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# Identification of the *Vibrio vulnificus cadC* and Evaluation of Its Role in Acid Tolerance

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**Abstract** An open reading frame encoding CadC, consisting of 526 amino acids, was identified from the upstream region of the *Vibrio vulnificus cadBA* operon. The deduced amino acid sequences of the *cadC* were 22 to 78% similar to those reported from other Enterobacteriaceae. Functions of *cadC* gene on acid tolerance were assessed by comparing acid tolerances of *V. vulnificus* and its isogenic mutant, whose *cadC* gene was inactivated by allelic exchanges. The results demonstrated that the gene product of *cadC* contributes to acid tolerance of *V. vulnificus*, and that its contribution is dependent on prior exposure of cells to moderately acidic pH. The cellular level of *cadB* and *cadA* transcripts decreased in the *cadC* mutant, indicating that CadC exerts its effect on acid tolerance of *V. vulnificus* by enhancing the expression of *cadBA* in a pH-dependent manner.

**Key words:** *Vibrio vulnificus*, *cadC*, acid tolerance

The pathogenic marine bacterium *Vibrio vulnificus* occurs in various raw seafoods, and has been identified as the causative agent of foodborne diseases, such as life-threatening septicemia and possibly gastroenteritis, in individuals with underlying predisposed conditions. Even otherwise healthy people are susceptible to serious wound infections after contact with shellfish or water contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death may occur within one to two days after the first signs of illness [14, 25].

Bacteria have evolved with elaborate protection systems to allow survival and/or growth during exposure to acidic environments [2, 5, 6]. The relationship between acid tolerance of pathogenic bacteria and their infectious dose in the pathogenesis has been well documented. One explanation proposed for the low infectious dose of the *Shigella* species is their ability to survive the acidic conditions of the stomach [7]. Acid-sensitive pathogens like *Salmonella* typically require $10^5$ to $10^{10}$ cells for infection while <500 *Shigella* can result in illness. It has been noted that *E. coli* O157:H7, whose infectious dose is low, perhaps as low as 10 to 100 cells, is also acid tolerant [1, 3]. This information indicates that acid tolerance is important to the pathogenicity of bacteria as well as their survival strategies in low pH environments.

Although, like many other foodborne pathogenic bacteria, *V. vulnificus* has to cope with ever-changing acidity in their growth environments to ensure developing illness, only a few studies have addressed the molecular mechanisms by which the bacterium can survive in low pH environments. Previously, we have cloned a 4.3-kb DNA fragment of *V. vulnificus*, which contains *cadBA* genes [21]. The *V. vulnifcius 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 [21].

However, until now, no definitive analysis of the mechanisms which modulate the expression of the *cadBA* operon has been reported. Accordingly, this study reports an investigation to characterize the regulation of the *cadBA* genes. Firstly, an open reading frame, *cadC*, was identified from upstream of the *cadBA*, and the nucleotide and deduced amino acid sequence of the *cadC* were analyzed. In addition, a *V. vulnificus* null mutant, in which the *cadC* gene was deleted, was constructed by allelic exchanges, and the possible roles of the CadC protein in regulation of the *cadBA* expression as well as in acid tolerance of *V. vulnificus* were explored.

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**Table 1.** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics[a] | Reference or Source |
|---|---|---|
| **Bacterial strains** | | |
| *V. vulnificus* | | |
| ATCC 29307 | Clinical isolate; virulent | Laboratory collection |
| MO6-24/O | Clinical isolate; virulent | Laboratory collection |
| JR309 | ATCC 29307 with *ΔcadC* | This study |
| *E. coli* | | |
| DH5α | *supE44 ΔlacU169 (ϕ80 lacZ* ΔM15*) hsdR17 recA1 endA1 gyrA96 relA1 relA1 thi-1 rel A1*; plasmid replication | Laboratory collection |
| SM10(*λpir*) | *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *λpir*; *oriT* of RP4 Km$^r$; host for π-requiring plasmids; conjugal donor | [18] |
| **Plasmids** | | |
| pGEM7zf(+) | pMB1 *ori*; Ap$^r$ | Promega |
| pLAFR3 | IncP *ori*; cosmid vector; Tc$^r$ | [24] |
| PDM4 | R6K *γori*; *sacB*; suicide vector; *oriT* of RP4; Cm$^r$ | [19] |
| pRK415 | IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc$^r$ | [11] |
| pJR990 | Cosmid containing *cadCBA* | This study |
| pJR9914 | 2.2-kb *cadA* fragment in pGEM7zf(+); Ap$^r$ | This study |
| pJR0311 | 1.3-kb *Nru*I, *Hin*dIII fragment containing of *cadC* in pGEM7zf(+); Ap$^r$ | This study |
| pJR0312 | 1.0-kb *ΔcadC* fragment in pGEM7zf(+); Ap$^r$ | This study |
| pJR0313 | 1.0-kb *ΔcadC* fragment in pDM4; Cm$^r$ | This study |
| pJR0012 | pRK415 with *cadC*; Tc$^r$ | This study |

[a]: Ap$^r$, ampicillin resistant; Cm$^r$, chloramphenicol resistant; Km$^r$, kanamycin resistant; Tc$^r$, tetracycline resistant.

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% (w/v) agar. Unless otherwise noted, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). For data analysis, averages and standard errors of the mean (SEM) were calculated from at least three independent determinations. The statistical significance of the difference among the *V. vulnificus* strains was evaluated using Student's unpaired *t* test (SAS software, SAS Institute Inc., Cary, NC, U.S.A.). Significance was accepted at $p<0.05$.

### Cloning and Identification of the *V. vulnificus cadC*

In a previous study, we identified and cloned *V. vulnificus cadBA* genes by a transposon-tagging method [21]. A cosmid library of *V. vulnificus* ATCC 29307 was screened using *cadB* DNA as a probe, and a colony showing a positive signal was isolated. The cosmid DNA, pJR990, was purified, and upstream of *cadB* was an open reading frame (ORF) (data not shown). The nucleotide sequences of the ORF in pJR990 were determined by primer walking (Korea Basic Science Research Center, Gwang-Ju, Korea). The nucleotide sequence revealed a coding region consisting of 1,581 nucleotides (data not shown). A database search for nucleotide sequences similar to those of the coding regions revealed a *cadC* gene cloned from *V. cholerae* with high levels of identity. The *cadC* from *V. cholerae* was 73% identical in nucleotide sequences with the coding region in pJR990 (data not shown). This suggested that the coding region is a homologue of the *cadC* gene reported from *V. cholerae*, a species closely related to *V. vulnificus*, and it was decided to name the coding region *cadC* of *V. vulnificus*.

### Amino Acid Sequence Analysis of *V. vulnificus* CadC

The amino acid sequence deduced from the *cadC* coding sequence revealed a protein, a CadC, as a putative transcription activator [17], composed of 526 amino acids with a theoretical molecular mass of 59,549 Da and a pI of 5.18. The amino acid composition and molecular weight of this CadC are similar to those of the CadC from other bacteria. The amino acid sequence of the *V. vulnificus* CadC were 22% to 78% identical to those of the CadC from *E. coli* and *V. cholerae*, respectively (Fig. 1). The predicted profile of the hydrophobicity proposed by Kyte and Doolittle [12, http://ca.expasy.org/cgi-bin/protscale.pl] is significantly similar to that of the CadC of *E. coli* and *V. cholerae* [26, 17], and is consistent with the fact that the CadC protein is a membrane spanning protein (data not shown). The two amino acid sequences of CadC from *V. vulnificus* and *V. cholerae* are similar at the higher level (78%, 420 out of 526 amino acids), and their identity appears constant throughout the entire protein (Fig. 1). However, the N-terminal extra 22 amino acid sequence is present within the 541-amino acid of CadC from *V. cholerae*.

It has been reported that the *V. cholerae* CadC protein is required for activation of *cadBA* transcription under conditions

**Fig. 1.** Sequence relatedness of CadC of *V. vulnificus*, *E. coli*, and *V. cholerae*.
Identical sequences are indicated with asterisks, and dashes represent missing sequences. Alignment was based on the amino acid sequences in the GenBank (NCBI) database and derived by the CLUSTRALW alignment program (http://www.ch.embnet.org/software/ClustalW.html).

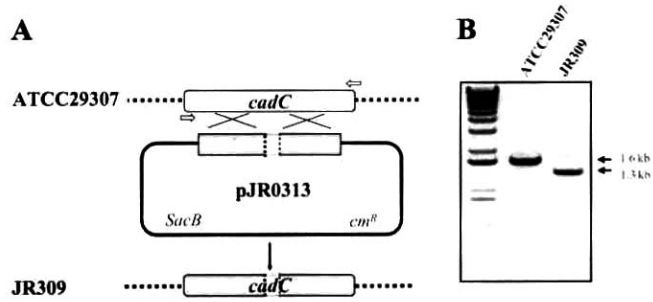


**Fig. 2.** Allelic-exchange procedure and construction of a *ΔcadC* isogenic mutant.
(A) Double homologous recombinations between strain ATCC 29307 and plasmid pJR0313 led to deletion of the *cadC* gene and resulted in construction of mutant JR309. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open boxes, the target *cadC* gene; open arrows, locations of the oligonucleotide primers used for confirmation of the *ΔcadC*; large X's represent genetic crossing over. Abbreviations: *SacB*, levansucrase gene; $Cm^R$, chloramphenicol resistance. (B) PCR analysis of ATCC 29307 and *ΔcadC* isogenic mutant generated by allelic exchange. Molecular size markers (1-kb ladder, GIBCO-BRL, Gaithersburg, MD, U.S.A.) appear in the end lane of the gel.

of low external pH [17]. The predicted DNA-binding domain of the CadC proteins resembles the DNA-binding domain of many prokaryotic transcriptional activators involved in environmental sensing [26]. Sequence analysis suggested that a DNA-binding domain in the amino-terminus of *V. vulnificus* CadC is separated from a periplasmic carboxy-terminal region by a single transmembrane segment (data not shown). Although the overall sequence between amino acids of *V. vulnificus* CadC and *E. coli* CadC was not substantially identical to each other, the amino acid sequences in the amino-terminal DNA-binding region seemed highly conserved (Fig. 1). It has been suggested that the *E. coli* CadC is also an acid-responsive, positive transcriptional regulator for the expression of *cadBA* and that it activates *cadBA* by directly binding to the promoter [16, 20, 26].

## Construction and Confirmation of the *V. vulnificus cadC* mutant

This study examined the role of the gene product of *cadC* gene in acid tolerance by constructing the *cadC* mutant of *V. vulnificus*. Construction of the mutant was carried out by the methods described previously [8, 9, 10, 13, 21], with only slight modifications. Briefly, a 1.6-kb DNA fragment carrying the whole *cadC* was amplified by PCR using primers CAD0001 (5'-CAACTGCAGCTAATGATTGGA-ATCTCTATTTTC-3') and CAD0002 (5'-CAGGATCCG-ATAAGAAAATGAACCGCTCTCA-3') and digested with *Nru*I and *Hin*dIII. The resulting 1.3-kb DNA fragment was isolated and ligated with pGEM7zf(+) to yield pJR0311. The *cadC* gene in pJR0311 was inactivated *in vitro* by deletion of one-fifth (300-bp) of the *cadC* open reading frame (Fig. 2, Table 1). The 1.0-kb *DcadC* from the resulting construct (pJR0312) was liberated and ligated with *Xho*I-*Sph*I-digested pDM4 [19], forming pJR0313.

Since pDM4 is a suicide vector containing the R6K γ origin of replication, SM10 (λ*pir*), *tra* (containing pJR0313) was used as a conjugal donor to generate the *ΔcadC* mutant of *V. vulnificus* ATCC 29307 by homologous recombination (Fig. 2A). The conjugation and isolation of the transconjugants were conducted using the methods previously described [8, 10, 21] and a double crossover, in which the wild-type *cadC* gene was replaced with the *ΔcadC* allele, was confirmed by PCR as shown in Fig. 2B. PCR analysis of genomic DNA from ATCC 29307 with primers CAD0001 and CAD0002 produced a 1.6-kb fragment (Fig. 2B), whereas genomic DNA from the *ΔcadC* mutant JR309 resulted in an amplified DNA fragment approximately 1.3-kb in length.

## Acid Tolerance of the *cadC* Mutant

Acid tolerance was assessed in a 10 mM sodium citrate buffer (pH 4.4) supplemented with 2.0% NaCl (SCBN) and filter sterilized. Cultures, grown for 4 h in LBS adjusted to a pH of either 5.8 or 7.6, were used to inoculate flasks containing 50 ml of SCBN to achieve a final concentration of ca. $10^5$ CFU/ml. Following inoculation, the flasks were incubated at 30°C with shaking (150 rpm) for 90 min. The samples were then removed and plated in duplicate on

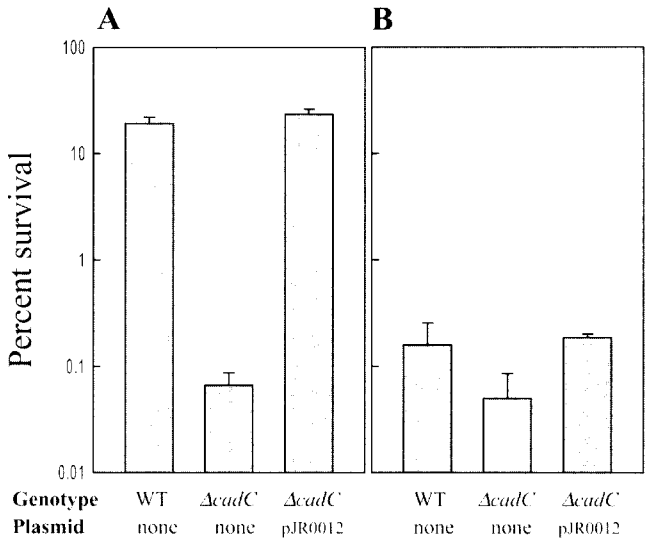


**Fig. 3.** Acid tolerance of ATCC 29307 and isogenic *ΔcadC* mutant.

Cultures, grown in LBS adjusted to a pH of 5.8 (A) or 7.6 (B), were used for acid challenge. The percentage of survivors was calculated using the CFU $ml^{-1}$ of wild-type (WT, ATCC 29307), and there was a 100% survival rate immediately after the challenge. Survivors of the *ΔcadC* mutant (JR301) complemented with a functional *cadC* (pJR0012) are also presented as indicated. Error bars represent the SEM.

LBS and incubated at 30°C. The percentage of survivors was calculated using the CFU/ml which was determined immediately after inoculation as 100% [21].

The survival of the wild-type (ATCC 29307) was significantly greater ($p<0.05$) than that of the *cadC* mutant (JR309) when cells grown at pH 5.8 were used for an acid challenge in SCBN (Fig. 3A). The parent strain decreased ca.1.0 $\log_{10}$ CFU $ml^{-1}$ (90%), while the JR309 strain decreased ca. 3.0 $\log_{10}$ CFU $ml^{-1}$ (99.9%) after a 90 min acid challenge. This indicated that the *cadC* mutant is more sensitive to acid than the wild-type, and that the CadC protein plays an important role in the acid tolerance of *V. vulnificus*.

To rule out the possibility that the decrease of acid tolerance by more than 100-fold resulted from polar effects of the *cadC* insertional mutation on downstream genes, the study examined if reintroduction of pJR0012 carrying recombinant *cadC* could complement the decrease in survival rate of JR309 cells. For this purpose, pJR0012 was constructed by subcloning the *cadC*, amplified by PCR using primers CAD0001 and CAD0002, and then digested with *Pst*I and *Bam*HI, and applied into the broad-host-range vector pRK415 [11] linearized with the same enzymes. The resulting plasmid was mobilized into *V. vulnificus* by conjugation. The survival rate of the JR309 (pJR0012) was restored to a level comparable to the wild-type level of ATCC 29307 (Fig. 3A). Therefore, the decreased acid tolerance of JR309 resulted from the inactivation of functional *cadC* rather than any polar effects on any genes downstream of *cadC*.

The survival of cells grown at pH 7.6 in acid challenges is shown in Fig. 3B. The survival of the wild-type and the *cadC* mutant is significantly lower (ca. 2 to 3-log CFU $ml^{-1}$ reduction) than the survival of cells grown at pH 5.8. Additionally, compared to the results with cells grown at pH 5.8, a smaller difference between the survival of the wild-type and the *cadC* mutant was observed (Fig. 3B). From these results, it was apparent that the contribution of the *cadC* for acid tolerance of *V. vulnificus* is substantial, but dependent on prior exposure to acidic pH.

**Effects of Mutation in *cadC* on the Expression of *cadBA***

The high level of identity found in the amino acid sequences and the close relatedness in hydrophobicity profiles for CadC protein from *V. vulnificus* and other Enterobacteriaceae indicated that they might perform a similar function in their physiological roles. To examine whether the expression of *V. vulnificus cadBA* is activated by CadC, the levels of *cadB* and *cadA* mRNA in the wild-type strain and JR309 were compared by Northern dot blot analysis.

For this purpose, two DNA probes, CADAP and CADBP, were labeled with [α-$^{32}$P]dCTP using the Prime-a-gene labeling system (Promega, Madison, WI, U.S.A.) and used for hybridizations [4, 10, 22]. The CADAP probe was prepared by labeling the 840-bp (base pairs) *Xba*I-*Eco*RI DNA fragment from pJR9914 (Table 1). The CADBP probe was prepared by labeling the 920-bp *Acc*I-*Hin*dIII DNA fragment internal to *cadB*. Total cellular RNAs from *V. vulnificus* strains were isolated using a Trizol reagent kit in accordance with the manufacturer's protocol (GIBCO-BRL, Gaithersburg, MD, U.S.A.). A series of reactions was performed according to standard procedures [23] with 20 μg of total RNA for Northern dot blot analysis.

When total RNA was isolated from cells grown at pH 5.8 and hybridized with the CadBP DNA probe, the *cadB* transcript was readily detectable only in the RNA of the wild-type strain, but not in JR309 (Fig. 4A). In a similar way, when CadAP was used as the DNA probe, the *cadA* transcript was not apparent in the RNA prepared from JR309. These results indicated that the expression of *cadBA* in *V. vulnificus* is under the positive control of CadC. However, when the level of *cadB* and *cadA* transcripts were looked for in the same amount of total RNA isolated from wild-type cells grown at pH 7.6, both transcripts were undetectable (Figs. 4A and 4B).

It has been previously observed that the activity of cadaverine/lysine antiporter and the lysine decarboxylase of *V. vulnificus* is induced by low pH, and reaches a maximum during the mid-exponential phase [21]. The results of the present study suggest that the pH-dependent variation of the activity of cadaverine/lysine antiporter and lysine decarboxylase resulted from changes in the level of *cadBA* transcription. In addition, it is apparent from the result that

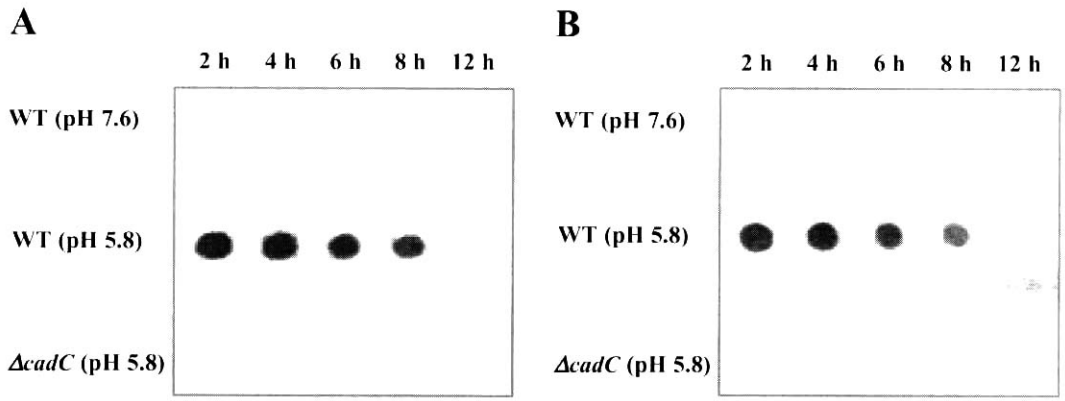


**Fig. 4.** Effects of mutation in *cadC* gene on the expression of *cadBA*.
For both panels, total RNAs were isolated at the indicated times from cultures of the wild type (WT) and *ΔcadC* mutant grown in LBS adjusted to a pH of 5.8 or 7.6 as indicated. The RNAs were transferred to nitrocellulose membrane (Roche, Indianapolis, IN, U.S.A.), and then hybridized to a [α-$^{32}$P] labelled DNA probe corresponding to the internal coding region of *cadB* (CadBP, panel A) or *cadA* (CadAP, panel B). The blots were visualized and quantified using a phosphorimage analyzer (BAS1500 model; Fuji Photo Film Co. Ltd, Tokyo, Japan) and the Image Gauge (version 3.12) program.

the CadC is not functional in cells grown at pH 7.6, and that the effect of CadC on the expression of *cadBA* is dependent on the pre-exposure of cells to acidic pH. Overall, these results lead us to propose that CadC exerted its effects on the production of lysine decarboxylase and cadaverine/lysine antiporter at the level of *cadBA* transcription and in a pH-dependent manner.

Previous studies with *E. coli* proposed that *cadA* is cotranscribed with *cadB* and that a promoter is responsible for pH-regulated expression of *cadBA* [15, 26]. Likewise, in *V. cholerae*, *cadBA* genes are transcribed as one transcriptional unit as observed in *E. coli*. However, *V. cholerae cadB* and *cadA* are also transcribed monocistronically, and the *cadA* also possesses an independent promoter [17]. In contrast to this, until now, no definitive analysis of the *V. vulnificus cadBA* expression has been reported at a molecular level. Neither the promoter(s) of the *cadBA* nor the numbers and types of transcript for *cadBA* have been previously identified. However, it was noted that variations in the level of *cadB* and *cadA* transcripts, which were observed in cells grown at different pHs, manifested a similar pattern (Figs. 4A and 4B). One possible explanation for this parallel variation in *cadB* transcript and *cadA* transcript is that the *cadBA* is one transcriptional unit, and the expression of *cadBA* is directed by the same promoter(s) which is elevated by CadC at a low pH. This assumption is in agreement with this study's previous observation that insertional mutation in *cadB* gives the effect of polarity on *cadA* [21].

The expression of the *E. coli cadBA* operon is regulated by CadC, as a function of pH [20, 26]. Likewise, CadC positively regulates the expression of *cadBA* genes in *V. cholerae* [17]. The data presented here suggest that the pH-dependent activity of CadC plays the major, if not sole, role in the induction of *V. vulnificus cadBA* transcription at acidic pH. However, the molecular mechanism by which *cadC* functions to activate the *cadBA* promoter is not clear. Until now, the question of whether CadC directly or indirectly affects the expression of *cadBA* has not yet been addressed. A working model, which explains how the transcription of *cadBA* is regulated by pH, also shows that the expression of *cadC* is altered at different pHs. Since neither the location nor the activity of the promoter of the *cadC* has been analyzed, this hypothesis needs further experimental tests. Another possible model is that the amino terminal domain of CadC is in the cytoplasm, with the other domain(s) localized in the outer face of the cytoplasmic membrane or periplasmic space. The outer domain(s) senses the changes in periplasmic pH and would result in a signal, such as conformational changes, transmittable to the cytoplasmic DNA binding domain. Then, the DNA-binding domain of CadC in turn would interact with the *cadBA* promoter. However, a direct interaction between CadC and *cadBA* promoter DNA has not yet been demonstrated.

The nucleotide sequence of the *cadC* gene of *V. vulnificus* ATCC 29307 was deposited into the GenBank under accession number AF324470.

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