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AphB Influences Acid Tolerance of *Vibrio vulnificus* by Activating Expression of the Positive Regulator CadC

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A mutant of *Vibrio vulnificus* that was more sensitive to low pH was screened from a library of mutants constructed by random transposon mutagenesis. By use of a transposon-tagging method, an open reading frame encoding a LysR homologue, AphB, was identified and cloned from *V. vulnificus*. The deduced amino acid sequence of AphB from *V. vulnificus* was 80% identical to that reported from *V. cholerae*. A mutational analysis demonstrated that the gene product of *aphB* contributes to acid tolerance of *V. vulnificus*. The lysine decarboxylase activity and cellular level of the *cadA* transcript were decreased in the *aphB* mutant, indicating that AphB exerts its effect on the acid tolerance of *V. vulnificus* by enhancing the expression of *cadBA*. Western blot analyses demonstrated that the cellular level of CadC, a transcription activator of the *cadBA* operon, was significantly reduced by *aphB* mutation, and a primer extension analysis revealed that the *cadC* promoter (P_{cadC}) activity was under the positive control of AphB. A direct interaction between AphB and the P_{cadC} DNA was demonstrated by gel mobility shift assays. The AphB binding site mapped by deletion analyses of the P_{cadC} regulatory region and confirmed by a DNase I protection assay was centered at the 61.5 bp upstream of the transcription start site. Accordingly, these results demonstrate that AphB and CadC function sequentially in a regulatory cascade to activate *cadBA* expression and that AphB activates the expression of *cadC* by directly binding to an upstream region of P_{cadC}.

Bacteria have evolved elaborate protection systems to allow survival and/or growth during exposure to acidic environments (for reviews, see references 2, 6, and 7). Among the bacterial acid protection systems, one type of acid pH neutralization mechanism is based on the production of cytoplasmic amino acid decarboxylases (4, 8, 21, 22, 30). Of the several amino acid decarboxylases known to be present in *Escherichia coli*, the *cadBA* genes encode a lysine/cadaverine antiporter and lysine decarboxylase, whose combined activity leads to the synthesis and excretion of cadaverine to counteract external acidification (22).

The relationship between the acid tolerance of pathogenic bacteria and their infectious dose in pathogenesis indicates that acid tolerance is important to the pathogenicity of bacteria and provides a survival strategy in low-pH environments (for a recent review, see reference 25). Recent studies using recombinase-based in vivo expression technology revealed that *Vibrio cholerae cadA* was induced during infection (23) and that mutations in genes essential for acid tolerance result in significant reduction in colonization in animal models (25). Like many other pathogenic bacteria, *Vibrio vulnificus*, a food-borne pathogenic bacterium, has to cope with ever-changing acidity in its growth environments to ensure developing illness. However, only a few studies have addressed the molecular mechanisms by which the bacterium can survive in low-pH environments. The present authors previously cloned a 9.3-kb DNA fragment of *V. vulnificus* which contains *cadBA* genes (32). It has been demonstrated that the gene products of *cadBA* contribute to the acid tolerance of *V. vulnificus* and that their contribution is dependent on prior exposure of the cells to moderately acidic pH (32).

The expression of the *E. coli cadBA* operon is regulated by CadC as a function of pH (29, 36). In a similar way, CadC positively regulates the expression of *cadBA* genes in *V. cholerae* (24). The present authors also recently demonstrated that *V. vulnificus cadBA* expression is activated by CadC in a pH-dependent manner and that CadC exerts its effects at a distance by directly binding to the upstream sequences of P_{cadBA} (34). However, the molecular mechanisms by which the bacterium modulates the expression of the *cadC* gene have not yet been characterized. Neither the promoter(s) of the *cadC* gene nor any *trans*-acting regulatory protein(s) required for the expression of the *cadC* promoter has been identified previously. Accordingly, here we extend our efforts to elucidate the regulation of *cadC* expression at a molecular level. For this purpose, an open reading frame (ORF) encoding a LysR homologue, AphB, was identified by a transposon-tagging method. A *V. vulnificus* null mutant in which the *aphB* gene was deleted was constructed by allelic exchange, and the possible roles of the AphB protein in the regulation of *cadC* expression as well as in acid tolerance of *V. vulnificus* were explored. As a result, it was found that AphB appeared to activate the expression of *cadC* by directly binding to the *cadC* promoter.

MATERIALS AND METHODS

Strains, plasmids, and culture media. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (wt/vol) agar. Unless noted otherwise, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (wt/vol) NaCl (LBS).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Bacterial strains		
V. vulnificus		
ATCC 29307	Clinical isolate; virulent	Laboratory collection
Δ*lacZ*	ATCC 29307 with Δ*lacZ*; Sm^r	1
JR312	ATCC 29307 with Δ*aphB*	This study
JR313	Δ*lacZ* with Δ*aphB*; Sm^r	This study
E. coli		
BL21(DE3)	F^- *ompT hsdS* (r_B^- m_B^-) *gal*(DE3)	Laboratory collection
SM10(λ*pir*)	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*; *oriT* of RP4; Km^r; host for π-requiring plasmids; conjugal donor	27
Plasmids		
Mini-Tn5 *lacZ1*	R6K γ *ori*; suicide vector; *oriT* of RP4; Ap^r	5
pDM4	R6K γ *ori sacB*; suicide vector; *oriT* of RP4; Cm^r	28
pET22b(+)	His tag protein expression vector	Novagen
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pGEX2T	GST fusion expression vector	Amersham
pHG0503	pET22b(+) with *aphB*; Ap^r	This study
pJR990	Cosmid containing *cadCBA*	33
pJR0308	pGEM-T Easy with *aphB*; Ap^r	This study
pJR0309	pRK415 with *aphB*; Tc^r	This study
pJR0325	0.6-kb Δ*aphB* fragment in pDM4; Cm^r	This study
pJR0530	pRKΩ*lacZ* with 501-bp fragment of *cadC* upstream region; Tc^r	This study
pJR0531	pRKΩ*lacZ* with 431-bp fragment of *cadC* upstream region; Tc^r	This study
pJR0533	pRKΩ*lacZ* with 386-bp fragment of *cadC* upstream region; Tc^r	This study
pJR0534	pRKΩ*lacZ* with 362-bp fragment of *cadC* upstream region; Tc^r	This study
pJR0535	pRKΩ*lacZ* with 301-bp fragment of *cadC* upstream region; Tc^r	This study
pRK415	IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc^r	12
pRKΩ*lacZ*	pRK415 with *lacZ*; Tc^r	1

When required, cultures of *V. vulnificus* strains were buffered at pH 5.8 with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma, St. Louis, MO) or pH 7.6 with 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Sigma).

Cloning of *V. vulnificus aphB*. A mutant that was more sensitive to low pH was screened from a library of *V. vulnificus* mutants generated by random transposon mutagenesis using mini-Tn5 *lacZ1* (5, 32). A DNA segment flanking the transposon insertion was amplified by PCR as described previously (13). Since a database search for homology to the amino acid sequence deduced from the resulting PCR product singled out *V. cholerae* AphB, a whole *aphB* ORF was amplified from the genomic DNA of *V. vulnificus* by PCR using a pair of oligonucleotide primers, APHB031 and APHB032 (Table 2). The primers were designed using the genomic sequence of *V. vulnificus* (GenBank accession number VV11998; www.ncbi.nlm.nih.gov). The amplified 889-bp *aphB* gene was ligated into pGEM-T Easy (Promega, Madison, WI) to result in pJR0308 (Table 1).

Generation of *aphB* deletion mutants. To inactivate *aphB* in vitro, one-third (300 bp) of the *aphB* ORF in pJR0308 was deleted and the resulting 0.6-kb Δ*aphB* construct was ligated with SphI/SacI-digested pDM4 (28), forming pJR0325. To generate the Δ*aphB* mutant by homologous recombination, *E. coli*

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence (5′ → 3′)[a]	Location[b]	Use(s)
APHB031	AACTGCAGGATGTGTCAGGAAATATG	Chromosomal DNA	Amplification of *aphB*
APHB032	CGGAATTCCTACATCGTTAGTGGATG	Chromosomal DNA	Amplification of *aphB*
APHB041	GGAATTCCATATGAAATTAGACGATC	Chromosomal DNA	Amplification of *aphB*
APHB042-1	CCGCTCGAGGTGGATGTTATAAG	Chromosomal DNA	Amplification of *aphB*
CAD0113	CGGGATCCATTGGAATCTATTTTCAAAT	Chromosomal DNA	Amplification of *cadC*
CAD0114	CGGAATTCTCGATTCGGTTTTTTTCTGTT	Chromosomal DNA	Amplification of *cadC*
CAD0101	GGAGACTTCCCTATCTTGTCGATACAGCTT	+117 to +146	Primer extension, gel mobility shift assay, DNase I footprinting
CAD0530	CTGCAGAATCGTTTTAGGCTTAGCAA	−152 to −133	Promoter deletion, DNase I footprinting
CAD0531	CTGCAGTGATTAAACCGCGAAACTTAG	−82 to −62	Promoter deletion, gel mobility shift assay
CAD0533	CTGCAGCTTAGGTGAACAGGCGTATC	−37 to −18	Promoter deletion
CAD0534	CTGCAGTTATAATCTCGCCCTCATTG	−13 to +7	Promoter deletion
CAD0535	CTGCAGCAAAGAGCGTTCTAATGATT	+49 to +68	Promoter deletion
CAD0414	GGATCCCGTTTTGGTACGGTCACCAC	+329 to +349	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 *cadC*.

SM10 λ*pir tra* (containing pJR0325) was used as a conjugal donor to *V. vulnificus* ATCC 29307 (27). For the construction of the *lacZ aphB* double mutant, Δ*lacZ*, an isogenic mutant of ATCC 29307 which lacks *lacZ*, was used as the recipient (34). The conjugation and isolation of the transconjugants were conducted using methods previously described (9, 11, 32).

Acid challenges and measurement of lysine decarboxylase activities. Acid tolerance was assessed in 10 mM sodium citrate buffer (pH 4.4) supplemented with 2.0% NaCl (SCBN) and filter sterilized. Cultures of *V. vulnificus* strains, buffered at pH 5.8, were grown at 30°C under aeration to an optical density at 600 nm (OD_{600}) of 0.8 and used to inoculate flasks containing 50 ml of SCBN to achieve a final concentration of ca. 10^5 CFU ml^{-1}. Following inoculation, the flasks were incubated at 30°C with shaking (150 rpm) for 90 min. The samples then removed were plated on LBS, and the percentages of survivors were calculated, using the CFU ml^{-1} determined immediately after inoculation as 100%.

Cultures grown to an OD_{600} of 0.8 as described above were used to determine the lysine decarboxylase activity based on procedures described previously (20, 31). A unit of the enzyme activity was defined as an increase in absorbance ($1{,}000 \times A_{340}$) of the reaction solution per min per cell density (OD_{600}) of cultures. Averages and standard errors of the means (SEM) were calculated from at least three independent trials.

Transcript analysis. Total cellular RNA was isolated from the *V. vulnificus* cultures grown at pH 5.8 by use of a Trizol reagent kit (Invitrogen, Carlsbad, CA). For Northern analyses, reactions were performed according to standard procedures (35) with 20 μg of total RNA. The DNA probe CADAP was prepared by labeling DNA fragments containing the *cadA* coding region with [α-^{32}P]dCTP as previously described (34) and used for the hybridizations.

For the primer extension experiments, an end-labeled 30-base primer, CAD0101 (Table 2), complementary to the coding region of *cadC*, was added to the RNA and then extended with SuperScript II RNase H^- reverse transcriptase (Invitrogen) as previously described (3, 9, 10). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated from pJR990 (Table 1) with the same primer used for the primer extension. Primer extension products and Northern hybridization products were visualized using a phosphorimager (model BAS1500; Fuji Photo Film Co. Ltd., Tokyo, Japan).

Purification of GST-CadCN fusion protein and Western blot analysis of CadC. The DNA fragment encoding the N-terminal 169 amino acids of CadC (CadCN) was amplified by PCR using the primers CAD0113 and CAD0114 (Table 2). The DNA fragment had BamHI and EcoRI sites at the ends that were used to make an in-frame gene fusion between the truncated *cadC* gene and the 3′ end of the *gst* gene encoding glutathione *S*-transferase (GST) in pGEX2T (Amersham, Uppsala, Sweden). The GST-CadCN protein was expressed and purified by affinity chromatography according to the manufacturer's procedures (Amersham). For Western blot analyses, the purified GST-CadCN was used to raise the anti-CadC antibody and immunoblotting was performed according to a procedure previously described by Jeong et al. (10).

Construction of set of *cadC-lacZ* transcriptional fusions. The primer CAD0414 (Table 2), with a BamHI restriction site followed by bases corresponding to the 5′ end of the *cadC* coding region, was used in conjunction with one of the following primers to amplify the DNA upstream of *cadC*: CAD0530 (for pJR0530), CAD0531 (for pJR0531), CAD0533 (for pJR0533), CAD0534 (for pJR0534), or CAD0535 (for pJR0535) (Table 2). The primers were designed to amplify the P_{cadC} promoter region extended up to −152, −82, −37, −13, and +49, respectively (see Fig. 5A). A PstI restriction site was added to these primers to facilitate cloning of the DNA fragments. The PCR products were digested with BamHI and PstI and inserted into pRKΩ*lacZ*, which carries the promoterless *lacZ* β-galactosidase gene (1), to create five *cadC-lacZ* reporter constructs. All constructions were confirmed by DNA sequencing. The $CHCl_3$-sodium dodecyl sulfate method described by Miller (26) was used to measure the β-galactosidase activity.

Overexpression and purification of *V. vulnificus* AphB. The *aphB* coding region was amplified by PCR using the primers APHB041 and APHB042-1 (Table 2) and then subcloned into a His_6 tag expression vector, pET22b(+) (Novagen, Darmstadt, Germany). The resulting plasmid, pHG0503, encoded AphB with a His_6 tag at the amino terminus. The His-tagged AphB protein was then expressed in *E. coli* BL21(DE3), and the protein was purified by affinity chromatography according to the manufacturer's procedure (QIAGEN, Valencia, CA).

Gel mobility shift assay and DNase I footprinting. For gel mobility shift assays, the 228-bp upstream region of *cadC*, extending from residues −82 to +146, was amplified by PCR using [γ-^{32}P]ATP-labeled CAD0101 and unlabeled CAD0531 as the primers (Table 2). The labeled 228-bp DNA (7 nM) fragment was incubated with various concentrations of purified His-tagged AphB protein for 30 min at 30°C in a 20-μl reaction mixture containing 1× binding buffer [50 mM HEPES (pH 7.6), 30 mM KCl, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 0.2% Tween 20] and 200 ng of poly(dI-dC) (Sigma). Following the incubation, the samples were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel (3). For competition analyses, various amounts of competitor DNA, the same but unlabeled 228-bp DNA, were added to the reaction mixture containing 7 nM of the labeled DNA prior to the addition of 80 nM of AphB.

For the DNase I protection assays, a 298-bp fragment of the *cadC* promoter region was generated by PCR amplification using a combination of [γ-^{32}P]ATP-labeled and unlabeled primers, CAD0530 and CAD0101, respectively (Table 2). The protein-DNA binding reactions with AphB were carried out as described above, except that 20 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ mix was added to the reaction mixtures, along with 1 μl of a DNase I solution (10 ng/μl; Sigma) (19). After incubation for 1 min at 25°C, the DNase I digestion was stopped by the addition of 80 μl of stop solution (35), and the DNA products were purified by ethanol precipitation. The purified DNA products were resolved on a sequencing gel alongside sequencing ladders of pJR990 generated using CAD0530 as a primer. Gels were processed as described for the primer extension analyses.

RESULTS

Identification of *aphB* gene and construction of *aphB* mutants of *V. vulnificus*. The amino acid sequence deduced from the *aphB* nucleotide sequence revealed a protein, a LysR homologue, AphB, composed of 291 amino acids with a theoretical molecular mass of 33,132 Da and a pI of 6.76. The amino acid sequence of *V. vulnificus* AphB was 80% identical to that of AphB of *V. cholerae* (data not shown; http://www.ebi.ac.uk/clustalw). The predicted profile of the hydrophobicity (http://www.expasy.ch) was significantly similar to that of AphB of *V. cholerae* and is consistent with the fact that the AphB protein is a cytosolic soluble protein (data not shown). To examine the role of AphB, *V. vulnificus aphB* isogenic mutants (Table 1) were constructed by allelic exchanges. Double crossovers, in which each wild-type *aphB* gene on the chromosome was replaced with the Δ*aphB* allele, were confirmed using previously described methods (10, 32) (data not shown). The mutants chosen for further analysis were named JR312 for the *aphB* mutant and JR313 for the *lacZ aphB* double mutant (Table 1).

Acid tolerance of the *aphB* mutant. The survival of the wild type (ATCC 29307) was significantly greater ($P < 0.05$) than that of the *aphB* mutant JR312 when challenged in SCBN adjusted to pH 4.4 (Fig. 1). The wild type decreased ca. 1.0 $\log_{10}$ CFU/ml (90%) whereas the JR312 strain decreased ca. 3.0 $\log_{10}$ CFU/ml (99.99%) after a 90-min acid challenge. This indicated that the *aphB* mutant is more sensitive to acid than the wild type and that AphB plays an important role in the acid tolerance of *V. vulnificus*. For the complementation of JR312, plasmid pJR0309 was constructed by subcloning the *aphB* coding region into pRK415 and under an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter (12). Complementation of the *aphB* gene in JR312 with a functional *aphB* gene (pJR0309) restored acid tolerance to a level equivalent to that of the wild type (Fig. 1). Therefore, the decreased acid tolerance of JR312 apparently resulted from the inactivation of functional *aphB* rather than any polar effects on the genes downstream of *aphB*.

Effect of mutation in *aphB* gene on lysine decarboxylase activity. For the wild type grown at pH 5.8, lysine decarboxylase was produced and reached a maximum of 2.0 U (Fig. 2A). The disruption of *aphB* in the mutant JR312 resulted in a reduced production of lysine decarboxylase activity ($P < 0.05$). The residual level of lysine decarboxylase activity in JR312 corresponded to less than 1/10 of that of the wild type (Fig.

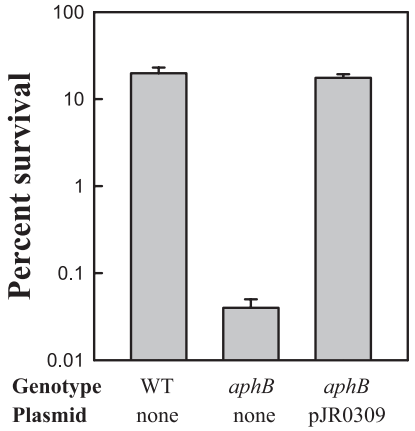

FIG. 1. Acid tolerance of ATCC 29307 and isogenic *aphB* mutant. Cultures grown in LBS adjusted to a pH of 5.8 were used for the acid challenge as described in Materials and Methods. The percentages of survivors were calculated, using the CFU/ml of ATCC 29307 (wild type [WT]) that survived immediately after the challenge as 100%. Survivors of the mutant complemented with a functional *aphB* gene (pJR0309) are also presented. All bars represent the mean numbers of survivors from three independent trials, and the error bars represent the SEM. *aphB*, *aphB* mutant.

2A). The *cadA* transcript was also decreased in the *aphB* mutant (Fig. 2B), indicating that the lysine decarboxylase expression was positively regulated by AphB at transcription level. Similarly to results from the acid tolerance tests, complementation of Δ*aphB* in JR312 with a functional *aphB* gene (pJR0309) restored the lysine decarboxylase activity and the *cadA* transcript to levels comparable to those of the wild type (Fig. 2A and B). Therefore, these results indicated that the reduced level of lysine decarboxylase could be the major, if not sole, cause for the decreased acid tolerance of the *V. vulnificus aphB* mutant.

Effect of AphB on *cadA* expression is mediated through CadC. There are several possible ways for AphB to affect *cadA* expression. One is by binding directly to the *cadA* promoter, P_{cadBA} (34). However, when examined by a gel mobility shift assay, AphB was not able to specifically bind to the *cadA* promoter region even after several attempts (data not shown). The other possibility is that AphB influences the accumulation of another regulator(s), which in turn is directly responsible for the activation of P_{cadBA}.

Since it has already been reported that CadC, a transmembrane protein, activates *cadBA* expression by directly binding to the upstream sequences of P_{cadBA} (34), the cellular levels of CadC were determined in the same amount of total protein isolated from the wild type and from the *aphB* mutant JR312 (Fig. 3A). Western blot analysis revealed that the cellular level of CadC in the *aphB* mutant was almost undetectable and significantly lower than that in the wild type, indicating that AphB influences the accumulation of CadC. From this result, it is likely that AphB indirectly activates the activity of P_{cadBA} by increasing the cellular level of CadC, which is required for P_{cadBA} activity.

In order to characterize the effect of AphB on the level of CadC in more detail, the levels of *cadC* transcription in the wild type and the *aphB* mutant were compared by primer extension analyses. A reverse transcript was identified from the RNA isolated from the wild-type cells (Fig. 3B). In contrast, no detectable reverse transcripts were apparent with the RNA from the *aphB* mutant, suggesting that AphB-dependent variation in the CadC level (Fig. 3A) resulted from changes in the level of *cadC* transcription. Determined based on the intensity of the bands of the reverse transcripts, the decreased *cadC* transcription in the *aphB* mutant was restored by the introduction of recombinant *aphB* (pJR0309) (Fig. 3B). Consequently, it appeared that AphB and CadC functioned sequentially in a regulatory cascade to activate P_{cadBA} activity.

The 5′ end of the *cadC* transcript was located 62 bp up-

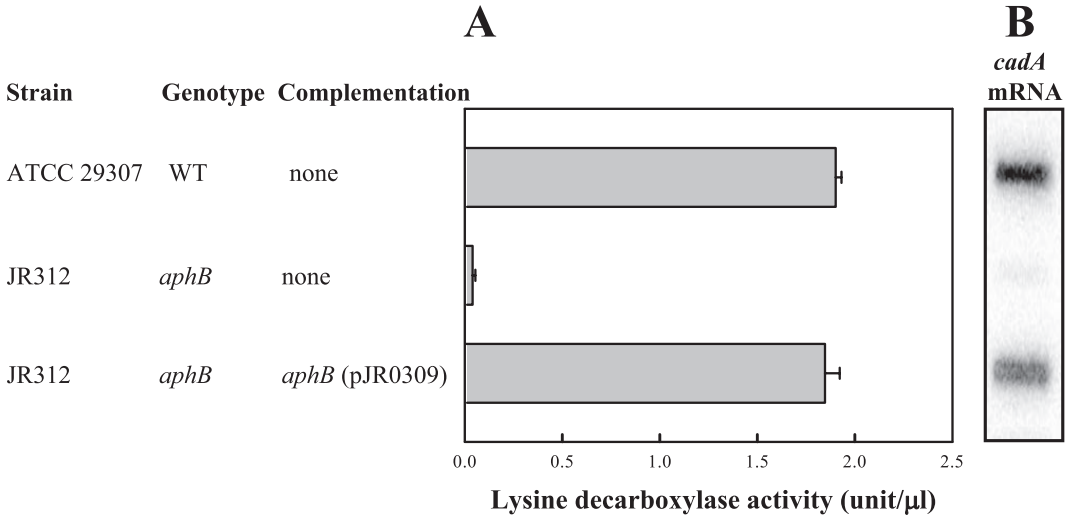

FIG. 2. Effect of mutations in *aphB* gene on lysine decarboxylase activity and *cadBA* transcription. Cultures were grown as described in the legend for Fig. 1, and then samples removed at an OD_{600} of 0.8 were analyzed for lysine decarboxylase activity (A) and *cadA* transcript (B). Complementation of the mutant with a functional *aphB* gene (pJR0309) is also presented. Relative activities of lysine decarboxylase were calculated as described in the text. Error bars represent the SEM. WT, wild type; *aphB*, *aphB* mutant.

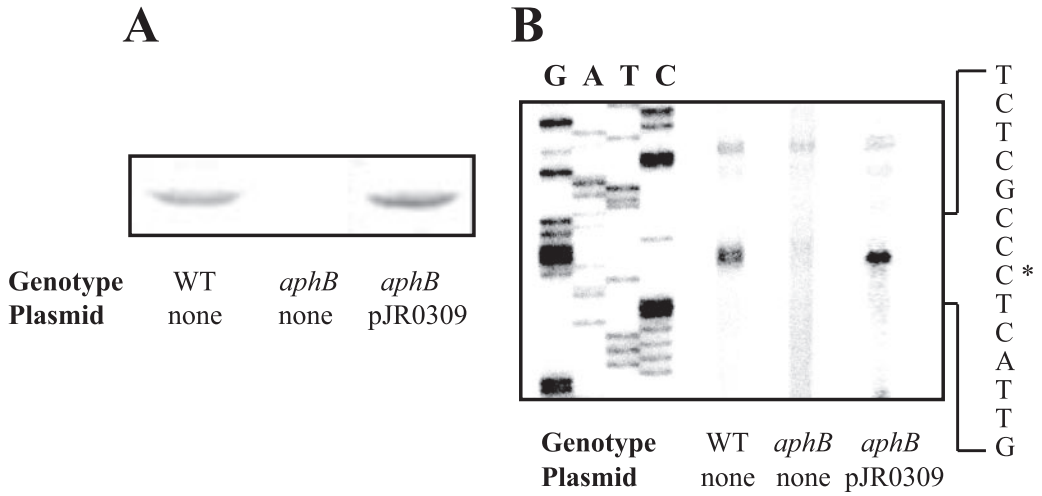

FIG. 3. Effects of *aphB* mutation on cellular level of CadC and P_{cadC} activity. Cultures were grown as described in the legend for Fig. 1 and then were analyzed for their CadC levels (A) and P_{cadC} activities (B). Complementations of the mutant with a functional *aphB* gene (pJR0309) are also presented. (A) CadC protein levels were determined by Western blot analyses using the immunoglobulin G fraction of rat anti-*V. vulnificus* CadC serum. (B) P_{cadC} activities were determined by primer extension of the RNA derived from each strain. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pJR990. The asterisk indicates the site of the transcription start. WT, wild type; *aphB*, *aphB* mutant.

stream of the translational initiation codon of CadC and subsequently designated +1 (Fig. 3B) (also see Fig. 6). The putative promoter constituting this transcription start site was named P_{cadC} to represent the *cadC* promoter.

AphB activates *cadC* expression by directly binding to P_{cadC}. The 228-bp DNA fragment encompassing the *cadC* regulatory region was incubated with increasing amounts of AphB and then subjected to electrophoresis. As seen in Fig. 4A, the addition of AphB at a concentration of 10 nM resulted in a shift of the 228-bp DNA fragment to a single band with a slower mobility. The binding of AphB was also specific, because assays were performed in the presence of 200 ng of poly(dI-dC) as a nonspecific competitor. In a second gel mobility shift assay, the same but unlabeled 228-bp DNA fragment was used as a self-competitor to confirm the specific binding of AphB to P_{cadC} (Fig. 4B). The unlabeled 228-bp DNA competed for the binding of AphB in a dose-dependent manner (Fig. 4B), confirming that AphB binds specifically to the *cadC* regulatory region.

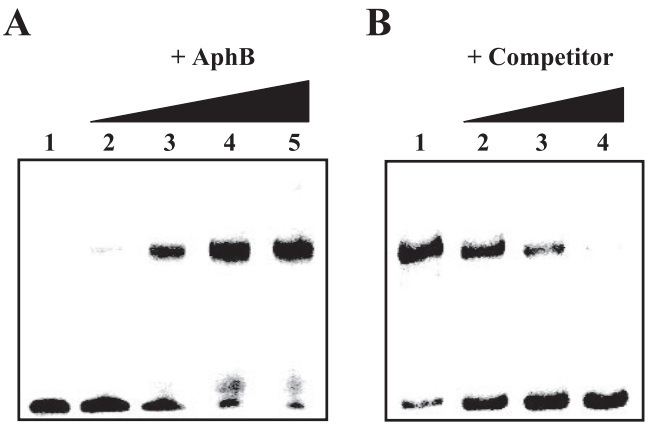

FIG. 4. Gel mobility shift assay for binding of AphB to *cadC* regulatory region. A 228-bp DNA fragment of the upstream region of P_{cadC} was radioactively labeled and then used as a probe DNA. The radiolabeled fragments (7 nM) were mixed with increasing amounts of AphB. (A) Lanes 1 to 5 show 0, 10, 20, 40, and 80 nM of AphB, respectively. (B) For competition analysis, the same but unlabeled DNA fragment was used as the competitor. Prior to the addition of AphB, various amounts of the competitor DNA were added to a reaction mixture containing 7 nM labeled DNA. Lanes 1 to 4, probe DNA incubated with 80 nM of AphB and 0, 7, 14, and 28 nM of the competitor DNA, respectively.

Deletion analysis of *cadC* promoter region. To delineate the *cis* DNA sequences in the P_{cadC} promoter region required for AphB activation, transcriptional fusions of the putative *cadC* regulatory region were made to the reporter gene *lacZ*. The pJR-reporter fusions are shown in Fig. 5A. The reporter constructs were transferred into the Δ*lacZ* strain and *lacZ aphB* double mutant JR313. β-Galactosidase activities were used to quantify the capacity of each *cadC* upstream fragment to activate P_{cadC}.

For the Δ*lacZ* strain containing pJR-reporter fusions with endpoints to −152 (pJR0530) and −82 (pJR0531), the β-galactosidase activities were about 2,200 U (Fig. 5B). However, β-galactosidase produced in JR313 carrying pJR0530 (or pJR0531) was significantly reduced, supporting the hypothesis that the expression of P_{cadC} is dependent on AphB. β-Galactosidase activities were reduced in the strains that carried reporter fusions with endpoints from −37 to −13 (pJR0533 and pJR0534, respectively), and the residual level was indistinguishable from the basal level (i.e., β-galactosidase activity with pJR0535 deleted up to +49) (Fig. 5A and B). Moreover, the levels of β-galactosidase activity in Δ*lacZ* (pJR0533) and JR313 (pJR0533) did not significantly differ. Similar results were observed when the β-galactosidase activities of the Δ*lacZ* and JR313 cells containing pJR0534 were compared (Fig. 5B). Therefore, these data indicate that the sequences necessary for the activation of P_{cadC} by AphB were absent from the *cadC* upstream regions carried in pJR0533 and pJR0534. Since the *cadC* upstream regions in pJR0531 and pJR0533 were deleted

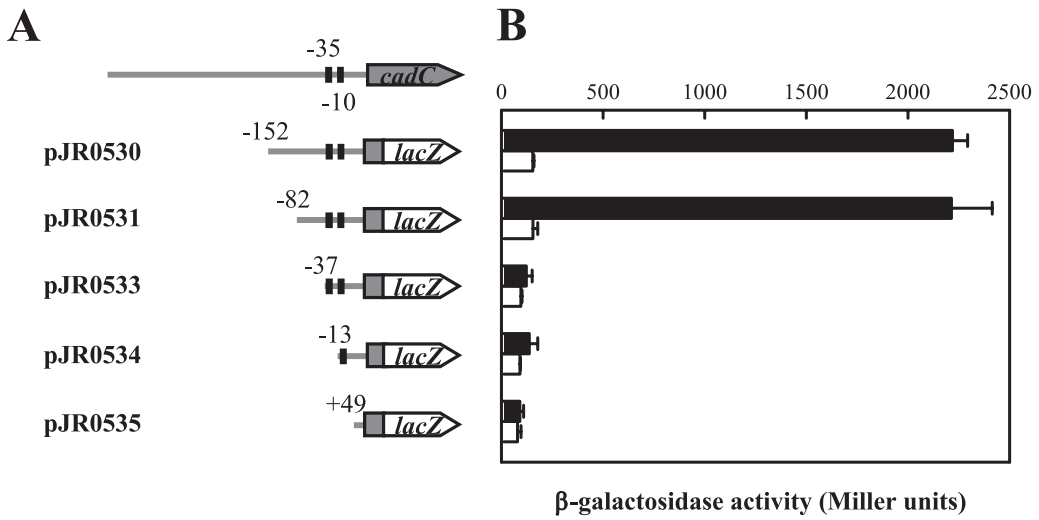

FIG. 5. Deletion analysis of the *cadC* promoter region. (A) Construction of *cadC-lacZ* fusion pJR plasmids. PCR fragments carrying the regulatory region of *cadC* with deletions were subcloned into pRKΩ*lacZ* to create each pJR-reporter fusion. Filled blocks, *cadC* coding regions; open blocks, *lacZ* DNA; solid lines, upstream region of *cadC*. The wild-type *cadC* regulatory region is shown at the top, with the proposed −10 and −35 regions marked. (B) β-Galactosidase activities were determined for the wild type (filled bars) and the *aphB* mutant (open bars) containing each pJR-reporter fusion as indicated. Cultures grown to an OD_{600} of 0.5 at pH 5.8 were used to measure the β-galactosidase activities. Error bars represent the SEM.

up to −82 and −37, respectively, it was reasonable to conclude that the important *cis*-acting element for the activation of P_{cadC} by AphB would range from 82 to 37 bp upstream of the P_{cadC} transcription start.

Identification of AphB binding site using DNase I protection analysis. To determine the precise location of the AphB binding site in the *cadC* regulatory region, a DNase I footprinting experiment was performed. DNase I footprinting revealed a clear protection pattern in the *cadC* upstream region between −82 and −41 (Fig. 6A). Several nucleotides also showed enhanced cleavages, which have been observed frequently in DNase I protection analyses of the binding sites of transcriptional regulatory proteins, such as CRP (cyclic AMP receptor protein) (10). The AphB binding site was centered 61.5 bp upstream from the transcriptional start site of *cadC* (Fig. 6B), confirming that AphB activates P_{cadC} directly by binding to the *cadC* upstream region.

In summary, the present study established that a LysR ho-

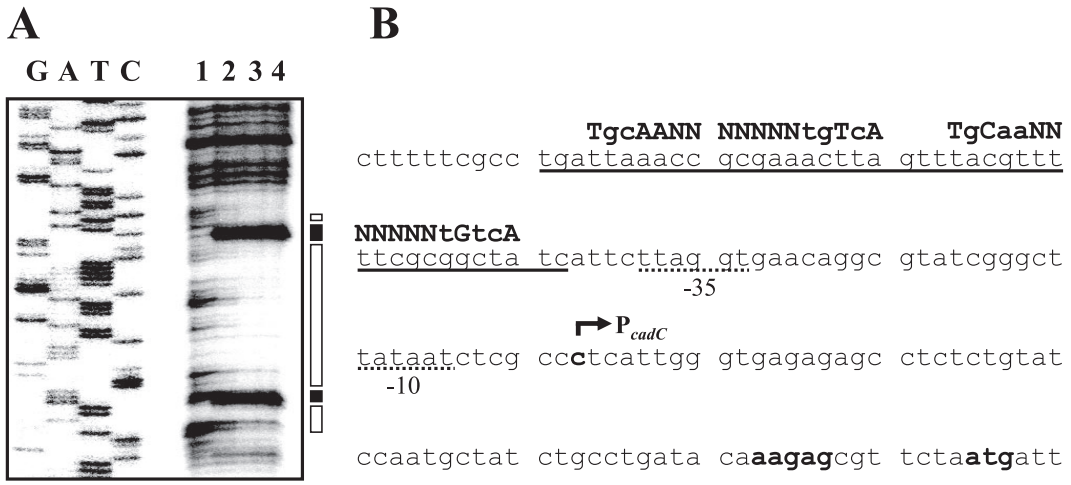

FIG. 6. Identification of AphB binding site using DNase I protection analysis (A) and sequence analysis of the *cadC* upstream region (B). (A) The ^{32}P-labeled 298-bp *cadC* regulatory region was incubated with increasing amounts of AphB and then digested with DNase I. Lane 1, no AphB added; lanes 2 to 4, AphB at 200, 300, and 400 nM, respectively. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pJR990. The hypersensitivity and protection in the presence of AphB are indicated by thick lines and open boxes, respectively. (B) The transcription start site is indicated by the bent arrow (P_{cadC}). The regions protected by AphB 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 continuous and broken lines, respectively. The possible *V. cholerae* AphB binding sequences suggested by Kovacikova and Skorupski (16, 17) are indicated above the *V. vulnificus* DNA sequence in uppercase letters. The ATG translation initiation codon and the putative ribosome binding site (AAGAG) are indicated in boldface type.

mologue, AphB, contributes to the acid tolerance of *V. vulnificus* by increasing the cellular level of CadC, which in turn activates the expression of the *cadBA* genes encoding cadaverine/lysine antiporter and lysine decarboxylase. AphB activates the expression of *cadC* by binding directly to an AphB binding site centered 61.5 bp upstream of P_{cadC}. Consequently, it would appear that AphB and CadC function sequentially in a regulatory cascade to activate P_{cadBA} activity.

DISCUSSION

Previous studies have proposed that *V. vulnificus cadBA* is a single transcriptional unit in which the transcription is directed by a promoter, P_{cadBA}, that is elevated by CadC at a low pH (32, 33, 34). In a similar way, CadC positively regulates the expression of the *cadBA* genes in *E. coli* and *V. cholerae* (24, 29, 36) in a pH-dependent manner. Recently, the present authors demonstrated that CadC activates the expression of *cadBA* by directly binding to a CadC binding site, which is centered 233.5 bp upstream of the transcription start site of P_{cadBA} (34). However, no definitive analysis of the regulatory mechanism of *cadC* expression has been reported yet at a molecular level. The data presented here demonstrated that *cadC* expression is activated by a LysR homologue, AphB, and that AphB exerts its effect by directly binding to the upstream region of P_{cadC}. Therefore, it appeared that AphB and CadC function sequentially in a regulatory cascade, that is, AphB increases the cellular level of CadC, which ultimately leads to the production of the lysine decarboxylase and lysine/cadaverine antiporter.

V. cholerae AphB is a member of the LysR family of transcriptional regulators and plays a central role in the activation of the ToxR virulence cascade (14). AphB initiates the expression of the ToxR virulence cascade by activating the expression of *tcpPH* in *V. cholerae* in response to as-yet-unknown environmental signals (14, 15). AphB cooperates with AphA, a second transcriptional activator, to synergistically activate *tcpPH* expression (14, 16, 17). AphB activates the expression of *tcpPH* by binding directly to the *tcpPH* promoter, and the AphB binding site extends from −78 to −43 (16). The observation that AphB is capable of rescuing some DNA binding-defective AphA mutants suggested that the proteins interact directly on the DNA (18). A search of the genome sequences of both *V. vulnificus* CMCP6 and YJ016 (GenBank accession numbers VV11998 and VV2418) revealed an *aphA* gene homologue with a high level of identity with *V. cholerae aphA* in nucleotide and amino acid sequences (S. H. Choi, unpublished data). An *aphA* null mutation did not significantly alter the lysine decarboxylase activity or the level of CadC, indicating that AphA is not required for the regulation of P_{cadC} (data not shown). Although the AphB proteins from *V. cholerae* and *V. vulnificus* exhibit high levels of identity (80% in amino acid sequences), the specific features of their functions clearly differ, such that the regulatory protein is dependent on AphA for the *tcpPH* promoter yet independent for the *cadC* promoter. Obviously, further studies are needed to clarify whether *V. vulnificus* AphB is really crucial for the overall success of the organism in pathogenesis, as suggested for *V. cholerae* AphB (14, 15, 17).

The present authors previously demonstrated that P_{cadBA} activity was not detectable in *V. vulnificus* at a neutral pH yet became apparent at an acidic pH (32, 34). However, this pH-responsive induction of *cadBA* in *V. vulnificus* was completely dependent on the presence of functional CadC, as no *cadBA* expression was detected with the *cadC* mutant under any of the conditions tested (34). These results suggested that pH-dependent activity of CadC plays the major role in the induction of *cadBA* transcription at an acidic pH. One working model explaining how *cadBA* transcription is regulated by pH is that the expression of *cadC* is altered at different pHs. However, transcription of *V. vulnificus* CadC is not influenced by pH (data not shown), indicating that the activities of AphB in cells grown at different pHs are not significantly different. Consistent with this, it has been reported previously that the expression of *V. cholerae aphB* is not influenced by environmental stimuli such as pH (14). Therefore, further understanding of the mechanism by which *V. vulnificus cadBA* is expressed differentially in response to pH variation awaits additional work.

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