The Journal of Microbiology (2009) Vol. 48, No. 1, pp. 00-00
DOI 10.1007/s12275-009-0227-2

Evidence That *Vibrio vulnificus* AhpC2 Is Essential for Survival Under High Salinity by Modulating Intracellular Level of ROS

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(Received June 27, 2009 / Accepted September 9, 2009)

Expression of *ahpC*2 encoding an alkyl hydroperoxide reductase of *Vibrio vulnificus*, a foodborne pathogen, was incrementally induced depending on NaCl concentrations in the culture. Growth of the *ahpC*2 mutant was significantly impaired with longer lag phase and lower growth rate when cultured under high salinity. ROS was accumulated in *V. vulnificus* cells when stressed by exposure to high salinity, and the *ahpC*2 mutant accumulated higher level of ROS as compared with the parental wild type. Consequently, the combined results suggest that AhpC2 contributes to the growth of *V. vulnificus* under high salinity by scavenging ROS in cells.

***Keywords*:** *V. vulnificus ahpC*2, salt stress, ROS

Bacteria have evolved with elaborate protection systems to allow survival and/or growth during exposure to environmental changes. Change in the external salinity is one of the most common environmental stresses that bacteria routinely encounter. *Vibrio vulnificus* is an opportunistic gram-negative pathogen that commonly contaminates raw oysters, and is the causative agent of food-borne diseases such as gastroenteritis and life-threatening septicemia (for a recent review, Jones and Oliver, 2009). Like many other food-borne pathogenic bacteria, *V. vulnificus* occurs in various environments having different salinity strength; it naturally inhabits coastal seawaters, contaminates shellfishes, and colonizes in the human body. This indicates that *V. vulnificus* has to cope with ever changing salinity in their growth environments.

However, until now a definitive analysis of the responsive adaptation of the pathogen to changes in saliniity has not been made, and the molecular mechanisms by which the bacterium can survive in hypersalinity environments have not yet been characterized. This lack of information on the mechanisms of hypersalinity tolerance makes it difficult to understand how the pathogen survive salt stress imposed not only by natural ecosystems but also by the present control practices, such as adding salt to suppress its growth in raw seafood. It is obvious that characterization of the hypersalinity tolerance system at molecular level is useful in delineating novel strategies to control the pathogen contaminated in raw seafood.

Accordingly, in the present study, as an effort to characterize the molecular mechanisms involved in hypersalinity stress resistance, a proteomic analysis was performed to screen and identify proteins of the *V. vulnificus* induced in response to high salinity of the culture media. A protein, AhpC2, an alkyl hydroperoxide reductase, was identified among the proteins specifically induced by exposure to high salinity. The possible roles of AhpC2 in hypersalinity stress resistance of *V. vulnificus* have been demonstrated by comparing the growth of an isogenic *ahpC*2 mutant with that of its parental wild type in the presence of high salinity. It is also apparent that the AhpC2 protein plays a role in hypersalinity stress resistance by scavenging intracellular reactive oxygen species (ROS) imposed by high salinity in culture media.

The strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, the *V. vulnificus* strains were grown in Luria-Bertani (LB) medium supplemented with 2.0% (w/v) NaCl (LBS). All the media components were purchased from Difco (Detroit, MI), and the chemicals were purchased from Sigma (St. Louis, MO). Averages and standard errors of the mean (SEM) were calculated from at least three independent determinations.

Proteomic analysis and identification of AhpC2

Proteins from the wild type *V. vulnificus* MO6-24/O cells grown in LB supplemented with 2% or 5% NaCl, respectively, were prepared, resolved by 2 D gel electrophoresis (2 DE), and silver stained as described elsewhere (Oh *et al*., 2009). The protein spots more abundant in *V. vulnificus* in the presence of 5% NaCl were excised, digested with trypsin (Promega, USA), and used for MS analysis with a MALDI-TOF mass spectrometer (Matrix-Assisted Laser Desorption ionization Mass Spectrometer, Voyager-DE STR Biospectrometry Workstation, Germany). Among the spots, one protein was identified as the *V. vulnificus* AhpC2 on the basis of the databases of the *V. vulnificus* CMCP6 genome which were retrieved from GenBank (accession number VV1_0453, www.ncbi.nlm.nih.gov) (Fig. 1A).

Transcription of *ahpC*2 is induced by hypersalinity in growth medium

When protein profiles of the *V. vulnificus* grown with 2% and 5% NaCl respectively were compared, AhpC2 was found

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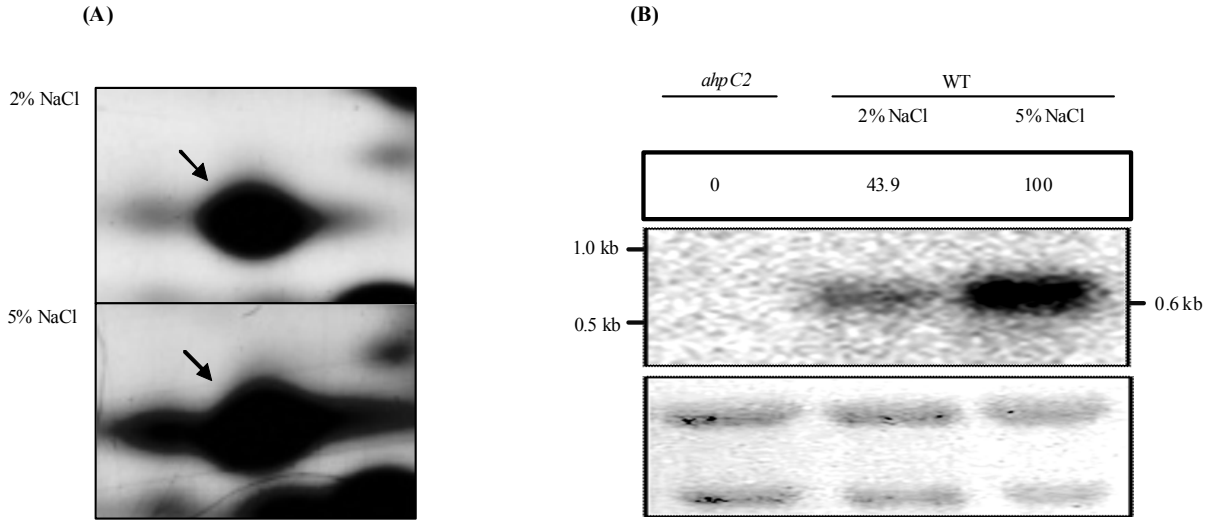

Fig. 1. Dependency of the *V. vulnificus ahpC*2 expression on high salinity. (A and B) The *V. vulnificus* strains were grown in LB supplemented with different levels of NaCl as indicated, then samples removed at an A_{600} of 0.6 were analyzed for protein profiles (A) and the *ahpC*2 transcript (B). (A) A protein spot whose abundance increased in the wild-type cells grown with 5% NaCl is indicated with arrows and was subjected to MALDI-TOF MS analysis. (B) Total RNAs were isolated as described previously (Jeong *et al*., 2009; Oh *et al*., 2009), separated (bottom) and then hybridized to AHPC2P, a ^{32}P DNA probe (top). The relative levels of the *ahpC*2 transcripts are presented relative to the level of the *ahpC*2 in the wild-type cells grown with 5% NaCl. The two bands represent rRNAs (bottom), and the molecular size markers (Invitrogen) and the *ahpC*2 transcripts are shown in kilobases. The 2 DE gels and Northern hybridization products were visualized and quantified using a phosphorimage analyzer (BAS1500, Fuji Photo Film Co. Ltd, Tokyo, Japan) and the Image Gauge (version 3.12) program.

more abundant in the culture grown with 5% NaCl (Fig. 1A). This variation of AhpC2 occurs possibly either at the transcriptional level or post-transcriptional level of the *ahpC*2 expression. To distinguish these two possibilities, changes in the level of the *ahpC*2 mRNA were monitored in the same amount of total RNA isolated from wild type and the *ahpC*2 mutant OH0505 cells grown in LB with different salinity. Construction of OH0505, in which *ahpC*2 was inactivated by insertion of *npt*I conferring resistance to kanamycin (Oka *et al*., 1981), was described elsewhere (Oh *et al*., 2008) (Table 1).

For Northern blot analysis, total cellular RNAs from the *V. vulnificus* strains were isolated, and a series of reactions was performed according to standard procedures (Sambrook and Russell, 2001) with 20 μg of total RNA. RNA was transferred to a nylon membrane, and hybridized as previously described (Lee *et al*., 2006; Rhee *et al*., 2006). The DNA probe AHPC2P was prepared by labeling the 0.6-kb DNA fragment containing the coding region of *ahpC*2 that was amplified by a PCR using primers TsaA01 (5'-AACGTCGATTGGCTCACAAA-3') and TsaA02 (5'-ACTTCGCCGTGCTTCT GGTG-3'). The relative levels of the *ahpC*2 mRNA increased as the NaCl concentration in the growth media increased from 2% to 5%, suggesting that the effect of hypersalinity on the level of AhpC2 was correlated with the increase in mRNA level of the *ahpC*2 gene (Fig. 1B).

Effect of *ahpC*2 mutation on the growth of *V. vulnificus* under various osmotic stresses

Since the expression of *ahpC*2 was induced by increasing the NaCl concentration in the growth media, we compared growth rates of the wild type and *ahpC*2 mutant OH0505 in the medium with different levels of NaCl. Cultures of the *V. vulnificus* strains were grown at 30°C under aeration, and the growth monitored by measuring the A_{600} of the cultures. It was

Table 1. Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics [a]	Reference or Source
Strains		
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	Laboratory collection
OH0505	MO6-24/O with *ahpC*2:: *nptI*; Km^r	Oh *et al*., 2008
Plasmids		
pJH0311	0.3-kb *NruI* fragment containing multi-cloning site of pUC19 cloned into pCOS5; Ap^r, Cm^r	Goo *et al*., 2006
pOH033	pJH0311 with *ahpC*2; Ap^r, Cm^r	Oh *et al*., 2008

[a]Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant

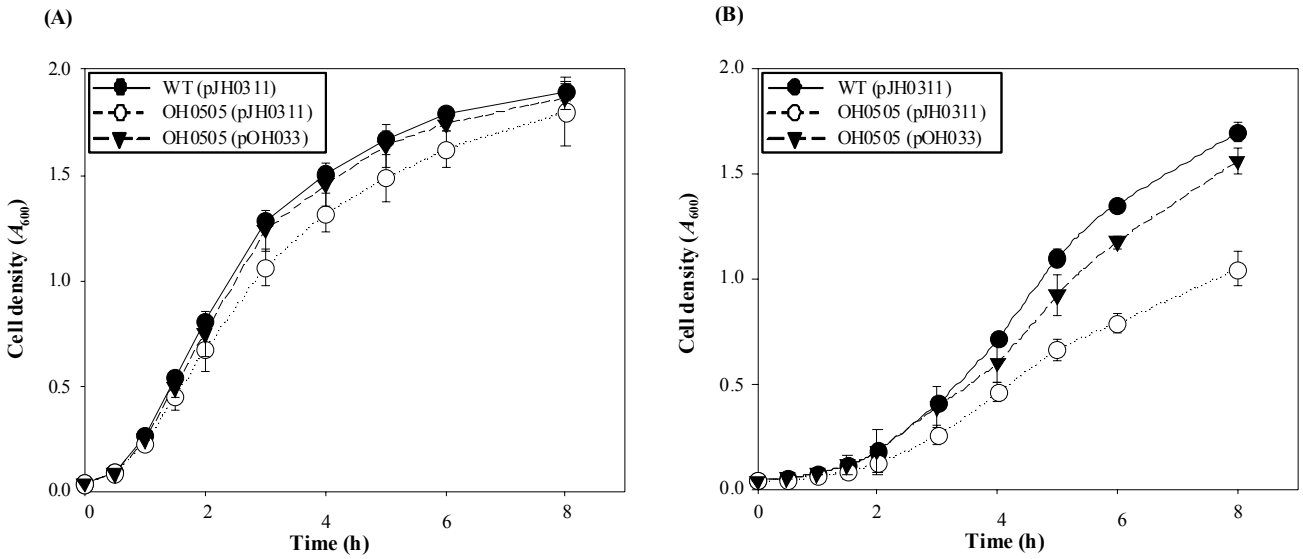

Fig. 2. Effects of the *ahpC*2 mutation on the growth of *V. vulnificus* under high salinity. Cultures of the wild type [WT (pJH0311)], *ahpC*2 mutat [OH0505 (pJH0311)], and complemented strain [OH0505 (pOH033)] were grown in LB supplemented with 2% (A) or with 5% NaCl (B) as indicated, and the growth monitored by measuring the A_{600} of the cultures. Details are described in text.

obvious that growth of both strains were impaired with a longer lag phase and lower growth rate when they were cultured with 5% NaCl (Fig. 2B). In the presence of 2% NaCl, the growth rate of the *ahpC*2 mutant was slightly but consistently lower than that of the parental wild type (Fig. 2A). However, the growth rate of the *ahpC*2 mutant in the LB supplemented with 5% NaCl was much lower than that of the wild type (Fig. 2B), indicating that AhpC2 is essential for growth and survival of *V. vulnificus* under adverse conditions that might be imposed by high level of NaCl.

To rule out the possibility that the impaired growth in LB with high salinity resulted from polar effects of the *ahpC*2 insertional mutation on downstream genes, we examined whether reintroduction of pOH033 carrying recombinant *ahpC*2 could complement the decrease in growth rate of OH0505 cells. The plasmid pOH033 was constructed by subcloning the *ahpC*2 as described elsewhere (Oh *et al*., 2008) (Table 1), and mobilized into *V. vulnificus* by conjugation. The growth rate of the OH0505 (pOH033) was restored to a level comparable to the wild-type level of MO6-24/O (Fig. 2A and B). Therefore, the decreased growth of OH0505 resulted from the inactivation of functional *ahpC*2 rather than any polar effects on any genes downstream of *ahpC*2.

To examine whether this decrease in growth rate of the *ahpC*2 mutant occurred due to increasing osmotic stress or due to the NaCl itself, the influence of other osmolytes on the growth of the wild type and *ahpC*2 mutant was analyzed. The growth rates of the *ahpC*2 mutant in LB supplemented with other types of osmolytes such as 0.6 M sucrose and 0.6 M KCl were not significantly different from those of the wild type (Fig. 3A). From this result, it is not likely that impaired growth of the *ahpC*2 mutant in the presence of 0.6 M NaCl resulted from the osmotic stress (Fig. 3B). The results combined suggested that AhpC2 could play a role in the growth and survival of *V. vulnificus* by contributing to reduction of a potential, yet unknown, stress(s) rather than directly counteracting the increased osmotic strengths.

Intracellular levels of ROS in *V. vulnificus* strains under high salinity

Intracellular levels of ROS were measured using the fluorescent dye 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) according to the procedures of Perez *et al*. (Perez *et al*., 2008). Briefly cultures were grown with 2% or 5% NaCl, respectively, to an A_{600} of 0.5, washed with potassium phosphate buffer (10 mM, pH 7.0), and incubated for 30 min in the same buffer containing 10 μM H_2DCF-DA. The cells were washed, suspended in the PBS, and disrupted by sonication. After clarified by centrifugation, the fluorescence intensity of 2',7'-dichlorofluorescein (DCF) in the supernatant was measured using a fluorescence spectrophotometer (M200, Tecan system, Switzerland) at excitation wavelength of 485 nm and emission wavelength of 535 nm. Fluorescence emission values were normalized to absorbance (A_{600}) of the cultures.

The DCF florescence was monitored in the cells grown with 2% or 5% NaCl. Both wild type and *ahpC*2 mutant cells grown with 5% NaCl showed consistent and significant increase in DCF fluorescence compared with those grown with 2% NaCl (Fig. 4A and B). This result indicated that both strains of *V. vulnificus* accumulated higher levels of ROS when they were stressed by high salinity. It is noteworthy that cells lacking the *ahpC*2 gene showed a significant increase in the DCF fluorescence as compared with the wild type, regardless of the levels of NaCl in the culture media, indicating that AhpC2 is responsible for reduction of ROS accumulated by high salinity in the growth conditions. Again, reintroduction of recombinant *ahpC*2 was able to complement the increase of intracellular level of ROS in OH0505 and the intracellular ROS in the complemented strain decreased to the level comparable to that of wild type (Fig. 4A and B).

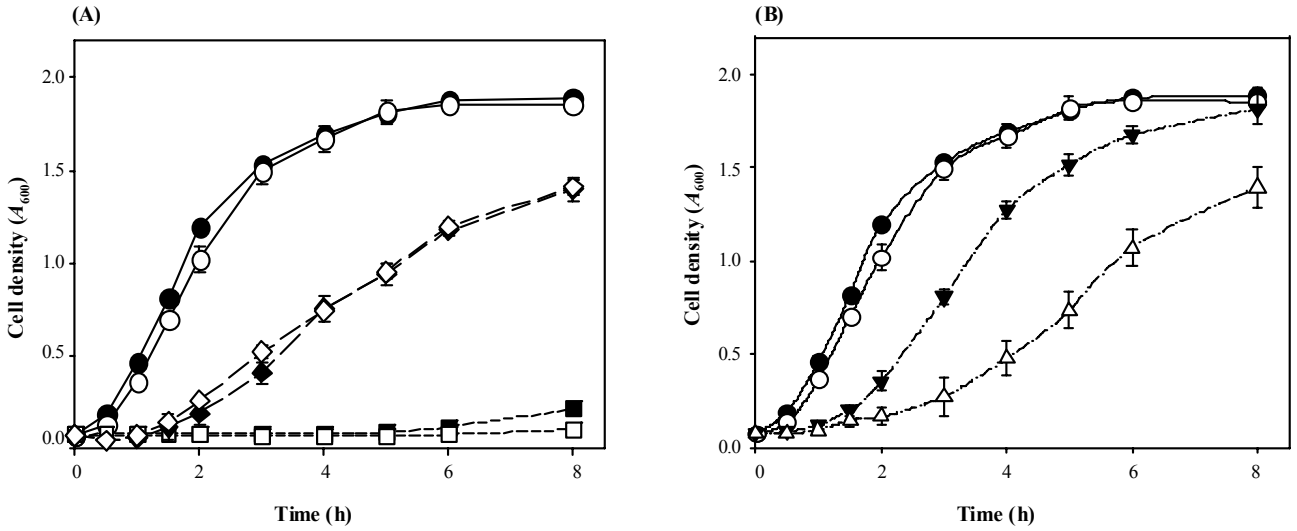

Fig. 3. Growth of the *V. vulnificus* strains under various osmotic stresses. Cultures of the wild type (filled symbols) or the *ahpC*2 mutant (open symbols) were grown in LBS media (A and B, ● and ○) and LBS supplemented with 0.6 M sucrose (A, and), with 0.6 M KCl (A, ■ and □) or with 0.6 M NaCl (B, ▼ and Δ) as a source of osmolytes, and the growth monitored by measuring the A_{600} of the cultures. Details are described in text.

Alkyl hydroperoxide reducase, AhpC, one of the best characterized peroxiredoxins, forming a novel NAD(P)H-dependent peroxide reductase system with AhpF, was originally identified from *Escherichia coli* and *Salmonella typhimurium* (Christman *et al*., 1985). Alkyl hydroperoxide reducase system, AhpCF, of Gram-negative bacteria, provides an important protection against peroxides in the environments and within host (Wood *et al*., 2003; Poole, 2005). In a previous study, *ahpC*1 and *ahpC*2 were identified among the *V. vulnificus* genes specifically induced by exposure to H_2O_2 (Lee, 2009. unpublished data). AhpC1, a homologue of *E. coli* AhpC, is 78% identical to those of the AhpC homologues from *E. coli* and *S. typhimurium* and constitutes an alkyl hydroperoxide reductase system with AhpF as a reductant in *V. vulnificus* (Baek *et al*., 2009).

AhpC2, an alternative alkyl hydroperoxide reductase of *V. vulnificus*, carries two conserved cysteinyl residues near its N- and C-termini, respectively (Oh, 2009. unpublished data). The amino acid composition and molecular weight of this AhpC2 are quite different from those of the *V. vulnificus* AhpC1 and AhpC homologues from other Gram-negative bacteria. The amino acid sequence of the AhpC2 was only 39% identical to that of the *V. vulnificus* AhpC1. Although this difference in amino acid sequences indicates that the function of AhpC2 would be different from that of AhpC1 in *V. vulnificus*, the purified AhpC2 was able to reduce H_2O_2 in the presence of thioredoxin A/ thioredoxin reductase and NADH as a hydrogen donor (Oh, 2009. unpublished data).

It is noteworthy that high salinity levels resulted in induction of AhpC2 of *V. vulnificus*, that is a halophilic organism and naturally inhabits in high-salinity environments. It has been reported that high concentration of salt protects bacteria from oxidative stresses such as paraquat and H_2O_2 (Kitzler and Fridovich, 1986; Antelmann *et al.*, 1996; Vattanaviboon *et al*., 2003; Yan *et al*., 2006). In the present study, high salinity appeared to increase the levels of ROS within *V. vulnificus* cells, whereby induce the expression of the antioxidant enzyme AhpC2 (Fig. 1 and 4). From this, it is likely that expression of other antioxidant enzymes such as catalase and superoxide dismutase of *V. vulnificus* would be also induced by high salinity. Although further understanding the molecular mechanisms by which high level of NaCl, not other osmolytes, increase the cellular ROS within *V. vulnificus* cells will await additional works, the induction of the antixoidant enzyme AhpC2 could play a role in the survival of this bacterium in

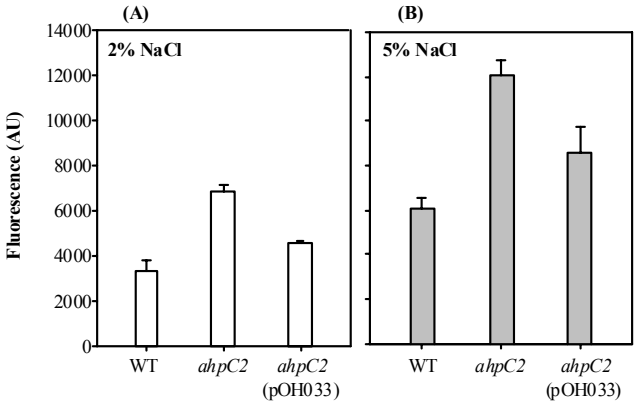

Fig. 4. Intracellular ROS levels in the *V. vulnificus* strains under high salinity. Cultures of the wild type (WT), *ahpC*2 mutat (*ahpC*2), and complemented strain [OH0505 (pOH033)] were grown in LB supplemented with 2% (A) or with 5% NaCl (B) as indicated. Exponential cultures grown to an A_{600} of 0.5 were harvested, and the intracellular ROS levels determined by the 2',7'-dichlorodihydrofluorescein (DCF) procedure as described elsewhere (Perez *et al*., 2008). Details are described in text. AU, arbitrary units.

the marine habitats.

In summary, it is apparent that expression of the *V. vulnificus ahpC*2 gene encoding an alkyl hydroperoxide reductase is incrementally induced with concentrations of NaCl. Growth of the *ahpC*2 mutant was significantly impaired with longer lag phase and lower growth rate when cultured with high salinity. ROS was accumulated in *V. vulnificus* when stressed by exposure to high salinity, and the *ahpC*2 mutant accumulated higher level of ROS as compared with the parental wild type. These results suggest that AhpC2 of *V. vulnificus* is essential for growth under high salinity by reducing ROS accumulated in cells.

Acknowledgments

This study was supported by grants to S.H.C from the Marine Biotechnology Program funded by the Ministry of Land, Transport, and Maritime Affairs; the World Class University project (R32-2008-000-10183-0); and the National Research Laboratory Grant (R0A-2007-000-20039-0), Republic of Korea.

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