

Non-coding sRNAs regulate virulence in the bacterial pathogen *Vibrio cholerae*

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Abbreviations: QS, quorum sensing; Qrr, quorum regulatory RNA; sRNA, small RNA; LCD, low cell density; HCD, high cell density; 5' UTR, 5' untranslated region; RBS, ribosome binding site; nt, nucleotide

Vibrio cholerae is the waterborne bacterium responsible for worldwide outbreaks of the acute, potentially fatal cholera diarrhea. The primary factors this human pathogen uses to cause the disease are controlled by a complex regulatory program linking extracellular signaling inputs to changes in expression of several critical virulence genes. Recently it has been uncovered that many non-coding regulatory sRNAs are important components of the *V. cholerae* virulence regulon. Most of these sRNAs appear to require the RNA-binding protein, Hfq, to interact with and alter the expression of target genes, while a few sRNAs appear to function by an Hfq-independent mechanism. Direct base-pairing between the sRNAs and putative target mRNAs has been shown in a few cases but the extent of each sRNAs regulon is not fully known. Genetic and biochemical methods, coupled with computational and genomics approaches, are being used to validate known sRNAs and also to identify many additional putative sRNAs that may play a role in the pathogenic lifestyle of *V. cholerae*.

Introduction

The bacterium *Vibrio cholerae* causes over 3,000,000 cases of the potentially fatal diarrheal disease cholera, resulting in approximately 100,000 deaths annually.¹ The lifestyle of this human pathogen includes an aquatic niche where it survives as either a free-living bacterium or in biofilm communities often associated with the surface of plankton. Once ingested via contaminated food or water, *V. cholerae* can gain access to the human intestine and replicate to large numbers while producing the cholera symptoms that aid in transmission back to the aquatic environment. The relative contribution to epidemics of person-to-person transmission, vs. naturally occurring environmental *V. cholerae* bacteria, remains a contentious issue, recently debated following the 2010 cholera outbreak in Haiti.²

The pathogenesis of *V. cholerae* within the human host is controlled by a complex signaling cascade of regulatory factors and

virulence genes, which have been the subject of several decades of intense study.³⁻⁵ During the last two decades, the identification of non-coding small RNAs (sRNAs) has revealed a new layer in bacterial gene regulation. Work in the model organism *Escherichia coli* led to the discovery of sRNAs as ubiquitous regulators of numerous cellular processes in many bacteria. In *V. cholerae*, several trans-acting sRNAs have recently been identified that regulate signaling pathways important in environmental settings, as well as in pathways that alter expression of genes affecting the ability of this pathogen to cause disease, the focus of this review. This field of research is likely to be a fecund area for future study with potential biomedical implications.

Bacteria encode both cis-acting and trans-acting non-coding sRNAs. Cis-acting sRNAs are perfectly complementary to an mRNA target expressed on the opposite strand of the DNA. In contrast, the trans-acting sRNAs described here are only partially complementary to one or more mRNA targets encoded elsewhere in the genome. In *E. coli* and *V. cholerae*, the RNA binding protein Hfq typically binds to both a trans-acting sRNA and its cognate mRNA, to lower the apparent dissociation constant between a sRNA and target mRNA,⁶⁻¹¹ to stabilize sRNAs,¹² and in some cases to facilitate degradation of the RNAs by recruiting RNase E.¹³ As Hfq is involved in many different sRNA/mRNA interactions, bacteria with an *hfq* gene deletion usually display pleiotropic defects.^{14,15} However, *V. cholerae* also encodes several trans-acting sRNAs, described below, that appear to interact with mRNAs without the necessity of Hfq,^{16,17} and also that act by titration of a protein rather than an Hfq-dependent base-pairing mechanism.¹⁸

Most bacterial trans-acting sRNAs regulate cognate target mRNAs through formation of a sRNA/mRNA duplex that negatively or positively alters the translation of the target gene. Repression is generally accomplished by base-pairing of the sRNA to nucleotides within the 5' untranslated region (5' UTR) of an mRNA that are adjacent to or within the ribosome binding site (RBS), thus preventing access by the translation machinery,¹⁹ or in some cases recruiting an RNase.¹³ In contrast, fewer examples of sRNA-dependent activation have been described, and typically occur as a consequence of sRNA pairing in an mRNA region distinct from the RBS. Activation results from an alteration of the secondary structure within a 5' UTR, that relieves an inhibitory

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structure in the mRNA, such as a hairpin, that blocks initiation or elongation of translation.²⁰

Initial discoveries of bacterial sRNAs grew out of genetic studies in which signaling pathways with missing components were shown to be controlled by non-coding regulatory sRNAs. More recently, algorithms have been developed to predict putative sRNAs and their potential targets based on DNA sequence features, coupled with current genomics methods that have likewise identified numerous putative regulatory sRNAs in *V. cholerae* and other bacteria.²¹ However, robust experimental validation of sRNA/mRNA base-pairing is required for a definitive demonstration of sRNA/mRNA base-pairing interactions. The benchmark for experimental evidence is obtained by the construction of a sRNA and an mRNA that both contain one or more nucleotide substitutions within the predicted pairing region. Often a native nucleotide(s) within the sRNA and the mRNA is replaced by the complementary base. This allows demonstration that pairing does not occur between a mutated sRNA and its native mRNA target, and vice versa; but that pairing is restored between a mutated sRNA and the mRNA target carrying a compensatory mutation. Computational predictions coupled with both in vivo and in vitro experimental methods have identified and validated in bacteria numerous sRNA/mRNA interactions and elegant regulatory mechanisms, with new discoveries invariably to follow. This review focuses on the relatively small group of trans-acting sRNAs currently known to play a role in the pathogenesis of the waterborne bacterial pathogen, *V. cholerae*.

***V. cholerae* Ecology and Pathogenesis**

V. cholerae in marine environments exists in a free living state and in association with surfaces (such as chitinous zooplankton and crab exoskeletons) as biofilms composed of bacterial cells, extracellular matrix material and DNA.²²⁻²⁵ Biofilm formation protects bacteria against environmental insults,²⁶ and biofilm-derived *V. cholerae* cells are also enhanced for virulence compared with free-living cells.²⁷⁻²⁹ Biofilm growth in standard nutrient-rich broth requires the production of exopolysaccharides, which are synthesized by the gene products of two VPS (*Vibrio* polysaccharide) operons, *vpsA-L* and *vpsK-Q*; though VPS production is not required for biofilms in sea water.^{23,30,31} Growth of *V. cholerae* in association with chitin also induces the bacteria to become naturally competent to take up extracellular DNA, which can be incorporated onto the chromosome allowing acquisition of genes encoding virulence factors and in antigenicity evolution.^{32,33} Thus, the environment may serve as a reservoir for potentially pathogenic *V. cholerae*, and also as a source of genetic diversity and an arsenal of virulence factors.

Following ingestion of bacteria in contaminated food or water, a subset of ingested *V. cholerae* cells survive passage through the acidic conditions of the stomach and gain access to the small intestine.³⁴ The two primary virulence factors responsible for colonization and subsequent disease are the cholera toxin (CT), encoded by *ctxAB*, and the toxin-co-regulated pilus (TCP), encoded by *tcpA-F*. CT is the canonical AB-type toxin, composed of five B subunits that bind as a pentameric ring to

the GM₁ ganglioside of intestinal epithelial cells to deliver the A subunit of the toxin into the cell. Within intestinal cells, the A subunit of CT ADP-ribosylates the G_s protein leading to activation of adenylate cyclase, resulting in a massive increase in cyclic AMP (cAMP) production. Accumulation of cAMP leads to the activation of the CFTR ion channel via phosphorylation, causing an efflux of chloride ions and water into the lumen of the small intestine, creating the diarrheal disease.³⁵

The second major virulence factor of *V. cholerae*, the type IV pilus TCP, is required for efficient colonization of the small intestine.^{36,37} The thread-like TCP pili promote cell-cell aggregation that aids in intestinal colonization,⁴ and also serve as a receptor for the CTX ϕ phage, which encodes the *ctxAB* genes for CT.³⁸ TCP is encoded by the *tcpA-F* operon located in a region of the genome called the *Vibrio* pathogenicity island (VPI). The VPI also contains other genes, including *acfA-D*, which are required for full colonization of a mouse intestine,³⁹ and *toxT*, the primary regulator of *tcpA-F* and *ctxAB*, the expression of which is coordinately regulated with TCP.

ToxT is the primary regulatory protein controlling transcription of both *ctxAB* and *tcpA-F* and a member of the AraC/XylS family of transcriptional activators (Fig. 1).⁴⁰ Degenerate 13 nucleotide (nt) sequences upstream of the -35 promoter element of ToxT-regulated genes, called “toxboxes,” serve as binding sites for the transcription factor.⁴¹ Transcription of ToxT is activated by ToxR, a transmembrane protein that contains a C-terminal DNA binding domain. ToxR stability requires ToxS, another inner membrane associated protein.⁴² TcpP, a second transmembrane protein, along with its partner protein TcpH, also activates ToxT transcription. Several environmental cues, including temperature, pH, osmolarity and bile salts alter expression of the virulence factors controlled by ToxT, via mechanisms both dependent and independent of TcpP/H and ToxR/S.^{43,44} Both ToxR and TcpP bind to the promoter of *toxT* to activate transcription.⁴⁵ While transcription of the *toxRS* operon is constitutively active, *tcpPH* transcription is controlled by two activator proteins, AphA and AphB, which both bind to the promoter of *tcpPH* to directly activate its transcription.^{46,47} This complex AphA regulon maintains control of ToxT, which is absolutely required for expression of *ctxAB* and *tcpA-F*, and thus virulence. With one exception, to date, the *V. cholerae* sRNAs described below that participate in virulence regulation either impinge on *toxT* expression, or are in turn controlled by ToxT (Fig. 1).

The Qrr sRNAs and the *V. cholerae* Quorum Sensing System

In 2002, collaborative studies by the Bassler group demonstrated that a cell-cell communication system, first described in the bioluminescent marine bacterium *Vibrio harveyi*, termed quorum sensing (QS), impinges on the ToxT virulence cascade of *V. cholerae*.⁴⁸⁻⁵⁰ The *V. cholerae* QS system described below allows for synchronization of gene expression in response to small chemical signal molecules (autoinducers or AIs) that are produced by *V. cholerae* and released into the extracellular environment. Importantly, multiple sRNAs are critical components of the *V.*

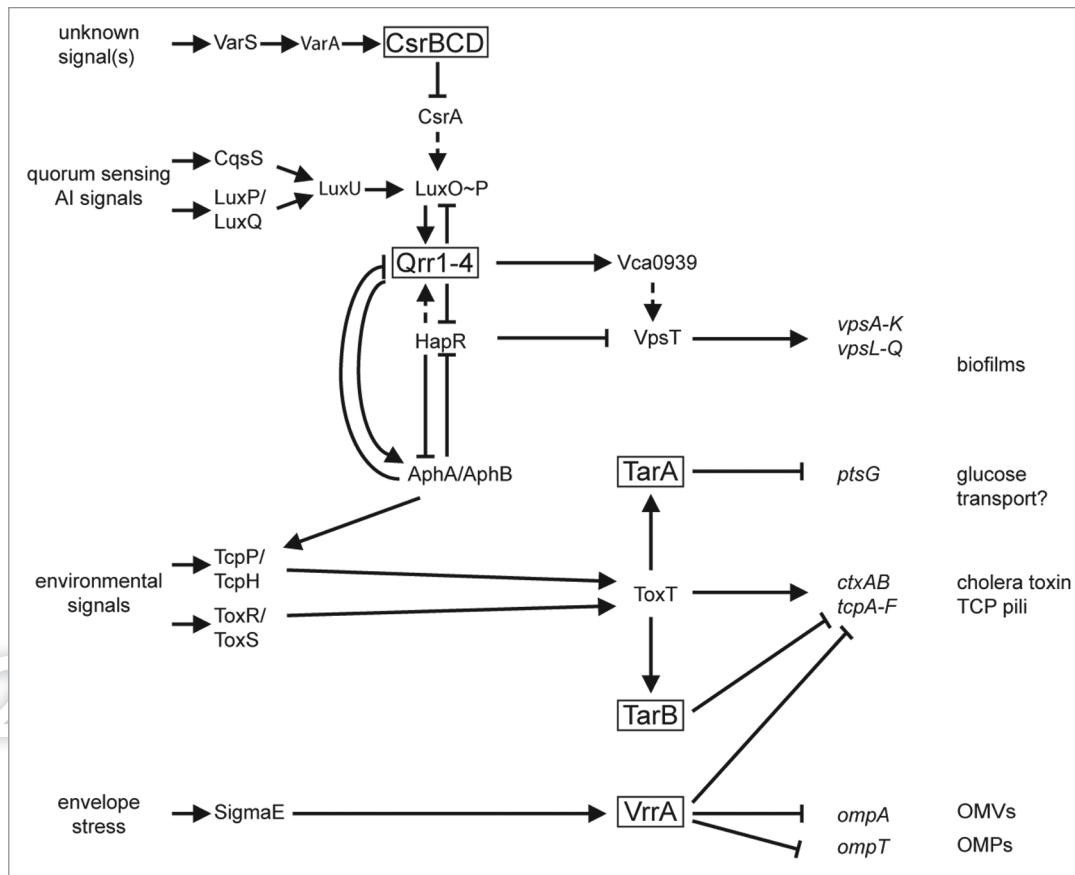


Figure 1. Regulatory network linking sRNAs to virulence in *V. cholerae*. Extracellular signal inputs coordinate regulatory cascades that connect the transcription of multiple sRNAs to the expression of genes that play a role in *V. cholerae* virulence. Sensory information is perceived by cognate receptors in or at the bacterial membrane (left), which transduce information into the regulatory network of the cell. Transcription of multiple sRNAs (boxed) is altered, resulting in changes in the expression of numerous genes involved in virulence and pathogenesis (right). The signal(s) sensed by VarS remains unclear. Dashed lines indicate indirect regulation. Solid lines indicate direct regulation. See text for additional details.

cholerae QS system and provided the first examples of non-coding sRNAs that regulate virulence gene expression in this pathogen (Fig. 1).

V. cholerae produces and responds to two QS autoinducers, CAI-1 (cholera autoinducer-1) and AI-2 (autoinducer-2), which are produced by CqsA and LuxS, respectively. CAI-1 is (S)-3-hydroxytridecan-4-one, produced and used by numerous vibrios.⁵¹ AI-2 is 4,5-dihydroxy-2,3-pentanedione (DPD), which is produced by many bacteria and described as a “universal” signaling molecule for inter-species communication.^{52,53} *V. cholerae* AIs are produced at a constant rate and thus serve as a proxy measure of bacterial number; with low AI levels at low cell density and high AI levels at high cell density.

Each AI has a cognate inner membrane receptor: CqsS for CAI-1 and the LuxP/Q heterodimer for AI-2. When *V. cholerae* experiences low cell density (LCD) conditions, and AI signals are scarce, the cognate AI receptors function as histidine kinases and transfer phosphate, via LuxU, to a response regulator, LuxO, which is a member of the NtrC family of transcription factors that activate transcription in conjunction with the alternate sigma factor, σ^{54} . Based on parallel genetic studies of the related QS system in bioluminescent *V. harveyi*, it was hypothesized that

phosphorylated LuxO (LuxO-P) repressed the activator of bioluminescence, LuxR (homologous to HapR in *V. cholerae*). This hypothesis was validated with a genetic screen that identified gene disruptions in *luxO* and *rpoN* (encoding σ^{54}), as expected, and also the previously described *hfq* gene.⁵⁵ A bioinformatics study followed, which identified the four *V. cholerae* sRNAs, Qrr1–4 (quorum-regulated RNA), that are transcriptionally activated at low cell density by LuxO-P in concert with σ^{54} .⁵⁵

The Qrr sRNAs act in concert with Hfq to regulate several different mRNAs, both positively and negatively. In *V. cholerae*, a strain carrying any single Qrr (with deletion of the other three *qrr* genes) exhibits WT QS behavior, thus the Qrr sRNAs were deemed functionally redundant for *hapR* regulation, which controls most QS phenotypes.⁵⁵ This is due to several feedback mechanisms that control Qrr transcription and maintain total Qrr levels even when one, two or three *qrr* genes are deleted.⁵⁶ The Qrr sRNAs vary in length from 96 to 108 nucleotides and are predicted by minimal free energy (MFE) calculations to form similar secondary structures to one another (Fig. 2).^{55,57} All sequenced pathogenic vibrios encode multiple Qrr sRNAs that each contain an absolutely conserved 32 nt sequence, which was predicted to be the site of interaction with mRNA targets.⁵⁵ When

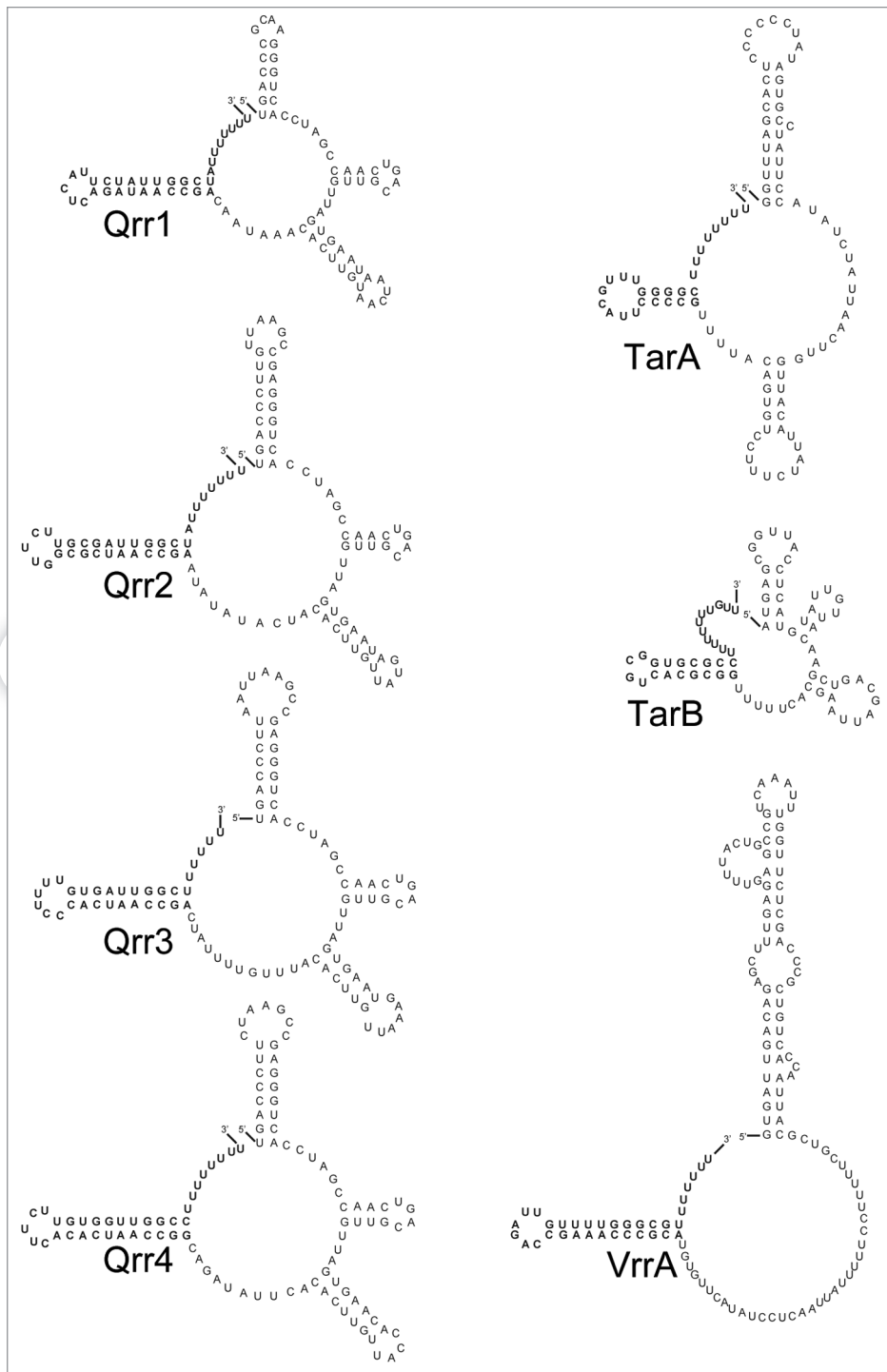


Figure 2. Predicted secondary structures of *V. cholerae* sRNAs that play a role in virulence gene expression. The lowest free energy structures of each sRNA are shown as predicted by mfold analysis (<http://mfold.rna.albany.edu/?q=mfold>). Qrr1 and Qrr4 structures appear different than described in 2004 in reference 55 using current mfold analysis tools. A predicted TarB secondary structure was not described in reference 87. To generate a single lowest free energy TarB structure, restrictions were imposed to prevent long range base-pairs by setting a maximum distance between paired bases at 30. The Rho-independent terminator of each sRNA is shown in bold.

V. cholerae reaches high cell density (HCD), and thus high AI levels, the AI-bound receptors switch to phosphatases, and as a result, unphosphorylated LuxO cannot activate Qrr transcription, initiating the QS response.

Several members of the Qrr regulon play a role in *V. cholerae* virulence, most prominently the transcriptional regulator, HapR,⁵⁸ which controls a variety of behaviors of *V. cholerae*, including virulence and biofilm formation.^{28,48,49,59,60} We have recently shown by in vivo and in vitro experiments with defined mutations in each RNA, that the Qrr sRNAs base-pair with *hapR* mRNA at the ribosome binding site (RBS), resulting in repression of *hapR* translation and a halt in HapR protein production (Fig. 3).^{11,55} The Qrr sRNAs may also facilitate destruction of the *hapR* mRNA by cellular RNases as increased levels of the sRNAs correlate with decreased levels of *hapR* mRNA.⁶¹ Repression of *hapR* translation by the Qrr sRNAs requires the RNA chaperone Hfq,⁵⁵ which also facilitates sRNA/mRNA interactions in *E. coli* as described in reference 6–10. *V. cholerae* Hfq serves a similar function by decreasing the apparent dissociation constant of *hapR* mRNA and Qrr sRNA in vitro.¹¹ Furthermore, the *V. cholerae* *hfq* gene is required for colonization of a suckling mouse intestine by the bacterium, a virulence defect presumably caused by a loss of function of many sRNAs.¹⁵ Indeed, microarray analysis indicated several important classes of genes with altered expression in a strain lacking Hfq, including genes encoding RpoE (stress response sigma factor), chemotaxis components, several histidine kinases, and multiple outer membrane proteins and periplasmic stress response proteins.¹⁵

QS regulation of the *V. cholerae* Qrr sRNAs plays a role in regulation of the virulence cascade described above. At HCD, in the absence of Qrr sRNAs, HapR directly represses transcription of *aphA* by binding to its promoter to block transcription.⁵⁰ Strains of *V.*

cholerae engineered to produce no Qrr sRNAs, do not make TcpP or CTX, and are severely impaired for colonization in an infant mouse model of *V. cholerae* pathogenesis.^{48,49} Virulence

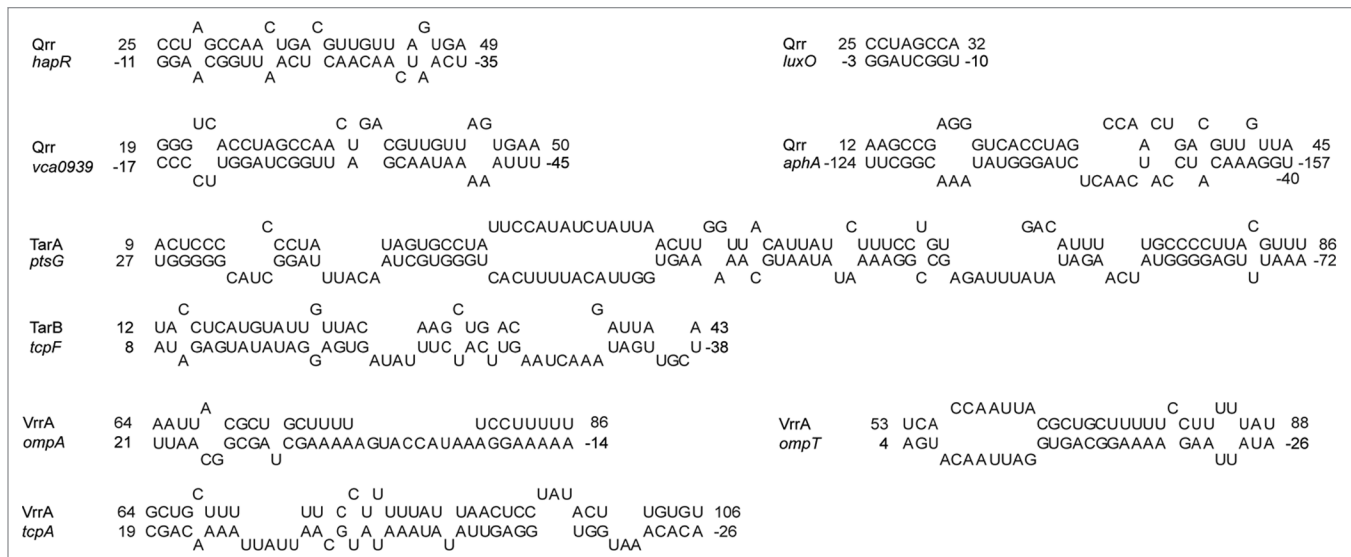


Figure 3. Predicted base pairing interactions between *V. cholerae* sRNAs and mRNA targets. Each sRNA/mRNA pairing shown was predicted with RNA hybrid program (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html>) analysis. The predictions shown were previously described for Qrr interactions with *hapR*, *luxO* and *vca0939* in reference 55, 56 and 72; TarA/*ptsG* in reference 86; VrrA/*ompA* and VrrA/*tcpA* in reference 17; and VrrA/*ompT* in reference 94. The Qrr/*aphA* and TarB/*tcpF* pairing predictions were performed for this review. For each predicted pairing shown, unpaired nucleotides were omitted from the ends for simplicity.

factor repression depends on AIs, as addition of synthetic AIs to a strain that cannot synthesize its own QS signals also causes a dramatic decrease in TCP production, indicating that production of the Qrr sRNAs is critical for negative regulation of HapR that enables CT and TCP production.⁶²

HapR accumulation at HCD controls additional phenotypes that may also play a role in virulence and/or biofilm formation. Notably, the AI-induced termination of Qrr synthesis and the resulting accumulation of HapR at HCD positively regulates expression of multiple competence genes, including *comEA*, required for *V. cholerae* to become capable of horizontally acquiring genetic material in the form of extracellular DNA.^{11,32,63} Natural competence in several other bacteria results in the acquisition of virulence factors and antibiotic resistance genes.^{64,65} QS-induced natural competence in *V. cholerae* can promote acquisition of the CTX ϕ prophage carrying *ctxAB*,⁶⁶ and alteration of genetic determinants of antigen specificity.⁶⁷ Indeed, the “mosaic structured” genome of *V. cholerae* is believed to be the result of numerous horizontal gene transfer events mediated by activities including chitin-induced natural competence.⁶⁸

V. cholerae biofilm formation is also controlled by Qrr/*hapR* interactions, as HapR serves as a repressor of *vps* transcription.^{28,59,60,69} HapR repression of *vps* transcription at HCD is directly mediated by binding of HapR to the promoter of *vpsT*, which encodes a positive regulator of the *vpsA-K* and *vpsL-Q* loci. QS, via HapR, also indirectly controls *vps* transcription and biofilm formation by modulating the accumulation of a secondary signaling molecule, cyclic di-GMP (c-di-GMP).⁶⁹⁻⁷¹ Interestingly, the Qrr sRNAs can directly activate translation of the mRNA of *vca0939*, a protein that contains an amino acid motif (GGDEF) indicative of proteins that directly synthesize c-di-GMP.^{72,73} Genetic evidence supports a model that at LCD, Hfq-dependent

base-pairing of the 32 nt interaction region of the Qrr sRNAs to the 5' UTR of *vca0939* mRNA prevents formation of an inhibitory secondary structure at the RBS (Fig. 3), thereby promoting translation in a manner similar to interactions in *E. coli* that promote translation of sigma factor RpoS by pairing with the RprA and DsrA sRNAs.^{74,75} Thus, the Qrr sRNAs appear to link QS to biofilm formation by multiple pathways: direct activation of *vca0939* translation, negative regulation of HapR resulting in high c-di-GMP and enhanced VPS-dependent biofilm formation at LCD, and low levels of c-di-GMP and biofilm repression at HCD.

Previous studies established that quorum sensing and the Qrr sRNAs play a role in *V. cholerae* pathogenesis through HapR-mediated effects on *aphA* transcription.⁷⁶ However, recently it was shown that *V. cholerae* Qrr sRNAs also directly regulate *aphA* mRNA translation independent of HapR.⁷⁷ Specifically, *aphA* expression is 3-fold increased in a *hapR* *V. cholerae* strain constitutively expressing the Qrr sRNAs. The Qrr sRNAs base-pair with the 5' UTR of *aphA* over 100 nt upstream of the ATG start codon and perhaps alter a putative secondary structure within the leader sequence that hinders translation (Fig. 3).⁷⁸ Interestingly, several Qrr nucleotides predicted to participate in *aphA* base-pairing lie outside of the absolutely conserved 32 nt sequence predicted to be important in the regulation of other direct Qrr targets (Figs. 2 and 3). Because *Vibrio* Qrr sRNAs show some sequence divergence outside of the conserved 32 nt region, it has been proposed that individual Qrr sRNAs may possess unique regulons not controlled by the other Qrr sRNAs. Indeed, in both *V. harveyi* and *V. cholerae*, individual Qrr sRNAs show different abilities to activate AphA translation due to sequence divergence that influences the ability of the Qrr sRNAs to bind *aphA* mRNA.^{77,78}

Finally, several regulatory feedback loops involving the *Vibrio* Qrrs also appear to maintain levels of the QS sRNAs in the cell. First, in *V. cholerae*, AphA represses transcription of both the *qrr* sRNAs and *hapR*, creating two feedback loops that refine the control of the quorum sensing system. In *V. harveyi*, AphA binds to the promoter of *qrr4* in a region that potentially occludes binding of the activator protein LuxO.⁷⁷ AphA appears to control several hundred genes at LCD in *V. harveyi*, however, it is unclear whether AphA controls those same genes in *V. cholerae* as the experiments to determine the AphA regulon were performed at HCD. Second, Qrr/*luxO* base-pairing establishes an additional feedback loop that is sufficient to downregulate *luxO* transcription by occluding the RBS of *luxOU* mRNA (Fig. 3).⁵⁶ Predicted Qrr/*luxO* pairing only represses *luxO* transcription several fold, compared with the more extensive Qrr/*hapR* pairing that halts HapR production (Fig. 3); presumably because full LuxO repression would in turn prevent Qrr transcription. Third, HapR itself activates Qrr transcription by an indirect mechanism that requires LuxO-P but does not involve direct interaction of HapR protein with the Qrr promoters.⁶¹ Presumably this feedback loop is important in a transition from HCD to LCD, since dense HCD cultures (devoid of Qrr sRNAs) rapidly accumulate the QS sRNA when transitioned to LCD conditions, but isogenic HapR⁻ strains incubated under similar conditions do not rapidly accumulate the sRNAs.⁶¹ Thus the levels of three proteins serve as inputs to control the levels of Qrr sRNAs in the *V. cholerae* cell: LuxO-P, HapR and AphA. Interestingly, each of these regulators participate in auto-inhibitory feedback loops, repressing their own transcription.^{56,61,77}

CsrBCD sRNAs and the VarA/S Signaling Pathway

The *V. cholerae* QS system described above controls multiple Qrr sRNAs in response to secreted AI signals. Remarkably, an additional second set of sRNAs also contributes to the *V. cholerae* QS system by altering the activity of the QS LuxO response regulator. A genetic screen to identify an additional regulator(s) of *V. cholerae* QS revealed that a two component regulatory system composed of a sensor kinase, VarS, and a response regulator, VarA, contributes to the regulation of *hapR* (Fig. 1).⁷⁹ A strain lacking VarA shows decreased expression of *hapR*, as measured by a luciferase-based reporter gene fusion that is activated by HapR. VarA had previously been identified and implicated in virulence; as a strain lacking *varA* show reduced production of CT and TcpA, the main structural pilus component of TCP, with a corresponding defect in colonization of infant mouse intestines.⁸⁰ Oddly, these experiments were performed in a HapR⁻ *V. cholerae* strain, suggesting that VarA has both QS-dependent and QS-independent roles in virulence regulation.

The *V. cholerae* VarS/VarA system is homologous to the GacS/GacA two-component system from *Pseudomonas* species that controls secondary metabolite production,^{81,82} and to the BarA/UvrA system in *E. coli*.^{18,83} The sensory information these signaling systems respond to is poorly understood. In *E. coli*, UvrA, activates transcription of two sRNAs (CsrB and CsrC) that control the activity of CsrA, an RNA binding protein, which

regulates a variety of processes in *E. coli* by destabilizing RNA.⁸⁴ The sRNAs control CsrA by directly binding to and titrating out CsrA protein, preventing CsrA from destabilizing its target RNAs.^{18,83} Since, the Csr sRNAs in *V. cholerae*, *E. coli* and other bacteria do not function by base-pairing interactions with other RNAs, these Csr sRNAs act by an Hfq-independent mechanism.

In *V. cholerae*, the VarS/VarA sensory system activates the expression of three sRNAs, CsrB-D, which are approximately 350 nt in length and functionally homologous to the CsrB sRNA from *E. coli*.⁷⁹ Predicted secondary structures indicate that each of the sRNAs contains numerous hairpins with the loop regions often containing the sequences AGGA/AGGGA, which are thought to facilitate CsrA binding.^{18,83} In *V. cholerae*, CsrA is required for the effect of VarS/VarA on HapR expression, consistent with the interpretation that the CsrB-D sRNAs function similarly in *V. cholerae* and *E. coli* by titrating CsrA. *V. cholerae* strains with a deletion in the *varS* or *varA* gene, or a triple mutant lacking all three CsrBCD sRNAs display an increase in Qrr levels and lower HapR production.⁷⁹ In contrast, a *csrA* mutation in each of these genetic backgrounds results in an opposite QS phenotype. CsrA appears to have a positive effect on LuxO activity, because a strain carrying an allele of *luxO* (*luxO* D47E) that mimics the phosphorylated active form of the protein shows low HapR levels and these levels are unaltered when the strain also carries the *csrA* mutation. Transcription of *luxO* is not altered by the VarS/VarA/CsrBCD/A system, therefore it is believed an unidentified factor links CsrA to LuxO.^{79,85}

ToxT-controlled TarA and TarB sRNAs

The seven *V. cholerae* sRNAs discussed above all regulate virulence by reducing the levels of HapR, a repressor of the AphA regulon that controls production of ToxT. Recently, two additional sRNAs, TarA and TarB (for ToxT activated RNAs) have been discovered in *V. cholerae* that are regulated by ToxT and appear to have effects on virulence in animal models. Both of these sRNAs are encoded within the VPI and are activated by ToxT.^{86,87} The 99 nt TarA sRNA (Fig. 2) is encoded upstream of *tcpI* and contains two toxboxes for ToxT binding within its promoter.⁸⁶ To date, the only target gene known to be controlled by TarA is *ptsG*, which is involved in regulating the uptake of glucose. Like the other Hfq-dependent sRNAs described here, TarA is a trans-acting sRNA. Although trans-encoded, TarA shows remarkable sequence complementarity with the 5' UTR of *ptsG*, with almost two thirds of its nucleotides predicted to base-pair with *ptsG* mRNA by RNAhybrid (Fig. 3). Repression of *ptsG* translation by the TarA sRNA is based in part on the observation that deletion of the *tarA* gene leads to increased levels of *ptsG* mRNA.⁸⁶ The reciprocal is also observed; *tarA* over-expression causes growth defects in glucose minimal medium that are similar to the phenotype of a *V. cholerae* *ptsG* deletion mutant.⁸⁶ This regulation appears to be dependent on Hfq, as TarA is unstable in a *V. cholerae* *hfq* mutant. However, the role of TarA in *V. cholerae* virulence is not entirely clear as different studies have documented conflicting results. The group that initially discovered TarA reported a slight disadvantage for a *tarA*

mutant when competed against wild type in an infant mouse colonization model.⁸⁶ However, a more recent study using a different *V. cholerae* strain documented no virulence defect for a *tarA* mutant.⁸⁷ It is also unclear how or if control of glucose transport is responsible for the modest virulence defect in the original study, or whether *ctxAB* or *tcpA-F* are also under TarA (and/or *ptsG*) control. Demonstration of TarA/*ptsG* pairing by mutational analysis of the sRNA/mRNA interaction and in vitro RNA interaction, as well as an analysis of the ToxT regulon in strains altered in TarA/*ptsG* pairing will likely shed light on the role of this new sRNA in the in vivo ecology of this pathogen.

An additional ToxT-regulated sRNA was recently identified that also appears to play a role in *V. cholerae* virulence. TarB, which is also encoded within the VPI, along with *tcpA-F*, is a relatively small 60 nt sRNA (Fig. 2) that is positively regulated by ToxT.⁸⁷ Discovered using a high-throughput sequencing approach to identify sRNAs regulated by ToxT, the TarB promoter contains two toxboxes shown to be bound by ToxT protein in gel-shift experiments, consistent with a model of direct transcriptional activation of *tarB* by ToxT.⁸⁷ Interestingly, *V. cholerae* strains lacking TarB outcompete wild type strains in an infant mouse model, suggesting that TarB has a negative effect on *V. cholerae* virulence. Specifically TarB targets the 5' UTR of *tcpF* (Fig. 3), the final gene in the *tcpA-F* operon, which encodes the secreted TcpF colonization factor. Surprisingly, TarB/*tcpF* interactions do not require Hfq, as levels of TarB sRNA and *tcpF* mRNA are unaltered in an Hfq⁻ compared with an Hfq⁺ genetic background.^{87,88} The regulation of TcpF appears to be responsible for the increased ability of TarB⁻ strain to colonize the mouse small intestine, as a strain carrying a *tcpF* allele with a mutation in the 5' UTR rendering it TarB-independent displays a colonization phenotype identical to a strain lacking *tarB*.⁸⁷ It remains unclear why a sRNA that is activated by ToxT, a positive regulator of the major *V. cholerae* virulence factors (CTX and TCP), would repress TcpF expression. It is proposed that TarB may be expressed only in certain microenvironments as a mechanism for fine-tuning expression of virulence factors, but no studies have yet addressed this hypothesis.

The VrrA sRNA and Tcp

Many sRNAs in other gram-negative species have been shown to directly target mRNAs encoding outer membrane proteins (OMPs).⁸⁹ In *V. cholerae* the sRNA, MicX, which was identified by bioinformatic methods, appears to interact with the mRNA of an uncharacterized OMP, *vc0972*, although this presumptive pairing does not have an effect on virulence.¹⁶ In contrast, another *V. cholerae* sRNA, VrrA, has been experimentally shown to interact with the mRNA of an OMP and in doing so alters virulence expression.¹⁷ VrrA is an approximately 140 nt sRNA (Fig. 2) that was identified following a transposon mutagenesis screen. Like the Qrr sRNAs, transcription of *vrrA* depends on an alternative sigma factor, in this case σ^E , indicating it may be expressed in response to stress.¹⁷ In both *E. coli* and *Salmonella typhimurium*, σ^E activates the MicA and RybB sRNAs, which regulate OMPs, consistent with the σ^E function of monitoring envelope stress.⁹⁰⁻⁹²

Deletion of *vrrA* causes an increase in the levels of OmpA, an outer membrane porin. So too, loss of OmpA due to overexpression of *vrrA* increases outer membrane vesicle production, which has been hypothesized to be involved in a variety of functions in gram-negative bacteria including pathogenesis and biofilm development.⁹³ VrrA binds to the 5' UTR of *ompA* mRNA in a region overlapping the translation initiation region (Fig. 3), as determined by in vitro toeprinting analysis. However, VrrA-mediated repression of *ompA* translation does not strictly require Hfq as overexpression of VrrA represses OmpA production even in a Δ *hfq* mutant.¹⁷ Interestingly, VrrA requires Hfq to bind to and repress the mRNA encoding another outer membrane porin, OmpT (Fig. 3).⁹⁴ Like TarB, VrrA may also negatively regulate virulence in *V. cholerae*, as a *vrrA* deletion strain out-competes WT *V. cholerae* for infant mouse colonization.¹⁷ An *ompA* deficient strain also shows a 10-fold defect in infant mouse colonization, consistent with the in vivo effect of the *vrrA* deletion.

The role of the VrrA sRNA in *V. cholerae* virulence may be more complex, however, as VrrA, like the Qrr sRNAs, may regulate a large set of mRNAs. Specifically, VrrA was shown to be complementary to the 5' UTR of a third mRNA, namely, *tcpA*, and thus may also directly alter its translation by a base-pairing mechanism (Fig. 3), although this has not yet been demonstrated.¹⁷ That VrrA negatively regulates *tcpA*, was demonstrated by overexpression of VrrA, which causes a decrease in TCP levels, while deletion of the VrrA sRNA results in a modest increase in TCP pilin levels.¹⁷ It is unclear what the relative contributions are of OmpA, OmpT and TcpA regulation by the VrrA sRNA to *V. cholerae* pathogenesis, as the regulation of each target has not been genetically separated.

Additional sRNAs

While a relatively small number of sRNAs have been identified that play a role in *V. cholerae* pathogenesis, it is likely that many more regulatory RNAs remain to be discovered and characterized in this pathogen. Suggestions of more sRNAs have come from both bioinformatic and high-throughput sequencing studies.^{87,95,96} Experimental validation of these new putative regulators will likely identify a role for many of these sRNAs in both in vivo and environmental settings.

A bioinformatic study relying on genomic DNA features such as Rho-independent terminators within intergenic regions of DNA identified 32 novel putative sRNAs.⁹⁵ Validation of expression by northern blot analysis showed that 6 of 9 predicted sRNAs tested were expressed under the conditions used. Bioinformatic approaches are still limited however, by a reliance on known sequence motifs and a need for validation of the expression of the sRNA.

Deep-sequencing studies have revealed a wealth of potential sRNAs in *V. cholerae*. Previously, direct cloning and sequencing of the bacterial transcriptome to search for sRNAs had been technically difficult due to the challenges of removing the housekeeping tRNA and rRNA that are similar in size to potential sRNAs. To resolve this dilemma, the Camilli group devised a technique termed sRNA-Seq.⁹⁶ Briefly, 10–200 nt RNAs gel-extracted from

total RNAs are bound to linkers and then annealed to DNA oligonucleotides that can be used to differentiate and exclusively degrade DNA complexes with rRNA and tRNA using RNase H. The remaining linkered RNAs can be reverse transcribed and subjected to high throughput sequencing. This novel study identified all of the sRNAs experimentally known at that time in *V. cholerae*, as well as more than 600 potentially novel sRNAs, from both antisense strands of coding sequences and putative intergenic regions.⁹⁶ A second sRNA-Seq study sought to specifically identify sRNAs involved in pathogenesis using a *V. cholerae* strain that overexpressed ToxT to activate the major *V. cholerae* virulence factors.⁸⁷ By cross-referencing with a set of sequence data of ToxT binding sequences, a putative list of sRNAs likely to be directly activated by ToxT was established. This method uncovered the previously discovered TarA and led to the identification of TarB, both of which appear to affect *V. cholerae* virulence, as described above. In addition, 16 other putative ToxT-activated sRNAs were discovered. Whether one or more of these sRNAs play a role in the virulence of *V. cholerae* remains to be determined. A third study examining the transcriptome of *V. cholerae* in animal models of infection detected the presence of several known sRNAs, including the previously mentioned Qrr sRNAs, TarA and CsrC, as well as over 70 potentially novel sRNAs.⁹⁷ Thus it is increasingly apparent that sRNAs are important, newly recognized regulatory, not only in *V. cholerae* virulence, but in the virulence of many other pathogens as well.

Concluding Remarks

The sRNAs discovered in the bioinformatic and deep sequencing studies described above could be involved in many aspects of bacterial physiology important for *V. cholerae* during infection or in the environment. Several *V. cholerae* sRNAs have been discovered, such as RyhB, which is potentially involved in iron acquisition, though is not required for mouse colonization.⁹⁸

MicX, like VrrA, regulates an OMP,¹⁶ but does not appear to play a role in virulence, as described above. The MtlX sRNA regulates carbon metabolism through post-transcriptional regulation of the mRNA of a mannitol transporter, MtlA.^{96,99} Finally, the TfoR sRNA activates translation of *tfoX* mRNA, which encodes the TfoX regulator required for natural competence in response to chitin.¹⁰⁰ Thus, regulatory networks are being uncovered that link multiple sensory inputs, such as chitin and QS AIs, via small RNAs, to the control of genes for behaviors important in the environment and in the human host.^{32,63,100,101}

It is increasingly appreciated that non-coding sRNAs are critical regulatory factors that play fundamental roles in bacteria. While the full extent of sRNA-based regulation in *V. cholerae* is not yet known, several sRNAs have already been identified that alter the production of the two main virulence factors of *V. cholerae* critical for causing the cholera disease CTX and TCP. Some of the sRNAs, such as the Qrr sRNAs, control both in vivo and environmental behaviors, while other sRNAs appear to have a role in fundamental bacterial physiology. The Qrr sRNAs and VrrA, also appear to directly interact with many mRNA target genes that participate in virulence gene expression, and it remains possible that multiple target genes will be found for each of the other sRNAs described here. Determining the specific signaling inputs that activate the transcription of these sRNAs will also aid in defining the molecular mechanisms by which these regulatory pathways are controlled. Ultimately, interdisciplinary methods that incorporate genetic, bioinformatic, genomic and biochemical approaches will continue to be essential for a comprehensive understanding of the contribution of sRNAs to virulence in *V. cholerae*.

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