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The Virulence of *Vibrio vulnificus* is Affected by the Cellular Level of Superoxide Dismutase Activity

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Abstract The virulence of superoxide dismutase (SOD) mutants of *Vibrio vulnificus*, as tested by intraperitoneal injection into mice, decreases in the order of *sodC* mutant, *sodA* mutant, and *sodB* mutant lacking CuZnSOD, MnSOD, and FeSOD, respectively. The survival of SOD mutants under superoxide stress also decreases in the same order. The virulence of *soxR* mutant, which is unable to induce MnSOD in response to superoxide, is similar to that of the *sodA* mutant, as the survival of the *soxR* mutant under superoxide stress is similar to that of the *sodA* mutant. Consistently, the lowered survival of the *soxR* mutant is complemented not only with *soxR* but also with *sodA*. Thus, the virulence of *V. vulnificus* is significantly affected by the cellular level of SOD activity, and an increase in SOD level through MnSOD induction by SoxR under superoxide stress is essential for virulence.

Keywords: Superoxide dismutase, virulence, *Vibrio vulnificus*

Vibrio vulnificus is a halophilic estuarine bacterium that can cause foodborne gastroenteritis or infect an open wound. It may get into the blood stream, causing a rapidly progressing, fulminating septicemia, which very often results in death among individuals with chronic liver disease [3].

Previously, we examined the ability of *V. vulnificus* to tolerate acidity [9], since the pathogen may travel through the acidic environment of the stomach to colonize the intestinal lumen for foodborne infections. Oxidative stress is generated when *V. vulnificus* is exposed to low pH, and MnSOD is induced by the control of SoxR under acidic conditions. Accordingly, mutations in *soxR* or *sodA* of *V. vulnificus* 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 [9].

Interestingly, MnSOD of *Staphylococcus aureus* is also found among acid-shock proteins when cells are exposed to low pH [4]. The precise mechanisms by which SOD of *S. aureus* is induced under the conditions and involved in acid tolerance are not known. Although the *sodA* mutant of *S. aureus* was less able to survive acid stress, it showed no alteration in pathogenicity in a mouse abscess model of infection compared with its parental strain [4].

In an effort to understand the contributions of SODs to the virulence of *V. vulnificus*, 50% lethal dose (LD_{50}) was examined after intraperitoneal (IP) injection of SOD mutants into mice. Unlike *S. aureus*, the virulence of *V. vulnificus* was significantly affected by the cellular level of SOD activity. An increase in SOD level through MnSOD induction by SoxR was essential for the virulence of cells, as observed for the cell survival under superoxide stress.

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 [20] supplemented with 2% (w/v) NaCl (LBS), as described previously [10, 12]. *Escherichia coli* was grown at 37°C in LB. Cell growth was monitored by measuring the culture absorbance at 600 nm (A_{600}). pRK415- and pDM4-derived plasmids were mobilized into *V. vulnificus* by conjugation as described previously [9].

For the quantification of SOD activity, cell extracts were prepared as described previously [2]. The cellular SOD activities in cell extracts were determined as described previously [13].

Tolerance to oxidative stress was examined essentially as described previously [6, 8, 9]. Cells were grown to late logarithmic phase (A_{600}, ~4.0) in LBS (pH 7.5), harvested, and washed with phosphate-buffered saline (PBS, pH 7.5) supplemented with 3 mM methyl viologen (MV), followed by suspension in the same buffer to a final concentration of 10^5 colony forming units (CFU)/ml. A control experiment was performed, in which PBS (pH 7.5) without MV was used. Cell suspensions were incubated at 30°C with shaking. Samples were taken intermittently for 90 min and viable

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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	Type strain, ATCC29307, Rif^r	[11]
SA1	AR, *sodA::aph*; Km^r	[9]
SB1	AR, *sodB::aph*; Km^r	[9]
SC1	AR, *sodC::aph*; Km^r	[9]
SR1	AR, *soxR::aph*; Km^r	[9]
E. coli		
S17-1	C600::RP4 2-(Tc::Mu)(Km::Tn7) *thi pro hsdR hsdM*$^+$ *recA*	[21]
S17-1λ*pir*	λ*pir* lysogen of S17-1	[21]
Plasmids		
pRK415	*ori* IncP Mob RP4 *lacZα*; Tc^r	[7]
pDM4	*ori* R6K Mob RP4; Cm^r	[14]
pRKSA	pRK415+0.8-kb fragment containing *sodA*; Tc^r	[9]
pRKSB	pRK415+1.1-kb fragment containing *sodB*; Tc^r	[9]
pRKSC	pRK415+1.1-kb fragment containing *sodC*; Tc^r	[9]
pRKSoxR	pRK415+2.8-kb fragment containing *soxR*; Tc^r	[8]

counts (CFU/ml) were determined by plating dilutions of cells on LBS (pH 7.5) agar plates. Survival was expressed as percentage of the initial CFU. Data shown are representatives of triplicate experiments, yielding similar results.

For LD_{50} determination, the exponentially growing *V. vulnificus* was harvested right before IP injection into the normal male ICR mice (specific pathogen free, 5–6 weeks old, weight between 60 and 65 g; Daehan Animal Co., Taejon, Korea), essentially as described previously [5, 15]. Six mice in each group were injected with 0.1 ml of serial dilutions (10^{10} to 10^6 CFU/ml) of each strain in PBS (pH 7.5). No prior loading of mice with iron dextran was employed before IP injection. The infected mice were observed for 48 h. The LD_{50} was calculated by the method of Reed and Muench [16].

Even though the cellular SOD level is important for the acid tolerance of *V. vulnificus* [9], the effect of acid stress on cell survival is mostly undiscovered, except for the induction of lysine decarboxylase [17–19]. Thus, the role of cellular SODs in cell survival under the superoxide stress at neutral pH may be different from that examined at low pH. Accordingly, the tolerance of SOD mutants to superoxide was determined at pH 7.5 in the presence of MV (3 mM) and compared with that of wild type (Fig. 1). The percent survival of the initial CFU of each mutant decreased in the order of *sodC* mutant SC1, *sodA* mutant SA1, and *sodB* mutant SB1 lacking CuZnSOD, MnSOD, and FeSOD, respectively (Fig. 1B1), which reflects the total cellular SOD activities of the mutants that had been determined previously [9]. The cytosolic SOD activity of SC1 was similar to that of wild type, whereas total cellular SOD activities of SA1 and SB1 were approximately 85% and 25% of the wild-type activity, respectively [9]. Thus, the tolerance of *V. vulnificus* to superoxide stress in neutral pH environment is also determined by the cellular level of SOD activity, as observed in acid environment. The sensitivity

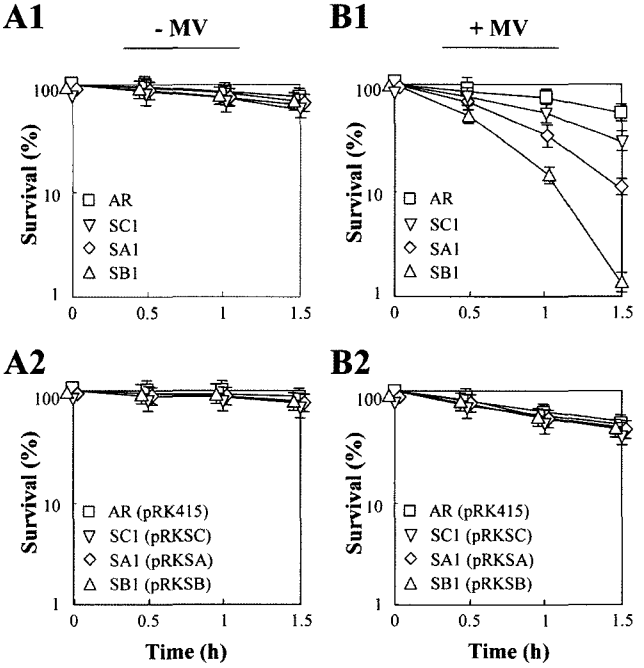

Fig. 1. Tolerance of SOD mutants to superoxide stress.
Cells grown in LBS (pH 7.5) were transferred to PBS (pH 7.5) containing 3 mM MV (**B**). Transfer to PBS (pH 7.5) without MV was included as a control (**A**). Viable cell counts of wild-type AR (□), *sodC* mutant SC1 (▽), *sodA* mutant SA1 (◇), and *sodB* mutant SB1 (△) were determined, and survival was expressed as the percentage of initial CFU/ml, which were approximately 10^5 (A1 and B1). The survival of SOD mutants complemented with corresponding genes was also determined (A2 and B2). The error bars correspond to the SD of the means.

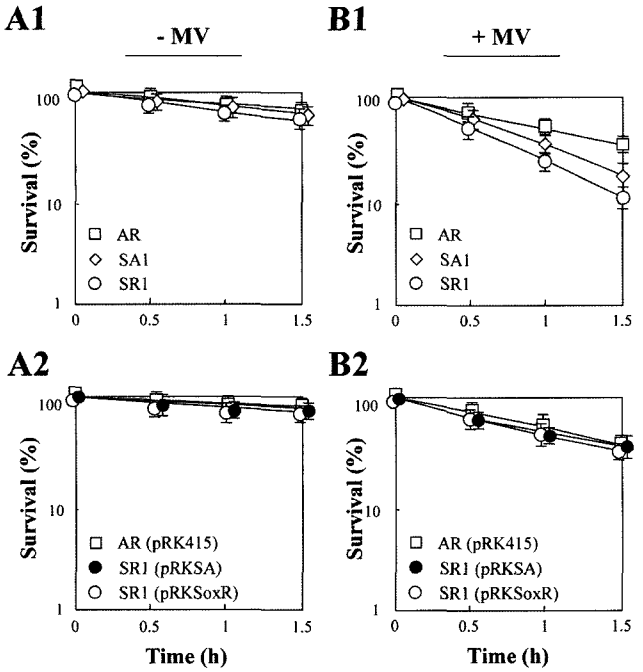

Fig. 2. Tolerance of SoxR mutant to superoxide stress.
Cells grown in LBS (pH 7.5) were transferred to PBS (pH 7.5) containing 3 mM MV (**B**). Transfer to PBS (pH 7.5) without MV was included as a control (**A**). Viable cell counts of wild-type AR (□), *sodA* mutant SA1 (◇), and *sodR* mutant SR1 (○) were determined, and survival was expressed as percentage of the initial CFU/ml, which were approximately 10^5 (A1 and B1). The survival of SoxR mutant complemented with *soxR* (○) or *sodA* (●) gene was also determined (A2 and B2). The error bars correspond to the SD of the means.

of the SOD mutants to superoxide was fully complemented with the genes corresponding to the mutations (Figs. 1A2 and 1B2). The SOD mutants showed survival similar to that of wild type in the absence of MV (Figs. 1A1 and 1A2).

The MnSOD expression of *V. vulnificus* is induced by SoxR [8, 9], possibly *via* another regulator such as SoxS as observed in *E. coli* [1]. However, no SoxS homolog of *V. vulnificus* has been found to date. The *soxR* mutant SR1 showed survival similar to that of wild type in the absence of MV (Fig. 2A1), whereas its survival was similar to that of *sodA* mutant SA1 in the presence of MV (Fig. 2B1). The sensitivity of SR1 to superoxide was complemented not only with *soxR* but also with *sodA* (Fig. 2B2), which indicates that the major role of SoxR is the induction of MnSOD under superoxide stress, and further confirms that SoxR-mediated induction of MnSOD is important for the survival of *V. vulnificus* under the oxidative stress conditions.

The virulence of *V. vulnificus* mutants defective in the expression of SOD was examined through the IP injection of the cells into mice. The LD_{50} of SA1 was approximately 110-fold higher than that of wild type (Table 2), which implies that the *in vivo* virulence is significantly attenuated by the lack of MnSOD expression. The more attenuated virulence was observed with SB1, which is consistent with

Table 2. The virulence of SOD mutants and the *soxR* mutant of *V. vulnificus* compared with that of wild type.

Strain	LD_{50} (CFU/mouse)	Relative fold
Wild type[a]	3.98×10^7	1.0
SR1	3.16×10^9	79.4
SA1	4.39×10^9	110.3
SB1	2.50×10^{10}	628.1
SC1	3.16×10^8	7.9

[a]Strain AR.

the lower SOD level in the cell and also with the marked decrease in percent survival under superoxide stress (Fig. 1B1). Consistently, SC1 showed the highest virulence compared with those of SA1 or SB1, albeit 8-fold higher LD_{50} than wild type.

The virulence of *soxR* mutant SR1 is similar to that of *sodA* mutant SA1 (Table 2). SoxR is also known to activate the transcription of lysine decarboxylase in response to superoxide [8]. Accordingly, no lysine decarboxylase is expressed in SR1 under superoxide stress [8], but that of SA1 should be induced under the conditions. The cadaverine, a product of lysine decarboxylase, acts as an antioxidant by scavenging superoxide radicals [6, 8]. However, the virulence of SR1 is not more attenuated compared with SA1, as illustrated by the LD_{50} of SR1 and SA1. Therefore, cadaverine appears to be less important in the virulence of *V. vulnificus* than would have been predicted by considering it as a superoxide scavenger in the cell in addition to SODs. The level of cadaverine in bacterial cells, however, may be largely limited by the availability of lysine in the host after IP injection. Although the involvement of cadaverine in virulence remains to be determined after oral infection, it is certain that the cellular SODs play a major role in the *in vivo* pathogenesis, under the infection conditions examined in this work, indicating that superoxide is one of the major stresses *V. vulnificus* should overcome in a host, and an increase in SOD level through MnSOD induction by SoxR under superoxide stress is essential for the virulence of *V. vulnificus*.

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