# DEFINING THE ROLES OF THE VC2 RIBOSWITCH AND TFOY IN THE C-DI-GMP REGULATORY NETWORK OF *VIBRIO CHOLERAE*

By

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**ABSTRACT**

DEFINING THE ROLES OF THE VC2 RIBOSWITCH AND TFOY IN THE C-DI-GMP REGULATORY NETWORK OF VIBRIO CHOLERAE

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The second messenger cyclic dimeric guanosine-monophosphate (c-di-GMP) is a central regulator of many different cellular activities in the bacterial domain and it plays an especially important role in the lifestyle transition of *Vibrio cholerae* between the marine environment and human infection. One of the primary effectors of this signal, the Vc2 c-di-GMP-binding riboswitch of *V. cholerae*, has been heavily studied *in vitro*, yet it remains poorly understood *in vivo*. Riboswitches have been traditionally characterized as *cis*-acting RNA elements that serve to regulate the gene expression of downstream coding sequences, but the relationship between the Vc2 element and its downstream gene, *tfoY*, is uncharacterized. In this work, we determine that *tfoY* is a vital component of the *V. cholerae* c-di-GMP program, specifically involved in motility, biofilm formation, and the direct genetic regulation of c-di-GMP metabolic enzymes. We also reveal that *V. cholerae* possesses both Vc2-dependent and Vc2-independent mechanisms for c-di-GMP regulation of *tfoY* expression. And finally, we present a novel paradigm for riboswitch and c-di-GMP gene regulation in which the stability and abundance of a small RNA is controlled by the ligand binding state of the Vc2 riboswitch aptamer domain.

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# CHAPTER 1

## A Review of Second Messenger Signaling

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Pursley, B.R., Hinshaw, K.C., Waters, C.M., Chandler, J.R., Antunes, L.C.M. (2016). Microbial Signaling. In: Bruce, TB and Trindade-Silva, AE (Eds). *Molecular Diversity of Environmental Prokaryotes*, CRC Press, Taylor & Francis Group. London, GB.

## INTRODUCTION

Second messenger signalling is an important feature of cellular behavior because it facilitates the communication of a local piece of information into a global message that can organize the actions of the cell at a holistic level. A single protein can serve as a receptor to sense an isolated stimulus, be it a stress event or the presence of a nutrient, and that protein can then produce a more broadly understood signal, the second message, for which there can be many different receptors in the cell. Those second messenger receptors can then all mount a response to the initial stimulus, and do so in a coordinated manner which improves the efficiency of the cellular reaction to the initial event. The specific second messengers of bacteria, their stimuli, their responses, and their roles and relationships to each other, are summarized below based on the current state of research in each field.

## SPECIFIC SIGNALS

### cAMP

Cyclic adenosine monophosphate (cAMP) was the first second messenger molecule identified, and it appears to be the most widely utilized among bacteria (Makman and Sutherland, 1965; Botsford and Harman, 1992). In *Escherichia coli*, cAMP is synthesized by the adenylate cyclase enzyme (Cya), and it is detected by the transcriptional regulator CRP (cAMP receptor protein), also commonly called CAP (catabolite activator protein). The CRP/cAMP signaling pathway allows bacteria to discriminate between different available carbon sources and utilize them in the most energy efficient manner possible. For example, glucose is an optimal carbon source for *E. coli* because it requires only a single modification to glucose-6-phosphate to initiate glycolysis. In contrast, a disaccharide sugar such as lactose must first be

broken down into its monosaccharide subunits in order to be metabolized, decreasing the energy obtained from each sugar subunit. Therefore the availability of glucose in *E. coli* represses utilization of all other sugars until the glucose is consumed, a process termed catabolite repression. This repression is mediated by Cya, which is inhibited for production of cAMP in the presence of glucose (Botsford and Harman, 1992). *E. coli* CRP relies on cAMP to be able to effectively bind DNA, so when glucose is depleted and Cya resumes production of cAMP, the cAMP-CRP complex binds DNA, recruiting RNA polymerase to target promoter sequences and inducing expression of alternative sugar utilization pathways. Although the specific outcomes of cAMP-CRP activity at a given promoter vary greatly based on the spatial organization of the CRP binding site relative to the transcriptional start site, the general trend is that cAMP-CRP activates gene expression (Botsford and Harman, 1992).

As one of the most highly expressed transcriptional regulators in the cell, CRP has evolved to control many processes other than catabolite repression. One of the most well studied alternative systems that relies on cAMP and CRP in *E. coli* is natural competence, the ability of the cell to uptake foreign DNA from the environment (Chandler, 1992; Dorocicz et al., 1993). This interrelationship of a catabolite repression system with natural competence makes sense when considering that DNA is relatively abundant in most environments and can be utilized as an alternative carbon source. Other cellular programs regulated by CRP include biofilm formation, motility, virulence factor expression, and cell division (Fong and Yildiz, 2008; Petersen and Young, 2002; D'Ari et al, 1988). The involvement of cAMP in each of these systems illustrates that central metabolism is a driving force in many bacterial behaviors. However, even though most bacteria produce cAMP, outside of the enteric bacteria most

catabolite repression systems are actually cAMP-independent (Postma et al., 1993). Understanding the role of cAMP in non-enteric bacteria is an active area of investigation.

**cGMP**

Although cyclic guanosine monophosphate (cGMP) has long been recognized as a critical signal in the eukaryotic domain, the early evidence of a role for cGMP in bacterial signaling was generally discounted for two main reasons. First, in bacteria where cGMP had been detected, it was usually present at significantly lower concentrations than cAMP, causing it to be regarded as an aberrant, nonfunctional product of the promiscuous activity of the adenylate cyclase enzyme (Bernlohr et al, 1974; Shibuya et al., 1977). Second, guanylate cyclases share very high amino acid sequence similarity with adenylate cyclases, making it difficult to identify true cGMP synthases in bacterial genomes by bioinformatic methods alone. In fact, as few as two amino acid changes at the active site motif are capable of switching the activity of these cyclase enzymes from one product to the other (Sunahara et al., 1998). Unfortunately, this relegates true cGMP-producing bacteria to be identified only through labor-intensive biochemical methods.

Because this field is only very recently emerging, bacterial cGMP utilization has only been explored in a few organisms. Cyanobacteria were the first target of study because, for unknown reasons, they were found to maintain unusually high levels of cGMP (Herdman and Elmorjani, 1998). The first genuine bacterial guanylate cyclase enzyme identified was from the cyanobacterium *Synechocystis* sp. PCC 6803 (Ochoa de Alda et al., 2000). *Synechocystis* sp. PCC 6803 is a freshwater photyosynthetic bacterium whose intracellular cGMP, cAMP, and c-di-GMP signal concentrations all fluctuate in response to stimulation by different light sources

(Herdman and Elmorjani, 1998; Terauchi and Ohmori, 2004; Savakis et al., 2012). The absorption of light energy during photosynthesis is a sensitive process, and over-stimulation by high energy UV-B radiation can cause damage to photosystem components. Normally in *Synechocystis* sp. PCC 6803, exposure to UV-B light causes a decrease in the intracellular concentration of cGMP, but a mutant strain with an inactivated cGMP phosphodiesterase was unable to lower the cGMP level in response to UV-B and was also shown to be deficient in its repair of photosystem damage (Cadoret et al., 2005).

Another up-and-coming player in the cGMP signaling field is *Rhodospirillum centenum*, a photosynthetic, nitrogen-fixing bacterium that is capable of forming metabolically inactive cysts as a survival mechanism to withstand extended periods of desiccation stress. Analogous to processes of spore formation in other bacteria, cyst formation requires a dramatic reprogramming of cellular functions, but is still poorly understood (Berleman and Bauer, 2003). In *R. centenum* cells grown under the starvation conditions that induce encystment, cGMP accumulates, and deletion of a guanylate cyclase cGMP synthesis enzyme abrogates cyst formation (Marden et al., 2011). A homologue of CRP named CgrA for "cyclic GMP receptor A," was found to have a much higher affinity for cGMP than cAMP, and deletion of this CRP homologue also prevented the cells from forming cysts (Marden et al., 2011).

**(p)ppGpp**

Another group of second messenger molecules that have been studied for decades are guanosine penta- and tetraphosphate [(p)ppGpp], collectively known as "magic spot" or the alarmones (Cashel and Gallant, 1969). (p)ppGpp has many roles in the cell, but the best understood is it's role managing the response to starvation in *E. coli*. During amino acid

starvation, uncharged tRNAs can enter the ribosome and cause translation to stall. Ribosome stalling serves as a signal for an enzyme that associates with the ribosomal complex, RelA, to synthesize (p)ppGpp (Haseltine and Block, 1973). Once (p)ppGpp is produced, it regulates RNA polymerase at numerous transcriptional start sites by interfering with transcription initiation. The mechanism of transcriptional regulation by (p)ppGpp involves a transcriptional elongation factor homologue called DksA, and they work together through physical contact with internal elements of the RNA polymerase to alter the stability of open complex formation during transcription initiation. Specifically, this second messenger shuts down transcription of new rRNAs and tRNAs and curtails energy expensive cellular processes such as DNA replication and cell division (Sands and Roberts, 1952; Magnusson et al., 2007). This effect is known as the stringent response, because it allows the cell to conserve its energy and resources while inducing the expression of proteins that can synthesize the missing amino acids. (p)ppGpp and DksA also effect global transcription by destabilizing the interaction of RNAP with the standard housekeeping sigma factor, $\sigma^{70}$, in favor of binding other alternative sigma factors (Jishage et al., 2002).

In *E. coli* and related bacteria, (p)ppGpp levels in the cell are also maintained by a protein called SpoT, which has both synthesis and hydrolysis activities, but normally only serves to convert (p)ppGpp back into GTP and GDP. SpoT is important because under certain other stress conditions such as fatty acid, iron, or phosphate starvation, it can become a net producer of (p)ppGpp and trigger the stringent response independently of RelA (Battesti and Bouveret, 2006; Vinella et al., 2005; Bougdour and Gottesman, 2007). A mutant lacking both *relA* and

*spoT* genes is historically called a “relaxed” mutant because it is unable to initiate the stringent response (Stent and Brenner, 1961).

It is important to note that no single mechanism accurately describes the (p)ppGpp systems of all bacteria. Different species vary greatly in the ratio of ppGpp produced vs pppGpp, and both of these alarmones have many direct and indirect effects. For example, in *B. subtilis*, (p)ppGpp impacts the entrance into sporulation. However, this effect it not mediated by the (p)ppGpp molecules directly, but rather by the depletion of intracellular GTP that occurs when (p)ppGpp is synthesized (Ochi et al., 1982). One commonality among (p)ppGpp systems is that many pathogenic bacteria appear to use the induction of the stringent response as a strategy for survival and persistence in the host during infection. *Pseudomonas aeruginosa* actually requires (p)ppGpp for virulence, both directly at the level of virulence factor expression and indirectly through the proper timing of its quorum sensing system (van Delden et al., 2001; Erickson et al., 2004).

**c-di-GMP**

Cyclic diguanosine monophosphate (c-di-GMP) is a recently appreciated, nearly ubiquitous second messenger that regulates a plethora of bacterial behaviors. In many bacteria c-di-GMP controls the switch between motility and a sessile biofilm state. These are two completely different lifestyle choices in that biofilms represent a surface-attached, sedentary state, and motility involves a free-swimming, planktonic state. Numerous additional behaviors are controlled by c-di-GMP including virulence factor expression, cell cycle progression, cell differentiation, RNA stability, and various stress responses (Römling et al., 2013).

Unlike the other second messenger systems that rely on only one or two enzymes to control signal synthesis and degradation, c-di-GMP metabolism is highly complex, with some bacterial genomes encoding dozens of different proteins dedicated to c-di-GMP turnover (Galperin, 2004). Diguanylate cyclases produce c-di-GMP and phosphodiesterases degrade c-di-GMP and both of these families of proteins are modular in nature. They typically contain the enzymatic domain at the C-terminus of the protein with a sensory domain fused to the N-terminus. These sensory domains detect external changes in the environment or internal changes in cellular metabolism, and they alter their c-di-GMP synthesis/degradation activity in response, thereby transmitting this information into the c-di-GMP pathway. The types of stimuli that effect internal c-di-GMP levels are often uniquely tuned to the lifestyles of particular bacteria such as cyanobacteria that respond to light, soil and marine bacteria that respond to nutrient availabilities, or plant and animal pathogens that respond to host factors (Cao et al., 2010; Hengst et al., 2010; Koestler and Waters, 2013; Tamayo et al., 2008; Yi et al., 2010).

*Vibrio cholerae*, the causative agent of the disease known as cholera, encodes the most well-studied c-di-GMP signal transduction system. Although *V. cholerae* receives a great deal of notoriety as a human pathogen, most wild strains are actually not virulent and *V. cholerae* thrives in the environment by scavenging chitin from the detritus of the copepods on which it can form biofilms (Nelson et al., 2009). c-di-GMP is an important regulator in *V. cholerae* that mediates the transition from a marine biofilm lifestyle to that of a pathogen in the human gut (Nelson et al., 2009). To that end, the *V. cholerae* genome encodes more than forty diguanylate cyclases, more than twenty c-di-GMP-specific phosphodiesterases, at least three different c-di-GMP-binding transcription factors with unique mechanisms, five c-di-GMP-binding PilZ domain-

containing proteins predicted to function through protein-protein interaction, and two c-di-GMP-binding riboswitch regulatory RNA elements (Römling et al., 2013). The general model of the *V. cholerae* life cycle is that marine environmental signals maintain c-di-GMP at a high level to induce biofilm formation and repress virulence factor expression while host factors inhibit c-di-GMP levels (Cotter and Stibitz, 2007).

**c-di-AMP**

The most recently discovered second messenger molecule, cyclic diadenosine monophosphate (c-di-AMP), is not as phylogenetically conserved as the other signaling systems, but it is notable for having been identified in many Gram-positive bacteria not known to use c-di-GMP (Woodward et al., 2010; Corrigan and Gründling, 2013). c-di-AMP is synthesized by diadenylate cyclase enzymes that are referred to as DAC domain proteins. Unlike the biochemical similarity that exists between the mononucleotide cyclases that synthesize cAMP and cGMP, discussed earlier, diadenylate and diguanylate cyclases (that synthesize c-di-AMP and c-di-GMP, respectively) bear no resemblance to each other (Corrigan and Gründling, 2013). Furthermore, most bacteria encode only one or a few diadenylate cyclase enzymes, and genetic experiments indicate that diadenylate cyclases are essential genes in some species (Corrigan and Gründling, 2013). This is consistent with the fact that c-di-AMP signaling has been associated with essential cellular processes such as fatty acid synthesis, cell wall homeostasis, the detection of DNA damage, the progression of sporulation, and the regulation of cell division (Zhang et al., 2013; Luo and Helmann, 2012; Witte et al., 2008; Oppenheimer-Shaanan et al., 2011; Corrigan et al., 2011).

Each new diadenylate cyclase enzyme identified appears to regulate a distinct pathway with its own exclusive effectors and phenotypic outputs. The Gram-positive bacterium *Bacillus subtilis* has three of these cyclases whose roles do not appear to overlap. One of them named "DNA integrity scanning protein A" (DisA), is a cytoplasmic diadenylate cyclase that also contains a DNA binding domain (Witte et al., 2008). DisA is expressed in cells coming out of exponential growth, before they have made a commitment to sporulation, a process whereby a subpopulation of *B. subtilis* encases daughter cells in an environmentally resistant coat to outlast harsh conditions. DisA physically scans the genome, stopping at sites of significant DNA damage, such as double-stranded breaks (Bejerano-Sagie et al., 2006). As it moves along the genome DisA actively produces c-di-AMP and the intracellular-concentration of c-di-AMP continually rises. If DisA encounters a DNA lesion site, it stops moving along the genome and halts the production of c-di-AMP (Witte et al., 2008). The pausing of DisA also causes activation of a separate c-di-AMP-specific phosphodiesterase enzyme which rapidly lowers the c-di-AMP concentration in the cell, and the resulting deficit of c-di-AMP prevents the activation of the master transcriptional regulator of sporulation (Oppenheimer-Shaanan et al., 2011).

**Future Research**

The largest gaps in the current understanding of second messengers regard the distribution and function of other types of nucleotide signals. Cyclic pyrimidine nucleotides, such as cCMP and cUMP, have been discovered in eukaryotes, and both of these nucleotides can be produced *in vitro* by some eukaryotic and bacterial adenylate cyclase enzymes, but it is unknown if they have a physiological role as signal molecules in bacteria (Hartwig et al., 2014; Göttle et al., 2010). Another curious development is the discovery of a hybrid signal, cyclic-

AMP-GMP, in *V. cholerae* (Davies et al., 2012). This hybrid is produced by the enzyme DncV which has no significant homology to known diadenylate or diguanylate cyclases, yet DncV can also generate c-di-AMP and c-di-GMP as minor products (Davies et al., 2012). Whether cyclic-AMP-GMP represents a unique signal transduction pathway unto itself or more of an interface between existing systems is not known. It is also not clear how well c-di-AMP and c-di-GMP receptors discriminate between these sorts of hybrid molecules and their usual substrates.

The ultimate fate of breakdown products of second messenger signals is yet another open question worthy of further consideration. The main pathway for the degradation of cyclic dinucleotides c-di-AMP and c-di-GMP generate the linear dinucleotides pApA and pGpG, respectively. Recent evidence indicates that monophosphorylated dinucleotides like these, termed nanoRNAs, are capable of priming RNA transcription in a promoter sequence-specific manner (Goldman et al., 2011; Nickels and Dove, 2011). The possibility that these dinucleotides control a network of genetic regulation unto themselves is an area of research yet to be explored.

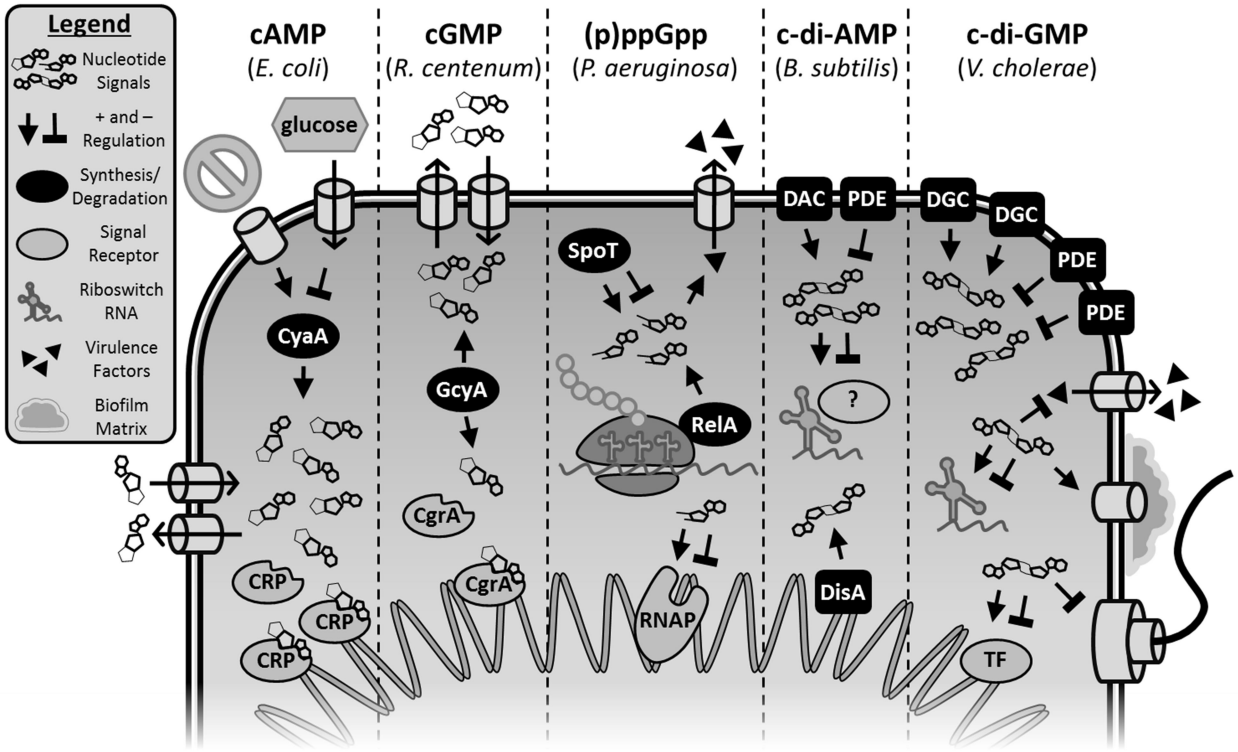



**Figure 1-1: Second Messenger Systems of Bacteria**

The major defining features of the second messenger signals, based on the current state of research in each respective field, are summarized in the figure above. The monomeric signals cAMP and cGMP are notable for their ability to accumulate both inside and outside the cell, and both have dedicated transcription factor effectors to influence gene regulation. The alarmone (p)ppGpp has the most unique system in that its metabolism and mode of gene regulation rely on direct interactions with the translational and transcriptional machinery of the cell, respectively. The dimeric signals c-di-GMP and c-di-AMP have metabolic enzymes which mostly are associated with the cell membrane where they serve to directly sense extracellular signals. The c-di-GMP and c-di-AMP systems also both utilize riboswitches as effectors in the control of gene regulation.

## INTERRELATIONSHIP OF SIGNALING SYSTEMS

### Signaling Specificity

A key feature of signal transduction pathways, including second messengers and quorum sensing systems, is the degree of signaling specificity with which they act. Systems can function at either low or high specificity, with low specificity referring to global regulation by a signal uniformly distributed throughout the cell, or high specificity referring to parallel signaling pathways with discreet subdomains of signaling within the cell. Whether a particular system functions at high or low specificity has less to do with the specific signaling molecule involved and more to do with how the regulation of that molecule has evolved in a particular organism.

Low specificity is based on a model in which one or more inputs feed into the same diffuse pool of signal, and changes in that signal are manifested as a global coordinated response. In other words, all environmental inputs affect all phenotypic outputs. (p)ppGpp signaling in *E. coli* is an example of a system that benefits from low specificity because when the cell detects the starvation of any one of a number of different nutrients, it can respond by simultaneously coordinating actions that conserve energy, such as shutting down cell division, stopping DNA replication, and turning off protein synthesis.

In contrast, high specificity signaling, in the strictest form, requires that each individual stimulus be detected by a unique receptor and only impact a specific phenotype. This could occur by generating discreet pools of signals within a cell. Many c-di-GMP regulated processes appear to benefit from some degree of high specificity, such as the regulation of asymmetric cell division in the environmental bacterium *Caulobacter crescentus*. *C. crescentus* reproduces through a process in which a sessile parent cell develops a single polar attachment apparatus at

one end called a “stalk,” and buds off a motile daughter cell with a single polar flagellum at the opposite end. This process requires a gradient of different local concentrations of c-di-GMP that spans the distance from one pole to the other. The gradient is maintained in part by a unique diguanylate cyclase that is localized to the stalk pole and a unique phosphodiesterase that is localized to the flagellar pole (Christen, et al., 2010).

There are many different strategies for generating high specificity signaling pathways. One strategy involves co-localizing the synthesis and degradation of the signal to a single cellular compartment. In fact, recent evidence indicates that some diguanylate cyclases and phosphodiesterases can directly interact with one another in ways that allow them to regulate each other’s enzymatic activity (Lindenberg et al., 2013). Another strategy is to bring signal synthesis enzymes and signal receptors into close contact with one another, forming a protein complex that can limit signal diffusion. Such is the case in *E. coli*, where a diguanylate cyclase and phosphodiesterase enzyme assemble into a ribonucleoprotein complex with polynucleotide phosphorylase, an RNA processing enzyme whose activity is regulated by c-di-GMP (Tuckerman, 2011). Additionally, such proteins could be encoded within the same operon on the genome so that they are co-transcribed and translated in close proximity to each other. One surprisingly common feature of c-di-GMP signaling systems are proteins with dual domains of diguanylate cyclase and phosphodiesterase activity contained within the same gene sequence.

**Second Messenger Integration**

It is important to remember that none of the second messengers discussed earlier exist in a vacuum, and there is potential for overlap to occur between these systems. The most

common point of intersection between the nucleotide signaling systems occurs at the level of gene expression. These interactions can be direct, through multiple second messenger transcription factors regulating the same promoters, or indirect, through broad shifts in cellular function. Biofilm formation is one trait where second messenger effectors can directly clash over differential regulation of the same genes. In *V. cholerae*, cAMP-CRP can antagonize c-di-GMP induction of exopolysaccharide synthesis, and in *E. coli*, (p)ppGpp can prevent exports of an adhesive polysaccharide that is synthesized in response to c-di-GMP (Fong and Yildiz, 2008; Boehm et al., 2009).

Direct interactions can even occur between second messengers at the molecular level when multiple signals converge on the same protein. Most diguanylate cyclases and phosphodiesterases are part of large multidomain proteins, and the majority of those domains remain functionally uncharacterized. Recently a diguanylate cyclase in *Xanthomonas campestris* was identified whose accessory domain can bind cGMP, and doing so increases the output of c-di-GMP from that enzyme (An et al., 2013). Likewise, an emerging trend for members of the cAMP receptor protein (CRP) family is the capacity for them to recognize nucleotides other than cAMP. The highly conserved cyclic nucleotide monophosphate binding domain of CRP homologues has now been shown to have an affinity for c-di-GMP and cGMP in different organisms, indicating the binding specificities of this domain are broader than previously appreciated (Leduc and Roberts, 2009; Marden et al., 2011). The consequences of this are not clear, as every CRP homologue described thus far has a definite preference for one nucleotide signal over all others, but it is important to not dismiss the possibility that the secondary affinity of these proteins for alternative signals might still play a physiological role.

Indirect interactions between nucleotide signals tend to occur at a global scale. For example, the role of (p)ppGpp in altering the sigma factor preference of the core RNA polymerase has indirect consequences for the cAMP and c-di-GMP systems, because both of them enlist alternative sigma factors in their downstream regulatory networks (Nagai et al., 1990; Lange and Hengge-Aronis, 1994; Srivastava et al., 2014). The most poorly understood interactions occur at the level of signal synthesis itself, where systems that use the same nucleotide share the same pool of nucleoside triphosphate substrate. Production of (p)ppGpp in *B. subtilis* is known to deplete the available cellular GTP, but the effect that has on c-di-GMP signaling remains an open question.

**Second Messengers that Function as Extracellular Signals**

Many bacteria have systems to actively export nucleotide signaling molecules, and even for those who don't have such systems the intracellular signals can be released as members of the population experience cell death and lysis. The potential for these signals to be detected by any nearby organisms has far-reaching implications, and underlies their ability to serve as external signals. The intracellular pathogen *Mycobacterium tuberculosis* has been shown to specifically release cAMP into the cytoplasm of host macrophage cells during infection as a strategy to disrupt host cell cAMP signaling and ensure its own survival (Agarwal et al., 2009). Similarly, cGMP, used by *Rhodospirillum centenum* to regulate the process of cyst formation, is heavily secreted and can induce encystment of neighboring cells (Marden et al., 2011).

Unlike other types of signaling systems in bacteria that require dedicated signal synthases that confer specificity to each species (e.g. quorum sensing), the systems involved in generating and recognizing nucleotide signals are effectively ubiquitous across the domains of

life. It was long thought that microbes and higher organisms had unique preferences for exclusive nucleotide signals, but modern research has dispelled those notions by identifying the capacity for both cyclic-mononucleotide and cyclic-dinucleotide signaling in all domains of life (Chen and Schaap, 2012). Interestingly, cells of higher eukaryotes react quite strongly to c-di-GMP and c-di-AMP by stimulating robust inflammatory responses, indicating that these molecules are recognized as Microbial Associated Molecular Patterns (MAMPs) (McWhirter et al., 2009; Woodward et al., 2010). Ongoing research is exploring the impact of these molecules on the immune system and the potential benefit of using them to modulate immune function (Chen et al., 2010).

**Second Messenger and Quorum Sensing Crosstalk**

Phenotypic programs regulated by quorum sensing often involve the production of public goods at significant metabolic cost to individual cells. Therefore, bacteria gain the most benefit from quorum sensing programs if they are engaged only when conditions are optimal. In *E. coli*, production of the universal quorum sensing molecule known as autoinducer-2 (AI-2) is regulated by cAMP-CRP, such that AI-2 synthesis is favored when glucose in present and AI-2 uptake is favored when glucose is absent (Wang et al., 2005). This provides *E. coli* tight control over quorum sensing in relation to the nutrient resources of its current environment. Also, because AI-2 is a signal recognized by a wide range of bacteria, this process could serve to communicate local information about the availability of carbon sources to neighboring organisms in a mixed species community.

Crosstalk between internal second messengers and external quorum sensing signals can also determine the participation of individual cells in cooperative multicellular behaviors.

*Agrobacterium tumefaciens* is a soil bacterium that uses a novel system of horizontal gene transfer to distribute a virulence plasmid throughout its population and into the root cells of plant hosts. This plasmid transfer system is induced by the accumulation of by a quorum sensing signal (Piper et al., 1993). However when the cells are experiencing carbon or nitrogen limitation, the (p)ppGpp system activates the expression of enzymes which degrade the quorum sensing signal (Zhang et al., 2004). This type of response to multiple inputs may ensure that a cell will not engage in the metabolically costly process of conjugation if doing so would squander its remaining resources and jeopardize its own individual survival.

**The Interdependence of Bacterial Signals**

One model that vividly illustrates the complexity of signaling systems working together is the life cycle of the plant pathogen *Xanthomonas campestris* pathovar campestris (*Xcc*). *Xcc* is agriculturally significant for is ability to cause black rot disease in cruciferous vegetables crops such as cabbage and broccoli, and related strains of *X. campestris* are industrially important as a source of the food additive xanthan gum. In nature, *Xcc* produces a xanthan exopolysaccharide reduces plant defenses against infection and contributes to disease (Yun et al., 2006). The timing of expression of the exopolysaccharide and other factors important for obtaining nutrients are coordinated by cGMP, c-di-GMP, a quorum sensing signal called “diffusible signal factor” (DSF), and a two-component system (An et al., 2013; Barber et al., 1997; Ryan et al., 2006). This complex integration of bacterial signals allows *Xcc* to thrive in multiple different host environments by coordinating survival on the plant surface, invasion throughout the plant tissue, and migration within the plant vasculature.

Among the genes required for the virulence cycle of *Xcc* is one coding for a recently discovered guanylate cyclase, XC_0250 . Although the steps between the initial contact of the bacterium with the plant and the induction of XC_0250 expression are not yet known, the cGMP generated by XC_0250 is critical for biofilm development and differentially regulates over 200 genes in *Xcc* (An et al., 2013). One of the regulated genes happens to be immediately upstream of XC_0250 on the genome, and its gene product, XC_0249, contains a cGMP binding domain. XC_0249 just so happens to be a diguanylate cyclase, and binding of cGMP to XC_0249 upregulates its production of c-di-GMP, inducing biofilm gene expression (An et al., 2013).

As a biofilm develops in the xylem of the plant, *Xcc* approaches a high cell density state. At a critical population density, the quorum sensing molecule DSF is sensed by the membrane receptor RpfC, which transfers a phosphate to the c-di-GMP-specific phosphodiesterase RpfG, stimulating hydrolysis of c-di-GMP (Ryan et al., 2006). Meanwhile, the dense growth of the *Xcc* biofilm in the vasculature of a plant creates an oxygen-poor environment, and in the RavS/RavR two-component pathway, RavS senses this low oxygen stress and activate RavR, which also happens to be a c-di-GMP phosphodiesterase (He et al., 2009).

The combined effect of RpfG and RavR activation is a decrease in the global pool of c-di-GMP, and this change is sensed by a CRP homologue named Clp. Instead of recognizing cAMP, Clp has an affinity for c-di-GMP (Chin et al., 2010). When bound to c-di-GMP, Clp poorly binds DNA, but when the intracellular concentration of c-di-GMP falls, the equilibrium of binding shifts and Clp becomes unbound. This free Clp can then activate transcription at a multitude of different sites across the genome, but most importantly, it turns on the expression of multiple virulence factors including extracellular proteases, cellulases, and a type-III secretion system (de

Crecy-Lagard et al., 1990; He et al., 2007). Thus, integration of both a cGMP second messenger and the DSF autoinducer impacts cellular c-di-GMP levels to enable *Xcc* infection of host plants.

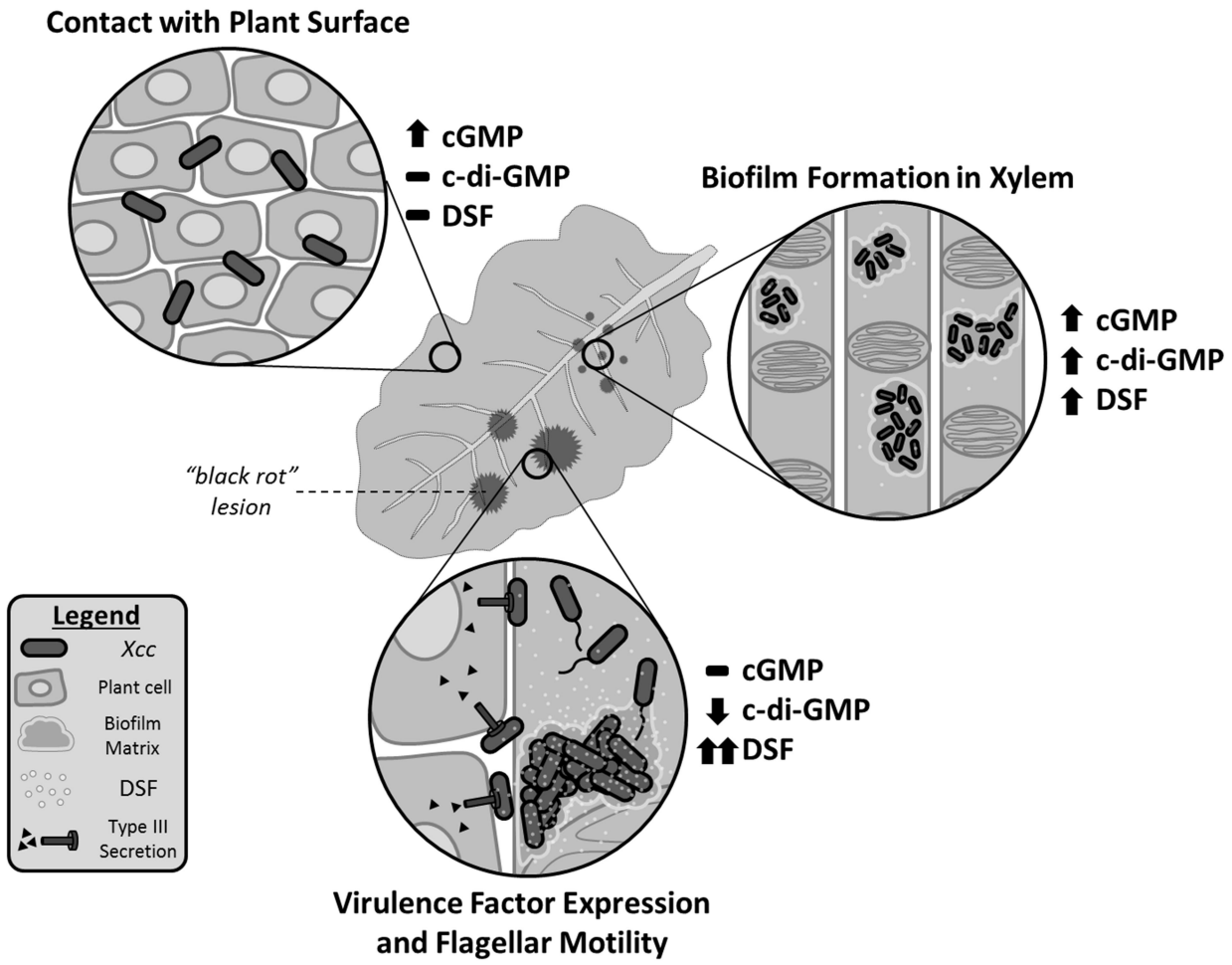



**Figure 1-2: Second Messenger Signaling in *Xcc***

The cycling of second messenger signals in the plant pathogen *Xcc*, as described in the text, is depicted in the figure above.

# CHAPTER 2

## Development of the Research Project

## THE VC2 C-DI-GMP RIBOSWITCH

At the time of its discovery, the Vc2 c-di-GMP riboswitch represented an impressive advancement in the field of c-di-GMP research (Sudarsan et al., 2008). Before 2008, there had been numerous reports of c-di-GMP involvement in biofilm formation, motility, and virulence in many different bacteria, yet there had been no explanation for how c-di-GMP directed these programs at the level of gene expression (Jenal and Malone, 2006; Cotter and Stibitz, 2007). The lone protein domain which had been shown to interact with c-di-GMP was the PilZ domain, but this domain was not associated with any known transcription factors, and was most commonly found by itself or in combination with cellulose synthase domains (Amikam and Galperin, 2005; Ryjenkov et al., 2006). Moreover, in *V. cholerae*, the function of the five PilZ domain containing proteins encoded in its genome was somewhat nebulous. Only two of them were found to bind c-di-GMP, and the effects of these proteins on c-di-GMP phenotypes when overexpressed or deleted were subtle (Pratt et al., 2007). This implied that none of the PilZ proteins were the master regulators of the primary c-di-GMP programs in *V. cholerae*, motility and biofilm formation.

If riboswitches were to be the master genetic regulators of c-di-GMP programs in all bacteria, the nature of that relationship was also not clear. Some of the organisms in which c-di-GMP riboswitches were found, such as *Geobacter uraniumreducens*, have upwards of two dozen of these elements within their genome, and situated at genetic loci with direct links to c-di-GMP phenotypes, such as upstream of flagellar biosynthesis operons (Sudarsan et al., 2008; Smith et al. 2009). However other c-di-GMP utilizing organisms, such as *Escherichia coli*, were not found to contain any c-di-GMP riboswitches, even though the importance of c-di-GMP in

those organisms was already well established (Galperin, 2004). The situation in *V. cholerae* falls between these two extremes, as it has a small number of riboswitches which are associated with genes not known to play a dominant or controlling role in its c-di-GMP programs.

The two class-I c-di-GMP binding riboswitches of *V. cholerae* are designated Vc1 and Vc2, each located on a different one of the two chromosomes of *V. cholerae*, and these are predicted to regulate the genes *gbpA* and *tfoY*, respectively (Sudarsan et al., 2008). *gbpA*, or glucose binding protein A, is an attachment factor expressed on the outer surface of the cell which has been shown to recognize specific sugar moieties in the both the lumen of the small intestine and on the surface of some chitinous organisms, explaining in part the host specificity for the targets of *V. cholerae* virulence (Kirn et al., 2005; Wong et al., 2012). Initial investigations into the function of *tfoY* focused on its relationship to its better known homologue, *tfoX*. *tfoX*, also referred to as *sxy* in *E. coli* and *Haemophilus influenzae*, is a transcriptional co-activator and regarded as the master regulator of bacterial competence in many gram-negative organisms (Cameron and Redfield, 2008) . Although *tfox*-domain containing proteins have never demonstrated a capacity to bind DNA on their own, *sxy* has been shown to modify DNA binding activity of CRP (Cameron and Redfield, 2008). In this way, *sxy* is able to control the expression of its own specific regulon which includes genes not only for DNA uptake, but also the heat shock response, and chemotaxis (Sinha et al., 2009). In *V. cholerae*, *tfoX* has been associated with the induction of natural competence during growth on chitinous surfaces, and is therefore considered more important during growth in the marine environment than in the human host (Meibom et al., 2005; Yamamoto et al., 2010)

Most organisms with a *tfoX*-domain containing protein have only one of these proteins, however, the *Vibrionaceae* family is unique in that all of its members have two, *tfoX* and *tfoY*, and in *V. cholerae* these are encoded on separate chromosomes (Pollack-Berti et al., 2010). A preliminary study in *Vibrio fischerii* linked *tfoY* to competence regulation, and claimed it was essential for this program in *Vibrio spp.* generally (Pollack-Berti et al., 2010). Specifically, the researchers showed that a strain with a transposon insertion in *tfoY* was deficient in an assay for natural competence (Pollack-Berti et al., 2010). However, other results presented in their work were contradictory to this claim and *V. cholerae* research groups have cast doubt on the notion that *tfoY* plays an important role in competence (Yamamoto et al., 2011). Surprisingly, there has been no previously published investigation into the nature of c-di-GMP regulation of *tfoY* or its function since the important discovery of the Vc2 riboswitch.

## THE VC2 RIBOSWITCH AS A C-DI-GMP BIOSENSOR

Our initial interest in the Vc2 riboswitch came from the idea that it could be used as a biosensor for the detection and measurement of c-di-GMP in natural systems. At the time our research began, the most reliable methods for quantifying the c-di-GMP content of a bacterial cell were only applicable *in vitro*, because they involved extraction of the molecule from the cell and destruction of the originating organism (Waters, 2010). This greatly limits the ability to study c-di-GMP changes in real-time or in a natural setting. A tool for measurement of c-di-GMP *in vivo* is of great interest to the field because of the model of c-di-GMP as the central regulatory switch for many different types of lifestyle changes in bacteria. In *V. cholerae* specifically, this switch is one between a marine organism growing in a biofilm on chitinous

surfaces and a deadly pathogen growing in the human gut during infection (Tischler and Camilli, 2004; Tischler and Camilli, 2005). Attempts have been made to characterize changes in c-di-GMP during human infection, and much can be inferred by identifying c-di-GMP metabolic enzymes or c-di-GMP-binding proteins that are involved at various stages of this process (Schild et al., 2007; Tamayo et al., 2008). However, the model that c-di-GMP directs these changes globally within the cell remains unproven as long as there is no way to observe the intracellular rise and fall of c-di-GMP concentrations directly.

To this end, some groups have developed transcriptional reporter biosensors which make use of c-di-GMP-responsive promoters (Rybtke et al., 2012). But this type of design adds multiple layers of abstraction to the measurement, since c-di-GMP is not being directly detected by the promoter, *per se*, it is being detected by a transcription factor protein, which then in turn regulates the promoter, and the promoter activity in and of itself relies on the cooperation of additional factors such as RNA polymerase. Riboswitch biosensors are attractive because they appear elegant in their mechanism of signal detection. Information (the ligand) is received (by the aptamer), transmitted (through the expression platform), and output (by the downstream coding sequence) all within the same molecule of mRNA. On the surface they seem to be free of interference by external factors that could add noise to the measurement. In truth, that is somewhat of an oversimplification, as the behavior of a riboswitch biosensor is still going to be affected by external factors, just different ones. It will be influenced by the sort of things that determine RNA structure, such as the speed of transcription by RNAP, the concentration of magnesium in the cell, or possibly even the temperature at which the cell is growing.

We undertook our own attempts at developing a biosensor with a plan to identify the natural mechanism of regulation by which c-di-GMP and the Vc2 riboswitch control *tfoY* and then adapt that mechanism to the control of an easily detectable reporter such as *gfp*. In the years that have passed since we began, multiple alternative strategies have emerged for using riboswitches to detect c-di-GMP *in vivo*: fusion of a c-di-GMP riboswitch to a RNA module which can bind an exogenously added fluorophore, engineering of a c-di-GMP riboswitch to control RNA cleavage by a hammerhead ribozyme, and a triple-tandem arrangement of c-di-GMP riboswitches controlling transcription of a fluorescent protein (Nakayama et al., 2012; Kellenberger et al., 2013; Gu et al., 2012; Zhou et al., 2016). Some of these biosensor designs have adapted the natural expression platform of non-Vc2 c-di-GMP riboswitches to control an output signal, and some have combined the natural aptamer domain of Vc2 with an artificial expression platform. Ironically, none of these methods employ the natural expression platform of the Vc2 riboswitch, even though Vc2 is, by far, the single most well-studied natural receptor for c-di-GMP, riboswitch or otherwise. What we now know, and what the subsequent chapters of this dissertation will reveal, is why that is the case. It is because the function and behavior of the Vc2 riboswitch in its natural environment, the *V. cholerae* cell, is far too complex to ever be useful as a simple, reliable biosensor.

# CHAPTER 3

# The Vc2 Cyclic di-GMP Dependent Riboswitch of *Vibrio cholerae* Regulates Expression of an Upstream Small RNA

**SUMMARY**

Cyclic di-GMP (c-di-GMP) is a bacterial second messenger molecule that is important in the biology of *Vibrio cholerae*, but the molecular mechanisms by which this molecule regulates downstream phenotypes have not been fully characterized. One such regulatory factor that may respond to c-di-GMP is the Vc2 c-di-GMP binding riboswitch, which is hypothesized to control the expression of a putative downstream transcription factor, *tfoY*. Although much is known about the physical and structural properties of the Vc2 riboswitch aptamer, the nature of its expression and function in *V. cholerae* has not been investigated. Here, we show that c-di-GMP binding to the Vc2 riboswitch is not required for c-di-GMP induction of *tfoY*. Rather, we identified four promoters upstream of *tfoY*, two of which initiate transcription downstream of Vc2, that ultimately lead to upregulation of *tfoY* transcription by c-di-GMP. Our results indicate that the primary function of c-di-GMP binding to Vc2 is to regulate the abundance and stability of an upstream non-coding small RNA (sRNA). The control of this sRNA is mediated by a novel mechanism of riboswitch function in which c-di-GMP binding to the Vc2 riboswitch located at the 3' end of this sRNA transcript protects it from degradation.

## INTRODUCTION

On a cellular level, bacteria translate information about external stimuli from their environment into internal signals that can serve to reprogram metabolic functions and behaviors. Second messengers are fundamental intracellular signals by which bacteria sense and respond to their environment. Cyclic dimeric guanosine monophoshate (c-di-GMP) is one such second messenger that is nearly ubiquitous among bacterial species and has been implicated in the control of virulence, biofilm formation, motility, cell cycle progression, and numerous other cellular programs (Römling et al., 2013). Whereas other nucleotide-based second messenger systems utilize only one or a few enzymes to synthesize and degrade the signal, the c-di-GMP system is highly complex. For example, the genome of *Vibrio cholerae* encodes 62 proteins involved in c-di-GMP turnover (Galperin et al., 2010).

Despite significant progress understanding c-di-GMP signaling, the regulatory mechanisms responsible for the transduction of the c-di-GMP signal have not been fully elucidated. Many of the protein effectors in this pathway that have been identified to date are species specific, and none of these factors are as ubiquitous among bacterial phyla as the molecule itself (Römling et al., 2013). In *Vibrio cholerae,* three c-di-GMP responsive transcription factors, VpsT, VpsR, and FlrA, have been identified, but their collective activity does not account for all of the changes in gene expression that are associated with c-di-GMP regulation (Krasteva et al., 2010; Srivastava et al., 2011; Srivastava et al., 2013). The discovery of a conserved c-di-GMP binding riboswitch revealed a new class of regulatory factors that could respond to c-di-GMP, and this riboswitch has been hypothesized to be an important missing link in the c-di-GMP signaling pathways of many bacteria (Sudarsan et al., 2008).

Riboswitches were originally described as allosterically regulated *cis*-acting mRNA elements (Nahvi et al., 2002). Traditional riboswitches function in the 5'-untranslated region of mRNAs through two separate but inter-related domains of secondary structure: an aptamer domain which is capable of binding to a specific metabolite or small molecule, and an expression platform which regulates either transcription or translation through formation of RNA secondary structures that control transcription termination or sequestration of the ribosome binding site, respectively (Serganov and Nudler, 2013). Upon binding to its ligand, the structural conformation of the aptamer domain changes, eliciting a complementary change in the secondary structure of the expression platform. In this way, the expression of the mRNA can switch between "on" or "off" states in direct response to concentrations of the molecule which it senses (Garst and Batey, 2009). Importantly, riboswitches are considered to function *in cis* to control expression of a gene located downstream on the same transcript.

Two classes of c-di-GMP binding riboswitches have been identified (Sudarsan et al., 2008; Lee et al., 2010). The most heavily investigated class-I c-di-GMP riboswitch is the Vc2 aptamer of *V. cholerae*, which has been thoroughly examined in multiple *in vitro* biochemical and structural studies (Kulshina et al, 2009; Smith et al., 2009; Smith et al., 2010; Fujita et al., 2012; Wood et al., 2012). The Vc2 riboswitch was originally characterized as a genetic "on-switch" based a set of experiments employing a translational reporter in a heterologous model organism, *Escherichia coli* (Sudarsan et al., 2008). However, no study has investigated the function of the Vc2 riboswitch in the native environment of a *V. cholerae* cell, nor has the molecular mechanism by which this riboswitch controls gene expression been determined. As a result, its physiological role in *V. cholerae* c-di-GMP regulation remains undefined.

The Vc2 riboswitch is located upstream of the gene *tfoY*, a putative transcription factor predicted to be involved in the regulation of genetic competence in *Vibrio spp.* (Pollack-Berti, 2010). Multiple reports have speculated that the proximity of the Vc2 riboswitch to *tfoY* indicates a role for the Vc2 riboswitch in the control of biofilm formation and motility in *V. cholerae* (Sudarsan et al., 2008; Shanahan and Strobel, 2012). Here, we show that *tfoY* expression is induced by increasing c-di-GMP in *V. cholerae*, but we demonstrate that this induction is independent of c-di-GMP binding to the Vc2 riboswitch and is due to regulation of transcription initiation. Although not impacting *tfoY* expression, mutations abolishing binding of c-di-GMP to the Vc2 apatmer drastically reduce the abundance of two sRNAs that contain the Vc2 apatmer at their 3' ends. Our results indicate that c-di-GMP binding to this 3' riboswitch stabilizes the upstream sRNA by preventing its degradation, a novel mechanism of riboswitch-mediated genetic control by c-di-GMP.

## RESULTS

### Induction of *tfoY* by increased c-di-GMP occurs independently of c-di-GMP binding to the Vc2 aptamer

To determine the mechanism of Vc2 riboswitch-mediated gene regulation, we created a GFP translational reporter, $P_1$-TL, for the gene *tfoY* containing the genomic region from -535 to +21 relative to the *tfoY* coding sequence (Fig. 3-3A). This reporter was introduced into *V. cholerae* cells harboring the *Vibrio harveyi* diguanylate cyclase enzyme, QrgB, under control of the IPTG-inducible $P_{tac}$ promoter. QrgB synthesizes c-di-GMP in *Vibrio cholerae* allowing for artificial modulation of the c-di-GMP concentration *in vivo* (Waters et al., 2008). As a control, an

active site mutant of QrgB termed QrgB* that cannot synthesize c-di-GMP was similarly expressed. As QrgB expression was increased, we observed a dose-dependent increase in fluorescence from our reporter, but expression of QrgB* had no effect, showing that this regulation is due to increased levels of c-di-GMP (Fig. 3-1A). To determine if this regulation was mediated by binding of c-di-GMP to the Vc2 riboswitch, we introduced mutations into the Vc2 aptamer sequence that disrupt binding of c-di-GMP. Specifically, the G20 and C92 sites of the Vc2 aptamer directly base pair with the c-di-GMP molecule when the riboswitch adopts its ligand bound state (Smith et al., 2009). Conversion of the C92 site to uracil has been shown to reduce the affinity of the Vc2 aptamer for c-di-GMP *in vitro* by more than three orders of magnitude, and conversion of both sites to uracil renders the aptamer unable to bind its ligand (Smith et al., 2009). Neither the single C92U mutation nor the double G20U/C92U mutation abrogated induction of $P_1$-TL by c-di-GMP, indicating that the induction of *tfoY* expression is independent of c-di-GMP binding to the Vc2 riboswitch (Fig. 3-1B and 3-1C).

**Multiple promoters regulate *tfoY* expression in a c-di-GMP dependent manner**

Based on these results, we hypothesized that the c-di-GMP-mediated induction of *tfoY* is due to control of transcription initiation of *tfoY* mRNA, however the promoters responsible for expression of the Vc2 riboswitch and *tfoY* have not been previously determined. Using 5'-Rapid Amplification of cDNA Ends (5'-RACE) to map the 5'-ends of transcripts encoding *tfoY*, we identified four potential transcriptional start sites we designated $P_{1\text{-}tfoY}$, $P_{2\text{-}tfoY}$, $P_{3\text{-}tfoY}$, and $P_{4\text{-}tfoY}$, with $P_{1\text{-}tfoY}$ being the furthest upstream from the coding sequence of *tfoY* (Fig. 3-3A). We constructed GFP transcriptional reporters encoding the genomic regions immediately upstream

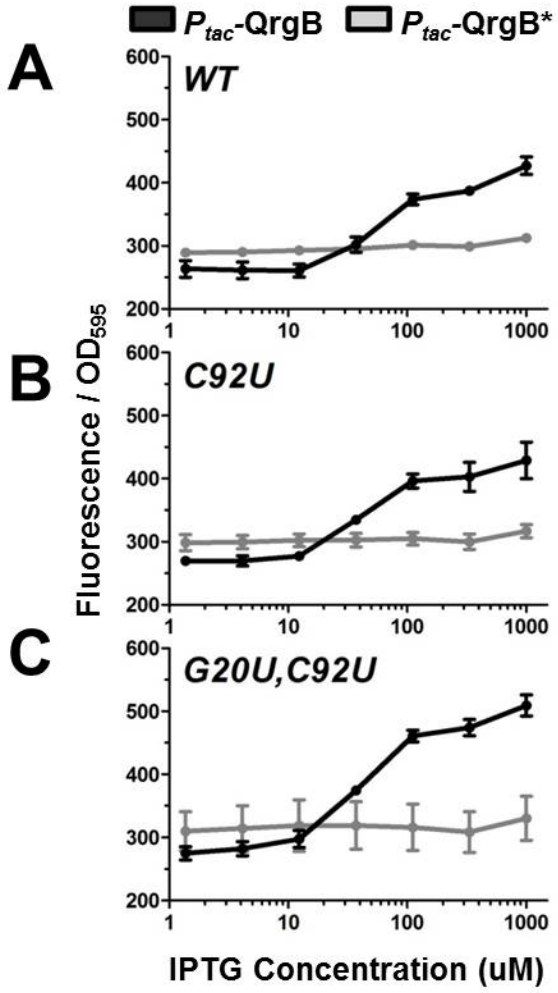



**Figure 3-1: c-di-GMP Controls *tfoY* gene Expression through Promoter Regulation**

**(A,B,C)** Lines depict fluorescence normalized by OD595 for *V. cholerae* strains harboring *tfoY* translational reporters corresponding to the sequence indicated in Fig. 3-3A. Reporter genotypes, relative to the Vc2 riboswitch aptamer are indicated by bold text within each graph. Overexpression vector genotypes are indicated by dark and light colors. QrgB is a *V. harveyi* diguanylate cyclase. QrgB* has a degenerate active site motif and is non-functional for cyclase activity (Waters et al.,2008).

of each of these sites and three of the four reporters (excluding $P_{4\text{-}tfoY}$) showed basal GFP expression in *V. cholerae* that was significantly greater than the vector control (Fig. 3-3B). The strength of transcription initiation from these start sites directly correlated with their distance

from the *tfoY* translation start site. This is consistent with the observation that the sequences of $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ strongly match the consensus -10 and -35 sites of $\sigma^{70}$-regulated promoters, with 7/12 and 10/12 identical bases, respectively, while the $P_{3\text{-}tfoY}$ region is less conserved with 3/12 (Fig. 3-2). Interestingly, the $P_{4\text{-}tfoY}$ encodes strong matches to the consensus $\sigma^{70}$ binding site with 10/12 identical bases, but the observed start site of $P_{4\text{-}tfoY}$ is a greater distance from the predicted -10 and -35, which may explain the relatively weak activity of this promoter. A recent study in *V. cholerae* identifying transcriptional start sites on a genome-wide scale using RNA-Seq also identified the locations of the $P_{1\text{-}tfoY}$ and $P_{3\text{-}tfoY}$ transcription start sites (Papenfort et al., 2015).

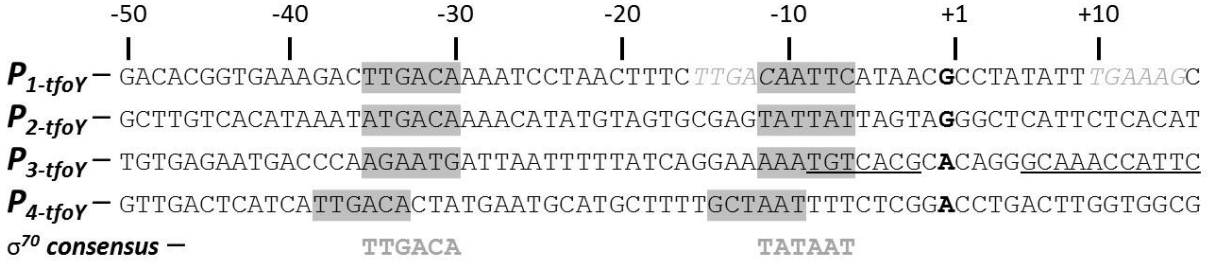



**Figure 3-2: Alignment of *tfoY* Promoters**

-35 and -10 regions of the promoters highlighted in grey; a second possible set of -35 -10 regions near $P_{1\text{-}tfoY}$ is indicated by grey italics. Transcriptional start sites determined by 5'-RACE are indicated in bold. Regions of the $P_{3\text{-}tfoY}$ promoter which overlap with the sequences that form the 5'-sides of the P1 and P2 structures of the Vc2 aptamer are underlined.

The location of the *tfoY* promoters indicates that only the two most upstream promoters, $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$, would produce transcripts that include the full sequence of the Vc2 riboswitch aptamer (Fig. 3-3A). The transcriptional start site of the $P_{3\text{-}tfoY}$ promoter is located at nucleotide position -194 relative to the start of the *tfoY* coding sequence, which corresponds to position

A16 of the riboswitch aptamer as described in Smith et al., 2009. This means that transcripts generated from the $P_{3\text{-}tfoY}$ promoter would not include the nucleotides that form the 5'-side of the P1 stem loop of the Vc2 aptamer (underlined in Fig. 3-2). A previous study showed that truncation of the bases on the 3'-side of the P1 stem lowers the affinity of the Vc2 aptamer for c-di-GMP by more than four orders of magnitude, implying that formation of the P1 stem loop is necessary for tight binding to c-di-GMP (Smith et al., 2010). Therefore transcripts from the $P_{3\text{-}tfoY}$ promoter would not contain a Vc2 riboswitch capable of effectively binding to c-di-GMP.

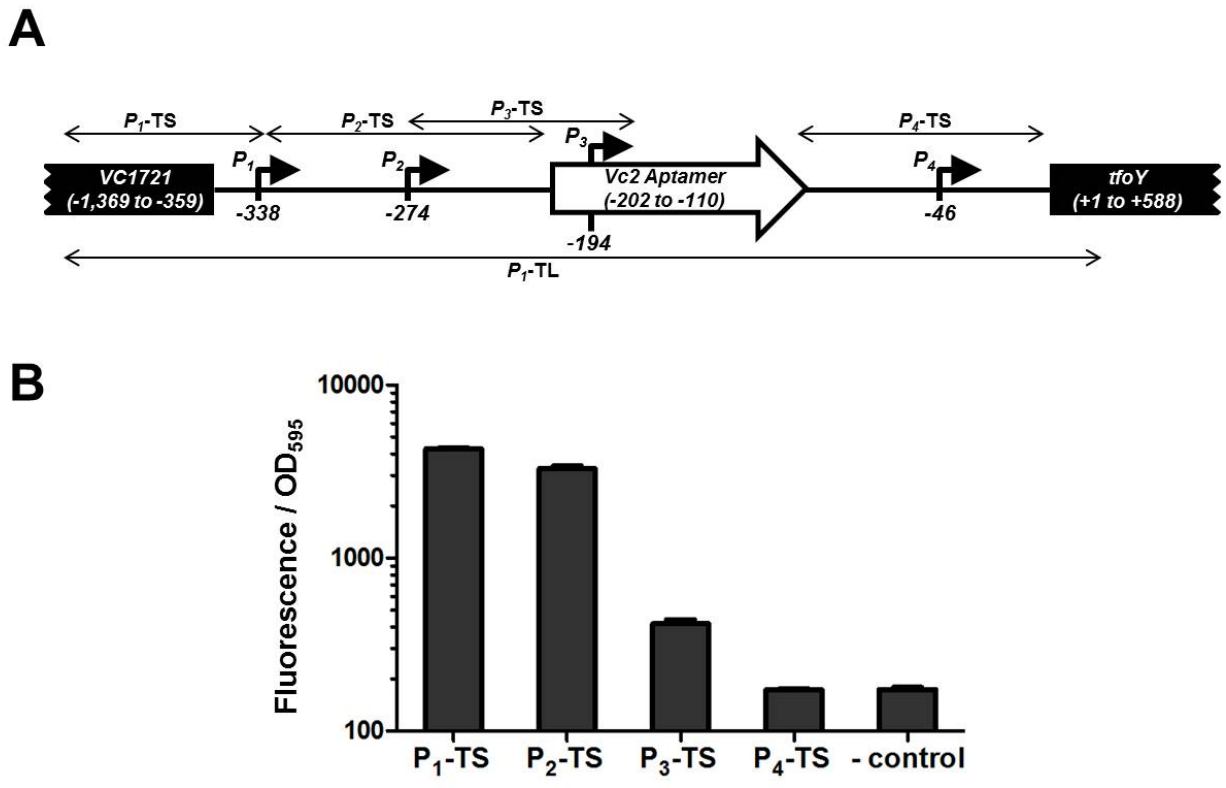



**Figure 3-3: Map of Vc2 Riboswitch Locus and the Relative Activity of Four tfoY Promoters**

**(A)** This map shows the four transcriptional start sites identified for *tfoY* transcripts and their location on *V. cholerae* Chromosome I. Numbering is relative to the start of the *tfoY* coding sequence. Sequences used for translational (TL) and transcriptional (TS) reporter fusions described in the text are indicated by bracketed arrows. **(B)** Bars depict fluorescence normalized by OD595 for *V. cholerae* strains harboring *tfoY* promoter transcriptional reporters. Reporter genotype is indicated on the Y-axis and corresponds to sequence indicated in panel A.

To determine the source of the riboswitch-independent induction of *tfoY* by c-di-GMP, we evaluated the response of the four *tfoY* promoter transcriptional fusions to both wild-type and elevated levels of c-di-GMP (Fig. 3-4A). At high c-di-GMP, the expression of the $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$ promoters was induced, the activity of $P_{2\text{-}tfoY}$ promoter was decreased, and the $P_{1\text{-}tfoY}$ promoter did not show a significant change in expression (Fig. 3-4A). Overall, we conclude that the combined transcriptional regulation at these four promoters explains the riboswitch-independent increase in *tfoY* expression we had initially observed with $P_1$-TL at high concentrations of c-di-GMP.

**Transcripts originating from $P_3$-*tfoY* and $P_4$-*tfoY* disproportionately encode *tfoY***

To further confirm the c-di-GMP regulation we had observed with our transcriptional fusions and to determine which promoters contribute to expression of *tfoY*, we conducted primer extension analysis. A biotin-labeled primer complementary to the +97 to +120 region of the *tfoY* coding sequence was used to exclusively reverse transcribe *tfoY* mRNA from total RNA extracts of *V. cholerae* cells. We expected to generate four major species of labeled ssDNA of sizes 458nt, 394nt, 314nt, and 166nt corresponding to the observed transcriptional start sites of the $P_{1\text{-}tfoY}$, $P_{2\text{-}tfoY}$, $P_{3\text{-}tfoY}$, and $P_{4\text{-}tfoY}$ promoters, respectively. These were indeed the most prominent bands observed (Fig. 3-4B, Lane 1). Sequence analysis of the $P_{1\text{-}tfoY}$ promoter region indicated multiple sets of overlapping $\sigma^{70}$ consensus -35 and -10 binding sites that may explain the doublet of bands present at the $P_{1\text{-}tfoY}$ size range (Fig. 3-2). The set of bands located between the $P_{2\text{-}tfoY}$ and $P_{3\text{-}tfoY}$ promoter regions may indicate degradation at the 5'-end of $P_{1\text{-}tfoY}$

and $P_{2\text{-}tfoY}$ transcripts. We also observed a band in the ~800nt size range, indicating a potential fifth promoter for *tfoY* located in the middle of the *VC1721* coding sequence, which was missed

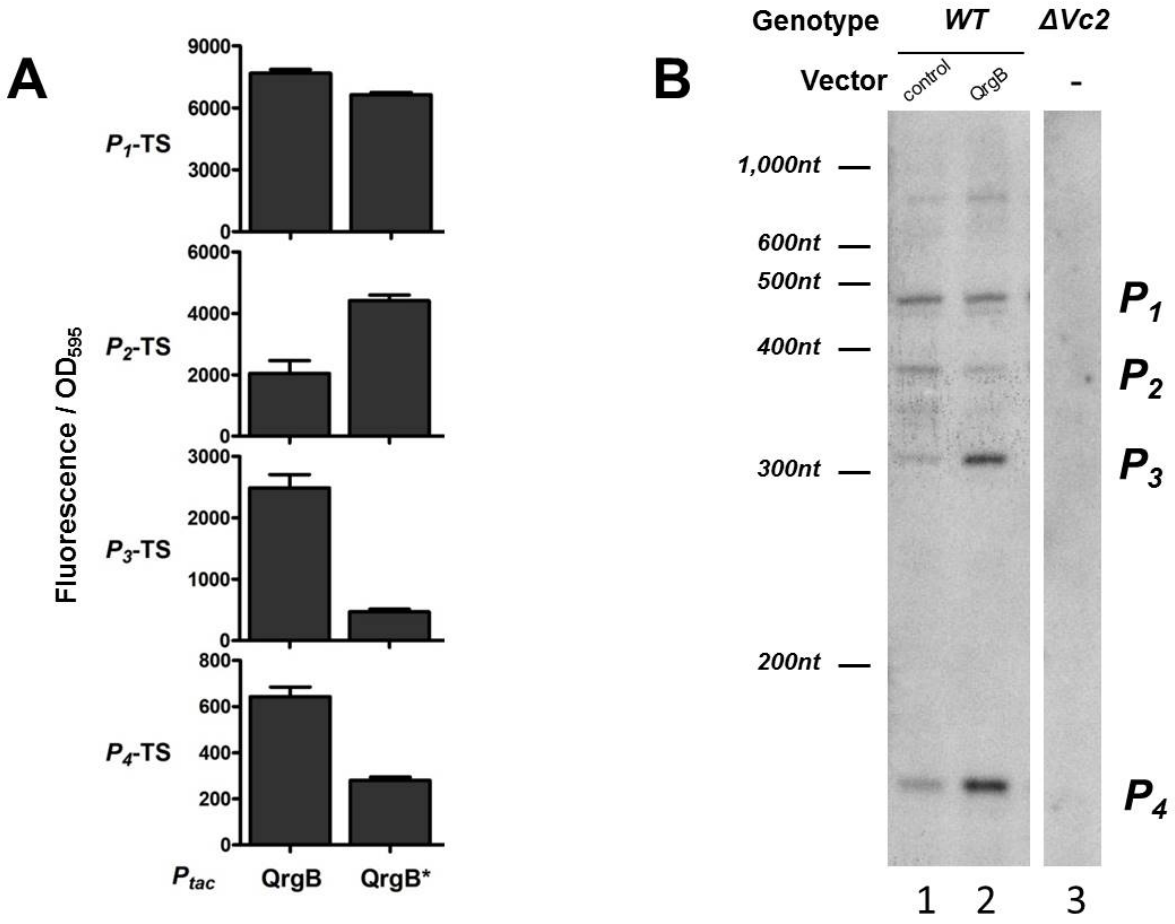



**Figure 3-4: c-di-GMP Regulates Transcriptional Activity of *tfoY* Promoters**

**(A)** Bars depict fluorescence normalized by OD595 for *V. cholerae* strains harboring *tfoY* promoter transcriptional reporters. Reporter genotype is indicated on the Y-axis and corresponds to sequence indicated in Figure 3-1. Overexpression vector genotype is indicated on the X-axis, and is the same as described in Figure 3-1. All bars depict cultures grown at a 1mM concentration of IPTG. **(B)** Primer extension analysis of tfoY transcripts. Lanes are as described in the main text. This figure is representative of multiple experiments. The film shown here is overexposed in order to aid the visualization of bands. Values reported in the text were quantified from blots exposed to film over a linear range of detection. *(Lane 1: CW2034 w/ pEVS141, Lane 2: CW2034 w/ pCMW75, Lane 3: BP22)*

by our initial 5'-RACE analysis. However, since the contribution of this putative promoter represents less than 10% of the total transcripts containing *tfoY* under all conditions tested and this transcript is not differentially regulated in response to changing c-di-GMP levels, we did not consider it further. Importantly, none of these bands were observed when samples from a mutant strain with a chromosomal deletion of the entire *VC1721-tfoY* intergenic region (*Δ*Vc2) was analyzed by primer extension, confirming the specificity of our assay.

We quantified the intensity of each transcript using image analysis to determine the relative contribution of each promoter to *tfoY* mRNA. Under wild-type conditions (Fig.3B, Lane 1), the upstream promoters, $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$, account for 67% of *tfoY* transcripts, while the downstream promoters, $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$, account for 27% of *tfoY* expression. This is surprising given that our transcriptional reporters indicated the combined activity of the upstream promoters is 14-fold greater than the combined activity of the downstream promoters. This implies that under wild-type conditions, the majority of mature transcripts initiated from the $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ promoters either do not reach or no longer contain the *tfoY* coding sequence.

We also determined the contribution of the promoters to *tfoY* expression during high c-di-GMP conditions (Fig. 3-4B, Lane 2). The total *tfoY* transcript levels are increased 1.9-fold upon expression of QrgB, with the contribution of the upstream promoters decreasing to 25%, and the downstream promoters responsible for 71% of *tfoY* expression (Fig. 3-4B, Lane 2). The transcripts produced by $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$ increased 6.7-fold and 4.0-fold, respectively, while $P_{2\text{-}tfoY}$ transcripts were reduced 2.3-fold. These results are consistent with our transcriptional fusion data concerning the behavior of the individual promoters at high c-di-GMP (Fig. 3-4A),

and it supports our hypothesis that induction of transcription from $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$ is responsible for the increase in expression from the *tfoY* translational reporter (Fig. 3-1).

### $P_{1\text{-}tfoY}$ produces transcripts that are truncated at the 3'end of the riboswitch aptamer

The results of our primer extension assay suggested that the majority of transcripts initiating from the two upstream *tfoY* promoters do not contain the *tfoY* coding sequence. Therefore, we sought to identify the location of the 3'-ends of transcripts originating from $P_{1\text{-}tfoY}$ by conducting a 3'-RACE assay. In total, we recovered 66 sequences which mapped to the *VC1721-tfoY* intergenic region (Fig. 3-5). We did not recover any transcripts with a 3'-end mapping downstream of the *tfoY* stop codon, however, we did recover multiple transcripts

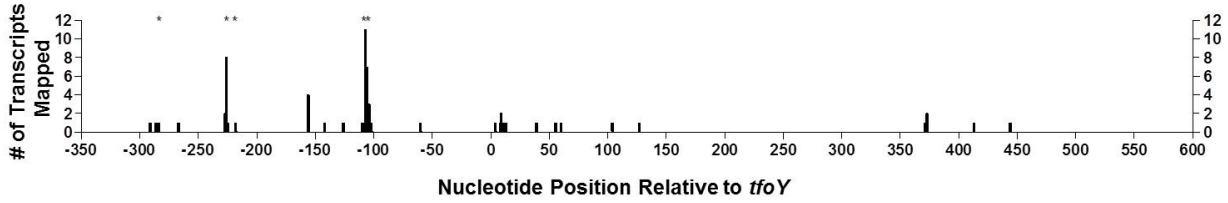



**Figure 3-5: 3'-RACE of $P_{1\text{-}tfoY}$ transcripts**

Figure is as described in the main text. Numbering of the X-axis corresponds to the genomic map in Figure 3-3A, except for position zero, which is included here for reference but does not represent a nucleotide position on the genome. Y-axis indicates the number of $P_{1\text{-}tfoY}$ transcript sequences recovered whose 3'-end mapped to the position indicated. Asterisks indicate sites where sequences contained 3'-end tails with extra-genomic nucleotides. 66 sequences are depicted in total.

whose 3'-ends were dispersed within the *tfoY* coding sequence, suggesting high amounts of message degradation. Surprisingly, the 3'-end of the majority of sequences was located upstream of the *tfoY* translational start site. The greatest number of 3'-ends we observed, 38%

of all recovered transcripts, mapped to the region -110 to -102 relative to the start of the *tfoY* coding sequence, which is the region immediately adjacent to the 3'-end of the stem-loop that forms the base of the Vc2 riboswitch aptamer (Smith et al., 2009). An additional 17% of the sequences contained a 3'-end located further upstream, between -227 to -225 relative to the start of the *tfoY* coding sequence. This region correlates with the 3'-end of a putative *rho*-independent terminator located between the $P_{2\text{-}tfoY}$ and $P_{3\text{-}tfoY}$ transcriptional start sites (Kingsford et al., 2007). 30% of the transcripts recovered featured 3'-end tails of between 1 and 4 extragenomic nucleotides, almost exclusively adenines, and the majority of these tailed transcripts mapped to the aforementioned sites of the Vc2 aptamer or the upstream *rho*-independent terminator. Combined with our previous observation that the $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ promoters contribute less to the production of *tfoY* mRNA than expected based on their transcriptional strength (Fig. 3-4B), these results led us to hypothesize that most of the transcripts initiated from $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ are sRNAs with the Vc2 riboswitch aptamer located at their 3' ends.

**Multiple sRNAs are transcribed from the *VC1721-tfoY* intergenic region**

To confirm that the expected sRNA species produced by the upstream *tfoY* promoters were present in *V. cholerae*, we performed a series of Northern blots with probes encompassing different segments of the *VC1721-tfoY* intergenic region (Fig. 3-6). Four probes were used that are complementary to the following regions: between the $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ start sites (probe $P_1$, -337 to -272), between the $P_{2\text{-}tfoY}$ and $P_{3\text{-}tfoY}$ start sites (probe $P_2$, -272 to -191), a

larger sequence overlapping the $P_{1\text{-}tfoY}$ to $P_{3\text{-}tfoY}$ start sites (probe $P_{1+2}$, -337 to -191) , and the sequence of the Vc2 riboswitch aptamer (probe Vc2, -211 to -94).

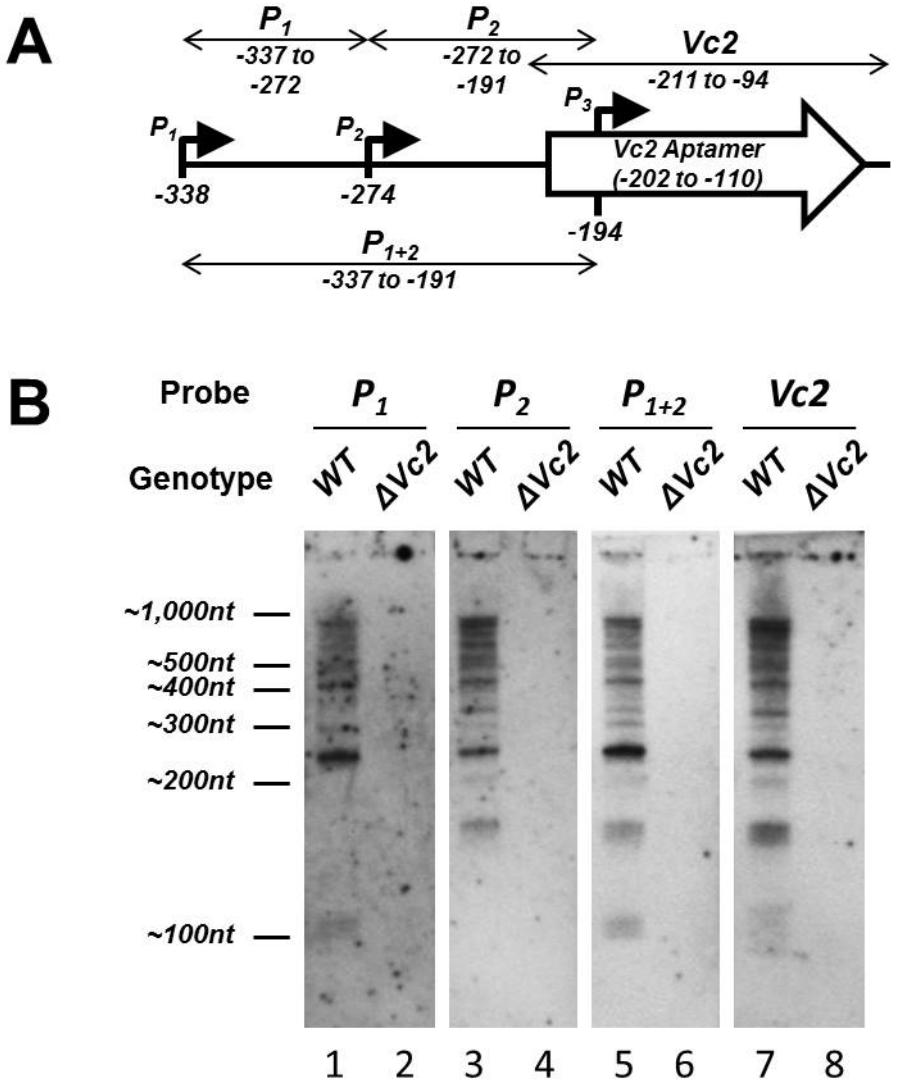



**Figure 3-6: Northern Blot Analysis of *VC1721-tfoY* Intergenic Region**

Figure is as described in the main text. The film shown here is overexposed in order to aid the visualization of bands.

Based on the results of the 3'-RACE assay, we expected to observe specific sRNA transcripts of ~231 nucleotides and ~167 nucleotides in size originating from the $P_{1\text{-}tfoY}$ and $P_{2\text{-}}$

$_{tfoY}$ promoters, respectively, with both ending at the immediate 3' edge of the Vc2 aptamer. The presence of the ~231 nucleotide band in all four blots (Fig. 3-6, Lanes 1, 3, 5, and 7), and the presence of the ~167 nucleotide band in all but the probe $P_1$ blot (Fig. 3-6, Lanes 3, 5, and 7), confirms the location of these sRNAs species relative to the promoters and the riboswitch aptamer. We refer to these ~231 nucleotide and ~167 nucleotide transcripts henceforth as the $P_1$-*Vc2* and $P_2$-*Vc2* sRNAs, respectively. We also expected to observe a sRNA ~112 nucleotides in size generated from the $P_{1\text{-}tfoY}$ promoter and ending at the predicted intrinsic terminator immediately downstream of the $P_{2\text{-}tfoY}$ start site. Though the signal is faint, the appearance of this band in the probe $P_1$ and $P_{1+2}$ blots and its absence from the probe $P_2$ blot confirmed the presence of this transcript as well.

Overall, the probes detected a large range of RNA species of different sizes. This is consistent with the results of multiple transcriptomic studies in *V. cholerae* which have reported multiple sRNAs that map to the *VC1721-tfoY* intergenic region and differences between the expression of the Vc2 riboswitch and the *tfoY* coding sequence (Fig. 3-7) (Liu et al., 2009; Bradley et al., 2011; Mandlik et al., 2011; Raabe et al., 2011; Papenfort et al., 2015). All the signals in the Northern blot analysis were lost in the *Δ*Vc2 mutant, showing that in fact these RNA species are specific to the Vc2 riboswitch locus. It is also worth noting that the coding sequence of *VC1721*, the nearest gene upstream of the riboswitch, is 1,011 nucleotides in length. Therefore, based on their size, all the transcripts detected here should have originated from within the *VC1721* intergenic region and do not represent run-off transcription from upstream *VC1721* mRNA.

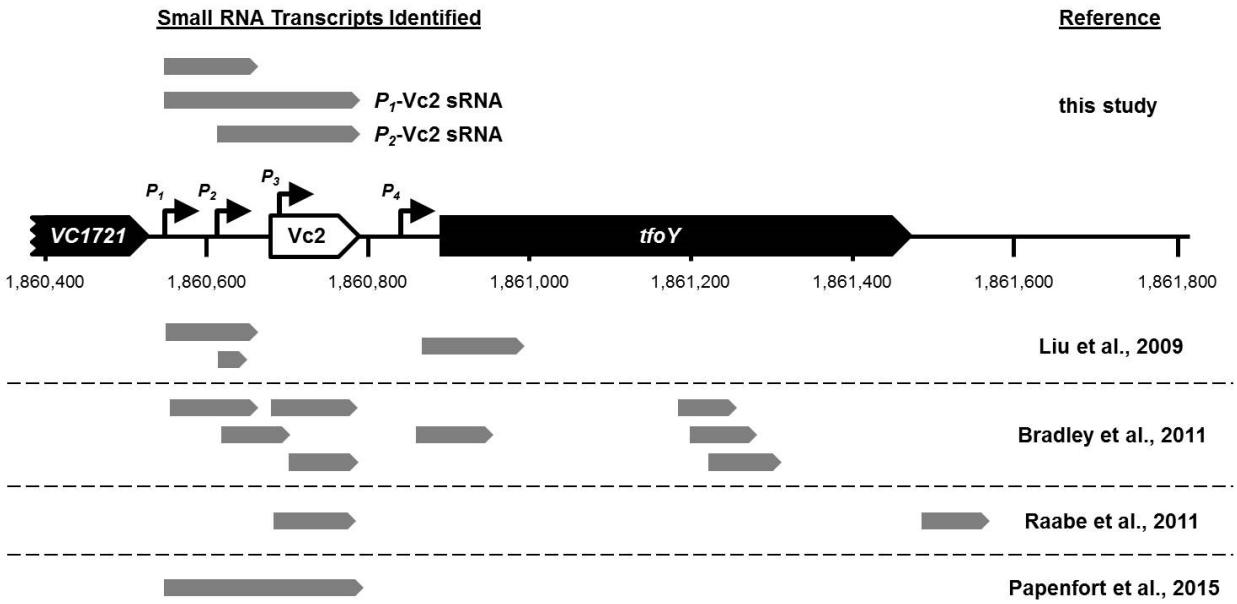



**Figure 3-7: Reported sRNAs at *tfoY* Locus**

RNA transcripts reported in the literature to date are indicated by gray arrows at the appropriate positions relative to the *V. cholerae* O1 biovar El Tor str. N16961 *tfoY* genomic locus. Position of sRNAs described in this study was determined by northern blotting and 5'- and 3'-RACE. Position of sRNAs described in previous studies was determined by various methods employing RNA-seq.

**The Vc2 riboswitch regulates the abundance of sRNAs**

To investigate the effect of changes in c-di-GMP on Vc2 riboswitch-containing transcripts *in vivo*, we performed a higher resolution Northern blot of *V. cholerae* RNA extracts with probe Vc2 (Fig. 3-8). An initial observation across all of our samples is that there are many different Vc2 riboswitch-containing transcripts between 300 and 1000 nucleotides in length (Fig. 3-8). The annotated coding sequence of *tfoY* is 588 nucleotides, therefore the minimum size of full-length *tfoY* mRNAs should be 926 nucleotides if originating from $P_{1\text{-}tfoY}$, or 782 nucleotides if originating from $P_{3\text{-}tfoY}$. This implies that a significant number of the transcripts detected contain only a fraction of the annotated *tfoY* open reading frame, again consistent

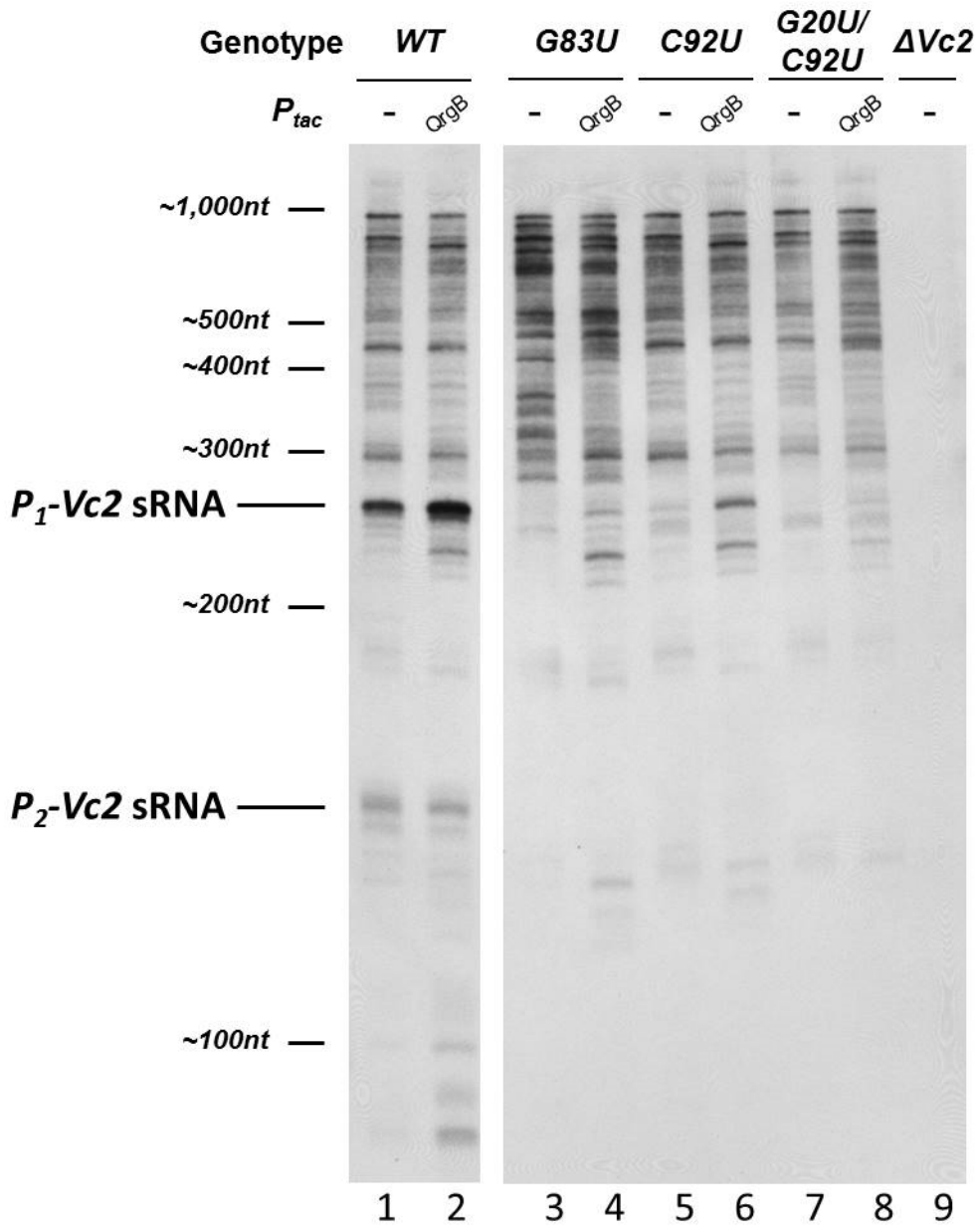



**Figure 3-8: Northern Blot Analysis of *V. cholerae* Riboswitch Mutants**

Lanes are as described in the main text. This figure is representative of multiple experiments. The film shown here is overexposed in order to aid the visualization of bands. Values reported in the text were quantified from blots exposed to film over a linear range of detection. *(Lanes 1,2: CW2034, Lanes 3,4: BP33, Lanes 5,6: BP34, Lanes 7,8: BP35, Lane 9: BP20; Odd Lanes: w/ pEVS141, Even Lanes: w/ pCMW75)*

with data from previous RNA-Seq studies of *V. cholerae* (Fig. 3-7) and our previous 3'-RACE analysis (Fig. 3-5). An integrity analysis of ribosomal RNA from our samples using an Agilent Bioanalyzer confirmed that our RNA was of high quality with little to no degradation occurring during extraction (data not shown). From this we conclude that most Vc2 riboswitch-containing transcripts are inherently unstable and subject a high rate of turnover *in vivo*.

Regardless of the transcriptional processes occurring downstream of the Vc2 aptamer, under wild-type conditions the $P_1$-*Vc2* sRNA is the most abundant single RNA species produced that contains the Vc2 aptamer sequence (Fig. 3-8, Lane 1). This is consistent with the disparity we had previously observed between the high activity of the $P_{1\text{-}tfoY}$ promoter and its low contribution to total *tfoY* mRNA (Fig. 3-4B). During QrgB overexpression, which leads to high levels of c-di-GMP, we observed a 2.5-fold increase in the abundance of the $P_1$-Vc2 sRNA (Fig. 3-8, Lane 2). The transcriptional reporter data and primer extension analysis discussed earlier had indicated that the activity of $P_{1\text{-}tfoY}$ is not significantly affected by induction of QrgB (Fig. 3-4), implying that the increase in $P_1$-Vc2 sRNA observed here is the result of a post-transcriptional mechanism. The abundance of the $P_2$-Vc2 sRNA was slightly decreased at high c-di-GMP, and that decrease could be attributed to c-di-GMP-mediated repression of the $P_{2\text{-}tfoY}$ promoter (Fig. 3-4).

We hypothesized that the post-transcriptional mechanism responsible for the increase in $P_1$-Vc2 sRNAs during QrgB overexpression is dependent on c-di-GMP binding to the Vc2 aptamer. To determine the effect of riboswitch mutations on production of $P_1$-*Vc2* sRNAs, we used a previously described vector system for allelic exchange to introduce individual point mutations onto the *V. cholerae* chromosome at the Vc2 riboswitch locus (Skorupski and Taylor,

1996). The G20 and C92 sites of the Vc2 riboswitch are the sites, which directly interact with the guanosine bases of c-di-GMP, and substitutions of uracil at either of these sites is significantly disruptive to ligand binding (Kulshina et al., 2009; Smith et al., 2009). Accordingly, in strains with either single or double mutations at the ligand binding sites we observed loss of the $P_1$-Vc2 sRNA (Fig. 3-8, Lanes 5-8). In the C92U mutant, the reduction in abundance from wild-type was 30-fold, and in the G20U/C92U mutant the amount of $P_1$-Vc2 sRNA remaining was below the limit of detection. We also observed complete loss of the $P_2$-Vc2 sRNA in both of these mutant backgrounds. Overexpression of QrgB is able to raise intracellular c-di-GMP concentrations 10-fold in *V. cholerae*, and we hypothesized that raising the c-di-GMP level of the cell should counteract the effect of the mutations. Indeed, when QrgB was overexpressed in the C92U and G20U/C92U strains we observed partial recovery of the abundance of the $P_1$-Vc2 sRNA in the C92U mutant but virtually no recovery in the G20U/C92U double mutant (Fig. 3-8, Lanes 6 and 8). The relative strength of this recovery in each strain was consistent with the previous biochemical data that the single binding site mutant retains a much higher c-di-GMP affinity than the double mutant (Smith et al., 2009).

The G83 site of the Vc2 riboswitch is essential to formation of a base pair with the C44 site which links together and stabilizes the P2 and P3 helices of the Vc2 aptamer around the ligand binding pocket (Kulshina et al., 2009; Smith et al., 2009). Replacing the G83 residue with uracil leads to a 250-fold decrease in the affinity of the Vc2 aptamer for c-di-GMP *in vitro*, but it also has significant effects on the global tertiary structure of the riboswitch, which is not the case for the mutations G20U and C92U (Smith et al., 2010; Wood et al., 2012). We constructed a *V. cholerae* strain with a single G83U point mutation at the Vc2 riboswitch genomic locus and

evaluated its effect on transcript production *in vivo*. This mutation results in the loss of the $P_1$-Vc2 and $P_2$-Vc2 sRNAs, and overexpression of QrgB in this background is sufficient to partially recover the loss (Fig. 3-8, Lanes 3 and 4). However, we also note that the G83U mutation causes significant changes to the pattern and abundance of riboswitch-containing transcripts >300nt in length, the transcripts containing variable amounts of the *tfoY* coding sequence, compared to the wild-type strain and binding site mutants (Fig. 3-8).

The traditional dogma for riboswitch-mediated gene regulation is that tertiary structure, ligand recognition, and regulatory function are all inextricably linked (Serganov and Nudler, 2013). Here we observe that the G83U and G20U/C92U mutations display the same phenotype with regard to the upstream transcript of the $P_1$-Vc2 sRNA, but display different phenotypes with regard to the downstream transcripts of *tfoY* mRNA, implying that Vc2 structural organization and Vc2 ligand recognition have mutually exclusive phenotypes. This result supports a model in which, at wild-type levels of c-di-GMP, the interaction of the Vc2 riboswitch with its ligand is important for regulation of the upstream sequence and yet serves no role in determining the expression of the downstream gene, *tfoY*.

**The Vc2 riboswitch is not sufficient for transcription termination *in vitro***

The observation that short transcripts are produced which contain the Vc2 aptamer at the 3'-end would be consistent with the hypothesis that the expression platform of the Vc2 riboswitch functions as a *rho*-independent transcriptional terminator. The only c-di-GMP riboswitches for which regulatory mechanisms have been experimentally verified are c-di-GMP binding aptamers connected to either *rho*-independent transcriptional terminators or ribozyme

expression platforms (Sudarsan et al., 2008; Furukawa et al. 2012; Lee et al., 2010). As other groups have reported, no readily apparent terminator structure is located adjacent to the Vc2 riboswitch, and bioinformatic analysis with *rho*-independent terminator prediction software indicates that the best candidate terminator nearby is located at the +193 to +235 region of the *tfoY* coding sequence, which is more than 300 nucleotides downstream of the 3'-end of the Vc2 aptamer (Sudarsan et al., 2008; Furukawa et al. 2012; Kingsford et al., 2007). Nevertheless, to experimentally test if the Vc2 aptamer stimulates transcription termination upon binding to c-di-GMP, we conducted an *in vitro* transcription termination assay with *E. coli* RNA polymerase complexed with $\sigma^{70}$ using a linear PCR template encompassing the genomic region -535 to +120 relative to the *tfoY* coding sequence.

Because our transcription template included all of the *tfoY* promoters, we expected to see full-length transcription products of sizes 458, 394, 314, and 166 nucleotides corresponding to the observed transcriptional start sites of the $P_{1\text{-}tfoY}$, $P_{2\text{-}tfoY}$, $P_{3\text{-}tfoY}$, and $P_{4\text{-}tfoY}$ promoters, respectively, and all of those transcripts were observed (Fig. 3-9A, Lane 1). Only two other major transcripts were detected, one >600 nucleotides, likely generated from end-to-end transcription of the template, and one at the ~180 nucleotides size range. Most notably, we did not observe any major transcript in the size range of the $P_1$-Vc2 sRNA, which is the most prominent riboswitch-containing transcript generated *in vivo* (Fig. 3-8).

We tested the possibility that Vc2 riboswitch could induce termination by adding exogenous c-di-GMP to the transcription reaction (Fig. 3-9A, Lane 2), yet no changes in the size or abundance of transcripts were observed. We also tested the effect of disrupting the structural integrity of the riboswitch by using transcription templates containing the same

mutations in the aptamer sequence which had earlier been investigated *in vivo*. Neither the G83U structural mutation nor the G20U/C92U binding site mutations described earlier had any detectable effect on the production of transcripts *in vitro*.

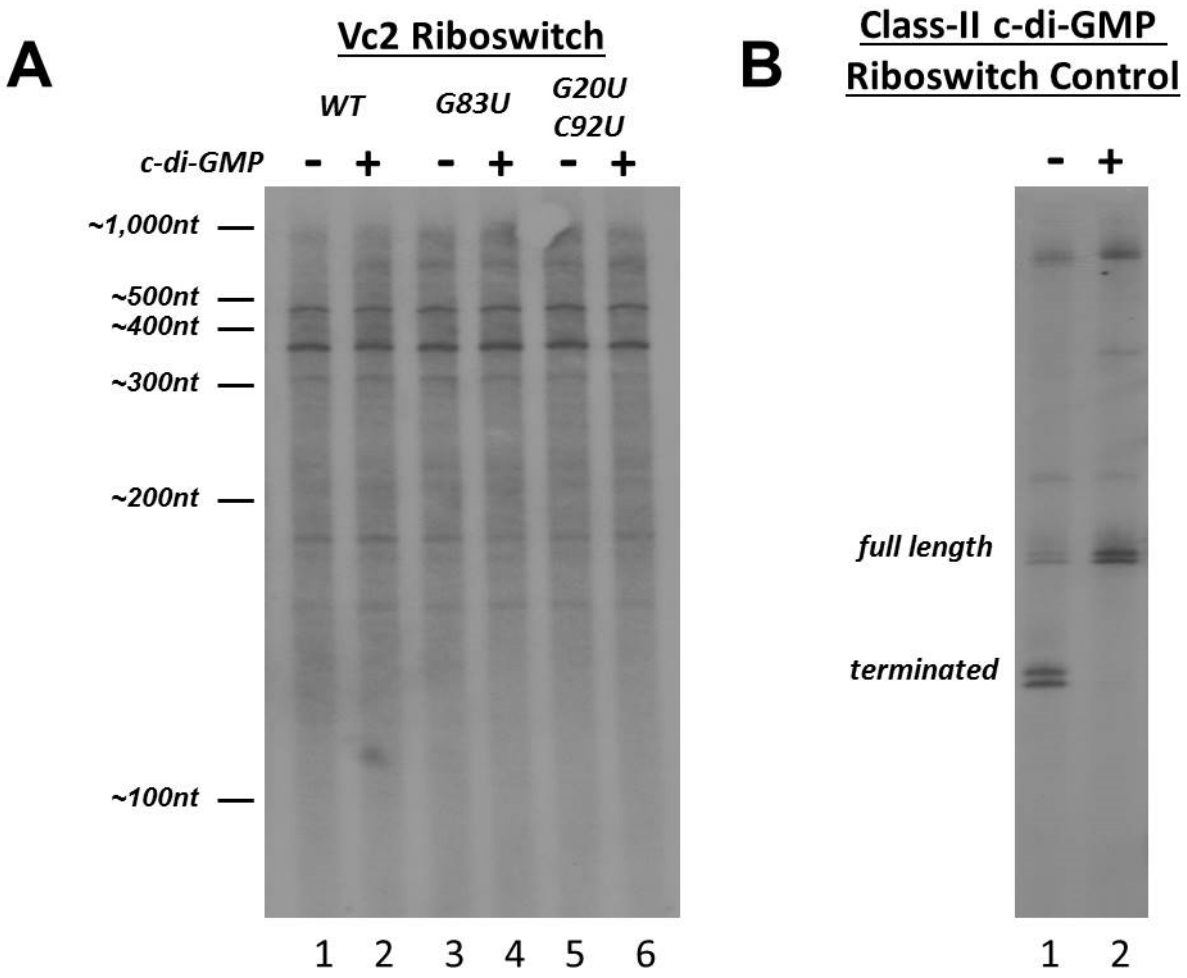



**Figure 3-9: *in vitro* Termination Assay with c-di-GMP**

Lanes are as described in the main text. This figure is representative of multiple experiments.

To ensure that our reaction conditions were sufficient for detecting potential riboswitch mediated termination, we tested a transcriptional template comprising the *ompR*-associated class-II c-di-GMP riboswitch of *Clostridium difficile*, for which a transcription termination

mechanism had previously been demonstrated *in vitro* (Lee et al., 2010). For this control experiment, addition of c-di-GMP inhibited transcription termination (Fig. 3-9B). Since we observed c-di-GMP regulation of termination with the control riboswitch but not with the Vc2 riboswitch, we conclude that the Vc2 riboswitch does not generate the $P_1$-Vc2 and $P_2$-Vc2 sRNAs through *rho*-independent termination.

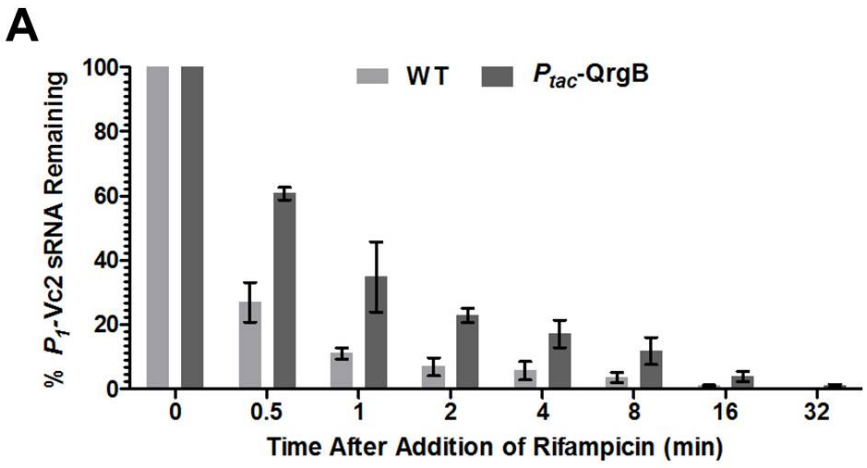
**Stability of the Vc2 sRNA is c-di-GMP dependent**

We next hypothesized that the Vc2 aptamer controls the post-transcriptional stability of the Vc2 sRNAs. To test this, we conducted a rifampicin RNA stability experiment. Rifampicin is an antibiotic that binds to RNA polymerase to prevent the initiation of new rounds of transcription for the majority of genes in the bacterial cell (Campbell et al., 2001). We added rifampicin at mid-log phase growth of *V. cholerae* cells under conditions of both wild-type and elevated c-di-GMP, and subsequently extracted RNA from the cells over a series of multiple time intervals. Transcripts containing the Vc2 aptamer sequence were detected by Northern blot with probe Vc2, and we then quantified the amount of $P_1$-Vc2 sRNA remaining at each time point after the addition of rifampicin relative to the amount of $P_1$-Vc2 sRNA at time zero (Fig. 3-10A).

Our analysis revealed that the $P_1$-Vc2 and $P_2$-Vc2 sRNA species are significantly more stable than all other transcripts originating from the *VC1721-tfoY* intergenic locus (Fig. 3-11). Surprisingly, this analysis also revealed that the $P_1$-Vc2 and $P_2$-Vc2 sRNAs are not single uniform RNA species, but instead are both represented by a doublet of transcripts with a 5 to 10 nucleotide difference in size (Fig. 3-11). An explanation for this doublet is postulated in the

discussion. For the purposes of our quantitative analysis of the amount of transcript remaining, both bands at the $P_1$-Vc2 sRNA size range were quantified together (Fig. 3-10A).

As was demonstrated earlier, the total population of $P_1$-Vc2 sRNAs is greater in the cell when c-di-GMP is elevated (Fig. 3-8). Our RNA stability assay indicated that the individual members of the $P_1$-Vc2 sRNA population, on average, also persist for longer in the cell when c-di-GMP is elevated (Fig. 3-11A). This difference in stability between conditions of low and high c-di-GMP is a sufficient mechanism to explain the difference in the abundance of the $P_1$-Vc2 sRNA between those same conditions (Fig. 3-8). However, the observed degradation of the $P_1$-Vc2 sRNA does not adhere to a simple one-phase decay model. Instead, this sRNA appears to be degraded rapidly at early time points and slowly at later time points. In order to compare the stability of the $P_1$-Vc2 sRNA transcripts over time, we calculated the effective half-lives




B

| Time Points | Relative $P_1$-Vc2 Half-Life (min) WT | $P_{tac}$-QrgB |
|---|---|---|
| 0-0.5 | 0.26 | 0.69 |
| 0.5-1 | 0.39 | 0.62 |
| 1-2 | 1.46 | 1.66 |
| 2-4 | 7.58 | 4.68 |
| 4-8 | 6.13 | 7.44 |
| 8-16 | 4.04 | 5.12 |
| 16-32 | * | 7.90 |

**Figure 3-10: RNA Stability Assay with Rifampicin**

**(A)** Stability assays were performed in triplicate for cells grown under both wild-type and elevated c-di-GMP conditions and the mean percent of $P_1$-Vc2 sRNA transcript remaining at each time point is depicted. **(B)** From the same data shown in A, the half-life of the $P_1$-Vc2 sRNA was calculated for the period between each time point.

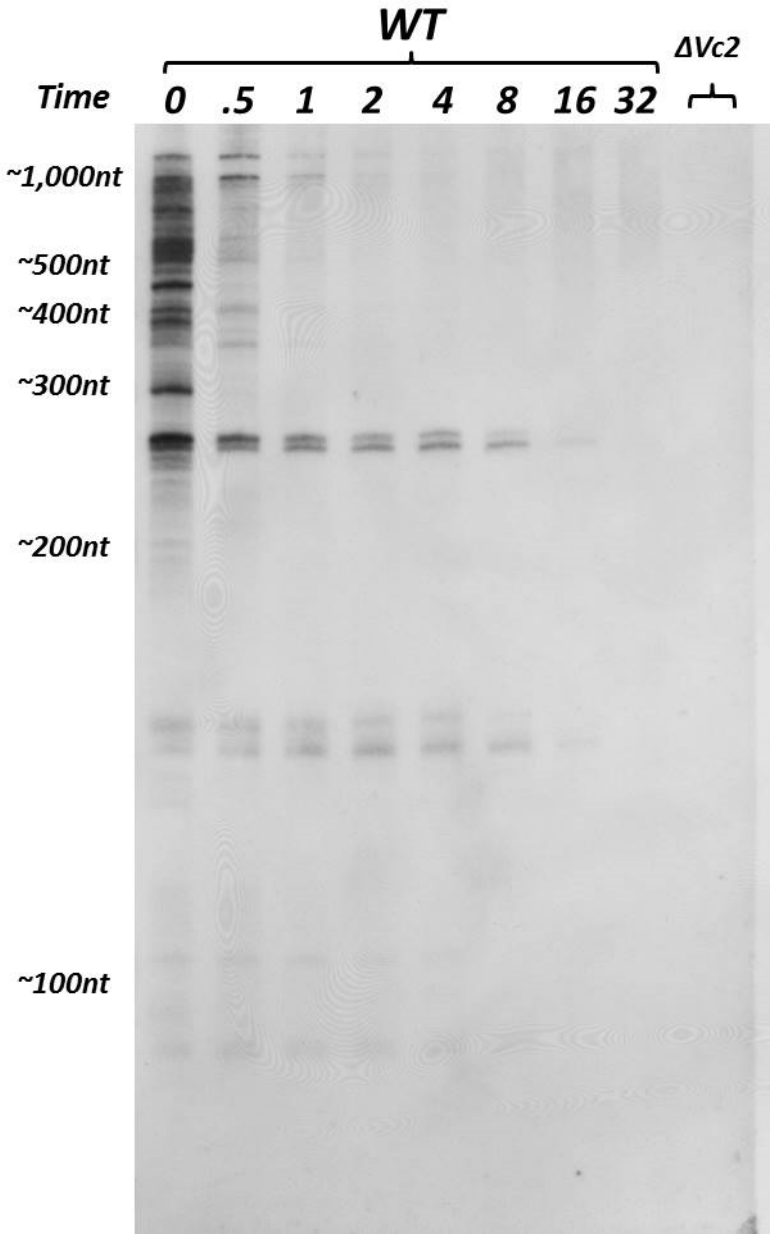



**Figure 3-11: RNA Stability Assay with Rifampicin**

A single exposure of a Northern blot from one of the replicates of the RNA stability assay. Measurements of RNA abundance were performed from a series of exposures in order to quantify the intensity of each band on the blot within its appropriate liner range.

observed for the transcripts relative to each set of time points (Fig. 3-10B). At the first two time points under wild-type conditions, the half-life of the $P_1$-Vc2 sRNA is less than half a minute,

while at later time points this half-life has risen to the range of 4 to 8 minutes. Under elevated c-di-GMP conditions, the half-life of the $P_1$-Vc2 sRNA shows a similar trend (Fig. 3-10B).

The observed pattern of increasing half-lives is consistent with there being multiple versions of the $P_1$-Vc2 sRNA with different rates of decay. Because the $P_1$-Vc2 sRNA contains a functional Vc2 aptamer domain, we hypothesize these different versions of transcript to be c-di-GMP-bound and -unbound forms of the $P_1$-Vc2 sRNA. From this interpretation, the 4 to 8 minute half-life measurement at later time points represents the closest approximation to the actual half-life of the c-di-GMP-bound form of the $P_1$-Vc2 sRNA, and the actual half-life of the c-di-GMP-unbound form of the $P_1$-Vc2 sRNA is less than the lowest measured half-life at the earliest time point, i.e. less than 15.6 seconds. When compared to studies on global RNA stability in other bacteria, this shift in the stability of the $P_1$-Vc2 sRNA is quite dramatic, as measurements in *E. coli* indicate the half-life of most RNAs to be in the range of 2 to 8 minutes (Pedersen et al., 1978; Bernstein et al., 2002; Selinger et al., 2003; Chen et al., 2015).

## DISCUSSION

The promise of riboswitches, when they were first described, is that they could provide an elegant means for genetic regulation in pathways where protein factors are not known to participate (Nahvi et al., 2002). Indeed, much of the original interest garnered by the discovery of the class-I c-di-GMP riboswitch was based on the fact that no c-di-GMP-binding transcription factors were known at the time (Sudarsan et al., 2008; Tamayo et al. 2007). However since then, numerous c-di-GMP responsive transcription factors have been identified across multiple bacterial phyla (Römling et al., 2013). Bioinformatic analyses of class-I c-di-GMP-dependent

riboswitches predict that they function in the 5'-untranslated region of mRNAs to transmit changes in c-di-GMP to the control of biofilm, motility, and other extracellular phenotypes (Weinberg et al., 2007; Sudarsan et al., 2008). However, little is actually known about the physiological role of this specific group of riboswitches because none have been explored in depth *in vivo*. To address this question, we examined the impact of the Vc2 riboswitch on expression of the *tfoY* gene in *V. cholerae*.

Our initial observations in *V. cholerae* suggested that the Vc2 riboswitch was a genetic "on" switch because a reporter containing the Vc2 sequence showed increased expression when c-di-GMP in the cell was elevated. Upon further examination we discovered that this induction is actually not dependent on binding of c-di-GMP to the Vc2 riboswitch, but is instead a consequence of the aggregate transcriptional regulation of four previously undescribed promoters located upstream of *tfoY* (Fig. 3-1, 3- 3, 3-4A). Noting the discrepancy between the strength of the two most upstream promoters and their relatively weak output of *tfoY* mRNA, we determined that the vast majority of Vc2 riboswitch-containing transcripts do not ultimately contain *tfoY* mRNA (Fig. 3-4B, 3-5). Instead, the upstream *tfoY* promoters produce a significant number of sRNA species with a diverse collection of sizes, the most prominent of which, designated as the $P_1$-Vc2 sRNA, originates at the $P_{1\text{-}tfoY}$ promoter and features the Vc2 aptamer domain at its 3' terminus (Fig. 3-6).

Superficially, the observation that increased c-di-GMP leads to production of short transcripts gives an impression that the Vc2 riboswitch serves as a transcriptional terminator. However, our results do not support that conclusion, as we did not observe transcription termination mediated by the Vc2 riboswitch *in vitro* (Fig. 3-9). Furthermore, termination at a

site downstream of the riboswitch would not explain how the $P_1$-Vc2 sRNA becomes truncated at the immediate 3'-end of the helical stem that forms the base of the Vc2 aptamer structure. Riboswitches that function by intrinsic termination employ an additional RNA structure, termed the expression platform, which is adjacent to, but separate from, the aptamer domain (Serganov and Nudler, 2013). For the Vc2 riboswitch, no such structure is predicted or readily identifiable downstream of the aptamer domain (Sudarsan et al., 2008; Furukawa et al., 2012). Moreover, if transcription termination were indeed driven by ligand binding to the riboswitch, then mutations which abrogate c-di-GMP binding to Vc2 should have significantly altered expression of *tfoY* in our translational fusion (Fig. 3-1). Finally, both primer extension analysis and Northern blotting indicates that transcription initiated at the $P_{1\text{-}tfoY}$ promoter is regularly able to proceed past the riboswitch. No evidence, therefore, supports the hypothesis that the Vc2 riboswitch functions by controlling transcription termination.

The most apparent phenotype for Vc2 riboswitch function is the changing abundance of the $P_1$-Vc2 sRNA. When intracellular c-di-GMP concentrations are elevated, more $P_1$-Vc2 sRNA is present, and when the Vc2 aptamer sequence is mutated on the genome less $P_1$-Vc2 sRNA is present (Fig. 3-8). Because the activity of the $P_{1\text{-}tfoY}$ promoter is not significantly affected by changes in c-di-GMP, the changing abundance of the $P_1$-Vc2 sRNA must be the consequence of post-transcriptional regulation. Our data indicates that the c-di-GMP-unbound $P_1$-Vc2 sRNA is subject to very rapid turnover by the RNA degradosome, but the c-di-GMP-bound $P_1$-Vc2 sRNA is significantly more resistant to degradation (Fig. 3-10B). Under normal conditions, most of the $P_1$-Vc2 sRNAs are unbound and unstable, but at high c-di-GMP a greater percentage of $P_1$-Vc2 sRNAs are ligand-bound and become highly stable, shifting the equilibrium of RNA turnover in

favor of a higher concentration of $P_1$-Vc2 sRNA transcripts (Fig. 3-12). Thus, we conclude that the Vc2 riboswitch functions by ligand-mediated aptamer protection from 3' degradation (Fig. 3-12). Here we report a novel mechanism of riboswitch-mediated gene regulation; control of upstream sequence stability via a 3'-aptamer. Considering that a characteristic of aptamer domains is to fold into a compact nucleotide structure around the target ligand we expect that this form of riboswitch mediated gene regulation may be widespread.

Our model of how the Vc2 riboswitch regulates RNA processing is unique from known mechanisms of riboswitch-mediated RNA degradation. The best characterized examples of riboswitches that both produce small RNA fragments and regulate RNA processing include the *glmS* riboswitch and ribozyme, the *yitJ* riboswitch and RNase Y, and the *lysC* riboswitch and RNase E (Collins et al., 2007; Shahbabian et al., 2009; Caron et al., 2012). All those systems have two things in common: (1) the primary regulatory function of the riboswitch is control of downstream gene expression, and (2) the effect of ligand-binding is destabilizing because it promotes degradation of riboswitch-containing transcripts. The Vc2 riboswitch differs from those previous examples on both accounts: (1) *tfoY* is not primarily regulated by the Vc2 riboswitch because two independent c-di-GMP-responsive promoters exist downstream of the Vc2 aptamer, and (2) c-di-GMP binding to the Vc2 riboswitch is stabilizing because it inhibits degradation of the $P_1$-Vc2 sRNA. The only previous example of 3'-end riboswitch-regulated RNA degradation comes from the plant domain. In that case, a thiamin pyrophosphate riboswitch that is located downstream of a thiamin biosynthetic gene controls RNA processing of the 3'-UTR in a manner that determines the expression level of the mature mRNA (Wachter et al., 2007). But again, whether ligand bound or not, the structure of the riboswitch aptamer is

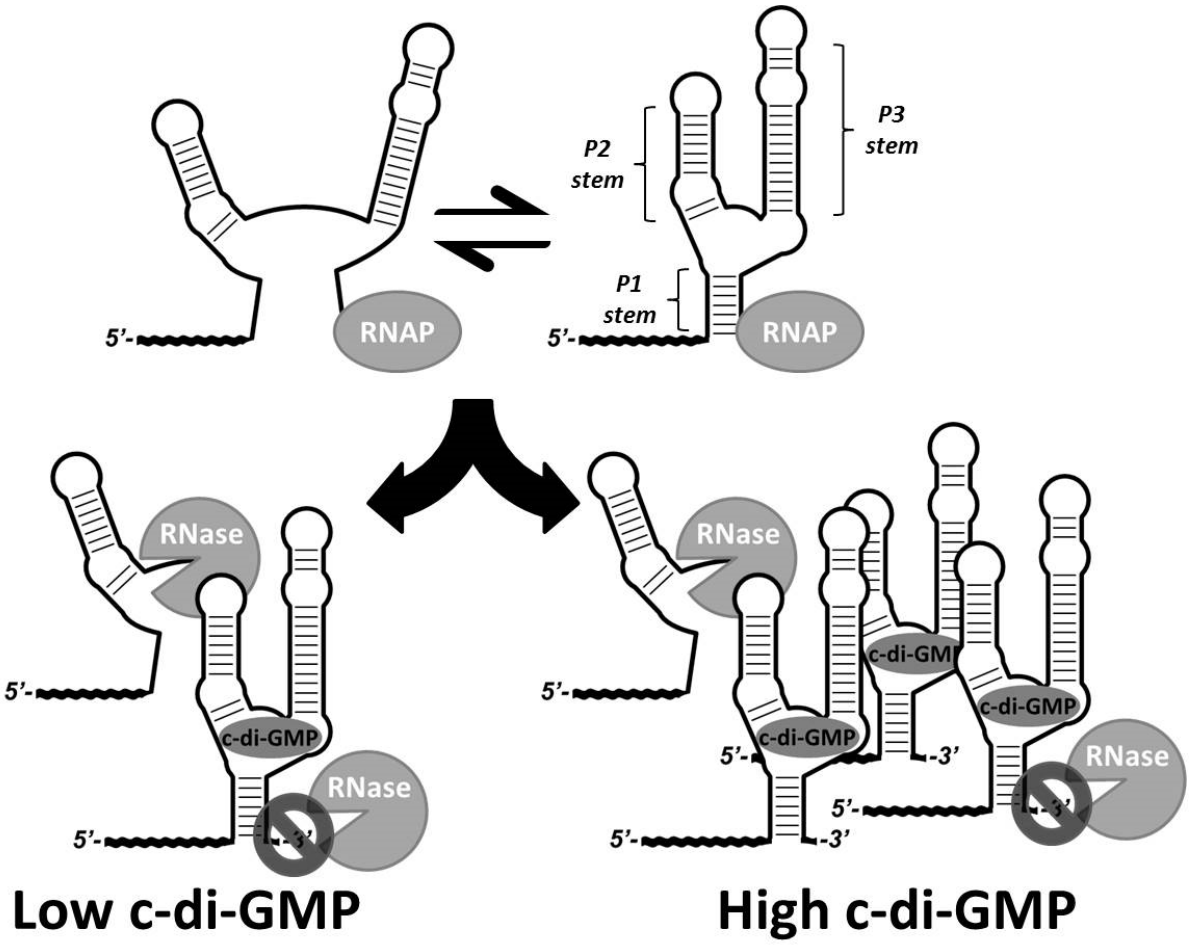



**Figure 3-12: Model of Vc2 Riboswitch Function**

During transcription and before ligand binding occurs, the P2 and P3 helices of the Vc2 aptamer pre-organize while the P1 helix is labile. After transcription the RNA is subject to the 3'-5' degradative regulation that affects all *tfoY* transcripts. If the riboswitch aptamer on the transcript never bound c-di-GMP, then the P1 helix was never stabilized and the RNA is quickly turned over. If the riboswitch aptamer on the transcript did bind c-di-GMP, then the closure of the P1 helix was stabilized and the RNA is protected from degradation by 3'-5' RNase activity.

sacrificed in either of the processing outcomes and the mature transcript does not contain a functional riboswitch (Wachter et al., 2007).

Stem-loop structures are common features at the 3’-end of bacterial mRNAs and have long been understood to provide protection against the 3’-5’ exonuclease activity of the RNA

degradosome, which has a preference for segments of single-stranded RNA (Rauhut and Klug, 1999). The initial biochemical work on the Vc2 riboswitch provides clues about how the aptamer structure could serve a role in 3'-end stabilization. Specifically, the P1 stem of the Vc2 riboswitch is predicted to be the actor in Vc2 riboswitch function because it is the most labile region of the aptamer structure which is stabilized in the c-di-GMP-bound state, and proper closure of the P1 stem requires ligand binding (Sudarsan et al., 2008; Smith et al., 2009). In fact, the other two helical stems of the Vc2 aptamer undergo relatively little structural change during ligand binding because their preorganization is a requirement for formation of the c-di-GMP binding pocket (Wood et al., 2012). It is no coincidence then that the 3'-end of the $P_1$-Vc2 sRNA is located immediately adjacent to the base of the P1 stem and that this was also the site of poly-adenylation for the sRNAs we recovered (Fig. 3-5). The doublet sizes of $P_1$-Vc2 sRNA transcripts and the pattern of their degradation we observed in our rifampicin stability assay are consistent with distinct populations of 3'-tailed and non-tailed transcripts, which is a hallmark of the 3'-end degradative processes described for many regulatory RNAs in bacteria (Fig. 3-2; Régnier and Marujo, 2000). We hypothesize that closure of the P1 stem of the Vc2 aptamer during a c-di-GMP binding event increases the stability of the $P_1$-Vc2 sRNA by making the nucleotides at the 3'-end of the riboswitch transcript inaccessible to 3'-5' exonucleases (Fig. 3-12).

Previous work with the Vc2 riboswitch has made note of the exceptionally strong, picomolar affinity of the c-di-GMP aptamer for its ligand as measured *in vitro*, despite the observation that c-di-GMP concentrations *in vivo* reside in the nanomolar to low micromolar range (Smith et al., 2009; Smith et al., 2010; Massie et al., 2012). Specifically, ligand binding at

the Vc2 aptamer experiences an unusually slow off-rate, so slow, in fact, that each c-di-GMP binding event should effectively be irreversible over the lifetime of the RNA transcript in the cell (Smith et al., 2009; Smith et al., 2010). For canonical riboswitches that employ transcriptional mechanisms of regulation, the on-rate of ligand binding is the more important feature of aptamer kinetics because a switching decision must be made in a brief window of time that is heavily constrained by the speed of the RNA polymerase (Garst and Batey, 2009). After initial ligand binding occurs, long-term retention of the ligand by the riboswitch aptamer is unimportant in transcriptional regulation because the RNA polymerase has long passed and a final decision about the "on" or "off" state of downstream gene expression has already been made. Biochemical study of the G20 and C92 sites of the Vc2 aptamer showed that the effect of those mutations was minimal on the c-di-GMP binding on-rate but had a great effect on the off-rate (Smith et al., 2009; Smith et al., 2010). This is consistent with our observations about the effect of those mutations *in vivo*. Downstream gene expression of *tfoY*, a process that should be on-rate-dependent, is unaffected by G20/C92 mutation (Fig. 3-1 and 3-6), yet the stability of the $P_1$-Vc2 sRNA, a process that, in our model, should be off-rate-dependent, is dramatically affected by G20/C92 mutation (Fig. 3-8). The slow off-rate of the Vc2 riboswitch is consistent with c-di-GMP-binding serving a continued function throughout the entire lifetime of the $P_1$-Vc2 sRNA in the cell by maintaining the Vc2 aptamer in a structure that would make the nucleotides of the aptamer inaccessible to RNAses.

An important outstanding question of this research regards the function of the $P_1$-Vc2 sRNA after expression. Trans-acting riboswitch-containing sRNAs are a controversial and very recently emerging topic for which only a handful of reports have been made. One of the first

reports came from *Listeria monocytogenes* in which an *S*-adenosylmethionine riboswitch was described as binding its ligand to induce transcription termination, and then discarding the ligand to allow the bases of the aptamer to rearrange and interact through complementarity with a target mRNA (Loh et al., 2009). This starkly contrasts our Vc2 riboswitch model in which the fate of the $P_1$-Vc2 sRNA is dependent on the binding and long-term retention of the ligand by the aptamer domain (Fig. 3-12). Another report, in *Bdellovibrio bacteriovorus*, describes a type-I c-di-GMP riboswitch transcript that was found to be more highly represented in an RNA-Seq data set than all other non-rRNA, non-tRNA transcripts, and was hypothesized to function as a storage bank for c-di-GMP that controls the transition between the growth and attack phases of the cell (Karunker et al., 2013). In contrast, our measurements on the abundance of the $P_1$-Vc2 sRNA indicate that it is not among the mostly highly expressed transcripts of *V. cholerae*, nor would it be capable of desaturating the global pool of c-di-GMP in *V. cholerae* (data not shown). Recently, an RNA-Seq study focusing on regulatory RNAs in *C. difficile* included an analysis of the expression of genes downstream of various riboswitches (Soutourina et al., 2013). Many different small transcripts and putative RNA degradation intermediates were identified originating from multiple c-di-GMP riboswitch loci, including one case where a ~160 nucleotide riboswitch-containing transcript was the most prominent RNA produced by a promoter 496 nucleotides upstream of the nearest gene (Soutourina et al., 2013). No function was identified for these short transcripts, and their regulation was not explored in depth. We are currently exploring the function of the Vc2 sRNAs in *V. cholerae*.

## EXPERIMENTAL PROCEDURES

### Strains and Growth Conditions

The wild-type *V. cholerae* strain used in this study is CW2034, an El Tor biotype C6706str2 derivative containing a deletion of the *vpsL* gene (Waters et al., 2008). Use of a *vpsL*- strain facilitates accurate spectrophotometric readings during transcriptional and translational reporter assays due to decreased biofilm formation. Propagation of DNA for genetic manipulation and mating of reporter plasmids was conducted in *E. coli* S-17-*λpir* (Simon et al, 1983). All growth of bacteria was performed at 35°C in Miller LB Broth (Acumedia), or on LB agar plates, with ampicillin (100 µg/mL), kanamycin (100 µg/mL), polymyxin B (10 IU/mL), streptomycin (500 µg/mL), and isopropyl-β-D-thiogalactoside (IPTG) at 100 µM when appropriate. Liquid cultures in tubes or flasks were continuously shaken at 220 RPM; microplate cultures were continuously shaken at 150 RPM.

### Reporter Assays

Each replicate was derived from an individual colony from a bacterial mating. Strains were grown overnight in test tubes then diluted 1:10,000 into fresh media containing IPTG, and inoculated at 100 µL volumes into in black, clear-bottom 96-well microplates. Plates were spectrophotometrically measured with a SpectraMax M5 (Molecular Devices) after reaching late log phase growth. GFP fluorescence was read with excitation at 475nm and emission at 510nm; absorbance was read at 595nm.

**Genetic Manipulations**

All genetic reporters were constructed in the pBRP31 background. pBRP31 was constructed by PvuII-EcoRI digestion of pMMB67EH (Fürste et al., 1986) to remove the *lacI*$^q$ and $P_{tac}$ elements, and inserting the SphI-BamHI promoterless GFP fragment from pCMW1 (Waters and Bassler, 2006), with some minor modifications to the multi-cloning site. Inserts for reporter vectors were generated by PCR with Phusion DNA Polymerase (NEB). For transcriptional reporters, the insert was ligated 29 bp upstream of the GFP coding sequence and thus utilized the consensus ribosome binding site featured in pCMW1. For translational reporters, the insert contained the first 21 bp of the *tfoY* coding sequence and was ligated to the second codon of the GFP coding sequence.

*V. cholerae* mutant strains were constructed using allelic exchange with vectors derived from pKAS32 (Skorupski and Taylor, 1996). Vectors containing mutant riboswitch alleles were generated with the QuickChange Site-Directed Mutagenesis Kit (Agilent) and mated into *V. cholerae* so as to create markerless strains with single point mutations on the genome.

**5'- and 3'-RACE**

The 5'-RACE was performed using the Kit, according to the manufacturer's instructions. 5'-RACE was preformed both with RNA from wild type *V. cholerae* using a *tfoY*-specific downstream primer, and RNA from *V. cholerae* cells carrying *tfoY* promoter reporter plasmids using a GFP-specific downstream primer in order to better isolate and identify the start sites of each individual promoter. The specific sequences of the primers used during amplification are listed in the Appendix.

The 3'-RACE method was adapted from Argaman et al., 2001. A DNA oligonucleotide adapter was synthesized with a monophosphate at the 5'-end and an inverted thymidine base at the 3'-end (IDT). 500 pmol of adapter was ligated to 10 µg *V. cholerae* RNA at 37°C with 20 units of T4 RNA ligase (NEB) in a 20 µL reaction containing 1X T4 RNA ligase buffer, 1mM ATP, 10% DMSO, and 20 units RNasin (Promega). Ligation products were reverse transcribed into cDNA and amplified with the Access RT-PCR Kit (Promega) using a primer complementary to the 3' adapter and a primer specific for the 5'-end sequence of transcripts initiated at the $P_{1\text{-}tfoY}$ promoter. The products generated were electrophoresed on an agarose gel, and DNA was excised and purified from all size ranges and recovered by TOPO TA cloning into pCR2.1 (Invitrogen).

**Northern Blotting**

RNA was extracted from mid-log phase cultures using TRIzol Reagent (Invitrogen). Samples were normalized to the same concentration using spectrophotometric measurement and evaluated for ribosomal RNA integrity using a Bioanalyzer (Agilent). RNA was separated by PAGE, transferred by semi-dry blotting onto a positively-charged nylon membrane, and baked in a vacuum oven. The membrane was hybridized with probe at 65°C for at least 4 hours in ULTRAhyb buffer (Ambion) and washed three times with 0.1x SSC at 65°C. The probe was detected by chemiluminescence using the Phototope-Star Detection Kit (NEB) and autoradiographic film. Film images were digitized by scanning at high resolution with a Typhoon FLA 9500 Imager (GE Healthcare), and quantitative measurements were using the image analysis software Fiji (Schindelin et al., 2012).

In all, four RNA probes named probe $P_1$, probe $P_2$, probe $P_{1+2}$, and probe Vc2 were used, which are complementary to the genomic regions -337 to -272, -272 to -191, -337 to -191, and -211 to -94, respectively, relative to the coding sequence of *tfoY*. Probes were labeled with Bio-11-UTP during *in vitro* transcription with the MAXIscript T7 Kit (Ambion) from PCR-derived DNA templates containing a consensus T7 promoter fused to the appropriate sequence.

**Primer Extension**

RNA was extracted from mid-log phase cultures using TRIzol Reagent (Invitrogen) and DNAse treated. Labeled ssDNA was produced from RNA samples using a 5'-biotinylated primer originating from position +120 of the *tfoY* coding sequence (Table A-3), and Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The ssDNA was electrophoresed, blotted, detected, and imaged using the same general methods described above for RNA samples.

***in vitro* Transcription**

DNA templates for in vitro transcription were generated by PCR from the vectors used for construction of *V. cholerae* genomic riboswitch mutants. Templates encompassed the genomic region from -535 nt to +120 nt relative to the *tfoY* coding sequence. The template for the *C. difficile* class-II riboswitch encompassed the region from -761 nt to -505 nt relative to the coding sequence of the gene *CD3267* of *C. difficile* 630, the same region investigated by Lee et al. 2010. Transcription reactions included 150 ng of DNA template, 2 mM DTT, 0.25mM NTPs, 0.1mM c-di-GMP when required, and 0.3 units of $\sigma^{70}$-saturated *E. coli* RNA polymerase

holoenzyme (Epicentre) in the transcription buffer recommended by the manufacturer. Bio-11-UTP (Ambion) was used in reactions at a concentration of 20% of total UTP to label transcripts after it had been determined not to interfere with the generation of transcription products (data not shown).

**RNA Stability Assay**

200 ml cultures were grown shaking in baffled flasks after being started with a 1:10,000 dilution of overnight culture into fresh media. When the optical density reached ~0.400-0.500, 10 mL of culture was withdrawn for a time zero reading and rifampicin was added to the culture for a final concentration of 250 μg/mL. Ten more mL of culture was removed at each subsequent time point, stabilized with 1 mL of RNA stop solution (10% phenol in 95% ethanol), and placed on ice, as described by Bernstein et al., 2002. RNA was promptly extracted from the cells using Trizol Reagent (Invitrogen), according to the manufacturer's instructions. RNA samples were normalized, electrophoresed, blotted, and detected as described above. For quantitative analysis, a series of film exposures over multiple lengths of time were collected, and once digitized, images were only compared between images for which the film exposure was not saturated.

# CHAPTER 4

# The *Vibrio cholerae* Vc2 Cyclic di-GMP Riboswitch Negatively Regulates Motility Through Repression of *tfoY*

**SUMMARY**

The second messenger molecule 3', 5'-cyclic diguanylic acid (c-di-GMP) induces biofilm formation and inhibits motility in the majority of bacteria. The *V. cholerae* c-di-GMP signal transduction network is complex encoding a myriad of c-di-GMP synthesis/degradation enzymes and three c-di-GMP binding transcription factors. *V. cholerae* also encodes two c-di-GMP binding riboswitch elements. Here we show that the Vc2 riboswitch functions as an off-switch by inhibiting expression of the downstream gene *tfoY* in response to c-di-GMP binding. We find that TfoY promotes dispersive motility in *V. cholerae* at low concentration of c-di-GMP. Epistasis analysis indicates that both Vc2 regulation of *tfoY* expression and c-di-GMP control of the flagellar biosynthesis master transcription regulator, FlrA, are necessary to prevent motility induction of *V. cholerae* when c-di-GMP is high. We further show that TfoY can induce expression of motility both through *flaA,* the major flagellin subunit of *V. cholerae,* and the *flaEDB* alternate flagellins, which were previously unknown to be sufficient for motility. Our results place Vc2 and TfoY as key regulators of motility in *V. cholerae* in response to changing concentrations of c-di-GMP.

## INTRODUCTION

On a cellular level, bacteria translate information about external stimuli from their environment into internal signals that can serve to reprogram metabolic functions and behaviors. Second messengers are fundamental intracellular signals by which bacteria sense and respond to their environment. 3', 5'-cyclic diguanylic acid (c-di-GMP) is one such second messenger that is nearly ubiquitous among bacterial species and has been implicated in the control of virulence, biofilm formation, motility, cell cycle progression, and numerous other cellular programs (Romling *et al.*, 2013). Whereas other nucleotide-based second messenger systems utilize only one or a few enzymes to synthesize and degrade the signal, the c-di-GMP system is highly complex. For example, the genome of *Vibrio cholerae* encodes 62 proteins involved in c-di-GMP turnover (Galperin *et al.*, 2010).

Despite significant progress in the field of identifying new c-di-GMP associated phenotypes, the regulatory mechanisms responsible for the transduction of the signal itself have not been fully elucidated. Many of the protein effectors of c-di-GMP that have been identified to date are species specific, and none of these factors are as ubiquitous among bacterial phyla as the molecule itself (Romling *et al.*, 2013). In *V. cholerae,* three c-di-GMP responsive transcription factors, VpsT, VpsR, and FlrA, have been identified, but their collective activity does not account for all of the changes in gene expression that are associated with c-di-GMP regulation (Krasteva *et al.*, 2010, Srivastava *et al.*, 2011, Srivastava *et al.*, 2013). The discovery of two classes of c-di-GMP binding riboswitches revealed a new category of regulatory factors that could respond to this molecule, and these riboswitches are hypothesized

to be important missing links in the c-di-GMP signaling pathways of many bacteria (Sudarsan *et al.*, 2008, Lee *et al.*, 2010).

The Vc2 riboswitch of *V. cholerae* is the most thoroughly examined class-I c-di-GMP riboswitch aptamer, having been the focus of numerous *in vitro* biochemical and structural studies (Kulshina *et al.*, 2009, Smith *et al.*, 2009, Smith *et al.*, 2010, Fujita *et al.*, 2012, Wood *et al.*, 2012, Inuzuka *et al.*, 2016). However, surprisingly, the molecular mechanism by which this riboswitch controls gene expression has never been fully demonstrated. Vc2 was originally defined as a genetic on-switch, but recent evidence indicates that those characterizations of Vc2 function were incorrect (Sudarsan *et al.*, 2008, Inuzuka et al., 2016). The physiological role of the Vc2 riboswitch in *V. cholerae* c-di-GMP regulation remains undefined because no study to date has investigated its behavior in the native context of a *V. cholerae* cell. The Vc2 riboswitch is encoded upstream of the gene *tfoY*, a putative transcription factor predicted to be involved in the regulation of genetic competence in *Vibrio spp.,* but recently described as contributing to regulation of Type IV secretion (Pollack-Berti *et al.*, 2010, Metzger *et al.*, 2016). Multiple reports have speculated that the proximity of the Vc2 riboswitch to *tfoY* indicates a role for the Vc2 riboswitch in the control of biofilm formation and motility in *V. cholerae*, but as of yet no data supports this speculation (Sudarsan et al., 2008, Shanahan & Strobel, 2012).

Many questions still remain about the raw mechanics of bacterial motility in *V. cholerae*. The *V. cholerae* chromosome encodes five flagellins, *flaABCDE*, which are all simultaneously expressed in wild-type cells (Klose and Mekalanos, 1998a; Klose and Mekalanos, 1998b). The FlaABCD flagellins have each been found to be incorporated into wild-type flagella, however, loss of a single flagellin gene, *flaA*, renders *V. cholerae* cells completely aflagellated

(Xicohtencatl-Cortés et al., 2006; Yoon and Mekalanos, 1998). Moreover, the alternative flagellin genes *flaBCDE* are not required for expression of a functional flagellum, as bacteria containing only *flaA* can still be flagellated and perform as well as the wild-type strain in a motility assay (Klose and Mekalanos, 1998a). Importantly, no study to date has described a motility mechanism for which the alternative flagellins can participate independently of *flaA*.

In this study, we show that *tfoY* expression is induced upon decreasing c-di-GMP in *V. cholerae*, and this induction requires a functional c-di-GMP binding site within the Vc2 riboswitch. Structural mutations to Vc2 also increase expression of *tfoY*, indicating that this riboswitch functions as an off-switch in its native context. We find that TfoY induces dispersive motility of *V. cholerae* in both a *flaA*-dependent and *flaA*-independent fashion. Finally, our results indicate that induction of motility at low c-di-GMP occurs through multiple regulatory pathways.

## RESULTS

### *tfoY* expression is induced at low intracellular concentrations of c-di-GMP in a Vc2 riboswitch-dependent manner

Recent studies have reported at least three potential transcriptional start sites for *tfoY* transcripts but have not evaluated the promoter activity at these sites or their relative contributions to *tfoY* expression (Papenfort et al., 2015, Inuzuka et al., 2016). We also performed a 5'-RACE analysis in this region and identified a fourth transcriptional start site. Two of these sites are located upstream of the Vc2 riboswitch ($P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$), one is located

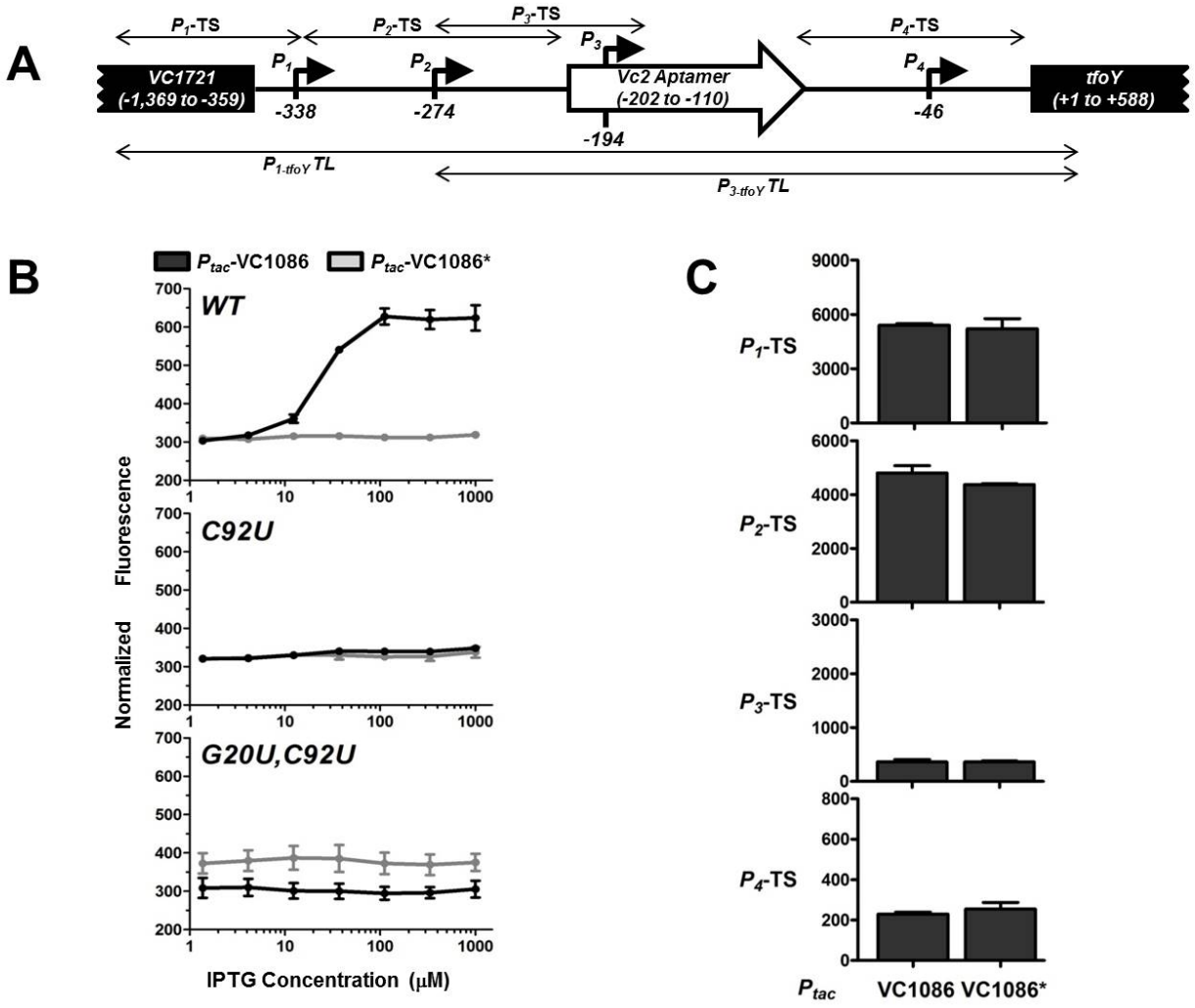



**Figure 4-1: Regulation of *tfoY* expression at low c-di-GMP is controlled by the Vc2 riboswitch**

**(A)** Four transcriptional start sites were identified upstream of the *tfoY* gene as indicated by the arrows. The regions of DNA used for the transcriptional fusions of each promoter to *gfp* are labeled by the bidirectional arrows above the map while the region used for the *tfoY* translational fusion to *gfp* is indicated by the bidirectional arrow below the map. (**B)** Expression of the $P_{1}$-*tfoY* translation fusion and mutations in the Vc2 riboswitch are shown as fluorescence divided by $OD_{595}$ in response to decreasing concentrations of c-di-GMP ($P_{tac}$-VC1086) or the active site mutant control ($P_{tac}$-VC1086). (**C)** The response of the Vc2 promoter transcriptional fusions to induction of VC1086 or VC1086* is indicated. Error bars indicate the standard deviation.

within the Vc2 aptamer domain ($P_{3\text{-}tfoY}$), and one is located downstream of Vc2 ($P_{4\text{-}tfoY}$) (Fig. 4-1A). We constructed transcriptional fusions of these four promoter regions to *gfp*, indicated in Fig. 4-1A, and we measured significant *gfp* expression above a vector control, confirming these are legitimate transcription start sites (Fig. 4-1C and data not shown).

To evaluate if the Vc2 riboswitch responds to changes in c-di-GMP, we constructed a *tfoY* translational reporter fusion to *gfp* that encodes all four *tfoY* promoters, $P_{1\text{-}tfoY}$TL, and examined this reporter in cells that contained either a native *V. cholerae* phosphodiesterase (PDE), VC1086, or its enzymatically inactive mutant, VC1086* (EAL mutated to AAL) expressed from a plasmid (Waters *et al.*, 2008) . Decreasing the c-di-GMP level in *V. cholerae* with VC1086 resulted in a dose-dependent increase in fluorescence from the translational reporter, reaching roughly a 2-fold change in expression at its maximum (Fig. 4-1B). To determine if this increase in expression of the reporter was elicited by the Vc2 riboswitch, we introduced the mutations G20U and C92U, which disrupt its ability of the riboswitch to bind c-di-GMP, into the riboswitch aptamer domain in $P_{1\text{-}tfoY}$TL (Smith et al., 2009). The VC1086-mediated increase in downstream gene expression was lost in both of these mutant backgrounds, indicating that a functional riboswitch aptamer domain is required for induction of *tfoY* at low concentrations of c-di-GMP.

To further confirm that the induction of *tfoY* at low c-di-GMP was not caused by differential regulation of any of the *tfoY* promoters, we measured the activity of *gfp* transcriptional fusions to the four *tfoY* promoters during WT and low c-di-GMP conditions (Fig. 4-1C). None of the transcriptional reporters showed any significant change in activity between conditions of VC1086 and VC1086* overexpression, confirming that the primary mechanism for regulation of *tfoY* at low c-di-GMP is the Vc2 riboswitch.

**Mutations to the Vc2 aptamer sequence have different effects on downstream gene expression**

Our results thus far suggest that the Vc2 riboswitch functions as an off-switch because at wild type levels of c-di-GMP, when the ligand concentration is favorable to the c-di-GMP bound state of the Vc2 aptamer, downstream gene expression is inhibited. Also, the G20U, C92U mutations that abolish c-di-GMP binding prevent induction of the downstream gene as the c-di-GMP concentration is lowered. Surprisingly, however, the basal expression of *tfoY* is the same whether the Vc2 riboswitch is intact or has these binding site mutations. If Vc2 functions as a canonical off-switch, we would expect disruptive mutations at the ligand binding site to induce downstream gene expression. We suspected that the specific binding site mutations that we employed might actually be locking the Vc2 riboswitch structure into an off-state that does not require ligand binding to reduce downstream gene expression, which is analogous to recently reported results for Vc1, the other c-di-GMP-dependent riboswitch in *V. cholerae* (Kariisa *et al.*, 2016).

It has been noted that the nucleotides which directly pair with the c-di-GMP ligand are actually not the most phylogenetically well-conserved nucleotides of the consensus class-I c-di-GMP riboswitch aptamer structure (Smith et al., 2009). Instead, sites such as A47 and the C44-G83 pair, which are crucial for stabilizing the three-dimensional arrangement of the aptamer domain around the ligand, hold the distinction of being the most highly conserved residues (see Fig. 4-2B) (Smith et al., 2010) . Additionally, the Vc2 aptamer has been described as exhibiting a

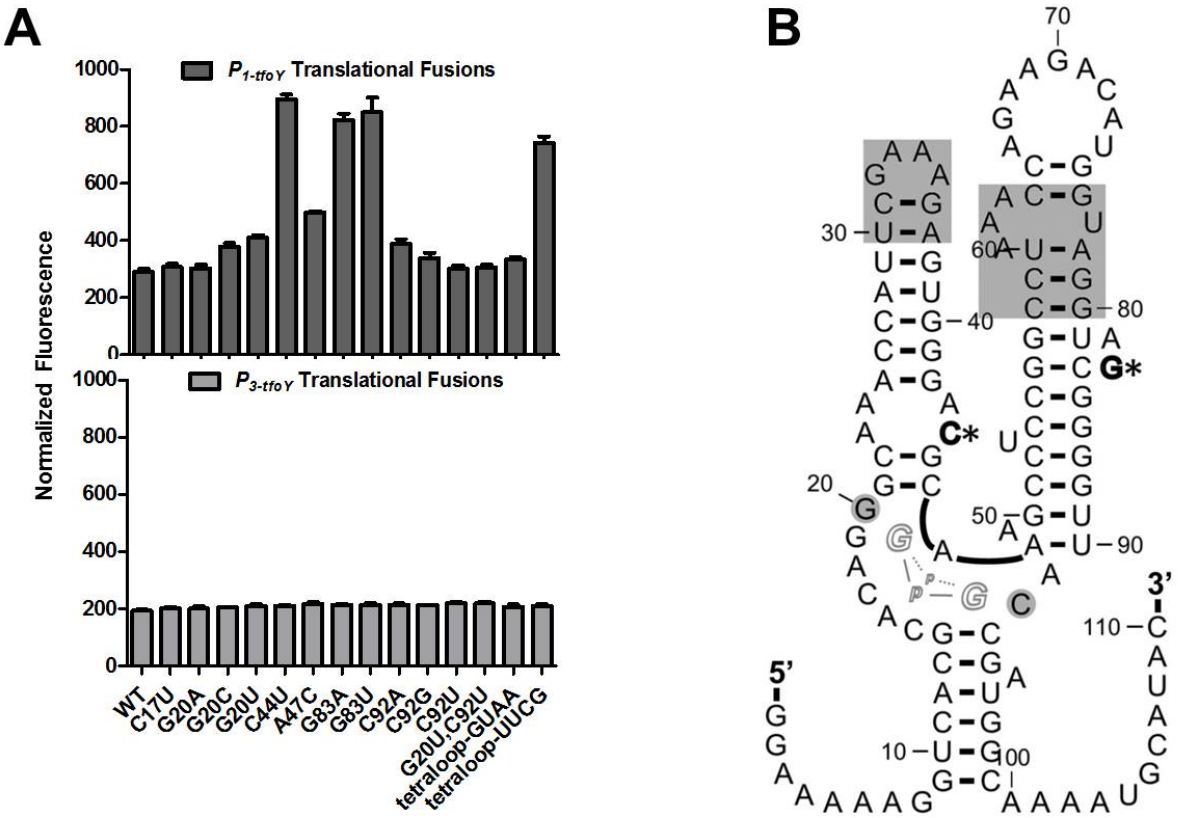



**Figure 4-2: Structural mutations to Vc2 increase downstream gene expression**

**(A)** A number of mutations were constructed in *tfoY* translational fusions to *gfp* encoding the entire upstream region ($P_{1\text{-}tfoY}$, top) and just the $P_3$ and $P_4$ promoters ($P_{3\text{-}tfoY}$, bottom), and the fluorescence per $OD_{595}$ is indicated at unaltered levels of c-di-GMP. Error bars indicate the standard deviation. (**B)** This secondary structure depiction of the 110-nt Vc2 riboswitch aptamer is based on previously published biochemical work (Smith et al., 2010, Smith et al. 2011, Wood et al. 2012). Gray circles indicate the bases which form nucleotide pairs with the c-di-GMP ligand, which is indicated by gray letters. Asterisks indicate the bases which form a pseudoknot pair, and the tetraloop and tetraloop receptor regions are indicated by gray boxes. This figure was generated in part using the software program R2R (Weinberg and Breaker, 2011)

high degree of pre-organization *in vitro*, such that structural elements, specifically the C44-G83 base pair and the tetraloop-receptor interaction, may still form even when c-di-GMP is not available (Wood et al., 2012). Therefore, we hypothesized that nucleotide mutations which directly prevent tertiary structure formation, such as G83U, should have a greater effect on

downstream gene expression than nucleotide mutations which primarily affect ligand recognition, such as G20U and C92U. To further explore this hypothesis, we introduced mutations into the $P_{1\text{-}tfoY}$TL reporter at bases in the Vc2 riboswitch sequence which are considered the most important for structural integrity of the aptamer domain. Specifically, we evaluated the impact of mutations around the ligand binding pocket at C17 and A47 which stabilize the position of the ligand, mutations to the C44-G83 base pairing which stabilizes the interaction between the P2 and P3 loops of the aptamer, and mutation of the tetraloop of the P2 stem, which associates with its receptor in the P3 stem (Smith et al., 2009, Smith et al., 2010, Smith *et al.*, 2011, Fujita et al., 2012). Consistent with our expectations, mutations which should fully disorganize the aptamer, such as C44U, G83U, and the whole tetraloop mutation to UUCG, caused the greatest change in the expression of $P_{1\text{-}tfoY}$TL (Fig. 4-2A). Conversely, mutating the G20 and C92 bases to additional nucleotides had a minimal effect on expression (Fig. 4-2A). Overall, we saw that increased disruption of the riboswitch structure leads to increased downstream gene expression, supporting the categorization of Vc2 as an off-switch.

The arrangement of the four independent *tfoY* promoters relative to the Vc2 aptamer indicates that only the two most upstream promoters, $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$, would be able to produce transcripts which include the full sequence of the Vc2 domain. This implies that transcripts from the $P_{3\text{-}tfoY}$ promoter would not contain a functional riboswitch. We predicted that if the Vc2-disruptive mutations were included in $P_{3\text{-}tfoY}$ transcripts, they would have no impact on downstream gene expression. To test this, we constructed another *gfp* translational reporter fusion for *tfoY*, $P_{3\text{-}tfoY}$TL, which excluded the $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ promoters from the reporter sequence (Fig. 4-1A). As expected, none of the Vc2 mutations impacted expression

when encoded into $P_{3\text{-}tfoY}$TL, confirming that only the $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ promoters can transcribe a functional riboswitch (Fig. 4-2A).

**_tfoY_ and the Vc2 riboswitch are necessary for _V. cholerae_ motility induction at low c-di-GMP**

One of the most prominent bacterial phenotypes regulated by c-di-GMP is motility. *V. cholerae* has a single polar flagellum which allows it to engage in swimming motility, and this behavior is inhibited by high intracellular levels of c-di-GMP (Beyhan *et al.*, 2006). We hypothesized that because the Vc2 riboswitch turns on *tfoY* expression at low c-di-GMP, *tfoY* might induce motility. A recent publication independently determined that *tfoY* can modulate motility and Type VI secretion in *V. cholerae* although the contribution of Vc2 to *tfoY* regulation was not addressed (Metzger *et al.*, 2016). Moreover, these authors concluded that c-di-GMP regulation of motility is not dependent on *tfoY,* which directly contrasts our results described below.

Motility can be measured by assaying the ability of the bacteria to swim through a semi-solid, low-percentage agar medium. For all the motility assays described in this study, we used a modified LB medium in which the concentration of nutrient components, both the tryptone and yeast extract, are reduced 10-fold while the concentration of sodium chloride is unchanged. With this media, we observed a biphasic pattern of motility that was not as readily seen when using standard LB (Fig. 4-3). In the first phase, which we term the “dense phase”, the bacterial colony exhibits dense growth and limited movement away from the site of inoculation. During the second phase, which we term the “dispersive phase”, the colony expands outward at a

rapid rate, but grows less densely in the new areas as it expands. In our assay, *V. cholerae* shifts from the dense to dispersive phase at about 13 hours after inoculation.

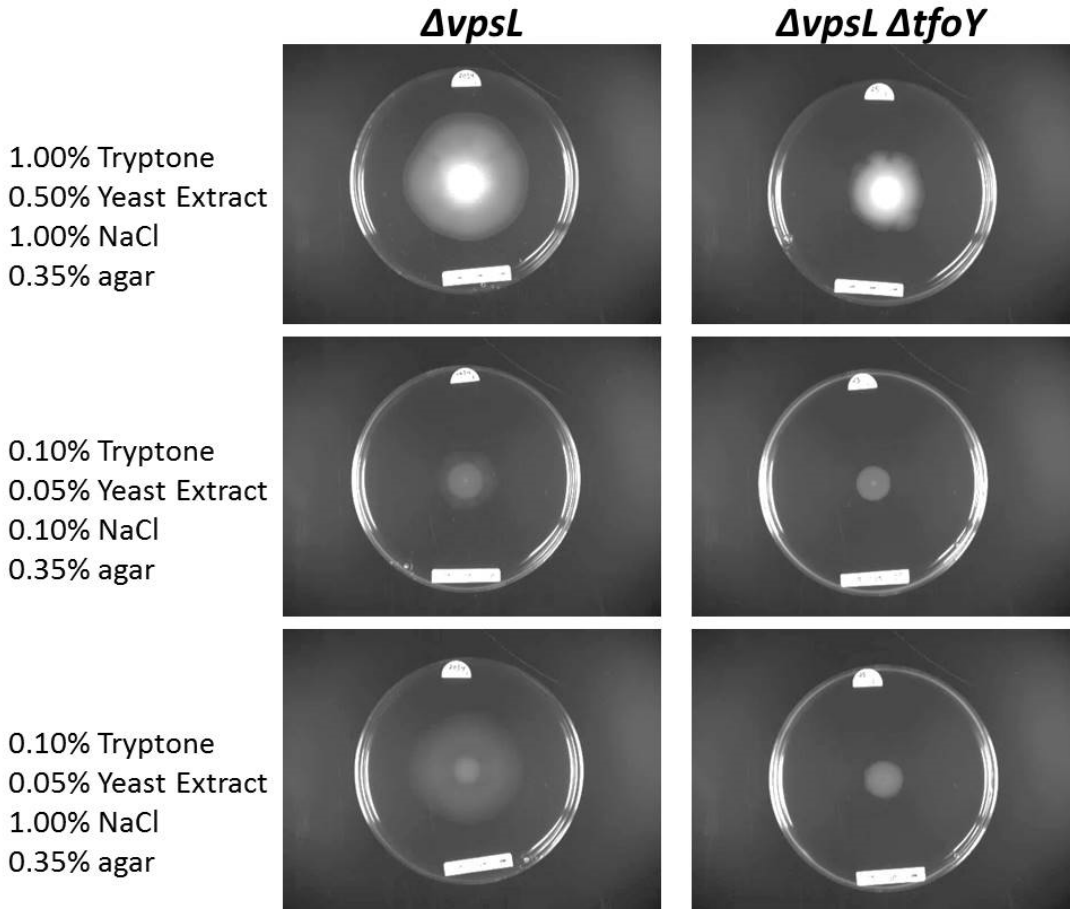



**Figure 4-3: Low nutrient agar amplifies *tfoY*-dependent bi-phasic motility of *V. cholerae***

Motility of *ΔvpsL* and *ΔvpsL ΔtfoY V. cholerae* in the indicated nutrient conditions is shown. Motility is dependent on NaCl due to the sodium driven flagellum of *V. cholerae,* but the impact of *tfoY* on dispersive motility is most evident at 10% tryptone and yeast extract concentrations. The final concentrations of nutrient components are indicated as weight per volume.

To test the impact of the riboswitch and *tfoY* on motility, we measured motility through semi-solid agar of three strains: *ΔvpsL*, *ΔvpsL ΔtfoY*, and *ΔvpsL Vc2(G20U,C92U)*. Mutation of *vpsL* prevents synthesis of the Vibrio Polysaccharide (VPS) required for biofilm formation in *V.*

*cholerae.* We used this genetic background because previous results indicate the production of VPS can negatively influence motility and we wanted to exclude this factor from these experiments (Srivastava et al., 2013). We assessed motility at two different time points, 11 hours and 25 hours, which are representative of the two distinct phases of motility (Fig. 4-4A). At 11 hours, all three strains had colonized the media extending from the site of inoculation, and there was no discernable difference between the motility of these strains (data not shown).

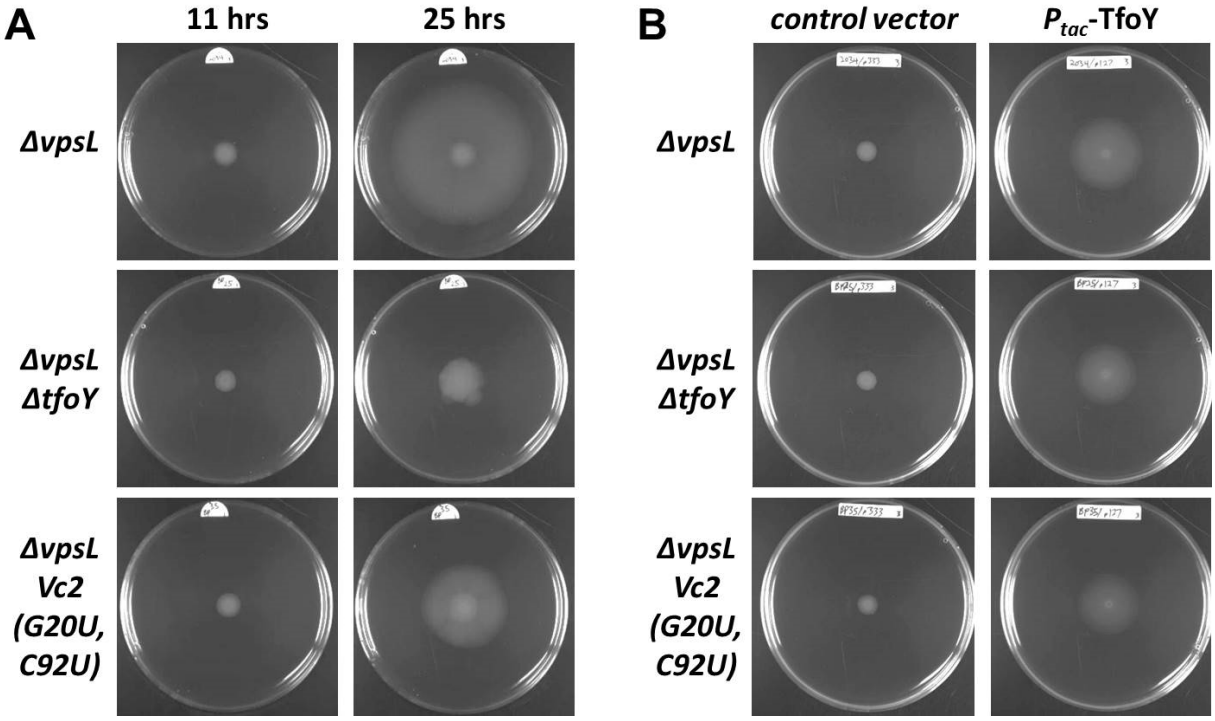



**Figure 4-4: TfoY induces *V. cholerae* swimming motility**

Representative motility assays of *V. cholerae* in reduced nutrient LB plates at 0.35% agar with regard to **(A)** *tfoY* and Vc2 riboswitch mutations at 11 and 25 hours and **(B)** TfoY overexpression in these mutations at 10 hours.

At 25 hours, the *ΔvpsL ΔtfoY* strain exhibited a significantly reduced area of motility, with the colony covering much less area on the plate than the *ΔvpsL* strain. Additionally, the *ΔvpsL ΔtfoY*

colony appeared homogenously dense, exhibiting a dense phase beyond the normal radius of that observed in the *ΔvpsL* strain (Fig. 4-4A). This indicates that the *ΔvpsL ΔtfoY* strain is unable to properly transition to the dispersive phase. It is also notable that the edge of the *ΔvpsL ΔtfoY* colony does not appear uniformly round. At multiple sites, areas of less-dense, faster-moving bacteria can be seen sectoring away from the rest of the colony. The incidence of these sectors increases with incubation time, and we hypothesize that they contain suppressor mutants which have recovered the ability to engage the dispersive phase (data not shown). The riboswitch mutant strain *ΔvpsL Vc2(G20U,C92U)* also displayed a reduced capacity for motility, but the result was intermediate between the phenotypes of the *ΔvpsL ΔtfoY* strain and the *ΔvpsL* control (Fig. 4-4A). This is consistent with the fact that the G20U, C92U variant of the Vc2 riboswitch locks the expression of *tfoY* at a low level that cannot be induced, but at a level of expression that is significantly greater than the *tfoY* null mutant.

**TfoY overexpression disrupts the timing of motility induction**

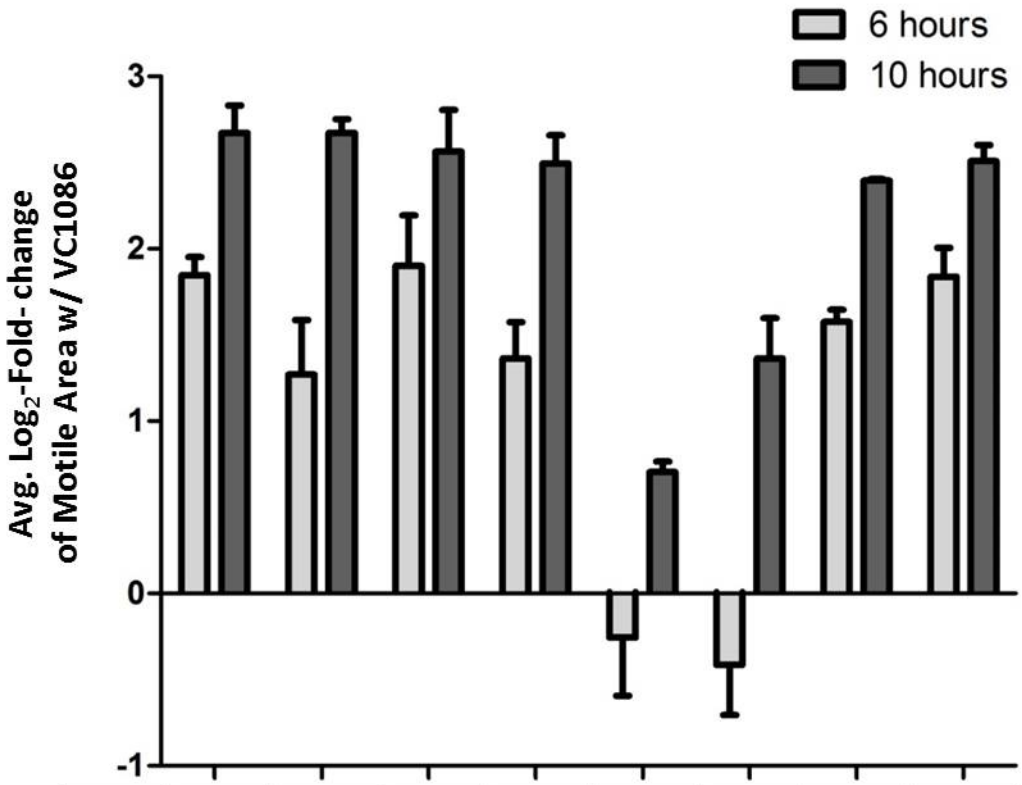
To confirm that the motility defects of the *ΔvpsL ΔtfoY* and *ΔvpsL Vc2(G20U,C92U)* strains resulted from a disruption of *tfoY* expression, we tested whether expression of TfoY protein from a plasmid was sufficient to recover the normal motility behavior. We transformed the strains with a plasmid containing TfoY under the control of the IPTG-inducible $P_{tac}$ promoter and evaluated their motility in semi-solid agar containing IPTG. After 10 hours, strains carrying an empty control vector displayed the normal pattern of motility that is characteristic of the dense phase. The strains carrying the TfoY overexpression vector, however, had colonized a much larger area of the plate within the same amount of time (Fig. 4-4B). For these strains, the

ring of dense phase growth was smaller than normal, and appeared to be the same diameter as the initial droplet of culture used to inoculate the plates, indicating that the bacteria had switched to dispersive phase growth much sooner after contact with the media. The *ΔvpsL*, *ΔvpsL ΔtfoY*, and *ΔvpsL Vc2(G20U,C92U)* strains all appeared similar in this regard, indicating that ectopic expression of TfoY was sufficient to overcome the motility defects of both mutant backgrounds.

**Induction of motility at low c-di-GMP is both *tfoY* and FlrA dependent**

We hypothesized that the two phases of motility we had been observing were indicative of two different c-di-GMP states within the bacteria. C-di-GMP levels in *V. cholerae* are, in part, controlled by its quorum sensing system, such that as a colony of bacteria ages and reaches a high cell-density state, the intracellular c-di-GMP level would be expected to decrease (Waters et al., 2008). To examine the relationship between *tfoY*, c-di-GMP, and the dense and dispersive phases of motile growth, we assayed the motility of strains carrying either the PDE VC1086 or the mutant control VC1086* expressed from a plasmid at both 6 and 10 hours (Fig. 4-5). The results are expressed as a fold-change of the motile area in strains expressing VC1086 versus VC1086*; thus a positive fold change is indicative of an induction of motility at low c-di-GMP levels.

At the 6 hour time point in the *ΔvpsL* background, we observed a 3.6-fold greater area of motility with VC1086 overexpression than with VC1086* overexpression, consistent with the differences in intracellular c-di-GMP concentration between those strains (Fig. 4-5) (Waters et al., 2008). For the *ΔvpsL ΔtfoY* mutant, the basal level of motility appeared reduced relative to




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *vpsL* | *Δ* | *Δ* | *Δ* | *Δ* | *Δ* | *Δ* | *Δ* | *Δ* |
| FlrA | WT | WT | WT | R176H | R176H | R176H | R176H | R176H |
| *tfoY* | WT | *Δ* | WT | WT | *Δ* | WT | *Δ* | WT |
| *Vc2* | WT | WT | G20U, C92U | WT | WT | G20U, C92U | WT | G20U, C92U |
| *lacZ* | WT | WT | WT | WT | WT | WT | *:tfoY* | *:tfoY* |

**Figure 4-5: *tfoY* and FlrA are required for induction of motility at low c-di-GMP**

Agar based motility in low nutrient agar was examined at 6 hours (light gray) or 10 hours (dark gray). The data are indicated as the fold change in motility of overexpression of the PDE VC1086 versus overexpression of its active site control VC01086* of the eight *V. cholerae* mutant strains indicated by the table under the graph. Error bars indicate the standard deviation.

*ΔvpsL*; however, overexpression of VC1086 significantly recovered this defect, increasing the motile area by 2.4-fold, implying that although the *ΔvpsL ΔtfoY* mutant has a lower underlying capacity for motility, VC1086 can still induce motility in this strain similar to the wild-type strain.

The *ΔvpsL Vc2 (G20U, C92U)* mutant also showed the same pattern - a slightly reduced capacity for motility, but a similar potential for induction of motility. These results indicate that the regulation of *tfoY* by the Vc2 riboswitch is not the lone c-di-GMP pathway determining the progression from the dense phase to the dispersive phase of motility, and other factors can compensate for the loss of, or decreased expression of, *tfoY*.

We hypothesized that *flrA*, the master transcriptional regulator of the flagellar biosynthesis genes in *V. cholerae*, would likely be the factor which maintains the inducibility of motility at low c-di-GMP and compensates for defects in *tfoY,* as we had previously demonstrated the ability of FlrA to directly bind c-di-GMP in a manner which prevents flagellar gene activation (Srivastava et al., 2013). That study also identified a mutant of FlrA, FlrA(R176H), which does not bind to c-di-GMP and instead promotes flagellar gene expression at a constitutive level even at high concentrations of c-di-GMP (Srivastava et al., 2013). We therefore tested VC1086 overexpression in a *ΔvpsL flrA(R176H)* mutant background because we expected motility to be uninducible as c-di-GMP decreased. Surprisingly, the *ΔvpsL flrA(R176H)* mutant showed a similar 2.5-fold induction of motility under VC1086 overexpression, mirroring the behavior of both the *ΔvpsL ΔtfoY* and *ΔvpsL Vc2 (G20U, C92U)* mutants (Fig. 4-5).

Since disruption of neither *tfoY* nor *flrA* alone could prevent the phosphodiesterase induction of motility, we questioned whether these genes might represent independent, redundant pathways for c-di-GMP regulation of motility. If that is the case, *V. cholerae* could compensate for the loss of c-di-GMP regulation of *tfoY* or *flrA* individually, but it should not be able to compensate for the loss of both. To test this, we constructed *ΔvpsL flrA(R176H) ΔtfoY* and *ΔvpsL flrA(R176H) Vc2(G20U, C92U)* triple mutant strains. Strikingly, overexpression of

VC1086 in these strains was unable to induce motility at 6 hours (Fig. 4-5). Restoration of *tfoY* by insertion of a wild type copy of *tfoY* containing the wild type Vc2 and $P_{1\text{-}4\text{-}tfoY}$ promoter elements at the *lacZ* locus of the *V. cholerae* chromosome was able to restore the induction of motility in both triple mutants (Fig. 4-5). These results show at that at this time point, the induction of motility seen upon decreased c-di-GMP is due to both induction of *tfoY* and inhibition of FlrA activity.

By 10 hours post-inoculation we observed a larger 6-fold induction of motility in the *ΔvpsL* and *ΔvpsL ΔtfoY* or *ΔvpsL Vc2 (G20U, C92U)* mutant strains, indicating these pathways were parallel and redundant at this time point. Surprisingly, the *ΔvpsL flrA(R176H) ΔtfoY* and *ΔvpsL flrA(R176H) Vc2(G20U, C92U)* triple mutant strains in which both *tfoY* induction and FlrA regulation by c-di-GMP were disrupted maintained a small but significant ~ 2-fold increase in motility in response to reduced c-di-GMP levels. The eventual recovery of motility in these strains was unexpected, and indicates that while *flrA* and *tfoY* might be the most dominant pathways for c-di-GMP regulation of motility in *V. cholerae*, at least one other unknown c-di-GMP-dependent pathway also exists, which may only be activated during a later stage of growth in a swimming assay. As seen at 6 hours, insertion of *tfoY* and the upstream Vc2 riboswitch fully recovered motility in the triple mutant strains.

**TfoY induction of motility does not require wild type flagella**

It is notable that neither the *ΔvpsL ΔtfoY* nor the *ΔvpsL flrA(R176H)* strains were effectively disrupted for c-di-GMP regulation of motility, and only when the mutations were combined did the bacteria become appreciably less responsive to PDE overexpression. This

suggested that the *flrA* and *tfoY* pathways function in parallel to control motility, but it is not clear if they utilize similar or different mechanisms. FlrA regulates motility by activating expression of flagellar biosynthesis genes, and because TfoY is a putative transcription factor, we tested whether it might impact expression of these genes as well (Prouty et al., 2001, Pollack-Berti et al., 2010). We selected representative genes from different classes of the flagellar gene expression hierarchy, and compared their fold-change in expression upon induction of FlrA or TfoY relative to a vector control. While FlrA overexpression strongly activated these flagellar biosynthesis genes, TfoY expression did not have a significant effect, with the only outlier being *flgM*, which was activated 3-fold and 1.7-fold by FlrA and TfoY respectively (Fig. 4-6).

Since TfoY did not have a significant effect on the expression of the flagellar genes we evaluated, we then examined whether the flagellar apparatus itself was important for the TfoY mediated induction of motility. The *V. cholerae* genome encodes five different flagellin subunit proteins separated into two loci, *flaAC* and *flaEDB*. Previous research has reported that only *flaA* is essential for swimming motility in a soft agar motility assay, and *V. cholerae* is aflagellated without it (Klose & Mekalanos, 1998a). We generated three different mutants by deleting each of the two flagellin loci individually, and also both together, and then the motility of these mutants was examined under the condition of TfoY overexpression (Fig. 4-7). There was no discernable difference in the motility behavior of the *ΔvpsL ΔflaEDB* mutant from the *ΔvpsL* control (Fig. 4-2). In both of these strains, *V. cholerae* was highly motile and TfoY induced the expected pattern of dispersive motility seen earlier. This result suggests that the alternate *flaEDB* flagellin locus is not required for TfoY induction of dispersive motility at this time point.

Similar to previously published results, both the *ΔvpsL ΔflaAC* and *ΔvpsL ΔflaABCDE* strains exhibited no significant movement from the site of inoculation at a 13-hour time point, reconfirming that *flaA* is the dominant driver of motility in this condition.

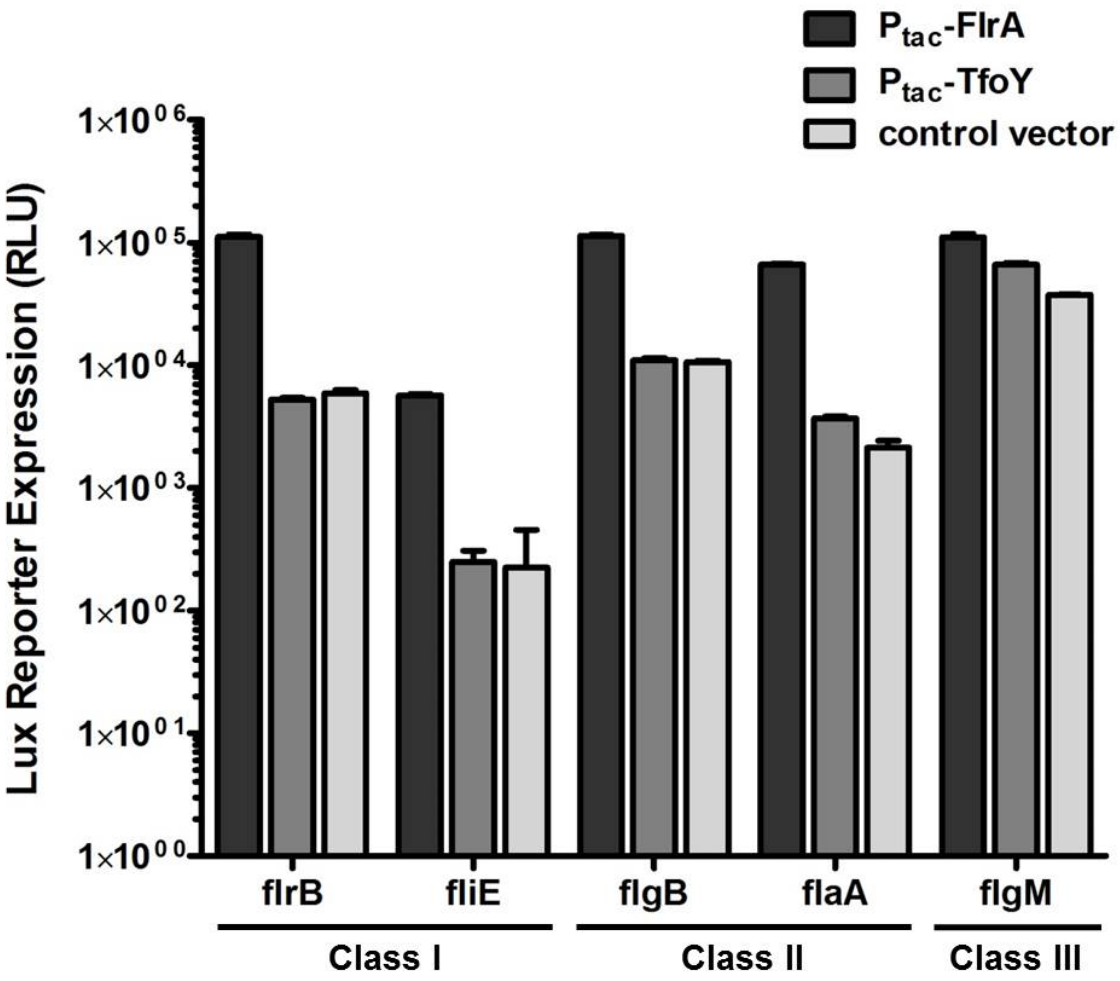



**Figure 4-6: TfoY does not induce expression of the flagellar biosynthesis genes**

The expression of transcriptional fusions of the indicated flagellar biosynthesis genes to *lux* as previously described (Srivastava et al., 2013) was examined upon overexpression of FlrA or TfoY relative to the empty control vector. Data are expressed as bioluminescence normalized to $OD_{595}$, and error bars represent the standard deviation. Flagellar gene class designations are as previously described (Prouty et al., 2001).

Because our previous results had demonstrated time dependent effects on motility, we hypothesized that a phenotype for the *flaEDB* flagellins might only be observable at later time

point and therefore extended the incubation time of the plates in excess of 1.5 days. At this later time point, we observed a significant difference in the ability of the flagellin knockout strains to travel through the agar. The *ΔvpsL ΔflaEDB* strain, which had already demonstrated a

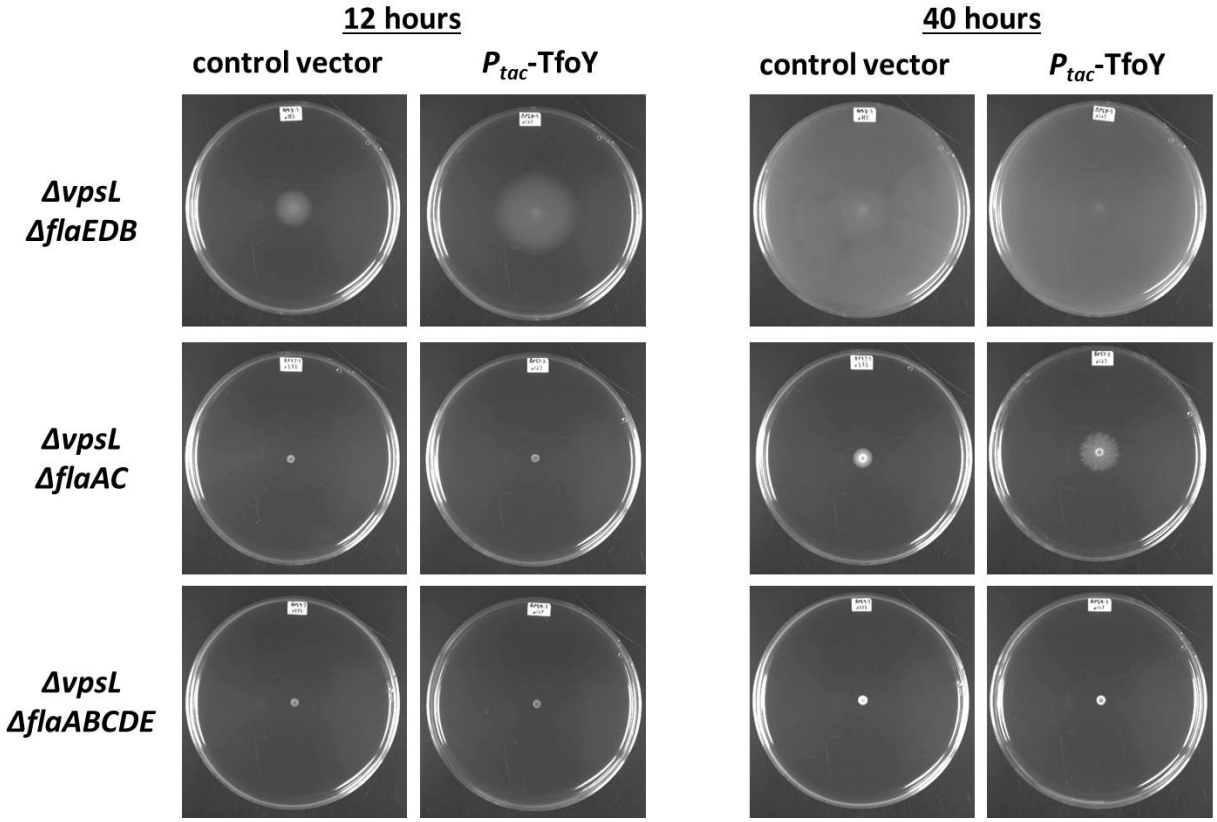



**Figure 4-7: TfoY can induce both *flaAC*- and *flaEDB*-dependent motility in *V. cholerae***

Motility of the three mutant strains indicated containing the control vector of the *tfoY* overexpression plasmid plated on standard motility agar with IPTG at 12 or 40 hours is shown.

level of motility comparable to the wild type, had easily colonized the entire plate before reaching the second time point. However, at this time point, the *ΔvpsL ΔflaAC* strain, which had appeared to be non-motile at 13 hours, now clearly exhibited a capacity for motility that was further induced by TfoY expression. Strikingly, the pattern of movement for the TfoY-induced colony was unlike that of either the control vector or the normal swimming behavior of *V.*

*cholerae*. Instead of moving diffusely through the media, the colony radiated outward along discrete, narrow paths and did not densely colonize the space in the media between these paths (see also Fig.4-8). In contrast, the complete flagellin knockout *ΔvpsL ΔflaABCDE* had extended only slightly beyond the site of inoculation. The extremely limited movement is likely the passive result of continued cell division and not the result of an active mechanism of locomotion, and we conclude that the *ΔflaABCDE* complete flagellin mutant of *V. cholerae* is

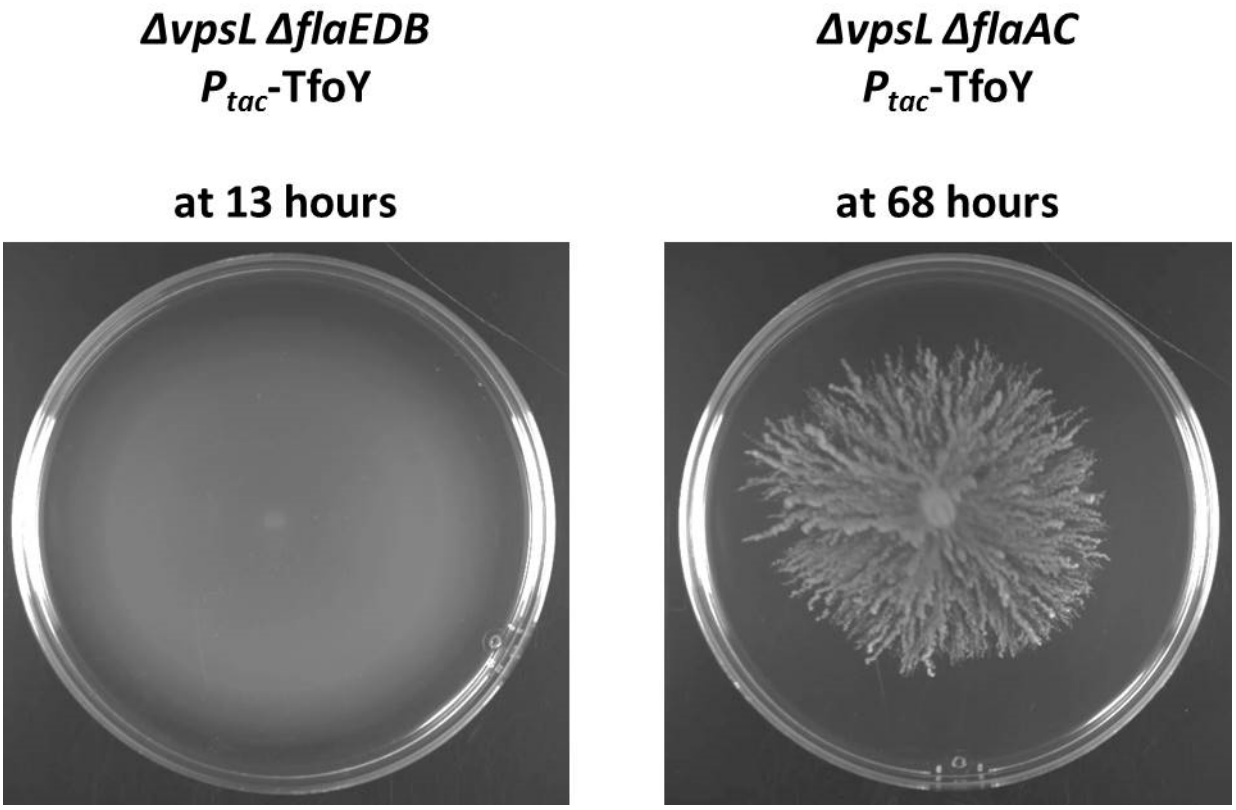



**Figure 4-8: Differences in colony morphology between *flaAC*- and *flaEDB*-dependent motility**

Morphology of strains overexpressing TfoY in motility media with 0.25% agar are presented for visual comparison at time points by which they have reached similar relative distances within the media.

truly non-motile in semi-solid agar. Also, overexpression of TfoY in this strain had no impact on motility. Our results show that *V. cholerae* encoding *flaAC* is capable of normal swimming,

whereas strains lacking *flaAC* but possessing an intact *flaEDB* locus are capable of an alternate form of motility. Both of these types of motility are positively impacted by TfoY.

Given the unexpected finding that the *ΔvpsL ΔflaAC* is capable of an alternate motility in a TfoY-inducible manner, we sought to determine the requirement of the alternative flagellins in this process. Starting with the *ΔvpsL ΔflaABCDE* strain carrying a TfoY overexpression vector, we complemented each of the four alternative flagellins *flaBCDE* and assayed the motility of these stains on semi-solid agar plates at a late time point (Fig. 4-9). Individually, none of the

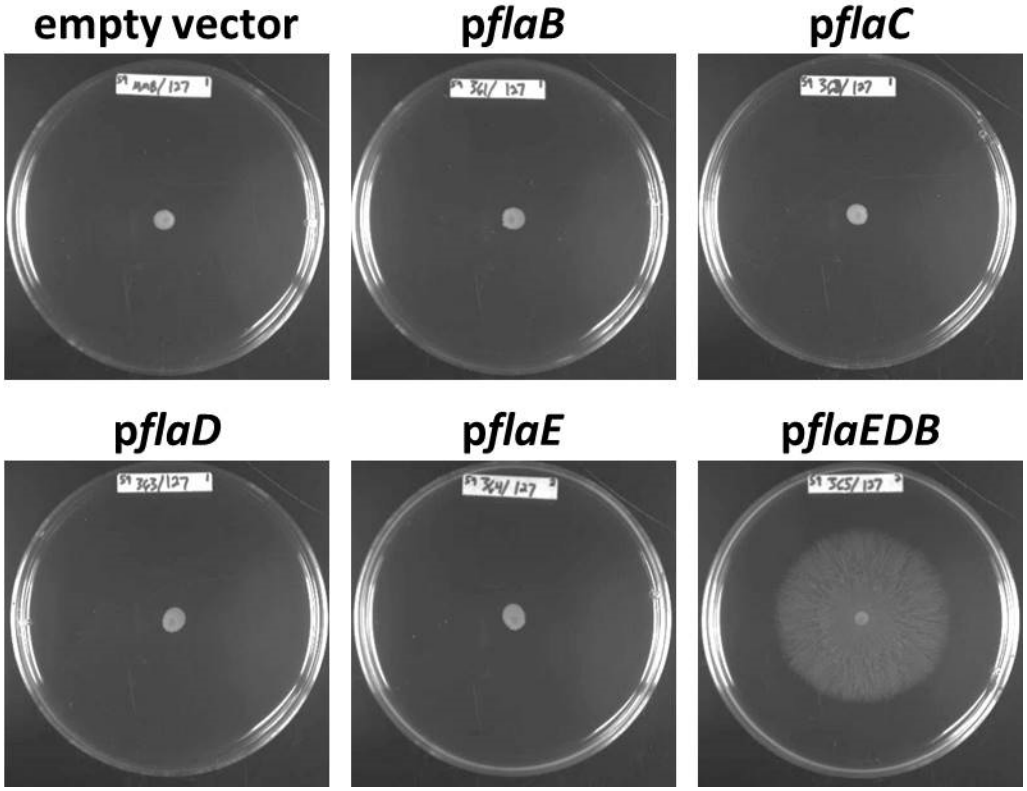



**Figure 4-9: Overproduction of *flaEDB* can complement TfoY-dependent late stage motility**

A Δ*vpsL* Δ*flaABCDE* double mutant containing the Ptac-TfoY overexpression plasmid grown on IPTG was complemented with the indicated *fla* genes expressed from a second plasmid. The 50 hour time point is shown.

alternative flagellins was able to restore motility. However, complementation of the entire *flaEDB* locus did restore this alternate motility, confirming the involvement of some combination of these flagellins in the TfoY-induced alternate motility of *V. cholerae*. We hypothesized that TfoY might induce transcription of these alternate flagellin genes; however, transcriptional fusions of these genes showed no response to TfoY expression (data not shown) indicating TfoY functions by a different mechanism.

## DISCUSSION

The Vc2 c-di-GMP riboswitch was discovered in 2008, but its role in the c-di-GMP signal transduction pathway of *V. cholerae* has not been described. Here we show that the Vc2 riboswitch is required for proper regulation of *tfoY* at low intracellular concentrations of c-di-GMP. This pattern of regulation, higher expression at lower concentrations of ligand, implies that the Vc2 riboswitch functions as on off-switch for the *tfoY* gene. This result contradicts previous experiments which have categorized the Vc2 riboswitch as an "on-switch" (Sudarsan et al., 2008, Fujita et al., 2012). Specifically, Sudarsan et al. constructed a *tfoY* translational reporter and showed that the introduction of mutations at sites within the riboswitch significantly decreased expression of their reporter. Fujita et al. also used this same reporter system and communicated similar results. In both cases, those groups reported the opposite of the results shown in Figure 4-2. Our explanation for this discrepancy is two-fold. First, the translational reporter used by Sudarsan et al. was constructed without knowledge of the promoter elements in the region identified here, and as a result, the sequence they chose excluded the $P_{1\text{-}tfoY}$ promoter. Second, their analysis was conducted in the heterologous

organism *E. coli*, not *V. cholerae*. Basal intracellular c-di-GMP concentrations are known to be different in these organisms, and given the putative involvement of a *V. cholerae*-specific transcription factor at the *tfoY* promoters, their experiments in *E. coli* do not reflect the native environment of the Vc2 riboswitch. Of note, a more recent *in vitro* mutational analysis of Vc2 also predicted that this riboswitch should function as an off-switch, consistent with our findings, however, that report also omitted the role of the $P_{1\text{-}tfoY}$ promoter in its analysis (Inuzuka et al., 2016).

Although the behavior of the wild-type Vc2 riboswitch in our expression assay mimics the function of an off-switch, the behavior of riboswitches with mutations in the c-di-GMP binding site in the same assay does not. If Vc2 was a canonical off-switch, mutations that disrupt c-di-GMP binding would lead to high expression. However, we observed the opposite; the C92U and G20U, C92U mutations locked the riboswitch in a state of low *tfoY* expression (Fig. 4-1). Our more comprehensive mutational analysis of the behavior of Vc2 shows that mutations which are disruptive to the structure of the Vc2 aptamer domain do result in higher *tfoY* expression, but mutations at the ligand binding sites do not (Fig. 4-2). Previous biochemical analysis of the Vc2 riboswitch found that the aptamer domain is highly pre-organized in the absence of c-di-GMP binding and does not undergo significant structural rearrangement upon ligand binding (Wood et al., 2012). From our data, it appears that only mutations which are disruptive to aptamer preorganization turn gene expression on *in vivo*. Therefore, in the context of *tfoY* mRNA, it could be hypothesized that the binding site mutants have a more stable pre-organized state and do not require ligand binding for stabilization. This finding is consistent with a recent study focusing on Vc1, a related c-di-GMP binding riboswitch that is

also encoded in *V. cholerae* genome. For Vc1, some of the mutations that blocked the ability of Vc1 to bind c-di-GMP mimicked the c-di-GMP bound state rather than the c-di-GMP unbound state (Kariisa et al., 2016).

Curiously, we determined that *tfoY* has four different promoters capable of driving its expression. Expression of *tfoY* from $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ mRNA would be dependent upon the Vc2 riboswitch, while expression from $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$ mRNA would be Vc2 independent. This leads us to hypothesize that there are additional important factors which control *tfoY* expression at the transcriptional level through these promoters, but is important to note that in the conditions tested here, mutation of the Vc2 riboswitch alone was sufficient to abolish expression and c-di-GMP regulation of *tfoY*.

In this work, we have expanded our understanding of the major pathways controlling motility by c-di-GMP in *V. cholerae* (Fig. 4-10). Strains with both *ΔvpsL* and FlrA(R176H) mutations, two components previously shown to connect c-di-GMP to motility, maintained a significant increase in motility during phosphodiesterase overexpression, on par with the induction of motility in strains with the *ΔvpsL* mutation alone. Only when c-di-GMP control of *tfoY* was additionally disrupted did the strains display an inability to properly engage motility in response to low c-di-GMP (Fig. 4-5). This dysregulation of motility was seen not just when *tfoY* was deleted, but also when single point mutations were made to inactivate the Vc2 riboswitch, demonstrating the necessity of Vc2 control of *tfoY* expression at low c-di-GMP for motility

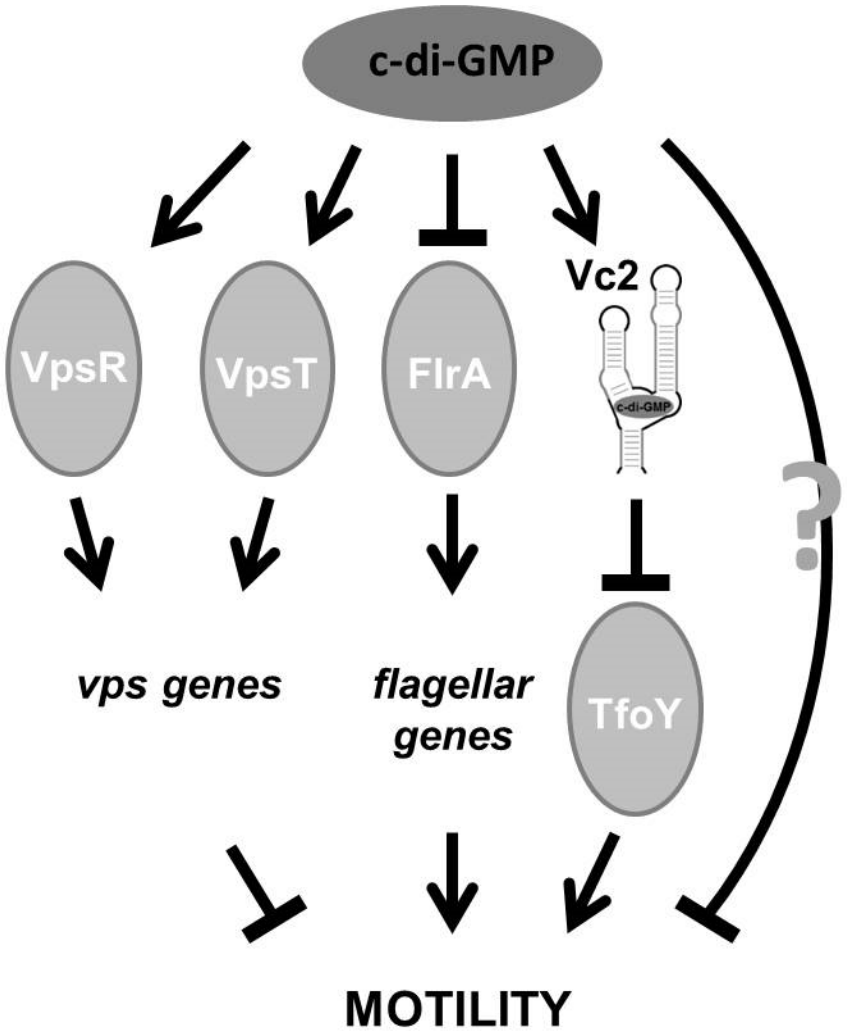



**Figure 4-10: Four pathways control c-di-GMP regulation of motility in *V. cholerae***

As previously shown (Srivastava et. al., 2013), induction of the *vps* extracellular polysaccharide biosynthesis genes and inactivation of the transcription factor FlrA by c-di-GMP inhibits motility. Here we show that inhibition of *tfoY* expression by the Vc2 riboswitch and a fourth unidentified pathway both contribute to the inhibition of motility by c-di-GMP in *V. cholerae*.

induction. Examination of motility at later time points suggests there is a third major pathway through which c-di-GMP can repress motility that remains to be identified. *V. cholerae* encodes five proteins with c-di-GMP binding PilZ domains (Pratt *et al.*, 2007), and perhaps one of these proteins is responsible for this third pathway, although none of these proteins show significant

similarity with the well characterized PilZ protein YcgR from *E. coli* that inhibits flagellar function upon c-di-GMP binding (Boehm *et al.*, 2010, Fang & Gomelsky, 2010, Paul *et al.*, 2010).

The molecular mechanism by which Vc2 regulates expression of *tfoY* still remains to be determined. Riboswitches typically function by modulating either transcription termination or translation efficiency. Sequence analysis of the RNA downstream of Vc2 does not indicate any Rho-independent termination structures, and we observe no differences during *in vitro* transcription assays of this region with and without c-di-GMP (Fig. 3-9). Therefore, our results are consistent with a recent study that predicted Vc2 negatively impacts translation of *tfoY*, and we are exploring this mechanism *in vivo* (Inuzuka et al., 2016).

Metzger et. al. recently reported that *tfoY* is not necessary for the induction of motility at low levels of c-di-GMP and concluded that it is therefore not important for this process (Metzger et al., 2016); however, we observe a clear reduction in motility at low c-di-GMP when *tfoY* is deleted or the Vc2 riboswitch is mutated. The differences in these conclusions stem from our use of low-nutrient agar, which enhances the dispersive motility phenotype induced by *tfoY*, highlighting the impact of *tfoY* on motility regulation. Indeed, Metzger et. al. observed only a minor effect on motility in a *tfoY* mutant in their conditions whereas mutation of *tfoY* in low nutrient agar dramatically reduces motility (Fig. 4-4A).

Major outstanding questions include the identity of the regulatory targets of *tfoY* and how those targets control the motility program of *V. cholerae*. Our results suggest that *tfoY* appears to play a vital role in managing motility, as having too little or too much TfoY disrupts the normal timing of motility engagement as a colony grows from a low cell-density to a high cell-density state. Deletion of *tfoY* renders *V. cholerae* unable to progress to the dispersive

phase of motility that is characteristic of late growth, and overexpression of TfoY causes *V. cholerae* to disperse during early growth. Our evidence indicates that *tfoY* can induce motility both through the well-described *flaA*-dependent bacterial swimming program that is commonly recognized as the primary form of *V. cholerae* locomotion and an alternate motility dependent upon *flaEDB*. *V. cholerae ΔflaA* mutants were observed to not have visible flagella (Klose & Mekalanos, 1998a), yet TfoY overexpression can induce motility in these strains. This alternative motility does not develop at a pace or in a pattern consistent with normal swimming behavior, but it does seem to require the alternative flagellin locus *flaEDB* (Fig. 4-7). This is the first demonstration of a motility behavior that is dependent on the alternative flagellin subunits of *V. cholerae*, and it will be interesting to understand the molecular mechanism behind this alternate motility.

## EXPERIMENTAL PROCEDURES

### Strains and Growth Conditions

All V. cholerae experiments were performed with El Tor biotype C6706str2 *ΔvpsL* derivative strains which are deficient for biofilm formation (Waters et al., 2008). The *ΔvpsL* mutation aids in the accuracy of spectrophotometric readings during reporter assays and reduces uncertainty about the effect of biofilm formation on the motility phenotypes examined. Propagation of DNA for genetic manipulation and mating of reporter plasmids was conducted in *E. coli* S-17-*λpir* (Simon *et al.*, 1983). Unless otherwise specified, bacteria were grown at 35°C in Miller LB Broth (Acumedia), or on LB agar plates, with ampicillin (100 μg/mL), kanamycin (100 μg/mL), chloramphenicol (10 μg/mL), polymyxin B (10 IU/mL ), streptomycin (500 μg/mL), and isopropyl-β-D-thiogalactoside (IPTG) at 100 μM as required. Liquid cultures in glass tubes or flasks were shaken at 220 RPM and cultures in microtiter plates were shaken at 150 RPM.

### Reporter Assays

Each replicate was derived from an individual colony from a bacterial mating. Strains were grown overnight in test tubes then diluted 1:5,000 into fresh media containing IPTG, and inoculated at 100 μL volumes into in black, clear-bottom 96-well microplates. Plates were spectrophotometrically measured with a SpectraMax M5 (Molecular Devices) after reaching late log phase growth. GFP fluorescence was read with excitation at 475 nm and emission at 510 nm; absorbance was read at 595 nm.

**Genetic Manipulations**

All *gfp* genetic reporters were constructed in the pBRP31 background. pBRP31 was constructed by PvuII-EcoRI digestion of pMMB67EH (Furste *et al.*, 1986) to remove the *lacI*$^q$ and $P_{tac}$ elements, and inserting the SphI-BamHI promoterless GFP fragment from pCMW1 (Waters & Bassler, 2006), with some minor modifications to the multi-cloning site. Inserts for reporter vectors were generated by PCR with Phusion DNA Polymerase (NEB). For transcriptional reporters, the insert was ligated 29 bp upstream of the GFP coding sequence and thus utilized the consensus ribosome binding site featured in pCMW1. For translational reporters, the insert contained the first 21 bp of the *tfoY* coding sequence and was ligated to the second codon of the GFP coding sequence. Luminescient reporters were constructed as previously descibed, respectively (Srivastava et al. 2013). Ptac-TfoY was constructed from the pEVS143 backbone (Dunn et al., 2006).

*V. cholerae* mutant strains were constructed using allelic exchange with vectors derived from pKAS32 (Skorupski & Taylor, 1996). Vectors containing mutant riboswitch alleles were generated with the QuickChange Site-Directed Mutagenesis Kit (Agilent) and mated into *V. cholerae* so as to create markerless strains with single point mutations on the genome. For complementation of the Vc2-tfoY locus, the complemented region was inserted into a pKAS32 derivative, flanked upstream and downstream by ~800bp regions of VC2338, a *V. cholerae* homologue of *lacZ* which contains a frameshift mutation in the sequenced strain N16961 (Heidelberg et al., 2000).

**5'-RACE**

The 5'-RACE (Invitrogen) was performed according to the manufacturer's instructions. 5'-RACE was preformed both with RNA from wild type *V. cholerae* using a *tfoY*-specific downstream primer, and RNA from *V. cholerae* cells carrying *tfoY* promoter reporter plasmids using a GFP-specific downstream primer in order to better isolate and identify the start sites of each individual promoter.

**Motility assays**

Unless otherwise specified, motility media consisted of 0.1% tryptone, 0.05% yeast extract, 1.0% sodium chloride and 0.35% agar, with antibiotics and IPTG added where appropriate. 100 mm diameter plates were prepared with 15 mL of media, and 1.5 µL of overnight culture was deposited onto the surface of the agar after solidification. Inoculation by stabbing of the media was unnecessary because at the concentration of agar used, *V. cholerae* sinks into the agar and does not grow on the surface. After inoculation, they were incubated for 1 hour in upright position at room temperature. The plates were then inverted and incubated at 35°C in a humid box to reduce desiccation and were photographed using an AlphaImager HP system (ProteinSimple). Quantitative measurements were made using the image analysis software Fiji (Schindelin et al., 2012).

# CHAPTER 5

## Future Directions and Conclusions

## INTRODUCTION

This chapter presents additional experiments aimed at answering some of the outstanding research questions which arose during the course of the work presented in Chapters 3 and 4. Many of these experiments were designed and performed concurrently with the previous research. While some of these experiments are fully-developed, others are more preliminary, in that they do not yet provide a complete story but still serve to establish a foundation for future studies to build upon.

## RESULTS

### Regulation of *tfoY* Promoters by c-di-GMP-binding Transcription Factors

Earlier, we demonstrated that the regulation of *tfoY* expression under high c-di-GMP conditions was both riboswitch-independent and *tfoY* promoter-dependent (Figure 3-1; Figure 3-3). This led us to hypothesize that regulation of the *tfoY* promoters relied on one or more of the known *V. cholerae* c-di-GMP-binding transcription factors. We first tested the involvement of VpsR, the major transcriptional activator of *Vibrio* polysaccharide biosynthesis genes in *V. cholerae* and a protein recently identified as binding c-di-GMP (Yildiz et al., 2001; Srivastava et al., 2011). Using the same *gfp* transcriptional reporters described in previous chapters, we measured promoter activity in a *ΔvpsR* strain and found that the *tfoY* promoters were no longer responsive to c-di-GMP (Figure 5-1). Specifically, the $P_{2\text{-}tfoY}$ promoter could not be repressed and the $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$ could not be activated by overexpression of the diguanylate cyclase QrgB.

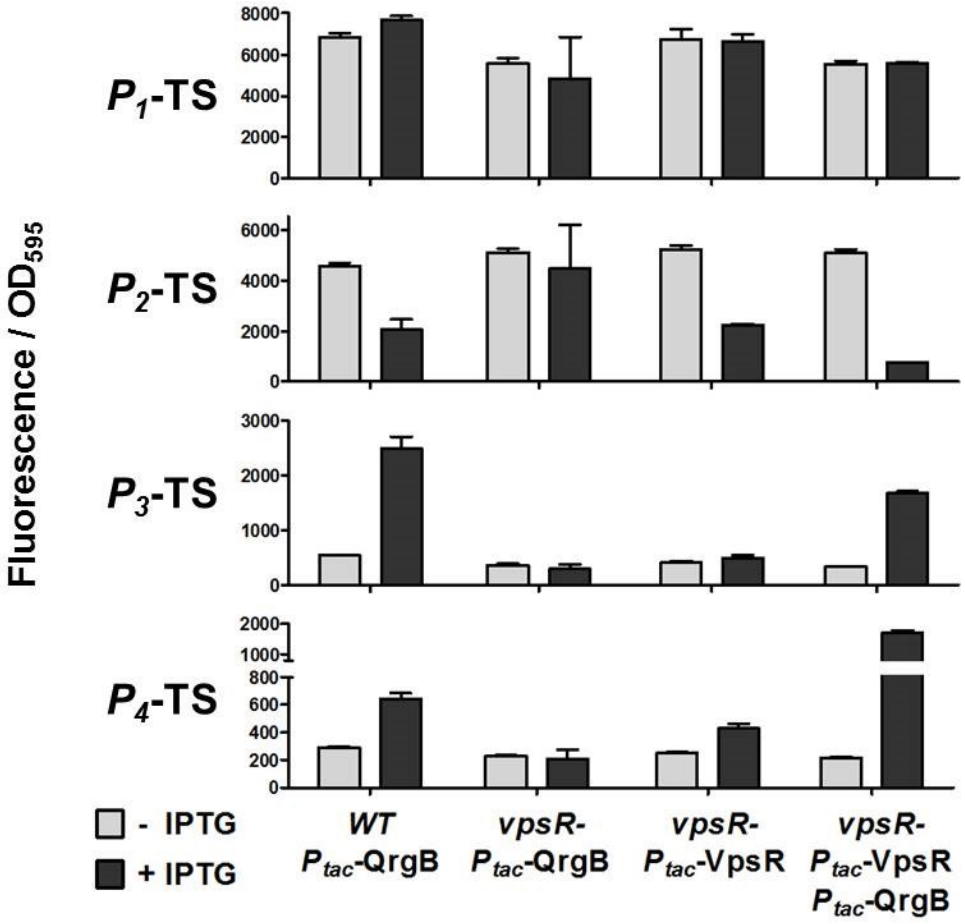



**Figure 5-1: c-di-GMP Regulation of *tfoY* Promoters is *vpsR*-dependent**

Activity of *gfp* transcriptional reporters measured at 24 hours. Promoter regions used in transcriptional reporters correspond to map in Figure 3-2. IPTG was used at 0.1mM concentration to induce $P_{tac}$ expression. Error bars indicate standard deviation.

The mechanism by which c-di-GMP modifies VpsR activity has yet to be described in the literature, so we sought to determine whether or not VpsR regulation *tfoY* promoters was dependent on the c-di-GMP state of the cell. When VpsR was complemented back and overexpressed in the *ΔvpsR* background, $P_{2\text{-}tfoY}$ was repressed, $P_{4\text{-}tfoY}$ was activated – although to a lesser extent than under wild-type conditions, and $P_{3\text{-}tfoY}$ still showed no response (Figure 5-1). Only when both QrgB and VpsR were dually overexpressed could expression of $P_{3\text{-}tfoY}$ be

induced in the *ΔvpsR* strain, implying a strict requirement for both c-di-GMP and VpsR in the activation of this promoter.

We also tested the potential regulation of the *tfoY* promoters by one of the other c-di-GMP-binding transcription factors of *V. cholerae*, VpsT, another regulator of *Vibrio* polysaccharide genes (Krasteva et al, 2010). Though subtle, loss of *vpsT* had an effect on the expression of the $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$ during QrgB overexpression, causing a slight decrease in the magnitude of induction (Figure 5-2). This result would be consistent with previous reports of VpsT playing a role in the activation of *vpsR* expression (Beyhan et al., 2007). Because activation of $P_{3\text{-}tfoY}$ and $P_{4\text{-}tfoY}$ is only completely lost in a *ΔvpsR* background, we believe that VpsT is not functioning directly at the *tfoY* promoters and only causes an effect in this assay because its absence reduces the extent of *vpsR* induction from the chromosome by QrgB. Notably, the repression of the $P_{2\text{-}tfoY}$ promoter was not reduced in magnitude by the absence of *vpsT*.

**Binding of VpsR at the *tfoY* Promoter Sequences**

We had confirmed that *vpsR* was necessary for the normal regulation of three of the four *tfoY* promoters, but we did not know if that regulation occurred directly through VpsR interacting with the genome in the region of the promoters. A previous study predicted a potential VpsR binding site upstream of the $P_{3\text{-}tfoY}$ promoter, and scanning of the DNA sequences in this region actually revealed two possible sites directly adjacent to one another (Yildiz et al., 2004; Figure 5-3A). Coincidentally, the spacing and directionality of these VpsR sites shows that they form an inverted repeat in the region immediately downstream of the transcriptional start site of $P_{2\text{-}tfoY}$, and this sequence was previously predicted to serve as a rho-

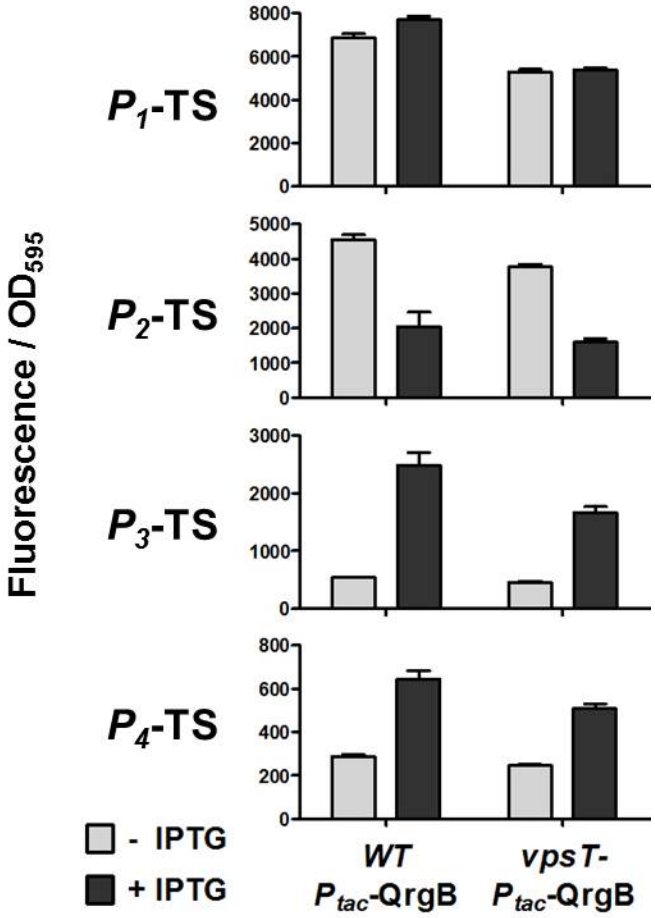



**Figure 5-2: c-di-GMP Regulation of *tfoY* Promoters is *vpsT*-independent**

Activity of *gfp* transcriptional reporters measured at 24 hours. Promoter regions used in transcriptional reporters correspond to map in Figure 3-2. IPTG was used at 0.1mM concentration to induce $P_{tac}$ expression. Error bars indicate standard deviation.

independent transcriptional terminator for the upstream gene *VC1721* (Kingsford et al., 2007; Figure 5-3B). We compared this sequence to a group of other known VpsR binding sites where direct interaction of VpsR and DNA had previously been demonstrated (Figure 5-3C; Lin et al., 2007). The *tfoy*-associated VpsR sites strongly matched with these other sites, and also

matched a computationally derived consensus motif for VpsR binding which was recently reported (Zamorano-Sanchez et al., 2015).

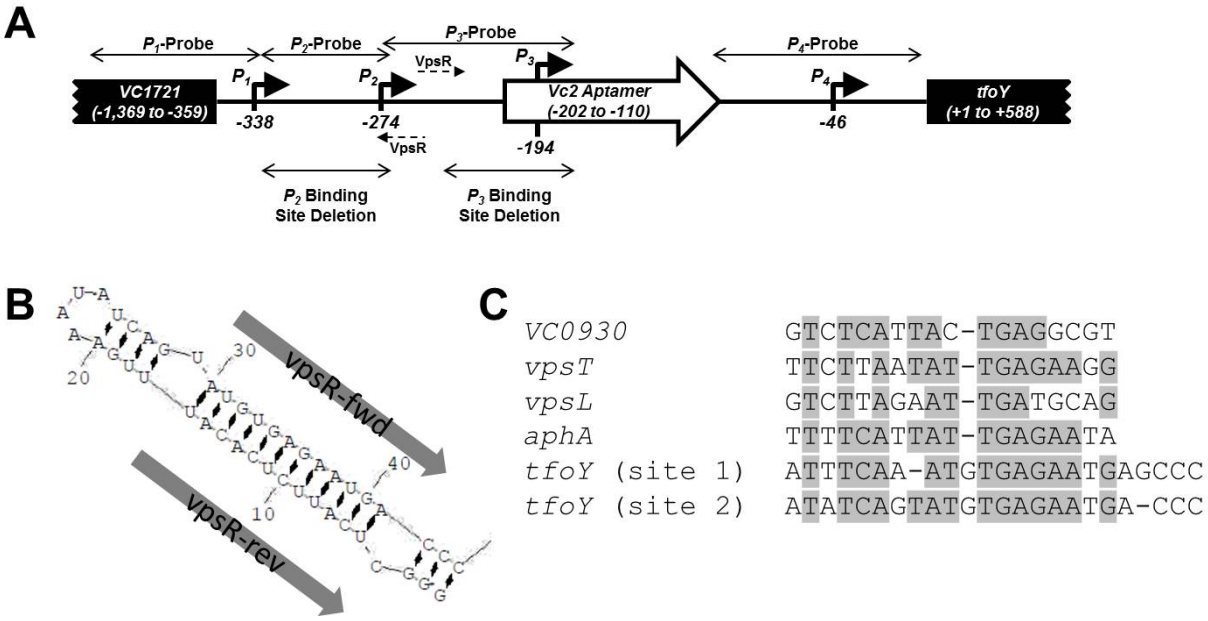



**Figure 5-3: VpsR Binding Sites Are Located Between P3-tfoY and P4-tfoY Promoters**

**(A)** Consecutive consensus VpsR binding sites located immediately downstream of the P2-tfoY transcriptional start site. Sequence regions used for electrophoretic mobility shift assay probes and transcriptional reporters are indicated with solid arrows. **(B)** The sequence of the VpsR binding sites at the tfoY locus form an inverted repeat that was previously predicted to serve as a rho-independent transcriptional terminator for the upstream gene VC1721 (Kingsford, 2007). **(C)** Nucleotide positions which share identity with *tfoY* sites are highlighted in gray.

We designed four fluorescently labeled DNA probes, one for each of the respective *tfoY* promoter regions indicated in Figure 5-3A, and tested their potential for VpsR binding in an electrophoretic mobility shift assay. As a positive control, we also tested a probe for a known VpsR-regulated sequence, the promoter for *vpsT* (Srivastava et al., 2011). In our experience with this assay VpsR has a relatively high affinity for DNA fragments regardless of sequence. The specificity of the interaction between VpsR and DNA is revealed not simply by its shifting but by

the pattern of bands that emerges when it shifts. At the highest concentration of VpsR used, we observed three shifted bands with the positive control *vpsT* promoter probe, and we observed these same three bands with the $P_{2\text{-}tfoY}$, $P_{3\text{-}tfoY}$, and $P_{4\text{-}tfoY}$ DNA probes (Figure 5-4A). Unlike the control, in which most of the shifted probe appeared to be in the two smallest shifted bands, for $P_{2\text{-}tfoY}$ and $P_{3\text{-}tfoY}$ most of the shifted probe was shifted to the largest size band. With the $P_{1\text{-}tfoY}$ probe, only a single shifted band was observed. Because the appearance of at least one shifted band in all of our samples creates some ambiguity about the results this assay, we sought to further test the specificity of VpsR binding by removing the putative VpsR binding site sequences from the $P_{3\text{-}tfoY}$ probe. When we tested a probe for the binding site deletion fragment of $P_{3\text{-}tfoY}$ we observed only a single shifted band, indicating that loss of the VpsR binding sites had significantly reduced the specificity of its interaction with VpsR.

When constructing the $P_{3\text{-}tfoY}$ binding site deletion probe, we had included a full 50 base pairs of the native sequence upstream of the $P_{3\text{-}tfoY}$ transcriptional start site. Technically, this only removed one and a half of the VpsR binding sites, but it kept intact the -35 and associated regions of the promoter sequence which should allow for proper recognition by RNA polymerase. We then used the same sequence to make a binding site deletion variant of the $P_{3\text{-}}$*tfoY gfp* transcriptional reporter. When we tested the expression of this reporter it showed much lower basal activity, near the minimum limit of detection, and it could not be activated by overexpression of QrgB (Figure 5-4B).

In our previous transcriptional reporter assays the DNA fragment referred to as $P_2$-TS actually included the VpsR binding site region immediately downstream of the $P_{2\text{-}tfoY}$ start site (Figure 3-2A; Figure 3-3A). Yet when designing our probes for the mobility shift assay we used

only the sequence upstream of the $P_{2\text{-}tfoY}$ transcriptional start site, effectively making it a VpsR binding site deletion variant of $P_{2\text{-}tfoY}$. We then examined the behavior of this same sequence as a *gfp* transcriptional reporter and found that it was not repressed by QrgB, however, it did show lower basal activity than the original $P_2$-TS reporter (Figure 5-4C).

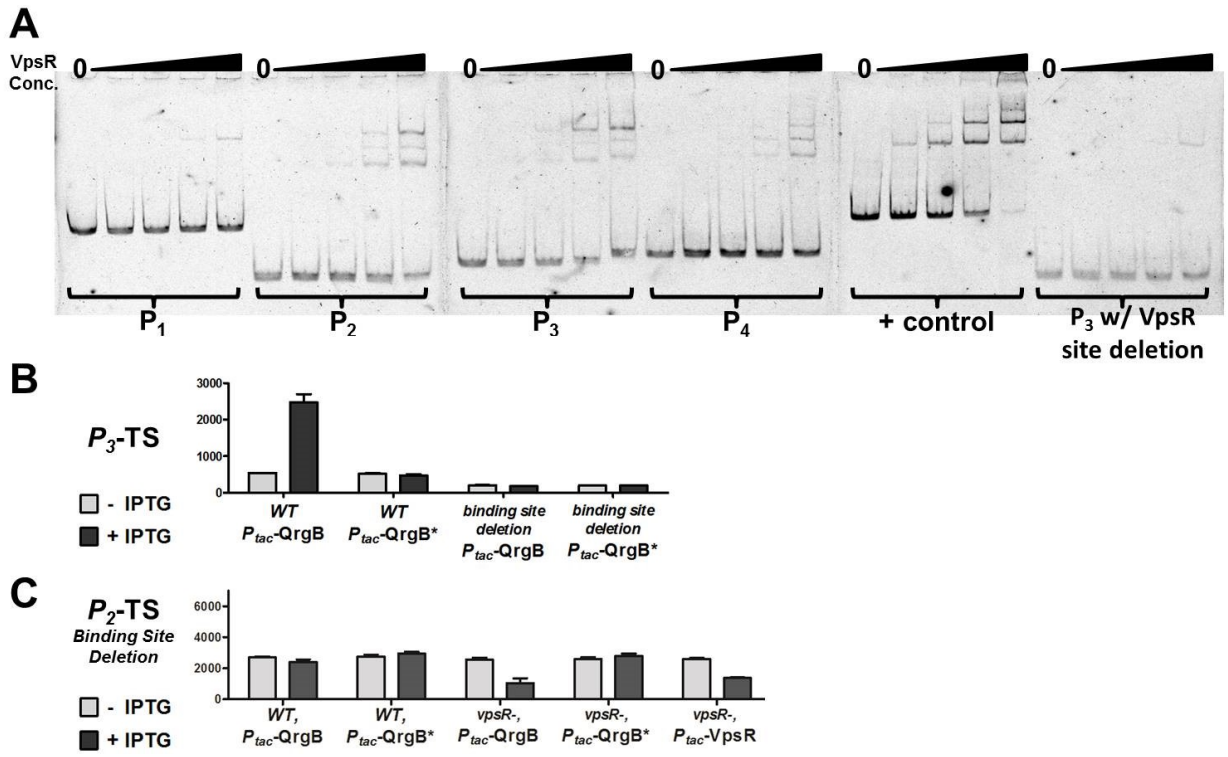



**Figure 5-4: VpsR Binds Specifically at P3-tfoY and P4-tfoY Promoters**

**(A)** Gel results of electrophoretic mobility shift assays. The leftmost lane of each sample group contains probe only and the subsequent lanes to the right contain increasing amounts of VpsR protein. Probe sequences correspond to map in Figure 5-3. **(B,C)** Promoter regions used in transcriptional reporters correspond to map in Figure 5-3. Error bars indicate standard deviation.

### Involvement of CRP at the *tfoY* Promoters

*tfoY* and its homologue *tfoX* have previously been identified as a members of the *N*-acetylglucosamine utilization regulon of *V. cholerae*, a system which in is known to be

controlled in part by CRP (Meibom et al., 2004; Blokesch, 2012). Moreover, direct regulation of the *tfoX* promoter by CRP has already been demonstrated, so we set out to test the behavior of the *tfoY* promoters in a *Δcrp* background (Wu et al., 2015). Deletion of *crp* had a dramatic effect of the expression of all four *tfoY* promoters (Figure 5-5A). The basal expression of the two most upstream promoters was increased and the basal expression of the two most downstream promoters was decreased, with the expression of $P_{3\text{-}tfoY}$ specifically being reduced to within the lower limit of detection in the assay. Additionally, the loss of *crp* interfered with the normal regulation of the promoters by c-di-GMP. QrgB overexpression was not able to induce $P_{3\text{-}tfoY}$

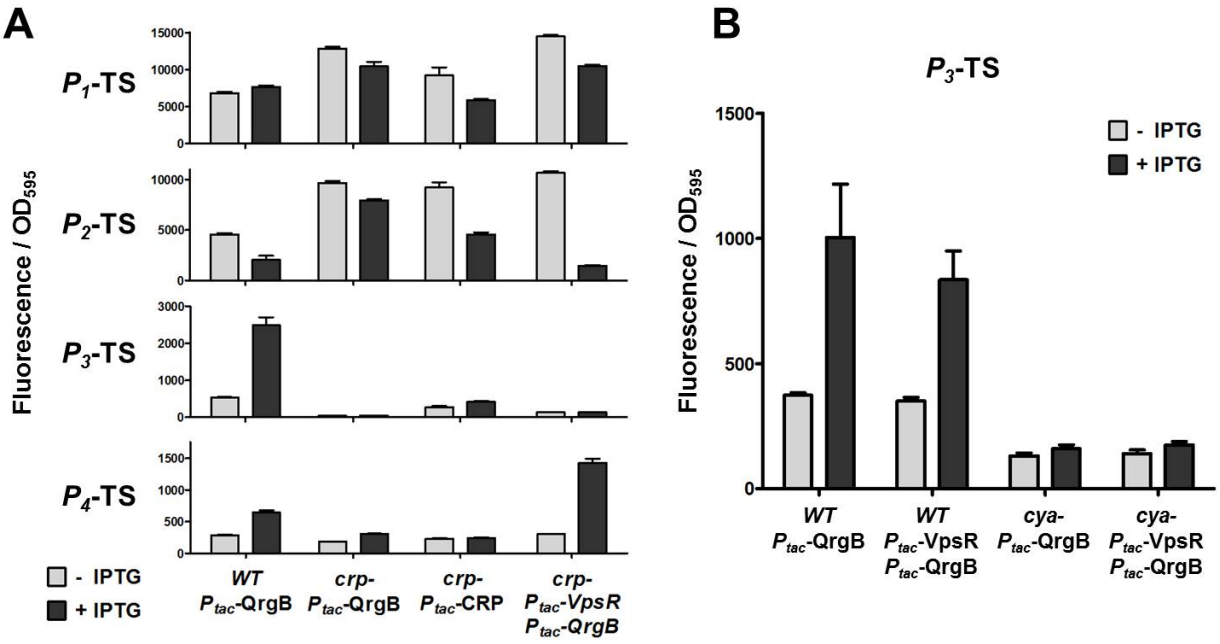



**Figure 5-5: c-di-GMP and cAMP Regulation Converge at *tfoY* Promoters**

Activity of *gfp* transcriptional reporters measured at 24 hours. Promoter regions used in transcriptional reporters correspond to map in Figure 3-2. IPTG was used at 0.1mM concentration to induce $P_{tac}$ expression. Error bars indicate standard deviation.

expression, and the magnitude of both the repression of $P_{2\text{-}tfoY}$ and the activation of $P_{4\text{-}tfoY}$ was also reduced.

To confirm that the effects we saw were due to the loss of *crp*, we introduced CRP back into the *Δcrp* strains on an IPTG-inducible expression vector. When overexpressed, CRP was able to repress the $P_{1\text{-}tfoY}$ and $P_{2\text{-}tfoY}$ promoters back down to near wild-type levels of activity, and it was also able to restore the basal expression of $P_{3\text{-}tfoY}$ to near the wild-type level (Figure 5-5A). To determine if the role of CRP at the *tfoY* promoters was essential for, or simply supportive to, c-di-GMP regulation, we simultaneously overexpressed VpsR and QrgB in the *Δcrp* mutant. This combination was able to strongly repress $P_{2\text{-}tfoY}$ and strongly activate $P_{4\text{-}tfoY}$, however, $P_{3\text{-}tfoY}$ activity still did not recover, indicating an essential role for CRP in its regulation. Because CRP relies on a second messenger, cAMP, for much of its regulatory behavior, we were curious if the loss of cAMP alone was sufficient to prevent the activation of $P_{3\text{-}tfoY}$ . We deleted the *V. cholerae* adenylate cyclase *cyaA* (*VC0122*) and tested the regulation of the $P_{3\text{-}tfoY}$ reporter (Figure 5-5B). In this cAMP-deficient background, overexpression of neither QrgB alone nor QrgB in combination with VpsR could induce expression of $P_{3\text{-}tfoY}$ . This indicates that activation of $P_{3\text{-}tfoY}$ expression carries a strict requirement for VpsR, CRP, c-di-GMP, and cAMP all present simultaneously and working cooperatively.

**Targets of Transcriptional Regulation by TfoY**

Having investigated the various transcription factors involved at the TfoY locus, we sought to turn our attention to identifying the genetic loci where TfoY, itself a predicted transcription factor, might be active. We started by screening a library of random *V. cholerae*

genomic fragments fused to luciferase (*lux* operon) to identify promoters whose activity was responsive to TfoY overexpression. To narrow our focus and identify genes specifically within the high-c-di-GMP regulon of TfoY, we also screened candidate promoters for their response to overexpression of QrgB. The screen yielded various putative promoters for genes of unknown function, but it also returned multiple leads for genes associated with c-di-GMP metabolism (Table 5-1). Due to previous work performed in our laboratory, we had available a *gfp*-reporter

| Screen Isolate | Gene Locus | Downstream Gene Putative Function | $Log_2$ Fold-Change w/ $P_{tac}$-TfoY | $Log_2$ Fold-Change w/ $P_{tac}$-QrgB |
|---|---|---|---|---|
| 7:F10 | VC_1177 | hypothetical protein CDS | -2.80 | -2.88 |
| 7:G4 | VCA0720 | guanylate cyclase-related protein CDS | -2.44 | -4.80 |
| 8:G7 | VCA0013 | maltodextrin phosphorylase CDS | -1.87 | -0.78 |
| 9:B6 | VC_0338 | transporter, putative CDS (sodium-dependent dicarboxylate transporter) | -2.80 | -1.03 |
| 9:B8 | VCA0798 | CbbY family protein CDS (citrate lyase subunit beta) | -2.72 | -1.46 |
| 9:H3 | VCA0939 | sensory box/GGDEF family protein CDS | -2.73 | -1.43 |
| 10:A10 | VCA0162 | hypothetical protein CDS | -0.84 | -0.31 |
| 10:A11 | VC_0478 | fructose-bisphosphate aldolase CDS | -1.58 | -1.53 |
| 10:B9 | VCA0988 | methyl-accepting chemotaxis protein | -4.33 | -2.72 |
| 10:F11 | VC_2224 | GGDEF family protein CDS | -0.43 | -0.45 |
| 10:H2 | VC_1349 | sensory box sensor histidine kinase/response regulator CDS | -3.07 | -1.78 |
| 11:F11 | VC_1353 | GGDEF family protein CDS | -4.41 | -2.81 |

**Table 5-1: Selected Results from TfoY-responsive Promoter Screen**

Information for selected TfoY-responsive promoters with regard to the nearest downstream coding sequence and its putative function. Rightmost columns indicate fold-change of gene expression as measured in *lux* reporter expression assays under the indicated protein overexpression conditions.

library for the promoters of all 61 of the diguanylate cyclases and c-di-GMP phosphodiesterases in *V. cholerae*. Screening of this library revealed that the expression of at least 17 of these c-di-GMP metabolic proteins is more than two-fold regulated by TfoY overexpression (Table 5-2).

| Reference Tag | Vector | Gene Locus | C-di-GMP Metabolic Domains | $Log_2$ Fold-Change w/ $P_{tac}$-TfoY |
|---|---|---|---|---|
| GG_1 | pCMW34 | VC_0900 | GGDEF | +2.45 |
| GG_2 | pCMW35 | VC_1029 | GGDEF | -1.14 |
| GG_5 | pCMW37 | VC_1185 | GGDEF | -2.57 |
| GG_7 | 11:F11 | VC_1353 | GGDEF | -4.41 |
| GG_9 | pCMW47 | VC_1370 | GGDEF | -2.27 |
| GG_13 | pCMW50 | VC_1599 | GGDEF | -2.80 |
| GG_21 | pCMW61 | VCA0217 | GGDEF | -1.68 |
| GG_22 | pCMW62 | VCA0557 | GGDEF | -1.53 |
| GG_24 | pCMW64 | VCA0697 | GGDEF | -3.28 |
| GG_25 | pCMW66 | VCA0848 | GGDEF | -1.12 |
| GG_26 | 9:H3 | VCA0939 | GGDEF | -2.73 |
| GG_29 | pCMW70 | VCA0965 | GGDEF | -1.73 |
| GG_31 | pCMW27 | VC_0072 | GGDEF/EAL | -1.12 |
| GG_36 | pCMW73 | VC_0703 | GGDEF/EAL | -2.06 |
| GG_39 | pCMW59 | VCA0080 | GGDEF/EAL | -1.04 |
| EAL_3 | pCMW82 | VC_1086 | EAL | -1.66 |
| EAL_9 | pCMW88 | VC_1851 | EAL | -1.62 |

**Table 5- 2: TfoY Regulates Expression of c-di-GMP Metabolic Enzymes**

Information for diguanylate cyclase and c-di-GMP phosphodiesterase enzymes whose promoters responded to TfoY overexpression. Rightmost column indicates fold-change of gene expression as measured in *gfp* reporter assays. In the case of GG_7 and GG_26 the reporter output was *lux,* as those promoters were identified from screen listed in Table 5-1.

With the surprising result that TfoY might actually be a powerful global regulator of c-di-GMP metabolism, we wanted to confirm that the changes in diguanylate cyclase gene

expression attributable to TfoY were occurring through direct transcriptional regulation by the TfoY protein and not simply pleotropic effects of its overexpression. If transcriptional regulation by TfoY is mechanistically similar to regulation by other *tfoX*-domain-containing proteins, then it should require the cooperation of CRP (Cameron and Redfield, 2008). We examined regulation of multiple DGC promoters in a *Δcrp* background, and found that loss of CRP had different effects on the different promoters, with some losing expression altogether while others developed constitutively high expression (Figure 5-6). But remarkably, in each case we tested, loss of CRP had abolished the capacity for regulation of the promoters by TfoY.

**Specific Control of the Diguanylate Cyclase *VCA0697* by TfoY**

Previous studies have shown that overexpression of VCA0697 can induce biofilm formation and deletion of *VCA0697* leads to increased motility as well as decreased virulence factor expression (Massie et al., 2012; Liu et al., 2010; Syed et al., 2009) These effects on multiple different c-di-GMP programs, indicate that *VCA0697* may play a globally important role through multiple different pathways of c-di-GMP signaling. For this reason we selected it for further analysis with regard to regulation by TfoY. In a wild-type cell, overexpression of QrgB, or VpsR, or TfoY is able to repress *VCA0697* expression, to about half of its normal level in the case of QrgB and VpsR or to less than an eighth in the case of TfoY (Figure 5-7). When *tfoY* is deleted, QrgB is unable to repress *VCA0697*, and the effectiveness of repression by VpsR is decreased by half. Complementation and overexpression of TfoY back into the *ΔtfoY* strain fully restores repression of *VCA0697*. When the Vc2 riboswitch is disabled by *Vc2 (G20U,C92U)* mutations,

there is no significant effect on the repression of *VCA0697* by QrgB or VpsR, but when VpsR is lost from the chromosome, QrgB is again unable to repress *VCA0697*.

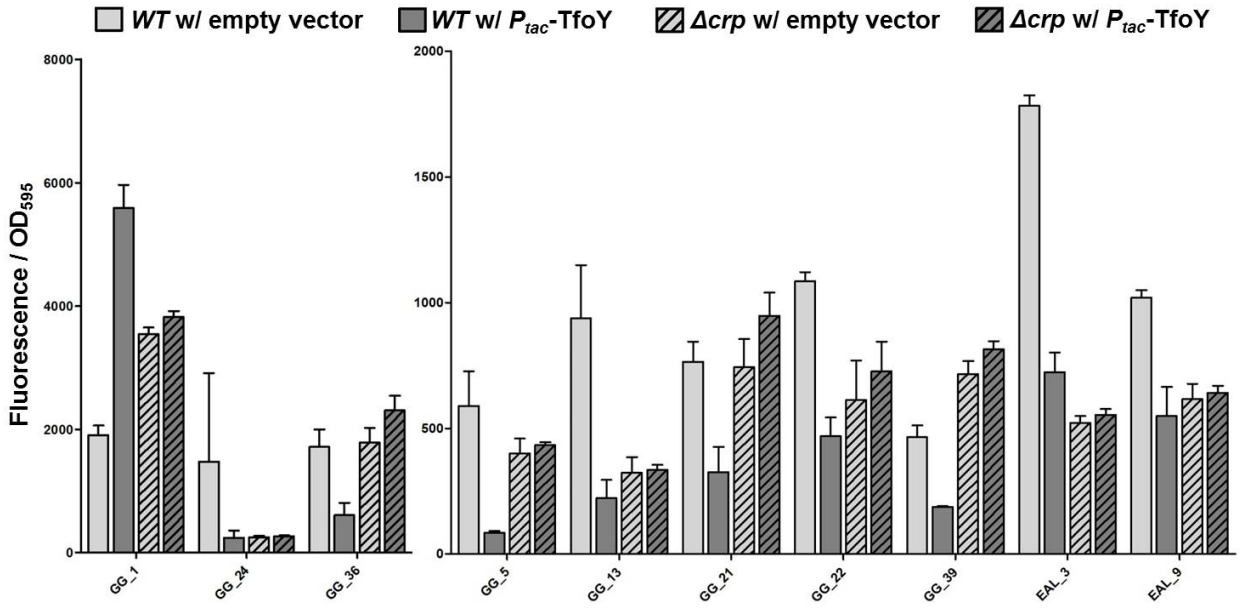



**Figure 5-6: TfoY Regulation of c-di-GMP Metabolic Enzymes is CRP-dependent**

Activity of *gfp* transcriptional reporters measured at 12 hours. X-axis labels correspond to Reference Tags listed in Table 5-2. All samples included IPTG at 0.1mM concentration to induce $P_{tac}$ expression. Error bars indicate standard deviation.

**Role of *tfoY* in Biofilm Formation**

Biofilm formation is one of the most prominent c-di-GMP-associated phenotypes in *V. cholerae*, and since we found that *tfoY* is regulated by c-di-GMP, specifically through VpsR, we have looked at biofilm formation in relation to *tfoY* on multiple occasions. Unfortunately, those prior analyses, mostly involving microplate format biofilm assays, have largely been inconclusive (data not shown). Recent results, however, looking at conditions of *tfoY* deletion and TfoY overexpression in a standing test tube format biofilm assay, have renewed our

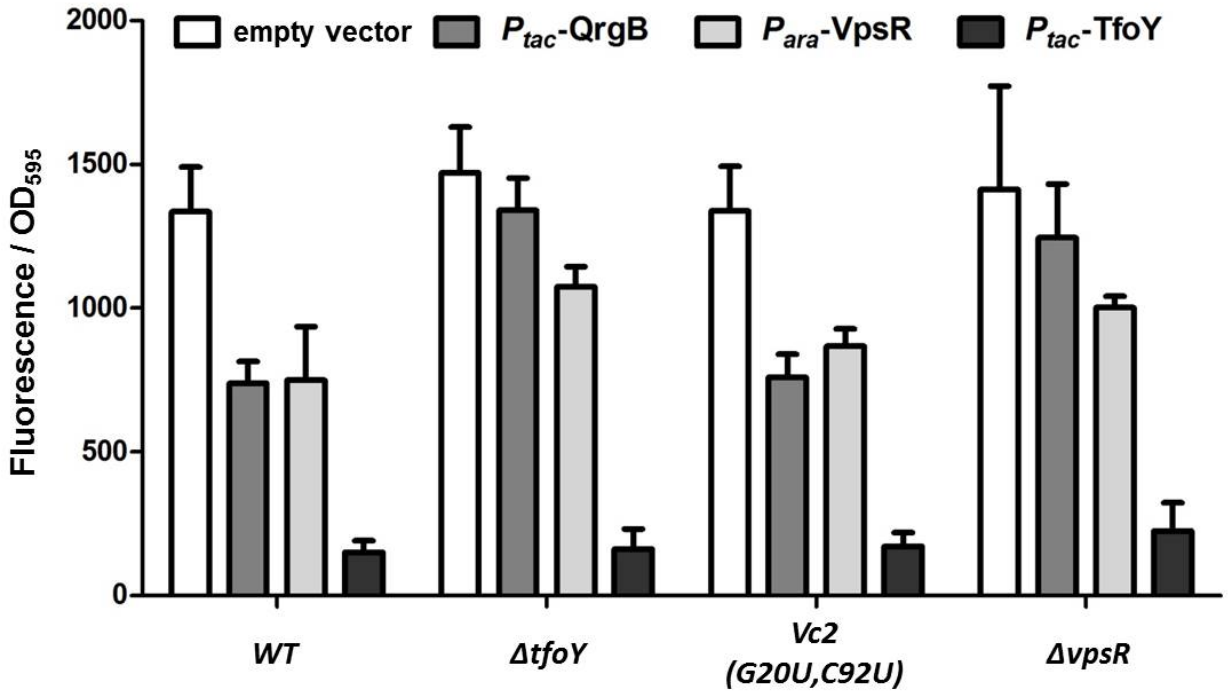



**Figure 5-7: c-di-GMP Regulation of *VCA0697* is *tfoY*-dependent and Vc2-independent**

Activity of *gfp* transcriptional reporters measured at 9 hours. All samples included either IPTG at 0.1mM concentration or arabinose at 0.02% to induce $P_{tac}$ or $P_{ara}$ expression, respectively. Error bars indicate standard deviation.

interest in the phenotype. We incubated test tube cultures unshaken at 35°C degrees for 12 hours, added IPTG to induce the TfoY expression, and measured biofilm formation at 30 hours. At that time point, the planktonic cultures were removed from the tubes, the glass was stained with crystal violet, and the tubes were gently washed before visualization. After staining we noticed striking differences in the appearance of biofilm residue deposited onto the surface of the glass tubes (Figure 5-8). Specifically, in the *wild-type* and *ΔtfoY* backgrounds, both of which are *vpsL*$^{+}$ strains, there was a dense, narrow band of crystal violet stain present in tubes where TfoY had been overexpressed. This band appeared to correlate with the location of the former

air-liquid interface when the tubes were still filled with culture, and it was noticeably absent in the *ΔvpsL* strains. Unfortunately in this experiment we were unable to accurately quantify the total amount of crystal violet present in each tube, as we had difficulty completely eluting it from the biofilm material adhered to the glass (data not shown).

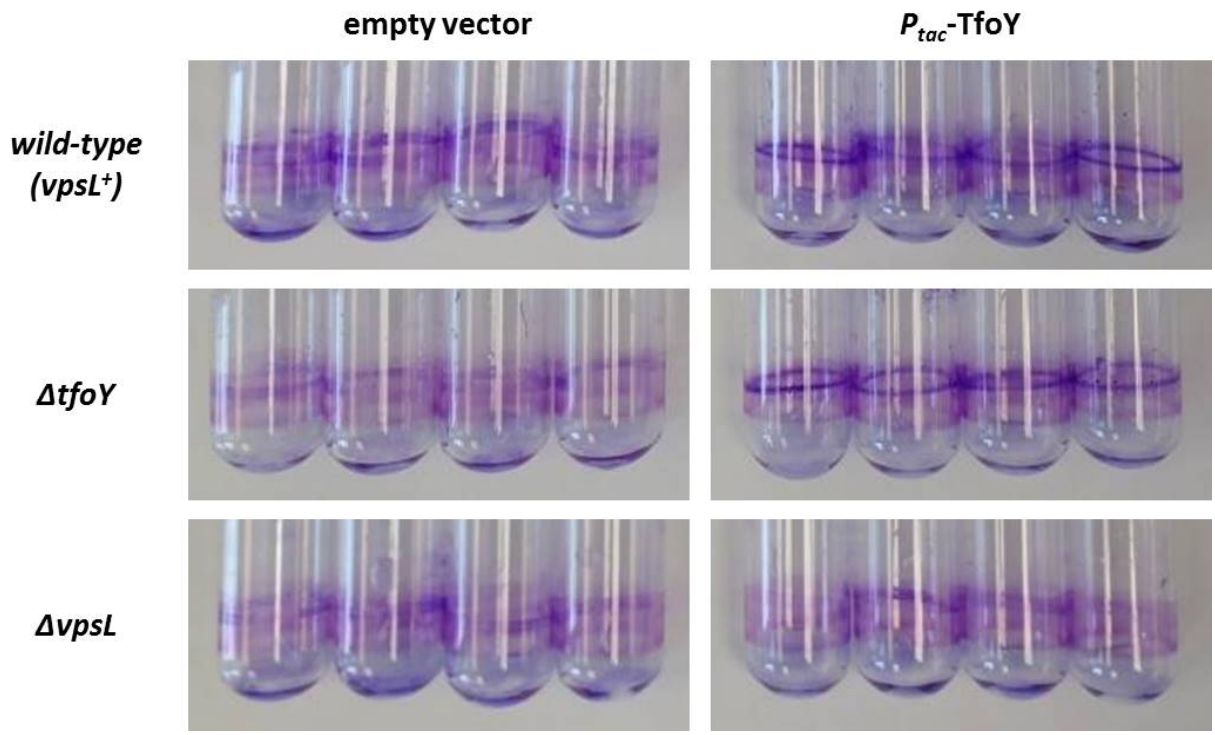



**Figure 5-8: *tfoY* Plays a Role in Biofilm Formation**

Shown are four biological replicates for each experimental group. Biofilm assay performed as described in the text.

**Role of *tfoY* in Bacterial Foam**

Another phenotype involving *tfoY*, discovered by sheer happenstance, is that of foam production. During growth of liquid cultures in baffled flasks, we repeatedly noticed that some of our *V. cholerae* strains accumulated a significant quantity of air bubbles at the liquid-air interface. Baffled flasks are commonly used to aerate media and can often introduce bubbles

into liquid culture, but the air bubbles normally dissipate after shaking of the flask is stopped and the liquid is allowed to settle. Instead, we noticed that in some of our cultures the bubbles amassed into a thick layer of foam atop the liquid phase which resisted decay even after the cultures had be given time to settle. We eventually ascertained that foam production correlated with induction of a high c-di-GMP state during QrgB overexpression and we subsequently undertook an analysis of the contribution of *tfoY* to this process.

Our initial experiments were performed in LB media with *V. cholerae* strains which were all disrupted for the gene *vpsL* and therefore lacking in the ability to synthesize *Vibrio* polysaccharide or produce a biofilm. 50 mL cultures were continuously shaken at 220 RPM and 35°C in 250mL deep-baffled flasks and stopped for 5 minutes of settling at regular intervals to allow for visualization of foam (Figure 5-9). Under these conditions, cultures overexpressing QrgB produced a copious amount of foam that would remain stable until about 24 hours of growth. Overexpression of the control protein QrgB* did not result in stable foam production at any point during growth. We then tested QrgB overexpression in various mutants of the Vc2 riboswitch-*tfoY* genetic locus and observed stable foam lasting to only 12 hours in the *ΔtfoY* mutant, to 16 hours in the *ΔVc2* mutant (deleted for the *VC1721-tfoY* intergenic region), and to 20 hours in the *Vc2 (G20U,C92U)* mutant. Notably, the *ΔtfoY* mutant does not completely lack foam, indicating that there are other c-di-GMP stimulated factors involved in this phenotype. However, the distribution of foam stabilities we saw correlates well with relative amount of TfoY we would expect to be present in the various mutants – the *ΔtfoY* mutant would have no TfoY, the *ΔVc2* mutant would have some TfoY because it retains the *tfoY* coding sequence but it

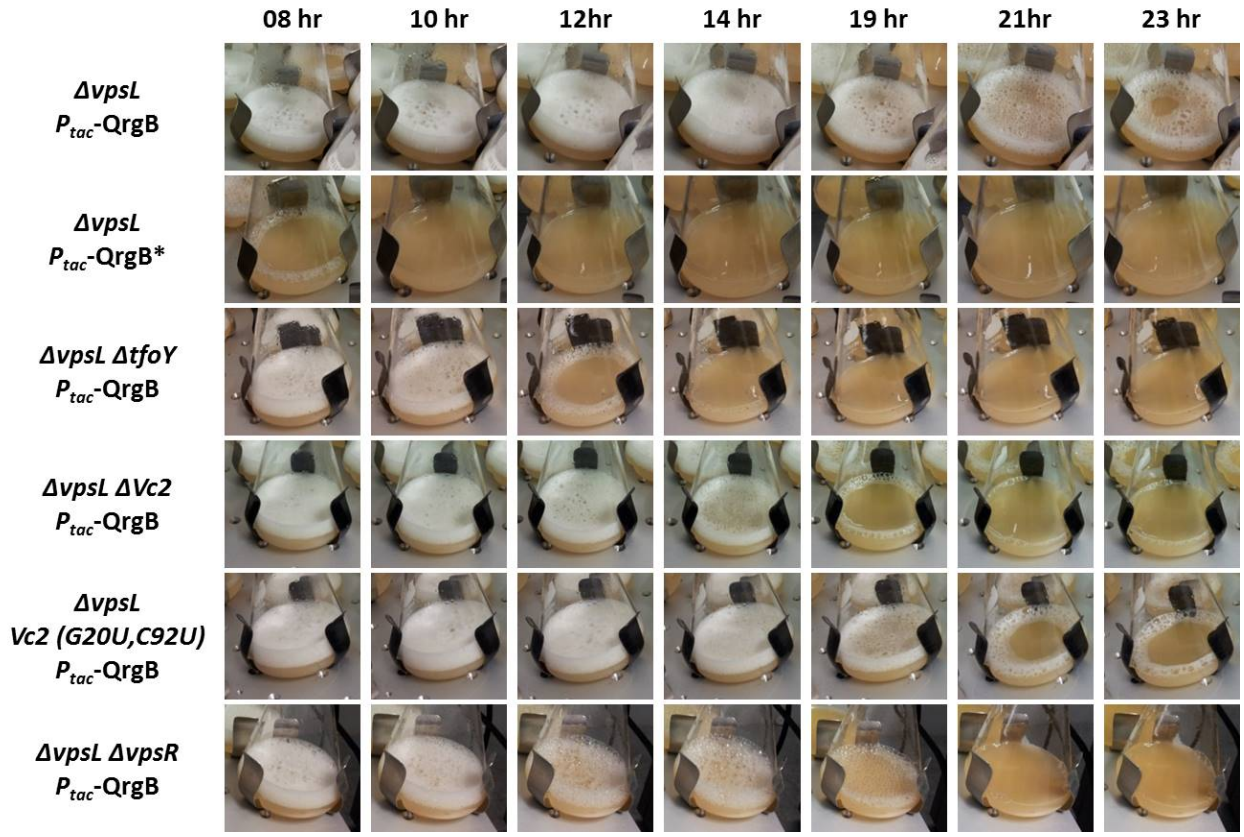



**Figure 5-9: *tfoY* Is Important for c-di-GMP Regulation of Foaming**

Visualization of foam production by *V. cholerae* strains during growth in LB medium. Experiment was performed with three biological replicates for each strain condition, but only one flask is shown as a representative sample. Same flask for each sample group is shown across the duration of the experiment.

lacks the *tfoY* promoters, and the *Vc2 (G20U,C92U)* mutant should have an amount of TfoY comparable to *wild-type*. These results indicate that foam, as a high c-di-GMP phenotype, is relatively *tfoY*-dependent and Vc2 riboswitch-independent, so we hypothesized that loss of VpsR, the transcriptional regulator of *tfoY* would also have reduced foam production. When we overexpressed QrgB in the *ΔvpsR* background, the foam it produced was stable to only about 18 hours, on par with that of the *ΔVc2* mutant.

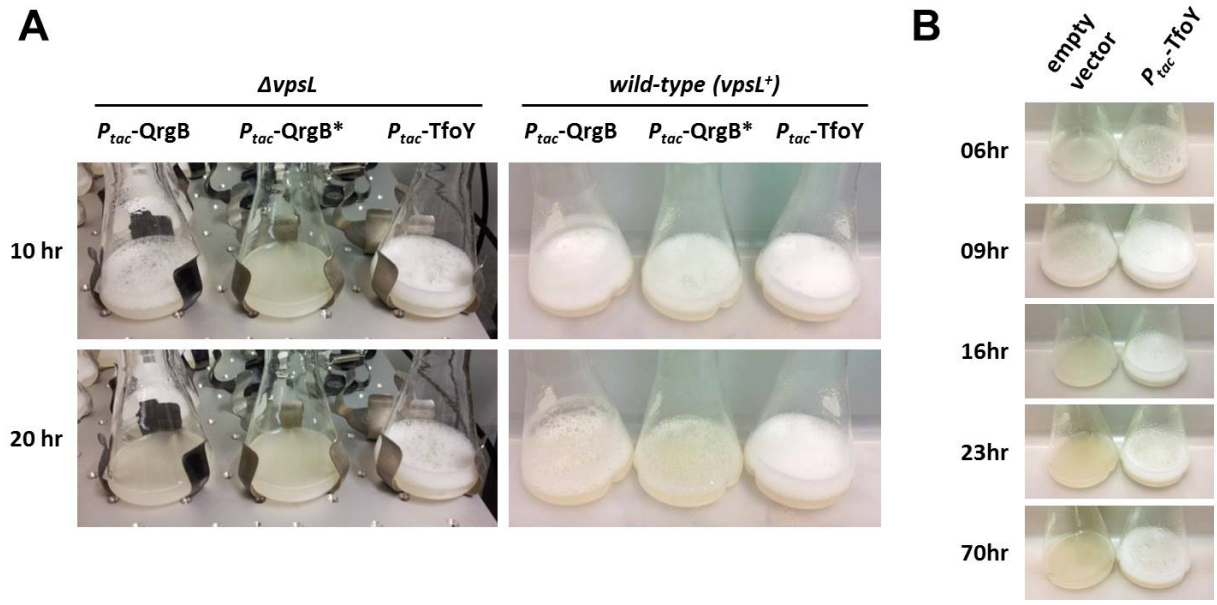



**Figure 5-10: TfoY Overexpression Induces Foaming**

Visualization of foam production by *V. cholerae* strains during growth in AB medium with 1% casamino acids. Experiment was performed with three biological replicates for each strain condition, but only one flask is shown as a representative sample. Same flask for each sample group is shown across the duration of the experiment

We also discovered a relationship between foam production and the type of growth media employed, ultimately deciding on the use of a defined AB medium with 1% casamino acids as the sole carbon source. In this media, the role of *tfoY* in foaming appeared to be dominant to c-di-GMP, as overexpression of TfoY produced a greater abundance and a longer-lasting quality of foam than overexpression of QrgB (Figure 5-10A). In fact, the foam induced by TfoY was stable for in excess of three days of continuous culture (Figure 5-10B). Having thus far only examined foam in the *ΔvpsL* background, we wanted to look at *wild-type (vpsL$^+$)* strains as well to determine if biofilm formation, a prominent high c-di-GMP program also had any effect on foaming. We found *vpsL$^+$* strains produced more foam across the board, with some foam detectable even in the QrgB* control strain, although TfoY overexpression still yielded the

greatest quantity and most stable variety of foam (Figure 5-10A). Notably, QrgB overexpression in the *wild-type* background produced a stickier type of foam that was able to coat much of inside of the flask. This indicates that foam production is likely a complex, multi-component phenotype, of which, *tfoY* might regulate foam volume or strength independently of other foam attributes.

## DISCUSSION

Though not definitive, the results of our electrophoretic mobility shift assay provide strong evidence for a direct interaction between VpsR and the *tfoY* promoters. A multiple-banding pattern in this experiment, as we saw, is an indicator that the protein involved binds DNA as on oligomeric complex and not as a monomer. The nature of c-di-GMP's effect on VpsR activity is not yet known, but an attractive theory is that c-di-GMP may control oligomerization of the protein. We tested the addition of c-di-GMP to the mobility shift assay, but it did not improve the strength of VpsR binding (data not shown). This is not entirely unexpected, as the results of the $P_{3\text{-}tfoY}$ reporter assays hint that the optimal interaction between VpsR and DNA in this region requires the additional cooperativity of CRP and cAMP. Our current hypothesis is that there are multiple types of VpsR binding sites, for example primary and secondary sites, throughout the *VC1721-tfoY* intergenic region. Primary sites would be those with a high degree of similarity to the consensus VpsR DNA binding motif, such as upstream of $P_{3\text{-}tfoY}$, where regulation would be dependent on c-di-GMP. Secondary sites would not strongly match the consensus sequence, but would still allow for gene regulation in a manner independent of the c-di-GMP binding state of the VpsR protein, such as at $P_{2\text{-}tfoY}$ and $P_{4\text{-}tfoY}$.

The requirement of both c-di-GMP and cAMP for $P_{3\text{-}tfoY}$ promoter regulation, and the implied cooperation of VpsR and CRP in the induction of *tfoY* expression is a rather unexpected finding. Previous work in *V. cholerae* has portrayed c-di-GMP and cAMP as antagonists with regard to various gene regulatory programs. The cAMP-CRP complex suppresses biofilm formation, in part through the repression of VpsR, but also through the repression of *cdgA*, a diguanylate cyclase (Fong and Yildiz, 2008). At the same time, cAMP-CRP promotes flagellar gene expression and motility, a process which c-di-GMP interferes with by disrupting the DNA binding activity of the master flagellar regulator FlrA (Liang et al., 2007; Srivastava et al, 2013).

Biofilm formation and motility are at different ends of the lifestyle spectrum, and we now have evidence that *tfoY* can impact both of these programs. But surprisingly, *tfoY* expression appears to be favorable to both motility and biofilm formation. One explanation for this is that *tfoY* might not be vital while either program is ongoing, but it could play a crucial role during the transitions between these programs. For example, if *V. cholerae* senses it has a preferred carbon source available it would be inclined to produce a biofilm. VpsR levels will increase, but cAMP will be low, so the $P_{3\text{-}tfoY}$ promoter will not be active. As soon as the cell starts to run out of its carbon source, VpsR levels will still be high, but cAMP will now be on the rise, and $P_{3\text{-}tfoY}$ will turn on just as the cell is making the decision to adjust its lifestyle. In Chapter 4 we witnessed a similar phenomenon with regard to motility. When we artificially induced a phosphodiesterase to immediately turn on motility, *tfoY* became dispensable, as its individual deletion did not significantly affect motility (Fig. 4-4, *ΔtfoY* strain). But when cells were inoculated onto motility agar without the benefit of an extra phosphodiesterase, and they

had to figure out for themselves how to transition from low to high motility, the cells lacking *tfoY* were unable to complete this process (Fig. 4-3A).

If *tfoY* does function at the pivot point between high and low c-di-GMP phenotypes, it may help explain why *tfoY* would be regulating so many c-di-GMP metabolic enzymes. The revelation that TfoY can repress more than a dozen diguanylate cyclases leads to the expectation that it could be involved in the maintenance of intracellular c-di-GMP homeostasis. However it is hard to predict how much of an effect transcriptional regulation alone will have on c-di-GMP levels because the majority of c-di-GMP metabolic proteins contain sensory domains which control the activity of their enzymatic domain (Romling et al., 2013). For example, if the enzymatic activity of a diguanylate cyclase requires activation by a specific stimulus, and that stimulus is not present, then the transcriptional repression of that diguanylate cyclase by TfoY would have no effect on intracellular c-di-GMP. So far, our measurements of c-di-GMP concentration during TfoY overexpression have not been conclusive (data not shown). Complicating these matters is the difficulty in attributing changes in global c-di-GMP to individual diguanylate cyclases. At this point, it is hard to verify the degree to which TfoY activation of GG1 and repression of EAL 3 and EAL 9 could compensate for the repression of the fourteen other diguanylate cyclases. Currently the most reliable biochemical method for measuring c-di-GMP, HPLC-mass spectroscopy, can only be used to determine an average global concentration from an extract of a large population of cells (Waters, 2010).

A long-term challenge in the study of *tfoY* will be parsing out how it can regulate a particular phenotype at high c-di-GMP, but not at low c-di-GMP, or vice versa. For instance, we have now discovered that TfoY overexpression can stimulate foam production, and foam

production appears to be a high-c-di-GMP phenotype. The most likely explanation for foaming is the presence of a secreted factor in high c-di-GMP and high TfoY cultures which is absent under normal conditions. The recent finding that TfoY is a primary regulator of Type-VI secretion might lend support to that hypothesis (Metzger et al., 2016). However, that study actually reported that Type-VI secretion was enhanced during phosphodiesterase expression but not during diguanylate cyclase overexpression, implying that Type-VI secretion is exclusively a low-c-di-GMP phenotype of TfoY (Metzger et al., 2016). It is unclear how these phenotypes can be locked to only one c-di-GMP state or the other when we know TfoY is highly expressed and functional during both. To help solve this problem with regard to foam, we sought to identify genes essential for foam production, and devised a microplate assay for scoring foam volume which we could use to screen a library of *V. cholerae* transposon mutants. However, after screening upwards of 4,000 mutants we were unable to find any which had reliably lost the ability to produce foam during TfoY overexpression (data not shown). This could indicate that foam production is a multifactorial phenotype, such that there are multiple inputs downstream of TfoY and disruption of a single one cannot stop foam production. It is likely that TfoY regulation of motility and biofilm formation will turn out to be just as complex.

## FINAL CONCLUSIONS

A riboswitch and its downstream gene are traditionally thought of as an indivisible genetic unit. That philosophy relies on a simple genetic model in which there is a single promoter, a single riboswitch and a single gene. The Vc2-*tfoY* relationship is not that simple. There are four promoters for *tfoY*, and they further separate into two behavioral groups. Each native regulatory element we identified that impacts *tfoY* expression is able to discriminate between these two groups. c-di-GMP activates the downstream promoters and represses an upstream one. VpsR activates the downstream promoters and represses an upstream one. cAMP and CRP allow expression from the downstream promoters and reduce expression from the upstream ones.

Riboswitches are inherently binary and designed to allow gene expression only under a single, specific scenario. Ligand is present - gene is on; ligand is absent - gene is off. Or vice versa. But *V. cholerae* appears to need *tfoY* under both conditions, at both extremes of ligand concentration. So it has evolved to use an RNA factor, the Vc2 riboswitch, at one end of the spectrum to sense when signal concentration has dropped too low, and it uses a protein factor, VpsR, at the other end of the spectrum to sense when signal concentration has risen too high.

The regulation at low c-di-GMP supports the traditional riboswitch model, that Vc2 and *tfoY* are indivisible. *tfoY* is important for motility under this condition, and disruption of c-di-GMP binding at the Vc2 riboswitch disrupts motility nearly as much as the outright deletion of *tfoY*. However, the regulation at high c-di-GMP does not support the traditional riboswitch model. Under this condition *tfoY* is important for regulation of c-di-GMP metabolic enzymes, foam production, and likely biofilm formation as well. Deletion of *tfoY* prevents the repression

of *VCA0697* by c-di-GMP. Disruption of c-di-GMP binding at the Vc2 riboswitch has no effect on repression of *VCA0697*. Deletion of *tfoY* greatly reduces foam production by c-di-GMP. Disruption of c-di-GMP binding at the Vc2 riboswitch has minimal effect on foam production.

At high c-di-GMP the Vc2 riboswitch is not simply decoupled from regulating *tfoY*. It has its own separate responsibility, which is the regulation of the upstream sRNA sequence. Without c-di-GMP, the Vc2-sRNAs are not expressed. Without the ligand binding sites of the Vc2 aptamer, the Vc2 sRNAs are not expressed. Because of the riboswitch, the Vc2-sRNAs are highly stable and only become more so as the concentration of ligand increases. While it is still unknown what function the Vc2-sRNAs serve within the cell, their discovery and regulation advances our understanding beyond the traditional model of how riboswitches can work.

**APPENDIX**

**Table A-1: Strain List**

| Name | Genotype | Chapter | Reference |
|---|---|---|---|
| BH1514 | *vpsL*$^{+}$ | 5 | Waters *et al*., 2008 |
| BP20 | *ΔvpsL ΔVc2* | 3 | this study |
| BP25 | *Δ*vpsL *Δ*tfoY | 4 | this study |
| BP32 | *vpsL*$^{+}$ *ΔtfoY* | 5 | this study |
| BP33 | *ΔvpsL* Vc2 (G83U) | 3 | this study |
| BP34 | *ΔvpsL* Vc2 (C92U) | 3 | this study |
| BP35 | *ΔvpsL* Vc2 (G20U,C92U) | 3,4 | this study |
| BP36 | *ΔvpsL ΔcyaA* | 5 | this study |
| BP57 | *ΔvpsL ΔflaAC* | 4 | this study |
| BP58 | *ΔvpsL ΔflaEDB* | 4 | this study |
| BP59 | *ΔvpsL ΔflaABCDE* | 4 | this study |
| BP60 | *ΔvpsL* FlrA (R176H) | 4 | this study |
| BP62 | *ΔvpsL* FlrA (R176H) *ΔtfoY* | 4 | this study |
| BP64 | *ΔvpsL* FlrA (R176H) Vc2 (G20U,C92U) | 4 | this study |
| BP69 | *ΔvpsL* FlrA (R176H) *ΔtfoY lacZ::tfoY* | 4 | this study |
| BP70 | *ΔvpsL* FlrA (R176H) Vc2 (G20U,C92U) *lacZ::tfoY* | 4 | this study |
| CW2034 | *ΔvpsL* | 3,4,5 | Waters *et al*., 2008 |
| DS05 | *ΔvpsL Δcrp* | 5 | this study |
| WN310 | *ΔvpsL ΔvpsR* | 5 | Srivastava *et al*., 2011 |

**Table A-2: Vector and Primer List**

For each vector constructed for this study, sequences are given for primers used in PCR to generate vector inserts. For primer sequences, restriction endonuclease sites are indicated by underline.

| Name | Description | Chapter | Reference |
|---|---|---|---|
| pCMW75 | **$P_{tac}$-QrgB (pEVS141 backbone)** | 3,4,5 | Waters *et al*., 2008 |
| pCMW98 | **$P_{tac}$-QrgB* (pEVS141 backbone)** | 3,4,5 | Waters *et al*., 2008 |
| pBRP102 | **$P_1$-TS; EMSA probe $P_1$ template**<br>*Fwd-5'-ATCAGGTACCGCAGAATACTTCTCTCCACC-3'*<br>*Rev-5'-ATCACTCGAGCGTTATGAATTGTCAAGAAAGTT-3'* | 3,4,5 | this study |
| pBRP34 | **$P_2$-TS**<br>*Fwd-5'-ATCAGGTACCTATATTTGAAAGCTTGTCAC-3'*<br>*Rev-5'-ATCACTCGAGCCTGATAAAAATTAATCATTCTTGG-3'* | 3,4,5 | this study |
| pBRP50 | **$P_3$-TS; RNA probe $P_2$ template; EMSA probe $P_3$ template**<br>*Fwd-5'-ATCAGGTACCGCTCATTCTCACATTTGAAATA-3'*<br>*Rev-5'-ATCACTCGAGCTGTGCGTGACATTTTTCCTG-3'* | 3,4,5 | this study |
| pBRP35 | **$P_4$-TS; EMSA probe $P_4$ template**<br>*Fwd-5'-ATCAGGTACCACACTTTGTTGACTCATCATTG-3'*<br>*Rev-5'-ATCACTCGAGCTGTTAGTCTCGGAGTATTG-3'* | 3,4,5 | this study |
| pBRP67 | **$P_1$-TL, Vc2 (WT)**<br>*Fwd-5'-ATCAGGTACCGCAGAATACTTCTCTCCACC-3'*<br>*Rev-5'-ATCAGCTAGCTTTTAATACTGGTTTATCCATGCTGT TAGTCTCGGAGTATTG-3'* | 3,4 | this study |
| pBRP107 | **$P_1$-TL, Vc2 (C92U)**<br>*Mutagenesis-5'-AGCGGGGTTATCGATGGCAA-3'* | 3,4 | this study |
| pBRP94 | **$P_1$-TL, Vc2 (G20U,C92U)**<br>*Mutagenesis-5'-ACGCACAGTGCAAACCATTC-3'* | 3,4 | this study |
| pBRP95 | **$P_3$-TL, Vc2 (WT); $P_3$-TL (used during 5'-R.A.C.E.)**<br>*Fwd-5'-ATCAGGTACCGCTCATTCTCACATTTGAAATA-3'*<br>*Rev-5'-ATCAGCTAGCTTTTAATACTGGTTTATCCATGCTGT TAGTCTCGGAGTATTG-3'* | 3,4 | this study |
| pBRP71 | **$P_2$-TS VpsR binding site deletion; RNA probe $P_1$ template;**<br>**EMSA probe $P_3$ template**<br>*Fwd-5'-ATCAGGTACCTATATTTGAAAGCTTGTCAC-3'*<br>*Rev-5'-ATCACTCGAGCCCTACTAATAATACTCGCAC-3'* | 3,5 | this study |
| pBRP91 | **$P_3$-TS VpsR binding site deletion; RNA probe $P_2$ template; EMSA probe $P_3$ template**<br>*Fwd-5'-ATCAGGTACCGTGAGAATGACCCAAGAATG-3'*<br>*Rev-5'-ATCACTCGAGCTGTGCGTGACATTTTTCCTG-3'* | 3,5 | this study |
| pMMB67EH | **empty vector control** | 4,5 | Fürste *et al*., 1986 |
| pEVS141 | **protein overexpression control** | 3 | Dunn *et al*., 2006 |
| pBRP31 | **promotorless GFP control** | 3 | this study |
| PBRP33 | **$P_{1+2}$-TS (used during 5'-R.A.C.E.)**<br>*Fwd-5'-ATCAGGTACCGCAGAATACTTCTCTCCACC-3'*<br>*Rev-5'-ATCACTCGAGCCTGATAAAAATTAATCATTCTTGG-3'* | 3 | this study |
| pBRP27 | **RNA probe Vc2 template**<br>*Fwd-5'-ATCAGGTACCAGGAAAAATGTCACGCAC-3'*<br>*Rev-5'-ATCACTCGAGCAAAGTGTATGCATTTTGC-3'* | 3 | this study |

**Table A-2 (cont'd)**

| Name | Description | Chapter | Reference |
|---|---|---|---|
| pBRP86 | **RNA probe $P_{1+2}$ template**<br>*Fwd-5'-ATCAGGTACCTATATTTGAAAGCTTGTCAC-3'*<br>*Rev-5'-ATCACTCGAGCTGTGCGTGACATTTTTCCTG-3'* | 3 | this study |
| pBRP78 | **PCR template for in vitro transcription of *C. difficile* class-II c-di-GMP riboswitch**<br>*Fwd-5'- ATCAGGTACCATCTTATATCTAAGAATATGGA AATATTG-3'*<br>*Rev-5'-ATCACTCGAGCTAATACTCTTATTTTCAAATTT TGCAAC -3'* | 3 | this study |
| pBRP172 | **allelic exchange vector, Vc2 (WT);**<br>**PCR template for in vitro transcription**<br>*Fwd-5'-ATCAGGTACCTCGCGACCAAATATC-3'*<br>*Rev-5'- ATCAGAGCTCCAATGTTTACCTTCGATTGC-3'* | 3 | this study |
| pBRP177 | **allelic exchange vector, Vc2 (G83U);**<br>**PCR template for in vitro transcription**<br>*Mutagenesis-5'-TGGTAGGTATCGGGGTTAC-3'* | 3 | this study |
| pBRP178 | **allelic exchange vector, Vc2 (C92U)**<br>*Mutagenesis-5'-AGCGGGGTTATCGATGGCAA-3'* | 3 | this study |
| pBRP179 | **allelic exchange vector, Vc2 (G20U,C92U);**<br>**PCR template for in vitro transcription**<br>*Mutagenesis-5'-ACGCACAGTGCAAACCATTC-3'* | 3 | this study |
| pCMW121 | **$P_{tac}$-VC1086 (pEVS141 backbone)** | 4 | Waters *et al.*, 2008 |
| pCMW126 | **$P_{tac}$-VC1086* (pEVS141 backbone)** | 4 | Waters *et al.*, 2008 |
| pBRP333 | **empty vector control (pEVS141 backbone)** | 4 | this study |
| pBRP127 | **$P_{tac}$-TfoY (pEVS141 backbone)**<br>*Fwd-5'-ATCAGAATTCAGGAGCTAAGGAAGCTA AAATGGATAAACCAGTATTAAAAG-3'*<br>*Rev-5'-ATCAGGATCCTCAGAGATGCCTTAATAGCTC-3'* | 4 | this study |
| pBRP183 | **$P_1$-TL, Vc2 (C17U)**<br>*Mutagenesis-5'-ATGTCACGCATAGGGCAAAC-3'* | 4 | this study |
| pBRP184 | **$P_1$-TL, Vc2 (G20A)**<br>*Mutagenesis-5'-ACGCACAGAGCAAACCATTC-3'* | 4 | this study |
| pBRP185 | **$P_1$-TL, Vc2 (G20C)**<br>*Mutagenesis-5'-ACGCACAGCGCAAACCATTC-3'* | 4 | this study |
| pBRP133 | **$P_1$-TL, Vc2 (G20U)**<br>*Mutagenesis-5'-ACGCACAGTGCAAACCATTC-3'* | 4 | this study |
| pBRP160 | **$P_1$-TL, Vc2 (C44U)**<br>*Mutagenesis-5'-AGAGTGGGATGCAAAGCCT-3'* | 4 | this study |
| pBRP159 | **$P_1$-TL, Vc2 (A47C)**<br>*Mutagenesis-5'-GAGTGGGACGCCAAGCCTC-3'* | 4 | this study |
| pBRP161 | **$P_1$-TL, Vc2 (G83A)**<br>*Mutagenesis-5'-TGGTAGGTAACGGGGTTAC-3'* | 4 | this study |
| pBRP68 | **$P_1$-TL, Vc2 (G83U)**<br>*Mutagenesis-5'-TGGTAGGTATCGGGGTTAC-3'* | 4 | this study |
| pBRP187 | **$P_1$-TL, Vc2 (C92G)**<br>*Mutagenesis-5'-AGCGGGGTTAGCGATGGCAA-3'* | 4 | this study |
| pBRP188 | **$P_1$-TL, Vc2 (tetraloop-GUAA)**<br>*Mutagenesis-5'-AACCATTCGTAAGAGTGGGA-3'* | 4 | this study |

**Table A-2 (cont'd)**

| Name | Description | Chapter | Reference |
|---|---|---|---|
| pBRP189 | ***$P_1$*-TL, Vc2 (tetraloop-UUCG)**<br>*Mutagenesis-5'-GCAAACCATTCTTCGGAGTGGGACGCA-3'* | 4 | this study |
| pBRP108 | ***$P_3$*-TL, Vc2 (C92U)**<br>*Mutagenesis-5'-AGCGGGGTTATCGATGGCAA-3'* | 4 | this study |
| pBRP97 | ***$P_3$*-TL, Vc2 (G20U,C92U)**<br>*Mutagenesis-5'-ACGCACAGTGCAAACCATTC-3'* | 4 | this study |
| pBRP195 | ***$P_3$*-TL, Vc2 (C17U)**<br>*Mutagenesis-5'-ATGTCACGCATAGGGCAAAC-3'* | 4 | this study |
| pBRP196 | ***$P_3$*-TL, Vc2 (G20A)**<br>*Mutagenesis-5'-ACGCACAGAGCAAACCATTC-3'* | 4 | this study |
| pBRP197 | ***$P_3$*-TL, Vc2 (G20C)**<br>*Mutagenesis-5'-ACGCACAGCGCAAACCATTC-3'* | 4 | this study |
| pBRP134 | ***$P_3$*-TL, Vc2 (G20U)**<br>*Mutagenesis-5'-ACGCACAGTGCAAACCATTC-3'* | 4 | this study |
| pBRP164 | ***$P_3$*-TL, Vc2 (C44U)**<br>*Mutagenesis-5'-AGAGTGGGATGCAAAGCCT-3'* | 4 | this study |
| pBRP163 | ***$P_3$*-TL, Vc2 (A47C)**<br>*Mutagenesis-5'-GAGTGGGACGCCAAGCCTC-3'* | 4 | this study |
| pBRP165 | ***$P_3$*-TL, Vc2 (G83A)**<br>*Mutagenesis-5'-TGGTAGGTAACGGGGTTAC-3'* | 4 | this study |
| pBRP96 | ***$P_3$*-TL, Vc2 (G83U)**<br>*Mutagenesis-5'-TGGTAGGTATCGGGGTTAC-3'* | 4 | this study |
| pBRP199 | ***$P_3$*-TL, Vc2 (C92G)**<br>*Mutagenesis-5'-AGCGGGGTTAGCGATGGCAA-3'* | 4 | this study |
| pBRP200 | ***$P_3$*-TL, Vc2 (tetraloop-GUAA)**<br>*Mutagenesis-5'-AACCATTCGTAAGAGTGGGA-3'* | 4 | this study |
| pBRP201 | ***$P_3$*-TL, Vc2 (tetraloop-UUCG)**<br>*Mutagenesis-5'-GCAAACCATTCTTCGGAGTGGGACGCA-3'* | 4 | this study |
| pLLP15 | ***$P_{tac}$*-FlrA (pEVS141 backbone)** | 4 | Srivastava *et al*., 2013 |
| pDS49 | ***flrB* transcriptional reporter** | 4 | Srivastava *et al*., 2013 |
| pDS## | ***fliE* transcriptional reporter** | 4 | Srivastava *et al*., 2013 |
| pDS73 | ***flgB* transcriptional reporter** | 4 | Srivastava *et al*., 2013 |
| pDS72 | ***flaA* transcriptional reporter** | 4 | Srivastava *et al*., 2013 |
| pDS74 | ***flgM* transcriptional reporter** | 4 | Srivastava *et al*., 2013 |
| pBRP361 | ***flaB* complementation**<br>*Fwd-5'-ACCTCTCGAGTATCGATGTGACTTCAGTTGG-3'*<br>*Rev-5'-ACCTGGATCCAATCCAGCCGAAGCTGGATT-3'* | 4 | this study |
| pBRP362 | ***flaC* complementation**<br>*Fwd-5'-ACCTCTCGAGACGTGGGTATGTAATGAAGG-3'*<br>*Rev-5'-ACCTGGATCCTTCCTATCAACTCGAACTAGC-3'* | 4 | this study |
| pBRP363 | ***flaD* complementation**<br>*Fwd-5'-ACCTCTCGAGTATGTCGATGGGCATCGCT-3'*<br>*Rev-5'-ACCTGGATCCAATCCAGCCGAAGCTGGATT-3'* | 4 | this study |
| pBRP364 | ***flaE* complementation**<br>*Fwd-5'-ACCTCTCGAGATGGTACAACGCTTTGTGC-3'*<br>*Rev-5'-ACCTGGATCCAGTTAGAGACGAAGTCGAGC-3'* | 4 | this study |

**Table A-2 (cont'd)**

| Name | Description | Chapter | Reference |
|---|---|---|---|
| pBRP365 | ***flaEDB* complementation**<br>*Fwd-5'-ACCTCTCGAGATGGTACAACGCTTTGTGC-3'*<br>*Rev-5'-ACCTGGATCCAATCCAGCCGAAGCTGGATT-3'* | 4 | this study |
| pBRP307 | **$P_{tac}$- TfoY (pMMB67EH backbone)**<br>*Fwd-5'-ATCAGAATTCAGGAGCTAAGGAAGCTA AAATGGATAAACCAGTATTAAAAG-3'*<br>*Rev-5'-ATCAGGATCCTCAGAGATGCCTTAATAGCTC-3'* | 5 | this study |
| pBRP1 | **$P_{tac}$- QrgB* (pMMB67EH backbone)** | 5 | Srivastava *et al*., 2013 |
| pBRP2 | **$P_{tac}$- QrgB (pMMB67EH backbone)** | 5 | Srivastava *et al*., 2013 |
| pBRP175 | **$P_{tac}$-VpsR, QrgB (pEVS141 backbone)** | 5 | this study |
| pCMW131 | **$P_{tac}$-VpsR (pEVS141 backbone)** | 5 | Srivastava *et al*., 2011 |
| pMLH17 | **$P_{ara}$-VpsR** | 5 | this study |
| pBRP174 | **$P_{tac}$-CRP (pEVS141 backbone)**<br>*Fwd-5'-ATCAGAATTCAGGAGCTAAGGAAGCTA AAATGGTTCTAGGTAAACCTCAAAC-3'*<br>*Rev-5'-ATCAGGATCCTTATCGGGGCACTTAGCGAG-3'* | 5 | this study |

**Table A-3: Miscellaneous DNA Sequences**

| Description | Sequence | Reference |
|---|---|---|
| Primer Extension Biotinylated Primer | 5'- /Biotin/-CACAAGCGCAAACATAGTTTCGTC | this study |
| 3'-R.A.C.E. adapter | 5'-P-*TCTAGAGGCCTGAATTCTCGAGCATGC*-idT-3' | this study |
| Primers for *in vitro* Transcription Template of Vc2 Region | *Fwd-5'-ATCAGGTACCGCAGAATACTTCTCTCCACC-3'*<br>*Rev-5'-ATCACTCGAGCTTCAGGTAGAGCGTAGTAC-3'* | this study |
| Primers for *in vitro* Transcription Template of *C. difficile* Riboswitch | *Fwd-5'-ATCAGGTACCATCTTATATCTAAGAATATGGA AATATTG-3'*<br>*Rev-5'-ATCACTCGAGCTAATACTCTTATTTTCAAATTT TGCAAC -3'* | this study |

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