Expression of the \textit{cpdA} Gene, Encoding a 3',5'-Cyclic AMP (cAMP) Phosphodiesterase, Is Positively Regulated by the cAMP-cAMP Receptor Protein Complex$^{\dagger}$

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The pathogenic bacterium \textit{Vibrio vulnificus} is a normal inhabitant of marine estuary environments and infects humans via ingestion of seafood or contact with seawater. Thus, the life cycle of this pathogen involves periods of stress when environmental parameters are fluctuated (25). \textit{V. vulnificus} must survive such conditions and be able to proliferate with high metabolic activity when the proper host conditions are encountered to cause fatal septicemia or gastroenteritis (32). This bacterium is therefore expected to be able to sense the fluctuations in the surrounding environment and to respond to conditions in its physicochemically distinct niches.\par

Cyclic 3',5'-AMP (cAMP) is a signaling molecule that mediates a variety of cellular processes, is finely modulated by the regulation of its synthesis, excretion, and degradation. In this study, cAMP phosphodiesterase (CpdA), an enzyme that catalyzes the conversion of cAMP to AMP, was characterized in a pathogenic bacterium, \textit{Vibrio vulnificus}. The \textit{cpdA} gene exists in an operon composed of \textit{mutT}, \textit{yqiB}, \textit{cpdA}, and \textit{yqiA}, the transcription of which was initiated at position −22 upstream of \textit{mutT}. A \textit{cpdA}-null mutant of \textit{V. vulnificus} contained significantly higher levels of cAMP than the wild type but showed no detectable cAMP when a multicopy plasmid of the \textit{cpdA} gene was provided in \textit{trans}, suggesting that CpdA is responsible for cAMP degradation. Cellular contents of the CpdA protein decreased dramatically in both \textit{cyA} and \textit{crp} mutants. In addition, levels of expression of the \textit{cpdA:luxAB} transcription fusion decreased in \textit{cyA} and \textit{crp} mutants. The level of expression of \textit{cpdA:luxAB} in the \textit{cyA} mutant increased in a concentration-dependent manner upon the exogenous addition of cAMP. The cAMP-CAMP receptor protein (CRP) complex bound directly to the upstream region of \textit{mutT}, which includes a putative CRP-binding sequence centered at position −95.5 relative to the transcription start site. Site-directed mutagenesis or the deletion of this sequence in the \textit{cpdA} transcription fusion resulted in the loss of regulation by cAMP and CRP. Thus, this study demonstrates that CpdA plays a crucial role in determining the intracellular cAMP level and shows for the first time that the expression of \textit{cpdA} is activated by the cAMP-CRP complex via direct binding to the regulatory region.

The pathogenic bacterium \textit{Vibrio vulnificus} is a normal inhabitant of marine estuary environments and infects humans via ingestion of seafood or contact with seawater. Thus, the life cycle of this pathogen involves periods of stress when environmental parameters are fluctuated (25). \textit{V. vulnificus} must survive such conditions and be able to proliferate with high metabolic activity when the proper host conditions are encountered to cause fatal septicemia or gastroenteritis (32). This bacterium is therefore expected to be able to sense the fluctuations in the surrounding environment and to respond to conditions in its physicochemically distinct niches.\par

Cyclic 3',5'-AMP (cAMP) is a cellular signaling metabolite that is involved in relieving the carbon catabolite repression of many genes and operons that encode diverse catabolic enzymes (10). It also mediates multiple global regulatory networks by controlling the expression of major transcription factors in a variety of microorganisms (4, 11). For example, bacterial global regulators such as sigma factor S (RpoS) or ferric uptake regulator ( Fur) have been shown to be regulated by cAMP complexed with another global regulator, CAMP receptor protein (CRP), which is itself regulated by cAMP (9, 13, 20). Recently, the cAMP-CRP complex has been reported to be involved in the regulation of numerous virulence factors in pathogenic bacteria. The production of the potent virulence factors of pathogenic \textit{Vibrio} species, cholera toxin (\textit{ctxAB}) and toxin-coregulated pilus (\textit{tcpPH}) in \textit{Vibrio cholerae} (31) and metalloprotease (\textit{vvpE}) and cytolytic hemolysin (\textit{vvhBA}) in \textit{V. vulnificus} (2, 8, 14), has been shown to be regulated by the cAMP-CRP complex. Thus, it has been suggested that cAMP is one of the key molecules for the timely expression of virulence factors in pathogenic bacteria (10).\par

Microorganisms are required to modulate cAMP levels to mediate various cellular processes in response to physiological demands (19). The final intracellular concentration of cAMP is determined by a fine-tuned regulatory system that includes its synthesis by adenylate cyclase, its extracellular excretion, and its cleavage into 5'-AMP by 3',5'-cAMP phosphodiesterase (5, 28). Although we have extensive knowledge about the synthesis and excretion of cAMP (5), the enzymatic hydrolysis of cAMP and the genes encoding these enzymes have been studied in only a limited number of bacterial species. The genes encoding the cytoplasmic 3',5'-cAMP phosphodiesterase (CpdA) have been isolated and characterized for only two bacterial species so far, \textit{Escherichia coli} (12) and \textit{Haemophilus influenzae} (23). Studies of some bacterial species, including \textit{E. coli} (12), \textit{Salmonella enterica} serovar Typhimurium (4), \textit{Bradyrhizobium japonicum} (7), and \textit{Mycococcus xanthus} (18), indicate that the CpdA proteins are involved in decreasing intracellular cAMP levels. The regulation of \textit{cpdA} expression, however, has not yet been studied.\par

We have isolated the \textit{cpdA} gene from the genomic library of
TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td>Strains</td>
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<tr>
<td><em>V. vulnificus</em></td>
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<tr>
<td>ATCC 29307</td>
<td>Clinical isolate; virulent</td>
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<tr>
<td>AR</td>
<td>ATCC 29307 but spontaneous rifampin resistance</td>
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<td>AR but ΔcpdA</td>
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<td>KP301</td>
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<td>DH5α</td>
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<td>Laboratory collection</td>
</tr>
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<td>SM10Δpir</td>
<td>thi-1 thr leu tonA lacY supE recA1:Rps-2:Te; Mupir; OrfT of RPs; Km'</td>
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</tr>
<tr>
<td>JM109</td>
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<td>Plasmids</td>
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<td>pUC19</td>
<td>Cloning vector; lacZ, Ap'</td>
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<td>pINE45</td>
<td>pUC19 with 1.72-kb Sau3AI fragment of <em>V. vulnificus</em> DNA containing the <em>cpdA</em> gene</td>
<td>This study</td>
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<td>pLAFR5</td>
<td>IncP Te'; derivative of pLAFR3 containing double cos cassettes</td>
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<tr>
<td>pHS51</td>
<td>pLAFR5 containing EcoRI and HindIII fragment of pINE45</td>
<td>This study</td>
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<tr>
<td>pOE30</td>
<td>Expression vector; Ap'</td>
<td>Qiagen</td>
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<td>pOE30-cpdA</td>
<td>pOE30 with <em>V. vulnificus</em> cpdA</td>
<td>This study</td>
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<tr>
<td>pHK0011</td>
<td>Transcriptional fusion plasmid with promoterless lacZB; Te'</td>
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<td>pSMK-cpdA-1</td>
<td>pHK0011 with cpdA upstream region (positions −1521 to +41 relative to cpdA IC)</td>
<td>This study</td>
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<td>pSMK-cpdA2mt</td>
<td>pSMK-cpdA2 but mutated CRP-binding site</td>
<td>This study</td>
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</tbody>
</table>

V. vulnificus (GenBank accession numberAY221025) (20).

When the putative amino acid sequence of *V. vulnificus* CpdA was aligned with those deduced from the corresponding genes of *E. coli* (GenBank accession number BAB09396.1) and *H. influenzae* (GenBank accession number NP_438561.1), it showed significant identities of 51 and 45%, respectively (see Fig. S1 in the supplemental material). In the present study, we investigated the role of this gene product in determining intracellular cAMP levels and examined the regulation of *cpdA* gene expression in *V. vulnificus*.

**MATERIALS AND METHODS**

**Strains, plasmids, and culture cultivation.** The strains and plasmids used in this study are listed in Table 1. *E. coli* strains used for plasmid DNA preparation and conjugal transfer were grown at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics. *V. vulnificus* strains were grown in LB medium supplemented with an additional 1.5% (wt/vol) NaCl (LBS) at 30°C unless stated otherwise. All medium components were purchased from Difco, and chemicals and antibiotics were obtained from Sigma.

**Determinant of CAMP concentrations.** Wild-type and Δγv and ΔcpdA mutant *V. vulnificus* strains were grown in LBS. The ΔcpdA mutant carrying either a broad-host-range vector (pLAFR5) (16) or *cpdA*-containing pLAFR5 (pHS15) was grown in LBS supplemented with 3 μg/ml tetracycline. Exponential-phase (the optical density at 595 nm [OD595] ranged from 0.6 to 0.7) stationary-phase (the OD595 ranged from 3.3 to 4.0) cells were harvested and lysed, and the amount of CAMP in the lysates was estimated using the CAMP Biotrack enzyme immunoassay system according to the manufacturer’s instructions (Amersham). To determine concentrations of secreted CAMP, the same procedure was applied to cell-free spent medium, which had been filtered through a 0.22-μm-pore-size membrane.

Western blot analysis of CpdA. A pair of oligonucleotides, cpdAexp-F (5'—CCCGATTCCTGTGCAATACATACATCAGTGATACG-3') and cpdAexp-R (5'—GGGTCGGTGTGGTAG GACGCGCTCATAGCTAACC-3') [underlined sequence indicates a BamHI restriction site] and cpdAexp-R (5'—GGGTCGGTGGTAG GACGCGCTCATAGCTAACC-3') [underlined sequence indicates a PstI restriction site], were used to amplify an 840-bp DNA fragment containing the full sequence of the cpdA open reading frame (ORF) from the genomic DNA of *V. vulnificus*. Recombinant CpdA (rCpdA) was overexpressed in *E. coli* JM109 cells carrying pQE30-cpdA with His6-tagged CpdA and purified using a Ni-nitrilotriacetic acid affinity column as directed by the manufacturer (Qiagen). Purified rCpdA was used to generate polyclonal antibodies by three immunizations of Sprague-Dawley rats (200 μg of CpdA protein per immunization) at 3-week intervals. Cell lysates of wild-type and ΔcpdA, crp, and Δγv mutant *V. vulnificus* were prepared by sonication in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.05% [vol/vol] Tween 20 [pH 8.0]) (29). One hundred microliters of each bacterial lysate was fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a Hybond P membrane (Amersham). The membrane was incubated with polyclonal antibodies against rCpdA (1:10,000 dilution) and then incubated with alkaline phosphatase-conjugated rabbit anti-rat immunoglobulin G (1:5,000; Sigma). Immunoreactive protein bands were visualized using a nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) system (Promega). To investigate the effect of added CAMP on CpdA levels, CAMP was added to the Δγv mutant at a final concentration of 1.0 mM for 1.5 h before extracts were prepared.

Northern blot analysis. Total RNA was extracted from wild-type *V. vulnificus* ATCC 29307 cells using Trizol reagent (Gibco BRL) according to the manufacturer’s instructions and quantified by spectrophotometric readings at 260 nm. Thirty micrograms of RNA was fractionated by 1% formaldehyde agarose gel electrophoresis in a running buffer (0.1 M MOPS [morpholinepropanesulfonic acid], 40 mM sodium acetate, and 5 mM EDTA), blotted onto a Hybond N membrane (Amersham) by capillary transfer in 20× SSPE (3 mM NaCl, 0.2 M NaH2PO4, 0.02 M EDTA), and immobilized using a UV cross-linker (CL-1000; UVP). Blots were incubated for 2 h at 42°C in a prehybridization solution (5× SSPE, 50% formamide, 5× Denhardt’s solution, 0.5% SDS, 200 μg/ml salmon...
sperrn DNA, and 10% dextran sulfate). Hybridization at 42°C was continued overnight in the presence of a labeled cpdA probe. For the preparation of the probe, cpdA gene-containing plasmid pNE145 was digested with EcoRI and HindIII, and the 1.7-kb inserted DNA fragment was isolated using the Gene-Clean kit (Biol01) and labeled with [α-32P]ATP using a Random Primer kit (Takara). The membrane was washed twice with 2× SSPE-0.1% SDS at room temperature for 15 min and twice with 0.1× SSPE-0.5% SDS at 60°C for 30 min and then exposed to X-ray film (29).

**Primer extension analysis.** A primer, yqi-R (5'-TTCCATCATTGCATTCC ACC-3'), was designed to be complementary to positions −976 to −956 with respect to the initiation codon (IC) of cpdA. The primer was labeled at the 5′ terminus with [γ-32P]ATP using T4 polynucleotide kinase (Takara), and then incubated with 150 μg RNA in hybridization buffer (10 mM Tris-HCl, 1 mM EDTA, 1.25 M KCl [pH 8.0]), and then incubated at 65°C for 5 min. RNA was converted to cDNA with SuperScript II reverse transcriptase (Invitrogen). The resultant cDNA products were precipitated and resolved on a sequencing gel. Sequencing gels were dried and then visualized with a phosphorimager (Personal Molecular Image FX; Bio-Rad).

**Complementation of the cpdA gene.** An intact cpdA gene was isolated as EcoRI and HindIII from pNE145, a pUC19-based plasmid with a 1.72-kb Sau3AI fragment containing the whole upstream region of the cpdA2. Next, cpdA-siteF (5'-GTCCCGTAAAACAAAAAATGA/H11032) and cpdA-siteR (5'-GGGGTACCAAATGAATTGTTTAAACCTAAA-3'/H9262) was cloned into pGEM-11zf(-) and HindIII from pINE45, a pUC19-based plasmid with a 1.72-kb Sau3AI fragment containing the whole upstream region of the cpdA2 (see above) was cloned into pGEM-11zf(-) and included as nonspecific DNA in the binding assay.

**RESULTS**

**Genetic organization of the cpdA gene in *V. vulnificus.* **V. vulnificus* genomic library plasmids containing the cpdA gene, pNE145 and pNE45-1, were obtained from a screening experiment to isolate the factors for which gene products caused the change in roos gene expression (20). Sequencing of insert DNA in these plasmids revealed the flanking regions of the cpdA gene. The upstream region of the cpdA gene contains two ORFs homologous to mutT and yqiB of *E. coli* that are transcribed in the same direction as cpdA (Fig. 1A). Further upstream, an ORF homologous to the tolC gene of *E. coli* was found, which is transcribed in the opposite direction from mutT-yqiB-cpdA. An ORF downstream of cpdA is homologous to *E. coli* yqiA. Downstream in the opposite direction of the mutT-yqiB-cpdA-yqiA gene cluster, an ORF homologous to the topoisomerase gene is present.

**Identification of the cpdA promoter.** To define the promoter of the cpdA gene, two experiments were performed. Northern blotting using a cpdA gene probe showed the presence of a single band approximately 2.5 kb long (Fig. 1B). Because the cpdA gene seems to be organized as an operon composed of mutT, yqiB, cpdA, and yqiA, this suggested that the cpdA gene was transcribed with its flanking genes from a promoter upstream of the first gene, mutT. To test this hypothesis, two different cpdA::luxAB transcriptional reporter fusions were constructed: pSMK-cpdA-1 includes the whole upstream region of the cpdA gene to the 3′ end of the tolC gene, and the other, pSMK-cpdA2, includes only the intergenic space between tolC and mutT.

**Electrophoretic mobility shift assay.** The *V. vulnificus* cRP protein was overexpressed in *E. coli* BL21 carrying pKH001 (15), a pRSETA (Invitrogen)-based expression plasmid, and purified by Ni-nitrotriacetic acid affinity chromatography according to the manufacturer’s instructions (Qiagen). The 373-bp upstream region of the mutT gene was PCR amplified using SMK-F1K and SMK-R2X (5'-GGCTCTAGAATCCGAGCATTGCTC-3') and labeled with [α-32P]ATP using T4 polynucleotide kinase. The labeled DNA fragment (225 nM) was incubated with various concentrations of purified cRP protein (50 to 200 nM) for 30 min at 37°C in a 20-μl reaction mixture containing 1× binding buffer (26) with 500 μM cAMP (Sigma). Following the addition of 3 μl of loading buffer, the samples were separated on a 6% nondenaturing polyacrylamide gel. For competition analyses, the identical but unlabelled sequence was included as a competitor. The competitor DNA from 22.5 to 675 nM was added to reaction mixtures containing 225 nM of labeled DNA prior to the addition of 200 nM CPR.

A 378-bp DNA fragment encompassing the promoter of the copA gene, encoding glyceraldehyde-3-phosphate dehydrogenase, was amplified from *V. vulnificus* genomic DNA using primers gap-F (5'-CATTAATCTGATATCATGTCCG-3') and gap-R (5'-AATCAGTGGATACAGCGG-3') and included as nonspecific DNA in the binding assay.

In conclusion, the cpdA promoter was defined as the sequences upstream of the *V. vulnificus* yqiA gene. Further studies are required to define the regulatory factors involved in the expression of the genes encoding the *V. vulnificus* cpdA gene.

**Acknowledgments**

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (no. 12045056) from the Ministry of Education, Science, Sports, and Culture of Japan and a Grant-in-Aid for Scientific Research (C) (no. 12680394) from the Ministry of Education, Science, Sports, and Culture.
FIG. 1. Genetic organization and transcription of the mutT-yqiB-
cpdA-yqiA operon in V. vulnificus. (A) Based upon the genetic orga-
nization of the cpdA gene, two different cpdA::luxAB transcriptional
fusions were constructed: one (pSMK-cpdA1) includes the upstream
region of mutT, the first gene of the tentative operon, and the other
(pSMK-cpdA-1) includes the entire upstream region of cpdA. (B) For
Northern blot analysis of cpdA mRNA, total RNA extracted from
stationary-phase V. vulnificus cells was hybridized with a 32P-labeled
cpdA probe and visualized by autoradiography. The left lane (lane 1)
is ethidium bromide-stained total RNA from wild-type V. vulnificus 
in a formamide agarose gel, and the right lane (lane 2) is a Northern
hybridization using the cpdA probe. Arrows and numbers on the left
side indicate the molecular sizes of 23S and 16S RNAs in kilobases
(kb). (C) Wild-type V. vulnificus carrying each fusion or the vector only
(pKHo011) was grown in LBS medium supplemented with 3 µg/ml
tetracycline, aliquots were sampled, and their cell masses (OD 595) and
intracellular cAMP levels (Table 2) were determined. Spent medium sampled from the
wild-type strain and a ΔcyA mutant deficient in the cAMP-
synthesizing enzyme adenylate cyclase. The intracellular concentrations of cAMP in wild-type cells grown in LBS medium at exponential and stationary phases were 26 and 6.3 pmol/mg protein, respectively (Table 2). The ΔcyA mutant had nonde-
tectable levels of cAMP in both growth phases. In contrast, the
ΔcpdA mutant showed highly elevated levels of cellular cAMP, estimated at 99 to ~137 and 37 to ~45 pmol/mg protein from exponential- and stationary-phase cells, respectively. When the intact cpdA gene was introduced into the ΔcpdA mutant on the multicopy plasmid pHS51, its intracellular cAMP level decreased to below the detection limit.

Similarly, the level of extracellular cAMP in cell-free spent medium from wild-type and ΔcpdA mutant cultures was also determined. Spent medium sampled from the ΔcpdA mutant culture showed a 1.7-fold-higher concentration of cAMP than that of the wild type (S.-M. Kim and K.-H. Lee, unpublished data). This implied that the higher intracellular cAMP level in the ΔcpdA mutant was not from a reduced excretion of cAMP but possibly from an absence of CAMP phosphodiesterase ac-
tivity. Therefore, this suggests that CpdA controls the level of 
intracellular cAMP through degradation.

Contents of CpdA in wild-type, ΔcyA mutant, and crp mutant
V. vulnificus strains. Since V. vulnificus CpdA appears to de-

sensus sequence (5'-AAATGTGATCTAGATCACATTT-3') (6) was discernible at position –95.5 relative to the transcriptional 
start site.

Role of CpdA in modulating cellular cAMP levels. To investi-
gate the function of CpdA, the level of cellular cAMP of a V. vulnificus ΔcpdA mutant (20) was compared to that of the

wild-type strain and a ΔcyA mutant deficient in the cAMP-
synthesizing enzyme adenylate cyclase. The intracellular concentrations of cAMP in wild-type cells grown in LBS medium at exponential and stationary phases were 26 and 6.3 pmol/mg protein, respectively (Table 2). The ΔcyA mutant had nonde-
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Contents of CpdA in wild-type, ΔcyA mutant, and crp mutant
V. vulnificus strains. Since V. vulnificus CpdA appears to de-
grade cellular cAMP via a cAMP phosphodiesterase activity, as is seen in other bacteria (12, 23), the effect of the substrate cAMP on the level of the CpdA protein was examined. Western blotting using polyclonal antibodies raised against the recombinant CpdA and then incubated with alkaline phosphatase-conjugated rabbit anti-rat immunoglobulin G. Upon incubation with the nitroblue tetrazolium-BCIP system, the CpdA protein appeared as an immunoreactive band as indicated by an arrow. (B) The intensities of bands corresponding to CpdA were estimated by densitometry, and the densitometric readings are presented in the plot as values relative to those of CpdA of the wild-type strain.

Effect of cAMP and CRP on cpaA gene expression. Because the upstream region of the mutT-yqiB-cpdA-yqiA operon includes a putative CRP-binding site (Fig. 2B) and the Δcya and crp mutants contained less CpdA protein than did the wild type (Fig. 3), the effect of cAMP on cpaA gene expression was further confirmed using a transcriptional reporter fusion. The cpaA::luxAB transcriptional fusion pSMK-cpdA2 showed reduced levels of expression in the Δcya or crp mutant, which were about three- to fourfold lower than the levels of expression in the wild type (Fig. 4). The degree of the decrease in the level of transcription fusion expression in the Δcya or crp mutant was similar to the extent of the decrease in the level of the CpdA protein in the same mutants as shown in Fig. 3.

To investigate whether the lowered level of expression of pSMK-cpdA2 in the Δcya mutant was caused by the absence of cAMP, various concentrations of cAMP were added, and the level of expression of pSMK-cpdA2 was measured after 1.5 h (Fig. 5A). The level of expression increased in a dose-dependent manner, with maximal expression observed in the Δcya mutant incubated with ≥0.5 mM cAMP. In a subsequent experiment, cAMP was exogenously added to the Δcya mutant carrying pSMK-cpdA2. In the presence of 1.0 mM CAMP in the medium, the mutant expressed the fusion at increasingly higher rates than the control in a time-dependent manner, peaking at 2 h after the addition of cAMP (Fig. 5B). Similarly, 1.0 mM CAMP was exogenously added to either the wild type or the Δcya mutant, and their cellular levels of CpdA protein were compared. A Western blot using polyclonal antibodies against CpdA showed that the wild type was not influenced by the exogenous addition of cAMP, but the Δcya mutant showed higher levels in the presence of added CAMP (Fig. 5C). All of the above-described results suggest that the cellular amount of CpdA is regulated at the transcriptional level via cAMP-CRP complex-mediated activation.

Direct interaction of the cAMP-CRP complex with the regulatory region of cpaA. The above-described results clearly indicate that both CAMP and CRP positively affect cpaA expression. To determine whether the cAMP-CRP complex acts by binding to the regulatory region of the mutT-yqiB-cpdA-yqiA operon, an electrophoretic mobility shift assay was performed using the V. vulnificus CRP protein and a 373-bp DNA encompassing the region used for the construction of pSMK-cpdA2. As shown in Fig. 6, the addition of CRP and CAMP resulted in a slower mobility of the DNA fragment in a dose-dependent manner. The binding of CAMP-CRP to the DNA was specific, because excess unlabeled DNA abolished the formation of the slower-moving band, although retarded mobility was retained.

### Table 2

<table>
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<tr>
<th>Strain</th>
<th>CpdA Level (wt)</th>
<th>CpdA Level (Δcya)</th>
<th>CpdA Level (crp)</th>
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### Figure 3

#### A

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#### B

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<tbody>
<tr>
<td>wt</td>
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</tr>
<tr>
<td>Δcya</td>
<td>0.3</td>
</tr>
<tr>
<td>crp</td>
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</table>

### Figure 4

**Comparison of cya and crp mutants in CpaA expression.** Wild-type (wt), crp, and Δcya V. vulnificus strains carrying pSMK-cpdA2 were grown to stationary phase in LBS medium supplemented with 3 μg/ml tetracycline, and aliquots were sampled and measured for cell mass (OD<sub>595</sub>) and bioluminescence (RLU). Luciferase activities are expressed as normalized values by dividing the RLU by the OD<sub>595</sub> of each sample. The activities of two independent experiments were averaged and are shown with their standard deviations. An asterisk indicates P values less than 0.005.

#### Figure 5

**Effect of cAMP and CRP on CpaA expression.** (A) Lysates of V. vulnificus strains grown to stationary phase (OD<sub>595</sub> of 1.5) were used to examine CpdA levels by Western blotting. One hundred micrograms of each bacterial lysate was fractionated by SDS-polyacrylamide gel electrophoresis. The blotted membrane was incubated with polyclonal antibodies raised against the recombinant CpdA and then incubated with alkaline phosphatase-conjugated rabbit anti-rat immunoglobulin G. Upon incubation with the nitroblue tetrazolium-BCIP system, the CpdA protein appeared as an immunoreactive band as indicated by an arrow. (B) The intensities of bands corresponding to CpdA were estimated by densitometry, and the densitometric readings are presented in the plot as values relative to those of CpdA of the wild-type strain.
in the presence of an unrelated sequence, namely, the \textit{V. vulnificus} gap promoter.

Site-directed mutagenesis of the CRP-binding site at the regulatory region of \textit{cpdA}. The sequence of the upstream region of the \textit{mutT-yqiB-cpdA-yqiA} operon was analyzed for the cAMP-CRP-binding sequence. A region with considerable homology to the \textit{E. coli} CRP-binding site (AAATGTGATCTAGATCACA TTT; underlining indicates highly conserved residues) was found in the upstream region centered at position 11002 relative to the transcription start site (Fig. 3B). To determine whether the putative binding site plays a role in \textit{cpdA} transcription, the site was modified by either site-directed mutagenesis or the deletion of the first 11 out of 22 nucleotides of the putative CRP-binding site (Fig. 7A and B). The altered DNAs were used to construct the \textit{luxAB} transcriptional fusions. pSMK-cpdA2mt is the same as pSMK-cpdA2 but includes the mutated CRP-binding site. pSMK-cpdA3 is missing 15 nucleotides at the 5' end of the pSMK-cpdA2 insert.

These mutant fusions showed similar basal levels of expression in the wild-type, \textit{crp}, and \Delta\textit{cyA} strains (Fig. 7C and D) compared to the level of pSMK-cpdA2 expression in the \textit{crp} and \Delta\textit{cyA} strains. In addition, to verify if cAMP-CRP binding to this putative site occurs, the DNA fragment used in Fig. 6 and the same DNA but with the mutagenized putative CRP-binding site were used for electrophoretic mobility shift assays. No binding of cAMP-CRP to the mutagenized probe was ob-
FIG. 7. Effect of mutating the putative CRP binding site on cpdA expression. (A) A putative CRP-binding site, based upon the conserved nucleotide sequences for CRP binding in E. coli (6), was found in the upstream region of mutT centered at position -95.5 upstream of the transcription start site (designated with a +1) and indicated as an open box in the pSMK-cpdA2 fusion. The same transcription fusion with an altered putative CRP-binding site, pSMK-cpdA2mt, was constructed by site-directed mutagenesis to change the nucleotides as shown in B (hatched box). The upstream region of mutT in pSMK-cpdA3 has a deletion of the first 11 of the 22 nucleotides that comprise the putative CRP-binding site. Wild-type, crp, and Δcya strains carrying pSMK-cpdA2mt (C) or pSMK-cpdA3 (D) were grown in LBS medium supplemented with 3 μg/ml tetracycline, and aliquots were sampled to estimate specific luciferase activities. Luciferase activities are expressed as described in the legend of Fig. 4. (E) Assay of binding of the cAMP-CRP complex to the DNA fragments carrying the original sequence (wt probe) (the same DNA used in Fig. 6) or the mutagenized sequence (mt probe) (as indicated in B) was performed as described in the legend of Fig. 6. Lane 1, wild-type probe without CRP; lane 2, wild-type probe with 200 nM CRP; lane 3, mutagenized probe without CRP; lanes 4, 5, and 6, mutagenized probe with 20, 100, and 200 nM CRP, respectively. The arrow on the left side indicates the unbound DNA probe, whereas the arrow on the right side indicates the DNA bound with CRP.
served (Fig. 7E, lanes 3 to 6), while the wild-type probe was efficiently bound by the cAMP-CRP complex (Fig. 6 and 7E, lanes 1 to 2). These results suggest that cpdA expression is activated by cAMP-CRP acting on the region between positions −106 and −85 relative to its transcription start site.

**DISCUSSION**

The activity of 3',5'-cAMP phosphodiesterase has been found to play a role in optimizing cAMP concentrations in some bacteria, to induce the starvation response, to regulate catabolite-sensitive operons, or to protect against a high influx of cAMP (1, 4). Using the cpdA gene isolated from *V. vulnificus*, we investigated the role of CpdA with respect to modulating cAMP concentrations, which may subsequently result in an adjustment of bacterial responses to diverse stimuli and the control of virulence factor expression within host environments.

Bacterial cells grown in LB-based media without the sugars transported by phosphotransferase systems such as glucose did not exhibit maximal cAMP levels when they entered stationary phase (20, 22). cAMP levels in *V. vulnificus* (20) were estimated to be approximately 20 to −50 pmol cAMP/mg of protein in the exponential phase, decreasing to about 5 pmol cAMP/mg of protein in stationary phase (20) (Table 2). When *V. vulnificus* was deficient in cAMP phosphodiesterase (CpdA), it showed highly elevated levels of cAMP compared to those of an isogenic wild-type strain during both exponential and stationary phases (Table 2). The increased level of cAMP in the ΔcpdA mutant has also been confirmed by measuring gene expression, which is tightly regulated by cAMP and thus might serve as an index of the intracellular level of cAMP. For example, the rpoS gene, encoding *V. vulnificus* sigma factor S, is known to be repressed by cAMP (20). We found approximately twofold-lower levels of expression of the rpoS gene in the ΔcpdA mutant than in the wild type (20). When the ΔcpdA mutant was supplied with the intact cpdA gene, its cAMP level was too low to be detected, similar to the ΔcytA mutant that lacks adenylate cyclase activity (Table 2). Therefore, CpdA appears to be an important factor in controlling the intracellular concentration of cAMP in *V. vulnificus*.

The expression of many enzymes is induced by the presence of their substrate molecules (33). This regulatory pattern for catalytic enzymes prompted us to study the effect of cAMP on the regulation of cpdA expression. cAMP expression at the transcriptional level was activated by the cAMP-CRP complex (Fig. 3 and 4). The regulatory region for cpdA includes a sequence homologous to the *E. coli* CRP consensus sequence (Fig. 2B), and we found that this sequence was bound by the cAMP-CRP complex (Fig. 6 and 7). This site is from positions −106 to −85 (centered at position −95.5) with respect to its transcription start site, which is considered an activation site for class III CRP-dependent promoters such as the araBAD promoter (33). Class III promoters have been reported to require a secondary regulator protein (e.g., the AraC protein for the araBAD promoter) for maximal induction. Regulation at class III promoters was also reported to involve the formation of a DNA loop (21).

Exponential-phase cells growing in LBS medium contained higher cAMP contents (Table 2), possibly due to a lowered expression or reduced activity of CpdA during this growth phase. Thus, it is required to search and identify another regulator for cpdA transcription and its possible interaction with the cAMP-CRP complex for optimal cpdA expression in *V. vulnificus*, as shown in the araBAD promoter (33). It is also possible that the presence of other factors acting at the post-transcriptional level finely adjusts the CpdA protein content or its enzymatic activity.

Both the Northern blot and primer extension experiments clearly suggest that the cpdA gene is organized as an operon with its upstream flanking genes mutT and yqiB as well as its downstream gene yqiA (Fig. 1 and 2). MutT is the nucleoside triphosphatase pyrophosphohydrolase that catalyzes a conversion of deoxyribonucleoside triphosphate to deoxyribonucleoside monophosphate (3). The predicted gene products of yqiB and yqiA have homology to a hypothetical phosphohydrolase from *Vibrio parahaemolyticus* (GenBank accession no. ZP_01993039.1) and a hypothetical esterase from *Yersinia pestis* (GenBank accession no. NP_404305.1), respectively. Their functions, however, have not been determined. Thus, it is currently not known why the cpdA gene, which encodes an enzyme to catalyze the conversion of cAMP to AMP, is co-regulated and coexpressed with the other three genes, although the gene products from this operon might be involved in the metabolism of nucleotides to produce nucleoside monophosphates or to remove undesired nucleotides. Further study is needed to elucidate their biological significance.

Cyclic phosphodiesterases have been shown to be involved in the stress response of *M. xanthus* against temperature and osmotic shocks (18). The ΔcpdA mutant of *V. vulnificus* strain shows additional phenotypes. For example, scanning electron microscopy of wild-type and ΔcpdA mutant *V. vulnificus* strains revealed that the cpdA gene product is required for normal cellular morphology (see Fig. S2 in the supplemental material). The elongated morphology of the ΔcpdA mutant might result from altered expression of the genes involved in cell division and/or cell shape caused by a failure to adjust cAMP levels (4, 5). In addition, proteomic screening of *V. vulnificus* proteins required for mature biofilm formation found many proteins whose levels of expression are expected to be regulated by the cAMP-CRP complex (17). These results imply that deficiency in functional CpdA results in pleiotropic effects on the pathogenic bacterium *V. vulnificus*. According to data described previously by Merrell et al. (24), cpdA is one of the most important colonization factors in *V. cholerae*. Therefore, it will be interesting to investigate the roles of CpdA in the pathogenesis of *V. vulnificus*.

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