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Cyclic Diguanylate Regulates *Vibrio cholerae* Virulence Gene Expression

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The cyclic dinucleotide second messenger cyclic diguanylate (c-diGMP) has been implicated in regulation of cell surface properties in several bacterial species, including *Vibrio cholerae*. Expression of genes required for *V. cholerae* biofilm formation is activated by an increased intracellular c-diGMP concentration. The response regulator VieA, which contains a domain responsible for degradation of c-diGMP, is required to maintain a low concentration of c-diGMP and repress biofilm formation. The VieSAB three-component signal transduction system was, however, originally identified as a regulator of *ctxAB*, the genes encoding cholera toxin (CT). Here we show that the c-diGMP phosphodiesterase activity of VieA is required to enhance CT production. This regulation occurred at the transcriptional level, and ectopically altering the c-diGMP concentration by expression of diguanylate cyclase or phosphodiesterase enzymes also affected *ctxAB* transcription. The c-diGMP phosphodiesterase activity of VieA was also required for maximal transcription *toxT* but did not influence the activity of ToxR or expression of TcpP. Finally, a single amino acid substitution in VieA that increases the intracellular c-diGMP concentration led to attenuation in the infant mouse model of cholera. Since virulence genes including *toxT* and *ctxA* are repressed by a high concentration of c-diGMP, while biofilm genes are activated, we suggest that c-diGMP signaling is important for the transition of *V. cholerae* from the environment to the host.

Cyclic diguanylate (c-diGMP) [bis(3′,5′)-cyclic diguanylic acid] is a prokaryotic cyclic dinucleotide second messenger that has been implicated in controlling properties of the cell surface in diverse bacterial species (8, 23). c-diGMP was originally characterized in *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*), an organism that synthesizes a cellulose extracellular matrix (44, 45). In *G. xylinus*, the c-diGMP concentration is controlled by six proteins that have competing diguanylate cyclase or phosphodiesterase activities to synthesize and degrade c-diGMP, respectively (49). Each of these cyclase and phosphodiesterase proteins may be activated by environmental signals that serve as the first messenger; for example, the *G. xylinus* PDEA1 phosphodiesterase is responsive to oxygen (6). The c-diGMP second messenger then controls production of the cellulose matrix by allosterically activating the cellulose synthase enzyme (54). The protein domains responsible for synthesis and degradation of c-diGMP, named GGDEF and EAL, respectively, for their conserved amino acid residues, are widespread in bacterial genomes (13). There is evidence that proteins with the GGDEF and EAL domains control production of extracellular polysaccharide matrices in several bacterial species, including *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Yersinia pestis*, and *Vibrio cholerae* (12, 14, 25, 48, 51).

In *V. cholerae*, the causative agent of the severe diarrheal disease cholera, c-diGMP concentration affects transcription of *Vibrio* exopolysaccharide synthesis (*vps*) genes that are required for biofilm formation (51). The ability of *V. cholerae* to form biofilms may contribute to persistence of the organism in environmental reservoirs between epidemics, since bacteria within a biofilm are more resistant to chemical stresses (53, 55). Although the mechanism has not yet been elucidated, increased concentration of the c-diGMP second messenger activates *vps* gene expression. The response regulator VieA contains an EAL domain that is a putative c-diGMP phosphodiesterase and that is required to maintain a low level of c-diGMP under growth conditions that do not favor biofilm formation (51). Mutations at conserved residues in the VieA EAL domain result in an elevated c-diGMP concentration and activation of *vps* transcription. In contrast, the VieA phosphoreceiver and helix-turn-helix domains are not necessary for regulation of *vps* transcription (51).

Although VieA negatively regulates expression of genes required for biofilm formation, it was originally identified as a putative positive regulator of virulence genes. In humans, *V. cholerae* causes disease by colonizing the small intestine and secreting cholera toxin (CT), an ADP-ribosylating toxin that elicits profuse watery diarrhea (24). Expression of CT and the toxin-coregulated pilus (TCP), the primary colonization factor, are controlled by a complex network of transcriptional activators (reviewed in references 30 and 43). Both *ctxAB*, the genes encoding CT, and the *tcp* operon are activated by an AraC family transcription factor, ToxT (2, 5, 18, 21). Expression of *toxT* is, in turn, controlled by two pairs of inner membrane proteins, ToxR/S and TcpP/H (10, 17, 31). TcpP and TcpH are encoded adjacent to the *tcp* operon, and their expression is activated by two proteins, AphA and AphB, that are responsive to environmental signals (28, 29). In addition to indirectly

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controlling *ctxAB* expression by activating transcription of *toxT*, ToxR has been shown to directly activate transcription from the *ctxAB* promoter in *Escherichia coli* (39). The transmembrane domain of ToxR was recently implicated in the ToxT-independent induction of CT expression by bile salts (22). The VieSAB three-component system was identified as an additional regulator of virulence genes in a screen for in vivo activators of *ctxAB* (34). The VieSAB system is required for maximal expression of *ctxAB* both in vivo and during growth under in vitro conditions that induce virulence factor expression, but the exact regulatory mechanism was not determined (52).

Here we show that the VieA response regulator is required in the *V. cholerae* classical biotype for maximal activation of CT production during growth in vitro under conditions that induce virulence gene expression. Specifically, the EAL domain of VieA must be functional to maintain the low intracellular c-diGMP concentration that enhances CT production. We show that transcription of both *ctxA* and *toxT* is repressed by an increased intracellular concentration of c-diGMP and that control of the c-diGMP concentration by VieA is necessary for wild-type levels of colonization in an animal model of infection. Since we have previously implicated the c-diGMP second messenger as an activator of *vps* gene expression and biofilm formation, we propose that c-diGMP modulates gene expression during the transition from environmental reservoirs to the human host.

MATERIALS AND METHODS

Growth conditions. Bacteria were grown in Luria-Bertani (LB) broth at 37°C with aeration unless otherwise noted. M9 salts plus 0.5% glycerol, trace metals (1 ml/liter of 5% $MgSO_4$, 0.5% $MnCl_2 \cdot 4H_2O$, 0.5% $FeCl_3$, 0.4% trinitriloacetic acid) (3), and L-asparagine, L-arginine, L-glutamate, and L-serine, each at 25 mM (M9 + NRES), was prepared as described previously (38). A similar medium with morpholinepropanesulfonic acid (MOPS) salts as the base (46) was used for ^{32}P labeling of nucleotides. Expression from the P_{araBAD} promoter was induced by addition of arabinose to a 0.1% final concentration. Antibiotics were used at the following concentrations: streptomycin (Sm), 100 μg/ml; ampicillin (Ap), 50 μg/ml; chloramphenicol (Cm), 5 μg/ml.

Strain and plasmid construction. All strains and plasmids used in this study are listed in Table 1. Plasmids with oriR6K were propagated in *E. coli* DH5αλpir; all other plasmids were propagated in *E. coli* DH5α. Oligonucleotide primers are listed in Table 2.

Plasmid pAT1276 was constructed by ligating *toxT*, *lacZ*, and *tcpJ* fragments into pCVD442. The *toxT* fragment (1.2 kbp) was amplified by PCR from O395 genomic DNA using primers toxTF1 and TZJR1; the *tcpJ* fragment (800 bp) was similarly amplified using primers 5JF1 and 3JR1. The *E. coli lacZ* gene was amplified with primers TZJF1 and CZBR1. All three products were cloned into pCR-Script and subsequently removed by digestion with the following enzymes: for *toxT*, XbaI/PstI; for *lacZ*, PstI/KpnI; for *tcpJ*, KpnI/SacI. The products were ligated to XbaI/SacI-digested pCVD442, and the construct was confirmed by enzyme digestions, PCR, and sequencing.

Plasmid pAT1809 for generation of *tcpP* RNA probe was constructed by PCR amplifying an internal fragment of *tcpP* (347 bp) from O395 genomic DNA, using primers TcpPF1 and TcpPR1 and cloning into pGEM-T. The template for in vitro transcription of the antisense probe was generated from this plasmid by PCR using primers TcpPF1 and T7R. Plasmid pAT1822 for complementation of the *vieA*(*E170A*) mutation was constructed by PCR amplification of the *vieA* gene and 1,517 bp 5′ to the *vieA* start codon using primers F38 and VieAR. The product was subcloned into pCR-Script, removed by digestion with SacI/SalI, and ligated into similarly digested pMMB67EH. Plasmid pAT1868 for generating plasmid integrations at the *vpsR* locus was constructed by PCR amplification of a 507-bp internal fragment of the *vpsR* gene using primers vpsRF2 and vpsRR2. The PCR product was digested with SphI/SacI and ligated into similarly digested pGP704.

Strains AC-V1296, AC-V1382, and AC-V1848 were generated by allelic exchange with plasmid pAT1276 in the appropriate strain background as previously described (33). Integration of *lacZ* downstream of *toxT* was confirmed by PCR with primers tcpEF2 and lacZR. Strain AC-V1302 was constructed by mating strain AC-V1296 with *E. coli* SM10λpir containing plasmid pAC396 and selecting Sm^r Ap^r exconjugates as described previously (33). Integration of the plasmid at the *toxR* locus was confirmed by colony PCR using primers oriR/toxRR2. Strains AC-V1869 and AC-V1870 were similarly constructed by mating strains AC-V61 and AC-V1596, respectively, with *E. coli* SM10λpir containing plasmid pAT1868 and selecting Sm^r Ap^r exconjugates. Integration of the plasmid at the *vpsR* locus was confirmed by colony PCR using primers oriR and vpsRF3. Strains AC-V1660, AC-V1661, AC-V1843, AC-V1109, AC-V1815, and AC-V1824 were constructed by electroporation of the appropriate plasmid into the indicated strain background. Strains O395, AC-V1386, and AC-V1596 were made electrocompetent as described previously (52).

Cholera toxin ELISA. Strains were grown overnight in M9 + NRES at 30°C with aeration. The final optical density at 600 nm (OD_{600}) was determined, cells were pelleted by centrifugation, and the supernatant was filter sterilized (0.45-μm polyvinylidene difluoride filter). The concentration of CT in supernatants was determined by GM_1 enzyme-linked immunosorbent assay (ELISA) using polyclonal goat antiserum to the CT B subunit (List Biolabs) and antigoat immunoglobulin G conjugated to alkaline phosphatase (Sigma) as described previously (20). A purified CT B subunit of known concentration (List Biolabs) was used to determine the amount of CT in the samples.

RNase protection assay (RPA) and c-diGMP quantitation. RPAs were performed essentially as described previously (52) using RNA isolated from strains grown in MOPS + NRES at 30°C with aeration to mid-exponential phase (OD_{600} = 0.6). The *rpoB* riboprobe was included in all hybridizations to serve as a loading control as described previously (52). Data were collected with a PhosphorImager and analyzed using ImageQuant (Molecular Dynamics). Band intensities were quantitated and normalized to the intensity of the *rpoB* band. The reported severalfold differences in transcript level are the average from three independent experiments. Determination of c-diGMP concentration was done using aliquots of the same cultures essentially as described previously (51) except that bacteria were labeled with [^{32}P]orthophosphate for 30 min. Quantitation of c-diGMP spots observed by two-dimensional thin-layer chromatography (2D-TLC) was done using ImageQuant. Values were corrected for background and were normalized to the intensity of the GDP spot. The intensity of the GDP spot did not vary appreciably (less than 1.5-fold differences) in the strain backgrounds tested, with the exception of the GGDEF-overexpressing strain, in which it was substantially reduced.

Immunoblots for TcpA and TcpP. Strains were grown in M9 + NRES at 30°C with aeration to an OD_{600} of 0.6. Cells were pelleted by centrifugation, resuspended in sodium dodecyl sulfate sample buffer, and boiled for 5 min. Equivalent volumes of each sample were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to Hybond nitrocellulose (Amersham) or Immobilon-P polyvinylidene difluoride (Millipore) membranes and blotted with rabbit polyclonal antisera against the TcpA or TcpP proteins. Donkey antirabbit immunoglobulin conjugated to horseradish peroxidase was used as a secondary antibody. Immunoreactive bands were detected with the ECL kit (Amersham) and exposure to film (Kodak).

β-Galactosidase assays. Strains containing the *toxT-lacZ-tcpJ* transcriptional fusion were struck for single colonies on LB plates and incubated overnight at 37°C. Colonies were resuspended in M9 + NRES, the OD_{600} was determined, and 6 ml M9 + NRES in 16- by 1.5-cm tubes was inoculated to a starting OD_{600} of 0.1. Cultures were incubated at 30°C with vigorous shaking. At 2-h intervals, the OD_{600} was determined and 0.5 ml was harvested for β-galactosidase assays, which were done according to the Miller method (37).

In vivo competition assays. Competition assays with the infant mouse model of cholera were performed essentially as described previously (33) except that plate-grown bacteria were used as the inocula. The *lacZ vie*(*AE170A*) mutant and wild-type O395 strains were struck for single colonies on LB plates and incubated overnight at 37°C. For each strain, approximately 30 colonies were scraped off plates into 1 ml of LB and the OD_{600} was determined. Equivalent amounts of each strain were mixed and adjusted to a final OD_{600} of 0.01 (approximately 10^7 CFU/ml). Five-day-old CD-1 infant mice were intragastrically inoculated with 50 μl of this mix. Competitions to assess complementation of the *vieA*(*E170A*) mutant were performed against strain AC-V1109. Similar competitions were performed in vitro by inoculating bacteria into LB and incubating overnight at 37°C with aeration. Care of and procedures done on mice complied with relevant federal guidelines as well as Tufts University animal use policies.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype	Source or reference
E. coli strains		
DH5α	F− Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	16, 27
DH5αλpir	F− Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* λ::*pir*	16, 27
SM10λpir	*thi recA thr leu tonA lacY supE* RP4-2-Tc::Mu λ::*pir*	Laboratory strain
AC-E1277	SM10λpir (pAT1276), Ap^r	This work
V. cholerae strains		
O395	Classical biotype, Sm^r	50
AC-V61	O395 *lacZ*::*res-tet-res*, spontaneous partial deletion of *lacZ*, Sm^r	4
AC-V1386	AC-V61 Δ*vieA*, Sm^r	51
AC-V1596	AC-V61 *vieA*(*E170A*), Sm^r	51
AC-V744	O395 Δ*toxR*, Sm^r	26
AC-V449	O395 Δ*tcpP*, Sm^r	57
AC-V1296	AC-V61 *toxT-lacZ-tcpJ*, Sm^r	This work
AC-V1382	AC-V1386 *toxT-lacZ-tcpJ*, Sm^r	This work
AC-V1848	AC-V1596 *toxT-lacZ-tcpJ*, Sm^r	This work
AC-V1302	AC-V1296 *toxR*::pGP704 Sm^r Ap^r	This work
AC-V1460	AC-V61 pBAD33, Sm^r Cm^r	51
AC-V1726	AC-V61 pAT1662, Sm^r Cm^r	51
AC-V1463	AC-V1386 pBAD33, Sm^r Cm^r	51
AC-V1464	AC-V1386 pAT1337, Sm^r Cm^r	51
AC-V1660	AC-V1386 pAT1568, Sm^r Cm^r	This work
AC-V1661	AC-V1386 pAT1615, Sm^r Cm^r	This work
AC-V1843	AC-V1386 pAT1647, Sm^r Cm^r	This work
AC-V1109	O395 pMMB67EH, Sm^r Ap^r	This work
AC-V1815	AC-V1596 pMMB67EH, Sm^r Ap^r	This work
AC-V1824	AC-V1596 pAT1822, Sm^r Ap^r	This work
AC-V1869	AC-V61 *vpsR*::pGP704 Sm^r Ap^r	This work
AC-V1870	AC-V1596 *vpsR*::pGP704 Sm^r Ap^r	This work
Plasmids		
pCVD442	*oriR6K mobRP4 sacB*, Ap^r	11
pAT1276	pCVD442::*toxT-lacZ-tcpJ*, Ap^r	This work
pGP704	*oriR6K mobRP4*, Ap^r	38
pAC396	pGP704::*'toxR'*, Ap^r	38
pAT1868	pGP704::*'vpsR'*, Ap^r	This work
pCR-Script	fl (+) *ori*, Ap^r	Stratagene
pGEM-T	fl *ori*, Ap^r	Promega
pAT856	pGEM-T::*'rpoB'*, Ap^r	52
pAT853	pGEM-T::*'ctxA'*, Ap^r	52
pDSM701	pGEM-T::*'toxT'*, Ap^r	52
pDSM702	pGEM-T::*'ompT'*, Ap^r	36
pDSM703	pGEM-T::*'ompU'*, Ap^r	36
pAT1809	pGEM-T::*'tcpP'*, Ap^r	This work
pMMB67EH	IncQ broad-host-range cloning vector, Ap^r	40
pAT1822	pMMB67EH::*vieA*, Ap^r	This work
pBAD33	pACYC184 *ori, araC* P_{araBAD}, Cm^r	15
pAT1337	pBAD33::*vieA*, Cm^r	48
pAT1568	pBAD33::VieA-EAL-His_6, Cm^r	51
pAT1615	pBAD33::VieAEAL(E170A)-His_6, Cm^r	51
pAT1647	pBAD33::'RocS-His_6, Cm^r	51
pAT1662	pBAD33::VCA0956-His_6, Cm^r	51

RESULTS

VieA regulates cholera toxin production. We have previously shown that the VieSAB three-component signal transduction system regulates production of CT in the *V. cholerae* El Tor biotype (52). Unlike the El Tor biotype, which requires a complex growth condition for in vitro induction of virulence genes, the *V. cholerae* classical biotype expresses virulence factors during growth at 30°C in either LB (pH 6.5) or a defined medium (M9) plus the L amino acids asparagine, arginine, glutamate, and serine (NRES) (38). Although the classical biotype does not require the VieSAB system for expression of CT under the LB (pH 6.5) inducing condition (data not shown), we decided to test whether the VieSAB system is necessary for CT production in M9 + NRES because the VieS sensor kinase contains two motifs in its predicted periplasmic domain with homology to amino acid binding proteins. While the VieB response regulator was dispensable (data not shown), VieA was necessary for maximal CT production in M9 + NRES as assessed by GM_1 ELISA (Fig. 1A). Specifically, the putative c-diGMP phosphodiesterase EAL domain of VieA

TABLE 2. Oligonucleotide primers used in this study[a]

Oligonucleotide name	Sequence 5′ - 3′
toxTF1	GCTCTAGATGTCAAACCATATCAGCCTAG
TZJR1	AACTGCAGAGGATCAAGTAAACGTATTCC
5JF1	GGGGTACCCTACAGTATTTAACTCAACCA
3JR1	GCGAGCTCCTTCATTTTAGCTCACATTAA
TZJF1	AACTGCAGTTTANGATACATTTTTATGACCATGATTACGGATTCA
CZBR1	GGGGTACCTTATTTTTGACACCAGACCAA
tcpPF1	TCAATTTCCCGATAACCTTT
tcpPR1	AGTCAGCTTCATCAACAACG
F38	GATGGAGATTTTGGCTTCA
vieAR	AACTGCAGTAGGTACAGCCATAACTCTCG
tcpEF2	GGAGTTATCTATGACCCTGTT
lacZR	GAGGGGACGACGACAGTATC
oriR	CAGCAGTTCAACCTGTTG
toxRR2	ATGGCATCGTTAGGGTTAG
vpsRF2	TGGCATGCTATGCCTATGAAGCGTTTG
vpsRR2	GCGAGCTCGTAGCACGATATCCGATGC
vpsRF3	CATGCTGAAGTTGGAAAA

[a] Restriction enzyme sites are indicated by underlining.

was required to enhance CT production. The E170A point mutation at a highly conserved residue of the EAL domain does not affect expression of VieA as assessed by immunoblotting with polyclonal antiserum against VieA (data not shown) but causes an eightfold reduction in the amount of CT produced, similar to the sixfold reduction observed for the in-frame deletion of *vieA* (Fig. 1A). These results suggest that VieA activates CT production indirectly by lowering the intracellular concentration of c-diGMP.

VieA regulates cholera toxin through control of c-diGMP. To confirm that VieA regulates CT production by controlling intracellular c-diGMP concentration, the c-diGMP concentration was ectopically altered by expression of proteins with the GGDEF or EAL domains from an arabinose-inducible promoter. To increase the c-diGMP concentration, the GGDEF protein VCA0956, which synthesizes c-diGMP both in vivo and in vitro (48; A.D. Tischler and A. Camilli, unpublished data), was expressed. Since c-diGMP is barely detectable in the wild-type strain, we used the Δ*vieA* mutant, which exhibits an elevated c-diGMP concentration (51), and attempted to complement the mutation by expressing either the VieA EAL domain or an N-terminally truncated form of the RocS protein (′RocS; VC0653) that includes an EAL domain. Both of these proteins have putative c-diGMP phosphodiesterase activity, since they were previously shown to complement the Δ*vieA* strain with respect to *vps* gene expression (51). As a negative control, the VieA EAL domain containing the E170A point mutation was expressed. All of these proteins were tagged with a His_6 epitope to facilitate determination of expression levels by immunoblotting. Each protein was undetectable in the absence of induction, and all were expressed at similar levels following induction with arabinose (data not shown).

Ectopic expression of the GGDEF and EAL proteins by arabinose induction from a P_{BAD} promoter had the expected effects on CT production. Expression of the GGDEF protein VCA0956 in wild-type *V. cholerae*, which, like mutations in *vieA*, causes an increase in the c-diGMP concentration, reduced CT production to a virtually undetectable level (Fig. 1B). Conversely, expression of either full-length VieA or the VieA EAL domain complemented the Δ*vieA* mutation; CT

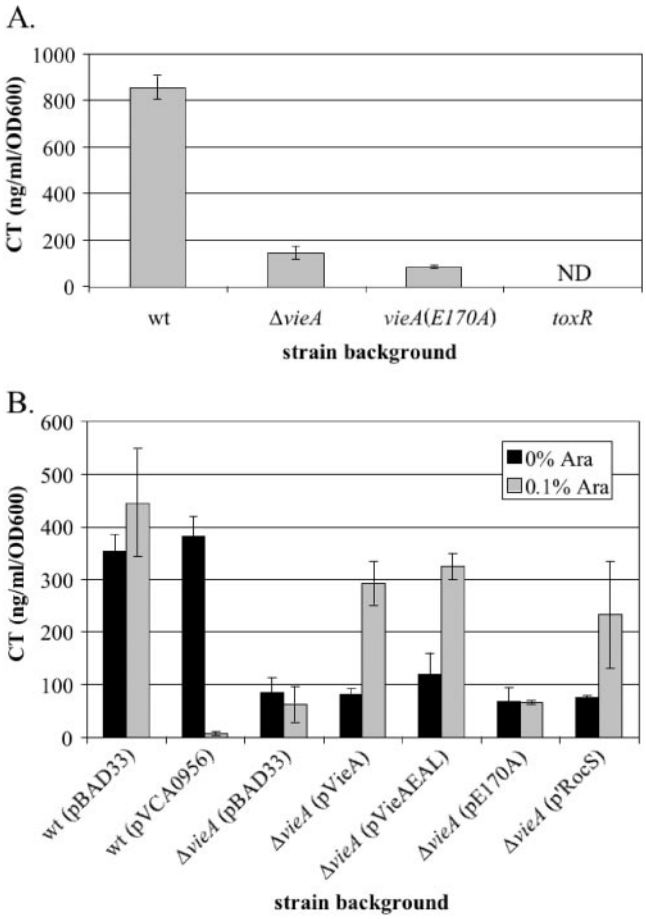

FIG. 1. CT production is regulated by c-diGMP. GM_1 ELISAs to quantify production of the CT B subunit were done with culture supernatants from the indicated strains grown overnight in M9 + NRES. Purified CT B subunit of known concentration was used to determine the concentration in the samples. ND, not detected. A. Strains AC-V61 (wild type), AC-V1386 (Δ*vieA*), AC-V1596 [*vieA*(*E170A*)], and AC-V744 (Δ*toxR*). B. Strains were grown in the absence (black bars) or presence (gray bars) of 0.1% arabinose. The wild-type and Δ*vieA* mutant strain backgrounds carrying the indicated plasmids (strains AC-V1460, AC-V1726, AC-V1463, AC-V1464, AC-V1660, AC-V1661, and AC-V1843).

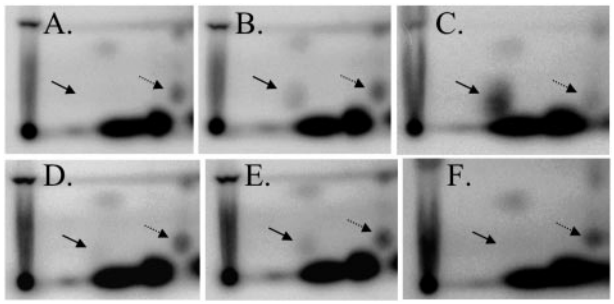

FIG. 2. 2D-TLC of ^{32}P-labeled nucleotide extracts to detect c-diGMP. Strains were grown in MOPS plus NRES to an OD_{600} of 0.6 and labeled for 30 min with [^{32}P]orthophosphate. Nucleotides were extracted with formic acid, spotted on TLC plates (origin at the bottom left corner), and developed in 0.2 M NH_4HCO_3, pH 7.8, in the first dimension (bottom to top) and 1.5 M KH_2PO_4, pH 3.65, in the second dimension (left to right). c-diGMP and GDP spots are indicated by the solid and dashed arrows, respectively. Only the relevant portion of the TLC plate is shown. A. AC-V61, wild type. B. AC-V1596, *vieA*(*E170A*). C-F. Strains were grown with 0.1% arabinose to induce expression from the pBAD promoter. C. AC-V1726, wild type (pVCA0956). D. AC-V1660, Δ*vieA* (pVieA-EAL). E. AC-V1661, Δ*vieA* (pE170A). F. AC-V1843, Δ*vieA* (p′RocS).

production was fully restored to the level for the wild-type vector control (Fig. 1B). In contrast, complementation was not observed when the VieA EAL domain containing the E170A point mutation was expressed (Fig. 1B). Finally, expression of ′RocS restored CT production to near the wild-type level (Fig. 1B). These results confirm that VieA regulates CT expression indirectly through its influence on the level of c-diGMP.

Cholera toxin is regulated at the transcriptional level. Since production of CT was decreased in strains with increased intracellular c-diGMP concentration, we tested whether transcription of the *ctxAB* genes was similarly affected. In order to monitor the c-diGMP concentration simultaneously with levels of *ctxA* transcription, these experiments were performed in a phosphate-limiting MOPS minimal medium that allows radioactive labeling of nucleotides with ^{32}P (51). Strains were grown in MOPS plus NRES to mid-exponential phase (OD_{600} = 0.6), and samples of each culture were taken for nucleotide labeling and RNA extraction. 2D-TLC of nucleotide extracts confirmed the predicted changes in the c-diGMP concentration. Specifically, the *vieA*(*E170A*) and Δ*vieA* mutations caused sixfold increases in the c-diGMP concentration, relative to that of the wild type (Fig. 2A and B and data not shown). Similarly, expression of the VCA0956 GGDEF protein resulted in a 28-fold increase in the level of c-diGMP relative to that of the uninduced control (Fig. 2C and data not shown). In contrast, expression of the VieA EAL domain or ′RocS in the Δ*vieA* mutant background lowered the c-diGMP concentration (Fig. 2D and F). While ′RocS expression resulted in a fivefold decrease in the c-diGMP level relative to that of the uninduced control, the VieA EAL domain reduced the c-diGMP concentration only two-fold. Finally, expression of the E170A mutant VieA EAL domain in the Δ*vieA* mutant background had no effect on the level of c-diGMP (Fig. 2E).

The amount of *ctxA* transcript was measured for each strain by RPA. A fourfold decrease in *ctxA* transcript was consistently observed for both the Δ*vieA* and *vieA*(*E170A*) mutants compared to the wild type, suggesting that *ctxA* transcription is repressed by an increased c-diGMP concentration (Fig. 3A). To confirm that the c-diGMP concentration affects transcription of *ctxA*, similar RPA experiments were performed using strains overexpressing either the VCA0956 GGDEF protein or the various EAL domains. In quantifying the level of *ctxA* transcript for these experiments, we compared the arabinose-induced sample to the relevant uninduced control for each strain rather than comparing all samples to the vector control due to differences in growth rates of the strains that may have influenced gene expression. We believe this is an appropriate comparison, since all proteins expressed from the pBAD33 vector were undetectable in the absence of arabinose induction (data not shown). Consistent with the results of the CT ELISAs, a sevenfold decrease in *ctxA* transcript was observed when the VCA0956 GGDEF protein was expressed (Fig. 4). Conversely, expression of the VieA EAL domain or ′RocS

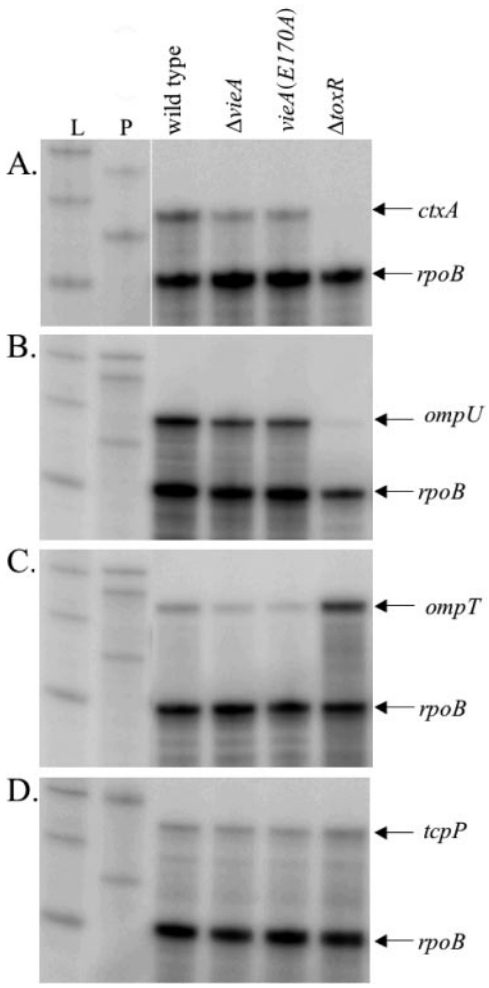

FIG. 3. VieA regulates transcription of *ctxA*. The wild-type (AC-V61), Δ*vieA* (AC-V1386), *vieA*(*E170A*) (AC-V1596), and Δ*toxR* (AC-V744) strains were grown to an OD_{600} of 0.6 in MOPS plus NRES, RNA was extracted, and indicated transcripts were detected by RNase protection assay. L indicates RNA ladder; P indicates undigested probes (only 0.1% of probe used in RPAs was loaded). All samples were normalized to the *rpoB* loading control and are the average for three independent experiments.

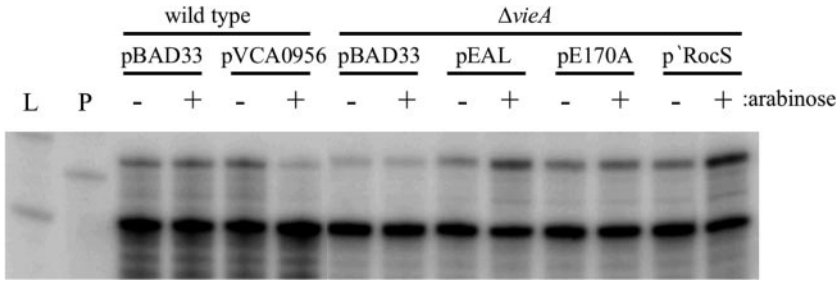

FIG. 4. Ectopic expression of the GGDEF or EAL protein affects *ctxA* transcription. Strains with the indicated plasmids were grown in MOPS plus NRES with or without 0.1% arabinose to an OD_{600} of 0.6, RNA was isolated, and the *ctxA* and *rpoB* transcripts were detected by RPA. L indicates RNA ladder; P indicates undigested probes (only 0.1% of probe used in RPAs was loaded). All samples were normalized to the *rpoB* loading control and are the average for three independent experiments.

restored *ctxA* transcription to near wild-type levels (3-fold and 4.5-fold increases, respectively) (Fig. 4). Overexpression of the E170A mutant VieA EAL domain, however, gave no increase in *ctxA* transcription relative to that for the uninduced control (Fig. 4).

c-diGMP concentration affects transcription of *toxT*. Our previous work in the El Tor biotype suggested that the VieSAB system might indirectly affect *ctxAB* transcription by controlling transcription of the positive regulator ToxT (52). To test whether *toxT* transcription is regulated by VieA in the classical biotype, RPAs were done on the same RNA samples used to assess *ctxA* regulation. In these mid-exponential-phase RNA samples, the level of *toxT* transcript was quite low, preventing accurate quantitation of the effect of c-diGMP on *toxT* regulation (data not shown).

As an alternative method to assess *toxT* expression, a nonpolar *toxT-lacZ* transcriptional fusion was constructed and introduced into the wild-type, Δ*vieA*, and *vieA*(*E170A*) strain backgrounds. Transcriptional activity was assessed for each of these strains over time during growth in M9 + NRES by performing β-galactosidase assays. A *toxR* plasmid insertion mutant was included in these experiments as a negative control. Although all strains grew at indistinguishable rates (data not shown), reporter fusion activity increased 10-fold during exponential-phase growth for the wild-type strain but only fourfold for the Δ*vieA* or *vieA*(*E170A*) mutant (Fig. 5). The activity of the fusion was significantly different from that of the wild type at 6, 8, and 10 h in both the Δ*vieA* and *vieA*(*E170A*) mutants as assessed by unpaired, two-tailed *t* tests ($P < 0.05$). These results suggest that the ability of VieA to lower the c-diGMP concentration is required for maximal expression of ToxT, which in turn activates *ctxA* transcription.

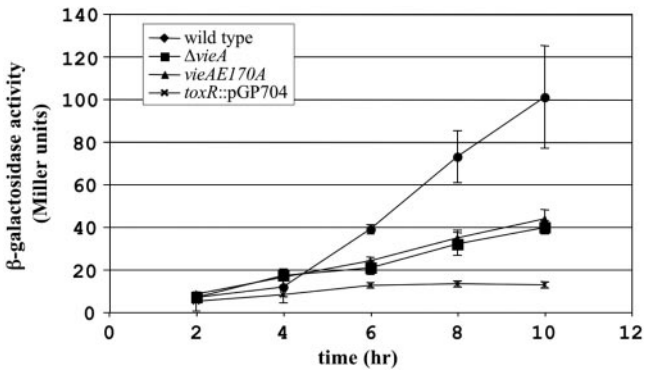

FIG. 5. *toxT-lacZ* fusion activity is reduced in *vieA* mutants. Strains containing the *toxT-lacZ* fusion were grown in M9 + NRES at 30°C with shaking. β-Galactosidase activity was determined at 2-h intervals by the Miller method. Each result presented is the average ± standard error from three independent experiments.

c-diGMP does not affect regulators above ToxT. ToxT is positively regulated by two DNA binding proteins, ToxR and TcpP, both of which localize to the inner membrane (10, 17, 31). In addition to regulating expression of virulence factors, ToxR is responsible for the inverse regulation of the outer membrane porins, OmpT and OmpU. ToxR is a repressor of *ompT* transcription (35), while it activates expression of *ompU* (7). Since ToxR is constitutively expressed under standard growth conditions (9), environmental signals that activate expression of virulence genes presumably affect the ability of ToxR to bind DNA. To examine whether the c-diGMP concentration alters *toxT* transcription by changing the activation state of ToxR, RPAs to examine *ompU* and *ompT* transcription were done. Although a slight (approximately 1.5-fold) decrease in *ompU* transcript was observed in the Δ*vieA* and *vieA*(*E170A*) mutants relative to the wild-type level, a similar 2.5-fold decrease was observed for the *ompT* transcript (Fig. 3B and C). If ToxR activity was affected by the c-diGMP concentration, an increase in the level of *ompT* transcript would be expected, concomitant with the decrease in *ompU* transcription. These results suggest that ToxR activation is not affected by the c-diGMP concentration.

TcpP is regulated at the transcriptional level by two activators, AphA and AphB (28, 29). To test whether decreased *toxT* expression might be caused by reduced *tcpP* transcription, RPAs were performed. Although the amount of *tcpP* transcript detected was low, it was reproducibly detected at levels similar to that for the wild type in both the Δ*vieA* and *vieA*(*E170A*) mutants (less that 1.5-fold reduction) (Fig. 3D). In addition, overexpression of EAL or GGDEF domains did not affect the level of *tcpP* transcript (data not shown).

It was recently reported that TcpP is a target for proteolysis in the absence of its partner protein, TcpH (1). The protease responsible for degradation of TcpP has not yet been identified, but we have observed that several secreted proteases are up-regulated in a *vieA* mutant (A.D. Tischler, J. T. Pratt, and A. Camilli, unpublished data). To test whether TcpP protein

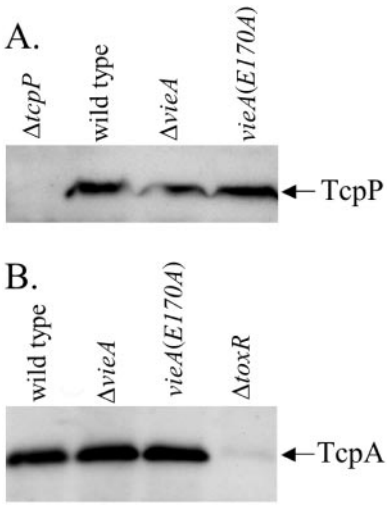

FIG. 6. TcpP and TcpA protein levels are not affected by *vieA* mutations. A. TcpP was detected by immunoblotting for strains AC-V61 (wild type), AC-V1386 (Δ*vieA*), AC-V1596 [*vieA*(*E170A*)], and AC-V449 (Δ*tcpP*) grown to an OD_{600} of 0.6 in M9 + NRES at 30°C. B. TcpA was detected by immunoblotting for strains AC-V61 (wild type), AC-V1386 (Δ*vieA*), AC-V1596 [*vieA*(*E170A*)], and AC-V744 (Δ*toxR*) grown to an OD_{600} of 0.6 in M9 + NRES at 30°C.

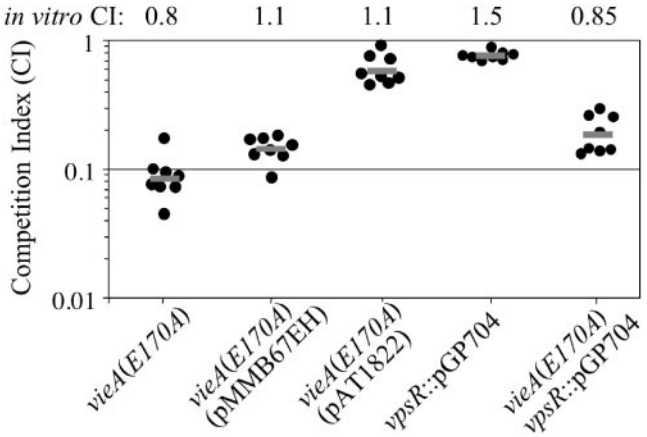

FIG. 7. The *vieA*(*E170A*) mutant is attenuated for colonization of the infant mouse. Competition assays were performed with the infant mouse model of cholera. Strains AC-V1596 [*vieA*(*E170A*)], AC-V1869 (*vpsR*::pGP704), and AC-V1870 [*vieA*(*E170A*), *vpsR*::pGP704] were competed against strain O395. Strains AC-V1815 and AC-V1824 [*vieA*(*E170A*) containing the pMMB67EH or pAT1822 complementing plasmid, respectively] were competed against strain AC-V1109 (O395 carrying pMMB67EH). The competition index is the ratio of mutant to wild-type bacteria recovered from the small intestine, corrected for the ratio present in the inoculum. Each dot represents an individual mouse; the gray line represents the geometric mean of the data. The competition indices from in vitro competitions in LB are indicated above the graph.

expression is affected by an increased c-diGMP concentration at either the level of translation or protein stability, immunoblots were performed on *vieA* mutant strains grown in M9 + NRES. We consistently observed similar levels of TcpP protein in the wild-type and *vieA* mutant strains (Fig. 6A), suggesting that translation and stability of TcpP are unaffected by c-diGMP.

c-diGMP does not affect TcpA production. ToxT is a master regulator of virulence gene expression, activating not only transcription of the *ctxAB* genes but also that of many other genes, including those required for synthesizing the TCP (2). To test whether other genes regulated by ToxT are also affected by the c-diGMP concentration, expression of TcpA, the major pilin subunit, was assessed by immunoblotting. Contrary to our expectations, TcpA production was not affected by either mutation in *vieA* (Fig. 6B).

Control of c-diGMP concentration by VieA is required for intestinal colonization. Because control of the c-diGMP concentration by VieA is required in the *V. cholerae* classical biotype for in vitro expression of *ctxA* and *toxT* but not for *tcpA* expression, it was unclear whether this regulation would affect virulence. To address this question, competition assays were performed in the infant mouse model of cholera. In contrast to the El Tor biotype, in which the VieSAB system is not required for colonization (33), the classical biotype requires the function of the VieA response regulator for full colonization. Specifically, the c-diGMP phosphodiesterase activity of VieA is necessary, since the *vieA*(*E170A*) mutant exhibited a 10-fold decrease in colonization fitness (Fig. 7).

To confirm that the colonization defect of the *vieA*(*E170A*) point mutant is due to the *vieA*(*E170A*) mutation and not a secondary mutation, the mutant was complemented with plasmid pAT1822, which encodes *vieA* on the low-copy-number vector pMMB67EH. While the pMMB67EH vector did not affect the competition index, the pAT1822 complementing plasmid restored the *vieA*(*E170A*) mutant to near wild-type levels of colonization (Fig. 7). We suspect that the competition index of the complemented strain is not restored to a value of 1 because the complementing plasmid is unstable. Although pAT1822 was present in 100% of colony isolates from the inoculum, it had been lost by an average of 32% of the isolates after growth within the animal, as determined by replica plating to medium containing Ap. In contrast, the pMMB67EH vector control was lost by less that 1% of the wild-type control strain after passage through the animal. This analysis implies that *vieA* expression from pAT1822 is slightly deleterious to strain fitness during infection, perhaps as a result of overexpression or incorrect temporal regulation.

Since we have previously implicated the c-diGMP phosphodiesterase activity of VieA in regulation of the *vps* genes that are required for exopolysaccharide synthesis and biofilm formation (51), we hypothesized that overexpression of the *vps* genes in the *vieA*(*E170A*) mutant might be responsible for the colonization defect. To test this possibility, a plasmid insertion mutation was constructed in *vpsR*, which encodes a positive regulator that is necessary for *vps* gene expression (56). This mutation abrogates *vps* gene expression as assessed by measuring β-galactosidase activity of a *vps-lacZ* fusion (data not shown). In addition, the *vpsR*::pGP704 mutation in an otherwise wild-type background does not affect colonization of the infant mouse small intestine in a competition assay (Fig. 7). The *vieA*(*E170A*) *vpsR*::pGP704 double mutant still exhibits a fivefold defect in colonization fitness (Fig. 7), suggesting that misregulation of *vps* gene expression cannot account for the colonization defect of the *vieA*(*E170A*) mutant. Taken together, the above results suggest that c-diGMP plays a role in

modulating in vivo expression of virulence factors that mediate colonization.

DISCUSSION

Virulence gene expression in *V. cholerae* is regulated by a complex cascade of transcriptional activators. Here we show that in addition to the known regulatory factors, the cyclic nucleotide second messenger c-diGMP negatively regulates transcription of the *toxT* and *ctxA* genes under conditions that stimulate virulence factor expression. Specifically, the putative phosphodiesterase activity of the VieA EAL domain is required for maximal expression of *ctxA* and *toxT* during growth under a minimal medium plus amino acids condition. The ability of VieA to control the c-diGMP concentration is also critical during infection, since the *vieA*(*E170A*) EAL domain point mutant was attenuated 10-fold in the infant mouse model of cholera. This represents the first demonstration with any organism that the c-diGMP second messenger regulates expression of virulence factors.

Our results implicate c-diGMP as a signal that represses expression of virulence factors and suggest possible mechanisms of transcriptional regulation by this second messenger. Since an increased concentration of c-diGMP repressed transcription of *toxT*, regulators of *toxT* may be directly affected by c-diGMP. We ruled out the possibility that ToxR is responsive to the c-diGMP concentration by examining expression of genes encoding the outer membrane proteins OmpU and OmpT, which are regulated directly by ToxR (7, 35). Transcription of *ompU* and *ompT* was only modestly affected by mutations in VieA that perturb the c-diGMP concentration. Additionally, levels of both the *ompU* and *ompT* transcripts were reduced in the *vieA* mutants, inconsistent with the inverse regulation of these genes by ToxR. ToxR cooperates with TcpP to activate transcription at the *toxT* promoter (31). We demonstrate that both *tcpP* transcript and TcpP protein levels are unchanged in response to the c-diGMP concentration. It is possible, however, that TcpP protein activity is affected by c-diGMP, resulting in reduced *toxT* transcription.

Alternatively, the activity of ToxT may be affected by the c-diGMP concentration. ToxT is a member of the AraC family of transcriptional regulators (18); like other members of this family, ToxT has an N-terminal domain that may function as a binding site for a small molecule effector such as c-diGMP. Indeed, it has been suggested that ToxT responds directly to environmental stimuli including temperature and bile (47). ToxT positively regulates its own expression by activating transcription from the *tcpA* promoter, which lies approximately 10.6 kb 5′ of *toxT*; the resultant read-through transcription is thought to contribute to sustained ToxT expression (57). Since we cannot distinguish between transcripts initiating at the *toxT* and *tcpA* promoters using the *toxT-lacZ* fusion, it is possible that the differences in fusion activity that we observe are due to changes in activation of the *tcpA* promoter by ToxT. However, it is unlikely that transcription from the *tcpA* promoter is negatively regulated in a specific manner by c-diGMP, since we observed no change in the production of TcpA in *vieA* mutant strains compared to that for the wild type.

A final hypothesis to explain virulence gene regulation by c-diGMP is that an as yet unidentified regulatory factor is responsible for sensing the intracellular concentration of c-diGMP and controlling virulence gene expression. This hypothesis is attractive given the unexpected differences we observed in the expression of *ctxA* and *tcpA*. Perhaps a repressor that acts specifically at the *toxT* and *ctxA* promoters controls transcription in response to c-diGMP.

Since we have shown that transcription of *toxT* is altered in *vieA* mutant strains that exhibit increased levels of c-diGMP, it is surprising that TcpA production was unaffected in these mutants. Although production of CT and TCP is generally coordinately regulated, since both the *ctxA* and *tcpA* promoters are activated by ToxT, differences in the regulation of these two promoters have previously been reported. For example, both *ctxA* and *tcpA* are repressed by H-NS, a histone-like nucleoid structuring protein, but repression is less stringent at the *tcpA* promoter (41). ToxT is thought to act, in part, by alleviating H-NS-mediated repression (58). Perhaps the concentration of the ToxT protein in strains with an increased concentration of c-diGMP is sufficient to derepress the *tcpA* promoter but not the *ctxA* promoter. In addition, differences in the timing of *ctxA* and *tcpA* transcription have been observed during infection (32). Specifically, while *tcpA* expression is induced within 1 h postinoculation, *ctxA* transcription is not induced until 4 h postinoculation. Perhaps changes in the concentration of ToxT within the cell account for these differences in the timing of transcriptional induction. Alternatively, it is possible that additional factors, including those that control and/or respond to the c-diGMP concentration, contribute to repression of *ctxA* early in the infection process.

In addition to regulating virulence gene expression under in vitro inducing conditions, control of the c-diGMP concentration by VieA is needed for wild-type levels of colonization of the host small intestine. In competition assays with the infant mouse model of cholera, we demonstrate that a VieA mutant that lacks c-diGMP phosphodiesterase activity is reduced 10-fold in colonization fitness. We were able to complement this defect in *trans*, confirming that it is the mutation in *vieA* that is responsible for the defect, rather than a secondary mutation. The reduced colonization fitness of the *vieA*(*E170A*) mutant was somewhat surprising, however, given that we observed no change in expression of the major colonization factor TcpA under in vitro growth conditions that induce virulence gene expression. In addition to affecting expression of the virulence factors *toxT* and *ctxAB*, mutations in *vieA* that increase the concentration of c-diGMP also result in increased expression of the *Vibrio* exopolysaccharide (*vps*) genes that are required for biofilm formation (51). To test whether misregulation of the *vps* genes is responsible for the colonization defect of the *vieA*(*E170A*) mutant, we constructed a mutation in *vpsR*, a positive regulator of *vps* gene expression. This mutation did not affect the ability of the *vieA*(*E170A*) mutant to colonize the small intestine of the infant mouse, suggesting that misregulation of the *vps* genes is not responsible for the colonization defect. It is possible that although mutations in *vieA* do not affect expression of TcpA in vitro, the timing of transcriptional activation or the assembly of TcpA subunits into a functional pilus may be altered in vivo. Finally, it is possible that VieA and the c-diGMP second messenger regulate expression of other factor(s) required for colonization.

In contrast to the *Vibrio* exopolysaccharide (*vps*) genes,

which we have previously demonstrated are activated by an increased intracellular concentration of c-diGMP (51), virulence genes are negatively regulated by c-diGMP. This inverse regulation of environmental and virulence genes by c-diGMP is intriguing. It suggests that the c-diGMP second messenger may play an important role in the transition between growth in the environment and survival within the host small intestine. Our data show that the *V. cholerae* classical biotype requires regulation of the c-diGMP concentration by VieA to achieve wild-type levels of colonization in the infant mouse model. Although the VieSAB three-component system is not necessary for colonization by El Tor *V. cholerae* (33), it is possible that proteins with redundant function can fulfill this role of VieA in the El Tor biotype. The *V. cholerae* genome encodes 22 proteins that are homologous to putative c-diGMP phosphodiesterases, and each of these may contribute to reducing the intracellular c-diGMP concentration upon entry into the host. In fact, the VC0130 gene, which encodes a putative c-diGMP phosphodiesterase, was recently identified in a screen for in vivo-induced genes in the El Tor biotype (42). Perhaps the VC0130 protein is required for regulation of the c-diGMP concentration to activate virulence gene expression in El Tor *V. cholerae*.

Although this is the first report that virulence genes are transcriptionally regulated in response to the c-diGMP concentration, it is likely that c-diGMP regulates virulence phenotypes in other bacterial species. Coding sequences for the conserved GGDEF and EAL protein domains that are responsible for synthesis and degradation of c-diGMP are present in the genomes of the majority of pathogens with completed sequences. There is preliminary evidence that proteins with these domains control virulence-related processes in *P. aeruginosa* (12) and *Y. pestis* (19, 25), but whether these proteins control the c-diGMP concentration remains to be determined. It is possible that regulation of virulence by c-diGMP will become a common theme in pathogenic bacterial species.

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ADDENDUM IN PROOF

After acceptance of our paper, a paper (**K. B. Hirst, M. MacCross, M. U. Shiloh, K. H Darwin, S. Singh, R. A. Jones, S. Ehrt, Z. Zhang, B. L. Gaffney, S. Gandotra, D. W. Holden, D. Murray, and C. Nathan**, Mol. Microbiol **56:**1234–1245, 2005) demonstrating that the *Salmonella* EAL domain protein CdgR is required for resistance to oxidative stress both in vitro and during growth in host pathogens was published. Mutation of *cdgR* also enhanced killing of macrophages by *Salmonella*. CdgR was required to lower the cellular level of cyclic diguanylate, suggesting that cyclic diguanylate also regulates virulence properties in *Salmonella*.

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