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Lysine Decarboxylase Expression by *Vibrio vulnificus* Is Induced by SoxR in Response to Superoxide Stress▽

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Lysine decarboxylase expression by *Vibrio vulnificus*, which is up-regulated by CadC in response to acid stress, is also induced by SoxR in response to superoxide stress. SoxR binds to the promoter region of the *cadBA* operon, coding for a lysine-cadaverine antiporter (CadB) and a lysine decarboxylase (CadA). The induction of *cadBA* transcription by SoxR is independent of CadC. Cadaverine, which neutralizes the external medium, also appears to scavenge superoxide radicals, since increasing cellular cadaverine by elevating the gene dosage of *cadBA* significantly diminished the induction of Mn-containing superoxide dismutase under methyl viologen-induced oxidative stress. Consistently, a lack of cadaverine caused by mutation in *cadA* resulted in low tolerance to oxidative stress compared with that of the wild type.

Vibrio vulnificus is a pathogen that may cause food-borne gastroenteritis. Resistance to acid stress is an important virulence factor of many enteric bacteria, including *V. vulnificus*, *Vibrio cholerae*, and *Escherichia coli*. Multiple effects of low external pH on physiological responses have been documented for *E. coli* (for a review, see reference 3). When exposed to low pH, one of the most striking responses is the induction of amino acid decarboxylases which form amines from their respective substrates (for a review, see reference 33). Glutamate decarboxylase and lysine decarboxylase are typical examples that have been studied in *E. coli.* Lysine decarboxylase is also required for the acid tolerance of *V. vulnificus* and *V. cholerae* (17, 26). The neutralization of external pH by amine is known to protect cells from acid stress. Polyamines are also associated with protection of cells from the toxic effects of oxygen (4, 6, 20, 34).

The transcription of *cadBA*, which codes for a lysine-cadaverine antiporter and an inducible lysine decarboxylase, respectively, is activated in acid environments. CadC, a membrane-bound protein whose gene lies upstream from *cadBA*, has been identified as a positive regulator of *cadBA* expression (18, 21). A lack of cadaverine caused by mutation in *cadA* resulted in low tolerance to low pH (26). A *cadC* mutant of *V. vulnificus*, whose lysine decarboxylase activity is significantly decreased at low pH, also showed low tolerance to low pH (25, 27).

Previously, we found that the cellular superoxide level is elevated when *V. vulnificus* is exposed to low pH (13). The transcription of *sodA*, coding for Mn-containing superoxide dismutase (MnSOD), is activated by SoxR in acid environments. Accordingly, mutations in *soxR* or *sodA* resulted in low tolerance to low pH. An increase of cytosolic SOD activity through MnSOD induction is essential for *V. vulnificus* to withstand the acid challenge (13).

Although *cadBA* expression by *V. vulnificus* is largely regulated by CadC, the lysine decarboxylase activity of a *cadC* mutant was still induced at low pH, to yield 25% of the wild-type level. This result indicates another regulatory mechanism for the enzyme induction, which is independent of CadC. Since superoxide stress was shown to build up in an acid environment, it was determined whether lysine decarboxylase of *V. vulnificus* is induced under superoxide stress. Indeed, *cadBA* transcription is increased by methyl viologen (MV) treatment, but no such response was observed in *soxR* mutant. SoxR binds to the promoter region of the *cadBA* operon. Thus, lysine decarboxylase expression by *V. vulnificus*, which is activated by CadC in response to acid stress, is also induced by SoxR in response to superoxide stress. Consistently, a *cadC soxR* double mutant barely showed lysine decarboxylase activity in an acid environment. CadC and SoxR regulate *cadBA* expression independently. Cadaverine appears to scavenge superoxide radicals, since MnSOD induction under superoxide stress was significantly reduced by an increase of cadaverine formation in *V. vulnificus*. The antioxidant role of cadaverine was further corroborated by the higher sensitivity of a *cadA* mutant to MV-induced oxidative stress compared with the wild type.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *V. vulnificus* was grown at 30°C in Luria-Bertani (LB) medium (28) supplemented with 2% (wt/vol) NaCl (LBS) (pH 7.5). *E. coli* was grown at 37°C in LB medium. When appropriate, antibiotics were added at concentrations described previously (13). Cell growth measurement and growth transition to LBS (pH 5.0) were carried out as described previously (13). The same transfer to LBS (pH 7.5) containing 3 mM MV was performed to examine *cadBA* expression in response to superoxide stress. The experiments were repeated at least three times, yielding similar results; the averages from three independent experiments are shown.

Conjugation. pRK415- and pDM4-derived plasmids were transformed into *E. coli* S17-1 and S17-1λ*pir*, respectively, and were subsequently mobilized into *V. vulnificus* by conjugation as described previously (13).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
V. vulnificus		
AR	ATCC 29307, Rif^r	10
JR203	ATCC 29307, *cadA*::*nptI* Km^r	26
JR309	AR, Δ*cadC*	25
SR1	AR, *soxR*::*aph* Km^r	13
CSR1	JR309, Δ*cadC soxR*::*aph* Km^r	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	8
S17-1	C600::RP4 2-(Tc::Mu) (Km::Tn7) *thi pro hsdR hsdM*$^+$ *recA*	30
S17-1λ*pir*	λ*pir* lysogen of S17-1	30
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)	Stratagene
Plasmids		
pDM4	*ori* R6K Mob RP4; Cm^r	19
pDMSXR	pDM4 + 3.1-kb fragment containing *soxR*::*aph*; Km^r Cm^r	13
pRK415	*ori* IncP Mob RP4 *lacZ*α; Tc^r	11
pRKCAT	pRK415 with *cat*; Sm^r/Sp^r Tc^r	13
pCB367	pRK415 + *cadB*::*cat* fusion; 456-bp *cadBA* DNA from −367 to +89; Sm^r/Sp^r Tc^r	This study
pCB097	pRK415 + *cadB*::*cat* fusion; 186-bp *cadBA* DNA from −97 to +89; Sm^r/Sp^r Tc^r	This study
pRKCadBA	pRK415 + 3.8-kb *cadBA* DNA from −10; Tc^r	This study
pRKCadC	pRK415 + 2.1-kb DNA containing *cadC*; Tc^r	This study
pRKSoxR	pRK415 + 0.6-kb DNA containing *soxR*; Tc^r	This study
pMAL-p2E	*ori* M13, MBP fusion vector; Ap^r	NEB
pMAL-SXR	pMAL-p2E + 0.6-kb DNA containing *soxR*; Ap^r	This study

Lysine decarboxylase activity and cadaverine determination. The lysine decarboxylase activity of *V. vulnificus* and the cadaverine level in culture medium were determined as described previously (13, 15, 26). The enzyme reaction was monitored by measuring the absorbance at 340 nm. Specific activities were calculated as $1{,}000 \times A_{340}$ per min (units) per A_{600} (15).

Detection and quantification of SOD activity. Preparation of cell extracts, electrophoresis on a native polyacrylamide (12%) gel, and staining of SOD activity were performed as described previously (2). The relative SOD activities between samples were also quantified by scanning the gel with the Tina 2.0 program of the BIO-Imaging analyzer (Fuji, Japan). Proteins were determined by a modified Lowry method using bovine serum albumin as a standard (16).

Construction of *cadB*::*cat* fusion and CAT assay. A 456-bp DNA fragment extending from position −367 to +89 (+1 is the 5′ end of the *cadBA* transcript [27]) was PCR amplified using forward primer F1 and reverse primer R1; F1 (5′-CCTAG**CTGCAG**CGCATTT-3′ [mutated sequence is underlined, unless noted otherwise]) contains a PstI site (boldface) and R1 (5′-CGACACC**TCTA GA**GGCAA-3′) contains an XbaI site (boldface). Another 186-bp DNA fragment spanning from position −97 to +89 was PCR amplified using forward primer F2 and reverse primer R1 described above; F2 (5′-AAT**CTGCAG**TTCG AA-3′) contains a PstI site (boldface). The PCR products were digested with PstI and XbaI and cloned into the PstI/XbaI sites of pRKCAT containing *cat* (13) to generate *cadB*::*cat* fusion constructs of pCB367 and pCB097 (Table 1). The plasmids contain transcription-translation stop Ω DNA (Sm^r/Sp^r) (24) at the border between the vector and *cadB* upstream DNA. The recombinant plasmids were mobilized into *V. vulnificus* by conjugation as described above. Chloramphenicol (Cm) acetyltransferase (CAT) assay was performed as described previously (13, 29). Activity was expressed as nanomoles of Cm acetylated $minute^{-1}$ $milligram\ of\ protein^{-1}$ (29).

RNA isolation and primer extension analysis. Total RNA was isolated from *V. vulnificus* as described previously (12). The primer 5′-CTCGCCAGTGTAGAA G-3′, representing the coding strand of *cadB* between codons 31 and 35, was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega, Madison, WI). The products of the extension reaction were analyzed on an 8.3 M urea–8% polyacrylamide sequencing gel. The nucleotide sequence was determined by the dideoxy termination reaction with a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Construction of *cadC soxR* double mutant. pDM4-derived recombinant plasmid pDMSXR (13) was used for *soxR* disruption in *V. vulnificus cadC* mutant JR309 (25). The plasmid was transformed into *E. coli* S17-1λ*pir* and mobilized into JR309 as described previously (13). Conjugants carrying a single crossover were obtained by selecting colonies on LBS containing Cm, kanamycin (Km), and rifampin (Rif). The *cadC soxR* double mutants showing indications of double crossover (Cm^s Km^r Rif^r) were isolated on LBS agar plates supplemented with 10% sucrose. The DNA replacement was confirmed by Southern hybridization analysis (28).

Overexpression and purification of SoxR by using a construct fused to maltose-binding protein (MBP). In order to clone *soxR* into the expression vector pMAL-p2E (New England Biolabs, Beverly, MA), the initiation and stop codons of SoxR were modified to have EcoRI and HindIII sites, respectively. Forward primer F3 (5′-GGAAGC**GAATTC**GACATC-3′ [mutated sequence is underlined, unless otherwise noted]), having the SoxR initiation codon changed into an EcoRI site (boldface), was used in PCR with reverse primer R2 (5′-GATA**AA GCTT**AGCTGGCTAAC-3′), containing a HindIII site (boldface) that had been mutated from its stop codon. The PCR product was digested with EcoRI and HindIII and cloned into pMAL-p2E to generate pMAL-SXR. Sequence analysis confirmed an in-frame insertion into the plasmid.

When *E. coli* BL21(DE3) containing pMAL-SXR grew to an A_{600} of between 0.6 and 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at 0.1 mM for the overexpression of MBP-SoxR fusion protein. Cells were harvested 2 h after IPTG induction, disrupted by sonication, and centrifuged at 4°C to obtain cell extracts. An MBP fusion protein of approximately 59 kDa was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) and purified using amylose resin (New England Biolabs, Beverly, MA) as described previously (28). *V. vulnificus* SoxR (17 kDa on SDS-PAGE) was further purified after digestion of the fusion protein with enterokinase, followed by MBP binding to amylose resin (28). The purified SoxR was assessed for purity and size by SDS-PAGE.

Gel mobility shift assay. The DNA fragments to be run for the gel mobility shift assay were labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega, Madison, WI). The DNA probes (approximately 10^4 cpm) were incubated for 10 min at 25°C with various amounts of purified SoxR in a previously described buffer comprising 12.5 mM Tris-HCl (pH 7.5), 5% glycerol, 62.5 mM KCl, 0.75 mM dithiothreitol, 5 mM $MgCl_2$, and 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech, Piscataway, NJ) (12, 14). The reaction mixtures were analyzed using 5% nondenaturing polyacrylamide gels as described previously (12, 14).

Survival under superoxide stress. Tolerance to oxidative stress was examined essentially in the same way as described for tolerance to low pH (13). Cells were

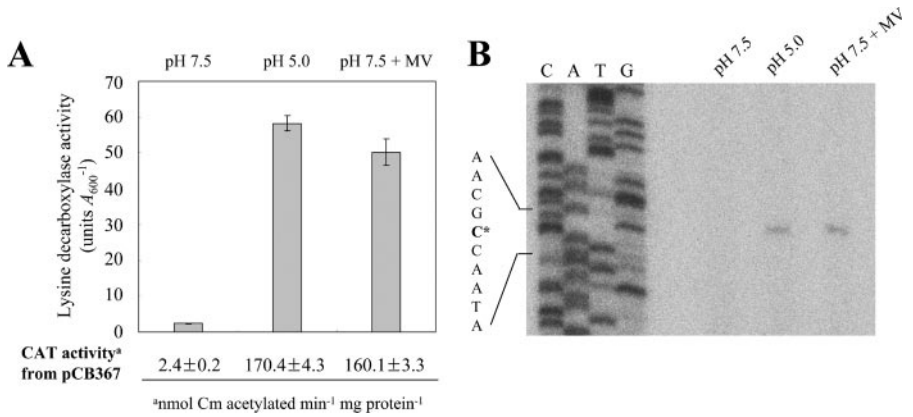

FIG. 1. Lysine decarboxylase expression by *V. vulnificus* under acid stress and superoxide stress. (A) Lysine decarboxylase activity of wild-type cells (AR) after growth transition to either LBS (pH 7.5) supplemented with 3 mM MV or LBS adjusted to pH 5.0. The growth transition to LBS (pH 7.5) was included as a control. Lysine decarboxylase activities were measured every hour, and only the maximum activities, which were observed 4 h, 2 h, and 4 h after transfer to LBS (pH 7.5), LBS (pH 5.0), and LBS (pH 7.5) supplemented with 3 mM MV, respectively, are shown. CAT activity from pCB367 containing a *cadB*::*cat* transcriptional fusion construct is shown. The error bars correspond to the standard deviations of the means, and CAT activity data represent means ± standard deviations. (B) Mapping of the 5′ end of the *cadBA* transcript of wild-type cells (AR) under acid stress and superoxide stress. Cells were harvested at 4 h, 2 h, and 4 h, after transfer to LBS (pH 7.5), LBS (pH 5.0), and LBS (pH 7.5) supplemented with 3 mM MV, respectively. The same ^{32}P-labeled oligonucleotide was used to generate the sequence ladder (lanes C, A, T, and G). The DNA sequence of the noncoding strand is illustrated on the left, with the 5′ end of the transcript marked with an asterisk.

grown to late logarithmic phase (A_{600}, ~4.0) in LBS (pH 7.5), and an aliquot (0.5 ml) was then harvested and inoculated into LBS (pH 7.5) supplemented with 3 mM MV. The initial A_{600} was close to 0.1. Cells were incubated for 4 h for the induction of lysine decarboxylase and then harvested and washed with phosphate-buffered saline (PBS) (pH 7.5) (28), followed by suspension to a final concentration of 10^5 CFU ml^{-1} in the same buffer containing 3 mM MV. A control experiment was performed with PBS (pH 7.5) without MV. Cell suspensions were incubated at 30°C with shaking (13). Samples were taken intermittently for 90 min, and viable counts (CFU/ml) were determined by plating dilutions of cells on LBS (pH 7.5) agar plates. Survival was expressed as the percentage of the initial CFU. The experiments were repeated three times, yielding similar results; data shown are representative of triplicate experiments.

RESULTS

Lysine decarboxylase expression by *V. vulnificus*, which is up-regulated under acid stress, is also induced under superoxide stress. The lysine decarboxylase induction of *V. vulnificus* in an acid environment is regulated at the level of *cadBA* transcription (27). CadC, a putative transmembrane protein, activates the transcription of *cadBA* in a pH-dependent manner (25, 27). Since superoxide stress was shown to build up at low pH (13), it was determined whether lysine decarboxylase induction was affected by oxidative stress. *V. vulnificus* grown exponentially was inoculated into fresh LBS (pH 7.5) supplemented with 3 mM MV or fresh LBS adjusted to pH 5.0 as described in Materials and Methods. Cells began to grow after lag phases of approximately 4 h in both transitions, whereas no lag occurred after inoculation into LBS (pH 7.5), as described previously (13). Cells were measured for lysine decarboxylase activity after transition, and the maximum ones are shown (Fig. 1A). As previously observed (13), lysine decarboxylase activity was highly induced during the lag period after the transfer to low pH (Fig. 1A). Remarkably, similar induction of the enzyme activity was also observed during the lag after transfer to medium (pH 7.5) supplemented with MV, whereas no induction was detected without MV (Fig. 1A). *cadA* expression was also examined using the *cadB*::*cat* transcriptional fusion construct pCB367 (Fig. 1A and 2), since *cadBA* genes of *V. vulnificus* are transcribed monocistronically (27). The plasmid contains a 367-bp regulatory DNA upstream from the 5′ end of the *cadBA* transcript (27), which also includes a CadC-binding domain centered at position −233.5 (+1 is the 5′ end of the *cadBA* transcript, unless stated otherwise). The CAT activity from the *cadB*::*cat* fusion construct pCB367 in the wild-type cells, which was up-regulated in an acid environment, was also induced in the presence of MV (Fig. 1A). Hydrogen peroxide (100 μM) treatment did not result in any induction of lysine decarboxylase expression (data not shown). The results argue that lysine decarboxylase expression by *V. vulnificus*, which is

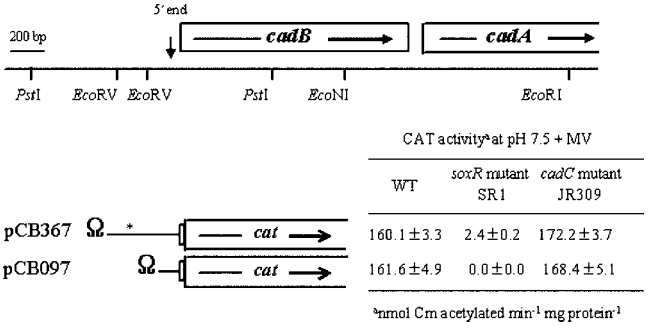

FIG. 2. CAT activity from a *cadB*::*cat* fusion. pCB367 has the 456 bp of *cadBA* DNA extending from −367 to +89 (+1 is the 5′ end of the *cadBA* transcript), whereas pCB097 harbors the 186 bp of *cadBA* DNA extending from −97 to +89. Transcription-translation stop Ω DNA (Sm^r/Sp^r) (24) is inserted at the border between the vector and *cadB* regulatory DNA. The CadC-binding domain is shown with an asterisk. The plasmids were mobilized into the wild type (AR) (WT), *soxR* mutant SR1, and *cadC* mutant JR309, and CAT activities were determined 4 h after growth transition to LBS (pH 7.5) supplemented with 3 mM MV. CAT activity data represent means ± standard deviations.

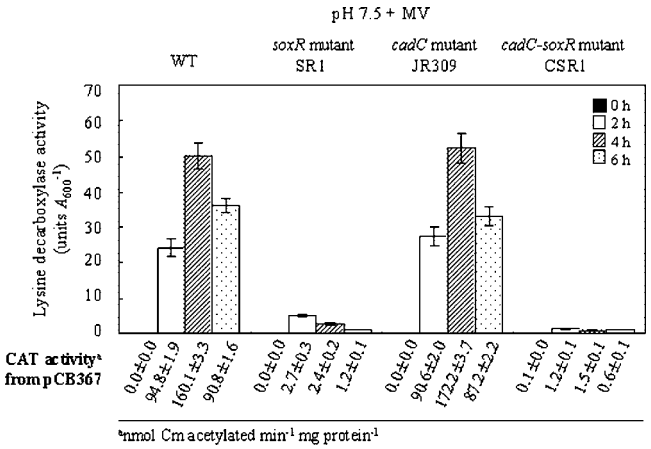

FIG. 3. Expression of lysine decarboxylase of the wild type (AR) (WT), *soxR* mutant SR1, *cadC* mutant JR309, and *cadC soxR* double mutant CSR1 after growth transition to LBS (pH 7.5) supplemented with 3 mM MV. Lysine decarboxylase activity was examined with cells harvested at 0 h, 2 h, 4 h, and 6 h after transfer. CAT activity from pCB367 was determined at the time points indicated. The error bars correspond to the standard deviations of the means, and CAT activity data represent means ± standard deviations.

regulated at the level of *cadBA* transcription, is highly induced in response to superoxide stress.

We examined whether *cadBA* transcription under superoxide stress employs the same promoter as that for transcription under acid stress (27). The 5′ end of the *cadBA* transcript from the total RNA of cells grown in the presence of MV was mapped at 54 nucleotides upstream from the CadB initiation codon (Fig. 1B), which is the same as determined with cells grown in an acid environment (Fig. 1B) (27). The results suggest the same promoter is used for the expression of *cadBA* under both acid stress and superoxide stress. No extension product was observed without MV at pH 7.5 (Fig. 1B).

Lysine decarboxylase induction in response to superoxide stress is regulated by SoxR. When *V. vulnificus* was treated with other superoxide generators, such as menadione (4 mM) and plumbagin (20 μM), the induced activities of lysine decarboxylase were similar to that observed with MV (3 mM) (data not shown). Lysine decarboxylase expression by *V. vulnificus* increased in proportion to the level of MV treatment; enzyme activities of 2.1, 23.4, 52.5, and 88.5 (units A_{600}^{-1}) were observed after treatment with MV at 0, 1, 3, and 6 mM, respectively. Thus, the level of superoxide stress determines the induced level of lysine decarboxylase.

It was determined whether CadC and/or the redox-responsive transcriptional regulator SoxR regulates *cadBA* transcription in response to superoxide stress. *cadC* mutant JR309 showed induction of lysine decarboxylase activity like that of the wild type after MV treatment (Fig. 3). Neither *soxR* mutant SR1 nor *cadC soxR* double mutant CSR1, however, showed such induction (Fig. 3). The lysine decarboxylase induction of mutants SR1 and CSR1 in response to superoxide stress was fully complemented with *soxR* DNA, whereas *cadC* DNA, which complemented *cadC* mutant JR309 (27), did not restore the enzyme induction in CSR1 (Table 2). The CAT activity from pCB367 reflects the lysine decarboxylase activity (Fig. 3). Therefore, SoxR induces *cadBA* transcription in response to superoxide stress, and the regulation is independent of CadC.

The *cadBA* upstream DNA responsible for SoxR regulation was narrowed down using another *cadB*::*cat* transcriptional fusion construct, pCB097 (Fig. 2). Its *cad* DNA contains 97 bp of regulatory DNA upstream from the 5′ end of the transcript and does not include the CadC-binding region. The plasmid was mobilized into the wild-type strain, *cadC* mutant JR309, and *soxR* mutant SR1, and CAT activities were measured under superoxide stress. The CAT activity from pCB097 was nearly the same as that from pCB367, and there were essentially no differences in the activity levels between the wild type and *cadC* mutant JR309. However, the CAT activities from the plasmids were barely detected in *soxR* mutant SR1. The results further corroborates that the SoxR-dependent *cadBA* transcription is independent of CadC, and the 97-bp regulatory DNA of pCB097 appears to be sufficient for SoxR regulation.

SoxR directly binds to the regulatory DNA of the *cadBA* operon. We examined whether SoxR regulates *cadBA* transcription by directly binding to the regulatory DNA of the operon or indirectly, possibly via another regulator such as SoxS, as observed in *E. coli*. No SoxS homolog, however, has been found in *V. vulnificus*. The gel mobility of the 161-bp *cadB* regulatory DNA (Fig. 4A, probe *a*) extending from position −97 to +64 was shifted by the purified SoxR from *V. vulnificus* (Fig. 4B). The retarded band was intensified in proportion to the amount of SoxR used. Neither the 128-bp DNA (from −166 to −38; probe *b*) nor the 129-bp DNA (from −10 to +119; probe *c*) was retarded by SoxR (Fig. 4A and B). Thus, the DNA region between −38 and −10 is required for SoxR binding. This result is consistent with that suggested by the CAT assays with pCB367 and pCB097 (Fig. 2).

Cellular cadaverine appears to acts as a superoxide scavenger. Polyamines are associated with many biochemical processes, such as the regulation of gene expression, the stabilization of chromatin, and the prevention of DNA damage (6, 33, 35). Polyamines, which may directly scavenge oxygen radicals (7), have been also known to protect cells from the toxic effects of reactive oxygen such as hydrogen peroxide, singlet oxygen, and oxygen radicals (4, 6, 20, 34). We examined whether cadaverine acts as a superoxide scavenger in *V. vulnificus*. The gene dosage of *cadBA* was increased by maintaining plasmid pRKCadBA in *trans* in wild-type cells, and the resulting cells were subjected to growth transition to LBS (pH 7.5)

TABLE 2. Complementation of lysine decarboxylase induction of *V. vulnificus* mutants with *cadC* and *soxR* DNA under superoxide stress

Strain	Plasmid	Lysine decarboxylase activity[a] (units A_{600}^{-1})
Wild-type AR	pRK415	55.5 ± 2.9
soxR mutant SR1	pRK415	1.1 ± 0.1
	pRKSoxR	59.7 ± 2.2
cadC-soxR mutant CSR1	pRK415	1.2 ± 0.1
	pRKSoxR	52.1 ± 4.8
	pRKCadC	5.0 ± 0.3

[a] Enzyme activity was measured 4 h after growth transition to LBS (pH 7.5) supplemented with 3 mM MV. Data represent means ± standard deviations.

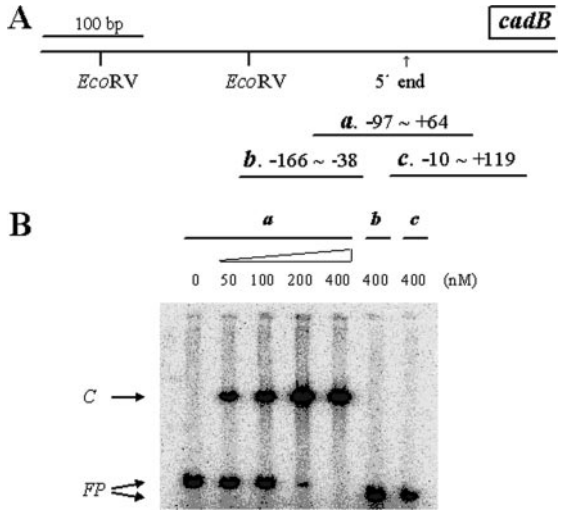

FIG. 4. Gel mobility shift assay of *cadB* regulatory DNA with purified SoxR. (A) Facing sets of 17-mers were used for PCR to obtain DNA probes *a* (from position −97 to +64), *b* (from −166 to −38), and *c* (from −10 to +119), which were then labeled with [γ-^{32}P]ATP. (B) Gel mobility shift assays were performed as described in Materials and Methods. Probe *a* was incubated with increasing concentrations of purified SoxR (0, 50, 100, 200, and 400 nM), whereas probes *b* and *c* were incubated with 400 nM purified SoxR protein. Free probes (FP) and binding complex (C) are indicated with arrows.

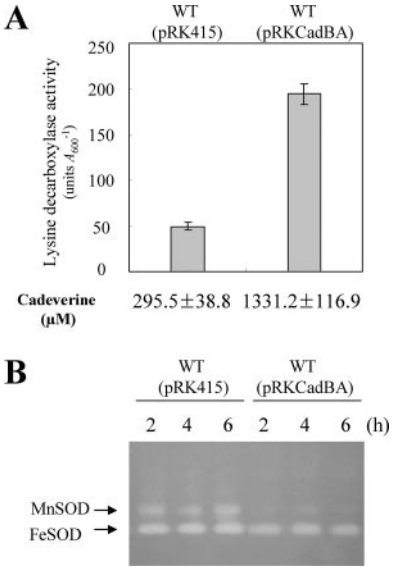

FIG. 5. Lysine decarboxylase, cadaverine excretion, and cytosolic SOD activities of wild-type (WT) cells (AR) harboring pRKCadBA in *trans* after growth transition to LBS (pH 7.5) supplemented with 3 mM MV. (A) The lysine decarboxylase activity of wild-type cells harboring either pRKCadBA or pRK415 was examined 4 h after transfer to LBS (pH 7.5) supplemented with 3 mM MV. Cadaverine in the culture supernatant was determined at the same time points. The error bars correspond to the standard deviations of the means, and cadaverine concentrations represent means ± standard deviations. (B) The cytosolic SOD activity of wild-type cells harboring either pRKCadBA or pRK415 was examined at 2 h, 4 h, and 6 h after the same transfer. The same amount (50 μg) of protein was loaded in each lane.

supplemented with 3 mM MV. A 3.8-kb insert DNA of pRKCadBA, which had been PCR amplified with a set of facing primers flanking *cadBA*, does not contain its own promoter, since the forward primer was the same as that used for probe *c* (Fig. 4). Thus, the lysine decarboxylase activity of wild-type cells containing pRKCadBA, whose insert was cloned in the same orientation as the *lac* promoter of pRK415, was not affected by MV treatment (data not shown) and was approximately four times as much as that of the control cells containing pRK415 (Fig. 5A).

Cadaverine concentrations in cell-free supernatants from cultures of wild-type cells containing pRKCadBA also increased approximately fourfold compared with that for control cells (Fig. 5A). The cadaverine in the culture supernatant should reflect its content in the cells. The MnSOD activity from the cells containing pRKCadBA was only 10 to 15% of that from the control cells under superoxide stress (Fig. 5B). Thus, the increase of cellular cadaverine of *V. vulnificus* decreased the induction of MnSOD under MV-induced oxidative stress (Fig. 5B), suggestive of radical scavenge by cadaverine in cells.

Although the increase of cadaverine formation in *V. vulnificus* decreased MnSOD induction under superoxide stress (Fig. 5), addition of exogenous cadaverine (2 mM) to the cultures of wild-type *V. vulnificus* and the *cadA* mutant did not affect MnSOD expression under superoxide stress (data not shown). The results implied that unlike the polyamine-deficient mutant of *E. coli*, where cadaverine is taken up into the cells by CadB at neutral pH (31), exogenous cadaverine may not go into *V. vulnificus* under the conditions examined in this work. It remains to be determined whether the cadaverine uptake would occur only in a polyamine-deficient mutant or whether there are any intrinsic differences in cadaverine uptake between *E. coli* and *V. vulnificus*.

Cadaverine is needed for superoxide stress tolerance of *V. vulnificus*. We examined whether cadaverine formation affects *V. vulnificus* survival under oxidative stress. The *cadA* mutant JR203 was more susceptible to MV-induced oxidative stress than the wild type (Fig. 6B), whereas no significant difference in survival between the wild type and the mutant was observed

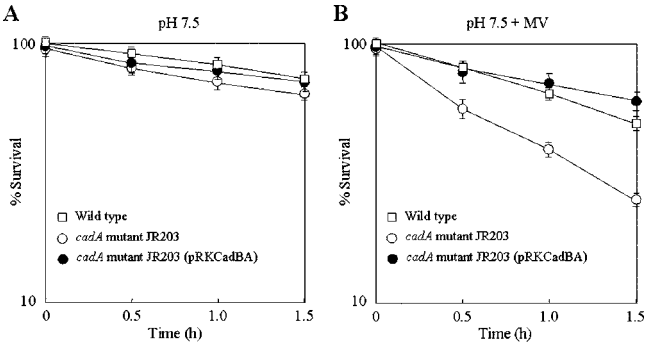

FIG. 6. Tolerance of a *cadA* mutant to superoxide stress. Cells grown in LBS (pH 7.5) supplemented with 3 mM MV were transferred to PBS (pH 7.5) containing 3 mM MV (B). Transfer to PBS (pH 7.5) without MV was included as a control (A). Viable cell counts of the wild type (AR) (WT) (□), *cadA* mutant JR203 (○), and *cadA* mutant JR203 containing pRKCadBA (●) were determined, and survival was expressed as percentage of the initial CFU. The error bars correspond to the standard deviations of the means.

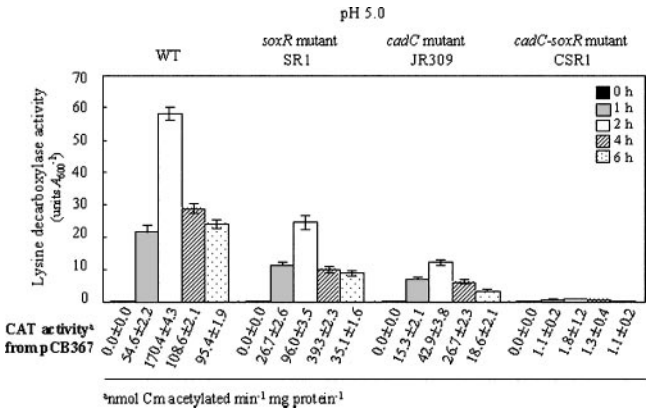

FIG. 7. Expression of lysine decarboxylase of the wild type (AR) (WT), *soxR* mutant SR1, *cadC* mutant JR309, and *cadC soxR* double mutant CSR1 after growth transition to LBS (pH 5.0). Lysine decarboxylase activity was examined with cells harvested 0 h, 1 h, 2 h, 4 h, and 6 h after transfer. CAT activity from pCB367 was determined at the time points indicated. The error bars correspond to the standard deviations of the means, and CAT activity data represent means ± standard deviations.

without MV (Fig. 6A). The low tolerance of the *cadA* mutant to MV-induced stress was complemented with pRKCadBA (Fig. 6B). Thus, cadaverine is needed for tolerance to superoxide stress in *V. vulnificus*.

Lysine decarboxylase induction at low pH is controlled by CadC and SoxR. When *V. vulnificus* is exposed to low pH, superoxide stress is generated in addition to the acid stress (13). Accordingly, it was determined whether the lysine decarboxylase expression by *V. vulnificus* in an acid environment is also regulated by SoxR. Indeed, the *soxR* mutant SR1 showed only 50% of the lysine decarboxylase activity of wild-type cells after a growth transition to pH 5.0, whereas the *cadC* mutant JR309 showed approximately 25% of the wild-type activity under the same conditions (Fig. 7). Thus, SoxR induces the enzyme expression in an acid environment, although CadC-mediated induction appears to be more significant. The enzyme activity was barely detected in the *cadC soxR* double mutant CSR1. The CAT activity from pCB367 also reflects the lysine decarboxylase activity. Therefore, the transcriptional induction of *cadBA* of *V. vulnificus* is regulated by both CadC and SoxR in acid environments.

DISCUSSION

As a mechanism to withstand acid challenge, the lysine decarboxylase of *V. vulnificus* is induced by CadC in response to acid stress. The production and excretion of cadaverine are essential for neutralization of the external pH, thus protecting cells from acid stress (17, 21, 26, 31). In this work we found that cadaverine also acts as an antioxidant to scavenge superoxide radicals. Furthermore, SoxR was shown to induce *cadBA* transcription, possibly by direct binding to the regulatory DNA of *cadBA*.

soxR and *soxS*, coding for two separate transcriptional activators, constitute a two-step activation cascade, in which SoxR activates *soxS* expression in response to superoxide and the increased level of SoxS induces target genes to protect cells from oxidative damage (1). The −10/−35 spacer of the *soxS* promoter, where a dimeric form of SoxR binds, constitutes the overlong 19-bp distance, in comparison with spacers of 17 ± 1 bp for most *E. coli* promoters. The topology of the *soxS* promoter is compensated by the conformational change of SoxR, which is accompanied by the oxidation of [2Fe-2S] clusters upon exposure to superoxide stress (5, 9, 22; for a review, see reference 23). The −10/−35 spacer of the *V. vulnificus cadBA* promoter is 20 bp (27), whereas that of its *sodA* promoter is 17 bp (13). Although both *sodA* and *cadBA* of *V. vulnificus* are induced by SoxR, only the *cadBA* promoter showed a gel electrophoretic mobility shift by the protein (Fig. 4); no such result was observed with the *sodA* promoter (13). The results indicate the direct involvement of SoxR in activating *cadBA* transcription.

However, the *cadBA* transcription by *V. vulnificus* at low pH is still significant in *soxR* mutant SR1, which means that CadC can activate *cadBA* transcription without SoxR. Considering that the CadC-binding domain is centered at −233.5, there should be a way for CadC to interact with the *cadBA* promoter. A putative integration host factor (IHF)-binding site was found between the CadC-binding domain and the *cadBA* promoter. The *cadBA* transcription by wild-type cells at low pH was reduced by approximately 40% in the IHF mutant (J.-S. Kim and J. K. Lee, unpublished results). The results suggest a possible interaction of CadC with RNA polymerase bound on the *cadBA* promoter through IHF-mediated DNA bending (for a review, see reference 32). The molecular basis for the interaction between CadC and RNA polymerase remains to be examined.

Unlike that of *V. vulnificus*, MnSOD of *E. coli* is not induced at low pH (13). When *E. coli* was treated with MV (3 mM), no significant induction of lysine decarboxylase was observed (Kim and Lee, unpublished results). Thus, the results in this work further support the intrinsic differences in acid tolerance response between *E. coli* and *V. vulnificus*.

When a spin trap, 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO), is included in the xanthine oxidase reaction, the most widely used biochemical source of superoxide, DMPO reacts with superoxide to reveal a signal characteristic of a DMPO-OH spin adduct, with a quartet signal showing intensity ratios of 1:2:2:1 and hyperfine coupling constants of $\alpha^N = \alpha\beta^H = 14.89$ G in electron paramagnetic resonance spectroscopy. The signal was significantly reduced by addition of *E. coli* MnSOD or cadaverine in the reaction mixture (Kim and Lee, unpublished results), thus confirming that cadaverine is capable of scavenging superoxide radicals. Consistently, the increase of cellular cadaverine decreased the expression of MnSOD under oxidative stress (Fig. 5), and a *V. vulnificus* mutant deficient in *cadA* is more susceptible to reactive oxygen than the wild type (Fig. 6). Thus, the results shown in this work further corroborate the antioxidant role of cadaverine.

Taken together, expression of *V. vulnificus* lysine decarboxylase, which is activated by CadC in response to acid stress, is also induced by SoxR in response to superoxide stress. Since superoxide stress is generated in acid environments, the lysine decarboxylase expression by *V. vulnificus* at low pH is also induced by SoxR. The cadaverine thus formed not only neutralizes the external medium (13, 26) but also scavenges superoxide radicals in *V. vulnificus*.

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