A QUORUM-SENSING-REGULATED SMALL RNA THAT CONTROLS PRODUCTION OF THE OUTER MEMBRANE PROTEIN OMPA IN VIBRIO HARVEYI

Alice K. Min

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ABSTRACT

Bacteria use a cell-cell communication process called quorum sensing (QS) to transition between individual and community lifestyles. QS relies on the production, secretion, detection, and response to extracellular signaling molecules called autoinducers (AI) that increase in concentration in step with increases in bacterial population density. In *Vibrio harveyi*, transitions into and out of QS mode are driven by five homologous small RNAs (sRNAs) called Qrr1-5 that control the production of AphA and LuxR, the master low-cell density and master high-cell density QS regulators, respectively. Using high-throughput whole genome microarrays, we discover a sRNA that we name QsrA (Quorum-sensing-regulated sRNA A) that is activated by LuxR and repressed by AphA. This regulatory arrangement ensures maximal production of QsrA at high density. QsrA, in turn, post-transcriptionally controls multiple mRNA targets. One target, VIBHAR_04892 encoding the outer membrane protein A (OmpA) is activated more than 60-fold by QsrA at high-cell density. Gene-fusion and epistasis analyses suggest there exists an intermediate factor through which QsrA controls *ompA*. 
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CHAPTER 1

Background
Quorum sensing in *Vibrio harveyi*

*Vibrio harveyi* is a Gram-negative marine bacterium that exists as free-swimming single cells, as a multicellular community as part of a biofilm, or in association with hosts as a pathogen (Nealson and Hastings, 1979; Ng and Bassler, 2009). In nature, *V. harveyi* is exposed to fluctuating environments, and thus must be able to rapidly alternate among its lifestyles. *V. harveyi* uses quorum sensing (QS) to transition between these growth modes. QS is a bacterial cell-cell communication process that relies on the production, secretion, detection, and response to extracellular signaling molecules called autoinducers (AIs). Group-wide detection of AIs allows bacteria to synchronously alter behavior in response to changes in cell-population density and species complexity.

*V. harveyi* produces and detects three different AIs, AI-1, AI-2, and CAI-1 (Henke and Bassler, 2004; Rutherford and Bassler, 2012). AI-1 is a *V. harveyi*-specific AI that is synthesized by the LuxM synthase. AI-1 is detected by the LuxN histidine sensor kinase receptor. AI-2 is used for interspecies communication, recognized not only by bacteria, but also across different kingdoms. In *V. harveyi*, AI-2 is synthesized by the LuxS synthase and detected by the periplasmic protein LuxP in complex with the LuxQ histidine sensor kinase receptor. CqsS is a vibrio-specific AI that is synthesized by the CqsA synthase and detected by the CqsS histidine sensor kinase receptor. Each AI encodes discrete information about the vicinal community such that different combinations of AI inputs lead to distinct patterns of QS-controlled gene expression.

In *V. harveyi*, the three different AI/receptor systems act in parallel to control collective behaviors (Fig. 1-1). At low cell density (LCD), the concentrations of AI-1, AI-2, and CAI-1 are low, such that their cognate receptors, LuxN, LuxPQ, and CqsS, respectively, are unliganded. In this state, the receptors act as kinases, shuttling phosphate through a phosphorylation cascade to the response regulator LuxO (Freeman and Bassler, 1999a, b; Lilley and Bassler, 2000; Neiditch et al., 2006; Swem et al., 2008). LuxO-P, together with the alternative sigma factor $\sigma^{54}$, activates the transcription of genes encoding five homologous small regulatory RNAs (sRNAs) called Qrr1-5. Qrr1-5 promote the translation of AphA, the master LCD QS regulator, and repress the translation of LuxR, the master high-cell density (HCD) QS
regulator (Feng et al., 2015; Lenz et al., 2004). Thus, AphA is made at LCD and LuxR is not. AphA controls genes that are beneficial for individual behaviors, including those required for biofilm formation (Rutherford et al., 2011; van Kessel et al., 2013a).

At HCD, AIs accumulate to the threshold level required for binding to their cognate receptors. Binding converts the receptors from kinases to phosphatases (Neiditch et al., 2006; Swem et al., 2008). This step reverses the phosphoryl flow through the regulatory cascade. Dephosphorylated LuxO is inactive so transcription of the qrr1-5 genes ceases. Under this condition, AphA translation is not activated and LuxR translation is derepressed. Thus, LuxR is made and AphA is not. LuxR regulates genes necessary for group behaviors, including the genes responsible for bioluminescence, the hallmark QS-regulated trait (Bassler et al., 1993; Rutherford and Bassler, 2012).
**Fig 1-1.** The *V. harveyi* QS circuit.

*V. harveyi* produces and detects three autoinducers (AIs), AI-1, AI-2, and CAI-1. Information encoded in the AIs is transduced internally through a shared phosphorelay cascade that converges to modulate the levels of five homologous trans-acting sRNAs called Qrr1-5. The Qrr sRNAs dictate the levels of AphA and LuxR that are made. AphA is the master QS regulator at low-cell density, and it regulates genes responsible for individual behaviors. LuxR is the master QS regulator at high-low density, and it regulates genes responsible for group behaviors.
**Regulatory small RNAs in bacteria**

Regulatory small RNAs (sRNAs) are increasingly recognized for their critical roles in bacterial physiology, including envelope homeostasis, iron regulation, carbohydrate utilization, pathogenesis, and QS (Caldelari et al., 2013; Gottesman and Storz, 2011; Lenz et al., 2004; Papenfort and Vogel, 2010; Vogel and Luisi, 2011). The advent of new technologies, such as high-throughput microarrays and RNA-sequencing (RNA-seq), has enabled hundreds of bacterial sRNAs to be identified and characterized. sRNAs range in length from approximately 50 to 500 nucleotides. Numerous classes of sRNAs have been described to date, including protein-binding sRNAs, base-pairing sRNAs, which are subdivided into cis-encoded antisense sRNAs, and trans-encoded regulatory sRNAs, and, more recently, CRISPR RNAs (Gottesman and Storz, 2011; Waters and Storz, 2009).

While the majority of sRNAs function by base-pairing with mRNAs, a few have been identified to regulate proteins. The 6S and the CsrB sRNAs are the most extensively studied protein-binding sRNAs. Protein-binding sRNAs typically antagonize the activity of their cognate proteins by mimicking other nucleic acids. For example, the *E. coli* 6S sRNA mimics an open promoter structure that specifically binds to and sequesters the $\sigma^{70}$-containing RNA polymerase (Wassarman, 2007). Consequently, at stationary phase, when 6S sRNA is abundant, transcription from many $\sigma^{70}$ promoters is inhibited, whereas increasing levels of stationary phase $\sigma^{S}$-bound RNA polymerase induce increased transcription from $\sigma^{S}$ promoters. On the other hand, the CsrB family of sRNAs simulates an mRNA element that binds to the CsrA protein and its homologs. The CsrA protein, which plays roles in carbon utilization, bacterial motility, and virulence, inhibits translation of mRNAs containing GGA-rich elements. The CsrB sRNAs contain multiple GGA-binding sites, thereby sequestering the CsrA protein away from native mRNA substrates (Gottesman and Storz, 2011; Waters and Storz, 2009).

Base-pairing sRNAs comprise the most abundant class of sRNAs. Two types exist, the cis-encoded antisense sRNAs and the trans-encoded regulatory sRNAs. Cis-sRNAs are transcribed from the antisense DNA strand immediately opposite of their target genes. Hence, each cis-sRNA shares an extensive complementarity with a single target mRNA that it regulates. Based on where they base-pair,
cis-sRNAs can be divided into 5’-overlapping, 3’-overlapping, and ORF-binding cis-sRNAs (Georg and Hess, 2011). Base-pairing cis-sRNAs change the secondary structure of target mRNAs, ultimately influencing stability and half-life, with variable outcomes. Cis-sRNAs were first identified in mobile elements such as bacteriophage, plasmids, and transposons where they function in copy-number regulation (Wagner et al., 2002). More recently, cis-sRNAs have been identified in bacterial chromosomes. One widely-studied class of chromosomal cis-sRNA acts as antitoxins by repressing the translation of toxic proteins. Another group of cis-sRNAs enhances translation by increasing the production or the half-life of the target mRNA. For example, in *E. coli*, a stationary phase induced GadY cis-sRNA base pairs with the bicistronic *gadXW* mRNA (Opdyke et al., 2004; Tramonti et al., 2008). The *gadXW* mRNA is cleaved into two separate *gadX* and *gadW* transcripts, which accumulate and are translated to activate the glutamate-dependent acid resistance system. In contrast to cis-encoded sRNAs, the trans-encoded sRNAs are transcribed at distant locations from their targets. Trans-encoded sRNAs can simultaneously regulate multiple targets, with whom they share limited complementarity. Trans-sRNAs will be described in greater detail in the following section.

A more recently discovered class of sRNAs participates in clustered regulatory interspaced short palindromic repeats/CRISPR associated (CRISPR/Cas) systems, which provide acquired immunity against viruses and plasmids in bacteria (Karginov and Hannon, 2010; Marraffini, 2015; Waters and Storz, 2009). Unlike restriction-based defense mechanisms that degrade DNA with a given restriction enzyme recognition site, CRISPR/Cas systems are highly specific, adaptive, and heritable. CRISPR loci are clusters of short, often palindromic, DNA repeats that are separated by variable short spacer regions derived from foreign DNA. Several *cas* genes are located proximal to the repeat-spacer cluster. Together, the CRISPR RNAs (crRNAs) transcribed from the incorporated foreign DNA spacer and the Cas proteins target complementary DNA for degradation. While the compositions of CRISPR/Cas loci vary greatly across bacterial species, the basic immune mechanism is shared among all types of CRISPR/Cas systems.
Trans-encoded regulatory small RNAs

Trans-encoded regulatory sRNAs are the most prevalent and extensively studied class of sRNAs (De Lay et al., 2013; Gottesman and Storz, 2011; Updegrove et al., 2015; Vogel and Luisi, 2011; Waters and Storz, 2009). Trans-sRNAs are characterized by several physical features: 1) Almost all have Rho-independent terminators comprised of a stem loop followed by a stretch of U residues; 2) They have a defined seed region, or a distinct region for base-pairing with target mRNAs; 3) Typically, the seed region forms limited base-pairing interactions with the target mRNAs, which allows them to simultaneously regulate multiple targets that are distantly located; 5) In Gram-negative bacteria, an RNA chaperone protein called Hfq, a homolog of eukaryotic Sm and Lm proteins, is necessary for the proper function of trans-sRNAs; and 6) All trans-sRNAs possess double-stranded stem loop regions that increase stability and allow appropriate orientation of the seed region, the Hfq binding site, and the binding sites for other accessory proteins. Besides these essential features, trans-sRNAs are structurally diverse, varying both in size (~50 to 250 nt) and structure.

The most widely described consequence of base-pairing trans-sRNAs is post-transcriptional repression resulting from translation initiation inhibition, target mRNA decay enhancement, or both. In all cases, sRNAs typically regulate target mRNAs by binding directly at or around the ribosomal binding site (RBS) in the target 5’ untranslated region (UTR) and they block translation (De Lay et al., 2013; Gottesman and Storz, 2011). The Hfq protein mediates base-pairing between sRNAs and target mRNAs, presumably increasing their local concentrations to enable robust regulation (De Lay et al., 2013; Vogel and Luisi, 2011). Hfq also protects unpaired sRNAs against degradation by the endoribonuclease RNAse E while recruiting RNAse E to initiate degradation of the sRNA, target mRNA, or both in the sRNA:mRNA complex. Other mechanisms of sRNA repression include sRNA binding within mRNA coding regions or within intergenic regions of polycistronic transcripts, promoting endonucleolytic cleavage (Desnoyers et al., 2009; Papenfort et al., 2010; Pfeiffer et al., 2009).

Trans-sRNAs can also activate target mRNA expression (Frohlich et al., 2013; Frohlich and Vogel, 2009; Papenfort et al., 2013). One well-known mechanism of activation involves the sRNA base pairing with the target mRNA 5’ UTR whose intrinsic translation inhibitory structure becomes unfolded, revealing
the RBS and promoting translation. Alternative activation mechanisms include endonucleolytic cleavage to make the RBS more accessible, and conversely, increasing the half-life of target mRNAs by protecting them from endonucleolytic destruction (Obana et al., 2010; Opdyke et al., 2011; Papenfort et al., 2013; Ramirez-Pena et al., 2010).

The prevalence of trans-sRNAs in highly regulated pathways that also involve transcriptional factors has raised questions about the evolutionary advantages of sRNA regulators. Several benefits have been proposed. First, the ability of trans-sRNAs to repress or enhance translation and to target mRNAs for endonucleolytic destruction while protecting other mRNAs facilitates a rapid on/off transitions in target protein synthesis (Updegrove et al., 2015; Waters and Storz, 2009). The metabolic cost of sRNA synthesis is lower relative to protein synthesis because sRNAs tend to be smaller in size and they do not require translation. Similarly, sRNAs are degraded more rapidly than proteins. In addition, some sRNAs are degraded along with their target mRNAs in a process called coupled degradation, allowing inactivation of the regulator, a process that is unique to sRNAs. sRNAs can also act in concert with transcriptional factors to add additional layers of regulation. For example, sRNAs can enhance target activation or repression by simultaneously activating or repressing target expression along with a transcription factor. On the other hand, sRNAs can reverse the direction of regulation by reciprocally repressing or activating target expression relative to the transcription factor. Hence, incorporation of sRNAs in regulatory circuits provides plasticity in the strength, speed, and reversibility of target regulation.
The Qrr sRNAs in Vibrio harveyi

In V. harveyi, the Qrr1-5 sRNAs drive the QS gene expression programs necessary for transitioning between individual and group lifestyles. The Qrr sRNAs belong to the family of trans-encoded regulatory sRNAs. Each Qrr sRNA is expressed at a different level. At LCD, the steady state level is highest for Qrr4, followed by Qrr2, Qrr3, Qrr1, and then Qrr5 (Ng and Bassler, 2009; Tu and Bassler, 2007). Unlike the four homologous Qrr sRNAs in Vibrio cholerae that are redundant and exhibit dosage compensation if any one of the qrr genes is not expressed, the Qrr sRNAs in V. harveyi are non-redundant and they do not dosage compensate (Lenz et al., 2004; Tu and Bassler, 2007). Instead, the V. harveyi Qrr sRNAs work additively to control QS. If any of the qrr is not expressed, V. harveyi displays a QS phenotype change stemming from reduced qrr expression. The relative strength of each Qrr sRNA in controlling QS-regulated genes mirrors its respective expression level: Qrr4 > Qrr2 > Qrr1 > Qrr5. Hence, the QS behavior of a V. harveyi mutants that only express qrr4 is the closest to the wild-type compared to V. harveyi mutants harboring any other single Qrr sRNA. On the other hand, V. harveyi mutants that only express qrr5 exhibit almost no QS behavior. The lack of dosage compensation in V. harveyi Qrr sRNA regulation suggests that each Qrr could play a unique role in the QS circuit. In fact, the five Qrr sRNAs are each controlled by unique promoters, indicating that each qrr gene is regulated by different transcription factors (Shao et al., 2013; Tu and Bassler, 2007). Differential regulation of each Qrr sRNA possibly adds greater versatility in QS gene regulation enabling fine-tuning of QS responses to distinct environmental cues (Ng and Bassler, 2009).

The Qrr sRNAs possess four stem loops (SL): SL1, SL2, SL3, and SL4 (Lenz et al., 2004; Shao and Bassler, 2012; Tu and Bassler, 2007). SL1 and SL2 are the most conserved regions and are important for base pairing with the mRNA targets. SL1 also bestows stability to the Qrr sRNAs by protecting them from RNase E-mediated degradation. SL3 plays an accessory role in base-pairing and stability. SL4 contains the Rho-independent terminator. Among the five Qrr sRNAs, Qrr1 is the most different because it lacks nine nucleotides that are conserved in Qrr2-5 near the 5’ end (Shao and Bassler, 2012). These missing nine nucleotides are important for base-pairing with particular mRNA targets, including AphA. Hence, Qrr2-5 activate AphA translation, whereas Qrr1 does not. Some mRNA targets,
such as LuxR, require other base-pairing regions of the Qrr sRNAs, and are therefore regulated by all five Qrr sRNAs.

In addition to the luxR and aphA mRNAs, the Qrr sRNAs regulate 18 other targets (Shao et al., 2013). 16 of these targets lie outside of the V. harveyi QS circuit. The two remaining are the luxO and luxM mRNAs, both of whose products are key for V. harveyi QS. The luxR, aphA, luxO, and luxM mRNAs are each regulated by the Qrr sRNAs through distinct mechanisms (Feng et al., 2015). The Qrr sRNAs represses LuxR translation through catalytic degradation, represses LuxM translation through coupled degradation, represses LuxO translation through sequestration, and activate AphA translation, during which the Qrr sRNAs are degraded. The mechanism of action is driven by the particular interaction and the strength of base-pairing between the Qrr sRNAs and the mRNA target. For example, mRNA targets that base pair with SL1 result in Qrr degradation. These targets include both repressed (luxM) and activated (aphA) targets. mRNA targets that base pair with SL2 do not cause Qrr degradation. Instead, these mRNAs sequester the Qrr sRNA if the binding is strong (luxO) or they are catalytically regulated if binding is weak (luxR) (Feng et al., 2015). The use of various regulatory mechanisms to control mRNA targets allows nuanced control of both the Qrr sRNAs and their target mRNAs, which, in turn, precisely governs the overall QS response.
CHAPTER 2

A Quorum-Sensing-Regulated Small RNA that Controls

Production of the Outer Membrane Protein OmpA in *Vibrio harveyi*
Introduction

As described in Chapter 1, *V. harveyi* uses QS to synchronously alter behaviors that enable it to transition between individual and collective lifestyles. These transitions are driven by five sRNAs called Qrr1-5 that control the production of AphA and LuxR, the master low-cell density (LCD) and high-cell density (HCD) QS regulators, respectively. Beyond controlling AphA and LuxR production, the Qrr sRNAs also post-transcriptionally regulate 18 other target mRNAs encoding proteins that function within and outside of the QS signal transduction cascade (Feng et al., 2015; Shao et al., 2013). Here, we find that the Qrr sRNAs also indirectly influence production of other sRNAs via their control of AphA and LuxR. We discover and characterize one of these sRNAs that we name QsrA (Quorum-sensing-regulated sRNA A). QsrA is an Hfq-dependent trans-sRNA that is directly activated by LuxR and repressed by AphA. Thus, *qsrA* is maximally expressed at HCD. QsrA controls several targets, one of which is *VIBHAR_04892* encoding the outer membrane protein A (OmpA). QsrA activates *ompA* more than 60-fold. Both the *ompA* mRNA transcript and the OmpA protein levels are significantly reduced in a *V. harveyi* ΔqsrA strain compared to the *V. harveyi* wild-type. Gene fusion and epistasis experiments suggest that QsrA acts indirectly to control *ompA* expression indicating that an additional factor exists that links QsrA to *ompA*. We propose that QsrA-mediated activation of *ompA* contributes to *V. harveyi* homeostasis. Fine-tuning OmpA levels could influence the composition of the outer membrane enhancing functions such as maintenance of cell morphology, surface adhesion, and colonization.
Results

High-throughput whole genome V. harveyi microarrays identify the QsrA sRNA.

The five homologous Qrr sRNAs drive V. harveyi QS dynamics. We wondered whether there exist additional sRNAs that are involved in QS. To explore this possibility, we used whole genome microarrays comparing V. harveyi ΔluxO and luxO D47E strains that are locked in HCD and LCD modes, respectively, for differences in gene expression. To avoid identifying all QS target genes, we restricted our analysis to intergenic regions of the genome. This strategy allowed us to identify roughly a dozen genes that presumably encode sRNAs. As a test of our method, we selected the most highly regulated of these candidates for analysis: a region ~150 bp downstream of and antisense to the VIBHAR_05179 gene, encoding a hypothetical protein (Fig. 2-1). The microarray results show that this region is 30-fold more highly expressed in the HCD-locked ΔluxO strain than in the LCD-locked luxO D47E strain (Fig. 2-1).

Using Northern blot analysis and oligo-probe tiling, we confirmed expression of a sRNA in the predicted region. No signal was detected when the opposite strand was probed, confirming the existence of an independent, strand-specific entity in this region (Figs. 2-2, 2-3). We used RNA-seq to establish that the region encodes a 67 nucleotide (nt) sRNA, which we name QsrA. Database analysis suggests that qsrA exists in only a few vibrio species: two sequenced V. harveyi strains, a V. campbellii strain, and a V. jasicida strain have the qsrA gene downstream of a VIBHAR_05179 ortholog.
**Fig 2-1.** *V. harveyi* whole genome microarrays identify QsrA.

A. Intergenic region between *VIBHAR_05179* and *VIBHAR_05180*. The top solid arrows depict the microarray probes that bind to the antisense DNA strand. The bottom dashed arrows depict probes that bind to the sense DNA strand. Microarray analysis identified two probes that were approximately 30-fold up-regulated in the *V. harveyi ΔluxO* strain compared to the *luxO* D47E strain.

B. Microarray analysis results comparing ΔluxO vs. luxO D47E strains and the corresponding up-regulated probes from (A).
Fig 2-2. Northern blot analysis of the qsrA genomic region.

Total RNA from *V. haveyi luxO* D47E (KM83) and ΔluxO (JAF78) strains were collected and resolved by gel electrophoresis and Northern blots were performed. Mixtures of radiolabeled single stranded oligonucleotide probes spanning the intergenic region between *VIBHAR_05179* and *VIBHAR_05180* were used to detect QsrA. The left two lanes were probed with mixed oligonucleotides that bind the sense strand. The right two lanes were probed with mixed oligonucleotides that bind the antisense strand. The white arrow on the right marks where the QsrA band is located.
Fig 2-3. Tiled oligonucleotide probing pinpoints the qsrA location.

A. Chromosomal location of qsrA downstream of and antisense to VIBHAR_05179. Solid lines A-E represent radiolabeled single-stranded oligonucleotide probes that bind the antisense DNA strand.

B. Total RNA from V. haveyi luxO D47E (KM83) and ΔluxO (JAF78) strains was collected and resolved by electrophoresis. Northern blots were performed. Each lane was probed using the designated probe. The lanes labeled X are blank.
*QsrA is an Hfq-dependent quorum-sensing-regulated sRNA.*

The cell density dependent expression of *qsrA* is a hallmark of a QS-regulated gene. To confirm this notion, we measured QsrA levels in wild-type and QS mutant *V. harveyi* strains using Northern blot analyses (Fig. 2-4A). Consistent with the initial microarray analysis, the *luxO* D47E strain had almost no detectable QsrA and significantly higher QsrA was present in the ∆*luxO* strain than in the wild-type. Relative to the wild-type, the ∆*luxR* mutant, which is locked at LCD, had low levels of QsrA RNA while increased QsrA was present in the ∆*aphA* mutant that mimics the HCD mode (Fig. 2-4A). Finally, the ∆*luxR, ∆aphA* double mutant had intermediate levels of QsrA, between those of the single ∆*luxR* and the single ∆*aphA* mutants. These results suggest that LuxR and/or AphA could regulate *qsrA*.

To test whether LuxR and/or AphA are involved in *qsrA* regulation, we used qRT-PCR to assess QsrA levels in *V. harveyi* wild-type, the ∆*luxR* and ∆*aphA* single mutants, and a ∆*luxR, ∆aphA* double mutant at a range of cell densities (Fig. 2-4B). QsrA levels increased with increases in cell density in the wild-type and the ∆*aphA* single mutant, and a modest cell density dependent increase occurred in the ∆*luxR* single mutant. QsrA levels were, however, always lower in the ∆*luxR* strain and always higher in the ∆*aphA* strain than in the wild-type. In the double ∆*luxR, ∆aphA* mutant, QsrA was present at a level intermediate between that of the ∆*luxR* and ∆*aphA* single mutants. Together, these results suggest that LuxR activates and AphA represses *qsrA* expression.

Based on its location in an intergenic region, QsrA is predicted to be a trans-regulatory sRNA. Trans-sRNAs often require the RNA chaperone Hfq to facilitate base-pairing with the 5' UTR of target mRNAs and to prevent RNase-mediated degradation (Vogel and Luisi, 2011). To test whether Hfq interacts with QsrA, we used Northern blot analysis to measure QsrA stability in the presence and absence of Hfq (Fig. 2-4C). In wild-type *V. harveyi*, the half-life of QsrA was > 32 min. At t=0, the *V. harveyi* ∆*hfq* mutant produced ~7-fold less QsrA and QsrA had a half-life of ~8 min. These results indicate that Hfq stabilizes the QsrA sRNA.
**Fig 2-4.** QsrA is an Hfq-dependent quorum-sensing-regulated sRNA.

A. QsrA levels as detected by Northern blot in *V. harveyi* wild-type (BB120), *luxO* D47E (KM83), *ΔluxO* (JAF78), *ΔluxR* (KM669), *ΔaphA* (JV48), and *ΔluxR, ΔaphA* (STR417) strains. Cells were harvested at the designated OD$_{600}$ levels. 5S rRNA is the loading control.

B. QsrA sRNA levels were measured by qRT-PCR in *V. harveyi* wild-type (BB120), *ΔluxR* (KM669), *ΔaphA* (JV48), and *ΔluxR, ΔaphA* (STR417) strains. Means and standard deviations for RNA collected from three independent replicates are shown. All *qsrA* transcript levels were calculated relative to the *V. harveyi* wild-type at OD$_{600}$=0.05. 5S rRNA was used as the endogenous control.

C. Northern blot to determine the half-life of QsrA in the *V. harveyi* wild-type (BB120) and *Δhfq* (DL2066) strains. Cells were collected at OD$_{600}$=1.0. At t=0, cells were treated with rifampicin (500 μg ml$^{-1}$) to stop transcription. Cells were collected 0-32 min thereafter. 5S rRNA is the loading control.
LuxR directly activates qsrA expression.

The above mutant analyses (Figs. 2-4A, B) suggest that LuxR activates qsrA expression. Regulation could be direct or indirect. The qsrA 5’ UTR does not contain a canonical LuxR consensus sequence (Pompeani et al., 2008; van Kessel et al., 2013b). However, not all LuxR-bound promoters have identifiable motifs (van Kessel et al., 2013b). To test if LuxR binds the qsrA promoter, we developed a heterologous two-plasmid system in E. coli in which one plasmid expresses luxR from the arabinose-inducible P_{BAD} promoter and a second plasmid expresses gfp from a 300 base-pair fragment harboring the P_{qsrA} promoter. Using this system, we found that 5-fold more GFP is produced when LuxR and the P_{qsrA}::gfp fusion are simultaneously induced than when either is induced alone (Fig. 2-5A, first four bars). This result suggests that LuxR does indeed bind to the qsrA promoter to activate its transcription.

To define the LuxR binding region in the qsrA promoter, we generated a set of P_{qsrA}::gfp fusions harboring increasingly shorter fragments of the qsrA promoter. LuxR ceased to activate transcription when fewer than 83 bp of the qsrA promoter were present (Fig. 2-5A). To determine if transcriptional activation correlates with the ability of LuxR to bind the qsrA promoter, we performed EMSAs using purified LuxR protein and 100 bp oligonucleotides harboring from as few as 53 bp to as many as 83 bp of the qsrA promoter. In addition to binding the oligonucleotide containing the -83 to +7 region of the qsrA promoter, LuxR also bound to oligonucleotides containing the -73 to +7 and the -63 to +7 region. No binding occurred to a fragment containing the -53 to +7 region (Fig. 2-5B). Thus, while LuxR-directed transcriptional activation of qsrA requires at least 83 bases of the promoter, a shorter region is sufficient for LuxR binding.

We also calculated the strength of LuxR binding to P_{qsrA} using variable LuxR protein concentrations and a fixed concentration of the qsrA promoter containing the -83 to +7 region. Binding to the P_{qsrA} oligonucleotide induced LuxR to aggregate at concentrations equal to and above 300 nM (Fig. 2-5C). Hence, the maximal P_{qsrA} oligonucleotide shift by a LuxR monomer occurred at approximately 60%. The K_d was calculated using data obtained from three independent electrophoretic mobility shift assays (EMSA). LuxR exhibited a relatively weak affinity for P_{qsrA} (K_d ≈ 120 nM) (Figs. 2-5C, D) compared to
previously studied LuxR regulated promoters (van Kessel et al., 2013b). For comparison, LuxR binds the VIBHAR_05222 promoter (encoding a putative thioesterase) promoter with a $K_d$ of 0.7 nM and the archetypal luxC (luciferase) promoter with a $K_d$ of ~103 nM.

The most highly conserved DNA sequences upstream of the *V. harveyi*, *V. campbellii*, and *V. jasicida* qsrA genes lie within the -84 to -1 region in *V. harveyi* and in *V. campbellii* and in the -83 to -1 region in *V. jasicida* (there exists a one base-pair deletion in the *V. jasicida* 5' region) (Fig. 2-6A) consistent with our above results showing that this is the region required for transcriptional activation. To define which nucleotides are most important within this region, we generated $P_{qsrA}$-gfp fusions in which we scrambled the sequences in conserved and nonconserved regions of the qsrA promoter. Altering conserved bases resulted in 2 to 8-fold reductions in $P_{qsrA}$-gfp expression, while changes in nonconserved bases had no effect (Fig. 2-6B). Because these conserved sequences do not encode a canonical LuxR binding site, we speculate that they have a topological function that facilitates LuxR binding and transcriptional activation.
Fig 2-5. LuxR directly activates qsrA.

A. Fluorescence measured from a P_{qsrA}-gfp fusion in *E. coli* without (white bars) and with (black bars) the addition of 0.2% arabinose to induce luxR expression. Values on the x-axis represent different lengths of P_{qsrA} DNA upstream of the qsrA +1 site driving gfp expression. The Ctl plasmid does not contain any promoter driving gfp. luxR is expressed from the arabinose-inducible P_{BAD} promoter via a second plasmid. Fluorescence values on the y-axis represent the measured GFP level per unit OD_{600} and are in arbitrary units. Mean and standard deviation values of triplicate cultures are shown.
B. EMSAs with radiolabeled $P_{qsrA}$ DNA (0.1 nM) in the presence of increasing LuxR protein concentrations (0-100 nM). All oligonucleotides are 100 nt long and they contain the designated regions of the $qsrA$ promoter.

C. Quantitative EMSA with radiolabeled $P_{qsrA}$ DNA (0.1 nM) containing the -83 to +7 $P_{qsrA}$ region in the presence of increasing LuxR concentration (0-500 nM). LuxR aggregates at concentrations equal to or greater than 300 nM.

D. DNA binding curves for three independent quantitative EMSAs for LuxR binding to the -83 to +7 $P_{qsrA}$ fragment. For each EMSA, $K_d$ was calculated by fitting data points with a nonlinear regression curve using GraphPad Prism software. The means and standard deviations for the three $K_d$ values are shown. Fig. 2-5C is a representative quantitative EMSA that is depicted by the curve with the circle symbols.
Fig 2-6. Conserved regions of the qsrA promoter are required for LuxR activation.

A. Sequence alignment of the qsrA promoter and coding regions in vibrio strains that possess qsrA genes. Conserved nucleotides are in red, nonconserved nucleotides are in black, and the missing nucleotide in the *V. jasicida* sequence is indicated as a hyphen. Conserved regions 1-5 were selected for mutation and are boxed in solid lines. Nonconserved regions A and B were selected for mutation and are boxed in dashed lines. Regions 1-5, A, and B were scrambled to generate the mutated P<sub>qsrA</sub> regions.

B. Fluorescence measured from the qsrA promoter with scrambled conserved and nonconserved regions from Panel A in *E. coli*. The Ctl plasmid does not contain any promoter driving *gfp*. The P<sub>qsrA</sub>-*gfp* plasmid has 300 bp of qsrA promoter fused to *gfp*. A second plasmid expressed *luxR* from the arabinose-inducible P<sub>BAD</sub> promoter. Fluorescence was measured without (white bars) and with (black bars) the addition of 0.2% arabinose. Fluorescence values as in Fig. 2-5A. Mean and standard deviation values of triplicate cultures are shown.
AphA directly represses qsrA expression.

In parallel with our study of LuxR activation of qsrA, we examined whether repression by AphA is direct or indirect. For this analysis, we used a two-plasmid system adapted from Feng et al. (2015) in which one plasmid expressed aphA constitutively from the PLtet-O1 promoter and a second plasmid expressed P_{qsrA}\text{-}\textit{gfp}. We found that P_{qsrA}\text{-}\textit{gfp} expression was repressed 5-fold in the AphA-expressing strain compared to the control strain carrying an empty vector (Fig. 2-7A, first four bars). Thus, we conclude that AphA repression of qsrA is direct. When both LuxR and AphA were simultaneously co-expressed in \textit{E. coli} along with the P_{qsrA}\text{-}\textit{gfp} containing plasmid, \textit{gfp} expression was repressed, indicating that AphA repression overpowers LuxR activation (Fig. 2-7B). To define the AphA binding region in the qsrA promoter, we generated truncated versions of P_{qsrA} fused to \textit{gfp} similar to what we did above for LuxR. AphA repressed transcription of the full length 300 bp qsrA promoter. However, repression was either weak or absent for constructs harboring fewer than 300 bp (Fig. 2-7A).

We also tested the requirements for AphA repression of qsrA expression. Mutations in conserved and nonconserved qsrA promoter regions diminished AphA repression of qsrA (Fig. 2-7C). There was one exception – mutation of conserved region 5 increased GFP production (Fig. 2-7C) perhaps due to the proximity of the mutated region to the RNA polymerase binding site, which increased RNA polymerase access or affinity. Together, these results suggest that AphA is sensitive to both the length and the sequence of the qsrA promoter fragment.

There is no known promoter consensus sequence to which AphA binds (Kovacikova and Skorupski, 2001; van Kessel et al., 2013b). Using the above LuxR binding studies as a guide, we performed EMSAs with purified AphA and 100 bp oligonucleotides harboring 53-83 bp of the qsrA promoter (Fig. 2-7D). Similar to LuxR, AphA bound to the oligonucleotide containing the -83 to +7, the -73 to +7, and the -63 to +7 regions. AphA did not bind to a fragment containing the -53 to +7 region. It was surprising that while a full-length qsrA promoter is required for transcriptional repression, AphA nonetheless bound to qsrA promoter fragments as short as 63 base-pairs.
Unlike LuxR, addition of increasing AphA protein did not produce a corresponding increase in shifted band intensity as judged by EMSA (Figs. 2-7D, E). As a result, we were unable to calculate the $K_d$ for AphA at the $P_{qsrA}$ promoter (Fig. 2-7E). Collectively, our results suggest that there could be two modes of transcription factor binding at the $qsrA$ promoter – tight (sequence specific) and loose (non-sequence specific) (Kalodimos et al., 2004; Slutsky and Mirny, 2004; von Hippel and Berg, 1986). Tight binding occurs when a protein recognizes a DNA sequence and consequently undergoes a conformational change, such as $\alpha$-helix formation, to facilitate proximal interaction with specific base-pairs via hydrogen bonds and salt bridges. Loose binding occurs when a protein electrostatically binds to a DNA fragment and does not undergo a conformational change in the process. We hypothesize that sequence specific tight binding occurs between LuxR and the $qsrA$ promoter because increasing protein concentration leads to a corresponding increase in band shift. Binding by AphA yields both tight and loose complexes, in which tight complexes remain intact when they are resolved by EMSA whereas loose complexes cannot be visualized by this method.
Fig 2-7. AphA directly represses qsrA.

A. Fluorescence measured from a \( P_{qsrA} \)-gfp fusion in \( E. coli \) in the presence of an empty vector (white bars) or the vector constitutively expressing \( aphA \) (black bars). The designated lengths of the \( qsrA \) promoter upstream of \( gfp \) are shown under the corresponding bars. The bars labeled Ctl represent measurements from strains lacking the \( gfp \)-expressing plasmid. Fluorescence values as in Fig. 2-5A. Mean and standard deviation values of triplicate cultures are shown.

B. Fluorescence measured from a \( P_{qsrA} \)-gfp fusion containing 300 bp of \( qsrA \) promoter in an \( E. coli \) strain co-expressing an empty vector (Ctl), a vector harboring \( aphA \), \( luxR \), or both \( luxR \) and \( aphA \). Fluorescence values as in Fig. 2-5A. Mean and standard deviation values of triplicate cultures are shown.

C. Fluorescence measured from the \( qsrA \) promoter with scrambled conserved and nonconserved regions (from Fig. 2-6A) in \( E. coli \) that also harbors an empty vector (white bars) or a vector that constitutively expresses \( aphA \) (black bars). The bars labeled Ctl represent measurements from strains lacking the \( gfp \)-expressing plasmid. Fluorescence values as in Fig. 2-5A. Mean and standard deviation values of triplicate cultures are shown.

D. EMSAs with radiolabeled \( P_{qsrA} \) DNA (0.1 nM) in the presence of increasing concentration of AphA protein (0-100 nM). All oligonucleotides are 100 nt and contain the designated regions of \( P_{qsrA} \).

E. Quantitative EMSA with radiolabeled \( P_{qsrA} \) DNA (0.1 nM) containing the -83 to +7 region in the presence of increasing AphA protein (0-500 nM).
Microarray analyses suggest possible QsrA targets.

To investigate what biological role QsrA plays in *V. harveyi*, we screened for QsrA mRNA targets by comparing whole transcriptome profiles of *V. harveyi* wild-type to that of a ΔqsrA strain. We identified seven genes that exhibited greater than 4-fold regulation. These targets encode: membrane proteins (*VIBHAR*_01082/predicted membrane protein, and *VIBHAR*_04892/outer membrane protein A), metabolic enzymes (*VIBHAR*_05638/nitrogen fixation NifW family protein, and *VIBHAR*_06097/NADH:ubiquinone oxidoreductase), a biofilm-associated enzyme (*VIBHAR*_02528/c-di-GMP phosphodiesterase), and hypothetical proteins (*VIBHAR*_02854, and *VIBHAR*_04801) (Table 2-1).

We also performed transcriptome analyses comparing gene expression in the ΔaphA strain to the ΔaphA, ΔqsrA double mutant *V. harveyi* strain. Our rationale was the following: Deletion of *aphA* locks *V. harveyi* in HCD mode causing maximal expression of *qsrA* (Figs. 2-4A, B), which could, in turn, drive maximum alterations in expression of QsrA-controlled targets. The majority of targets identified by this strategy corresponded to the targets identified in the analysis of the wild-type vs. ΔqsrA study (Table 2-1). In addition, *VIBHAR*_05179, the gene immediately adjacent to *qsrA*, was found to be activated by QsrA using the second strategy.
### Table 2.1. Putative QsrA targets identified by microarray analyses

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Predicted function</th>
<th>Microarray</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT vs. ΔqsrA</td>
</tr>
<tr>
<td>VIBHAR_01082</td>
<td>Predicted membrane protein</td>
<td>+6.75</td>
</tr>
<tr>
<td>VIBHAR_04801</td>
<td>Hypothetical protein</td>
<td>+4.38</td>
</tr>
<tr>
<td>VIBHAR_04892</td>
<td>OmpA-OmpF porin, OOP family</td>
<td>+5.81</td>
</tr>
<tr>
<td>VIBHAR_05179</td>
<td>Hypothetical protein</td>
<td>n/a</td>
</tr>
<tr>
<td>VIBHAR_02528</td>
<td>c-di-GMP phosphodiesterase</td>
<td>-4.74</td>
</tr>
<tr>
<td>VIBHAR_02854</td>
<td>Hypothetical protein</td>
<td>-6.26</td>
</tr>
<tr>
<td>VIBHAR_05638</td>
<td>Nitrogen fixation NifW family protein</td>
<td>-4.73</td>
</tr>
<tr>
<td>VIBHAR_06097</td>
<td>NADH: ubiquinone oxidoreductase</td>
<td>-8.38</td>
</tr>
</tbody>
</table>

+, targets activated by QsrA. These genes exhibit reduced expression in the ΔqsrA and ΔaphA, ΔqsrA strains.

−, targets repressed by QsrA. These genes exhibit increased expression in the ΔqsrA and ΔaphA, ΔqsrA strains.
n/a, targets whose fold-regulation did not change.

### Table 2.2. QsrA control of target genes as determined by qRT-PCR

<table>
<thead>
<tr>
<th>Gene number</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT vs. ΔqsrA</td>
</tr>
<tr>
<td>VIBHAR_01082</td>
<td>n/a</td>
</tr>
<tr>
<td>VIBHAR_04801</td>
<td>n/a</td>
</tr>
<tr>
<td>VIBHAR_04892</td>
<td>+65.49</td>
</tr>
<tr>
<td>VIBHAR_05179</td>
<td>n/a</td>
</tr>
<tr>
<td>VIBHAR_02528</td>
<td>-2.04</td>
</tr>
<tr>
<td>VIBHAR_02854</td>
<td>-5.09</td>
</tr>
<tr>
<td>VIBHAR_05638</td>
<td>-8.38</td>
</tr>
<tr>
<td>VIBHAR_06097</td>
<td>-2.71</td>
</tr>
</tbody>
</table>

+, targets activated by QsrA. These genes exhibit reduced expression in the ΔqsrA and ΔaphA, ΔqsrA strains.

−, targets repressed by QsrA. These genes exhibit increased expression in the ΔqsrA and ΔaphA, ΔqsrA strains.
n/a, targets whose fold-regulation did not change.
**VIBHAR_04892 (OmpA) is regulated by QsrA.**

We confirmed the most promising targets from the microarray analyses using qRT-PCR (Table 2-2). **VIBHAR_04892** was the most highly regulated among all QsrA targets, exhibiting > 60-fold lower expression in the \( \Delta \text{qsrA} \) strain compared to the wild-type. **VIBHAR_04892** encodes the outer membrane protein A (OmpA). A genome search revealed that *V. harveyi* has two other genes, **VIBHAR_06262** and **VIBHAR_01273**, which encode OmpA homologs. However, qRT-PCR demonstrates that QsrA only regulates **VIBHAR_04892** (Fig. 2-8A). The 5' UTR of the three **ompA** genes have no significant overlap, consistent with our finding that QsrA regulates only a single **ompA** gene.

Western blot analyses confirmed that the *V. harveyi* \( \Delta \text{qsrA} \) strain produces almost no detectable **VIBHAR_04892** protein compared to the wild-type both at LCD (OD\(_{600} = 0.1\)) and at HCD (OD\(_{600} = 1.0\)) (Figs. 2-8B, C). We examined **VIBHAR_04892** production in all the combinations of \( \Delta \text{qsrA} \), \( \Delta \text{aphA} \), and \( \Delta \text{luxR} \) single, double, and triple mutants. Only the \( \Delta \text{aphA} \), \( \Delta \text{qsrA} \) double mutant made less **VIBHAR_04892** than wild-type and only at LCD (Fig. 2-8B). qRT-PCR confirmed the Western blot analyses (Fig. 2-9). These results suggest that AphA represses **VIBHAR_04892** indirectly by repressing **qsrA**, and also via another mechanism that is independent of **qsrA**. Presumably, other regulators also control **VIBHAR_04892** production, which masks the effects of the QS mutants at HCD. OMP proteins are typically controlled by multiple regulators (Frohlich et al., 2012; Guillier et al., 2006; Johansen et al., 2006; Papenfort et al., 2006; Song et al., 2008; Vogel and Papenfort, 2006).

To determine whether QsrA directly regulates **VIBHAR_04892**, we used *E. coli* carrying a plasmid expressing **qsrA** from the constitutive PLlac-O1 promoter and a second plasmid expressing **VIBHAR_04892** 5' UTR-GFP translational fusions driven by the PLtet-O1 promoter. sRNA regulation can occur in the 5' UTR or in the coding sequence of a gene. Therefore, we constructed multiple **VIBHAR_04892-GFP** translational fusions, ranging from 400 bp upstream of the AUG codon (the +1 transcription start site) to 150 bp into the **VIBHAR_04892** coding sequence. Production of QsrA had no effect on expression of any of the **VIBHAR_04892-GFP** fusions (Fig. 2-8D). Hence, QsrA must indirectly
regulate *VIBHAR_04892*. We note that *VIBHAR_04892* was not identified as a target in the \( \Delta \text{aphA} \) vs. \( \Delta \text{aphA}, \Delta \text{qsrA} \) microarray analysis (Table 2-1), and consistent with this, we find that *VIBHAR_04892* expression is similar in the \( \Delta \text{aphA} \) and \( \Delta \text{aphA}, \Delta \text{qsrA} \) strains (Figs. 2-8E, 2-9). We speculate that both AphA and QsrA control *VIBHAR_04892* via an unknown intermediate factor and AphA control is stronger than QsrA control. Therefore, deletion of *aphA* masks the effect of QsrA on *VIBHAR_04892*. 
Fig 2-8. QsrA activates expression of VIBHAR_04892 (ompA).

A. Relative ompA transcript levels in V. harveyi wild-type (BB120; white bars) and the ΔqsrA (AKM150; black bars) strains measured by qRT-PCR. Cells were harvested at OD₆₀₀=1.0. The ompA transcript levels from the ΔqsrA strain were calculated relative to V. harveyi wild-type. Error bars are standard deviations of data from triplicate samples and technical duplicates. 5S rRNA was used as the endogenous control.

B. OmpA protein levels in V. harveyi wild-type (AKM404), ΔqsrA (AKM405), ΔaphA (AKM413), ΔaphA, ΔqsrA (AKM414), ΔluxR (AKM416), ΔluxR, ΔqsrA (AKM418), ΔluxR, ΔaphA (AKM420), and ΔluxR, ΔaphA, ΔqsrA (AKM421) strains at low-cell density, OD₆₀₀=0.1.

C. As in panel B but at high-cell density, OD₆₀₀=1.0.

In panels B and C, C-terminal 3XFLAG-tagged OmpA was probed using anti-FLAG M2-Peroxidase (HRP) antibody. RpoA was used as the loading control.

D. Fluorescence measured from ompA 5’ UTR-GFP fusions in E. coli that also harbored an empty control vector (white bars) or a plasmid expressing qsrA from a constitutive PLlac-O1 promoter (black bars). The translational GFP fusions contained 400 bp of 5’ UTR plus 30~150 nucleotides of the ompA coding sequence (CDS). Fluorescence values as in Fig. 2-5A. Mean and standard deviation values of triplicate cultures are shown.

E. Relative transcript levels of ompA in V. harveyi ΔaphA (JV48) and ΔaphA, ΔqsrA (AKM345) mutants measured by qRT-PCR. The ompA transcript level from the ΔaphA, ΔqsrA strain was calculated relative to the single ΔaphA mutant. Error bars are standard deviations of data from triplicate samples and technical duplicates. 5S rRNA was used as the endogenous control.
Fig 2-9. qRT-PCR analysis of ompA transcript levels in V. harveyi.

Relative ompA transcript levels were measured in V. harveyi wild-type (AKM404), ΔqsrA (AKM405), ΔaphA (AKM413), ΔaphA, ΔqsrA (AKM414), ΔluxR (AKM416), ΔluxR, ΔqsrA (AKM418), ΔluxR, ΔaphA (AKM420), and ΔluxR, ΔaphA, ΔqsrA (AKM421) strains at low-cell density, OD$_{600}$=0.1 (white bars), and at high-cell density, OD$_{600}$=1.0 (black bars). Mean and standard deviation values of triplicate samples and technical duplicates are shown. All ompA transcript levels were calculated relative to the V. harveyi wild-type at OD$_{600}$=0.1. 5S rRNA was used as the endogenous control.
Discussion

Bacterial sRNAs are important post-transcriptional regulators of processes including envelope homeostasis, iron regulation, carbohydrate utilization, pathogenesis, and QS (Caldelari et al., 2013; Gottesman and Storz, 2011; Lenz et al., 2004; Papenfort and Vogel, 2010; Vogel and Luisi, 2011). sRNAs are diverse, varying in size and in secondary structure. The trans-acting regulatory sRNAs comprise the most prevalent class. Trans-sRNAs are characterized by their distant chromosomal locations relative to the genes encoding their mRNA targets, with whom they form short, imperfect base-pairing interactions. The five Qrr trans-sRNAs play a prominent role in *V. harveyi* QS by driving transitions between its free-swimming single cell state, its multicellular lifestyle in biofilms, and its association with hosts as a pathogen (Nealson and Hastings, 1979; Ng and Bassler, 2009; Rutherford et al., 2011).

Using high-throughput whole genome microarrays, here we discovered a dozen intergenic non-coding sRNAs that could play roles in *V. harveyi* QS. One of them, which we name QsrA, acts immediately downstream of LuxR and AphA. This finding establishes that there are four tiers of QS gene regulation (Fig. 2-10). Tier 1 is comprised of Qrr1-5 that control 20 targets within and outside of the QS circuit. Tier 2 harbors the transcription factors AphA, LuxR, and the other immediate targets of the Qrr sRNAs. Tier 3 consists of hundreds of genes regulated by AphA and LuxR, now including QsrA. The identification of QsrA suggests that there exists a fourth tier in the QS circuit, consisting of QsrA targets, which include VIBHAR_04892 and several other genes identified in this study. Presumably, incorporating QsrA downstream of LuxR and AphA in the QS cascade provides *V. harveyi* additional plasticity to diversify the timing of QS-controlled gene expression.
Fig 2-10. The *V. harveyi* QS circuit has four tiers of gene regulation.

Tier 1 harbors Qrr1-5. Tier 2 includes the master QS low-cell density and high-cell density regulators, AphA and LuxR, respectively, and other direct targets of the Qrr sRNAs. Tier 3 is composed of direct targets of AphA and LuxR, including QsrA. Tier 4 is a newly discovered regulatory level that consists of targets controlled by QsrA.
In this realm, different combinations of autoinducer inputs lead to distinct levels of qrr1-5 expression, discrete levels of AphA and LuxR production, and consequently, unique patterns of QS-controlled gene expression. We know that the precise gene regulation pattern that occurs at each tier of the QS circuit culminates in the global QS response. Nonetheless, the relationship between autoinducer input and the time-dependent gene expression profile for each QS tier has not been fully defined. What is known is that, in V. harveyi, QS-regulated promoters display specific affinities for LuxR and AphA (Rutherford et al., 2011; van Kessel et al., 2013a). As LuxR production ramps up in step with cell density increases, high affinity LuxR-regulated promoters (Tier 3, Fig. 2-10), even if they lie in a low regulatory tier, can be activated prior to promoters in the tier above (Tier 2, Fig. 2-10). Likewise, as AphA production rises as cell density decreases, high affinity AphA-regulated promoters can be regulated prior to low affinity promoters irrespective of their positional hierarchy. Furthermore, feedback loops and cooperativity are embedded in the circuit that also disrupt a simple regulatory tier to gene expression timing relationship. For example, LuxR activates and AphA represses qrr1-5 expression, Qrr sRNAs activate AphA and repress LuxR production, and AphA and LuxR repress each other's expression (Fig. 2-10) (Rutherford et al., 2011; Waters and Bassler, 2006). While these features make disentangling these relationships on a global scale difficult, the discovery of QsrA hints at widespread interconnections between transcriptional and post-transcriptional regulators that act in concert to fine-tune QS gene regulation.

QsrA regulates multiple mRNA targets, one of which is VIBHAR_04892 encoding OmpA. This is a common theme, as numerous studies in enterobacteria have shown that trans-acting sRNAs control the expression of outer membrane proteins (OMPs) (Frohlich et al., 2012; Guillier et al., 2006; Johansen et al., 2006; Papenfort et al., 2006; Song et al., 2008; Vogel and Papenfort, 2006). Indeed, almost a third of all characterized E. coli/Salmonella sRNAs regulate OMPs including OmpA, OmpC, OmpF, OmpW, and OmpX. Many OMPs, such as OmpC and OmpF, act as general porins that promote passage of beneficial nutrients into the cells while acting as barriers to entry of harmful molecules. Some OMPs, including OmpA, have been implicated in functions beyond their porin roles, such as serving structural roles as physical links between the outer membrane and the underlying peptidoglycan layer, acting as recognition elements for phage infection, mediating surface adhesion, and promoting colonization that leads to biofilm
formation and/or host infection (Smith et al., 2007). We constructed an \textit{ompA} \textit{V. harveyi} null mutant but did not identify any obvious role for OmpA in salt tolerance, nutrient acquisition, or biofilm formation. Thus, while additional experiments are necessary to define the function of \textit{VIBHAR_04892}, this study demonstrates the involvement of QS in OmpA production. QsrA indirectly activates \textit{VIBHAR_04892} presumably through an intermediate factor.

Numerous \textit{ompA}-regulating sRNAs have been identified in enterobacteria, including MicA, RybB, RseX, and VrrA (Douchin et al., 2006; Guillier et al., 2006; Johansen et al., 2006; Papenfort et al., 2006; Song et al., 2008; Vogel and Papenfort, 2006). Notably, in the four previous cases, expression of the genes encoding the regulatory sRNAs is controlled by the alternative sigma factor, $\sigma^E$, that, itself, is activated during stationary phase and upon accumulation of misfolded OMPs. All four of these sRNAs repress \textit{ompA}, presumably to maintain homeostatic OMP levels which prevents toxic OmpA buildup. By contrast, QsrA is not regulated by $\sigma^E$ and QsrA is an activator, not a repressor of \textit{ompA}.

Several studies have examined the regulatory roles and benefits of sRNA regulators in relation to OMPs. For example, in \textit{E. coli}, the transcription factor OmpR and the OmpR-regulated sRNAs, MicC and MicF control OmpC and OmpF, respectively. OmpR represses \textit{ompF} transcription, and OmpR-activated MicF also post-transcriptionally represses \textit{ompF}. By contrast, OmpR activates transcription of \textit{ompC} and OmpR represses \textit{micC} transcription. MicC post-transcriptionally represses \textit{ompC}. In both cases, incorporation of the sRNA enables simultaneous transcriptional and post-transcriptional control of target OMP genes to tighten inhibition of OmpF production and to enhance activation of OmpC production under appropriate conditions. Analogous to this scenario, simultaneous repression of \textit{qsrA} and \textit{VIBHAR_04892} by AphA at LCD ensures both transcriptional repression and that there is no post-transcriptional activation of OmpA. In addition, QsrA acting in concert with LuxR should strengthen and perhaps accelerate activation of \textit{ompA} expression during the transition from LCD to HCD mode.

\textit{V. harveyi} has two other \textit{ompA} genes, \textit{VIBHAR_06262} and \textit{VIBHAR_01273}. The three 5' UTRs upstream of the \textit{ompA} genes vary considerably, possibly explaining why QsrA only regulates \textit{VIBHAR_04892} (Fig.
VrrA is a sRNA that regulates the *ompA* gene (VC2213) in *V. cholerae*, a close relative of *V. harveyi* (Song et al., 2008). VrrA is a direct repressor of *ompA*, and it does not require Hfq. Genomic scanning suggests that VrrA exists in *V. harveyi* and that it could regulate all three *V. harveyi* *ompA* genes (Fig. 2-12). This finding suggests that *V. harveyi* has the capacity to use sRNAs to regulate all three of its *ompA* genes, presumably in response to appropriate stimuli or under appropriate environmental circumstances.

In summary, QsrA is an Hfq-dependent trans-sRNA that acts in the *V. harveyi* QS network. In contrast to previously studied *ompA*-regulating sRNAs in other enterobacteria, QsrA is an activator of *ompA* expression. QsrA is maximally produced at HCD and presumably exerts its major effects on its targets when *V. harveyi* launches its multicellular lifestyle. Intriguingly, QsrA is poorly conserved across bacterial species. It exists only in two sequenced *V. harveyi* strains, and two other species closely related to *V. harveyi*: *V. campbellii* and *V. jasicida*. In all four cases qsrA is located immediately downstream of *VIBHAR_05179*. QsrA bears no sequence similarity to other known sRNAs. This limited conservation suggests that QsrA may play a specific QS function in these vibrio species. Indeed, QsrA adds a new tier of regulation to the known QS network by acting simultaneously with and sequentially to the QS master transcription factors, AphA and LuxR.
Fig 2-11. Predicted interaction between *V. harveyi* QsrA and *VIBHAR_04892* mRNA.

RNAhybrid (Rehmsmeier et al, 2004) was used to predict RNA-duplex formation between QsrA and the 5’ UTR regions of the three *V. harveyi ompA* mRNAs. QsrA was predicted to only base-pair with the 5’ UTR of *VIBHAR_04892* mRNA. No significant complementarity was predicted to exist between QsrA and the 5’ UTR of *VIBHAR_01273* and *VIBHAR_06262* mRNAs so those alignments are not shown.

Fig 2-12. Predicted interaction between *V. harveyi* VrrA and the three *V. harveyi ompA* mRNAs.

RNAhybrid (Rehmsmeier et al, 2004) was used to predict RNA-duplex formation between VrrA and the 5’ UTR regions of the three *V. harveyi ompA* mRNAs. VrrA was predicted to base-pair all three *ompA* mRNAs. Start codons are shown in red.
Materials and Methods

Bacterial strains and growth conditions.

V. harveyi strain BB120 (Bassler et al., 1997) and derivatives were grown in Luria-marine (LM) medium with shaking at 30°C. E. coli strains S17-1λpir (de Lorenzo and Timmis, 1994), DH10B (Electromax; Invitrogen) and derivatives were grown in Luria-Bertani (LB) medium with shaking at 37°C. Strains used in this study are described in Table 2-3. Antibiotics (Sigma) were used at the following concentrations: ampicillin, 100 μg ml⁻¹; kanamycin, 100 μg ml⁻¹; chloramphenicol, 10 μg ml⁻¹; tetracycline, 10 μg ml⁻¹; gentamicin, 100 μg ml⁻¹; and polymyxin B, 50 U ml⁻¹. Constructs harboring the P_BAD promoter were induced with 0.2% arabinose (Sigma). Plasmids were introduced into electrocompetent E. coli S17-1λpir using 0.2 μM cuvettes (USA Scientific) and a Bio-Rad MicroPulser. Plasmids were transferred from E. coli S17-1λpir into V. harveyi by conjugation. Exconjugants were isolated on LM plates containing polymyxin B and the appropriate antibiotic for plasmid maintenance.

DNA manipulations and mutant construction.

E. coli strain S17-1λpir and DH10B were employed for cloning using standard procedures as in Sambrook et al. (1989). Polymerase chain reactions (PCR) were performed using iProof High-Fidelity DNA polymerase (Bio-Rad). Restriction enzymes, calf intestinal phosphatase (CIP), T4 DNA ligase, and Gibson Assembly Master Mix were purchased from New England Biolabs (NEB). Plasmids used in this study are listed in Table 2-4. Plasmids were constructed using primers listed in Table 2-5 from Integrated DNA Technologies (IDT). The High-Speed Plasmid Mini Kit (IBI) was used for plasmid extractions and the Zymoclean Gel DNA Recovery Kit (Zymogen) for PCR purifications. All plasmid constructs were confirmed by sequencing by Genewiz, Inc. The arabinose-inducible luxR gene was blunt cloned under the araC-pBAD promoter in the pBAD myc/His A (Invitrogen) plasmid. To examine whether LuxR activates qsrA transcription, the qsrA promoter was cloned upstream of the RBS and gfp gene encoded in pCMW-1 (Waters and Bassler, 2006). aphA was driven by the PLtet-O1 promoter from pZA31-lucNB (Feng et al., 2015). To study whether AphA represses qsrA transcription, the P_qsrA-gfp fusion from pCMW-1 was introduced into pZE12G. The 5’ UTRs of VIBHAR_04892 were cloned under the PLtetO-O1 promoter of the pXG-10 plasmid (Urban and Vogel, 2007). The qsrA gene was cloned under the PLlacO-O1 promoter.
of pZE12G plasmid for studies requiring constitutive expression. *V. harveyi* mutants (Table 2-3) were constructed using λ red recombineering in *E. coli* S17-1λpir::pKD46 (Datsenko and Wanner, 2000). Mutations were introduced into *V. harveyi* genomic DNA that had been cloned into the pLAFR2 cosmid, followed by homologous recombination into genome (Rutherford et al., 2011). Sequences encoding 3XFLAG tag were introduced at the 3’ end of VIBHAR_04892 using the Cm<sup>R</sup> suicide vector pRE112 (Edwards et al., 1998).

GFP reporter assay.
Overnight cultures of *E. coli* strains were diluted 1:1000 in triplicate, and subsequently, grown with shaking at 37°C in LB medium with appropriate antibiotics. GFP fluorescence and OD<sub>600</sub> were measured after 16 h using an Envision 2103 Multilabel Reader (Perkin Elmer).

Northern blotting.
Overnight cultures of *V. harveyi* were diluted 1:1000 into fresh LM medium and grown to the indicated cell densities (OD<sub>600</sub>). Transcription and translation were terminated by adding STOP Mix [95% (vol/vol) EtOH and 5% (vol/vol) phenol]. Bacteria were pelleted by centrifugation (15 min, 4000 rpm, 4°C) and stored in -80°C until RNA isolation. Total RNA was isolated using the Trizol method. Northern blotting was carried out as previously described (Urban and Vogel, 2007). In brief, 5 μg of total RNA was loaded and electrophoresed on 6% polyacrylamide gels (7M urea) followed by transfer to Hybond-XL membranes (GE Healthcare). For QsrA sRNA detection, membranes were hybridized with the QsrA riboprobe at 68°C in Rapid-hyb buffer (GE Healthcare). Three washing steps with SSC wash buffers (2x, 1x, 0.5x, in this order) were performed. For control 5S rRNA detection, membranes were hybridized with a 5’ end labeled DNA probe at 42°C in Rapid-hyb buffer (GE Healthcare) and washed in three steps with SSC wash buffers (5x, 1x, 0.5x, in this order). Wash buffers were supplemented with 0.1% SDS. Blots were exposed to a PhosphorImager screen (GE Healthcare), scanned with Typhoon 9410 (GE Healthcare), and band intensities were quantified with Image J (http://imagej.nih.gov/ij/). The riboprobe was synthesized by T7-mediated in vitro transcription of 100 ng template DNA in the presence of [α-<sup>32</sup>P]UTP with the MAXIscript
Kit (Ambion). The 5’ end-labeled DNA probe was synthesized using T4 polynucleotide kinase (NEB) in the presence of $[^{32}\text{P}]\text{ATP}$.

**qRT-PCR.**

RNA used for quantitative RT-PCR (qRT-PCR) was isolated from *V. harveyi* at the specified OD$_{600}$ levels using the RNeasy Mini Kit (Qiagen) followed by DNase treatment (Ambion). cDNA was generated with SuperScript III reverse transcriptase (Invitrogen) using 2 μg of RNA. Real-time PCR analyses were performed on an ABI Prism Quantstudio 6 Flex Sequence Detection System using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosciences). Triplicate biological samples were measured and analyzed by a comparative Ct method (Applied Biosystems) in which the relative amount of target RNA was normalized to the internal control 5S rRNA first, and subsequently, to each other.

**EMSA.**

LuxR and AphA were purified as described (van Kessel et al., 2013a; van Kessel et al., 2013b). DNA substrates generated by PCR were purified using Zymoclean Gel DNA Recovery Kit (Zymogen). DNA was labeled with $[^{32}\text{P}]\text{ATP}$ in 20 μl reaction mixtures containing T4 polynucleotide kinase (NEB) for 30 min at 37°C and cleaned on ProbeQuant G-50 microcolumns (GE Healthcare). Labeled DNA was incubated for 30 min in a 15 μl reaction mixture containing binding buffer (10 mM HEPES [pH 7.5], 100 mM KCl, 2 mM dithiothreitol [DTT], 200 μM EDTA), 10 ng/μl poly(dIdC), 100 μg/ml bovine serum albumin (BSA), and the desired concentration of protein (LuxR or AphA) diluted in dilution buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol). Reaction mixtures were separated on 6% TGE (25 mM Tris, 0.25 M glycine, 1 mM EDTA)-polyacrylamide native gels. Gels were dried at 80°C for 45 min, exposed overnight to a PhosphorImager screen (GE Healthcare), scanned with Typhoon 9410 (GE Healthcare), and analyzed by Image J (http://imagej.nih.gov/ij/).

**Western blotting.**

Overnight cultures of *V. harveyi* wild-type and QS mutants harboring C-terminal 3XFLAG-tagged VIBHAR_04892 were diluted 1:1000 into fresh LM medium and grown to the indicated cell densities (OD$_{600}$). Cells totaling an OD$_{600}$ of 1.0 were collected and pelleted. The cells were resuspended in 100 μl
of 1x Bugbuster (Novagen) containing 50 μg ml⁻¹ Lysozyme (Sigma) and 10 U ml⁻¹ Benzonase Nuclease (Novagen), and diluted into 4x SDS-PAGE protein loading buffer. Samples were incubated in 37°C water bath for 1 h followed by immediate electrophoresis on 4-15% Mini-Protein Gels (Bio-Rad). Proteins were transferred to PVDF membranes and blotted with monoclonal anti-FLAG M2-Peroxidase (HRP) antibody produced in mouse (Sigma) and with monoclonal RNA Polymerase-α (RpoA) antibody produced in mouse (Neoclone). Chemiluminescence was visualized by ImageQuant LAS 4000 (GE Healthcare).

**Microarray analysis.**

Microarrays contained three 60-mer probes per ORF in the *V. harveyi* genome (GenBank strain BAA-1116), up to one probe per 100 bp of intergenic sequence and no probe for intergenic sequences below 60 bp (Amadid design ID 021087). Each probes were spotted in duplicate. RNA from *V. harveyi* strains was isolated from cells grown in LM medium to an OD₆₀₀ of 1.0 in triplicate using the Trizol method. Subsequent cDNA synthesis and microarray hybridization, washing, and scanning were performed according to the Low Input Quick Amp WT Labeling Kit, Two-Color (Agilent) protocol using 50 ng of total RNA input. Data were extracted with Agilent Feature Extractor and analyzed using the Princeton University Microarray Database (PUMAdb) ([http://puma.princeton.edu/](http://puma.princeton.edu/)). Data were retrieved for probes that were above background (p<0.0001) and differed more than 2-fold. Probes were averaged for each gene. At least three arrays were performed comparing three independent cultures for each strain.
## Table 2-3. Strains used in this study

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>S17&lt;sub&gt;pir&lt;/sub&gt;</td>
<td>Wild-type</td>
<td>(de Lorenzo and Timmis, 1994)</td>
</tr>
<tr>
<td>DH10B</td>
<td>Wild-type</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>V. harveyi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB120</td>
<td>Wild-type</td>
<td>(Bassler et al., 1997)</td>
</tr>
<tr>
<td>JAF78</td>
<td>ΔluxO::cm</td>
<td>(Freeman and Bassler, 1999a)</td>
</tr>
<tr>
<td>KM83</td>
<td>luxO D47E::cm</td>
<td>(Tu and Bassler, 2007)</td>
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<td>ΔaphA</td>
<td>(Rutherford et al., 2011)</td>
</tr>
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<td>ΔluxR</td>
<td>(Pompeani et al., 2008)</td>
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<td>(van Kessel et al., 2013a)</td>
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<td>Δhfq</td>
<td>(Lenz et al., 2004)</td>
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<tr>
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<td>ΔqsrA</td>
<td>This study</td>
</tr>
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<td>ΔaphA, ΔqsrA</td>
<td>This study</td>
</tr>
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<td>Wild-type / VIBHAR_04892::3XFLAG</td>
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<td>AKM421</td>
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<td>Plasmid</td>
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<td>-------------------------------------------------------</td>
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<td>pLAFR2</td>
<td>Empty vector</td>
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<td>pLAFR2 ΔaphA::cm</td>
<td>(Rutherford et al., 2011)</td>
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<tr>
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<td>This study</td>
</tr>
<tr>
<td>pCMW-1</td>
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<td>(Waters and Bassler, 2006)</td>
</tr>
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<td>pqsrA::gfp</td>
<td>Transcriptional reporter qsrA</td>
<td>This study</td>
</tr>
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<td>pZA31-lucNB</td>
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</tr>
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<td>This study</td>
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<td>This study</td>
</tr>
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</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
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<td>(Urban and Vogel, 2007)</td>
</tr>
<tr>
<td>pompA::gfp</td>
<td>GFP reporter plasmid. Carries the 400 bp VIBHAR_04892 (ompA) 5’ UTR and 30-150 bp of ompA coding sequence</td>
<td>This study</td>
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<tr>
<td>pTL18</td>
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### Table 2-5. Primers used in this study

Sequences are provided in the 5' → 3' direction. 5'P denotes 5' monophosphate.

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<td>TTTTTCTAGATTTAAATCAGAAGGC</td>
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<td>KPO-0677</td>
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AMO808  TTTTTTATGCATGAGATTTACGTCACAAA  AGTTATCTAATCT  pompA::gfp (insert )
AMO653  TTTTGCTAGCGTAGCCACTGCAAGAGC  GCGATTTC  pompA::gfp (insert – 5’UTR to 30bp CDS)
AMO944  TTTTTGCTAGCAGTACGCAAGAGCG  ACATTCGA  pompA::gfp (insert – 5’UTR to 75bp CDS)
AMO954  TTTTTGCTAGCGTAGCCACTGCAAGAGCACATTCGA  GCGC  pompA::gfp (insert – 5’UTR to 150bp CDS)
AMO776  GAGAAGCGGTGTAAGTGAACTGCATAGGC  CATGCTAGAAACTGGC  pRE112 VIBHAR_04892:: 3XFLAG
AMO778  AGACGCTGCTTTAAAAAGCTACGACTTTCCATGCTGCAAGAGCG  TTATGCAGCGGACTGTAAGTGAACTGCATAGGC  GCGCATGCTAGAAACTGGC  pRE112 VIBHAR_04892:: 3XFLAG
AMO779  TAGTCGGGTCACCTTCGCATTAAAGAAGTACGCATGCTGCAAGAGCGTACGACTTTCCATGCTGCAAGAGCG  TCAGTGAACGTAGACCCAGCGCTCT  pRE112 VIBHAR_04892:: 3XFLAG
AMO780  TGAATAAGTGATAGGGCCCGATCCCAGTGACATCTTAGCACTGGC  Northern blot riboprobe and qRT-PCR primer for 5S rRNA
AMO104  CCATTCCGAACTCAGAAGTGAA  Northern blot riboprobe primer for 5S rRNA
AMO105  TACTCTCACATGGGGAAGGCC  qRT-PCR primer for 5S rRNA
AMO106  GTTTTTTAATACGACTCACTATAGGGA  GGATTCTTCACATGGGGAAGGCC  Northern blot riboprobe primer for 5S rRNA
AMO19  TGCAATCCTTAACGCTCTGAGC  Northern blot riboprobe primer for QsrA
AMO20  GTTTTTTAATACGACTCACTATAGGGA  GGATTCTTCACATGGGGAAGGCC  Northern blot riboprobe primer for QsrA
AMO358  TCCTTAACGCTCTGACTGAGCTT  qRT-PCR primer for QsrA
AMO359  CTAGCCTGCATTGTCGCTAGG  qRT-PCR primer for QsrA
AMO73  AAGCAATGTGTGGTTGGTTC  qRT-PCR primer for VIBHAR_01082
AMO74  CCTCGATAGTGCGATAAG  qRT-PCR primer for VIBHAR_01082
AMO91  CACGGTAACTAGCTGCAAA  qRT-PCR primer for VIBHAR_04801
AMO92  GAAGCGTACAAGCGACCTTTTC  qRT-PCR primer for VIBHAR_04801
AMO75  GTTCTAGCTGGTGGCGTAAT  qRT-PCR primer for VIBHAR_04892
AMO76  TAGGTGACATGCGGTCTTTAAC  qRT-PCR primer for VIBHAR_04892
AMO732  CGCCTTCATCGCTGGTATTA  qRT-PCR primer for VIBHAR_05179
AMO733  CGGACTTGATGAGCAGAGATTCA  qRT-PCR primer for VIBHAR_05179
AMO55  CGCCTTCCTCGAACTCTCATACAT  qRT-PCR primer for VIBHAR_02528
AMO56  ATGCGCGGACAGTAGTATAAT  qRT-PCR primer for VIBHAR_02528
AMO51  CGTTAAGCAAGGTCGAGCATA  qRT-PCR primer for VIBHAR_02854
AMO52  GGCTAATGTATGACGCAGAAGAG  qRT-PCR primer for VIBHAR_02854
AMO63  AGCCAAGATGCCCCAGTT  qRT-PCR primer for VIBHAR_05638
AMO64  TCACTAATTCCACTCTGTGTATTC  qRT-PCR primer for VIBHAR_05638
AMO47  ATCGACAGATCATCCATCAAAC  qRT-PCR primer for VIBHAR_06097
AMO48  CTGTGGAACGCGAAGCTA  qRT-PCR primer for VIBHAR_06097
AMO854  CATTAGGTGCCAGTAGACTATAC  qRT-PCR primer for VIBHAR_06262
AMO855  CGAAGAAGTTTGCATCGATGTC  qRT-PCR primer for VIBHAR_06262
AMO858  CTGTCACTCAGTCTGCAGCTA  qRT-PCR primer for VIBHAR_01273
AMO859  GTCGTCTTTACGTCGTTCAT  qRT-PCR primer for VIBHAR_01273
AMO224  TACTAACCATTCCATCATACCTAAAC  EMSA probe primer for 83bp P_qsrA
AMO281  GCCTGCTGAGATGACTTAC  EMSA probe primer for 83bp P_qsrA
AMO473  TCCATCACCCTTACGGTCATGGGTG  EMSA probe primer for 73bp P_qsrA
AMO474  CCTAATCATGGCTGAGTC  EMSA probe primer for 73bp P_qsrA
AMO475  CAAAGGTCAATGTGATGAGCAAG  EMSA probe primer for 63bp P_qsrA
AMO476  ATGTATATCTCTAATAGGCCTG  EMSA probe primer for 63bp P_qsrA
AMO227  GTTGTGTGATGGCAAATAGTGAA  EMSA probe primer for 53bp $P_{qsrA}$
AMO284  TGCTAGCCATATGTATATCTCCTTAA  EMSA probe primer for 53bp $P_{qsrA}$
STR1087 CTACAACTCTGAAAGAAAGGCTAT  Antisense probe A to detect QsrA
STR1085 CTCTTTAGTCTTTTATTAGTCA GTA  Antisense probe B to detect QsrA
STR1071 CAGCTTTGTGTGTCTGTGCAACCC  Antisense probe C to detect QsrA
STR1069 TGGCTCAGGCTAGGCGAAGCTCAG  Antisense probe D to detect QsrA
STR1067 CAGGAGTTACTATATGCAATTGG  Antisense probe E to detect QsrA
Portions of Chapter 2 have been presented at the following meetings:

- Gordon Research Conference on Marine Microbes, Girona, Spain, 2016 (Poster)
- Zing Conference on Regulating with RNA in Bacteria and Archaea, Cancun, Mexico, 2015 (Poster)
- Rutgers University RWJMS/Princeton University MD-PhD Symposium, Piscataway, NJ 2015 (Poster)
- Princeton University Molecular Biology Departmental retreat, Princeton, NJ 2015 (Poster)
- The 5th ASM Conference on Cell-Cell Communication in Bacteria, San Antonio, TX, 2014 (Poster)
- Rutgers University RWJMS/Princeton University MD-PhD Symposium, Piscataway, NJ 2014 (Poster)
- Princeton University Molecular Biology Departmental retreat, Princeton, NJ 2014 (Poster)

I designed all experiments, constructed all strains, conducted all biological assays, and performed all analyses with input from Bonnie L. Bassler and Kai Papenfort.
CHAPTER 3

Conclusion
Five homologous trans-regulatory sRNAs called the Qrr sRNAs lie at the heart of the *V. harveyi* QS circuit. The Qrr sRNAs use multiple regulatory mechanisms to precisely control the levels of several key components in the QS circuit. Two of these components are AphA and LuxR, the master LCD and HCD QS regulators, respectively. The precise levels and activities of AphA and LuxR combine to execute appropriate QS gene expression programs. The goal of this work was to identify additional sRNAs that play roles in the *V. harveyi* QS circuit. Using high-throughput whole genome microarrays, we discovered dozens of intergenic sRNAs in *V. harveyi* that were differentially regulated in the LCD-locked *luxO D47E V. harveyi* strain compared to the HCD-locked Δ*luxO* *V. harveyi* strain. Here, we have studied the most highly regulated of these sRNAs, which we name QsrA.

QsrA is an Hfq-dependent trans-regulatory sRNA that is 30-fold more highly expressed in the Δ*luxO V. harveyi* strain compared to the *luxO D47E V. harveyi* strain. Promoter-reporter fusion assays showed that QsrA is directly activated by LuxR while it is directly repressed by AphA. This regulatory relationship allows maximal production of QsrA at HCD. Using whole genome microarrays, we identified eight mRNA targets of QsrA. The most highly regulated target is VIBHAR_04892 encoding the outer membrane protein A (OmpA). qRT-PCR analyses show that VIBHAR_04892 mRNA levels are more than 50-fold reduced in the *V. harveyi ΔqsrA* strain compared to the wild-type, indicating that QsrA activates expression of VIBHAR_04892. In line with the VIBHAR_04892 transcript level, western blots show that a *V. harveyi ΔqsrA* strain produces significantly less VIBHAR_04892 protein compared to the wild-type. Gene fusion and epistasis experiments suggest that QsrA control of VIBHAR_04892 is not direct and that an additional factor exists that links QsrA to VIBHAR_04892.

OmpA proteins generally function in the maintenance of cell morphology, nutrient acquisition, surface adhesion, colonization, and biofilm formation (Smith et al., 2007). Several sRNAs have been identified to repress the production of OmpA in other bacteria, including MicA and RseX in *E. coli*, MicA and RybB in *S. typhimurium*, and VrrA in *V. cholerae*. (Douchin et al., 2006; Guillier et al., 2006; Johansen et al., 2006; Papenfort et al., 2006; Song et al., 2008; Vogel and Papenfort, 2006). In contrast, QsrA is a sRNA activator of OmpA production in *V. harveyi*. *V. harveyi* also contains a VrrA homolog...
(Song et al., 2008). Hence, QsrA could potentially counteract the action of VrrA to precisely control the homeostatic level of OmpA. Going forward, gene fusion and competition assays could decipher the exact relationship between QsrA, VrrA, and OmpA in *V. harveyi*. Along these lines, further studies are necessary to define the specific function of VIBHAR_04892 as well as why *V. harveyi* has two other OmpA proteins in addition to VIBHAR_04892. Transcriptome profiling, phenotypic analyses, and microscopy, both at the single-cell and at the population levels, performed in all combination of single, double and triple mutants could be used to define the unique role of each OmpA, and perhaps their combined roles.

QsrA is poorly conserved; it is present in only a few closely related vibrio species. For that reason, we predict that QsrA plays a specific QS function in *V. harveyi*. Indeed, our identification of QsrA adds yet another tier in the QS circuit, further diversifying the timing of QS-controlled genes (Fig. 2-9). Different combinations of AI input lead to distinct levels of qrr1-5 expression, discrete levels of LuxR production, and unique patterns of QS-controlled gene expression. We know that gene regulation occurs at each level of the QS circuit, in which the Qrr sRNAs, LuxR, and AphA control discrete targets to contribute to the global QS response. However, the relationship between AI input and the time-dependent gene expression profile at each tier is largely unknown. What is known is that there exist different classes of QS-regulated promoters that display variable affinities for LuxR. Presumably the same is true for AphA, although it has not yet been studied. This gradient in LuxR-driven promoter strength (and presumably a companion, but reciprocal gradient in AphA-driven promoter strength) enables some genes in the bottom-most tier to be activated prior to others during QS transitions. Gene expression profiles of the Qrr sRNAs, LuxR, AphA, QsrA, and their targets at increasing cell densities could establish a temporal hierarchy for the four different tiers of the QS circuit and show how the QS program consisting of hundreds of genes is established in *V. harveyi*.
CHAPTER 4

References


