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The Small RNA Chaperone Hfq and Multiple Small RNAs Control Quorum Sensing in *Vibrio harveyi* and *Vibrio cholerae*

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Summary

Quorum-sensing bacteria communicate with extracellular signal molecules called autoinducers. This process allows community-wide synchronization of gene expression. A screen for additional components of the *Vibrio harveyi* and *Vibrio cholerae* quorum-sensing circuits revealed the protein Hfq. Hfq mediates interactions between small, regulatory RNAs (sRNAs) and specific messenger RNA (mRNA) targets. These interactions typically alter the stability of the target transcripts. We show that Hfq mediates the destabilization of the mRNA encoding the quorum-sensing master regulators LuxR (*V. harveyi*) and HapR (*V. cholerae*), implicating an sRNA in the circuit. Using a bioinformatics approach to identify putative sRNAs, we identified four candidate sRNAs in *V. cholerae*. The simultaneous deletion of all four sRNAs is required to stabilize *hapR* mRNA. We propose that Hfq, together with these sRNAs, creates an ultrasensitive regulatory switch that controls the critical transition into the high cell density, quorum-sensing mode.

Introduction

Quorum sensing is a process of cell-to-cell communication that bacteria use to assess their population density in order to coordinate the gene expression of the community (Miller and Bassler, 2001). Quorum sensing requires the production, secretion, and detection of extracellular signal molecules termed autoinducers. Diverse behaviors are controlled by quorum sensing, but, typically, these behaviors are ones that would be ineffective if only a small group of cells carried them out. Often, bacteria produce and detect multiple autoinducers, some of which are used for intraspecies communication, while others promote interspecies communication (Federle and Bassler, 2003; Fuqua et al., 2001; Xavier and Bassler, 2003).

The marine bacterium *Vibrio harveyi* produces and detects two autoinducers, AI-1 and AI-2, and these signals control the expression of multiple genes, including those for bioluminescence (luciferase) (Bassler et al., 1993, 1994a; Cao and Meighen, 1989; Chen et al., 2002b), siderophore production (Lilley and Bassler, 2000), colony morphology, metalloprotease production (Mok et al., 2003), and type III secretion (Henke and Bassler, 2004).

In *V. harveyi*, AI-1 and AI-2 are produced by the synthases LuxM and LuxS, respectively (Bassler et al., 1993; Surette et al., 1999). LuxN detects AI-1, and LuxPQ detects AI-2 (Figure 1A) (Bassler et al., 1993, 1994a; Chen et al., 2002b; Freeman et al., 2000). LuxN and LuxQ are membrane bound two-component hybrid sensor-kinase proteins. LuxP, which binds AI-2 in the periplasm, is required with LuxQ for the response to AI-2 (Bassler et al., 1994a; Chen et al., 2002b). Sensory information from both systems converges at the phosphorelay protein LuxU, and LuxU transmits the signal to the response regulator LuxO (Bassler et al., 1994b; Freeman and Bassler, 1999, 2000). A transcriptional activator called LuxR is also required for expression of *lux* and other quorum sensing-controlled genes (Henke and Bassler, 2004; Martin et al., 1989; Miyamoto et al., 1994; Showalter et al., 1990).

The human pathogen *Vibrio cholerae* possesses quorum-sensing systems analogous to the two described above for *V. harveyi* (Miller et al., 2002). The *V. cholerae* autoinducers CAI-1 and AI-2 are synthesized by CqsA and LuxS and detected by CqsS and LuxPQ, respectively (Figure 1B). *V. cholerae* has an additional system (System 3) that remains to be identified (Miller et al., 2002). Sensory information from all three systems converges at LuxO. The *V. cholerae* LuxR homolog is called HapR (Jobling and Holmes, 1997). Quorum sensing controls virulence and biofilm formation in *V. cholerae* (Hammer and Bassler, 2003; Kovacikova and Skorupski, 2002; Miller et al., 2002; Vance et al., 2003; Zhu and Mekalanos, 2003; Zhu et al., 2002).

The *V. harveyi* and *V. cholerae* quorum-sensing circuits operate similarly (Miller et al., 2002). At low cell density, i.e., in the absence of autoinducers, the sensors act as kinases and transfer phosphate via LuxU to LuxO. LuxO-phosphate (LuxO-P) is active and negatively regulates *lux*. At high cell density, i.e., when the autoinducers are present, the sensors act as phosphatases. Phosphate flow through the circuit is reversed, resulting in dephosphorylation and inactivation of LuxO (Freeman and Bassler, 1999, 2000; Freeman et al., 2000). Under this condition, the transcriptional regulators LuxR in *V. harveyi* and HapR in *V. cholerae* bind the *lux* promoter and activate transcription (Figure 1).

LuxO-P-mediated repression of *lux* is indirect (Lilley and Bassler, 2000). LuxO is homologous to members of the NtrC family of response regulators, which can act either as transcriptional activators or repressors. Those that are activators require the alternative sigma factor σ^{54} for function, while those that are repressors do not (Benson et al., 1994; North et al., 1996; Reitzer and Magasanik, 1985; Wingrove and Gober, 1994; Wu and Newton, 1997). LuxO is a member of the activator class of NtrC homologs. We have suggested that, at low cell

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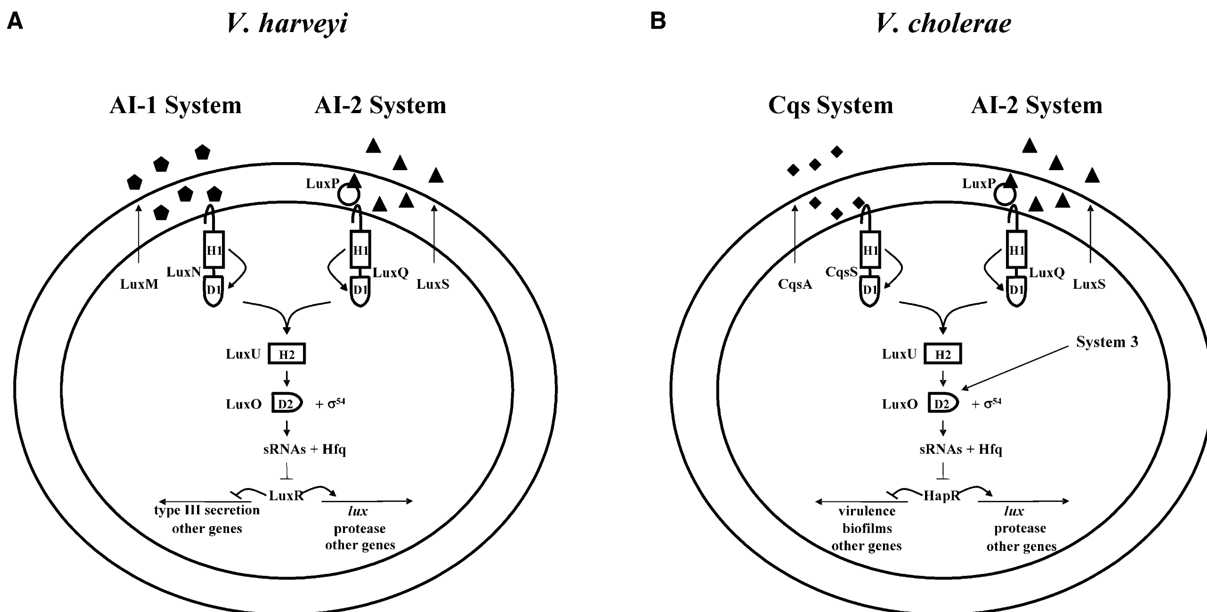

Figure 1. Models of the *V. harveyi* and *V. cholerae* Quorum-Sensing Circuits

(A) Two quorum-sensing systems function in parallel to regulate gene expression in *V. harveyi*. Pentagons and triangles represent AI-1 and AI-2, respectively.

(B) Three quorum-sensing systems function in parallel to regulate gene expression in *V. cholerae*. The functions making up the third circuit (denoted System 3) remain to be identified. Diamonds and triangles represent CAI-1 and AI-2, respectively. In both circuits, phosphate flows in the direction indicated by the arrows at low cell density and in the opposite direction at high cell density.

density, LuxO-P activates the expression of a repressor that controls the downstream target genes. We show that multiple, redundant small regulatory RNAs (sRNAs), together with the sRNA binding protein Hfq, fulfill this repressor role. Specifically, at low cell density, the Hfq-sRNA repressor complexes destabilize the *V. harveyi luxR* and *V. cholerae hapR* mRNAs (Figure 1).

Results

A Genetic Screen for the *V. harveyi* Quorum-Sensing Repressor Reveals Hfq

To identify the putative repressor that acts downstream of LuxO, we used a previously characterized LuxO allele, *luxO* D47E (Freeman and Bassler, 1999). The D47E mutation alters the site of phosphorylation, and "locks" the LuxO D47E protein into a state mimicking LuxO-P. LuxO-P activates transcription of the putative repressor of *lux* at low cell density. Consistent with the model, *V. harveyi luxO* D47E strains are dark, presumably due to constitutive expression of the *lux* repressor. We mutagenized a *V. harveyi luxO* D47E strain with the transposon Mini-Mu*lacZ* and screened for colonies that had acquired a bright phenotype, indicating that they had obtained a mutation bypassing the dark LuxO D47E phenotype.

Of the 40,000 transposon insertion mutants generated, 85 were bright. The majority of these (82) contained transposon insertions in either *luxO* or *rpoN*, the gene encoding σ^{54}. Three bright mutants did not harbor mutations in either of these genes. A *V. harveyi* genomic cosmid library was introduced into one of these mutants (BNL211) and screened for restoration of the dark phenotype. All cosmids conferring a dark phenotype contained overlapping regions of DNA, suggesting that a single locus was responsible. One cosmid, pBNL2014, was mutated with Tn*5lacZ* to pinpoint the region responsible for *lux* repression. The region identified was cloned and sequenced and found to contain the gene *hfq* (Figure 2A). The *V. harveyi hfq* gene displays high homology to *hfq* genes from other vibrio species, including *Vibrio parahaemolyticus*, *V. cholerae*, and *Vibrio vulnificus*, with 100%, 95%, and 94% identity, respectively (NCBI Blast: http://www.ncbi.nlm.nih.gov/BLAST/).

Hfq Is Required for Quorum-Sensing Repression

To verify that Hfq has a role in quorum-sensing regulation in *V. harveyi*, *hfq* null mutations were introduced onto the chromosomes of the *V. harveyi* wild-type and *luxO* D47E strains. The Lux phenotypes of the single *hfq* and double *luxO* D47E, *hfq* mutants were examined and compared to those of the wild-type, *luxO*, and *luxO* D47E *V. harveyi* strains (Figure 2B). Wild-type *V. harveyi* displays typical quorum-sensing behavior (squares): it is very bright immediately following dilution into fresh medium, but, early in the assay, luminescence decreases precipitously (~1000-fold) due to dilution of the autoinducers to a level below that required for activation of *lux*. However, as the cells grow, endogenously produced autoinducers accumulate to the level required for detection. Light production commences and increases 1000-fold, ultimately reaching the predilution level. The *luxO* null strain (diamonds) is constitutively bright because, in the absence of LuxO, no *lux* repressor is produced. Conversely, the *luxO* D47E strain (open triangles) is dark. The *hfq* mutant (circles) has a phenotype indistinguishable from the *luxO* mutant, demonstrating that Hfq is required for repression of *lux* expression at low cell

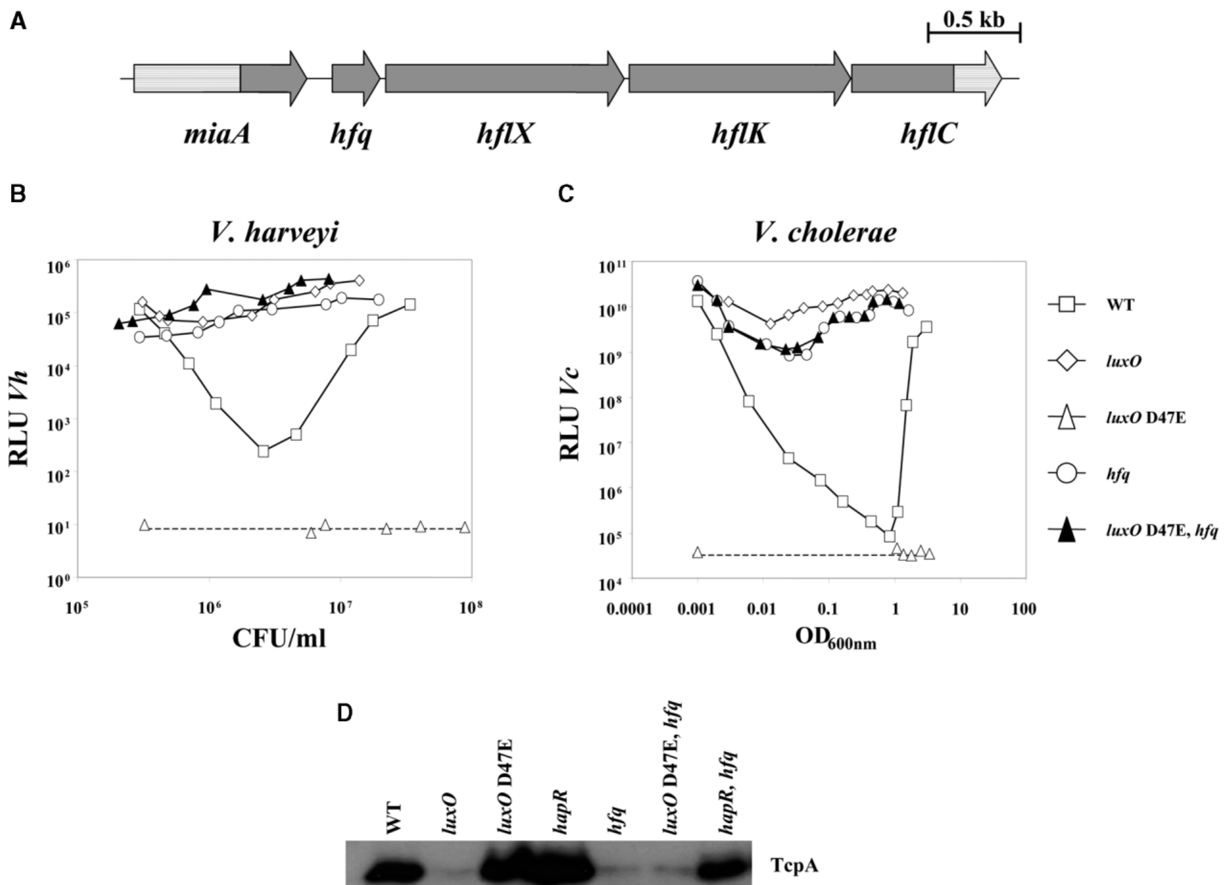

Figure 2. Hfq Is Required for Quorum-Sensing Repression in *V. harveyi* and *V. cholerae*

(A) The *hfq* locus in *V. harveyi*. *miaA* and *hflC* were not fully sequenced (unsequenced regions are denoted by light-colored shading). (B) Bioluminescence assays for *V. harveyi* strains are: BB120 (WT, squares), JAF78 (Δ*luxO*::cm^r, diamonds), JAF548 (*luxO* D47E, open triangles), BNL258 (*hfq*::Tn*5lacZ*, circles), and BNL211 (*luxO* D47E, *hfq*::Mini-Mu*lacZ*, closed triangles). Relative light units for *V. harveyi* are defined as counts min^{-1} $ml^{-1} \times 10^3$/cfu ml^{-1}. (C) Bioluminescence assays for *V. cholerae* strains are: MM227 (WT, squares), MM349 (Δ*luxO*, diamonds), BH48 (*luxO* D47E, open triangles), DL2078 (Δ*hfq*, circles), and DL2378 (*luxO* D47E, Δ*hfq*, closed triangles). Relative light units for *V. cholerae* are defined as counts min^{-1} ml^{-1}/OD_{600nm}. In (B) and (C), the dotted lines represent the limit of detection for light. (D) *V. cholerae* strains analyzed for TcpA production by Western blot are: C6706str2 (WT), MM307 (Δ*luxO*), BH38 (*luxO* D47E), MM194 (Δ*hapR*), DL2066 (Δ*hfq*), DL2146 (*luxO* D47E, Δ*hfq*), and DL2607 (Δ*hapR*, Δ*hfq*).

densities. The *luxO* D47E, *hfq* double mutant (closed triangles) is also constitutively bright, showing that repression of *lux* by Hfq occurs downstream of LuxO.

We confirmed that Hfq has the identical role in *V. cholerae* that it has in *V. harveyi* quorum sensing by measuring density-dependent light production from *V. cholerae* strains carrying *V. harveyi lux*. The Lux phenotypes of the wild-type (squares), *luxO* (diamonds), *luxO* D47E (open triangles), *hfq* (circles), and *luxO* D47E, *hfq* (closed triangles) single and double *V. cholerae* mutants mimic the corresponding *V. harveyi* mutant phenotypes (Figure 2C).

Hfq Is Required for Regulation of Virulence Gene Expression in *V. cholerae*

In *V. cholerae*, quorum sensing promotes virulence gene expression at low cell density (Figure 1B). To show that the quorum-sensing activity of Hfq is not restricted to the nonnative *lux* target in *V. cholerae*, we measured TcpA (the major subunit of the toxin-coregulated pilus) production using Western blots (Figure 2D) (Taylor, 1991). TcpA is present in the wild-type strain because, at low cell density, quorum sensing initiates the cascade leading to TcpA production, which enables its detection at high cell density (Zhu et al., 2002). No TcpA is observed in the *luxO* strain, because LuxO-P is required at low cell density to initiate the production of the TcpA observed in the wild-type. In contrast, high levels of TcpA are observed in the *luxO* D47E strain. Similarly, the *hapR* mutant that is also locked in low cell density mode produces high levels of TcpA. Importantly, low TcpA is detected in the *hfq* mutant, demonstrating that Hfq is indeed required for virulence-factor expression. TcpA production in the double *luxO* D47E, *hfq* and *hapR*, *hfq* mutants demonstrates that Hfq acts downstream of LuxO and upstream of HapR in the quorum-sensing regulatory cascade.

Predictions for Hfq Involvement in Quorum Sensing

In *Escherichia coli* and other bacteria, Hfq binds a variety of small regulatory RNAs (sRNAs) and promotes interac-

tion between the sRNAs and their target mRNAs (Masse et al., 2003b; Valentin-Hansen et al., 2004). These Hfq-sRNA complexes alter the stability/translation of the target mRNAs. Our finding that Hfq is required for quorum-sensing repression in *V. harveyi* and *V. cholerae* led us to two predictions: first, quorum-sensing repression occurs posttranscriptionally, and, second, there must be one or more sRNAs involved. The finding that LuxO-P and σ^{54} do not control transcription of *hfq* (data not shown) led us to predict that, at low cell density, the LuxO-P-σ^{54} complex activates the transcription of the gene(s) encoding the sRNA(s). The remainder of the experiments presented here test these predictions.

Hfq Affects the Stability of *luxR/hapR* mRNA

LuxR and HapR appear to be the master regulators of their respective quorum-sensing regulons (Henke and Bassler, 2004; Zhu et al., 2002). Knowing this suggested two possible methods by which Hfq could repress quorum sensing-controlled gene expression. First, Hfq could directly act on the mRNA encoding each of the known target genes of the quorum-sensing regulons. Second, Hfq could act on the mRNAs encoding LuxR in *V. harveyi* and HapR in *V. cholerae*. We reasoned this latter possibility was more likely, because, in this scenario, Hfq need only act on a single mRNA in each species.

Northern blots were used to determine the effect of *hfq* mutations on *luxR* and *hapR* mRNA stability in *V. harveyi* and *V. cholerae*. Rifampicin was added to cultures to terminate transcription, after which the *luxR* and *hapR* transcripts were monitored over time. The analysis was performed in *luxO* D47E strains to assess the fate of the *luxR* and *hapR* transcripts at low cell density. Figure 3A shows that, under these conditions, both the *luxR* and *hapR* transcripts disappear immediately following termination of transcription (panels labeled *luxO* D47E). However, in the *luxO* D47E, *hfq* double mutants, the transcripts show significantly increased longevity (Figure 3A, panels labeled *luxO* D47E, *hfq*). The control shows that mutation of *hfq* has no effect on the stability of *rpsL* mRNA (Figure 3A, four lower panels). Western blots show that the increased stability of the *luxR* and *hapR* mRNAs in the *hfq* mutants leads to increased levels of the LuxR and HapR proteins (Figure 3B). These results demonstrate that, at low cell density, Hfq destabilizes the *luxR* and *hapR* mRNA in *V. harveyi* and *V. cholerae*, respectively, which leads to reduced LuxR and HapR protein in the cells.

LuxO-P Regulation of *hapR* Is Posttranscriptional and Requires Hfq

Previous analyses have suggested that LuxO-P controls transcription of *hapR* in *V. cholerae* (Zhu et al., 2002). Our inability to detect *hapR* mRNA in the Northern blots at time zero does not allow us to distinguish between transcriptional and posttranscriptional regulation of *hapR*. We constructed chromosomal *hapR-lacZ* transcriptional, translational, and promoter fusions and measured their activities in the *V. cholerae* wild-type, *luxO* D47E, *hfq*, and *luxO* D47E, *hfq* strains. The transcriptional and translational fusions are repressed in the *luxO* D47E strain, and repression requires Hfq (Figures 4A and 4B, respectively). In contrast, LuxO D47E does not repress the *hapR-lacZ* promoter fusion in which *lacZ* is fused to the predicted site of transcription initiation (+1 site) (Figure 4C). These results suggest that LuxO-P regulation of *hapR* is posttranscriptional and show that the region between the predicted +1 site and the *hapR* coding region is required for Hfq control.

Identification of sRNAs Involved in Quorum-Sensing using Bioinformatics

Our findings point toward a LuxO-σ^{54}-regulated sRNA in the quorum-sensing signal-transduction circuits of *V. harveyi* and *V. cholerae*. Because these small genes are very difficult to identify by traditional genetic approaches, we developed a method to scan the *V. cholerae* genome for candidate sRNA loci. We could not perform this analysis in *V. harveyi*, because its genome has not been sequenced. We used the following parameters in our analysis: (1) expression of the sRNA is activated by the LuxO-P-σ^{54} complex, thus the upstream region of the locus must contain a σ^{54} binding site; (2) most sRNAs identified to date have Rho-independent terminators, and we assumed this to be the case for the putative sRNAs in our analysis (Argaman et al., 2001; Chen et al., 2002a; Wassarman et al., 2001); (3) most sRNAs are located in intergenic regions, so we restricted our search to regions between annotated genes (Argaman et al., 2001; Wassarman et al., 2001); and (4) the sRNA must be conserved in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The completed genome sequences of these vibrios show that they possess homologs of *luxR/hapR*, *luxO*, *luxU*, and *hfq*, suggesting that they have a conserved quorum-sensing regulatory mechanism (Chen et al., 2003; Heidelberg et al., 2000; Makino et al., 2003).

Using PATSER, we scanned the *V. cholerae* genome for potential σ^{54} binding sites with a weight matrix constructed from a compiled set of ~180 σ^{54} binding sites from multiple bacterial species (Barrios et al., 1999; Dombrecht et al., 2002; Hertz and Stormo, 1999; van Helden, 2003). We considered all hits above a cutoff score chosen to include all binding sites upstream of genes in *V. cholerae* that are known to be regulated by σ^{54}. In a parallel procedure, the upstream regions of the known *V. cholerae* σ^{54}-regulated genes were extracted, and we used the program CONSENSUS to search for a 16 bp motif in these sequences (Hertz and Stormo, 1999). The motif so obtained corresponded perfectly to the known σ^{54} binding sites in *V. cholerae*, with the consensus sequence 5′-TGGCAC-N_5-TTGCA/T-3′. The aligned set of binding sites was used to construct a weight matrix for σ^{54} sites specific to *V. cholerae*. Because the weight matrices obtained by these two procedures were quite similar, the final result did not depend on the weight matrix used to scan the genome.

Our analysis of *V. cholerae* identified several predicted σ^{54} binding sites in intergenic regions. We examined these regions for conservation across the specified vibrio genomes and for the presence of Rho-independent terminators. These constraints narrowed the search to four intergenic regions. The sequences and alignment of these four regions are shown in Figure 5A, along with the corresponding regions from *V. parahaemolyticus* and *V. vulnificus*. These four loci are highly homologous to one another. They all contain the signature σ^{54} binding

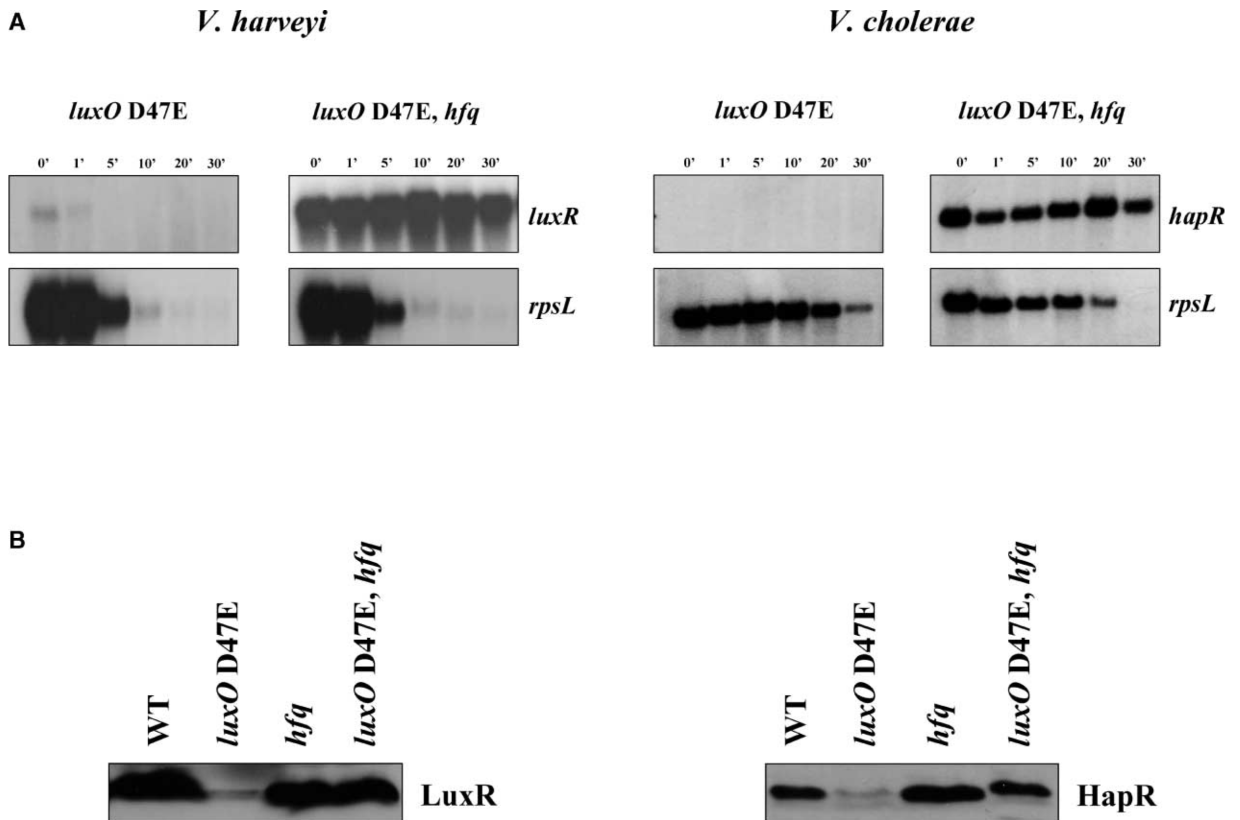

Figure 3. Hfq Regulates the Expression of *luxR* and *hapR* Posttranscriptionally

Non-steady-state Northern blots were used to analyze *luxR*/*hapR* transcript stability in the following: (A) *V. harveyi* JAF548 (*luxO* D47E) and BNL211 (*luxO* D47E, *hfq*::Mini-Mu*lacZ*); and (B) *V. cholerae* BH38 (*luxO* D47E) and DL2146 (*luxO* D47E, Δ*hfq*). (C) Western blots on lysates of *V. harveyi* BB120 (WT), JAF548 (*luxO* D47E), BNL258 (*hfq*::Tn*5lacZ*), BNL211 (*luxO* D47E, *hfq*::Mini-Mu*lacZ*), and (D) *V. cholerae* C6706str2 (WT), BH38 (*luxO* D47E), DL2066 (Δ*hfq*), DL2146 (*luxO* D47E, Δ*hfq*) measured LuxR and HapR protein, respectively.

site and a terminator, suggesting that these elements are independently transcribed loci. We name these loci *qrr*1, *qrr*2, *qrr*3, and *qrr*4 (for ***q***uorum ***r***egulatory ***r***na 1–4). Interestingly, one of the sRNA loci, *qrr*1, is located immediately upstream of *luxO*. In *V. parahaemolyticus* and *V. vulnificus*, a fifth putative sRNA locus was identified that fulfills all of our search criteria (denoted *qrr*5).

Using the RNAFOLD program, we found the predicted secondary structures of the candidate sRNAs (Figure 5B) (Hofacker, 2003). The predicted structures of Qrr2 and Qrr3 are very similar, as is the structure of Qrr4 if the three nucleotide pairs joining the two center loops are melted (and note these three nucleotide pairs are not conserved across species). Thus, only the predicted structure of Qrr1 is obviously distinct. Qrr2 and Qrr3 show a site similar to the proposed Hfq binding site, which is an 8–12 nucleotide AU-rich region adjacent to stem loops (Moll et al., 2003). The composition of the loops is variable across species, but the stems are highly conserved, supporting the folding predictions. Many small regulatory RNAs act by base pairing to complementary regions in the 5′ untranslated region of the mRNA. Using the program LALIGN, which finds the best local alignment of the input sequences, we aligned the complement of the *hapR* untranslated upstream region with all four *V. cholerae* sRNAs and the *luxR* upstream region with *V. harveyi* Qrr1 (Figures 5C and 5D, respectively). Interestingly, the region we identify as being potentially involved in the complementary base pairing is absolutely conserved among all four sRNA candidates, with a single base difference in *V. harveyi qrr*1 (Figure 5A). The highly conserved region overlaps the *hapR* and *luxR* putative ribosome binding sites (AAGGAUAU for *hapR* and AAGGAAAA for *luxR*). Finally, analysis of the upstream regions of *hapR* and *luxR* and their orthologs across all the sequenced vibrios indicates that this putative region of interaction with the sRNAs is strongly conserved.

We propose that the four putative sRNA loci in *V. cholerae*, and possibly five in *V. parahaemolyticus*, *V. vulnificus*, and *V. harveyi* (see Discussion), are regulated by LuxO-P together with σ^{54}. Analysis of the upstream regions of the candidate sRNA loci shows a highly conserved region upstream of the σ^{54} site. This site has dyad symmetry, and the consensus sequence is TTGCAW$_3$TGCAA (where W corresponds to A/T). We hypothesize that this region could be important for LuxO-P binding.

LuxO-P-σ^{54} Controls the Expression of the sRNA Loci

To ascertain whether any of the candidate sRNA loci is a target of LuxO-P-σ^{54} regulation in *V. cholerae*, Northern blot analysis was used to quantify transcript levels. The DNA encoding the putative sRNAs was amplified by PCR and used to probe identical Northern blots con-

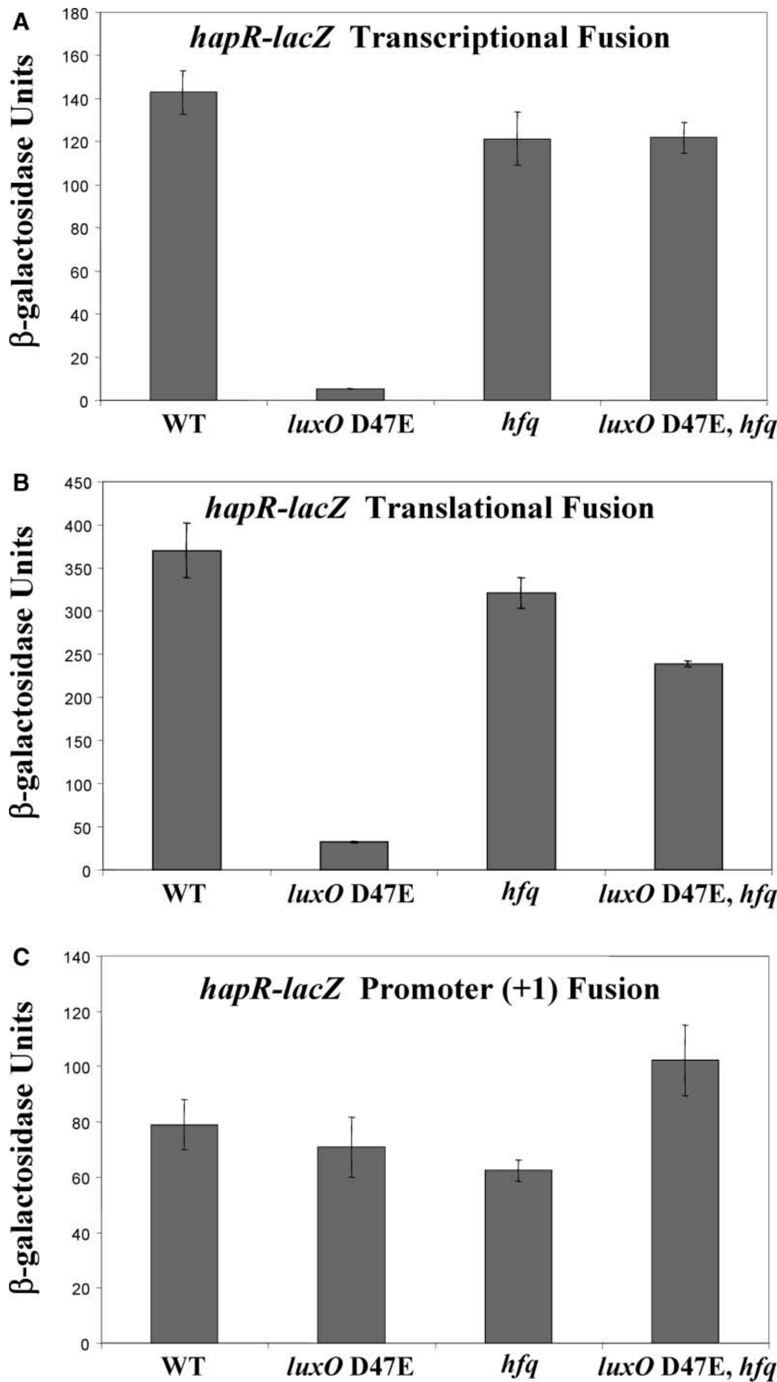

Figure 4. LuxO-P and Hfq Regulate *hapR* Posttranscriptionally

β-galactosidase activity of (A) *hapR-lacZ* transcription in *V. cholerae* DL2106 (WT, Δ*lacZ*), DL2099 (*luxO* D47E, Δ*lacZ*), DL2523 (Δ*hfq*, Δ*lacZ*), and DL2441 (*luxO* D47E, Δ*hfq*, Δ*lacZ*). (B) *hapR-lacZ* translation in *V. cholerae* DL2543 (WT, Δ*lacZ*), DL2542 (*luxO* D47E, Δ*lacZ*), DL2531 (Δ*hfq*, Δ*lacZ*), and DL2533 (*luxO* D47E, Δ*hfq*, Δ*lacZ*). (C) *hapR-lacZ* promoter activity in *V. cholerae* DL2748 (WT, Δ*lacZ*), DL2771 (*luxO* D47E, Δ*lacZ*), DL2703 (Δ*hfq*, Δ*lacZ*), and DL2698 (*luxO* D47E, Δ*hfq*, Δ*lacZ*).

taining RNA isolated from low cell density cultures of wild-type, *luxO*, *luxO* D47E, and *rpoN V. cholerae* strains. Figure 6A (*hapR*$^+$ panel) shows that, surprisingly, only Qrr4 is obviously regulated by LuxO-P-σ^{54}. A very small amount of this sRNA is detected in wild-type cells, whereas high levels are present in the *luxO* D47E strain. Importantly, Qrr4 is undetectable in both the *luxO* and *rpoN* mutants, consistent with a requirement for both LuxO-P and σ^{54} for activating the expression of the locus encoding Qrr4 at low cell density. We did not detect the other three *V. cholerae* sRNAs in this analysis.

Recently, it was shown in *E. coli* that, upon binding its mRNA target, the sRNA RyhB is degraded along with the target by RNaseE (Masse et al., 2003a). In the absence of the mRNA targets, increased stability of RyhB is observed (see Discussion). We wondered if the Qrr sRNAs were being degraded along with the *hapR* target mRNA. To test this, we deleted *hapR* in the wild-type, *luxO*, *luxO* D47E, and *rpoN V. cholerae* strains, prepared RNA, performed Northern blots, and probed them for all four sRNAs (Figure 6A, *hapR*$^-$ panel). In the absence of *hapR* mRNA, an increase in the level of Qrr4 is observed in the wild-type and *luxO* D47E strains. We also detect minor amounts of Qrr2 and Qrr3 in the *luxO*

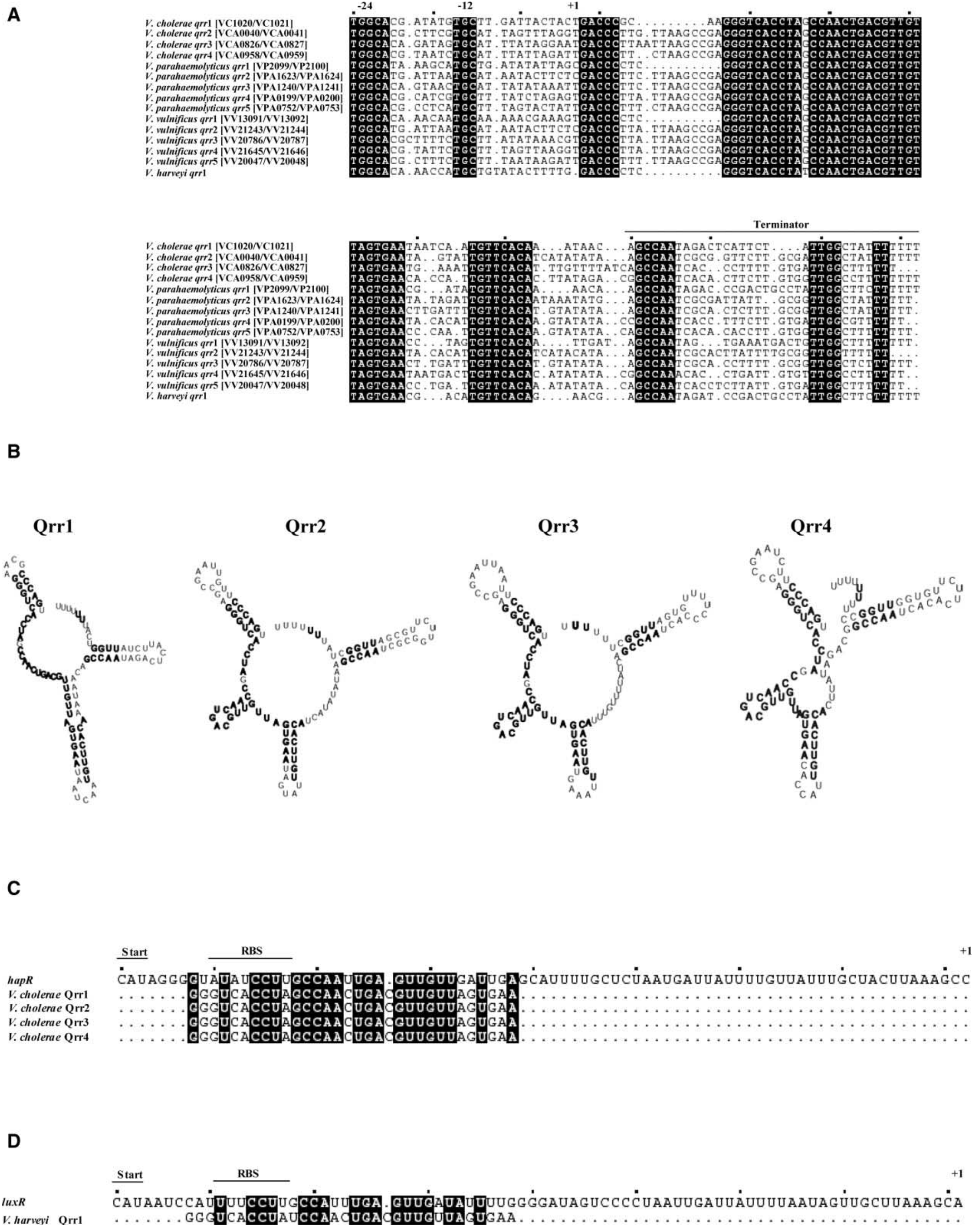

Figure 5. Bioinformatic Analysis of the Qrr sRNAs in *V. cholerae, V. parahaemolyticus, V. vulnificus*, and *V. harveyi*

(A) Multiple sequence alignment of the *qrr* genes encoding the sRNAs identified in *V. cholerae, V. parahaemolyticus, V. vulnificus*, and *V. harveyi*. Annotations for the genes flanking each sRNA are given in the brackets. Numbering of sRNAs is based on orthology of flanking genes. Nucleotides in black indicate perfect alignment. The putative σ^{54} binding site is marked as −12 and −24, the predicted start of transcription is labeled as +1, and the terminator is noted by the line over the sequence. (B) Lowest-energy secondary-structural predictions for the Qrr sRNAs identified in *V. cholerae*. Bold typeface indicates the regions conserved across all sRNAs in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. (C) Alignment of the complement of the *hapR* UTR with a portion of the Qrr sRNAs identified in *V. cholerae*. (D) Alignment of the complement of the *luxR* UTR with a portion of sRNA Qrr1 identified in *V. harveyi*. In (C) and (D), the translational start site (Start), the ribosome binding site (RBS), and the transcriptional start site (+1) are indicated. (A), (C), and (D) were produced using CLUSTALW (Thompson et al., 1994) and ESPript (Gouet et al., 1999).

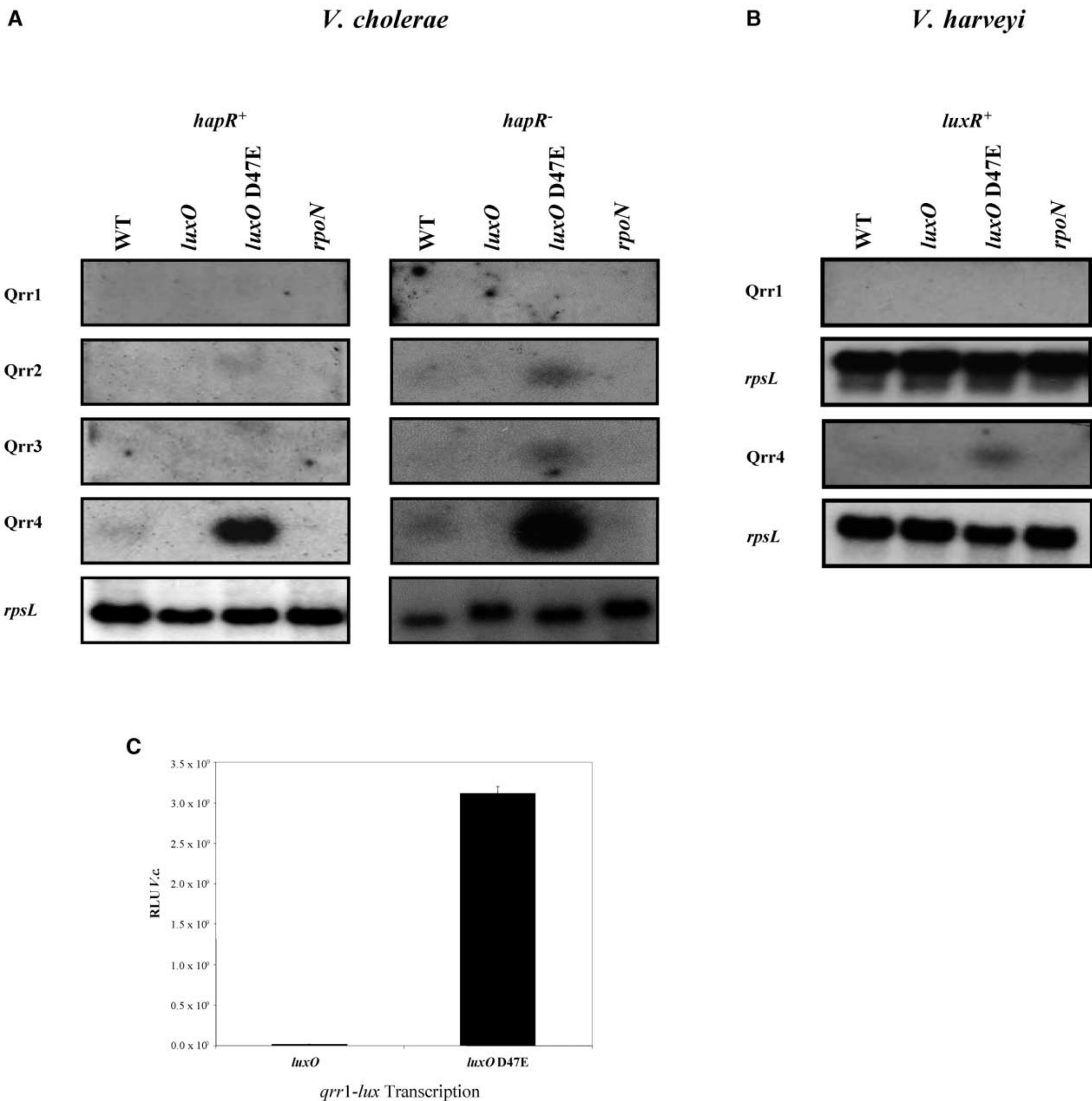

Figure 6. Regulation of Expression of the sRNAs by Quorum Sensing

RNA isolated from (A) *V. cholerae* C6706str2 (WT), MM307 (Δ*luxO*), BH38 (*luxO* D47E), BH76 (Δ*rpoN*) was probed for sRNAs Qrr1, Qrr2, Qrr3, and Qrr4, and *V. cholerae rpsL* is shown as the loading control. (B) RNA isolated from *V. harveyi* BB120 (WT), BB721 (*luxO*::Tn*5lacZ*), JAF548 (*luxO* D47E), and BNL240 (*rpoN*::cm^r) was probed for sRNA Qrr1 with a probe made against *V. harveyi qrr*1 and for sRNA Qrr4 with a probe made against *V. cholerae qrr*4. *V. harveyi rpsL* is shown as the loading control. (C) Single time point RLU for *V. cholerae* strains DL3212 (*luxO*) and DL3213 (*luxO* D47E) containing the *qrr*1-*lux* transcriptional fusion in trans.

D47E strain, showing that they are indeed regulated by LuxO-P and that their levels increase in the absence of *hapR* mRNA. However, we could not detect the sRNA Qrr1.

We know from our sequencing that *qrr*1 resides upstream of *luxO* in *V. harveyi*; however, we could not detect this sRNA by Northern blot (Figure 6B). Because the genome of *V. harveyi* has not been sequenced, we do not know whether genes corresponding to *qrr*2, *qrr*3, *qrr*4, and/or *qrr*5 of *V. parahaemolyticus* and *V. vulnificus* are present. We successfully detected Qrr4 from *V. harveyi* by probing total RNA with the DNA probe made from the *V. cholerae qrr*4 PCR product, showing that such an sRNA exists in *V. harveyi*, and its expression is induced by LuxO D47E (Figure 6B).

The inability to detect *V. cholerae* and *V. harveyi* Qrr1 could be a consequence of extremely low expression of *qrr*1 or instability of the *qrr*1 transcript, coupled with the insensitivity of the Northern blot procedure. Alternatively, expression of *qrr*1 might not be controlled by LuxO-P; however, this seemed unlikely, based on the bioinformatics analysis. To eliminate this latter possibil-

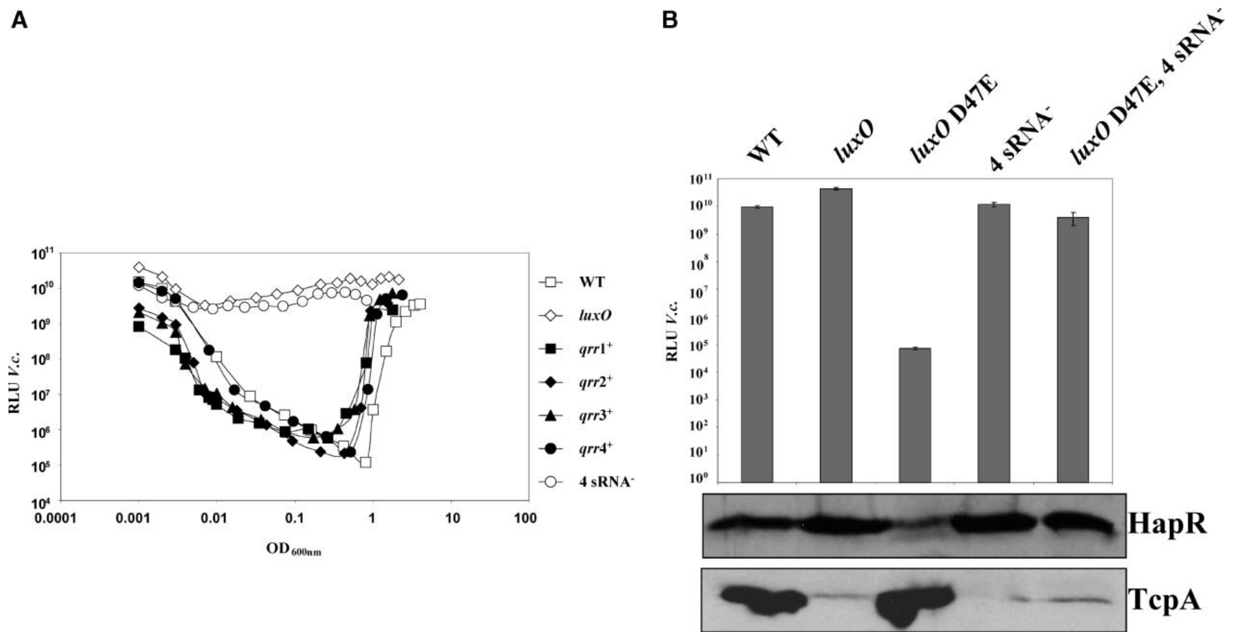

Figure 7. Simultaneous Deletion of the Four sRNAs Is Required to Affect Quorum-Sensing in *V. cholerae*

(A) Bioluminescence assays were performed on *V. cholerae*: MM227 (WT, open squares), MM349 (Δ*luxO*, open diamonds), DL2998 (Δ*qrr*2, Δ*qrr*3, Δ*qrr*4, closed squares), DL2996 (Δ*qrr*1, Δ*qrr*3, Δ*qrr*4, closed diamonds), DL2955 (Δ*qrr*1, Δ*qrr*2, Δ*qrr*4, closed triangles), DL2997 (Δ*qrr*1, Δ*qrr*2, Δ*qrr*3, closed circles), DL2956 (Δ*qrr*1, Δ*qrr*2, Δ*qrr*3, Δ*qrr*4, open circles).

(B) Single time point RLU for *V. cholerae* strains MM227 (WT), MM349 (Δ*luxO*), BH48 (*luxO* D47E), DL2956 (Δ*qrr*1, Δ*qrr*2, Δ*qrr*3, Δ*qrr*4), and DL3024 (*luxO* D47E, Δ*qrr*1, Δ*qrr*2, Δ*qrr*3, Δ*qrr*4). Western blots probed for HapR and TcpA from *V. cholerae* strains C6706str2 (WT), MM307 (Δ*luxO*), BH38 (*luxO* D47E), DL2953 (Δ*qrr*1, Δ*qrr*2, Δ*qrr*3, Δ*qrr*4), and DL3020 (*luxO* D47E, Δ*qrr*1, Δ*qrr*2, Δ*qrr*3, Δ*qrr*4).

ity, we constructed a *qrr*1 transcriptional reporter by fusing the upstream region of *V. cholerae qrr*1 to the *luxCDABE* (luciferase) operon and tested whether this construct was regulated by LuxO-P. We measured the expression of the *qrr*1-*lux* fusion in the *luxO* null and *luxO* D47E *V. cholerae* strains. The presence of LuxO D47E causes a 220-fold increase in expression from the *qrr*1 promoter, verifying that, indeed, *qrr*1 is regulated by LuxO-P (Figure 6C). We conclude that transcription of all four *qrr* genes is regulated by LuxO-P.

Four sRNAs Are Involved in Quorum-Sensing Repression in *V. cholerae*

To gain insight into the individual roles of the four sRNAs in quorum sensing, single, double, triple, and the quadruple *qrr* sRNA deletions were constructed in *V. cholerae*. Subsequently, we measured density-dependent light production and found that only the simultaneous deletion of all four sRNAs affected bioluminescence expression (Figure 7A). The results are shown only for each triple mutant and the quadruple mutant. Single and double sRNA deletion mutants behaved similarly to the triple mutants. Remarkably, the results show that if any one of the four sRNAs is present, *V. cholerae* expresses density-dependent bioluminescence similar to the wild-type. However, deletion of all four sRNA genes together results in a constitutive *lux* phenotype identical to the *luxO* null mutant. Thus, all four sRNAs participate in quorum-sensing repression, although any one alone is sufficient.

Because deletion of the sRNAs eliminates quorum-sensing repression in *V. cholerae*, it follows that overexpression of the sRNAs should result in constitutive repression. We tested this by overexpressing *V. cholerae qrr*1 in various *V. cholerae* and *V. harveyi* strains and examining the impact on light production. Compared to the vector-alone control, when *V. cholerae qrr*1 is overexpressed in *V. cholerae*, light production is reduced to 21% in the wild-type, 10% in the *luxO* null strain, and 1% in the quadruple sRNA mutant (Table 1). Overexpression of *V. cholerae qrr*1 in *V. harveyi* reduces light production to 12% in the wild-type and to 3% in the *luxO* null strain. Thus, the *V. cholerae* sRNA Qrr1 functions in both *V. cholerae* and *V. harveyi* to repress quorum sensing. We suspect that identical results would be obtained with any of the other three sRNAs identified in this work.

All of the data presented here suggest that multiple sRNAs act downstream of LuxO-P to destabilize *luxR*/

Table 1. Overexpression of *V. cholerae qrr*1 in *V. cholerae* and *V. harveyi*

Strain	Percent Light Remaining Compared to Vector Alone
V. cholerae wt	21
V. cholerae Δ*luxO*	10
V. cholerae 4 sRNA⁻	1
V. harveyi wt	12
V. harveyi Δ*luxO*	3

These experiments are representative of several trials that did not differ significantly.

hapR mRNA and regulate quorum-sensing dependent gene expression in *V. harveyi* and *V. cholerae*. As a final verification of this model, we performed an epistasis test in *V. cholerae*. We measured light production and HapR and TcpA protein levels in the *V. cholerae* wild-type strain, the *luxO* null mutant, the *luxO* D47E mutant, the quadruple sRNA deletion mutant, and the *luxO* D47E mutant containing the quadruple deletion of the *qrr* genes (Figure 7B). Maximal light is produced and a corresponding high level of HapR protein is observed in the high cell density wild-type and *luxO* strains. Both light and HapR protein levels are severely reduced in the *luxO* D47E strain. However, deletion of the four sRNAs alone or in the *luxO* D47E background restores maximal light production and maximal HapR protein production, showing that the four sRNAs are required for repression and act downstream of LuxO. Because *lux* and *tcpA* are regulated in an opposite manner by quorum sensing, TcpA levels are expected to vary reciprocally with those of *lux* expression and HapR concentration in the quorum-sensing mutants. Figure 7B shows this is the case and, importantly, that the four sRNAs are epistatic to LuxO-P in regulation of *tcpA*.

Discussion

Both *V. harveyi* and *V. cholerae* use quorum sensing to regulate gene expression in response to changes in cell density. Many of the regulatory components making up these vibrio communication systems have been identified and their roles characterized (Federle and Bassler, 2003; Xavier and Bassler, 2003). Genetic analyses have shown that one such component, LuxO, is phosphorylated at low cell density, and, in this form, interacts with the alternative sigma factor σ^{54} to activate a putative downstream repressor (Lilley and Bassler, 2000). Here we show that, in *V. cholerae*, the repressor is the sRNA chaperone Hfq and four sRNAs. LuxO-P, together with σ^{54}, activates the expression of the loci encoding all four sRNAs, and repression occurs via Hfq-sRNA-mediated destabilization of the *hapR* mRNA transcript (Figure 1). Surprisingly, while four sRNAs are involved, any one is sufficient for complete quorum-sensing repression. In *V. harveyi*, most probably five sRNAs work in conjunction with Hfq to destabilize the *luxR* mRNA (see below).

Small, untranslated RNA molecules have roles in controlling gene expression in bacteria (Masse et al., 2003b) and eukaryotes (Carrington and Ambros, 2003). In eukaryotes, astonishingly complex regulation is carried out by microRNAs (miRNAs). For example, in *Caenorhabditis elegans* and *Drosophila melanogaster*, some untranslated regions of mRNAs contain multiple sites to which miRNAs can bind. In other instances, one miRNA can control the expression of another miRNA. Finally, in *D. melanogaster*, distinct miRNAs can affect a single process (such as apoptosis), but each miRNA acts on a different function in the pathway (Carrington and Ambros, 2003). In bacteria, sRNAs regulate a variety of cellular processes, including carbon storage and utilization (Romeo, 1998), response to iron limitation (Masse et al., 2003a; Masse and Gottesman, 2002), response to oxidative stress (Zhang et al., 1998), and transition to stationary phase (Repoila et al., 2003). sRNAs regulate gene expression at a variety of levels, including but not limited to mRNA stability and translation (Masse et al., 2003b). Regulation occurs via RNA-protein interactions and RNA-RNA base pairing. Bacterial sRNAs are usually on the order of 100 nucleotides in length, and they can act either positively or negatively on their targets, depending on the location within the message of the RNA-RNA interaction (Masse et al., 2003b). For example, the sRNA OxyS binds to the *rpoS* mRNA and prevents its translation (Zhang et al., 1998, 2002). In contrast, the sRNAs DsrA and RprA enhance translation of *rpoS* mRNA by binding to an upstream leader region and eliminating the formation of a particular secondary structure within the message that inhibits translation (Majdalani et al., 1998, 2002). RyhB binds within the mRNA specifying the succinate dehydrogenase (*sdhCDAB*) operon and facilitates degradation of the transcript (Masse et al., 2003a; Masse and Gottesman, 2002). In many known cases, the sRNA chaperone protein Hfq is required to enhance the interaction between sRNAs and their target mRNAs (Moller et al., 2002; Zhang et al., 2002). In addition, many newly identified sRNAs that have no known regulatory function can bind to Hfq directly, suggesting that Hfq is an RNA chaperone for a large number of sRNAs (Wassarman et al., 2001; Zhang et al., 2003). Hfq has long been known to be a global regulator of gene expression, and it is now believed that this property of Hfq stems from its interaction with numerous regulatory sRNAs.

Our analysis predicted four highly homologous sRNAs as potential partners to Hfq in quorum-sensing regulation in *V. cholerae*, (Figures 5–7). While experimental verification is required, bioinformatics suggests five sRNAs are involved in the analogous *V. parahaemolyticus* and *V. vulnificus* quorum-sensing circuits (Figure 5). Since *V. harveyi* is more closely related to *V. parahaemolyticus* than to *V. cholerae* (Rowe-Magnus et al., 2001), we suspect that five sRNAs are likely to partner with Hfq in *V. harveyi* quorum-sensing regulation.

Interestingly, the gene encoding sRNA Qrr1 is located immediately upstream of the *luxOU* operon in all the analyzed vibrios (Figure 5). Conservation of gene order across species is generally indicative of functions that act in the same process (Dandekar et al., 1998). We hypothesize that the *qrr*1-*luxOU* locus represents an ancient evolutionary unit. This sRNA is noticeably different from the other three in that it is predicted to fold into a stem-loop structure with a loop that is much smaller than those predicted for the other three sRNAs (Figure 5B). This is partially accounted for by the fact that Qrr1 is missing several stretches of nucleotides that are conserved in Qrr2, Qrr3, and Qrr4 (see Figure 5A). Preliminary phylogenetic analysis indicates that the four sRNAs are paralogs, i.e., they derive from duplications of a single sRNA gene in an ancestral organism (presumably the sRNA linked to *luxOU*) followed by speciation into the family of vibrios. This evolutionary scenario leaves open the question of why multiple sRNAs are used to control quorum sensing in these organisms.

Extensive redundancy exists among the four sRNAs: the simultaneous inactivation of all four is necessary to eliminate Hfq-mediated quorum-sensing repression (Figure 7A), and, consistent with this, overexpression of only one sRNA is sufficient for repression (Table 1). In

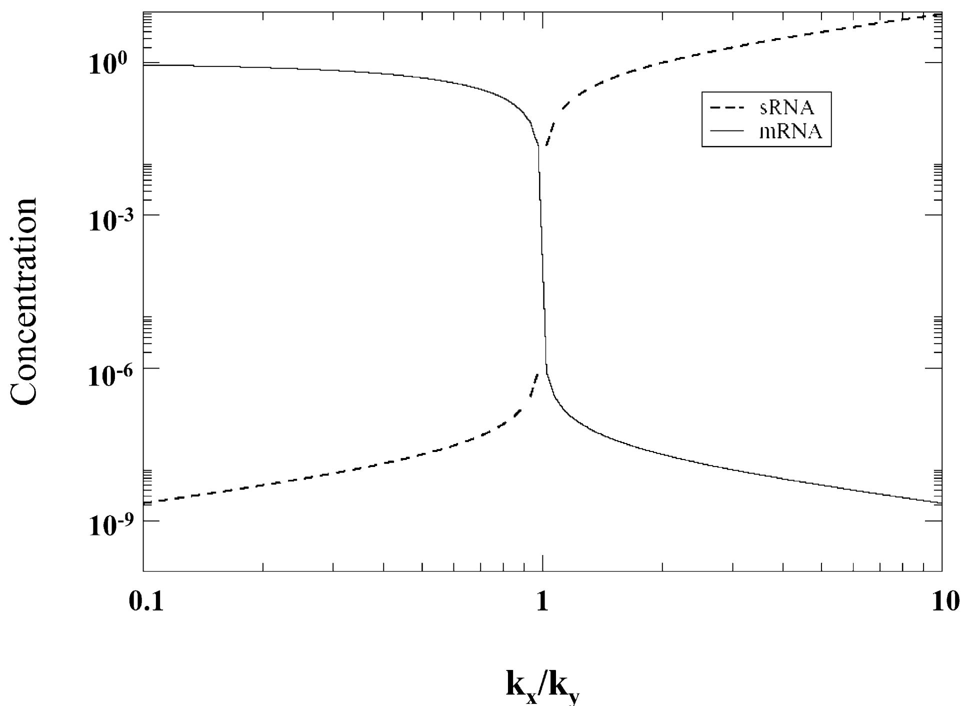

Figure 8. Mutual Destruction of sRNA and Target mRNA Produces an Ultrasensitive Response to the Rate of sRNA Synthesis.

If the rate of sRNA synthesis, k_x, drops below the rate of target mRNA synthesis, k_y, the steady-state pool of target message rises abruptly. In the quorum-sensing circuit, this implies that an ultrasensitive increase in *hapR/luxR* mRNA levels occurs with decreasing levels of LuxO-P as cell density increases. The curves are generated from Equation 7 of Elf et al. (2003), which describes the production of two chemical species (the sRNA and the mRNA) that undergo mutual destruction with a second-order rate constant k_{md} ($= v_{max}/K_x K_y$) and also undergo intrinsic, first-order degradation at a rate μ. In the curves shown, we have set the one adjustable parameter $k_{md} k_y/\mu^2$ to equal 5×10^7, which is in the regime where degradation of the minority species is primarily due to mutual-destruction processes. Concentrations are given in units of k_y/μ.

principle, a single sRNA activated by LuxO-P could accomplish the transition between low and high cell density states. One possibility is that the presence of multiple sRNAs is important to fine tune this transition. If so, the presence of four sRNAs may allow additional regulatory inputs (e.g., metabolic) to influence when the transition occurs. Indeed, we have identified conserved motifs, consistent with regulatory-factor binding sites, in the upstream regions of the *qrr*2 and *qrr*3 genes, in addition to the putative LuxO-P binding sites located upstream of all four sRNAs (R.V.K., unpublished data).

We cannot detect sRNAs Qrr1, Qrr2, or Qrr3 in the *luxO* D47E strain by Northern analysis (Figure 6A, *hapR*$^+$ panel). Nonetheless, their inactivation is required to eliminate Hfq-directed quorum-sensing repression (Figure 7), so they must be present, and they definitely act under our experimental conditions. Recently, it was reported that Hfq, together with the sRNA RyhB, facilitates the degradation of the mRNA encoding SodB in *E. coli* (Masse et al., 2003a). The sRNA RyhB when paired to its target mRNA is rapidly degraded by RNaseE. Other sRNAs were also shown to be stabilized in the absence of their specific target mRNAs, suggesting that this regulatory mechanism is generalizable (Masse et al., 2003a). We found that the levels of sRNA Qrr2, Qrr3, and Qrr4 increased in the *luxO* D47E strain in the absence of *hapR* mRNA (Figure 6). This finding suggests that, first, like *qrr*4, *qrr*2 and *qrr*3 are controlled by LuxO-P and, second, that as in *E. coli*, the presence of the target mRNA reduces the stability of these three sRNAs. If messages specifying genes other than *hapR* are also targets of regulation by this set of sRNAs, it might be necessary to eliminate these mRNAs to observe increased stability and/or abundance of sRNA Qrr1 and further increased stability and/or abundance of sRNAs Qrr2 and Qrr3. A transcriptional reporter fusion to the *qrr*1 promoter demonstrates that it too is controlled by LuxO-P (Figure 6C). We suspect that we cannot detect this sRNA by Northern blot, because the technique does not have the sensitivity required to detect RNAs with very low abundance. In one additional attempt to study Qrr1, we hypothesized that the absence of the Qrr2–4 sRNAs might lead to enhanced expression of the remaining *qrr* gene, *qrr*1. However, no change in transcription of *qrr*1-*lux* occurred in the triple mutant, suggesting that the presence or absence of *qrr*2–4 does not affect the expression of *qrr*1 (data not shown).

Why does LuxO regulate *hapR/luxR* via sRNAs rather than directly? One reason may be that the sRNAs allow a simple "inversion" of regulatory control, so that the activator LuxO can repress *hapR/luxR*. However, this inversion could also be accomplished in other ways, e.g., by switching the regulation pattern of *hapR/luxR* with respect to its target genes. A more fundamental motivation for control via sRNAs may be to achieve an ultrasensitive (switchlike) response to the level of

LuxO-P. As base pairing of an sRNA with its target message is known to promote degradation of both the sRNA and the message, this "mutual destruction" provides an elegant mechanism for ultrasensitivity. Specifically, as shown in Figure 8, if the rate of synthesis of a particular sRNA exceeds the rate of synthesis of its target message, even if only slightly, then the sRNA can accumulate in the cell, and target message levels can be reduced to very low levels. In contrast, if the rate of synthesis of a particular target message exceeds that of its regulatory sRNA, then the message can accumulate (Figure 8). The ultrasensitive mechanism described here also applies in the case of multiple sRNAs interacting with one or more mRNA targets (Paulsson and Ehrenberg, 2001). The use of sRNAs to accomplish an ultrasensitive response may be particularly apt for processes such as quorum sensing in which an all-or-nothing response is indicated. Similarly, this all-or-nothing requirement could explain why sRNAs control the entry into stationary phase. A different kind of sRNA switch was highlighted by Masse et al. (2003a) for the RyhB system. In this case, the sRNA RyhB was shown to mediate a rapid, reversible switch in time in response to a *large* change in input (e.g., addition of iron to the medium). In the quorum-sensing circuit, by contrast, the switch occurs in response to a *small* change in input (e.g., LuxO-P levels) that is strongly amplified into a transition between two discrete states (e.g., low and high cell density). How fast this switch occurs in time will depend on the rate of change of LuxO-P levels as well as on the rate of accumulation and/or degradation of *luxR/hapR* mRNA and LuxR/HapR protein. Interestingly, an ultrasensitive response to LuxO-P via the rate of sRNA production puts a premium on precisely controlling the transcription rate of the sRNAs, consistent with our hypothesis that the presence of multiple sRNAs represents a mechanism for fine tuning the transition between low and high cell density states.

The decision to transition from acting alone to participating in a group activity is a critical one for bacteria. In vibrios, sophisticated regulatory devices are located at different positions in the quorum-sensing signal-transduction relay to ensure that this decision occurs under the appropriate set of circumstances and with high fidelity. Previously, we showed that a coincidence detector regulates entry into high cell density mode: the simultaneous presence of multiple autoinducers is required to reverse the direction of phosphoflow through the system and thus to initiate the critical transition from the individualistic to the group lifestyle (Mok et al., 2003). The coincidence-detection scheme likely protects the quorum-sensing circuit from molecules in the environment that resemble the bona fide autoinducers. In the present work, we show that an ultrasensitive switch involving multiple sRNAs exists to make the commitment step into quorum-sensing mode definitive. We argue that this transition is not graded but rather an on/off switch, which turns off behaviors that are useful when carried out alone and turns on behaviors that are productive when carried out as a community.

Experimental Procedures

Bacterial Strains and Media

V. harveyi strains are derived from BB120 (Bassler et al., 1997) and were grown at 30°C with aeration in Luria-marine (LM), heart infusion (HI), or autoinducer bioassay (AB) broth (Bassler et al., 1994b; Freeman and Bassler, 1999). *V. cholerae* strains are derivatives of El Tor strain C6706str2 (Thelin and Taylor, 1996) and were grown at 30°C with aeration in Luria-Bertani (LB) or SOC broth (Sambrook et al., 1989). For studies of toxin coregulated pilus (TCP), *V. cholerae* was grown at 37°C in AKI medium under AKI conditions (Iwanaga et al., 1986). *E. coli* S17-1λpir (de Lorenzo and Timmis, 1994) and JM109 (Yanisch-Perron et al., 1985) were used to propagate plasmids at 37°C in LB. The following antibiotics were used: ampicillin (amp), 100μg/ml; tetracycline (tet), 10μg/ml; kanamycin (kan), 100μg/ml; chloramphenicol (cm), 10μg/ml; and gentamicin (gent), 100μg/ml. Streptomycin (strep) was used at 1 mg/ml and polymyxcin B (pb) at 50 units/ml.

DNA Manipulations

All DNA manipulations were performed according to Sambrook et al. (1989). PFU turbo polymerase (Stratgene) was used for PCR reactions used in cloning, whereas Taq polymerase (Roche) was used for all other PCR reactions. dNTPs, restriction endonucleases, and T4 ligase were obtained from New England Biolabs. DNA purification kits were obtained from QIAGEN. Primer sequences are available upon request. *V. harveyi* deletions were constructed using the method of Datsenko and Wanner (2000). Constructions were placed on the *V. harveyi* chromosome by allele replacement (Bassler et al., 1993). In-frame deletions were constructed by the method of Skorupski and Taylor (1996). *hapR-lacZ* reporter fusions were constructed via the method of Kalogeraki and Winans (1997). *qrr*1 from *V. cholerae* was overexpressed from plasmid pKK177-3RI (gift of G. Storz). For *V. harveyi*, a kan resistance cassette was also incorporated into pKK177-3RI. The *qrr*1-*lux* transcriptional fusion plasmid was constructed by ligating a *Pac*I fragment from pCS26-*Pac* (Bjarnason et al., 2003) into an engineered *Pac*I site in pBBR1MCS (Kovach et al., 1994). The vector was digested with *Bam*HI, which eliminated a roughly 2 kb DNA fragment, and a PCR-amplified fragment containing the promoter region of *V. cholerae qrr*1 was cloned into the *Bam*HI site.

Bioluminescence Assays

V. harveyi cultures were grown in AB broth for 14 hr at 30°C with aeration. The cultures were diluted 1:5000 prior to bioluminescence assays, which were performed as described (Bassler et al., 1993). Relative light units for *V. harveyi* are defined as counts min^{-1} ml^{-1} × 10^3/cfu ml^{-1}. *V. cholerae* bioluminescence assays were performed following 10 hr growth at 30°C in SOC containing tet to maintain the plasmid pBB1 carrying *V. harveyi luxCDABE*. OD_{600nm} for each culture was measured, and the cultures were diluted such that each culture was at the same cell density (~1:1000 dilution). Light and OD_{600} were measured every 45 min as described (Miller et al., 2002). Relative light units for *V. cholerae* are defined as counts min^{-1} ml^{-1}/OD_{600nm}.

β-Galactosidase Assays

β-galactosidase assays were performed in triplicate as in Slauch and Silhavy (1991). β-galactosidase units are defined as [V_{max}][dilution factor]/OD_{600nm}.

Western Blot Analysis and Antibody Preparation

Western blot analysis was performed as described (Henke and Bassler, 2004), the membranes were exposed to anti-TcpA antibody, and chemiluminescence detection (Amersham) was used (Sun et al., 1991). To analyze HapR and LuxR protein levels, HapR and LuxR were purified (Chen et al. 2002b), and polyclonal antibodies were generated (Henke and Bassler, 2004). Polyclonal antisera were adsorbed to both *E. coli* pGEX-4T-1 lysates and either a *V. cholerae hapR* mutant lysate or a *V. harveyi luxR* mutant lysate prior to use.

Northern Blot Analysis

Cultures used for RNA preparations were grown to OD_{600nm} of 0.5. Rifampicin was added at 100 μg/ml, and each culture was further incubated with aeration at 30°C. Aliquots were taken at the appropriate times, and RNA was extracted with TRIzol (Invitrogen) and chloroform. RNA was precipitated with isopropanol, washed with 75% ethanol, and resuspended in DEPC water. Northern blots were

performed as described (Martin et al. 1989). Steady-state Northern blots were performed as above except that no rifampicin was added.

Genetic Screen to Identify *hfq*

V. harveyi strain JAF548 (*luxO* D47E kan^r^) was mutagenized with Mini-Mu*lacZ* (cm^r^) as described (Martin et al., 1989). Bright colonies were isolated, and insertions in *luxO* and *rpoN* were identified by PCR and complementation. Tn*5lacZ* mutagenesis of *hfq* in cosmid pBNL2014 was carried out as described previously (Showalter et al., 1990). Transposon insertions were mapped by restriction analysis and sequencing. Cosmid pBNL2031, containing a Tn*5lacZ* insertion in *hfq*, was used in the allelic replacement procedure to generate BNL258 (*hfq*::Tn*5lacZ*).

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