AphA and LuxR/HapR reciprocally control quorum sensing in vibrios

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Bacteria cycle between periods when they perform individual behaviors and periods when they perform group behaviors. These transitions are controlled by a cell–cell communication process called quorum sensing, in which extracellular signal molecules, called autoinducers (AIs), are released, accumulate, and are synchronously detected by a group of bacteria. AI detection results in community-wide changes in gene expression, enabling bacteria to collectively execute behaviors such as bioluminescence, biofilm formation, and virulence factor production. In this study, we show that the transcription factor AphA is a master regulator of quorum sensing that operates at low cell density (LCD) in *Vibrio harveyi* and *Vibrio cholerae*. In contrast, LuxR (*V. harveyi*)/HapR (*V. cholerae*) is the master regulator that operates at high cell density (HCD). At LCD, redundant small noncoding RNAs (sRNAs) activate production of AphA, and AphA and the sRNAs repress production of LuxR/HapR. Conversely, at HCD, LuxR/HapR represses *aphA*. This network architecture ensures maximal AphA production at LCD and maximal LuxR/HapR production at HCD. Microarray analyses reveal that 300 genes are regulated by AphA at LCD in *V. harveyi*, a subset of which is also controlled by LuxR. We propose that reciprocal gradients of AphA and LuxR/HapR establish the quorum-sensing LCD and HCD gene expression patterns, respectively.

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Quorum sensing is a mechanism of bacterial cell–cell communication. This process relies on the production, release, and detection of extracellular signaling molecules called autoinducers (AIs) that drive gene expression programs underlying collective behaviors such as virulence factor production, biofilm formation, and bioluminescence (Davies et al. 1998; Hammer and Bassler 2003; Novick 2003; Ng and Bassler 2009). The bioluminescent marine bacterium *Vibrio harveyi* produces three AIs that are detected by cognate two-component membrane-bound receptors (Ng and Bassler 2009). At low cell density (LCD), the extracellular concentration of AIs is low and the unliganded receptors function as kinases, shuttling phosphate to the response regulator called LuxO (Freeman and Bassler 1999). LuxO~P activates transcription of five genes encoding homologous small noncoding RNAs (sRNAs) (Lenz et al. 2004; Tu and Bassler 2007). These sRNAs are called the Qrr sRNAs (Fig. 1). At LCD, with the assistance of Hfq, the Qrr sRNAs base-pair with the mRNA encoding the master quorum-sensing regulator LuxR and prevent its translation (Fig. 1; Tu and Bassler

2007). In the absence of LuxR, genes required for individual behaviors are expressed, while genes required for group behaviors are not. At high cell density (HCD), the extracellular concentration of AIs is high and the AIs are bound by their cognate receptors. AI binding switches the receptors from kinases to phosphatases (Neiditch et al. 2006; Swem et al. 2008), and this reverses the flow of phosphate through the circuit, leading to dephosphorylation of LuxO and cessation of *qrr* transcription (Fig. 1). In the absence of the Qrr sRNAs, *luxR* mRNA is translated, LuxR protein is produced, and LuxR controls the cascade of genes underlying collective behaviors (Swartzman et al. 1992; Henke and Bassler 2004; Waters and Bassler 2006; Ng and Bassler 2009).

The Qrr sRNA pool dictates whether the LCD or HCD gene expression program is executed, due to the principal role the Qrr sRNAs play in regulating production of the master quorum-sensing regulator LuxR. Underscoring their importance, several regulatory feedback loops exist to precisely maintain the pool of Qrr sRNAs at appropriate levels. First, LuxR activates *qrr* expression, which increases Qrr sRNA production (Svenningsen et al. 2008; Tu et al. 2008). The Qrr sRNAs feed back to repress *luxR* mRNA translation, causing reduced LuxR production. This, in turn, reduces Qrr sRNA production. Second, the Qrr sRNAs inhibit translation of *luxO*, which decreases Qrr levels because *qrr* expression requires

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Figure 1. Model for reciprocal quorum-sensing control of AphA and LuxR. (*Left*) At LCD, low concentrations of AIs lead to phosphorylation of LuxO (LuxO~P), which activates expression of the *qrr1–5* genes encoding five redundant sRNAs (Qrr1–5). The Qrr sRNAs promote translation of the mRNA encoding AphA and inhibit translation of the mRNA encoding LuxR. AphA represses *luxR*, and AphA feeds back to repress *qrr* expression. (*Right*) At HCD, high concentrations of AIs reverse the phosphate flow in the circuit, leading to dephosphorylation of LuxO and cessation of Qrr sRNA production. AphA translation ceases and LuxR translation occurs. LuxR represses *aphA*, and LuxR feeds back to activate *qrr* expression. This network architecture results in reciprocal gradients of production of AphA and LuxR: Maximal AphA and minimal LuxR production occur at LCD, while minimal AphA and maximal LuxR production occur at HCD. AphA and LuxR are global transcriptional regulators, and they, individually and together, affect the expression of hundreds of target genes.

LuxO~P (Svenningsen et al. 2009; Tu et al. 2010). Finally, the Qrr sRNAs also repress expression of genes encoding the AI synthases and cognate receptors (Mehta et al. 2009; S Teng, JN Schaffer, KC Tu, P Mehta, W Lu, NP Ong, BL Bassler, and NS Wingreen, in prep.). This loop fine-tunes AI:receptor ratios in response to changing AI concentrations, again altering LuxO~P levels and changing the Qrr sRNA pool. Together, these feedback loops adjust the levels of Qrr sRNAs such that they precisely track with changes in AI levels.

We hypothesized previously that, in addition to LuxO~P and LuxR, other regulators could control *V. harveyi* *qrr* gene expression (Tu and Bassler 2007; Tu et al. 2008). This idea came from our findings that the five *qrr* genes exhibit different expression profiles, and distinct DNA sequence motifs indicative of transcription factor-binding sites exist upstream of each *qrr* gene (Tu and Bassler 2007). Here, to identify putative regulatory factors, we performed a Tn5 mutagenesis screen for insertions altering *qrr4* expression. This analysis identified AphA as a repressor of *qrr4* (Fig. 1). AphA is a winged-helix transcription factor that controls virulence factor production in the closely related pathogen *Vibrio cholerae* (Skorupski and Taylor 1999; De Silva et al. 2005). We show that, in addition to repressing *qrr4*, AphA represses *luxR*. This finding, coupled with previous re-

ports demonstrating that LuxR represses *aphA* (Lin et al. 2007; Pompeani et al. 2008), establishes that AphA and LuxR mutually repress each other. We also demonstrate that the Qrr sRNAs activate *aphA*. The regulatory architecture linking the Qrr sRNAs, LuxR, and AphA causes AphA to be maximally produced at LCD and LuxR to be maximally produced at HCD, with the Qrr sRNAs acting as the switch governing which of these transcription factors is expressed (Fig. 1). Microarray analyses show that AphA controls nearly 300 genes at LCD in *V. harveyi*, some of which overlap with known targets of LuxR. Targets of AphA include genes involved in type III secretion, flagella synthesis, and pilus production, and members of the quorum-sensing circuit (*qrrs*, *luxR*, and *aphA*). Finally, we show that AphA plays an analogous role in the quorum-sensing cascade of *V. cholerae*, further solidifying the link between quorum sensing and virulence (Kovacikova and Skorupski 2002; Kovacikova et al. 2003).

Results

AphA regulates qrr expression

Previous work suggested that factors in addition to LuxO~P and LuxR could regulate expression of the *qrr* genes in *V. harveyi* (Tu and Bassler 2007; Tu et al. 2008). To identify such factors, we performed a random Tn5 mutagenesis screen in *V. harveyi* and assessed the mutants for altered fluorescence from a *qrr4-gfp* transcriptional fusion. Because the *qrr* genes are expressed at LCD (Lenz et al. 2004; Tu and Bassler 2007), we used a *V. harveyi* mutant locked in the LCD state as the parent strain for the mutagenesis (TL45). We reasoned that insertions inactivating repressors of *qrr4* expression would result in mutants exhibiting increased GFP production, while mutants harboring insertions in activators of *qrr4* expression would exhibit decreased GFP production. Importantly, the strain used for this experiment also lacked *luxR*, which enabled us to avoid identifying components that function indirectly through LuxR, a known activator of *qrr4* (Tu et al. 2008).

We isolated 50 Tn5 insertion mutants displaying altered *qrr4-gfp* expression (~200,000 mutants were screened). Fourteen insertions caused increased Qrr4-GFP production, and 36 insertions caused decreased fluorescence. Here, we focus on one transposon insertion mutant exhibiting increased Qrr4-GFP production (Fig. 2A). This Tn5 insertion mapped to *V. harveyi* gene VIBHAR_00046. This gene, called *aphA*, is conserved among vibrios and encodes a winged-helix transcription factor (Skorupski and Taylor 1999; De Silva et al. 2005). Introduction of a plasmid carrying the *aphA* gene together with its 380-base-pair (bp) upstream region decreased *qrr4-gfp* expression to the level observed in the parent strain (Fig. 2A). This result confirms that transposon disruption of *aphA* caused the observed increase in *qrr4-gfp* expression, suggesting that AphA is a repressor of *qrr4*. We examined whether AphA also affects expression of the other *V. harveyi* *qrr* genes by measuring *qrr* expression in strains

Figure 2. AphA regulates *qrr* gene expression. (A) Fluorescence from a chromosomal *qrr4-gfp* transcriptional fusion was measured in a locked LCD *V. harveyi* strain (TL45: Δ *luxM*, Δ *luxS*, Δ *cqsS*, Δ *luxR*, and Δ *qrr4::gfp*) and in the same strain carrying a Tn5 insertion in *aphA* (STRVh0095: TL45, *aphA::Tn5*). The insertion mutant was complemented with a plasmid carrying *aphA*, denoted *paphA* (pSTR0502). Means and SEMs of measurements from quadruplicate overnight cultures are shown. (B) *Qrr1*, *Qrr2*, *Qrr3*, *Qrr4*, and *Qrr5* sRNA levels were measured by quantitative RT-PCR (qRT-PCR) in locked LCD *V. harveyi* strains lacking *luxR* and containing *aphA* (KM810: *luxO* D47E, Δ *luxR*) (white bars) or lacking *aphA* (JV55: KM810, Δ *aphA*) (black bars). Means and SEMs for RNA collected from three independent cultures are shown. (C) Fluorescence from a *qrr4-gfp* transcriptional fusion on a plasmid (pKT1136) was measured in *E. coli* MC4100 carrying *luxO* D47E on the chromosome at the λ *att* site (STR0018). This strain contained an empty vector (pASK75) or a vector carrying *aphA*, denoted *paphA* (pSTR0538). Means and SEMs for triplicate overnight cultures are shown.

containing and lacking *aphA*. Similar to the logic we used in the initial Tn5 screen, this experiment was performed in a locked LCD *V. harveyi* strain lacking *luxR*. Deletion of *aphA* increased expression of *qrr2*, *qrr3*, and *qrr4* (Fig. 2B). Thus, AphA represses *qrr2*, *qrr3*, and *qrr4* at LCD and repression is LuxR-independent. Expression of *qrr1* remained unchanged in the presence and absence of *aphA*, indicating that AphA does not regulate *qrr1*. Although *qrr5* expression is normally low (Tu and Bassler 2007), expression decreased modestly in the Δ *aphA* strain, indi-

cating that AphA could activate *qrr5* expression. However, this decrease did not occur in the Δ *aphA* strain containing LuxR (Supplemental Fig. S1). We are currently investigating the role of AphA (and potentially other factors) in regulation of *qrr1* and *qrr5*.

The above results show that, at LCD, AphA represses *qrr2*, *qrr3*, and *qrr4*. We used two strategies to determine whether AphA acts directly or indirectly on the *qrr4* promoter. First, *qrr4* expression was measured in the presence and absence of AphA in *Escherichia coli* carrying the *V. harveyi luxO* D47E gene on the chromosome. LuxO D47E is a LuxO~P mimic that constitutively activates transcription of the *qrr* promoters (Freeman and Bassler 1999). We reasoned that, if AphA repressed *qrr4-gfp* expression in this *E. coli* strain, it would indicate that AphA acts directly on the *qrr4* promoter, because no quorum-sensing components other than LuxO D47E are present in *E. coli*. Indeed, when AphA was introduced into *E. coli*, threefold repression of *qrr4-gfp* occurred, consistent with the results in *V. harveyi* (Fig. 2C). Second, we purified the AphA protein and examined binding to *qrr4* promoter DNA. Five ~50-bp fluorescently labeled probes covering

Figure 3. AphA binds to the *qrr4*, *luxR*, and *aphA* promoters. (A) Five fluorescently labeled DNA fragments were used to assess AphA binding. The schematic illustrates the *qrr4* promoter, but the positions are similar for *luxR* and *aphA*. (B) Representative fluorescence anisotropy binding curves for the *qrr4* promoter probe set from A are shown. Each point displays the mean and SEM of three separate binding reactions at each concentration of AphA. GraphPad software was used to fit one-site-specific non-linear binding curves. (C) Summary of binding assays for AphA binding to probes in the *qrr4*, *luxR*, and *aphA* promoters. The promoter, the probe number, the position of the probe in base pairs relative to the predicted transcription start sites, and the calculated K_d in nanomolar for probes exhibiting binding are shown (see Supplemental Fig. S3 for individual binding curves). The K_d s and SDs are interpolated from curves fit to the averages of three separate samples tested at each concentration.

the *qrr4* promoter from -180 bp to $+50$ bp relative to the transcription start site were tested (Fig. 3A). Specific binding occurred only to a fragment carrying DNA overlapping the LuxO~P-binding site (-126 to -76 relative to the transcription start site) (Fig. 3B). AphA bound this fragment with a K_d of ≈ 19 nM (Fig. 3C). Together, the above data show that AphA binds directly to the *qrr4* promoter to repress transcription. We expect that AphA uses the same mechanism to repress *qrr2* and *qrr3*.

AphA regulates *luxR* expression

The *V. harveyi* quorum-sensing circuit contains numerous regulatory feedback loops involving the Qrr sRNAs (Fig. 1; Ng and Bassler 2009). Above, we demonstrated that AphA represses the *qrr* genes (Fig. 2), and we know that the Qrr sRNAs repress *luxR* (Tu and Bassler 2007). Thus, we wondered what consequence altering AphA levels would have on *luxR*. Our prediction was that increased AphA would decrease *qrr* expression, promoting increased LuxR production. Quite to the contrary, when we overexpressed *aphA*, *luxR* expression decreased (Supplemental Fig. S2A). This result suggests that AphA represses *luxR*, and, furthermore, this repressive effect overrides any increased LuxR production stemming from AphA-directed repression of the *qrr* genes. *luxR* expression exhibited a similar decrease when *aphA* was overexpressed in a *V. harveyi* strain lacking the Qrr sRNAs ($\Delta qrr1-5$) (Fig. 4A), indicating that AphA repression of *luxR* does not depend on the Qrr sRNAs. AphA repression of *luxR* requires a functional AphA protein because, when a DNA-binding-defective AphA mutant protein (AphA K63E) (Kovacikova et al. 2004) was overexpressed, *luxR* expression was not altered (*aphA**) (Fig. 4A).

To examine whether AphA repression of *luxR* is direct, we measured AphA binding to DNA fragments spanning the *luxR* promoter region. Specific AphA binding ($K_d \approx 137$ nM) occurred immediately downstream from the *luxR* transcription start site ($+6$ to $+37$ relative to the transcription start site) (Fig. 3C; Supplemental Fig. S3B). Together, these data indicate that AphA represses *V. harveyi* *luxR* expression by binding to the *luxR* promoter.

AphA represses its own expression

One mechanism bacteria commonly use to maintain appropriate levels of transcription factors is autorepression (Beckstein and Serrano 2000; Rosenfeld et al. 2002; Nevozhay et al. 2009). Indeed, both of the known quorum-sensing transcription factors, LuxR and LuxO, display autorepression (Fig. 1; Chatterjee et al. 1996; Tu et al. 2010). Likewise, AphA autorepresses itself in *V. cholerae* (Lin et al. 2007). To address whether this is the case for AphA in *V. harveyi*, we measured *aphA* transcript levels using probes complementary to the *aphA* 5' untranslated region (UTR). This strategy allowed us to exclusively follow chromosomally encoded *aphA*, since this upstream DNA was not present on the *aphA* overexpression construct. Overexpression of *aphA* in the locked LCD strain lacking *luxR* resulted in a twofold reduction in chromosomally

Figure 4. AphA represses expression of *luxR* and *aphA*. (A) *luxR* expression was monitored by qRT-PCR in a $\Delta qrr1-5$ (KT282: $\Delta qrr1-5$) strain carrying an empty vector (pJV17), a vector expressing *V. harveyi* *aphA* driven by an IPTG-inducible pTac promoter (*paphA*; pSTR0504), or the same vector expressing an *aphA* mutant defective for DNA binding (*paphA**; pSTR0615) (Kovacikova et al. 2004). Averages and SEMs are shown. (B) Expression of *V. harveyi* chromosomal *aphA* was monitored by qRT-PCR in a locked LCD strain lacking *luxR* (KM812: *luxO* D47E, $\Delta luxR$). The primers used in the qRT-PCR reactions were specific to the 5' UTR, which is not encoded on the overexpression vector *paphA* (pSTR0504). Means and SEMs for three independent cultures are shown.

encoded *aphA* expression (Fig. 4B), indicating that AphA modestly represses its own expression independently of LuxR. AphA autorepression also occurred in a strain lacking the Qrr sRNAs ($\Delta qrr1-5$) (Supplemental Fig. S2B), indicating that AphA repression of *aphA* does not occur through the Qrr sRNAs. Repression did not occur when the DNA-binding-defective *aphA* mutant (*aphA**) was overexpressed (Fig. 4B; Supplemental Fig. S2B). We confirmed that AphA autorepression is direct using DNA-binding analyses. AphA specifically bound only to a fragment of DNA harboring the putative RNA polymerase binding site (-36 to $+14$ relative to the *aphA* transcription start site) with a K_d of ≈ 60 nM (Fig. 3C; Supplemental Fig. S3C).

LuxR and the Qrr sRNAs regulate *aphA* expression

LuxR has been shown previously to bind to the *aphA* promoter to repress *aphA* expression (Fig. 1; Lin et al. 2007; Pompeani et al. 2008). We confirmed this LuxR function by comparing *aphA* expression in *V. harveyi* strains producing low and high levels of LuxR. To test the effects of low levels of LuxR, we used a locked LCD strain (*luxO* D47E) in which the Qrr sRNAs are constitutively expressed and prevent production of LuxR. To test the

Our genetic analyses revealed four targets in the *V. harveyi* quorum-sensing circuit (*qrr2*, *qrr3*, *qrr4*, and *luxR*) whose transcription is controlled by AphA (Fig. 1). Given that AphA is a transcription factor, we wondered whether it controls the expression of additional *V. harveyi* genes. We performed microarray analyses to test this possibility. Our above findings demonstrate that AphA is maximally produced at LCD. For this reason, we compared the gene expression profiles of locked LCD *V. harveyi* strains possessing and lacking *aphA*. Again, as in our above analyses, we used $\Delta luxR$ strains to eliminate complications from indirect effects of LuxR. We identified 296 genes that were expressed at levels significantly above background ($P < 0.0001$) and whose expression changed more than twofold (Supplemental Table S1). Our results suggest that AphA activates 99 of these genes and represses the other 197 genes. Among the repressed group were *qrr2*, *qrr3*, and *qrr4*, confirming our above observations (Fig. 2). About half of the AphA-controlled genes (156) are classified as hypothetical genes. The remaining 140 genes have predicted functions, including in type III secretion, reductases/dehydrogenases, pilus

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production, flagellar structure, and gene expression (Table 1; Supplemental Table S1).

Several of the AphA-controlled genes, such as the *qrr* genes and genes involved in type III secretion, are especially interesting to us because they are known to be regulated by LuxR (Henke and Bassler 2004; Waters and Bassler 2006; Tu et al. 2008). However, *luxR* was deleted in the strains used for the microarrays. Thus, we validated the microarray results and tested the role of LuxR by measuring AphA and/or LuxR regulation of a number of candidate targets at LCD (Fig. 6). One group of genes is regulated exclusively by AphA (for example, VIBHAR_06904) (Fig. 6A). A second class of genes is repressed by both AphA and LuxR (for example, VIBHAR_01726) (Fig. 6B). Finally, we identified a set of genes that is regulated by both AphA and LuxR, but by opposing means: One transcription factor represses expression, while the other activates expression (for example, VIBHAR_05035) (Fig. 6C). These results show that, first, AphA controls a large repertoire of genes at LCD that are not controlled by LuxR, and second, AphA and LuxR jointly regulate another set of genes.

AphA has a role in the V. cholerae quorum-sensing circuit

In *V. cholerae*, AphA is involved in the regulatory cascade promoting virulence. Specifically, AphA activates *tcpPH*, and TcpPH is required for expression of *toxT*, which encodes a major virulence regulator in *V. cholerae* (Kovacicova and Skorupski 1999, 2001; Skorupski and Taylor 1999). *V. cholerae* AphA is 86% identical to *V. harveyi* AphA. Both *V. harveyi* and *V. cholerae* use the Qrr sRNAs to control expression of their corresponding master quorum-sensing regulators, LuxR and HapR, respectively (Lenz et al. 2004; Tu and Bassler 2007). In addition, LuxR/HapR represses *aphA* in both species (Kovacicova and Skorupski 2002; Lin et al. 2005; Pompeani et al. 2008). Given our above results demonstrating the central location of AphA in the *V. harveyi* quorum-sensing cascade, we wondered whether *V. cholerae* AphA plays an analogous role. That is, in *V. cholerae*, does AphA regulate *qrr* and *hapR* expression, and do the Qrr sRNAs regulate *aphA* expression? We test these possibil-

Table 1. Classes of genes regulated by AphA more than twofold as determined by microarray analysis

Function	Number of genes
Type III secretion apparatus	38
Metabolism	38
Oxidoreductases	16
Flagellar apparatus	14
Membrane-associated	11
Stress-related	9
Gene expression	8
Quorum-sensing	3
Pilus-related	3
Hypothetical	156

See Supplemental Table S1 for a complete list of the genes.

Figure 6. AphA and LuxR individually and jointly control target genes. RNA encoding candidate target genes was measured by qRT-PCR in the LCD-locked *V. harveyi* strains containing *aphA* (JAF548: *luxO* D47E), lacking *aphA* (JV50: JAF548, Δ*aphA*), lacking *luxR* (KM810: JAF548, Δ*luxR*), and lacking *aphA* and *luxR* (JV55: KM810, Δ*aphA*). Primers specific to VIBHAR_06904 (A), VIBHAR_01726 (B), and VIBHAR_05035 (C) were used. RNA was collected from three independent cultures, and means and SEMs are shown.

ities below. First, as a control, we verified that, in our hands, *V. cholerae* AphA can activate a known target, *vpsT* (Yang et al. 2009), by measuring expression of a *vpsT-lux* reporter following overexpression of *V. cholerae aphA*. Overproduction of AphA resulted in dramatically increased *vpsT-lux* expression (~12-fold) (Fig. 7A), consistent with previous findings that AphA activates *vpsT* expression (Yang et al. 2009).

To examine the effect of AphA on *V. cholerae qrr* gene expression, we used the same strategy that we used above in *V. harveyi*. Specifically, to maximize *qrr* gene expression and eliminate indirect effects caused by HapR, we used an LCD-locked *V. cholerae* strain lacking *hapR*. In this strain, deletion of *aphA* increased expression of *qrr2*, *qrr3*, and *qrr4* (Fig. 7B), showing that, in *V. cholerae*, as in *V. harveyi*, AphA represses *qrr2*, *qrr3*, and *qrr4* expression independently of HapR. To test whether AphA controls *hapR* expression, we overexpressed *aphA* and measured *hapR* expression at HCD (when HapR levels are at their maximum). Indeed, overexpression of *aphA* repressed *hapR* expression (Fig. 7C). Finally, we measured *aphA* expression in *V. cholerae* strains lacking *hapR* that were locked at HCD or LCD. As a reminder, in the HCD strain, Qrr levels are low, while in the LCD strain, Qrr levels are high. This strategy allowed us to determine whether *V. cholerae aphA* is regulated by the Qrr sRNAs in the absence of HapR. Indeed, expression of *aphA* was threefold higher in the LCD-locked mutant than in

Figure 7. *V. cholerae* AphA represses expression of *hapR* and *qrr4*, and the *V. cholerae* Qrr sRNAs activate expression of *aphA*. (A) Bioluminescence from a *vpsT-lux* transcriptional fusion on a plasmid (pDL1711) was assayed in a *V. cholerae* locked HCD strain (WN865: Δ *luxO*) in the presence of an empty vector (pJV17) or a vector carrying *V. cholerae* *aphA* (pSTR0612). (B) *Qrr2*, *Qrr3*, and *Qrr4* sRNA levels were measured by qRT-PCR in locked LCD *V. cholerae* strains lacking *hapR* and containing *aphA* (JC1796: *luxO* D47E, Δ *hapR*) (white bars) or lacking *hapR* and *aphA* (STRVc0044: JC1796, Δ *aphA*) (black bars). Means and SEMs for RNA collected from three independent cultures are shown. (C) *hapR* expression was measured directly using the QuantiGene Plex Reagent System (Panomics) (Tu et al. 2010). RNA was collected from an HCD-locked *V. cholerae*

strain containing *hapR* (WN865: Δ *luxO*). The strain carried an empty vector (pJV17) or a vector overexpressing *V. cholerae* *aphA*, denoted *paphA*(Vc) (pSTR0612). (D) *aphA* RNA was measured by the same procedure in a locked HCD strain lacking *hapR* (WN868: WN865, Δ *hapR*) and a locked LCD strain lacking *hapR* (JC1796: *luxO* D47E, Δ *hapR*). In all cases, RNA from at least three independent cultures was analyzed. Means and SEMs are shown.

the HCD-locked mutant (Fig. 7D), demonstrating that the Qrr sRNAs likely activate expression of *V. cholerae* *aphA*. We therefore conclude that AphA controls the flow of information through both *V. harveyi* and *V. cholerae* quorum-sensing circuits.

Discussion

The quorum-sensing bacterium *V. harveyi* produces, detects, and responds to three AIs to control collective behaviors. At LCD, LuxO~P activates transcription of the *qrr1*–5 genes, encoding five redundant sRNAs (*Qrr1*–5), which repress translation of the master quorum-sensing regulator LuxR. Here we show that, at LCD, the Qrr sRNAs simultaneously activate production of another transcription factor, AphA. At HCD, the detection of AIs results in dephosphorylation of LuxO. Qrr sRNA production stops, which eliminates activation of *aphA* expression, and, in contrast, allows LuxR production to commence. Further reinforcement of this pattern comes from mutual repression of *aphA* and *luxR*. Specifically, AphA represses *luxR* expression at LCD and LuxR represses *aphA* expression at HCD. Additionally, AphA and LuxR both feed back to control *qrr* expression. The consequence of this network architecture is reciprocal control of AphA and LuxR. At LCD, AphA predominates: The Qrr sRNAs are present and activate translation of *aphA*, and AphA represses *luxR* (Fig. 1). At HCD, LuxR predominates: The Qrr sRNAs are not present to repress *luxR* or activate *aphA*, and, furthermore, LuxR represses *aphA* (Fig. 1). This same network architecture reciprocally controls the levels of AphA and HapR (a homolog of LuxR) in the *V. cholerae* quorum-sensing circuit.

Whether AphA, LuxR, or both are produced is determined by the pool of five Qrr sRNAs. The primary

mechanism controlling Qrr sRNA production is via the cascade that phosphorylates or dephosphorylates LuxO in response to the absence or presence of AIs, respectively (Freeman and Bassler 1999). We predict that, at each particular concentration of LuxO~P, there is a corresponding pool size of the Qrr sRNAs. This pool, in turn, establishes that particular amounts of AphA and LuxR are present. We are interested in understanding the mechanisms underlying Qrr sRNA activation (*aphA*) and repression (*luxR* and *luxO*) of translation of mRNA targets. We performed some studies of Qrr-directed repression of *luxR* and *luxO* translation (Lenz et al. 2004; Tu and Bassler 2007; Svenningsen et al. 2009; Tu et al. 2010). Repression occurs by Hfq-dependent Qrr sRNA base-pairing with the target mRNA over the ribosome-binding site and translation initiation codon. Binding at this site occludes the ribosome and exposes the target mRNAs to degradation. Regarding Qrr sRNA-directed activation of *aphA* expression, secondary structure predictions of the *aphA* mRNA indicate that stem-loops exist in the *aphA* 5' UTR (data not shown). One of these putative stem-loops contains the predicted ribosome-binding site and start codon. This scenario is analogous to the 5' UTR of sRNA-activated target mRNAs in other bacterial systems (Frohlich and Vogel 2009). At these targets, sRNAs base-pair with an mRNA region that does not overlap with the ribosome-binding site. Base-pairing disrupts inhibitory stem-loop structures, allowing the ribosome access to the mRNA, stabilizing the mRNA, or both (Majdalani et al. 1998; Hammer and Bassler 2007; Prevost et al. 2007; Landt et al. 2010).

Qrr sRNA control, coupled with mutual AphA–LuxR repression, likely allows *V. harveyi* to generate a variety of AphA:LuxR concentration ratios. Each blend of these two transcription factors will, likewise, drive a precise

pattern of gene expression. If AphA and LuxR did not jointly control genes, AphA could repress or activate genes at LCD and LuxR could repress or activate genes at HCD. However, the AphA and LuxR regulons overlap, as shown by our microarray analyses, providing the cell with access to more sophisticated regulatory programs. Genes controlled by both LuxR and AphA can theoretically fall into four categories: AphA and LuxR both repress, AphA and LuxR both activate, AphA activates and LuxR represses, and AphA represses and LuxR activates. Additionally, each factor will have a specific strength of regulation at each promoter. Having two regulators of varying strength impinging on the same promoters could establish a combinatorial pattern of gene regulation in response to changing AI concentrations and different cell densities.

Type III secretion genes stand as one example of coregulation by LuxR and AphA. In this case, both regulators repress expression (VIBHAR_01726) (for example, Fig. 6B; Supplemental Table S1; Henke and Bassler 2004; Waters and Bassler 2006). Thus, production of the type III secretion apparatus is repressed at LCD and HCD, but by AphA in the first case and by LuxR in the second case. When, then, are type III secretion genes expressed? We propose that, as cells transition from LCD to HCD, at a specific concentration of extracellular AIs, there will simultaneously exist low levels of both AphA and LuxR, and this is the window of cell densities during which the type III secretion system is maximally expressed. Another gene coregulated by LuxR and AphA is the hypothetical protein VIBHAR_05035, which is repressed by AphA and activated by LuxR (Fig. 6C). This gene is repressed by two mechanisms at LCD (repression by AphA and lack of activation by LuxR). The gene is activated at HCD when AphA levels are low and LuxR levels are high. This regulatory wiring ensures that VIBHAR_05035 is expressed only at HCD. We identified two other genes likely to exhibit the VIBHAR_05035 pattern of regulation (VIBHAR_06262 and VIBHAR_06741) (Waters and Bassler 2006). We are currently characterizing the AphA and LuxR regulons to define the mode and strength of regulation by AphA, LuxR, and the two transcription factors together at each promoter. We anticipate that future experiments will identify examples of all four regulatory combinations described above.

The newly appreciated role of AphA as an LCD master regulator begins to account for some puzzling aspects of the quorum-sensing network. One outstanding puzzle is why the circuit uses five highly similar sRNAs (Tu and Bassler 2007). Obviously, genetic amplification of sRNAs immediately implies an increase of total sRNA production. Among other effects, this increased sRNA production accelerates the transition of cells from HCD to LCD (Mehta et al. 2008). We focus on this transition because other features of the network also seem engineered to achieve rapid sRNA production upon a switch to LCD. Specifically, phosphorylation of LuxO following removal of AIs is rapid, while the decrease of the sRNA activators LuxO and LuxR is slow, implying that maximal sRNA production occurs immediately following a switch to

LCD (Tu and Bassler 2007; Teng et al. 2010; Tu et al. 2010). Physiologically, the transition from HCD to LCD can be as fast as expulsion of cells from a host or shedding from a biofilm, and so a rapid change in gene expression in this case may be highly advantageous. In contrast, the LCD-to-HCD transition presumably depends on cell growth and is therefore likely to be slow, requiring at least several cell generations. Rapid production of sRNAs following a switch to LCD is beneficial only if these sRNAs can, in turn, rapidly affect protein levels. This evident fact has presented a puzzle: The known targets of the sRNAs—e.g., LuxO and LuxR—are negatively regulated, implying that rapid production of sRNAs can only halt protein production, leaving the existing pools of protein to decay slowly via dilution by growth, as no proteolysis of LuxO or LuxR has been observed. The answer may lie in our discovery that *aphA* is positively regulated by the sRNAs: sRNA activation of *aphA* translation, with silent *aphA* transcripts that presumably already exist in the cell at HCD, likely enables a rapid, many-fold increase in the level of AphA immediately following the switch to LCD. Thus, positive regulation of AphA may provide the missing piece of the puzzle of why the multiple redundant sRNAs exist in the *V. harveyi* quorum-sensing network; namely, multiple sRNA genes means a higher total sRNA peak production rate, and, consequently, a faster accumulation of AphA at the HCD-to-LCD transition. What about the additional regulatory links we found to AphA? First, the negative regulation of *aphA* expression by LuxR, while reducing AphA production at the switch to LCD, is unlikely to affect the fold increase of AphA, and may be required to keep AphA levels low at HCD. Second, we expect that the negative feedback of AphA on sRNA production is present to ensure that the rapid production of sRNAs at the switch to LCD is reduced to a tolerable steady-state level once AphA accumulates (and notice that the decrease of LuxR at LCD, by reducing sRNA activation, has a parallel effect) (Tu et al. 2008). Third, repression of *luxR* by AphA helps keep LuxR levels low at LCD. Finally, the negative feedback of AphA on itself is likely to play a similar homeostatic role for AphA levels that LuxR autorepression plays for LuxR levels (Tu et al. 2008). Interestingly, the presence of AphA at LCD may contribute to the asymmetrically slow response of cells following addition of AI, since not only must LuxR accumulate, but AphA must also be diluted by growth for cells to transition to the HCD gene expression program. Overall, the *V. harveyi* network architecture seems to be strongly influenced by the dynamics of quorum-sensing transitions. We will directly explore these dynamics, in both wild-type cells and mutants engineered to change network architecture, in future experiments.

AphA has been well-studied for its role in controlling virulence in *V. cholerae*. Specifically, AphA, together with AphB, activates *tcpPH* expression, and TcpPH subsequently activates *toxT*, encoding a major *V. cholerae* virulence regulator (Kovacikova and Skorupski 1999, 2001; Skorupski and Taylor 1999; Kovacikova et al. 2004). In *V. cholerae*, AphA controls at least 15 additional target

genes, some of which are involved in biofilm formation or motility (Kovacikova and Skorupski 2001; Kovacikova et al. 2003, 2005; Yang et al. 2009). Our results indicate that AphA controls a much larger regulon of genes than this at LCD in *V. harveyi* (and thus possibly also in *V. cholerae*). These target genes were revealed using *V. harveyi* strains locked at LCD and lacking *luxR*. Previous studies of AphA in *V. cholerae* examined cells under quite different conditions than the ones we used here. Specifically, the earlier *V. cholerae* microarray analyses were performed at a relatively HCD in strains possessing and lacking *aphA* (Kovacikova et al. 2005). Furthermore, HapR was wild type in these strains. Based on our results with *V. harveyi* and our preliminary results with *V. cholerae*, we suspect that HapR was the transcription factor that primarily functioned under the previous experimental conditions, and this could account for why so few genes were identified to be controlled by AphA (Supplemental Fig. S4). We predict that, at LCD, AphA regulates many additional *V. cholerae* genes. Some of these genes are likely to be critical for pathogenicity, given that *V. cholerae* produces its suite of virulence factors only at LCD (Kovacikova and Skorupski 2002; Miller et al. 2002).

We made progress in determining how *V. harveyi* LuxR controls gene expression by defining its DNA-binding site and decoding its regulon (Waters and Bassler 2006; Lee et al. 2008; Pompeani et al. 2008), but we understand comparatively little about AphA in *V. harveyi*. However, the mechanisms of AphA repression and activation have received attention in *V. cholerae*, which should guide our continued analyses (Kovacikova and Skorupski 2001; Kovacikova et al. 2003, 2004, 2005, 2010; De Silva et al. 2005; Yang et al. 2009). The preliminary results described here suggest that, in *V. harveyi*, AphA uses at least two mechanisms for repression. First, we take the case of *qrr4*: AphA binds to the *qrr4* promoter where LuxO~P binds. LuxO~P binding is required for activation (Lenz et al. 2004; Tu and Bassler 2007), and thus AphA binding likely excludes LuxO~P binding. Interestingly, AphA does not repress *qrr1* or *qrr5* expression. Differences in positioning of the LuxO~P site at each of these promoters (Tu and Bassler 2007) and the strength of AphA binding could account for our findings. Second, we take the case of the *luxR* and *aphA* promoters: AphA binds in close proximity to the transcription start sites near where RNA polymerase binds. AphA occupancy likely prevents RNA polymerase from binding. This second mechanism is analogous to how AphA represses the *alsD* promoter in *V. cholerae* (Kovacikova et al. 2005).

Understanding LuxR/HapR and its regulon will provide insight into the HCD lifestyle of vibrios, presumably relevant when they occupy hosts. Likewise, understanding AphA and its regulon should give clues about the LCD lifestyle of vibrios, presumably relevant when they are free-living in the environment. Based on the present findings, we predict that AphA-activated genes are beneficial for individual behaviors, while AphA-repressed genes are useful for collective behaviors. Additionally, placing AphA, an LCD regulator, at the center of the quorum-sensing circuit likely allows vibrios to optimally adjust from high expression of genes associated

with a stressful stationary-phase environment to high expression of genes that adapt the bacteria to the nutrient-abundant rapid-growth phase. Consistent with this notion, we identified numerous AphA-repressed oxidases and reductases that could be involved in alleviating oxidative stress during stationary phase (Supplemental Table S1; Nystrom and Gustavsson 1998; Chang et al. 2002). Likewise, we identified an AphA-repressed protease and an oligopeptidase that could be involved in preventing protein accumulation during stationary phase (Supplemental Table S1; Gottesman 2003; Weichart et al. 2003). We suggest that AphA is used at LCD to promote individual behaviors and prevent expression of genes required for growth under stressful environmental conditions, whereas LuxR is present at HCD to promote collective behaviors and control the genetic program useful for nutrient-limiting situations.

Materials and methods

Bacterial strains and media

V. harveyi strain BB120 (BAA-1116) (Bassler et al. 1997) and derivatives were grown in Luria-Murine (LM) medium with shaking at 30°C. *E. coli* strains S17-1 λ pir (de Lorenzo and Timmis 1994), BL21(DE3) (Invitrogen), MC4100 (Casadaban 1976), and derivatives, and *V. cholerae* C6706 biovar El Tor (Thelin and Taylor 1996) were grown in Luria-Bertani (LB) medium with shaking at 30°C unless otherwise noted. Strains used in this study are described in Supplemental Table S2. Antibiotics (Sigma) were used at the following concentrations: 100 μ g/mL ampicillin, 10 μ g/mL chloramphenicol, 100 μ g/mL kanamycin, 100 μ g/mL gentamicin, 50 μ g/mL polymyxin B, and 10 μ g/mL tetracycline (except 5 μ g/mL in liquid medium for *V. harveyi*). The Ptac promoter constructs were induced with 0.2 mM IPTG (in *V. harveyi*) or 0.05 mM IPTG (in *V. cholerae*). Levels of induction for wild-type *aphA* and the DNA-binding-defective mutant *aphA* (AphA K63E, *aphA*^{*}) (Kovacikova et al. 2004) were similar (Supplemental Fig. S5). *hfq* mRNA levels were not significantly affected by overexpression of wild-type *aphA* or the DNA-binding-defective *aphA* mutant (data not shown). Induction of *aphA*(Vc) in *V. cholerae* with higher IPTG concentrations resulted in a growth defect that we cannot explain; however, this growth defect was not observed when *aphA* was overexpressed in *V. harveyi*. *qrr4* on plasmid pSTR0227 was induced with 1 mM rhamnose. Plasmid constructs were introduced into electrocompetent *E. coli* S17-1 λ pir using 0.2 μ M cuvettes (USA Scientific) using a Bio-Rad MicroPulser. Plasmids were transferred into *V. harveyi* and *V. cholerae* by conjugation between *E. coli* S17-1 λ pir and *V. harveyi* and *V. cholerae* cultures on LB plates, followed by isolation of exconjugants on LM or LB plates containing polymyxin B and the appropriate antibiotic selection for plasmid maintenance.

DNA manipulations and mutant construction

E. coli S17-1 λ pir was used for all cloning procedures. DNA manipulations were performed as in Sambrook et al. (1989). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Antarctic phosphatase were purchased from New England Biolabs. PCR reactions used iProof DNA polymerase (Bio-Rad). Plasmids were constructed as described in Supplemental Table S3 using primers listed in Supplemental Table S4 that were purchased from Integrated DNA Technologies (IDT). QuickChange

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mutagenesis (Stratagene) was used to introduce the DNA-binding-defective mutation onto pSTR0504 (resulting in pSTR0615) using primers listed in Supplemental Table S4. All plasmids were confirmed by sequencing at Genewiz. *luxO* D47E was moved onto the *E. coli* chromosome using the λ InCh method (Boyd et al. 2000) and contained a promoter mutation rendering it unresponsive to Qrr sRNA regulation (Tu et al. 2010). Mutants in *V. harveyi* were constructed using λ red recombineering in *E. coli* S17- λ pir::pKD46 (Datsenko and Wanner 2000) on the pLAFR2 cosmid, which contained regions of the *V. harveyi* genome. The mutant cosmids (see Supplemental Table S3) were conjugated into *V. harveyi*, and mutant alleles were incorporated into the *V. harveyi* genome by homologous recombination (Bassler et al. 1993). Antibiotic markers were removed using FLP-mediated recombination by expressing FLP from a plasmid, pTL18 (Long et al. 2009). *V. cholerae* in-frame deletions were constructed as described (Skorupski and Taylor 1996).

Screen for regulators of *qrr4* expression

Locked LCD *V. harveyi* (TL45) (Long et al. 2009) was conjugated with *E. coli* S17 λ pir containing pRL27 (Larsen et al. 2002) overnight on LB plates, and the mixture was subsequently plated onto LM agar plates containing kanamycin and polymyxin B to select for exconjugants with Tn5 transposon insertions. A total of 2000–4000 colonies from at least six separate matings were pooled from the agar surface and resuspended in ~4 mL of LM medium supplemented with kanamycin and polymyxin B. Aliquots were added to glycerol and frozen at -80°C or immediately used for FACS analysis (Waters and Bassler 2006). A 0.5-mL aliquot of the well-suspended mutant pool was diluted into 4 mL of LM supplemented with kanamycin and polymyxin B and grown for 1–2 h at 30°C with aeration. At least three separate 100- μL aliquots of the culture were diluted into 1 mL of filtered $1\times$ PBS, and the cells were sorted using fluorescence-assisted cell sorting (Becton Dickinson FACSaria cell sorter). Approximately 200,000 events were screened corresponding to 200,000 cells containing Tn5 insertions, although not all insertions were unique. To screen for mutants in repressors of *qrr4-gfp*, cells exhibiting the highest levels of fluorescence were isolated (the top 1%). To screen for activators, cells exhibiting lowest fluorescence were isolated (the bottom 4%). These initial sorted populations were enriched by resorting. Dilutions of the final sorted population were plated onto LM agar supplemented with kanamycin and polymyxin B and incubated overnight at 30°C . Individual colonies were arrayed into 150 μL of LM supplemented with kanamycin and polymyxin B in separate wells of 96-well plates and grown with shaking overnight at 30°C . GFP was measured from the overnight cultures, and cultures exhibiting increased GFP were retained. A total of 50 mutants were obtained. The locations of the Tn5 insertions were mapped as described in the Supplemental Material.

RNA isolation

RNA used for microarray analyses and quantitative RT-PCR (qRT-PCR) was isolated from *V. harveyi* cultures at an $\text{OD}_{600} = 1.1$ – 1.2 using Trizol (Invitrogen) or by following the QIAgen RNeasy Minikit with RNeasy Protect protocol (Qiagen). See the Supplemental Material for details.

qRT-PCR

Following RNA isolation from at least three independent cultures, cDNA was generated as described (Tu and Bassler 2007)

with SuperScript III reverse transcriptase (Invitrogen) using 1–3 μg of RNA. Real-time PCR analyses were also performed as described (Tu and Bassler 2007) on an ABI Prism 7900HT Sequence Detection System using Sybr Green mix (ABI). The primers used are listed in Supplemental Table S4. Triplicate biological samples were measured and analyzed by a comparative C_T method (Applied Biosystems) in which the relative amount of each target RNA was normalized to the same internal control RNA (*hfq*) and then to each other to determine the relative changes in RNA levels.

Direct RNA measurements

RNA levels were directly measured using the QuantiGene Plex 2.0 Reagent system (Panomics) as described (Tu et al. 2010). RNA from 100 μL of culture was processed following the manufacturer's protocol and analyzed with beads carrying probes specific to the targets indicated in the figure legends. RNA levels were normalized to the levels of *hfq* or *recA* RNA (both remained unchanged during the course of the experiments). qRT-PCR and analysis with QuantiGene Plex Reagent System gave similar results.

GFP and bioluminescence assays

OD_{600} , GFP, and bioluminescence were all measured as described previously (Bassler et al. 1993; Lenz et al. 2004; Tu and Bassler 2007). Values reported are the mean and SEM of at least three biological replicates at an OD_{600} of 1.0–1.2, unless otherwise noted.

AphA purification

AphA was purified after overexpression from plasmid pSTR0606 encoding a 6-His tag, a thrombin cleavage sequence, and *V. harveyi* *aphA* under the T7 promoter as described in the Supplemental Material.

Fluorescence anisotropy

DNA binding was assessed by fluorescence anisotropy. Oligonucleotides (Supplemental Table S4) containing a 5' fluoroscein tag were purchased from IDT to be used as substrates, and fluorescence anisotropy analyses were performed as described (see the Supplemental Material for details; Pompeani et al. 2008). The concentrations of AphA protein added are indicated in each graph. One-site-specific nonlinear binding curves were fit to the data using GraphPad software, and the K_d were interpolated from the plots. Three samples were averaged to obtain each point, and the K_d and SD for the fit line are shown.

Microarray analysis

Microarrays contained three 60-mer probes per ORF in the *V. harveyi* genome (GenBank strain BAA-1116), each spotted in duplicate, for a total of six probes per gene (Agilent custom array 2521087). RNA preparation, cDNA synthesis, hybridization conditions, and data acquisition are described in the Supplemental Material. Data were extracted with Agilent Feature Extractor and analyzed on the Princeton University Microarray Database (PUMAdb, <http://puma.princeton.edu>) based on Gollub et al. (2003). These data are publically available on the PUMAdb. Data were retrieved for probes that were above background ($P < 0.0001$) and differed more than twofold. Probes were averaged for each gene. Four arrays were performed comparing three independent cultures of each strain as well as a dye-swap comparison for one set of strains.

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