

Quorum Sensing Controls Biofilm Formation in *Vibrio cholerae* through Modulation of Cyclic Di-GMP Levels and Repression of *vpsT*[▽]

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Two chemical signaling systems, quorum sensing (QS) and 3',5'-cyclic diguanylic acid (c-di-GMP), reciprocally control biofilm formation in *Vibrio cholerae*. QS is the process by which bacteria communicate via the secretion and detection of autoinducers, and in *V. cholerae*, QS represses biofilm formation. c-di-GMP is an intracellular second messenger that contains information regarding local environmental conditions, and in *V. cholerae*, c-di-GMP activates biofilm formation. Here we show that HapR, a major regulator of QS, represses biofilm formation in *V. cholerae* through two distinct mechanisms. HapR controls the transcription of 14 genes encoding a group of proteins that synthesize and degrade c-di-GMP. The net effect of this transcriptional program is a reduction in cellular c-di-GMP levels at high cell density and, consequently, a decrease in biofilm formation. Increasing the c-di-GMP concentration at high cell density to the level present in the low-cell-density QS state restores biofilm formation, showing that c-di-GMP is epistatic to QS in the control of biofilm formation in *V. cholerae*. In addition, HapR binds to and directly represses the expression of the biofilm transcriptional activator, *vpsT*. Together, our results suggest that *V. cholerae* integrates information about the vicinal bacterial community contained in extracellular QS autoinducers with the intracellular environmental information encoded in c-di-GMP to control biofilm formation.

Vibrio cholerae, the causative agent of the diarrheal disease cholera, exists primarily in marine environments, and infection of humans usually occurs through ingestion of contaminated water (45). After traversing the stomach, *V. cholerae* expresses an array of virulence factors that enable colonization of the host intestinal epithelium. The major colonization factor, toxin coregulated pilus, promotes adherence to the intestinal lining, and the subsequent secretion of cholera toxin leads to severe diarrhea and release of the bacterium into the environment (14). Critical to its infection cycle is *V. cholerae*'s ability to alternate between expression of virulence traits essential for survival inside the host and expression of traits such as biofilm formation that are necessary for survival in its ex vivo marine environment (13, 22, 23, 51, 59). Two chemical signaling systems, quorum sensing (QS) via autoinducer (AI) molecules (see below) and 3',5'-cyclic diguanylic acid (c-di-GMP) signaling, control the transition between these two lifestyles.

QS is a cell-cell communication process involving the production, secretion, and detection of chemical signal molecules known as AIs that allows bacteria to synchronize the behavior of the population (54). *V. cholerae* produces two AIs and responds to them by using parallel phosphorelay signaling systems (36). The two AI molecules are termed CAI-1, (S)-3-hydroxytridecan-4-one (19), and AI-2, (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (5, 36). In the low-cell density state (i.e., when AI levels are low), the AI receptors function as kinases, and funnel phosphate to the response regulator, LuxO (Fig. 1). LuxO~P activates the expression of

four genes encoding the Qrr small regulatory RNAs (sRNAs) (31, 52). The Qrr sRNAs destabilize the mRNA encoding a major regulator of QS, HapR (25, 31). This relay culminates in the expression of low-cell density specific genes, including genes required for biofilm formation and virulence factor production (17, 36, 59, 60) (Fig. 1, left side). When the cell density increases, the AIs accumulate, bind their cognate receptors, and switch the receptors to phosphatases. Phosphatase activity leads to dephosphorylation of LuxO and termination of *qrr* expression. The mRNA encoding HapR is stabilized, and HapR protein is produced (31, 36). HapR is a DNA-binding transcription factor that initiates a program of gene expression that switches the cells from the individual, low-cell-density state to the high-cell-density state (Fig. 1, right side). When HapR is produced, biofilm and virulence genes are repressed, which is proposed to promote dispersal of *V. cholerae* (17, 36, 59). The molecular mechanism connecting QS to virulence factor expression is well defined (27, 28). However, the regulatory mechanism linking QS to repression of genes controlling biofilm formation has not been established.

The intracellular second messenger molecule c-di-GMP regulates the transition from a motile lifestyle to a sessile state in numerous bacteria (reviewed in references 7, 24, 39, and 42). Proteins containing domains with GGDEF motifs synthesize c-di-GMP by the cyclization of two GTP molecules and the loss of two pyrophosphate moieties. Degradation of c-di-GMP is carried out by proteins containing domains with EAL or HD-GYP motifs. *V. cholerae* possesses 62 genes that encode proteins with domains predicted to be involved in governing c-di-GMP levels (16). Consistent with what is known in other bacteria, in *V. cholerae*, high levels of c-di-GMP enhance biofilm formation and repress virulence factor expression and motility, while low levels of c-di-GMP repress biofilm forma-

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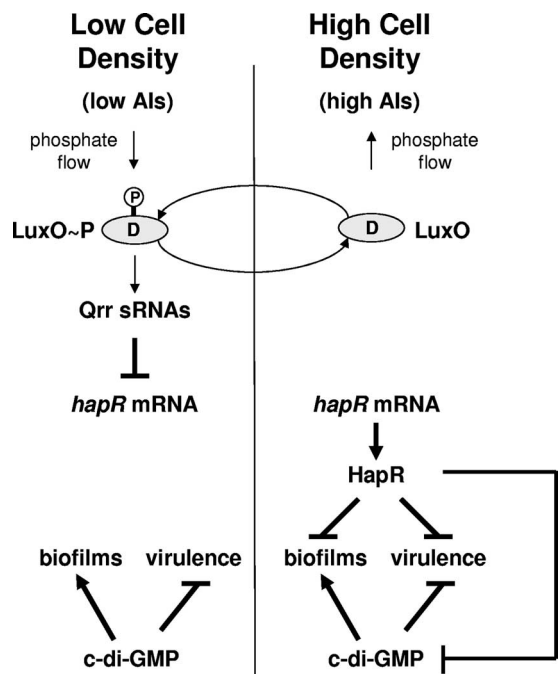


FIG. 1. Simplified model of the interaction between QS and c-di-GMP in the regulation of gene expression in *V. cholerae*. When concentrations of AIs are low (left side), the response regulator LuxO is phosphorylated, resulting in expression of multiple genes encoding the Qrr sRNAs that repress translation of the master transcriptional regulator, HapR. When concentrations of AIs are high (right side), LuxO is dephosphorylated which leads to termination of *qrr* expression. In the absence of the Qrr sRNAs, HapR is produced. HapR represses both biofilm formation and virulence factor expression. Like HapR, c-di-GMP also represses virulence factor expression, but unlike HapR, c-di-GMP activates biofilm formation. Here, HapR is shown to repress biofilm formation both directly (via control of *vpsT*) and indirectly by reducing the levels of c-di-GMP.

tion and induce virulence factor expression and motility (2, 50, 51) (Fig. 1). Therefore, c-di-GMP is proposed to mediate the transition of *V. cholerae* from the in vivo (low c-di-GMP levels) to the ex vivo (high c-di-GMP levels) lifestyle (50, 51).

Here, we show that HapR production at high cell density in *V. cholerae* represses biofilm formation through two distinct mechanisms. First, HapR alters the expression of 14 genes encoding proteins with GGDEF and/or EAL domains. QS control of these genes results in increased intracellular levels of c-di-GMP at low cell density and decreased c-di-GMP levels at high cell density. The major consequence of increased c-di-GMP in the low-cell-density state is induction of the biofilm transcriptional activator *vpsT* (4). Second, we show that HapR directly binds to and represses the expression of *vpsT*, contributing to the reduction in biofilm formation that occurs at high cell density. Artificial production of c-di-GMP in high-cell-density strains to levels that naturally occur in the low-cell-density state induces biofilm production, while artificial reduction of c-di-GMP in the low-cell-density state represses biofilm formation, indicating that c-di-GMP is epistatic to QS in the control of biofilm formation in *V. cholerae*. We propose that the integration of the c-di-GMP and QS relays allows *V. cholerae* to combine information about the surrounding bacterial population contained in extracellular chemical QS signals with

information regarding local environmental conditions contained in the intracellular chemical signal c-di-GMP to control biofilm formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All *V. cholerae* strains used in the present study were derived from the wild-type (WT) El Tor biotype strain C6706str2 (49). The strains carrying mutations in the QS circuit are *luxO(D47E)* (SLS340) and $\Delta luxO$ (SLS349). $\Delta vpsL$ mutants were constructed as previously described (48) and are designated CW2034 ($\Delta vpsL$), CW2035 [*luxO(D47E)* $\Delta vpsL$], CW2036 ($\Delta hapR \Delta vpsL$), and CW2037 ($\Delta luxO \Delta vpsL$). *V. cholerae* was grown in Luria-Bertani medium (LB) at 37°C with shaking in all cases except in bioluminescence experiments when 30°C growth with shaking was used. Antibiotics were obtained from Sigma and used at the following concentrations (in $\mu\text{g/ml}$): ampicillin, 100; kanamycin, 100; and chloramphenicol, 10 (unless stated otherwise). *qrgB*, *qrgB** (GGDEF \rightarrow AAEEF), VC1086, and VC1086* (EAL \rightarrow AAL) were induced with 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside; Sigma). The *E. coli* strains S17- λ pir (8) was used for general cloning procedures, while BL21 (35) was used to determine QrgB toxicity in *E. coli* (data not shown).

DNA manipulations. DNA manipulation was performed by using standard procedures (44). T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (NEB). PCRs were carried out with iProof DNA polymerase (NEB). *qrgB* was PCR amplified from *V. harveyi* WT strain BB120, and VC1086 was amplified from WT *V. cholerae*. Both were cloned into the EcoRI and BamHI sites of pEV5143 (12). *gfp* transcriptional fusions were constructed in the plasmid pCMW1 (55) by insertion into the SphI and SalI restriction sites. The *qrgB** (AAEEF) and VC1086* (AAL) alleles were constructed by using a QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The sequences and designations of all plasmids and primers used are available on request.

***gfp* and bioluminescence measurements.** *V. cholerae* strains CW2035 and CW2037 carrying the GGDEF- and/or EAL-*gfp* transcriptional fusions were grown overnight and diluted 1/100 in triplicate in LB broth with kanamycin. *gfp* expression was determined by using a BD FACSAria (Becton Dickinson) flow cytometer at 6 h (prestationary phase, optical density = 1.0) and 20 h (stationary phase, optical density = 2.5) by diluting 40 μl of the culture in 1 ml of phosphate-buffered saline. The flow cytometer settings were as follows: forward scatter (log) voltage 400, side scatter (log) voltage 505, and fluorescein isothiocyanate (log) voltage 626. Only genes that showed reproducible twofold changes in regulation between the low- and high-cell-density locked mutants were considered QS regulated. The expression analyses of *vpsT*, *vpsR*, and *vpsL* were performed in Costar 96-well black-bottom plates (catalog no. 3904). The vector control was the plasmid pEV5141 (12). Overnight cultures were diluted 1/100 in triplicate in LB broth with kanamycin (10 $\mu\text{g/ml}$), chloramphenicol (1 $\mu\text{g/ml}$), and IPTG, and bioluminescence was determined on an EnVision Multilabel 2103 plate reader (Perkin-Elmer) after 7 h of growth.

Purification of HapR and gel mobility shift assays. HapR was purified with the IMPACT protein purification system (NEB) using the expression plasmid pTYB11 and the protocol described in the manufacturer's instructions. Purified HapR was stored in 20 mM Tris (pH 7.5), 1 mM EDTA, 10 mM NaCl, and 0.1 mM dithiothreitol with 20% glycerol as previously described (33). DNA probes for gel mobility shift analyses were generated by using 5'-tagged fluorescent primers in a standard PCR and purified by using the ZymoClean gel DNA recovery kit (Zymo Research). Each probe (10 nM) was incubated with the indicated amount of HapR (42 to 1,000 nM) and 1 μl of 1 μg of poly(dI-dC)/ml in a final volume of 20 μl at 30°C for 15 min. Gel mobility shifts were performed on a 5% TAE-polyacrylamide gel and visualized by using a Storm 860 imaging system (Molecular Dynamics). For the experiment shown in Fig. 8, purified c-di-GMP, or an equivalent amount of 5% methanol was preincubated with 200 nM HapR at the indicated concentrations for 15 min at 30°C prior to the addition of the DNA probe.

Determination of intracellular c-di-GMP levels. c-di-GMP levels were determined in *V. cholerae* WT and *luxO(D47E)* strains containing the vector pEV5141 (12) or overexpressing *qrgB*, *qrgB**, VC1086, and VC1086*. Each strain was inoculated from a frozen stock in 3 ml of LB with kanamycin in an 18X150 mM glass tube and grown overnight with shaking at 37°C. Inoculation of overnight cultures with a previous overnight culture showed lower fold differences in c-di-GMP than direct inoculation from the frozen stock culture (~3-fold versus 20-fold, respectively). This difference between inoculating techniques may indicate that the c-di-GMP levels decrease over time in stationary phase. C-di-GMP was also measured at early times in the growth curve (optical density at 600 nm

of 0.1 to 0.5), but only low level c-di-GMP was detected, and no significant differences were observed between strains. Analysis at low cell densities requires a long centrifugation step that may result in a reduction in c-di-GMP signal. Frozen stock cultures were prepared by diluting 750 μ l of an overnight culture with 250 μ l of 80% glycerol. After 16 h of growth, 1.5 ml of each culture was pelleted in a benchtop microcentrifuge at 16,100 relative centrifugal force for 30 s. The resulting supernatant was removed, 300 μ l of lysis buffer (40% acetonitrile, 40% methanol, 0.1% formic acid) (38) was added, and the pellet was resuspended with a pipette and mixed for 30 s by vortexing, followed by 15 min of incubation on ice. Insoluble material was removed by benchtop centrifugation at 16,100 relative centrifugal force for 5 min at 4°C. The resulting supernatant was collected and analyzed by using liquid chromatography-tandem mass spectrometry on a Finnigan TSQ Quantum DiscoveryMax triple quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA), coupled with an LC-20AD HPLC system (Shimadzu, Columbia, MD). c-di-GMP was detected by using selected reaction monitoring analyses (SRM) in negative ionization mode. Two SRM were monitored simultaneously, m/z 689 \rightarrow 344 at 32 eV and m/z 689 \rightarrow 150 at 45 eV, which gave a signal ratio of 1:0.4 (Fig. 4A). The mass spectrometry parameters were as follows: spray voltage, 3,000 V; nitrogen as sheath gas at 30 lb/in² and auxiliary gas at 10 lb/in²; argon as the collision gas at 1.5 mtorr, and capillary temperature of 325°C. The scan time for each SRM was 0.1 s with a scan width of 1 m/z .

Chromatography conditions for c-di-GMP analyses were reverse phase with the ion pairing agent tributylamine (34). Liquid chromatography separation was achieved on a Synergi Hydro-RP column (4- μ m particle size, 150 by 2 mm; Phenomenex, Torrance, CA) at an acidic pH. Solvent A was 10 mM tributylamine plus 15 mM acetic acid in 97:3 water-methanol. Solvent B was methanol. The gradient was as follows: $t = 0$, 0% solvent B; $t = 5$ min, 0% solvent B; $t = 10$ min, 20% solvent B; $t = 15$ min, 20% solvent B; $t = 30$ min, 65% solvent B; $t = 33$ min, 95% solvent B; $t = 37$ min, 95% solvent B; $t = 38$ min, 0% solvent B; and $t = 45$ min, 0% solvent B. Each sample had a running time of 45 min. Under this chromatography condition, c-di-GMP had a retention time of 27.5 min. Other liquid chromatography parameters were as follows: autosampler temperature, 4°C; column temperature, 25°C; injection volume, 10 μ l; and solvent flow rate, 200 μ l/min. The signal for c-di-GMP in biological samples was defined as the observed peak area on the corresponding chromatography trace. All of the samples had 1 μ g of isotopically labeled ATP/ μ l (>98% ¹³C, >98% ¹⁵N [Medical Isotopes, Pelham, NH]; SRM m/z 521 \rightarrow 423 at 21 eV) as the internal standard; no systematic variation in the internal standard was observed. Chemically synthesized c-di-GMP (GLSynthesis, Inc., Worcester, MA, using a modification of methods described previously [26, 40]) was used to determine the retention time and fragmentation pattern of genuine c-di-GMP.

This method had a limit of detection of 5 ng/ml. Running the c-di-GMP standard dissolved in 50:50 methanol-H₂O in a series of concentrations (5 ng/ml, 10 ng/ml, 100 ng/ml, 1 μ g/ml, and 10 μ g/ml) showed that the assay is linear from 5 ng/ml to 10 μ g/ml with an R^2 of 0.999. In Fig. 4A, representative chromatography traces for the c-di-GMP purified standard and for a typical biological sample are shown. In the biological sample, in addition to c-di-GMP (retention time, 27.6 min), there is also a peak at 26.1 min corresponding to an unknown interfering compound. Accurate mass measurement of the biological sample in positive ion mode was made on an Orbitrap mass spectrometer (Thermo Electron Corp.) and revealed masses for two peaks of 691.1356 and 691.1011 with associated mass errors relative to protonated c-di-GMP (C₂₀H₂₅N₁₀O₁₀P₂⁺; m/z 691.1021) of 48 and 1.5 ppm (the typical mass error on the Orbitrap is <2 ppm) (21). These mass data confirm that the latter peak corresponds to c-di-GMP, while the first peak does not match the chemical formula of c-di-GMP.

Biofilm formation. Static cultures were grown for 24 h from a 1/100 dilution of an overnight culture that was previously grown shaking at 37°C. Glass tubes (18 by 150 mm) containing 5 ml of LB with kanamycin and IPTG were used, and biofilm formation was monitored by the generation of pellicles at the air-liquid interface.

RESULTS

QS regulates the expression of 14 genes encoding proteins with GGDEF and EAL domains. Two genes encoding proteins with GGDEF domains, *qrgA* and *qrgB* (quorum-regulated GGDEF, denoted promoters 275 and 342 [55]; gene peptide identifications ABU70180.1 and ABU69224.1, respectively), were identified as repressed at high cell density in the model QS bacterium *Vibrio harveyi*. *V. harveyi* only poorly produces

biofilms, so although the initial link between QS and c-di-GMP regulators was made in *V. harveyi*, examining their inputs into biofilm formation was difficult to pursue. For this reason, we chose to explore the link between QS, c-di-GMP, and biofilm formation in the related bacterium, *V. cholerae*, which produces robust biofilms (17, 53, 58, 59).

We inferred, based on the *V. harveyi* finding, that QS might influence the transcription of genes involved in the production of c-di-GMP in *V. cholerae*. *V. cholerae* possesses 31 genes encoding proteins containing GGDEF domains, 10 genes encoding proteins containing GGDEF and EAL domains, and 12 genes encoding proteins with only EAL domains. In addition, *V. cholerae* has nine genes encoding proteins with HD-GYP domains, a family of proteins that have also been shown to degrade c-di-GMP (11, 16, 41). To determine the extent of QS regulation of genes involved in c-di-GMP signaling in *V. cholerae*, *gfp* transcriptional fusions were constructed to 52 of the 53 genes encoding GGDEF- or EAL-containing proteins. Multiple attempts to PCR amplify the promoter of VC0515 were unsuccessful, which suggests it is either not present or is altered in the genome of the *V. cholerae* strain used here (El Tor biotype strain C6706str2) compared to the sequenced strain (El Tor N16961). For genes encoded in operons, the promoter preceding the first gene was cloned and analyzed. Analysis of the regulation of HD-GYP proteins by QS will be reported elsewhere (B. Hammer and B. Bassler, unpublished data).

Flow cytometry was used to measure expression of each *gfp* promoter fusion in a *V. cholerae luxO(D47E)* mutant that constitutively mimics the low-cell-density state versus the expression in a *V. cholerae Δ luxO* mutant that constitutively mimics the high-cell-density state. The *V. cholerae luxO(D47E)* mutant (15) locks the cells at low cell density because it harbors a gain of function allele of *luxO* that constitutively mimics LuxO~P, leading to constitutive Qrr sRNA expression and repression of *hapR* (31) (see Fig. 1, left side). In contrast, the *V. cholerae Δ luxO* mutant is locked in high cell density because the Qrr sRNAs are not transcribed and, consequently, the *hapR* mRNA is not destabilized (31) (Fig. 1, right side). The *gfp* expression of each promoter fusion was determined at two time points, and 14 of the 52 promoters examined exhibited QS regulation (Fig. 2). QS regulation of each of these promoters depended on HapR (data not shown), suggesting that all of these genes lie downstream of the known QS machinery. VCA0074 was previously identified in a microarray analysis aimed at identifying QS regulated genes (59). VCA0074 was also identified in experiments examining the *V. cholerae* smooth-rugose colony transition and was named *cgdA* (32).

HapR both directly and indirectly controls gene expression (17, 27, 28, 33, 36, 59). For this reason, gel mobility shift assays were used to determine which of the QS-regulated promoters of genes encoding GGDEF and EAL proteins are directly controlled by HapR. Regions of the *aphA* promoter containing a known HapR binding site (*aphA*⁺) and lacking the binding site (*aphA*⁻) were used as controls for HapR binding specificity (28). As expected, purified HapR bound and shifted the *aphA* upstream DNA region containing the HapR binding site but not the DNA lacking the site (Fig. 3). Six of the fourteen QS-regulated promoters driving genes encoding proteins containing GGDEF and/or EAL domains interacted with HapR. Five promoters bound HapR at levels similar to those required

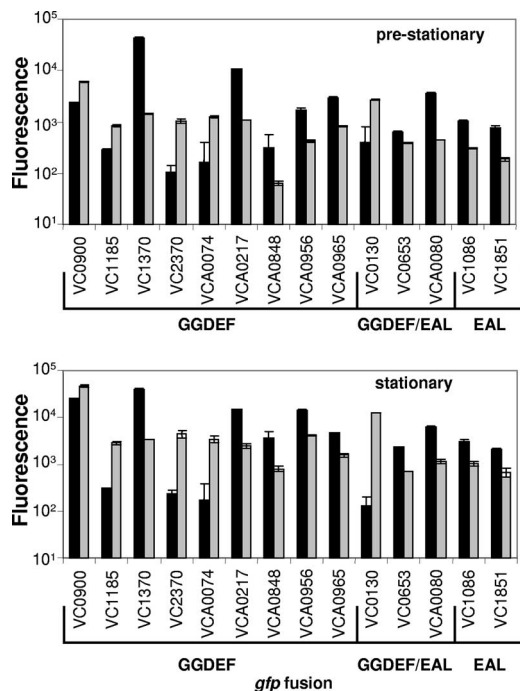


FIG. 2. QS regulates 14 genes involved in the control of c-di-GMP. The expression of *gfp* transcriptional fusions to the promoter of each predicted GGDEF and EAL encoding gene was measured in the high-cell-density $\Delta luxO$ strain (■) and the low-cell-density *luxO(D47E)* strain (□) at two time points (prestationary, top; and stationary, bottom). Fourteen genes showed twofold or greater regulation. The QS-controlled genes containing only a GGDEF motif (GGDEF), a GGDEF and EAL motif (GGDEF/EAL) and only an EAL motif (EAL) are indicated on the x axis. The error bars show the standard deviation of three independent cultures.

for binding to the *aphA* promoter (right panels, VC2370, VC0900, VC1851, VC1086, and VCA0074), while one promoter required higher levels of HapR and also showed larger, less-specific mobility shifts (left panel, VCA0080). Both QS-activated and QS-repressed promoters were directly bound by HapR. There was no correlation between the fold regulation of transcription and direct interaction with HapR. For example, VC1370 is strongly induced by QS (30.1- and 11.9-fold at the late-exponential phase and stationary phase, respectively) but shows no HapR binding.

These results indicate that QS, either directly or indirectly through HapR, significantly influences the expression of multiple genes encoding proteins with GGDEF and/or EAL domains. Based on previous studies showing that QS and c-di-GMP reciprocally control biofilm formation in *V. cholerae* (2, 17, 50, 59) (Fig. 1), we would predict that GGDEF-containing proteins that synthesize c-di-GMP would be repressed by QS and therefore expressed at low cell density (to promote biofilm formation), whereas EAL-containing proteins that degrade c-di-GMP would be activated by QS and dominate at a high cell density (to repress biofilm formation). However, an overall pattern of regulation is not obvious. Of the nine genes encoding proteins with exclusively GGDEF domains, four are repressed by QS, while five are activated. Likewise, one gene encoding a protein with both GGDEF and EAL domains was repressed, while two were activated. Consistent with our pre-

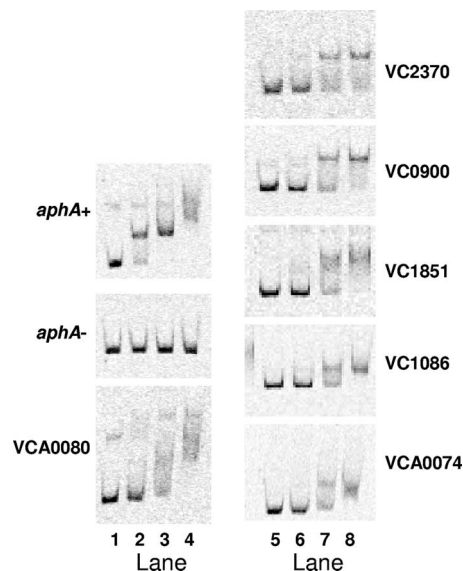


FIG. 3. HapR binds six promoters preceding genes encoding GGDEF and EAL proteins. Gel mobility shifts are shown for the promoters of genes encoding GGDEF and EAL containing proteins that interacted with HapR. HapR concentrations are as follows: lanes; 1 and 5, 0 nM; 2, 80 nM; 3, 420 nM; 4, 1,000 nM; 6, 40 nM; 7, 200 nM; and 8, 500 nM. *aphA*⁺, contains the HapR binding site; *aphA*⁻, lacks the HapR binding site.

diction, two genes encoding proteins containing only EAL domains were activated by QS. A *V. cholerae* gene encoding a GGDEF protein (VCA0939) was recently shown to be directly activated posttranscriptionally by the Qrr sRNAs acting independently of HapR (18). QS regulation of the VCA0939-*gfp* fusion was not observed in the present study, highlighting that the *gfp* fusions used here report transcriptional, not posttranscriptional, control.

QS controls the intracellular level of c-di-GMP in *V. cholerae*. Although HapR-mediated regulation of the transcription of genes encoding GGDEF- and EAL-type proteins did not exhibit a clear pattern, the net result of the transcriptional alterations we observed could translate into modulation of the intracellular pool of c-di-GMP, which in turn could control biofilm formation. To examine this possibility, we developed a high-pressure liquid chromatography-tandem mass spectrometry method to measure the relative concentration of c-di-GMP in bacterial lysates. We measured c-di-GMP in the *V. cholerae* WT strain to determine the level present at high cell density and in the *luxO(D47E)* locked low-cell density mutant to determine the level present at low cell density. The retention time and fragmentation pattern of authentic c-di-GMP was determined by using a chemically synthesized standard (a generous gift from George O'Toole). A peak with identical properties to the c-di-GMP standard was observed in each strain (Fig. 4A). Importantly, the *luxO(D47E)* locked low-cell-density mutant contained 20-fold more c-di-GMP than the WT grown to high cell density (Fig. 4B, vector control). Thus, QS control of transcription of genes encoding GGDEF and EAL proteins indeed alters the intracellular level of c-di-GMP. This result agrees with a previous finding that shows a $\Delta hapR$ rugose colony morphology of *V. cholerae* (i.e., that also mimics low

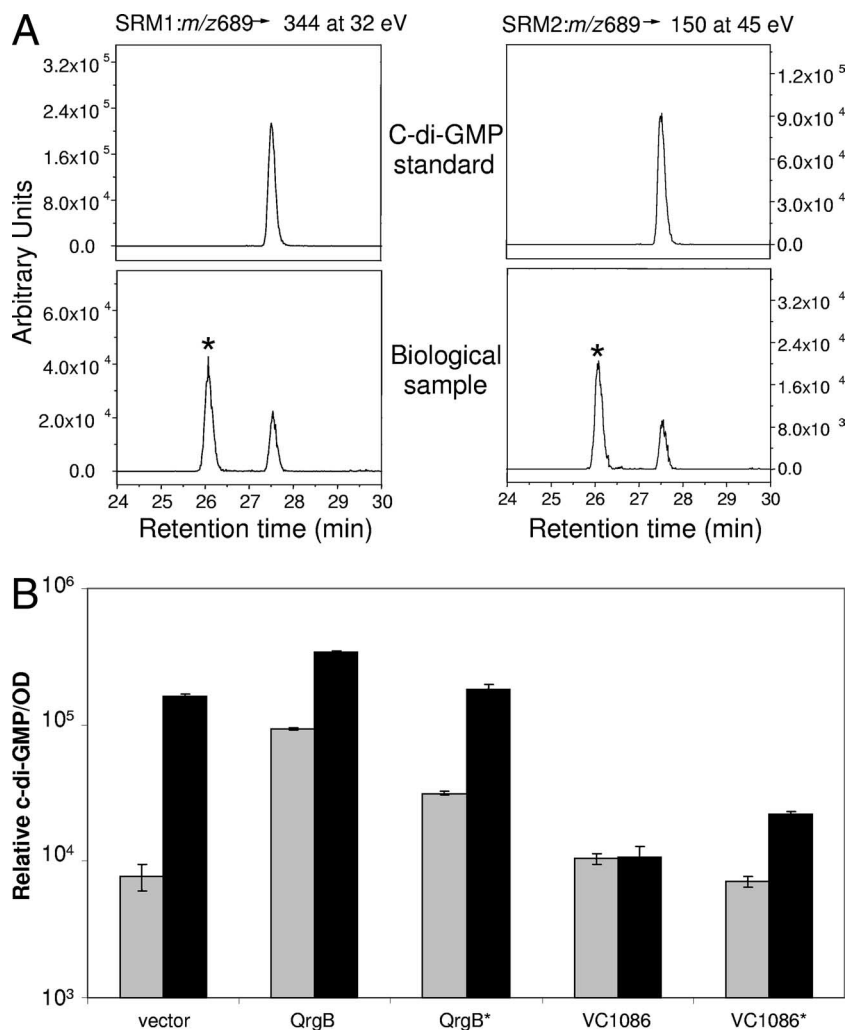


FIG. 4. c-di-GMP levels in *V. cholerae* strains. (A) In vivo c-di-GMP levels were determined by measuring ionization fragments with SRM1 (m/z 689→344 at 32 eV [left]) and SRM2 (m/z 689→150 at 45 eV [right]). The top panels show the purified standard at 2 $\mu\text{g/ml}$ in 50:50 methanol- H_2O , and the bottom panels show the biological sample. c-di-GMP has a retention time of 27.5 min. The peak labeled with an asterisk in the biological sample is an unknown interfering compound that is neither a conformer nor an isomer of c-di-GMP. (B) Relative levels of c-di-GMP in *V. cholerae* WT (□) and the *luxO(D47E)* mutant (■) containing a vector control or overexpressing QrgB, QrgB* (GGEEF→AAEEF), VC1086, or VC1086* (EAL→AAL) were determined by tandem high-pressure liquid chromatography–mass spectrophotometric analysis. Each strain was analyzed in triplicate. Error bars indicate the standard errors of the mean.

cell density) has higher levels of c-di-GMP than the smooth morphotype (32). High levels of c-di-GMP at the low-cell-density QS state are consistent with biofilm formation, while decreased c-di-GMP concentrations at the high-cell-density state correspond to reduced biofilm formation.

c-di-GMP is epistatic to QS in control of biofilm formation in *V. cholerae*. Increased c-di-GMP at the low-cell-density state relative to the high-cell-density state suggests that QS could control biofilm formation in *V. cholerae* through modulation of c-di-GMP levels. If so, we reasoned that increasing c-di-GMP levels in *V. cholerae* at a high cell density by overexpression of an active GGDEF type protein could reestablish biofilm formation under this condition. To test this, we used the GGDEF protein, QrgB, which we had previously studied in *V. harveyi* (identified as AI-regulated clone 342, ABU69224.1) (55). QrgB was chosen for two reasons. First, overexpression of QrgB was toxic to the *E. coli* strain BL21, indicating that QrgB

functions as a diguanylate cyclase under laboratory conditions (43). Mutation of the QrgB active site (QrgB*, GGEEF→AAEEF) alleviated toxicity, showing that, indeed, toxicity is due to c-di-GMP production (data not shown). Second, *V. cholerae* strain C6706str2 does not possess a homolog for *qrgB*. Thus, we reasoned that phenotypes associated with overexpression of *qrgB* would most likely result from c-di-GMP production rather than another property of the protein, such as interaction with other cellular components.

To examine what effect increasing c-di-GMP has on *V. cholerae* biofilm formation at high cell density, we assayed pellicle formation in the WT strain grown to high cell density and in the locked low-cell-density *luxO(D47E)* mutant with or without *qrgB* overexpression (Fig. 5). *V. cholerae* WT does not normally produce pellicles (17); however, overexpression of *qrgB* induced biofilm formation in this strain (Fig. 5). This activity was dependent on c-di-GMP production by QrgB because overex-

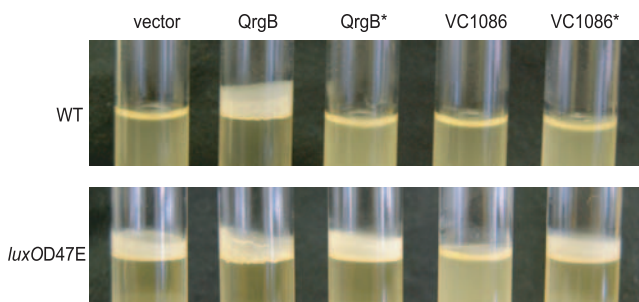


FIG. 5. c-di-GMP is epistatic to QS in the control of biofilms. Biofilm formation in overnight static cultures of *V. cholerae* WT and *luxO(D47E)* strains containing either the vector alone (denoted vector) or overexpressing QrgB, QrgB* (GGEEF→AAEEF), VC1086, or VC1086* (EAL→AAL).

pression of the QrgB* active site mutant did not induce biofilm formation (Fig. 5). As expected, the *luxO(D47E)* strain formed biofilms under each of these conditions.

Using the same logic, we hypothesized that reducing c-di-GMP levels in the *luxO(D47E)* mutant strain through overexpression of an EAL-type protein should inhibit biofilm formation. For these experiments, the EAL encoded by the locus VC1086 was overexpressed. VC1086 was chosen for two reasons. First, expression of VC1086 is activated directly by HapR (Fig. 2 and 3), suggesting it may be involved in the natural reduction of c-di-GMP levels that occurs at high cell density. Second, VC1086 encodes the key amino acid residues that are predicted to be critical in enzymatically active EAL domains (46). As predicted, overexpression of VC1086 resulted in poor biofilm formation in the *luxO(D47E)* mutant (Fig. 5). The biofilm reduction caused by VC1086 depended on its c-di-GMP degradation activity because overexpression of an active site mutant of VC1086 (VC1086*, EAL→AAL) did not eliminate biofilm formation (Fig. 5). No biofilms were formed under any condition in a $\Delta vpsL$ mutant strain, suggesting the biofilms observed require the known *vps* extracellular polysaccharide biosynthetic operon (data not shown). Because the production of c-di-GMP at high cell density or removal of c-di-GMP at low cell density overrides QS control of biofilm formation, we conclude that c-di-GMP is epistatic to QS in the control of biofilm formation in *V. cholerae*.

We measured the corresponding alterations in c-di-GMP that occur after the overexpression of QrgB and VC1086 in the WT strain and the *luxO(D47E)* mutant (Fig. 4B). The amount of c-di-GMP produced after QrgB overexpression in the WT strain is roughly equivalent to that produced endogenously in locked low-cell-density strains (see second gray bar in Fig. 4B). Thus, we infer that the effect of increasing c-di-GMP on biofilm formation through overexpression of QrgB that we observe at high cell density (Fig. 5) is physiologically relevant and not due to unnaturally high levels of c-di-GMP. A modest but reproducible increase in c-di-GMP levels occurred when the QrgB* active site mutant protein was overexpressed in the WT strain, but this change was significantly less than that after overexpression of WT QrgB. Although we do not understand this result, we note that QrgB encodes a RXXD product inhibition feedback motif (6), which would likely remain functional in the QrgB* allele. One possibility is that QrgB* could bind

c-di-GMP at the RXXD site reducing the free intracellular c-di-GMP pool. This reduction in free c-di-GMP may alleviate product inhibition in other GGDEF proteins in the cell, slightly increasing their diguanylate cyclase activities. Overexpression of VC1086 in the *luxO(D47E)* mutant reduced the c-di-GMP concentration consistent with the ability of VC1086 to repress biofilm formation in this strain. Overexpression of the active site allele of VC1086 (VC1086*) in the *luxO(D47E)* low-cell-density mutant only partially restored normal c-di-GMP levels. Apparently, this level of c-di-GMP is sufficient to promote biofilm formation in the *luxO(D47E)* mutant (Fig. 5). Importantly, Fig. 4B shows that similar levels of c-di-GMP are present when the QrgB* allele is overexpressed in the WT strain, and the VC1086* allele is overexpressed in the *luxO(D47E)* strain. However, the biofilm phenotypes of these two strains are dramatically different (Fig. 5). One notable difference between these two strains is that the WT produces HapR, whereas the *luxO(D47E)* mutant does not (Fig. 1). This result suggests that, in addition to modulating c-di-GMP levels through the activation and/or repression of transcription of genes encoding GGDEF and EAL proteins, HapR could control biofilm formation through additional mechanisms.

HapR and c-di-GMP reciprocally control *vpsT* expression. Biofilm formation in *V. cholerae* is primarily controlled by two transcriptional activators, VpsR and VpsT, which are required for the expression of the *Vibrio* polysaccharide biosynthesis operons, *vpsA-K* and *vpsL-Q* (4, 56). We previously showed that QS regulates biofilm formation specifically through repression of *vpsT* (30). QS repression of *vpsR* has also been reported (57), but we have not observed this (17). Increased c-di-GMP is reported to activate both *vpsT* and *vpsR* (2, 50). Because HapR reduces c-di-GMP levels via altering overall GGDEF and EAL activity and because c-di-GMP is epistatic to HapR in the control of biofilm formation (Fig. 5), we predict that HapR and c-di-GMP must function in the same regulatory pathway. Furthermore, because c-di-GMP and HapR have opposite effects on biofilm formation (Fig. 1), we predict that HapR and c-di-GMP must act reciprocally. Given previous studies, the obvious candidate(s) for the point at which QS and c-di-GMP integrate information into biofilm formation is *vpsT* and/or *vpsR*.

To examine these hypotheses, we analyzed expression of *vpsR*, *vpsT*, and *vpsL* transcriptional fusions to luciferase in both WT (Fig. 6, gray bars) and the *luxO(D47E)* mutant (Fig. 6, black bars) *V. cholerae* strains. We also measured their transcription in the presence of high levels of c-di-GMP (via overexpression of QrgB) and low c-di-GMP levels (via overexpression of VC1086). Overexpression of the QrgB* and VC1086* active-site mutant alleles had no effect on the expression of the *vps* genes compared to the vector control (data not shown). We note that increasing c-di-GMP levels in the *luxO(D47E)* mutant causes *V. cholerae* to aggregate, making quantification of gene expression difficult. For this reason, each of the strains used in this set of experiments carried an in-frame deletion of *vpsL* to eliminate polysaccharide production and subsequent aggregation.

Consistent with our previous work showing that QS represses biofilm formation, *vpsT* and *vpsL* expression are repressed in the WT strain grown to high cell density relative to the *luxO(D47E)* locked low-cell-density mutant, whereas no

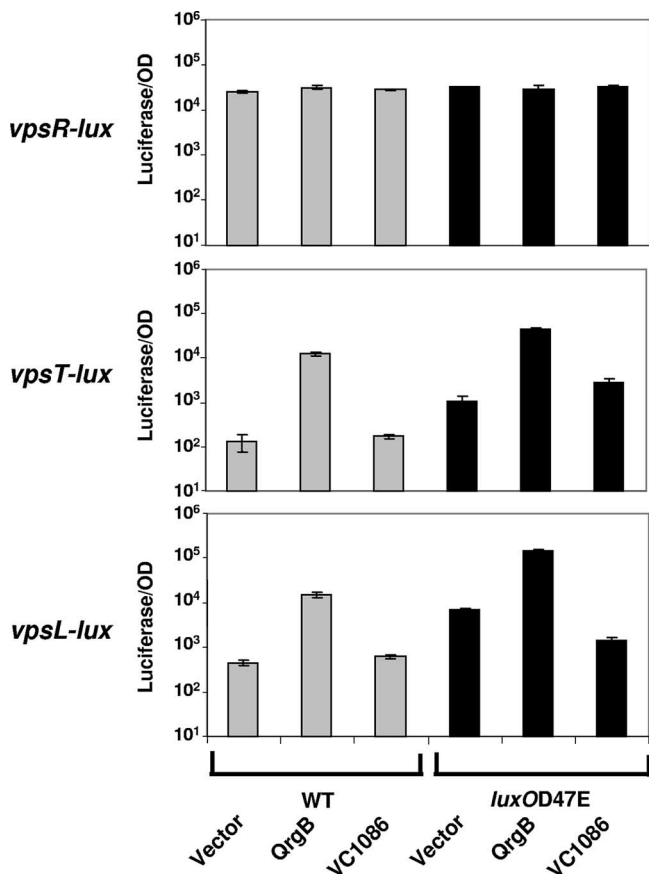


FIG. 6. QS and c-di-GMP reciprocally regulate *vpsT* and *vpsL*. Expression of *vpsR-lux* (top), *vpsT-lux* (middle), and *vpsL-lux* (bottom) was determined in WT (gray bars) and *luxO(D47E)* (black bars) *V. cholerae* strains containing a vector control or overexpressing QrgB or VC1086. Error bars indicate the standard deviations of three independent cultures.

QS regulation of *vpsR* occurred (Fig. 6, vector controls). In contrast, increasing c-di-GMP activated expression of *vpsT* and *vpsL* in both the WT and the *luxO(D47E)* genetic backgrounds. In the reciprocal experiment, overexpression of VC1086 reduced expression of *vpsL-lux* (fivefold) in the *luxO(D47E)* mutant strain, a finding consistent with a reduction in biofilm formation under this condition. Interestingly, overexpression of VC1086 had no effect on *vpsT-lux* expression. No difference in *vpsL* or *vpsT* expression occurred upon overproduction of VC1086 in the WT. This final result is not unexpected since the WT strain has naturally low levels of c-di-GMP (Fig. 4B). Unlike previously published results (2), we observed no differences in *vpsR-lux* expression upon changes in c-di-GMP levels. From these results, we conclude that the major influence of QS and c-di-GMP on the regulation of biofilm formation occurs through transcriptional changes in *vpsT* and *vpsL*. Since VpsT activates the expression of *vpsL*, the QS and c-di-GMP effects on *vpsL* transcription are likely due, in a large part, to downstream consequences of regulation of *vpsT*. However, because overexpression of VC1086 reduced *vpsL* but not *vpsT* expression, c-di-GMP levels may also control expression of *vpsL* through a *vpsT*-independent mechanism.

We considered two possible mechanisms for how HapR and

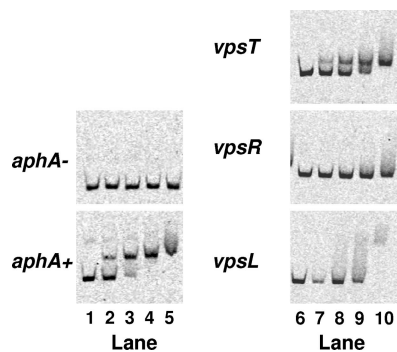


FIG. 7. HapR binds the *vpsT* promoter. Gel mobility shift analyses of the *vpsT*, *vpsR*, and *vpsL* promoters with purified HapR. HapR amounts are as follows: lanes 1 and 6, 0 nM; 2 and 7, 40 nM; 3 and 8, 120 nM; 4 and 9, 250 nM; and 5 and 10, 500 nM. *aphA-*, no HapR binding site; *aphA+*, contains the HapR binding site.

c-di-GMP regulate *vpsT*. First, the effect of HapR on *vpsT* expression could be exclusively through control of c-di-GMP levels. In this scenario, HapR-controlled reductions in c-di-GMP levels at high cell density are translated (by an unknown mechanism) into transcriptional repression of *vpsT* expression. However, our results demonstrate that artificially reducing c-di-GMP in the absence of HapR is not sufficient to fully repress *vpsT* (Fig. 4B and 6). A second possibility is that HapR controls c-di-GMP levels which impinge on *vpsT* expression, and HapR also regulates the *vpsT* promoter. To investigate this second possibility, we determined whether HapR could bind to the *vpsT* (and *vpsR* and *vpsL*) promoter by using gel mobility shift assays. Indeed, HapR directly binds the *vpsT* promoter (Fig. 7). At high concentrations of HapR, we also observed a shift of the *vpsL* promoter, but we do not know whether this interaction is physiologically relevant. HapR showed no interaction with the *vpsR* promoter at any concentration examined. From these data, we conclude that, in addition to modulating the levels of c-di-GMP, HapR directly represses *vpsT* expression. We note that these results support the findings in Fig. 6 that QS regulates biofilm formation exclusively through the repression of *vpsT* (and not via the repression of *vpsR*).

HapR is a member of the TetR family of DNA repressors. This family of proteins typically possesses an N-terminal DNA-binding domain and a C-terminal sensory domain that interacts with a small molecule to modulate its DNA-binding activity (10, 20, 37, 47). Consistent with these findings, the recently reported crystal structure of HapR shows it contains a water-soluble channel in its C-terminal sensory domain that could bind a small molecule ligand (9). A mechanism for reciprocal regulation of *vpsT* by HapR and c-di-GMP that incorporates these new structural results and our above findings is that HapR binds to c-di-GMP, and this prevents HapR from binding the *vpsT* promoter. To test this possibility, binding of HapR to the *aphA* and *vpsT* promoters was examined in the presence or absence of chemically synthesized c-di-GMP. No difference in DNA binding to either promoter occurred at any c-di-GMP concentration examined (Fig. 8). Therefore, we conclude that HapR represses *vpsT* expression both by reducing the cellular concentration of c-di-GMP via altering transcription of genes

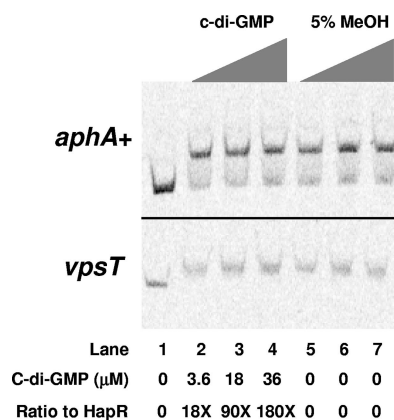


FIG. 8. C-di-GMP does not alter HapR DNA binding. HapR binding to the *aphA* (top panel) and *vpsT* promoter (bottom panel) was determined in the presence of increasing amounts of c-di-GMP (lanes 2 to 4) or a 5% methanol buffer control (lanes 5 to 7). The concentration of c-di-GMP and the molar ratio of c-di-GMP to HapR are indicated below the panels. Lane 1 contains no HapR, while lanes 2 to 7 have 200 nM HapR.

encoding GGDEF and EAL proteins and by directly binding to the *vpsT* promoter.

DISCUSSION

Bacteria use both intra- and extracellular chemical signals to couple information about their physiology, the surrounding environment, and the vicinal bacterial community into changes in gene expression (3). Detection of this sensory information is vital to direct proper responses to fluctuating environmental conditions. For *V. cholerae*, this response can be simplified to a transition between in vivo (virulence) or ex vivo (biofilm) developmental pathways. Although virulence factors such as the toxin-coregulated pilus and cholera toxin promote adhesion and nutrient acquisition inside the host and subsequently, dissemination into the environment at high numbers, biofilm formation enables *V. cholerae* to survive in nutrient-poor conditions outside of the host (22, 23, 59). Biofilm formation also increases the infectivity of *V. cholerae*, but, importantly, dispersal of biofilms is thought to occur once the bacteria colonize the host (13, 59). Two chemical signaling pathways converge to control the transition between these two critical developmental fates, QS and c-di-GMP (17, 36, 50, 51).

Based on our previous discovery of a link between QS and transcriptional regulation of GGDEF proteins in *V. harveyi* (55), we hypothesized that QS might regulate biofilm formation in *V. cholerae* through modulation of intracellular c-di-GMP levels. A number of lines of evidence suggest that this model is correct. First, HapR regulates the transcription of 14 genes encoding proteins harboring GGDEF and/or EAL domains. Second, the intracellular concentration of c-di-GMP is higher in locked low-cell-density strains than in locked high-cell-density strains. Third, production of c-di-GMP at high cell density at levels analogous to those present in the low-cell-density state induces biofilm formation, showing that c-di-GMP is epistatic to HapR in the control of biofilms. Finally, HapR and c-di-GMP converge to control the identical regulatory component in the biofilm transcriptional cascade, *vpsT*. In

addition to regulating *vpsT* through the modulation of c-di-GMP levels, HapR directly represses *vpsT* expression.

The influence of HapR on biofilm formation through the transcriptional regulation of GGDEF- and EAL-type proteins likely depends heavily on environmental stimuli that control GGDEF and EAL activities. In contrast, direct repression of *vpsT* by HapR is likely only responsive to changes in the surrounding bacterial population and thus less sensitive to prevailing environmental conditions. Repression of *vpsT* requires the simultaneous presence of HapR and low c-di-GMP levels because either the absence of HapR or high c-di-GMP levels induces expression of *vpsT* (Fig. 6). As mentioned above, c-di-GMP signaling is epistatic to QS signaling because high c-di-GMP levels, which presumably reflect ex vivo conditions, override HapR-directed repression of *vpsT* (Fig. 6), and ultimately biofilm formation (Fig. 5). This final finding suggests that, under environmental conditions favoring high c-di-GMP levels, *V. cholerae* could produce biofilms irrespective of cell density.

How changes in c-di-GMP concentrations are coupled to changes in the transcription of target genes in bacteria is not known. We examined whether HapR could be a component of this unknown regulatory cascade, but our results showed no alteration in HapR DNA binding in the presence of c-di-GMP. It remains possible that HapR does bind c-di-GMP and that binding modulates the activity of HapR without disrupting gross DNA binding, which would not be detected in our assay. The *bet* and *mer* systems in *E. coli* both contain transcriptional repressors whose activities are modulated by interaction with cognate ligands while DNA binding is not affected (1, 29).

Although QS regulates the transcription of 14 GGDEF- and/or EAL-encoding proteins, we predict that this is but one layer of regulation modulating intracellular c-di-GMP concentrations. Most of these proteins have N-terminal sensory domains that are hypothesized to recognize specific environmental cues (7, 42). Recognition of these cues elicits changes in the enzymatic activities of the corresponding C-terminal GGDEF or EAL domains. Therefore, environmental conditions greatly influence c-di-GMP levels. Our results demonstrate that global HapR transcriptional control of genes encoding GGDEF- and EAL-type proteins indeed alters c-di-GMP levels, suggesting that, at least in some cases, changes in transcription lead directly to changes in net c-di-GMP biosynthesis or degradation. This finding implies that at least some of the cues recognized by the 14 GGDEF/EAL proteins controlled by QS are present in our experimental setup. However, our experiments do not sample changes in the local environment that would normally be experienced by *V. cholerae* transitioning between aquatic reservoirs in the environment and in vivo niches inside of a host. These environmental changes, coupled with QS-controlled transcriptional regulation of genes encoding GGDEF and EAL proteins, could uniquely set the optimal intracellular c-di-GMP concentrations required for survival in various environments, as well as during the transitions between them. Because we are not currently able to simulate these environments, many of the cues detected by proteins controlling c-di-GMP levels are likely not present under laboratory conditions. Thus, we suspect that the results we show here, and specifically the changes in c-di-GMP we observe in cells, likely underestimate the full range of c-di-GMP concentrations, and thus the

full range of phenotypic changes, the cells naturally experience.

QS and c-di-GMP are two important chemical signaling relays in *V. cholerae*. Each of these relays alternates between two distinct states. Low- and high-cell-density QS states reflect the density and complexity of the surrounding bacteria in the local environment, while low and high intracellular levels of c-di-GMP potentially reflect in vivo versus ex vivo environments (51). These two signaling relays converge to regulate biofilms and virulence factor expression, processes fundamental for *V. cholerae* survival ex vivo and in vivo, respectively. Interestingly, although each signaling relay transmits information into both processes, QS and c-di-GMP function antagonistically to control biofilm formation, while they act synergistically to repress virulence. Likewise, although high c-di-GMP levels override QS control of biofilm formation, it is not known which process dominates during virulence factor expression. Apparently, *V. cholerae* parses the unique information carried by these two chemical signaling processes appropriately to regulate particular behaviors depending on the relative importance of cell density and environmental inputs. The plasticity with which *V. cholerae* can convert these two chemical inputs into unique gene expression patterns could provide *V. cholerae* tremendous flexibility in adapting to a fluctuating environment.

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