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# Characterizing a novel direct target of the quorum-sensing controlled small RNAs in *V. cholerae*

Undergraduate Honors Thesis

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## Introduction

The bacterial genus *Vibrio* represents an abundant and ubiquitous component of marine, brackish, and freshwater microbial ecosystems. *Vibrio* bacteria can live planktonically as free-living organisms, or in communities attached to surfaces in biofilms. *Vibrio* biofilms are commonly formed in association with chitinous animals such as copepods, or on abiotic surfaces. Some *Vibrios*, including *Vibrio cholerae*, are also capable of colonizing the human digestive system. *V. cholerae*, the causative agent of the deadly diarrheal disease cholera, endemic to parts on Africa, India, and South East Asia. Following the 2010 earthquake in Haiti, an cholera epidemic emerged afflicting over 600,000 people and resulting in over 7,500 deaths in the first two years (Katz *et al.*, 2013).

*V. cholerae* is able to communicate with nearby bacteria to regulate gene expression by a process termed quorum sensing (QS) (Reviewed in Ng & Bassler, 2009). Quorum sensing is a strategy employed by a majority of extant bacteria that enables changes in behavior based on the cell density of their population. Bacteria synthesize, release, and then sense small molecules called autoinducers (AI) that are often specific to a particular species. In an environment at low cell density, when AI concentrations are low, the bacteria do not sense a quorum. At high cell density, when AI concentrations are high, the bacterial population senses the molecules by binding of the AIs to specific membrane bound receptors. Tethering the presence or absence of the AI signal to gene regulation allows the bacteria to differentially express genes as a function of cell density (Ng & Bassler, 2009). *V. cholerae* uses quorum sensing to regulate a large number of genes, including those for virulence factors, biofilm formation, and natural competence (Zhu *et al.*, 2002, Hammer & Bassler, 2003, Meibom *et al.*, 2005)

In *V. cholerae*, the quorum sensing circuit includes four small RNAs (sRNAs), which are non-coding RNAs that are transcribed but not translated. sRNAs were first discovered in Eukaryotes where they regulate target genes post-transcriptionally, typically by pairing with mRNA targets in the 3'UTR or sometimes within the coding sequence of the message itself (Rana, 2007). Bacterial sRNAs are typically 50-250 nucleotides in length and also regulate target genes post-transcriptionally. The typical mechanism for regulation is base-pairing to the 5' untranslated region (UTR) of target mRNA transcripts, which can either act to promote translation or, more commonly, repress it (Storz *et al.*, 2005). The sRNAs in the quorum sensing pathway of *V. cholerae* are called Qrrs (quorum regulatory RNAs). The Qrr sRNAs are regulated in response to cell density. When concentrations of *V. cholerae* AIs (CAI-1 and AI-2) are low (at low cell density), the AI receptor proteins (CqsS and LuxP/Q, respectively) act as histidine kinases, donating phosphate to LuxU, a phosphotransfer protein, which then donates the phosphate to LuxO, a response regulator. Phosphorylated LuxO can then bind the Qrr promoter regions and recruit RNA polymerase to activate transcription of the Qrr sRNAs (See Figure 1). All four Qrr sRNAs contain a completely conserved 21 nucleotide sequence that base pairs with several direct mRNA targets (Bardill & Hammer, 2012)

One of the Qrr sRNA targets, *hapR*, codes for a transcription factor that regulates many genes including virulence factors, biofilms, and natural competence. It has been shown empirically that base pairing occurs between the Qrr sRNAs and *hapR* (Figure 2A), and that this interaction is dependent on an RNA binding protein called Hfq (Bardill *et al.*, 2011). Specifically, the Qrr sRNAs base pair to *hapR* mRNA overlapping the ribosome binding site, which prevents translation. Initially, *hapR* was the only known target of the Qrr sRNAs. However, a genetic

screen of a random promoter library (Hammer & Bassler, 2009) identified additional target mRNAs controlled by the Qrr sRNAs. One target, *vca0939*, was predicted to code for a GGDEF-type protein (Galperin *et al.*, 2001), which can synthesize cyclic-di-GMP, a secondary messenger molecule used in many regulatory circuits. Most notably, c-di-GMP regulates biofilms, important both in *V. cholerae's* aquatic life and its ability to cause disease (Srivastava & Waters, 2012). It has been shown recently that Vca0939 synthesizes c-di-GMP and that activation of *vca0939* by Qrr sRNAs positively impacts *V. cholerae's* ability to produce biofilms (Zhao *et al.*, 2013).

To identify additional *V. cholerae* genes directly regulated by Qrr interactions, a random plasmid-based library of luciferase gene fusions (described in Hammer & Bassler, 2009) was introduced into an *E. coli* strain, in which expression of a Qrr sRNA was under control of the arabinose inducible $P_{BAD}$ promoter. The use of the *E.* coli system uncoupled any regulation by *V. cholerae* specific factors, because the *V. cholerae* quorum sensing pathway is not present in *E. coli*. The library was screened for Qrr-controlled genes by comparing luciferase expression without and with the addition of arabinose. Several candidate genes were identified, including VC0939 (unpublished results, Bardill & Hammer). One novel candidate gene, VC1831, is the focus of my thesis research and will be described here.

VC1831 is annotated as a histidine kinase protein, similar to the two QS AI receptors described above. It has also been shown that the QS LuxU phosphotransferase protein is capable of donating phosphate to VC1831, as well as the known QS AI receptors, CqsS and LuxQ (see Fig. 1) (Shikuma *et al.*, 2009). As a result, we hypothesize that VC1831 is part of a feedback loop that may be responsible for fine tuning the quorum sensing response. Quorum sensing feedback

loops have been elucidated previously (Svenningsen *et al.*, 2008, Tu *et al.*, 2010), showing that the regulatory circuit in *V. cholerae* is highly evolved and is apparently a more complex network than was first imagined. It is important to also characterize VC1831 and determine its role in regulating the quorum sensing response in *V. cholerae*. Since the quorum sensing pathway regulates cholera toxin and other virulence factors, any factors in the pathway are also potential targets for medical intervention

## Results

### *The Qrr sRNAs repress vc1831 in vivo*

A screen described above using a luciferase-based reporter identified *vc1831* as a repressed target of the Qrr sRNAs. To demonstrate direct interactions between *vc1831* and the Qrrs, the simple *E. coli* system was again employed to uncouple any *V. cholerae*-specific factors. A plasmid (pQrr) was constructed that placed one Qrr (Qrr1) under control of a constitutive promoter. An empty vector (pControl) was used as a control Because the luciferase-reporter was designed as a promoter trap and not to identify sRNA target genes (Hammer & Bassler, 2009), a second plasmid (pVC1831-FLAG) was constructed that carried the *vc1831* gene under control of its own promoter (Bardill & Hammer, unpublished). Importantly, the plasmid was engineered to include FLAG epitope tag at the C-terminus of VC1831. This permitted the presence of VC1831-FLAG protein to be visualized by western blot, using anti-FLAG antibodies.

Overnight cultures of *E. coli* carrying pVC1831-FLAG and pControl express VC1831 protein, which is detectable as a single band on the western blot (Figure 3, lane 1). In contrast, no band was observed for the *E. coli* culture carrying pVC1831-FLAG and pQrr (Figure 3, lane 2). These

results were consistent with those obtained in the genetic screen and consistent with the model that Qrr sRNAs directly interact with *vc1831* at the RBS and repress translation (Figure 2B).

*vc1831-Qrr interaction dependent on RNA chaperone protein Hfq*

Hfq is an RNA binding protein known to stabilize many RNA-RNA interactions, including the interactions between Qrr sRNAs and known target mRNAs such as hapR and vca0939 (Bardill et al., 2011, Lenz *et al.*, 2004, Zhao et al., 2013). All known direct targets of the Qrr sRNAs are regulated only when Hfq protein is present. To test whether this was also the case with *vc1831,* pVC1831-FLAG and either pQrr or pControl were introduced into an *E. coli hfq-* mutant strain. *E.coli hfq-* cells carrying pVC1831-FLAG and pControl express VC1831 (Figure 4, lane 3). VC1831 protein was also expressed in *hfq-* cells with the pQrr plasmid (Figure 4, lane 4) indicating that Qrr1 was unable to repress translation of *vc1831* in this background. These results were consistent with the model that Hfq mediates direct interaction between the Qrr sRNAs and *vc1831* mRNA.

*Mutations in the 5'UTR do not prevent regulation by Qrr2*

In the case of *hapR,* a single nucleotide was found that, when mutated, prevented Qrr sRNAs from base pairing to the mRNA (Bardill et al., 2011). This same method worked for *vca0939*; a nucleotide substitution was found that prevented Qrr sRNAs from regulating *vca0939* (Zhao et al., 2013). These results validate that Qrr regulation of these two targets is due to direct base pairing. To test if this was also true for *vc1831*, mutations were made in the 5'untranslated region (5'-UTR) of *vc1831* at nucleotides predicted to participate in interactions with Qrr sRNA. All possible single-nucleotide mutations were made in the 5'UTR between the predicted RBS and the

115 start codon. By Western blot, E. coli carrying pQrr fully repressed each of the plasmids that
116 carried VC1831-FLAG with a single nucleotide mutation in its 5'UTR (data not shown).
117
118 These results suggested that it may be necessary to disrupt the interaction more substantially as
119 described in several studies of *E. coli* sRNAs (Coornaert *et al.*, 2010, Mandin & Gottesman,
120 2009, De Lay & Gottesman, 2009). Larger mutations, ranging from two to fourteen nucleotides
121 were made and tested; unexpectedly, even when all fourteen base pairs between the predicted
122 ribosome binding site and the start codon were mutated, the presence of Qrr sRNA still
123 prevented the accumulation of VC1831 protein (Figure 5). This result indicated to us that
124 changes to the predicted site of interaction were not sufficient to cause deregulation. The same
125 prediction tool (TargetRNA, Tjaden, 2008) had yielded predictions for *hapR* (Figure 2A) and
126 *vca0939* (not shown) that were verified experimentally, but so far the same methods have not
127 worked for *vc1831*.
128
129 *Mutant vc1831 RNA fragments do not bind with Qrr sRNA* in vitro
130 In prior studies of Qrr targets (Bardill et al., 2011, Zhao et al., 2013) direct base pairing was
131 determined by purifying Qrr sRNA and the target mRNA and conducting and electrophoretic
132 mobility shift assay (EMSA) with the addition of purified Hfq protein. This decoupled the
133 interaction from any other factors present in a living cell. Thus to test whether Qrr RNA was
134 indeed capable of base pairing to *vc1831* RNA, Dr. Bardill, a post-doc in the lab, engineered
135 plasmids to conduct similar experiments. First, a plasmid to express a 106 base pair fragment of
136 the *vc1831* mRNA, starting with the +1 of transcription was created. (Full-length *vc1831* mRNA
137 is over 2,200 base pairs long, and would not have been able to be seen on the same gel as the 108

base pair Qrr sRNA due to size differences). The same mutations tested *in vivo* (5 nucleotides and larger) were introduced to the 5'UTR of the *vc1831* fragment. *vc1831* RNA was purified from cells containing these plasmids. These RNA molecules were used in electrophoretic mobility shift assays with radioactively labeled Qrr2 and purified *V. cholerae* Hfq protein. It was observed that Qrr2 formed a complex with Hfq protein (Figure 6). The addition of the wild type *vc*1831 fragment caused a supershift, indicating that Qrr2 was binding to *vc1831.* In all mutant fragments tested, this supershift was not observed. These data were consistent with the model that the 5'UTR was base pairing with Qrr sRNA molecules in a sequence specific manner. However, this data did not match the *in vivo* data using the same mutations to the 5'UTR.

*Computational methods predict that Qrr sRNA may pair with vc1831 at multiple sites*

Because of the disconnect between the *in vivo* Western Blot data and the *in vitro* EMSA data, it was hypothesized that there could be another Qrr site downstream of the 106 base pairs tested *in vitro*. Though multiple sRNA binding sites are more common in eukaryotic systems, it is not unprecedented for a bacterial sRNA to bind to multiple sites in a single mRNA transcript (Rice et al., 2012).

RNAup (Lorenz *et al.*, 2011), a program that predicts RNA-RNA interactions using free energy calculations, was used to determine whether the mRNA of *vc1831* contained one or more additional sites for the Qrr sRNAs in addition to the site in the 5' UTR. The *vc1831* transcript is 2,226 nucleotides (nt), and a decision was made to interrogate sequence fragments of 50-100 nts from RNAup, since testing larger fragments from *hapR* and *vca0939* for putative Qrr interaction sites had generated false positives in the (data not shown). A script was written to test every

possible fragment of *vc1831* mRNA corresponding to sliding windows between 50 and 100 base pairs long, constituting over 120,000 RNAup calls (details provided in Methods). These fragments were tested with a 33 base pair fragment of the Qrr2 which included the 21 base pair conserved region known to interact with all of its targets (Figure 2). As a control, ***hapR*** was tested using the same script, since it is known empirically that only one region is sufficient for interaction with the Qrr sRNAs. The outputs from the RNAup calls were consolidated and visualized (Figure 7). In the case of ***hapR***, only one region was clearly predicted to interact with the Qrr fragment by RNAup, and the region mapped to the 5'UTR shown by Bardill et. al. to pair with Qrrs *in vitro*. In contrast, the RNAup analysis of *vc1831* generated many potential binding sites for the Qrr sRNAs. The site with the highest score was the region predicted by TargetRNA in the 5'UTR (Figure 2B). However, there were other sites in the *vc1831* coding sequence that scored much higher than any region seen in the ***hapR*** coding sequence. This indicated to us the possibility of one or more additional sites being acted upon by the Qrr sRNAs in the message of *vc1831*.

*Replacing the native 5' UTR of vc1831 appears to prevent regulation by the Qrr sRNAs*

Because the region of interaction within the 5'UTR extended into the predicted ribosome binding site and the start codon (Figure 2), mutations extending 3' were not be made in the native 5'UTR due to concern that they would adversely affect translation. As an alternative test to determine the necessity of the native 5'UTR for Qrr pairing, the entire promoter and 5'UTR were replaced with the IPTG-inducible ptac promoter and 5'UTR. This construct (ptac-VC1831-FLAG) was moved into *E.coli* cells containing either pQrr or pControl described above. These cells were induced with concentrations of IPTG ranging from 500μM to 4μM, and VC1831FLAG was

visualized by Western blot (Figure 7). VC1831 protein was produced in detectable quantity with 20μM IPTG induction (Figure 7 lane 7), and it was still detected in cells with pQrr (Figure 7 lane 8). This data is, on the surface, consistent with there being only one Qrr site. An alternative interpretation of this data is provided in the Discussion section.

*VC1831 up-regulates Qrr transcription in a LuxU dependent manner*

The current model of the quorum sensing pathway in *V. cholerae* suggests that only CqsS and LuxPQ can transduce phosphate to LuxU (Figure 1) (Miller *et al.*, 2002). This implies that these two sensory systems are the only input into the quorum sensing pathway via LuxU. Because of the *in vitro* evidence that VC1831 protein was capable of dephosphorylating LuxU (Shikuma et al., 2009), and that VC1831 is predicted to be a kinase by functional group analysis, we hypothesized that VC1831 could positively regulate Qrr sRNA transcription in a manner similar to CqsS and LuxPQ.

To test this, two plasmids were mated into a *V. cholerae ΔcqsS ΔluxQ* double sensor mutant. One plasmid (pQrrLux) had a Qrr-luciferase transcriptional fusion, which was used as a reporter. The other plasmid either contained VC1831 under control of the ptac promoter ($pP_{tac}$VC1831-FLAG), or was an empty vector ($pP_{tac}$Control). Compared to the control, double sensor mutant cells with $pP_{tac}$VC1831-FLAG had higher levels of Qrr, indicated by an increase in bioluminescence (Figure 9, comparing bars 1 and 2). To test whether this positive regulation was dependent on luxU, the same plasmids were moved into a *ΔcqsS ΔluxQ ΔluxU* triple mutant. Cells with $pP_{tac}$Control and pVC1831 had comparable light levels (Figure 9, comparing bars 3

and 4). These data were consistent with the model that VC1831 acts by phosphorylating LuxU to positively regulate the Qrr sRNAs.

## Discussion

The quorum sensing pathway of *V. cholerae* controls many genes important for both environmental behaviors and pathogenesis. Many of the known genes regulated by quorum sensing are dependent on HapR, which was originally believed to be the only target of the Qrr sRNAs (Miller et al., 2002). It is now known that the Qrr sRNAs regulate other targets directly: LuxO, VCA0939, and the focus of this study, VC1831.

The *E. coli* experiments shown here are consistent with a model of direct interaction between *vc1831* mRNA and the Qrr sRNAs. Expressing a Qrr sRNA from a plasmid in *E. coli* prevented the translation of *vc1831*. This effect was shown to be dependent on the presence of Hfq. In order to show empirically that this result was due to direct base pairing between Qrr sRNAs and *vc1831* mRNA, further experiments were carried out. *In vitro* experiments showed that Qrr2 sRNA could bind directly to a fragment of *vc1831* containing the first 106 base pairs of the message. When this fragment included mutations to the predicted site of interaction, binding of Qrr2 to the fragment was not observed.

It was unexpected that extensive changes in the 5' UTR of the *vc1831* gene would still allow Qrr sRNAs to repress *vc1831* translation *in vivo*, especially considering these same changes prevented binding *in vitro* (compare Figures 5 and 6). This disconnect led to the hypothesis that there might be one or more Qrr interaction sites downstream of the 106 basepairs tested *in vitro*.

Computational analysis showed that there may indeed be additional sites in the *vc1831* transcript to which the Qrr sRNAs are binding. However, when the 5'UTR was completely replaced with the ptac 5'UTR, the repression appeared to be abolished. Taken at face value, this result is consistent with there only being one Qrr site in the 5'UTR. It is possible that *in vivo*, interactions with the chaperone protein Hfq facilitate pairing between the mutated 5'UTR and the Qrr sRNAs in an unexpected manner, particularly when multicopy plasmids are used and heterologous promoters express RNAs at levels that may not reflect native conditions. Indeed, wild type Qrrs expressed from a multi-copy plasmid can repress *hapR* from a multicopy plasmid, and the same regulation is observed when both are in single copy (Zhao, Bardill, Hammer ref). However, multicopy wild type Qrrs can also repress *hapR* with a point mutation in the 5'UTR site; and it is believed that such results are a consequence of altering the relative ratios of the RNA by non native expression (Bardill, unpublished results). It is possible that expression of the Qrrs and vc1831 from the multicopy plasmids chosen here and used in *E. coli* do not reflect the conditions under which both are expressed in *V. cholerae*.

Another similar experimental variable may also explain the discrepancies observed here. One interpretation of the ptac-VC1831 experiment (Figure 8) is that excessive *vc1831* was not transcribed uniformly in each cell in the population. Ptac is an "all-or nothing" promoter due to regulation of LacY permease (Jensen *et al.*, 1993). LacY permease, responsible for active transport of IPTG into the cell, is itself induced by the presence of IPTG through inactivation of LacI. Therefore at low concentrations of IPTG, a small population of cells in the culture will import a majority of the IPTG in the media. This results in some cells in a population expressing a ptac-regulated target maximally, while other cells do not express the ptac-regulated target. So,

even though the 20μM bands in Figure 7 (Lanes 7 and 8) are fainter than the control bands (Lanes 1 and 2), these bands represent a population average of the bacterial culture and the levels of *vc1831* observed simply represent an average of very different cell responses.

Two experiments are now being performed to further test whether there are multiple Qrr sites on *vc1831* mRNA. First, it has been shown that deleting the lactose permease *lacY* can prevent the all-or-nothing response of a ptac-inducible system (Jensen et al., 1993). The same ptac-VC1831 plasmid will be moved into an *E. coli ΔlacY* strain carrying pQrr or pControl, and tested using a similar range of IPTG concentrations as used before. If the results observed in Figure 8 were indeed due to variation in response to IPTG, then we would expect to see pQrr repress ptac-*vc1831* in the *ΔlacY* strain at concentrations of IPTG yielding appropriate levels of *vc1831*.

Second, translational fusions of *vc1831* fragments, with both wild type and mutant 5'UTRs, will be engineered in frame to *nusA* sequence. NusA protein is highly soluble and commercially available antibodies can be used to detect it. the These *vc1831* fragments will be made in sizes ranging from the 106 base pairs tested *in vitro* to full length, using the RNAup results as a guide. We will use NusA translational fusions, rather than FLAG fusions because of concerns that some of the *vc1831* fragments, when translated, may have exposed hydrophobic residues that would flag the peptide for degradation without being fused to a highly stable peptide. Another benefit of using NusA fusions is that it will increase the size considerably, which should make the smaller *vc1831* fragments easier to visualize by Western. These fragments will then be expressed in the presence or absence of Qrrs, and anti-NusA antibodies will be used to detect accumulation of the protein. We expect the mutant fragment with the same number of nucleotides as the *in vitro*

construct used in the EMSA experiments. If no additional sites for Qrr binding exist in the coding region of vc1831, then we expect that all of the VC1831-NusA fusions will be repressed in the presence of pQrr. Conversely, if additional sites do exist, then we expect that the smallest *vc1831* fragment with the mutant 5'UTR (corresponding to the 14-1 lane in Figure 6) will not be repressed by the Qrr sRNAs, and that one of the larger mutant fragments will be repressed. It would then follow that the additional sequence in the smallest repressed construct, compared to the largest unregulated construct, should contain an additional Qrr sRNA interaction site.

The bioluminescence data from the *V. cholerae* experiments are consistent with VC1831 acting through LuxU to regulate the Qrr sRNAs, and therefore regulating quorum sensing dependent phenotypes such as biofilm formation, natural competence, and virulence. VC1831 is predicted to have trans-membrane domains. It is possible based on the current evidence that VC1831, a predicted sensor kinase, is acting as a third input into the quorum sensing circuit of *V. cholerae*. This possibility is currently being worked on by our research group. This would be an exciting result, as it could help researchers better understand the quorum sensing system, which impacts many behaviors including virulence. Potentially this information may prove useful to identifying new anti-quorum sensing strategies for preventing or treating cholera.

## Materials and Methods

*Western blot analysis*

LB liquid with the appropriate antibiotics was inoculated with cells from single colonies on LB plates. These cultures were allowed to grow over night shaking at 37 C. These cultures were diluted 1:100 the next day and grown for 3 hours. IPTG was added to cultures to create varying

final concentrations (500μM, 100μM, 20μM, 4μM, and no IPTG) and the cells grew for 2 more hours. These cultures were then normalized to equal cell densities, pelleted by centrifugation, and resuspended in Laemmli sample buffer, The samples were boiled for 10 minutes, and then subjected to SDS-PAGE. The samples in the gel were then transferred to a PVDF membrane, and then probed with anti-FLAG antibodies produced in mice. The signal was amplified using an anti-mouse antibody, and visualized using a chemiluminescent detection kit and a ChemiDoc XRS HQ system (Bio-Rad).

*High throughput automated RNAup analysis*

A script was written in the Perl programming language to call RNAup for each input sequence and write the output to a file. This file was then parsed using JavaScript. Scores were assigned to each nucleotide in the sequence by adding up the predicted Gibbs Free Energy for each predicted interaction in which a given nucleotide appeared. To visualize the output, these scores were transformed using a linear scale to a color scale from Red to Blue. This color information was then used to build an HTML page of the sequence using the d3.js data visualization library. The source code for all scripts used in this analysis is available to view and download at https://gist.github.com/elsherbini/7904238

*Bioluminescence assays*

*V. cholerae* cultures were grown overnight (~16 hours) and then diluted to a common $OD_{600}$ of 0.005 in media either with IPTG or without. Light production was measured after 4 hours and 30 minutes using a liquid scintillation counter (Wallac Model 1409). The $OD_{600}$ was measured using

320 a spectrophotometer. Relative Light Units were calculated by dividing the scintillation counts by
321 the $OD_{600}$.

## References


Bardill, J. P. & B. K. Hammer, (2012) Non-coding sRNAs regulate virulence in the bacterial pathogen Vibrio cholerae. *RNA Biol* **9**: 392-401.

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

Coornaert, A., A. Lu, P. Mandin, M. Springer, S. Gottesman & M. Guillier, (2010) MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol* **76**: 467-479.

De Lay, N. & S. Gottesman, (2009) The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. *J Bacteriol* **191**: 461-476.

Galperin, M. Y., A. N. Nikolskaya & E. V. Koonin, (2001) Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* **203**: 11-21.

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

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

Jensen, P. R., H. V. Westerhoff & O. Michelsen, (1993) The use of lac-type promoters in control analysis. *Eur J Biochem* **211**: 181-191.

Katz, L. S., A. Petkau, J. Beaulaurier, S. Tyler, E. S. Antonova, M. A. Turnsek, Y. Guo, S. Wang, E. E. Paxinos, F. Orata, L. M. Gladney, S. Stroika, J. P. Folster, L. Rowe, M. M. Freeman, N. Knox, M. Frace, J. Boncy, M. Graham, B. K. Hammer, Y. Boucher, A. Bashir, W. P. Hanage, G. Van Domselaar & C. L. Tarr, (2013) Evolutionary dynamics of Vibrio cholerae O1 following a single-source introduction to Haiti. *MBio* **4**.

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

Lorenz, R., S. H. Bernhart, C. Honer Zu Siederdissen, H. Tafer, C. Flamm, P. F. Stadler & I. L. Hofacker, (2011) ViennaRNA Package 2.0. *Algorithms Mol Biol* **6**: 26.

Mandin, P. & S. Gottesman, (2009) A genetic approach for finding small RNAs regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system. *Mol Microbiol* **72**: 551-565.

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

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

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

Rana, T. M., (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol* **8**: 23-36.

Rice, J. B., D. Balasubramanian & C. K. Vanderpool, (2012) Small RNA binding-site multiplicity involved in translational regulation of a polycistronic mRNA. *Proc Natl Acad Sci U S A* **109**: E2691-2698.

Shikuma, N. J., J. C. Fong, L. S. Odell, B. S. Perchuk, M. T. Laub & F. H. Yildiz, (2009) Overexpression of VpsS, a hybrid sensor kinase, enhances biofilm formation in Vibrio cholerae. *J Bacteriol* **191**: 5147-5158.

Srivastava, D. & C. M. Waters, (2012) A tangled web: regulatory connections between quorum sensing and cyclic Di-GMP. *J Bacteriol* **194**: 4485-4493.

Storz, G., S. Altuvia & K. M. Wassarman, (2005) An abundance of RNA regulators. *Annu Rev Biochem* **74**: 199-217.

Svenningsen, S. L., C. M. Waters & B. L. Bassler, (2008) A negative feedback loop involving small RNAs accelerates Vibrio cholerae's transition out of quorum-sensing mode. *Genes Dev* **22**: 226-238.

Tjaden, B., (2008) TargetRNA: a tool for predicting targets of small RNA action in bacteria. *Nucleic Acids Res* **36**: W109-113.

Tu, K. C., T. Long, S. L. Svenningsen, N. S. Wingreen & B. L. Bassler, (2010) Negative feedback loops involving small regulatory RNAs precisely control the Vibrio harveyi quorum-sensing response. *Mol Cell* **37**: 567-579.

Zhao, X., B. J. Koestler, C. M. Waters & B. K. Hammer, (2013) Post-transcriptional activation of a diguanylate cyclase by quorum sensing small RNAs promotes biofilm formation in Vibrio cholerae. *Mol Microbiol* **89**: 989-1002.

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

## Figures

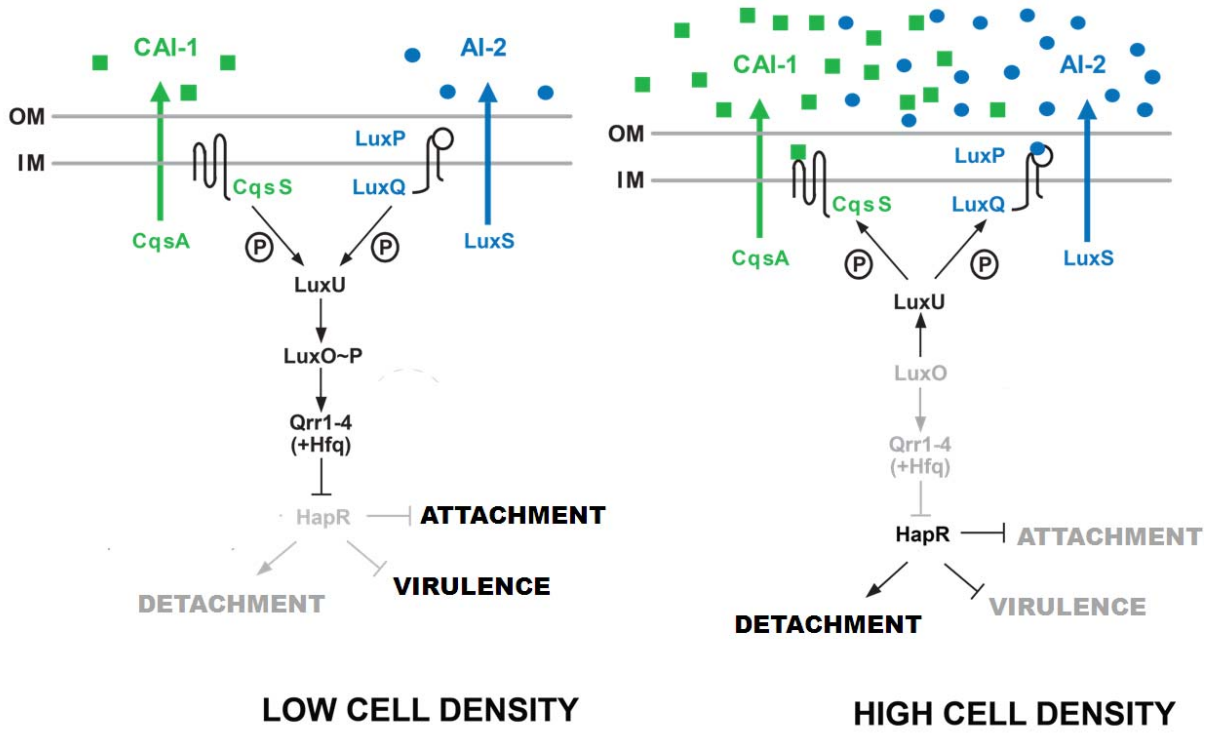
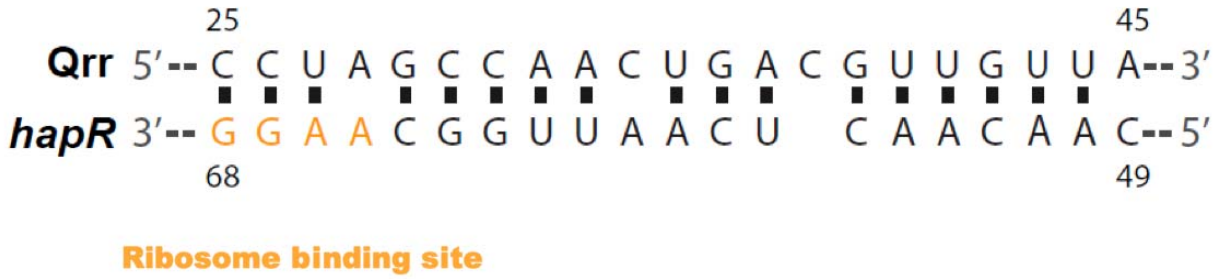



**Figure 1**. A model of the quorum sensing system in *V. cholerae*. At low cell density (left) AI concentrations are low. The AI receptors are not bound to AI, and act as histidine kinases, donating phosphate to LuxU, which donates phosphate to LuxO. Phosphorylated LuxO (LuxO-P) can then activate transcription of the 4 Qrr sRNAs. The sRNAs act with Hfq to regulate genes posttranscriptionally by base pairing with target mRNAs. HapR is repressed, and genes important for attachment and virulence are turned on. At high cell density (right), AI concentration is high. The AI receptors are bound and act as phosphatases, reversing the flow of phosphate. LuxO is not phosphorylated and no longer activates transcription of the Qrr sRNAs. HapR is no longer repressed, and genes important for natural competence are on.

**A**




**B**


**Figure 2.** Predicted binding site of the Qrr sRNAs with *hapR*(A) and *vc1831*(B). The ribosome binding site is highlighted in yellow, Watson Crick base-pairing is represented by black squares, and G-U base-pairing is represented by red squares.

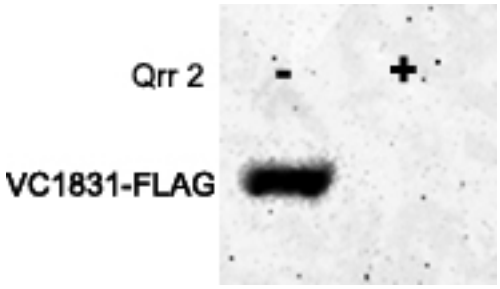



**Figure 3.** Western Blot analysis of VC1831-FLAG without or with Qrr2 in *E. coli*. One Qrr (Qrr2) was expressed from a plasmid under control of a constitutive promoter. A second plasmid carried the *vc1831* gene under control of its own promoter that was engineered with a C-terminal FLAG epitope tag. The presence of VC1831-FLAG protein was visualized by western blot, using anti-FLAG antibodies. Using this system it was observed that the presence of Qrr prevented translation of VC1831.

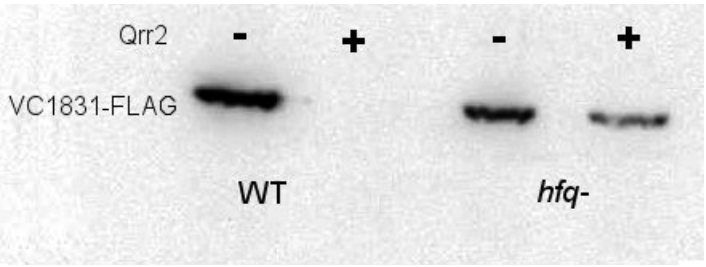



**Figure 4.** Western blot analysis of VC1831 in an *hfq*- mutant. The accumulation of VC1831-FLAG was visualized in the presence or absence of Qrr2 in both a wild type background and an *hfq*- strain. VC1831-FLAG accumulates in the *hfq*- strain in the presence of Qrr2, indicating that the Qrr-*vc1831* interaction is Hfq dependent.

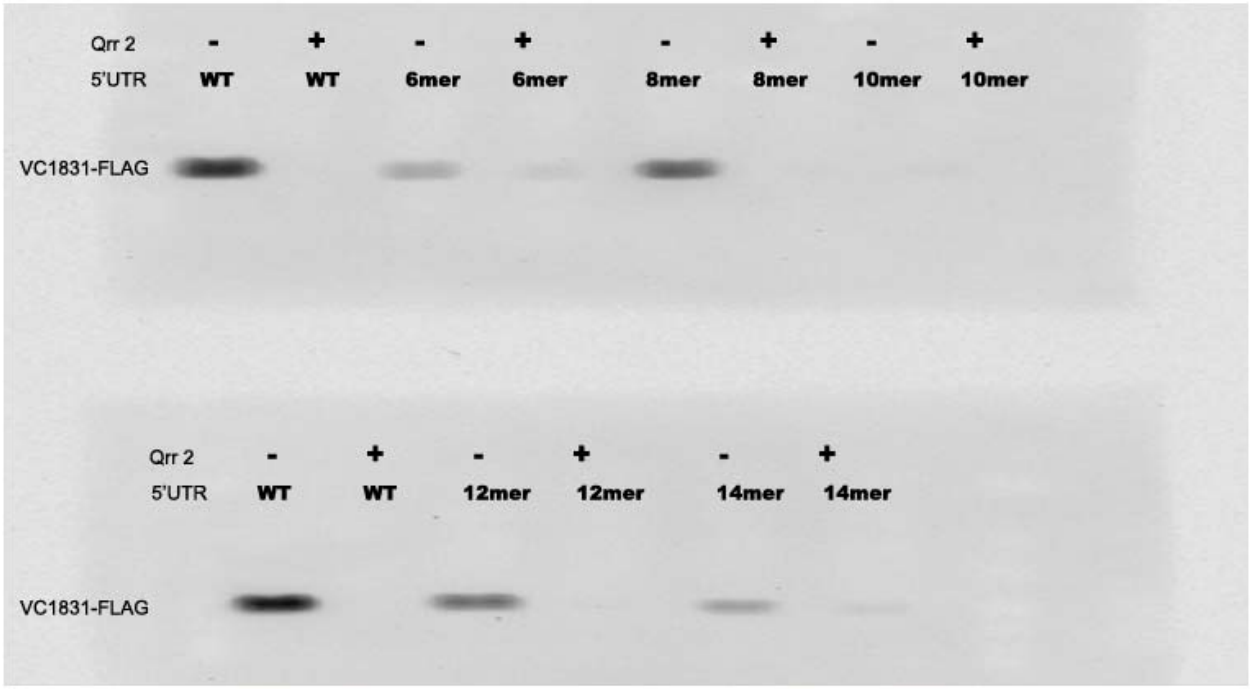



**Figure 5** Western Blot analysis of VC1831 with different mutations in the 5'UTR without or with Qrr2 in *E. coli*. With the wild type 5'UTR, Qrr2 prevented the accumulation of VC1831-FLAG. In all of the tested mutants, Qrr2 still repressed VC1831-FLAG, despite extensive changes to the predicted binding site.

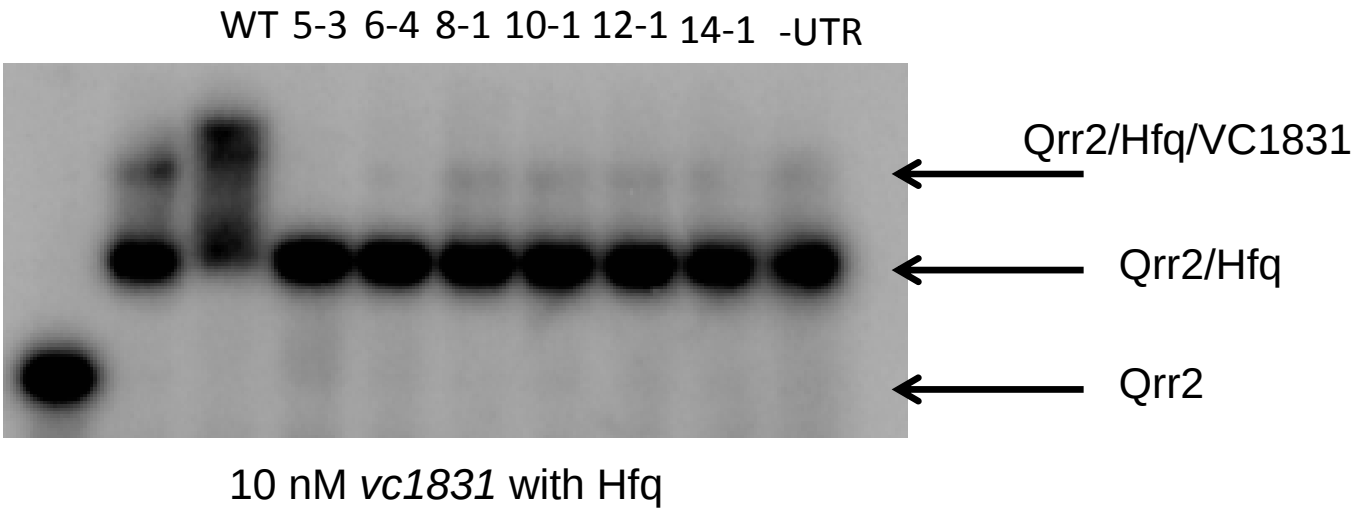



**Figure 6.** Electrophoretic mobility shift assay (EMSA) with radio-labeled Qrr2 (conducted by Dr. Bardill). When Hfq is present, the Qrr2 band shifts. With wild type VC1831, a super shift is observed, indicating sRNA mRNA interaction. In all tested mutants, no supershift is observed, indicating that the mutations abolish the sRNA-mRNA interaction.

**A. *hapR***


**B. *vc1831***


**Figure 7.** A visualization of the automated calls to RNAup to predict sites of Qrr sRNA interaction with a target. The scores for each nucleotide were calculated based on the number of predictions which included that nucleotide, and the average free energy for each of those interactions. These scores were transformed into color using a linear scale from blue for 0 and red for the max score from the data set. **A** *hapR* sequence was used as a control. Only one clear site appeared, which mapped to the site already shown to bind *in vitro* by Bardill et. al. **B** For

*vc1831*, many potential binding sites appeared. This may indicate that the Qrr sRNAs are binding in more than one place on the *vc1831* mRNA, and could explain the disconnect between the *in vivo* and *in vitro* mutant 5'UTR results.

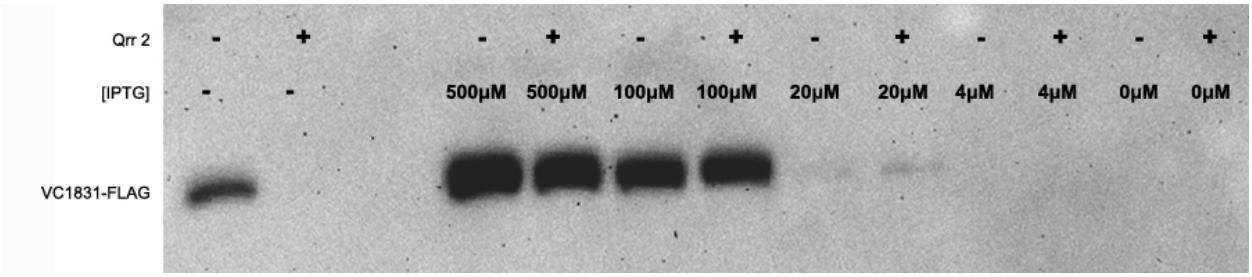



**Figure 8.** Western Blot analysis of VC1831 under control of the $P_{tac}$ promoter and 5' UTR with varying concentrations of IPTG, with the first two lanes having wild-type VC1831-FLAG as a control. The smallest concentration of IPTG where the accumulation of VC1831 is observed is 20μM. This accumulation was seen both in the presence and absence of Qrr, indicating that this construct was no under Qrr control.

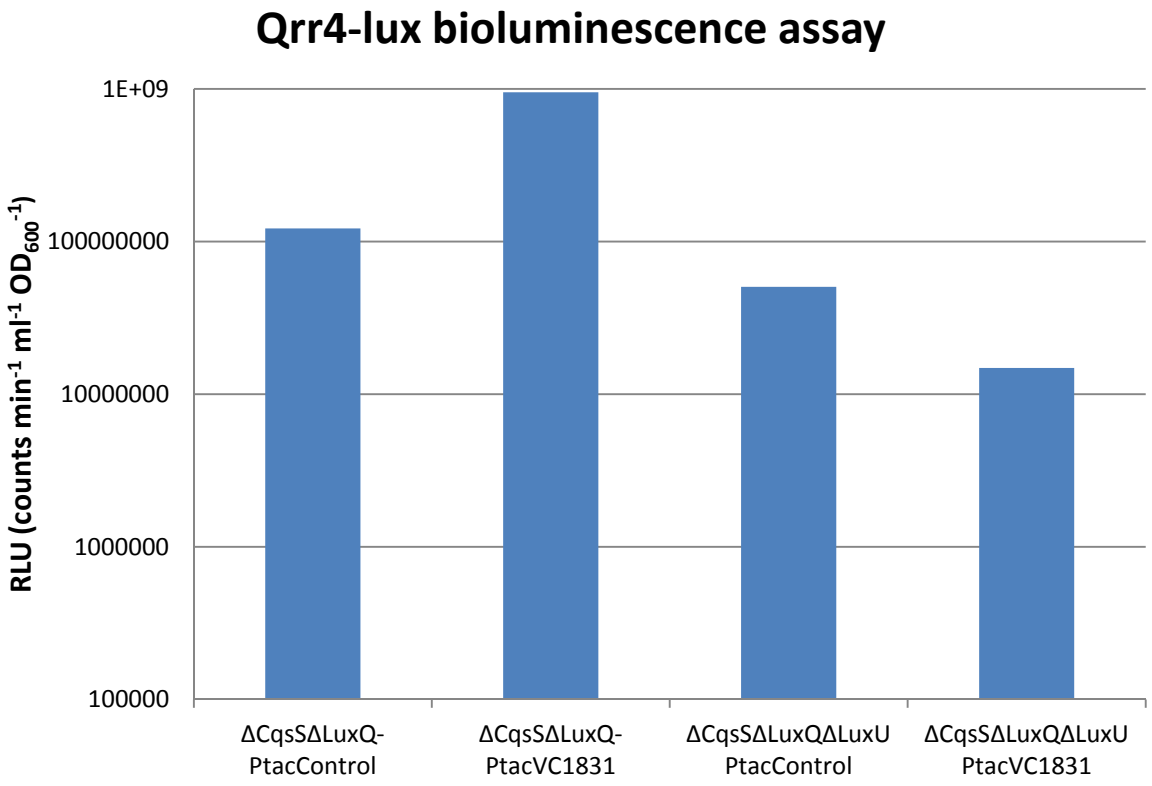



**Figure 9.** Qrr4-lux bioluminescence assay with or without VC1831 in different backgrounds of *V. cholerae*. In a *ΔcqsS ΔluxQ* double sensor mutant, PtacVC1831 increased transcriptional levels of Qrr4 (bars 1 and 2). This effect was not seen in the *ΔcqsS ΔluxQΔluxU* triple mutant, implying that VC1831 is dependent on LuxU to positively regulate the Qrr sRNAs.

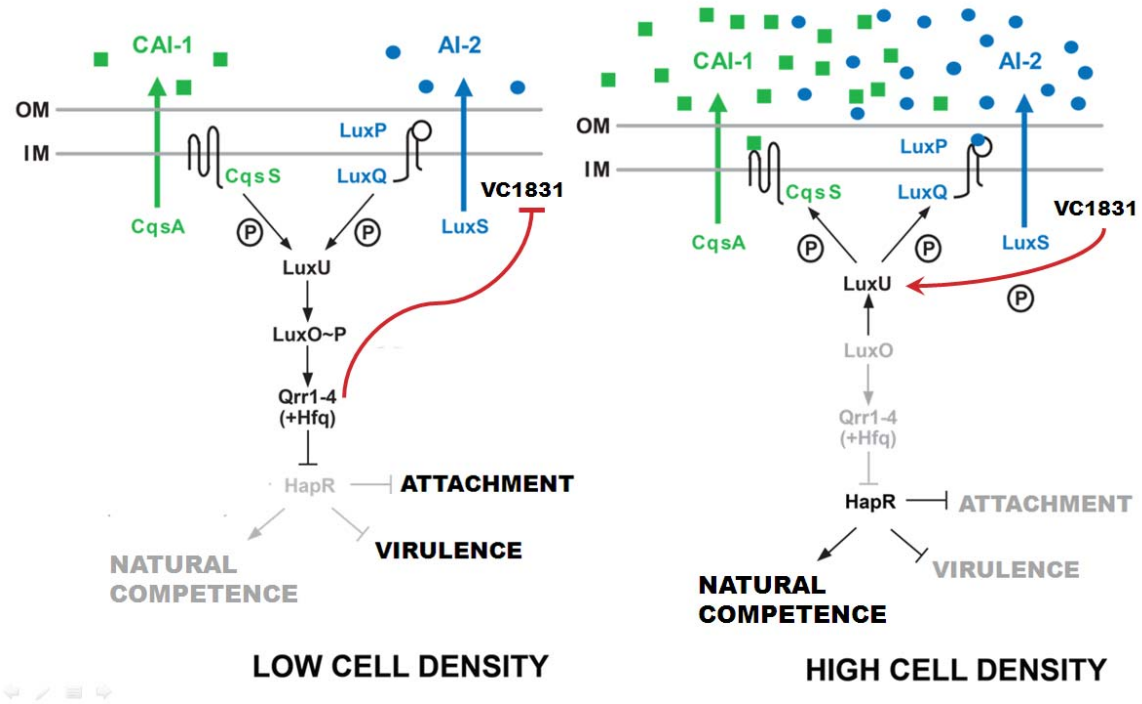



**Figure 10** The quorum sensing model in *V. cholerae* including VC1831. At low cell density, the Qrr sRNAs are present, which repress VC1831. At high cell density, VC1831 is translated, which is capable of donating phosphate to LuxU. This current model implies that VC1831 could be important in the transition from high cell density to low cell density by phosphorylating LuxU, jumpstarting Qrr sRNA production.