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Individual and Combined Roles of the Master Regulators AphA and LuxR in Control of the *Vibrio harveyi* Quorum-Sensing Regulon

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Bacteria use a chemical communication process called quorum sensing to control transitions between individual and group behaviors. In the *Vibrio harveyi* quorum-sensing circuit, two master transcription factors, AphA and LuxR, coordinate the quorum-sensing response. Here we show that AphA regulates 167 genes, LuxR regulates 625 genes, and they coregulate 77 genes. LuxR strongly controls genes at both low cell density and high cell density, suggesting that it is the major quorum-sensing regulator. In contrast, AphA is absent at high cell density and acts to fine-tune quorum-sensing gene expression at low cell density. We examined two loci as case studies of coregulation by AphA and LuxR. First, AphA and LuxR directly regulate expression of the genes encoding the quorum-regulatory small RNAs Qrr2, Qrr3, and Qrr4, the consequence of which is a specifically timed transition between the individual and the group life-styles. Second, AphA and LuxR repress type III secretion system genes but at different times and to different extents. The consequence of this regulation is that type III secretion is restricted to a peak at mid-cell density. Thus, the asymmetric production of AphA and LuxR coupled with differences in their strengths and timing of target gene regulation generate a precise temporal pattern of gene expression.

Bacteria use quorum-sensing-mediated communication to monitor population density and to alternate between individual and group behaviors. Quorum sensing relies on the production of and response to extracellular signaling molecules called autoinducers (AIs) that encode information about the cell density and the species composition of the bacterial community. *Vibrio harveyi* is a focus of quorum-sensing studies because it has provided the parts list and the regulatory design principles for bacterial communication. *V. harveyi* produces and detects three AIs, AI-1, CAI-1, and AI-2, that specify intraspecies, intragenus, and interspecies communication, respectively (1). The AIs are detected by three cognate membrane-bound two-component sensor kinase receptors, LuxN, CqsS, and LuxQ (1, 2). LuxQ works in conjunction with LuxP, a periplasmic binding protein (3).

At low cell density (LCD), when AI concentrations are low, the receptors act as kinases, shuttling phosphate through the quorum-sensing cascade to the response regulator LuxO. Phosphorylated LuxO (LuxO~P) activates the transcription of genes encoding five regulatory small RNAs (sRNAs), called Qrr1 to Qrr5 (4–9). The Qrr sRNAs control the production of the two quorum-sensing master transcription factors, AphA and LuxR (Fig. 1). The base pairing of the Qrr sRNAs to *aphA* mRNA induces its translation (10, 11), whereas the base pairing of the Qrr sRNAs to *luxR* mRNA causes its degradation (7, 9). Thus, at LCD, AphA is maximally produced, and LuxR is minimally produced (10). This pattern is reinforced by the AphA repression of *luxR* transcription at LCD. Under this condition, *V. harveyi* cells act as individuals.

At high cell density (HCD), when AIs have accumulated, the AIs bind to their cognate receptors, causing them to switch from kinases to phosphatases, which results in the dephosphorylation of LuxO. In the absence of LuxO~P, the transcription level of the *qrr* genes is reduced, and this relieves the repression of *luxR* and decreases the activation of *aphA* (Fig. 1). Consequently, maximal LuxR production and minimal AphA production occur, a pattern that is reinforced by LuxR repressing the transcription of *aphA* (12, 13).

LuxR and AphA can both activate and repress gene expression (10, 13). LuxR and its homologs, for example, HapR in *Vibrio cholerae* and SmcR in *Vibrio vulnificus*, are members of the TetR family of transcriptional regulators (12). At HCD, LuxR controls genes including *luxCDABE* (luciferase), the type III secretion system (TTSS) genes, *qrr*2, *qrr*3, *qrr*4, and *aphA* (10, 12–17). A previous reporter screen revealed that LuxR regulates over 50 promoters at HCD in *V. harveyi* (13). This screen was not saturated because known targets, such as *luxCDABE*, were not identified. AphA is a winged-helix transcription factor that controls virulence factor production in *V. cholerae* (18, 19). In *V. harveyi*, AphA was recently shown to regulate the expression of 296 genes at LCD, including *luxR*, *qrr*2, *qrr*3, and *qrr*4 (10).

Here we determine the individual and combined contributions of AphA and LuxR to quorum-sensing target gene expression in *V. harveyi*. Our biochemical analysis shows that, consistent with previous genetic predictions, the AphA and LuxR proteins exhibit reciprocal production patterns in response to changes in AI levels. These production profiles are, however, asymmetric: AphA is produced at LCD, but no AphA protein can be detected at HCD. LuxR is, in contrast, present throughout the growth curve but at higher concentrations at HCD than at LCD. Thus, AphA functions exclusively at LCD, while LuxR functions to different extents throughout growth. Microarrays and quantitative real-time PCR (qRT-PCR) demonstrate that AphA and LuxR control genes in distinct regulons, and they also coregulate 77 genes. To under-

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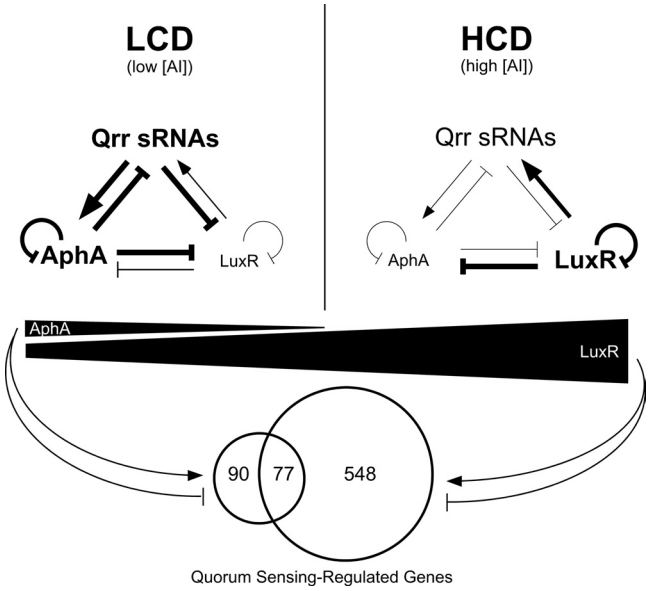

FIG 1 Model for the alternating pattern of LuxR and AphA control of the quorum-sensing regulon. (Left) At LCD, a low AI concentration results in the production of LuxO~P, which activates *qrr* gene expression. The Qrr sRNAs repress the translation of LuxR and activate the translation of AphA. AphA feeds back to repress the expression of the *qrr* genes and *luxR*. (Right) At HCD, a high AI concentration results in the dephosphorylation of LuxO, which terminates the activation of *qrr* gene expression. Low Qrr levels allow the translation of *luxR* mRNA and cause decreased *aphA* expression levels. LuxR feeds back to repress *aphA* transcription and to activate *qrr* gene transcription. This regulatory arrangement produces maximal AphA at LCD and maximal LuxR at HCD. AphA controls 167 genes, and LuxR controls 625 genes. Seventy-seven of these genes are coregulated.

stand coregulation by AphA and LuxR, we examined two sets of these shared targets: the *qrr* sRNA genes and the TTSS genes. We find that LuxR and AphA both bind to and control the expression of the *qrr* promoters. The activation of the *qrr* genes by LuxR and the repression of the *qrr* genes by AphA precisely control Qrr sRNA levels during quorum-sensing transitions. Second, LuxR and AphA both bind to and repress the expression of TTSS genes, resulting in minimal TTSS gene expression at both LCD and HCD and a spike of TTSS gene expression at mid-cell density. Thus, the asymmetric production profiles of AphA and LuxR coupled with their individual and combined control of downstream regulatory targets establish a finely choreographed pattern of quorum-sensing gene expression.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* strains S17-1λ*pir*, BL21(DE3) (Invitrogen), and derivatives (see Table S1 in the supplemental material) were grown with shaking at 37°C in Luria-Bertani (LB) medium unless otherwise stated. *V. harveyi* strain BB120 (BAA-1116) and derivatives (see Table S1 in the supplemental material) were grown with shaking at 30°C in Luria-Marine (LM) (20) medium. Antibiotics (Sigma) were used at concentrations described previously (10). Conjugation was used to transfer plasmids from *E. coli* into *V. harveyi* (10). Protein production from plasmids containing the P_{tac} promoter and the theophylline riboswitch was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 1 mM theophylline, respectively.

Molecular methods. *E. coli* strain S17-1λ*pir* was used for cloning procedures. Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and calf intestinal phosphatase were purchased from New England BioLabs (NEB). PCR mixtures contained iProof DNA polymerase (Bio-Rad). Plasmids containing the P_{tac} promoter, *lacI*, and the theophylline riboswitch were constructed by using pSAL12.1, a kind gift of the Gallivan laboratory (21), as described in Table S2 in the supplemental material. Oligonucleotides (Integrated DNA Technologies) are listed in Table S3 in the supplemental material. Plasmid constructs were introduced into electrocompetent *E. coli* S17-1λ*pir* cells by using 0.2-cm Bio-Rad cuvettes. Sequences were confirmed by Genewiz, Inc., with plasmid-specific primers. The construction of mutations in *V. harveyi* was performed as previously described (10).

RNA was isolated from bacterial cultures by using the TRIzol (Invitrogen) method, as described previously (10). cDNA synthesis and qRT-PCR were performed as described previously (10), using primers listed in Table S3 in the supplemental material. Reactions were normalized to an internal standard (*hfq*) and analyzed by using the standard curve or $^{\Delta\Delta}C_T$ method. Western blot experiments were conducted as previously described (11, 17). Methods describing AphA and LuxR protein purification and DNA binding assays are included in the supplemental material.

Electrophoretic mobility shift assays (EMSAs). Oligonucleotides (see Table S3 in the supplemental material) were purchased from Integrated DNA Technologies (PAGE purified). Complementary substrates were annealed in annealing buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl) at 95°C for 1 min, followed by cooling 1°C per min for 70 min. Substrates generated by PCR were purified by gel extraction followed by QIAquick columns (Qiagen). Substrates were labeled with [γ-^{32}P]ATP in 30-μl reaction mixtures containing T4 polynucleotide kinase (NEB) for 30 min at 37°C and cleaned on ProbeQuant G-50 microcolumns (GE Healthcare). Labeled DNA was incubated for 30 min in a 15-μl reaction mixture containing binding buffer (10 mM HEPES [pH 7.5], 100 mM KCl, 2 mM dithiothreitol [DTT], 200 μM EDTA), 10 ng/μl poly(dI-dC), 100 μg/ml bovine serum albumin (BSA), and the desired protein (AphA or LuxR) diluted in dilution buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol). The reaction mixtures were separated on 6% TGE (25 mM Tris, 0.25 M glycine, 1 mM EDTA)-polyacrylamide native gels, and the gels were dried at 80°C for 45 min and exposed overnight on a storage phosphor screen. The screen was scanned by using a Typhoon scanner and analyzed both qualitatively and quantitatively by using ImageQuant software (GE Healthcare).

Time course analyses of gene expression. Cultures of *V. harveyi* strains grown overnight were diluted ~1:1,000 in LM medium, and aliquots were collected at the time points noted. Samples were also collected from the cultures grown overnight to determine RNA levels at the initial time points. Transcript levels were analyzed by Quanti-gene Plex technology, as described previously (22), or RNA was collected and analyzed by qRT-PCR or microarrays.

AI-1 titration experiments. Cultures of strains TL25 (Δ*luxM* Δ*luxPQ* Δ*cqsS*), YS10 (Δ*luxM* Δ*luxPQ* Δ*cqsS* Δ*luxR*), YS50 (Δ*luxM* Δ*luxPQ* Δ*cqsS* Δ*aphA*), or YS51 (Δ*luxM* Δ*luxPQ* Δ*cqsS* Δ*aphA* Δ*luxR*) grown overnight were diluted to an optical density at 600 nm (OD_{600}) of 0.001 with various concentrations of AI-1 added. Cells were collected at an OD_{600} of 1.0 for RNA extraction and protein sample preparation.

Inducible expression of *luxR* and *aphA*. The inducible expression of *luxR* and *aphA* was accomplished by constructing a plasmid (pJV025) that contained regulatory elements to control transcription and translation. The plasmid harbors *lacI*^{q} and contains the P_{tac} promoter with a LacI^q operator site that is sensitive to IPTG. The theophylline riboswitch aptamer (21) from pSAL12.1 was inserted into the 5′ untranslated region (5′-UTR), which results in the repression of translation in the absence of theophylline. FLAG-*luxR* and FLAG-*aphA* were cloned under the control of this "$P_{tactheo}$" promoter to yield pJV057 and pSTR738, respectively. To induce *aphA* and *luxR* expression, *V. harveyi* strains were diluted 1:1,000, grown to an OD_{600} of ~0.2, and induced with 1 mM IPTG and 1 mM theophylline, and samples were collected after 3 h. To monitor gene expression at LCD, cultures grown overnight in the presence of 1 mM IPTG

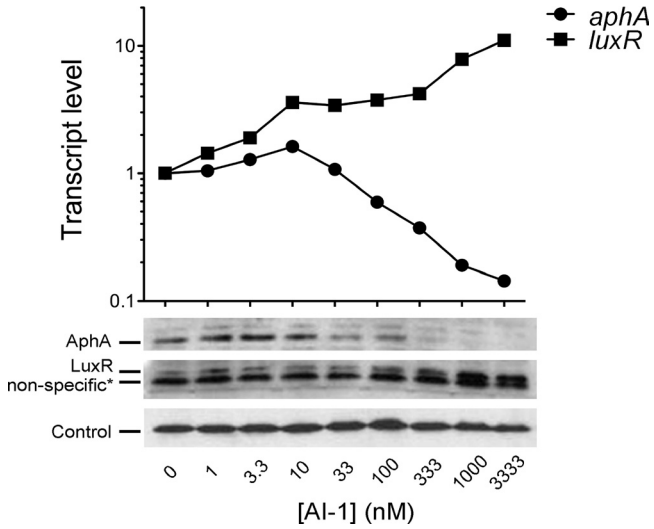

FIG 2 AphA and LuxR production profiles during quorum sensing. Shown are data for qRT-PCR of *aphA* and *luxR* and Western blot analyses of AphA and LuxR in *V. harveyi* TL25 (Δ*luxM* Δ*luxPQ* Δ*cqsS*) cells grown in the presence of various concentrations of AI-1. The loading control is a nonspecific band (~50 kDa) detected by the AphA antibody. A nonspecific band (*) was also detected by the LuxR antibody.

were diluted 1:1,000 and grown to an OD_{600} of ~0.02 in 1 mM IPTG and 1 mM theophylline.

Microarray analyses. RNA from *V. harveyi* strains was collected from cells grown in LM medium to an OD_{600} of ~1.2 in triplicate. The synthesis of cDNA and the microarray protocol were described previously (10). *V. harveyi* BB120 microarrays (Agilent) were designed as custom gene expression arrays (Amadid design ID 037644), using the melting temperature (T_m) method and the best-probe method. The arrays contained two 60-mer probes per open reading frame. Gene expression data were averaged from probes that were above background levels, as determined by Feature Extractor (Agilent); had P values of <0.0001; and differed by more than 2-fold in three out of four of the arrays. Data analysis was performed with the Princeton University Microarray Database (PUMAdb) (http://puma.princeton.edu/). These data are publically available at PUMAdb.

RESULTS

LuxR and AphA have asymmetric production profiles. AphA and LuxR are the two master transcriptional regulators of quorum sensing. Thus, the precise patterns of AphA and LuxR production should govern the downstream pattern of quorum-sensing gene expression. To investigate AphA and LuxR production throughout quorum sensing, we measured the relative *aphA* and *luxR* transcript and AphA and LuxR protein production profiles at different AI concentrations. In this experiment, we used a *V. harveyi* strain (TL25 [Δ*luxM* Δ*luxPQ* Δ*cqsS*]) that does not produce AI-1 but responds to it when it is supplied exogenously. We applied different AI-1 concentrations to *V. harveyi* TL25 cells to mimic specific quorum-sensing states. The reason why we used strain TL25 rather than the wild type is because it is not possible to collect enough protein under LCD conditions to reliably perform Western blot analyses. We found that *aphA* levels decreased and *luxR* levels increased with increasing AI-1 concentrations (Fig. 2). Protein levels tracked with their corresponding mRNA levels (Fig. 2). LuxR could be detected even in the absence of AI-1, whereas AphA decreased to undetectable levels at AI-1 concentrations above 333 nM. These experiments demonstrate that AphA and LuxR exhibit opposite, but nonequivalent, alterations in production during *V. harveyi* quorum sensing, and this asymmetry is controlled by AIs. Importantly, and we return to this point below, AphA and LuxR are both present at LCD.

LuxR and AphA are master regulators of quorum sensing. To define the individual and combined contributions of AphA and LuxR to quorum-sensing target gene expression, we used microarray analyses. We compared profiles from *V. harveyi* mutant strains that constitutively mimic the LCD and HCD states. The LCD *V. harveyi* strain harbors a LuxO~P mimic, LuxO D47E (6, 9). The HCD *V. harveyi* strain is a Δ*luxO* strain (9). Microarray analyses revealed that levels of 365 transcripts differed by 2-fold or more in these two strains, and we refer to this set of genes as the "quorum-sensing regulon" (see Table S4 and Fig. S1 in the supplemental material). To identify the LCD genes controlled by AphA, we compared the expression profile of a *luxO*-D47E Δ*luxR* strain to that of a *luxO*-D47E Δ*luxR* Δ*aphA* strain. This "AphA LCD regulon" contains 167 genes (Fig. 1, left circle; see also Table S4 and Fig. S1 in the supplemental material). To identify the HCD genes controlled by LuxR, we compared the profile of a Δ*luxO* Δ*aphA* strain to that of a Δ*luxO* Δ*aphA* Δ*luxR* strain and discovered 625 genes. We call this set the "LuxR HCD regulon" (Fig. 1, right circle; see also Table S4 and Fig. S1 in the supplemental material). Interestingly, the AphA LCD regulon and the LuxR HCD regulon each include genes that are not members of the quorum-sensing regulon. Likewise, not all of the quorum-sensing regulon genes are contained in the AphA or LuxR regulons. Possible explanations for these results are provided in the Discussion.

A comparison of the AphA LCD regulon with the LuxR HCD regulon showed that 77 genes with a variety of predicted functions are controlled by both transcription factors (Table 1 and Fig. 1). Within this set of genes and the individual AphA and LuxR regulons, we identified all eight possible patterns of gene regulation by AphA and/or LuxR. There are genes activated exclusively by LuxR (306 genes, e.g., *luxC*), repressed exclusively by LuxR (242 genes, e.g., VIBHAR_05222, a putative thioesterase), activated exclusively by AphA (45 genes, e.g., VIBHAR_05936, a hypothetical protein), or repressed exclusively by AphA (45 genes, e.g., VIBHAR_02308, a hypothetical protein). There are also genes activated by LuxR and AphA (7 genes, e.g., VIBHAR_03648, *pyrI*, a putative aspartate carbamoyltransferase regulatory subunit), genes repressed by LuxR and AphA (52 genes, e.g., *exsD*, a TTSS transcription regulator), genes repressed by LuxR and activated by AphA (8 genes, e.g., VIBHAR_04884, a hypothetical protein), and

TABLE 1 Functional classes of genes coregulated by AphA and LuxR[b]

Function	No. of genes
Type III secretion apparatus	32
Stress response	4
Metabolism	3
Qrr sRNAs[a]	3
Oxidoreductases	2
Proteases	2
Membrane/transport	2
Hypothetical	32

[a] The regulation of *qrr2* to *qrr4* by AphA and LuxR was demonstrated previously (10, 16).
[b] See Table S4 in the supplemental material for a complete list of genes regulated by AphA and LuxR.

genes activated by LuxR and repressed by AphA (10 genes, e.g., VIBHAR_02509, a homolog of *V. cholerae hapA*). A representative of each class of gene is shown in Fig. S1 in the supplemental material. To confirm these results, we performed complementation experiments by expressing either *aphA* or *luxR* from an inducible promoter in a *V. harveyi* Δ*aphA* Δ*luxR* mutant strain (STR417), followed by qRT-PCR of the target genes (see Fig. S2 in the supplemental material). In most cases, complementation occurred. However, in a few cases, regulatory patterns different than expected occurred, suggesting that factors in addition to AphA and LuxR must be involved in controlling these genes (for example, *hapA* [see Fig. S2 in the supplemental material]).

As noted above, asymmetry exists in the LuxR and AphA protein production profiles (Fig. 2). Specifically, although LuxR is maximally produced at HCD, it is also present at LCD, whereas little or no AphA is present at HCD. The microarrays showed that LuxR regulates the expression of 82 genes at LCD, while AphA has no influence on gene regulation at HCD (see Table S4 and Fig. S1 in the supplemental material). Collectively, our results favor a model in which LuxR controls genes at both HCD and LCD, while AphA regulates genes predominantly at LCD.

LuxR and AphA direct global gene expression patterns. A curious finding was that over 50 genes are repressed by AphA at LCD and repressed by LuxR at HCD (see, for example, the TTSS genes, represented by *exsD*, in Fig. S1 in the supplemental material). Thus, we wondered, When are such genes expressed? To explore this and other possible interesting coregulation patterns, we used microarrays to monitor the expression levels of all 77 target genes that are controlled by both AphA and LuxR using *V. harveyi* strains possessing and lacking *luxR* and/or *aphA* (see Fig. S3 in the supplemental material). We found that genes repressed by both AphA and LuxR are maximally expressed in the window between LCD (OD_{600} of ~0.01) and HCD (OD_{600} of ~1.0). For example, consider the TTSS operons (see *exsD* in Fig. 3A). The full repression of *exsD* at LCD requires both AphA and LuxR at LCD, whereas only LuxR is necessary for repression at HCD. In other instances, the opposing effects of AphA and LuxR offset each other; for example, take the case of *hapA* (Fig. 3B). In this example, AphA repression is significantly stronger than is LuxR activation, leading to overall increasing *hapA* expression levels over growth. Because of this difference, the expression profile of *hapA* in the *V. harveyi* Δ*aphA* Δ*luxR* mutant strain is more similar to that in the Δ*aphA* single mutant than to that in the Δ*luxR* single mutant. For genes that are controlled roughly equally but in opposite directions by AphA and LuxR, the deletion of either *aphA* or *luxR* results in altered gene expression, but the deletion of both factors restores expression to nearly the wild-type pattern (VIBHAR_02311, a hypothetical protein, is AphA repressed and LuxR activated) (Fig. 3C). Thus, employing both AphA and LuxR in the quorum-sensing cascade enables patterns of gene expression that would not be possible if only a single transcription factor was involved.

Direct coregulation of the *qrr*4 promoter by LuxR and AphA. Because the Qrr sRNAs are central quorum-sensing regulators responsible for conveying the information encoded by AIs to the target gene output, we examined their control by AphA and LuxR in some detail. Using *qrr*4 as the representative, we investigated whether regulation requires both AphA and LuxR. We engineered a *V. harveyi* strain lacking both *aphA* and *luxR*, reintroduced inducible *aphA* and inducible *luxR* individually on plasmids, and measured *qrr*4 expression levels. LuxR activated *qrr*4 expression

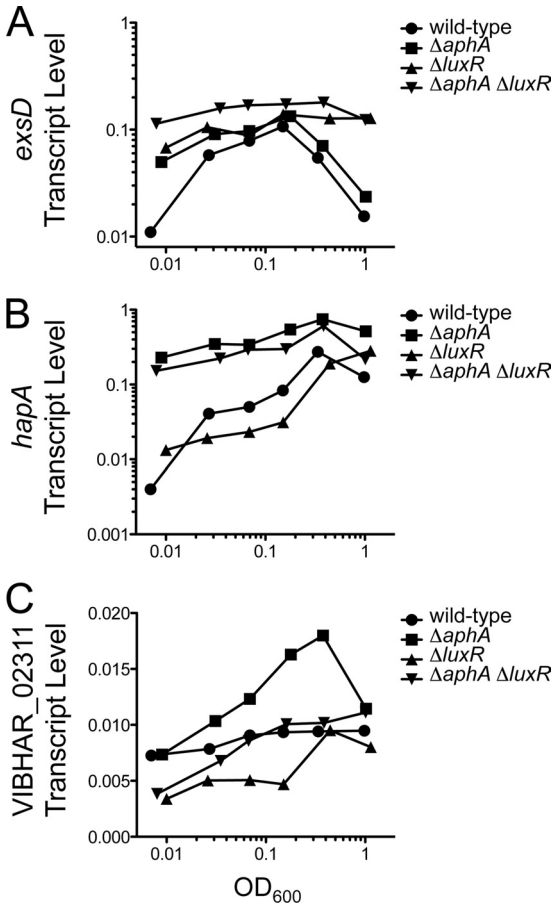

FIG 3 AphA and LuxR coregulate genes during transitions between LCD and HCD. Expression profiles of representative genes at the indicated cell densities are shown. qRT-PCR was used to compare RNA samples collected from the wild-type (BB120), Δ*aphA* (JV48), Δ*luxR* (KM669), and Δ*aphA* Δ*luxR* (STR417) strains. The graphs show data from two independent experiments.

2.4-fold independently of AphA, and AphA repressed *qrr*4 expression 2.9-fold independently of LuxR (Fig. 4A).

The finding that LuxR and AphA can individually regulate *qrr*4 expression does not exclude the possibility that they influence each other's function at the *qrr*4 promoter when both are present. Therefore, we examined whether both transcription factors could simultaneously bind to the *qrr*4 promoter. LuxR binds to the 21-bp palindrome TTCTGATAAATGTATTAGTAG located 166 bp upstream of the *qrr*4 transcription start site (Fig. 4B) (12). We found that AphA binds the 16-bp sequence TTGCATCATTTT GCAT located 125 bp upstream of the *qrr*4 transcription start site (Fig. 4B; see also Fig. S4 in the supplemental material). This AphA binding sequence shares a low level of identity (6 identical base pairs) with the AphA site characterized previously for *V. cholerae* (23, 24). We performed EMSAs with a probe harboring both the LuxR and AphA binding sites. LuxR and AphA each bound this DNA fragment (Fig. 4C), which is consistent with their ability to individually control *qrr*4 expression (Fig. 4A). In the presence of both LuxR and AphA, a DNA supershift occurred, indicating that both transcription factors can simultaneously bind the fragment (Fig. 4C).

To determine how LuxR and AphA together affect *in vivo qrr*4

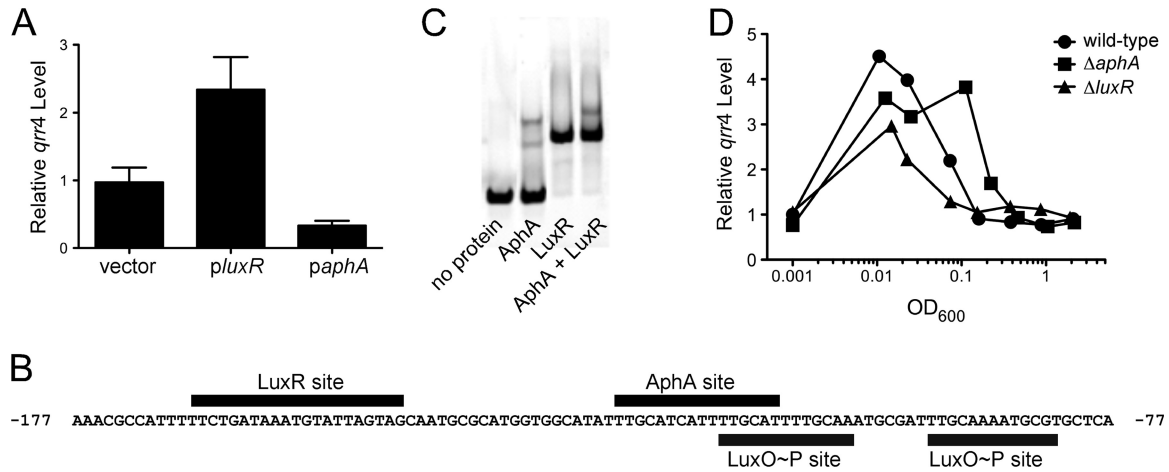

FIG 4 Regulation of *qrr4* by AphA and LuxR. (A) *qrr4* levels were measured by qRT-PCR. Samples were collected from strains expressing FLAG-*luxR* (pJV057) or FLAG-*aphA* (pSTR738) in a *V. harveyi* Δ*luxR* Δ*aphA* strain (STR417). RNA measurements were normalized to values for a control strain containing the empty vector (pJV139). Error bars are standard deviations of data from triplicate biological samples and technical duplicates. (B) LuxR, AphA, and LuxO~P binding sites at the *qrr4* promoter are indicated. Numbers denote positions relative to the transcription start site. (C) EMSAs with radiolabeled *qrr4* promoter DNA (positions −170 to −109 relative to the transcription start site) in the presence of 600 nM LuxR, 600 nM AphA, or both. (D) *qrr4* expression was monitored by qRT-PCR as cells transitioned from LCD (OD_{600} of ~0.01) to HCD (OD_{600} of ~1.0) in wild-type (BB120), Δ*aphA* (JV48), and Δ*luxR* (KM669) *V. harveyi* strains. The data shown represent four independent experiments.

expression, we monitored *qrr4* expression as wild-type, Δ*aphA*, and Δ*luxR V. harveyi* cells transitioned from LCD to HCD. In the wild-type strain, following dilution into fresh medium, the *qrr4* expression level increased ~5-fold at LCD (OD_{600} of ~0.01), and the expression level subsequently decreased 6-fold as cells transitioned to HCD (OD_{600} of ~1.0) (Fig. 4D). The deletion of *aphA* prolonged the maximal expression of *qrr4* relative to that in wild-type *V. harveyi*. This result shows that AphA is responsible for setting the timing of the transition to HCD by repressing *qrr* expression. The deletion of *luxR*, in contrast, resulted in only modestly reduced levels of *qrr4* compared to those of the wild-type strain at LCD (Fig. 4D). This result shows that in wild-type *V. harveyi*, sufficient LuxR is present at LCD to activate *qrr4* expression immediately after the transition to LCD occurs, suggesting that LuxR is responsible for the rapid entrance into the LCD mode.

AphA and LuxR directly control regulation of the TTSS genes. A second interesting case of coregulation by AphA and LuxR concerns the TTSS genes. We showed that TTSS genes are repressed by both AphA and LuxR, which confines their expression to a peak at mid-cell density (Fig. 3A; see also Table S4 and Fig. S5 in the supplemental material) (10, 13, 17). Here we investigate how this regulatory pattern is coordinated by AphA and LuxR. There are four TTSS structural operons in *V. harveyi*, TTSS.I to TTSS.IV (Fig. 5A), and these are activated by the regulators ExsA and ExsB (17). *exsA* and *exsB* lie upstream of the TTSS.IV operon (Fig. 5A) (13, 17). We first examined whether LuxR and AphA directly bind the promoters driving the expression of the TTSS genes using EMSAs with purified LuxR and AphA proteins. Using the LuxR position weight matrix reported previously (12), we predicted that two LuxR binding sites exist in P_B (the *exsB* promoter) (positions −63 and +5 relative to the transcription start site) and that three exist in P_A (the *exsA* promoter) (positions −115, +187, and +259 relative to the transcription start site) (Fig. 5A), and indeed, LuxR bound DNA fragments containing each of the predicted binding sites (Fig. 5B). A previous study did not find that LuxR bound at the site predicted at position −63 in P_B (25). We assume that this discrepancy is due to different EMSA reaction conditions. LuxR did not bind to the TTSS.IV, TTSS.II/III, or TTSS.I promoter fragments (see Fig. S6 in the supplemental material). We do not yet have a position weight matrix to predict AphA binding sites. However, using fragments encompassing the entire promoter regions, we found that AphA also bound the P_A and P_B promoter fragments (Fig. 5C). Additionally, AphA bound the TTSS.IV and TTSS.I promoter fragments (Fig. 5C) but not the TTSS.II/III promoter fragment (see Fig. S6 in the supplemental material). Complementation experiments showed that the expression of either *aphA* or *luxR* causes the repression of both *exsA* and *exsB* (Fig. 5D) and all four TTSS structural operons in *V. harveyi* (see Fig. S5 in the supplemental material). Collectively, our data suggest that AphA (at LCD) and LuxR (at both LCD and HCD) directly repress *exsA* and *exsB* expression, and this leads to decreased expression levels of the four TTSS structural operons. In addition, AphA directly represses TTSS.I and TTSS.IV at LCD. The combined effect of this repression is a peak of TTSS expression levels between LCD and HCD.

The ultimate output of the TTSS operons is a set of secreted proteins. To show that the transcriptional regulatory pattern generated by LuxR and AphA culminates in corresponding changes in protein levels, we monitored the production of the TTSS effector protein VopD using the AI-1-sensing strain (TL25) in the absence of AI-1 (LCD) or in the presence of saturating AI-1 concentrations (HCD). VopD is present at LCD, and it is undetectable at HCD (Fig. 5E). The deletion of *aphA* resulted in increased VopD levels only at LCD, whereas the deletion of *luxR* increased VopD levels at both LCD and HCD (Fig. 5E).

DISCUSSION

V. harveyi uses two master transcription factors, AphA and LuxR, to regulate target genes underpinning individual and group behaviors. Here we demonstrate that AphA and LuxR individually and jointly regulate ~700 genes to specify these alternative lifestyles. In response to AI, the first transcripts to change are those of

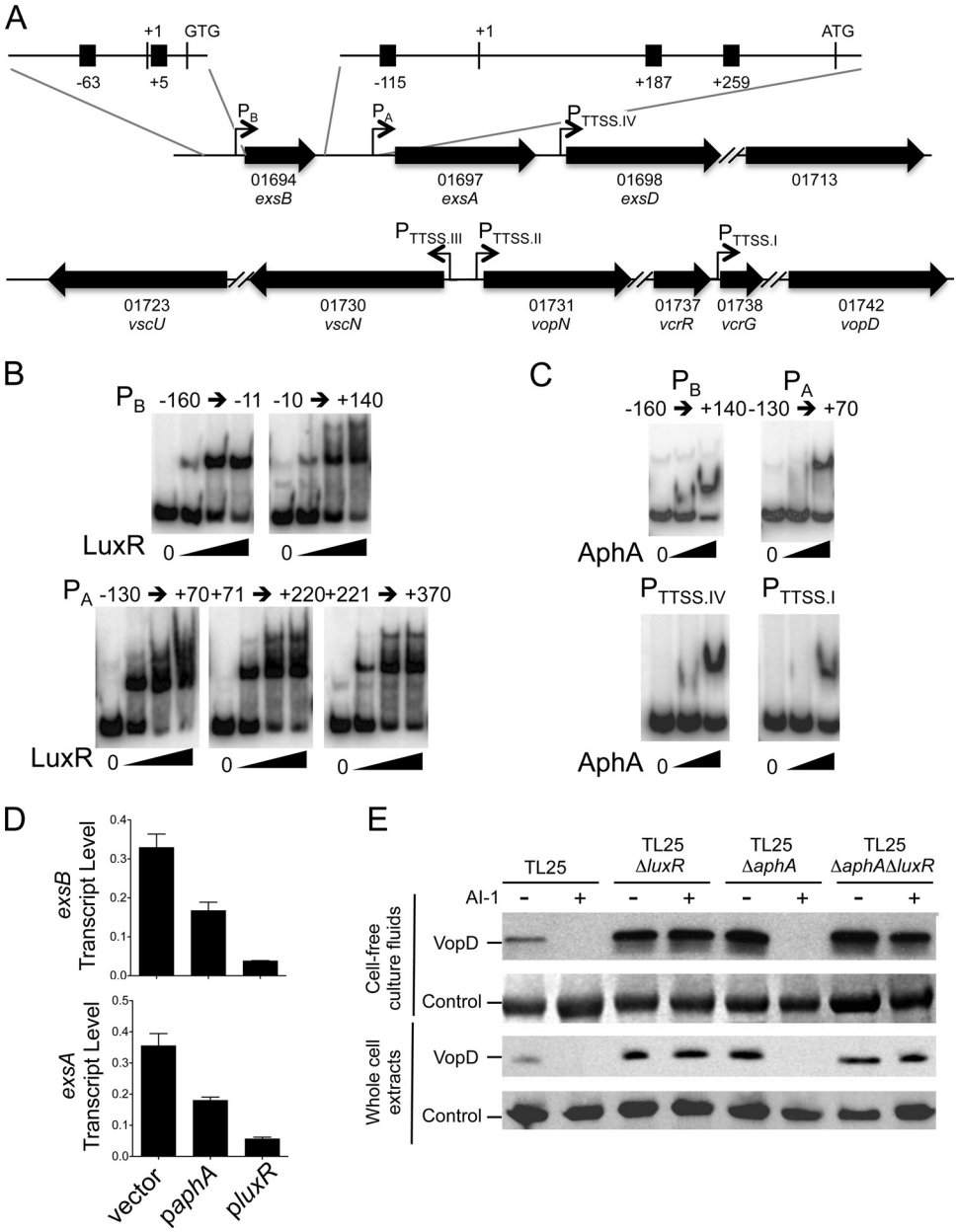

FIG 5 AphA and LuxR directly repress TTSS genes and protein secretion. (A) Schematic of the TTSS operons in *V. harveyi*. Promoters (P) are indicated for the two regulators *exsA* (P_A) and *exsB* (P_B) and the four structural operons ($P_{TTSS.I}$ to $P_{TTSS.IV}$): TTSS.I (VIBHAR_01738-01742), TTSS.II (VIBHAR_01731-01737), TTSS.III (VIBHAR_01730-01723), and TTSS.IV (VIBHAR_01698-01713). Predicted LuxR binding sites in P_A and P_B are indicated by black boxes. Their locations relative to the transcription start sites are indicated below. (B) EMSAs are shown for reaction mixtures containing LuxR (0, 25, 100, and 250 nM) in the presence of P_A or P_B promoter fragments (1 nM). (C) EMSAs are shown for reaction mixtures containing AphA (0, 900, and 1,800 nM) and the indicated promoter DNA fragments (1 nM). For P_A or P_B, the coordinates indicate the distance to the transcription start site shown in panel A. The $P_{TTSS.I}$ and $P_{TTSS.IV}$ fragments correspond to 300 bp upstream of the translation start codons. (D) Relative transcript levels determined by qRT-PCR for *exsA* and *exsB* following the overproduction of AphA (p*aphA*) (pSTR738) or LuxR (p*luxR*) (pJV057). Error bars represent standard deviations of data from triplicate biological samples. (E) Western blot analysis of VopD (whole-cell extracts and cell-free culture fluids) from *V. harveyi* strain TL25 (Δ*luxM* Δ*luxPQ* Δ*cqsS*) and the TL25 Δ*aphA*, TL25 Δ*luxR*, and TL25 Δ*aphA* Δ*luxR* strains grown in the absence or presence of 1 μM AI-1.

aphA and *luxR* (data not shown). Specifically, the *aphA* expression level decreases and the *luxR* expression level increases within 15 min. This finding suggests that a priority in quorum sensing is to alter the levels of these two master transcription factors in order to promote the subsequent pattern of downstream gene expression. Several putative transcription factors exist among the AphA and LuxR target genes, and these secondary regulators could be responsible for linking the immediate AphA and LuxR responses to gene expression changes that occur later in the quorum-sensing cascade.

AphA acts at LCD, and LuxR acts at both LCD and HCD. AphA is present at LCD and at the transition to HCD, during which time it controls the expression of 167 genes. Ninety of these genes are regulated exclusively by AphA (i.e., not by LuxR), indi-

cating that these genes encode functions that must be activated or repressed when there are few cells in the population. LuxR is present at both LCD and HCD. Eighty-two genes are controlled by LuxR at LCD, and 75 of these are also controlled by LuxR at HCD. We predict that these 82 LuxR targets harbor the highest-affinity LuxR binding sites because they are controlled by LuxR when it exists at its lowest levels. These 82 genes and the additional 543 HCD LuxR targets likely encode functions that are important for carrying out group behaviors (activated) or that are detrimental to collective activities (repressed).

Quorum-sensing genes that are regulated independently of AphA and LuxR. A comparison of the LCD-locked strain (*luxO*-D47E) to the HCD-locked strain (Δ*luxO*) shows that the entire quorum-sensing regulon consists of 365 genes. Regulation by AphA and/or LuxR accounts for 78% of the differences between these two strains. However, 79 genes in the quorum-sensing regulon are not controlled by LuxR or AphA (see Fig. S1 in the supplemental material). This result suggests that additional factors are involved in quorum-sensing gene regulation. The most obvious candidates for carrying out this regulation are the Qrr sRNAs. Currently, the expression levels of only four genes are known to be controlled by the Qrr sRNAs in *V. harveyi* (*luxR*, *aphA*, *luxO*, and *luxM*), and one additional target has been identified in *V. cholerae* (*vca0939*) (9, 10, 22, 26, 27). Some of the 79 genes that we identified could be under Qrr control, and we are currently investigating this possibility.

AphA- and LuxR-controlled targets that are not members of the quorum-sensing regulon. We identified 429 genes that are under LuxR and/or AphA control that are not members of the quorum-sensing regulon (see Fig. S1 in the supplemental material). There are at least two possible explanations for these results. First, the regulation of some genes might be uncovered only in the complete absence of the transcription factor, i.e., in the Δ*aphA* or the Δ*luxR* deletion strain. If low levels of LuxR or low levels of AphA are sufficient to control gene expression, such genes will not be revealed in microarrays comparing the Δ*luxO* and *luxO*-D47E strains because residual LuxR and AphA exist. An example of this type of target is VIBHAR_06500 to VIBHAR_06505 (VIBHAR_06500-06505), which are activated 9-fold by LuxR at LCD and 6-fold by LuxR at HCD (see Table S4 in the supplemental material). A second explanation for our identification of AphA- and LuxR-controlled genes that are not members of the quorum-sensing regulon is that some genes are controlled by LuxR or AphA only during the quorum-sensing transition, so their steady-state LCD and HCD levels are roughly identical. Again, these genes would not be revealed in our microarray experiments. An example is VIBHAR_01372, which is repressed 4-fold by LuxR at HCD and 3-fold by AphA at LCD (see Table S4 in the supplemental material).

Two unequal master regulators. We identified 77 genes that are regulated by both AphA and LuxR. Because each transcription factor can act as a repressor and as an activator, there are four possible combinations of coregulation, and we identified at least one example of each: both factors repress, both factors activate, AphA activates and LuxR represses, and AphA represses and LuxR activates. The largest class of genes controlled by both AphA and LuxR is the repressed/repressed class, which contains the TTSS operons.

Generally, LuxR regulation is stronger than that of AphA. For example, consider the TTSS.I operon, which is repressed 3-fold by AphA at LCD and repressed 21-fold by LuxR at HCD. If the role of LuxR overwhelms that of AphA, why employ AphA? We propose that AphA regulation ensures fine-tuned expression at LCD. Another interesting set of genes are VIBHAR_02040-02042, which are activated ~7.5-fold by LuxR at both LCD and HCD. If the expression of these genes must be modulated, another factor, such as AphA, must accomplish this task. Indeed, AphA represses VIBHAR_02040-02042 3-fold at LCD. Thus, AphA can serve to control genes at specific points in the *V. harveyi* life cycle when regulation by LuxR is insufficient.

Regulation of the TTSS genes by AphA and LuxR. TTSSs encode important virulence factors in pathogens, including *Vibrio parahaemolyticus*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas aeruginosa* (17, 28, 29). We found that maximal TTSS gene expression in *V. harveyi* is confined to the window of growth between LCD and HCD (Fig. 3A). Our model is that at LCD, high levels of AphA combined with low LuxR levels are required for repression. Conversely, at HCD, a high LuxR concentration is sufficient for the repression of the TTSS operons. Precise temporal control of virulence gene expression may be critical to optimize infectivity, in this case by limiting expression to particular cell densities. We do not know why maximal TTSS gene expression at the LCD-to-HCD transition is optimal for *V. harveyi* pathogenesis. Interestingly, another virulence factor, HapA, is also regulated by both AphA and LuxR.

Generation of global patterns of gene expression. Multiple feedback loops involving the Qrr sRNAs control the flow of information through the quorum-sensing circuit (9, 16, 22). AphA represses and LuxR activates *qrr* expression. Additionally, AphA and LuxR repress each other's expression (Fig. 1). These feedback loops are critical to maintain the appropriate levels of the Qrr sRNAs during the transitions from HCD to LCD and from LCD to HCD. We propose a model for how these feedback loops could affect the transitions. First, consider the HCD-to-LCD transition when there is an immediate need to terminate group behaviors. The activation of the *qrr* genes is a function of LuxO~P and LuxR. LuxO phosphorylation occurs on a time scale of seconds, so *qrr* activation by LuxO~P is immediate. Residual LuxR protein that accumulated at HCD (Fig. 2) further activates *qrr* gene expression. The surge in Qrr production represses *luxR* expression, thus ending the HCD program. The Qrr sRNAs activate *aphA* expression, which initiates the LCD program. AphA ensures that the LCD program is undertaken by transcriptionally repressing *luxR*.

During the opposite transition from LCD to HCD, AIs lead to decreased concentrations of LuxO~P and thus to decreased levels of Qrr sRNA production. Therefore, the level of LuxR production increases and the level of AphA production decreases. Residual AphA continues to repress *qrr* expression, causing a more rapid termination of the LCD program than if AphA was not a part of the program. The LuxR repression of *aphA* ensures a commitment to HCD. This model predicts that the deletion of *aphA* should extend Qrr sRNA production and slow the progression to HCD, which is indeed borne out by our results (Fig. 4D).

We suspect that the main advantage of having two rather than one master regulator concerns response kinetics. That said, once the system possesses both AphA and LuxR, adding additional binding sites for either or both of these transcription factors is an economical mechanism to achieve an even finer regulation of particular targets. Furthermore, employing two regulators could allow the integration of environmental cues, in addition to the quo-

rum-sensing autoinducers, into the control of gene expression. Ultimately, because maximal productions of AphA and LuxR occur at distinct times (AphA at LCD and LuxR at HCD), individual regulation and coregulation by AphA and LuxR establish complex gene expression patterns that could not be achieved if only one of these regulators was a member of the quorum-sensing cascade.

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