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Induction of Manganese-Containing Superoxide Dismutase Is Required for Acid Tolerance in *Vibrio vulnificus*

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The manganese-containing superoxide dismutase (MnSOD) of *Vibrio vulnificus*, normally detected after the onset of the stationary phase, is expressed during the lag that immediately follows the transfer of cells grown exponentially to a fresh medium acidified to pH 5.0, whereas Fe-containing SOD is constitutively expressed. The signal triggering the growth lag and MnSOD induction therein is not low pH but intracellular superoxide accumulated under these conditions, since addition of a superoxide scavenger not only shortened the lag but also abrogated the MnSOD induction. If the lysine decarboxylase reaction proceeds in the presence of sufficient lysine, the broth is rapidly neutralized to abolish the generation of oxidative stress. Accordingly, the acid tolerance response was examined without the addition of lysine. SoxR regulates MnSOD induction. Lack of MnSOD caused by mutations in *soxR* or *sodA* resulted in low tolerance to low pH. The *fur* mutant derepressing MnSOD showed better tolerance than the wild type. Thus, an increase in total cytosolic SOD activity through MnSOD induction is essential for the cell to withstand the acid challenge. The contribution of cuprozinc-containing SOD to acid tolerance is not significant compared with those of cytosolic SODs.

Vibrio vulnificus, a halophilic estuarine bacterium, is a virulent pathogen which can cause food-borne gastroenteritis or infect an open wound that is exposed to seawater harboring the organism. Among individuals with chronic liver disease, it can infect the bloodstream, causing a severe illness characterized by fever and chills, decrease in blood pressure (septic shock), and skin breakdown. A rapidly progressing, fulminating septicemia can result in death (for a review, see reference 3). *V. vulnificus* may travel through the acidic environment of the stomach to colonize the intestinal lumen. The bacterium, however, is sensitive to the acidic gastric fluid. Thus, the ability to tolerate gastric acidity is necessary for the pathogen to cause food-borne infections.

Upon exposure of enteric bacteria to low pH, several physiological responses ensue to cope with the acid stress. These responses contribute to the virulence of pathogens, including *Escherichia coli* and *Vibrio cholerae* (26, 30, 45). Multiple effects of acid stress on gene expression have been documented in *E. coli* (for a review, see reference 4). One of the most striking responses to low external pH is the generation of amines (γ-aminobutyrate, cadaverine, agmatine, and putrescine) and CO_2 by amino acid decarboxylases in the presence of their respective substrates (glutamate, lysine, arginine, and ornithine, respectively). Concomitant with the production and excretion of amines is some neutralization of the external pH, thus protecting cells from the acid stress (34, 48). The inhibition of porins by excreted cadaverine, and thereby porin-mediated outer membrane permeability as well, has been proposed as another mechanism that provides *E. coli* with the ability to survive in an acidic environment (41, 42). The induction of *cadBA* coding for a lysine-cadaverine antiporter (CadB) and a lysine decarboxylase (CadA) has been well illustrated as an acid pH-dependent response in *E. coli* and *V. cholerae* (30, 48). CadC, a membrane-bound protein whose gene lies upstream from *cadBA*, has been identified as a positive regulator of *cadBA* expression (31, 34). A contribution of CadBA to acid tolerance has also been demonstrated in *V. vulnificus* (40).

The system responsible for pH homeostasis of *Salmonella enterica* serovar Typhimurium during exposure to low pH is composed of a complex cascade of acid shock proteins (ASPs) that are thought to contribute to survival in an acidic environment (10). Several inducible amino acid decarboxylases are also included among the ASPs. Lysine decarboxylase, which contributes to pH homeostasis, is required for acid survival of *S. enterica* serovar Typhimurium (38). The acid tolerance response of *S. enterica* serovar Typhimurium is under the positive control of two global regulators, the iron-regulatory protein Fur (ferric uptake regulation) and an alternative sigma factor, σ^s (11, 24).

Interestingly, manganese-containing superoxide dismutase (MnSOD) is found among ASPs when bacterial cells, such as those of *Staphylococcus aureus*, *Streptococcus oralis*, and *Streptococcus mutans*, are exposed to low pH (7, 50, 51). An *S. aureus sodA* mutant lacking MnSOD was less able to survive acid stress (7). The precise mechanisms by which MnSOD is induced and involved in acid tolerance are not known. MnSOD and Fe-containing superoxide dismutase (FeSOD), key enzymes in the defense against the oxidative damage by superoxide (6), often have been found in the cytoplasm of prokary-

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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	Type strain	21
AR	ATCO 29307, Rif^r	23
HLM101	MO6-24/O, Δ*fur, fur*::*aph*; Km^r	23
FSA1	MO6-24/O, Δ*fur*, Δ*sodA*; Km^r	This study
KPR101	AR, Δ*rpoS*	37
SA1	AR, *sodA*::*aph*; Km^r	This study
SB1	AR, *sodB*::*aph*; Km^r	This study
SC1	AR, *sodC*::*aph*; Km^r	This study
SR1	AR, *soxR*::*aph*; Km^r	This study
JR203	ATCC 29307, *cadA*::*npt*; Km^r	40
E. coli		
DH5α	*supE*44 Δ*lacU*169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	12
S17-1	C600::RP4 2-(Tc::Mu)(Km::Tn7) *thi pro hsdR hsdM*$^+$ *recA*	46
S17-1λ*pir*	λ*pir* lysogen of S17-1	46
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)	Stratagene
Plasmids		
pRK415	*ori* IncP Mob RP4 *lacZa*; Tc^r	16
pSODAC	pRK415 + *sodA*::*cat* fusion; Tc^r	This study
pDM4	*ori* R6K Mob RP4; Cm^r	32
pDMSAKm	pDM4 + 3.0-kb fragment containing *sodA*::*aph*; Km^r, Cm^r	This study
pDMSAid	pDM4 + Δ*sodA*; 0.6-kb *Xba*I-*Xho*I fragment of pSAid; internally deleted (*Bgl*II-*Stu*I) from *sodA*; Cm^r	This study
pDMSB	pDM4 + 3.1-kb fragment containing *sodB*::*aph*; Km^r, Cm^r	This study
pDMSC	pDM4 + 3.1-kb fragment containing *sodC*::*aph*; Km^r, Cm^r	This study
pDMSXR	pDM4 + 2.8-kb fragment containing *soxR*::*aph*; Km^r, Cm^r	This study
pRKSA	pRK415 + 0.8-kb fragment containing *sodA*; Tc^r	This study
pRKSB	pRK415 + 0.8-kb fragment containing *sodB*; Tc^r	This study
pRKSC	pRK415 + 1.1-kb fragment containing *sodC*; Tc^r	This study

otic cells (49), whereas copper- and zinc-containing superoxide dismutase (CuZnSOD) has been found in the periplasm of several gram-negative bacteria, such as *E. coli* (14).

FeSOD of *V. vulnificus* is constitutively expressed, but its MnSOD is detected when iron is limited in culture medium, suggestive of Fur-mediated expression of MnSOD (19). We found that MnSOD is also induced under acidic conditions. When growing cells of *V. vulnificus* were transferred to medium acidified to pH 5.0, cell growth temporarily ceased (ca. 2- to 3-h lag). The cellular superoxide level was increased during the lag, and MnSOD was induced by the control of SoxR. In an effort to understand the contributions of SODs to acid tolerance of this pathogen, *sod* expression and the survival of *sod* mutants were examined under acidic conditions. The increase in cytosolic SOD activities through MnSOD induction appears to be important for cell survival at acidic pH.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *V. vulnificus* was grown at 30°C in Luria-Bertani (LB) medium (43) supplemented with 2% (wt/vol) NaCl (LBS), whose pH had been adjusted to pH 7.5. *E. coli* was grown at 37°C in LB. Cells harboring antibiotic resistance genes were selected using appropriate antibiotics; chloramphenicol (Cm), kanamycin (Km), tetracycline (Tc), and rifampin (Rif) were used at 2, 150, 2, and 50 μg/ml, respectively, for *V. vulnificus*, whereas ampicillin, streptomycin (Sm), spectinomycin (Sp), Cm, Km, and Tc (final concentrations of 50, 25, 20, 25, 25, and 10 μg/ml, respectively) were used for *E. coli*. Cell growth was monitored by measuring the culture absorbance at 600 nm (A_{600}). If the A_{600} of the *V. vulnificus* culture was larger than 0.7, it was diluted prior to measurement.

For growth transition, cells were first grown in LBS (pH 7.5) with sufficient aeration (35-ml culture in 300-ml flask at 250-rpm shake) up to logarithmic growth phase (A_{600}, ~2.0). An aliquot (0.5 ml) was then harvested and inoculated into LBS (pH 5.0) after a brief wash with the medium. The initial A_{600} value was generally close to 0.1. Cells were incubated with shaking as described above, and aliquots were taken intermittently for the measurement of cell growth, pH, SOD activity, and MnSOD expression, using *sodA*::*cat* fusion (see below). The same transfer to LBS (pH 7.5) was included as a control. The experiments were repeated three times, yielding similar results; a typical experiment is shown. In this work a pH of 5.0 was chosen deliberately to monitor the adaptive responses of cells to an acidic environment wherein they could still grow. At a pH less than 4.0, cell death occurs.

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 with minor modification (37). *E. coli* and *V. vulnificus* were grown exponentially until the A_{600} values reached 1.0 and 2.0, respectively. The donor (100 μl) and recipient (200 μl) were mixed, washed two times with LB, spotted on LB agar, and incubated at 37°C for 8 h. The mixture was suspended in an appropriate volume of LBS following a brief wash with the medium and plated on thiosulfate-citrate-bile salts-sucrose agar (5) to select *V. vulnificus* or on LBS containing Rif to select *V. vulnificus* AR.

Detection and quantification of SOD activity. Preparation of cell extracts, electrophoresis of a native polyacrylamide (12%) gel, and staining of SOD activity were performed as described previously (2). Cytosolic SOD activities in cell extracts were determined as described previously (29). KCN (2 mM) was included in the reaction mixture to inhibit CuZnSOD. The relative SOD levels between samples were also quantified by scanning the activity-stained gel using the Tina 2.0 program of a BIO-Imaging analyzer (FUJI, Japan). Proteins were determined by a modified Lowry method using bovine serum albumin as a standard (28).

Construction of *sodA*::*cat* fusion and chloramphenicol acetyltransferase (CAT) assay. A 1.6-kb XbaI/BamHI DNA fragment containing *cat* from the pCAT-basic vector (Promega, Madison, WI) was cloned into pRK415 to generate pRKCAT. A 452-bp DNA fragment extending from −407 to 45 relative to

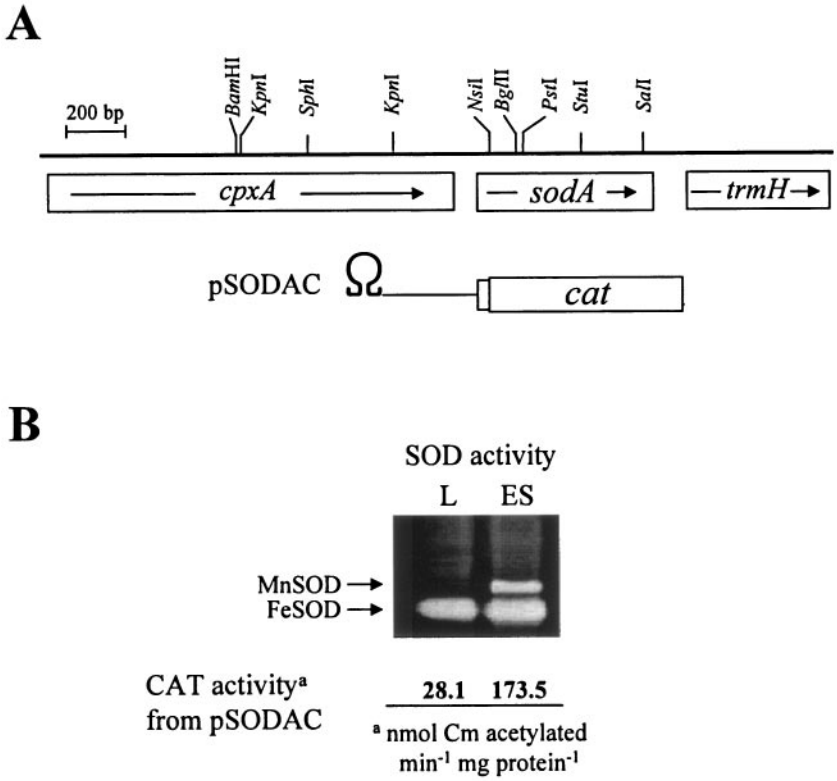

FIG. 1. CAT activities from *sodA*::*cat* fusion and expression of cytosolic SODs of *V. vulnificus*. (A) pSODAC has an insert in which *sodA* regulatory DNA is transcriptionally fused to *cat* and cloned downstream from transcription-translation stop Ω DNA (Sm^r/Sp^r) (36). *cpxA* coding for a sensor of the Cpx two-component system perceiving cell envelope stress and *trmH* coding for tRNA methyltransferase are shown. (B) Activities of MnSOD and FeSOD in extracts of *V. vulnificus* cells at logarithmic (L) (A_{600}, ~2.0) and early stationary (ES) (A_{600}, ~7.0) growth phases. The same amount of protein (50 μg) was loaded in each lane unless stated otherwise. CAT activities from pSODAC in the wild type harvested at the growth phases indicated are illustrated below the gel.

the MnSOD initiation codon was PCR amplified using two primers: the forward primer, 5′-CTAAG**CTGCAG**ATGAGC-3′ (mutated sequence underlined, unless otherwise noted), containing the PstI site (in bold), and the reverse primer, 5′-GTATGGC**TCTAGA**GCATCG-3′, containing the XbaI site (in bold). The PCR product was examined through DNA sequence analyses, digested with PstI and XbaI, and cloned into PstI/XbaI sites of pRKCAT to generate a *sodA*::*cat* fusion of pSODAC. The recombinant plasmid was mobilized through conjugation into *V. vulnificus* as described above.

The CAT assay was performed as described previously (44). The reaction mixture consisted of 0.1 mM acetyl-coenzyme A, 1 mM 5,5′-dithiobis-2-nitrobenzoic acid, 0.25 mM Cm in 1 ml 100 mM Tris-HCl (pH 7.8). The reaction was initiated by adding 20 μl cell extracts, followed by incubation at 30°C for 3 min, and monitored by measuring the absorbance at 412 nm. Activity was expressed as nmol Cm acetylated min^{-1} mg $protein^{-1}$ (44).

RNA isolation and Northern (RNA) hybridization analysis. Total RNA from *V. vulnificus* was extracted using a TRI reagent kit (MRC, Cincinnati, Ohio) according to the manufacturer's procedure. RNA quantification, electrophoretic separation of denatured RNA, blot transfer, labeling of the strand-specific RNA probe using [α-^{32}P]CTP, and hybridization with the probe were performed as described previously (17, 18).

Primer extension analysis. The primer 5′-GATGCTCAAGCTCACTG-3′, representing the coding strand of *sodA* between the 45th and 51st codons, was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega, Madison, WI). Total RNA from *V. vulnificus* at early stationary phase (A_{600}, ~7.0) was used for the extension reaction (17), and the products 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, Cleveland, Ohio).

Construction of mutants. (i) DNA for *sodA* disruption. A 761-bp DNA fragment extending from −114 to 647 relative to the MnSOD initiation codon was PCR amplified from genomic DNA using forward (5′-GTTGCGAGTCACCATTAC-3′) and reverse (5′-CGATAAGCGCACGGTGCTC-3′) primers and cloned into pGEM-T (Promega, Madison, WI) to generate pBSA. The PstI site (66th residue) within *sodA* was interrupted with 2.2-kb Ω DNA (Km^r) (8), and the resulting 3.0-kb XbaI/XhoI fragment was cloned into the suicide vector pDM4 (32) to generate pDMSAkm.

For internal deletion of *sodA* on the chromosome of HLM101 (*fur*::*aph*; Km^r) (23), a 173-bp BglII-StuI DNA (residues between 55th and 113th) within *sodA* was deleted from pBSA, followed by fill-in and ligation, and the resulting 0.6-kb XbaI/XhoI fragment was cloned into pDM4 to yield pDMSAid.

(ii) DNA for *sodB* disruption. A 909-bp DNA fragment extending from −284 to 625 relative to the FeSOD initiation codon was PCR amplified from genomic DNA using a forward (5′-GCTTCCTCGTTCACGG-3′) and reverse (5′-CTAA ATTGGAACTTAAG-3′) primers and cloned into pGEM-T. The XbaI site (57th residue) of *sodB* was interrupted with Ω DNA (Km^r), and the resulting 3.1-kb SacI/XhoI DNA fragment was cloned into pDM4 to yield pDMSB.

(iii) DNA for *sodC* disruption. From the cosmid library of *V. vulnificus* genomic DNA, a 1.1-kb BglII-MboI DNA fragment encompassing *sodC* coding for CuZnSOD was cloned into pGEM-T. The NsiI site (122nd residue) of *sodC* was interrupted with Ω DNA (Km^r), and the resulting 3.1-kb SacI/XbaI DNA fragment was cloned into pDM4 to yield pDMSC.

(iv) DNA for *soxR* disruption. A 585-bp DNA fragment extending from −80 to 505 relative to the SoxR initiation codon was PCR amplified from genomic DNA using a forward (5′-GCTCAACTTAACTTGAGG-3′) and reverse (5′-CTTGC CACCGCAAACGC-3′) primers and inserted into pGEM-T. The HincII site (17th residue) of *soxR* was interrupted with Ω DNA (Km^r), and the resulting 2.8-kb XbaI/XhoI DNA fragment was cloned into pDM4 to generate pDMSXR.

(v) Mutant selection. pDM4-derived recombinant plasmids were mobilized into *V. vulnificus* AR (for pDMSAkm, pDMSB, pDMSC, and pDMSXR) and *V. vulnificus* HLM101 (for pDMSAid) through conjugation as described above. Conjugants carrying a single crossover were selected using Cm. The mutants showing indications of double crossover (Cm^s) were isolated in the presence of

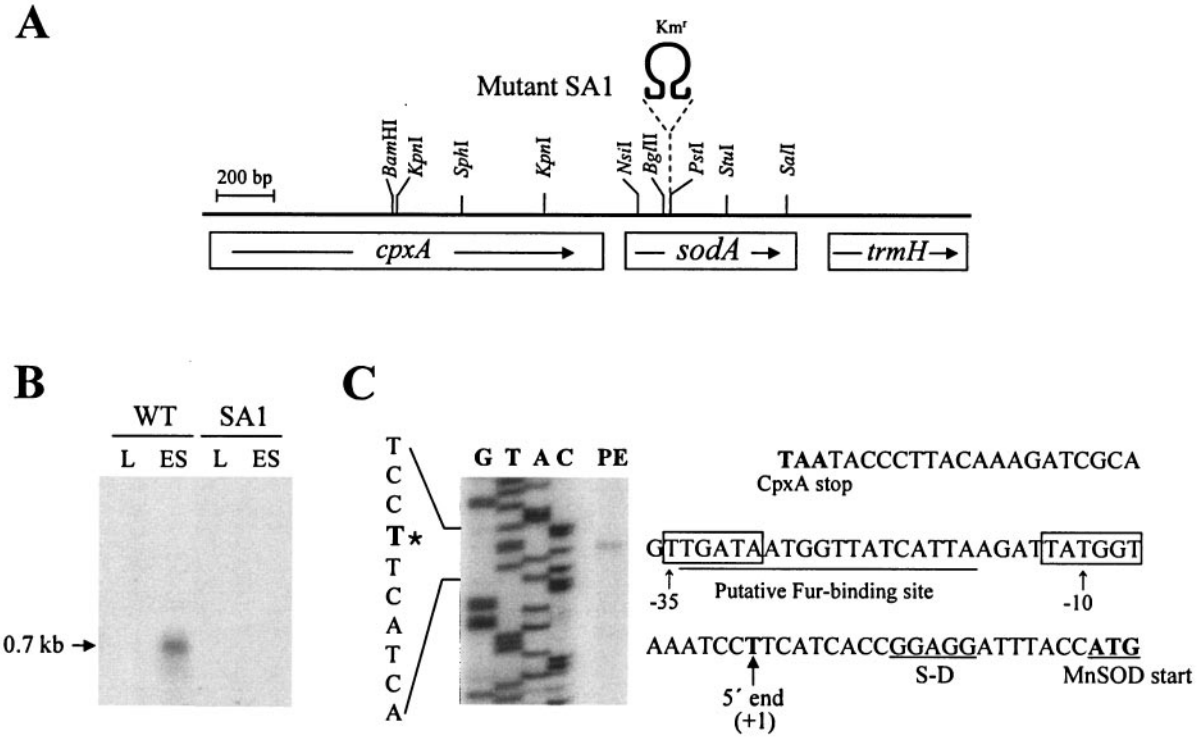

FIG. 2. *sodA* transcription. (A) *sodA* coding for MnSOD was interrupted with Ω DNA (Km^r) to generate mutant SA1. A 0.7-kb *sodA* transcript is indicated by an arrow below the restriction map. (B) Northern blot hybridization analysis with [α^{32}P]CTP-labeled RNA probes corresponding to a 520-bp NsiI-SalI DNA fragment specific to *sodA*. RNA was prepared from the wild type and SA1. L and ES denote the growth phases, as described in the Fig. 1 legend. (C) Mapping of 5′ end of *sodA* transcript by primer extension. Extension with RNA from the wild type at ES growth phase is shown in lane PE. The same ^{32}P-labeled oligonucleotide was used to generate the sequence ladder (lanes G, T, A, and C). DNA sequence is illustrated on the left, with the 5′ end of the transcript marked with an asterisk. The *sodA* regulatory DNA between the CpxA stop and MnSOD initiation codons is shown on the right. The −35 and −10 sequences typical of σ^{70}-type promoter (5′ end [**T**] of the transcript: +1) are boxed, and a putative binding site of the ferric uptake regulator (Fur) is underlined. A putative ribosome-binding site (S-D) is also shown.

10% sucrose. The chromosomal structures of the mutants were examined by Southern hybridization analysis (43).

Measurement of intracellular superoxide level. Cells were harvested, washed with phosphate-buffered saline (PBS) (43), and suspended in 50 mM potassium phosphate buffer (pH 7.3) supplemented with 2% NaCl. Cells (20 μl) were put in 0.4 ml of buffer containing 20 μl 2 mM bis-*N*-methylacridinium nitrate (lucigenin) (Sigma, St. Louis, MO), and luminescence was immediately measured using MicroLumatPlus LB 96V (Berthold Technologies GmbH & Co. KG) as described previously (27). The instrument was operated with a 5-s delay and 10-s integration time.

Lysine decarboxylase activity and cadaverine determination. The lysine decarboxylase activity of *V. vulnificus* and the cadaverine excreted in culture medium were assayed as described previously (25, 40). The enzyme reaction was monitored by measuring the absorbance at 340 nm. Specific activities were calculated as 1,000 × A_{340} per min (units) per A_{600} (25).

Survival at acid pH. Acid tolerance was examined as described previously (40). Cells were grown to logarithmic phase (A_{600}, ~2.0) in LBS (pH 7.5), harvested, washed with 10 mM sodium citrate buffer (pH 5.0) supplemented with 2% NaCl (SCBN) (40), and suspended in the same buffer to a final concentration of 10^5 CFU ml^{-1}. A control experiment was performed at pH 7.5, in which PBS (pH 7.5) was used instead of SCBN. Cell suspensions were incubated at 30°C with shaking as described above for cell growth. 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

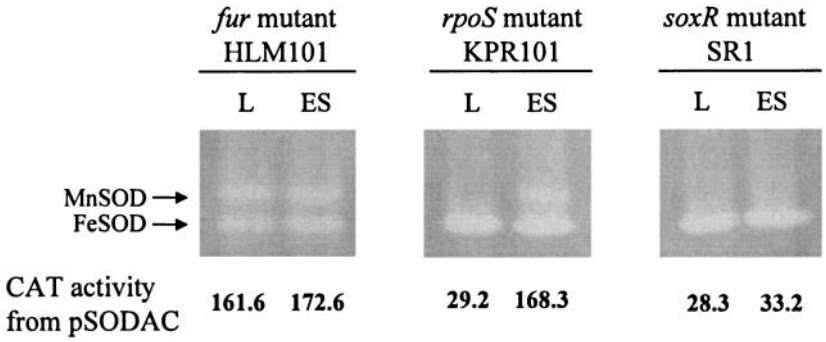

FIG. 3. Expression of cytosolic SODs and CAT activities from *sodA*::*cat* fusion in HLM101, KPR101, and SR1. L and ES denote the growth phases, as described in the Fig. 1 legend. The CAT activity unit is the same as in Fig. 1 unless otherwise specified.

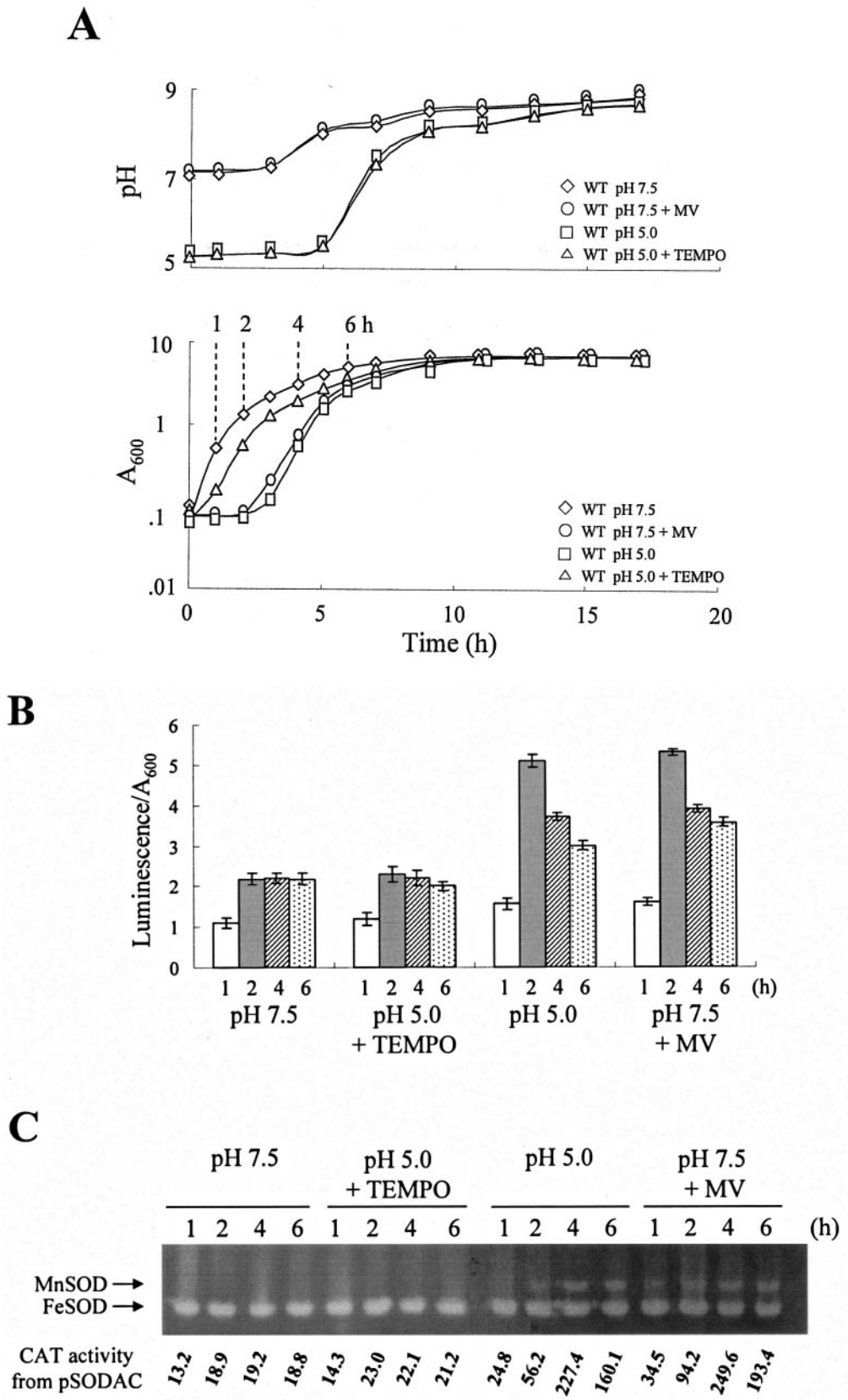

FIG. 4. Growth, cellular superoxide level, and SOD expression of the wild type after transition to LBS adjusted to pHs 7.5 and 5.0. (A) Cell growth and pH curves of the culture broth after transition to LBS (pH 7.5) (◇), LBS (pH 7.5) containing 3 mM MV (○), LBS (pH 5.0) (□), or LBS (pH 5.0) supplemented with 1 mM TEMPO (△). Time points (1, 2, 4, and 6 h) to harvest cells for the assay of superoxide level and MnSOD expression are indicated. (B) Detection of cellular superoxide using lucigenin. Cells were harvested at 1 (white bar), 2 (shaded bar), 4 (hatched bar), and 6 h (dotted bar). The standard deviations of the luminescence are shown on each bar. (C) Expression of cytosolic SODs and CAT activity from *sodA*::*cat* fusion were determined with cells harvested at the time points indicated.

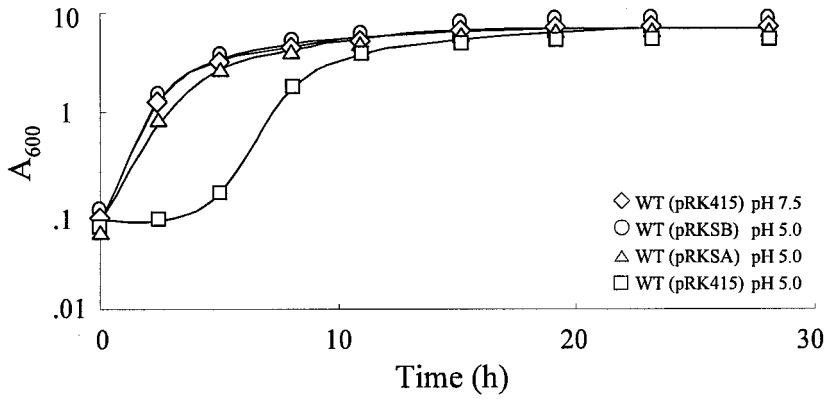

FIG. 5. Growth of the wild type containing *sod* genes in *trans* after transition to LBS (pH 5.0). Growth of the wild type containing *sodB* (○), *sodA* (△), and vector (pRK415) (□) in *trans*. The wild type containing pRK415 transferred to LBS (pH 7.5) (◇) is included as a control.

a percentage of the initial CFU. Data shown are representatives of triplicate experiments.

RESULTS

MnSOD expression of *V. vulnificus* is regulated transcriptionally. A constitutively expressed FeSOD is found in the cytoplasm of *V. vulnificus* together with an inducible MnSOD, which is expressed from the onset of stationary phase (Fig. 1B). Unlike *E. coli*, no MnSOD activity is found during the exponential growth of *V. vulnificus*. The bacterium also has a CuZnSOD in its periplasm, but its level is not high enough to appear on native gel with the amounts of proteins loaded. The *sodA*::*cat* transcriptional fusion construct, pSODAC (Fig. 1A), was maintained in *trans* in wild-type cells, and CAT activity was measured (Fig. 1B). The CAT activity from pSODAC at early stationary phase was approximately sixfold higher than in logarithmic phase. Thus, the growth-dependent expression of MnSOD appears to be regulated transcriptionally.

A 0.7-kb transcript was detected after hybridization of a *sodA*-specific probe to the total RNA from cells harvested at early stationary phase, whereas it was barely detectable from exponentially growing cells (Fig. 2B). No such transcript was found in the *sodA* mutant SA1 (Fig. 2A and B). The 5′ end of the transcript was mapped at 21 nucleotides upstream from the MnSOD initiation codon (Fig. 2C). Canonical sequences typical of σ^{70}-type promoters were found, and a putative binding site of ferric uptake regulator (Fur) (35), a repressor for *sodA* transcription in the presence of the Fe^{2+} ion, is located in the promoter region (Fig. 2C).

TABLE 2. Lysine decarboxylase activities of the wild type and *cadA* mutant JR203 in LBS (pH 7.5) and LBS (pH 5.0) after growth transition

Strain	pH[a]	Lysine decarboxylase activity (units A_{600}^{-1}) at[b]:			
		1	2	4	6
WT[d]	7.5	0.0	2.6	1.2	0.4
WT	5.0	22.0	55.2	26.2	21.6
WT[c]	5.0	35.2	123.0	51.8	44.2
JR203	7.5	0.0	0.1	0.0	0.0
JR203	5.0	0.0	1.8	0.2	0.2
JR203	5.0	0.0	1.7	0.2	0.2

[a] Medium pH after transition.
[b] 1, 2, 4, and 6 denote the time points (h) following the transition.
[c] Lysine (15 mM) was added to the medium right after the transition.
[d] WT, wild type.

MnSOD expression, positively regulated by SoxR and negatively regulated by Fur, is not affected by RpoS. MnSOD activity was not detected in extracts of the *soxR* mutant SR1 (Fig. 3), which indicates that SoxR is required as a transcriptional activator as in *E. coli* (52). Likewise, the CAT activity from pSODAC in the *soxR* mutant was not induced (Fig. 3). It was tested whether the alternative sigma factor σ^s, a globular regulator (20) for stress resistance in many bacteria, including *V. vulnificus* (37), affects *sodA* expression. The CAT activities from pSODAC and MnSOD expression in the *rpoS* mutant KPR101 (Fig. 3) were the same as those in the wild type (Fig. 1B), which suggests that RpoS does not affect *sodA* expression even though MnSOD of *V. vulnificus* is expressed in stationary phase. FeSOD levels were unaffected by mutations in *soxR* and *rpoS*. In the *fur* mutant HLM101, *sodA* expression was derepressed even during exponential growth, as revealed by the activities of CAT and MnSOD (Fig. 3). The FeSOD activity of HLM101 was reduced to approximately 40% of that of the wild type. Thus, Fur, a repressor for *sodA* transcription, appears to act as an activator for *sodB* transcription.

Growth lag and MnSOD induction therein are triggered by superoxide accumulated upon cell exposure to low pH. Fresh LBS whose pH had been adjusted to 5.0 was inoculated with *V. vulnificus* grown exponentially at pH 7.5, as described in Materials and Methods. The cells showed a growth lag of approximately 3 h, whereas no lag was observed after transition to LBS (pH 7.5) (Fig. 4A). The medium pH increased toward alkalinity during late exponential growth (Fig. 4A). The cellular superoxide level, as determined using lucigenin, increased after transition to the low pH, which was approximately twofold higher than that measured after transition to pH 7.5 (Fig. 4B). MnSOD activity, once induced during the lag (Fig. 4C), increased up to a level comparable to that observed at the early stationary growth phase (compare Fig. 4C with Fig. 1B), whereas it was barely detectable at pH 7.5 (Fig. 4C). The CAT activity from pSODAC reflecting transcriptional control of MnSOD expression paralleled enzyme activity (Fig. 4C). Addition of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl

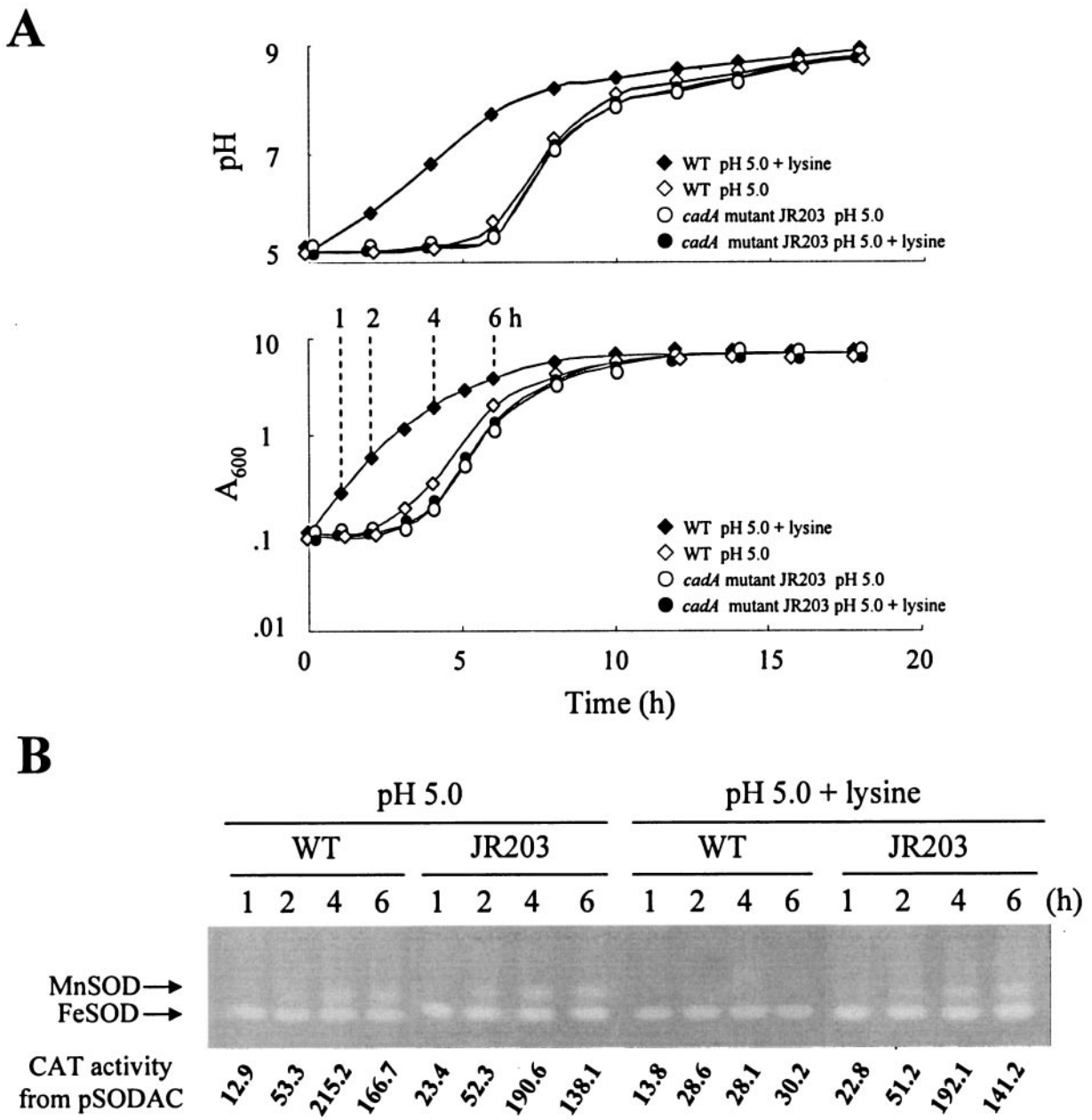

FIG. 6. Growth and SOD expression of the wild type and a *cadA* mutant after transition to LBS (pH 5.0) supplemented with lysine (15 mM). (A) Cell growth and pH curves of the culture broth of the wild type (◇, ◆) and JR203 (○, ●) without (open) or with (closed) lysine. Time points (1, 2, 4, and 6 h) to harvest cells for the assay of MnSOD expression are indicated. (B) Expression of cytosolic SODs and CAT activities from *sodA*::*cat* fusion in cells harvested at the time points indicated.

(TEMPO), a superoxide scavenger that can freely pass into the cell (22, 47), lowered the superoxide level (Fig. 4B). The scavenger not only shortened the lag (Fig. 4A) but also abolished MnSOD induction (Fig. 4C). The broth pH was not affected by TEMPO (Fig. 4A). The MnSOD expression was induced at pH 7.5 in the presence of a superoxide generator, methyl viologen (MV) (Fig. 4C), which also resulted in growth lag (Fig. 4A). The broth pH was not changed by MV (Fig. 4A). Thus, it appears that MnSOD induction of *V. vulnificus* is not controlled by low pH but by superoxide accumulated in cells under acidic conditions.

If superoxide accumulated at low pH inhibits cell growth, the growth lag should disappear by increasing SOD activities in the cytosol. *sodA* and *sodB* were cloned in downstream of the *lac* promoter of pRK415 and were maintained in *trans* in the wild type. A significant level of MnSOD, comparable to that found in the wild type at the early stationary phase, was expressed in cells containing pRKSA even during exponential growth, and an approximately twofold-higher level of FeSOD was constitutively expressed in cells containing pRKSB (data not shown). No growth lag was observed when cells containing pRKSA and pRKSB were transferred to low pH (Fig. 5). The broth pH was

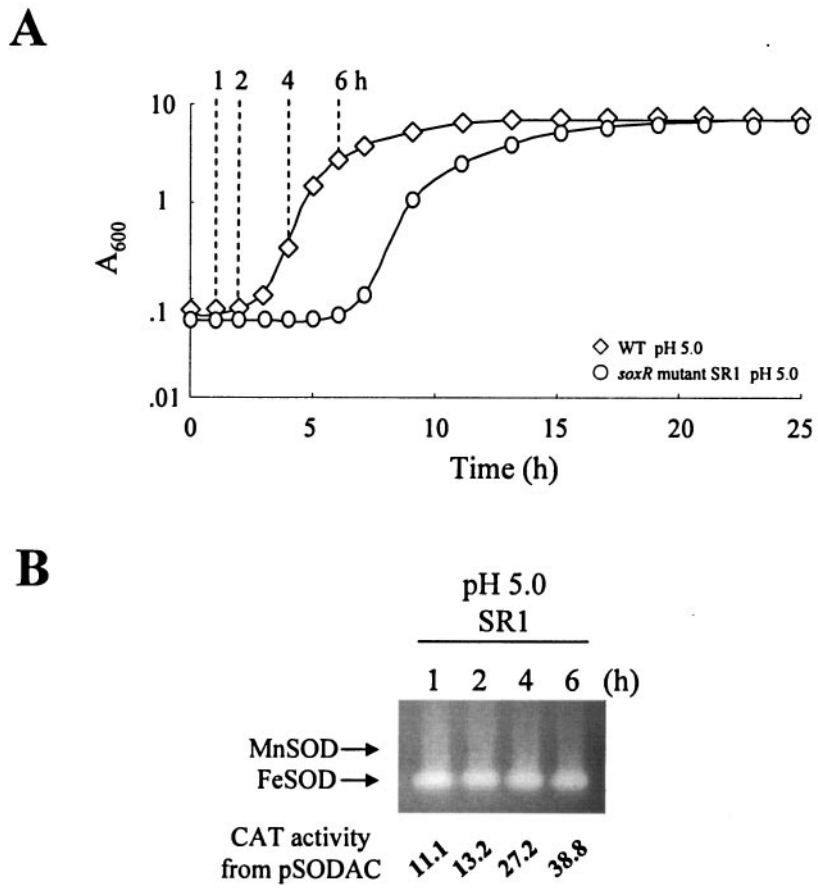

FIG. 7. Growth and SOD expression of *soxR* mutant after transition to LBS (pH 5.0). (A) Cell growth of SR1 (○). The wild type (◇) after transition to the same medium is included for comparison. Time points (1, 2, 4, and 6 h) to harvest cells for the assay of MnSOD expression are indicated. (B) Expression of cytosolic SODs and CAT activities from *sodA*::*cat* fusion in SR1 harvested at the time points indicated.

not affected by *sod* genes in *trans* (data not shown). Therefore, temporary cessation of cell growth at low pH is due to intracellular superoxide accumulated under the acidic conditions.

Lysine decarboxylase reaction abolishes acid stress by rapid neutralization of the culture broth. It was determined whether MnSOD expression is affected by the reaction catalyzed by lysine decarboxylase, since the activity is associated with neutralization of the culture broth (34, 48). Lysine decarboxylase activity increased up to approximately 55 units at its maximum level at 2 h after transfer to low pH (Table 2). However, approximately 20-fold-lower activity was detected with transfer to pH 7.5 (Table 2). Thus, the enzyme was induced during the lag after transition to low pH.

Lysine decarboxylase activity was barely detectable in the *cadA* mutant JR203 (Table 2), which indicates that the activity is specific to CadA, although another enzyme (or homolog) was proposed from the residual activity in JR203 (40). The mutant at low pH showed a 3- to 4-h lag, which is similar to that of the wild type (Fig. 6A, pH 5.0). The pH curves and the MnSOD expressions of the mutant were not different from those of the wild type (Fig. 6A and B, pH 5.0). However, the effect of the lysine decarboxylase reaction on pH homeostasis was valid when lysine (15 mM), known to induce the enzyme in *E. coli* via the periplasmic domain of CadC (32), was added to LBS (pH 5.0). The maximum level of enzyme activity of the wild type increased approximately twofold over that measured without lysine addition (Table 2). The production and excretion of cadaverine, whose concentration increased by approximately fourfold (1,186 ± 18 μM), was accompanied by rapid neutralization of the culture broth (Fig. 6A, pH 5.0, plus lysine). The cellular superoxide level of the wild type did not increase under the conditions (data not shown), which resulted in neither growth lag nor MnSOD induction (Fig. 6A and B, pH 5.0, plus lysine). Such results were not observed with JR203. Thus, the lysine decarboxylase reaction prevents generation of oxidative stress by rapid neutralization of the culture broth. The acid tolerance response was examined without lysine addition. A pH rise during late exponential growth of JR203 also suggests a lysine decarboxylase-independent mechanism(s) for neutralization of the extracellular medium, which remains to be determined.

MnSOD induction at low pH is regulated through SoxR. When SoxR, the positive regulator for *sodA* expression (52), was deleted, the mutant showed a growth lag extended to 7 h following the transfer to low pH (Fig. 7A). The medium pH increased toward alkalinity during late exponential growth (data not shown). A similar lag was observed with the *sodA* mutant SA1 (data not shown). Thus, the lack of MnSOD

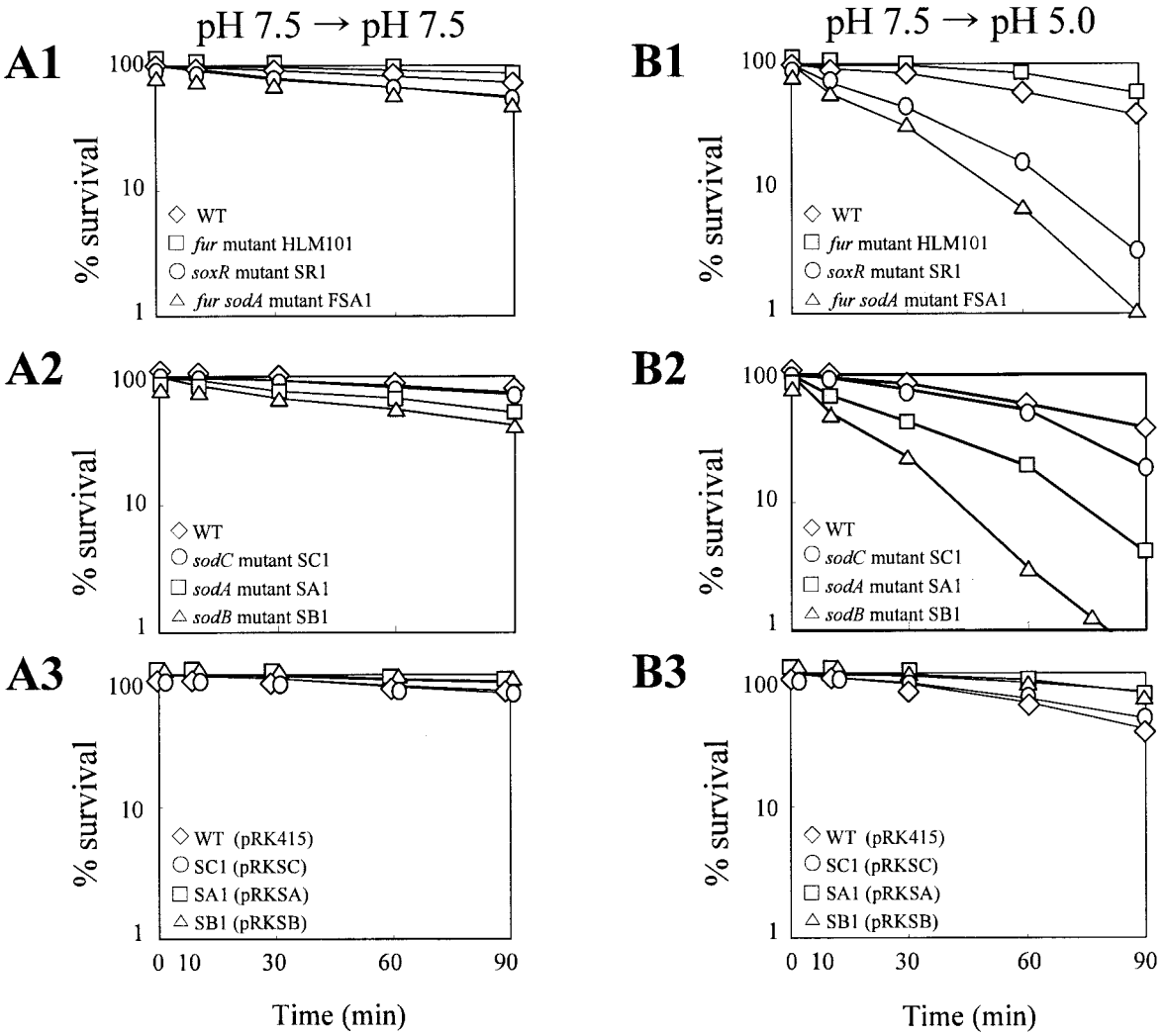

FIG. 8. Acid tolerance of regulatory mutants affecting *sodA* expression, *sod* mutants, and *sod* mutants complemented with corresponding genes. Cells grown in LBS (pH 7.5) with transition to PBS (pH 7.5) (A) and SCBN (pH 5.0) (B). (A1 and B1) Regulatory mutants HLM101 (□), SR1 (○), and FSA1 (△); (A2 and B2) *sod* mutants SC1 (○), SA1 (□), and SB1 (△); (A3 and B3) *sod* mutants complemented with corresponding genes, SC1 containing pRKSC (○), SA1 containing pRKSA (□), and SB1 containing pRKSB (△). The wild type (◇) and the wild type containing pRK415 (◇) are included as a control in each experiment. Survival was expressed as percentages of the initial numbers of CFU ml^{-1}, which were approximately 10^5. Data shown are representative of triplicate experiments.

expression extended the growth lag at low pH. Neither the CAT activity from pSODAC nor MnSOD was induced in the *soxR* mutant SR1 (Fig. 7B), illustrating that MnSOD induction at low pH is regulated by SoxR.

Survival of *V. vulnificus* defective in cytosolic SOD expression is significantly affected at low pH. Although the lag period at low pH was significantly affected by oxidative stress, it cannot be a measure for acid tolerance, as illustrated by the *cadA* mutant JR203. Although the lag of JR203 at low pH was comparable with that of the wild type (Fig. 6A), its acid tolerance was approximately 10-fold lower than that of the wild type (40). Thus, percent survival was determined as described in Materials and Methods for quantitative measurement of acid tolerance.

The *soxR* and *sodA* mutants SR1 and SA1 showed survival similar to that of the wild type at pH 7.5 (Fig. 8A1 and A2) but increased killing at pH 5.0 (Fig. 8A1 and B2). The results confirm that MnSOD expression is crucial for survival of *V. vulnificus* in acidic environments.

The survival of the *fur* mutant HLM101 derepressing MnSOD (Fig. 3) was similar to that of the wild type at pH 7.5 (Fig. 8A1). As expected, the mutant endured acidity better than the wild type (Fig. 8B1). The higher tolerance could be ascribed to the elevated level of cytosolic SODs in the mutant, since the total cytosolic SOD activity, measured in the presence of KCN as described in Materials and Methods, of HLM101 grown in LBS is always higher than that of the wild type by approximately 10 to 50% irrespective of the pH of the medium (data not shown). Iron is more soluble at acid pH than it is at alkaline pH (53), and excess intracellular iron can be detrimental to the cell (9). Accordingly, it was considered possible that the lethal effect at low pH could be enhanced by the

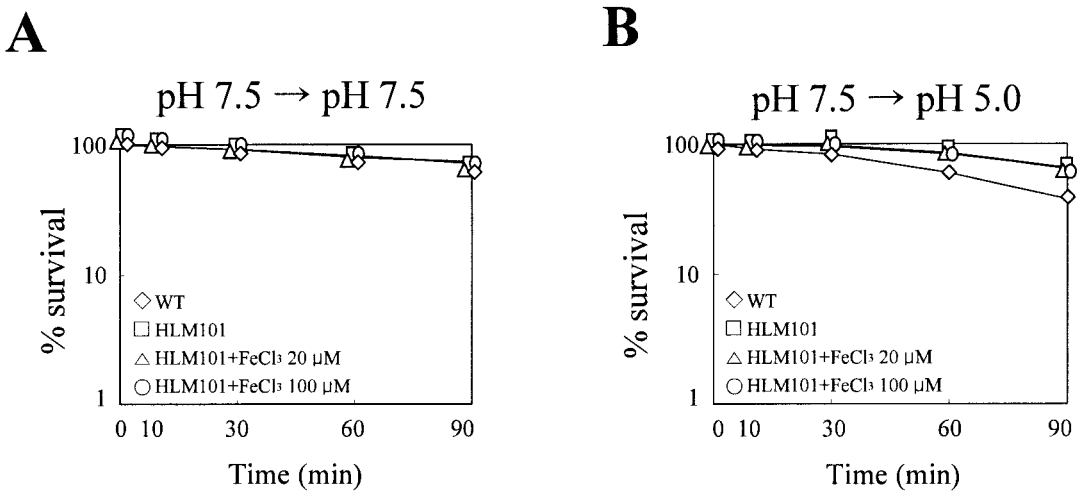

FIG. 9. Acid tolerance of *fur* mutant in the presence of $FeCl_3$. The wild type (◇) and HLM101 grown in LBS (pH 7.5) were transited to PBS (pH 7.5) (A) and SCBN (pH 5.0) (B). $FeCl_3$ was added to the buffer; none (□), 20 (△), and 100 μM (○). Survival was expressed as percentages of the initial numbers of CFU ml^{-1}, which were approximately 10^5. Data shown are representative of triplicate experiments.

unregulated delivery of iron into the mutant. Contrary to expectations, the addition of $FeCl_3$ up to 100 μM did not change the survival phenotype (Fig. 9A and B). This suggests that potential iron toxicity through the delivery of iron, possibly unregulated by the lack of Fur, into the mutant may not be a factor affecting acid tolerance in *V. vulnificus*. The tolerance endurance was abolished by introduction of a *sodA* mutation in HLM101 (see FSA1 in Fig. 8B1). Thus, it is the derepressed MnSOD expression of the *fur* mutant that is related to acid tolerance.

Cellular level of cytosolic SODs is important for cell survival at low pH. *sodB* and *sodC*, coding for FeSOD and CuZnSOD, respectively, of *V. vulnificus*, were interrupted to generate mutants SB1 and SC1 (Fig. 10). MnSOD of SB1 is derepressed from the exponential growth phase (Fig. 10A2). The expression of MnSOD and FeSOD was not changed by the

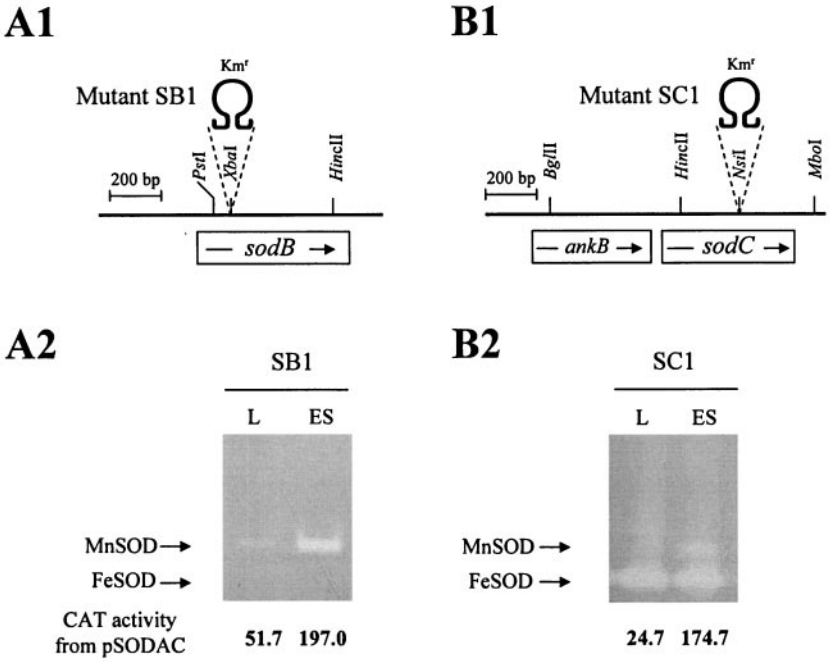

FIG. 10. Chromosomal structures of *sodB* and *sodC* mutants and their expression of cytosolic SODs. (A1 and B1) *sodB* and *sodC* were interrupted with Ω DNA (Km^r) to generate mutants SB1 (A1) and SC1 (B1), respectively. *ankB* coding for an ankyrin-like protein is shown (B1). (A2 and B2) Expression of cytosolic SODs and CAT activity from *sodA*::*cat* fusion in SB1 (A2) and SC1 (B2). L and ES stand for the growth phases, as described in the Fig. 1 legend.

mutation in *sodC* (Fig. 10B2). Total cytosolic SOD activities in cell extracts from SC1, SB1, and SA1 grown to logarithmic phase were measured in the presence of KCN. The cytosolic SOD activity of SC1 is similar to that of the wild type, whereas those of SA1 and SB1 amounted to 85 and 25% of the wild-type activity, respectively, illustrating that the total cytosolic activity decreased in the order of SC1, SA1, and SB1. Acid survival of *sod* mutants also decreased in the order of SC1, SA1, and SB1 compared with the wild type (Fig. 8B2). The mutational effects were significant at acid pH (compare Fig. 8B2 with Fig. 8A2) and were complemented with corresponding *sod* genes in *trans* (Fig. 8A3 and B3). Thus, the higher level of cytosolic SOD activities appears to support the better tolerance to acid pH. The contribution of CuZnSOD to acid tolerance was not significant compared with those of cytosolic SODs.

Taken together, when *V. vulnificus* is exposed to low pH, superoxide is accumulated in cells. MnSOD expression is then induced, which results in an increase of cytosolic SOD activity. Superoxide removal by the cytosolic SODs is essential for acid tolerance of *V. vulnificus*.

DISCUSSION

In this work, acid-specific stress could be separated from oxidative stress by TEMPO, a scavenger for superoxide. Since the scavenger abolishes MnSOD induction at low pH, the stress responsible for *sodA* transcription is not pH but superoxide. Fe^{2+} of heme in acid pH may undergo spontaneous oxidation to Fe^{3+} with concomitant generation of superoxide (33). Acid also could affect all the cell components from the membrane to the cytosol, and the proteins therein could be partly unfolded but still work to generate reactive oxygen species including superoxide under aerobic conditions. Interestingly, there have been reports of increased expression of both DnaK and GroEL/ES classes of chaperones in response to growth at low pH (15, 36). By the same token, the experimental results shown in this work can also suggest that a lack of either MnSOD or FeSOD of *V. vulnificus* may result in intracellular buildup of superoxide, which causes cellular damage that renders the cell more sensitive to the acid stress.

E. coli MnSOD is also induced by MV (13), as in *V. vulnificus*. However, unlike the case with *V. vulnificus*, a substantial level of MnSOD activity is detected in *E. coli* during exponential growth. Neither derepressed MnSOD activity nor a growth lag was observed after transition of *E. coli* to LB acidified to pH 4.0 or 5.0 (J.-S. Kim and J. K. Lee, unpublished results). The results suggest that there are intrinsic differences in the acid tolerance response between *E. coli* and *V. vulnificus*. Superoxide may not be generated *in E. coli* under acidic conditions, or the superoxide level, if generated, may be too low to induce MnSOD.

V. vulnificus SoxR (17 kDa on a sodium dodecyl sulfate-polyacrylamide gel) was purified after tag removal from the fusion protein MBP-SoxR, which had been overexpressed in *E. coli*. The purified SoxR protein displayed binding affinity for *E. coli soxS* regulatory DNA (from −116 to +64; +1, 5′ end of the *soxS* transcript) in a gel mobility shift. However, the mobility of *V. vulnificus sodA* regulatory DNA (from −87 to +51; +1, 5′ end of the *sodA* transcript) was not affected by SoxR (J.-S. Kim and J. K. Lee, unpublished results). Thus, the SoxR-mediated *sodA* expression of *V. vulnificus* appears to be exerted indirectly, possibly via another regulator, such as SoxS, as observed with *E. coli* (1). In *V. vulnificus*, however, no SoxS homolog has been found to date.

SoxR regulates MnSOD induction at acid pH, which is required for an acid tolerance response (Fig. 8). *rpoS* and *cadC* mutants of *V. vulnificus* also revealed higher levels of acid killing than the wild type (37, 39). For both *rpoS* and *cadC* mutants, however, the acid pH-dependent MnSOD inductions are the same as those for the wild type (J.-S. Kim and J. K. Lee, unpublished results). Thus, *rpoS* and *cadC* mutants are acid sensitive for reasons other than defects in acid pH-dependent MnSOD induction. Therefore, acid resistance of *V. vulnificus* appears to be a multifactorial phenomenon that includes stress responses to acidity as well as superoxide accumulated under acidic conditions.

From the survival of *sod* mutants, it was concluded that the higher SOD level in the cytosol supports better survival at acid pH. The same was true for a lethality assay following intraperitoneal injection of the cells into mouse (J.-S. Kim and J. K. Lee, unpublished results), reflecting that superoxide is one of the major stresses that *V. vulnificus* should overcome in vivo.

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