The Journal of Microbiology, October 2009, p. 624-632
DOI 10.1007/s12275-009-0130-x

Vol. 47, No. 5

Identification of the *Vibrio vulnificus ahpC*1 Gene and Its Influence on Survival under Oxidative Stress and Virulence

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(Received April 22, 2009 / Accepted May 27, 2009)

Pathogens have evolved sophisticated mechanisms to survive oxidative stresses imposed by host defense systems, and the mechanisms are closely linked to their virulence. In the present study, *ahpC*1, a homologue of *Escherichia coli ahpC* encoding a peroxiredoxin, was identified among the *Vibrio vulnificus* genes specifically induced by exposure to H_2O_2. In order to analyze the role of AhpC1 in the pathogenesis of *V. vulnificus*, a mutant, in which the *ahpC*1 gene was disrupted, was constructed by allelic exchanges. The *ahpC*1 mutant was hypersusceptable to killing by reactive oxygen species (ROS) such as H_2O_2 and *t*-BOOH, which is one of the most commonly used hydroperoxides *in vitro*. The purified AhpC1 reduced H_2O_2 in the presence of AhpF and NADH as a hydrogen donor, indicating that *V. vulnificus* AhpC1 is a NADH-dependent peroxiredoxin and constitutes a peroxide reductase system with AhpF. Compared to wild type, the *ahpC*1 mutant exhibited less cytotoxicity toward INT-407 epithelial cells *in vitro* and reduced virulence in a mouse model. In addition, the *ahpC*1 mutant was significantly diminished in growth with INT-407 epithelial cells, reflecting that the ability of the mutant to grow, survive, and persist during infection is also impaired. Consequently, the combined results suggest that AhpC1 and the capability of resistance to oxidative stresses contribute to the virulence of *V. vulnificus* by assuring growth and survival during infection.

Keywords: *V. vulnificus ahpC*1, oxidative stress, virulence

Oxidative stress caused by increased levels of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) can lead to the damage of all cellular components including protein, DNA and membrane lipid. Although incomplete reduction of oxygen during respiration and aerobic metabolism is the main source of endogenous ROS for bacterial cells, exposure to metals and redox-active chemicals also cause increased levels of the ROS (Storz and Zheng, 2000). In addition, pathogenic bacteria are inevitably exposed to ROS that are crucial to host defense for the optimal microcidal activity of neutrophils and other phagocytes (Miller and Britigan, 1997). Therefore, pathogens have evolved sophisticated mechanisms to survive oxidative stresses imposed by not only enodogenous sources but also host defense systems, and the mechanisms are closely linked to their virulence (Storz and Zheng, 2000).

The mechanisms of bacterial defense against oxidative stresses include highly specific and effective enzymatic pathways such as those catalyzed by superoxide dismutase, catalase, peroxidase, and glutathione peroxidase in combination with glutathione reductase and glutathione (Miller and Britigan, 1997). Among these, peroxiredoxins are a family of cysteine-based peroxidases able to reduce hydrogen peroxide and alkyl hydroperoxides by the use of reducing equivalents (or reductants) derived from thiol-containing donor molecules such as glutathione, thioredoxin, and alkyl hydroperoxide reductase subunit F (AhpF). AhpC (alkyl hydroperoxide reductase subunit C), 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). AhpC and its homologues carrying two conserved cysteinyl residues near its N- and C-termini, respectively, are ubiquitous proteins and expressed in a wide range of eubacteria (Wood *et al*., 2003; Poole, 2005).

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases such as life-threatening septicemia and possibly gastroenteritis in individuals with underlying predisposing conditions (for recent reviews, see Linkous and Oliver, 1999; Strom and Paranjpye, 2000; Jones and Oliver, 2009). Although, like many other pathogenic bacteria, *V. vulnificus* has to cope with oxidative stresses in host environments to ensure developing illness, only a few studies have addressed the molecular mechanisms by which the bacterium can survive under oxidative stresses (Park *et al*., 2004; Kang *et al*., 2007). Accordingly, as an effort to characterize the molecular mechanisms involved in oxidative stress resistance, a transcriptome analysis was performed using the *V. vulnificus* Whole Genome Twin-Chip in the present study. An open reading frame (ORF), *ahpC*1, a homologue of *E. coli ahpC*, was identified among the genes specifically induced by exposure to H_2O_2. The purified AhpC1 reduced H_2O_2 in the presence of AhpF and NADH

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Table 1. Plasmids and bacterial strains used in this study

Strains or plasmids	Relevant characteristics[a]	Reference or source
Strains		
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	Laboratory collection
OH0701	MO6-24/O with *ahpC*1::*nptI*; Km^r	This study
E. coli		
DH5α	*supE44 D lacU169 (f80 lacZ* DM15*) hsdR17 recA1 endA1 gyrA96 thi-1 relAI*	Laboratory collection
SM10 λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pir*; km^r; host for π-requiring plasmids; conjugal donor	Miller and Mekalanos (1988)
BL21 (DE3)	F^-, *ompT*, *hsdS* (r_B^-, m_B^-), *gal* (DE3)	Laboratory collection
Plasmids		
pDM4	R6K *γ ori*; s*acB;* suicide vector; *oriT* of RP4; Cm^r	Milton *et al*. (1996)
pRSET A	His-tag protein expression vector	Invitrogen
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	Pharmacia
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pJH0311	0.3-kb NruI fragment containing multi-cloning site of pUC19 cloned into pCOS5; Ap^r, Cm^r	Goo *et al*. (2006)
pMJ0701	0.9-kb fragment carrying part of *ahpC*1 in pGEM-T Easy; Ap^r	This study
pMJ0702	pMJ0701 with *ahpC*1::*nptI*; Ap^r, Km^r	This study
pMJ0703	pDM4 with *ahpC*1::*nptI*; Cm^r, Km^r	This study
pWK0702	pJH0311 with *ahpC1F*; Ap^r, Cm^r	This study
pWK0704	pRSET A with *ahpC*1; Ap^r	This study
pWK0802	pRSET A with *ahpF*; Ap^r	This study

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

as a hydrogen donor. The function of the AhpC1 protein during an infectious process was further accessed by constructing an isogenic *ahpC*1 mutant of *V. vulnificus* and applying the molecular version of Koch's postulates (Falkow, 1988). The possible roles of AhpC1 in virulence of *V. vulnificus* have been demonstrated by comparing the virulence of the mutant with that of its parental wild type in *in vitro* cell culture and in mice.

Materials and Methods

Strains, plasmids, and 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 (USA), and the chemicals were purchased from Sigma (USA).

Identification and cloning of *V. vulnificus ahpC*1

To identify genes induced upon exposure to oxidative stress, the *V. vulnificus* cells grown to OD_{600} of 0.5 in LBS were exposed to 250 μM hydrogen peroxide (H_2O_2) for 30 min. A transcriptome analysis was performed using the *V. vulnificus* Whole Genome Twin-Chip as described previously (Jeong and Choi, 2008), and then transcription profiles from the *V. vulnificus* cells exposed either to H_2O_2 or LBS alone were compared. Among the genes of which expression was more induced in the cells exposed to H_2O_2 (data not shown), *ahpC*1 encoding a peroxiredoxin was selected for further characterization. A whole *ahpC*1 ORF was amplified from the genomic DNA of *V. vulnificus* by a PCR using a pair of oligonucleotide primers, AhpC0701 and AhpC0704 (Table 2). The primers were designed using the genomic sequence of *V. vulnificus* CMCP6 (GenBank accession no. AE016795 and AE016796, www.ncbi.nlm.nih.gov). The amplified 928-bp *ahpC*1 was ligated into pGEM-T easy (Promega, USA) to

Table 2. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence (5'→3'[a])	Use
AhpC 0701	TCAAATAACGCCTTTAGTCCCTCAGG	Mutant construction
AhpC 0704	ATCTAGGCTGACCACTAAACGGAC	Mutant construction
AhpC-CP01	GAGCTCCGATGCCAAACCTAACAAACT	Complementation of *ahpC*1
AhpC-CP02	CCCGGGACATGCCTTTGCTG	Complementation of *ahpC*1
HIS-AHPC01	CTCGAGATGATTAACACTACTATCAAACCA	Amplification of *ahpC*1
HIS-AHPC02	AAGCTTTTAGATTTTGCCAACTAGGTC	Amplification of *ahpC*1
HIS-AHPF01	CTCGAGATGCTAGACCAAGCGATC	Amplification of *ahpF*
HIS-AHPF02	AAGCTTTTAGCCTTGCTTACGAATCAA	Amplification of *ahpF*

[a] Regions of oligonucleotides not complementary to corresponding genes are underlined

result in pMJ 0701 (Table 1).

Generation of the *ahpC*1 mutant by allelic exchange

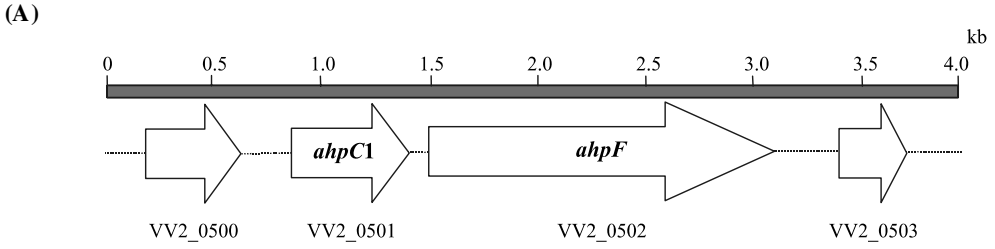
The *ahpC*1 gene in pMJ0701 that was inactivated *in vitro* by inserting the 1.2-kb DNA fragment carrying *nptI* encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin (Oka *et al*., 1981) into a unique *Bam*HI site present within the ORF of *ahpC*1. The 2.1-kb *ahpC*1::*nptI* cartridge from the resulting construction (pMJ 0702) was liberated and ligated with *Sac*I-*Sph*I digested pDM4 (Milton *et al*., 1996), forming pMJ0703. *E. coli* SM10 *λpir*, *tra* (Miller and Mekalanos, 1988) harboring pMJ0703 was used as a conjugal donor to *V. vulnificus* MO6-24/O. The conjugation and isolation of the transconjugants were conducted using the methods previously described (Lee *et al*., 2007; Oh *et al*., 2009).

Survival under oxidative stresses

The ability of the *V. vulnificus* wild type, *ahpC*1 mutant, and complemented strain to survive under oxidative stresses was assayed by measuring growth on the LBS agar medium containing either 250 μM H_2O_2 or 60 μM *tert*-butyl hydroperoxide (*t*-BOOH). H_2O_2 and *t*-BOOH were prepared and added to the LBS agar medium by using previously described procedures (Jeong *et al*., 2000). Equal number of the strains grown to log phase (OD_{600} of 0.5) were serially diluted from 10 to 1×10^4 folds and then 10 μl of the diluted cultures were spotted onto the medium, and then the growth was measured after 12 h.

Overexpression and purification of *V. vulnificus* AhpC1 and AhpF

The coding region of *ahpC*1 was amplified using the chromosomal DNA of *V. vulnificus* MO6-24/O as a template and oligonucleotide primers, HIS-AHPC01 and HIS-AHPC02 (Table 2). The 0.6 kb PCR product was subcloned into a 6× Histidine tagging expression vector, pRSET A (Invitrogen, USA). The resulting plasmid, pWK0704 encoded AhpC1 with a 6× His tag at the amino terminus (Table 1). The His-tagged AhpC1 protein was then expressed in *E. coli* BL21 (DE3), and the protein was purified by affinity chromatography according to the manufacturer's procedure (QIAGEN, USA). In a similar way, the expression and purification of His-tagged AhpF were fulfilled using pWK802 (Table 1), carrying the *V. vulnificus ahpF* gene amplified using oligonucleotide primers, HIS-AHPF01, HIS-AHPF02 (Table 2).



(B)

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S. typhimurium MSLINTKIKPFKNQAFKNGEFIEVTEKDTEGRWSVFFFYPADFTFVCPTELGDVADHYEE 60
E. coli        MSLINTKIKPFKNQAFKNGEFIEITEKDTEGRWSVFFFYPADFTFVCPTELGDVADHYEE 60
V. vulnificus  --MINTTIKPFSATAYKDGKFVDITEQDVLGKWSVFFFYPADFTFVCPTELGDLADHYEE 58
                  *** ****   * * * *   ** *  * ********************** ******

S. typhimurium LQKLGVDVYSVSTDTHFTHKAWHSSSETIAKIKYAMIGDPTGALTRNFDNMREDEGLADR 120
E. coli        LQKLGVDVYAVSTDTHFTHKAWHSSSETIAKIKYAMIGDPTGALTRNFDNMREDEGLADR 120
V. vulnificus  LQSRGVEVYSVSTDTHFTHKAWHDSSDTIGKIKYFMVGDQTGNITNNFGVMRPGQGLADR 118
               **  ** ** ************* ** ** **** * ** **  * **  **   *****

S. typhimurium ATFVVDPQGIIQAIEVTAEGIGRDASDLLRKIKAAQYVAAHPGEVCPAKWKEGEATLAPS 180
E. coli        ATFVVDPQGIIQAIEVTAEGIGRDASDLLRKIKAAQYVASHPGEVCPAKWKEGEATLAPS 180
V. vulnificus  ATFVIDPQGVIQAVEITAEGIGRDAEDLLRKIKAAQYVAAHPGEVCPAKWKEGEQTLAPS 178
               **** **** *** * ********* ************** ************** *****

S. typhimurium LDLVGKI 187
E. coli        LDLVGKI 187
V. vulnificus  LDLVGKI 185
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Fig. 1. Physical map of the *V. vulnificus ahpC*1 gene and sequence relatedness of AhpC1 proteins reported from other bacteria. (A) The arrows represent the coding regions of *ahpC*1, *ahpF*, and part of the less characterized genes encoding a putative isopropylmalate synthase (VV2_0500) and a cold shock protein (VV2_0503). The figure was derived using the nucleotide sequences of *V. vulnificus* CMCP6 (accession number NC_004460) genomes in the GenBank databases (NCBI). The gene IDs are shown below each coding region. (B) Identical sequences are indicated with asterisks, and dashes represent missing sequences. Two conserved cysteine residues are boxed in the N-terminal and C-terminal regions, respectively. Alignment was based on the amino acid sequences in the GenBank database and derived by the CLUSTALW alignment program (http://www.ch.embnet.org/software/ClustalW.html).

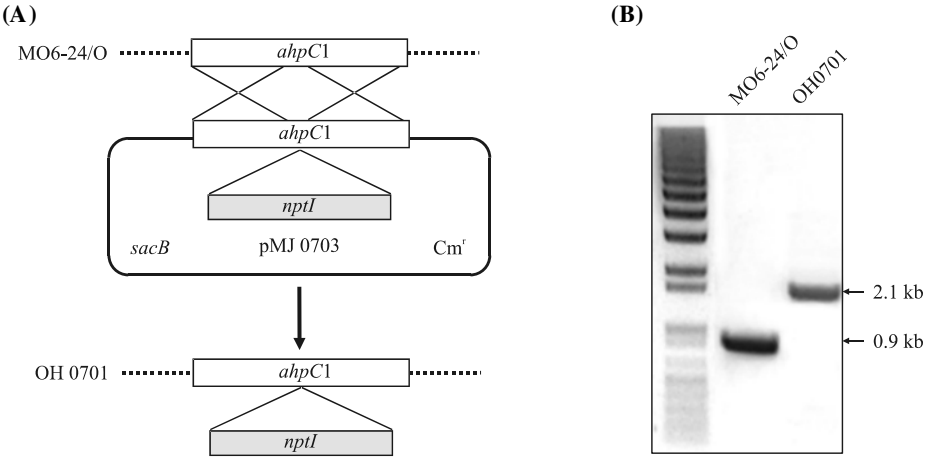

Fig. 2. Allelic exchange procedure and construction of the *ahpC*1::*nptI* isogenic mutant. (A) Double homologous recombinations between strain MO6-24/O and plasmid pMJ0703 led to an interruption of the *ahpC*1 gene and resulted in the construction of the mutant OH0701. The dashed lines represent the bacterial chromosome; the full line, the plasmid DNA; the open box, the target *ahpC*1 gene; the shaded box, the *nptI* gene; and the large Xes, genetic crossing over. Abbreviations; *sacB*, levansucrase gene; Cm^r, chloramphenicol resistance gene. (B) PCR analysis of MO6-24/O and the isogenic mutant OH0701 generated by allelic exchange. Molecular size markers (1 kb plus DNA ladder, Invitrogen) and PCR products are indicated.

Peroxide reductase activity of AhpC1

The peroxide reductase (peroxidase) activity of purified AhpC1 was determined according to the method described elsewhere (Wang *et al*., 2005). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 150 mM ammonium sulfate, 100 μM peroxides (H_2O_2 or *t*-BOOH), and either 2 μM AhpC1, 0.2 μM AhpF, or both proteins. The reaction was initiated by adding NADH (200 μM) to 200 μl of the mixture and carried out at room temperature. The oxidation of NADH in the resulting mixture was determined by measuring the decrease of absorbance at 340 nm (A_{340}) for time intervals. The protein concentrations were determined by the method of Bradford with bovine serum albumin as the standard.

Cytotoxicity assay

Cytotoxicity assays were performed using INT-407 (ATCC CCL-6) human intestinal epithelial cells. The preparation of the INT-407 cells and infection with the bacterial cultures were performed in a 96-well tissue culture plate (Nunc, Denmark) as previously described (Park *et al*., 2006). The cytotoxicity was then determined by measuring the activity of lactate dehydrogenase (LDH) released in the supernatant using a Cytotoxicity Detection kit (Roche, Mannheim, Germany), and expressed using the total LDH activity released from the cells completely lysed by 1% Triton-X 100 as 100%. Morphologic studies were also carried out using INT-407 cells, which were seeded onto glass coverslips placed at the bottom of the tissue culture plate and infected with the *V. vulnificus* strains. The cells were fixed in methanol, stained with 0.4% Giemsa, and examined under a light microscope (Park *et al*., 2006).

LD_{50} determination

The 50% lethal doses (LD_{50}s) of wild type and the *ahpC*1 mutant were compared using ICR mice (Specific Pathogen-Free; Seoul National University), as described elsewhere (Park *et al*., 2006; Lee *et al*., 2007). The infected mice were observed for 24 h, and the LD_{50}s were calculated using the method described by Reed and Muench (1938). Mice were injected intraperitoneally with 250 μg of iron dextran for each gram of body weight immediately before injection with bacterial cells. All manipulations of mice were approved by the Animal Care and Use Committee at Seoul National University.

Bacterial growth rates during infection

The INT-407 cells were infected using the wild type, *ahpC*1 mutant or complemented strain at a multiplicity of infection (MOI) of 10, and growth rates of the bacterial strains during the infection were monitored. For this purpose, samples of the supernatant of the INT-407 cells were removed at regular intervals and bacterial cells in the supernatant were determined by counting colony forming units (CFU) on LBS agar plates.

Data analysis

Averages and standard errors of the mean (SEM) were calculated from at least three independent determinations. The statistical significance of the difference among the *V. vulnificus* strains was evaluated using Student's unpaired *t* test (SAS software, SAS Institute Inc., USA). Significance was accepted at $P<0.05$.

Results

Organization and sequence relatedness of *V. vulnificus ahpC*1

In the course of a search for *ahpC*1 in the *V. vulnificus* genome sequence database (GenBank accession no. AE016795

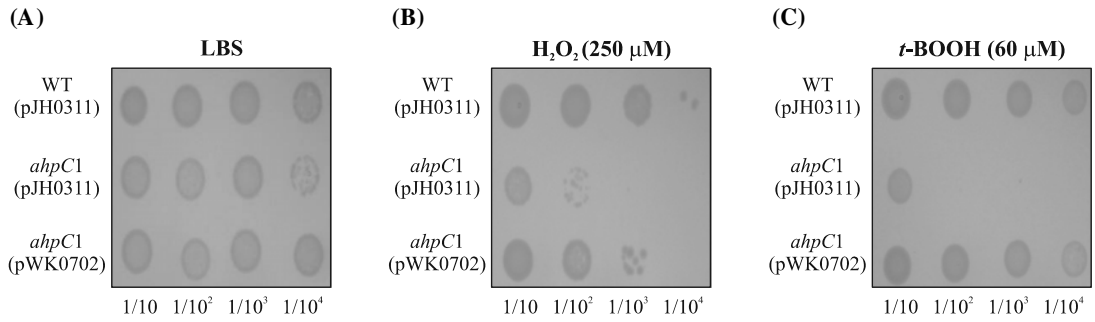

Fig. 3. Survival of the *V. vulnificus* strains under oxidative stress. Wild-type [WT (pJH0311)], *ahpC*1 mutant [*ahpC*1 (pJH0311)], or complemented strain [*ahpC*1 (pWK0701)] were compared for their ability to grow on LBS plates supplemented without oxidants (A) or with 250 μM H_2O_2 (B) or 60 μM *t*-BOOH (C). Serial 10-fold dilutions of each culture grown to an OD_{600} of 0.5 were spotted on plates as indicated and photographed after 12 h growth by using a UMAX digital imaging system (UTA-1100, UMAX Technologies, Inc.). Each experiment was repeated at least twice.

and AE016796), a homology of *ahpF* encoding a subunit F that constitutes alkyl hydroperoxide reductase system with another subunit C, AhpC1, was found. The *ahpF* gene is located downstream of *ahpC*1 and the two coding regions of *ahpC*1 and *ahpF* are transcribed in the same direction (Fig. 1A). The *ahpC*1 and *ahpF* genes of *E. coli* and *S. typhimurium*, which are well characterized at molecular level, are organized in the same orientation as in *V. vulnificus ahpC* and *ahpF* (data not shown).

The amino acid sequence deduced from the *ahpC*1 coding sequence revealed a protein, AhpC1 composed of 185 amino acids with a theoretical molecular mass of 20,424 Da and pI of 4.94. The amino acid composition and molecular weight of this AhpC1 are quite similar to those of the AhpC homologues from other Gram-negative bacteria. The amino acid sequence of the AhpC1 was 78% identical to those of the AhpC homologues from *E. coli* and *S. typhimurium* and their identity appeared evenly throughout the whole proteins (Fig. 1B). This similarity found in genetic organization and deduced amino acid sequences indicates that the *ahpC*1 gene also encodes a subunit required to form an alkyl hydroperoxide reductase system of *V. vulnificus*.

Construction and confirmation of the *V. vulnificus ahpC*1 mutant

To further characterize the function of Vv-AhpC1, an isogenic *V. vulnificus ahpC*1 mutant was constructed by allelic exchange (Fig. 2A). A double crossover, in which the wild-type *ahpC*1 gene was replaced with the *ahpC*1::*nptI* allele, was confirmed by analyzing the DNA segment encompassing *ahpC*1::*nptI*, which was amplified using a pair of PCR primers, AhpC 0701 and AhpC 0704 (Table 2). The PCR analysis of the genomic DNA from MO6-24/O with the primers produced a 0.9-kb fragment (Fig. 2B); whereas, the genomic DNA from the *ahpC*1::*nptI* mutant resulted in an amplified DNA fragment approximately 2.1 kb in length.

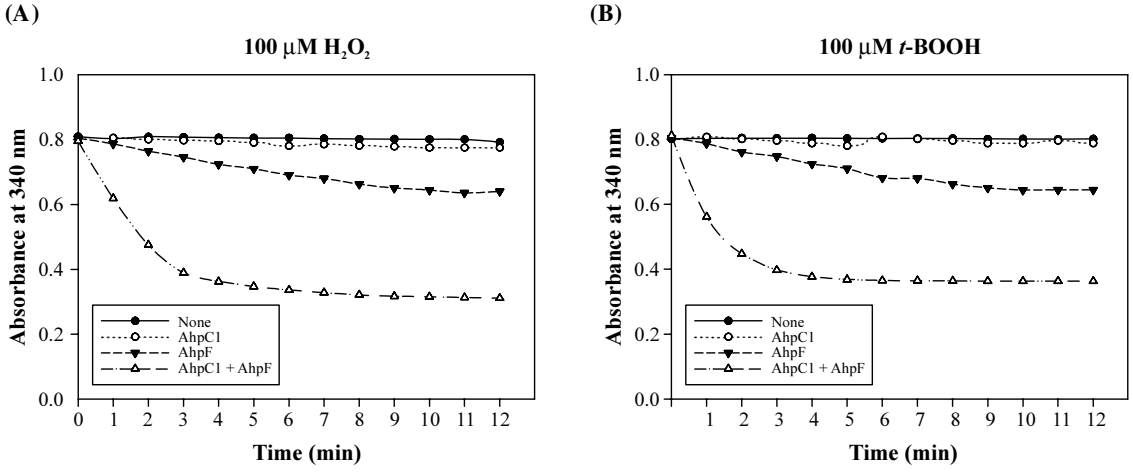

Fig. 4. NADH and AhpF-dependent peroxide reductase assay of AhpC1. The peroxide reductase activity of AhpC1 was determined by monitoring NADH oxidation measured as the decrease in absorbance at 340 nm in the presence of H_2O_2 (A) and *t*-BOOH (B) as indicated. NADH, AhpC1, and AhpF were omitted from the reaction mixture as a negative control (None, ●). Either AhpC1 (○) or AhpF (▼) alone, or both proteins (△) are added to the reaction mixture as indicated. Details are in 'Materials and Methods'. Each experiment was repeated at least three times and represents mean of the experiments.

The 2.1 kb fragment was in agreement with the predicted size of the DNA fragment containing the wild-type *ahpC*1 (0.9 kb) and the *nptI* gene (1.2 kb). The *V. vulnificus ahpC*1 mutant chosen for further analysis was named OH0701 as shown in Fig. 2B.

Effects of the *ahpC*1 mutation on the growth of *V. vulnificus* under oxidative stresses

The growth of the *ahpC*1 mutant OH0701 under oxidative stresses was compared to that of parent strain. When spotted on LBS plates supplemented with 250 μM of H_2O_2, growth of the *ahpC*1 mutant OH0701 was significantly impaired, suggesting that AhpC1 is responsible for optimum growth in the presence of H_2O_2 (Fig. 3A). In a similar way, the growth rate of the wild-type is much higher than that of the *ahpC*1 mutant when the strains are cultured on the LBS plates in the presence of 60 μM *t*-BOOH (Fig. 3B). These results suggested that the gene product of *ahpC*1, AhpC1 is responsible for the growth and survival of *V. vulnificus* under various oxidative stresses.

Reintroduction of recombinant *ahpC*1 could not complement the decrease of growth rate of OH0701 under oxidative stresses (data not shown). Therefore, to complement the *ahpC*1 mutation, pWK0702 (Table 1) was constructed by subcloning *ahpC*1*F* amplified by PCR using primers AhpC-CP01 and AhpC-CP02 (Table 2) into the broad host-range vector pJH0311 (Goo *et al*., 2006). The impaired growth of OH0701 under oxidative stresses was restored by the re-introduction of pWK0702 (Fig. 3A and B). These results indicated that the presumed *V. vulnificus ahpC*1 and *ahpF* are organized as a single transcriptional unit as in the *ahpCF* genes of *E. coli* (Zheng *et al*., 2001), and that both AhpC1 and AhpF are required for survival under oxidative stresses.

Alkyl hydroperoxide reductase activity of AhpC1

The peroxide reductase activity of AhpC1 was determined by measuring its ability to reduce H_2O_2 and *t*-BOOH with NADH as the reducing agent. When AhpF is omitted from the reaction mixture, AhpC1 alone is not able to reduce the peroxides, and thereby no change in the oxidation of NADH (A_{340}) was observed (Fig. 4A and B). In contrast, AhpF alone had NADH oxidation activity, demonstrating steady decrease in A_{340}. However, the rate of NADH oxidation increased when AhpC1 and AhpF were present together in the reaction mixture, indicating that the AhpC1 activity of reducing peroxides is dependent on AhpF. When H_2O_2 and *t*-BOOH

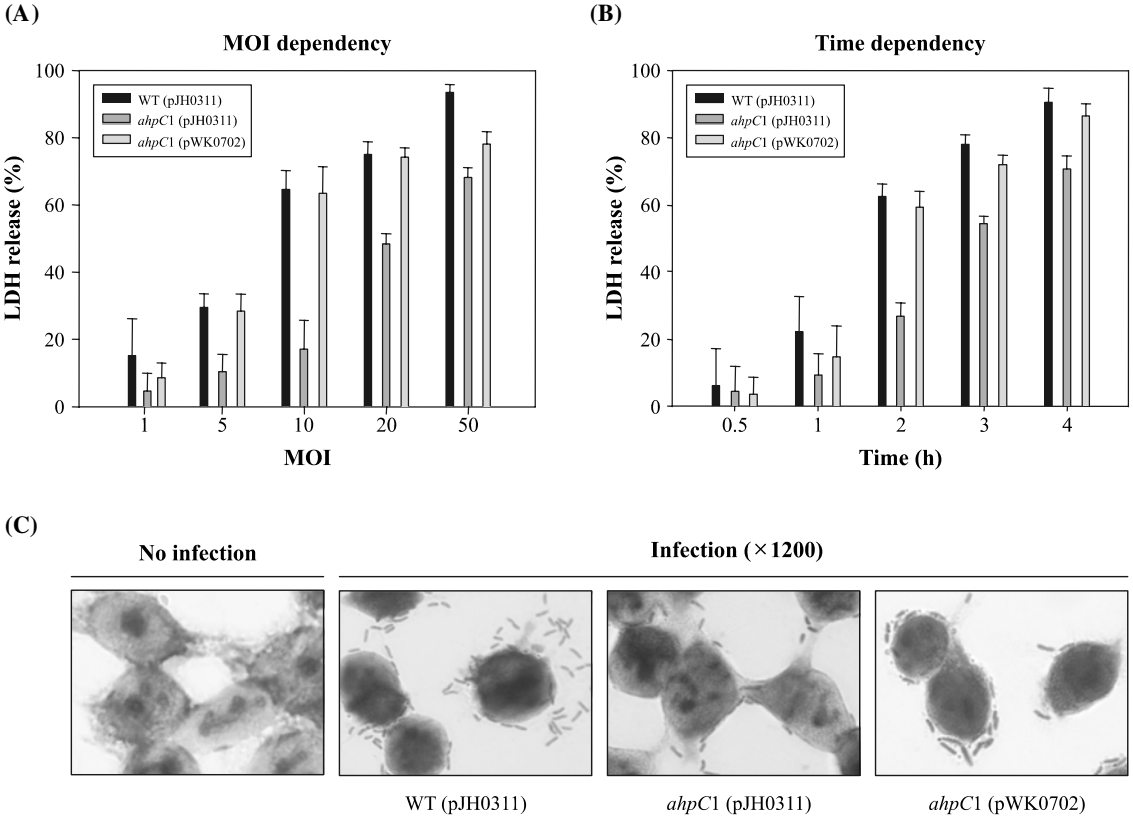

Fig. 5. Effect of *ahpC*1 mutation on virulence of *V. vulnificus* towards INT-407 cells. INT-407 cells were infected with the *V. vulnificus* strains at various MOIs for 1.5 h (A), or at an MOI of 10 for various incubation times (B). Thereafter, the cell cytotoxicity was determined by an LDH release assay. The data represent the mean±SEM from three independent experiments. (C) Morphologic studies were also carried out using INT-407 cells, which were seeded onto glass cover slips placed at the bottom of the tissue culture plate and infected with the *V. vulnificus* strains as described in 'Materials and Methods'. The cells were morphologically observed using a light microscope (×1,200) after Giemsa staining. For both panels, wild type, WT (pJH0311); *ahpC*1 mutant, *ahpC*1 (pJH0311); complemented strain, *ahpC*1 (pWK0702).

Table 3. Effects of the *ahpC*1 mutation on the lethality of *V. vulnificus* to mice[a]

Strain	Intraperitoneal LD_{50} (CFU)
MO6-24/O (n=6)	4.3×10^{0}
*ahpC*1 mutant (n=6)	2.5×10^{4}

[a] n, number of iron-treated mice for each inoculation group, ranging from 1 to 10^{7} CFU in 10-fold increments

were tested with two proteins together, no substrate preference was observed. (Fig. 4A and B). Similar results were obtained in independent experiments using NADPH (data not shown). These results indicated that AhpC1 is a peroxiredoxin and constitutes an alkyl hydroperoxide reductase system with AhpF as a reductant in *V. vulnificus*.

AhpC1 is required for cytotoxicity toward epithelial cells *in vitro*

In order to examine the effect of the *ahpC*1 mutation on the virulence of *V. vulnificus*, LDH activities from monolayers of INT-407 cells that were infected with 100 μl of suspension of the wild type and OH0701 strains at a different multiplicity of infection (MOI) and incubated for 1.5 h were determined (Fig. 5A). The *ahpC*1 mutant OH0701 exhibited much less LDH releasing activity and the level of LDH activity released from INT-407 cells infected with OH0701 at a MOI of 10 was almost 3-fold less than that from the cells infected with wild type. In a similar way, INT-407 cells were infected at an MOI of 10, and LDH activity released from the cells was compared at different incubation times as indicated (Fig. 5B). The cells infected with OH0701 released lower level of LDH activity than that of the cells infected with wild type while the cells were incubated with bacterial suspension as long as 4 h. The lower LDH activities were restored to the level released from the cells infected with wild type, when the cells were incubated with OH0701 (pWK0702).

Morphologic studies were also carried out using INT-407 cells infected with the *V. vulnificus* strains at an MOI of 10 for 1 h (Fig. 5C). The stained cells were assessed for size, regularity of the cell margin, and the morphological characteristics of the nuclei. As shown in Fig. 5C, many Giemsa-stained INT-407 cells exhibited marked cellular damage after infection with the wild type and OH0701 (pWK0702). Cytoplasmic loss and nuclear material condensation, typical phenotypes of cell death, were observed in the intestinal cells infected with the wild type and OH0701 (pWK0702). In contrast, fewer dead cells were observed after incubation with OH0701. The cells infected with OH0701 exhibited a less damaged surface and less cytoplasmic loss. These results suggest that AhpC1 is important as regards the ability of *V. vulnificus* to infect and injure host cells.

Virulence in mice is dependent on *ahpC*1

Role of the *V. vulnificus ahpC*1 gene in virulence was further examined using a mouse model. The LD_{50}s in iron-

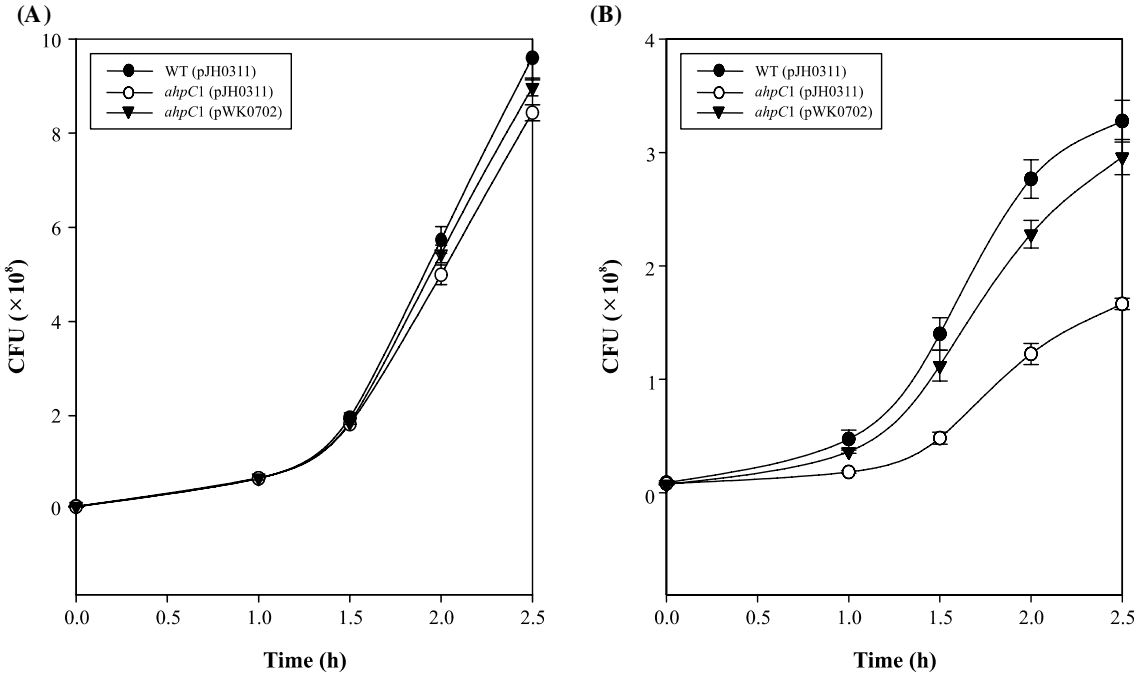

Fig. 6. Growth rates of the wild type and *ahpC*1 mutant during infection. (A) Growths of the *V. vulnificus* strains in the MEM medium supplemented with 1% FBS, in the absence of the INT-407 cells, were determined by enumerating CFU on LBS agar plates at time intervals as indicated. (B) Growths of the strains during the infection of INT-407 cells were monitored. The strains were used to infect the INT-407 cells at an MOI of 10 and then bacterial cells during infection were enumerated as described above. The data represent the mean from three independent experiments. For both panels, wild type, WT (pJH0311); *ahpC*1 mutant, *ahpC*1 (pJH0311); complemented strain, *ahpC*1 (pWK0702).

overloaded mice after intraperitoneal infections of *V. vulnificus* strains are shown in Table 3. The LD_{50} of OH0701 is greater than 10^4 CFU, compared with an LD_{50} lower than 10^1 CFU for wild type. Therefore, in the mouse model of intraperitoneal infection, in which the *ahpC*1 mutant showed approximately 4-log increase in LD_{50} over the wild type, the *ahpC*1 mutant appeared to be significantly less virulent than its parental wild type. Taking these results together, it is reasonable to conclude that the *ahpC*1 is essential for virulence of *V. vulnificus* in mice as well as *in vitro* cell culture.

Growth rates of the *V. vulnificus* strains during infection

It is not easy to imagine that AhpC1 could directly injure host (or host cells) as proposed for hemolysin, elastolytic protease, and RtxA (Shinoda *et al*., 1985; Hase and Finkelstain, 1993; Lee *et al*., 2007). To examine whether reduced cytotoxicity of the *ahpC*1 mutant (Fig. 5) resulted from defects in its growth, we compared the growth rate of the *ahpC*1 mutant with that of the wild type. The growth rate of the *ahpC*1 mutant in MEM with 1% FBS, in which INT-407 cells were absent, was not significantly different from that of the wild type (Fig. 6A). During the infection, however, the growth rate of the *ahpC*1 mutant in the INT-407 tissue cultures was significantly lower than that of the wild type (Fig. 6B), indicating that AhpC1 could play a role in the pathogenesis of *V. vulnificus* by assuring growth of the pathogen during infection.

Discussion

It has been generally accepted that virulence factors of infecting microorganisms include all those factors contributing to survival and multiplication on or within host as well as to disease (Mekalanos, 1992). Plants and animals possess mechanisms to specifically generate ROS as a defense against microbial invasion (Storz and Zheng, 2000), and phagocyte-derived ROS and their role in host defense have been biochemically and cytologically well characterized (Miller and Brightigan, 1997). Microorganisms have developed elaborate protection system not only to avoid contact with phagocyte-derived ROS but also to defend themselves from injury once ROS are encountered. The relationship between ROS resistance of pathogenic bacteria and their virulence in the pathogenesis has been well documented. As an example, *S. typhimurium* carrying mutations in genes essential for protecting against the toxicity of ROS are hypersusceptable to macrophage killing, and show attenuated virulence in mice (Fang *et al*., 1999). Therefore, it is conceivable that factors contributing to protecting against the deleterious effects of ROS are important for virulence of pathogenic bacteria.

The alkyl hydroperoxide reductase system, AhpCF, of bacteria provides an important protection against peroxides in the environment and within the host (Master *et al*., 2002; Brenot *et al*., 2005). AhpCF consists of a catalytic subunit C (AhpC1, peroxiredoxin) and a reductase subunit (AhpF). The *ahpC*1 gene has been highly conserved and can be found in the sequence databases of most completed genomes (Poole, 2003), indicating that protection against ROS by AhpC and its homologues are critical to many organisms. Bacterial AhpCs in *E. coli* and *S. typhimurium* are two cysteinyl peroxiredoxins and their catalytic properties are well characterized biochemically (Poole, 2005). The enzymes utilize a cysteinyl residue at the catalytic site in the N-terminal region (Fig. 1B) for hydroperoxide reduction, leading to formation of a sulfenic acid derivative of the cysteine. The sulfenic acid then reacts with a second cysteine in the C-terminal region to generate a intersubunit disulfide bond in a dimeric form of AhpC. This disulfide bond in AhpC is subsequently reduced to regenerate the active AhpC by a pair of cysteines of AhpF. The resulting oxidized AhpF is reduced by electron transfer cascade from an AhpF-bound flavin moiety, and then finally from NADH (Jönsson *et al*., 2007). In the present study, *V. vulnificus* AhpC1 carries two cysteines at the 45^{th} and 164^{th} residues and shows 78% similarity in amino acid sequences with the AhpC of *E. coli* and *S. typhimurium* (Fig. 1B). In addition, *V. vulnificus* AhpC1 requires AhpF and NADH as reductants for optimum peroxide reduction (Fig. 4). All these combined indicates that catalytic properties of AhpC1 are also similar with those of the *E. coli* and *S. typhimurium* AhpC.

The diseases resulting from infection with *V. vulnificus* are remarkable as regards their invasive nature, ensuing severe tissue damage, and rapidly fulminating course (Linkous and Oliver, 1999; Strom and Paranjpye, 2000; Jones and Oliver, 2009). This multifaceted nature of pathology of the diseases indicates that numerous virulence factors are typically involved in pathogenesis of the organism. To investigate whether capability of ROS resistance plays an important role as a virulence factor in the pathogenesis of *V. vulnificus*, the *V. vulnificus ahpC*1 mutant showing increased sensitivity to peroxides was constructed. When compared to wild type, the *ahpC*1 mutant is less toxic to intestinal epithelial cells *in vitro*, and also shows significantly diminished virulence in mice as measured by their abilities to cause death. In addition, the growth rate of the *ahpC*1 mutant in the INT-407 tissue cultures was significantly lower than that of the wild type (Fig. 6B). As a conclusion, these results combined suggest that AhpC1 and the capability of protection against ROS toxicity could contribute to the pathogenesis of *V. vulnificus* by assuring survival and multiplication during infection rather than directly aggravating damage or injury of the host.

Acknowledgements

This study was supported by grants to S.H.C from the Basic Science Research Project (R-01-2007-000-20590-0), the World Class University project (R32-2008-000-10183-0), and the National Research Laboratory Grant (R0A-2007-000-20039-0) of the Korea Science and Engineering Foundation, Republic of Korea.

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