INAUGURAL ARTICLE

Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*

Brian K. Hammer* and Bonnie L. Bassler*†‡

*Department of Molecular Biology, Princeton University, and †Howard Hughes Medical Institute, Princeton, NJ 08544-1014

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected on April 25, 2006.

Contributed by Bonnie L. Bassler, April 26, 2007 (sent for review April 20, 2007)

Using a process called quorum sensing (QS), bacteria communicate with extracellular signal molecules called autoinducers (AIs). Response to AIs allows bacteria to coordinate gene expression on a population-wide scale and thereby carry out particular behaviors in unison, much like multicellular organisms. In *Vibrio cholerae* El Tor, the etiological agent of the current cholera pandemic, AI information is transduced internally through a phosphorelay circuit that impinges on the transcription of multiple small regulatory RNAs (sRNAs). These RNAs base-pair with, and repress the translation of, the mRNA encoding the master transcriptional regulator HapR. In *V. cholerae*, HapR controls virulence factor expression and biofilm formation. Here we identify a sRNA-dependent, HapR-independent QS pathway in which the sRNAs base-pair with a new target mRNA and activate translation by preventing formation of a translation-inhibiting stem-loop structure. We show that the classical *V. cholerae* strain, which caused previous pandemics and is reportedly incapable of QS because of a nonfunctional HapR, nonetheless exhibits QS-controlled gene expression through this new HapR-independent pathway.

autoinducer | sRNA | virulence | HapR

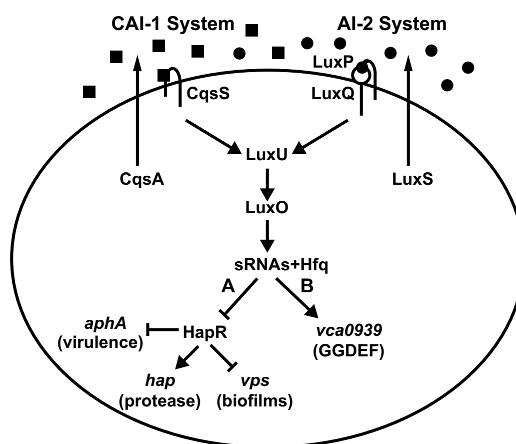

Fig. 1. The *V. cholerae* QS circuit. HapR-dependent (A) and HapR-independent (B) pathways are controlled by Qrr sRNAs and Hfq. See Introduction for details. Squares, CAI-1; circles, AI-2.

Quorum sensing (QS) is a process of cell–cell communication that enables bacterial populations to collectively control gene expression and thus coordinate group behaviors (1). QS is achieved by the synthesis, secretion, and detection of signal molecules called autoinducers (AIs) that accumulate in proportion to increasing cell density. The human pathogen *Vibrio cholerae* uses two AIs, CAI-1 and AI-2, to control QS target genes (Fig. 1). CqsA produces the intragenera signal CAI-1 (a compound of unknown structure), which is detected by the cognate sensor kinase protein CqsS (2, 3). LuxS synthesizes AI-2, a furanosyl borate diester, which fosters interspecies communication. AI-2 is detected by the LuxPQ receptor complex (4–7).

At low cell density, in the absence of AIs, CqsS and LuxQ act as kinases, transferring phosphate to the response regulator LuxO via the phosphotransfer protein LuxU. LuxO~P activates the transcription of genes encoding four small regulatory RNAs (sRNAs) called Qrr1–4 (quorum regulatory RNAs). The Qrr sRNAs, along with the sRNA chaperone, Hfq, base-pair with and destabilize the mRNA encoding *hapR*, and so no HapR is produced (8). Under this condition, HapR-repressed genes are expressed whereas HapR-activated genes are not (Fig. 1, pathway A). At high cell density, binding of AIs to their cognate sensors switches the sensors from kinases to phosphatases, reversing the phosphorylation cascade. Dephosphorylated LuxO is inactive, so transcription of the *qrr* genes terminates, HapR is produced, and the pattern of HapR-dependent gene regulation is inverted. Genes controlled by QS include those for virulence factor expression and biofilm formation (2, 9–13). Importantly, every *V. cholerae* QS target gene identified to date (>70 genes) requires HapR for regulation (Fig. 1).

V. cholerae El Tor (*V. cholerae*^{El}) is responsible for the current cholera pandemic, whereas classical *V. cholerae* (*V. cholerae*^{Cl}) was responsible for previous pandemics (14). *V. cholerae*^{Cl} and some other *V. cholerae* strains carry frameshift mutations in *hapR*. Canonical HapR-controlled reporters are not properly regulated in these strains, and, because of this, these strains have been deemed incapable of QS (15).

Results

Identification of a HapR-Independent QS Target Gene. We designed a genetic screen to identify *V. cholerae* AI-regulated target genes. A library of random *V. cholerae*^{El} genomic fragments fused to a promoterless *luxCDABE* cassette was introduced into Δ*cqsA*, Δ*luxS* (CAI-1$^-$, AI-2$^-$) *V. cholerae*^{El}. After parallel coculture with either a *V. cholerae*^{El} AI-producing (CAI-1$^+$, AI-2$^+$) or AI-nonproducing (CAI-1$^-$, AI-2$^-$) strain (referred to below as AI$^+$ donor and AI$^-$ control, respectively), the library was screened for altered *lux* expression. Twenty-five unique promoters displayed differential regulation; five fusions were repressed, and 20 were activated by AIs.

Here we focus on one AI-repressed gene (*vca0939*) that exhibited a particularly interesting pattern of QS regulation. *vca0939* is predicted to encode a GGDEF domain-containing protein. Bacterial GGDEF proteins synthesize the intracellular small-molecule second messenger cyclic di-GMP, which regulates numerous processes including biofilm formation (16). We

Author contributions: B.K.H. and B.L.B. designed research; B.K.H. performed research; B.K.H. contributed new reagents/analytic tools; B.K.H. and B.L.B. analyzed data; and B.K.H. and B.L.B. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: QS, quorum sensing; AI, autoinducer; sRNA, small regulatory RNA.

‡To whom correspondence should be addressed. E-mail: bbassler@princeton.edu.

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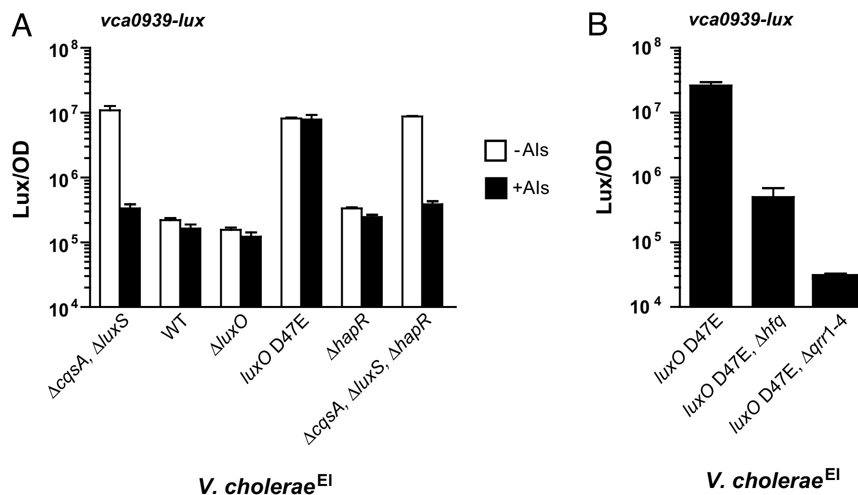

Fig. 2. HapR-independent expression of *vca0939*. Shown is *vca0939-lux* expression in *V. cholerae*^{El} strains incubated in a 1:1 coculture with either a *V. cholerae*^{El} CAI-1$^-$, AI-2$^-$ strain (−AIs, open bars) or a *V. cholerae*^{El} CAI-1$^+$, AI-2$^+$ strain (+AIs, filled bars) (*A*) and *V. cholerae*^{El} monocultures (*B*). Each panel shows the mean bioluminescence/OD (Lux/OD) and the standard deviations of triplicate cultures.

found that, in the *V. cholerae*^{El} Δ*cqsA*, Δ*luxS* strain, *vca0939-lux* is expressed, and addition of exogenous AIs reduces the expression ≈30-fold (Fig. 2*A*). Consistent with repression by AIs, at high cell density, in the wild-type strain (i.e., CAI-1$^+$, AI-2$^+$) and in the Δ*luxO* mutant that is "locked" in high cell density mode, there is only low-level expression of *vca0939-lux* irrespective of the presence of additional AIs. Again, as expected, in the locked low cell density *luxO* D47E mutant, which carries a constitutive LuxO∼P mimic, *vca0939-lux* expression is activated ≈30-fold relative to the wild type, irrespective of the presence of exogenous AIs. However, *vca0939-lux* expression is not activated in the Δ*hapR* mutant. This result was unanticipated because both the Δ*hapR* and *luxO* D47E mutants simulate the low cell density state. Thus, the Δ*hapR* result suggests that AIs repress expression of *vca0939-lux* in the absence of HapR, and, as mentioned, all *V. cholerae* AI-responsive genes identified to date require HapR for their regulation (2, 9–13). To explore this peculiar expression pattern, *vca0939-lux* expression was measured in a Δ*cqsA*, Δ*luxS*, Δ*hapR*, triple mutant (CAI-1$^-$, AI-2$^-$, HapR$^-$) cocultured with the AI$^+$ donor or AI$^-$ control. *vca0939-lux* expression in the Δ*cqsA*, Δ*luxS*, Δ*hapR* triple mutant with or without AIs is identical to that in the Δ*cqsA*, Δ*luxS* double mutant (Fig. 2*A*), showing that indeed the AI-mediated repression of *vca0939* expression occurs in the absence of HapR.

***vca0939* Expression Is Controlled by Qrr sRNAs and Hfq.** Our results show that *vca0939* expression requires LuxO but not HapR. Hfq and the Qrr sRNAs lie between LuxO and HapR in the QS circuit (Fig. 1) (8). To examine their roles in *vca0939* regulation, we deleted either *hfq* or the four *qrr* genes and measured *vca0939-lux* expression. Deletion of *hfq* causes a ≈100-fold reduction in *luxO* D47E-dependent expression of *vca0939-lux*, and elimination of the Qrr sRNAs causes an even more severe reduction (Fig. 2*B*), showing that both Hfq and the Qrr sRNAs are required for QS regulation of *vca0939*. We note that higher residual *vca0939-lux* expression occurs in the Δ*hfq* mutant than in the Δ*qrr*1–4, mutant suggesting that the sRNAs may possess a low level ability to activate *vca0939-lux* in the absence of Hfq.

The only previously predicted function for the Qrr sRNAs is to destabilize a single target mRNA, that encoding *hapR* (8). To determine whether the Qrr sRNAs function directly or indirectly to control *vca0939* expression, we measured *vca0939-lux* expression in *Escherichia coli* in the absence and the presence of constitutively produced Qrr1. *E. coli* encodes an Hfq homolog but lacks the other *V. cholerae* QS components (17). Expression of *vca0939-lux* is activated >1,000-fold by Qrr1 in *E. coli* (left bars in Fig. 3*A*). This transcription pattern reflects VCA0939 protein production, because C-terminally FLAG-tagged VCA0939 is detected in the presence of Qrr1 but cannot be detected when Qrr1 is absent (data not shown). Thus, in addition to directly repressing *hapR* expression, Qrr1 and, we infer, the other Qrr sRNAs also directly activate *vca0939* expression.

The *V. cholerae* Qrr sRNAs Base-Pair with Multiple Target mRNAs. Because Qrr1 directly regulates *vca0939* expression (Fig. 3*A*), we speculated that the Qrr sRNAs base-pair with the mRNA encoding *vca0939*. To test this hypothesis, an MFOLD algorithm was used to compare the predicted secondary structures of Qrr/*hapR* and Qrr/*vca0939* mRNA hybrids (Fig. 3*B*) (18). In the case of Qrr/*hapR*, the algorithm predicts the exact base-pairing identified previously (8), giving us confidence in this approach. In the case of Qrr/*vca0939*, identical interactions between *vc0939* mRNA and each of the four Qrr sRNAs are predicted, and, importantly, the critical base-pairing region in each sRNA is identical to that required for the Qrr/*hapR* interactions. However, a distinct region of the *vca0939* mRNA appears to participate in base-pairing to the sRNAs. Specifically, the ribosome binding site of *hapR* is occluded in the Qrr/*hapR* duplex but not in the predicted interactions between the Qrr sRNAs and *vca0939* (Fig. 3*B*). Rather, analysis of the 5′ UTR of the *vca0939* mRNA suggests that, in the absence of the Qrr sRNAs, a single inhibitory stem-loop structure exists in a region overlapping both the RBS and the putative sRNA binding site (19). Qrr sRNA interaction with the *vca0939* mRNA apparently prevents formation of this inhibitory structure, allows access to the ribosome, and promotes translation. These predictions suggest a molecular explanation underlying the different outcomes (i.e., activation versus inhibition of translation) of Qrr/mRNA target base-pairing interactions.

The *V. cholerae* Qrr sRNAs Disrupt an Inhibitory Stem Loop Structure in the *vca0939* mRNA. To test the validity of the above predictions, we altered sites in the *vca0939* 5′ UTR predicted to be crucial for the interaction with the Qrr sRNAs or for the formation of the inhibitory stem-loop structure. We subsequently measured their effects on *vca0939-lux* expression. We disrupted the putative sRNA binding site in *vca0939* by changing an AA doublet to a UU (see arrow in Fig. 3*C*) while leaving the nucleotides required

Fig. 3. Base-pairing between Qrr sRNAs and *hapR* and *vca0939* mRNAs. (*A*) *E. coli* carrying either a vector control (open bars) or a *qrr*1-expressing vector (filled bars), along with a plasmid containing *vca0939-lux* or the mutagenized reporters *vca0939*(aa→uu)-*lux* or *vca0939*(cc→gg)-*lux*. (*B*) MFOLD predictions (18) for mRNA hybrids containing the Qrr sRNA sequence fused by a six-base linker (denoted n) to *hapR* (Qrr/*hapR*) or *vca0939* (Qrr/*vca0939*) mRNA. (*C*) Predicted Qrr/*vca0939* duplex showing the location of the *vca0939*(aa→uu)-*lux* mutation (arrow under the boxed nucleotides). (*D*) The predicted inhibitory stem-loop structure in the mRNA of *vca0939* showing the location of the *vca0939*(cc→gg)-*lux* mutation (arrow adjacent to the boxed nucleotides) (19). In *B*–*D* the RBSs are shown in red, the initiation codons are underlined, and the predicted sRNA binding site in the *vca0939* mRNA is in blue.

for the formation of the putative inhibitory stem-loop structure intact. The AA to UU alteration reduced expression of *vca0939-lux* in *E. coli* carrying Qrr1 to background levels [Fig. 3*A*, center bars, denoted *vca0939*(aa→uu)-*lux*], suggesting that Qrr1 no longer binds to *vca0939* mRNA and promotes its translation. In the reciprocal experiment, we mutated the site predicted to be required for forming the inhibitory stem-loop by changing a CC pair to GG (see arrow in Fig. 3*D*). This disruption was engineered into *vca0939* in a region outside of the site proposed to be required for sRNA binding. This mutation caused maximal expression of *vca0939-lux* in the presence and absence of the Qrr sRNA [Fig. 3*A*, right bars, denoted *vca0939*(cc→gg)-*lux*]. Thus, disruption of the stem-loop structure alleviates any requirement for a Qrr sRNA in activation of *vca0939* expression. Together, our results demonstrate that indeed the Qrr sRNAs directly base-pair with *vca0939* mRNA, and, furthermore, they function to activate its expression by disrupting an inhibitory stem-loop structure at the RBS.

QS Occurs in *V. cholerae* Strains Carrying *hapR* Null Mutations. Many previously characterized clinical isolates of *V. cholerae* have been deemed incapable of QS based on DNA sequence data documenting the absence of a functional HapR master regulator. For example, *V. cholerae* classical strain O395 (*V. cholerae*^{Cl}) possesses a frameshift mutation in *hapR* generating a truncated polypeptide (79 aa, compared with the 204-aa protein of *V. cholerae*^{El}) (15). Our inspection of the *V. cholerae*^{Cl} genome shows that all of the other components of its QS circuit are intact and that a homolog of *vca0939* is present. Based on the above results, we predict that *V. cholerae*^{Cl} is capable of QS (i.e., responding to AIs) through the HapR-independent pathway identified here. To verify that the overall *V. cholerae*^{Cl} QS circuit functions, we introduced *qrr-lux* and *vca0939-lux* fusions into wild-type, Δ*luxO*, and *luxO* D47E *V. cholerae*^{Cl} strains. Fig. 4*A* shows that the expression patterns of both reporters exactly mimic those in the corresponding *V. cholerae* El Tor strains (see Fig. 2*A*) (8). Specifically, both the *qrr* sRNA and *vca0939* genes are maximally expressed in the low cell density state (*luxO* D47E mutant) and minimally expressed in the high cell density state (WT and Δ*luxO* mutant).

To establish that the *V. cholerae*^{Cl} biotype is capable of a QS response, we assayed whether a *V. cholerae*^{Cl} Δ*cqsA*, Δ*luxS* (i.e., CAI-1$^-$, AI-2$^-$) mutant could regulate *qrr-lux* and *vca0939-lux* expression in response to the exogenous addition of AIs. To do this, as in Fig. 2, we used coculture, this time with either a *V. cholerae*^{Cl} AI$^+$ donor or *V. cholerae*^{Cl} AI$^-$ control strain. Expression of *qrr-lux* and *vca0939-lux* is repressed 100- and 6-fold respectively, by the presence of AIs (Fig. 4*B*). Thus, *V. cholerae* isolates, including classical strains, like O395, that lack functional HapR proteins, nonetheless use QS to regulate expression of *vca0939* and very likely other target genes.

Discussion

HapR has been designated the master regulator of QS in *V. cholerae* because all QS target genes identified to date require it for their control (2, 9–13). Previous studies supported a model in which all QS AI information was channeled exclusively to HapR via the four Qrr sRNAs (8). Here we show that another target, *vca0939*, encoding a putative GGDEF protein, is also regulated by AIs and by the Qrr sRNAs. Critically, however, unlike the *hapR* mRNA that is negatively controlled by the Qrr sRNAs, the *vca0939* mRNA is positively controlled by the Qrr sRNAs and in a HapR-independent manner. Together, our results show that the Qrr sRNAs, in association with the mRNA chaperone Hfq, act as both positive and negative regulators of mRNA translation, and, furthermore, both HapR-dependent and HapR-independent QS pathways exist in *V. cholerae*.

Only two other Hfq-dependent sRNAs (DsrA and RyhB, both in *E. coli*) are known to have dual functions as posttranscriptional activators and repressors. Base-pairing of the DsrA sRNA to the *rpoS* mRNA, encoding the stationary phase σ factor RpoS, activates translation by preventing formation of an inhibitory secondary structure (20). DsrA also prevents translation by binding to the mRNA encoding the nucleoid protein H-NS (21). Likewise, the RyhB sRNA controls iron scavenging by base-

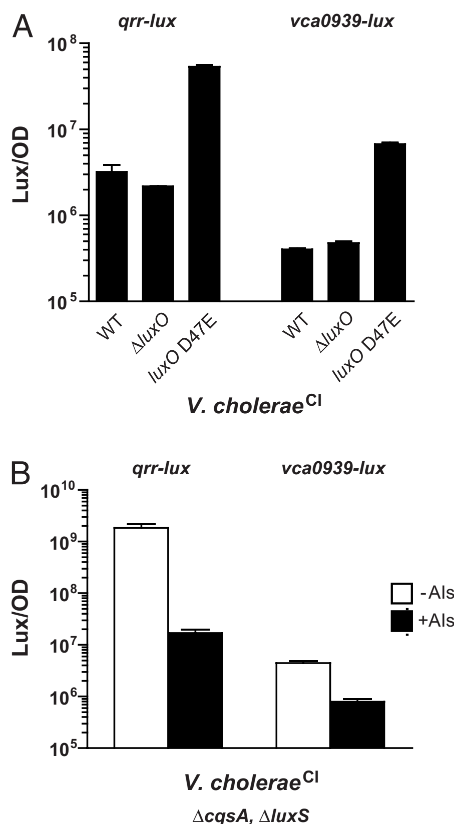

Fig. 4. QS occurs in *V. cholerae*^{Cl} strain O395 despite a nonfunctional HapR. (*A*) *qrr-lux* (left bars) and *vca0939-lux* (right bars) expression in *V. cholerae*^{Cl} monocultures. (*B*) *V. cholerae*^{Cl} CAI-1$^-$, AI-2$^-$ mutant carrying either *qrr-lux* (left bars) or *vca0939-lux* (right bars) in a 1:1 coculture with either a *V. cholerae*^{Cl} CAI-1$^-$, AI-2$^-$ strain (−AIs, open bars), or a *V. cholerae*^{Cl} CAI-1$^+$, AI-2$^+$ strain (+AIs, filled bars). Mean bioluminescence/OD (Lux/OD) and the standard deviations of triplicate cultures are shown.

pairing to and activating translation of the *shiA* mRNA, which encodes a permease. RyhB also functions in the *E. coli* stress response by binding to, and triggering the degradation of, the *sodB* mRNA encoding superoxide dismutase (22, 23).

The target of Qrr regulation defined here, *vca0939*, encodes a protein containing a GGDEF motif. GGDEF proteins are guanylate cyclases that synthesize the intracellular second messenger c-di-GMP. Another set of proteins, containing EAL or HD-GYP motifs, are phosphodiesterases responsible for breaking down c-di-GMP. Increased c-di-GMP levels, among other things, induce expression of exopolysaccharide biosynthetic genes required for biofilm formation in *V. cholerae* (14). Deletion of *vca0939* in *V. cholerae* El Tor did not alter expression of exopolysaccharide biosynthesis genes or biofilm formation (data not shown). This result was not unexpected because *V. cholerae* is predicted to possess 41 GGDEF proteins and 31 EAL and HD-GYP proteins (24). Significant redundancy exists in the roles these proteins play in controlling c-di-GMP levels in *V. cholerae*, and, frequently, deletion of the gene encoding one GGDEF or EAL protein, as in the case of *vca0939*, does not result in an observable phenotype.

The genomes of all sequenced *Vibrio* species show that they possess QS circuits extremely similar to that of *V. cholerae* El Tor strain C6706 (1). *V. cholerae* classical strain O395 and *V. cholerae* El Tor strain N16961 harbor nonsense mutations in their *hapR* genes (15), but, by contrast, these strains have only conservative mutations in genes encoding the other components of their QS circuitry. Strikingly, all four *qrr* DNA sequences in these *V. cholerae* strains are 100% identical to those in *V. cholerae* El Tor strain C6706, suggesting that, irrespective of the presence or absence of HapR, maintenance of functional Qrr sRNAs is critical. We suggest that this is because the Qrr sRNAs play an essential regulatory role in HapR-independent QS pathways that make HapR$^-$ *V. cholerae* strains capable of cell–cell communication.

We propose that, at low cell density (low AI levels), LuxO~P activates expression of the genes encoding the Qrr sRNAs, which, along with Hfq, bind to target mRNAs and alter their fates (Fig. 1). In the case of the *hapR* mRNA, the Qrr sRNAs base-pair over the RBS and destabilize the message. This interaction alters HapR-dependent gene expression (Fig. 1, pathway A). In the case of *vca0939*, base-pairing of the Qrr sRNAs antagonizes an inhibitory stem-loop and allows translation of VCA0939 (Fig. 1, pathway B). In *V. cholerae* strains containing a functional HapR (e.g., pandemic El Tor C6706), both pathways are used (Fig. 1, pathways A and B). In strains lacking HapR (e.g., pandemic classical O395 and El Tor N16961), only the latter pathway is relevant (Fig. 1, pathway B). Our screen was performed in a HapR$^+$ strain. Fortuitously, it allowed us to identify one HapR-independent target gene. We predict that additional target mRNAs like *vca0939* exist and are regulated directly by the Qrr sRNAs in *V. cholerae* strains. We expect this to be the case in other *Vibrio* species because, as mentioned, they also contain *V. cholerae*-like QS systems. We are currently performing genetic screens (in HapR$^-$ *Vibrio* strains) and *in silico* analyses to identify additional targets of this HapR-independent, AI-responsive QS pathway.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. Bioluminescence was measured in the following *V. cholerae*^{El} C6706str strains carrying the *vca0939-lux* reporter: WT (BH2028); Δ*cqsA*, Δ*luxS* (BH1842); Δ*luxO* (BH2029); *luxO* D47E (BH2031); Δ*hapR* (BH2030); Δ*cqsA*, Δ*luxS*, Δ*hapR* (BH2111); *luxO* D47E, Δ*hfq* (BH2183); and *luxO* D47E, Δ*qrr*1–4 (BH2133). Bioluminescence was measured in the following *V. cholerae*^{Cl} O395str strains carrying a *vca0939-lux* reporter: WT (BH2341); Δ*luxO* (BH2332); *luxO* D47E (BH2323), and Δ*cqsA*, Δ*luxS* (BH2386). Bioluminescence was measured in the following *V. cholerae*^{Cl} O395str strains carrying a *qrr-lux* reporter: WT (BH2302); Δ*luxO* (BH2330); *luxO* D47E (BH2325); and Δ*cqsA*, Δ*luxS* (BH2385). *E. coli* DH10B strains used in Fig. 3*A* carry either pKK*qrr*1 (BH2130, BH2470, and BH2472) or a control vector pKK177 (BH2128, BH2469, and BH2471), as described in ref. 8, along with the *vca0939-lux* (BH2130, BH2128), *vca0939*(aa→uu)-*lux* (BH2470, BH2469), or *vca0939*(cc→gg)-*lux* (BH2472, BH2471) reporter plasmids, respectively. The QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce the aa→uu and cc→gg mutations into the *vca0939-lux* reporter plasmid. *V. cholerae* strains were constructed as described (25). *V. cholerae*^{El} C6706str WT (BH1718) and *V. cholerae*^{Cl} O395str WT (BH2388) strains and the corresponding Δ*cqsA*, Δ*luxS* (BH1720) and Δ*cqsA*, Δ*luxS* (BH2387) strains carrying the vector pBBR*lux* (described in ref. 8) served as AI$^+$ donors and AI$^-$ controls, respectively. Cultures were grown in LB medium at 30°C with aeration. Chloramphenicol (10 mg·liter^{-1}); and ampicillin (100 mg·liter^{-1}) were used to maintain plasmids.

Bioluminescence Assays. Overnight cultures were diluted 1:100 in LB. In coculture experiments, donor strains were mixed at a 1:1

ratio with recipients. *V. cholerae*^{El} cocultures were incubated for 8 h, and *V. cholerae*^{Cl} cocultures were incubated for 6 h; monocultures were incubated overnight. Absorbance and bioluminescence were quantified thereafter. Lux/OD is defined as counts per $min^{-1} \cdot ml^{-1}/OD_{600}$.

We thank the members of the B.L.B. laboratory for insightful discussions. We are grateful to G. Chimalakonda for help with the screen and J. Irgon for bioinformatics. This research was supported by the Howard Hughes Medical Institute (B.L.B.) and by National Institutes of Health grants (to B.L.B. and B.K.H.).

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