Non-coding small RNAs in the *Vibrio cholerae* quorum sensing system control the expression of important genes for associations in the environment and in the human host

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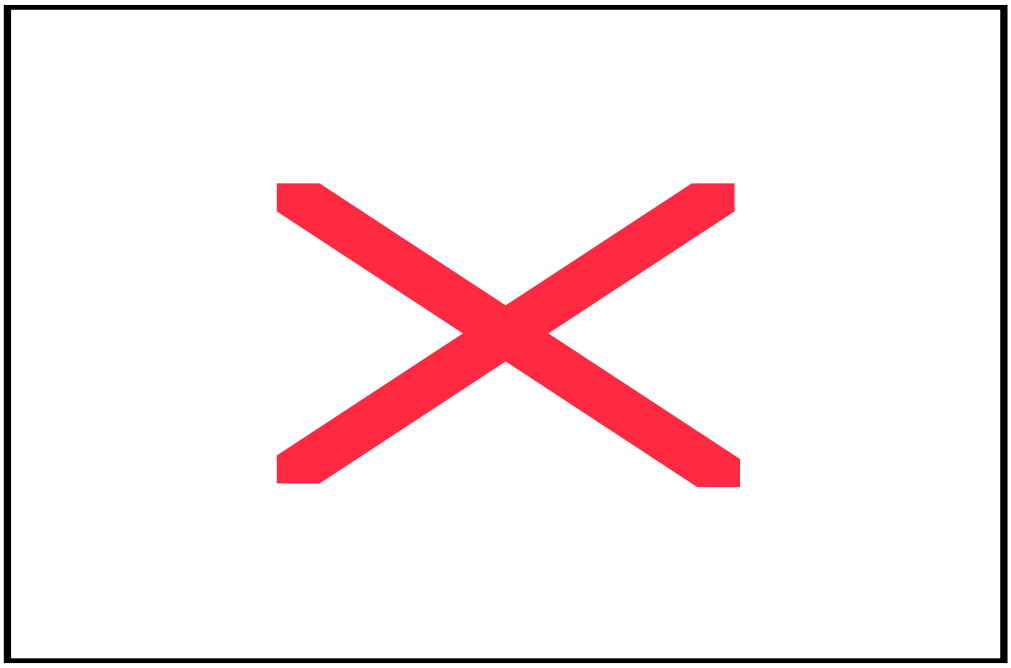
Introduction

Bacteria produce and then respond to signal molecules termed autoinducers (AI) in a process of cell-cell communication called quorum sensing (QS). Extracellular AI levels increase with increasing cell density, so AI detection allows bacteria to monitor their population numbers. When AIs reach a critical concentration, the bacteria in the group synchronize their gene expression to orchestrate behaviors, such as virulence, biofilm formation, and DNA uptake, which are more productive for bacterial groups than individuals (Ng and Bassler 2009).



Vibrios are ubiquitous marine bacteria that secrete polysaccharides (VPS) to form biofilms on chitinous surfaces of plankton and other marine animals (Yildiz and Visick 2009), where *Vibrio cholerae* can acquire extracellular DNA to facilitate horizontal gene transfer (Meibom, Blokesch et al. 2005). *V. cholerae* is also a human pathogen that can colonizes the intestine using the TCP pilus and then secretes CTX toxin to cause the disease cholera (Ng and Bassler 2009).

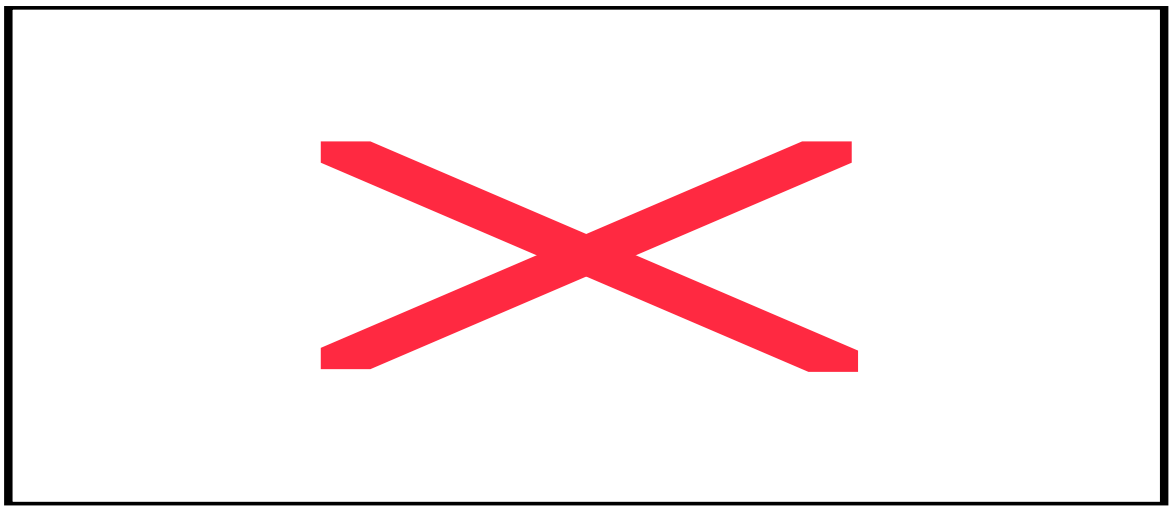
V. cholerae and other *Vibrios* communicate using a QS system with two AIs: CAI-I and AI-2 (Fig. 1). At low cell density, when sensors CqsS and LuxP/Q are not bound by AIs, a signaling cascade transfers phosphate to response regulator LuxO, via LuxU. LuxO~P activates transcription of four ~100 bp non-coding small RNAs (sRNAs) that are thought to base-pair with the leader regions of several mRNAs altering translation with the aid of RNA-binding protein, Hfq. Specifically, binding of the Quorum regulatory RNAs (Qrr1-4) is predicted to activate a biofilm promoting gene (*vca0939*) (Hammer and Bassler 2007), and to repress the genes for the Qrr activator (*luxO*) (Svenningsen, Waters et al. 2008) and a transcription factor (*hapR)* (Lenz,

Mok et al. 2004), permitting expression of virulence and biofilm factors. At high cell density, AI binding reverses the signaling cascade, inactivating LuxO and no Qrrs are made. Thus, HapR is produced, repressing virulence and biofilm genes and permitting expression of natural competence genes (*comEA*) (Zhu *et al*., 2002; Hammer & Bassler, 2003; Meibom *et al.,* 2005). Genetic evidence supports the model that sRNA/*mRNA* interactions play a critical role in coordinating the QS response in both pathogenic and environmental settings; however, Qrr/mRNA binding has not been demonstrated experimentally. We are using a combination of *in silico*, *in vivo*, and *in vitro* approaches to determine the molecular mechanism of action for these QS non-coding sRNA regulators.

Results

In silico *predictions*

Based on previous genetic studies, each sRNA target gene was tested for Qrr base-pairing potential using the TargetRNA algorithm (Tjaden 2008). For each Qrr, the identical 21 bp region, which is absolutely conserved among the Qrrs of all sequenced clinical and environmental *Vibrios*, is predicted to form a duplex with the leader region of each mRNA at a position indicated in Fig. 2A relative to the start codon. We hypothesize that the presumptive Qrr/*hapR* and Qrr/*luxO* interactions repress translation by overlapping the ribosome binding sites (Fig. 2A, circles), while the Qrr/*vca0939* interaction activates translation by binding to an inhibitory structure in the mRNA (Hammer and Bassler 2007). Each Qrr/*mRNA* interaction contains multiple mismatches, so we are developing *in vivo* and *in vitro* methods and tools to quantify the contribution of individual bps to each pairing.

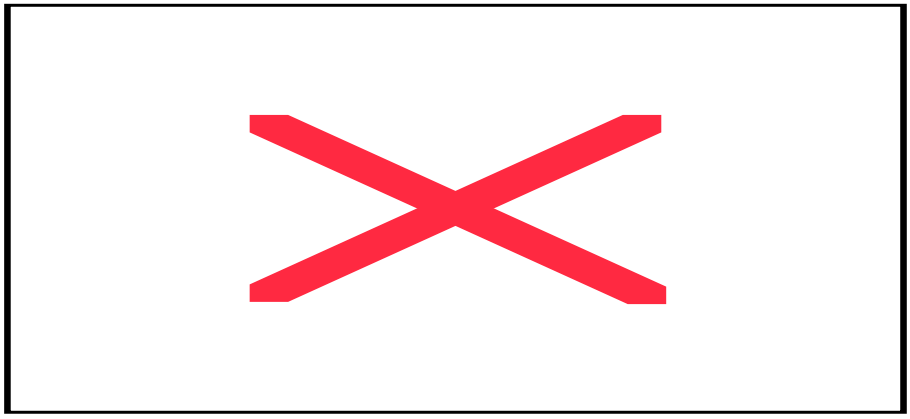


In vivo *analysis*

Any one Qrr is sufficient to produce the QS response (Lenz, Mok et al. 2004), thus a sRNA expression vector (pQrr) was engineered to express a Qrr under control of a heterologous promoter. Reporter plasmids were also made with the promoter and leader sequence of mRNA targets fused to the gene for green fluorescent protein (*gfp*) or operon for bioluminescence (*lux*). In *V. cholerae* carrying both plasmids, the Qrr sRNA represses *hapR-gfp* and activates *vca0939-lux* as measured by GFP or Lux levels per cell (per OD_{600}). A plasmid ($pQrr^{\Delta 6}$) expressing a Qrr with a 6 bp deletion in a critical region (Fig. 2A, BOLD) functioned as poorly as the vector control (Figs. 2B and 2C). A *luxO-gfp* fusion has been made, and single bp substitutions in pQrr and the reporter plasmids are being tested to measure the base-pairing requirements.

In vitro *quantification of sRNA/mRNA binding*

The Qrr sRNAs and Hfq are required for proper QS regulation of *hapR*, *luxO* and *vca0939* (Fig. 2 and (Lenz, Mok et al. 2004) (Hammer and Bassler 2007); however, it has not been determined whether the Qrr RNA form an RNA duplex with target mRNAs; and whether Hfq aids in Qrr/mRNA binding. We *in vitro* synthesized each Qrr and *hapR* RNA, and also purified *V. cholerae* Hfq protein. By quantifying on a native PAGE gel, the mobility shift of a radiolabeled Qrr incubated with increasing *hapR* levels, we show that each Qrrs form a complex with *hapR* (Fig. 3A), and that Hfq enhances duplex formation (Fig 3B) by ~15-fold (compare dissociation constants, or K_ds -/+ Hfq). We are completing the analyses with Qrr3, and with *luxO* and *vca0939* RNA, as well as *in vitro* synthesizing mutated RNAs to complement the *in vivo* studies.



Discussion

The marine pathogen *Vibrio cholerae* uses multiple non-coding sRNAs to coordinate the expression of virulence, biofilm, and natural competence genes in response to extracellular QS signals. The Qrr sRNAs are absolutely conserved among *Vibrios* at base pairs that we demonstrate here are important for *in vivo* regulation of three mRNA targets: *hapR*, *vca0939*, and *luxO*. *In vitro* binding studies bolster the *in vivo* results and show that indeed each of the four Qrrs can form a duplex with *hapR* mRNA and that the Hfq protein aids in this pairing, consistent with its designation as an RNA chaperone in *E. coli*.

We anticipate that our current systematic mutational analyses of both the Qrrs and each target mRNA will define the base pairs critical for sRNA function. Because strains lacking the Hfq protein, the Qrr sRNAs, or the sRNA activator protein (LuxO) are avirulent, biofilm deficient, and altered in DNA uptake, understanding the molecular mechanism of Qrr control is essential for defining the role of *V. cholerae* QS plays in marine and clinical settings.

References

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