J. Microbiol. Biotechnol. (2009), **19**(10), 1122–1126
doi: 10.4014/jmb.0903.03012
First published online 4 May 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cadaverine is Transported into *Vibrio vulnificus* Through its CadB in Alkaline Environment

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Received: March 17, 2009 / Revised: April 2, 2009 / Accepted: April 7, 2009

The exogenously added cadaverine is effective in protecting *Vibrio vulnificus* from methyl viologen (MV)-induced superoxide stress at pH 8.5. Such a protective effect by cadaverine was not observed at pH 7.5. Consistently, the accumulated level of intracellular cadaverine at pH 8.5 is approximately four times as much as that of the control cell at pH 7.5. Cadaverine accumulation is not affected by MV. The protection of *V. vulnificus* by cadaverine from superoxide stress was abolished when *cadB* coding for the lysine–cadaverine antiporter was interrupted. However, the cadaverine-mediated protection was complemented with *cadB* DNA. Therefore, CadB of *V. vulnificus* not only acts as a lysine–cadaverine antiporter at acid pH to neutralize the external medium, but also mediates cadaverine uptake at alkaline pH to result in cell protection from superoxide stress.

Keywords: *Vibrio vulnificus*, CadB, cadaverine uptake, alkaline pH, superoxide stress

The ability to tolerate gastric acidity is necessary for *Vibrio vulnificus* to cause foodborne gastroenteritis. When *V. vulnificus* is exposed to low pH, superoxide stress is generated in the cell to cause the induction of MnSOD [6]. Another striking response to low external pH is the generation of cadaverine (1,5-pentanediamine) and CO_2 by lysine decarboxylase in the presence of lysine. The production and excretion of cadaverine is accompanied by some neutralization of the external pH, thus protecting cells from the acid stress [15, 21]. The acid pH-dependent induction of *cadBA* coding for a lysine–cadaverine antiporter (CadB) and a lysine decarboxylase (CadA) has been well illustrated in *E. coli*, *V. cholerae*, and *V. vulnificus* [11, 12, 15, 17, 18, 21]. The expression of *V. vulnificus cadBA* is not only upregulated by CadC in response to acid stress [16, 18] but also induced by SoxR in response to superoxide stress [7]. Thus, cadaverine, which neutralizes the external medium, also relieves the cellular oxidative stress by scavenging superoxide [4, 7]. Polyamines are known to be associated with the protection of cell from the toxic effects of oxygen [1, 2, 14, 23].

Cadaverine uptake at neutral pH, which requires proton-motive force, is illustrated with *Escherichia coli* CadB [21], implying its transport by cadaverine/proton symport. The physiological significance for cadaverine uptake at neutral pH, however, remained to be determined. The amino acid residues of *E. coli* CadB, which affect excretion and uptake of cadaverine, have been investigated [22].

We tested whether the exogenously added cadaverine exerts any effect on the protection of *V. vulnificus* from oxidative stress. The exogenously added cadaverine protects cells from superoxide stress at pH 8.5. Consistently, cadaverine was found to be accumulated in the cell through CadB under the conditions. Unlike *E. coli*, such effect was not observed at pH 7.5. Thus, CadB of *V. vulnificus*, which acts as a lysine–cadaverine antiporter at acid pH to neutralize the medium, also mediates cadaverine uptake at alkaline pH to protect cells from superoxide stress.

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 [19] supplemented with 2% (w/v) NaCl (LBS, pH 7.5). *E. coli* was grown at 37°C in LB. When appropriate, antibiotics were added at concentrations as described previously [6].

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 [6].

Construction of *cadA–sodA* Double Mutant

A suicide plasmid pDMSAid, in which a 173-bp BglII-StuI DNA (residues between 55th and 113th) within *sodA* was deleted from a

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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	ATCC29307, Rif^r	[8]
JR203	ATCC29307, *cadA::nptI*; Km^r	[17]
SA1	AR, *sodA::aph*; Km^r	[6]
SACA1	AR, *sodA* internally deleted, *cadA::aph*; Km^r	This study
JR202	AR, *cadB::nptI*; Km^r	[17]
E.coli		
DH5α	*supE*44 Δ*lacU*169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	[3]
S17-1	C600::RP4 2-(Tc::Mu)(Km::Tn7) *thi pro hsdR hsdM*$^+$ *recA*	[20]
S17-1λ*pir*	λ*pir* lysogen of S17-1	[20]
Plasmids		
pDM4	*ori* R6K Mob RP4; Cm^r	[13]
pRK415	*ori* IncP Mob RP4 *lacZ*α; Tc^r	[5]
pDMSAid	pDM4+0.6-kb *sodA* DNA with 173-bp BglII-StuI internal deletion; Cm^r	[6]
pRKCadB	pRK415+2.5-kb *cadB* DNA; Tc^r	This study

761-bp DNA extending from −114 to 647 relative to the MnSOD initiation codon [6], was mobilized into *V. vulnificus* JR203 (*cadA::aph*; kanamycin [Km]^r) [17] through conjugation as described above. Conjugants carrying a single crossover were selected using chloramphenicol (Cm). The mutant showing indication of double crossover (Cm^s) was isolated in the presence of 10% sucrose. The chromosomal structure of the mutant was examined by Southern hybridization analysis [19].

Survival Under Superoxide Stress and Protection from Cell Death by Cadaverine

Tolerance to oxidative stress was examined essentially in the same way as described for the tolerance to low pH [6]. Cells were 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 for the induction of *cadBA*. The initial A_{600} value was close to 0.1. Cells were incubated for 4 h, and

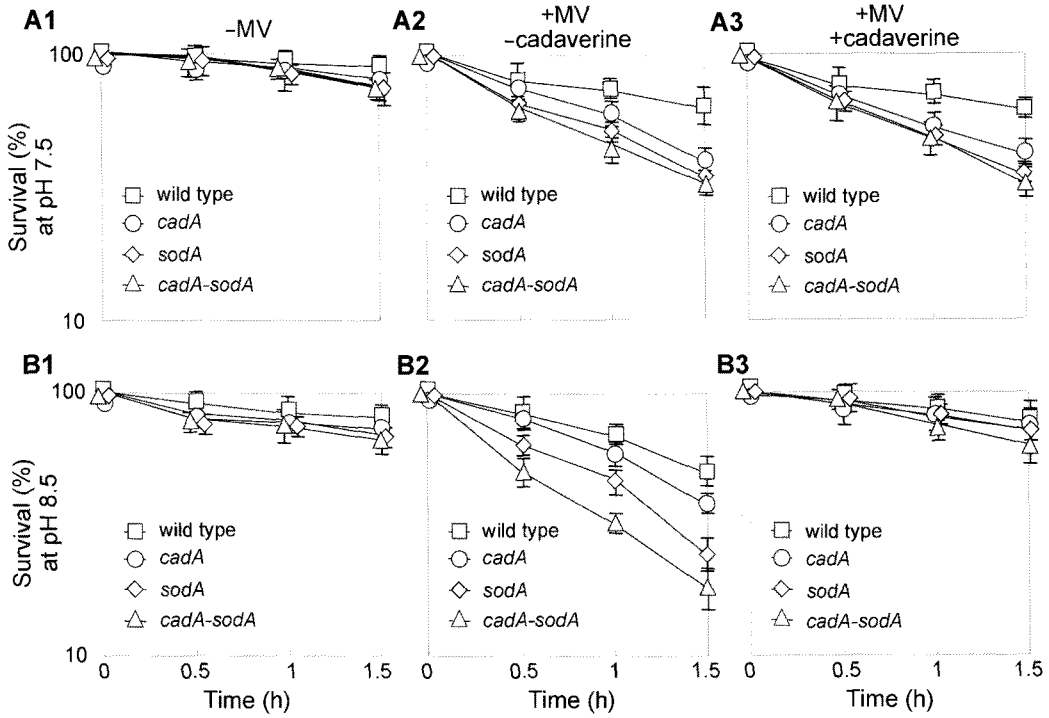

Fig. 1. Effect of the exogenously added cadaverine on cell tolerance to superoxide stress at pH 7.5 and pH 8.5.
Cells grown in LBS (pH 7.5) supplemented with 3 mM MV were transferred to PBS (pH 7.5) (**A**) and PBS (pH 8.5) (**B**) containing the same concentration of MV in the presence (**A3** and **B3**) and absence (**A2** and **B2**) of 5 mM cadaverine. Transfer to PBS without MV and cadaverine was included as a control (**A1** and **B1**). Viable cell counts of wild type (□), *cadA* mutant (○), *sodA* mutant (◇), and *cadA-sodA* mutant (△) were determined, and survival was expressed as the percentage of initial CFU/ml, which was approximately 10^5. The error bars correspond to the SD of the means.

then harvested and washed with phosphate-buffered saline (PBS) (pH 7.5 and pH 8.5) [19], followed by suspension to a final concentration of 10^5 CFU/ml in the same buffer containing 3 mV MV. A control experiment was performed in PBS (pH 7.5 and pH 8.5) without MV. The cadaverine-mediated protection from oxidative stress was examined in parallel with PBS (pH 7.5 and pH 8.5) supplemented with 5 mM cadaverine. Cell suspensions were incubated at 30°C with shaking. 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 a percentage of the initial CFU. The experiments were repeated three times, yielding similar results; data shown are representatives of triplicate experiments.

Cadaverine Determination

The cadaverine level in culture medium was determined as described previously [6, 9, 17]. To determine the cellular cadaverine, approximately 10^5 colony-forming units (CFU) were harvested, washed two times with 20 mM potassium phosphate buffer (pH 7.4), and suspended in 0.2 ml of the same buffer. Cells were broken by sonication in a Branson 250 Sonifier operated at 4°C for 2 min (pulse mode, 50% duty cycle, output 2.0), followed by centrifugation (10,000 ×*g* for 5 min) at 4°C. Fifty μl of cell-free extracts was mixed with the same volume of 1 M Na_2CO_3 to stop any endogenous lysine decarboxylase activity. Then, 100 μl of 10 mM 2',4',6'-trinitrobenzylsulfonic acid was added and the mixture was incubated for 5 min at 40°C. The reaction product *N,N'*-bistrinitrophenylcadaverine was extracted with 1 ml of toluene after vigorous vortex for 30 sec and centrifugation at 10,000 ×*g* for 1 min. The toluene extract was measured spectrophotometrically at 340 nm. Another mixture, in which cell-free extracts had not been treated with 2',4',6'-trinitrobenzylsulfonic acid, was extracted with toluene in the same way and used as a blank. Protein in cell-free extracts was determined by a modified Lowry method [10] using bovine serum albumin as a standard.

Results and Discussion

Exogenously Added Cadaverine is Effective in Protecting *V. vulnificus* from Superoxide Stress at pH 8.5

Since cadaverine scavenges superoxide radicals [4, 7], the *V. vulnificus cadA* mutant [17], which does not synthesize cadaverine from lysine because of the lack of lysine decarboxylase, shows lower survival under superoxide stress compared with wild type (Figs. 1, A1, A2, B1, and B2). MnSOD, which is induced under superoxide stress [6], is a major enzyme that detoxifies the cellular superoxide. Accordingly, the *sodA* mutant and *cadA-sodA* mutant are also sensitive to superoxide stress (Figs. 1, A1, A2, B1, and B2).

When *V. vulnificus* is exposed to low pH, the expression of the *cadBA* operon is induced by the concerted activation by CadC [16, 18] and SoxR [7]. Superoxide stress was shown to build up intracellularly in acid environment [6, 7]. Lysine under this condition is decarboxylated to form cadaverine, which not only acts as an antioxidant in the cell but also neutralizes the external medium. As cadaverine comes out of the cell, lysine simultaneously goes into the cell through an antiporter CadB to result in forming more cadaverine. It was examined whether cadaverine, when added exogenously, was effective in protecting cells from superoxide stress. No protective effect by the exogenously added cadaverine (5 mM) was observed at pH 7.5 with both wild-type and the mutant strains (Fig. 1, A3). However, significant protection from superoxide stress was observed with the mutants and even the wild type by cadaverine (5 mM) at pH 8.5 (Fig. 1, B3). The results not only corroborate the antioxidant role of cadaverine but also suggest its accumulation in the cell at alkaline pH.

Cadaverine is Accumulated in Cells at pH 8.5

It was determined whether the cadaverine-mediated protective effect was exerted through its accumulation in cells. Cells from survival experiments were harvested at time 0 and 1.5 h, broken by sonication, and analyzed for their cadaverine contents. Since the wild type and *sodA* mutant harbor lysine decarboxylase activity, the cadverine level was analyzed only with cells defective in lysine decarboxylase; *cadA* and *cadA–sodA* mutants. The cellular cadaverine at pH 7.5 was not increased by the exposure of cells to the exogenously added cadaverine (Fig. 2A). Thus, it appears that cadaverine does not move into the cell at pH 7.5. However, cellular cadaverine at pH 8.5 is elevated 2-fold after exposure to

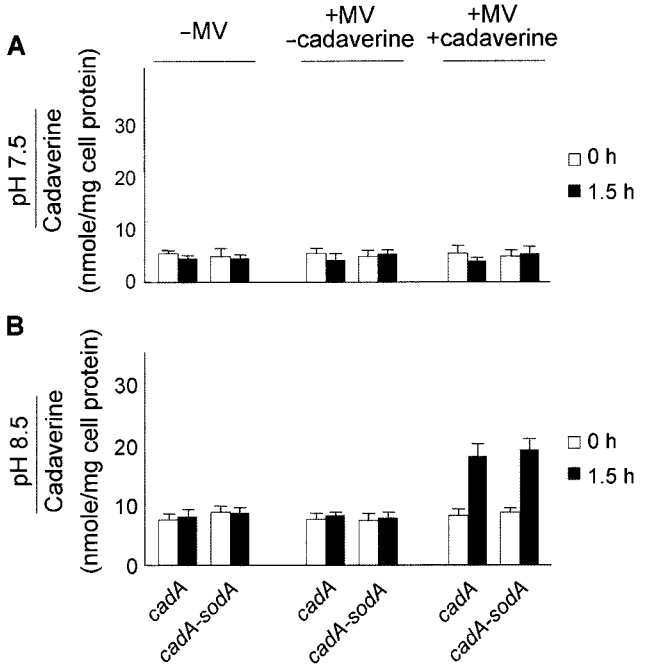

Fig. 2. Determination of cellular cadaverine after exposure of *cadA* mutant and *cadA–sodA* mutant to the exogenously added cadaverine at pH 7.5 and pH 8.5.

Approximately 10^5 CFU of *cadA* mutant and *cadA–sodA* mutant from the tolerance experiments (Fig. 1) were harvested at times 0 and 1.5 h and examined for cadaverine level. The error bars correspond to the SD of the means.

cadaverine (5 mM) (Fig. 2B, +MV +cadaverine), implying its accumulation in the cell at pH 8.5.

Cells that had not been exposed to cadaverine at pH 8.5 showed higher basal levels of cadaverine compared with those measured at pH 7.5. Since the mutants do not synthesize lysine decarboxylase, the basal level should reflect other polyamines that could be generated by other amino acid decarboxylases. Previously, neutralization of the acidic medium was observed by the *cadA* mutant of *V. vulnificus* during its late exponential growth, and it was suggested that the pH rise under the condition is mediated by a CadA-independent mechanism [6].

Effect of Exogenously Added Cadaverine on *V. vulnificus* Survival Under Superoxide Stress is Exerted by CadB

Since cadaverine appears to be transported into the cell at pH 8.5, it was determined whether CadB is involved in its uptake. The survival of the *cadB* mutant was examined and compared with wild type. The exogenously added cadaverine did not show any protective effect on the survival of *cadB* mutant at pH 7.5 (Fig. 3A). The same result was observed at pH 8.5 (Fig. 3B). However, complementation of the *cadB* mutant with *cadB* DNA *in trans* completely restored the cadaverine-mediated protection at pH 8.5 (Fig. 3, B3). Consistently, the *cadA* mutant is protected by the exogenously added cadaverine at pH 8.5 (Fig. 1, B3 and Fig. 3, B3). Therefore, cadaverine is transported into the cell through CadB at pH 8.5. The *cadB*-complemented strain after 1.5-h exposure to cadaverine showed a 2-fold higher level of cellular cadaverine compared with that of the *cadB* mutant (Fig. 4). The cellular cadaverine level of the *cadB*-complemented strain is similar to that of *cadA* mutant measured under the same conditions (compare Fig. 4 with that of Fig. 2B). The cadaverine accumulation in the cell is not affected by the presence of MV (Fig. 4). Although the primary structure of *V. vulnificus* CadB is highly similar (82%) to that of *E. coli* CadB, which requires proton-motive force for the cadaverine transport [21], cadaverine accumulation in *V. vulnificus* at only lower proton concentration (higher pH) strongly suggests the cadaverine uniport by its CadB.

V. vulnificus faces the slightly alkaline environment in the intestine when the pathogen comes out of the acidic juice of the stomach. Cadaverine, once produced at low pH, may be around and moves into the cell through CadB at the anterior intestine. *V. vulnificus* may then have a higher resistance to oxidative stress, due to cadaverine accumulated in the cell. Thus, *V. vulnificus* CadB is

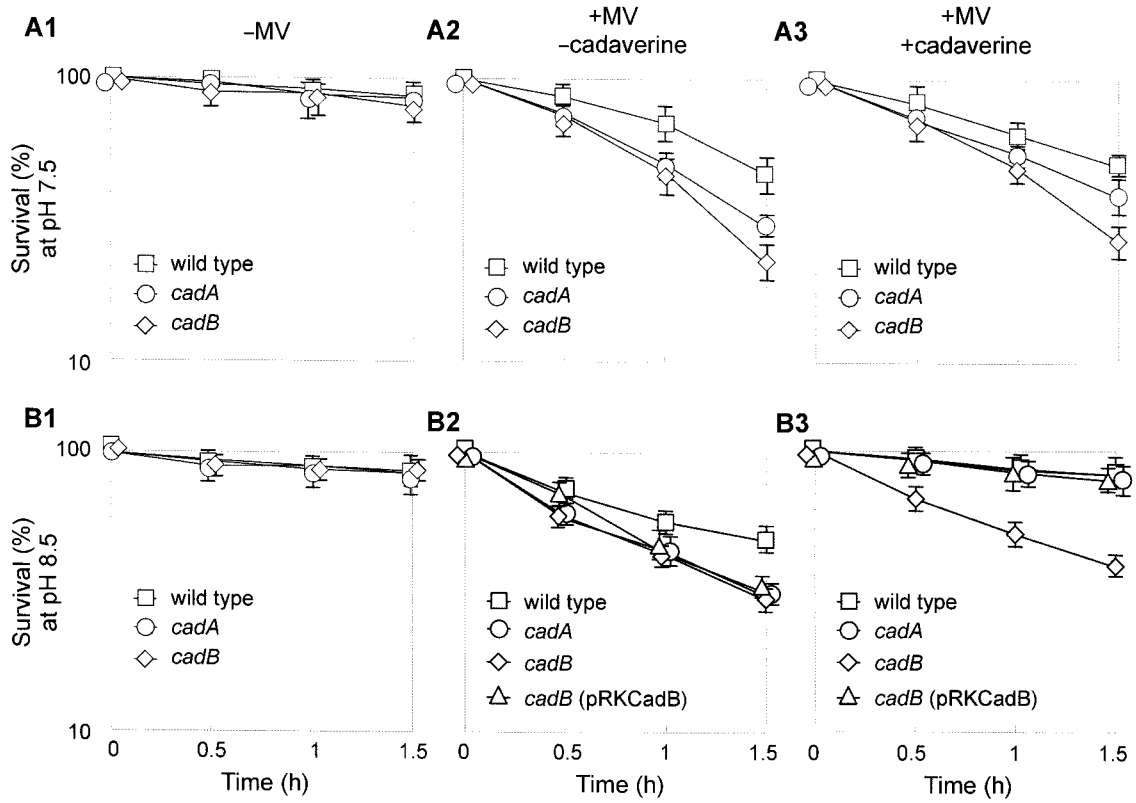

Fig. 3. Involvement of CadB in cell tolerance to superoxide stress in the presence of exogenously added cadaverine.
Cells were grown and transferred to PBS as described in the Fig. 1 legend. Viable cell counts of wild type (□), *cadA* mutant (○), *cadB* mutant (◇), and *cadB* mutant containing pRKCadB (△) were determined, and survival was expressed as the percentage of initial CFU/ml, which was approximately 10^5. The error bars correspond to the SD of the means.

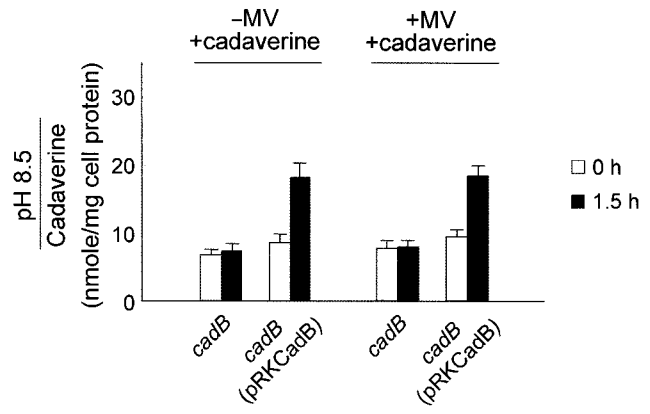

Fig. 4. Determination of cellular cadaverine after exposure of the *cadB* mutant and its *cadB*-complemented strain to the exogenously added cadaverine at pH 8.5.

Approximately 10^5 CFU of the *cadB* mutant and its complemented strain from the tolerance experiments (Fig. 3) were harvested at times 0 and 1.5 h and examined for cadaverine level. Mutants, which had been treated under the same conditions but without MV, were also included as a control. The error bars correspond to the SD of the means.

important for the tolerance to oxidative stress by mediating the transport of the exogenously present cadaverine into the cell.

Acknowledgment

This work was supported by the Seoul R&BD program (10580).

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