Roles of Alkyl Hydroperoxide Reductase Subunit C (AhpC) in Viable but Nonculturable *Vibrio parahaemolyticus*

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Alkylhydroperoxide reductase subunit C (AhpC) is the catalytic subunit responsible for the detoxification of reactive oxygen species that form in bacterial cells or derived from host, and facilitate the survival of pathogenic bacteria under environmental stresses or during infection. This study investigates their role in the induction and maintenance of a viable but nonculturable (VBNC) state in *Vibrio parahaemolyticus*. In this investigation, *ahpC1* (VPA1683) and *ahpC2* (VP0580) were identified in chromosomes II and I of this pathogen, respectively. Deletion mutants of these two *ahpC* genes and their complementary strains were constructed from a parent strain KX-V231. The growth of these strains was monitored on Tryptic Soy Agar-3% NaCl in the presence of extrinsic peroxides, H$_2$O$_2$ and tert-butyl hydroperoxide (t-BOOH), at different incubation temperatures. Results revealed that both *ahpC* genes were protective for t-BOOH, while *ahpC1* was protective for H$_2$O$_2$. The protective function of *ahpC2* at 4°C was higher than that of *ahpC1*. The times to induce the VBNC state (4.7 weeks) at 4°C in a modified Morita mineral salt solution with 0.5% NaCl and then...
maintain the VBNC state (4.7 week) in *ahpC2* and *ahpC1-ahpC2* double

mutants were significantly shorter than in the parent strain (induction 6.2

weeks, maintenance 7.8 weeks) and the *ahpC1* mutant (induction 6.0

weeks, maintenance 8.0 weeks) (*p* < 0.03). Complementation with an

*ahpC2* gene reverted the effects of the *ahpC2* mutation on shortening the

induction and maintenance of the VBNC state. This investigation

identified the different functions of the two *ahpC* genes and confirmed

the particular role of *ahpC2* in the VBNC state of *V. parahaemolyticus*.


Key words: *Vibrio parahaemolyticus*; AhpC; viable but nonculturable

state; oxidative stress; resuscitation
INTRODUCTION

Vibrio parahaemolyticus is a halophilic Gram-negative bacterium which causes foodborne gastroenteritis worldwide and is prevalent in some Asian countries (2). This pathogen exhibits global significance since the occurrence of the first pandemic O3:K6 strains in 1996 (35). It also inhabits brackish water, where a lot of those shellfish are found (48). Similar to other vibrios in the marine environment, a unique physiological state known as viable but nonculturable (VBNC) state in V. parahaemolyticus can be induced by incubation at low temperature in a mineral salt starvation medium (5, 20, 49), and cells in this state are viable while unable to form colonies on common agar medium, while they can be resuscitated by a temperature upshift treatment (50).

The VBNC state may represent a dormant state that improves the survival of non-sporulating bacteria in an adverse environment (38). Drastic morphological and physiological changes have been demonstrated in the VBNC state of V. parahaemolyticus (47, 49). Virulence usually decreases markedly as cells enter the VBNC state (47), nevertheless, virulence genes and pathogenic potential are maintained (38,
42). However, the genetic control of the VBNC state of this pathogen or other bacteria has not been fully clarified.

The involvement of antioxidative factors in the VBNC state has been investigated (7, 32, 46). Adding extrinsic catalase or other antioxidative factors to the culture media improves the culturability of VBNC cultures in *Aeromonas hydrophila* (46), *Escherichia coli* (32) and *V. vulnificus* (7). In the VBNC suppression mutant of *V. vulnificus*, glutathione S-transferase is identified; this is a cytosolic, mitochondrial, and microsomal protein that detoxifies endogenous compounds, such as peroxidized lipids (1). This enzyme is highly expressed by the VBNC-suppressing mutant (about 10-fold changes in 6 to 24 h) under stress by low temperature (1). These studies suggest that upon exposure to high nutrient level, the VBNC cells form reactive oxygen species (ROS), while these cells are not able to detoxify these harmful radicals and thus fail to replicate and grow in such nutrient media (6, 7).

An *oxyR* mutant of *V. vulnificus* that lacks catalase activity has been constructed, and it is nonculturable on solid media (24). This study suggests that the cold-induced loss of catalase activity attributes to the induction of VBNC state. However, OxyR regulates the expression of
both catalases and alkylhydroperoxide reductase subunit C (AhpC) in several Gram-negative bacteria in response to elevated levels of ROS (11, 16), and AhpCs are the catalytic subunits of these enzymes (33). The alkylhydroperoxide reductases are members of a family of peroxidases, collectively called peroxiredoxins, or thiol peroxidases (TPx family), which can be divided into two subgroups, depending on the number of conserved cysteines. These ubiquitous enzymes constitute an important component of defense against a wide range of substrates, including H₂O₂, organic peroxides and peroxynitrite. They are involved in both the control of endogenous peroxides and the inducible defense response to exogenous peroxides or general stresses (17). The roles of catalases and AhpCs in VBNC state need to be differentiated.

Searching through the genome of V. parahaemolyticus RIMD2210633 (28) revealed the presence of several putative AphC factors (VP0580, VPA1681, VPA1683, VPA1684, VPA1293). Using BLAST analysis (http://blast.ncbi.nlm.nih.gov/) with homologous genes of V. cholerae, V. vulnificus and Salmonella enterica (21), the VPA1683 and VP0580 genes of V. parahaemolyticus are designated as ahpC1 and ahpC2, respectively.

In our previous study, the induction of the VBNC state in V.
protein death AlphC2 was about 10-fold increased as determined by 2-D gel electrophoresis (25). Based on the above-mentioned functions of antioxidative factors in VBNC state, we hypothesize that AhpCs play important roles in conquering the low level oxidative stress generated in cells which are incubated at low temperature in starvation medium and consequentially slow down the loss of culturability during the induction of VBNC state and maintain the viability of cells which have entered the VBNC state. In this study, the specific roles of ahpC1 and ahpC2 in the induction and maintenance of the VBNC state in V. parahaemolyticus were investigated by means of deletion mutations and gene complementation, while the gene expressions of various antioxidative factors and their regulators (RpoS and OxyR) in these mutants and the wild-type strain were monitored.

MATERIALS AND METHODS
**Bacterial strains and culture conditions.** *V. parahaemolyticus* strain KX-V231 (KP+, serotype O3:K6, lab no. 1173), isolated in Thailand from a clinical specimen, was used in this study (Table 1). It was stored frozen at -85°C in beads in Microbank cryovials (PRO-LAB Diagnostics, Austin, TX, USA). It was cultured at 37°C on Tryptic Soy Agar (Becton-Dickinson Diagnostic Systems, Sparks, MD, USA) that was supplemented with 3% sodium chloride (TSA-3% NaCl), or in Tryptic soy broth-3%NaCl (TSB-3%NaCl) in a 50 ml tube, which was shaken at 160 rpm. A 50 μl aliquot of the 16 h broth culture was inoculated into 10 ml of fresh TSB-3% NaCl and incubated at 37°C with shaking for 2 h, to enter the exponential phase (around 10⁸ CFU/ml) and this culture was used as the inoculum in the following experiments. *Escherichia coli* was cultured in Luria Broth (LB, Becton-Dickinson) at 37°C and shaken at 160 rpm. Chloramphenicol (final concentration of 6 μg/ml) or chloramphenicol (20 μg/ml)/ampicillin (50 μg/ml) was added to the media as required for the cultivation of some of the *V. parahaemolyticus* or *E. coli* strains, respectively.

**Construction of deletion mutants.** Deletion mutants of the *ahpC* genes were constructed following the published method with
modifications (37). PCR-amplified DNA fragments that were used to construct the in-frame deletion mutation of *ahpC1* were generated by means of overlap PCR as described previously (40). Two DNA fragments were amplified by PCR with *V. parahaemolyticus* KX-V231 chromosomal DNA as the template – one with the primer pair ahpC1-1 and ahpC1-2 and the other with the primer pair ahpC1-3 and ahpC1-4 (supplementary Table S1). Phusion high-fidelity DNA polymerase (Finnzymes Oy, Vantaa, Finland) was used in this PCR reaction. These two amplified fragments were then used as templates for a second PCR with the primers ahpC1-1 and ahpC1-4, resulting in the construction of a fragment with a deletion in the *ahpC1* gene. Such a fragment, that contained the deletion was purified, reacted with *Taq* at 72°C for 30 min to add 3’ A-overhangs to the blunt ends, and cloned into the pGEMT-easy vector and transformed into *E. coli* XL1 blue, following the protocol of the manufacturer (Promega Co., Madison, WI, USA). The inserted sequence was verified by sequencing. This fragment was then removed from the pGEMT-easy vector by digestion using SacI and SphI and cloned into a suicide vector, pDS132, which contained the chloramphenicol-resistant gene and the *sacB* gene, conferring sensitivity.
to sucrose. This plasmid (pDS132-ahpC1-deletion) was introduced into *E. coli* SM10-pir and then mated with *V. parahaemolyticus* strain KX-V231. Thiosulfate-citrate-bile-sucrose (TCBS) agar that contained chloramphenicol was used to screen the *V. parahaemolyticus* cells containing the inserted plasmid. The *V. parahaemolyticus* clones were isolated and cultured in Luria-Bertani (LB, Becton-Dickinson) broth that was supplemented with 2% NaCl and chloramphenicol. DNA was extracted from these cultures and the inserted sequence was detected by PCR using the ahpC1-1 and ahpC1-4 primers. The culture that contained pDS132-ahpC1-deletion plasmid was incubated at 37°C for 3 hours in the LB broth that contained 2% NaCl and then plated on an LB agar plate that contained 2% NaCl and 10% sucrose. The colonies isolated that were unable to grow on LB agar plate that contained chloramphenicol were selected, and the homologous recombination of the deleted fragment was verified by PCR using primers ahpC1-0 and ahpC1-5. Following the same procedures using different primers, the *ahpC2* deletion mutant was also constructed (Table 1). Amplification of the *ahpC1* and *ahpC2* genes by PCR with the primers ahpc1-0 and ahpc1-5 or ahpc2-0 and ahpc2-5 yielded amplicons of 1,849bp or 2,004bp, respectively, while the in-frame
deletion mutants formed amplicons of 1,336 bp or 1,494 bp, respectively. The mutated genes were also verified by the nucleotide sequencing of the amplified fragments. Sequencing service was provided by Genomics BioSci & Tech, Inc., Taipei, using Sanger’s method with Applied Biosystems 3730 analyzer.

Construction of complementary strains. The entire lengths of $ahpC1$ and $ahpC2$ genes were amplified by PCR with *V. parahaemolyticus* KX-V231 chromosomal DNA as the template using primer pairs ($ahpC1$-salI and $ahpC1$-sphI for $ahpC1$, $ahpC2$-salI and $ahpC2$-sphI for $ahpC2$) with restriction enzyme linkers (salI, sphI) (Table S1). The amplicons were digested with SalI and SphI, and ligated to the shuttle vector pSCB01 which had been digested with the same enzymes. Shuttle vector pSCB01 (8,123 bp) was constructed by ligating the $mobRP4$ fragment that was recovered from the HindIII-digested fragments of pDS132 to pBR328 that had been digested by HindIII (Table 1). The plasmids, pSCB02 and pSCB03, containing the entire lengths of $aphC1$ or $ahpC2$ genes, respectively, were propagated in *E. coli* SM10 $\lambda$-pir and conjugated to the corresponding $ahpC$ mutants to generate complementary strains, which were selected by their
chloramphenicol resistance (Table 1). The presence of entire lengths of

*ahpC1* and *ahpC2* genes in these strains was verified by PCR.

**Effects of peroxides and incubation temperatures on growth and survival of the *ahpC* mutants.** Cultures of different *V. parahaemolyticus*

strains in the exponential phase, with an absorbance at 600 nm of 0.5-0.6,

were $10^{-1}$ to $10^{-5}$-fold (-1 to -5 log$_{10}$) diluted by TSB-3% NaCl. Ten

microliter aliquots of the diluted suspensions were spotted on TSA-3%

NaCl plates with or without the addition of 250 μM H$_2$O$_2$ or 80 μM

tert-butyl hydroperoxide (t-BOOH). The plates were incubated at 25°C

for 16 h, and the colony-forming ability of the inoculums was observed

(4).

Aliquots (100 μl) of different strains ($10^8$ CFU/ml) were also spread

on TSA-3% NaCl plates and incubated at 22, 30 or 37°C for 24, 15 and

15 h, respectively, and the diameters of colonies were measured. In

another experiment, cultures in exponential phase in TSB-3% NaCl were

incubated at 4°C and the survivors were counted by standard plate count

method for 9 days.

**Induction of VBNC state and resuscitation of the VBNC cells.**

Different strains of *V. parahaemolyticus* were induced to enter the VBNC
state by a previously described method (25, 49). Briefly, bacterial cells in
the exponential phase were harvested, washed twice using modified
Morita mineral salt solution (MMS-0.5%NaCl) and resuspended in the
same medium at a concentration of 10^7 cells/ml, before being incubated at
4°C in a static state to induce the VBNC state.

Culturable cells of the cultures were counted at weekly intervals by
the standard plate count method. When the cultures entered the VBNC
state, the cultures were resuscitated every week by temperature upshift
treatment at 25°C for 48 h prior to plate counting (49, 50). The levels of
culturable cells (cfu/ml) were expressed in log_{10} values.

**Quantitative reverse transcription polymerase chain reaction.** The
expressions of antioxidative genes and related regulators were determined
by real-time quantitative reverse transcription-polymerase chain reaction
(RT-qPCR)(13). Briefly, cells were lysed with TRIzol®Reagent
(Invitrogen, UK) and RNA samples were extracted using an RNApure kit
(Genesis Biotech Inc., Taipei, Taiwan), following the manufacturer's
instructions. RNA samples were treated with DNase I (Takara Bio Inc.,
Shiga, Japan) and then reverse-transcribed using a SuperScript® III
First-Strand Synthesis SuperMix (Invitrogen, UK), following the
instructions of the manufacturer. Primers (Supplementary Table S2) were designed using the Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and 16S rRNA was used as the internal control. Real-time PCR was performed using the ABI Prism 7300 Sequence Detection System (Perkin-Elmer Applied Biosystems) with an SYBR Green PCR Master Mix and RT-PCR reagents. All the data were normalized with the 16S gene expression levels of the culture at each time point and the normalized values for each gene were compared. The expression of each gene was compared to its expression in the exponential phase and the relative values were expressed in log_{10} folds (log fold) following the instructions of the manufacturer (Perkin-Elmer Applied Biosystems).

The quantity and quality of the RNA samples were assayed by determining the 260/280 nm absorbance ratio. The integrity of the RNA samples, before and after the DNase treatment, was assessed by 1% (w/v) agarose gel electrophoresis with ethidium bromide staining. The absence of genomic DNA contamination was verified by PCR amplification using total RNA as the template and primers that were designed for amplification of a 500 bp DNA fragment (F:
5’-CAGGCCTAACACATGCAAGTC; R: 5’-ATTACCGCGGCTGCTGG) from the 16S rRNA gene and a 72 bp DNA fragment (F: 5’-AGCATCAACCTATTCGTGTT; R: 5’-TAATTCGTAGCGATTGTCTG) from the VP2468 (L,D-Carboxypeptidase) gene (supplementary Fig. S1). Also, a recombinant plasmid for VP2468 gene was created to be used as a calibration standard, and a strong correlation (R²=0.999) between the concentration of this target gene and Ct values was observed using the protocol herein (supplementary Fig. S2) (27, 29).

**Statistical analysis.** Triplicate experiments were performed for the induction of VBNC state and resuscitation of the VBNC cultures. Replicate experiments on gene expression were performed. Statistical significance of data was assessed by Student’s t-test or ANOVA with Duncan’s multiple test at a significance level of α = 0.05, using SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL, USA).

**RESULTS**

It is hypothesized that the change of culturability of the *V.*
parahaemolyticus cells incubated at the VBNC induction conditions depends on the presence of toxic ROS and function of the conquering antioxidative AhpCs. In this study, we only demonstrated the antioxidative properties of these AhpCs, and its role in determining the induction time and maintenance of the VBNC state.

**Antioxidative activity of the ahpC mutants.** The deletion mutants of *ahpC1* and *ahpC2* and their corresponding complementary strains were examined for their colony-forming abilities on an agar medium under the influences of extrinsic peroxides. The extents of growth of the wild-type, mutants and complementary strains when spotted at different dilutions were similar on TSA-3% NaCl with no extrinsic peroxide added (Fig. 1A). In the presence of inorganic H$_2$O$_2$, the colony-forming abilities of the *ahpC1* mutant (ΔahpC1) and *ahpC1-ahpC2* double mutant (ΔahpC12) were reduced, and such inhibition was reversed by complementation of the *ahpC1* gene (ΔahpC1/C1). The colony-forming abilities of the *ahpC2* mutant and its complementary strain were similar to that of the wild-type strain (Fig. 1B). The presence of 80 μM of t-BOOH reduced the colony-forming abilities of the wild-type strain and such inhibition was further enhanced in the *ahpC1, ahpC2* and double mutants. Presence of
the complementary *ahpC* genes in the single *ahpC* mutants substantially reverted such inhibition (Fig. 1C). These results demonstrated the antioxidative function of these two *ahpC* genes.

**Growth and survival of *ahpC* mutants at different incubation temperatures.** When the wild-type KX-V231, *ahpC* mutants and their complementary strains were spread on rich agar medium (TSA-3% NaCl) and incubated at 22, 30 or 37°C, the *ahpC1-ahpC2* double mutant (strain ΔahpC12) formed the smallest colonies at these temperatures, while the *ahpC1* mutant (strain ΔahpC1) formed significantly smaller colonies than those of the *ahpC2* mutant (strain ΔahpC2) or the wild-type strain (*p* < 0.05). The influence of *ahpC1* on the diameter of the colonies was enhanced at incubation temperatures of 22 and 30°C, but not at 37°C. Chloramphenicol was added to the agar medium to maintain the complementary plasmids or cloning vector, and it affected the size of the colonies. The double mutant that contained the cloning vector (ΔahpC12V) that was grown on agar medium that contained chloramphenicol was markedly smaller than that of ΔahpC12, while the mutants with the complementary *ahpC1* or *ahpC2* gene relieved the inhibition of growth and formed larger colonies on the same agar medium.
When the *V. parahaemolyticus* cultures in TSB-3% NaCl were incubated at 4°C, the culturability of the KX-V231 and *ahpC* mutants (strains ΔahpC1, ΔahpC2) gradually declined at similar rates (about 22-28% survivals in nine days), while the culturability of the double mutant (strain ΔahpC12) declined rapidly to less than 1% in nine days (Fig. 2A). When the double mutants that contained the cloning vector or complementary plasmids in broth that contained chloramphenicol were incubated at 4°C, the culturability of the double mutant (strain ΔahpC12V) declined rapidly; the slow rate of decline (about 24% survivals in nine days) was restored in the presence of complementary *ahpC2* gene (strain ΔahpC12/C2). Complementation with *ahpC1* (strain ΔahpC12/C1) did not alter the culturability of this double mutant (Fig. 2B). These results demonstrated that *ahpC2* was important in the survival of *V. parahaemolyticus* at refrigerating temperature.

**Induction of VBNC state and resuscitation in the *ahpC* mutants.**

This study demonstrated that *ahpC2* has a greater role than *ahpC1* in the VBNC state in *V. parahaemolyticus* and the maintenance of viability in that state by gene deletion and complementation (Figs. 3, 4). The VBNC
state was successfully induced in the wild-type strain in approximately six weeks, while the induction times in \textit{ahpC2} mutant (strain \(\Delta\text{ahpC2}\)) and \(\text{ahpC1-ahpC2}\) double mutant (strain \(\Delta\text{ahpC12}\)) were significantly shorter in about 4.6 weeks \((p \leq 0.05)\) (Figs. 3, 4). The VBNC induction time of the \(\text{ahpC2}\) mutant with the presence of its complementary gene (strain \(\Delta\text{ahpC2/C2}\)) was significantly longer (about 5.5 weeks) than strain \(\Delta\text{ahpC2}\) and was approximately equal to that in the wild-type strain \((p < 0.05)\). The induction time of the VBNC state in the \(\text{ahpC1}\) mutant (strain \(\Delta\text{ahpC1}\)) was not significantly different from that of the wild-type strain \((p > 0.05)\).

Within 5 to 7 weeks in the VBNC state, the levels of culturable cell of the resuscitated cultures (about 7 log cfu/ml) were similar to those at the beginning of the VBNC induction (Fig. 3). The wild-type strain remained viable and maintained in the VBNC state for an average of 7.8 weeks could be resuscitated, and this time was significantly shortened to 4.7 weeks for the \(\text{ahpC2}\) mutant (strain \(\Delta\text{ahpC2}\)) and \(\text{ahpC1-ahpC2}\) double mutant (strain \(\Delta\text{ahpC12}\)) \((p \leq 0.05)\). The times to maintain the cultures in the VBNC state were not significantly different in the \(\text{ahpC1}\) mutant (strain \(\Delta\text{ahpC1}\)) (8.0 week), the complementary strain of the \(\text{ahpC2}\)
mutant (strain $\Delta$ahpC2/C2) (7.5 week) and wild-type strain (7.8 week) (Fig. 4) ($p > 0.05$). These results revealed that $ahpC2$ was markedly associated with the induction and maintenance of the VBNC state in $V. parahaemolyticus$.

**Gene expression.** The gene expressions of antioxidative genes (4 catalases and 2 AhpCs) and related regulators (RpoS and OxyR) in the induction of the VBNC state and the resuscitation of VBNC cultures were monitored by RT-qPCR. The relative quantities of $ahpC1$ and $ahpC2$ transcripts in the wild-type strain declined rapidly during the first two weeks (from 0 to -2.38 log fold changes for $ahpC1$, from 0 to -1.75 log fold changes for $ahpC2$) of induction of the VBNC state and thereafter, they declined gradually (-3.01 log fold changes at 12 week for $ahpC1$, -3.25 log fold changes for $ahpC2$) (Fig. 5). The relative quantity of the $ahpC1$ transcript in the $ahpC2$ mutant (strain $\Delta$ahpC2) (-2.88 to -3.06 log fold changes) was significantly lower than that in the wild-type strain (-1.99 to -2.58 log fold changes) upon incubation under the VBNC induction condition for 6-8 weeks ($p < 0.05$) (Fig. 5A). The relative quantities of the $ahpC2$ transcript in the wild-type and $ahpC1$ mutant (strain $\Delta$ahpC1) were not significantly different throughout the
monitoring period \((p > 0.05)\) (Fig. 5B). These results suggested that expression of \(ahpC\) genes were markedly affected in the \(ahpC2\) mutant. The expression of four catalase genes (VPA0305, VPA0453, VPA0768 and VPA1418) was monitored and the results reveal two patterns of changes in the relative quantity of their transcripts. Patterns for VPA0305 and VPA1418 were similar, while patterns for VPA0453 and VPA0768 were similar, and the patterns of VPA0305 and VPA0453 were presented in Fig. 6. Relative quantities of the transcripts of all these catalase genes declined rapidly in the first two weeks and declined gradually in the rest of time. The relative quantities of catalase VPA0305 in the wild-type (-1.68 to -2.63 log fold changes) and \(ahpC1\) mutant (-1.67 to -3.25 log fold changes) were not significant different \((p > 0.05)\) and were significantly higher than those of the \(ahpC2\) mutant (-2.99 to -4.12 log fold changes) and \(ahpC1-ahpC2\) double mutant (-4.45 to -5.51 log fold changes) from 4 to 8 weeks \((p <= 0.05)\) (Fig. 6A). Expression patterns of VPA0453 in different strains were not significantly different, whereas the relative quantity of the transcripts of VPA0453 declined to about -2 log fold changes in two weeks and gradually declined in the rest of time \((p > 0.05)\) (Fig. 6B). These results suggested the expression of
some catalase genes were also markedly affected in the \textit{ahpC2} and the \textit{ahpC1-ahpC2} mutants.

The relative quantities of \textit{rpoS} and \textit{oxyR} regulators declined at different rates in different mutant strains incubated under the VBNC induction condition. At 6 to 8 weeks, the expressions of both regulators in the wild-type strain (-1.03 to -1.89 log fold changes for \textit{rpoS}, -2.44 to -2.99 log fold changes for \textit{oxyR}) and \textit{ahpC1} mutants (-1.12 to -2.05 log fold changes for \textit{rpoS}, -2.62 to -3.14 log fold changes for \textit{oxyR}) were significantly higher than those of the \textit{ahpC2} mutant (-3.14 to -3.51 log fold changes for \textit{rpoS}, -3.78 to -3.93 log fold changes for \textit{oxyR}) and the double \textit{ahpC1-ahpC2} double mutant (-2.79 to -2.98 log fold changes for \textit{rpoS}, -3.91 to -4.21 log fold changes for \textit{oxyR}) ($p < 0.05$) (Fig. 7). These results suggested the expression of these two regulator genes were also markedly affected in the \textit{ahpC2} and the \textit{ahpC1-ahpC2} mutants.

The expressions of these genes were also monitored during the resuscitation of the VBNC cultures. After temperature-upshift treatment, the levels of the transcripts for all of these genes in the mutant strains were increased to levels similar to those of the wild-type strain (0 to 2 log fold in relative to that of exponential phase), and remained at low level
(-3 to -4 log fold) when the VBNC cultures failed to be resuscitated (data not shown).

Under the VBNC induction conditions for several weeks, the relative quantities of transcripts for these antioxidants and regulator genes remained low (-3 to -5 log fold changes) and, in most cases, these transcripts remained detectable when the VBNC cultures could no longer be resuscitated (Fig. 4-6).

DISCUSSION

The VBNC state is typically induced in vibrios and some other Gram-negative bacteria in a nutrient-limited medium at low temperature (39, 47, 51). As a survival strategy, bacterial cells in the VBNC state undergo substantial changes in their morphology and metabolic activities (10, 13), while the pathogenic potential is maintained in this state (38, 42). Thus the VBNC state of pathogenic vibrios remains as consistent threat to public health. A microarray was utilized to analyze a total of 3707 genes of V. cholerae, and 100 genes were found to be expressed in the VBNC state, and the distribution of genes among the various functional groups in
the VBNC state was similar to that obtained for the stationary cells, suggesting that the VBNC bacteria may retain the functions that are essential for viability (3). The metabolic activities of the VBNC bacteria generate endogenous ROS, which damage the cell (14) and may cause a loss of culturability and viability, especially in cells with weakened antioxidative activity, whereas the sodC gene (VC1583) was the only antioxidative gene expressed among the SOD, catalase and AhpC genes (3).

Our previous proteomic study suggested the specific role of ahpC2 in the VBNC state of *V. parahaemolyticus* (25), and in this study the involvement of ahpC2 was further supported by gene deletion and complementation (Figs. 3, 4). The role of ahpC2 in the VBNC state was also indicated by the change of gene expression in the mutant strains. The expression of ahpC genes, catalase genes and related regulators declined rapidly by three to five orders of magnitude as the culturability declined and remained low in *V. parahaemolyticus* under the VBNC state-induction conditions, while the expression of these genes was even lower in the ahpC2 and ahpC1-ahpC2 double mutant than in the ahpC1 mutant (Figs. 5-7).
Different species of peroxiredoxins have been identified in bacteria, including the fusion protein of thiol peroxidase and glutaredoxin in *V. cholerae* (9). Functions of different AhpC species have been reported in a few studies. *Legionella pneumophila* and *Coxiella burnetii* each contain two *ahpC* genes, *ahpC1* and *ahpC2*. The *ahpC2* is adjacent to an alkyl hydroperoxide reductase partner (*ahpD*), which is also found in *Mycobacterium* species. *ahpC1* is expressed following the exponential phase and *ahpC2* is expressed during the early exponential phase. The *ahpC1* mRNA levels are approximately seven to ten times those of *ahpC2D* mRNA. The expression of *ahpC2D* is significantly increased in the *ahpC1* mutant, whereas *ahpC1* expression is unchanged in the *ahpC2D* mutant, indicating the compensatory activity of the *ahpC2D* system in response to oxidative stress (22). In *V. vulnificus*, AhpC1 is a typical NADH-dependent peroxiredoxin and forms a peroxide reductase system with AhpF (4), while the AhpC2 helps the growth of *V. vulnificus* under high salinity by scavenging ROS in cells (23). This study shows that both *ahpC1* and *ahpC2* of *V. parahaemolyticus* were active against t-BOOH, which is commonly used to mimic lipid hydroperoxides, and *ahpC1* was more effective than *ahpC2* against H$_2$O$_2$ (Fig. 1).
The unique role of \textit{ahpC2} in inducing and maintaining the VBNC state of \textit{V. parahaemolyticus} may be associated with its enhanced expression/activity at low incubation temperatures in a starvation medium, and/or the inhibition of \textit{ahpC1} under such conditions. In \textit{Anabaena} sp., \textit{ahpC} is associated with protection against high temperature (47°C), high salinity, toxic pesticides (carbofuran), heavy metals and ultraviolet irradiation (30). However, in \textit{Xanthomonas campestris} \textit{ahpC} performs no protective function against heat treatment (8). The expression of AhpC proteins in \textit{Acidithiobacillus ferrooxidans} (34) and \textit{Shewanella putrefaciens} (26) increases when they are incubated at low temperatures of 2-15°C. In another study, the promoter of the putative \textit{ahpC} gene in the \textit{Shewanella} species is highly responsive to incubation at a low temperature of 4°C (31). These investigations reveal that different species of AhpC in these bacteria may respond differently to high and low incubation temperatures.

The data herein reveal that the functions of \textit{ahpC1} and \textit{ahpC2} in \textit{V. parahaemolyticus} may be influenced by incubation temperature and the richness of the culture medium. AhpC1 is probably the chief functional peroxidase in \textit{V. parahaemolyticus} that is cultured in a rich medium at
appropriate incubation temperatures (Table 2). When the cultures were incubated at 4°C in rich media, the \textit{ahpC1} and \textit{ahpC2} mutants and the parent wild-type strain exhibited similar survival rates (Fig. 2A), suggesting the compensatory activity of these two \textit{ahpC} genes in a rich medium. However, when these mutants were cultured in a starvation medium at 4°C (under VBNC state inducing conditions), \textit{ahpC1} failed to compensate for the normal function of \textit{ahpC2} in the \textit{ahpC2} mutant (Fig. 4, 5). We postulate that the \textit{ahpC2} may be activated at 4°C regardless of the culture medium, whereas \textit{ahpC1} may be activated only in the rich medium. Different regulators may be critical for sensing the peroxides and regulating the expression of these \textit{ahpC} genes.

Two regulators (\textit{rpoS}, \textit{oxyR}) associated with the function of peroxidases in \textit{V. parahaemolyticus} were identified. The catalase genes and \textit{ahpC} genes have been demonstrated to exhibit a compensatory expression pattern in bacteria under the influence of the OxyR regulon (11), whereas \textit{rpoS} is a general stress response regulator (18, 45). However, in this study, the expression of catalase genes was reduced in the \textit{ahpC2} mutant, rather than being stimulated through a typical compensatory expression pattern (Fig. 7). The compensatory function of
catalases may fail at low temperature, under starvation or under a combination of both of these stresses, which are commonly applied to induce the VBNC state.

In addition to rpoS and oxyR, other regulators may also be involved in the temperature-regulated activity. The native ahpC1 gene compensated for the normal function of ahpC2 in the ahpC2 mutant when V. parahaemolyticus was incubated at 4°C in a rich medium (Fig. 2A), while the extrachromosomal complementary ahpC1 gene did not (Fig. 2B). The function of the native ahpC1 at low temperature may depend on a regulator that is associated with it. A searching of the genome of V. parahaemolyticus (28) shows that VPA1682, which is adjacent to ahpC1 (VPA1683), may be an ohrR-like regulator that is associated with temperature-regulated activities (22). VPA1682 is a putative MarR family protein and homologous to the organic peroxide sensor and transcriptional regulator of both Bacillus subtilis (15) and some Gram-negative bacteria (12, 36). OxyR and OhrR are commonly known as the positive (19) and negative (44) regulators, respectively, of the functions of catalases and AhpCs. Coordinated activity of these regulators may be responsible for the normal functioning of the peroxyredoxins in
the VBNC state of *V. parahaemolyticus*.

Since the transcripts of both *ahpC1* and *ahpC2* similarly declined to low levels by approximately three orders of magnitude in the induction of the VBNC state (Fig. 4), while enhanced quantity of AhpC2 protein was detected (25), enhanced translation efficiency and protein stability may also result in the increased quantity of this antioxidative protein that was produced. Certain *ahpC* genes may be better expressed than other antioxidative genes and function better at low temperatures (31, 34). The lowered expression and weaker function of catalases under VBNC-inducing conditions (24) may also strengthen the involvement of AhpCs, since both detoxify peroxides.

In conclusion, this investigation demonstrated the antioxidative activities of *ahpC1* and *ahpC2* against H$_2$O$_2$ and organic peroxide in *V. parahaemolyticus*, whereas *ahpC2* alone played a significant role in the induction and maintenance of the VBNC state in this pathogen. This study facilitates the understanding of the roles of AhpCs in the growth and survival of *V. parahaemolyticus* under environmental stresses. Direct evidences, such as quantifications of ROS and antioxidative activities during induction of VBNC state, may be needed in further study to define
the role of ROS in this state.

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### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics/sequence</th>
<th>Source/Reference</th>
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<tr>
<td>V. parahaemolyticus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KX-V231</td>
<td>Wild type, serotype O3:K6, KP⁺, clinical isolate</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC1</td>
<td>KX-V231 ΔahpC1</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC2</td>
<td>KX-V231ΔahpC2</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC12</td>
<td>KX-V231ΔahpC1 ΔahpC2</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC1/C1</td>
<td>Strain ΔahpC1 containing pSCB02</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC2/C2</td>
<td>Strain ΔahpC2 containing pSCB03</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC12/C1</td>
<td>Strain ΔahpC12 containing pSCB02</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC12/C2</td>
<td>Strain ΔahpC12 containing pSCB03</td>
<td>This study</td>
</tr>
<tr>
<td>ΔahpC12V</td>
<td>Strain ΔahpC12 containing pSCB01</td>
<td>This study</td>
</tr>
<tr>
<td>KX-V231V</td>
<td>KX-V231 containing pSCB01</td>
<td>This study</td>
</tr>
</tbody>
</table>

| E. coli | | |
| XL1 blue | recA1 endA1 gyrA96 thi-1 hsdR17 | Stratagene |
| | supE44 relA1 lac [F’ proAB lacF’] | |
\[ \text{ZAM15 Tn10 (Tet^r)} \]

\[ \text{SM10}\lambda\text{-pir} \quad \text{thi thr leu tonA lacY supE} \quad (43) \]

\[ \text{recA::RP4-2-Tc::Mu } \lambda \text{ pirR6K; Km}^r \]

**Plasmid**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM T-easy</td>
<td>Cloning vector, Ap^r</td>
<td>Promega</td>
</tr>
<tr>
<td>pBR328</td>
<td>Vector derived from pBR325 and pMB1, Ap^r, Cm^r, Tc^r</td>
<td>Roche</td>
</tr>
<tr>
<td>pDS132</td>
<td>R6K ori, mobRP4, sacB, Cm^r</td>
<td>(41)</td>
</tr>
<tr>
<td>pSCA01</td>
<td>pDS132 with ahpc1-deletion</td>
<td>This study</td>
</tr>
<tr>
<td>pSCA02</td>
<td>pDS132 with ahpc2-deletion</td>
<td>This study</td>
</tr>
<tr>
<td>pSCB01</td>
<td>Derived from pBR328 and pDS132, mobRP4, Ap^r, Cm^r, Tc^r</td>
<td>This study</td>
</tr>
<tr>
<td>pSCB02</td>
<td>pSCB01 with Tc::ahpc1</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>promoter-ahpc1</td>
<td></td>
</tr>
<tr>
<td>pSCB03</td>
<td>pSCB01 with Tc::ahpc2</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>promoter-ahpc2</td>
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</table>
TABLE 2. Growth of *V. parahaemolyticus* strains on agar medium upon incubation at different temperatures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diameter of colonies (mm) at, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22</td>
</tr>
<tr>
<td>KX-V231</td>
<td>5.8 ± 0.84 a</td>
</tr>
<tr>
<td>ΔahpC1</td>
<td>3.8 ± 0.45 b</td>
</tr>
<tr>
<td>ΔahpC2</td>
<td>5.2 ± 0.45 a</td>
</tr>
<tr>
<td>ΔahpC12</td>
<td>1.6 ± 0.55 c</td>
</tr>
<tr>
<td>ΔahpC12/C1</td>
<td>1.2 ± 0.45 cd</td>
</tr>
<tr>
<td>ΔahpC12/C2</td>
<td>1.2 ± 0.45 cd</td>
</tr>
<tr>
<td>ΔahpC12V</td>
<td>0.67 ± 0.06 d</td>
</tr>
</tbody>
</table>

Different strains of *V. parahaemolyticus* were spread on TSA-3% NaCl and incubated at 22, 30 or 37 °C for 24, 15 or 15 h, respectively. Medium for the culture of strains ΔahpC12/C1, ΔahpC12/C2 and ΔahpC12V contained chloramphenicol. Sizes of colonies were measured. Sizes at various temperatures were analyzed separately by ANOVA with Duncan’s multiple range test, and those indicated by different letters were significantly different (p<0.05).
Figure Legends

Fig. 1. Colony forming ability of wild-type and *ahpC* mutant strains of *Vibrio parahaemolyticus*. Wild-type strain (KX-V231), *ahpC1* mutant (strain ΔahpC1) and its complementary strain (strain ΔahpC1/C1), *ahpC2* mutant (strain ΔahpC2) and its complementary strain (strain ΔahpC2/C2), and the *ahpC1-ahpC2* double mutant (strain ΔahpC12) were 10\(^{-1}\) to 10\(^{-5}\)-fold diluted (-1 to -5 in log scale) and spotted on TSA-3% NaCl that contained no extrinsic peroxides (A), 250 μM H\(_2\)O\(_2\) (B), or 80 μM t-BOOH (C), and incubated at 25°C for 16 h.

Fig. 2. Survival of different *V. parahaemolyticus* strains in TSB-3% NaCl incubated at 4°C for different periods. A, wild-type strain (KX-V231), *ahpC1* mutant (strain ΔahpC1), *ahpC2* mutant (strain ΔahpC2) and *ahpC1-ahpC2* double mutant (strain ΔahpC12); B, strains that contained cloning vector pSCB01 (KX-V231V, SCA03V) or complementary *ahpC1* gene (strain ΔahpC12/C1) or *ahpC2* gene (SCA03C2).

Fig. 3. Induction of VBNC state in different strains of *Vibrio parahaemolyticus* and their resuscitation. *V. parahaemolyticus*
strains in exponential phase were incubated at 4°C in MMS-0.5% NaCl medium and culturable cells were enumerated at different intervals. A, wild-type KX-V231; B, \textit{ahpC1} mutant (strain Δ\textit{ahpC1}); C, \textit{ahpC2} mutant (strain Δ\textit{ahpC2}) (●, ○) and its complementary strain (strain Δ\textit{ahpC2/C2}) (▼, △); D, \textit{ahpC1-ahpC2} double mutant; solid symbols, level of culturable cells during induction of VBNC state; open symbols, level of culturable cells of the VBNC culture after temperature-upshift treatment.

Fig. 4. Time required by different strains of \textit{Vibrio parahaemolyticus} to enter the VBNC state and the maximum length of time of the VBNC culture that could be resuscitated. Strain KX-V231, wild-type; Δ\textit{ahpC1}, \textit{ahpC1} mutant; Δ\textit{ahpC2}, \textit{ahpC2} mutant; Δ\textit{ahpC12}, \textit{ahpC1} and \textit{ahpC2} double mutant; Δ\textit{ahpC2/C2}, \textit{ahpC2} mutant with complementary \textit{ahpC2} gene; solid bars, times required to enter the VBNC state; open bars, maximum length of time of the cells in VBNC state that could be resuscitated. Asterisks indicate significant difference from corresponding values for wild-type strain at \( p < 0.05 \).
Fig. 5. Expression of *ahpC* genes in different strains of *Vibrio parahaemolyticus* incubated under VBNC-inducing conditions. *V. parahaemolyticus* cultures in exponential phase were incubated at 4°C in MMS-0.5% NaCl medium. Relative quantities of transcripts of *ahpC1* (A) and *ahpC2* (B) were determined by RT-qPCR and their calculated quantities were compared to those in exponential phase specified as -1 week in the graph and presented on log scale. Solid arrow indicates the time to enter VBNC state, while open arrow indicates the time after which the VBNC cells failed to be resuscitated.

Fig. 6. Expression of catalase genes VPA0305(A) and VPA0453(B) in different strains of *Vibrio parahaemolyticus* under VBNC-inducing conditions. *V. parahaemolyticus* strains in exponential phase were incubated at 4°C in MMS-0.5% NaCl medium. Relative quantities of transcripts of catalase gene VPA0305 were determined by RT-qPCR and their calculated quantities were compared to those in exponential phase specified as -1 week in the graph and presented on log scale. Solid arrow indicates time to enter VBNC state, while open arrow indicates
the time after which the VBNC cells failed to be resuscitated.

Fig. 7. Expression of regulators rpoS (A) and oxyR (B) in different strains of *Vibrio parahaemolyticus* under VBNC-inducing conditions. *V. parahaemolyticus* strains in exponential phase were incubated at 4°C in MMS-0.5% NaCl medium. Relative quantities of transcripts of *rpoS* and *oxyR* in different strains were determined by RT-qPCR and their calculated quantities were compared to those in exponential phase specified as -1 week in the graph and presented on log scale. Solid arrow indicates time to enter VBNC state, while open arrow indicates the time after which the VBNC cells failed to be resuscitated.
Fig. 1.
Fig. 2

(A) Survival of KX-V231 and ΔahpC1, ΔahpC2, ΔahpC12.

(B) Survival of KX-V231V and ΔahpC12V, ΔahpC12/C1, ΔahpC12/C2.

Time, day

Survival %
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.