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CadC Activates pH-Dependent Expression of the *Vibrio vulnificus cadBA* Operon at a Distance through Direct Binding to an Upstream Region

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The *Vibrio vulnificus cadBA* genes were transcribed as a transcriptional operon by a single promoter, P_{cadBA}, which was activated by CadC in a pH-dependent manner. A direct interaction between CadC and the P_{cadBA} DNA was demonstrated, and a CadC binding site centered at −233.5 was mapped by deletion analyses of P_{cadBA} and confirmed by a DNase I protection assay.

Bacteria have developed elaborate protection systems to allow survival and/or growth during exposure to acidic environments (2, 6). Among such bacterial acid protection systems, acid pH neutralization mechanisms are based on the production of cytoplasmic amino acid decarboxylases (8, 12). Of the several amino acid decarboxylases known to be present in *Escherichia coli*, the *cadBA* genes encode a lysine/cadaverine antiporter and a lysine decarboxylase, whose combined activity leads to the synthesis and excretion of cadaverine to counteract external acidification (12, 13). Previous studies have noted that the lysine decarboxylase of *E. coli* is induced at an acidic pH (16, 24). Mutational analyses have proposed that the expression of the *E. coli cadBA* operon is regulated by CadC, an activator, and LysP, a repressor (17, 24).

The pathogenic marine bacterium *Vibrio vulnificus* occurs in raw seafood and has been identified as a causative agent of food-borne diseases (7, 11). We have recently cloned a 4.3-kb DNA fragment of *V. vulnificus* containing the *cadBA* genes (20). It was demonstrated that gene products of *cadBA* contribute to the acid tolerance of *V. vulnificus* and that their contribution is dependent on prior exposure of cells to a moderately acidic pH (20). Recently, an open reading frame, *cadC*, was also identified upstream of *cadBA*, and it has been proposed that CadC is essential for the survival of *V. vulnificus* upon exposure to an acidic pH and acts as a positive regulator for the expression of *cadBA* (21). However, the question of whether CadC directly or indirectly affects *cadBA* expression has not yet been addressed. Neither the promoter(s) of the *cadBA* genes activated by CadC nor the sequences upstream of the *cadBA* required for activation by CadC have been previously identified. Accordingly, here we extend our efforts to elucidate the regulation of the *cadBA* expression at a molecular level.

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The strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, *V. vulnificus* strains were grown in Luria-Bertani medium supplemented with 2.0% (wt/vol) NaCl (LBS). The lysine decarboxylase activity in the cells and cadaverine excretion in the supernatants were determined according to procedures previously described (19, 20). For the statistical analysis, Student's *t* test was used to evaluate the differences between the enzyme activities for the various strains (SAS software; SAS Institute Inc., Cary, NC). The averages and standard errors of the means (SEM) were calculated from at least three independent trials.

Transcription of *cadBA* as a single transcriptional unit. Total RNA was isolated from the cultures of the wild-type and JR202 strains grown to an optical density at 600 nm (OD_{600}) of 0.8 with LBS buffered to pH 5.8 and used for Northern blot analyses (22). The DNA probes CADBP and CADAP were prepared by labeling DNA fragments containing the coding region of either *cadB* or *cadA* (Fig. 1A), respectively, with [α-^{32}P]dCTP and used for the hybridizations (4, 10).

When hybridized with the CADBP DNA probe, only a single transcript, approximately 3.7 kb, was detected in the RNA of the wild-type strain (Fig. 1B). Based on the DNA sequence of *cadBA*, it was anticipated that the *cadB* mRNA would be approximately 1.3 kb in length. The cotranscription of *cadB* and *cadA* was predicted to produce a 3.7-kb transcript. CADAP also hybridized to 3.7-kb RNA (Fig. 1B), thereby demonstrating that the *cadBA* genes are transcribed as a transcriptional operon rather than as two independent genes. Strain JR202 is a null mutant in which the chromosomal *cadB* is replaced with *cadB*:*nptI* as previously described (20). Neither the *cadA* transcript nor the *cadB* transcript was apparent in the RNA prepared from JR202 (Fig. 1B). This lack of the *cadA* transcript in JR202 indicates that the insertional mutation of *cadB* has a polar effect on *cadA*, thus supporting the present assumption that *cadBA* is one transcriptional unit.

Effect of a *cadC* mutation on lysine decarboxylase activity and cadaverine excretion. To inactivate *cadC* in vitro, 1.2-kb *nptI* DNA conferring resistance to kanamycin (18) was inserted into a unique SalI site present within the *cadC* open reading frame to result in pJR0011 (Table 1). The *cadC* mutants

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
V. vulnificus strains		
ATCC 29307	Clinical isolate; virulent	Laboratory collection
*Vv*Δ*lacZ*	ATCC29307 with Δ*lacZ*; Sm^r	1
JR201	ATCC29307 with *cadC*::*nptI*; Km^r	This study
JR202	ATCC29307 with *cadB*::*nptI*; Km^r	20
JR243	*Vv*Δ*lacZ* with *cadC*::*nptI*; Km^r Sm^r	This study
Plasmids		
pRKΩ*lacZ*	pRK415 with *lacZ*; Tc^r	1
pJR990	Cosmid containing *cadCBA*	20
pJR991	4.3-kb EcoRI fragment containing *cadB*, part of *cadC*, part of *cadA* in pGEM7zf(+); Ap^r	20
pJR0011	pCVD442 with *cadC*::*nptI*; Ap^r Km^r	This study
pJR0012	pRK415 with *cadC*; Tc^r	This study
pJR0515	pRKΩ*lacZ* with 623-bp fragment of *cadBA* upstream region; Tc^r	This study
pJR0516	pRKΩ*lacZ* with 605-bp fragment of *cadBA* upstream region; Tc^r	This study
pJR0517	pRKΩ*lacZ* with 555-bp fragment of *cadBA* upstream region; Tc^r	This study
pJR0518	pRKΩ*lacZ* with 505-bp fragment of *cadBA* upstream region; Tc^r	This study
pJR0519	pRKΩ*lacZ* with 305-bp fragment of *cadBA* upstream region; Tc^r	This study

(Table 1) were constructed by allelic exchanges and double crossovers, in which each wild-type *cadC* gene on the chromosome was replaced with the *cadC:nptI* allele on pJR0011 using previously described methods (4, 10).

For the wild-type strain grown at pH 5.8, lysine decarboxylase was produced and reached a maximum of 2.0 units (Fig. 2A). The disruption of *cadC* in the *cadC* mutant JR201 resulted in a reduced production of lysine decarboxylase activity ($P < 0.05$). The residual level of lysine decarboxylase activity in JR201 corresponded to less than 1/10 of that of the wild type. As to the cadaverine excretion in the *cadC* mutant, it was significantly decreased with a trend similar to that for the lysine decarboxylase (Fig. 2B). When pJR0012 carrying recombinant *cadC* (Table 1) was reintroduced, the lysine decarboxylase activity and excretion of cadaverine in JR201 (pJR0012) were restored to levels comparable to the wild-type levels (Fig. 2A and B). Therefore, the decreased lysine decarboxylase activity and excretion of cadaverine in JR201 were proved to result from the functional inactivation of *cadC* rather than any polar effects on genes downstream of *cadC*. These results suggested that CadC acts as a positive regulator in the expression of *cadA* and *cadB* of *V. vulnificus*.

Identification of a transcription start site of the *cadBA* operon. For the primer extension experiments, RNA was prepared from the wild type and the *cadC* mutant JR201 grown at pH 7.6 and 5.8, respectively. An end-labeled 24-base primer,

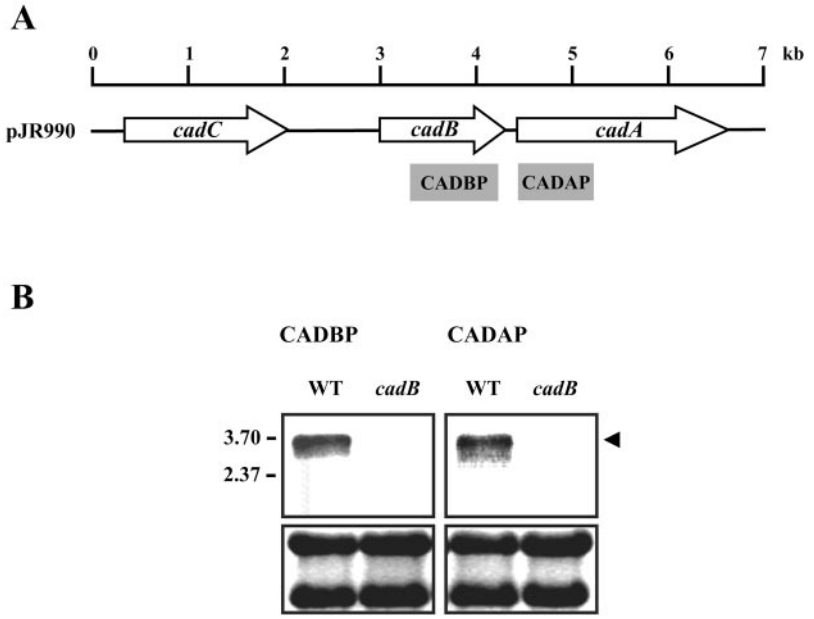

FIG. 1. Schematic representation of the *V. vulnificus cad* genes and Northern blot analysis of the *cadBA* operon. (A) The arrows represent the transcriptional directions and the coding regions of *cad* genes. The DNA probes, CADBP and CADAP, used for the Northern blot analyses are depicted by shaded bars. (B) For Northern blot analyses, total RNA from wild type (WT) and JR202 (*cadB*) was separated and hybridized to a DNA probe as indicated. The molecular size markers (Invitrogen, California) and the *cad* transcript are shown in kilobases.

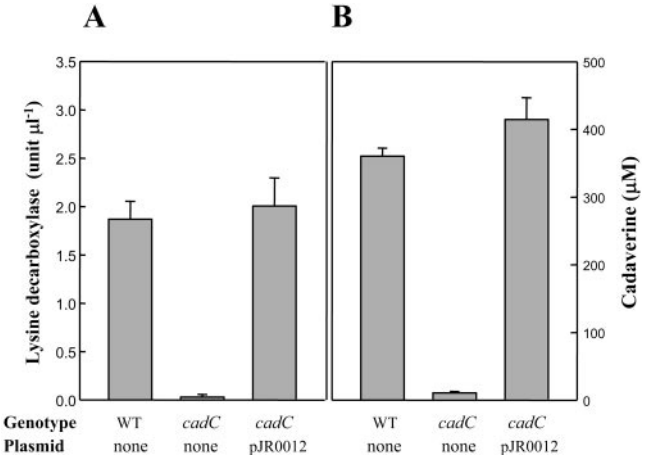

FIG. 2. Effect of a *cadC* mutation on lysine decarboxylase activity and cadaverine excretion. For both panels, cultures of the wild type (WT) and JR201 (*cadC* mutant) were grown at a pH of 5.8. Samples were removed at an OD_{600} of 0.8 and analyzed for lysine decarboxylase activity (A) and for cadaverine excretion (B). Complementations of the *cadC* mutant with functional *cadC* (pJR0012) are also presented as indicated. The error bars represent the SEM.

CAD9902, complementary to the coding region of *cadB* was added to the RNA and then extended with SuperScript II RNase H^- reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) as previously described (4, 9). A reverse transcript was identified from the RNA isolated from the wild-type cells grown at pH 5.8 (Fig. 3). In contrast, no detectable reverse transcripts were apparent with the RNA from JR201 grown at pH 5.8, suggesting that the CadC-dependent variation in the lysine/cadaverine antiporter and lysine decarboxylase activity (Fig. 2A and B) resulted from changes in the level of *cadBA* transcription. Primer extension analyses performed with the RNA prepared from cells of the wild type as well as the *cadC* mutant grown at pH 7.6 failed to produce a visible product

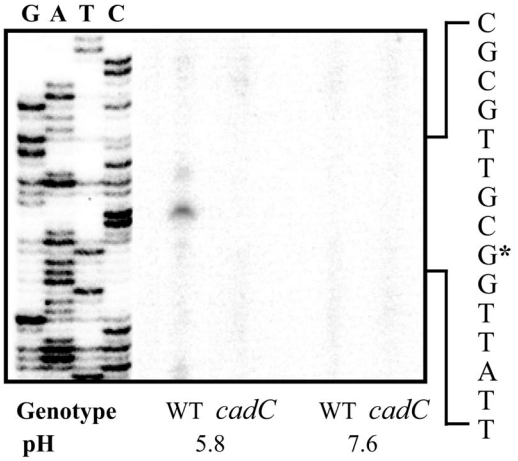

FIG. 3. Primer extension analysis of *cadBA* transcripts. The transcription start site was determined by the primer extension of the RNA derived from the wild type (WT) and JR201 (*cadC* mutant), grown at pH 5.8 or pH 7.6 as indicated. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pJR991. The asterisk indicates the site of the transcription start.

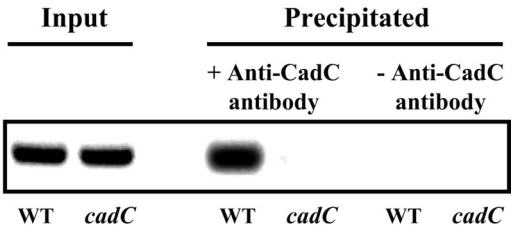

FIG. 4. Analysis of CadC binding to the upstream region of *cadBA*. The cells were cross-linked, washed, and then sonicated to produce sheared chromatin as described elsewhere (23). The DNA was purified from the sheared chromatins before precipitation (input, positive control) and after precipitation with protein A-Sepharose in the presence (+) or absence (−) of the anti-CadC antibody. The DNA was then amplified by a PCR using primers specific to the *cadBA* promoter. WT, wild type; *cadC*, *cadC* mutant.

(Fig. 3). Thus, it was apparent that CadC functioned as a positive regulator only when cells were grown at pH 5.8, and hence that the effect of CadC on the transcription of *cadBA* is dependent on the preexposure of the cells to an acidic pH.

The 5′ end of the *cadBA* transcript was located 54 bp upstream of the translational initiation codon of *cadB* and subsequently designated +1 (Fig. 3). The putative promoter constituting this transcription start site was named P_{cadBA} to represent the *cadBA* promoter. Despite several attempts, no other transcription start sites were identified by primer extension analyses using different sets of primers hybridizing to the coding region of *cadA* as well as *cadB* (data not shown). This indicated that a single promoter, P_{cadBA}, is used for the transcription of both *cadB* and *cadA* genes and that *cadBA* is expressed as a transcriptional operon.

CadC directly binds to the *cadBA* promoter. To determine whether CadC binds to the *cadBA* promoter in vivo, the cross-linked chromatin from the wild-type and *cadC* mutant JR201 cells was immunoprecipitated using the anti-CadC antibody (Fig. 4). The chromatin immunoprecipitation experiments were performed using formaldehyde cross-linking as described by Shang et al. (23). As positive controls, the input chromatin from both the wild type and JR201 appeared to carry the *cadBA* promoter DNA (Fig. 4). After the cross-links were reversed, the *cadBA* promoter fragment was detected in the chromatin precipitate from the wild type, induced with the anti-CadC antibody, based on a PCR using the primers CAD0305 and CAD0306 (Table 2). The primers were designed to specifically amplify the *cadBA* promoter DNA, which is 360 bp in length. The presence of the *cadBA* promoter DNA in the precipitated chromatin was caused by the specific binding of the CadC protein to the DNA, since no *cadBA* promoter DNA was detected in the precipitate induced in the absence of the anti-CadC antibody. Consistent with this, no detectable level of the *cadBA* promoter fragment was detected in the anti-CadC immunoprecipitate of the *cadC* mutant (Fig. 4), indicating that the CadC protein directly binds to the *cadBA* promoter in *V. vulnificus*.

Deletion analysis of *cadBA* promoter region. To delineate the *cis* DNA sequences in the P_{cadBA} promoter region required for CadC activation, transcriptional fusions of the putative *cadBA* regulatory region were made to the reporter gene *lacZ*.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence (5′→3′)[a]	Location[b]	Use
CAD9902	GCAATTAGGCCGATTTTCTTAGTA	66 to 89	Primer extension
CAD0305	GGCTTGGATAAATTCGATATC	−348 to −328	In vivo binding assay
CAD0306	GAATAAATAACCGCAACGC	12 to −7	In vivo binding assay
CAD0503	CTTAATGGCAAGTTGATTGAC	−307 to −287	DNase I footprinting
CAD0504	GATGAGACAAAAATGAGTTTC	−165 to −145	DNase I footprinting
CAD0515	AACTGCAGGAGCAGGTTGATAATTTT	−270 to −253	Promoter deletion
CAD0516	AACTGCAGATTGCTATGAGTTTATGTTT	−252 to −233	Promoter deletion
CAD0517	AACTGCAGTGATTTATGTATTGGGTG	−202 to −185	Promoter deletion
CAD0518	AACTGCAGGTCTCATCATTTTATTGATA	−152 to −133	Promoter deletion
CAD0519	AACTGCAGGATGTTATGTCATCTGATAC	49 to 68	Promoter deletion
CAD0112-1	TAGGATCCGCTAAGTTGCCAATCCAGTTC	333 to 353	Promoter deletion

[a] Regions of oligonucleotide not complementary to the corresponding genes are underlined.
[b] Shown are the oligonucleotide positions, where +1 is the transcription start site of *cadBA*.

The primer CAD0112-1 (Table 2) was used in conjunction with one of the following primers for PCR amplification of the DNA upstream of *cadBA*: CAD0515 (for pJR0515), CAD0516 (for pJR0516), CAD0517 (for pJR0517), CAD0518 (for pJR0518), and CAD0519 (for pJR0519) (Table 2). The primers were designed to amplify the P_{cadBA} promoter region extending up to −270, −252, −202, −152, and +49, respectively (Fig. 5A). The PCR products were inserted into pRKΩ*lacZ*, which carries the promoterless *lacZ* β-galactosidase gene (1), to create five *cad-lacZ* reporter constructs (Fig. 5A). The reporter constructs were transferred into the *Vv*Δ*lacZ* strain and *lacZ cadC* double mutant JR243, and the β-galactosidase activities were used to quantify the ability of each *cadBA* upstream fragment to activate the transcription of the *cadBA* operon (Fig. 5B).

For the *Vv*Δ*lacZ* strain containing pJR0515, the β-galactosidase activity was about 1,000 units (15) (Fig. 5B). This level of β-galactosidase activity was comparable to that of the *Vv*Δ*lacZ* strain carrying pJR0516; however, the β-galactosidase produced in the *cadC* mutant JR243 carrying pJR0516 (or pJR515) was significantly reduced to approximately 10% of the wild-type level (Fig. 5B). These results indicate that the *cadBA* upstream region deleted up to −252 is sufficient for the CadC activation of P_{cadBA}.

The β-galactosidase activity was reduced in the strains that carried pJR0517, and the levels of β-galactosidase activity in the *Vv*Δ*lacZ* (pJR0517) and JR243 (pJR0517) strains did not significantly differ (Fig. 5B). Similar results were observed when the β-galactosidase activities were compared between *Vv*Δ*lacZ* and JR243 cells containing pJR0518 and pJR0519. Therefore, these data indicate that the sequences necessary for the activation of P_{cadBA} by CadC were absent from the *cadBA* upstream regions present in pJR0517, pJR0518, and pJR0519. Since the *cadBA* upstream region in pJR0517 was deleted up to −202, it is reasonable to conclude that the *cis*-acting element important for the activation of P_{cadBA} by CadC ranges from 252 to 202 bp upstream of the P_{cadBA} transcription start site.

CadC binding site for the *cadBA* promoter. The greatest limitation in determining CadC binding to the *cadBA* promoter in vitro was the purification of the CadC protein, as several attempts to overexpress and purify CadC in *E. coli* were unsuccessful (data not shown). Accordingly, we determined the binding site of CadC for the upstream region of *cadBA*

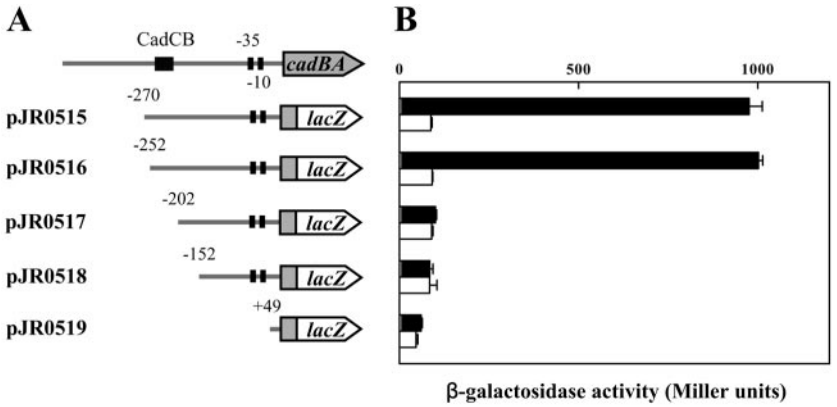

FIG. 5. Localization of the CadC binding site in the P_{cadBA}. (A) PCR fragments carrying the regulatory region of *cadBA* with deletions were subcloned into pRKΩ*lacZ* (1) to create each pJR reporter. The 5′ ends of the deletions are shown on the left as indicated. Shaded blocks, *cadBA* coding regions; open blocks, *lacZ* DNA; solid lines, upstream region of *cadBA*. The wild-type *cadBA* regulatory region is shown on top with the proposed −10 region, −35 region, and CadC binding site (CadCB). (B) The β-galactosidase activities were determined in the wild type (solid bars) and *cadC* isogenic mutant JR201 (open bars) containing each pJR reporter. Cultures grown to an OD_{600} of 0.5 at pH 5.8 were used to measure β-galactosidase activities. The error bars represent the SEM.

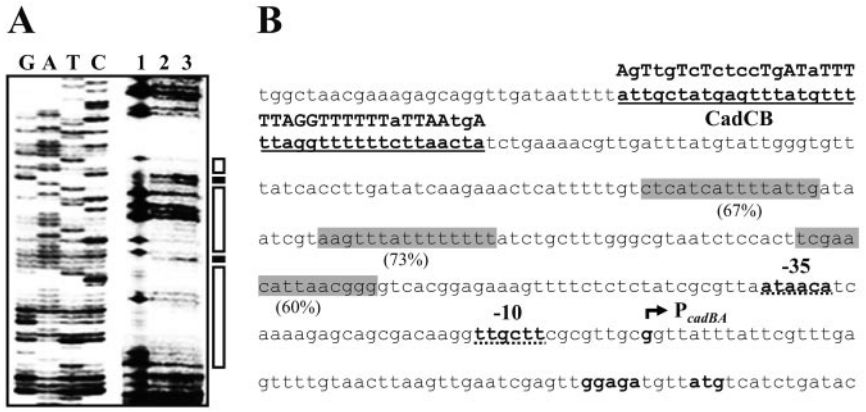

FIG. 6. Identification of CadC binding site using DNase I protection analysis. (A) DNase I protection analysis of CadC binding to wild-type *cadBA* regulatory region. Lane 1, 10 μg of membrane fraction of *cadC* mutant; lanes 2 and 3, 5 μg and 10 μg of membrane fraction of wild type, respectively. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pJR991. The hypersensitivity and protection in the presence of CadC are indicated by the thick lines and open boxes, respectively. (B) Sequence analysis of the *cadBA* upstream region. The transcription start site is indicated by the bent arrow (P_{cadBA}). The regions protected by CadC in a DNase I protection analysis and the −10 and −35 regions assigned on the basis of homology to a consensus sequence from *E. coli* are underlined with solid and broken lines, respectively. The possible *V. cholerae* CadC binding sequences are identified by homology to the *V. vulnificus* CadC binding sequences, while conserved nucleotides are indicated above the *V. vulnificus* DNA sequence in uppercase. The assigned sequences for the Lrp binding sites are shaded, and below them is shown the percentage of identity to the consensus sequence YAGHAWATTWTDCTR (5) (Y = C/T, H = "not G," W = A/T, D = "not C," R = A/G). The ATG translation initiation codon and putative ribosome binding site (GGAGA) are indicated in boldface.

using a crude extract of *V. vulnificus* as a source of the CadC protein. The total cellular proteins of *V. vulnificus* were separated into the cytoplasmic and membrane fractions and then analyzed by a Western blot assay using an anti-CadC antibody to determine the cellular location of the CadC protein. For this purpose, the rat polyclonal antibody specific to CadC was raised and purified according to the procedure previously described by Jeong et al. (10). The CadC protein was primarily found in the membrane fraction (data not shown), which was consistent with the prediction that the CadC protein is a membrane-spanning protein, proposed based on the hydrophobicity profile of the protein (21). The transmembrane region of the CadC was searched using protein topology prediction programs (http://www.sacs.ucsf.edu/Links/transmem.html). The search predicted a transmembrane region spanning between 175 and 203 amino acids from the N terminus.

A DNase I footprinting experiment was performed to determine the precise location of the CadC binding site in P_{cadBA}. The 163-bp DNA fragment of the *cadBA* promoter region, extending from residues −307 to −145, was amplified by PCR using [γ-^{32}P]ATP-labeled CAD0504 and unlabeled CAD0503 (Table 2) as primers and using DNA as a template. Binding of CadC to the labeled DNA and DNase I digestion of the protein-DNA complexes were carried out following the procedures described previously (4, 10). In this experiment, the membrane fraction was used as the protein source for CadC, and the 20-μl reaction mixture contained 1× binding buffer (20 mM morpholineethanesulfonic acid [pH 5.8], 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol), 10 mM lysine, and 1 μg of poly(dI-dC) (Sigma, St. Louis, MO). The DNase I footprinting revealed a protection pattern in the *cadBA* upstream region between −214 and −252 (Fig. 6A). Several nucleotides also showed enhanced cleavage, which has been frequently observed in DNase I protection analyses of the binding sites of transcriptional regulatory proteins (10). The CadC binding site was centered 233.5 bp upstream from the transcriptional start site of *cadBA* (Fig. 6B), confirming that CadC activates P_{cadBA} directly by binding to the *cadBA* upstream region.

The expression of the *E. coli cadBA* operon is regulated by CadC as a function of the pH (16, 24). In a similar way, CadC positively regulates the expression of the *cadBA* genes in *Vibrio cholerae* (14). However, until now, the question of whether CadC directly or indirectly affects the expression of *cadBA* in *E. coli* and *V. cholerae* has not been addressed. The data presented here demonstrated a direct interaction between CadC and P_{cadBA} in *V. vulnificus* (Fig. 4 and 6), and a DNase I protection assay revealed the CadC binding site centered at −233.5. The sequences assigned for the CadC binding site in *V. vulnificus* are also found in the *cadBA* upstream region in *V. cholerae* (Fig. 6B), indicating that the CadC binding site sequences are conserved between these bacteria.

It would seem that CadC binding at −233.5 is unusually distant for the activation of bacterial promoters. For activation of *papBA* in *E. coli*, cyclic AMP receptor protein binding at −215.5 is the most distant binding as reported previously (25). However, in this example, the leucine-responsive regulatory protein (Lrp) binds to multiple sites extended between the cyclic AMP receptor protein binding and the RNA polymerase (RNAP) binding. In the present study, the three sequences of the intervening region revealed reasonable homologies to the Lrp binding consensus sequences (Fig. 6B) (5). Lrp is a small nucleoid-structuring protein that binds and bends DNA at specific sequences. Lrp-induced bending (forming a DNA loop) facilitates a protein-protein interaction between an upstream activator and RNAP (3, 5). We have recently identified the Lrp homolog in *V. vulnificus* and deposited its sequence (GenBank accession number AY160773). However, additional studies are needed to clarify whether these regions act as Lrp recognition sites and whether the Lrp is in-

volved in the activation of P_{cadBA} by forming a DNA loop between CadC and RNAP.

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