

**NON-CODING SMALL RNAS REGULATE MULTIPLE MRNA
TARGETS TO CONTROL THE *VIBRIO CHOLERAE*
QUORUM SENSING RESPONSE**

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To Ting, Mom, Dad, and my dearest Grandma.

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LIST OF SYMBOLS AND ABBREVIATIONS

AI	autoinducer
AI-2	autoinducer-2
Amp	ampicillin
Bap1	biofilm-associated protein 1
bp	base pair
CAI-1	cholera autoinducer-1
CT	cholera toxin
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
DNA	deoxyribonucleic acid
DGC	diguanylate cyclase
EMSA	electrophoretic mobility shift assay
GFP	green fluorescent protein
HCD	high cell density
IPTG	isopropyl- β -D-thiogalactopyranoside
Kan	kanamycin
LCD	low cell density
LB	Luria-Bertani broth
mM	millimolar
μ M	micromolar
mL	milliliter
nt	nucleotide
OD	optical density
PCR	polymerase chain reaction
PDE	phosphodiesterase
Qrr	quorum regulatory RNA
QS	Quorum sensing
RBS	ribosome binding site
RbmA	rugosity and biofilm structure modulator A
RLU	relative light units
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
sRNA	small ribonucleic acid
TCP	toxin-coregulated pilus
UTR	untranslated region
VPS	<i>Vibrio</i> exopolysaccharides
WT	wild type

SUMMARY

The waterborne bacterial pathogen *Vibrio cholerae* uses a process of cell-to-cell communication called quorum sensing (QS) to coordinate transcription of four sRNAs (Qrr1-4; quorum regulatory RNAs) in response to changes in extracellular QS signals that accumulate with cell density. Genetic evidence indicates that the Qrr sRNAs regulate gene expression post-transcriptionally by altering the translation of several mRNA targets to which they are predicted to bind. The Qrr sRNAs are predicted to negatively control translation of several mRNAs, including *hapR*, which encodes the master QS transcription factor that controls genes for virulence factors, biofilm formation, protease production, and DNA uptake. The Qrr sRNAs are also predicted to positively control *vca0939*, which encodes a GGDEF family protein that promote biofilm formation by elevating intracellular levels of the second messenger molecule c-di-GMP. Using complementary *in vivo*, *in vitro*, and bioinformatic approaches, I showed that Qrr sRNAs base-pair with and repress translation of the mRNA encoding HapR. A single nucleotide mutation in Qrr RNA abolishes *hapR* pairing and thus prevents cholera toxin production and biofilm formation that are important in disease, and also alters expression of competence genes required for uptake of DNA in marine settings. I also demonstrated that base-pairing of the Qrr sRNAs with *vca0939* disrupts an inhibitory structure in the 5' UTR of the mRNA. Qrr-activated translation of *vca0939* was sufficient to promote synthesis of c-di-GMP and early biofilm formation in a HapR-independent manner. Thus, these studies define the non-coding Qrr sRNAs as a critical component allowing *V. cholerae* to sense and respond to environmental cues to regulate important developmental processes such as biofilm formation.

CHAPTER 1

INTRODUCTION

1.1. *Vibrio cholerae*, a marine bacterium and human pathogen

Members of the genus *Vibrio*, including *Vibrio cholerae*, are indigenous inhabitants of marine and brackish water environments, where they exist in both a free living state and in association with marine animals and plants (Thompson *et al.*, 2006). *V. cholera* is also an important human pathogen responsible for pandemic outbreaks of the fatal diarrheal disease cholera in developing countries where inadequate sanitation leads to consumption of contaminated water (Sack *et al.*, 2004). Like all bacteria, *Vibrios* sense numerous environmental inputs and respond by regulating expression of genes tailored to particular conditions. Members of the genus *Vibrio* use a cell-cell communication process called quorum sensing (QS) to monitor cell density of the bacterial population (Ng & Bassler, 2009). This is achieved because QS bacterial populations produce and then respond to chemical signal molecules called autoinducers (AIs) that accumulate in proportion to the population density. The response to AIs is transduced to downstream transcriptional regulators to alter gene expression. Thus, global QS allows bacterial populations to behave like multi-cellular organisms (Bassler & Losick, 2006). Much of what is known about the *Vibrio* QS system has been described in *V. cholerae*, the focus of this dissertation.

Vibrio cholerae achieves QS using two autoinducers (AIs); CAI-1 and AI-2. CAI-1, a (S)-3-hydroxytridecan-4-one, is made exclusively by *Vibrios* and acts as an intraspecies signal (Henke & Bassler, 2004). AI-2, a furanosyl borate diester, is produced by hundreds

of bacterial species and facilitates inter-species communication between *Vibrios* and other bacteria (Xavier & Bassler, 2005a, Federle & Bassler, 2003, Chen *et al.*, 2002). *V. cholerae* uses these extracellular AIs to coordinate the expression of >100 genes in response to changes in the bacterial population density (Zhu *et al.*, 2002c, Miller *et al.*, 2002, Yildiz *et al.*, 2004, Hammer & Bassler, 2007, Hammer & Bassler, 2009b). Numerous studies have detailed the role of *V. cholerae* QS in the regulation of genes important in both the environmental and host niches occupied by this microbe (for review see (Ng & Bassler, 2009)). These studies support a model that, following ingestion by humans, *V. cholerae* colonizes the small intestine, and secretes factors at low cell density (low AI levels) that promote virulence; namely the cholera toxin (CT), toxin coregulated pilus (TCP), and biofilm-promoting *Vibrio* exopolysaccharide (VPS) (Hammer & Bassler, 2003, Miller *et al.*, 2002, Zhu *et al.*, 2002c). Once at high cell density when AIs are at high levels, biofilm and virulence factor expression is greatly reduced while expression of a putative detachment factor (HapA) is induced that promotes transmission of *V. cholerae* back into the environment (Higgins *et al.*, 2007, Zhu & Mekalanos, 2003, Kovacikova & Skorupski, 2002, Kovacikova *et al.*, 2003, Waters *et al.*, 2008a, Liu *et al.*, 2008, Hammer & Bassler, 2009b). QS regulation is critical for virulence factors expression *in vivo* as described in more detail below. In the marine settings, accumulation of AIs at high cell density, such as in biofilms, triggers expression of genes that promote natural competence for the uptake of extracellular DNA (Meibom *et al.*, 2005, Udden *et al.*, 2008, Antonova & Hammer, 2011). Incorporation of the acquired DNA, the process of natural transformation, is one mechanism for horizontal transfer of genetic material between bacteria. Thus QS is thought to play critical roles for *V. cholerae* in both clinical and marine settings.

1.2. Regulation of the *Vibrio cholerae* Quorum-Sensing System

The *Vibrio cholerae* QS response is initiated in response to recognition of secreted AI signals (Ng and Bassler, 2009). At low cell density (LCD), in the absence of CAI-1 and AI-2, the cognate membrane bound two-component sensors (CqsS and LuxP/Q, respectively) autophosphorylate and transfer phosphate to the phosphotransfer protein LuxU (Fig. 1.1A), which in turn transfers phosphate to the response regulator LuxO. LuxO~P, in conjunction with the alternate sigma factor, σ^{54} , activates the transcription of genes encoding four small regulatory RNAs (sRNAs) called Qrr1-4 for Quorum Regulatory RNAs (Bassler *et al.*, 1993b, Bassler *et al.*, 1994a, Bassler *et al.*, 1994b, Freeman & Bassler, 1999a, Freeman & Bassler, 1999c, Freeman *et al.*, 2000, Miller *et al.*, 2002). The Qrr sRNAs, along with the sRNA chaperone, Hfq, are thought to destabilize the mRNA of *hapR*, preventing translation of the transcription factor, HapR (Lenz *et al.*, 2004, Svenningsen *et al.*, 2008). Recently, *vca0939*, which is predicted to encode a GGDEF protein that participates in the synthesis of the intracellular second messenger molecule bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) and in biofilm formation, was found to be activated by the Qrr sRNAs (Hammer & Bassler, 2007). The Qrr sRNAs also repress *luxO*, the Qrr sRNA activator, forming a LuxO-Qrr feedback loop to precisely calibrate the level of Qrr activity and ensure proper timing of QS regulated behaviors (Svenningsen *et al.*, 2009). Finally, the Qrr sRNAs positively regulate translation of the gene encoding AphA, a positive regulator of virulence gene expression (Rutherford *et al.*, 2011). Thus, at LCD when the Qrrs are transcribed, LuxO protein levels are repressed, AphA is activated, Vca0939 is made, and HapR protein is not produced.

At high cell density (HCD), the binding of AIs to their cognate sensors switches the two-component sensors from acting as kinases to acting as phosphatases (Fig. 1.1B).

Phosphate is removed from LuxO, via LuxU, and dephosphorylated LuxO no longer activates *qrr* transcription. In the absence of the Qrr sRNAs, *luxO* is no longer repressed, *vca0939* is not translated, and *hapR* mRNA is translated into HapR protein. HapR was shown to bind directly to and represses the promoter of *aphA*, the product of which is required for expression of the cholera toxin (Miller et al., 2002) and toxin co-regulated pilus genes (Kovacikova et al., 2004, Kovacikova & Skorupski, 2001, Kovacikova & Skorupski, 2002, Zhu & Mekalanos, 2003, Sack et al., 2004, Faruque et al., 2003). HapR also indirectly represses the expression of the *vps* biofilm biosynthesis genes that are predicted to play a role in biofilm formation and intestinal attachment (Hammer & Bassler, 2003, Hammer & Bassler, 2009b, Waters et al., 2008b, Zhu & Mekalanos, 2003). Finally, HapR also activates transcription of *hapA*, which encodes the HapA protease proposed to aid in detachment from the intestine (Finkelstein et al., 1992, Jobling & Holmes, 1997). In marine biofilms, HapR indirectly activates transcription of *comEA*, which encodes a DNA binding protein required for DNA uptake in marine settings (Meibom et al., 2005, Antonova & Hammer, 2011).

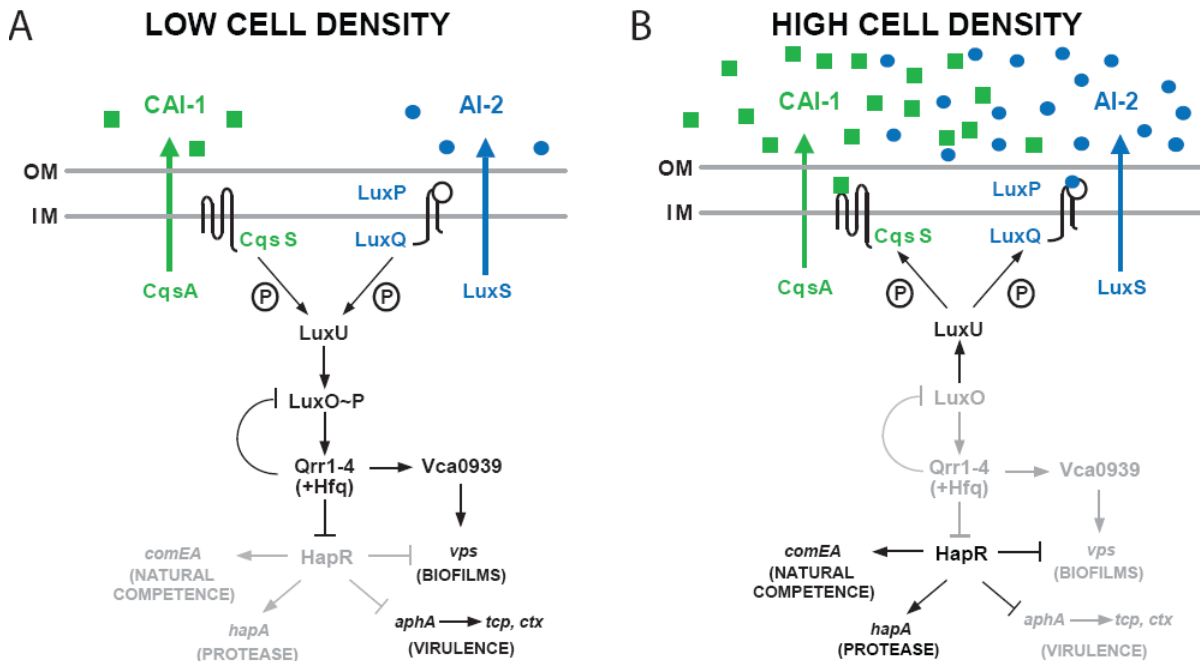


Figure. 1.1 The *V. cholerae* quorum sensing Circuit. Squares and circles represent CAI-1 and AI-2, respectively.

Several QS mutants display characteristics that “lock” *V. cholerae* into a state that mimics a particular cell density. A *V. cholerae luxO* D47E mutant strain is “locked” at LCD for it carries an active allele of *luxO* that mimics LuxO~P, and therefore constitutively expresses the *qrr* genes, which repress *hapR* mRNA (Fig. 1.1). A $\Delta hapR$ mutant, which produces no HapR, is also “locked” in the LCD mode. Conversely, a $\Delta luxO$ mutant is “locked” in the HCD mode because the *qrr* genes are not expressed, and HapR is produced constitutively. These mutants serve as useful tools to characterize QS target genes as QS-activated or QS-repressed.

Remarkably, although *V. cholera* encodes four Qrrs, the sRNAs appear to be functionally redundant. Previous analyses of single, double, triple, and quadruple *qrr* gene deletion mutations showed that *V. cholerae* strains with deletions of any three of the *qrr* genes (containing only one of the Qrrs) express the canonical HapR-activated luciferase operon of bioluminescent Vibrio, *Vibrio harveyi*, in a density dependent manner, like a WT *V. cholerae* strain (Lenz et al., 2004). Only the $\Delta qrr1-4$ strain is locked at HCD and constitutively produces HapR like the *luxO* strain (Lenz et al., 2004, Svenningsen et al., 2009). Demonstration of functional redundancy has allowed for *in vivo* studies of Qrr control using a single sRNA expressed in both *E. coli* and *V. cholerae* (Hammer & Bassler, 2007, Svenningsen et al., 2009, Tu et al., 2008).

Many *Vibrios*, including the human pathogens *V. vulnificus* and *V. parahaemolyticus*, utilize a QS system with multiple Qrrs and a circuit similar to that of *V. cholerae* (Fig 1). Importantly, all of the ~100 nt pathogenic *Vibrio* Qrr homologs have an absolutely conserved 21nt region, which in *V. cholerae* is predicted to interact with each mRNA target (Hammer & Bassler, 2007, Svenningsen et al., 2009, Tu et al., 2008, Lenz et al.,

2004). Although this region was predicted to be essential for each Qrr/mRNA base-pairing, it has not been determined experimentally and is the focus of this dissertation.

1.3. Regulatory mechanisms of sRNAs

The *V. cholerae* Qrrs are members of a class of sRNAs first described in *E. coli* and since identified in a wide range of bacteria that act by base-pairing with target mRNAs to modify mRNA translation or stability (Majdalani *et al.*, 2005). Bacteria encode both cis-acting and trans-acting non-coding RNAs. *Cis*-acting sRNAs are perfectly complementary to an mRNA target expressed on the opposite strand of the DNA. In contrast, the *trans*-acting sRNAs are only partially complementary to one or more mRNA targets encoded elsewhere in the genome. Unlike *cis*-encoded sRNAs, the Qrrs are *trans*-encoded RNAs, which share only limited complementarity with their target mRNAs (Aiba, 2007). *Trans*-encoded sRNAs, like the Qrrs, typically base-pair with multiple mRNAs by making limited contacts with target mRNAs in discontinuous patches, and thus necessitating the assistance of the RNA chaperone protein, Hfq (Valentin-Hansen *et al.*, 2004, Jousselin *et al.*, 2009). Hfq is thought to promote sRNA/mRNA base-pairing by protecting RNAs from degradation, recruiting both sRNAs and their mRNA targets, and remodeling RNA structure (Moller *et al.*, 2002). The dependence of the *V. cholerae* Qrrs on Hfq is reflected in the identical “locked” HCD phenotype observed with $\Delta qrr1-4$ and $\Delta luxO$ mutant strains, as described above (Lenz *et al.*, 2004).

Bacterial Hfq-dependent *trans*-encoded sRNAs can both positively and negatively impact translation of target genes. Generally, sRNAs regulate negatively by binding to the 5' untranslated region (UTR) of mRNAs and most often occlude the ribosome binding site (RBS), leading to repression of protein levels through translational inhibition, mRNA degradation, or both (Aiba, 2007, Gottesman, 2005, Sharma *et al.*, 2007, Prevost *et al.*,

2007, Urban & Vogel, 2008). Genetic evidence supports this regulatory mechanism for Qrr/hapR interactions in *V. cholerae*; as a $\Delta qrr1-4$ mutant expresses HapR constitutively, while a strain constitutively producing the Qrr sRNAs makes no HapR (Lenz, et al, 2004). In contrast, sRNAs may also act as post-transcriptional activators that release self-repression of certain mRNA targets, which can form a stem loop in the 5' UTR blocking ribosome access (Brown & Elliott, 1997). Prior studies supported a model that Qrr/vca0939 interaction in *V. cholerae* follow a similar regulatory mechanism (Hammer & Bassler, 2007). Specifically, constitutive Qrr expression allows for vca0939 expression, while a $\Delta qrr1-4$ strain does not express vca0939 (Hammer & Bassler, 2007).

In *E. coli*, to validate predicted sRNA/mRNA interaction *in vivo*, a system utilizing two plasmids is often used (Mandin & Gottesman, 2009). Here, the sRNAs are expressed from one plasmid under a strong constitutive promoter, for example, P_{Tac}. Plasmid-borne translational gene fusions to the *gfp* gene, encoding Green Fluorescent Protein, are commonly used to assay post-transcriptional regulation of the target gene. Using these two plasmids, single nucleotide substitutions can be easily introduced in both the sRNA and mRNA target. For *in vitro* study of sRNA/mRNA binding, expression plasmids are also used to transcribe RNAs. These biochemical experiments include determination of binding affinity by electrophoretic mobility shift assay (EMSA) of *in vitro* transcribed RNAs. Such approaches have successfully demonstrated the regulation of mRNA targets by sRNAs in *E. coli* (Urban & Vogel, 2007, Mandin & Gottesman, 2009), however, such methods have not been utilized to document binding of Qrr RNA to any of the four target genes (*hapR*, *vca0939*, *luxO*, and *aphA*) predicted by genetic analyses to interact with the Qrr sRNAs. Similar, it has not been determined in *V. cholerae* whether these direct Qrr/mRNA interactions control the phenotypes regulated by the mRNA target, for example HapR-dependent phenotypes, as well as HapR-independent phenotypes potentially controlled by *vca0939*.

1.4. Quorum sensing and cyclic diguanylate (c-di-GMP) regulates

Vibrio cholerae biofilm formation

Vibrio cholerae is found naturally in environmental aquatic reservoirs either existing as a member of the planktonic community or attached as a biofilm to biotic or abiotic surfaces, such as shellfish, aquatic plants, and chitinous surfaces of copepods and other zooplankton (Faruque *et al.*, 1998). Biofilm formation plays an important role for survival of *V. cholerae* in the environment and is also thought to contribute during association with the human host (Watnick & Kolter, 1999, Yildiz & Schoolnik, 1999) (White, 1938). The biofilm mode of growth is now thought to be the preferred lifestyle of microbes since attached bacteria are more resistant to antibiotics; immune clearance; and osmotic, acid, and oxidative stresses as compared with planktonic cells (Leid *et al.*, 2002, Zhu & Mekalanos, 2003, Costerton *et al.*, 1999, Hall-Stoodley & Stoodley, 2005, Parsek & Singh, 2003, Stewart & Costerton, 2001, Mah *et al.*, 2003, Anderson & O'Toole, 2008, Reisner *et al.*, 2005).

Biofilm formation begins with the transport and attachment of bacteria to surfaces. In *V. cholerae*, flagella are involved in initial stages of biofilm formation. Loss of flagellar genes generally results in decreased attachment, indicating that flagella promote not only motility but also participation in interactions with a surface (Watnick & Kolter, 1999, Watnick *et al.*, 2001, Lauriano *et al.*, 2004). Recently, three matrix proteins (Bap1, RbmA, and RbmC), which contain carbohydrate-binding domains, have been identified that are involved in biofilm formation (Fong & Yildiz, 2007, Berk *et al.*, 2012, Fong *et al.*, 2006, Fong *et al.*, 2010). Bap1 (biofilm-associated protein 1; VC1888) aids in initial adherence to surfaces, which contributes to early colony corrugation (Fong & Yildiz, 2007, Berk *et al.*, 2012). After initial attachment, cells secrete RbmA (rugosity and biofilm structure modulator A), which accumulates to facilitate cell-cell adhesion and

colonization of a surface. Following the formation of initial cell clusters, *V. cholerae* continue to produce RbmC and Bap1, which allow the formation of envelopes that encapsulate cell clusters, which are often surrounded by extrapolymeric substances (Moorthy & Watnick, 2005). Further growth of bacteria and continued production of *Vibrio* polysaccharide (VPS) lead to the development of mature biofilms (Watnick & Kolter, 1999, Yildiz & Schoolnik, 1999). VPS (*Vibrio* polysaccharide), encoded by the *vps* genes, is a major component of mature *Vibrio cholerae* biofilms. The *vps* genes, which are organized into two clusters (*vpsA-K* and *vpsL-Q*) on the large chromosome, along with the matrix proteins, are required for the generation of two morphologically different variants, termed smooth and rugose (Yildiz & Schoolnik, 1999). Cells in the mature biofilms can return to the planktonic stage through detachment, thus completing the cycle of biofilm development (Watnick & Kolter, 1999, O'Toole *et al.*, 2000), although the detachment process is still poorly understood.

In *V. cholerae*, biofilm formation is usually regulated by a regulatory pathway that includes quorum sensing and signaling via the intracellular second messenger cyclic di-GMP (Bis-(3'-5')-cyclic dimeric guanosine monophosphate) (Galperin, 2004, Hengge, 2009). Expression of the *vps* genes and the switch between smooth and rugose colony morphology are positively regulated by the transcriptional regulators VpsR and VpsT, which are both homologous to two-component response regulators (Yildiz *et al.*, 2001, Casper-Lindley & Yildiz, 2004). Quorum sensing controls the transition from a planktonic to biofilm-forming lifestyle through two major regulators, HapR, the QS master regulator, and AphA, which controls gene expression at low cell density when HapR is absent. HapR represses VpsT by directly binding to the *vpsT* promoter. AphA, which is repressed by HapR at high cell density, positively regulates VpsR (Waters *et al.*, 2008b, Casper-Lindley & Yildiz, 2004). Thus, at low cell density, VpsT and VpsR are expressed to induce the VPS production (Beyhan *et al.*, 2007). Additional regulatory

mechanisms remain to be discovered as VpsT and VpsR are also positively regulated by the increase in c-di-GMP.

Intracellular c-di-GMP, predicted to be present in 85% of all bacteria, is believed to play an important role in the transition between a planktonic and sessile lifestyle (Galperin, 2004, Hengge, 2009). C-di-GMP is synthesized from two molecules of GTP by diguanylate cyclase (DGC) enzymes containing an approximately 170-amino-acid-long protein domain, GGDEF (or GGEEF) (Kulasakara *et al.*, 2006), named after the amino acids conserved in the active site of the enzyme (Ryjenkov *et al.*, 2005). C-di-GMP degradation is catalysed by phosphodiesterase (PDE) enzymes that have either an HD-GYP or EAL domains, which are approximately 250 amino acids in length (Schmidt *et al.*, 2005) (Fig. 1.2). These domains are often found in association with other signaling domains that may directly sense environmental cues. Thus far, the sensory domains identified include various transmembrane or periplasmic domains that may be involved in sensing small molecules. PAS domains, for example, sense flavin or haem and may also sense molecular oxygen, light or redox potential (Qi *et al.*, 2009, Chang *et al.*, 2001). In some cases, multiple sensory input domains are found on the same protein (Bernier *et al.*, 2011, Carlson *et al.*, 2010, Kanazawa *et al.*, 2010, Karatan *et al.*, 2005, Merritt *et al.*, 2007, Tuckerman *et al.*, 2009). *Vibrio cholerae* encodes 40 predicted GGDEF domains, 20 EAL domains, and 9 HD-GYP domains, although some may be enzymatically inactive (Galperin, 2004). The multiple inputs observed in c-di-GMP signaling networks have led to questions regarding whether this system functions via high or low specificity, i.e., whether c-di-GMP exists within the cell as a single general pool, or in several discrete pools, or both. The available evidence supports a model that within the same cell, several c-di-GMP pools may act together to regulate some common phenotypes, while other phenotypes may be under control of a dedicated regulatory pathway (Massie *et al.*, 2012). Strategies that could accommodate this model include spatial separation of signaling

systems, where the genes are only expressed in a certain cellular location, or temporal separation of signaling systems, where the structural genes are only expressed during certain growth phases (Tuckerman *et al.*, 2011, Abel *et al.*, 2011, Paul *et al.*, 2004, Christen *et al.*, 2010).

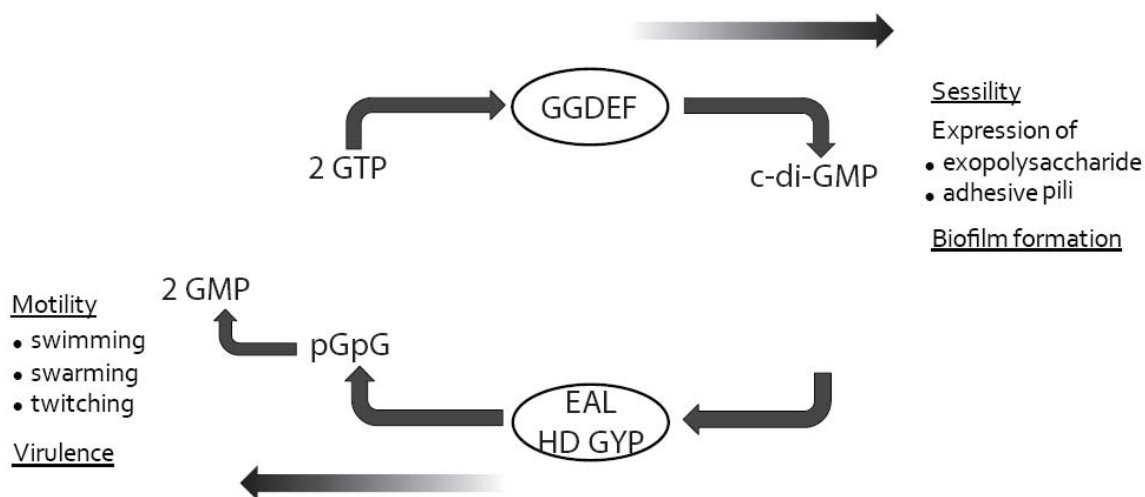


Figure. 1.2 Regulatory mechanism of c-di-GMP metabolism and signalling. Adapted from Romling & Amikam. *Curr Opin Microbiol* 2006 9:218-228.

In *V. cholerae*, quorum sensing and c-di-GMP signaling are integrated to allow information about local cell density to be merged with other environmental cues to control biofilm formation. At high cell density, expression of HapR indirectly represses *vpsT* gene expression by regulating the transcription of 14 different GGDEFs and EALs, which in turn lead to a decrease in c-di-GMP levels and lower *vps* expression (Waters *et al.*, 2008b, Casper-Lindley & Yildiz, 2004). QS also converges with the c-di-GMP signaling machinery to control the expression of AphA and VpsT. Activation of transcription of both *aphA* and *vpsT* by c-di-GMP requires the transcriptional activator VpsR, which binds to c-di-GMP. AphA expression is repressed by HapR by direct binding of the *aphA* promoter at high cell density. Interestingly, this binding site for HapR overlaps with the binding site of VpsR in the *aphA* promoter (Srivastava *et al.*, 2011, Waters *et al.*, 2008b). Similarly, VpsT is also regulated by HapR and VpsR using

overlapping DNA binding sites (Srivastava & Waters, 2012). QS control of c-di-GMP levels may also occur independently of HapR. The Qrr sRNAs directly activate expression of the gene for a putative GGDEF protein, Vca0939, which is consistent with a high intracellular concentration of c-di-GMP at low cell density (Hammer & Bassler, 2007). Recently, overexpression of several DGCs was shown to promote biofilm formation in *V. cholerae*, and *vca0939* expressed from an inducible promoter and lacking its native 5'UTR was able to increase biofilm formation. It remains to be determined whether Qrr expression can activate translation of Vca0939 protein, whether Vca0939 (which is annotated as a “GGDEF family member) indeed has DGC activity that can produce c-di-GMP, and whether Qrr-activated Vca0939 can promote biofilm formation.

1.5. Significance

In this dissertation, I focus on defining the molecular mechanisms that the Qrr sRNAs use for base-pairing with the mRNA targets, *hapR* and *vca0939*. Specifically I characterize single nucleotide substitutions within a *V. cholerae* Qrr sRNA that abolish regulation of each mRNA, and then measure the consequence to QS-regulated genes and corresponding phenotypes. In studying Vca0939, we also define a HapR-independent pathway that regulates biofilm formation through Qrr sRNAs, which is important for *V. cholerae* to thrive both in the human host and in the natural environment. Because sRNAs are ubiquitous regulators of fundamental developmental processes in bacteria and higher organisms, defining the molecular mechanisms by which these sRNAs act in *V. cholerae* will have fundamental implications for understanding regulatory circuits of all cellular systems. Bacteria with environmental, clinical, and industrial significance also use QS to control the expression of a diverse range of gene products (Miller et al., 2002, Zhu et al., 2002c, Hammer & Bassler, 2003, Zhu & Mekalanos, 2003). Thus, these QS studies may lead to the development of pro- and anti-QS strategies for the production

of useful products. For example, it may be possible to rationally engineer QS systems to promote the production of commercially important products by beneficial QS bacteria, or prevent infection by harmful QS bacteria. Successful translation of fundamental research to applied product development will require a thorough dissection of the QS regulatory pathways.

CHAPTER 2

THE *VIBRIO CHOLERAE* QUORUM SENSING RESPONSE

IS MEDIATED BY HFQ-DEPENDENT SRNA/MRNA

BASE-PAIRING INTERACTIONS

2.1. Summary

Vibrio cholerae quorum sensing controls expression of four redundant sRNAs, Qrr1-4. The Qrr sRNAs are predicted to alter the translation of several mRNAs, including, *hapR*, which encodes a transcription factor that controls genes for virulence factors, biofilm formation, protease production, and DNA uptake. Each Qrr contains a 21 nucleotide region absolutely conserved among pathogenic *Vibrios*, and predicted to base-pair with mRNA targets, like *hapR*, aided by the RNA chaperone Hfq. This molecular mechanism was not experimentally tested previously, and we provide here both *in vivo* and *in vitro* evidence to validate this model. In *Escherichia coli*, Qrr expression repressed a HapR-GFP translational fusion, and a specific nucleotide substitution in the 21 nucleotide region eliminated HapR control, while a compensatory mutation in *hapR* restored it. In *V. cholerae*, the identical mutations also deregulated HapR-dependent gene expression and corresponding QS phenotypes by altering HapR protein levels. We calculated *in vitro* binding affinities of a Qrr/*hapR* complex and show that Hfq stabilizes complex formation. Finally, the Qrr mutation with *in vivo* defects also prevented Qrr/*hapR* binding, while the compensatory *hapR* mutation restored binding. These results demonstrate that the *V. cholerae* QS response is mediated by base-pairing interactions between Qrr sRNAs and *hapR* mRNA.

2.2. Introduction

Bacteria communicate with one another by small secreted signal molecules, called autoinducers (AIs), using a process termed quorum sensing (QS). Because the levels of AIs increase in proportion to bacterial density, when the AIs reach a threshold concentration the bacterial population responds by synchronously altering gene expression. A variety of group behaviors are regulated by QS, including bioluminescence, biofilm formation, sporulation, DNA uptake and virulence (Ng & Bassler, 2009), suggesting that QS allows bacterial populations to behave like multicellular organisms.

Vibrio cholerae is a marine bacterium that grows primarily in brackish water in both a free living state and in association with marine animals and plants (Thompson & Swings, 2006). When ingested by humans, *V. cholerae* is able to colonize the small intestine, where it secretes the potent cholera toxin (CT) that causes the potentially fatal cholera diarrhea (Sack et al., 2004). Recently, it has been shown that QS in *V. cholerae* controls expression of numerous traits, including several virulence factors. Multiple studies support a model that *V. cholerae* enters the small intestine at low cell density and expresses factors that promote virulence; namely CT, the colonization factor called the toxin co-regulated pilus (TCP), and biofilm-promoting *Vibrio* exopolysaccharides (VPS) (Hammer & Bassler, 2003, Miller et al., 2002, Zhu *et al.*, 2002b, Zhu & Mekalanos, 2003). AI accumulation at high cell density triggers repression of CT and attachment factors, suggesting that modulation of *V. cholerae* QS may be a potential therapeutic strategy (Duan & March, 2010, Higgins et al., 2007). High levels of AI also stimulate the production of the secreted hemagglutinin/protease, HapA, which promotes detachment of *V. cholerae* from intestinal cells, and is thought to enhance the transmission of the organism from the host back into the environment (Finkelstein et al., 1992).

The components of the *V. cholerae* quorum sensing system are conserved among many members of the genus *Vibrio*. Specifically, *V. cholerae* produces two different AI signaling molecules called CAI-1 and AI-2 (Xavier & Bassler, 2005a, Chen et al., 2002, Henke & Bassler, 2004, Higgins et al., 2007, Miller et al., 2002). At low cell density, in the absence of CAI-1 and AI-2 signals, the two cognate periplasmic two-component sensors, CqsS and LuxP/Q, respectively, autophosphorylate and transfer phosphate to a response regulator LuxO, through the LuxU phosphotransfer protein (Bassler et al., 1993b, Bassler et al., 1994a, Bassler et al., 1994b, Freeman & Bassler, 1999a, Freeman & Bassler, 1999c, Freeman et al., 2000, Miller et al., 2002). Phosphorylated LuxO (LuxO-P), along with the sigma factor, σ^{54} , activates the transcription of four regulatory small RNAs (sRNAs), called Qrr1-4 (quorum regulatory RNAs) (Lenz et al., 2004). Genetic studies support the hypothesis that the Qrr sRNAs repress the translation of the transcription factor HapR, presumably by binding to the 5' untranslated region (5'-UTR) of *hapR* mRNA and occluding the ribosome binding site (Lenz et al., 2004) (Fig. 2.1A). This process requires the RNA chaperone Hfq (Lenz et al., 2004), which has also been shown to facilitate sRNA/mRNA interactions in *Escherichia coli* (Sledjeski et al., 2001, Lease & Woodson, 2004, Udekwu et al., 2005, Updegrave et al., 2008, Soper et al., 2010) and other bacteria (Vogel, 2009). The levels of *hapR* mRNA and the Qrr sRNAs are inversely related, suggesting that the Qrr sRNAs may also facilitate the degradation of *hapR* mRNA by cellular RNases (Svenningsen et al., 2008). The Qrr sRNAs alter translation of targets in addition to *hapR* mRNA. Specifically, the Qrr sRNAs negatively regulate the mRNA of *luxO*, establishing a feedback loop to control Qrr production (Svenningsen et al., 2009). The Qrr sRNAs also positively regulate the mRNA of *vca0939*, a gene involved in regulating levels of the secondary signaling molecule cyclic di-GMP (Hammer & Bassler, 2007). Finally, the Qrr sRNAs activate production of AphA, a positive regulator of virulence gene expression (Rutherford et al., 2011).

At high cell density, in the presence of the AIs, the flow of phosphate through the quorum sensing system is reversed. AI binding converts the cognate sensors from kinases to phosphatases, which removes phosphate from LuxO, preventing activation of *qrr* transcription (Freeman & Bassler, 1999a, Freeman et al., 2000, Lenz et al., 2004, Miller et al., 2002). In the absence of sRNAs, *hapR* mRNA is stabilized and translated. The transcription factor, HapR, activates and represses multiple genes, including regulators of virulence and biofilm formation. Specifically, HapR controls virulence regulation by repressing transcription of the *aphA* gene, which encodes the AphA activator of the *toxT* regulon that upregulates CT and TCP (Miller et al., 2002, Zhu et al., 2002b, Kovacikova & Skorupski, 2002, Kovacikova et al., 2004, Kovacikova & Skorupski, 2001). HapR also indirectly represses transcription of the *vpsL-N* exopolysaccharide biosynthesis operon by binding directly to the promoter of the biofilm transcription factor *vpsT* and also by altering the intracellular levels of cyclic di-GMP (Hammer & Bassler, 2003, Hammer & Bassler, 2009a, Waters et al., 2008b). Thus, at high cell density, both virulence and biofilm formation are repressed, presumably because no Qrr sRNAs are present to bind *hapR* mRNA. Consistent with this model, strains of *V. cholerae* with mutations that prevent expression of the Qrr sRNAs are unable to form biofilms, produce no TCP or CT, and are avirulent in infant mouse colonization assays (Hammer & Bassler, 2003, Miller et al., 2002, Zhu et al., 2002b, Zhu & Mekalanos, 2003). Recent studies have shown that AI accumulation in environmental biofilms leading to increased HapR levels also promotes natural competence of *V. cholerae* by inducing the competence regulatory gene, *comEA* which is required for uptake of extracellular DNA (Meibom et al., 2005) (Antonova and Hammer, 2011).

V. cholerae-like QS systems in many pathogenic *Vibrios*, such as *V. vulnificus* and *V. parahaemolyticus*, include orthologs of the four Qrr sRNAs (Lenz et al., 2004, Tu & Bassler, 2007, Zuker, 2003). Importantly, each of the *Vibrio* Qrrs possesses an absolutely

conserved 21 nucleotide sequence, which is predicted to facilitate imperfect base-pair interactions with the 5'-UTR of each Qrr target gene identified thus far, including *hapR* (Fig. 2.1B). Each pathogenic *Vibrio* encodes four or five Qrr sRNAs, and in *V. cholerae*, the four Qrr sRNAs are functionally redundant; a mutant possessing only one of the Qrr sRNAs is able to engage in QS-like behavior like the isogenic wild type strain (Lenz et al., 2004). This is due to multiple regulatory feedback loops that serve to normalize the total amount of Qrr sRNAs in the cell (Svenningsen et al., 2009, Svenningsen et al., 2008, Lin et al., 2005, Rutherford et al., 2011).

While genetic data support a model that RNA duplex formation between a Qrr and *hapR* mRNA, with the assistance of Hfq, is required for repression of *hapR* translation and alteration of HapR-dependent phenotypes in *V. cholerae*, this has not been tested experimentally. Here we define a single nucleotide substitution within a *V. cholerae* Qrr sRNA that prevents repression of *hapR* translation and as a consequence, alters HapR-regulated genes and corresponding phenotypes. QS-dependent gene expression and behaviors are restored by a compensatory mutation in the 5'-UTR of *hapR*. Additionally, we show *in vitro* that Qrr RNA forms a complex with *hapR* RNA that is stabilized by the RNA chaperone Hfq. The Qrr mutation also inhibits the binding of the sRNA to the *hapR* RNA target, which is fully restored with a compensatory mutation in the *hapR* mRNA target. We predict that the Qrr sRNA nucleotides defined here may also play a role in duplex formation with other recently described target RNAs that participate in the QS response.

2.3. Results

2.3.1. *In vivo* identification of nucleotides important for Qrr/hapR regulation

Previous genetic studies in *V. cholerae* support a model that at low cell density, the Qrr sRNAs, along with the sRNA chaperone, Hfq, destabilize the mRNA of *hapR* (Lenz et al., 2004, Svenningsen et al., 2008). Close inspection of the 5'-UTR of the *hapR* mRNA shows significant complementarity to an absolutely conserved 21 nucleotide region of the Qrr sRNAs (Fig. 2.1). To test the hypothesis that the Qrr sRNAs and *hapR* directly interact, we used a simplified *E. coli* system, which is uncoupled from the *V. cholerae* QS pathway and has been used previously to demonstrate the function of *Vibrio* sRNAs *in vivo* (Tu et al., 2010, Hammer & Bassler, 2007, Svenningsen et al., 2009). Specifically in *E. coli*, we expressed from one plasmid a single Qrr under control of a heterologous promoter and measured the effect on *hapR* mRNA using a HapR-GFP translational fusion encoded on another plasmid. In the absence of Qrr, *hapR-gfp* is maximally expressed, and when WT Qrr is present, *hapR-gfp* is repressed approximately 10-fold (Fig. 2.2, 1st and 2nd bars). A Qrr with a single nucleotide substitution at position 28 (Qrr_{A28C}), a position not predicted to base-pair with *hapR* (Fig. 2.1B), still repressed *hapR-GFP* like the WT Qrr (Fig. 2.2, 3rd bar). Nearly identical results were obtained with the other two substitutions at position 28 (Qrr_{A28U}, and Qrr_{A28G}) (data not shown). However, a Qrr with a mutation in a nucleotide that is predicted to base-pair with *hapR* mRNA, Qrr_{C30G} (Fig. 2.1B), no longer represses *hapR-gfp*. Indeed, *hap-gfp* levels in the presence of Qrr_{C30G} are comparable to conditions when no Qrrs are expressed (Fig. 2.2, compare 4th bar to 1st bar). To demonstrate that this loss in Qrr repression of HapR is due to impairment of Qrr/*hapR* mRNA base-pairing, we engineered a compensatory mutation in *hapR-gfp* fusion (*hapR_{G63C}*) (Fig. 2.1B) and introduced it into *E. coli* expressing either WT Qrr or Qrr_{C30G}. The WT Qrr was unable to repress *hapR_{G63C}-gfp* (Fig. 2.2, 5th bar), presumably due to a failure to bind to the mutated *hapR* mRNA. However, Qrr_{C30G} was able to

effectively repress *hapR_{G63C-gfp}* (Fig. 2.2, 6th bar). Together, these results in *E. coli* suggest that, in *V. cholerae*, *Qrr₂₈* is not important for *hapR* pairing, while base-pairing between *Qrr_{C30}* and *hapR_{G63}* is indispensable for *hapR* repression (Fig. 2.1).

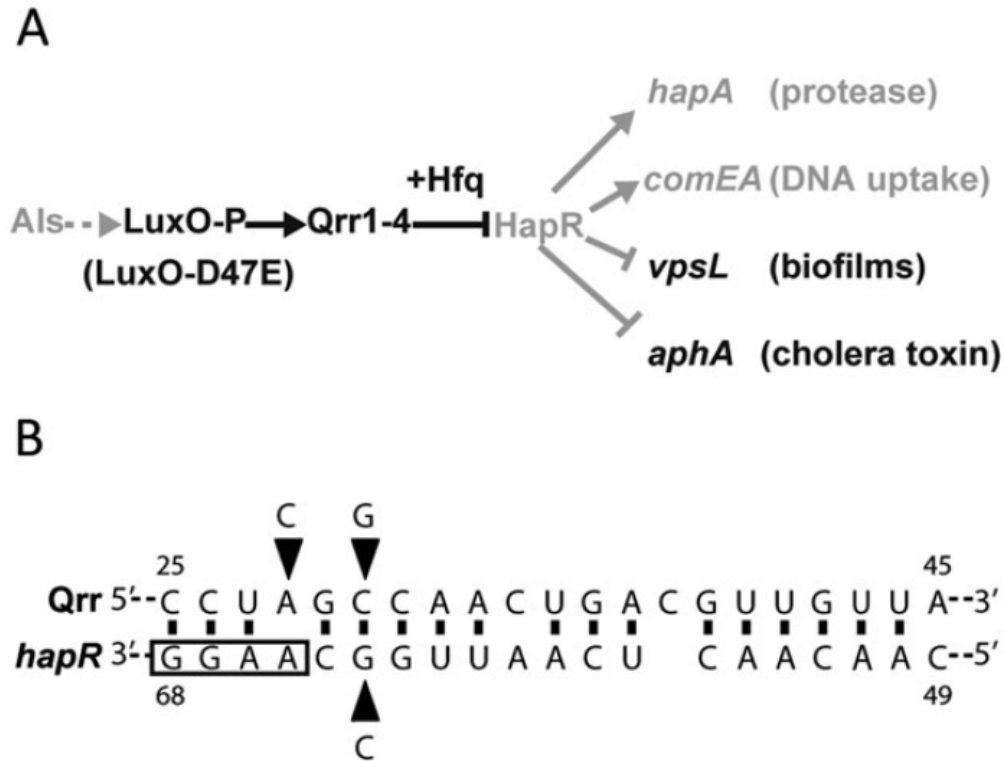


Figure 2.1. Model of HapR repression by the Qrr sRNAs at low cell density in the *V. cholerae* quorum sensing circuit. A. The lack of AIs at LCD results in phosphorylation of LuxO (mimicked by the *luxO* D47E allele), which activates transcription of the Qrr sRNAs. Qrr sRNA represses *hapR* translation and the absence of HapR alters expression of HapR-dependent genes and their associated phenotypes (in parentheses). B. Putative base-pairing between Qrr sRNAs to *hapR* mRNA. Predicted pairing of the absolutely conserved 21 nucleotide region of the Qrr sRNAs to the *hapR* 5'-UTR. Nucleotide positions are indicated relative to the +1 of transcription of each RNA. Substitutions made in this study are indicated. The boxed sequence is a portion of the *hapR* ribosome binding site.

2.3.2. Alteration of base-pairing between Qrr and *hapR* RNA alters HapR-dependent gene expression

The results from *E. coli* showed that Qrr_{C30G} was unable to repress WT HapR-GFP when both are expressed from multi-copy plasmids. Therefore, we next sought to determine Qrr/*hapR* base-pairing interactions in *V. cholerae* where the Qrr and *hapR* genes are each under control of its native promoter on the chromosome. Because, it has been shown previously in *V. cholerae* that a single chromosomally-encoded Qrr sRNA represses HapR as efficiently as all four Qrr sRNAs due to functional redundancy (Lenz et al., 2004), we used a single Qrr sRNA for our analyses in *V. cholerae*. Using standard genetic techniques we integrated either WT Qrr or the Qrr_{2C30G} mutant under control of the native *qrr2* promoter at the *lacZ* locus of a *V. cholerae* strain deleted for all four native Qrr sRNAs ($\Delta qrr1-4$). We also replaced the WT *luxO* allele in this strain with the *luxO* D47E allele that is “locked” at low cell density (Freeman & Bassler, 1999a, Lenz et al., 2004) permitting the constitutive expression of the Qrr (either WT or C30G) integrated at *lacZ* irrespective of AI levels. We then determined the effect of chromosomal sRNA expression on a variety of promoters known to be regulated by HapR in *V. cholerae* strains engineered to contain either WT *hapR* or *hapR*_{G63C} at the native *hapR* locus.

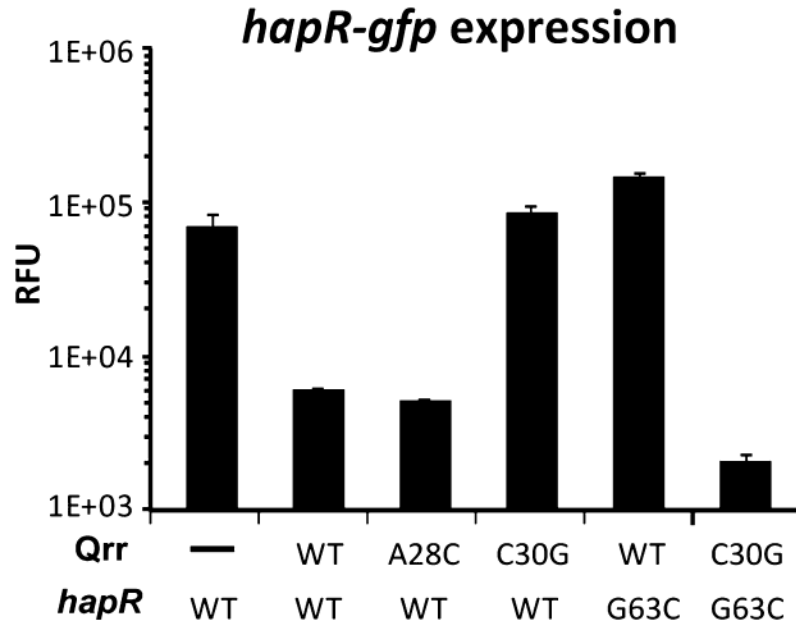
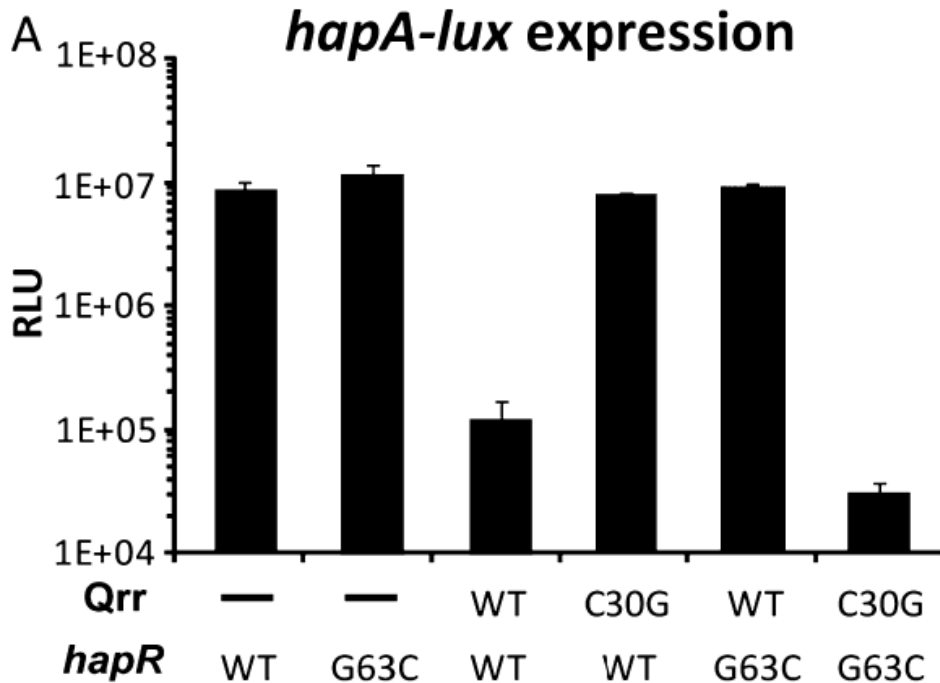
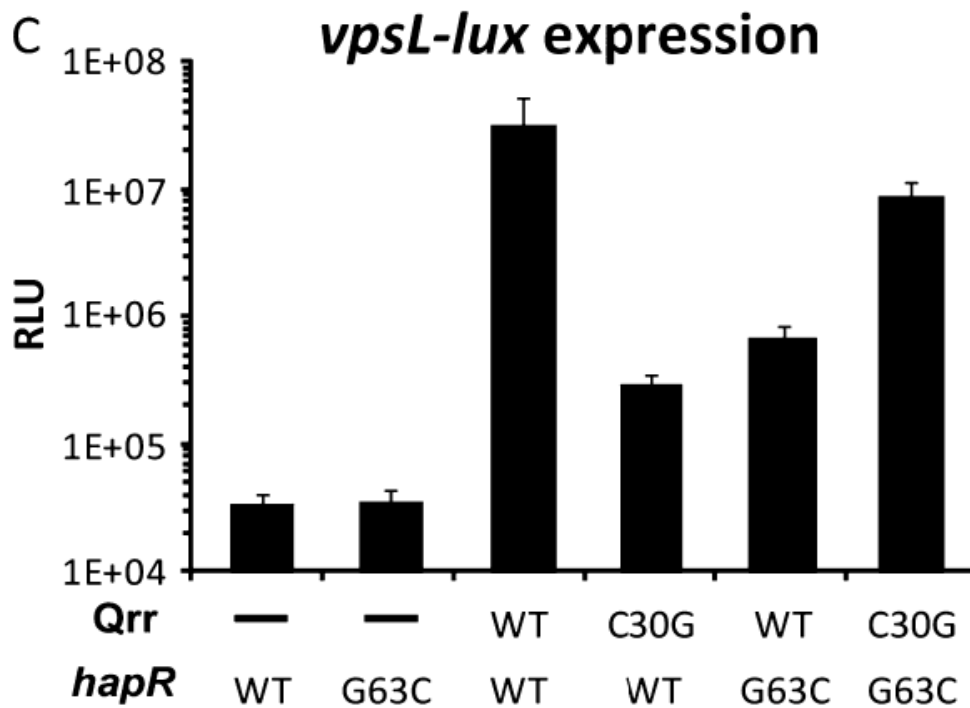
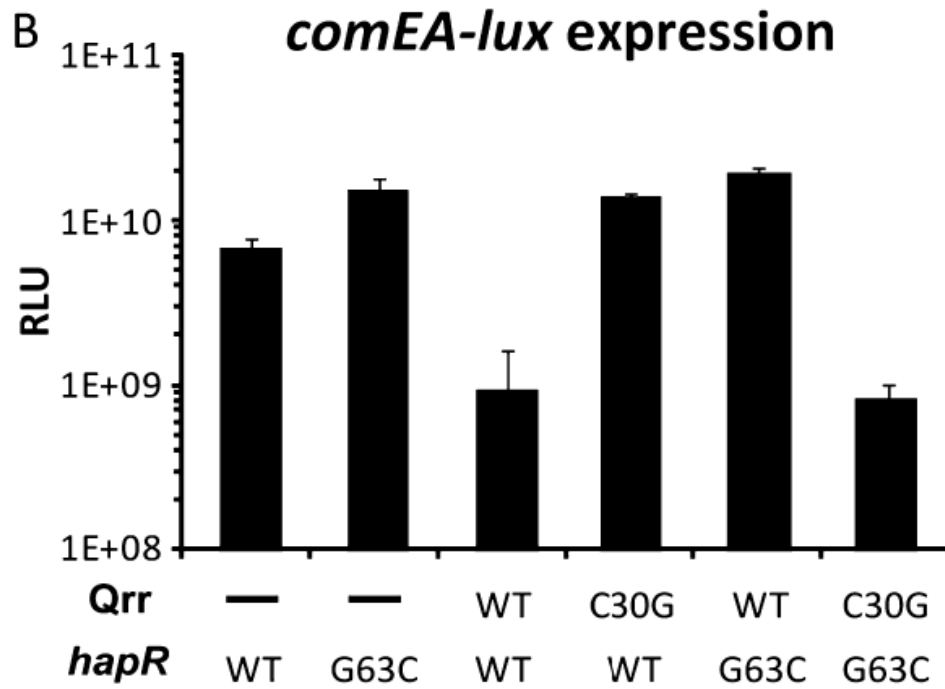


Figure 2.2. Effect of Qrr_{C30G} mutation on the expression of *hapR-gfp* in *E. coli*. *E. coli* strains expressing the indicated Qrr and *hapR-gfp* alleles were grown as triplicate cultures and analyzed for fluorescence using a multi-well plate reader. GFP expression is quantified as fluorescence per OD₆₀₀ (described in *Experimental Procedures*). Data shown are mean values +/- standard deviation for the triplicate cultures from one representative experiment of three performed.

To test the consequence of disruptions in Qrr/*hapR* pairing on the regulation of both direct and indirect targets of HapR, we used plasmid-borne transcriptional fusions of the promoters of *hapA*, *comEA*, *vpsL* and *aphA* to the luciferase gene (*lux*). Transcription of *hapA* and *comEA* are activated by HapR, while *vpsL* and *aphA* are repressed by HapR (Fig. 2.1A). Therefore, in a *V. cholerae* $\Delta qrr1-4$, *luxO* D47E mutant, *hapR* is constitutively expressed, so that *hapA* and *comEA* are expressed while *aphA* and *vpsL* are repressed (Fig. 2.3, A-D, 1st bar). The expression pattern of each *lux* fusion is unaltered when the *hapR* allele carries the G63C mutation (Fig. 2.3, A-D, 2nd bar). However, when the WT Qrr sRNA is expressed with WT *hapR*, the pattern of transcription for each reporters is reversed; *hapA* and *comEA* are repressed, while *aphA* and *vpsT* are expressed (Fig. 2.3, A-D, 3rd bars). Qrr_{C30G}, which can not repress WT *hapR* in *E. coli* when both were plasmid-encoded (Fig. 2.2), is also unable to repress *hapR* in *V. cholerae* (Fig. 2.3),

producing an expression pattern of the HapR-regulated reporters similar to that of a strain carrying no Qrr sRNA (Fig. 2.3, A-D, compare 1st and 4th bars). The *hapR*_{G63C} mutant cannot be repressed by WT Qrr as well, producing a similar pattern of expression seen when no Qrr is expressed (Fig. 2.3, A-D, compare 2nd and 5th bars). However, when both mutant alleles are expressed together, Qrr_{C30G} is able to repress *hapR*_{G63C} and proper regulation of each *lux* reporter is restored (Fig. 2.3 A-D, compare 3rd and 6th bars), presumably because the compensatory mutation in *hapR* (*hapR*_{G63C}) restores pairing with Qrr_{C30G} sRNA. These results are consistent with the *hapR-gfp* expression pattern seen in *E. coli* (Fig. 2.2) and suggest that Qrr/*hapR* base-pairing between Qrr_{C30} and *hapR*_{G63} is critical for mediating multiple QS-dependent regulatory pathways.





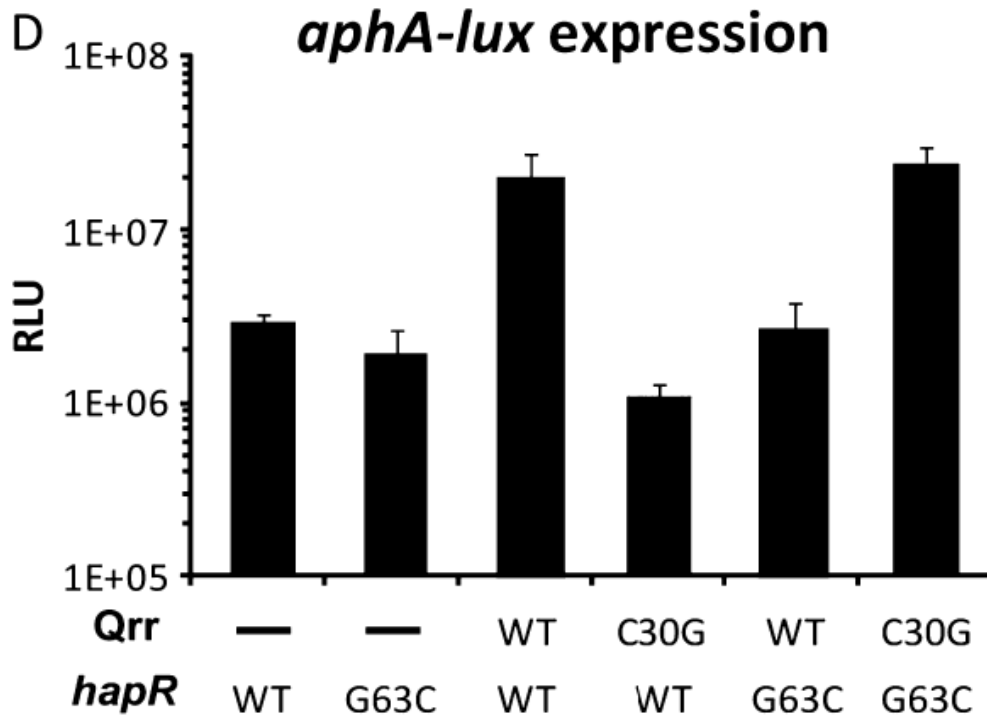


Figure 2. 3. Effect of Qrr_{C30G} mutation on HapR-regulated promoters. *V. cholerae* strains expressing the indicated Qrr and *hapR* were analyzed for expression of bioluminescence from *hapA-lux* (A), *comEA-lux* (B), *aphA-lux* (C), and *vpsL-lux* (D) transcriptional fusions. Bioluminescence is defined as relative light production per OD₆₀₀. Quadruplicate cultures were grown and analyzed for light. Data shown are mean values +/- standard deviation for the quadruplicate cultures from one representative experiment of three performed.

2.3.3. Alteration of base-pairing between Qrr and *hapR* RNA disrupts multiple *V. cholerae* phenotypes

The *V. cholerae* quorum sensing circuit regulates diverse phenotypes including extracellular protease production, natural competence, biofilm formation, and virulence (Fig. 2.1A) (Hammer & Bassler, 2003, Miller et al., 2002, Zhu et al., 2002b, Kovacicova & Skorupski, 2002, Meibom et al., 2005). Because the data described above indicate the Qrr_{C30}/*hapR*_{G63} pairing is critical for control of HapR-regulated promoters, we predicted that mutations disrupting Qrr/*hapR* pairing would manifest by changes in *V. cholerae* QS phenotypes. To test this hypothesis, we examined multiple *in vivo* phenotypes controlled

by QS-mediated regulation of HapR. Again, we exploited the *V. cholerae* $\Delta qrr1-4$, *luxO* D47E mutant “locked” at low cell density that expresses a single Qrr (either WT of the Qrr_{C30G}) and WT *hapR* or *hapR*_{G63C} as described above.

The first phenotype examined was the production of the secreted hemagglutinin/protease, HapA (the product of *hapA* described in Fig. 2.4A), which is upregulated by HapR (Jobling & Holmes, 1997). Cell-free supernatants of cultures were assayed for protease activity using a photometric azocasein assay described previously (Denkin & Nelson, 1999). Strains lacking any Qrr, but expressing either WT *hapR* or *hapR*_{G63C}, showed maximal protease activity (Fig. 2.4A, 1st and 2nd bar). Expression of WT Qrr resulted in minimal protease levels in the strain that expressed WT *hapR* (Fig. 2.4A, 3rd bar). However, Qrr_{C30G} was unable to lower protease production (Fig. 2.4A, 4th bar). Furthermore, *hapR*_{G63C} was unresponsive to WT Qrr, also showing the maximum level of protease production (Fig. 2.4A, 5th bar). Finally, the strain coexpressing both Qrr_{C30G} and *hapR*_{G63C} produced a similar level of protease as a strain expressing both WT RNA molecules (Fig. 2.4A, compare 3rd and 6th bars). Thus protease production requires proper Qrr/*hapR* mRNA base-pairing.

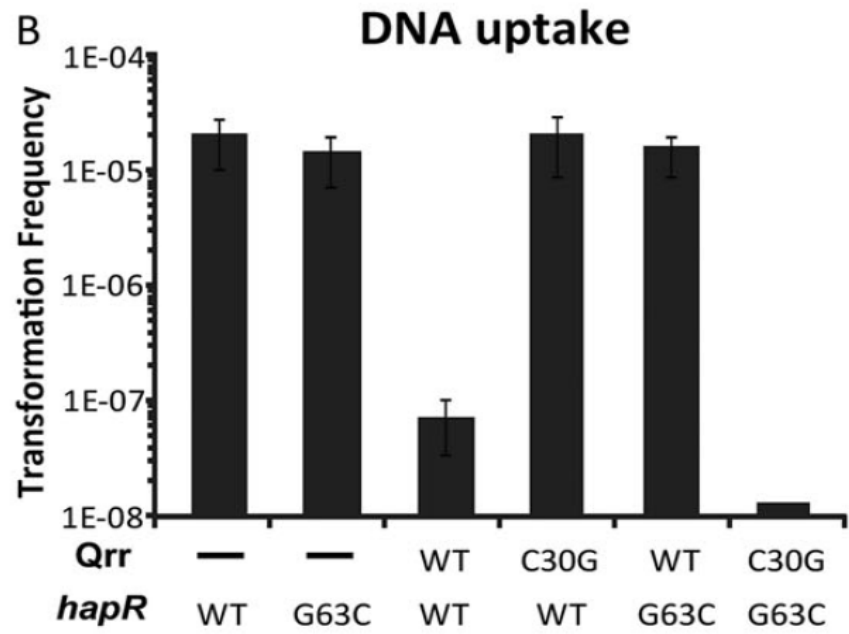
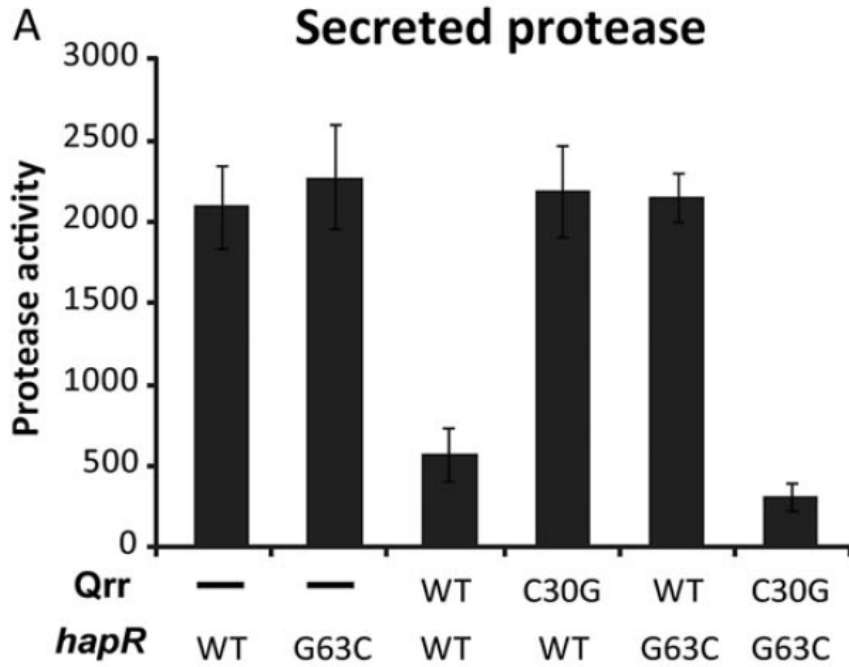
HapR is required for maximal expression of the competence gene, *comEA*, encoding a DNA-binding protein required for the uptake of extracellular DNA in response to QS AIs (Meibom et al., 2005) (Antonova & Hammer, 2011) Expression of *comEA* also requires activation by the regulator Tfox, which is induced in the presence of chitin, although it is currently unknown whether HapR and TfoX directly control *comEA* transcription and whether there are additional regulatory inputs to *comEA* not yet identified. To determine whether Qrr_{C30G} also disregulated natural competence in *V. cholerae*, we used a previously defined assay for *V. cholerae* natural competence (Meibom et al., 2005). Briefly, kanamycin-sensitive *V. cholerae* cells were incubated on crab shells in artificial

seawater in the presence of *V. cholerae* DNA containing a kanamycin resistance (*kanR*) marker. Following incubation, the cells were plated on LB containing kanamycin. Strains without Qrr sRNAs, but carrying WT *hapR* or *hapR*_{G63C} take up and integrate the marked DNA into their genomes at a frequency of $\sim 10^{-5}$, similar to previous *V. cholerae* studies (Meibom et al., 2005), and thus produce colonies on the antibiotic selection plates (Fig. 2.4B, 1st and 2nd bars). Production of the WT Qrr decreases the competence of the bacteria by approximately 1000-fold (Fig. 2.4B, 3rd bar). Strains expressing either Qrr_{C30G} in the presence of WT *hapR*, or WT Qrr in the presence of *hapR*_{G63C}, are also fully competent, comparable to levels when no Qrr is expressed (Fig. 2.4B, compare 4th and 5th bars). However, expression of both Qrr_{C30G} and *hapR*_{G63C} together decreases the competence of the bacteria to similar levels as the strain expressing both wild type alleles (Fig. 2.4B, 4th and 6th bars). No kanamycin resistant colonies were obtained in the absence of marked DNA (data not shown).

HapR controls biofilm formation by repressing the *vpsT* transcription factor and controlling expression of genes for products that alter intracellular c-di-GMP second messenger molecules (Hammer & Bassler, 2003, Waters et al., 2008b, Hammer & Bassler, 2009a). Based on the *V. cholerae* QS model (Fig. 2.1), biofilm formation requires Qrr-dependent repression of *hapR* translation to permit expression of the *vpsL-N* exopolysaccharide biosynthesis genes. To determine whether Qrr_{C30G} altered biofilm production, we incubated strains of *V. cholerae* statically at room temperature for 24 hours, and observed biofilms that adhered to the sides of the test tubes and formed a pellicle at the air–broth interface. In the absence of Qrr, no biofilms are formed (Fig. 2.4C, 1st and 2nd tubes) as observed previously for strains making no Qrr sRNAs (Hammer & Bassler, 2003, Zhu et al., 2002b). Strains expressing WT Qrr and WT *hapR* produce a visible pellicle, indicating significant biofilm formation (Fig. 2.4C, 3rd tube). However, strains expressing Qrr_{C30G} with WT *hapR* or WT Qrr, with *hapR*_{G63C}, form no

visible biofilms (Fig. 2.4C, 4th and 5th tubes), consistent with constitutive *hapR* translation in these cells. Biofilm formation was restored upon expression of both Qrr_{C30G} and *hapR*_{G63C}, resulting in a strain that behaves like WT, producing a visible pellicle (Fig. 2.4C, 6th tube).

V. cholerae virulence expression is regulated by many sensory inputs, including QS (Zhu et al., 2002b, Higgins et al., 2007). HapR protein represses *aphA*, an activator of the ToxT regulon that promotes CT production, thus strains with no Qrr sRNAs constitutively express *hapR* and do not produce CT (Higgins et al., 2007, Zhu et al., 2002b). To determine whether Qrr_{C30G} regulates CT production we measured the levels of CT in cell-free culture fluids from the strains described above. Strains expressing *hapR* alone produce low levels of CT (Fig. 2.4D 1st and 2nd bars). Expression of WT Qrr, however, increases the amount of CT produced by ~ 500 fold (Fig. 2.4D, 3rd bar). Strains expressing Qrr_{C30G} and WT *hapR* or WT Qrr and *hapR*_{G63C} produce low levels of CT, ~1-4 fold more than a strain expressing no Qrr sRNAs (Fig. 2.4D, 4th and 5th bars). In contrast, a strain expressing both mutant alleles produces a high level of CT, ~ 500 fold above background, similar to a strain expressing WT Qrr and *hapR* (Fig. 2.4D, 3rd and 6th bars). Taken together, these data indicate that base-pairing between Qrr sRNAs and *hapR* mRNA facilitates QS-mediated regulation of HapR-dependent phenotypes important in the environment and the human host.



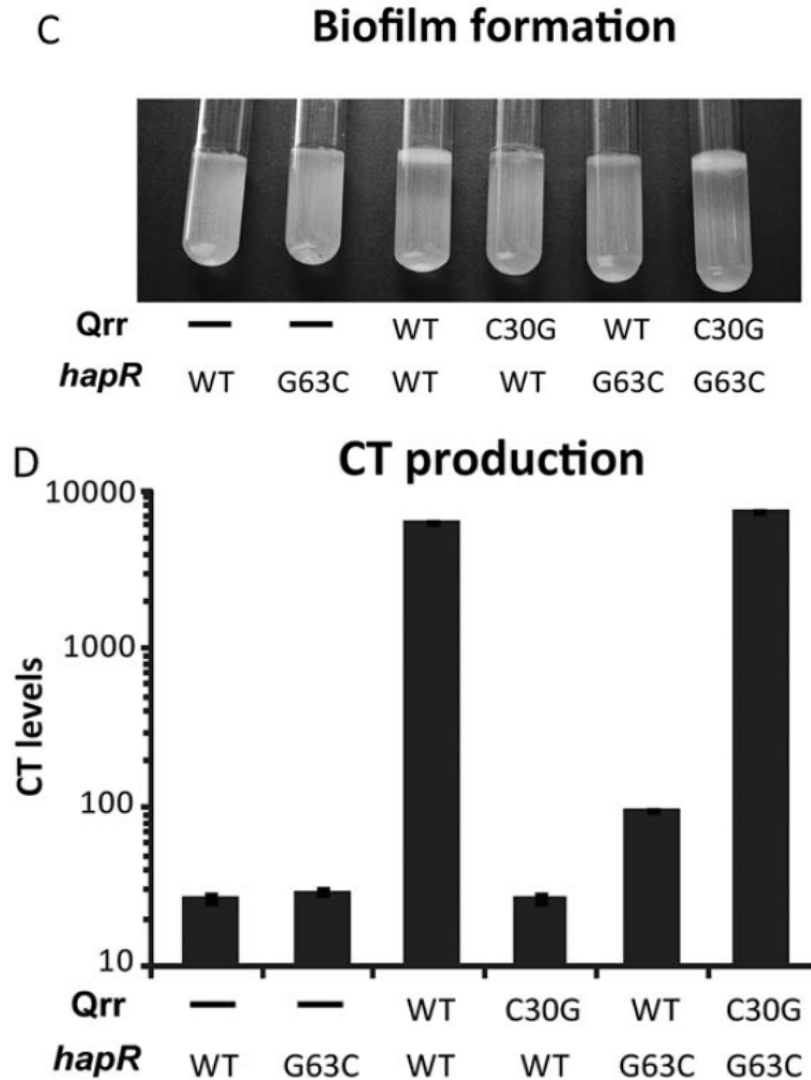


Figure 2.4. Effect of Qrr_{C30G} mutation on HapR-regulated *V. cholerae* phenotypes. A. Extracellular protease activity assays. Cell-free supernatants of cultures were assayed for protease activity using a photometric azocasein assay following 6 h of growth. The activity of the secreted protease per OD₆₀₀ is defined in *Experimental Procedures*. B. Crab shell natural competence assay. *V. cholerae* strains were incubated in ASW containing sterilized crab exoskeletons and purified *kanR* DNA and plated in the presence and absence of kanamycin to calculate Transformation Frequency. C. Biofilm formation. Strains were grown statically in test tubes and biofilms evaluated by the appearance of the pellicle the air-medium interface. D. CT production assay. Strains were incubated under CT-inducing conditions and levels of CT in cell free culture supernatant were determined by ELISA. Values in A and B are the mean of at least 3 independent experiments and error bars indicate standard error. Image shown in C is a representative image of 3 independent experiments. Values are the mean of at least 3 independent experiments and error bars indicate standard error. D shows the mean values +/- standard

deviation obtained from triplicate cultures from one representative experiment of three performed.

2.3.4. Alterations in HapR-based phenotypes are caused by changes in HapR protein levels

To determine whether the changes in HapR-based reporters and phenotypes in *V. cholerae* were a consequence of changes in HapR protein levels, we performed western blots on extracts of the “locked” low cell density strains carrying a WT or mutated Qrr and *hapR* using an antibody to *V. cholerae* HapR. In the absence of Qrr sRNAs, a high level of HapR expression was observed (Fig. 2.5, 1st and 2nd lanes). The presence of a single Qrr sRNA reduced the level of HapR protein (Fig. 2.5, 3rd lane). Expression of Qrr_{C30G} did not lower the level of WT HapR within the cells (Fig. 2.5, 4th lane), nor did expression of WT Qrr lower the expression of HapR_{G63C} (Fig. 2.5, 5th lane). However, expression of Qrr_{C30G} lowered the level of HapR_{G63C} in a strain expressing both RNAs (Fig. 2.5). Thus the dysregulation of QS phenotypes and reporters seen in strains expressing the Qrr_{C30G} or *hapR*_{G63C} alleles is caused by changes in HapR protein levels.

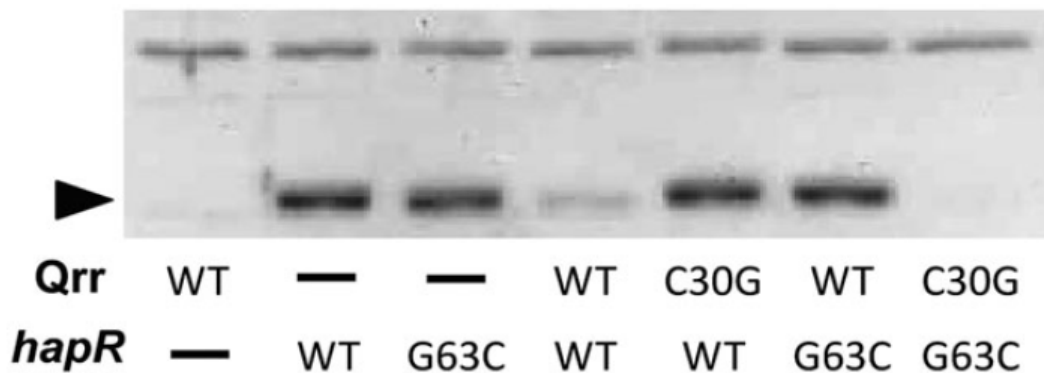


Figure 2.5. The effect of Qrr/*hapR* base-pairing on HapR protein levels. Liquid cultures of the strains indicated were pelleted, resuspended in sample buffer, subjected to SDS-PAGE and transferred to PVDF membrane. Blots were probed with an antibody to HapR and visualized. Triangle indicates HapR. Higher molecular weight band is a non-specific reactive protein that serves as a loading control.

2.3.5. Qrr binds to *hapR* RNA *in vitro*

The data presented above demonstrate that base-pairing is the likely mechanism for Qrr-mediated regulation of HapR and QS phenotypes. Genetic data support the model that the Qrr sRNAs regulate *hapR* mRNA translation by binding to and occluding the ribosome binding site of the mRNA. Furthermore, *V. cholerae* Hfq is thought to stabilize the putative complex formed between the two RNAs, as a *V. cholerae* Δhfq mutant displays constitutive *hapR* expression identical to that of $\Delta qrr1-4$ strain (Lenz et al., 2004). To test experimentally this model *in vitro*, we used an electrophoretic mobility shift assay (EMSA) to examine the ability of purified Qrr sRNA and *hapR* mRNA to bind to one another, both in the presence and absence of Hfq. A fixed concentration of radiolabeled Qrr2 was incubated with increasing amounts of *hapR* RNA with or without purified *V. cholerae* Hfq. Following a one hour incubation, the reactions were subjected to native polyacrylamide gel electrophoresis, which preserved macromolecular complexes and the RNA was visualized using a phosphorimager. Addition of unlabeled full length *hapR* RNA produced a shift in the mobility of the labeled Qrr2, causing it to migrate at a position consistent with a much higher apparent molecular weight (Fig. 2.6A). However, this binding was relatively weak, with only about 40% of the labeled sRNA migrating as a complex at the highest concentration of *hapR* RNA tested (Fig. 2.6A and C).

Binding reactions were also performed in the presence of Hfq to more closely simulate *in vivo* conditions within *E. coli* and *V. cholerae*. The labeled Qrr RNA bound to the Hfq protein, in the absence of *hapR* RNA, producing a shift in mobility (Fig. 2.6B, compare 1st and 2nd lanes). When increasing amounts of unlabeled *hapR* RNA were added, Hfq stabilized the interaction between the two RNAs, promoting full binding of the labeled sRNA to much lower concentrations of target RNA than observed in the absence of Hfq (Fig. 2.6A and 2.6B). Specifically, by quantifying the relative amount of Qrr2 in complex

with *hapR* RNA at the concentrations of target RNA tested, we calculated the apparent K_d of the interactions using a one-site specific binding model. Without Hfq, the K_d of the interaction between the two RNAs was 315 +/- 71 nM. Addition of Hfq lowered the K_d approximately 40-fold to 7.1 +/- 1.8 nM (Fig. 2.6C and D). Thus, consistent with the genetic evidence that Hfq is essential *in vivo* for the QS response (Lenz et al., 2004), addition of Hfq in the purified system enhances Qrr/*hapR* mRNA binding. No binding was observed between Qrr2 and *hapR* RNA lacking the 5'-UTR indicating the specificity of the binding reaction (data not shown). The other *V. cholerae* Qrr sRNAs also bound to *hapR* mRNA with similar K_d values (data not shown), consistent with their functional redundancy *in vivo*.

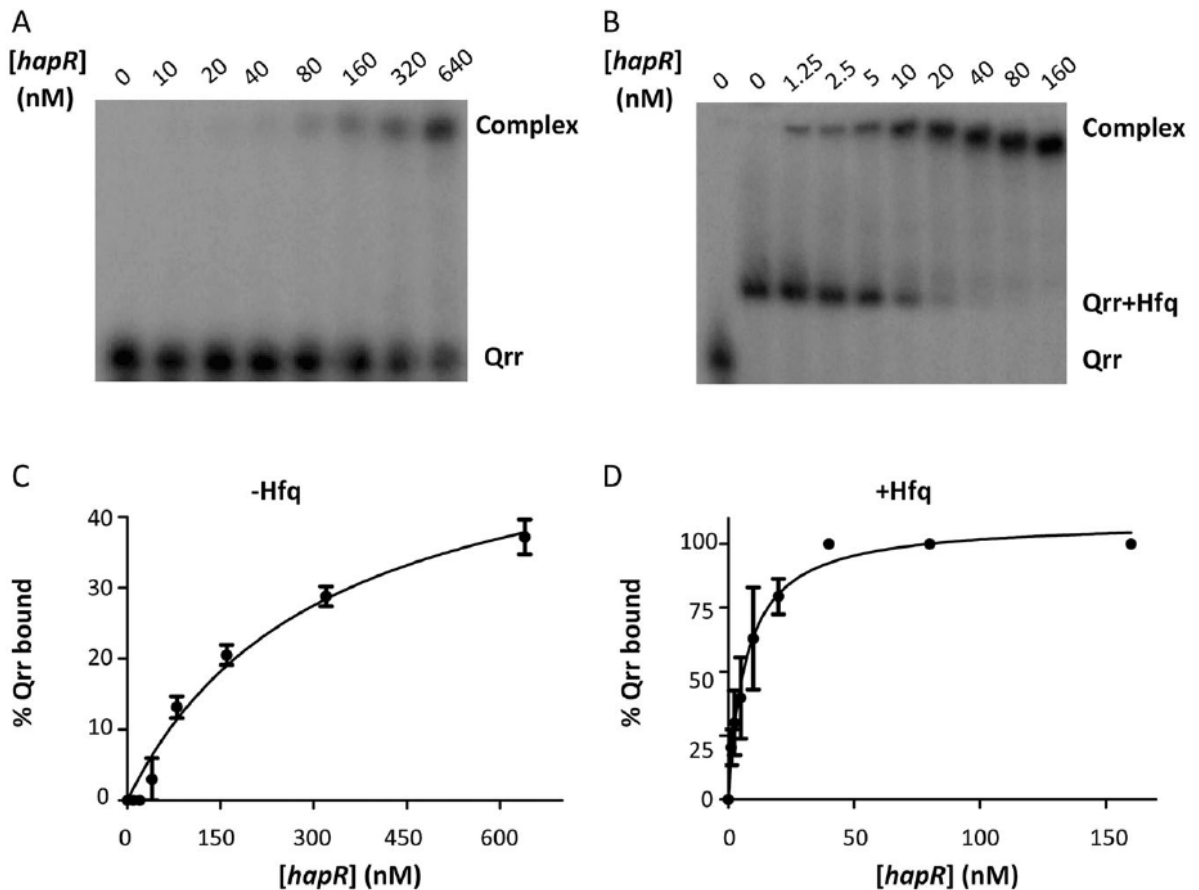


Figure 2.6. Binding of Qrr2 to *hapR* mRNA in the presence or absence of *V. cholerae* Hfq. Radiolabeled Qrr2 was incubated with increasing amounts of *hapR* mRNA without (A) or with 300 nM Hfq (B) as described in Experimental procedures. Concentration of *hapR* mRNA (in nM) is indicated above each gel. Complex refers to the

Qrr2-HapR complex with or without Hfq. The fraction of Qrr bound to *hapR* mRNA (the band labeled “Complex”) as a percentage of the total Qrr RNA in a given lane was quantified and plotted against the concentration of *hapR* RNA for reactions lacking (C) or containing Hfq (D). Values are the mean of at least 3 independent experiments and error bars indicate standard error. Best fit lines were generated using non-linear regression analyses to determine the apparent K_d of the interactions.

2.3.6. Mutation of a single nucleotide abolishes binding of Qrr to *hapR* RNA

As the Qrr_{C30G} mutant was unable to properly regulate *hapR*, we examined the ability of Qrr_{C30G} to bind purified *hapR* mRNA. Both Qrr_{C30G} and *hapR*_{G63C} RNAs were generated by *in vitro* transcription and used in EMSAs with WT Qrr and WT *hapR* in the presence of saturating amounts of Hfq as described above. Addition of WT *hapR* RNA caused an apparent shift in the molecular weight of the labeled WT Qrr sRNA, however, addition of *hapR*_{G63C} RNA did not alter the mobility of WT Qrr RNA (Fig. 2.7 2nd and 3rd lane). Similarly, labeled Qrr_{C30G} RNA migrates at a similar rate in the presence or absence of WT *hapR* RNA (Fig. 2.7 4th and 5th lanes). However, when the two mutated RNAs were incubated together, the interaction was restored (Fig. 2.7, 6th lane). In the presence of Hfq, the K_d of the Qrr_{C30G}/*hapR*_{G63C} complex (3.5 +/- 0.5 nM) is similar to the WT Qrr/WT *hapR* complex (7.1 +/- 1.8 nM) (Fig. 2.6D, and data not shown). This demonstrates that the *in vivo* defects seen with Qrr_{C30G} are caused by an inability to bind to *hapR* RNA and that restoration of the *in vivo* QS-dependent transcriptional responses and phenotypes with the compensatory *hapR*_{G63C} allele are due to re-establishment of Qrr/*hapR* binding.

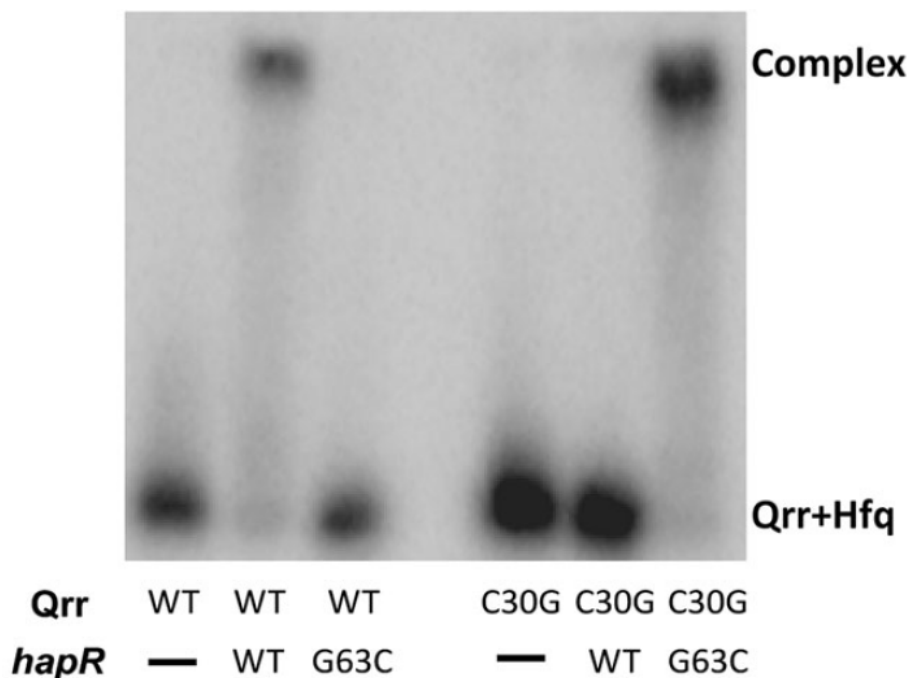


Figure 2.7. The Effect of Qrr_{C30G} on *hapR* binding. Radiolabeled WT Qrr or Qrr_{C30G} was incubated with 10 nM WT *hapR* mRNA or *hapR*_{G63C} RNA in the presence of Hfq. Reactions were run on a 6% native polyacrylamide gel and radiolabeled Qrr was visualized using a phosphorimager. Complex refers to the Qrr2/*hapR*/Hfq complex.

2.4. Discussion

The QS pathway of *V. cholerae* controls expression of many genes critical in the environmental setting and in pathogenesis. The transcriptional reprogramming caused by QS is dependent on four small regulatory RNAs, the Qrr sRNAs. The Qrr sRNAs were identified by genetic and bioinformatic methods and predicted to function redundantly by base-pairing with the mRNA of the transcription factor *hapR*, aided by the RNA accessory protein, Hfq. Specifically, a 21 nucleotide absolutely conserved sequence was predicted to base-pair with *hapR* mRNA and overlap the ribosome binding site of the mRNA. This proposed mechanism had not previously been experimentally tested.

Here we present both *in vivo* and *in vitro* results of nucleotide substitution experiments to examine the base-pairing requirements for Qrr action. We show that mutation of a Qrr nucleotide predicted to be in an unpaired bulge of the putative Qrr/*hapR* mRNA complex has no effect on Qrr activity. However, a single substitution in either the Qrr or *hapR* mRNA of nucleotides predicted to be involved in base-pairing abolishes the ability of the Qrr to control HapR protein levels, and therefore *hapR*-regulated promoters and corresponding phenotypes. When both mutant alleles are expressed, however, Qrr regulation of *hapR* is restored. We demonstrate that Qrr sRNA binds to the RNA chaperone Hfq and to *hapR* RNA and that the RNA-RNA complex is stabilized by the presence of Hfq. Furthermore, we demonstrate that a mutation, which alters Qrr regulation *in vivo*, also abolishes binding *in vitro* to *hapR* RNA. These defects, both *in vivo* and *in vitro*, are restored by a compensatory mutation in *hapR* mRNA. This establishes the molecular mechanism of Qrr sRNA action as a base-pairing between the sRNA and its target, *hapR* mRNA.

Trans-acting bacterial sRNAs generally work through a base-pairing mechanism. The complementarity between a sRNA and its target mRNA is often imperfect, commonly including mismatches, bulges or unpaired nucleotides. This imperfect base-pairing may be partly compensated for by the chaperone Hfq, which has been shown to increase the stability of some RNA-RNA complexes (Jousselin et al., 2009, Updegrove et al., 2008, Lease & Woodson, 2004, Soper et al., 2010). Despite the presence of Hfq, however, nucleotide pairing clearly plays a role in sRNA-based regulation. In the *E. coli* sRNA regulator SgrS, a 6 nucleotide sequence is crucial for regulation of *ptsG*, despite extensive predicted base-pairing between the two RNA molecules extending beyond this region (Kawamoto *et al.*, 2006). Similar *E. coli* studies have also identified single nucleotide substitutions in sRNAs that disrupt potential base-pairing and alter the sRNA based regulation, and that may be restored by compensatory mutations in the

corresponding mRNA target (Altuvia *et al.*, 1998, Sledjeski *et al.*, 1996, Geissmann & Touati, 2004, Chen *et al.*, 2004, Rasmussen *et al.*, 2005, Udekwu *et al.*, 2005, Balbontin *et al.*, 2010). Thus, certain key nucleotides appear to drive the formation of sRNA-target complexes. Our data indicate that the C30 is one such nucleotide for the Qrr sRNAs and is the first critical nucleotide for sRNA regulation identified in *V. cholerae*. Interestingly, different nucleotides are predicted to be involved in base-pairing between the Qrr sRNAs and their other targets, the mRNAs of *luxOU* and *vca0939* (Hammer & Lo Svenningsen, 2011).

The *E. coli* experiments presented here (Fig. 2.2) showed that Qrr_{C30G} fully abolished base-pairing with WT *hapR*, and provided a simplified system to assess the effects of mutations in the Qrr sRNAs prior to construction of *V. cholerae* strains engineered to carry alleles mutated at the candidate nucleotides. However, overproduction of both RNAs in *E. coli* likely produces non-physiological levels of sRNA and target RNAs and has the potential to alter the molar ratio of the two RNAs relative to the levels in *V. cholerae*. Thus, it was appreciated that this system may not necessarily reflect conditions when each RNA is under control of its native promoter. Indeed, alterations in the expression of a sRNA or target mRNA can have dramatic effects on sRNA/mRNA regulation. In *E. coli*, overexpression of either a sRNA or its target mRNA can disregulate not only the specific RNA pair tested, but also other Hfq-dependent sRNA regulatory circuits by limiting Hfq availability (Hussein & Lim, 2010, Papenfort *et al.*, 2009). The *V. cholerae* experiments (Figs. 2.3-5) were designed so that both the sRNA and mRNA target were expressed at endogenous levels each under control of its native promoter, and utilized *V. cholerae* Hfq, thus reflecting the regulatory conditions encountered by these QS circuits *in vivo*. The fact that these *V. cholerae* experiments are entirely consistent with the *E. coli* experiments verified the importance of Qrr_{C30G} in *hapR* regulation, and also the importance of using similar levels of both sRNA and target

mRNAs when analyzing regulation. Indeed, we observed in *V. cholerae* that expression of a WT Qrr over-expressed from a multi-copy plasmid was able to repress the *hapR_{G63C}* mutant expressed from the chromosome, and likewise plasmid-borne Qrr_{C30G} was able to repress chromosomal WT *hapR* (data not shown). Importantly, when both RNAs were expressed from the chromosome, these artifacts were not observed (Fig. 2.3-5). Thus caution should be taken when interpreting results *in vivo* when the levels of the sRNAs and mRNA targets are artificially elevated disproportionately.

We have also shown that *in vitro* synthesized Qrr2 sRNA can interact with *hapR* RNA. By measuring the apparent K_d of this interaction in the absence of Hfq we were able to determine the stability of the sRNA/*hapR* mRNA target. The K_d value obtained, approximately 370 nM, was similar to K_d values observed for the binding of the *E. coli* sRNAs RprA, ArcZ, and DsrA to target *rpoS* mRNA (Lease & Woodson, 2004, Soper et al., 2010, Updegrave et al., 2008). Hfq stabilized the Qrr/*hapR* RNA complex by about a 40-fold, which is also similar to the effect of Hfq on *E. coli* sRNA/mRNA complexes (Soper et al., 2010, Updegrave et al., 2008). Hfq appears to participate in sRNA/*rpoS* interactions by increasing the stability of the sRNA/mRNA complex, though it does also affect the kinetics of complex formation (Soper et al., 2010, Kawamoto et al., 2006, Fender *et al.*, 2010). It remains to be determined whether *V. cholerae* Hfq is altering the kinetics of binding of the sRNAs to the *hapR* RNA.

Despite being able to dramatically increase the stability of the sRNA/mRNA complex, Hfq was not able to compensate for the loss of base-pairing caused by either Qrr_{C30G} or *hapR_{G63C}* and restore complex formation. This is consistent with the loss of function of the Qrr_{C30G} mutant *in vivo*. The Qrr_{C30G} mutant retains the ability to bind to Hfq (Fig. 2.7) indicating that the defect is caused by RNA-RNA interactions and not an inability to bind to the RNA chaperone. Additionally, Qrr_{C30G} forms a complex with *hapR_{G63C}* RNA.

Surprisingly, this Qrr_{C30G}/*hapR*_{G63C} complex has a slightly lower K_d than the complex between WT RNAs. This modestly enhanced binding is consistent with the slightly more stringent repression of HapR seen in several of the phenotypic assays (Fig. 2.4). Specifically the strain in which the Qrr_{C30G}/*hapR*_{G63C} complex forms shows slightly lower protease expression, less DNA uptake, possibly slightly higher levels of biofilm and CT production than a strain that is wild-type for both sRNA and *hapR*. Additional studies are being performed to determine the cause of this increased stability of the sRNA/mRNA complex and its potential *in vivo* consequences to the QS response.

The *V. cholerae* Qrr sRNAs possess several interesting features, including the conserved 21 nucleotide region that we show here base-pairs with *hapR* mRNA (Fig. 2.1B). It is unclear why all of the 21 nucleotides within this region are absolutely conserved not only within *V. cholerae*, but in other pathogenic *Vibrio* species as well (Lenz et al., 2004). Given the functional redundancy of the *V. cholerae* Qrr sRNAs (Lenz et al., 2004, Svenningsen et al., 2009), it could be expected that the one or more of the Qrr sRNAs in a given strain would have diverged, yet all sequenced pathogenic *V. cholerae* strains are absolutely conserved at the 21 nucleotide interaction region. Perhaps the presence of a single mutated Qrr sRNAs could have a dominant negative effect on the others. In particular, this may occur by titration of Hfq which is required for Qrr sRNA based regulation of *hapR* mRNA.

Interestingly, several of these absolutely conserved 21 nucleotides are not predicted to pair with *hapR* mRNA (Fig 2.1B). These non-base-pairing nucleotides may be required to preserve the characteristic structure of the Qrr sRNAs (Lenz et al., 2004). However, our results here show that alteration of at least one of these nucleotides (A28) does not affect *hapR* regulation, apparently altering neither Qrr/*hapR* base-pairing nor the predicted structure of the Qrr sRNA as the mutated sRNA remains functional. However, some Qrr

nucleotides that are not predicted to participate in base-pairing with *hapR* mRNA, such as A28, are predicted to pair with other known targets of the Qrr sRNAs, the mRNAs of *luxOU*, *vca0939* and *aphA* (Hammer & Bassler, 2007, Svenningsen et al., 2009, Hammer & Lo Svenningsen, 2011, Rutherford et al., 2011). It is possible that proper regulation of all of the Qrr target mRNAs requires all 21 nucleotides, but some nucleotides are critical for one mRNA target and expendable for others. Currently we are performing systematic analyses to assay the contribution of each Qrr nucleotide to the regulation of each QS-controlled mRNA target.

2.5. Experimental procedures

2.5.1. Strains, plasmids and culture conditions

Standard microbiological techniques were used for growth of *V. cholerae* and *E. coli*. Liquid cultures of LB broth were incubated at 37° C with shaking unless noted otherwise. Antibiotics were added as appropriate to maintain plasmid selection. The genotypes of all strains used in this study are in Table 2.1. *V. cholerae* strains are derived from strain C6706str2 (Thelin & Taylor, 1996). *E. coli* strains DH5 α , S17 λ -pir, and EC100D (Epicentre) were used for cloning. Allelic exchange plasmids were made using the pKAS32 vector (Skorupski & Taylor, 1996). Vectors for integration at the *lacZ* locus of *V. cholerae* were engineered as previously described (Hammer & Bassler, 2009a). All plasmids for expressing RNA by *in vitro* transcription were constructed using pUC18. Plasmids for expressing Qrr sRNAs and *hapR* in *E. coli* were constructed in pEVS141 and pLAFR respectively (Lenz et al., 2004, Miller et al., 2002). Plasmid for expressing *tfox* was constructed in pEVS 141. The plasmid for expression and purification of *V. cholerae* Hfq was made in pTYB11 (NEB). Single nucleotide substitutions were introduced by PCR-based methods or the QuikChange XL kit (Stratagene). Primer

sequences used for PCR amplification and additional cloning details are available on request. All constructs were sequenced to ensure that they did not possess random errors.

Table 2.1. Strains and plasmids used in this study

<i>V. cholerae</i>		
<u>Strain</u>	<u>Genotype</u>	<u>Reference</u>
BH1543	$\Delta hapR$	This study
BH2126	$\Delta qrr1-4, luxO$ D47E	This study
PB105	$\Delta qrr1-4, luxO$ D47E, $hapR_{G63C}$	This study
EZ402	$\Delta qrr1-4, luxO$ D47E, $lacZ::qrr2$	This study
PB107	$\Delta qrr1-4, luxO$ D47E, $lacZ::qrr2$, $hapR_{G63C}$	This study
EZ426	$\Delta qrr1-4, luxO$ D47E, $lacZ::qrr2_{C30G}$	This study
EZ427	$\Delta qrr1-4, luxO$ D47E, $lacZ::qrr2_{C30G}$, $hapR_{G63C}$	This study
<i>Plasmids</i>		
<u>Plasmid</u>	<u>Description</u>	<u>Reference</u>
pBBRlux-hap	Luciferase fusion to <i>hapA</i> promoter	This study
pBBRlux-vpsL	Luciferase fusion to <i>vpsL</i> promoter	(Lenz <i>et al.</i> , 2005)
pBBRlux-aphA	Luciferase fusion to <i>aphA</i> promoter	This study
pBBRlux-comEA	Luciferase fusion to <i>comEA</i> promoter	Antonova & Hammer, unpublished
pEZ126	Ectopic WT <i>qrr</i> expression	This study
pEZ329	Ectopic Qrr_{C30G} expression	This study
pEZ346	Ectopic Qrr_{A28C} expression	This study
pSLS73	HapR-GFP reporter	(Svenningsen <i>et al.</i> , 2008)
pEZ424	HapR _{G63C} -GFP reporter	This study
pPB004	<i>Qrr2</i> <i>in vitro</i> transcription	This study
pPB014	<i>hapR</i> <i>in vitro</i> transcription	This study
pPB096	$Qrr2_{C30G}$ <i>in vitro</i> transcription	This study
pPB104	$hapR_{G63C}$ <i>in vitro</i> transcription	This study
pPB007	Hfq expression plasmid	This study
pEZ190	<i>qrr2</i> integration at <i>lacZ</i>	This study
pEZ422	$qrr2_{C30G}$ integration at <i>lacZ</i>	This study
pPB103	$hapR_{G63C}$ integration plasmid	This study
pEA201	<i>tfox</i> expression plasmid	Antonova & Hammer, 2011

2.5.2. RNA purification and labeling

RNAs were synthesised by run-off transcription using a MEGAscript kit (Ambion) following manufacturers instructions. RNAs were precipitated with LiCl and resuspended in RNase free water. The quality of the RNA was verified by both denaturing and non-denaturing polyacrylamide gel electrophoresis followed by visualization with SYBR Green, and RNA concentrations were determined spectrophotometrically. RNA-end labeling was performed using the KinaseMax 5' End-Labeling Kit (Ambion). Briefly, Qrr sRNA was dephosphorylated using calf-intestine alkaline phosphatase and then the dephosphorylated RNA was labeled using [γ - 32 P]ATP and T4 polynucleotide kinase and the labeled RNA was purified using NucAway spin columns (Ambion).

2.5.3. Hfq purification

Hfq purification was performed using an affinity tag intein system (IMPACT Kit, New England Biolabs) following manufacturers instructions. Briefly, the *E. coli* strain expressing *V. cholerae hfq* from a plasmid was grown to exponential phase and induced with IPTG overnight at 16 °C. The culture was pelleted and lysed. Lysate was bound to chitin beads and washed and then intein cleavage was performed using buffer containing 1,4 dithiothreitol (DTT). Following elution, Hfq was treated with micrococcal nuclease (NEB) to remove any contaminating nucleic acids. The reaction was passed through a 30,000 d molecular weight cutoff filter to remove the nuclease, and the buffer was exchanged to a calcium free buffer. Quality of protein was assayed by PAGE followed by Coomassie staining, and protein concentration was determined spectrophotometrically.

2.5.4. EMSA and analysis

All binding assays were carried out in buffer containing 20 mM Tris HCl pH 8, 100 mM NH₄Cl, 50 mM NaCl, 50 mM KCl and 5% glycerol. ³²P-labelled Qrr2, either 4 nM (without Hfq), or 0.4 nM (with Hfq), was mixed with varying concentrations of either Hfq alone, or Hfq and *hapR* mRNA and incubated at 25° C for one hour. Following incubation, the reactions were subjected to non-denaturing PAGE at 4° C. The gels were then exposed to a storage phosphor screen that was scanned and analyzed with a Typhoon Phosphorimager (GE).

Calculation of binding constants (K_d) was performed by first determining the percentage of Qrr bound as a percentage of the total Qrr in a given reaction. Quantification of RNA levels was performed using the program Multiguage(FUJI). Apparent K_d values were calculated using a one-site, specific binding model with the program PRISM (Graphpad).

2.5.5. GFP expression analysis

To monitor *hapR-gfp* expression in *E. coli*, triplicate cultures were incubated overnight. Each culture was diluted 1:1000, grown for an additional 10 h and fluorescence and optical density of the culture was measured at various dilutions using a Synergy™ 4 Biotek multi-mode microplate reader. Relative Fluorescence Units (RFUs) are defined as arbitrary fluorescence units/OD₆₀₀.

2.5.6. Bioluminescence assays

V. cholerae bioluminescence expression was assayed as described previously (Miller et al., 2002, Zhu et al., 2002b). Briefly, quadruplicate *V. cholerae* cultures were grown for ~

14 h. Each culture was diluted 1:1000, grown for 10 h and light production was measured using a liquid scintillation counter (Wallac Model 1409). The optical density of each culture was determined spectrophotometrically. Relative Light Units (RLU) are defined as $\text{counts min}^{-1} \text{ ml}^{-1}/\text{OD}_{600}$. The strain used to measure *comEA-lux* activity also contained a plasmid that expressed *tfox* driven by an inducible promoter and assayed in the presence of 1 mM IPTG (Antonova and Hammer, 2011).

2.5.7. Protease assays

Triplicate *V. cholerae* cultures were grown overnight for ~14 h. Each culture was diluted 1:100, grown for 6 h and then protease activity was determined by the azocasein reaction described previously (Denkin & Nelson, 1999). Protease activity is defined as $(\text{OD}_{442}/\text{OD}_{600}) * 1000$.

2.5.8. Biofilm pellicle assays

V. cholerae biofilm pellicles were observed as described previously (Zhu et al., 2002b, Hammer & Bassler, 2003). Overnight cultures of *V. cholerae* were grown to OD_{600} about 2.0 and diluted 1:100 into LB broth and then incubated statically in test tubes for 24h at 30° C.

2.5.9. CT assays

V. cholerae strains were incubated statically in AKI medium for 4 h at 37° C, then transferred a shaking 37° C incubator for 16 h (Iwanaga *et al.*, 1986). Cell-free culture fluids were subjected to GM1 ganglioside enzyme-linked immunosorbent assay (ELISA)

CT assays described previously (Mekalanos, 1988). Cholera toxin produced are expressed as $\text{ng ml}^{-1}/\text{OD}_{600}$.

2.5.10. Chitin-induced natural transformation assay

Triplicate overnight cultures of *V. cholerae* were diluted 1:100 and incubated to $\text{OD}_{600} \sim 0.3$ as previously described (Meibom et al., 2005). Each culture was dispensed into one well of a 12-well microtiter plate containing sterile pieces of crab-shell in artificial sea water (ASW, Instant Ocean). After static incubation for 24 h at 30° C, 2 μg of genomic DNA marked with a kanamycin resistance gene at the *lacZ* locus was added to each well with fresh ASW. 24 h later, bacteria were released from the crab shell by vortexing and then plated onto LB medium with or without kanamycin. Transformation Frequency is defined as kanamycin resistant $\text{CFU ml}^{-1}/\text{total CFU ml}^{-1}$. The limit of detection of this assay is approximately 1×10^{-8} .

2.5.11. Western Blots

Mid-logarithmic cultures of *V. cholerae* were normalized to equal cell densities, pelleted by centrifugation and resuspend in Laemmli sample buffer. The samples were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were then probed with a previously described rabbit antibody to *V. cholerae* HapR (Henke & Bassler, 2004) followed by a peroxidase-conjugated secondary antibody (Sigma). Visualization was performed using a chemiluminescent detection kit (Thermo-Fisher) and a ChemiDoc XRS HQ system (Bio-Rad).

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CHAPTER 3

***VIBRIO CHOLERAE* QUORUM SENSING SRNAS ACTIVATE TRANSLATION OF A DIGUANYLATE CYCLASE THAT PROMOTE C-DI-GMP-DEPENDENT BIOFILM DEVELOPMENT**

3.1. Summary

Biofilm formation by the waterborne pathogen *Vibrio cholerae* aids attachment in aquatic ecosystems, is thought to promote transmission to the human host, and contribute to colonization of the small intestine where this bacterium causes the disease cholera. At low cell density, biofilm formation occurs in *V. cholerae* because the quorum sensing (QS) sRNAs (Qrr sRNAs) base-pair with and post-transcriptionally repress *hapR*, which encodes the QS master regulator. In the absence of HapR the intracellular second-messenger c-di-GMP accumulates, which activates transcription of the *Vibrio* exopolysaccharide genes (*vps*) required for biofilm development. The Qrr sRNAs were also predicted to base-pair with another mRNA, *vca0939*, and activate its translation. Since *Vca0939* was predicted to be a diguanylate cyclase (DGC) that can produce c-di-GMP, it was proposed that the Qrr sRNAs also promote biofilm formation at low cell density by HapR-dependent and HapR-independent c-di-GMP accumulation. Here we demonstrate *Vca0939* behaves as a DGC, and that direct Qrr/*vca0939* base-pairing activates translation of *Vca0939* to promote biofilm development. These results

demonstrate that *V. cholerae* QS regulates biofilm formation through Qrr-activated *vca0939* independent of the QS “master regulator” HapR.

3.2. Introduction

Quorum sensing (QS) is a process that bacteria use to communicate with one another by producing, secreting, and detecting small extracellular signal molecules, called autoinducers (AIs), which accumulate in proportion to bacterial density. Binding of the AIs to membrane-bound receptors initiates a signal transduction cascade that alters gene expression. QS coordinates expression of a variety of genes for group behaviors, including bioluminescence, biofilm formation, sporulation, antibiotic production, virulence factor expression, and competence for DNA uptake (Ng & Bassler, 2009, Miller & Bassler, 2001).

Vibrio cholerae secretes two AIs, CAI-1 and AI-2 (Schauder *et al.*, 2001, Higgins *et al.*, 2007, Xavier & Bassler, 2005b, Chen *et al.*, 2002, Henke & Bassler, 2004). At low cell density, in the absence of AIs, their cognate sensors CqsS and LuxP/Q act as kinases, shuttle phosphate through the phosphotransferase LuxU to the response regulator LuxO (Bassler *et al.*, 1994b, Bassler *et al.*, 1994a, Bassler *et al.*, 1993a, Freeman & Bassler, 1999a, Freeman & Bassler, 1999b, Freeman *et al.*, 2000, Miller *et al.*, 2002). Together with σ^{54} -loaded RNA polymerase, phosphorylated LuxO activates the transcription of genes encoding four non-coding small RNAs (sRNAs) called Qrr1-4 (quorum regulatory RNAs) (Lenz *et al.*, 2004). Genetic evidence supported a model that with the assistance of the RNA chaperone Hfq, the Qrr sRNAs regulate gene expression at low cell density

by altering translation of several mRNA targets to which they are predicted to bind. Recently, it was shown that the Qrrs negatively regulate *hapR* by direct base-pairing with the 5' untranslated region (5' UTR) of *hapR* mRNA, which encodes the "master regulator" of QS (Bardill *et al.*, 2011). Specifically the site to which the Qrrs bind overlaps the ribosome binding site (RBS).

At high cell density (HCD), when the concentrations of AIs are high, binding of each AI to its cognate receptor switches the receptors from kinases to phosphatases. Phosphate flow in the signal transduction pathway is reversed, resulting in dephosphorylation and inactivation of LuxO (Freeman & Bassler, 1999a, Freeman *et al.*, 2000). Therefore, the Qrr sRNAs are not transcribed, *hapR* mRNA is stabilized, and HapR protein is produced (Lenz *et al.*, 2004). HapR acts as both an activator and a repressor of gene expression. Specifically, AI-stimulated production of HapR activates the transcription of *hapA*, encoding the secreted hemagglutinin/protease (Finkelstein *et al.*, 1992, Jobling & Holmes, 1997), and *comEA*, which is required for natural competence of *V. cholerae* (Meibom *et al.*, 2005, Antonova & Hammer, 2011). HapR also represses transcription of the *aphA* gene, which encodes an activator of the ToxT regulon that includes the virulence factors cholera toxin (CT) and toxin co-regulated pilus (TCP) (Sack *et al.*, 2004, Zhu *et al.*, 2002a, Faruque *et al.*, 2003, Kovacicova & Skorupski, 2002, Kovacicova *et al.*, 2004, Kovacicova & Skorupski, 2001). HapR also indirectly represses transcription of the *vpsL-N* exopolysaccharide biosynthesis operon by binding directly to the promoter of the biofilm transcription factor *vpsT* (Zhu & Mekalanos, 2003, Hammer & Bassler, 2003, Hammer & Bassler, 2009a, Waters *et al.*, 2008b).

The Qrr sRNAs also alter translation of several targets in addition to *hapR* mRNA. Specifically, the Qrr sRNAs negatively regulate the mRNA of *luxO*, establishing a feedback loop to control Qrr production (Svenningsen et al., 2009), and activate production of AphA (Rutherford et al., 2011). Finally, the Qrr sRNAs also positively regulate the mRNA of *vca0939*, annotated as a "GGDEF family protein" (Hammer & Bassler, 2007). GGDEF family proteins harbor a GGDEF or GGEEF motif, and act as diguanylate cyclases (DGCs), which synthesize the intracellular second messenger cyclic di-GMP (bis-(3'-5')-cyclic dimeric guanosine monophosphate, or c-di-GMP). While genetic evidence supports a model that Qrr sRNAs regulate these genes post-transcriptionally, direct binding of the Qrrs to *luxO*, *aphA*, or *vca0939* has not been documented experimentally.

Many bacteria use intracellular c-di-GMP as a signaling molecule to facilitate transition between a sedentary and planktonic lifestyle (Galperin, 2004, Hengge, 2009). C-di-GMP is synthesized from two molecules of GTP by DGCs that contain GGDEF motif, and is broken down into 5-phosphoguanylyl-(3'-5')-guanosine (pGpG) by specific phosphodiesterases (PDEs) that contain either an EAL or HD-GYP motif (Ryjenkov et al., 2005, Schmidt et al., 2005, Tal *et al.*, 1998). In general, c-di-GMP promotes the biosynthesis of attachment factors and exopolysaccharide matrix in biofilms and inhibits motility by a variety of mechanisms (Jenal, 2004, Jenal & Malone, 2006, Romling *et al.*, 2005, Romling & Amikam, 2006, Ryan *et al.*, 2006, Wolfe & Visick, 2008, Cotter & Stibitz, 2007). Numerous fundamental bacterial behaviors such as cell cycle progression,

antibiotic production, pilin synthesis, type III secretion, RNA modulation, stress response, bacterial predation, and virulence are also c-di-GMP controlled (Duerig *et al.*, 2009, Fineran *et al.*, 2007, He & Zhang, 2008, Hobley *et al.*, 2012, Jenal & Malone, 2006, Kuchma *et al.*, 2005, Tamayo *et al.*, 2007, Weber *et al.*, 2006).

In *V. cholerae*, it is proposed that QS cooperates with c-di-GMP signaling pathways to regulate the transition between a motile, virulent state within the host and a sessile, biofilm state in aquatic environmental reservoirs (Srivastava & Waters, 2012). *Vibrio cholerae* encodes 40 DGC domains, 20 EAL domains, and 9 HD-GYP domains (Galperin, 2004). The QS regulator HapR represses the biofilm regulatory gene *vpsT* both directly and indirectly by controlling the transcription of 14 different GGDEF and EAL domain-encoding genes (Waters *et al.*, 2008b, Casper-Lindley & Yildiz, 2004) and also four HD-GYPs (Hammer & Bassler, 2009a). The consequence is that biofilm formation is reduced at high cell density. This is consistent with the observations of low intracellular concentration of c-di-GMP at HCD, and high concentrations of c-di-GMP at LCD (Fig. 3.1) (Waters *et al.*, 2008b, Hammer & Bassler, 2009a).

Previous genetic data support a model that in the absence of the Qrr sRNAs, *vca0939* mRNA forms an inhibitory stem-loop structure in its 5' UTR that is adjacent to the RBS and contains a portion of a putative sRNA binding site (Hammer & Bassler, 2007) (Fig. 3.1). It was hypothesized that with assistance of the RNA chaperone Hfq, Qrr sRNAs form an RNA duplex with the 5' stem of the putative inhibitory structure (Fig. 3.1), analogous to sRNAs in other bacteria, such as RprA and DsrA in *Escherichia coli* that

positively control *rpoS* by an “anti-antisense” mechanism (Majdalani *et al.*, 1998, Majdalani *et al.*, 2002, Frohlich & Vogel, 2009). Induction of Vca0939 by the Qrrs was proposed to contribute to elevated c-di-GMP levels and biofilm (*vps*) gene expression at LCD; and while a $\Delta vca0939$ is not impaired biofilm formation (Hammer & Bassler, 2007), overexpression of the coding region of Vca0939 enhances biofilms consistent with this model (Massie *et al.*, 2012). It remains unknown whether Vca0939 is indeed a DGC as the active site GGEEF domain has not been studied, nor has it been determined whether Qrr-dependent activation of *vca0939* translation is sufficient to increase c-di-GMP levels and enhance biofilm development.

Here we demonstrate post-transcriptional activation of *vca0939* results from base-pairing with Qrr sRNA, which is stabilized by the RNA chaperone Hfq. A single nucleotide substitution within a *V. cholerae* Qrr sRNA prevents activation of *vca0939* translation and as a consequence, alters Vca0939-regulated c-di-GMP production and HapR-independent biofilm formation. Qrr/*vca0939* base-pairing and *in vivo* phenotypes were restored by a compensatory single nucleotide mutation in the *vca0939*. We also show that the GGEEF domain of Vca0939 is necessary for synthesis of c-di-GMP and enhancement of biofilm development in *V. cholerae*. These results demonstrate that the *V. cholerae* Qrrs regulate biofilm formation via c-di-GMP levels using not only a HapR-dependent but also a HapR-independent mechanism.

3.3. Results

3.3.1. The Qrr sRNAs activate Vca0939 protein levels

Previously, *vca0939* was identified in *V. cholerae* as a gene positively regulated by the QS-dependent Qrr sRNAs, independent of HapR (Hammer & Bassler, 2007). Expression of a single plasmid-borne Qrr driven by a constitutive promoter was sufficient to promote expression of a gene fusion of *vca0939* to the luciferase operon (*lux*). Similar studies have since documented Qrr-based control of several additional target genes (Bardill et al., 2011, Tu et al., 2010, Svenningsen et al., 2009, Rutherford et al., 2011). Because it was predicted that direct Qrr base-pairing to *vca0939* mRNA positively controlled translation of Vca0939 protein, we tested whether observations made with the *vca0939-lux* reporter reflected changes in Vca0939 protein concentration. We constructed on a plasmid a flag-tagged version of *vca0939*, which remained under control of the native promoter and 5' UTR. An additional plasmid was made that expressed a Qrr under control of the P_{tac} promoter; vector control plasmids lacking the entire Qrr sequence or the entire *vca0939* sequence were used as negative controls. The levels of Vca0939 protein in *E. coli* were determined by western blot of extracts derived from strains carrying the *vca0939-flag* plasmid along with the Qrr-expressing plasmid or either vector controls. No Vca0939 protein was observed when only Qrr was expressed along with the vector control (Fig. 3.2, lane 1). Likewise, Vca0939 was not detected when *vca0939* was transcribed in the absence of Qrr induction (Fig. 3.2, lane 2). However, Vca0939 protein was observed when both *vca0939* was transcribed and the Qrr was induced, consistent with the model that translation of *vca0939* mRNA requires activation by direct Qrr interaction (Fig. 3.2, lane 5).

We used single nucleotide substitutions previously to validate that interaction of Qrr with *hapR* mRNA repressed *hapR* translation (Bardill et al., 2011). Thus, to determine here whether Qrr interactions with *vca0939* also activate *vca0939* translation, we engineered mutations in both Qrr and in *vca0939* that were predicted to disrupt Qrr/*vca0939* duplex formation (Fig. 3.1C). In the absence of Qrrs, Mfold (Zuker, 2003) predicts that the 5'UTR of *vca0939* mRNA forms a stem loop structure that does not contain, but was adjacent to, a putative RBS; and it has been proposed that this stem loop structure prevents translation in the absence of Qrr interaction (Fig. 3.1B) (Hammer & Bassler, 2007). This is in contrast to the 5'UTR of *hapR* mRNA which is predicted to be relative unstructured at the Qrr binding site (Fig. 3.8). Consistent with this model, deletion of either the sequence comprising the left or right stem of the putative inhibitory structure of *vca0939*, as well as deletion of the sequence for the entire stem loop, resulted in Qrr-independent production of Vca0939 (Fig. 3.9). These results confirmed that the sequence comprising the putative structure indeed inhibits translation, and also that the RBS of *vca0939* is not contained within the sequence of the putative inhibitory structure.

To define a nucleotide pair that is involved in the Qrr/*vca0939* interaction, we engineered a mutation in a nucleotide (nt) within the 5'UTR of *vca0939* (*vca0939_{A43C}*), that was not expected to be involved in pairing within the *vca0939* inhibitory stem loop structure (Fig. 3.1B), but was still predicted to interact with the Qrr RNA (Fig. 3.1C). In the absence of Qrr expression, like wild type (WT) *vca0939*, *vca0939_{A43C}* was not expressed (Fig. 3.2, lane 3), consistent with an intact inhibitory structure. However, whereas in the presence

of WT Qrr promoted WT *vca0939* translation (Fig. 3.2, lane 5), WT Qrr was unable to activate translation of *vca0939*_{A43C} (Fig. 3.2, lane 6), presumably due to disruption of the Qrr/*vca0939* interaction. To demonstrate that loss of Qrr-dependent *vca0939* activation was due to impairment of the Qrr/*vca0939* interaction, we engineered a compensatory mutation in the *qrr* (Qrr_{U27G}) and expressed it in *E. coli* carrying either WT *vca0939* or *vca0939*_{A43C}. No Vca0939 protein was observed when Qrr_{U27G} was expressed with WT *vca0939* (Fig. 3.2, lanes 4 and 7). However, expression of Qrr_{U27G} was able to effectively activate Vca0939_{A43C} translation (Fig. 3.2, lane 6), presumably because the mutations in each RNA restored the Qrr/*vca0939* interaction. These experiments in *E. coli* suggest that Qrr-dependent activation of Vca0939 translation in *V. cholerae* requires base-pairing between Qrr₂₇ and *vca0939*₄₃.

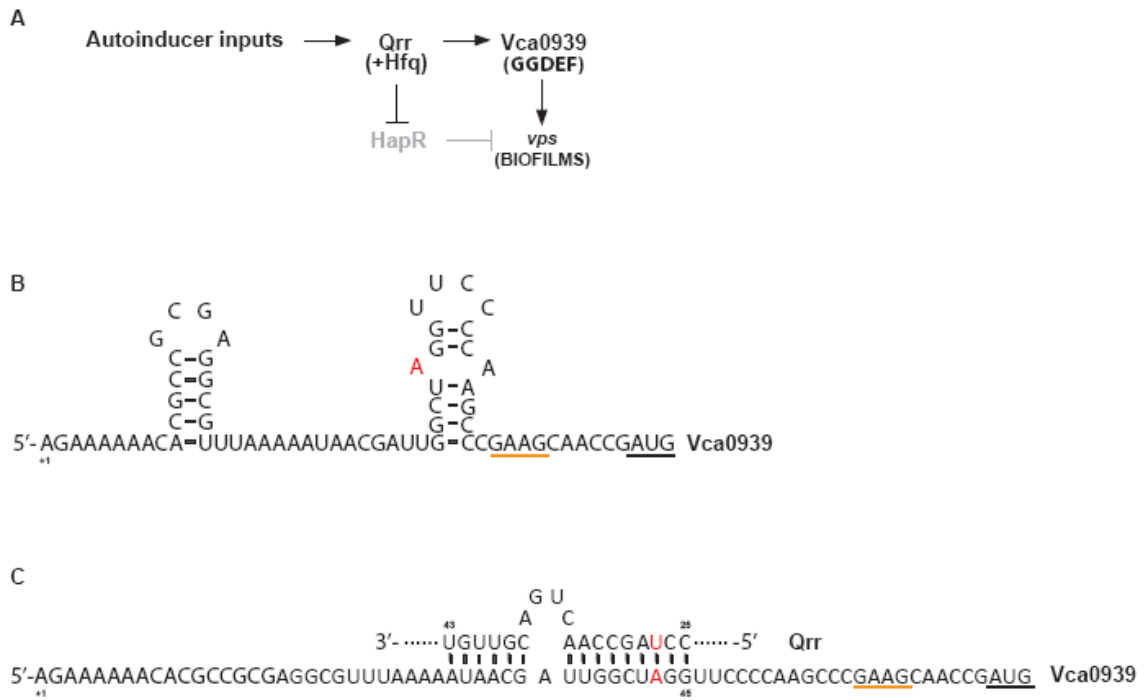


Figure 3.1. Model of biofilm formation by the Qrr sRNAs regulation at low cell density in the *V. cholerae* quorum sensing circuit. A. The lack of AIs at LCD results

in activation of transcription of the Qrr sRNAs. Qrr sRNA represses *hapR* translation and the absence of HapR is unable to repress *vps* gene expression. Qrr sRNA also activates *vca0939* expression, which serves as a GGDEF family protein to promote *vps* gene expression and biofilm formation. B. Putative secondary structure of the 5'UTR of *vca0939* mRNA. The underlined sequence in orange is the predicted *vca0939* ribosome binding site. The underlined sequence in black is the translation start site of *vca0939*. C. Putative base-pairing between Qrr sRNAs to *vca0939* mRNA. Predicted pairing of the Qrr sRNAs to the predicted inhibitory stem loop structure of *vca0939* 5'-UTR. Nucleotide positions are indicated relative to the +1 of transcription of each RNA. Substitutions made in this study are in red. The underlined sequence in orange is the predicted *vca0939* ribosome binding site. The underlined sequence in black is the translation start site of *vca0939*.

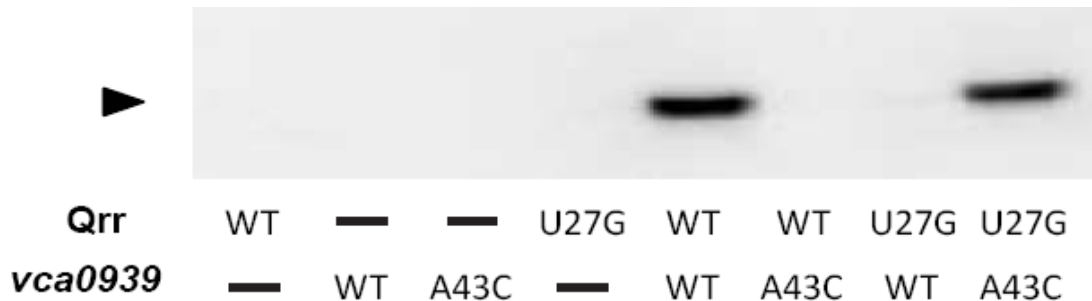


Figure 3.2. Effect of Qrr_{U27G} mutation on the expression of Vca0939-Flag in *E. coli*. *E. coli* strains expressing the indicated Qrr and *vca0939-flag* alleles were grown as triplicate cultures and analyzed for protein production using Western blot. Liquid cultures of the strains indicated were pelleted, resuspended in sample buffer, subjected to SDS-PAGE and transferred to PVDF membrane. Blots were probed with an antibody to Flag tag and visualized. Triangle indicates Vca0939-Flag. Reference bands indicates the location of each lane.

3.3.2. Qrr sRNA binds to *vca0939* mRNA *in vitro*

Previous genetic studies in *V. cholerae* showed that deletion of the *hfq* gene, or of all four *qrr* genes severely impaired *vca0939-lux*, consistent with both Hfq and Qrr sRNA involvement in *vca0939* activation (Hammer & Bassler, 2007). We sought to determine

whether *vca0939* activation by Qrr sRNA was indeed due to direct binding, as recently shown for Qrr/*hapR* (Bardill et al., 2011), and whether Hfq facilitates this process. Electrophoretic mobility shift assays (EMSA) were used to measure binding of purified, ³²P-radiolabelled Qrr RNA and *vca0939* RNA, both in the presence and absence of Hfq. Full length Qrr RNA was prepared as described (Bardill et al., 2011). 5' RACE identified the +1 of transcription and verified the length of the 5' UTR of *vca0939* was 67 nt (Fig. 3.1), and a 270 nt *vca0939* RNA was *in vitro* transcribed to allow detection of a shift on a native polyacrylamide gel that preserves macromolecular complexes. In the absence of Hfq, ³²P-radiolabelled Qrr incubated with increasing *vca0939* RNA Qrr sRNA resulted in a band shift only with a high *vca0939* RNA concentration (750 nM), indicative of weak binding (Fig. 3.3A). In the presence of purified *V. cholerae* Hfq (150 nM), ³²P-radiolabelled Qrr RNA displayed a shift in mobility (Fig. 3.3B, lane 2), and then with increasing amount of unlabeled *vca0939* RNA, resulted in a supershift consistent with the formation of a complex that may contain both RNAs and Hfq (Fig. 3.3B, lane 3-10). Although Qrr RNA can bind *hapR* RNA fully (Bardill et al., 2011) the same Qrr RNA never fully bound to *vca0939* RNA. These results are consistent with RNAup predictions (Gruber *et al.*, 2008) that the “opening energy” required to disrupt the inhibitory stem loop structure of *vca0939* for Qrr binding is greater than that required for *hapR* (Fig. 3.8).

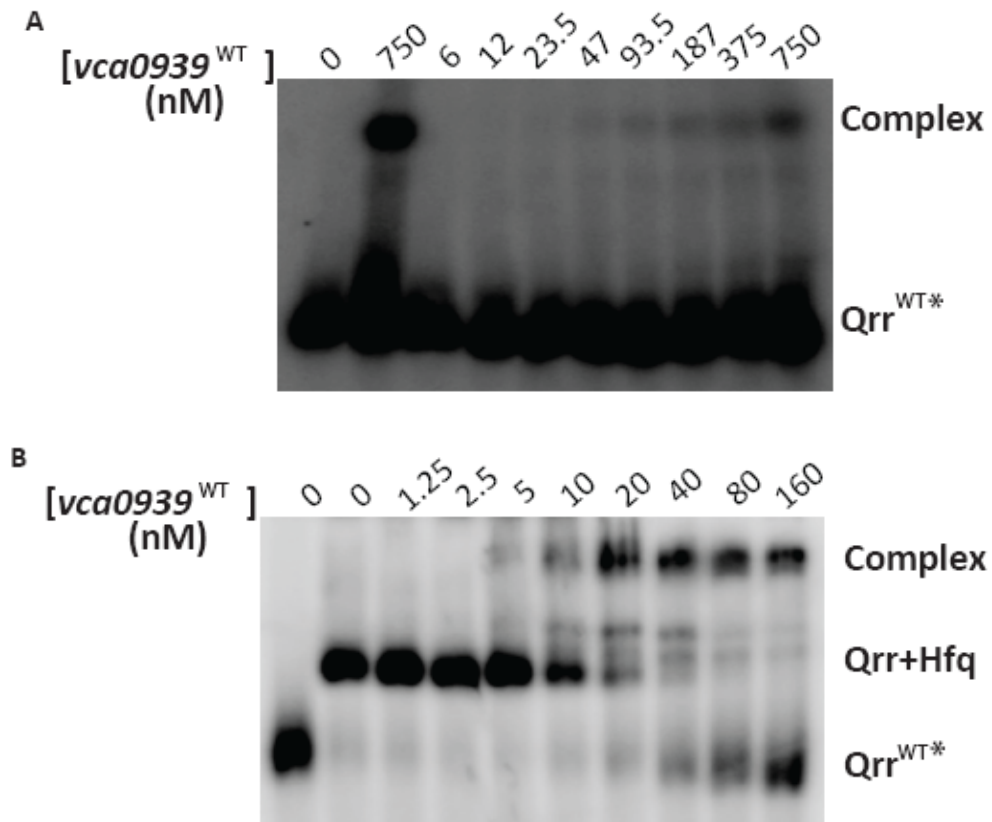
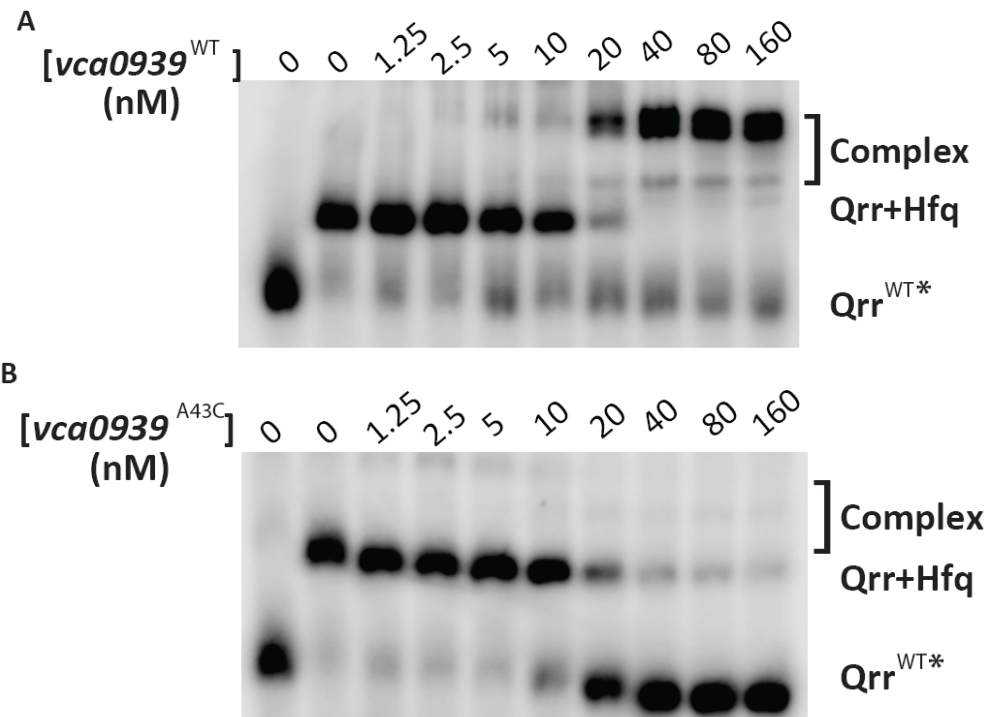


Figure 3.3. Binding of Qrr to *vca0939* mRNA in the presence or absence of *V. cholerae* Hfq. 0.4 nM of the radiolabeled Qrr was incubated with increasing amounts of *vca0939* mRNA without (A) or with 150 nM Hfq (B) as described in Experimental procedures. Concentration of *vca0939* mRNA (in nM) is indicated above each gel. Complex refers to the Qrr/*vca0939* complex with or without Hfq. See text for details.

3.3.3. Mutation of a single nucleotide abolishes binding of Qrr to *vca0939* RNA

In *E. coli*, Vca0939_{A43C} was not translated in the presence of WT Qrr, nor was WT Vca0939 translated in the presence of Qrr_{U27G} (Fig. 3.2, lanes 6 and 7, respectively). To test whether this loss of activation *in vivo* was a consequence of binding defects, we purified and radiolabelled Qrr_{U27G} RNA, and also purified *vca0939*_{A43C} RNA for EMSA. As in Fig. 3.3B, WT *vca0939* RNA binds WT Qrr RNA in the presence of Hfq, resulting

in a supershift with ~20 nM *vca0939* (Fig. 3.4A). However, no supershift was observed with addition of WT *vca0939*_{A43C} RNA to WT QrrRNA (Fig. 3.4B), or addition of WT *vca0939* RNA to Qrr_{U27G} RNA (Fig. 3.4C). When unlabeled *vca0939* RNA exceeded ~20 nM, the band representing the Qrr bound to Hfq (Qrr+Hfq) diminished while a presumptive unbound Qrr band increased in intensity, consistent with titration of Hfq away from radiolabelled Qrr by excessive unlabelled *vca0939* RNA (Fig. 3.4B and 3.4C). Importantly, Qrr_{U27G} and *vca0939*_{A43C}, which together reestablished translational activation in *E. coli* (Fig. 3.2, lane 8), showed a restoration of base-pairing and formation of a supershift complex by EMSA (Fig. 3.4D, see Discussion for details).



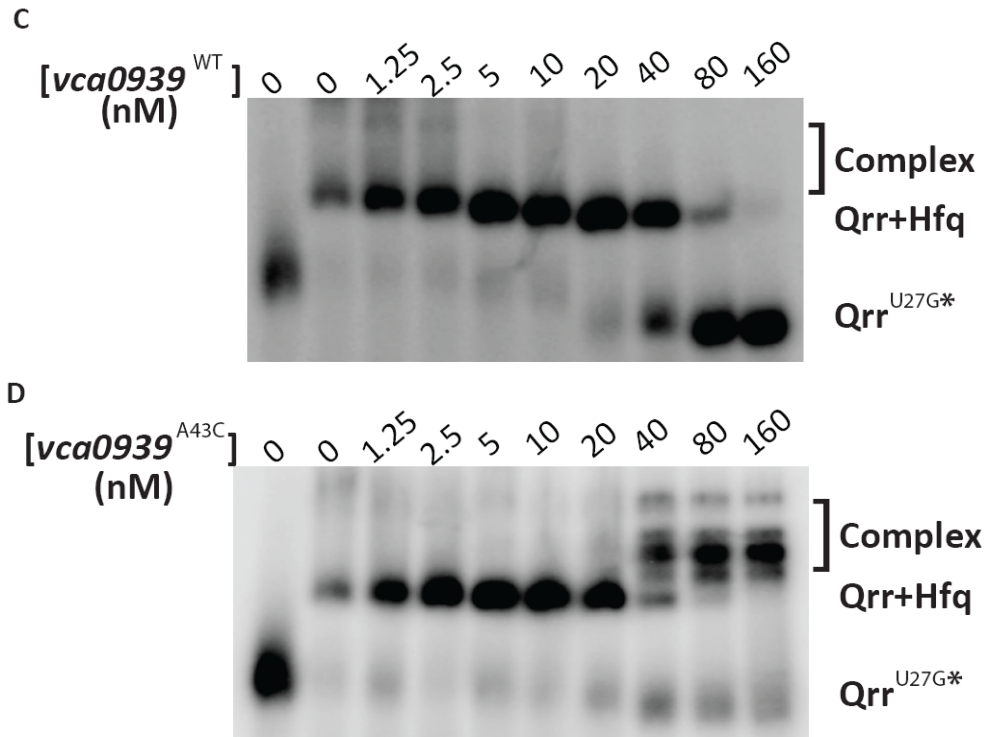


Figure 3.4. The Effect of Qrr_{U27G} on *vca0939* binding in the presence of *Vibrio cholerae* Hfq. 0.4 nM of each radiolabeled Qrr was incubated with increasing amount of *vca0939* RNA. Concentration of *vca0939* RNA (in nM) is indicated above each gel. Reactions were run on a 8% native polyacrylamide gel and radiolabeled Qrr was visualized using a phosphorimager. Complexes refers to the Qrr/*vca0939*/Hfq complex. A. Radiolabeled WT Qrr was incubated with WT *vca0939* RNA. B. Radiolabeled Qrr_{U27G} was incubated with WT *vca0939* RNA. C. Radiolabeled WT Qrr was incubated with *vca0939*_{A43C} RNA. D. Radiolabeled Qrr_{U27G} was incubated with *vca0939*_{A43C} RNA.

3.3.4. *Vca0939* is a diguanylate cyclase, which promotes early biofilm formation in *V. cholerae*

In *V. cholerae* c-di-GMP binds VpsR, which in turn activates VpsT; and c-di-GMP also binds to VpsT which in turn directly activates the transcription of the *vps* biosynthesis genes required for biofilm development (Srivastava et al., 2011, Krasteva et al., 2010). While *E. coli* and *in vitro* measurements confirmed Qrr/*vca0939* interaction, the physiological consequences of Qrr-dependent activation of *Vca0939* in *V. cholerae* had

not yet been tested. Since Vca0939 is annotated as a “GGDEF family protein” capable of synthesizing c-di-GMP, we tested whether Qrr-dependent activation of Vca0939 was sufficient to enhance biofilm formation. Since *V. cholerae* Qrr sRNAs are only expressed at low cell density, we introduced into a *V. cholerae* $\Delta qrr1-4$ strain a Qrr-expression plasmid and a Vca0939-FLAG plasmid similar to those utilized in *E. coli* (Fig. 3.2). We then observed biofilm formation on solid growth medium and measured c-di-GMP levels for each strain tested.

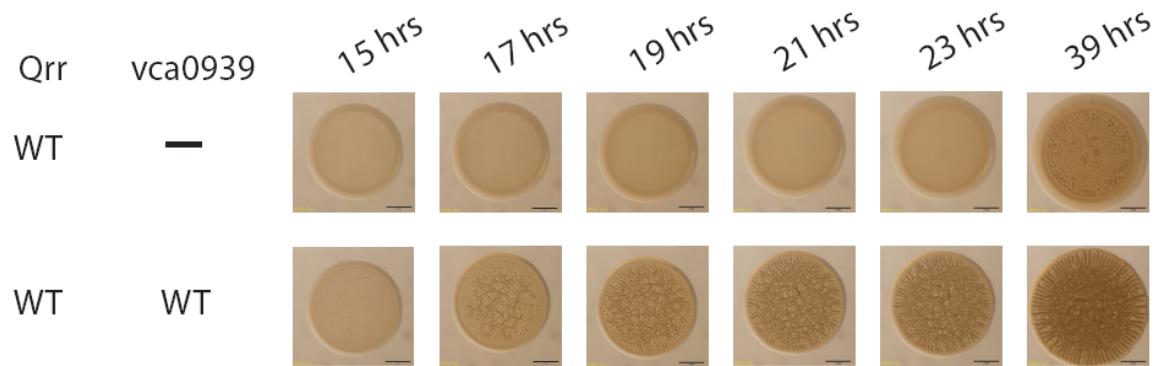


Figure 3.5. The GGDEF protein Vca0939 promotes biofilm formation in *V. cholerae*. Biofilm formation is observed at the time points indicated during 40 h growth on solid medium under dissecting microscope in a *V. cholerae* low-cell-density mutant (*luxO* D47E) carrying only WT Qrr (upper panel), and WT Vca0939-Flag with WT Qrr (lower panel).

To determine whether Qrr-activation of Vca0939 alters biofilm formation, aliquots of a diluted overnight culture were spotted onto solid growth medium to document biofilm development as described elsewhere (Ray *et al.*, 2012). Images of each colony formed were captured at multiple intervals over ~ 40 h growth. *V. cholerae* expressing Qrrs but not transcribing Vca0939 display a smooth colony morphology until after 39 h when a modest wrinkly (rugose) morphology appeared, which is indicative of *vps* production (Yildiz & Schoolnik, 1999) (Fig. 3.5). In contrast, when both Qrrs were expressed and

vca0939 was transcribed, rugose colony morphology was detected by 17 h (Fig. 3.5). Western blots of cell cultures from each colony confirmed Vca0939-FLAG expression when the Qrrs were expressed but not when Qrrs were not expressed (data not shown). These results demonstrate that activation of *vca0939* by the Qrrs promotes biofilm formation in *V. cholerae*.

Vca0939 is annotated as a “GGDEF family protein” due to a conserved amino acid motif (Tal et al., 1998), and thus predicted to act as a DGC capable of c-di-GMP synthesis. Indeed, Vca0939 has a GGEEF domain, which is a common variant of the GGDEF signature motif, and is typically enzymatically active (Kulasakara et al., 2006). To test whether the *V. cholerae* biofilm phenotype observed (Fig. 3.5, 17 h) was due to the DGC activity of Vca0939, we altered the GGEEF motif, to AAEEF or GGEAF (Waters et al., 2008b) (and Waters unpublished data) on the plasmid expressing Vca0939-FLAG. In *E. coli* and in *V. cholerae*, levels of Vca0939-FLAG_{AAEEF} and Vca0939-FLAG_{GGEAF} protein were comparable to WT Vca0939-FLAG by western blot (data not shown). In control strains, where *V. cholerae* expressed no Qrrs, so that *vca0939* or *vca0939*_{A43C} was not activated, colonies appeared smooth, as expected (Fig. 3.6A, panels 1 and 2). In contrast, *V. cholerae* expressing the WT Qrr and transcribing WT *vca0939*, formed rugose colonies, presumably due to its DGC activity (Fig. 3.6A, panel 3). However, when *V. cholerae* expresses Qrrs, and either Vca0939_{AADEF} or Vca0939_{GGDAF}, no rugosity was observed (Fig. 3.6A, panels 4 and 5), consistent with the hypothesis that the GGEEF motif was responsible for synthesis of c-di-GMP.

To confirm that the defect in biofilm formation observed with each GGEEF mutant in Fig. 3.6A was due to the loss of DGC activity for each protein, we quantified c-di-GMP levels by LS/MS-MS (Massie et al., 2012) for each strain in Fig. 3.6A. Strains lacking Qrrs that do not produce Vca0939 protein (Fig. 3.2, lanes 2 and 3) showed low levels of c-di-GMP (Fig. 3.6B bars 1 and 2), consistent with the biofilm phenotype observed (Fig. 3.6A, panels 1 and 2). The strain that carried both WT Qrr and WT *vca0939*, and produced Vca0939 protein (Fig. 3.2, lane 5), had > 10-fold higher c-di-GMP levels consistent with enhanced biofilms (Fig. 3.6A, panel 3). However, each biofilm-defective strain that carried a putative GGEEF active site mutation produced lower levels of c-di-GMP (Fig. 3.6B, bars 4 and 5), confirming that Vca0939 is a DGC capable of c-di-GMP synthesis.

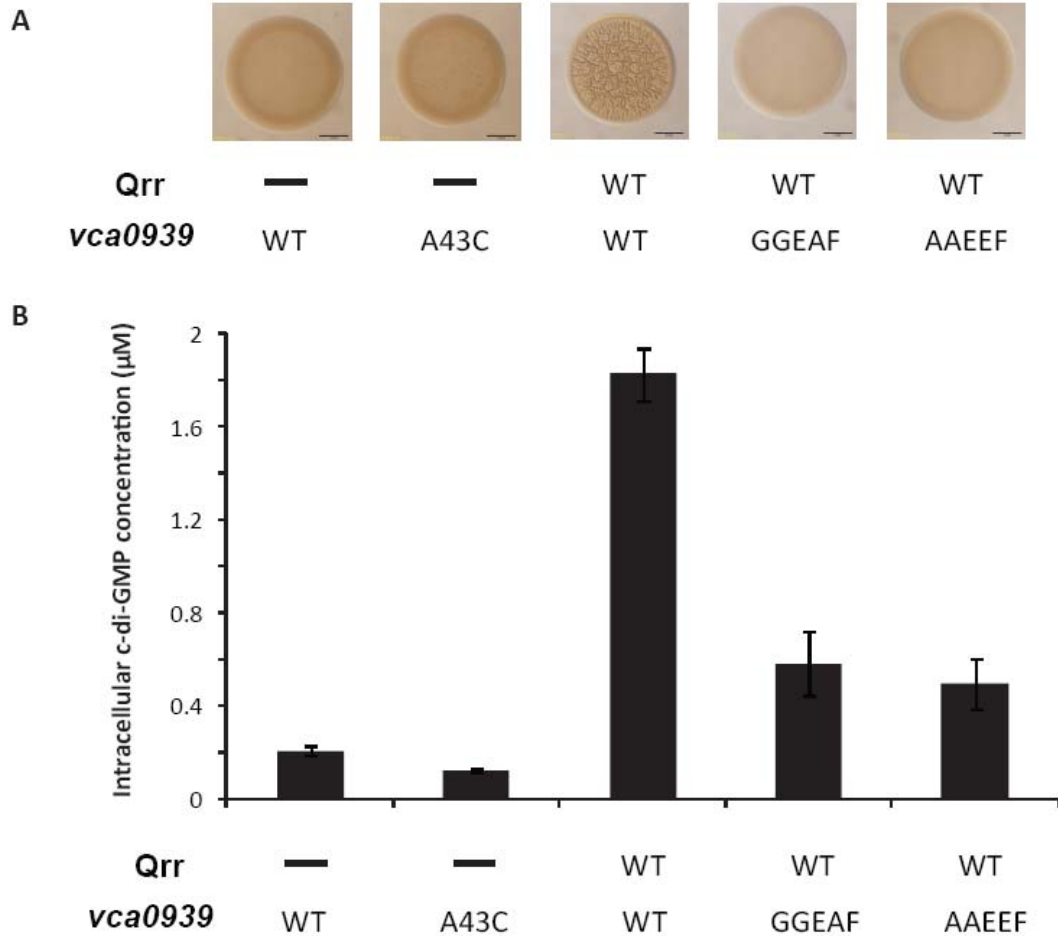


Figure 3.6. The active site of the DGC Vca0939 is required for enhanced c-di-GMP levels and biofilm formation in *V. cholerae*. A. Biofilm formation is observed under dissecting microscope in a *V. cholerae* low-cell-density mutant (*luxO* D47E) carrying WT Vca0939-Flag with empty vector of Qrr (panel 1), Vca0939_{A43C}-Flag with empty vector of Qrr (panel 2), WT Vca0939-Flag with WT Qrr (panel 3), Vca0939_{GGEAF} with WT Qrr (panel 4), and Vca0939_{AAEEF} with WT Qrr (panel 5). B. C-di-GMP concentrations of the strains shown in panel A. Data shown are mean values +/- standard deviation for the triplicate cultures from one representative experiment of three performed.

3.3.5. Base-pairing between Qrr and *vca0939* promotes *V. cholerae* biofilm formation

Since we confirmed that *vca0939* translation is activated by Qrrs via base-pairing interaction (Fig. 3.2 and 4), we wanted to examine whether the single nucleotide

mutations in *Qrr* and *vca0939* that disrupt pairing were sufficient to alter biofilm formation and c-di-GMP production. We introduced the *Qrr*_{U27G} and *vca0939*_{A43C} alleles into both plasmids used in Fig. 3.6, and introduced each into *V. cholerae* again to measure rugose colony formation and c-di-GMP levels. As with the control strains that do not express *Qrrs* (Fig. 3.7A, panels 1 and 2), a smooth colony morphology was observed in the strain carrying *Qrr*_{U27G} and WT *vca0939* and the strain carrying WT *Qrr* and *vca0939*_{A43C} (Fig. 3.7B, panels 4 and 5). Similar to the positive control rugose strain that expresses both WT *Qrr* and WT *vca0939*, the strain carrying *Qrr*_{U27G} and *Vca0939*_{A43C} was restored for production of a rugose colony morphology (compare Fig. 3.7A, panels 3 and 6). These results are consistent with both the western blots and *in vitro* EMSA results (Fig. 3.2 and 4). Indeed, the levels of c-di-GMP were consistent with the biofilm phenotypes, as strains with a rugose colony morphology had high c-di-GMP levels, while each smooth strains produced c-di-GMP at background levels (Compare Fig. 3.7A and 3.7B). Taken together, these data indicate that base-pairing of *Qrr* sRNAs and the mRNA of *vca0939* activates DGC activity that promotes biofilm formation.

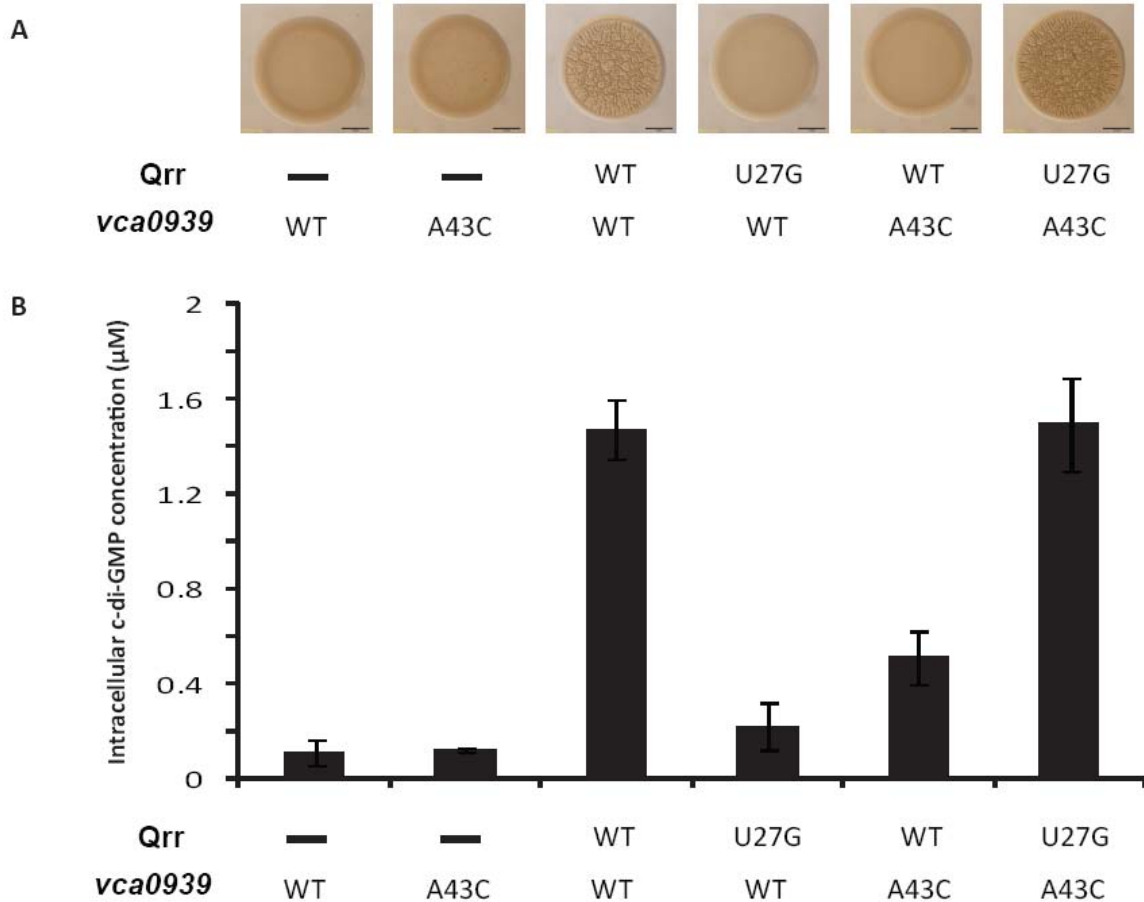


Figure 3.7. Effect of Qrr_{U27G} mutation on Vca0939-regulated c-di-GMP production and biofilm formation. A. Biofilm formation is measured in a *V. cholerae* low-cell-density mutant (*luxO* D47E) carrying the combinations of WT Vca0939-Flag and empty vector of Qrr (panel 1), Vca0939_{A43C}-Flag with empty vector of Qrr (panel 2), WT Vca0939-Flag with WT Qrr (panel 3), WT Vca0939-Flag with Qrr_{U27G} (panel 4), Vca0939_{A43C} with WT Qrr (panel 5), and Vca0939_{A43C} with Qrr_{U27G} (panel 6). B. C-di-GMP concentrations of the strains shown in panel A. Data shown are mean values +/- standard deviation for the triplicate cultures from one representative experiment of three performed.

Finally, because rugose colony morphology often occurs due the effect of elevated c-di-GMP levels on expression of two *vps* vibrio polysaccharide biosynthesis operons, we measured transcript abundance of the *vpsA-K* and the *vpsL-Q* operons by RT-PCR in strains producing or not Vca0939. As expected, higher levels of *vpsA-K* and *vpsL-Q* transcription (4.3-fold and 5-fold, respectively, data not shown) were observed in the *V.*

cholerae strain (Fig. 3.5 lower panels), which carried WT Qrr and WT Vca0939, compared to the strain that carried WT Qrr but was not transcribing *vca0939* (Fig. 3.5 upper panels). Similar higher levels of 2-fold and 3-fold were observed of *vpsA-K* and *vpsL-Q* mRNA, respectively, in a rugose *V. cholerae* $\Delta hapR$ strain relative to WT *V. cholerae*. Thus the DGC Vca0939, when post-transcriptionally activated by Qrr sRNA expression, independent of HapR, produces c-di-GMP that upregulates *vps* genes for biofilm formation.

3.4. Discussion

Vibrio cholerae populations sense and respond to environmental cues to control important developmental processes such as biofilm formation (Srivastava & Waters, 2012). Specifically, *V. cholerae* utilize QS as one of several signaling systems to control transcription of the *vps* (Vibrio polysaccharide) genes required for biofilm development (Hammer & Bassler, 2003, Casper-Lindley & Yildiz, 2004, Tischler & Camilli, 2004, Hammer & Bassler, 2009a, Martinez-Wilson *et al.*, 2008). Much research has focused on the contribution of the HapR “QS master regulator” to *vps*-dependent biofilms, as HapR abundance is inversely related to that of c-di-GMP. At low cell density when Qrr accumulation prevents HapR expression, c-di-GMP levels are high and biofilms form; and at high cell density when the lack of Qrrs allows HapR production, c-di-GMP levels are low and biofilm formation is halted (Waters *et al.*, 2008b, Hammer & Bassler, 2009a). Prior studies identified *vca0939* as a gene that is positively regulated by the Qrr sRNAs independent of HapR, and that was predicted to behave as a DGC capable of synthesizing c-di-GMP and promoting biofilms (Hammer & Bassler, 2007, Hammer &

Bassler, 2009a). Indeed, we recently demonstrated that overexpression of the *vca0939* coding region independent of the native 5' UTR enhanced biofilms in *V. cholerae* (Massie et al., 2012). In this paper, we have defined the regulation and consequences of *vca0939* expression in *V. cholerae*. Specifically we showed that *vca0939* translation is positively controlled by base-pairing of Qrr sRNA to its native 5' UTR, and that Vca0939 requires DGC activity to increase c-di-GMP levels and to enhance biofilm formation in *V. cholerae*.

V. cholerae encodes 40 DGCs based on the appearance of a conserved “GGDEF” or “GGEEF” domain, as well as 29 PDEs that include 20 EAL domain proteins and 9 HD-GYP proteins. All of these enzymes have the potential to alter c-di-GMP levels. Here we have shown that Qrr-dependent expression of *vca0939* enhanced biofilm development, by increasing expression of the *vps* genes, as observed in a $\Delta hapR$ strain, presumably by the cumulative action of c-di-GMP on VpsR and VpsT (Krasteva et al., 2010, Srivastava et al., 2011).

Accumulation of c-di-GMP in *V. cholerae* has also been shown to not only enhance biofilm formation, but inhibit motility (albeit modestly), by hampering transcription of flagellar genes including *flrB* and *flrC* (Beyhan *et al.*, 2006, Liu *et al.*, 2010, Krasteva et al., 2010). However, we observed no defect in motility and no significant change in transcript abundance of *flrB* and *flrC* by RT-PCR of the same strains tested in Fig. 3.5 and 3.6 (data not shown). It remains possible that levels of Vca0939, when induced by Qrr expression, although sufficient to enhance biofilms were insufficient to repress

motility under the conditions tested. It is also reasonable that Vca0939 may show “high specificity signaling” and regulates only a subset of dedicated targets involved in biofilms but not motility (Massie et al., 2012). Further investigation is warranted.

The Qrr “anti-antisense” mechanism described here is similar to how DsrA and RprA sRNAs were shown in *E. coli* to positively control translation of the *rpoS* mRNA; by preventing formation of an inhibitory secondary structure in the 5'UTR of *rpoS* that prevents translation in the absence of sRNA (Majdalani et al., 1998, Majdalani et al., 2002). By binding to the left stem of the putative inhibitory stem loop in *vca0939*, Qrrs RNAs appear capable of competing with the right stem thereby preventing formation of the putative inhibitory stem loop structure (Fig. 3.1). While deletion of the left, right or entire sequence of the inhibitory structure enabled Qrr-dependent *vca0939* expression (Fig. 3.9), single nt mutations in the right arm, but not left arm, prevented translation despite preserving the putative structure by Mfold (data not shown). This is not due to disruption of the RBS, since deletion of the entire right arm still allowed for Vca0939 production (Fig. 3.9). It is interesting that the Qrr binding site in the 5'UTR of *hapR* is primarily single stranded (14/17 nt), while nearly half of the Qrr binding site within *vca0939* (6/14 nt) is predicted to be contained in the inhibitory structure, with the remainder (8/14 nt) accessible for Qrr pairing (Fig. 3.8) (Bardill et al., 2011). RNAup calculations also predict that Qrr pairing to *vca0939* requires additional “opening energy” to disrupt the inhibitory structure that is not required for Qrr pairing to *hapR* (Fig. 3.8). These predictions are consistent with the observation that the apparent K_d of Qrr/*vca0939* pairing (23 nM) shows weaker binding than for Qrr/*hapR* pairing (7

nM). It will be interesting to compare the Qrr binding affinity determined here for *vca0939* and prior for *hapR* (Bardill et al., 2011), with the remaining mRNAs predicted to be under Qrr control, and with other sRNA/mRNA pairs identified in *V. cholerae* (Rutherford et al., 2011, Svenningsen et al., 2009) (Bardill and Hammer, 2012).

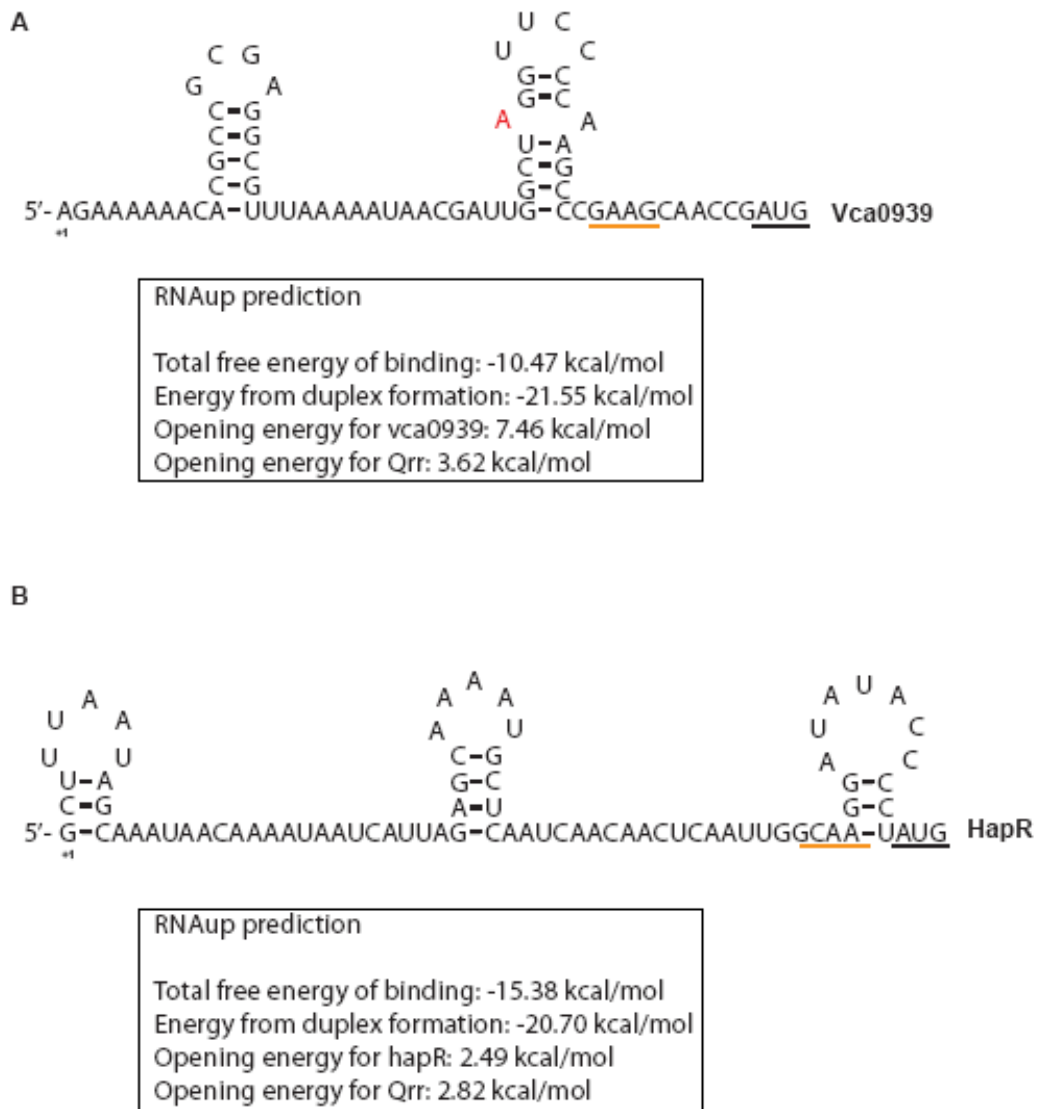


Figure 3.8. The 5'UTR of *vca0939* contains a putative inhibitory stem loop structure near the Qrr binding site that is not present in the 5'UTR of *hapR*. The Mfold algorithm was used to predict the minimal free energy structure of the *vca0939* 5'UTR (A, upper panel, as in Fig. 3.1) and also the *hapR* 5' UTR (B, upper panel). For Qrr/*vca0939* binding (A, lower panel) and Qrr/*hapR* binding (B, lower panel), RNAup

was used to determine the predicted total free energy of the interaction, which equals the energy of duplex formation, less the opening energy of the Qrr and the *vca0939* target.

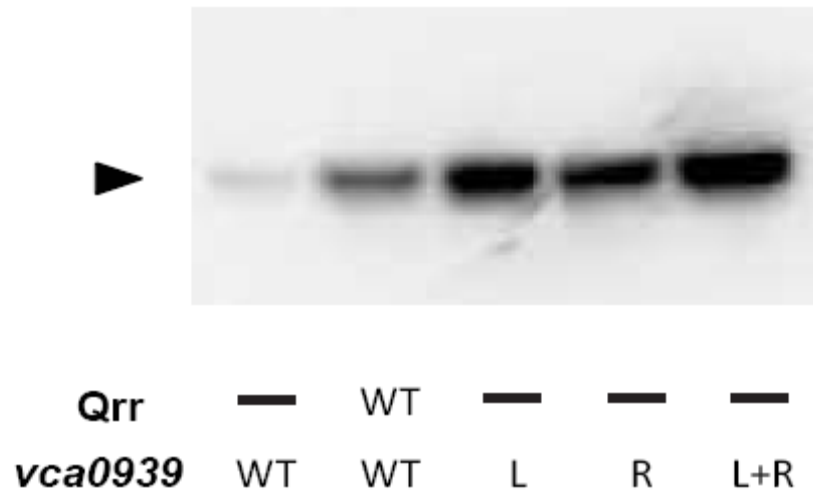


Figure 3.9. Effect of the inhibitory stem loop structure on the expression of Vca0939-Flag in *E.coli*. *E. coli* cultures were grown in triplicate and analyzed for protein production using Western blot. *E. coli* strains expressing WT *vca0939-flag* with empty vector of Qrr (lane 1) and WT Qrr (lane 2) serve as controls. In the absence of Qrr expression plasmid, expression of Vca0939_L-Flag (lane 3), Vca0939_R-Flag (lane 4), and Vca0939_{L+R}-Flag (lane 5) are measured. Reference bands indicate the location of each lane.

We demonstrate *in vitro* that Hfq facilitates the transition from the inhibitory *vca0939* structure to the activated form in which WT Qrr RNA is paired to the sequence corresponding to the left arm of WT *vca0939* (Fig. 3.1 and Fig. 3.5). It is curious that the Qrr_{WT}/*vca0939*_{WT} complex appeared to supershift to a location that may be distinct from the supershift observed for the Qrr_{U3G}/*vca0939*_{A43C} complex (Fig. 3.4). There are several possible explanations for this apparent change in the supershift complex. Firstly, there may be conformational changes in the *vca0939* mRNA not observable by computational methods, as Mfold predicted maintenance of the putative inhibitory structure despite single nt changes in the right stem that prevented translation (data not shown). Secondly,

the nature of Hfq's contribution to Qrr/*vca0939* pairing is poorly understood. Indeed, whether Hfq remains associated with an RprA/*rpoS* duplex or rapidly dissociates following RNA pairing is still not entirely clear (Fender et al., 2010, Soper *et al.*, 2011). Thus, it is possible that two species of a supershift could result from complexes formation of Qrr/*vca0939* with and without Hfq. We note that the only AU-rich region of the 67 nt 5'UTR of *vca0939* extends into the Qrr-binding site (Fig. 3.1B and 3.1C), thus it remains possible that Hfq and the Qrr sRNA compete for binding, such that after Qrr/*vca0939* association, Hfq no longer associates with the mRNA or with the sRNA/mRNA complex. Analysis of *vca0939* RNA structure could begin to address whether additional complexity exists in the activation of *vca0939* by the QS sRNAs.

Vca0939 was annotated as one of 40 putative GGDEF proteins in *V. cholerae* that acts as DGCs to synthesize c-di-GMP (Galperin, 2004). DGC enzymes are modular proteins encoding a variety of signal reception domains in the N terminus, and it has been proposed that certain GGDEFs may be activated in response to particular signals and tailored to control a defined set of c-di-GMP-controlled processes (Massie et al., 2012). Like other DGCs described, Vca0939 is predicted to carry an N-terminal PAS domain, which may be used to respond to oxygen or redox conditions (Qi et al., 2009, Chang et al., 2001). As such it is possible that Vca0939 regulates biofilm formation by integrating extra environmental and cellular signals with a QS response (Hengge, 2009).

Finally, the *vca0939* gene is one of four genes shown to be under Qrr control by genetic analysis. Each of the other three genes (*hapR*, *luxO*, *aphA*) participates in a feedback

loop with other components of the *V. cholerae* QS pathway (Tu et al., 2010, Svenningsen et al., 2008, Rutherford et al., 2011). Although not yet shown to participate in QS feedback, Vca0939 and HapR do participate together in regulation of biofilm development (Fig. 3.1), indicating that QS utilizes multiple Qrr-dependent pathways to control attachment. It remains possible that additional target genes under direct Qrr control remain to be identified in *V. cholerae*, as many sRNAs in *E. coli* have now been shown to control many mRNA targets (Soper et al., 2010, De Lay & Gottesman, 2012). Current and future studies of the *V. cholerae* sRNAs reveal that the QS response indeed utilizes a regulatory network of that includes as a critical component non-coding sRNAs.

3.5. Experimental procedures

3.5.1. Strains, plasmids and culture conditions

Standard microbiological techniques were used for growth of *V. cholerae* and *E. coli*. Liquid cultures of LB broth were incubated at 37° C with shaking unless noted otherwise. Antibiotics were added as appropriate to maintain plasmid selection. The genotypes of all strains used in this study are in Table 1. *V. cholerae* strains are derived from strain C6706str2 (Thelin & Taylor, 1996). *E. coli* strains DH5 α , S17 λ -pir, and EC100D (Epicentre) were used for cloning. All plasmids for expressing RNA by *in vitro* transcription were constructed using pUC18. Plasmids for expressing Qrr sRNAs and *vca0939* in *E. coli* were constructed in pEVS141 and pKK respectively (Lenz et al., 2004, Miller et al., 2002). Plasmids for expressing Qrr sRNAs and *vca0939* in *V. cholerae* were constructed in pKK and pEVS141 respectively (Lenz et al., 2004, Miller et al., 2002). The plasmid for expression and purification of *V. cholerae* Hfq was made in

pTYB11 (NEB). Single nucleotide substitutions were introduced by PCR-based methods or the QuikChange XL kit (Stratagene). Primer sequences used for PCR amplification and additional cloning details are available on request. All constructs were sequenced to ensure that they did not possess random errors.

Table 3.1. Strains and plasmids used in this study

<i>V. cholerae</i>		
<u>Strain</u>	<u>Genotype</u>	<u>Reference</u>
BH2126	$\Delta qrr1-4$, <i>luxOD47E</i>	This study
<i>Plasmids</i>		
<u>Plasmid</u>	<u>Description</u>	<u>Reference</u>
pKK	Control vector	Lenz et al., 2004
pEVS141	Control vector	Dunn et al., 2006
pEZ126	Ectopic WT <i>qrr</i> expression	Bardill et al., 2011
pEZ468	Ectopic <i>Qrr</i> _{U27C} expression	This study
pEZ702	Ectopic <i>Qrr</i> _L expression	This study
pEZ713	Ectopic <i>Qrr</i> _R expression	This study
pEZ701	Ectopic <i>Qrr</i> _{L+R} expression	This study
pEZ558	<i>Vca0939</i> -FLAG reporter	This study
pEZ718	<i>Vca0939</i> _{A43C} -FLAG reporter	This study
pEZ983	<i>Vca0939</i> _{AAEEF} -FLAG reporter	This study
pEZ982	<i>Vca0939</i> _{GGEAF} -FLAG reporter	This study
pPB004	<i>Qrr</i> <i>in vitro</i> transcription	Bardill et al., 2011
pEZ637	<i>Vca0939</i> <i>in vitro</i> transcription	This study
pEZ731	<i>Qrr2</i> _{U27G} <i>in vitro</i> transcription	This study
pEZ774	<i>Vca0939</i> _{A43C} <i>in vitro</i> transcription	This study
pPB007	Hfq expression plasmid	Bardill et al., 2011

3.5.2. Western Blotting

Bacterial cultures were grown to mid-logarithmic phase, normalized to cell density, pelleted by centrifugation and resuspended in Laemmli sample buffer. These samples were subjected to SDS-PAGE and transferred to PVDF membrane. Even protein loading was checked using Ponceau S stain. Membranes were blocked in a 5% milk tris buffered

saline solution and probed with a monoclonal anti-FLAG antibody (Sigma) followed by a peroxidase conjugated secondary antibody (Sigma). A chemiluminescent detection kit (Thermo-Fisher) was used to visualize the proteins on a ChemiDoc XRS HQ system (Bio-Rad).

3.5.3. Hfq and RNA purification and labeling

V. cholerae Hfq protein was purified as previously described (Bardill et al., 2011) using the IMPACT affinity tag intein kit (New England Biolabs). Protein quality was assessed by SDS-PAGE and concentration was determined spectrophotometrically.

Qrr sRNAs and *vca0939* mRNAs were generated by run-off transcription using a MEGAscript kit (Ambion). Following transcription, the RNAs were precipitated with LiCl and resuspended in RNase-free water. Both denaturing and non-denaturing PAGE were used to assess RNA quality following staining with SYBR Gold. RNA concentrations were determined spectrophotometrically. Dephosphorylated RNA was labeled using [γ -³²P]ATP and T4 polynucleotide kinase (Ambion). Labelled RNA was purified with NucAway spin columns (Ambion).

3.5.4. Electrophoretic mobility shift assays

Binding assays were conducted as described previously (Bardill et al., 2011). Briefly labeled Qrr RNA was mixed with varying concentrations of *vca0939* RNA in the presence or absence of Hfq. Final concentration of Qrr RNA was 0.4 nM with or without Hfq. Hfq concentration was 150 nM. Before mixing, the RNAs were denatured

by incubation at 70° C and quick-cooled on ice. After mixing, the reactions were incubated at 25° C for 1 hr and subjected to non-denaturing PAGE at 4° C. Gels were then used to expose a storage phosphor screen that was scanned and analyzed with a Typhoon Phosphorimager (GE). All reactions were carried out in buffer containing 20 mM Tris-HCL pH 8, 100 mM NH₄Cl, 50 mM NaCl, 50 mM KCl and 5% glycerol.

3.5.5. Biofilm development assays

Analysis of rugose colony morphology development were conducted as described previously (Fong et al., 2010, Ray et al., 2012). Cultures grown overnight at 30 ° C with shaking (250 rpm) were serially diluted with LB medium and 10 µl aliquots of the diluted cultures were spotted onto LB agar medium with Amp and Kan. Spot development was followed over a course of 40 h at 30 ° C. Images of the spotted cultures were acquired at the indicated time points using a dissecting microscope.

3.5.6. Determination of the Intracellular Concentration of c-di-GMP.

To quantify c-di-GMP, overnight culture of each strain of interest is back diluted for 1:100 with 3 biological replicates, 1.5 mL cultures were grown until mid-exponential phase. Cells were then pelleted by centrifugation and resuspended in 100 µL cold extraction buffer (40% Acetonitrile, 40% Methanol, 0.1 N Formic Acid). The supernatant was concentrated using a centrifugal evaporator and the product was resuspended in 100 µL water. Quantification of c-di-GMP was performed as previously described (Massie 2012, 22802636) using an Acquity Ultra Performance liquid chromatography system coupled with a Quattro Premier XE mass spectrometer. The concentration of c-di-GMP

was determined by quantifying an 8-point standard curve of chemically synthesized c-di-GMP (Biolog) ranging from 250 nM to 1.9 nM.

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CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

The ability to sense and respond to environmental cues is critical for *Vibrio cholerae* to control important developmental processes such as virulence and biofilm formation in the human host, and natural competence for DNA uptake in environmental settings. Specifically, *V. cholerae* integrates QS with c-di-GMP signaling to coordinate gene expression required for both the aquatic and host-associated phases of this pathogen. The transcriptional reprogramming caused by QS is dependent on four small regulatory RNAs, the Qrr sRNAs. The Qrr sRNAs were identified by genetic and bioinformatic methods and predicted imperfectly base-pairing with multiple mRNA targets using a region of 21 absolutely conserved nucleotides, and aided by the RNA chaperone protein, Hfq. While genetic evidence supports a model that Qrr sRNAs regulate *hapR*, *luxO*, *aphA*, or *vca0939* post-transcriptionally, direct binding of the Qrrs to these genes has not been documented experimentally prior to the work presented here.

This dissertation focused on the post-transcriptional regulatory mechanism used by the Qrr sRNAs to negatively control the master regulator HapR, as well as to positively control the GGDEF family protein Vca0939. Chapter 2 presented both *in vivo* and *in vitro* results of nucleotide substitution experiments to identify the base-pairing between Qrr and *hapR* mRNA. It also demonstrate that this base-pairing interaction overlaps the RBS of *hapR* mRNA, alters HapR protein levels, and results in HapR-dependent gene expression and corresponding QS phenotypes of virulence factors,

biofilm formation, protease production, and DNA uptake. In Chapter 3, I showed that Qrr sRNAs activate *Vca0939* expression by base-pairing with and disrupting an inhibitory stem loop structure in the 5' UTR of *vca0939* mRNA that otherwise prevents *Vca0939* translation. I also demonstrated that Qrr-dependent *Vca0939* expression promotes accumulation of c-di-GMP levels that enhance biofilm development. These results are consistent with a model that *V. cholerae* QS promotes biofilm formation at low cell density through Qrr-activated *vca0939* translation, independent of the “master QS regulator”, HapR.

It remains to be determined whether the QS sRNAs regulate additional mRNA not yet identified. Nonetheless, the research detailed in this dissertation sheds light on understanding the molecular mechanism of *V. cholerae* Qrr sRNAs and identifies them as critical regulatory factors for controlling fundamental processes important for *V. cholerae* survival in the environment and human host. This work also opens several future research directions that are worth further investigation:

1. A total of four mRNA targets have been described for the *V. cholerae* Qrrs. Absolute conservation of the 21 nts in the Qrr sRNAs is shown to be critical for proper pairing with *hapR* and *vca0939*, although different nts appear critical for each Qrr/mRNA interaction. Recently, the *aphA* gene was shown to be regulated by Qrr sRNAs using a region of the Qrr that extends beyond the 21 conserved nts. This raises the possibility that each Qrr sRNA may regulate one or more specific targets separately and also function together to regulate common targets. Comprehensive analysis of Qrr nts, using both our

in vivo methods and *in vitro* RNA binding experiments will be used to dissect the molecular mechanisms of additional sRNA/mRNA interactions that coordinate the QS response in this human pathogen.

2. In this study binding of Qrr RNA with *vca0939* RNA *in vitro* was documented. It is interesting to observe that single nucleotide mutations in RNA altered the migration of the “complex” in polyacrylamide gel. There are several explanation for this apparent change in the complex. One could be conformational changes in the RNAs, which cannot be predicted by current computational methods. Another could be the rapid dissociation of the Hfq from the RNAs after Qrr/*vca0939* base-pairing, as the nature of Hfq's contribution to sRNA/mRNA interactions is still poorly understood. Analysis of *vca0939* RNA structure could begin to address whether additional complexity exists in the activation of *vca0939* by the QS sRNAs.

3. *Vca0939* was shown to regulate biofilm formation in a HapR-independent manner. *Vca0939* is predicted to carry an N-terminal PAS domain, which may be used to respond to oxygen or redox conditions. It is unknown whether *Vca0939* regulates biofilm formation by integrating extra environmental and cellular signals with a QS response, and what molecular mechanisms *Vca0939* uses to regulate biofilm formation. Further studies using genome-wide analyses such as RT-PCR and RNA-seq will elucidate the signaling pathway used by *Vca0939* to regulate biofilm development.

In conclusion, we defined the molecular mechanisms that the Qrr sRNAs use for base-pairing with the mRNA targets, *hapR* and *vca0939*, and the impact on QS regulated gene expression and phenotypes. This study will have fundamental implications for understanding regulatory circuits of all cellular systems. Moreover, my QS investigations may lead to the development of pro- and anti-QS strategies for the production of useful products.

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