Transcriptome and Phenotypic Responses of *Vibrio cholerae* to Increased Cyclic di-GMP Level†

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***Vibrio cholerae*, the causative agent of cholera, is a facultative human pathogen with intestinal and aquatic life cycles. The capacity of *V. cholerae* to recognize and respond to fluctuating parameters in its environment is critical to its survival. In many microorganisms, the second messenger, 3',5'-cyclic diguanylic acid (c-di-GMP), is believed to be important for integrating environmental stimuli that affect cell physiology. Sequence analysis of the *V. cholerae* genome has revealed an abundance of genes encoding proteins with either GGDEF domains, EAL domains, or both, which are predicted to modulate cellular c-di-GMP concentrations. To elucidate the cellular processes controlled by c-di-GMP, whole-genome transcriptome responses of the El Tor and classical *V. cholerae* biotypes to increased c-di-GMP concentrations were determined. The results suggest that *V. cholerae* responds to an elevated level of c-di-GMP by increasing the transcription of the *vps*, *eps*, and *msh* genes and decreasing that of flagellar genes. The functions of other c-di-GMP-regulated genes in *V. cholerae* are yet to be identified.**

The facultative human pathogen, *Vibrio cholerae*, is the causative agent of the severe diarrheal disease Asiatic cholera. This pathogen, which alternates between intestinal and aquatic life cycles, is found in coastal, estuarine, and riverine environments in either a free-living state or a biofilm state attached to surfaces (9, 14, 38). The adaptation of *V. cholerae* to the ever-changing parameters of its ecosystem is critical to its survival. At present, relatively little is known about how the physical, chemical, and biological factors encountered by *V. cholerae* in its natural habitats are perceived and transduced into physiological changes and how, in turn, these changes affect its adaptation to the natural ecosystems.

Recent studies have indicated the importance in these processes of an intracellular signaling molecule termed 3',5'-cyclic diguanylic acid (c-di-GMP), which acts as a second messenger for integrating environmental signals that affect cellular physiology (11, 25). These studies have shown the cellular levels of c-di-GMP are controlled by diguanylate cyclases (DGCs) and phosphodiesterases (PDEAs), which are proteins that contain conserved domains termed GGDEF and EAL, respectively (8, 36, 44–48).

Proteins containing DGC and/or PDEA domains are widespread among bacteria (16, 17). Genome analysis of *V. cholerae* revealed that it has 31 genes that encode DGC proteins, 12 that encode PDEA proteins, and 10 that encode proteins with both the DGC and PDEA domains on the same polypeptide (17). The prevalence of genes encoding DGC- and PDEA-containing proteins in *V. cholerae* and the evidence that c-di-GMP is an essential modulator of biofilm formation in many

microorganisms prompted us to examine the c-di-GMP regulation and associated phenotypic changes in *V. cholerae* (3, 11, 25, 26, 39, 48).

To analyze the consequences of elevated c-di-GMP concentration in the cytoplasm of *V. cholerae*, we modulated the c-di-GMP level by overexpressing proteins with DGC or PDEA domains from an arabinose-inducible promoter. We then determined the whole-genome expression profiles of these cells. The study revealed that 4.5% of *V. cholerae* genes are differentially expressed in response to an increased level of c-di-GMP. The main responses were an increase in the expression of *vps* genes that are required for *Vibrio* polysaccharide (VPS) biosynthesis, *eps* genes involved in the extracellular protein secretion system (EPS), and *msh* genes required for the mannose-sensitive hemagglutinin (MSHA) type IV pilus biogenesis as well as a decrease in the expression of *fla* genes required for flagellum biogenesis.

To further understand the response of pathogenic *V. cholerae* to c-di-GMP, we performed transcriptome analysis in two *V. cholerae* O1 biotypes: El Tor and classical. The classification of *V. cholerae* strains is based on the lipopolysaccharide O antigen, which identifies over 200 serogroups in the species. Among these serogroups, only O1 and O139 cause epidemic cholera, as they harbor genes encoding cholera toxin and the colonization factor toxin-coregulated pilus in their genomes. The O1 serogroup can be further divided into the classical and El Tor biotypes on the basis of biochemical properties and phage susceptibility (14).

Although the responses to changes in c-di-GMP level were similar between the classical and El Tor biotypes with respect to the expression of the *vps*, *eps*, *msh*, and *fla* genes, there were significant differences in the expression patterns of other genes of unknown function. These results demonstrate that *V. cholerae* responds to changes in the level of c-di-GMP by modulating production of its cell surface properties, which in turn

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype	Source
<i>E. coli</i> strains		
CC118 λ pir	$\Delta(ara-leu)$ <i>araD</i> $\Delta lacX74$ <i>galE galK phoA20 thi-1 rpsE rpoB argE</i> (Am) <i>recA1</i> λ pir	21
S17- λ pir	<i>recA thi pro r_K⁻ m_K⁺ RP4:2-Tc:MuKm Tn7</i> λ pir	12
<i>V. cholerae</i> strains		
FY-Vc-1	<i>Vibrio cholerae</i> O1 El Tor A1552, smooth variant, Rif ^r	56
AC-V61	O395 <i>lacZ::res-tet-res</i> , spontaneous partial deletion of <i>lacZ</i> , Sm ^r Tc ^r	5
AC-V1724	FY-Vc-1 pBAD33, Rif ^r Cm ^r	This work
AC-V1725	FY-Vc-1 pAT1662, Rif ^r Cm ^r	This work
AC-V1884	FY-Vc-1 pAT1568, Rif ^r Cm ^r	This work
AC-V1460	AC-V61 pBAD33, Sm ^r Cm ^r	48
AC-V1726	AC-V61 pAT1662, Sm ^r Cm ^r	48
FY-Vc-237	FY-Vc-1 mTn7-GFP, Gm ^r	This work
FY-Vc-513	FY-Vc-237 pBAD33, Rif ^r Gm ^r Cm ^r	This work
FY-Vc-505	FY-Vc-237 pAT1662, Rif ^r Gm ^r Cm ^r	This work
FY-Vc-508	FY-Vc-237 pAT1568, Rif ^r Gm ^r Cm ^r	This work
FY-Vc-231	FY-Vc-1 $\Delta vpsI$, Rif ^r	This work
FY-Vc-499	FY-Vc-231 mTn7-GFP, Gm ^r	This work
FY-Vc-1024	FY-Vc-499 pAT1662, Rif ^r Gm ^r Cm ^r	This work
FY-Vc-1076	FY-Vc-1 $\Delta mshA$, Rif ^r	This work
FY-Vc-1073	FY-Vc-1076 mTn7-GFP, Gm ^r	This work
FY-Vc-1077	FY-Vc-1073 pAT1662, Rif ^r Gm ^r Cm ^r	This work
FY-Vc-987	FY-Vc-1 $\Delta VCA0956$, Rif ^r	This work
Plasmids		
pBAD33	pACYC184 ori, <i>araC</i> P _{araBAD} , Cm ^r	19
pAT1568	pBAD33::NTF3 <i>vieA</i> -His ₆ , Cm ^r	48
pAT1662	pBAD33::VCA0956-His ₆ , Cm ^r	48
pGP704- <i>sac28</i>	pGP704 derivative; <i>mob/oriT sacB</i> Ap ^r	G. Schoolnik
pFY-331	pGP704- <i>sac28</i> :: $\Delta VCA0956$, Ap ^r	This work
pAJH5	<i>vpsI</i> operon deletion construct	20
p28- <i>mshA</i>	pGP704- <i>sac28</i> :: $\Delta mshA$, Ap ^r	31
pMCM11	pGP704::mTn7-GFP, Gm ^r Ap ^r	M. Miller and G. Schoolnik
pUX-BF13	oriR6K helper plasmid, <i>mob/oriT</i> , provides the Tn7 transposition function in <i>trans</i> ; Ap ^r	2

leads to altered motility and biofilm formation. The functions of many potentially important c-di-GMP-regulated genes have yet to be identified.

MATERIALS AND METHODS

Growth conditions. Bacterial strains and plasmids are listed in Table 1. *V. cholerae* cultures were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) or MOPS (morpholinepropanesulfonic acid) medium, which was prepared by the addition of MOPS salts (40 mM MOPS [pH 7.4], 4 mM Tricine, 0.01 mM FeSO₄ · 7H₂O, 9.5 mM NH₄Cl, 0.28 mM KCl, 0.53 mM MgCl₂ · 6H₂O, 0.05 mM NaCl), minimal essential medium (MEM) vitamins (10 ml/liter [Gibco]), trace metals (1 ml/liter of 5% MgSO₄, 0.5% MnCl₂ · 4H₂O, 0.5% FeCl₃, 0.4% trinitroloacetic acid), L-asparagine (25 mM), L-arginine (25 mM), L-glutamate (25 mM), L-serine (25 mM), KH₂PO₄ (0.75 mM), and 0.5% glycerol (42). *V. cholerae* cultures were grown with aeration at 30°C, unless otherwise noted. Arabinose was added to a final concentration of 0.2% to induce expression from the P_{araBAD} promoter. Antibiotics were added at the following concentrations unless otherwise noted: for chloramphenicol (Cm), 5 µg/ml or 1.5 µg/ml; for rifampin (Rif), 100 µg/ml; for ampicillin (Ap), 100 µg/ml; and for gentamicin (Gm), 50 µg/ml.

Construction of the VCA0956 deletion mutant. We used a PCR method to generate an in-frame deletion mutant of the GGDEF protein VCA0956 by utilizing previously published methods (15, 24, 30). A 437-bp 5' flanking sequence of VCA0956 (including 6 bp of the coding sequence) was PCR amplified with primer A (5'-CGAGCTCCGGTTAGTCATAGCCCTGA-3') and primer B (5'-TGACTCGATTACATCTGTTTCGGATACGC-3'). Primer C (5'-AC AAGTGATGAATCGAGTCATGCCGCTCTAA-3') and primer D (5'-CTAG TCTAGATGGACGTCATAGTGGCTTGA-3') were used to amplify the last 18 bp of the 3' region of the gene plus 476 bp of the downstream flanking sequence. The two PCR products were joined using the splicing overlap extension tech-

nique, and the resulting PCR product, lacking most of the internal coding sequence of the gene, was digested with SacI and XbaI and cloned into similarly digested pGP704*sac28* suicide plasmid. The PCR products were sequenced to ensure that no errors were introduced during PCR amplification. The *Escherichia coli* CC118 λ pir strain was used to maintain the pGP704*sac28* plasmid carrying the deletion construct pFY-331. Biparental mating was carried out between recipient wild-type *V. cholerae* and the *E. coli* S17- λ pir strain harboring the deletion construct. Ampicillin- and rifampin-resistant *V. cholerae* strains resulting from a single homologous recombination were identified, grown without ampicillin, and then subjected to sucrose selection. Strains with properties of a double recombination event (ampicillin-sensitive and sucrose-resistant) were identified and further analyzed by PCR to confirm that the VCA0956 gene was deleted. In-frame deletion mutants of *mshA* and the *vpsI* operon (*vpsI*) were generated by following the same method described above, using p28-*mshA* and pAJH5 plasmids (Table 1), respectively.

GFP tagging of *V. cholerae* strains. The insertion of the green fluorescence protein (GFP) gene into the *V. cholerae* genome was performed by triparental mating among *V. cholerae*, *E. coli* S17- λ pir carrying the helper plasmid pUX-BF13, and *E. coli* S17- λ pir carrying pMCM11, which encodes a Tn7 cassette with the GFP gene. Exconjugants were selected on thiosulfate-citrate-bile salts-sucrose agar plates containing gentamicin (50 µg/ml). The mini Tn7 cassette inserts at a specific site in the *V. cholerae* genome, between the genes encoding VC0487 and VC0488 (nucleotides 519257 to 519261), causing a 5-bp duplication. The confirmation of GFP insertion was performed by colony PCR using primers GFP_1 (5'-CGATGTTGACCAGCCTCGTA-3') and GFP_2 (5'-CGGATCTC GACACAAGCGTA-3'). The growth rate of each GFP-tagged strain was compared to the corresponding parental background to ensure that the GFP insertion did not interfere with growth. Arabinose-inducible vectors were then introduced to GFP-tagged *V. cholerae* strains via electroporation.

RNA isolation. *V. cholerae* strains were grown on LB agar plates containing chloramphenicol (5 µg/ml) at 30°C overnight. For the VCA0956 overexpression

studies, colonies from semiconfluent plates of each strain were resuspended in 2 ml MOPS medium. The optical density of each culture at 600 nm (OD_{600}) was determined, and cultures were then inoculated to a starting OD_{600} of 0.05 in MOPS medium. Cultures were grown with aeration at 30°C, and c-di-GMP production was induced by the addition of arabinose to a final concentration of 0.2% when the OD_{600} of the cultures reached 0.30 to 0.35. Samples were harvested, and RNA was isolated as described previously (55).

Gene expression profiling. Whole-genome expression analysis was performed by using a common reference RNA that was isolated from uninduced *V. cholerae* AC-1724 cells grown to an OD_{600} of 0.30 to 0.35. RNA samples from test and reference samples were used in a reverse transcription reaction as follows: 3 µg of RNA and 5 µg of random hexamers were hydrolyzed at 80°C for 8 min and then chilled on ice for 5 min. The reaction mixture, containing first-strand buffer, 0.01 M dithiothreitol, aminoallyl (aa) dNTP labeling mix with aa-dUTP in a 3 (dUTP)-to-2 (aa-dUTP) ratio, and 400 U SuperScript III (Invitrogen), was added to cooled samples and incubated at 42°C for 3 to 4 h. In order to hydrolyze the RNA in the cDNA-RNA mixture, samples were incubated at 65°C for 10 min in the presence of 100 mM NaOH and 10 mM EDTA. After hydrolysis, reactions were neutralized by the addition of 1 M HEPES [pH 7.0], at a final concentration of 500 mM HEPES. Reactions were cleaned up with the QIAquick PCR purification kit (QIAGEN) by using phosphate wash and phosphate elution buffers and dried. The samples were indirectly labeled by covalent coupling of the *N*-hydroxysuccinimide esters of the cyanine fluorophores to the aminoallyl-labeled cDNAs as follows. Cleaned and dried samples were resuspended in freshly prepared 50 mM Na-bicarbonate buffer, pH 9.0. One vial of Cy3/Cy5 fluor (Amersham CyDye postlabeling reactive dye pack) was mixed with each sample and incubated for 1 h in the dark at room temperature. Unincorporated dye molecules were removed by using the QIAquick PCR purification kit (QIAGEN). Dye incorporation and cDNA concentrations were determined by measuring optical density at 260, 550, and 650 nm. The amount of nucleotides in each sample was determined by using following formula: amount of nucleotides (pmol) = ($OD_{260} \cdot \text{volume} [\mu\text{l}] \cdot 37 \text{ ng}/\mu\text{l} \cdot 1,000 \text{ pg}/\text{ng}$)/324.5 pg/pmol. The amount of incorporated dye molecules was determined by using following formula: pmol Cy3 = ($OD_{550} \cdot \text{volume} [\mu\text{l}]/0.15$); pmol Cy5 = ($OD_{650} \cdot \text{volume} [\mu\text{l}]/0.25$). The nucleotides-to-dye ratio was then calculated by dividing cDNA picomoles by Cy dye picomoles. Samples with >100 pmol dye incorporation and <10 nucleotides per dye ratio were used in the hybridizations (this method is modified from the Institute for Genomic Research's microbial RNA aminoallyl labeling for microarrays protocol). For hybridizations, dye-coupled test samples were mixed with the corresponding reference sample and dried. Amine-silane slides containing 70-mer oligonucleotides representing most of the open reading frames present in the *V. cholerae* genome were UV cross-linked at 250 mJoules and prehybridized in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 1% bovine serum albumin for at least 45 min at 42°C. The hybridization mix was prepared by resuspending dried samples in a solution containing 15 µg salmon sperm DNA, 15 µg tRNA, 3× SSC, and 0.1% sodium dodecyl sulfate. Concentrated hybridization mix was applied to the slides and incubated at 65°C for 12 to 20 h. After hybridization, slides were washed, dried, and scanned by using an Axon scanner to determine the fluorescence in each open reading frame-specific spot. The raw data was obtained by using the software package GenePix 4.1 (Axon). Normalized signal ratios were obtained with LOWESS print-tip normalization by using Bioconductor packages (<http://www.bioconductor.org>) (18) in an R environment. Differentially regulated genes were determined (with two biological and two technical replicates for each data point) with the significance analysis of microarrays (SAM) package (50) using twofold differences in gene expression and 1% false discovery rate (FDR) as a cutoff value.

Biofilm assays. The biofilm-forming capacities of the *V. cholerae* strains were determined by crystal violet staining assay after 2, 4, 6, and 12 h of incubating cultures in polyvinyl chloride microtiter plates under static conditions in LB and MOPS medium at 30°C (55, 57). For flow cell experiments, biofilms were grown at room temperature in flow chambers (individual channel dimensions of 1 by 4 by 40 mm) supplied with half-strength LB (0.5% peptone, 0.25% yeast extract, and 1% NaCl) containing chloramphenicol (3 µg/ml) at a flow rate of 13.5 ml h⁻¹. Arabinose was added at 0.2% final concentration to induce expression from the *P_{araBAD}* promoter. The flow cell system was assembled and prepared as described previously (22). *V. cholerae* cultures used to inoculate flow channels were prepared by inoculating 6 to 10 single colonies from a plate into flasks containing LB medium and growing them with aeration at 30°C for 20 h. Cultures were diluted to an OD_{600} of 0.1 in LB and used for inoculation as described previously (55). Image acquisition was done with a Zeiss Axiovert 200 M laser scanning microscope. IMARIS personal software and Adobe Photoshop were used for image processing.

Motility assays. LB and MOPS soft agar plates (0.3% agar) were used to determine the motility of bacterial strains. The diameter of the zone of motility was measured after 20 and 48 h of incubation at 30°C for LB- and MOPS-grown cells, respectively.

Two-dimensional TLC. The amount of c-di-GMP was quantified by using two-dimensional thin-layer chromatography (TLC) as previously described (48).

RESULTS AND DISCUSSION

An increase in intracellular c-di-GMP level alters the transcriptome of *V. cholerae*. The abundance and spatial distribution of *V. cholerae* proteins containing DGC and/or PDEA domains (53 proteins in total) indicate the importance of the c-di-GMP second messenger to the biology of the pathogen. A genomic approach was undertaken to gain insights into the responses of *V. cholerae* to changes in the intracellular c-di-GMP level. Relatively little is known about DGC and/or PDEA domain-harboring proteins in *V. cholerae*. Whereas the overexpression of such proteins has not, for the most part, proven effective in modulating the c-di-GMP level in *V. cholerae* (data not shown), the overexpression of VCA0956, a cytoplasmic protein composed of a single DGC domain, was successfully used to increase the c-di-GMP level in the *V. cholerae* classical biotype (48). Thus, in this study, we chose VCA0956 to increase the cytoplasmic c-di-GMP level in the *V. cholerae* El Tor biotype for modulating the expression of the VCA0956 message level via an arabinose-inducible system. The *V. cholerae* strain expressing VCA0956 was denoted E-DGC, and a control strain carrying only the plasmid vector (pBAD33) was denoted E-pBAD33. Both strains were grown in MOPS medium, and c-di-GMP accumulation before and after VCA0956 DGC induction with 0.2% arabinose was determined by using two-dimensional TLC. There was no observable c-di-GMP accumulation in E-DGC grown in the absence of arabinose (Fig. 1A). In contrast, a spot corresponding to c-di-GMP according to its R_f values was observed in E-DGC after 30 min of culture in the presence of arabinose (Fig. 1B) (40). No change in c-di-GMP level due to arabinose induction was observed in the E-pBAD33 strain (Fig. 1C). The c-di-GMP level, which was expressed as the ratio of c-di-GMP/GDP, in the E-DGC strain increased 4.53- and 6.96-fold following arabinose inductions lasting 15 and 30 min, respectively (Fig. 1C). Growth rates of the E-DGC strain in the presence or absence of arabinose did not differ from those of the wild type (data not shown).

Having identified conditions for modulating the cellular c-di-GMP level, we next performed whole-genome transcriptome analysis. To this end, E-DGC and E-pBAD33 strains were grown in MOPS medium until mid-exponential phase (OD_{600} , 0.3 to 0.35). The cultures were then supplemented with arabinose to a final concentration of 0.2%. Total RNA was isolated from both cultures 0, 15, and 30 min after arabinose addition and used for transcriptome analysis. Genes differentially regulated in the two strains were determined by using SAM software (50) using a ≥ 2.0 -fold change in gene expression and an FDR of ≤ 0.01 as criteria. The analysis identified 79 differentially expressed genes (63 genes were induced and 13 were repressed in the E-DGC strain relative to the control pBAD33 strain) after 15 min of arabinose induction. After 30 min, we identified 173 differentially regulated genes (151 induced and 22 repressed). The abundance of the

FIG. 1. Modulation of the c-di-GMP level using an inducible system. Analysis of total nucleotides from (A) uninduced and (B) arabinose-induced E-DGC cultures. Nucleotides were extracted from E-DGC grown in MOPS medium with [³²P]orthophosphate, spotted on TLC plates (lower left corner), and developed in 0.2 M NH₄HCO₃ (pH 7.8), in the first dimension (bottom to top of plate) and 1.5 M KH₂PO₄ (pH 3.65), in the second dimension (left to right of plate). The white arrow indicates the spot corresponding to c-di-GMP. (C) Quantification of c-di-GMP. Amounts of c-di-GMP and GDP were quantified using ImageQuant. The c-di-GMP/GDP ratio was used as an indication of the change in c-di-GMP concentration.

VCA0956 transcript, whose expression was modulated by arabinose to increase cellular c-di-GMP level, increased 59- and 38-fold after 15 and 30 min of induction, respectively. This observation, along with that of the increased cellular c-di-GMP concentration, validates our experimental design.

Based on annotated functions provided by the *V. cholerae* genome sequencing project, the genes regulated by c-di-GMP are predicted to participate in various cellular functions (Fig. 2). Below we discuss a selected subset of the differentially regulated genes and their significance. The complete list of differentially expressed genes is provided as supplemental material.

The expression of *vps* genes is increased by c-di-GMP. *V. cholerae* strains need to produce VPS to form well-developed biofilms. The expression of *vps* genes, required for the biosynthesis of VPS, increased two- to threefold in response to increased c-di-GMP concentration. The *vps* genes are located on the large chromosome (one of the two chromosomes of *V. cholerae*) and are predicted to be organized into two regions (57): the *vpsI* region, which is 11,464 bp in length, harbors 11 genes (VC0917 to VC0927) and the *vpsII* region, 6,550 bp, contains 6 genes (VC0934 to VC0939). The two regions are separated by an 8,321-bp region containing six additional genes (54, 55, 57). We previously showed that the expression of all 24 “*vsp* region” genes, including VC0916, is coordinately regulated (55). In this study, we observed that the expression of *vps* region genes is also correlated with c-di-GMP concentration. The expression of 17 out of 24 genes increased by 2.4- to

FIG. 2. Functional categories of differentially expressed genes in response to an increased c-di-GMP level. The number of genes whose expression is induced or repressed in response to the increased c-di-GMP level is presented according to the functions assigned to them by The Institute for Genomic Research.

FIG. 3. Expression of *vps*, *eps*, and *msh* genes and biofilm-forming capacity are modulated by the c-di-GMP level. (A) Expression profiles of *vps*, *msh*, and *eps* genes. Differences in the abundance of transcripts between the E-DGC and E-pBAD33 strains and the C-DGC and C-pBAD33 strains are presented by using the color scale shown at the bottom of the panels (red, induced; green, repressed). Differentially regulated genes were identified by SAM analysis using a 1.3-fold change and an FDR of $\leq 1\%$ as criteria. (B and C) Quantitative analysis of biofilm formation in LB (B) and in MOPS (C). Biofilm phenotypes of E-pBAD33, E-DGC and E-PDEA in the presence (+) and absence (–) of 0.2% arabinose are presented. Biofilms were formed under static conditions at 30°C. After 2, 4, 6, and 12 h of incubation, attached bacteria were stained with crystal violet, and the staining intensity was determined by absorbance of ethanol solubilized biofilms at 595 nm. Error bars indicate standard deviations.

7.8-fold after 15 min of arabinose induction. Twelve of these genes also exhibited increased expression (2.1- to 3.2-fold) after 30 min of induction (Fig. 3A). The expression of *vps* genes is positively regulated by the transcriptional regulatory proteins VpsR and VpsT, which belong to the response regulator family of two-component regulatory systems (6, 54). Thus, we determined whether the expression of *vpsR* and *vpsT* is also modulated by c-di-GMP. After 15 min of induction, we observed a 2.8-fold increase in

vpsT transcript abundance but no significant change in *vpsR* transcript. In contrast, we observed a 1.7-fold increase in the expression of *vpsR* after 30 min of VCA0956 DGC induction but no significant change in the expression level of *vpsT*. We have previously shown that VpsR and VpsT positively regulate each other's expression (6, 55). Temporal differences in the expression levels of these positive regulators suggest that the increase in *vpsT* transcription may be a direct response to increased c-di-GMP concentration, whereas the increase in the

abundance of *vpsR* message is a secondary effect due to increased expression of *vpsT*.

Taken together, our results show a positive correlation between the intracellular concentration of c-di-GMP and VPS production in *V. cholerae*. This finding is congruent with our previous observation that the *V. cholerae* classical biotype *vieA* deletion mutant, which accumulates more c-di-GMP than does the wild-type strain, expresses *vpsR*, *vpsA*, *vpsL*, and VC0928 at higher levels (48). In contrast, the same *vieA* deletion mutation in the *V. cholerae* El Tor biotype causes neither an increase in c-di-GMP concentration nor an altered *vps* gene expression (data not shown).

Expression of other factors required for biofilm formation is induced by c-di-GMP. The *V. cholerae* EPS system (also known as the type II secretion system), is responsible for the secretion of proteins through the outer membrane, including cholera toxin, hemagglutinin/protease, chitinase, neuraminidase, and lipase. Whether *eps* genes are coordinately regulated with those encoding proteins that are secreted by the EPS system has yet to be determined. Coordinate expression of the *vps* and *eps* genes was first observed in the rugose variant of the *V. cholerae* El Tor biotype, which is capable of producing high levels of VPS (55). In our study, the expression of 3 *eps* genes located in the 12-gene operon harboring genes *epsC-epsN* was induced in response to increased c-di-GMP (Fig. 3A) (43). Two products of the EPS pathway, EpsD and EpsE, are predicted to be important for the secretion of either VPS itself or a protein(s) that is involved in the transport or assembly of VPS (1). Our findings suggest that the expression of the *eps* and *vps* genes that are required for VPS production and extracellular protein secretion processes may be transcriptionally coordinated by the signaling molecule c-di-GMP.

Expression of the genes *mshF*, *mshG*, *mshI*, *mshJ*, and *mshN*, which encode proteins required for the production and function of the MSHA type IV pilus, increased by 1.4- to 1.8-fold in response to elevated c-di-GMP concentrations. MSHA facilitates the attachment of *V. cholerae* to both biotic and abiotic surfaces, and the biofilm development dynamics of an *mshA* mutant are markedly different from those of the wild type (7, 33, 51, 52). Since the expression of *msh* genes increases in response to an increased cellular concentration of c-di-GMP, we show for the first time a correlation between *vps* and *msh* gene expression.

An increase in the cytoplasmic c-di-GMP level alters biofilm development dynamics. MSHA and VPS are required for the development of mature biofilms by *V. cholerae* (27, 51, 52, 54, 57, 59). To determine the physiological consequences of increased *vps* and *msh* gene transcription, we compared the biofilm-forming capacities of the E-DGC and E-pBAD33 strains. Initially, we quantified the ability of cells grown both in LB and MOPS at 30°C under static conditions to form biofilms on the surface of microtiter plates. The results presented in Fig. 3B (in LB) and C (in MOPS) reveal that there is a significant increase in the capacity of E-DGC to form biofilms upon induction with arabinose. In LB-grown cultures, at 4 h of biofilm formation, the biofilm-forming capacity of induced E-DGC increased fourfold compared to that of uninduced culture. Similarly, at 6 h and 12 h into biofilm development, E-DGC biofilms were increased by 3.7- and 35-fold, respectively. In MOPS-grown cultures, at 2, 4, 6, and 12 h of biofilm

formation, the biofilm-forming capacity of induced E-DGC increased 11.5-, 40-, 43-, and 7.6-fold, respectively.

Transcriptome analysis revealed that the expression of *vps* genes increased in E-DGC within 15 min of induction with arabinose. However, an increase in the cells' capacity to form biofilms was not observed as rapidly. Biofilms are multicellular structures requiring the synthesis and export of large polymers. The time required for both the production of the exopolysaccharide and synthesis/assembly of the transport system may be one of the reasons for the delay in biofilm formation. Similarly, the differences in physiology and basal transcriptome states of free-living and attached bacteria could result in different phenotypic consequences.

We also attempted to modulate the c-di-GMP level in the strain *V. cholerae* O1 El Tor by overexpressing the PDEA domain of the VieA protein, which has c-di-GMP phosphodiesterase function, under the control of an arabinose-inducible promoter (47, 48). This strain, referred to as E-PDEA, showed decreased biofilm formation when grown in LB at 2 and 4 h after induction with arabinose (Fig. 3B). However, no significant change in biofilm formation was observed at later time points (6 and 12 h). When grown in MOPS, E-PDEA showed a decrease in biofilm formation after 12 h of induction with arabinose (Fig. 3C). The modest effect on biofilm formation upon induction of VieA expression is consistent with the fact that c-di-GMP levels are already very low (undetectable by two-dimensional TLC [data not shown]) in cells under the conditions used in this experiment. We did not observe a significant increase in biofilm formation upon arabinose induction in the control strain, E-pBAD33 (Fig. 3B and C).

Although these experiments showed that increasing the intracellular c-di-GMP concentration in *V. cholerae* O1 El Tor alters its biofilm-forming capacity, they did not provide any information about the architecture of the biofilms formed. To investigate the influence of c-di-GMP on biofilm morphology, we first generated a *V. cholerae* O1 El Tor strain harboring the gene encoding GFP on the large chromosome (E-GFP). The plasmids pBAD33 and pBAD33::VCA0956 were then introduced into E-GFP to obtain strains E-GFP-pBAD33 and E-GFP-DGC, respectively. Each strain formed biofilms in a flow cell system, and images were acquired by confocal scanning laser microscopy over the course of biofilm development. COMSTAT software was used to quantify biofilm features, including average thickness, maximum thickness, and total biomass (22).

A remarkable change in the dynamics of biofilm formation was observed in E-GFP-DGC upon arabinose induction. During the initial stages of biofilm development, there was no significant difference between induced and uninduced E-GFP-DGC cultures (data not shown). However, at 7 and 11 h postinduction with arabinose, E-GFP-DGC formed larger microcolonies than uninduced cultures, presumably because it accumulates a higher level of c-di-GMP. At 25 h, E-GFP-DGC biofilms exhibited full-surface coverage and formed well-developed biofilms, reaching average and maximum thicknesses of 38 μm and 49 μm , respectively. In contrast, the biofilms of uninduced cells were unable to fully cover the substratum and had average and maximum thicknesses of 32 μm and 43 μm , respectively. After 45 h of biofilm development, biofilm detachment characteristics were also markedly different between

FIG. 4. Biofilm development dynamics are altered in response to changes in c-di-GMP level. Biofilm structures of E-DGC in the absence (A) and presence (B) of 0.2% arabinose are shown. Biofilms were grown in flow chambers, and images were acquired with confocal laser scanning microscopy. Top-down and orthogonal views of biofilms are given in large panels and side panels, respectively. Bar, 30 μ m.

induced and uninduced cells. Whereas biofilms of the induced cells did not detach, those of uninduced cells showed a marked detachment from the substratum (Fig. 4). In addition, total biomass significantly increased at the 25- and 45-h time points when cells were grown in the presence of 0.2% arabinose. The E-GFP-pBAD33 did not exhibit significant changes in biofilm formation capacity following induction with arabinose (data not shown). These results indicate that an increase in the intracellular c-di-GMP level leads to the development of more compact biofilms, alters biofilm development kinetics, and prevents biofilm detachment.

The transcriptome analysis revealed that message abundance of *msh* and *vps* genes was higher in arabinose-induced E-DGC cells. To determine the contribution of MSHA and VPS to c-di-GMP-induced biofilm morphology, we introduced the plasmid pBAD33::VCA0956 into GFP-tagged $\Delta mshA$ and $\Delta vpsI$ mutant strains and examined the biofilm development dynamics in a flow cell system. The results shown in Fig. 5 revealed that the surface colonization capacity of the $\Delta mshA$ mutant and the mature biofilm-forming capacity of the $\Delta vpsI$ mutant were drastically reduced relative to those of the wild type, and this phenotype did not change in the presence of arabinose. This observation suggests that both MSHA and VPS are required for c-di-GMP-induced biofilms, and their requirement for biofilm formation cannot be compensated for by solely increasing c-di-GMP levels in the cell.

Expression of genes involved in flagellum biogenesis is reduced by an increased cytoplasmic c-di-GMP level. *V. cholerae* is motile by means of a single polar flagellum. The genes required for flagellum biogenesis and flagellar motility fall into four temporally distinct classes (I through IV) (37). Transcriptional profiling studies revealed that the expression of several class III and IV genes, which encode many of the flagellins and flagellar basal body rod proteins (*flgB*, *flgC*, *flgF*, *flgM*, *flaC*, and *flaD*), decreased by 2.1- to 2.5-fold in response to an increased level of c-di-GMP (Fig. 6A). The expression of many genes located in the flagellar transcriptional units (*flgD*, *flgE*, *flgG*, *flgI*, *fliF*, *fliH*, *fliG*, *fliN*, *fliS*, *fliA*, *fliG*, and *fliF*) was also reduced (between 1.5- and 1.9-fold). Similarly, the expression level of the transcription factor sigma-28 (*fliA*), which controls the transcription of class IV genes, was also lower. The decrease in expression of some of these flagellar genes (including *flaA*, *flgC*, *flgE*, *flgG*, *fliF*, *fliI*, *fliG*, and *fliN*) was seen within 15 min of VCA0956 DGC induction. Thus, the increased intracellular c-di-GMP level leads to a decrease in the expression of genes that are required for flagellum biosynthesis.

Further evidence that c-di-GMP regulates motility comes from an examination of the motility behaviors of the E-pBAD33, E-DGC, and E-PDEA strains. Motility phenotypes were compared on LB and MOPS soft agar plates supplemented with or without 0.2% arabinose (Fig. 6B). There were notable differences in the motility of these strains under these conditions. When tested on LB, strain E-DGC exhibited a significant decrease in motility when grown in the presence of arabinose (a 54% reduction in motility diameter relative to the uninduced control). In contrast, E-PDEA exhibited a significant increase (by 147%) in motility when induced with arabinose (Fig. 6C). The E-PDEA strain grown in the absence of induction also showed an increase in motility (although not significant) due to the leakiness of the *P_{araBAD}* promoter under this growth condition. In

contrast, the motility of the E-pBAD33 strain was not altered under any of the conditions tested. When tested on MOPS, motility behaviors of the strains E-DGC and E-pBAD33 were similar. However, E-PDEA exhibited a significant increase (by 176%) in motility when induced with arabinose (Fig. 6D). These results indicate that both expression of genes encoding proteins required for flagellum biogenesis and motility of E-DGC are negatively regulated by increased intracellular c-di-GMP concentration. An inverse relationship between the expression of genes required for flagellar biosynthesis and those for exopolysaccharide biosynthesis has previously been described for both *V. cholerae* and *Pseudomonas aeruginosa* (28, 29, 35, 53). In this study, we show that this inverse regulation can be mediated by a change in the cellular c-di-GMP concentration.

In addition to genes that encode components of the flagellum, certain genes predicted to encode proteins involved in chemotaxis were differentially regulated in response to increased c-di-GMP levels. The *V. cholerae* genome contains 43 genes encoding methyl-accepting chemotaxis proteins (MCP). The amount of messages corresponding to two genes that encode MCPs, VCA1034 and VC1405, increased by 2.0- and 1.7-fold, respectively. Conversely, the expression of VC1898, VCA0773, VC2439, VC1298, VCA0176, and VC2161 decreased by 1.4- to 2.9-fold. Thus, it is possible that the decreased motility behavior discussed above could be due to both altered chemotaxis and flagellar motility. We believe that c-di-GMP plays an important role in regulating flagellar motility rather than chemotaxis because the induction of DGC expression caused a reduction in the percentage of motile cells from $\geq 80\%$ to $\leq 10\%$ (data not shown).

The processes discussed thus far, VPS production and MSHA pili and flagellar motility, are critical to biofilm development (51, 52, 57). Biofilm formation by aquatic microorganisms is well documented (10). It has been proposed that the attachment of bacteria to a surface and subsequent biofilm growth exemplify a survival strategy. Although the steps involved in biofilm formation and some of the necessary structural and regulatory genes involved have been identified, relatively little is known about the signals and genetic networks that control gene expression during biofilm development. Our study highlights the importance of the c-di-GMP second messenger in linking the processes required for *V. cholerae* biofilm formation, although how the c-di-GMP level changes during biofilm development remains to be determined. Furthermore, in this study, we determined that the expression of VC1888, a protein recently shown to be involved in biofilm development (34), increases in response to elevated c-di-GMP levels. Thus, it is likely that we have identified additional genes that are critical for biofilm development in *V. cholerae*.

As the overexpression of VCA0956 altered the dynamics of biofilm development, we tested whether a mutation in the gene that encodes VCA0956 (termed *cdgF* for cyclic-diguanylate protein F) would cause a decrease in the biofilm-forming capacity of the El Tor biotype. The *cdgF* null mutant did not exhibit any phenotypic differences relative to the wild-type strain with respect to colony morphology, motility, and biofilm formation (data not shown). As the El Tor strain utilized in this study does not have a level of c-di-GMP detectable by two-dimensional TLC (B. Lim, S. Beyhan, J. Meir, and F. H. Yildiz,

FIG. 5. Biofilm development dynamics of *mshA* and *vpsI* deletion mutants are not altered in response to changes in the c-di-GMP level. Biofilm structures of *SΔmshA* and *SΔvpsI* strains in the absence (A and C) and presence (B and D) of 0.2% arabinose are shown. Biofilms were grown in flow chambers, and images were acquired with confocal laser scanning microscopy. Top-down and orthogonal views of biofilms are given in large panels and side panels, respectively. Bar, 30 μm.

FIG. 6. C-di-GMP modulates motility behavior. (A) Expression profile of flagellum biosynthesis genes in E-DGC and C-DGC after 30 min of DGC induction compared to E-pBAD33 and C-pBAD33, respectively. Genes were identified by SAM analysis using a 1.3-fold change and an FDR of $\leq 1\%$ as criteria. Expression ratios of significant genes are presented according to the color scheme shown at the bottom of the panel. (B) Motility phenotypes of E-pBAD33, E-DGC, and E-PDEA on LB and MOPS soft agar plates containing chloramphenicol with or without arabinose. The diameters of the motility zones of E-pBAD33, E-DGC, and E-PDEA were measured after 20 and 48 h of incubation at 30°C for LB- and MOPS-grown cells, respectively. (C and D) Changes in the diameters of the motility zones in the absence and presence of induction on LB (C) and MOPS (D) soft agar plates.

unpublished data), further decreasing the already low c-di-GMP levels by deleting VCA0956 may not contribute to any phenotypic changes under the conditions tested. Alternatively, since there are 40 other genes in *V. cholerae* that encode proteins with predicted DGC activity, it is possible that these proteins can compensate for the loss of VCA0956.

The increased concentration of c-di-GMP alters the expression of purine biosynthesis genes. The VCA0956 protein is a DGC that condenses two molecules of GTP to produce c-di-GMP. Hence, we surmised that the overexpression of the DGC-domain of VCA0956 could lead to changes in purine nucleotide biosynthetic pathways. Indeed, this was the case. Following VCA0956 DGC induction in the E-DGC strain expression of VC1190 (*purC*), which encodes a putative phosphoribosylaminoimidazole-succinocarboxamide (SAICAR) synthetase, decreased by 1.7-fold. SAICAR synthetase catalyzes the ATP-dependent conversion of 5'-phosphoribosyl-5-aminoimidazole-4-carboxylic acid and aspartic acid to SAICAR. In contrast, the expression of VC0767 (*guaB*), which encodes IMP dehydrogenase, required for the synthesis of GMP from IMP, increased in response to a higher concentration of c-di-GMP. Similarly, the expression of VC1992 (*purU*), which encodes 10-formyl-THF hydrolase, an enzyme that provides the formate for the synthesis of the purine ring used by 5'-phosphoribosylglycinamide transformylase, also increased.

Purine nucleotides can be synthesized either by de novo or salvage pathways. Synthesis of 5'-phosphoribosyl-1-pyrophosphate (PRPP), which is a precursor of purine and pyrimidine biosynthesis, is catalyzed by PRPP synthase using ATP and ribose-5'-phosphate as substrates. Synthesis of the nicotinamide nucleotides histidine and tryptophan requires 10 to 15% of PRPP for cells growing in minimal medium (58). In our transcriptome analysis, seven of the histidine biosynthesis genes (which are located in an eight-gene operon [VC1132 to VC1139]), were expressed 1.8-fold higher, on average, in response to increased c-di-GMP levels. Four of the genes involved in thiamine biosynthesis (*thiC*, *thiE*, *thiF*, and *thiS*), which uses an intermediate of the de novo purine biosynthesis pathway (5'-aminoimidazole ribonucleotide) as a reactant, were also expressed at higher levels. These observations may suggest that fluxes in the levels of precursors of the metabolic pathways using either GTP or its intermediates may alter the expression of the genes indicated above.

Increased concentrations of c-di-GMP alter expression of a large number of genes encoding hypothetical proteins. The most striking finding was that 50.9% (88 out of 173) of the genes that were differentially regulated in response to increased c-di-GMP concentration are predicted to encode hypothetical or conserved hypothetical proteins. These genes are organized into 74 different transcriptional units, and 68% of them are located on the small chromosome, while the remaining 32% are on the large chromosome. The identification of a large set of hypothetical proteins regulated by c-di-GMP underlines how little we know of c-di-GMP-regulated physiology and behavior. It is of particular interest that one of these genes, VC0928, which encodes a hypothetical protein whose expression was increased by 4.5-fold after 15 min of VCA0956 DGC induction, is required for the development and stability of biofilm structures in *V. cholerae* (J. C. N. Fong and F. H. Yildiz, unpublished data).

Analysis of early and late responses to an increased c-di-GMP level. To better understand the immediate and late responses of *V. cholerae* to c-di-GMP, we compared the transcriptome profiles acquired 15 and 30 min after VCA0956 DGC induction. Differences in transcription profiles between these two time points were determined by using scatter plot analysis. In the graph shown in Fig. 7, the *x* and *y* axes denote the changes (*n*-fold) in gene expression after 30 min and 15 min of VCA0956 DGC induction, respectively, whereby each spot represents a gene that was differentially regulated by two-fold in at least one experiment. Genes in E-DGC that were differentially regulated at only 30 min, at only 15 min, and at both time points are indicated. The graph allowed us to divide genes according to their regulation state into four quadrants (I through IV) as follows: quadrant I represents genes whose expression increased at both 15 and 30 min; II represents genes expressed at higher levels at the 15-min time point but lower levels at the 30-min time point; III represents genes expressed at lower levels at both time points; and IV represents genes expressed at higher levels at the 30-min time point but lower levels at the 15-min time point. The genes that were differentially regulated at both time points are found in only quadrants I and III, indicating that their expression is not temporally regulated in response to increased c-di-GMP concentration. Quadrant I contains mostly *vps* genes (40%) and genes encoding hypothetical proteins (40%), while quadrant III contains the *flgC* gene and those encoding additional hypothetical proteins. Genes differentially expressed at both time points comprised 16.4% of all the differentially expressed genes, whereas 20.4% and 63.2% were differentially expressed at only 15 or 30 min, respectively. Altogether the results suggest that there are initial and secondary responses to an increase in cellular concentrations of c-di-GMP. The detailed molecular mechanism of the timing of these responses is being investigated.

The response of the *V. cholerae* classical strain to increased c-di-GMP concentration. There are marked differences in the signals regulating virulence factor production between the classical and El Tor biotypes (13). As c-di-GMP is predicted to be important for connecting environmental signals to cellular responses, including biofilm formation and virulence gene expression (49), we sought to compare the responses of classical and El Tor strains to increased c-di-GMP concentration. To this end, we introduced plasmids pBAD33::VCA0956 and pBAD33 into the classical biotype, creating strains C-DGC and C-pBAD33, respectively. After 30 min of DGC induction, a 28-fold increase in c-di-GMP level was observed in C-DGC (49).

Transcriptome changes due to increased c-di-GMP levels were determined by using the growth conditions described above. SAM analysis revealed genes exhibiting changes in expression of at least twofold and FDRs less than 0.01. Using these criteria, 139 genes were up-regulated and 43 were down-regulated in C-DGC relative to C-pBAD33 after 30 min of induction with arabinose. The complete list of differentially expressed genes is provided as supplemental material. Below we discuss selected cellular processes that are altered by increased c-di-GMP concentration in the classical biotype.

One of the main cellular processes that was differentially regulated in the classical biotype in response to c-di-GMP is VPS production. Twenty-two of the 24 genes found in the "vps region" were up-regulated by 1.7- to 26-fold. The transcript

FIG. 7. Analysis of temporal transcriptome responses to c-di-GMP. Comparison of gene expression profiles of E-DGC after 15 and 30 min of DGC induction. Plot area is divided into four regions (I through IV) based on the expression patterns before and after induction. Black triangles indicate genes that were differentially expressed (change of at least twofold; FDR, $\leq 1\%$) only at 15 min, orange triangles indicate genes that were differentially expressed only at 30 min, and purple circles indicate genes that were differentially expressed at both time points.

abundance of *vpsR* and *vpsT*, the genes encoding two positive regulators of other *vps* genes, was increased by 7.7- and 9.1-fold, respectively. The magnitude of the increase in *vps* gene expression, including the positive regulators, *vpsT* and *vpsR*, in response to increased c-di-GMP concentration was greater in C-DGC than E-DGC (Fig. 3A). This observation suggests that, in the classical biotype, there is either a higher level of expression of a positive regulator of *vpsT* and/or *vpsR* or a lower level of expression of a negative regulator of these genes. We favor the latter explanation, since the classical strain harbors a frame shift mutation in the *hapR* gene, which encodes a quorum-sensing transcriptional regulator that negatively controls *vps* gene expression. However, it is also possible that yet-to-be-identified components of the network that regulates *vps* gene expression contribute to the observed difference.

In C-DGC, as in E-DGC, the expression of genes encoding MSHA (*mshD*, *mshE*, *mshI*, and *mshJ*) and EPS (*epsC*, *epsI*, and *epsN*) increased on average by 1.5-fold and 1.4-fold, respectively, further evidence of a correlation between *vps*, *eps*, and *msh* gene expression and increased c-di-GMP concentration (Fig. 3A). Similarly, the expression of a set of flagellar biosynthesis genes (*flgF*, *flgG*, *flhF*, *fliH*, *fliE*, and *motY*) decreased two- to threefold in C-DGC following induction with arabinose. When expression of all the genes involved in chemotaxis and motility was analyzed, we observed a 1.7-fold average decrease in the expression of most of the flagellar biosynthesis genes (*flaA*, *flaB*, *flaC*, *flaG*, *fliB*, *fliC*, *flgB*, *flgC*, *flgD*, *flgI*, *flhA*, *fliA*, *fliF*, *fliG*, and *fliN*) (Fig. 6D). Some of the

chemotaxis genes (*cheA-2*, *cheY-3*, *cheW-2*, and *cheD*) and four MCPs (VC1289, VC2161, VCA0658, and VCA0773) also exhibited decreased gene expression (1.5- to 1.7-fold). In contrast, the expression of two chemotaxis genes (*cheV-3* and a putative *cheW*) and five MCPs (VC0098, VC1859, VCA0864, VCA0988, and VCA1034) increased by 1.4- to 4-fold.

The expression of eight genes encoding DGC-family proteins (VC0703, VC1029, VC1185, VC1216, VC1934, VCA0074, VCA0697, and VCA0785) increased in C-DGC following induction, indicating that the c-di-GMP signal can lead to increased transcription of genes capable of further modulating the c-di-GMP concentration. Among these DGC-family proteins, VC0703, VC1934, and VCA0785 also contain a PDEA domain. In E-DGC, the expression of two genes with both DGC and PDEA domains (VC1934 and VCA0785) increased upon DGC induction. The role of each of the differentially regulated genes encoding DGC- and/or PDEA-family proteins in modulating the intracellular c-di-GMP concentration needs to be determined in order to understand differences in the c-di-GMP response in the two O1 biotypes. At present, only *mbaA*, which encodes VC0703, has been characterized in detail and found to be a negative regulator of biofilm formation in *V. cholerae* (4).

The expression of some of the pathogenesis genes (*acfB*, *tagE-1*, *tcpE*, *tcpJ*, *tcpT*, and VC1621) located in the *Vibrio* pathogenicity island increased by 1.6- to 2.7-fold in the classical biotype in response to an increase in the c-di-GMP level. However, the expression of *Vibrio* pathogenicity island genes

and *ctx* genes was markedly reduced in the classical biotype *vieA* deletion mutant (data not shown). This mutant produces a high level of c-di-GMP relative to the wild type when grown under a condition that induces virulence gene expression in the classical biotype, in M9 minimal medium supplemented with asparagine, arginine, glutamate, and serine (32). In light of these apparently contradictory findings, the exact mechanism by which c-di-GMP controls the expression of virulence genes in *V. cholerae* remains to be determined. It should be noted, however, that the observed transcriptional differences between the classical and El Tor biotypes could not be attributed to differences in the nucleotide sequence of VCA0956 between the two strains.

The ability of an organism to modulate the transcriptome in response to extracellular and intracellular signals will greatly influence its overall fitness. In this study, we determined genome-wide transcriptional responses and resulting phenotypic responses of the *V. cholerae* O1 El Tor biotype to changes in the concentration of the intracellular second messenger c-di-GMP. The results indicated that significant changes to the transcriptome occur in response to an increased c-di-GMP level, which in turn alter cell physiology and lead to alterations in biofilm development and motility behavior. Similar phenotypic changes were observed when the gene *adrA* (which regulates c-di-GMP concentration) was overexpressed in *Salmonella enterica* serovar Typhimurium (45), suggesting that different microorganisms respond to cellular increases in c-di-GMP in similar manners. Recent studies in *P. aeruginosa* indicate that an increased cellular c-di-GMP level can cause large changes in gene expression patterns (23), further suggesting that, in addition to being an allosteric activator (40, 41), c-di-GMP performs a general role as a second messenger (11, 25). The present study also revealed that the classical and El Tor biotypes of *V. cholerae* exhibit markedly different transcriptional profiles in response to increased c-di-GMP concentration. This finding may be important in light of the reported differences in the regulation of virulence factor production between the two biotypes.

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