *vpsR* and *flrA* mutants have implicated each of the regulators in controlling *gbpA* expression, with VpsT and VpsR suggested to act as activators and FlrA acting as a repressor [20, 55].

We tested the effect of *vpsT, vpsR* or *flrA* mutation on *gbpA* expression in response to c-di-GMP. The pPDE and control plasmids were introduced into the ∆*vpsT*, ∆*vpsT* and ∆*flrA* mutants, and *gbpA* expression was assessed by qRT-PCR and by western blot. Transcript analysis showed that the *flrA* mutant had somewhat (1.4-fold) higher *gbpA* transcript levels, and the *vpsT* and *vpsR* mutants had lower levels (55% and 46% decreased, respectively), than the parental strain (Figure 3.2B). These differences were not statistically significant, but the trends were supported by the results of the western blots comparing GbpA protein abundance in the mutants compared to the wild type with vector (Figure 3.2A). Furthermore, depletion of c-di-GMP (pPDE) in the ∆*flrA*, ∆*vpsT* and ∆*vpsR* mutants resulted in 4.8-, 9.6- and 11.0-fold increased *gbpA* transcript, respectively, compared to the mutants with unmodified c-di-GMP (Figure

3.2B). Inhibition of *gbpA* expression by c-di-GMP in the mutants and the wild type was apparent at the protein level as well (Figure 3.2A). The observed changes were equivalent to the increase seen in the wild type background. Thus, none of the known c-di-GMP sensing transcription factors regulate *gbpA* expression in response to this second messenger.

c-di-GMP inhibits *gbpA* transcription in a nutrient dependent manner.

GlcNAc and GlcNAc-containing mucin have been shown to regulate *gbpA* expression, and to date the only known transcriptional regulator of *gbpA* is NagC, a repressor of genes involved in GlcNAc utilization and of *gbpA* transcription [36-38]. We sought to determine how c-di-GMP impacts regulation of the *gbpA* promoter in the presence of N-acetylglucosamine (GlcNAc). We reasoned that GlcNAc, and perhaps other carbohydrates, may affect c-di-GMP inhibition of *gbpA* expression, and that NagC may participate in this process. Specifically, we postulated that c-di-GMP promotes NagC inhibition of *gbpA* expression, such that depletion of c-di-GMP results in NagC de-repression.

To test the effect of GlcNAc on regulation of *gbpA* expression by c-di-GMP, we generated *V. cholerae* strains with a translational fusion of *lacZ* to the *gbpA* promoter and 5'UTR, with either pPDE or the vector control. These strains were grown in a defined medium (M9 minimal medium, MM) supplemented with 0.5% (w/v) GlcNAc, glucose, maltose, sucrose, fructose or casamino acids. The additional carbohydrates were included to address whether the potential effect of GlcNAc is specific to this sugar,

and casamino acids were included as a non-carbohydrate carbon source control. The cultures were grown to mid-exponential phase (OD_{600} 0.45-0.6), and expression of *gbpA* was measured by β-galactosidase assay, allowing normalization to the optical density of the culture to adjust for differences in growth due to the carbon source. Expression of *gbpA* was variable in the media tested (Figure 3.3, black bars), possibly due to differences in growth rate with the different carbon sources. Compared to *V. cholerae* with wild type c-di-GMP levels, reduction of c-di-GMP led to significantly increased reporter activity in *V. cholerae* grown in GlcNAc (1.35-fold), glucose (1.73-fold), maltose (1.59-fold) and casamino acids (1.67-fold), but not in bacteria grown in sucrose or fructose (Figure 3.3, grey bars). The results could not be attributed to growth rate, as depleting c-di-GMP (pPDE) did not affect overall growth (data not shown). These results indicate that growth on GlcNAc does not interfere with c-di-GMP regulation of *gbpA* expression.

To test whether NagC is required for c-di-GMP inhibition of *gbpA* expression, pPDE and the control vector were introduced into *V. cholerae* with an in-frame deletion of *nagC* (Δ*nagC*). Consistent with previous reports [37, 38], deletion of *nagC* resulted in 2.2-fold higher GbpA protein (Figure 3.4A) and 1.8-fold higher *gbpA* transcript abundance (Figure 3.4B) than in the wild type. Upon lowering c-di-GMP levels through PDE gene expression, we observed 20-fold and 2.4-fold increases in *gbpA* transcript and GbpA protein, respectively, comparable to those seen in the wild type background (Figures 3.4B and 3.4A, respectively). Thus, NagC is not required for *gbpA* inhibition by c-di-GMP, corroborating the lack of an effect of growth with GlcNAc as the sole carbon source.

Low c-di-GMP induces *gbpA* transcription in a cAMP-CRP-dependent manner.

In *V. cholerae*, GlcNAc, sucrose and fructose are solely imported via a phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS), and maltose is taken up via a PTS-independent mechanism [31]. Glucose is a PTS-substrate that can also be imported via a PTS-independent pathway, and GlcNAc has been reported to be a PTS dependent carbohydrate in *V. cholerae* [31]. However, a *V. cholerae nagE* mutant, which does not produce the GlcNAc PTS transporter component, is able to grow, albeit at a reduced rate, with GlcNAc as the sole carbon source, suggesting that an alternate GlcNAc uptake pathway(s) exists [38]. We observed that c-di-GMP inhibition of *gbpA* expression did not occur during growth relying on the strictly PTS-dependent carbohydrates sucrose and fructose (Figure 3.3), suggesting a dependence of c-di-GMP regulation on carbon source availability and the mechanism of uptake. Limitation of PTS-carbohydrates results in increased cAMP biosynthesis and CRP activation, allowing the bacterium to activate alternate metabolic pathways [30]. Our results suggest that c-di-GMP inhibits *gbpA* transcription when cAMP-CRP levels are elevated. We thus considered the possibility that cAMP-CRP regulates *gbpA* transcription in a mechanism that is influenced by c-di-GMP levels.

To test the role of CRP in regulating *gbpA* in response to c-di-GMP, we generated a *V. cholerae* Δ*crp* mutant and examined it for *gbpA* production. By western blot, the Δ*crp* mutant reproducibly produced ~40% less GbpA than the parent strain, supporting a role for CRP in upregulating GbpA production (Figure 3.4A). The *gbpA* transcript was also reduced by 53% in the Δ*crp* mutant (Figure 3.4B). We next

determined the role of CRP in mediating c-di-GMP regulation of *gbpA* by assessing

expression in the Δ*crp* mutant bearing pPDE. Unlike in wild-type *V. cholerae* with the

control plasmid, when intracellular c-di-GMP levels were lowered by PDE production in

the Δ*crp* strain, there was no change in *gbpA* transcript or GbpA protein levels (Figures

3.4B and 3.4A, respectively), indicating that CRP is required for activation of *gbpA*

expression in response to decreased c-di-GMP concentrations.

We next evaluated the role of cAMP in the regulation of *gbpA* in *V. cholerae.* We

generated a strain with an in-frame deletion of *cya*, which encodes the adenylate

cyclase that synthesizes cAMP, yielding a strain that is incapable of CRP activation [56].

The *cya* mutant, like the CRP mutant, produced less GbpA, with an 80% decrease in

protein compared to the wild type (Figure 3.5A). A significant 80% reduction in *gbpA*

transcript abundance was also observed in the *cya* mutant using qRT-PCR (Figure 5B).

V. cholerae Δ*cya* was then transformed with pPDE or the control vector, allowing us to

determine the interplay between cAMP and c-di-GMP in controlling *gbpA* expression.

When intracellular c-di-GMP was lowered by PDE production in the Δ*cya* background,

there was no change in *gbpA* transcript or GbpA protein levels (Figures 3.5A and 3.5B),

mirroring the effect seen with the *crp* mutation.

Additionally, we tested the effect of CRP activation on GbpA production by

mutating *cpdA*, which encodes the cAMP phosphodiesterase. In this mutant, cAMP

cannot be degraded, and cAMP-CRP complex is constitutively active [52]. In the *cpdA*

mutant the levels of *gbpA* transcript and GbpA production were comparable to the wild

type parent (Figures 3.5B and 3.5A). Reducing c-di-GMP (pPDE) in the *cpdA* mutant

increased *gbpA* transcript and GbpA protein levels by 10.5-fold and 3.2-fold,

respectively, compared to the same strain with unmodified c-di-GMP (Figures 3.5B and 3.5A). These data further support that cAMP-CRP activates the expression of *gbpA*, and indicate that cAMP and CRP are required for c-di-GMP inhibition of *gbpA* expression.

CRP production and activity are not directly affected by c-di-GMP.

We next sought to understand how c-di-GMP influences CRP-dependent activation of *gbpA* expression; c-di-GMP could regulate *crp* transcription, CRP protein levels or CRP regulatory activity. To assess the effect of c-di-GMP on *crp* expression, *crp* transcript levels were measured in *V. cholerae* with wild type (vector) or reduced c-di-GMP levels (pPDE). Transcript levels of *crp* were not affected by altering c-di-GMP (Figure 3.6A), suggesting that c-di-GMP directly or indirectly affects CRP protein levels or activity. The effect of c-di-GMP on CRP protein levels was determined by western blot. Lysates from wild type *V. cholerae* with vector or pPDE, as well as a *crp*-null control strain containing vector, were probed with anti-CRP antibodies. No differences in CRP abundance were apparent in *V. cholerae* with wild type or low c-di-GMP (Figure 3.6B).

The identification of a consensus CRP binding site (GTGAGAGCTTGATTCCACATAT) upstream of *gbpA* (and upstream of the Vc1 sequence) using Virtual Footprint software [57] suggests that CRP interacts directly with the *gbpA* promoter. We postulated that c-di-GMP may interfere with the DNA-binding activity of CRP. To test this, we used electrophoretic mobility shift assays (EMSAs) to

evaluate the interactions between CRP, c-di-GMP and the *gbpA* promoter. A 293 bp

DNA fragment encompassing the *gbpA* promoter and C-terminally tagged CRP were

used, and a non-specific 133 bp DNA fragment was included as a negative control in

each sample. As observed previously for other promoters [58, 59], CRP alone was

unable to bind the *gbpA* promoter fragment, but upon addition of the CRP ligand cAMP,

CRP bound and shifted the *gbpA* promoter fragment (Figure 3.6C). In contrast, the

presence of c-di-GMP did not promote an interaction between CRP and the promoter

fragment. Moreover, the addition of 10-fold excess c-d-GMP did not interfere with

cAMP-CRP binding to the *gbpA* promoter. Therefore, *in vitro*, c-di-GMP does not

influence the binding of cAMP–CRP to the *gbpA* promoter.

The c-di-GMP and cAMP-CRP signaling pathways act together on the *gbpA*

promoter, but not other cAMP-CRP regulatory targets.

To determine if c-di-GMP impacts the regulatory function of CRP, we assessed

the regulation of additional CRP regulatory targets by c-di-GMP. We predicted that if c-

di-GMP was having a global effect on CRP activity, additional CRP targets would be

regulated in a fashion similar to *gbpA*. We tested three metabolic genes that are

predicted targets of cAMP-CRP, VC1046, VC2013, and VC2544. All three genes were

identified in a transcriptome analysis identifying CRP and cAMP regulated genes [33],

and we additionally selected them because they each have a predicted CRP binding

site in their promoter (Virtual Footprint) [57]. Our results showed that relative to the wild

type parent strain, transcript levels of VC1046, VC2013, and VC2544 were reduced by

65% or more in the Δ*crp* background, suggesting that these genes are positively

regulated by CRP (Figure 3.7A). Next, we compared the abundance of these transcripts in wild type *V. cholerae* with vector or pPDE to determine whether manipulation of c-di-GMP affected expression of these genes. Whereas c-di-GMP depletion resulted in an 18.5-fold increase in *gbpA* transcript abundance, we observed no difference in the abundance of these transcripts in the strain with low c-di-GMP, as compared to wild type c-di-GMP levels (Figure 3.7B). Together, these results suggest that the effect of low c-di-GMP on CRP activity at the *gbpA* promoter is not a global regulatory effect and that an additional factor mediates the impact of c-di-GMP on *gbpA* transcription initiation.

Discussion

In response to extracellular stimuli, bacteria manipulate the levels of intracellular second messengers to drive behavioral changes that promote survival. Herein, we describe the combined effects of two nucleotide second messengers, c-di-GMP and cAMP, on transcriptional regulation of *gbpA,* a gene encoding a *V. cholerae* colonization factor produced in aquatic and host environmental conditions. Whereas cAMP promotes *gbpA* transcription via CRP binding to the *gbpA* promoter, c-di-GMP has a negative effect of *gbpA* promoter activity, and cAMP-CRP is epistatic to c-di-GMP regulation (Figure 8). As distinct extracellular cues trigger the c-di-GMP and cAMP signaling pathways, GbpA production may be modulated in response to multiple environmental parameters encountered by *V. cholerae*.

Having determined that c-di-GMP negatively affects *gbpA* promoter activity, we sought to identify the c-di-GMP effector that acts on the *gbpA* promoter. In the process,

we determined that CRP activates *gbpA* transcription. The CRP protein binds the *gbpA* promoter in a cAMP-dependent manner *in vitro*, and a *crp* mutant has somewhat decreased production of GbpA. Moreover, in the *crp* mutant, depletion of c-di-GMP did not result in increased *gbpA* expression. The same effect was apparent in a *cya* mutant, which lacks the adenylate cyclase responsible for cAMP synthesis (eliminating the CRP activating signal). The observation that a cAMP phosphodiesterase (*cpdA*) mutant, with constitutively activated CRP, showed the same increase in GbpA upon c-di-GMP depletion as the parental strain indicates that cAMP-CRP promotes *gbpA* transcription, and only under CRP-activating conditions is the inhibitory effect of c-di-GMP on *gbpA* transcription apparent. Consistent with this, c-di-GMP inhibition of *gbpA* expression was observed during growth on carbon sources that do not rely on a PTS for uptake (glucose, maltose and casamino acids)—conditions in which cAMP is produced and CRP is active [31]. In contrast, growth in media with the PTS-dependent sugars sucrose and fructose, whose uptake does not stimulate cAMP production and CRP activation [31], did not reveal an effect of c-di-GMP. Thus, c-di-GMP inhibition of *gbpA* transcription is observable when cAMP-CRP levels are high. That *gbpA* transcription was comparable in the presence of PTS-dependent and independent nutrient sources was a surprising result given that we anticipated that growth with PTS carbohydrates and the resulting low cAMP-CRP would decrease *gbpA* expression. However, it is possible that expression is affected by growth rate, which varies between growth media.

Given the role of CRP in c-di-GMP regulation of *gbpA* expression, we explored the possibility that c-di-GMP influences CRP at various levels. We excluded effects of c-di-GMP on CRP gene transcription and protein abundance. In other bacterial species, c-di-

GMP has a direct role in controlling the activity of CRP-like proteins [60, 61]. For example, c-di-GMP has been shown to bind CAP (catabolite activation protein)-like protein CLP from *Xanthomonas campestris*, which inhibits its ability to bind DNA and thus to regulate virulence gene expression [60]. Our data indicate that, while CRP does bind the *gbpA* promoter in a cAMP-dependent manner, c-di-GMP does not affect cAMP-CRP binding. Finally, c-di-GMP did not affect CRP regulatory function, because c-di-GMP did not broadly affect the expression of other CRP-regulated genes. Together these findings indicate that c-di-GMP does not regulate CRP, but regulates another effector that co-regulates with *gbpA* expression.

Several other potential mediators of c-di-GMP inhibition of *gbpA* transcription were considered. First, we examined the role of NagC. Not only was NagC the only known regulator of *gbpA* expression [38], the NagC and CRP regulons were previously linked in *E. coli*, in which the NagC and CRP orthologues co-regulate the expression of genes in the chitobiose operon [62]. While our results support previous reports indicating that NagC negatively regulates *gbpA*, this GlcNAc-responsive regulator had no impact on c-di-GMP regulation of *gbpA* expression. GlcNAc was previously suggested to be a PTS-dependent sugar in *V. cholerae*, in which case growth with GlcNAc as the sole carbon source would not be expected to activate the cAMP-CRP pathway [31]. However, upregulation of *gbpA* was observed upon c-di-GMP depletion during growth with GlcNAc, mirroring the results obtained during growth with PTS-independent nutrients. One possible explanation for these results is that *gbpA* is regulated via multiple mechanisms during growth in GlcNAc (cAMP-CRP, NagC, c-di-GMP, possibly others), making the net *gbpA* transcription level difficult to predict. Alternatively, GlcNAc, like

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glucose, may not strictly rely on a PTS for uptake in *V. cholerae*. Indeed, although certain PTS components are essential for GlcNAc utilization in *V. cholerae*, a strain deficient in the EIIB^{GlcNAc} transporter (NagE) retains the ability to grow with GlcNAc as the sole carbon source [38], suggesting that an additional GlcNAc transporter exists in this bacterium. Thus, an alternative pathway for GlcNAc transport would alleviate any impact on the cAMP-CRP pathway.

We directly evaluated three previously defined transcription factors known to bind c-di-GMP in *V. cholerae*, VpsT, VpsR and FlrA, as potential mediators of *gbpA* inhibition by c-di-GMP. These regulators are compelling candidates, because *gbpA* (VCA0811) appeared in transcriptional profiling studies of the respective mutants [20, 55]. The FlrA protein was recently shown to directly sense c-di-GMP, and c-di-GMP binding inhibits the interaction of FlrA with a target flagellar gene promoter (*flrBC*) [24]. Similarly, VpsT, a well-known activator of biofilm exopolysaccharide genes in *V. cholerae* [28], was recently shown to bind c-di-GMP, resulting in enhanced binding to the EPS gene promoters [22]. Finally, VpsR, another positive regulator of *V. cholerae* exopolysaccharide gene expression and biofilm production [26], appears to interact directly with c-di-GMP; unlike VpsT and FlrA, c-di-GMP binding did not influence VpsR binding to target promoters (*vpsT* and *aphA*) [23]. However, none of these regulators were confirmed to regulate *gbpA* expression or affected c-di-GMP regulation of *gbpA*. The effector that senses c-di-GMP and impinges on *gbpA* expression remains unidentified.

It is possible that carbon source influences c-di-GMP levels in *V. cholerae*. Earlier studies showed cross-talk between cAMP and c-di-GMP signaling pathways in *V.*

cholerae. Fong *et. al* have demonstrated that cAMP-CRP signaling can impact c-di-GMP production by repressing the expression of the DGC *cdgA*, and accordingly, a *crp* mutant behaves like a strain with elevated c-di-GMP levels [33]. To our knowledge, our findings are the first to link the regulatory effects of both cAMP and c-di-GMP at a single promoter. Increasing cAMP levels by inactivating the phosphodiesterase CpdA did not alter *gbpA* expression, suggesting that when CRP is maximally active, c-di-GMP can still inhibit CRP activation of *gbpA*. We speculate that direct interplay between cAMP-CRP and a c-di-GMP regulated factor may impact *gbpA* transcription such that maximal CRP-dependent activation of *gbpA* is apparent under conditions in which cAMP is abundant and c-di-GMP is low. Alternatively, the two regulatory events may be independent, and the effect of c-di-GMP is only observable when cAMP-CRP is present. Additional studies are needed to determine how nutrient availability, cAMP-CRP and c-di-GMP interact to control *gbpA* expression, and perhaps expression of other genes.

Previous studies have demonstrated a link between the available carbon source and surface colonization by *V. cholerae*. The ability to respond to PTS carbohydrates is important for binding to chitin, chitin degradation, and chitin-induced competence [52, 63]. The presence of PTS sugars, mutation of the adenylate cyclase gene *cyaA*, and mutation of *crp*, each of which reduce or eliminate cAMP-CRP, diminish the ability of *V. cholerae* to interact with chitin [63]. As GbpA also plays a role in colonization of chitin and intestinal surfaces by *V. cholerae* [35, 36], it is tempting to speculate that cAMP-CRP links *gbpA* expression with expression of genes encoding chitin utilization and chitin-induced competence components. In addition, in a germ-free mouse model, *V. cholerae* requires a functional PTS system to persist in the intestine, indicating that this

bacterium relies on PTS carbohydrates during infection [31]. cAMP-CRP also influences expression of numerous other virulence factors of *V. cholerae* [32, 56, 64, 65]. Thus GbpA is part of the larger cAMP-CRP regulated program central to surface colonization by *V. cholerae*.

Genetic evidence suggests that *V. cholerae* modulates intracellular c-di-GMP levels during transitions between its native aquatic environment and the host intestine. Biofilm formation is positively regulated by c-di-GMP, which may enhance *V. cholerae* survival on chitin and other aquatic surfaces [18, 22, 48]. Indeed, c-di-GMP regulates the production of at least one other chitin binding protein, the hemagglutinin FrhA, and has been demonstrated to promote attachment of *V. cholerae* to chitin beads [20]. Reduction of c-di-GMP is required to promote bacterial motility and increase expression of virulence factors [21, 47, 66-68] , and there is evidence suggesting that *V. cholerae* may increase c-di-GMP at later stages of infection [69]. Dysregulation of c-di-GMP signaling likely affects the ability of *V. cholerae* to persist in and transition between these environments.

The data presented here and in previous reports point to an exceedingly complex regulation of GbpA. We have determined that c-di-GMP augments GbpA production via a *cis*-acting element, Vc1, which lies in the *gbpA* 5' untranslated region (Chapter 2). The action of c-di-GMP through Vc1 appears to oppose the inhibitory effect of c-di-GMP at the *gbpA* promoter. Based on these findings, we propose a model in which low c-di-GMP levels promote *gbpA* expression by allowing activation of *gbpA* transcription by cAMP-CRP, but relatively few transcripts are stabilized by c-di-GMP. Conversely, high c-di-GMP interferes with CRP activation of *gbpA* transcription, but a higher proportion of

those transcripts are stabilized by c-di-GMP. Thus, the net level of GbpA produced would depend upon the levels of cAMP and the c-di-GMP regulated factor(s) that impact CRP-dependent activation of *gbpA*, and different threshold levels of c-di-GMP may mediate inhibition of *gbpA* transcription initiation versus message stabilization.

Another layer of GbpA regulation occurs through post-translational hydrolysis of GbpA by two quorum sensing regulated proteases, HapA and PrtV [70]. Cell density-dependent hydrolysis of GbpA may also be linked to second messenger levels. The quorum sensing pathway of *V. cholerae* influences c-di-GMP levels at least in part through regulation of c-di-GMP metabolism genes [71, 72]. Quorum sensing is also linked to the cAMP-CRP signaling pathway in *V. cholerae*. CRP impacts the production of the quorum sensing regulator HapR, as well as synthesis of cholera autoinducer 1 [32, 59, 73]. Thus, the c-di-GMP, cAMP and quorum sensing signaling networks are intricately intertwined, leading to complex regulation of GbpA production.

Conclusions

In sum, the transcriptional, post-transcriptional and post-translational regulation of GbpA may allow *V. cholerae* to fine-tune GbpA production in response to a wide range of intracellular c-di-GMP concentrations. Importantly, numerous extracellular signals that impact intracellular c-di-GMP and cAMP levels could regulate GbpA production, influencing the ability of *V. cholerae* to colonize aquatic and host surfaces.
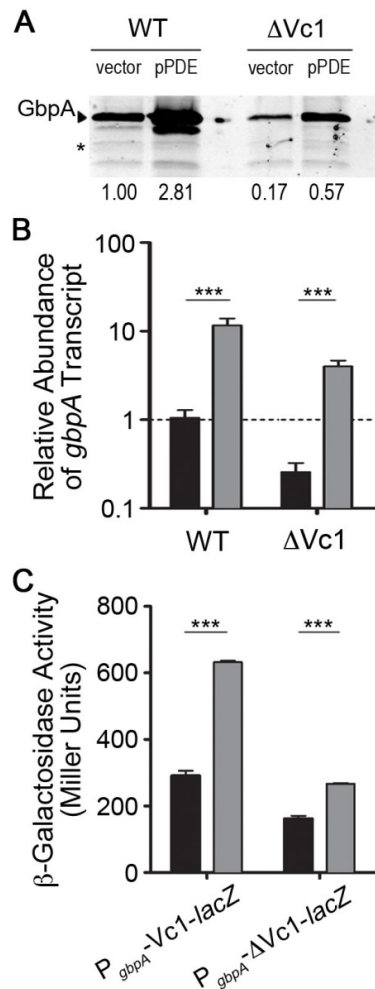
Figure 3.1. c-di-GMP inhibits *gbpA* expression independently of the Vc1 riboswitch. (A) GbpA production by *V. cholerae* wild type and ΔVc1 strains, each with wild type c-di-GMP (vector) or reduced c-di-GMP (pPDE), was determined by western blot. PDE gene expression was induced as described in the Materials and Methods. The image shown is a representative of three independent experiments. Densitometry analyses were done by normalizing the intensities of the bands corresponding to GbpA to the intensities of a cross-reactive band in the same lane (indicated by an asterisk), then comparing the normalized value to that of wild type *V. cholerae* with vector only. The fold change relative to wild type is indicated below each lane. (B) qRT-PCR was used to measure *gbpA* transcript levels in the strains described in (A), with black bars representing wild type c-di-GMP levels (vector) and grey bars representing decreased c-di-GMP (pPDE). The data were normalized relative to the wild-type containing vector only, using *rpoB* as the reference gene. Shown are the mean values and standard deviations. (C) P$_{gbpA}$-Vc1-*lacZ* or P$_{gbpA}$-ΔVc1-*lacZ* fusions were each introduced into *V. cholerae* with wild type c-di-GMP (vector, black bars) or reduced c-di-GMP (pPDE, gray bars), and the β-galactosidase activity in culture lysates of these strains was measured. Shown are the means and standard deviations of three independent experiments. (B and C) *** *P* < 0.001 by unpaired t-test comparing the indicated sets of data.

A

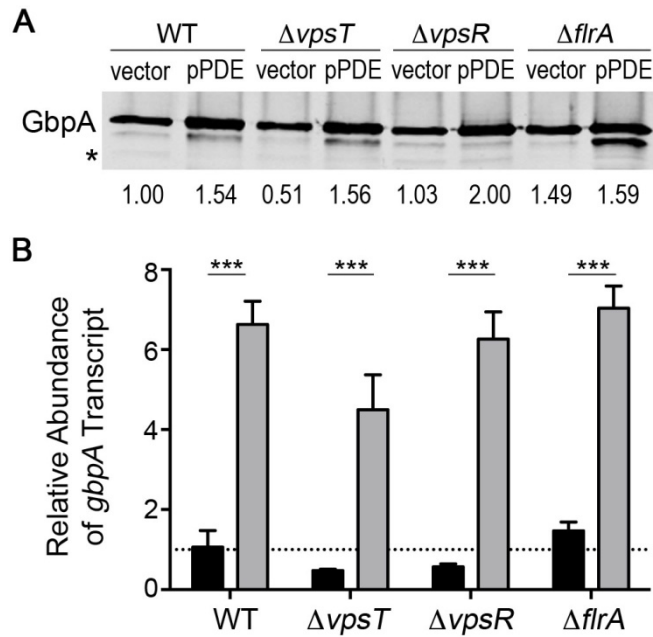|	WT		ΔvpsT		ΔvpsR		ΔflrA	
	vector	pPDE	vector	pPDE	vector	pPDE	vector	pPDE
GbpA								
*	1.00	1.54	0.51	1.56	1.03	2.00	1.49	1.59

B

Figure 3.2. Known c-di-GMP effectors FlrA, VpsT and VpsR do not regulate *gbpA* in response to c-di-GMP. (A) GbpA levels in the supernatants of wild type *V. cholerae*, Δ*flrA,* Δ*vpsT* and Δ*vpsR* strains, each with wild type (vector) and reduced levels of c-di-GMP (pPDE), were measured by western blot. PDE gene expression was induced as described in the Materials and Methods. The image shown is a representative of three separate experiments. Densitometry analyses were done by comparing the intensities of the GbpA bands to the intensities of a cross-reactive band in the same lane (indicated by an asterisk), then normalizing the value to that of wild type *V. cholerae* with vector. The fold change relative to the wild type is indicated below each lane. (B) qRT-PCR was used to measure the *gbpA* transcript abundance in wild type, Δ*flrA,* Δ*vpsT* and Δ*vpsR* strains of *V. cholerae,* each with wild type (vector, black bars) and reduced levels of c-di-GMP (pPDE, grey bars). The data were normalized relative to the wild-type containing vector only, using *rpoB* as the reference gene. Shown are the means and standard deviations from at least three independent samples. For the indicated comparisons, *** *P* < 0.001 by unpaired t-test.
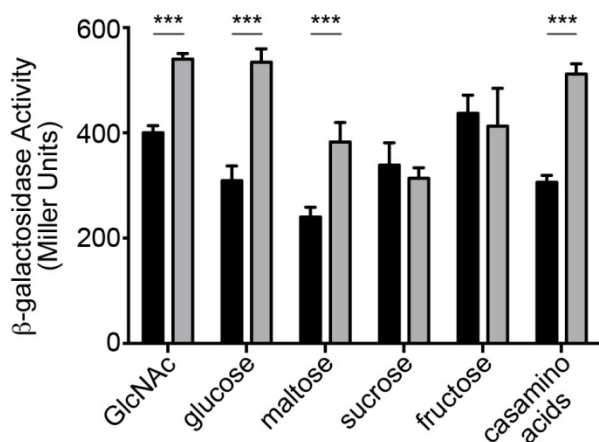
Figure 3.3. c-di-GMP inhibition of *gbpA* expression is influenced by carbon source availability. *V. cholerae* strains containing the P*gbpA*-Vc1-*lacZ* reporter fusion, with wild type c-di-GMP (vector, black bars) or reduced c-di-GMP (pPDE, grey bars), were grown in M9 minimal medium with 0.5% (w/v) N-acetylglucosamine (GlcNAc), glucose, maltose, sucrose, fructose or casamino acids to mid-logarithmic phase. PDE gene expression was induced as described in the Materials and Methods. Transcription was measured using β-galactosidase assays. Shown are the means and standard deviations from at least three independent experiments. For the indicated comparisons, *** $P <$ 0.001 by unpaired t-test.
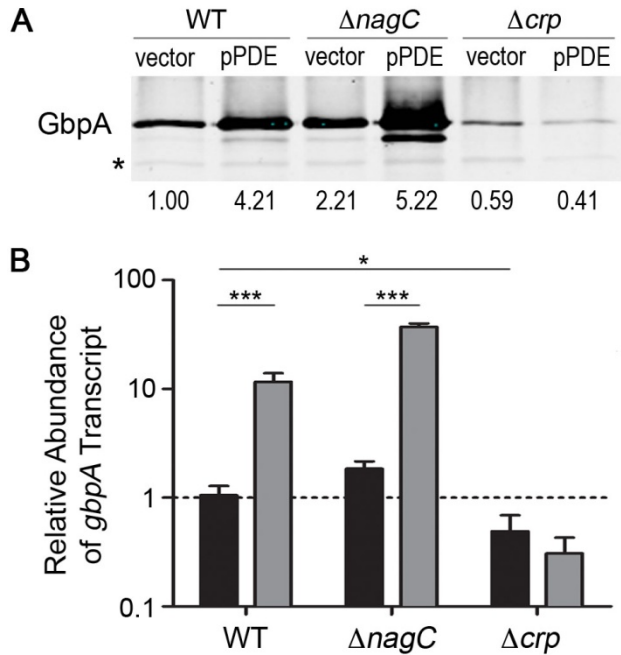
Figure 3.4. Reduction of c-di-GMP induces *gbpA* expression in a CRP dependent manner. (A) GbpA levels in the supernatants of wild type *V. cholerae*, Δ*nagC* and Δ*crp* strains, each with wild type (vector) and reduced levels of c-di-GMP (pPDE), were measured by western blot. PDE gene expression was induced as described in the Materials and Methods. The image shown is a representative of three separate experiments. Densitometry analyses were done by comparing the intensities of the GbpA bands to the intensities of a cross-reactive band in the same lane (indicated by an asterisk), then normalizing the value to that of wild type *V. cholerae* with vector. The fold change relative to the wild type is indicated below each lane. (B) qRT-PCR was used to measure the *gbpA* transcript abundance in wild type, Δ*nagC* and Δ*crp* strains of *V. cholerae,* each with wild type (vector, black bars) and reduced levels of c-di-GMP (pPDE, grey bars). The data were normalized relative to the wild-type containing vector only, using *rpoB* as the reference gene. Shown are the means and standard deviations from at least three independent samples. For the indicated comparisons, * $P < 0.05$, *** $P < 0.001$ by unpaired t-test.

A

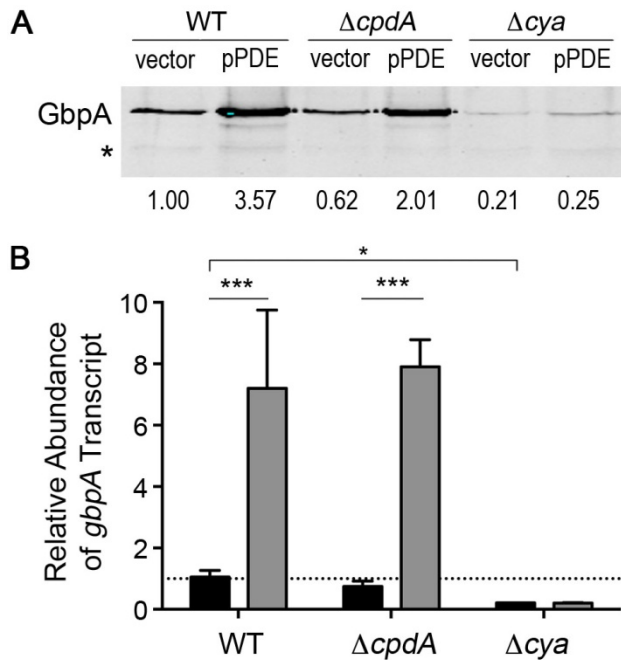|	WT		ΔcpdA		Δcya	
	vector	pPDE	vector	pPDE	vector	pPDE
GbpA						
*						
	1.00	3.57	0.62	2.01	0.21	0.25

Figure 3.5. Inactivation of the cAMP-CRP signaling pathway prevents c-di-GMP inhibition of *gbpA* expression. (A) GbpA levels in the supernatants of wild type *V. cholerae*, Δ*cpdA* (constitutively active CRP) and Δ*cya* (constitutively inactive CRP) strains, each with wild type (vector) and reduced levels of c-di-GMP (pPDE), were measured by western blot. PDE gene expression was induced as described in the Materials and Methods. The image shown is a representative of three separate experiments. Densitometry analyses were done by comparing the intensities of the GbpA bands to the intensities of a cross-reactive band in the same lane (indicated by an asterisk), then normalizing the value to that of wild type *V. cholerae* with vector. The fold change relative to the wild type is indicated below each lane. (B) qRT-PCR was used to measure the *gbpA* transcript abundance in wild type, Δ*cpdA* and Δ*cya* strains of *V. cholerae,* each with wild type (vector, black bars) and reduced levels of c-di-GMP (pPDE, grey bars). The data were normalized relative to the wild-type containing vector only, using *rpoB* as the reference gene. Shown are the means and standard deviations from at least three independent samples. For the indicated comparisons, * *P* < 0.05, *** *P* < 0.001 by unpaired t-test.
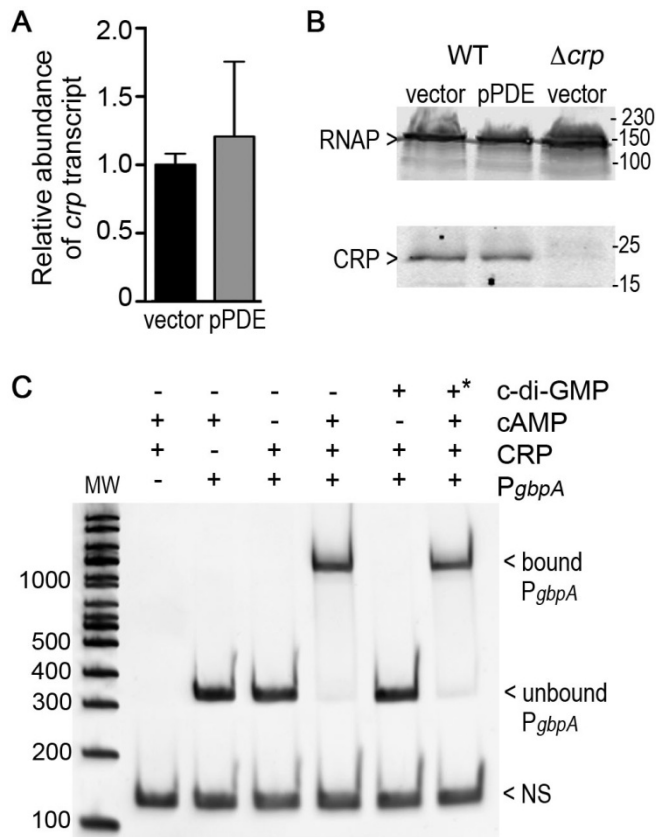
122

Figure 3.6. c-di-GMP does not regulate CRP gene transcription, protein stability or DNA binding. (A) Transcript levels for *crp* in *V. cholerae* with wild type (vector) or reduced c-di-GMP (pPDE) were measured by qRT-PCR. PDE gene expression was induced as described in the Materials and Methods. The data were normalized relative to the wild-type containing vector only, using *rpoB* as the reference gene. Shown are the means and standard deviations from at three independent samples. (B) CRP protein (23.6 kDa) levels in lysates of *V. cholerae* with wild type (vector) or reduced c-di-GMP (pPDE) were measured by western blot. *V. cholerae* Δ*crp* containing vector was included as a negative control. RNA Polymerase was detected on the same blot as a loading control. The images shown are from a representative of three independent experiments. Densitometry analyses were done by comparing the intensities of the GbpA bands to the intensities of the RNAP band in the same lane, then normalizing the value to that of wild type *V. cholerae* with vector. The fold change relative to the wild type is shown below each lane. (C) Using electrophoretic mobility shift assays, purified recombinant CRP was tested for the ability to bind and shift a DNA fragment encompassing the *gbpA* promoter in the presence or absence of cAMP and/or c-di-GMP. As a control, a non-specific DNA (indicated by "NS") fragment was added to all binding reactions and was confirmed not to be shifted by CRP. *In the final lane, c-di-GMP was added in 10-fold excess of cAMP.
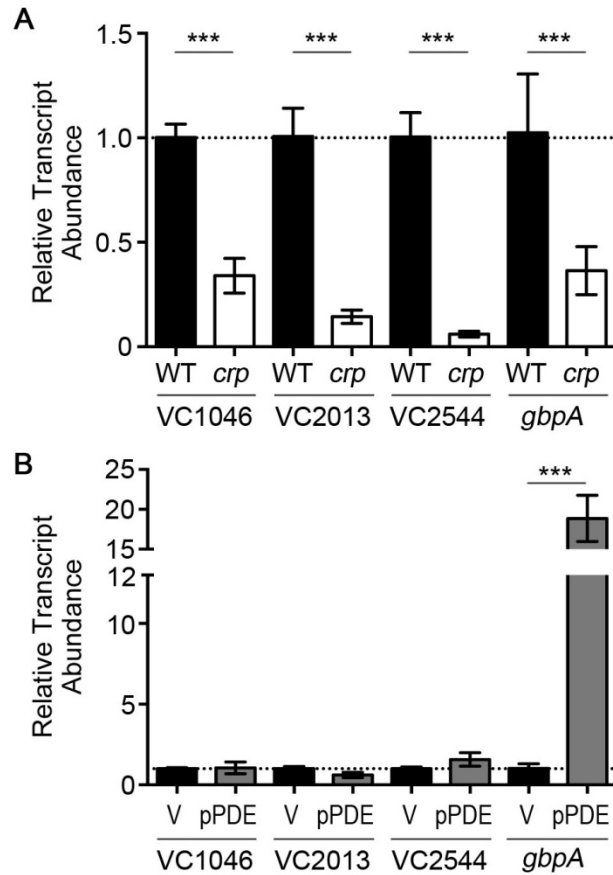
Figure 3.7. cAMP-CRP specifically impacts *gbpA* expression in response to c-di-GMP. (A) Putative CRP-regulated genes were selected for analysis by qRT-PCR to assess transcript abundance in wild type (grey bars) and Δ*crp* (black bars) strains. The data were normalized relative to the wild-type, using *rpoB* and *gyrA* as the reference genes. (B) The transcript abundance for the genes analyzed in (A) was determined for *V. cholerae* with wild type (vector, grey bars) and reduced levels of c-di-GMP (pPDE, white bars). The data were normalized relative to the wild-type containing vector only, using *rpoB* and *gyrA* as the reference genes. (A and B) Shown are the means and standard deviations from at least three independent samples. For the indicated comparisons, *** P < 0.001 by unpaired t-test.
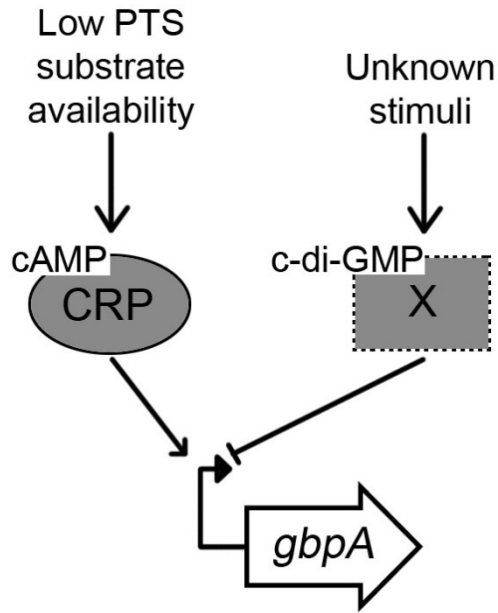
Figure 3.8. Model of c-di-GMP and cAMP regulation of GbpA production. During growth in conditions in which PTS carbohydrates are limiting, cAMP stimulates CRP to activate *gbpA* transcription. In response to an unknown stimulus (or stimuli), c-di-GMP inhibits *gbpA* transcription initiation via an unknown effector (factor "X"). c-di-GMP does not broadly impact the cAMP-CRP regulon, suggesting that the two signaling pathways converge on the *gbpA* promoter rather than act via cross-talk between the pathways. Because the effect of c-di-GMP depletion occurs only when CRP is present, cAMP-CRP is epistatic to c-di-GMP regulation.

Table 3.1. Strains used in this study.

Strain	Strain Description	Reference
Escherichia coli		
DH5α	F⁻ φ80*lacZ* ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (rK-, mK+) *phoA supE44 thi-1 gvrA96 relA1 λ-tonA*	Invitrogen; (77)
DH5αλpir	F⁻ Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17 supE44 thi-1 gvrA96 relA1 λpir*	(77)
SM10λpir	*thi thr leu tonA lacY supE recA*::RPA-2-Te::Mu *λpir*R6K, Km^r	(78)
Vibrio cholerae		
	C6706 (O1 El Tor)	(79)
	C6706 Δ*gbpA* (VCA0811)	This study
	C6706 Δ*lacZ*	(46)
	C6706 Δ*lacZ* pP$_{gbpA}$-Vc1-*lacZ*	This study
	C6706 Δ*lacZ* pP$_{gbpA}$-DVc1-*lacZ*	This study
	C6706 pBAD33	This study
	C6706 pBAD33::*vieA* (pPDE)	This study
	C6706 ΔVc1	This study
	C6706 ΔVc1 pBAD33	This study
	C6706 ΔVc1 pBAD33::*vieA* (pPDE)	This study
	C6706 Δ*nagC*	This study
	C6706 Δ*nagC* pBAD33	This study
	C6706 Δ*nagC* pBAD33::*vieA* (pPDE)	This study
	C6706 Δ*crp*	This study
	C6706 Δ*crp* pBAD33	This study
	C6706 Δ*crp* pBAD33::*vieA* (pPDE)	This study
	C6706 Δ*cpdA*	This study
	C6706 Δ*cpdA* pBAD33	This study
	C6706 Δ*cpdA* pBAD33::*vieA* (pPDE)	This study
	C6706 Δ*cya*	This study
	C6706 Δ*cya* pBAD33	This study
	C6706 Δ*cya* pBAD33::*vieA* (pPDE)	This study
	C6706 Δ*vpsT*	This study
	C6706 Δ*vpsT* pBAD33	This study
	C6706 Δ*vpsT* pBAD33::*vieA* (pPDE)	This study
	C6706 Δ*vpsR*	(47)
	C6706 Δ*vpsR* pBAD33	This study
	C6706 Δ*vpsR* pBAD33::*vieA* (pPDE)	This study
	C6706 Δ*flrA*	This study
	C6706 Δ*flrA* pBAD33	This study
	C6706 Δ*flrA* pBAD33::*vieA* (pPDE)	This study

Table 3.2. Plasmids used in this study.

Plasmid Name	Plasmid Description	Reference
pP*lac*thiM#2-*lacZ*	The TPP riboswitch encoded in the 5' UTR of *E. coli thiM* was mutated to function as an ON switch and cloned into pP*lac*-*lacZ*. Origin of replication *colEI*. Amp^R	(74)
pP$_{gbpA}$-Vc1-*lacZ*	*gbpA* promoter and 5'UTR cloned into plasmid pP*lac*thiM#2-*lacZ*; translational fusion	This study
pP$_{gbpA}$-ΔVc1-*lacZ*	*gbpA* promoter and 5'UTR, minus the Vc1 sequence (Δ 17-202 bp) cloned into plasmid pP*lac*thiM#2-*lacZ*; translational fusion	This study
pBAD33	Expression vector, P$_{ara}$ promoter, Cm^R	(75)
pBAD33::*vieA*	*vieA* in pBAD33 ("pPDE")	(48)
pBAD33::*crp*-his6	Expression vector for purification of CRP	This study
pCVD442	*oriR6K* plasmid with a polylinker, *mobRP4*, *bla*, and *sacB*	(76)
pCVD442::Δ*gbpA*	Allelic exchange vector for in-frame deletion of *gbpA*	This study
pCVD442::ΔVc1	Allelic exchange vector for in-frame deletion of Vc1	This study
pCVD442::Δ*nagC*	Allelic exchange vector for in-frame deletion of *nagC*	This study
pCVD442::Δ*crp*	Allelic exchange vector for in-frame deletion of *crp* (VC2614)	This study
pCVD442::Δ*cya*	Allelic exchange vector for in-frame deletion of *cya* (VC0122)	This study
pCVD442::Δ*cpdA*	Allelic exchange vector for in-frame deletion of *cpdA*	This study
pCVD442::Δ*vpsT*	Allelic exchange vector for in-frame deletion of *vpsT*	This study
pCVD442::Δ*vpsR*	Allelic exchange vector for in-frame deletion of *vpsR*	(47)
pCVD442::Δ*flrA*	Allelic exchange vector for in-frame deletion of *flrA*	This study

Table 3.3. Primers used in this study.

Primer Name	Oligonucleotide sequence (5' to 3') *	Reference
pCVDseqF	CTGTTGCATGGGCATAAAGTTGCC	This study
pCVDseqR	ACACAGGAACACTTAACGGCTGAC	This study
Vc1F1	CCTCTAGATACTCGTCAGGTCTTTGG	This study
Vc1R1	TTGCATGCTTGCCAAAGTGTGACCGAGAG	This study
Vc1F2	TTGCATGCCAGCTAACAAAGAAGAGTCTGTG	This study
Vc1R2	AAGAGCTCCGAGTGTACACGGTATCG	This study
gbpAF1	TTGCATGCTACTCGTCAGGTCTTTGG	This study
gbpAR1	TTGGTACCCATCACAGACTCTTCTTTG	This study
gbpAF2	TTGGTACCTAAGTTATCCTCCCTCTTAC	This study
gbpAR2	TTGAGCTCTTTCTCTGGATGGGAGTC	This study
gbpAF0	GCAAACGGTAGCAAGAAG	This study
nagCF1	GAGCATGCCTGACGATACGATGATTGATAC	This study
nagCR1	GACCATGGCATTATCAATTCTGCTCGTATTGTC	This study
nagCF2	GACCATGGGAAGACTGAGCTTTTTAGTTAAGC	This study
nagCR2	GAGAGCTCGAGCATCATCCCTAAGATCAG	This study
nagCF0	CGATACCATGCACAAAGC	This study
crpF1	CCTCTAGATTTATGAAGGCTTACACGGC	This study
crpR1	CCGCATGCCATAATAATCTCACTTCCTCTGCAG	This study
crpF2	CCGCATGCTAAGTGCCCCGATAACCC	This study
crpR2	CCGAGCTCTTGAACATCCCGATCCTTTG	This study
crpF0	CCCTACTTACTGGCGATGAT	This study
cyaF1	CCGAGCTCCATTGACGGCGTAAACTG	This study
cyaR1	CCGAATTCCAAGTTTGCTTCCCTGATATG	This study
cyaF2	CCGAATTCTAACTCGTTGACGTCTCAG	This study
cyaR2	CCTCTAGACATAAAGCGGTGTACCGTAC	This study
cyaF0	CAAGCTTACTTAGGCGAGTC	This study
cpdAF1	CCTCTAGAGCTGACTGTAGGCAGAATTG	This study
cpdAR1	GGGCATGCCAAAATCGGTAAACCTAACTCTG	This study
cpdAF2	GGGCATGCTGATATGACTGCTCGTCCTG	This study
cpdAR2	AAGAGCTCGTCTAAGGGCAACATAGTCTG	This study
cpdAF0	GTCGAGCGGGAAATGTTTG	This study
vpsTF1	CCGCATGCGCGAAGTTTCACGTACTCG	This study
vpsTR1	CCGGTACCCATTTCACCCCTCCTAACAC	This study
vpsTF2	CCGGTACCTAATTCGTTGTGTAATGTCTCTTCG	This study
vpsTR2	CCGAGCTCCATCCCACACATACCAACC	This study
vpsTF0	CGATGATATCTTGGCTCAACTC	This study
vpsTF1	TGCATGCCTACAACCCAAATCACGC	This study
vpsTR1	AATCAGCAAAACTTACATGAACCTATATTCCTT	This study
vpsTF2	GAATATAGGTTCATGTAAGTTTTGCTGATTTAC	This study
vpsTR2	TTTCTAGAGGTAAACTCAAGCCGATT	This study
vpsTF0	CTCTGTGGCGTTAGAAG	This study
FLRAF1	CCGCATGCCAACTTGTGGAACAGATGCAG	This study

FLRAR1	CC<u>GGTACC</u>CTGCATAGGTGAGATTATTTGCC	This study
FLRAF2	CC<u>GGTACC</u>AACATGCAACGCTAGGG	This study
FLRAR2	CC<u>GAGCTC</u>TCGACCATAGGCATAAATTCG	This study
flrAF0	GTTAGGCTATTTGGCCGAG	This study
RPB2F	CTGTCTCAAGCCGGTTACAA	(51)
RPB2R	TTTCTACCAGTGCAGAGATGC	(51)
gyrAqF	AATGTGCTGGGCAACGACTG	This study
gyrAqR	GAGCCAAAGTTACCTTGGCC	This study
gbpAqF	CAGTGGATTAGCGTATGGACAC	This study
gbpAqR	GTATTGAATCGCGCCACAGT	This study
crpqF	GGTGAGAAAGCGGAAACGCTGTACTA	This study
crpqR	CTTCAAACAAGCCAAGCTCACCGA	This study
VC1046qF	GCCTTGATCGAGCGGATTAT	This study
VC1046qR	TCACAGGCGATGATGTTGAG	This study
VC2013qF	CTATCGTCGGTTACGGCATTAT	This study
VC2013qR	ACCCAGATATTCAGGCAGTTG	This study
VC2544qF	TTCGCTTGGCACCTTCTATC	This study
VC2544qR	GTCCCTCTTCTGGCACATTT	This study
CRPeF	cc<u>GAATTC</u>GCAGAGGAAGTGAGATTATTATGG	This study
CRPeH6R	cc<u>GTCGAC</u>TTA*ATGGTGATGGTGATGGTG*GCGAGTGCCGTAAACCA	This study

* The underlined sequences represent restriction enzyme recognition sites. Italicized sequences encode a 6-histidine tag.

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CHAPTER 4: DISCUSSION

SUMMARY OF RESULTS

Each year, the facultative human pathogen *V. cholerae* causes millions of cases of severe cholera diarrheal disease, resulting in substantial morbidity and mortality. During outbreaks, in the absence of treatment, mortality rates can exceed 20% (1-3). Despite being primarily an aquatic organism, *V. cholerae* is adept at colonizing and flourishing in the human small intestine. *V. cholerae* has acquired the distinct ability to persist and transition between disparate environments by controlling the production of colonization, metabolic and virulence factors (3). Therefore, *V. cholerae* naturally inhabiting aquatic environments can serve as a continuous source of *V. cholerae* for ingestion and subsequent contamination of humans. In this thesis, we describe the regulation of *gbpA* by the combined contribution of two nucleotide second messengers, c-di-GMP and cAMP. GbpA is a colonization factor that is important for both persistence of *V. cholerae* in the aquatic environment and for colonizing the host intestinal epithelium (4). We first examine posttranscriptional regulation of *gbpA* by c-di-GMP through Vc1, a putative c- di-GMP riboswitch in *V. cholerae* (5). Vc1 is located in the 5' untranslated region of *gbpA*, and we provide evidence that in response c-di-GMP sensing, it positively regulates GbpA production. Furthermore, we provide evidence that cAMP promotes *gbpA* transcription via cAMP-CRP binding to the *gbpA* promoter, however c-di-GMP has a negative effect on cAMP-CRP activation of *gbpA* (Figure 4.1).

Collectively, these data suggest that distinct extracellular cues that signal to intracellular c-di-GMP and cAMP-CRP pathways, help modulate GbpA production in multiple environments encountered by *V. cholerae*.

The bacterial second messenger c-di-GMP relays iinformation about the extracellular environment to intracellular effectors. Although various types of c-di-GMP effectors have been indentified, many remain poorly defined, particularly the c-di-GMP riboswitch. We examine the function of the putative c-di-GMP riboswtich Vc1, and provide strong evidence that Vc1 responds to c-di-GMP *in vivo*. Specifically, we find that introducing the Vc1 sequence into a translational reporter construct (P*lac*UV5-Vc1UTR-*lacZ*), by fusing it to a reporter gene (*lacZ*) and placing the fusion under the transcriptional control of a heterologous, constitutive promoter, renders reporter activity regulatable by c-di-GMP. Whereas depletion of c-di-GMP through the ectopic expression of a c-di-GMP PDE gene reduces reporter activity, expression of a DGC gene augments reporter activity. These regulatory effects are attributable specifically to altered c-di-GMP, as expression of a mutant PDE gene incapable of c-di-GMP hydrolysis does not affect expression. Moreover, mutating a single residue in the Vc1 sequence, the G12 nucleotide predicted to be involved in c-di-GMP binding, eliminates regulation of downstream expression by c-di-GMP. These findings support our results showing that the same mutation at the native *gbpA* locus similarly inhibits downstream gene expression. SHAPE and equilibrium dialysis studies using Vc1 RNA corroborate a direct interaction between Vc1 and c-di-GMP. SHAPE analysis indicates that the reactivity of Vc1 changes in the presence of c-di-GMP, specifically in regions predicted to be contact points between c-di-GMP and Vc1. Moreover, the G12 mutation that

reduces downstream gene expression similarly diminishes the Vc1-c-di-GMP interaction observed through SHAPE analysis. Furthermore, *in vivo* experiments using the P*lac*UV5-Vc1UTR-*lacZ* reporter reveal that induction of a c-di-GMP PDE using a broad range of inducer concentrations, that reflect a range of intracellular c-di-GMP levels, promotes downstream gene expression in a dose-dependent manner. The ability of Vc1 to respond to a range of c-di-GMP levels is consistent with the predicted functions of a riboswitch. Together these results support a role for the Vc1 element in regulation of *gbpA* expression in response to c-di-GMP, and point to Vc1 functioning as an "on" switch in response to c-di-GMP.

Despite strong evidence that Vc1 functions as a c-di-GMP responsive element, some of our results suggest that Vc1 differs from Vc2, the canonical c-di-GMP riboswitch. For example, despite conservation of key residues in Vc1, we suspect the interaction of c-di-GMP with Vc1 RNA is much weaker than that with Vc2 RNA. We attempted to use equilibrium dialysis to determine the affinity of c-di-GMP for Vc1, but found that the maximum amount of binding observed, even with high concentrations of RNA, is ~70%. This severely limits the range of c-di-GMP concentrations we can work with, making it impossible to determine an accurate affinity measurement. Another reason for apparent weak binding may be the improper folding of Vc1. Indeed, when folded Vc1 RNA is run on a native gel, we observe several bands indicating multiple species of folded Vc1 RNA. In addition, the equilibrium dialysis data suggest that the rate of dissociation of c-di-GMP from Vc1 and Vc2 differs. The addition of excess, non-radiolabelled competitor c-di-GMP to the equilibrium chambers leads to displacement of labeled c-di-GMP from Vc1, but not from Vc2. These results suggest that binding of c-

di-GMP by Vc1 is reversible, which may allow for more rapid adaptation and modulation of GbpA production. Furthermore, previous analyses of Vc2 show that mutating G20 (equivalent to G12 in Vc1) reduces binding of c-di-GMP by 30,000-fold, essentially abolishing the interaction of Vc2 with c-di-GMP (23). However, using equilibrium dialysis, we show that the Vc1 G12 mutation reduces, but does not eliminate, the interaction with c-di-GMP *in vitro*. We confirm the interaction of Vc1 with c-di-GMP using SHAPE analysis, and find that regions in Vc1 adjacent to G12 and A39 have significantly altered reactivity in the presence of c-di-GMP, suggesting that those residues participate in c-di-GMP sensing. However, C104, which is equivalent to C92 in Vc2 and an essential residue for binding c-di-GMP, does not show a change in SHAPE reactivity, suggesting it is not essential for c-di-GMP binding to Vc1.

In addition to positive regulation of GbpA production by c-di-GMP through the Vc1 riboswitch, we also find that *gbpA* transcription is repressed by c-di-GMP. Specifically, we find that lowering intracellular c-di-GMP significantly increases *gbpA* transcription and GbpA production. We sought to determine the c-di-GMP responsive regulator that acts on the *gbpA* promoter, and in the process, identified CRP as a positive regulator *gbpA* transcription. The CRP protein binds the *gbpA* promoter in a cAMP-dependent manner *in vitro*, and a *crp* mutant has somewhat decreased production of GbpA. Moreover, in the *crp* mutant, depletion of c-di-GMP does not result in increased *gbpA* expression. The same effect is apparent in a *cya* mutant, which lacks the adenylate cyclase responsible for cAMP synthesis (eliminating the CRP activating signal). Interestingly, a cAMP phosphodiesterase (*cpdA*) mutant, with constitutively activated CRP, does not show elevated GbpA production and has the same increase in

GbpA upon c-di-GMP depletion as the parental strain. This observation indicates that when active, cAMP-CRP is poised to promote *gbpA* transcription, but it is only maximally active on the *gbpA* promoter when c-di-GMP levels are low. Consistent with the contribution of cAMP-CRP, c-di-GMP inhibition of *gbpA* expression is observed during growth on carbon sources that do not rely on PTS for uptake (glucose, maltose and casamino acids)- conditions in which cAMP is produced and CRP is active (6). In contrast, growth in media with the PTS-dependent sugars sucrose and fructose, whose uptake does not stimulate cAMP production and CRP activation, does not reveal an effect of c-di-GMP (6). Thus, c-di-GMP inhibition of *gbpA* transcription is observable when cAMP-CRP levels are high.

In order to determine effect of c-di-GMP on CRP activation of *gbpA* expression, we tested the possibility that c-di-GMP influences CRP levels or CRP activity *in vivo*. We find that c-di-GMP does not impact CRP gene transcription and protein abundance. Furthermore, c-di-GMP does not affect CRP regulatory activity *in vivo*, since manipulating intracellular c-di-GMP does not impact the expression of other CRP-regulated genes. Lastly, we show that CRP does bind the *gbpA* promoter in a cAMP-dependent manner, and c-di-GMP does not affect cAMP-CRP binding *in vivo.* Together these findings indicate that c-di-GMP does not directly regulate CRP. We hypothesize that c-di-GMP controls the levels or activity of another effector, which along with cAMP-CRP co-regulates *gbpA* expression. We directly evaluated three previously defined transcription factors known to bind c-di-GMP in *V. cholerae*, VpsT, VpsR and FlrA, as potential mediators of *gbpA* inhibition by c-di-GMP (7-9). However, none of these factors regulate *gbpA* expression in c-di-GMP dependent manner. To our knowledge,

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our findings provide the first link of the regulatory effects of both cAMP and c-di-GMP at a single promoter. We speculate that direct interplay between cAMP-CRP and a c-di-GMP regulated factor may impact *gbpA* transcription such that maximal CRP-dependent activation of *gbpA* is apparent under conditions in which cAMP is abundant and c-di-GMP is low. The effector that senses c-di-GMP and impinges on *gbpA* activation remains unidentified, but may reveal a larger regulon shared by c-di-GMP and cAMP pathways. Alternatively, the two regulatory events may be independent, and the effect of c-di-GMP is only observable when cAMP-CRP is present. Additional studies are needed to determine how nutrient availability, cAMP-CRP and c-di-GMP interact to control *gbpA* expression, and perhaps expression of other genes.

BIOLOGICAL IMPACT OF *gbpA* REGULATION

In this thesis, we propose a complex model of *gbpA* regulation by c-di-GMP. We determine that c-di-GMP augments GbpA production via Vc1, a putative c-di-GMP riboswitch, which lies in the *gbpA* 5' untranslated region. Positive regulation of c-di-GMP through Vc1 appears to oppose the inhibitory effect of c-di-GMP at the *gbpA* promoter. Based on these findings, we expect that in environmental conditions when c-di-GMP levels are low, *gbpA* expression is maximally activated by cAMP-CRP, however the proportion of *gbpA* transcripts bound to c-di-GMP via Vc1 are relatively few. Conversely, conditions where c-di-GMP levels are high prevents maximal activation of *gbpA* transcription by cAMP-CRP, but a higher proportion of *gbpA* transcripts are bound by c-di-GMP. Thus, under high and low c-di-GMP, GbpA production is promoted via posttranscriptional and transcriptional mechanisms, respectively. As such, the net

accumulation of GbpA relies heavily the levels of intracellular c-di-GMP, and the

threshold amount that mediates inhibition of *gbpA* transcription initiation and promotes

GbpA production through interactions with Vc1.

Several lines of evidence suggest that c-di-GMP levels change in *V. cholerae*

during transitions between its native aquatic environment and the host intestine. For

example, *V. cholerae* forms biofilms on surfaces in the environment, such as chitin, and

numerous studies have shown that biofilm formation is positively regulated by c-di-GMP

(7, 10, 11). Furthermore, increased intracellular c-di-GMP enhances the binding of *V.*

cholerae to chitin *in vitro* in a process dependent on the hemagglutinin FrhA (12).

Conversely, reduction of c-di-GMP is required to promote bacterial motility and increase

expression of virulence factors in the intestinal tract (7, 10, 11). Genetic evidence

suggests that c-di-GMP levels again increase during later stages of infection (13). In the

context of *V. cholerae* biology, the effect of Vc1 sensing of c-di-GMP to augment GbpA

production may contribute to colonization of environmental (chitin) surfaces, when c-di-

GMP levels may be elevated. Conversely, the effect of low c-di-GMP on increasing

gbpA transcription may contribute to attachment of host (small intestine) surfaces when

c-di-GMP levels may be low.

Defining the role of c-di-GMP in controlling GbpA production was further

complicated by the finding that *gbpA* transcription is positively regulated by cAMP-CRP.

Previous studies have suggested a link between the cAMP-CRP pathway and GbpA in

V. cholerae. Specifically, it was observed that reducing or eliminating cAMP-CRP

activity, diminished the ability of *V. cholerae* to interact with chitin, degrade chitin and

participate in chitin-induced competence (14). As GbpA also plays a role in colonization

of chitin, it is tempting to speculate that cAMP-CRP links *gbpA* expression with expression of genes encoding chitin utilization and chitin-induced competence components (4, 15). In addition, in a germ-free mouse model, *V. cholerae* requires functional cAMP-CRP to persist in the intestine, presumably by influencing expression of virulence factors important for nutrient utilization and colonization (16-19). Therefore, positive regulation of *gbpA* by cAMP-CRP may also contribute to intestinal colonization during infection. Interestingly, cAMP-CRP also controls the expression of ten genes predicted to encoded enzymes with DGC and PDE activity, suggesting that cAMP-CRP signaling can influence intracellular c-di-GMP turnover (20). Deletion of one of the predicted DGCs, *cdgA*, eliminated the elevated biofilm production observed in a *crp* mutant (20). Furthermore, cholera toxin and flagella, which are negatively regulated by c-di-GMP, are positively regulated by cAMP-CRP in *V. cholerae* (16, 17, 21, 22). Therefore, it appears that in *V. cholerae*, some pathways normally repressed by c-di-GMP signaling, are positively regulated by cAMP-CRP. These opposing modes of regulation employed by cAMP-CRP and c-di-GMP are reflected in our findings presented in this thesis.

CAVEATS AND FUTURE DIRECTIONS

We came across several caveats when characterizing the function of Vc1 in *V. cholerae.* First, analysis of the interaction between Vc1 and c-di-GMP using equilibrium dialysis showed that Vc1 RNA binds c-di-GMP to a lesser degree than Vc2 RNA. These results may reflect a lower binding affinity of Vc1 for c-di-GMP compared to the affinity of Vc2, which is extremely high (10 pM) (23). The equilibrium dialysis assay lacks the

sensitivity necessary to determine the K_D of Vc1 and c-di-GMP. However, unlike Vc2, the affinity of Vc1 for c-di-GMP may fall closer within the biological range of intracellular c-di-GMP concentrations, or Vc1 may not directly sense c-di-GMP. Using a more sensitive binding assay would better allow us to assess the affinity of c-di-GMP for Vc1.

Another caveat to consider with the Vc1 studies is that we were unable to determine the mode of regulation by c-di-GMP. We hypothesize that Vc1 binding stabilizes *gbpA* transcript, thus promoting gene expression. Unlike the c-di-GMP riboswitches described to date, the sequence immediately 3' of the predicted Vc1 aptamer does not indicate a likely mechanism of gene regulation. There are no obvious Rho-independent transcription terminator or anti-Shine Delgarno sequences. The same is true for the region 3' of the Vc1 aptamer. Quantitative reverse transcriptase PCR analysis of the entire *gbpA* transcript, which involved probing for the aptamer as well as four additional regions along the length of the mRNA, showed that the G12 mutation in Vc1 results in significantly reduced transcript abundance uniformly across the *gbpA* mRNA (data not shown). Additional work is needed to determine whether this occurs as a result of Vc1 controlling transcriptional read-through or transcript stability, or as an indirect effect on transcript stability in the absence of translation elongation. The mechanism by which the canonical GEMM riboswitch Vc2 regulates VC1722 expression also remains unknown.

Lastly, determining the role of Vc1 in controlling chitin binding or host colonization would thus require testing Vc1 mutants for altered phenotypes. Unfortunately, despite using the same *V. cholerae* strain and numerous approaches, we were unable to reproduce the effects of an in-frame deletion of *gbpA* on *V. cholerae* binding to chitin or

host colonization, so we could not assess the role of Vc1 specifically in these processes. Future work will define the roles of Vc1 and Vc2 in modulating c-di-GMP-regulated processes in response to extracellular cues in *V. cholerae*.

Our findings looking at the regulation of *gbpA* transcription initiation cAMP-CRP also presented with some caveats. Specifically, we could not unequivocally rule out the possibility that c-di-GMP impacts *gbpA* regulation by affecting cAMP-CRP activity. The effect of c-di-GMP on cAMP levels were not directly tested, therefore a negative impact of c-di-GMP on cAMP production could serve as an explanation of our findings. However, we considered this to be unlikely given the data we obtained in the *cpdA* background, which has elevated cAMP, but shows similar regulation of *gbpA* by c-di-GMP as the parent strain.

In the future, we plan to determine the role of non-conserved residues in Vc1 in c-di-GMP binding, and what role alternate contacts play in downstream gene expression. Furthermore, we plan to compare *in vivo* kinetics of Vc1 and Vc2 by comparing P*lac*UV5-Vc1UTR-*lacZ* reporter to the P*lac*UV5-Vc2UTR-*lacZ* reporter. Relating to the cAMP-CRP studies, it is possible that growth on different carbon source influences c-di-GMP levels in *V. cholerae*. Earlier findings showed cross-talk between cAMP and c-di-GMP signaling pathways in *V. cholerae* (20). Therefore, we plan to measure intracellular c-di-GMP concentrations in *V. cholerae* strains grown in PTS and non-PTS nutrient sources. We also hope to indentify the c-di-GMP responsive regulator important for cAMP-CRP activation of *gbpA*.
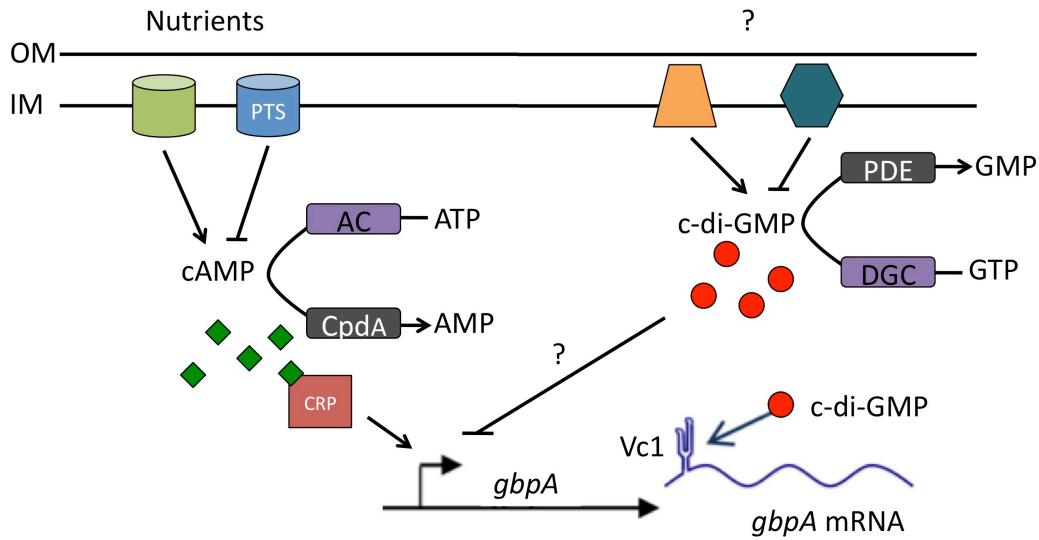
Figure 4.1. GbpA regulation by c-di-GMP and cAMP. Extracellular cues and nutrient availability can regulation intracellular levels of c-di-GMP (red circle) and cAMP (green diamond), respectively. Under low intracellular c-di-GMP conditions, cAMP-CRP promotes *gbpA* transcription, resulting in increased levels of GbpA at the cell surface. In contrast, under conditions of high intracellular c-di-GMP, *gbpA* levels are posttranscriptionally regulated through binding of c-di-GMP to the Vc1 riboswitch.

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[1]APPENDIX: A SYSTEMATIC ANALYSIS OF THE *IN VITRO* AND *IN VIVO* FUNCTIONS OF THE HD-GYP DOMAIN PROTEINS OF *VIBRIO CHOLERAE*

Summary

Background: The second messenger cyclic diguanylate (c-di-GMP) plays a central role in bacterial adaptation to extracellular stimuli, controlling processes such as motility, biofilm development, cell development and, in some pathogens, virulence. The intracellular level of c-di-GMP is controlled by the complementary activities of diguanylate cyclases containing a GGDEF domain and two classes of c-di-GMP phosphodiesterases containing an EAL or HD-GYP hydrolytic domain. Compared to the GGDEF and EAL domains, the functions of HD-GYP domain family proteins are poorly characterized. The human diarrheal pathogen *Vibrio cholerae* encodes nine putative HD-GYP domain proteins. To determine the contributions of HD-GYP domain proteins to c-di-GMP signaling in *V. cholerae*, we systematically analyzed the enzymatic functionality of each protein and their involvement in processes known to be regulated by c-di-GMP: motility, biofilm development and virulence.

Results: Complementary *in vitro* and *in vivo* experiments showed that four HD-GYP domain proteins are active c-di-GMP phosphodiesterases: VC1295, VC1348, VCA0210 and VCA0681. Mutation of individual HD-GYP domain genes, as well as combinatorial mutations of multiple HD-GYP domain genes, had no effect on motility or

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biofilm formation of *V. cholerae* under the conditions tested. Furthermore, no single HD-GYP domain gene affected intestinal colonization by *V. cholerae* in an infant mouse model. However, inactivation of multiple HD-GYP domain genes, including the four encoding functional phosphodiesterases, significantly attenuated colonization.

Conclusions: These results indicate that the HD-GYP family of c-di-GMP phosphodiesterases impacts signaling by this second messenger during infection. Altogether, this work greatly furthers the understanding of this important family of c-di-GMP metabolic enzymes and demonstrates a role for HD-GYP domain proteins in the virulence of *V. cholerae*.

Introduction

Cyclic diguanylate (c-di-GMP) is a bacterial second messenger first identified as an activator of cellulose synthesis in *Gluconacetobacter xylinus* [1]. Since its discovery, the number of processes known to be regulated by c-di-GMP in bacteria has expanded. c-di-GMP signaling has been shown to regulate numerous processes including, but not limited to, motility and biofilm production in numerous bacterial species (reviewed in [2-4]). In certain pathogens, c-di-GMP also influences virulence properties [5-17].

The concentration of c-di-GMP is controlled by the competing actions of two classes of enzymes: diguanylate cyclases, which are responsible for the synthesis of c-di-GMP from two molecules of GTP, and phosphodiesterases, which hydrolyze c-di-GMP forming two molecules of GMP. Diguanylate cyclase activity has been demonstrated in proteins containing GGDEF domains, and c-di-GMP

phosphodiesterase activity has been observed in two unrelated protein domains, the EAL and HD-GYP domains [18-24]. EAL domain phosphodiesterases were the first to be described and have been more extensively studied in terms of structure and biochemical and biological function. Comparatively little is known about the functions of HD-GYP domain proteins.

The first protein containing an HD-GYP domain shown to act as a c-di-GMP phosphodiesterase was RpfG from plant pathogenic *Xanthomonas spp.* [21]. RpfG is a response regulator containing a phosphoreceiver (REC) domain and an HD-GYP domain. Along with the sensor histidine kinase RpfC, RpfG responds to extracellular diffusible signal factor (DSF), a cell-to-cell signaling factor. Evidence suggests that, in response to DSF, RpfC phosphorylates the REC domain of RpfG, triggering the phosphodiesterase activity of the HD-GYP domain [25, 26]. The consequent decrease in intracellular c-di-GMP leads to derepression of Clp, a transcription factor inhibited by binding of c-di-GMP, activating transcription of genes necessary for virulence factor production [27-30]. Deletion of *rpfG* or amino acid substitutions in conserved residues of the HD-GYP domain, both of which abrogate c-di-GMP hydrolysis, resulted in decreased virulence factor secretion, and virulence factor secretion was restored in bacteria complemented with an EAL domain phosphodiesterase, indicating that c-di-GMP hydrolysis by RpfG is responsible for this phenotype [21, 25, 31, 32].

In *Pseudomonas aeruginosa*, two genes encoding HD-GYP domains, PA4108 and PA4781, are necessary for full virulence in the Greater wax moth *Galleria mellonella* and for optimal swarming motility [33-35]. The HD-GYP phosphodiesterase PdeB of *Borrelia burgdorferi* plays a role in motility and contributes to survival of the

bacterium in the tick vector *Ixodes scapularis* and to transmission of the bacterium to mice [13, 36].

The human diarrheal pathogen *Vibrio cholerae* genome contains numerous genes encoding confirmed or putative c-di-GMP metabolic enzymes: 31 genes encoding GGDEF domains, 12 genes encoding EAL domains, 10 genes encoding tandem GGDEF-EAL genes, and 9 genes encoding HD-GYP domains [37, 38]. A handful of diguanylate cyclases and EAL domain phosphodiesterase enzymes have been shown to impact motility, biofilm formation and virulence in animal models [14, 15, 39-46]. HD-GYP domain phosphodiesterases similarly have the potential to impact motility, biofilm formation and virulence of *V. cholerae* through modulation of c-di-GMP. However, relatively little is known about the function(s) of HD-GYP domain proteins in *V. cholerae*. The expression of four of these genes (VC2340, VCA0210, VCA0681, VCA0895) was upregulated in the presence of the quorum sensing autoinducers [47]. Ectopic over-expression of VCA0681 resulted in a reduced intracellular c-di-GMP concentration, indicating that VCA0681 possesses c-di-GMP phosphodiesterase activity [47]. Mutation of either VCA0681 or VCA0895 had no effect on biofilm formation in that study. Recently, bile acids, an extracellular signal encountered by *V. cholerae* in the intestine, were shown to activate and repress expression of the HD-GYP domain genes VC2497 and VC1295, respectively [48]. Furthermore, a VC1295 mutant has somewhat increased c-di-GMP and biofilm formation in the presence of bile acids, consistent with PDE function [48]. Beyond these experiments, not much is known about the function of HD-GYP containing proteins in *V. cholerae* or whether these proteins have phosphodiesterase activity.

Herein, we systematically analyze the biochemical and biological functions of the putative HD-GYP phosphodiesterases encoded by *V. cholerae.* We present *in vitro* and *in vivo* evidence that a subset of the HD-GYP domain proteins are enzymatically active and assess the roles of each HD-GYP domain gene in motility, biofilm formation and virulence of *V. cholerae.* This work greatly furthers the understanding of this important family of c-di-GMP metabolic enzymes and demonstrates a role for HD-GYP domain proteins in the virulence of *V. cholerae*.

Methods

Bacterial strains and growth conditions. V. cholerae C6706 and isogenic mutant strains (Table 1) were cultured in Luria-Bertani (LB) broth containing 100 µg/ml streptomycin (Sm) at 37 °C with aeration. *Escherichia coli* strains were grown in LB broth. When needed to select for plasmids, 50 µg/ml ampicillin (Amp) was added to the *V. cholerae* and *E. coli* cultures.

DNA manipulations and strain construction. Primers used in the construction of in-frame deletions of genes encoding putative HD-GYP domain proteins are listed in Table 3. Primers for deletion constructs are labeled with the locus tag of the target gene followed by F1, R1, F2 or R2 (e.g. VC1087F1 and VC2340R2). Deletions were made using standard allelic exchange methods [49]. Briefly, regions of homology upstream and downstream of the gene of interest were PCR amplified from *V. cholerae* C6706 genomic DNA using the geneF1+ geneR1 primers for the upstream fragment and geneF2 + geneR2 primers for the downstream fragment. The primers introduced

restriction sites (Table 3, underlined) that allowed cloning of the two PCR products to each other and into the suicide vector pCVD442. Ligation reaction products were transformed into DH5α λpir by electroporation, which were then selected by growth on LB-Amp agar. The desired clones were identified by screening by PCR with geneF1 + geneR2 primers and CVDseqF + CVDseqR primers that flank the multiple cloning site. Plasmids were purified from confirmed clones and transformed into Sm10λ*pir* cells by electroporation. Sm10λ*pir* strains containing the pCVD442 deletion constructs and *V. cholerae* recipient strains were grown on LB agar with Amp or Sm, respectively, then mated on LB agar lacking antibiotics for 6 hours at 37 °C. Growth was collected from the agar plate and streaked onto LB-Sm-Amp agar to select for *V. cholerae* transconjugants. Single colonies were grown in 1 mL LB broth lacking antibiotics for 8 hours. Dilutions were plated onto LB-Sm agar containing 10% sucrose and incubated for 16 hours at 30 °C. Sucrose-resistant, Amp-sensitive colonies were screened for deletion of the target gene by PCR using geneF0 and geneR2 primers.

We were unable to inactivate VC2497 by allelic exchange, so VC2497 was instead mutated by plasmid disruption with pGP704. An internal portion of VC2497 was amplified by PCR from *V. cholerae* C6706 genomic DNA using primers 2497koF and 2497koR. The product was digested with *Bgl*II and *Eco*RI and ligated into similarly digested pGP704. Ligation reaction products were transformed into DH5αλ*pir* cells by electroporation, followed by selection on LB-Amp agar. Insert-positive clones were identified by PCR using 2497koF + 2497koR. The resulting pGP704::'VC2497' plasmid was introduced into *V. cholerae* C6706 by a triparental mating with this recipient strain,

DH5αλ*pir* (pGP704::'VC2497'), and *E. coli* (pRK2013::Tn9). The integration of pGP704 into the VC2497 locus was confirmed by PCR using 2497koF + CVDseqR.

For the "DHDGYP7" strain, the following genes were sequentially inactivated as above, in order: VCA0681, VCA0210, VC2340, VC1348, VCA0895, VC1295, and VCA0931.

Construction of strains for overexpression. The genes encoding the 9 putative HD-GYP-domain containing proteins were amplified by PCR from *V. cholerae* C6706 using the geneF and geneHR primers listed in Table 2 (e.g. VC1087F and VC1087HR primers were used to amplify VC1087). The exception is VC1295, which was amplified from *V. cholerae* N16961, because VC1295 from strain C6706 contains a mutation encoding a premature stop codon. The geneHR primers introduce a sequence encoding six histidine residues at the C-terminus of the translated protein product. The PCR products for each gene were digested with the restriction enzymes indicated in the table and ligated into pMMB67EH. The ligations were transformed into *E. coli* DH5α, with subsequent selection on LB-Amp agar. Clones were screened using primers geneF + geneHR and primers 67EHF and 67EHR, which flank the multiple cloning site of pMMB67EH. The correct plasmids were introduced into *E. coli* BL21 via electroporation.

To generate a construct for expressing an inactive allele of VC1348, we replaced the codons for the HD motif (amino acids 217 and 218) with two codons for alanine. Using C6706 genomic DNA as a template, regions upstream and downstream of (and including) the targeted sequence were amplified with primers VC1348F + VC1348R1-AA and VC1348F2-AA + VC1348HR, respectively. The two fragments, which overlap by

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21 nucleotides including HD to AA mutation, were spliced together and the full product was amplified by PCR with primers VC1348F + VC1348HR. The resulting product was digested with *Sac*I and *Xba*I, ligated into similarly digested pMMB67EH, and transformed into *E. coli* DH5a. Correct clones were identified by PCR and sequencing, yielding pMMB67EH::VC1348AA-His6.

All expression plasmids were introduced into *V. cholerae* by conjugation.

In vitro c-di-GMP phosphodiesterase assays. Purified His_6-WspR protein from *Pseudomonas aeruginosa*, which contains a catalytically active GGDEF domain, was used to synthesize the radiolabelled c-di-GMP substrate for the phosphodiesterase enzymatic assays [50]. Purified His_6 –WspR (10 µL) was incubated overnight at 37 °C with 5 µL of $[\alpha\text{-}^{32}P]$GTP (3000 Ci/mmol; PerkinElmer Life Sciences) and 5 µL of 100 mM GTP buffer containing 75 mM Tris, pH 8, 250 mM NaCl, 25 mM KCl, and 10 mM $MgCl_2$. Subsequent steps were carried out as described [24].

E. coli BL21 containing either HD-GYP overexpression plasmid or vector were grown in LB-Amp broth overnight at 37 °C with aeration, then diluted 1:100 in 12 mL of LB-Amp containing 1 mM IPTG. Cultures were grown at 30 °C until an OD_{600} of 0.6-0.7 was reached. Cells were collected by centrifugation at 5000 x g for 10 minutes at 4 °C. The cells were suspended in 1 mL of phosphodiesterase buffer containing 75 mM Tris, pH 8, 25 mM KCl, 25 mM $MnCl_2$, and 10% glycerol. The cells were lysed by three rounds of freeze-thaw followed by sonication. Samples were centrifuged at 5,000 × g for 10 minutes at 4 °C. Supernatants (soluble material) were removed and stored at –20 °C prior to use in phosphodiesterase enzymatic assays. Reaction products were

identified based on previously determined migration in KH_2PO_4 buffer [51].

Assay for in vitro phosphodiesterase activity. Soluble fractions from *E. coli* BL21 overexpressing HD-GYP genes were tested for phosphodiesterase activity by incubating 18 μL of cell lysates with 2 μL of [^{32}P]c-di-GMP for 0 min to 30 min at room temperature as previously described [14]. As negative controls, cell lysates from similarly treated *E. coli* with empty vector and a buffer-only control were tested. The reactions were stopped by spotting 0.5 μL of the reactions on PEI-cellulose and allowing the spots to air-dry. Nucleotides were separated by thin layer chromatography in 1.5 M KH_2PO_4, pH 3.65. PEI-cellulose plates were air-dried. Reaction products were visualized by phosphorimagery using a Storm Phosphorimager (GE Healthcare).

Phenotype assays. Biofilm production was assayed by crystal violet staining as described previously [52, 53]. Briefly, biofilms of *V. cholerae* were grown statically in LB broth in 13 mm diameter glass culture tubes at room temperature (~23 °C) for 24 hours. Where indicated, 1 mM IPTG was added to induce phosphodiesterase gene expression. Unattached cells were removed, and the remaining biofilms were rinsed and stained with 0.1% crystal violet. The stained biofilms were washed with water, and then the stain was solubilized with 2 ml of 50% v/v ethanol. The biofilm material was dispersed using a sonicating water bath. Biofilm formation (*sugar, water and of course purple*) was determined by measuring the absorbance at 540 nm. At least 3 independent samples were assayed.

Motility was measured using soft agar assays as described [54]. Strains were grown overnight at 37 °C on LB + 1.5% agar. Single colonies were inoculated into motility plates (1% tryptone, 0.5% NaCl, 0.3% agar). Where indicated, 1 mM IPTG was added the plates to induce phosphodiesterase gene expression. Motility plates were incubated for 16 hours at 30 °C, and colony swarm diameters were measured. Three independent experiments of at least 4 replicates of each strain were performed.

Western blots. BL21 and *V. cholerae* strains containing HD-GYP expression vectors were grown in LB-Amp broth overnight at 37 °C with aeration, then diluted 1:100 in LB-Amp containing 1 mM IPTG. Cultures were grown at 30 °C with aeration to an OD_{600} of ~0.7. Cells were collected by centrifugation at 12,000 x g and suspended in 100 µL of 2x Laemmli sample buffer [55]. Samples were separated by electrophoresis on a 12% polyacrylamide SDS gel then transferred to a nitrocellulose membrane. Western blots were carried out according to standard procedures. Primary mouse His-6 antibody (ThermoScientific) was used at a 1:1000 dilution. Dylight 800 goat α-mouse secondary antibody (ThermoScientific) was used at a 1:15000 dilution. Blots were imaged using an Odyssey imager (LI-COR Biosciences).

In vivo *competition*: The animal experiments were done in accordance with protocol 12-239 approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Mouse *in vivo* competition experiments were carried out in 5 day old CD-1 mice as described previously [56]. Briefly, HD-GYP mutant strains were mixed 1:1 with WT Δ*lacZ* mice in 0.85% NaCl. To determine the ratio of

mutant to WT in the inocula, these mixtures were plated on LB-Sm agar containing 40

µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), which allows

differentiation of mutant and Δ*lacZ* bacteria (blue versus white colonies, respectively).

Mice were inoculated intragastrically with ~10^5 total bacteria. Animals were sacrificed 21

hours post-inoculation. Small intestines were harvested and homogenized, then

dilutions were plated on LB-Sm agar containing X-Gal. The competition index was

determined by dividing the ratio of mutant / Δ*lacZ* bacteria in the homogenates (output)

by the ratio of mutant / Δ*lacZ* bacteria in the inocula. In parallel, the mixtures used to

inoculate mice for *in vivo* competition experiments were also diluted 1:1,000 into 2 ml of

fresh LB broth and incubated overnight to determine the *in vitro* competition index. At

least 5 mice were used for each competition. Data were analyzed by the Wilcoxon

signed-rank test, with values compared to a hypothetical value of 1.

Results

  A subset of the 9 genes of V. cholerae *is predicted to encode enzymatically*

active HD-GYP domains. V. cholerae N16961 encodes 9 putative HD-GYP domain c-di-

GMP phosphodiesterases with variable lengths, domain architectures and predicted

cellular localizations. Most of the proteins are estimated to be approximately 400-500

amino acids in length, though VCA0895 is considerably larger, with 981 amino acids.

Two HD-GYP domain proteins, VC1295 and VCA0895, have predicted transmembrane

domains and are predicted to be localized in the inner membrane. As is common of c-di-

GMP metabolic enzymes, all 9 HD-GYP domain proteins contain additional domains

potentially involved in regulating protein function. Three HD-GYP domain proteins,

VC1087, VC1348 and VCA0210, possess a phosphoreceiver (REC) domain, suggesting that they may be modulated by phosphorylation. Two of the proteins, VC1295 and VCA0895, contain a domain present in Histidine kinases, Adenylate cyclases, Methyl-accepting proteins and Phosphatases (HAMP), which is a linker domain suggested to function in intramolecular signal transduction [57-59]. Three contain additional domains of unknown function: VC2497, DUF3391; VCA0210, DUF3369; and VCA0931, DUF3392. Interestingly, VCA0681 contains tandem HD and HD-GYP domains. Together, these 9 putative c-di-GMP phosphodiesterases have the potential to respond to myriad signals to modulate intracellular c-di-GMP.

Previous studies have used genetic and biochemical approaches to identify the residues of HD-GYP domain proteins that are important for catalytic activity [21, 60-62]. Mutational analysis of *Pm*GH, an HY-GYP domain phosphodiesterase from *Persephonella marina* for which the c-di-GMP cocrystal structure has been solved, identified at least 11 amino acids required for full phosphodiesterase activity [60]. Based on conservation of residues deemed critical for activity of the HD-GYP domain phosphodiesterase *Pm*GH, four of the 9 putative HD-GYP domain proteins encoded by *V. cholerae* are predicted to be enzymatically active: VC1295, VC1348, VCA0210 and VCA0931 (Figure 1B). VC2497, VC2340 and VCA0681 (predicted to have tandem HD and HD-GYP domains, labeled "a" and "b" in Figure 1, respectively) contain one non-conserved residue required for activity of *Pm*GH. Two, VC1087 and VCA0895, lack an intact HD motif and are expected to be enzymatically inactive.

Four HD-GYP domain proteins of V. cholerae *are enzymatically active* in vivo. To assess the enzymatic activity of each putative HD-GYP domain phosphodiesterase, we used motility and biofilm formation, processes well-known to be inhibited and activated by c-di-GMP in *V. cholerae*, respectively, as phenotypic indicators of *in vivo* enzymatic activity [14, 40, 43, 63]. Each HD-GYP domain-encoding gene was ectopically expressed in *V. cholerae* under the regulation of an inducible promoter. We used alleles containing a C-terminal sequence encoding a 6-histidine tag, allowing us to confirm expression by western blot. Each strain produced a His6-tagged protein of approximately the anticipated molecular weight, though the abundance of the proteins varied (Figure 2A, left). Expression of VCA0895-His6 was relatively poor; visualization required loading twice the amount of sample and a longer exposure of the membrane (Figure 2A, right).

The strains were then assayed for altered motility and biofilm formation compared to the parental strain carrying vector only, each with and without IPTG to induce gene expression. *V. cholerae* expressing the characterized EAL domain phosphodiesterase VC1592 was included as a positive control for both experiments [46]. Expression of VC1592, VC1295, VC1348, VCA0210, and VCA0681 significantly increased motility of *V. cholerae* through 0.3% agar medium, as evinced by expanded diameters of motility (Figure 2B). Expression of the same genes significantly decreased biofilm formation in rich medium on glass (Figure 2C). These results are consistent with reduced intracellular c-di-GMP upon expression of c-di-GMP phosphodiesterases and suggest that VC1295, VC1348, VCA0210 and VCA0681 encode active HD-GYP

domain c-di-GMP phosphodiesterases. As expected, VC1087 and VCA0895, which lack an intact HD motif, had no effects on motility or biofilm formation.

A subset of HD-GYP domain proteins of V. cholerae *are enzymatically active* in vitro. Because the HD-GYP domain proteins could affect biofilm formation and swimming motility independently of c-di-GMP hydrolytic activity, we also assayed their enzymatic activity *in vitro*. The same plasmids used for expression in *V. cholerae* were transformed into *E. coli* BL21. These strains were grown in the presence of IPTG to induce gene expression; *E. coli* BL21 with vector only was included as a negative control. Production of His6-tagged HD-GYP domain proteins was confirmed by western blot (Figure 3A, left). As in *V. cholerae*, *E. coli* expressing VCA0895 produced less protein, and loading 2-fold more sample was needed to detect VCA0895-His6 (Figure 3A, right). Culture lysates were tested for the ability to hydrolyze c-di-GMP by incubating them with radiolabeled c-di-GMP. The reaction products were separated by TLC and visualized by phosphorimagery. *In vitro*, VC1295, VCA0210 and VCA0681 reproducibly hydrolyzed c-di-GMP ($R_f \sim 0.3$) at levels above that of the vector-only negative control. Notably, in these reactions, both linearized pGpG ($R_f \sim 0.55$) and GMP ($R_f \sim 0.65$) were apparent. VC1348, which showed activity *in vivo*, failed to hydrolyze c-di-GMP above background levels. VC1087 and VCA0895, as expected, were inactive.

The effect of VC1348 expression on swimming motility require(s) c-di-GMP phosphodiesterase activity. For eight of the nine HD-GYP domain proteins, the *in vivo* over-expression and *in vitro* enzymatic assays were in agreement, indicating that

VC1295, VCA0210 and VCA0681 are functional c-di-GMP phosphodiesterases. Expression of the remaining HD-GYP domain protein, VC1348, increased motility and decreased biofilm formation of *V. cholerae*, consistent with reduced c-di-GMP in this strain. However, no c-di-GMP phosphodiesterase activity could be detected *in vitro*. To determine if the phenotypic changes upon VC1348 expression in *V. cholerae* were due to phosphodiesterase activity, the effect of expressing a mutant allele of VC1348 on *V. cholerae* motility was assessed as described above. The resulting mutant protein, herein named VC0398AA, contains alanine substitutions in the HD motif (amino acids 217 and 218), which has been shown to be required for c-di-GMP phosphodiesterase activity [21]. Expression of the positive control gene VC1592 and wild type VC1348 significantly increased motility of *V. cholerae* through 0.3% agar, while expression of VC1348AA did not (Figure 4). These results indicate that the HD motif of VC1348, and therefore c-di-GMP hydrolysis by this protein, is required for function in vivo.

Expression of the HD-GYP domain genes in V. cholerae during planktonic and biofilm growth. We postulated that the expression of the HD-GYP domain-encoding genes may be differentially expressed during growth of *V. cholerae* in a biofilm compared to in planktonic culture. For instance, one or more of the genes might be down-regulated, leading to increased intracellular c-di-GMP and enhanced biofilm formation. To get a picture of the transcriptional profiles of the HD-GYP domain genes, we used qRT-PCR to determine the relative expression of each HD-GYP domain gene in biofilm and planktonic culture. The *vpsT* gene, previously determined to be upregulated in a biofilm, was included as a positive control [64]. Several transcripts,

VC1348, VCA0210, VCA0681, and VCA0895, were significantly more abundant in biofilm cells than in planktonic cells (Figure 5). Only one, VC2497, was significantly reduced in biofilms. These data suggest that c-di-GMP phosphodiesterases can be upregulated in biofilms, for example to counter the activities of diguanylate cyclases during biofilm development.

Analysis of the roles of HD-GYP domain phosphodiesterases in motility, biofilm and virulence of V. cholerae. To assess the functions of the HD-GYP domain phosphodiesterases in *V. cholerae*, in-frame deletions were made in each gene, and the mutants were tested for altered biofilm formation and motility. None of the mutants showed phenotypes different from the WT parental strain (Figure 6A and 6C). To address the possibility that two or more of the HD-GYP proteins have redundant functions, additional mutants were made in which multiple HD-GYP encoding genes were deleted. None of these mutants, including strain DHDGYP7 (ΔVCA0681 ΔVCA0210 ΔVC2340 ΔVC1348 ΔVCA0895 ΔVC1295 and ΔVCA0931), which contains mutations in all HD-GYP genes encoding functional enzymes, were phenotypically different from the WT parent (Figures 6B and 6D).

Previous work with the infant mouse model has shown that c-di-GMP inhibits the ability of *V. cholerae* to effectively colonize the intestine. Mutation of the EAL domain phosphodiesterase VieA reduced fitness of *V. cholerae* O395 in the infant mouse [15]. We thus tested each of the HD-GYP mutants for colonization of the infant mouse small intestine using a competition assay. In this study, equivalent numbers of differentially labeled WT (*lacZ*+) and mutant (Δ*lacZ*) bacteria were co-inoculated into infant mice.

After 21 hours, the small intestines were harvested, homogenized and plated on media containing X-gal to enumerate the WT and mutant bacteria. Competition indices were determined by dividing the ratio of mutant: WT bacteria in the tissues to the ratio of mutant: WT bacteria in the input mixtures. None of the individual HD-GYP gene mutations affected the ability of *V. cholerae* to colonize the infant mouse small intestine (Figure 7). Interestingly, the additive mutation of 7 HD-GYP domain genes (DHD-GYP7), including all those that are enzymatically active PDEs, led to a significant decrease in bacterial burden in the small intestine (mean CI = 0.135, *P* < 0.001). This may be attributable in part to overall reduced fitness of the mutant, as the DHD-GYP7 strain was slightly attenuated for growth in LB compared to the Δ*lacZ* control (mean CI = 0.639, *P* < 0.05). These results indicate that two or more HD-GYP genes in combination are important for virulence of *V. cholerae*. Alternatively, the cumulative effects of multiple PDE on intracellular c-di-GMP levels during infection may impair host colonization.

Discussion

The c-di-GMP signaling system of *V. cholerae* is complex, including a large number of c-di-GMP synthases, hydrolases, effectors and regulatory targets. This work presents a methodical analysis of the functions of all 9 HD-GYP domain proteins encoded by *V. cholerae*.

Using *in vitro* and *in vivo* approaches, we found that a subset of the HD-GYP domain proteins possesses c-di-GMP phosphodiesterase activity. Activity correlates well with conservation of residues previously determined to be required for activity and

with the overall consensus sequence of the HD-GYP domain [61, 65]. We expected that VC1295, VC1348, VCA0210 and VCA0931 would be enzymatically active proteins; each contains conserved residues previously shown to be required for activity of *Pm*GH [61]. In addition, each contains conserved residues in the GYP motif region and other residues involved in recognition of c-di-GMP [61, 66]. We consistently observed *in vivo* and *in vitro* activity for VC1295 and VCA0210. VC1348 caused increased motility and reduced biofilm formation when expressed in *V. cholerae*, suggesting that it is able to hydrolyze c-di-GMP *in vivo*, but we did not detect enzymatic activity *in vitro*. Mutating the HD residues required for phosphodiesterase activity of HD-GYP domain proteins ablated the effect of VC1348 expression on motility of *V. cholerae*. These results indicate that VC1348 is indeed a functional c-di-GMP phosphodiesterase in *V. cholerae*. We suspect that expression of VC1348 in *V. cholerae* allows VC1348 to be post-translationally activated, likely though its REC domain, but that the activating partner is absent in *E. coli* BL21. The adjacent, divergently transcribed gene VC1349 encodes a putative sensor kinase/response regulator and may regulate VC1348 in *V. cholerae*. VCA0931 failed to demonstrate c-di-GMP hydrolysis, neither affecting biofilm formation or motility of *V. cholerae* nor hydrolyzing c-di-GMP *in vitro*. VCA0931 possesses an N-terminal domain of unknown function, which may post-translationally regulate the HD-GYP domain in response to specific cues lacking in our assays, or mask activity of the HD-GYP domain [33].

As we anticipated, VC1087 and VCA0895, which lack the HD dyad characteristic of HD metal-dependent phosphohydrolases, showed no evidence of c-di-GMP hydrolytic activity *in vivo* and *in vitro*. The activities of a subset of the HD-GYP domain

proteins could not be easily predicted due to lack of conservation of one or more residues expected to be required for activity: VC2497, VC2340 and VCA0681 [21, 61]. While VC2497 and VC2340 were inactive in all assays used, VCA0681 consistently showed phosphodiesterase activity. VCA0681 is one of the few HD-GYP proteins studied to date. Its enzymatic activity was previously demonstrated and its crystal structure was solved [47, 60]. The differences between the inactive VC2497 and VC2340 and the active HD-GYP domain proteins that determine enzymatic functionality are not readily apparent. Indeed, VCA0681 is a functional PDE despite lacking an aspartic acid required for activity of *Pm*GH (D308 in *Pm*GH, Q373 in VCA0681). The substitution of an aspartic acid with an asparagine in VCA0681 may preserve enzymatic activity, or the requirements of *Pm*GH may not be universal to all HD-GYP domain PDEs. Further characterization of structure-function relationships of HD-GYP domain phosphodiesterases from a variety of organisms will be necessary to define the features required for activity.

Analysis of the expression of the HD-GYP domain genes in *V. cholerae* showed that VC1348, VCA0210, VCA0681 and VCA0895 transcripts are significantly higher in biofilm-derived *V. cholerae* than in planktonic cells. Interestingly, three of these genes, VCA0210, VCA0681 and VCA0895 were among the four HD-GYP domain genes regulated by quorum sensing [47]. Thus, high cell density in the *V. cholerae* biofilm may be the signal controlling expression of these genes.

We found that mutation of individual HD-GYP domain genes did not affect c-di-GMP regulated phenotypes of *V. cholerae* under the conditions tested. Even removal of all active (and many inactive) HD-GYP domain proteins in a single strain had no effect

on motility or biofilm production. There are several possible reasons for the lack of phenotypic differences from the parent strain. First, it remains possible that HD-GYP domain phosphodiesterases, individually or as a class, impact c-di-GMP regulated processes other than those investigated here. Second, not all HD-GYP domain proteins are likely to be produced or enzymatically active under the conditions tested, so eliminating them may not have an observable effect. Our qRT-PCR studies of HD-GYP gene expression in planktonic and biofilm cultures support this possibility—expression of each HD-GYP domain gene is variable within those conditions. The observation that a VC1295 mutant produces somewhat more biofilm in the presence of bile acids further indicate that specific extracellular cues may be necessary to stimulate HD-GYP production and/or activity [48].

There may also be strain-dependent differences in the panel of c-di-GMP metabolic enzymes used, and different results might be obtained using a different *V. cholerae* strain. For example, mutation of the EAL domain phosphodiesterase VieA in the classical biotype of *V. cholerae* (strain O395) causes marked effects on motility, biofilm formation and virulence gene expression [15, 40]; the same mutation in the El Tor strain C6709 has no effect on these processes [14, 47].

HD-GYP domain proteins can also perform their regulatory function via physical interactions with other proteins, independently of c-di-GMP phosphodiesterase activity. In *Xanthomonas spp.*, for example, the HD-GYP phosphodiesterase RpfG interacts with two diguanylate cyclases to regulate motility [21, 66, 67]. The HD-GYP domain protein ECA3548 from *Pectobacterium atrosepticum* lacks apparent c-di-GMP phosphodiesterase activity (and lacks the conserved HD motif), yet still impacts c-di-

GMP regulated processes, possibly via direct interactions with other proteins [68]. The HD-GYP domain proteins of *V. cholerae* may also rely on protein-protein interactions to mediate their effects, and the necessary interactions may not have been achieved through over-expression or mutagenesis.

Finally, there is potential for phenotypic redundancy among the HD-GYP domain phosphodiesterases, as well as with EAL domain phosphodiesterases. Upon mutation of each of the GGDEF and EAL domain genes in *V. cholerae*, and only some mutants showed altered motility and/or biofilm development [41, 45]. Therefore the chance of observing an effect of mutating the HD-GYP subset of c-di-GMP phosphodiesterases may be low. This is supported by our finding that individually, mutation of HD-GYP genes had no effect on the ability of *V. cholerae* to colonize the infant mouse small intestine, but a combination of mutations (DHDGYP7) significantly impaired colonization. It is possible that a combination of two or more HD-GYP domain proteins, or that the additive effects of multiple c-di-GMP PDEs, influence virulence of *V. cholerae*. These findings suggest that HD-GYP domain proteins as a family influence c-di-GMP levels during infection, which has previously been shown to impact *V. cholerae* virulence by regulating the virulence gene regulator ToxT [14, 15].

In the context of an infection, discrete extracellular signals may trigger the production or activity of a specific c-di-GMP phosphodiesterase (or diguanylate cyclase), with cumulative but targeted effects on virulence gene expression. Strictly speaking, the c-di-GMP phosphodiesterases involved would not be mechanistically redundant (for example, having different interacting partners or subcellular localization), but work together toward the same phenotypic output. Such a regulatory system may

allow *V. cholerae* to induce virulence gene expression in the host despite the absence or mitigation of one specific extracellular signal. A different set of c-di-GMP metabolic enzymes could modulate biofilm or motility in response to a distinct set of signals.

Conclusions

Compared to GGDEF domain diguanylate cyclases and EAL domain phosphodiesterases, the HD-GYP domain phosphodiesterases have been understudied. This may be partly due to the fact that genes encoding HD-GYP domains are less widespread in bacterial genomes than those encoding GGDEF and EAL domains [38, 65]. In *V. cholerae*, only a subset of the HD-GYP domain genes encoded are functional c-di-GMP PDEs. The individual HD-GYP domain genes are dispensable for motility and biofilm formation under the conditions tested, but collectively two or more of the genes are important for virulence of *V. cholerae*. Additional studies of HD-GYP domain proteins from other bacteria are needed to gain a full understanding their roles in c-di-GMP signaling.
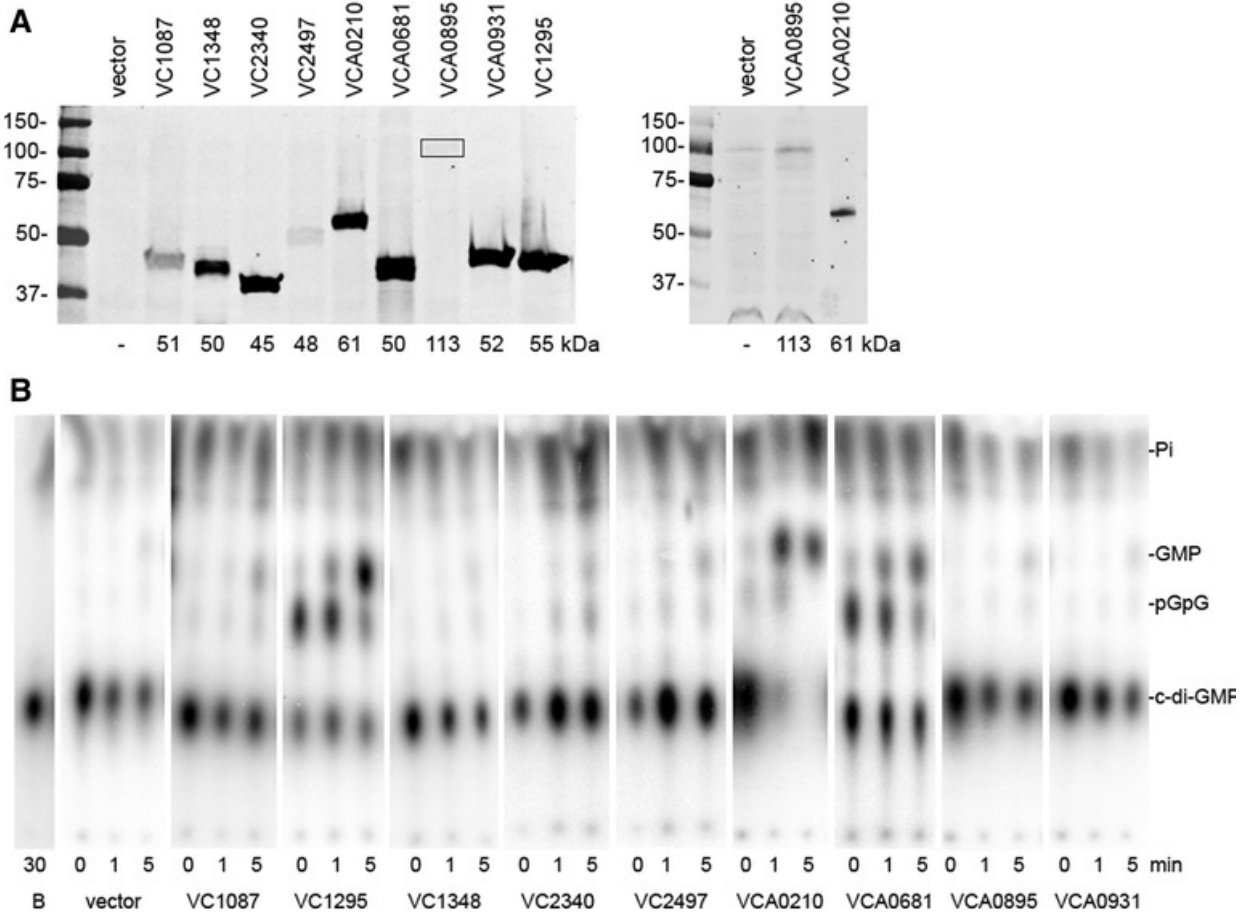
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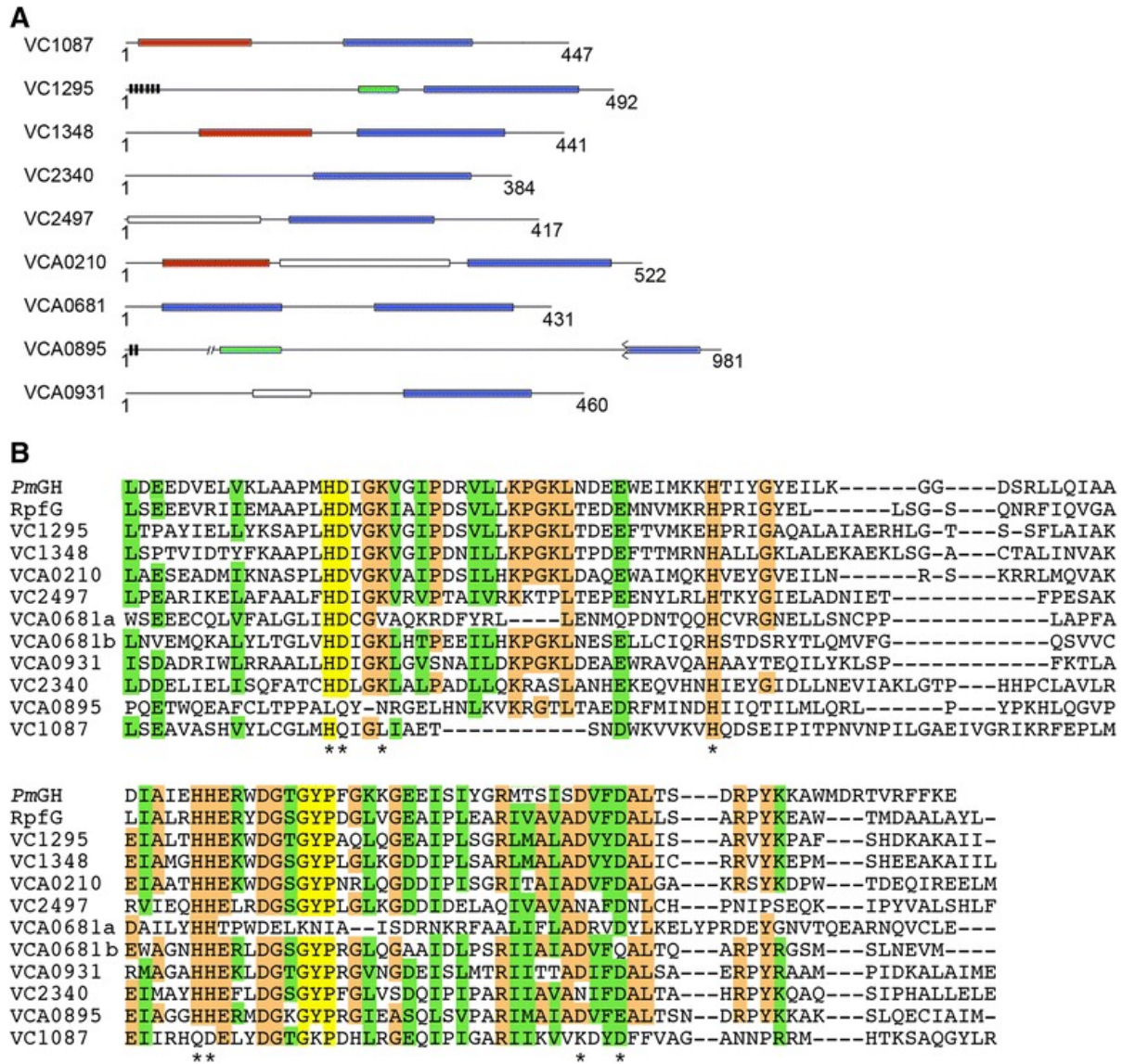
Figure 1. Domain architectures and sequence conservation of putative HD-GYP domain proteins of *V. cholerae*. (A) Predicted domains of the nine HD-GYP domain proteins encoded by *V. cholerae*. Shown are HD-GYP domains (blue), phosphoreceiver domains (red), HAMP domains (green), domains of unknown function (white) and putative transmembrane domains (black). The lengths of the proteins (number of amino acids) are indicated. (B) Alignment of *V. cholerae* HD-GYP domains. The HD-GYP domain amino acid sequences from *V. cholerae*, including the two tandem domains in VCA0681, were aligned to the HD-GYP domains of RpfG from *X. campestris* and *Pm*GH from *Persephonella marina* using Clustal Omega software. Highlighted are the HD and GYP motifs (yellow), identical amino acid residues (orange) and conserved amino acid residues (green). Asterisks indicate residues previously shown to be required for enzymatic activity of *Pm*GH [61].

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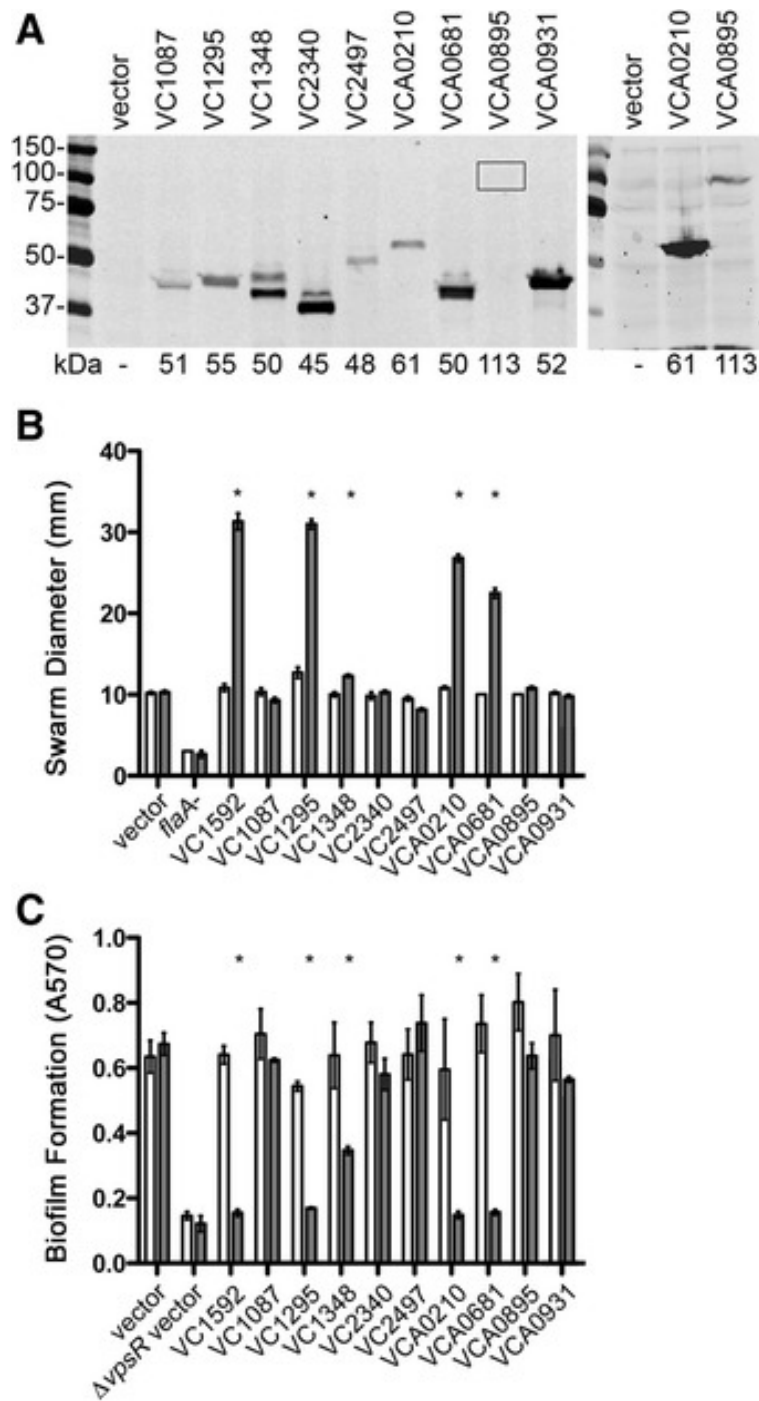
Figure 2. Ectopic over-expression of HD-GYP genes in *V. cholerae.* Each gene encoding a putative HD-GYP domain protein, tagged with a 6-histidine sequence at the 3' end, was expressed ectopically in *V. cholerae* using an IPTG-inducible promoter. (A) Production of each protein in *V. cholerae* after ~3 hours growth in the presence of 1 mM IPTG was assessed by western blot using anti-His6 antibodies. The predicted molecular weights of each protein, in kDa, are indicated at the bottom. Right: Expression of the large, predicted membrane protein VCA0895 was relatively poor; detection of the

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recombinant protein required loading more sample. (B) Motility of *V. cholerae* expressing each of the HD-GYP domain genes was assayed in soft agar medium as an indication of c-di-GMP hydrolysis, which augments flagellar motility of *V. cholerae*. Each strain was inoculated into motility medium with (grey bars) or without (white bars) 0.5 mM IPTG. The diameter of each motility swarm was measured after 20 hours incubation at room temperature. Shown are means and standard deviations for three biological replicates. (C) Biofilm formation by *V. cholerae* expressing each of the HD-GYP domain encoding genes was assessed as an indication of c-di-GMP hydrolysis, which decreases biofilm formation by *V. cholerae*. Each strain was grown in LB broth with (grey bars) or without (white bars) 0.5 mM IPTG to induce gene expression. Biofilm was assayed using standard crystal violet methods. Shown are means and standard deviations for three biological replicates. * *P* < 0.05 by two-way ANOVA with Bonferroni's post-test.

Figure 3. Heterologous expression of HD-GYP genes in *E. coli*. Each gene encoding a putative HD-GYP domain protein, tagged with a 6-histidine sequence at the 3' end, was expressed ectopically in *E. coli* BL21. (A) Production of each protein after ~3 hours growth in the presence of 1 mM IPTG was assessed by western blot using anti-His6 antibodies. The predicted molecular weights of each protein, in kDa, are indicated at the bottom. Right: Expression of the large, predicted membrane protein VCA0895 was relatively poor; detection of the recombinant protein required loading more sample. (B) Lysates of *E. coli* BL21 expressing each of the HD-GYP domain-encoding genes were tested for the ability to hydrolyze c-di-GMP as described in the Materials and Methods. Buffer only ("B") and lysates from *E. coli* BL21 with vector only were included as negative controls. The radiolabeled c-di-GMP substrate has a relative mobility (R_f) of ~0.3 and appears for all samples at t = 0, when the reaction was initiated by addition of [^{32}P]-c-di-GMP. The GMP reaction product appears at R_f ~ 0.65.
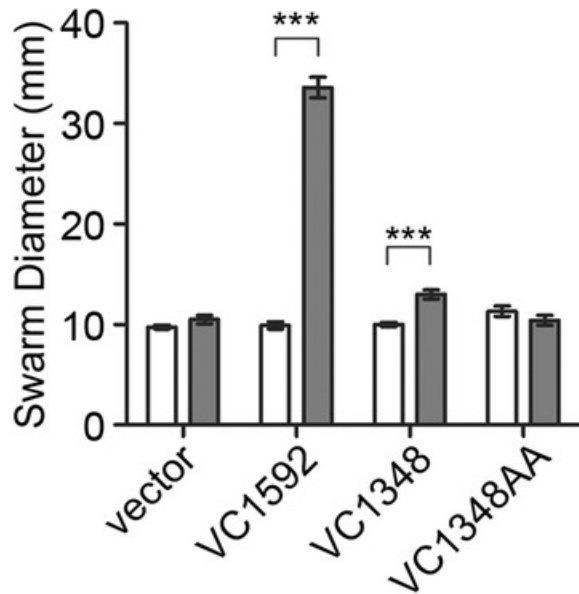
Figure 4. The HD residues of VC1348 are required for *in vivo* function. The genes encoding wild type VC1348 and VC1348 with alanine substitutions in the HD motif (VC1348AA) were expressed ectopically in *V. cholerae* using an IPTG-inducible promoter. The EAL domain PDE VC1592 was included as a positive control. The motility of *V. cholerae* bearing expression plasmids or vector control was assayed in soft agar medium as an indication of c-di-GMP hydrolysis, which augments flagellar motility of *V. cholerae*. Each strain was inoculated into motility medium with (grey bars) or without (white bars) 0.5 mM IPTG. The diameter of each motility swarm was measured after 20 hours incubation at room temperature. Shown are the means and standard deviations for six biological replicates. *** $P < 0.001$ by two-way ANOVA with Bonferroni's post-test.
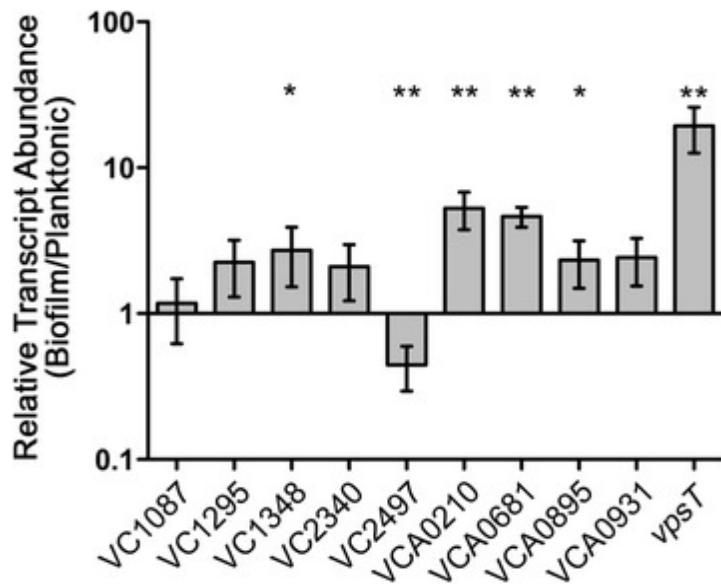
Figure 5. Abundance of mRNA encoding HD-GYP domain proteins in *V. cholerae* biofilms. qRT-PCR was used to measure the transcript abundance for each HD-GYP mRNA, the reference housekeeping gene *rpoB*, and the biofilm-induced control gene *vpsT*. The relative differences in transcript abundance between biofilm and planktonic *V. cholerae* cells were determined as described in the Materials and Methods. * *P* < 0.05 by unpaired t-test comparing biofilm to planktonic values, indicating a significant change in transcript abundance in biofilm relative to planktonic cells.
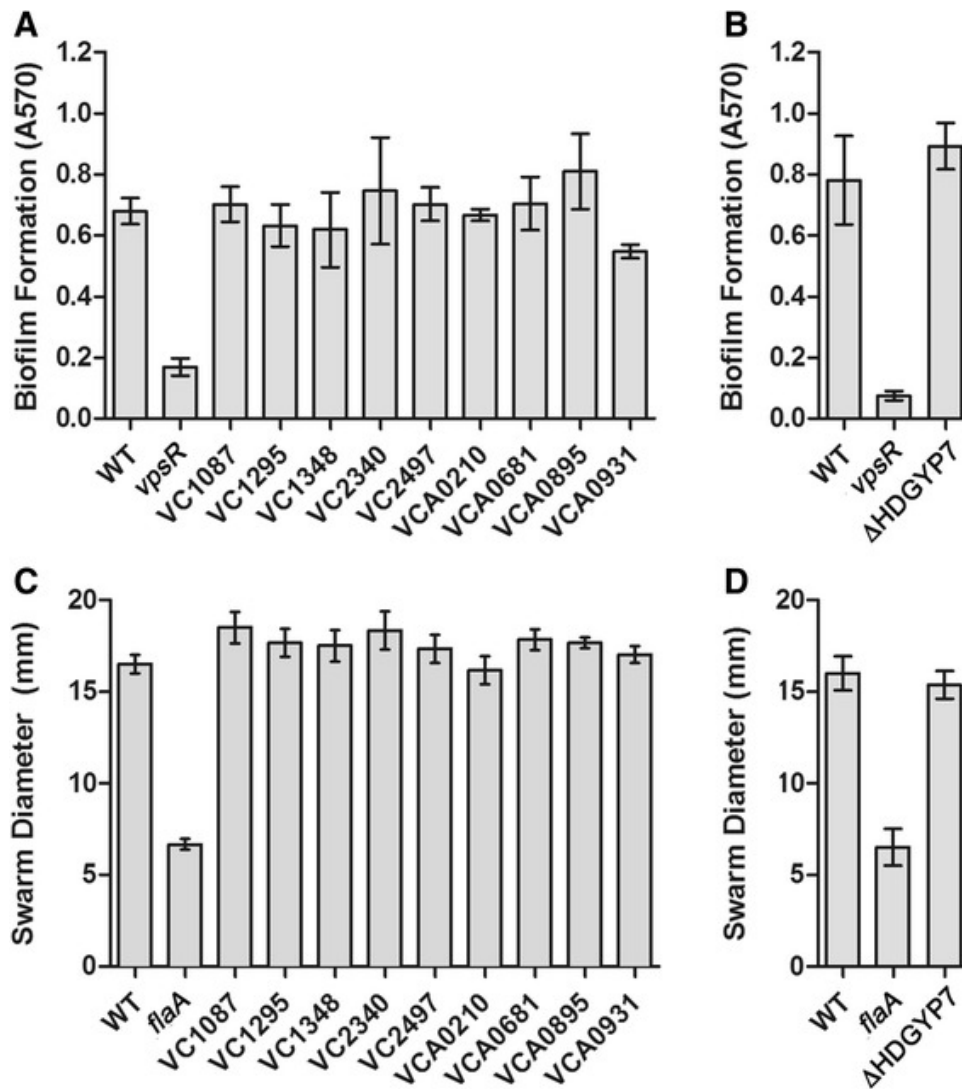
Figure 6. Biofilm and motility phenotypes of *V. cholerae* strains containing mutations in one or more HD-GYP domain encoding genes. *V. cholerae* containing mutations in single (A) or multiple (B) HD-GYP genes were assayed for biofilm formation after 48 hours static growth in rich medium using crystal violet staining. A *vpsR* mutant was included as a negative control. Shown are the means and standard deviations for three biological replicates. *V. cholerae* containing mutations in single (C) or multiple (D) HD-GYP genes were assayed for motility in soft agar medium. A *flaA* mutant was included as a negative control. Swarm diameters were measured after 20 hours growth at room temperature. Shown are the means and standard deviations for three biological replicates.
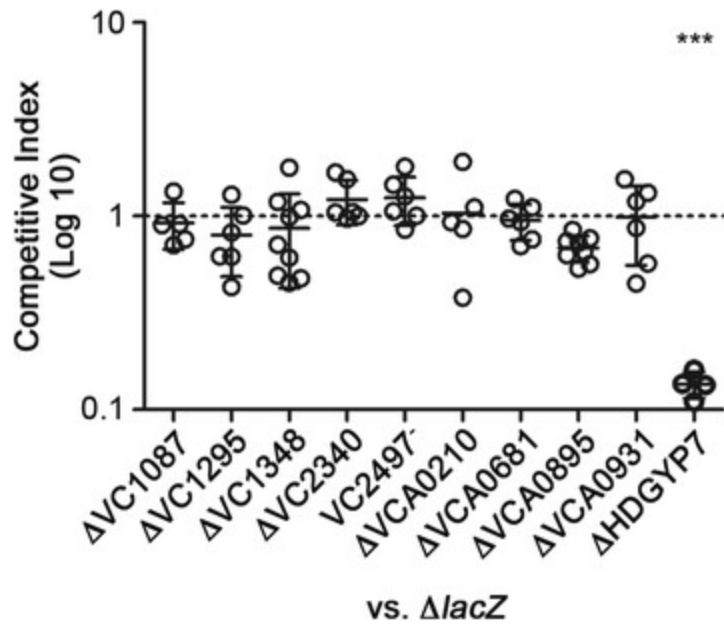
Figure 7. Assessment of virulence phenotypes *V. cholerae* HD-GYP gene mutants using the infant mouse model. Competition experiments were performed using the infant mouse model. Each mutant was co- inoculated in equal numbers with *V. cholerae* Δ*lacZ* (1:1 ratio) into 5-day-old CD1 mice. The bacteria present in the small intestine after 21 hours were enumerated on LB agar plates containing X-gal to differentiate mutant bacteria (blue colonies) from Δ*lacZ* bacteria (white colonies). The competition index (CI) was calculated as [(mutant CFU / Δ*lacZ* CFU)output] / [(mutant CFU / Δ*lacZ* CFU)input]. Each circle represents the CI from an individual animal, and the bars indicate the median. At least 4 mice were used per competition. *** $P < 0.001$, one sample t-test comparing values to a hypothetical value of 1 (*i.e.*, no difference between mutant and wild-type) and by one-way ANOVA.

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