Role of Cyclic Di-GMP during El Tor Biotype *Vibrio cholerae*
Infection: Characterization of the In Vivo-Induced Cyclic Di-GMP Phosphodiesterase CdpA

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In *Vibrio cholerae*, the second messenger cyclic di-GMP (c-di-GMP) positively regulates biofilm formation and negatively regulates virulence and is proposed to play an important role in the transition from persistence in the environment to survival in the host. Herein we describe a characterization of the infection-induced gene cdpA, which encodes both GGDEF and EAL domains, which are known to mediate diguanylate cyclase and c-di-GMP phosphodiesterase (PDE) activities, respectively. CdpA is shown to possess PDE activity, and this activity is regulated by its inactive degenerate GGDEF domain. CdpA inhibits biofilm formation but has no effect on colonization of the infant mouse small intestine. Consistent with these observations, cdpA is expressed during in vitro growth in a biofilm but is not expressed in vivo until the late stage of infection, after colonization has occurred. To test for a role of c-di-GMP in the early stages of infection, we artificially increased c-di-GMP and observed reduced colonization. This was attributed to a significant reduction in toxT transcription during infection. Cumulatively, these results support a model of the *V. cholerae* life cycle in which c-di-GMP must be down-regulated early after entering the small intestine and maintained at a low level to allow virulence gene expression, colonization, and motility at appropriate stages of infection.

*Vibrio cholerae*, the causative agent of the diarrheal disease cholera, is a highly motile gram-negative bacterium that is indigenous to aquatic environments and can survive in both marine water and freshwater. There is evidence showing that *V. cholerae* lives in association with other aquatic organisms, including cyanobacteria, copepods, and insect egg masses (18, 49). Furthermore, the ability of *V. cholerae* to survive in the environment likely depends on the ability to form biofilms (52, 56). A large-scale shift in gene expression occurs upon entering and exiting the human gastrointestinal tract (24, 32, 46), but the triggering signals and the mechanism of regulation of these switches are unknown. It is unclear how *V. cholerae* adapts to and grows within these very disparate environments. Bis-(3’-5’)-cyclic-di-GMP (c-di-GMP), first identified as an allosteric activator of cellulose synthase in *Glucanacetobacter xylinus* (40, 41, 53), has been recognized as an important bacterial second messenger involved in the regulation of a number of processes. Examples include extracellular polysaccharide biosynthesis in *Salmonella enterica* serovar Typhimurium, *Yersinia pestis*, *Pseudomonas aeruginosa*, and *V. cholerae* (14, 15, 20, 47, 50); motility in *S. enterica* serovar Typhimurium, *P. aeruginosa*, *Escherichia coli*, *Caulobacter crescentus*, and *V. cholerae* (1, 17, 21, 29, 47); differentiation in *C. crescentus* (1, 36); and virulence in *S. enterica* serovar Typhimurium, *P. aeruginosa*, *Y. pestis*, and *V. cholerae* (16, 20, 23, 51).

Biosynthesis and degradation of c-di-GMP are performed by three protein domains. Diguanylate cyclases (DGCs) containing a GGDEF domain, named for conserved residues, synthesize c-di-GMP from two GTPs (4, 36, 43). In turn, c-di-GMP is hydrolyzed by EAL domain phosphodiesterases (PDEs), including VieA, which is described below (9, 10, 45, 48). Recent evidence shows that the protein domain HD-GYP can also degrade c-di-GMP (42). Many bacteria encode multiple GGDEF, EAL, and HD-GYP domains, which are commonly found in conjunction with regulatory and sensory domains, as well as together on the same protein. For example, *V. cholerae* encodes 12 EAL, 30 GGDEF, 9 HD-GYP, and 10 GGDEF-EAL domain proteins (12, 13).

We propose that c-di-GMP plays a key role in regulating the changes in *V. cholerae* gene expression that occur during the shift from aquatic to host environments. Previous work by our laboratory studying the dual function protein VieA led to a model by which c-di-GMP inversely regulates biofilm formation and virulence of classical-biotype *V. cholerae*. High c-di-GMP resulting from inactivation of VieA PDE results in increased expression of vps (vibrio exopolysaccharide synthesis) genes and increased biofilm formation (48, 50). A similar effect is seen for *V. cholerae* overproducing the DGC encoded by VCA0956 (50). Conversely, c-di-GMP inhibits motility: mutation of *vieA* abrogates motility in soft agar medium (37). In addition, VieA PDE activity is necessary for virulence in the infant mouse model of *V. cholerae* colonization (51). VieA positively regulates in vitro expression of the major virulence gene transcriptional activator *toxT* and the genes encoding cholera toxin, *ctxAB* (51). Based on these data, c-di-GMP is poised to coordinate changes in gene expression during the shift from the aquatic environment to the host. This model is bolstered by the fact that the operon encoding VieA, *vieSAB*, is induced during infection (7) and contributes to *ctxAB* expression in the infant mouse (27), providing a mechanism by...
which c-di-GMP levels can be reduced upon entry into the host. Expression of vieSAB occurs at an early stage of infection (26), indicating that c-di-GMP must be reduced rapidly and early to allow maximal virulence gene expression.

Interestingly, VieA does not regulate biofilm formation, motility, or virulence in the El Tor biotype of V. cholerae (2, 27; R. Tamayo and A. Camilli, unpublished data), which is the biotype that causes present-day cholera; the classical biotype upon which the above-described model was based is essentially extinct in nature. However, c-di-GMP does control biofilm formation and motility in the El Tor biotype (3). There is also evidence that one putative PDE, CdgC (VCA0785), regulates virulence gene expression in vitro in an El Tor strain (30); however, VCA0785 is dispensable for virulence in an infant mouse model of colonization (Tamayo and Camilli, unpublished). Therefore, the role of c-di-GMP in virulence of El Tor biotype V. cholerae is unclear.

We hypothesize that c-di-GMP inversely regulates biofilm and virulence genes in El Tor as in the classical V. cholerae biotype. This could be accomplished by 1 or more of the other 21 EAL domain or 9 HD-GYP domain proteins, besides VieA, encoded by V. cholerae El Tor. Here, we characterize the activity, expression, and function of the GGDEF-EAL domain gene VC0130, herein named cdpA. We further define the role of c-di-GMP in regulation of virulence in El Tor by ectopically producing a DGC during infection. This work has allowed us to better understand the role of c-di-GMP in the virulence of V. cholerae at early and late stages of infection.

**MATERIALS AND METHODS**

**Bacterial growth conditions.** Unless otherwise noted, E. coli and V. cholerae El Tor biotype strain C6709, and derivative strains were grown in Luria-Bertani (LB) broth at 37°C with aeration. Antibiotics were used where appropriate as follows: 100 μg/ml streptomycin (Sm), 50 μg/ml ampicillin (Ap), 50 μg/ml

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**E. coli strains**

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* An internal fragment of cdpA was cloned in order to make the plasmid insertion.
the fusion within the EAL domain of the chromosomal copy of VC0130 was
by conjugation between wild-type (WT) AC51 and AC2129. Integration of

\[
\begin{align*}
&3287qR \quad \text{..................ATTTGCAGCAACGTCACCTGATGG} \\
&0130EAS \quad \text{..................TTTCTAGATTTAGGATACATTTTTGCGG} \\
&0130GAS \quad \text{...............GCGATTGCTGTCGGTGCT} \\
&A0956F1 \quad \text{................CCGAGCTCTTTAGGATACATTTTTGTGATG} \\
&vpsRF2 \quad \text{ ..................GAATATAGGTTCATGTAAGTTTTGCTGAT} \\
&vpsRR1 \quad \text{ ..................AATCAGCAAAACTTACATGAACCTATATT} \\
&0130EAL \quad \text{...............TTCTAGATTTAGGATACATTTTTATGGCGG} \\
&0130CGR \quad \text{...........TTGCATGCCTAAGC} \\
&0130GAR \quad \text{...........CCAAGC} \\
&ACCGAC \quad \text{TGGGCAACGGTTTTTGCT} \\
&A0956R \quad \text{...........GGTCTAGAGCCGGCTCAAACGAGTATAG} \\
&67EHR \quad \text{..................TTCACTTCTGAGTTCGGCAT} \\
&67EHF \quad \text{...................CGACATCATAACGGTTCTGG} \\
&oriR \quad \text{ ...............CAGCAGTTCAACCTGTTG} \\
&\text{Primer name} & \text{Sequence} & (5’ to 3’) (reference) \\ 
orIR & \text{CAGCAGTTCAACCTGTTG} \\
67EHF & \text{CGACATCATAACGGTTCTGG} \\
67EHF & \text{TTTCTAGATTTAGGATACATTTTTGCGG} \\
0130FX & \text{GTTTGAAGGGAAGCCGTTTTAATGTGCATTAGCATTACAG} \\
0130RX & \text{GGTCTAGAGCCGGCTCAAACGAGTATAG} \\
0130PF & \text{TAATGCGCTGAAGTGGAC} \\
C0130F & \text{TTCATAGTTAGGATACATTTTTGCGG} \\
C0130R & \text{GTTCATGCCTAAGC} \\
0130EAL & \text{TTTCTAGATTTAGGATACATTTTTATGGCGG} \\
0130CGR & \text{TTGCATGCCTA} \\
0130GAR & \text{CCAAGC} \\
ACCGAC & \text{TGGGCAACGGTTTTTGCT} \\
A0956F1 & \text{................CCGAGCTCTTTAGGATACATTTTTGTGATG} \\
vpsRF2 & \text{ ..................GAATATAGGTTCATGTAAGTTTTGCTGAT} \\
vpsRR1 & \text{ ..................AATCAGCAAAACTTACATGAACCTATATT} \\
0130EAL & \text{...............TTCTAGATTTAGGATACATTTTTATGGCGG} \\
0130CGR & \text{TTGCATGCCTA} \\
0130GAR & \text{CCAAGC} \\
ACCGAC & \text{TGGGCAACGGTTTTTGCT} \\

* Restriction sites are underlined; sequence encoding the His \(6\) tag is both
underlined and in boldface; boldface text without underlining indicates codons changed to obtain desired amino acid mutations.

kanamycin (Km), and 1 \(\mu\)g/ml tetracycline (Tc). Strains used in this study were
disrupted by plasmid insertion. An internal fragment of the gene was amplified using 0130FX and 0130RX and cloned into suicide vector pGPM704. This construct was introduced into \(V.\) cholerae by conjugation between wild-type (WT) AC51 and AC2129. Integration of the fusion within the EAL domain of the chromosomal copy of VC0130 was confirmed by PCR with primers orIR and 0130PF.

For complementation and overexpression studies, the full coding sequence of VC0130 was amplified by PCR using 0130F and 0130R, which introduces a six-histidine (His) tag to allow detection of the protein by Western blotting. Primers 0130F/0130CR were used to amplify the CGO3287-GGDE domain of VC0130, omitting the EAL domain. Residues G493 and E496 were next to the multiple cloning site. The incorporation of point mutations in GVGEW was confirmed by sequencing with primer 0130GAS.

The \(\beta\)-galactosidase activity of the resulting strains was confirmed to contain the plasmid by PCR using primers 67EHF and 67EHR.

For expression in \(V.\) cholerae, plasmids were transformed into C6709 (WT AC51) or the lacZ mutant strain AC66, as appropriate by electroporation. The resulting strains were confirmed to contain the plasmid by PCR using primers 67EHF and 67EHR.

Assays for enzymatic activity. Stationary-phase cultures of relevant strains were diluted 1:100 in 25 ml LB broth and incubated at 37°C with aeration until mid-exponential phase. IPTG was added to a final concentration of 0.5 mM to induce gene expression and the cultures were incubated 3 h more. Aliquots were collected for Western blot analysis using mouse anti-pentahistidine primary antibodies (Qiagen) and horseradish peroxidase-conjugated sheep anti-mouse secondary antibodies (Amersham). Western blots were developed with ECL detection reagents (Amersham).

For PDE assays, the pellets were resuspended in 0.5 ml reaction buffer (75 mM Tris, pH 8, 25 mM KCl, 10 mM MgCl\(_2\) containing 10% glycerol). For DGC reactions, the pellets were resuspended in cyclase buffer (75 mM Tris, pH 7.8, 250 mM NaCl, 25 mM KCl, 10 mM MgCl\(_2\) containing 10% glycerol). For both assays, bacteria were lysed by sonication.

PDE activity was assessed by incubating lysate with radiolabeled c-di-GMP, enzymatically synthesized as described previously (48). Aliquots were spotted on cellulose-polyethyleneimine thin-layer chromatography (TLC) plates 2 min after the addition of the substrate. Reaction products were separated in 1.5 M KH\(_2\)PO\(_4\), pH 3.65, and visualized by phosphorimaging. DGC reactions were performed as described above, i.e., 19.5 \(\mu\)l of lysate and 0.5 \(\mu\)l of substrate were combined (10-\(\mu\)Ci\(\gamma\)-GTP, 3,000 Ci/mmol; Perkin-Elmer).

2D-TLC of nucleotide extracts. Two-dimensional TLC (2D-TLC) assays were performed on radiolabeled nucleotide extracts as described previously (50). Where indicated, 1 mM IPTG was added at early exponential phase (optical density at 600 nm [OD\(_{600}\)] of 0.35) to induce gene expression. To quantify relative levels of c-di-GMP, the amount of c-di-GMP was normalized to the level of GMP in the sample (3, 50). At least two independent experiments were done.

In vitro phenotype assays. Biofilm and chemotaxis/motility assays were done essentially as described previously (37). Where indicated, 50 \(\mu\)M or 1 mM IPTG was added to induce gene expression.

In vivo competition assays. Standard in vivo competition assays were done as described previously (7). For competition assays using strains with plasmid-borne enzymes, strains were grown overnight at 37°C on LB agar plates containing Ampicillin (50 \(\mu\)g/ml) and IPTG. In vitro competition experiments were performed in parallel. Competition indices were determined as the ratio of output mutant to WT CFU to that ratio in the input.

RNA isolation and cDNA synthesis. For bacteria grown in LB broth, RNA was isolated from mid-exponential-phase cultures (OD\(_{600}\) of 0.5) incubated with 22 h postinfection (p.i.). The ileal samples from three mice were pooled into a single sample to obtain sufficient RNA for subsequent experiments.

For quantitative real-time PCR (qPCR) analysis of cdpA expression during infection, infant mice were inoculated intragastrically with 10\(^6\) WT bacteria or WT CFU to that ratio in the input. After suspension in LB broth to an OD\(_{600}\) of 0.20, IPTG was added to a final concentration of 0.20 mM to induce gene expression, and then cultures were incubated for 15 min at 37°C with aeration immediately prior to inoculation. Subsequently the competing strains were combined at 1:1 ratios in LB broth containing 50 \(\mu\)M IPTG. In vitro competition experiments were performed in parallel. Competition indices were determined as the ratio of output mutant to WT CFU to that ratio in the input.

RNA isolation and cDNA synthesis. For bacteria grown in LB broth, RNA was isolated from mid-exponential-phase cultures (OD\(_{600}\) of 0.5) incubated with aeration at 24°C (for biofilm experiments) or 37°C (for in vivo sample comparisons) as described previously (44).

For bacteria grown in biofilms, the biofilms were washed with phosphate-buffered saline to remove unattached bacteria. Biofilm-associated bacteria were recovered by vortexing in 1 ml Trizol and with 0.1-mm zirconia beads (BioSpec). RNA was purified as described previously (44). Aqueous phases from six separate biofilms were pooled into a single sample to obtain sufficient RNA for subsequent experiments.

For quantitative real-time PCR (qPCR) analysis of cdpA expression during infection, infant mice were inoculated intragastrically with 10\(^7\) WT bacteria or phosphate-buffered saline only (mock). At 22 h postinfection (p.i.), the ilea (most distal 3 cm of the small intestine) were dissected and snap-frozen in a dry ice-ethanol bath. The ileal samples from three mice were pooled into a single preparation and RNA was isolated as described previously (44).

cDNA was made as described previously (44). For broth culture and biofilm qPCR studies, 100 ng RNA was used in each reaction. For RNA obtained from...
mouse small intestines, as well as broth culture controls, 1 µg RNA was used in each reaction.

qPCR. Reactions were done with Brilliant SYBR green qPCR master mix (Stratagene) using Stratagene Mv3005P equipment and MxPro qPCR software. Reaction mixtures contained 10 ng or 100 ng template for biofilm or in vivo expression experiments, respectively, and reactions were done as described previously (44). qPCR primer sequences are listed in Table 2. All primer pairs showed efficiencies of 97% or greater (data not shown). At least five samples were tested for each condition and each template sample was tested in triplicate. Controls lacking reverse transcriptase were included. Data were analyzed as described previously (44).

Resolution assays. Expression of cdpA during infection was monitored using recombinase-based in vivo expression technology (RIVET) (6). The res-res

lacZ

cassette was stably integrated in the lacZ gene by conjugation between AC2528 containing pRes and C6709, yielding C6709 Res (35). The transcriptional fusion cdpA:tnpR was made in C6709 Res by mating with E. coli containing pVVET5nccdpA (35, 44), placing production of the TnpR resolvase under the control of the cdpA promoter.

The cdpA:tnpR strain was inoculated intragastrically into six 5-day-old CD-1 mice as described for competition assays. At 7 and 21 h p.i., three mice were sacrificed, and bacteria were recovered from the small intestines and plated on LB agar containing Sm-Ap and Sm-Km. The percentage of CFU that became Km sensitive was determined at each time point for each mouse. As a control, a strain with a tnpR fusion to ctxA was analyzed in parallel (27). Expression of the fusions in stationary-phase (10-h, 37°C) LB broth culture was analyzed as a control by plating dilutions and quantifying the percent loss of Km resistance as described above.

The effect of ectopically increasing c-di-GMP on virulence gene expression during infection was assessed by expressing vdcA or vdcA(I) during infection in a C6709 derivative previously used to measure in vivo transcription of the major virulence gene regulator toxT (26). This RIVET strain contains a transcriptional fusion of tnpR to toxT and a Tc resistance gene flanked by res sites stably integrated into lacZ. pVdcA and pVdcA(I) were transformed into this strain by electroporation. The amount of IPTG used, 50 µM, was experimentally determined to induce gene expression and protein detectable by Western blotting but not to affect the stability of the plasmid in the absence of antibiotic selection (data not shown). The pVdcA and pVdcA(I) RIVET strains were prepared for inoculation as described for the competition assays above, except inocula consisting of 10 µl of the IPTG-induced bacterial suspension were added to 990 µl LB containing 50 µM IPTG. At multiple time points p.i., bacteria were recovered from the small intestines of the mice (three per time point per strain) and plated on LB agar plates containing Sm and Sm-Tc to measure rates of Tc sensitivity and on Sm-Ap to test for retention of the plasmid. Two independent experiments were done; a representative experiment is shown. For all resolution assays, data were analyzed using the Mann-Whitney U test and a P value of <0.05 was considered significant.

RESULTS

Identification of a PDE that decreases the global concentration of c-di-GMP in V. cholerae. Because the c-di-GMP PDE VieA did not regulate the biofilm formation, motility, or virulence of V. cholerae, we took an unbiased approach to identify another EAL domain PDE that could substitute for VieA and modulate the transition from biofilm to colonization of a host by decreasing the c-di-GMP level. We focused on VC0130, because like vieA, it was identified as an in vivo-induced gene in mice and in humans by use of RIVET (31, 35). VC0130 is annotated as encoding a GGDEF family protein but also encodes an EAL domain (Fig. 1A). Interestingly, the GG(D/E)EF motif in the VC0130 protein is degenerate, having the sequence GVGEW. Structural analysis of the DGC PleD from C. crescentus showed that the second glycine is required for catalysis (8). Since this residue is absent from the VC0130 protein, this was expected to preclude DGC activity. The EAL motif is also divergent (ECL); however, other sequences conserved among EAL domains, such as the IDDFGTG and LKLD motifs, are present. We have found that the alanine residue of the VieA EAL motif could be mutated with no effect on PDE activity (Tamayo and Camilli, unpublished). We thus predicted that the VC0130 protein has c-di-GMP PDE activity and so could function similarly to VieA during infection, i.e., up-regulation of VC0130 during infection could reduce intracellular c-di-GMP, resulting in increased virulence factor expression and decreased expression of vps.

To determine whether the VC0130 gene encodes a DGC, a c-di-GMP PDE, or both, the full-length gene product was marked with a hexahistidine tag at the C terminus and expressed from an IPTG-inducible promoter in E. coli. Production of the proteins by E. coli was confirmed by Western blotting using anti-pentahistidine antibodies (Fig. 1B). Lysates

![FIG. 1. cdpA encodes a c-di-GMP PDE. (A) The domain structure of the VC0130 protein, CdpA, is shown. CdpA is a three-domain protein containing an N-terminal domain of unknown function (COG3287), a GGDEF, and an EAL domain. The divergent amino acid sequences present in cdpA at the GG(D/E)EF and EAL motifs are indicated in the relevant domains. The amino acids bounding each domain are noted below. (B) Lysates from AC1817 [VieA]; AC1835 [VieA(E170A)]; AC2576 [CdpA]; and AC2642 [CdpA(AVGAW)] were assayed for protein expression by Western blot analysis. The WT and respective mutant proteins were comparably expressed. No His6-tagged protein was detected in vector-only controls (not shown). (C) Lysates containing the indicated proteins as well as buffer- and vector-only controls were tested for the ability to hydrolyze radiolaabeled c-di-GMP. The reactions were analyzed by TLC; products and respective Rf values are indicated on the right.](http://iai.asm.org/article-pdf/82/11/1620/4062392/i821101620.pdf)
from *E. coli* producing the VC0130 protein along with VieA, VieA(E170A), and vector-only controls were tested for the ability to hydrolyze c-di-GMP. Whereas the VieA(E170A) and vector negative controls showed no hydrolysis of c-di-GMP, VC0130 protein-containing lysates showed degradation of c-di-GMP (R_f ~ 0.3) to GMP (R_f ~ 0.6) (Fig. 1C).

To detect DGC activity, VC0130 protein- or VdcA-containing lysates, as well as a vector-only control, were assessed for the ability to synthesize c-di-GMP using radiolabeled GTP as the substrate. VdcA was chosen as a control for these studies because it was previously shown to have in vitro DGC activity (48, 50). Whereas the reaction products of VdcA showed the presence of c-di-GMP, the VC0130 protein was unable to produce c-di-GMP (data not shown). Thus, as predicted by sequence analysis of the GGDEF and EAL domains of VC0130, the VC0130 protein possesses c-di-GMP PDE activity and so was named CdpA, for cyclic diguanylate PDE A.

Because cdpA has in vitro PDE activity, inactivation of cdpA was predicted to increase the global intracellular concentration of c-di-GMP in *V. cholerae*. 2D-TLC of radiolabeled nucleotide extracts showed that the cdpA mutant has a level of c-di-GMP almost fourfold higher than that of the respective parent strain (Table 3). The phenotype could be partially complemented by the introduction of *cdpA* on a low-copy-number, IPTG-inducible expression vector, pMMBneo. Overexpression of *cdpA* in the mutant by the addition of 1 mM IPTG led to undetectable levels of c-di-GMP. These data further support the assignment of *cdpA* as a c-di-GMP PDE gene.

**The GGDEF domain is necessary for PDE activity of CdpA.**

CdpA contains a degenerate GGDEF domain in addition to the EAL c-di-GMP hydrolytic domain. Another divergent GGDEF motif (GEDEF) was previously shown to modulate the activity of the tandem EAL domain in *CC3396* of *C. crescentus* (10). We hypothesized that the GGDEF domain could play a similar regulatory role in CdpA. To test this, we assayed the effect of the divergent GGDEF domain (GVGEW) on the PDE activity of CdpA.

A CdpA derivative in which the GVGEW sequence was mutated to AVGAW was made [CdpA(AVGA GW)]; the altered residues were shown to be involved in GTP binding in the DGC PleD (8). The ability of CdpA(AVGA GW) to hydrolyze c-di-GMP in vitro was determined. *E. coli* lysate containing CdpA(AVGAW) degraded less c-di-GMP than did that containing WT CdpA (Fig. 1C); both WT and mutant proteins were comparably produced by *E. coli* (Fig. 1B). Because this method is not quantitative and because it may not reflect the regulation of activity by *V. cholerae*, other methods were used to determine the role of the GGDEF domain in CdpA activity.

A low concentration of c-di-GMP is necessary for motility in *V. cholerae* (3, 37). WT cdpA, cdpA(AVGAW), and cdpA lacking the EAL domain [cdpA(ΔEAL)] were expressed from pMMBneo in the *cdpA* strain and assessed for the ability to increase the motility of *V. cholerae* on soft agar plates compared to that of the full-length genes. Expression of WT cdpA increased motility fourfold compared to what was seen for the parent strain containing empty vector (Fig. 2A). The vector-only control showed motility comparable to that of the WT parent (compare to WT in Fig. 3B). Overexpression of cdpA(ΔEAL) did not alter motility, supporting the previous findings that the CdpA GGDEF domain does not possess DGC activity and the EAL domain is required for PDE activity. This is in contrast to the reduction of motility seen for the strain containing active DGC (see Fig. 5C below). Expression of cdpA(AVGAW) caused a twofold increase in motility compared to what was seen for the vector-alone strain but was not as effective at enhancing motility as WT cdpA. This suggests that only partial EAL domain activity remains in this mutant derivative.

**FIG. 2.** The degenerate GGDEF domain of CdpA is required for optimal PDE activity of the EAL domain. Altered PDE activity of the mutated proteins was determined based on their effects on in vitro c-di-GMP-regulated phenotypes in the *cdpA* strain, namely, motility and biofilm production. (A) The mean diameters of motility are given on soft agar plates containing 1 mM IPTG to induce gene expression and Km to maintain the plasmids. (B) The amount of biofilm formed by the above-described strains grown under static conditions in the presence of 50 μM IPTG is shown. In both figures, asterisks indicate significant changes from WT. The pCdpA and pAVGAW strains had significantly different motility (*P* = 9 × 10⁻⁵) and biofilm formation (*P* = 6 × 10⁻⁴) levels.
The importance of the cdpA GGDEF domain in PDE activity was mirrored when the above strains were tested for V. cholerae biofilm formation, a process that requires a high c-di-GMP concentration (50). Overexpression of cdpA in the cdpA background dramatically inhibited biofilm formation (20-fold) compared to what was seen for the vector control, which produced a WT amount of biofilm (compare to WT in Fig. 3A). Indeed, the expression of cdpA resulted in biofilm production similar to that for the VPS-deficient vpsR mutant, indicating that the c-di-GMP concentration has been dramatically depleted. In contrast, the cdpA(ΔEAL) domain had no effect on biofilm formation (Fig. 2B). Again, these findings are consistent with CdpA being a c-di-GMP hydrolase and with the EAL domain being required for activity. Expression of cdpA-(AVGAW) inhibited biofilm production fourfold, indicating that the GVGEW sequence of CdpA is required for full PDE activity of the EAL domain. The effect of cdpA(AVGAW) expression was intermediate between those of the vector control and cdpA-expressing strains, suggesting that partial PDE activity remains in the CdpA(AVGAW) mutant.

**CdpA affects only a subset of c-di-GMP-regulated phenotypes.** For the section above, we utilized overexpression of cdpA and mutant derivatives to show PDE activity and corresponding effects on motility and biofilm formation. To better assess the physiologic role of CdpA, we tested the cdpA mutant for motility and biofilm formation.

Like mutation of vieA in the classical biotype, mutation of cdpA in El Tor resulted in increased (threefold) biofilm formation (Fig. 3A). Ectopic expression of cdpA complemented the phenotype. CdpA thus functions as a repressor of biofilm formation in the El Tor biotype. This is likely due to CdpA PDE activity, which reduces intracellular c-di-GMP and in this way could impair vps gene expression and biofilm formation.

The effect of CdpA on biofilm formation could occur at the level of gene transcription and/or protein activity. To examine if cdpA expression is increased during biofilm formation, we used qPCR to assess transcript levels. The cdpA transcript level was 4.8-fold higher in biofilm than in broth culture, indicating that cdpA is indeed induced by V. cholerae during biofilm formation (Table 4). For comparison, vpsR, a positive regulator of vps gene expression and biofilm formation (55), was expressed 7.7-fold in biofilm relative to what was seen for broth culture. In addition, RIVET using a cdpA::tnpR transcriptional fusion was adapted for use in measuring cdpA transcription during growth in a biofilm. This method corroborated the result that cdpA transcription is up-regulated in a biofilm compared to growth in broth culture (data not shown).

In the classical biotype, elevated c-di-GMP level reduces motility (37). Consistent with c-di-GMP repressing motility in the El Tor biotype, overexpression of cdpA dramatically increased motility (Fig. 2A). Therefore, we predicted that a null mutation in cdpA would result in reduced motility. However, this was not the case, as the cdpA mutant showed motility identical to that of the parent strain (Fig. 3B).

A vieA mutation causes a 10-fold reduction in colonization of the infant mouse in the classical biotype (51) but has no effect on colonization in the El Tor biotype. An El Tor CVD110 cdpA mutant was previously shown to have reduced virulence in mice (35); however, this virulence-attenuated vaccine strain has significant genotypic and phenotypic differences from other El Tor strains. To determine whether cdpA plays a role in the virulence of WT El Tor CVD709, competition assays were done to compare the colonization of cdpA with WT. The mutant showed a negligible defect (median confidence interval.

### Table 4. Induction of transcription in biofilm and during infection

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Comparison</th>
<th>Fold change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>vpsR</td>
<td>Biofilm/broth 24°C</td>
<td>7.0 ± 1.4$^*$</td>
</tr>
<tr>
<td>cdpA</td>
<td>Biofilm/broth 24°C</td>
<td>3.9 ± 1.0$^*$</td>
</tr>
<tr>
<td>cdpA</td>
<td>In vivo/broth 37°C</td>
<td>8.1 ± 6.0$^{**}$</td>
</tr>
</tbody>
</table>

$^a$ Cycle thresholds for cdpA and vpsR transcripts were normalized to those for rpoB in each sample.

$^b$, $^c$, $^{**}$, $^{***}$, $^{****}$: $P < 0.05$, $P = 0.03$ by Mann-Whitney U test; $^{**}$, $^{***}$, $^{****}$: $P = 0.008$ by Mann-Whitney U test.
[CI] = 0.7) in colonization compared to the WT (Fig. 3C). The colonization phenotype could be complemented by expressing cdpA in trans. However, this slight decrease in colonization was attributed to a slight growth deficit of the cdpA strain (in vitro CI ~ 0.8).

cdpA expression is not regulated by c-di-GMP concentration. Since c-di-GMP activates the expression of known biofilm genes, namely, vps genes, we asked whether c-di-GMP, which is expected to be at a high level in biofilm, could act as a positive regulator of cdpA expression. This would result in the hydrolysis of c-di-GMP by CdpA and thus constitute a negative-feedback loop to limit vps expression and biofilm formation.

To test this, qPCR was used to measure the levels of cdpA transcript in V. cholerae containing WT, high, and low levels of c-di-GMP. To achieve this, V. cholerae strains ectopically expressing vdcA (AC1902, high c-di-GMP), veeA PDE (AC1901, low c-di-GMP), or vector alone (AC1903, WT c-di-GMP) were used. All strains were grown with aeration at 37°C in LB broth containing IPTG to induce protein production. No differences were seen in cdpA expression under high and low c-di-GMP levels; thus, c-di-GMP is not a regulator of cdpA transcription under the condition examined (data not shown). In contrast, expression of the vpsR control, which is known to be activated by c-di-GMP, was found to be 2-fold higher in the high c-di-GMP condition and 14-fold lower in the low-c-di-GMP condition.

cdpA expression is induced during a late stage of infection of the infant mouse. cdpA was originally identified in a RIVET screen for genes induced during infection of the infant mouse (35). The identification of cdpA as a gene induced during growth in a biofilm ostensibly is contradictory to this, as growth in the host and biofilm environments are considered distinct. In addition, we failed to observe a requirement for cdpA in colonization of the mouse.

Because a different strain of V. cholerae was used in the original study, we first sought to confirm that cdpA transcription is induced during infection in the current strain, C6709. qPCR analysis showed that at 24 h p.i., cdpA was induced 7.1-fold in the mouse small intestine compared to what was seen for the mid-log-phase broth culture. Thus, cdpA is induced during infection in this strain.

A genetic screen done in our lab suggested that cdpA expression is induced late during the infection process (44). If so, this may explain the lack of a virulence phenotype, since the mouse model of colonization highlights early stages of infection. To address this, the temporal expression of cdpA during infection of the mouse was investigated using the RIVET reporter system. The cdpA::tnpR strain, as well as a ctxA-tnpR control strain, was inoculated into infant mice. At 7 and 21 h p.i., the percentages of Km-sensitive CFU present in the mouse small intestines were enumerated, indicating the percentages of bacteria that had expressed cdpA during infection. At 7 h p.i., only 4% of CFU were Km sensitive, indicating that cdpA was not expressed at early stages of infection (Fig. 4). However by 21 h, 47% of CFU were Km sensitive. In contrast, ctxA, which previously was shown to be expressed early during infection (28), showed high levels of expression (97% Km sensitive) by 7 h. Neither cdpA nor ctxA were expressed during growth in vitro (0.3% or 8% Km sensitive, respectively). These results show that cdpA transcription is activated at a late stage during colonization of the mouse.

c-di-GMP regulates virulence of V. cholerae biotype El Tor. Mutation of VieA and loss of its c-di-GMP hydrolytic activity results in reduced virulence in the classical biotype (51) but not the El Tor biotype (27) of V. cholerae. Thus, it was unclear if c-di-GMP regulates virulence in the El Tor biotype. Because cdpA is expressed during a late stage of infection, a cdpA mutant is not amenable for use in the analysis of the effect of high c-di-GMP on the virulence of El Tor biotype V. cholerae. Therefore, we further characterized the role of c-di-GMP in the regulation of virulence in the El Tor biotype by use of an alternative strategy. The effect of increased intracellular c-di-GMP on the ability of V. cholerae to colonize the mouse small intestine was assessed by artificially increasing c-di-GMP through the expression of vdcA, previously shown to activate biofilm formation and inhibit motility (3, 50).

To directly test whether expression of vdcA increases intracellular c-di-GMP, 2D-TLC was used to compare the amounts of c-di-GMP in nucleotide extracts from bacteria expressing vdcA and vdcA(I) to that from bacteria with vector alone in the presence of 1 mM IPTG. VdcA(I) is expected to lack DGC activity due to an E258A mutation in the predicted catalytic site. VdcA dramatically increased c-di-GMP relative to the vector control, whereas VdcA(I) did not (Fig. 5A). These experiments confirmed that VdcA increases c-di-GMP in V. cholerae and that the G258A mutation in VdcA(I) abolishes DGC activity.

Assays were performed to ensure that the ectopic overexpression of vdcA, and the consequent increase in c-di-GMP, result in the expected downstream in vitro effects, namely, increased biofilm formation and lowered motility (3, 37, 50).
Strains containing pVdcA, pVdcA', or vector were tested for the ability to form biofilm. Expression of vdcA caused a fourfold increase in biofilm formation compared to what was seen for vector and vdcA(I) controls, indicating the proper activation of biofilm formation by a high concentration of c-di-GMP (Fig. 5B). Motility, on the other hand, is repressed by c-di-GMP (3, 29, 37). VdcA but not VdcA(I) reduced the motility of C6709 compared to the vector control (Fig. 5C). Thus, VdcA modulates c-di-GMP and affects downstream phenotypes appropriately, and VdcA(I) serves as a control for protein levels without modifying c-di-GMP concentration.

V. cholerae with elevated c-di-GMP was analyzed for virulence using competition experiments in an infant mouse model of colonization. Bacteria containing pVdcA showed a threefold decrease in colonization relative to the vector control (Fig. 6A); the pVdcA' strain showed no such virulence defect. In fact, when the pVdcA strain was competed directly against the pVdcA' strain to control for any defects due to protein level, fivefold-reduced colonization was seen for the bacteria with increased c-di-GMP. Importantly, these defects were not seen when the strains were competed in vitro (data not shown), so attenuation of virulence was not due to a general reduction in growth. Furthermore, the plasmids were maintained at greater than 98% during the course of infection.

One possible cause of the virulence defect of the pVdcA strain is the high amount of VPS produced by these bacteria. Such increased VPS synthesis could physically impede colonization or metabolically slow replication in the small intestine. To address this possibility, pVdcA was transformed into V. cholerae lacking vpsR and tested in competition experiments. Deletion of vpsR by itself did not affect colonization (25; also data not shown). The vpsR(pVdcA) strain showed the same attenuation as the pVdcA strain, suggesting that the colonization defect of the pVdcA strain was independent of VPS production.

FIG. 5. Ectopic modulation of intracellular c-di-GMP by expression of vdcA. (A) To directly test the ability of vdcA expression to increase intracellular c-di-GMP, 2D-TLC was done to detect intracellular c-di-GMP in strains containing pVdcA (AC1902), pVdcA' (AC2389), or vector alone (AC1903). The c-di-GMP and GMP spots are indicated. (B) The pVdcA, pVdcA', and vector-only strains were tested for the ability to activate biofilm formation. The mean intensities of crystal violet staining of the biofilms (A570) are shown. (C) The pVdcA, pVdcA', and vector strains were tested for decreased motility. The mean diameters of motility in mm from three independent assays are shown. In panels B and C, asterisks indicate significant differences in biofilm and motility, respectively, compared to WT as determined by Student’s t test.

FIG. 6. The effects of increased c-di-GMP on virulence of V. cholerae. (A) The pVdcA and pVdcA' strains were competed against WT containing vector alone in mice. In addition, the VdcA and VdcA(I) strains were competed directly to control for any effects of protein levels. Each symbol represents the CI obtained from an individual mouse. The horizontal bars indicate the geometric mean CI. Asterisks denote statistically significant differences in colonization \((P < 1 \times 10^{-4})\). (B) Transcription of toxT during infection of the mouse in the presence of WT (pVdcA') and ectopically increased c-di-GMP (pVdcA) was measured using RIVET. The percentage of CFU that were Tc sensitive, and therefore the percentage of bacteria that have expressed toxT, at the indicated time points p.i. are shown. Each data point represents the mean from at least three mice. Asterisks indicate significant differences in resolution rate at that time point as determined by Student’s t test.
c-di-GMP inhibits virulence gene transcription during infection of the infant mouse. Because increased VPS was excluded as a cause for reduced colonization of the mouse small intestine by bacteria with high c-di-GMP, we hypothesized that the attenuation of virulence in the pVdcA strain was a result of reduced virulence gene transcription. In vitro studies using the classical biotype have shown that high c-di-GMP due to loss of VieA PDE activity inhibits the transcription of *V. cholerae* virulence genes, including ctxAB and the major virulence gene transcriptional regulator toxT (31). However, the effect of c-di-GMP on virulence gene expression during infection has not been directly tested.

VdcA and VdcA(I) were used to investigate the effect of c-di-GMP on virulence gene transcription in vivo. For these experiments, pVdcA and pVdcA’ were transformed into an El Tor strain that was previously constructed to monitor toxT transcription using RIVET (28). In this strain, the resolvase gene trpR is fused to toxT and a res-tet-res cassette is stably integrated in the lacZ locus. Previous studies using this strain have shown that toxT is not expressed during in vitro growth but is induced during infection, leading to production of TnpR, resolution of the res-tet-res cassette, and loss of Tc resistance.

The toxT-trpR strains containing pVdcA and pVdcA’ were determined to produce VdcA and VdcA(I) at comparable levels detectable by Western blotting (data not shown). In addition, these strains were tested for proper regulation of c-di-GMP by use of biofilm assays. Whereas VdcA dramatically increased biofilm formation compared to the vector control, VdcA(I) did not, indicating that c-di-GMP was increased only in the pVdcA toxT-trpR strain (data not shown).

Expression of toxT-trpR during infection was determined by measuring the rate of resolution at different time points p.i. At 2 h p.i., toxT was not yet expressed in the pVdcA and pVdcA’ toxT-trpR strains, since almost all bacteria isolated from the small intestines were still Tc resistant (Fig. 6B). By 4 h, toxT was beginning to be expressed, and by 6 h p.i., the pVdcA strain showed a twofold reduction in toxT transcription compared to the pVdcA’ strain, which allows the normal regulation of c-di-GMP. Whereas 63% of the VdcA’ bacteria had expressed toxT and lost Tc resistance, only 26% of the pVdcA strain, which has artificially high c-di-GMP during infection, had expressed toxT. Inhibition of toxT transcription by c-di-GMP was seen at subsequent time points as well. The plasmids were maintained in more than 98% of the CFU during infection. In addition, toxT was not expressed by either strain under in vitro growth conditions (data not shown). Thus, elevated c-di-GMP caused a reduction in transcription of toxT during infection.

**DISCUSSION**

We have proposed a model for *V. cholerae* in which intracellular c-di-GMP is elevated during persistence in aquatic reservoirs, particularly when *V. cholerae* is associated in a biofilm, and is reduced upon entry into the host small intestine to allow proper expression of virulence genes. This model was developed based on studies of the PDE VieA in classical-biotype *V. cholerae* (37, 50, 51). However, a vieA mutation causes neither biofilm, motility, nor virulence phenotype in the El Tor biotype, which causes cholera today. This may be due to differences in levels of vieA expression in the classical and El Tor biotypes. Whole-genome expression studies showed that vieA is expressed at fivefold-higher levels in the classical biotype than the El Tor when grown in vieA-inducing conditions (2). Moreover, VieA regulates 401 genes in the classical biotype but does not significantly affect gene expression in the El Tor biotype (2). VieA thus appears to have lost its role as a major c-di-GMP PDE in the El Tor biotype, at least under the conditions that have been examined.

While VieA, specifically its c-di-GMP PDE activity, in the classical biotype has been shown to be essential for colonization of the small intestine and to activate virulence gene transcription in vitro, the effect of c-di-GMP on virulence and virulence factor expression during infection has not been addressed directly for *V. cholerae*, specifically El Tor. Herein we investigated the role of c-di-GMP early and late during infection by the El Tor biotype.

The first approach undertaken involved the analysis of cdpA. Like the vieSAB operon containing vieA, cdpA was identified as a gene induced during infection of the mouse (35). In addition, cdpA transcription was found to be induced during infection of humans (31). In contrast to vieC in the classical biotype, cdpA in the El Tor biotype was necessary only for the inhibition of biofilm formation and had no effect on motility or colonization. This was observed for El Tor strain N16961 as well. A cdpA mutation in classical-biotype *V. cholerae* O395, however, had no significant effect on motility or biofilm (data not shown). This underscores key differences in the ways the two biotypes regulate c-di-GMP and downstream processes.

We show that CdpA possesses PDE but not DGC activity by direct enzymatic assays, as well as in vitro phenotype assays, despite the presence of a GGDEF domain. However, the GGDEF domain was required for the full PDE activity of CdpA. This has been observed previously for other c-di-GMP PDEs that contain tandem GGDEF and EAL domains, including CC3396 of *C. crescentus* and *P. aeruginosa* FilX and, more recently, Bf10 (19, 22). For CC3396, it was demonstrated that rather than catalyzing c-di-GMP biosynthesis, the degenerate GEDEF sequence bound GTP and decreased the Km for c-di-GMP hydrolysis by the EAL domain (10). We propose that this occurs for CdpA as well.

Because cdpA was identified as being induced during infection (35), it was surprising that it regulated biofilm but not colonization. We demonstrated that cdpA is expressed at high levels during growth in a biofilm compared to in shaking culture. That cdpA is expressed during biofilm formation and that there is elevated biofilm formation in the cdpA mutant suggest that c-di-GMP PDE induction plays a role in limiting c-di-GMP and the production of biofilm. Such a control mechanism limiting biofilm formation has yet to be described for any biofilm-forming bacterial species.

In addition, analysis of the temporal expression of cdpA showed that cdpA transcription occurs only at a late stage of infection, in contrast to ctxA, which was highly expressed soon after entry into the small intestine. Recent work from our laboratory has found that *V. cholerae* expresses a set of genes specifically at a late stage of infection and that many of these genes confer an advantage for survival in conditions representative of aquatic environments (44). Several putative GGDEF domain genes predicted to encode DGCs, as well as cdpA,
were among the late in vivo-induced genes (44). It is possible that cdpA acts to counteract the increased c-di-GMP that would occur as a result of increased DGC activity. We suspected that induction of cdpA expression might occur as a direct response to increasing c-di-GMP in both biofilm and late infection conditions. However, this was not the case, as transcription levels of cdpA were identical in high and low c-di-GMP concentrations.

The identification of cdpA as a gene induced in parallel with putative DGCs late in infection suggests an interesting role for CdpA in the small intestine. Increased production of VPS while in the host may be detrimental, and expression of cdpA may keep VPS production in check until the bacteria have exited the host. Alternatively, or in addition, the purpose of limiting c-di-GMP at this time of infection may be to allow induction of motility and chemotaxis genes. Planktonic V. cholerae is motile in human rice water stool, and recently V. cholerae has been shown to become motile as part of a “mucosal escape response” during which the bacteria detach from the epithelial surface (34). In fact, the expression of chemotaxis and motility genes is up-regulated during this process in rabbit ileal loops (54). This model is supported by the finding that cdpA transcription is up-regulated in human rice water stool as well (31).

These experiments underscore an important mechanism of regulation of c-di-GMP in the cell, namely, differential regulation of transcription of c-di-GMP metabolic genes. Indeed, regulation of enzyme production explains the differences in phenotypes observed as a result of mutating cdpA from what is seen for previously characterized PDE genes such as vieA and bifA. BifA represses biofilm formation and enhances motility of P. aeruginosa, as VieA does in the classical biopsyte of V. cholerae (22, 50, 51). These results suggest that VieA and BifA are expressed in their respective species under the conditions in which biofilm and motility are assayed. Notably, vieA has been shown to be transcribed in LB broth, as well as in vivo (26). The transcription of cdpA, on the other hand, is regulated differently. Resolution assays monitoring cdpA transcription showed that it is not appreciably expressed in LB broth (data not shown). As the medium used in the motility experiments is a similarly rich medium, it is possible that cdpA is not expressed in that medium, thus resulting in the lack of a motility phenotype for the cdpA mutant. Moreover, the biofilm assays used in this study were done in LB broth, so the induction of cdpA transcription was dependent on static growth as a biofilm, not on the medium.

Because cdpA is not expressed early during infection and has no discernible role in V. cholerae colonization of the small intestine, mutation of this gene was not a suitable strategy for investigating the effect of increased c-di-GMP on early stages of infection by the El Tor biotype. As an alternative approach, c-di-GMP was artificially increased by ectopic expression of vdcA. The advantage of this strategy is that it mimics the deletion of PDE(s) critical to reducing c-di-GMP upon entry into the host. V. cholerae with high c-di-GMP showed reduced colonization of the mouse small intestine. Decreased colonization was attributed to reduced expression of the major virulence gene regulator tosT during infection. These data provide the first direct evidence that high c-di-GMP in El Tor biotype V. cholerae is deleterious to colonization and virulence gene expression during infection of the small intestine, as predicted by the vieA studies with the classical biopsyte. The limitation of these studies is that, as the level of c-di-GMP that occurs naturally in V. cholerae during infection is unknown, the concentration of c-di-GMP achieved by vdcA expression during infection may not be representative of physiological fluctuations in c-di-GMP.

This work shows that c-di-GMP is inhibitory to the virulence of El Tor biopsyte V. cholerae at early stages of infection and suggests it may be deleterious to pathogenesis at later stages as well. Which of the 31 EAL or HD-GYP domain putative c-di- GMP PDE(s) mediates the reduction of c-di-GMP in the El Tor biopsyte upon entry into the small intestine remains unknown, but VieA likely serves this function in the classical biopsyte. It is also possible that PDEs are already present in V. cholerae and that signals from the host gastrointestinal tract activate enzymatic function. Later during infection, CdpA, and perhaps other PDEs, are produced. PDE expression at this stage may serve to counteract the activities of DGCs made at this time, with the purpose of minimizing VPS production during infection and/or allowing expression of motility and chemotaxis genes.

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