

## Basic Study

## Characterization of two alkyl hydroperoxide reductase C homologs alkyl hydroperoxide reductase C\_H1 and alkyl hydroperoxide reductase C\_H2 in *Bacillus subtilis*

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### Abstract

**AIM:** To identify alkyl hydroperoxide reductase subunit C (AhpC) homologs in *Bacillus subtilis* (*B. subtilis*) and to characterize their structural and biochemical properties. AhpC is responsible for the detoxification of reactive oxygen species in bacteria.

**METHODS:** Two AhpC homologs (AhpC\_H1 and AhpC\_H2) were identified by searching the *B. subtilis* database; these were then cloned and expressed in *Escherichia coli*. AhpC mutants carrying substitutions of catalytically important Cys residues (C37S, C47S, C166S, C37/47S, C37/166S, C47/166S, and C37/47/166S for AhpC\_H1; C52S, C169S, and C52/169S for AhpC\_H2) were obtained by site-directed mutagenesis and purified, and their structure-function relationship was analyzed. The *B. subtilis* *ahpC* genes were disrupted by the short flanking homology method, and the phenotypes of the resulting AhpC-deficient bacteria were examined.

**RESULTS:** Comparative characterization of AhpC homologs indicates that AhpC\_H1 contains an extra C37, which forms a disulfide bond with the peroxidatic C47, and behaves like an atypical 2-Cys AhpC, while AhpC\_H2 functions like a typical 2-Cys AhpC. Tryptic digestion analysis demonstrated the presence of intramolecular

Cys37-Cys47 linkage, which could be reduced by thioredoxin, resulting in the association of the dimer into higher-molecular-mass complexes. Peroxidase activity analysis of Cys→Ser mutants indicated that three Cys residues were involved in the catalysis. AhpC\_H1 was resistant to inactivation by peroxide substrates, but had lower activity at physiological H<sub>2</sub>O<sub>2</sub> concentrations compared to AhpC\_H2, suggesting that in *B. subtilis*, the enzymes may be physiologically functional at different substrate concentrations. The exposure to organic peroxides induced AhpC\_H1 expression, while AhpC\_H1-deficient mutants exhibited growth retardation in the stationary phase, suggesting the role of AhpC\_H1 as an antioxidant scavenger of lipid hydroperoxides and a stress-response factor in *B. subtilis*.

**CONCLUSION:** AhpC\_H1, a novel atypical 2-Cys AhpC, is functionally distinct from AhpC\_H2, a typical 2-Cys AhpC.

**Key words:** Cysteine-dependent peroxidase; Thioredoxin; Thiol peroxidase; Peroxiredoxin; Alkyl hydroperoxide; Ortholog; *Bacillus subtilis*; Oxidative stress

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**Core tip:** Two alkyl hydroperoxide reductase subunit C (AhpC) homologs (AhpC\_H1 and AhpC\_H2) were identified by searching the *Bacillus subtilis* database. Sequence homology and phylogenetic analyses revealed that AhpC\_H1 is an ortholog of *Escherichia coli* (*E. coli*) AhpC, a representative of bacterial AhpC. AhpC\_H1 forms dimers consisting of atypical 2-Cys subunits, while AhpC\_H2 behaves like a typical 2-Cys AhpC. These AhpC homologs may perform their respective physiological functions at different peroxide levels. Structural and catalytic differences between the enzymes indicate that AhpC\_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC.

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## INTRODUCTION

Peroxiredoxins (Prxs) are versatile and predominantly thiol-dependent peroxidases (EC 1.11.1.15) that detoxify hydrogen peroxide and various hydroperoxides<sup>[1,2]</sup>. They are ubiquitously expressed, with multiple isoforms present in most organisms (e.g., three isoforms in *Escherichia coli*, five in *Saccharomyces cerevisiae*, six in *Homo sapiens*, and nine in *Arabidopsis thaliana*)<sup>[3-13]</sup>. Prxs exhibit high reactivity with hydrogen peroxide and

organic hydroperoxides and are involved in peroxide detoxification and signal transduction<sup>[14-20]</sup>.

All Prxs share a common catalytic mechanism, in which an active-site cysteine (the peroxidatic cysteine) is oxidized to a sulfenic acid by a peroxide substrate, forming a disulfide linkage with a second resolving Cys, which is reduced back by the thioredoxin system consisting of thioredoxin 1 (Trx1), Trx reductase, and NADPH<sup>[1,2]</sup>. According to the number of functional Cys residues, Prxs are divided into two families: 1-Cys Prxs and 2-Cys Prxs<sup>[11,15]</sup>.

The 2-Cys family contains typical and atypical groups of enzymes<sup>[21]</sup>. The disulfide bridge formed in typical 2-Cys Prxs after hydroperoxide reduction is intermolecular, whereas in atypical Prxs, it is intramolecular<sup>[22]</sup>. The founding members of the 2-Cys Prx family, the Prx1/alkyl hydroperoxide reductase C (AhpC) subfamily, including human Prxs I-IV and bacterial AhpC proteins are well-studied and broadly distributed enzymes that form inter-subunit disulfide bonds during catalysis. The amino acid sequence and physiological function of this type of thioredoxin-dependent peroxidases (thiol-specific antioxidants) were first determined in *Saccharomyces cerevisiae*<sup>[23]</sup>, and later in humans<sup>[24]</sup> and *E. coli*<sup>[3]</sup>. The members of these subfamilies are typically dimeric and form intra-subunit disulfide bonds. Another subfamily (the BCP/PrxQ group) is predominantly bacterial, although present in some eukaryotes and plants, but is absent in animals<sup>[6,12]</sup>. Most enzymes of the BCP/PrxQ group are reportedly monomeric<sup>[25]</sup>, but there are two examples of dimeric proteins containing an A-type dimer (the TPx and Prx5 subfamilies). Current information suggests that BCP-PrxQ subfamily members can function as either atypical 2-Cys (α-group) or 1-Cys Prxs<sup>[26]</sup>.

Moreover, there are also high molecular weight multimeric enzymes<sup>[27]</sup>. One atypical 2-Cys Prx exists as a hexamer in oxidized conditions, but dissociates to dimers upon reduction<sup>[28]</sup>; the presence of the peroxidatic Cys is critical for hexamer formation, whereas substitution of the resolving Cys does not affect oligomerization<sup>[29]</sup>. By analogy with the dimer-decamer transition of the typical 2-Cys Prx previously reported<sup>[27]</sup>, the dimer-hexamer transition in atypical Prx displays a functional switch, which could be involved in signaling<sup>[28,30]</sup>.

All aerobic bacteria have evolved a variety of antioxidant enzymes such as superoxide dismutases, catalases, and Prxs to cope with damaging endogenous reactive oxygen species (ROS) generated by the aerobic respiratory chain and metal-catalyzed oxidative reactions<sup>[31]</sup>. *E. coli* contains three Prxs: AhpC (Prx1/AhpC subfamily)<sup>[9]</sup>, TPx (p20)<sup>[4,5]</sup>, and BCP (BCP/PrxQ subfamily)<sup>[6,25]</sup>.

Gene sequencing and phylogenetic studies have revealed that *B. subtilis* encodes four putative Prx enzymes with two conserved Cys residues, suggesting that they belong to different subgroups of the 2-Cys

**Table 1** Oligonucleotides used for amplifying the genes expressing *Bacillus* peroxiredoxins proteins and its Cys residue-mutated proteins

	Sequence of primer for point mutation
AhpC_H1	
C37S F	AGCGTATTCTCTTTCTACCCA
C37S R	TGGGTAGAAAGAGAATACGCT
C47S F	TTCTCTTTCTGATCTCCAACGAGCTT
C47S R	AAGCTCAGTTGGAGATACGAAAGAGAA
C166S F	CCAGGTGAAGTTTCTCCGGCTAAATGG
C166S R	CCATTAGCCGGAGAACTTCACCTGG
AhpC_H2	
C52S F	TTCACCTTTGTTTCTCCGACAGAAATT
C52S R	AATTTCTGTCGGAGAAAACAAAGTGAA
C169S F	ACTGGCGGACTCTCTCCGGCTAACTGG
C169S R	CCAGTTAGCCGGAGAGAGTCCGCCAGT
	Sequence of primer for protein expression
AhpC_H1	
Forward	CCGCATATGCTTTAATCGGTAAAGAAGATC
Reverse	CCGGTCGACTTAGATTTTACCTACTAGATCAAGG
AhpC_H2	
Forward	CCGCATATGGCAGAACGTATGGTAGGTAA
Reverse	CCGGTCGACTTAAAGTGTTTTGGCCTGGTTT
B_BCP	
Forward	CCGCATATGACTATAGAAATCGGACAAAAAC
Reverse	CCGGTCGACTTACTTTTCTAGACATTTTGAGG
B_TPx	
Forward	CCGCATATGGCTGAAATTACATTCAAAGGC
Reverse	CCGGTCGACTTACTTTCTAATGCAGCAGC

Prx family: there are two genes encoding putative AhpC-like proteins (AhpC\_H1 and AhpC\_H2), and two encoding TPx and BCP, respectively. AhpC\_H1 has an additional N-terminal Cys residues at positions 37 (Cys37) besides two conserved Cys47 and Cys166, while ahpC\_H2 possesses two conserved Cys residues (Cys52 and Cys169).

The present comparative study was designed to characterize two AhpC homologs and reveal the biological significance of the existence of two homologous AhpC enzymes in *B. subtilis*. This paper presents the first report of a detailed structure-functional analysis of the bacillus 2-Cys AhpC homologs AhpC\_H1 and AhpC\_H2.

## MATERIALS AND METHODS

### Materials

Bacterial culture media were purchased from Difco Laboratories (Detroit, MI, United States). Restriction enzymes and DNA ligase were obtained from Promega (Madison, WI, United States). Acrylamide-Bis (40% solution) was purchased from Bio-Rad (Hercules, CA, United States). Sodium dodecyl sulfate (SDS), ultrapure glycine, EDTA, dithiothreitol (DTT), Tris base, NADPH, ampicillin powder, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), H<sub>2</sub>O<sub>2</sub>, *tert*-Butyl hydroperoxide (*t*-BOOH) and cumene hydroperoxide (CMOOH) were from Sigma Aldrich (St. Louis, MO, United States). Iodoacetamide (IAA), N-ethyl maleimide (NEM), *tris* (2-carboxyethyl) phosphine (TCEP), 4-acetamide-4'-maleimidylstilbene-

2,2'-disulfonate (AMS), and sulfhydryl-reactive DyLight 405 maleimide were purchased from Thermo Scientific, Pierce (Rockford, IL, United States).

### Sequence and phylogenetic analyses

The NCBI BLAST tool (<http://www.ncbi.nlm.nih.gov>) was used to search for Prx amino acid homologs in the updated GenBank/EMBL and Swiss-Prot databases. Multiple sequence alignments of *B. subtilis* and *E. coli* Prxs were performed using the ClustalW 2.1 program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 6. A phylogenetic tree was generated by using the Maximum Likelihood method based on the JTT matrix-based model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches).

### Bacterial strains

*B. subtilis* Cu1065 (*subtilis* strain 168 *attSP $\beta$*  *trpC2*, a derivative of *B. subtilis* subsp. *subtilis* str. 168) and *E. coli* XL1-blue and BL21 (DE3) strains were grown in Luria Broth (LB) medium under vigorous agitation at 37 °C. Bacterial growth was monitored by the absorbance at 600 nm (OD<sub>600</sub>), and exponentially growing *B. subtilis* cultures were exposed to different stress conditions for 30 min.

### Cloning, site-directed mutagenesis, expression, and purification of recombinant proteins

The entire coding regions of *B. subtilis* genes encoding AhpC\_H1, AhpC\_H2, B\_BCP, and B\_TPx were amplified by PCR using the High Expand Fidelity kit (Roche Life Science, United States) and primers listed in Table 1. The entire coding region of the *E. coli* *ahpC* gene was amplified by PCR using the following primers: forward, 5'-GGG ATC CCA TAT GTC CTT AAT TAA CAC-3'; reverse, 5'-CCT CGA GTT AGA TTT TAC CAA CCA GGT-3'. To obtain Cys→Ser substitutions in AhpC\_H1 and AhpC\_H2, point mutations in the *ahpC\_H1* and *ahpC\_H2* genes were generated by site-directed mutagenesis by using complementary primers (Table 1). Gel-purified *ahpC* genes were digested with *Nde*I and *Xho*I, ligated into the pET-21a(+) expression vector by using T4 DNA ligase (Promega), and used to transform competent *E. coli* XL1-blue. Bacteria were selected on LB agar containing ampicillin (100  $\mu$ g/mL), and purified plasmids were used for the sequencing of the cloned coding region by automated DNA sequencing.

The plasmids containing the confirmed sequences were used to transform *E. coli* BL21 (DE3) cells, which were then cultured at 37 °C overnight in 100  $\mu$ g/mL ampicillin-containing LB. For protein expression, bacteria were diluted 1:250 in fresh medium, grown until OD<sub>600</sub> = 0.4, and induced with 0.5 mmol/L isopropyl-L-D-thiogalactose (IPTG) for 4 h. The soluble recombinant proteins were produced in *E. coli*. Cells

**Table 2** Oligonucleotides used for amplifying the DNA fusion construct to knockout *Bacillus* Prx genes

Prx null mutants	Primer names	Sequences of paired degenerated primers (5'→3')
ΔAhpC_H1	H1_Up F	CCTTATTTCACAGATAAGCTCCA
	Kan/H1 R'	CCTATCACCTCAAATGGTTCGCTG AATGTATATTCCTCCTAAAAATGTAT
	Kan/H1 F'	CGAGCGCTACGAGGAATTTGTATGGAGTGCATTCAATTGGTACTTG
ΔAhpC_H2	H1_Down R	ACGTGCAGTGTAGATCGCTGC
	H2_Up F	AAGTCGCGAATTGAGTTGTCC
	Kan/H2 R'	CCTATCACCTCAAATGGTTCGCTGTATATCCCTCCAATTTATTGTTTG
	Kan/H2 F'	CGAGCGCTACGAGGAATTTGTAT TTTTTCCTCAAGAACGAAAAGCGG
ΔB_BCP	H2_Down R	ATCTAAAATTGAGGAAAAACATCAA
	BCP_Up F	AATGGTACTGTGATCACTTCGTTT
	Kan/BCP R'	CCTATCACCTCAAATGGTTCGCTG TACGTTACCTCCGGATGTTTTTTT
	Kan/BCP F'	CGAGCGCTACGAGGAATTTGTAT ATCTCTATGAGCCTATGCTTACTT
ΔB_TPx	BCP_Down R	AGTAGTCGATATGTGCATGCATT
	TPx_Up F	TTTTTCGCTTTGGACATGCTAT
	Kan/TPx R'	CCTATCACCTCAAATGGTTCGCTGTATAATTCCTCCCTTTTGTATGTAT
	Kan/TPx F'	CGA GCGCTACGAGGAATTTGTAT GCAGGGAAAAAAGCTCCAGGC
	TPx_Down R	CGTCAGCTTTGAGCTCAAACG

Prx: Peroxiredoxins.

were harvested by centrifugation, suspended in 50 mmol/L Tris-HCl (pH 7.4) containing 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 mmol/L EDTA, disrupted by sonication, and centrifuged at 16000 × g for 30 min. The clarified supernatant was subjected to ammonium sulfate fractionation, size exclusion chromatography, and anion exchange chromatography. Size exclusion chromatography was performed using a Pharmacia chromatography system (Pharmacia Biotech). Protein solution was loaded onto a Sephacryl S-200 column equilibrated with 200 mmol/L NaCl-containing Tris-HCl, pH 8.0, at a flow rate of 0.2 mL/min at 4 °C, and protein elution was monitored by the absorbance at 280 nm. Anion exchange chromatography was performed using DEAE-cellulose DE52 equilibrated with Tris-HCl, pH 8.0; protein was eluted with a linear salt gradient (0–500 mmol/L NaCl) in Tris-HCl, pH 8.0. Protein concentration was determined using the Bradford protein assay kit (Bio-Rad, United States).

### Gene disruption by fusion PCR

The *B. subtilis* Prx-encoding genes were disrupted by the short flanking homology method<sup>[32]</sup>. The 5' and 3' regions of the *prx* genes were amplified using primers designed to obtain 0.2-kb DNA fragments (Table 2). A kanamycin-resistance determinant used as a selection marker for ΔPrx strains was amplified from the pDG780 vector using specific primers (forward, 5'-CAG CGA ACC ATT TGA GGT GAT AG-3' and reverse, 5'-CGA GCG CCT ACG AGG AAT TTG TAT-3'). The marker was flanked by the two 0.2-kb terminal regions of the Prx-encoding genes using two-step fusion PCR. The first step was performed by fusing the 5'-terminal Prx gene fragment to the kanamycin-resistance gene. The amplified product was gel-purified and used as a template for the second round of PCR to fuse the 3' terminal Prx gene fragment. The final PCR product was gel-purified and used for the disruption of each Prx-encoding gene in *B.*

*subtilis* Cu1065. The null mutants were selected on 20 µg/mL kanamycin-containing LB agar, and chromosomal DNA was isolated and analyzed by PCR to confirm the replacement of the target gene.

### Electrophoresis and Western blot analyses

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 14% acrylamide gels. In reducing conditions, proteins were dissolved in loading buffer containing 100 mmol/L DTT and boiled for 3 min prior to loading; in non-reducing conditions, proteins were loaded in DTT-free buffer without boiling. After electrophoresis, gels were stained with Coomassie Brilliant Blue (Sigma-Aldrich) or processed for Western blotting. For this, PAGE-separated proteins were transferred to nitrocellulose membranes (Bio-Rad), blocked with 5% (w/v) skim milk in PBS containing 0.1% Tween 20 (PBST) at room temperature for 2 h, and probed with a manufactured rabbit polyclonal anti-AhpC\_H1 (1:3000) antibodies (Young In Frontier, Seoul, South Korea) for 3 h. After three washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h at room temperature, washed three times with PBST, and developed using the TMB immunoblotting detection system (Kem-En-Tec).

Further protein characterization was performed by 2D electrophoresis. Briefly, the *B. subtilis* protein extract was loaded onto 7-cm immobilized pH gradient (IPG) strips (Bio-Rad, United States) with 4–7 pH gradient (left to right), and the first-dimension separation was conducted; in the second-dimension separation, 16% reducing SDS-PAGE gels were used. For protein detection, the gels were stained with Coomassie Brilliant Blue G-250 or subjected to immunoblotting.

### High-performance liquid chromatography

AhpC\_H1 was purified from *B. subtilis* and a 25-µg



sample in phosphate buffer, pH 7.4, containing 150 mmol/L KCl was loaded onto a ZORBAX® Pro 10/300 GF450 column equilibrated in phosphate buffer using an automatic injector (SIL-20AC, Shimadzu, Japan). Chromatography was performed on a Shimadzu high-performance liquid chromatography (HPLC) system at the flow rate of 0.5 mL/min at room temperature, and the absorbance was monitored using a diode array detector (SPD M10A, Shimadzu). The column was calibrated using protein molecular mass standards under the same conditions, and a calibration curve was obtained by plotting the  $V_e/V_o$  ratio (where  $V_e$  is the elution volume and  $V_o$  is the void volume) vs the logarithms of the molecular masses.

#### Chemical modification of cysteine sulfhydryl groups

For the reduction of protein disulfide groups, protein was reacted with chemical reducing agents such as DTT or TCEP or subjected to enzymatic reduction with *E. coli* Trx1, Trx1 reductase, and NADPH. After 30-min reaction at 30 °C, thiol-specific modification reagent 4-acetamide-4'-maleimidylstilbene-2,2'-disulfonate (AMS) was added for another 30 min at 30 °C.

#### Tryptic digestion and peptide identification

For HPLC analysis of Cys-containing peptides, sulfhydryl-reactive DyLight 405 maleimide (excitation and emission wavelengths 557 and 572 nm, respectively) was used to fluorescently label Cys residues in the intact or TCEP-denatured AhpC\_H1. Proteins digested with trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK, an inhibitor of contaminating chymotryptic activity) in the presence of 2 mol/L urea were analyzed by reversed-phase HPLC. Briefly, proteins were incubated in 50 mmol/L  $\text{NH}_4\text{CO}_3$  containing 2 mol/L urea and trypsin at the ratio 1:20 (w/w, trypsin/protein) at 30 °C for 12 h, and then loaded into a C18 HPLC column (2.1 mm × 25 cm). Peptides were eluted with a 5%-100% gradient of acetonitrile containing 0.2% trifluoroacetic acid (TFA) at the flow rate of 1 mL/min for 120 min. The elution of fluorescent peptides was monitored at 572 nm using a fluorescence detector (RF-10AXL, Shimadzu).

#### Enzyme assays

Enzymatic assays with the Trx system (NADPH, *E. coli* Trx reductase, and Trx1) and peroxides as substrates were conducted to detect peroxidase activity of the purified AhpC proteins by the decrease of absorbance at 340 nm. Briefly, the enzymes were added to the reaction mixture containing 0.14 mmol/L NADPH, 100 nmol/L *E. coli* Trx reductase, 5  $\mu\text{mol/L}$  *E. coli* Trx1, 50 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EDTA, and hydroperoxide substrates ( $\text{H}_2\text{O}_2$  and *t*-butyl hydroperoxide) at room temperature and the absorbance was continuously monitored at 340 nm using a diode array spectrophotometer (HP 7452A). Stock solutions of *t*-butyl hydroperoxide were prepared in DMSO prior to the reaction.

#### General methods

Plasmid and chromosomal DNA isolation, restriction enzyme digestion, *E. coli* transformation, and DNA ligation were performed according to standard protocols<sup>[32]</sup>. The transformation of naturally competent *B. subtilis* cells was carried out as previously described<sup>[33]</sup>.

## RESULTS

#### Bioinformatic analysis of *B. subtilis* putative Prx homologs

Four *prx*-homologous sequences in *B. subtilis* genome were identified by searching the Data Release R16.1 (the SubtiList World-Wide Web Server, <http://genolist.pasteur.fr/SubtiList/>). Figure 1 shows multiple sequence alignment of three *E. coli* Prxs (E\_AhpC, E\_BCP, and E\_TPx) and four *B. subtilis* Prxs using the ClustalW 2.1 program, and a resulting phylogenetic tree. The hallmark of Prxs is a conserved peroxidatic Cys residue located near the N-terminus. The analysis of multiple protein alignment revealed that all *B. subtilis* Prxs possessed a highly conserved peroxidatic Cys, suggesting that these enzymes belong to the Prx family. The cladogram reveals that, with the exception of B\_AhpC\_H2, all *Bacillus* Prxs displayed sequence similarity with the corresponding *E. coli* Prxs and were therefore named after the respective *E. coli* enzymes. Thus, E\_TPx and B\_TPx showed 41.5% identity in 164 overlapping amino acids and E\_BCP and B\_BCP demonstrated 39.7% identity in 151 overlapping amino acids. E\_AhpC and B\_AhpC\_H1 had 65.24% identity in a 187-amino acid region, while E\_AhpC and B\_AhpC\_H2 had 38.4% identity in a 159-amino acid sequence. Between B\_AhpC\_H1 and B\_AhpC\_H2, there was 37.0% identity in a 181-amino acid sequence.

Sequence similarity analysis indicates that AhpC\_H1 is a homologue of *E. coli* AhpC, a representative of the AhpC/Prx1 subfamily. Sequence alignment of AhpC homologs revealed two conserved Cys residues, catalytic and resolving, in the N-terminal and C-terminal regions, respectively, suggesting that B\_AhpC\_H2 also belongs to the AhpC/Prx1 family, although its similarity to E\_AhpC (38.4%) is much lower than that of B\_AhpC\_H1 to E\_AhpC (65.2%).

Taken together, these data suggest that *B. subtilis* encodes one member of the BCP/PrxQ, one member of the bacterial TPx, and two members of the AhpC/Prx1 subfamilies.

#### Structural analysis of *B. subtilis* recombinant AhpC proteins

AhpC\_H2 and AhpC\_H1 each contain a pair of conserved Cys residues (Cys52/Cys169 and Cys47/Cys166, respectively); in addition, AhpC\_H1 possesses an extra Cys37 (Figure 1). The members of the AhpC/Prx1 subfamily (including *E. coli* AhpC) are typical 2-Cys Prxs, which exist as homodimers formed via an intermolecular disulfide bond between two subunits.



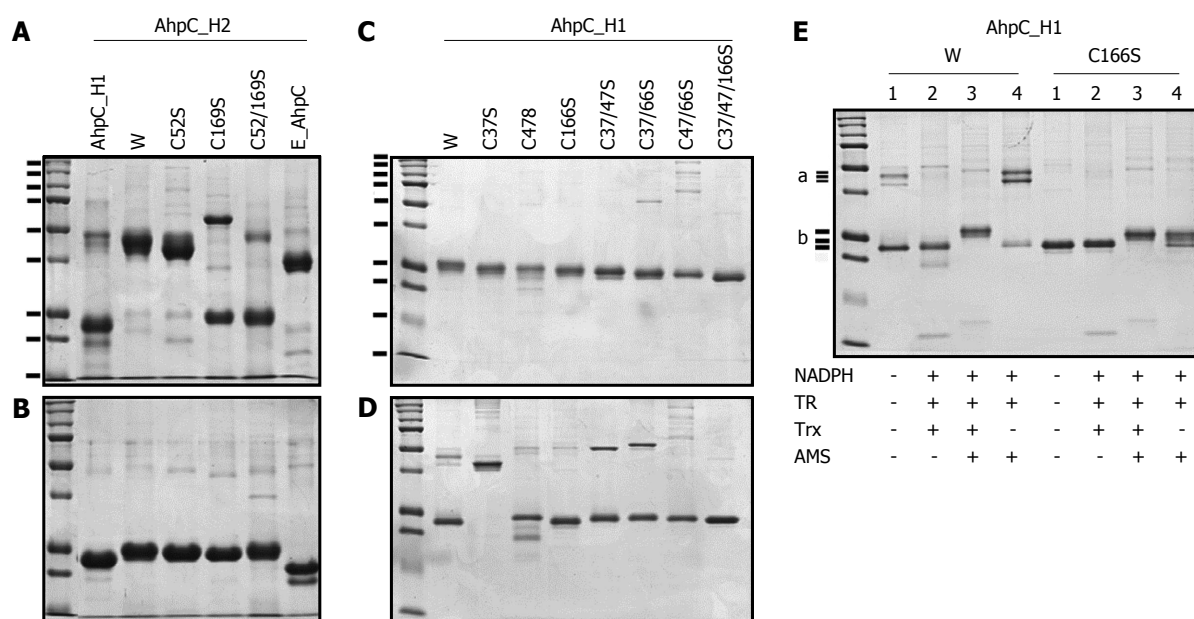
**Figure 1** Amino acid sequence alignment of *Bacillus subtilis* and *Escherichia coli* peroxiredoxins. *B. subtilis* database was searched using amino acid sequences around conserved N-terminal Cys residues as a query. In addition to reported bacterial genes encoding BCP and TPx enzymes, two putative AhpC-encoding genes were identified, and four *B. subtilis* and three *E. coli* Prx sequences were aligned. The two conserved Cys residues, N-terminal peroxidatic and C-terminal resolving Cys, among the AhpC proteins are shown as shaded bold letters. Proteins homologous to *E. coli* AhpC were identified using the ClustalW 2.1 multiple sequence alignment. The phylogenetic tree was generated by using MEGA version 6. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 7 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 143 positions in the final dataset. Position of Cys residues in AhpC proteins is shown at the bottom right panel. E\_BCP, *E. coli* BCP (accession number AAC75533); E\_p20 (TPx), *E. coli* TPx (accession number EDV68400); E\_AhpC, *E. coli* AhpC (accession NP\_415138); B\_ahpC\_H1, *B. subtilis* AhpC homolog 1 (accession number BAA11268); B\_AhpC\_H2, *B. subtilis* AhpC homolog 2 (accession number WP\_019258276); B\_BCP, *B. subtilis* BCP (accession number AAC75533); and B\_p20 (TPx), *B. subtilis* TPx (accession NP\_390827).

To examine the possible formation of a disulfide bond between the two conserved Cys residues in *B. subtilis* AhpC enzymes, Cys was replaced with Ser by site-directed mutagenesis and the purified mutant AhpC\_H1 and AhpC\_H2 proteins were analyzed by their migration patterns in SDS-PAGE.

In non-reducing SDS-PAGE gels, wild-type AhpC\_H2 and E\_AhpC appeared as single bands with the molecular masses about 48 kDa and about 40 kDa, respectively (Figure 2A), whereas single approximately 24.5-kDa and 22-kDa bands, respectively, were observed in reducing gels (Figure 2B), suggesting that in natural conditions, these enzyme exist as dimers

likely held together by disulfide linkages. However, most AhpC\_H1 appeared as a single band with a molecular mass of the monomer (approximately 23-kDa) under both non-reducing and reducing conditions (Figure 2A and B, respectively), suggesting the presence of monomers even in the oxidized state.

The migration pattern of AhpC\_H2 mutant proteins (C52S, C169S, and C52/169S) in non-reducing SDS-PAGE gels were quite different from that of the wild-type counterpart (Figure 2A). C169S mostly existed in a monomeric form, presenting the evidence of intermolecular disulfide linkage between the two identical subunits. In non-reducing conditions, the



**Figure 2** SDS-PAGE analyses of AhpC\_H1 and AhpC\_H2 proteins and their mutants carrying Cys→Ser substitutions. The wild-type (lane W) and mutated AhpC\_H2 and AhpC\_H1, and *E. coli* AhpC proteins were separated in non-reducing (A) and reducing (B) 12% SDS-PAGE gels; first lanes show molecular weight markers (15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa). TCEP/AMS-treated (C) and non-treated (D) wild-type (lane W) and mutated AhpC\_H1 proteins were separated in non-reducing 14% SDS-PAGE gels. AhpC\_H1 and its C166S mutant reduced or not with the Trx system containing Trx 1, Trx reductase (TR), and NADPH were modified with a sulfhydryl group-specific reagent AMS and separated in non-reducing 14% SDS-PAGE gels (E). a marks the position of a single AhpC\_H1 band (W, lane 1) and double bands (W, lane 4). b marks three positions of the AhpC\_H1 band after the treatments indicated at the bottom. First lanes in B1, B2, and C show molecular weight markers (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa).

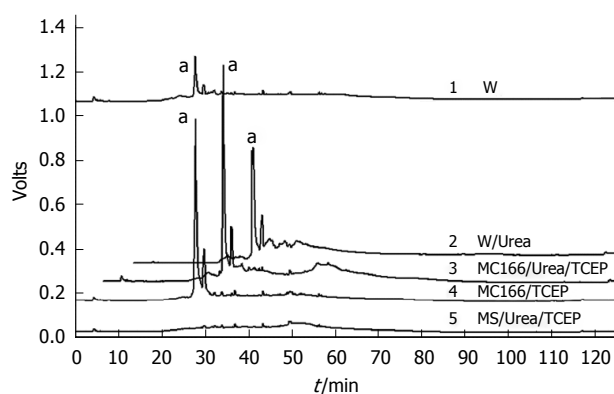
migration of the wild-type dimer appeared to be different from that of the mutant C52S and C169S dimers, suggesting the formation of inter-subunit disulfide bonds characteristic for typical 2-Cys Prxs such as E\_AhpC.

In contrast to AhpC\_H2 and E\_AhpC, most of the AhpC\_H1 proteins existed as monomers even in the oxidized state. The migration patterns of AhpC\_H1 Cys→Ser mutants (C37S, C47S, C166S, C37/47S, C37/166S, C47/166S, and C37/47/166S) indicated that the distance of protein migration in reducing conditions depended on the number of substituted Cys residues (Figure 2C), thus confirming that all three Cys residues are susceptible to AMS modification. In non-reducing conditions, the C37S protein migrated as a dimer (Figure 2D). Considering that, in non-reducing conditions, the wild-type protein was predominantly monomeric (lane W in Figure 2D), dimerization of the C37S mutant suggests that Cys37 could participate in the formation of an intramolecular disulfide bond. In non-reducing gels, most of the proteins carrying C47S and C166S substitutions were detected at the positions slightly higher than that of the wild-type protein; however, a single C166S mutant migrated similarly to the wild-type protein, suggesting the existence of intramolecular disulfide linkage between Cys37 and Cys47 in both proteins.

Taken together, these results indicate a possibility that AhpC\_H1 is an atypical 2-Cys enzyme, which forms the intramolecular disulfide linkage between Cys37 and Cys47 rather than between Cys47 and

Cys166. To examine the presence of Cys37-Cys47 intramolecular disulfide bond, the wild-type AhpC\_H1 and its C166S mutant were reduced using the Trx system, and analyzed by non-reducing SDS-PAGE. As shown in Figure 2E, Trx-treated C166S occupied a slightly higher position than the wild-type protein; AMS modification shifted the position of both proteins toward higher molecular weight (Figure 2E, lane 3). These results reveal that the disulfide bond can be specifically reduced by the Trx system. Furthermore, AMS modification of the wild-type AhpC\_H1 in the absence of Trx1 (Figure 2E, lane 4) or TCEP (data not shown) resulted in the formation of two different dimers, but did not cause dimerization of C166S, which confirms the presence of free Cys166 in AhpC\_H1. Other sulfhydryl-modifying agents such as NEM and IAA also induced AhpC\_H1 dimerization (data not shown). Overall, the effect of thiol-specific modification of free Cys166 on the tertiary structure of AhpC\_H1 is worth noting, although such modification may not occur *in vivo*.

Finally, we directly demonstrated the presence of free Cys166 residue in the oxidized AhpC\_H1. AhpC\_H1 was modified with fluorescent sulfhydryl-reactive maleimide and digested with trypsin in the presence or absence of 2 mol/L urea. The reversed-phase HPLC chromatogram of trypsin-digested native AhpC\_H1 demonstrates a unique peak of a fluorescent peptide (a in Figure 3, W), which was significantly increased in the trypsin-digested denatured (2 mol/L urea-treated) AhpC\_H1 (a in Figure 3, W/Urea). The



**Figure 3** Reversed-phase high-performance liquid chromatography analysis of fluorescently labeled trypsin-digested AhpC\_H1. AhpC\_H1 was modified with sulfhydryl-reactive DyLight 405 maleimide, digested with TPCK-treated trypsin in the presence or absence of 2 M urea, and analyzed by reversed-phase HPLC; fluorescence was monitored at 527 nm. Fluorescent peak (a) corresponds to a peptide containing free Cys residue conjugated with the fluorescent dye. W: Wild-type AhpC\_H1; MC166: C37/47S double mutant; MS: Cys37/47/166S triple mutant.

same peak appeared in the trypsin-digested denatured and native C37/47S double mutant (Figure 3, MC166/Urea/TCEP and M166/TCEP, respectively), which has only one free Cys166 residue. The triple mutant C37/47/166S (MS) did not produce any fluorescent peak. Taken together, these data suggest that the peak represents the peptide with free Cys166 residue, indicating the existence of free Cys166 and the intramolecular disulfide linkage between Cys37 and Cys47.

#### Enzymatic reduction of the intramolecular disulfide bond between Cys37 and Cys47

The modification of Trx-preincubated AhpC\_H1 and AhpC\_H2 with AMS, a thiol-specific reagent, resulted in the shift of the monomer to a slightly higher position (Figure 4A and B, respectively), suggesting that the Trx system (Trx1, Trx reductase, and NADPH) is able to reduce protein disulfide bonds in both AhpC\_H1 and AhpC\_H2.

Pre-incubation of AhpC\_H1 with the Trx system containing increasing concentrations of *E. coli* Trx1 resulted in an increase of the AMS-modified AhpC\_H1 in a Trx 1 concentration-dependent manner (Figure 4C), indicating that the intramolecular disulfide bond can be reduced by the Trx treatment. The dimerization of AMS-modified AhpC\_H1 was also confirmed (Figure 4C). Similarly, the dimeric AhpC\_H2 was gradually converted to a corresponding AMS-modified monomer in a Trx1 dose-dependent manner (Figure 4D).

To ensure functional activity of the Trx1 system to reduce disulfide bonds, we tested the Trx system in the reaction with *Bacillus* B\_BCP and B\_TPx as well as with Trx-supported peroxidases from other species such as *E. coli* AhpC and human Prx1 and Prx2 (Figure 4E-I, respectively). The results indicate that the reduced forms of all Prxs increased in a

Trx1 concentration-dependent manner, indicating that all the tested enzymes were sensitive to the Trx system. More importantly, in non-reducing conditions, AhpC\_H2, but not AhpC\_H1, demonstrated a pattern very similar to that of *E. coli* AhpC (E\_AhpC) (Figure 4G), suggesting that AhpC\_H2 is structurally similar to E\_AhpC in that they exist as intermolecular dimers linked by a disulfide bond, although amino acid sequence similarity between AhpC\_H1 and E\_AhpC is much higher than that between AhpC\_H2 and E\_AhpC (Figure 1). Taken together, these results indicate the presence of a redox-sensitive disulfide linkage between Cys37 and Cys47 of AhpC\_H1, characterizing it as a member of the atypical 2-Cys subfamily.

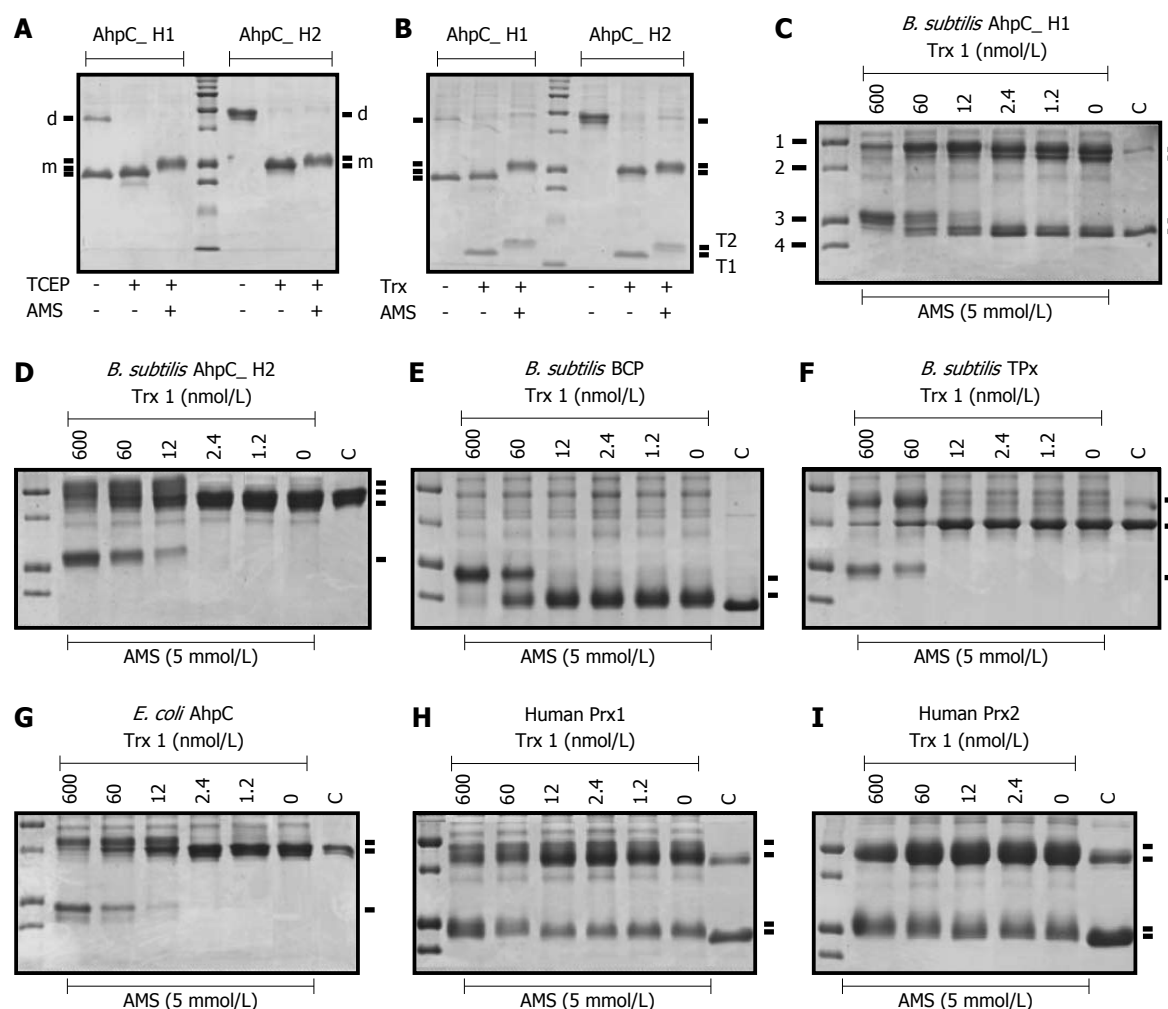
#### Identification of Cys37-Cys47 intramolecular disulfide linkage in the native AhpC\_H1 protein

In the previous experiments, we demonstrated that, unlike AhpC\_H2, AhpC\_H1 formed the intramolecular Cys37-Cys47 disulfide bond. However, it may be possible that it is characteristic only for the recombinant protein and not for the native *B. subtilis* AhpC\_H1. To test this possibility, we examined the presence of the disulfide linkage in the native *B. subtilis*-purified AhpC\_H1. Crude protein extract was fractionated using ammonium sulfate gradient (20% to 60%). Figure 5A shows that a major protein band of approximately 22-kDa was detected in the 40% and 45% fractions. Western blot analysis with the AhpC\_H1 antibody demonstrated that the predominant band was AhpC\_H1 (Figure 5B). In the 40% fraction, AhpC\_H1 was reduced with TCEP, and the resulting product was specifically modified with AMS (Figure 5C). The analysis of the modified proteins by non-reducing SDS-PAGE and western blotting (Figure 5D) revealed that AMS could modify only TCEP-reduced AhpC\_H1 (Figure 5D). Taken together, these results demonstrate that *B. subtilis* expresses a significant amount of oxidized AhpC\_H1 with the Cys37-Cys47 intramolecular disulfide linkage.

#### AhpC\_H1 oligomerization

Previous studies have demonstrated that the Cys37-Cys47 bond in AhpC\_H1 is redox-sensitive. AhpC\_H1 molecular mass was determined by HPLC. Figure 6A demonstrates that AhpC\_H1 was eluted between ovalbumin (OVA, 44 kDa) and carbonic anhydrase (CA, 29 kDa); therefore, the apparent AhpC\_H1 molecular mass was estimated to be approximately 38.6 kDa. Given that a theoretical AhpC\_H1 molecular mass is approximately 20.5 kDa, this result indicates that AhpC\_H1 exists as a homodimer. Considering that AhpC\_H1 mostly appears as a 22-kDa band in non-reducing SDS-PAGE gels, this result suggests that the dimer is likely formed *via* non-covalent interactions. On the other hand, AhpC\_H2 was eluted at 9.116 mL (data not shown). Given that the elution volume of apoferritin (AF, 443 kDa) was 9.514 mL, it suggests





**Figure 4** Non-reducing SDS-PAGE of peroxiredoxins reduced with the Trx system. (A, B) AhpC\_H1 and AhpC\_H2 were incubated without or with 2 mmol/L TCEP (A) or Trx system (B) for 30 min at 30 °C, followed by incubation with AMS for 30 min at 30 °C. The reaction was terminated by adding SDS sample buffer, and proteins were separated in 16% non-reducing SDS-PAGE gels. Protein migration pattern in A is the same as in B, suggesting that the Trx system reduced disulfide bonds in both proteins; d and m mark the position of dimeric and monomeric, respectively, AhpC\_H1 and AhpC\_H2. T1 and T2 denote the oxidized and reduced/modified Trx 1 proteins, respectively. Middle lanes show molecular weight markers (10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa). Trx, the Trx system; (C-F) Conversion of different Prxs from the oxidized to reduced form by the Trx system. 1, 2, 3, and 4 indicate molecular weight markers of 50, 37, 25, and 20 kDa, respectively.

that AhpC\_H2 oligomer has a higher molecular mass compared to AhpC\_H1.

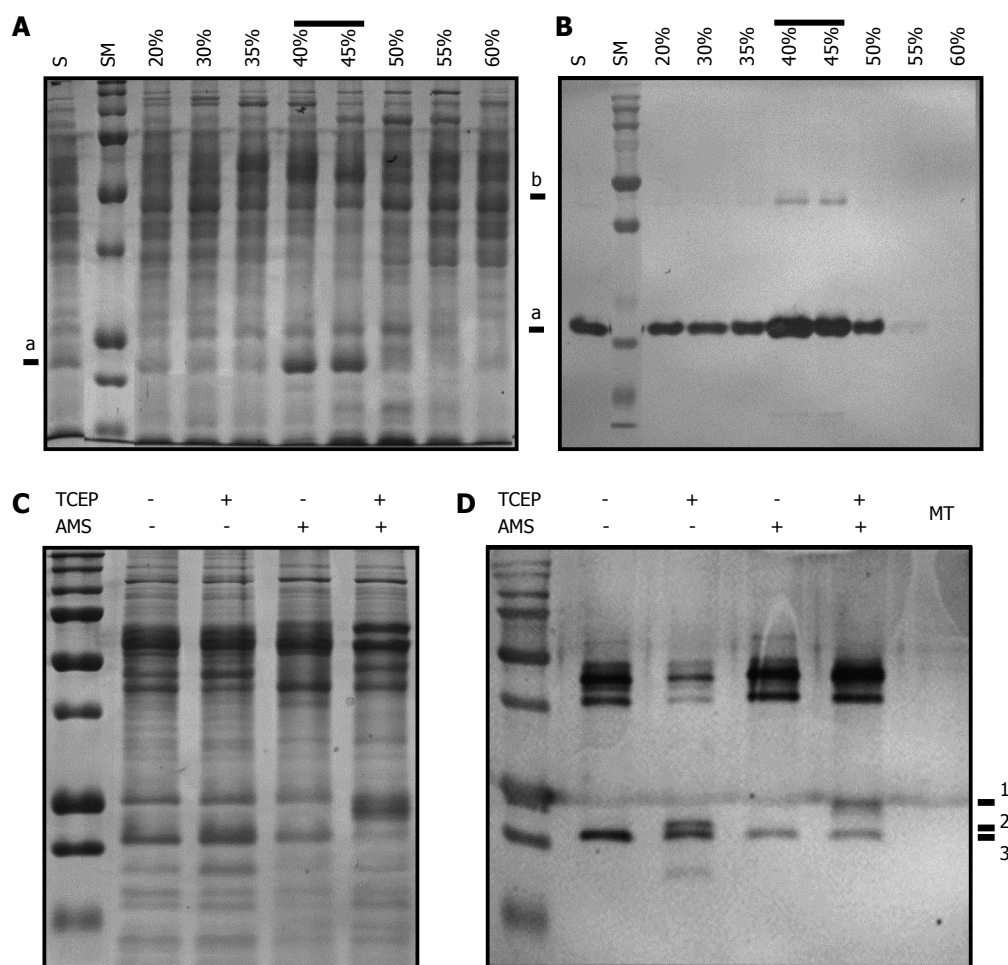
It has been reported that in some cases, Prx oligomerization is reversible and depends on the redox status of Cys residues<sup>[27-30]</sup>. To examine AhpC\_H1 redox-dependent oligomerization, the molecular mass of TCEP-reduced AhpC\_H1 was determined by HPLC. Figure 6B shows that the elution volume of reduced AhpC\_H1 was 9.379 mL (Figure 6B, AhpC\_H1/TCEP), which, considering the elution volume of 443-kDa apoferritin (9.514 mL) indicates that the apparent molecular mass of reduced AhpC\_H1 was above 443 kDa. AhpC\_H1 incubation with H<sub>2</sub>O<sub>2</sub> did not significantly affect the molecular mass (Figure 6B, AhpC\_H1/H<sub>2</sub>O<sub>2</sub>).

Taken together, these results suggest that AhpC\_H2 exists as a high molecular mass oligomer, while AhpC\_H1 is a homodimer. Oxidized AhpC\_H1 monomers are dimerized *via* noncovalent interactions; however, after

reduction, the dimers tend to assemble into high molecular mass oligomers.

### Substrate-mediated inactivation of AhpC\_H1 and AhpC\_H2

Because some Prxs, especially of eukaryotic origin, are known to be inactivated by high substrate concentrations<sup>[1,2]</sup>, we tested the response of AhpC\_H1 and AhpC\_H2 to elevated concentrations of their preferred substrate H<sub>2</sub>O<sub>2</sub> by using the Trx1 system. Peroxidase activity was continuously monitored by the absorbance at 340 nm indicating NADPH concentration. Figure 7A and B shows a time-dependent decrease in NADPH concentration due to the peroxidase activity of AhpC\_H1 and AhpC\_H2, respectively. At low H<sub>2</sub>O<sub>2</sub> concentrations (< 100 μmol/L), AhpC\_H2 was more active than AhpC\_H1, but was significantly inhibited by higher H<sub>2</sub>O<sub>2</sub> concentrations, whereas AhpC\_H1 was resistant even to high H<sub>2</sub>O<sub>2</sub> levels, which completely



**Figure 5 Identification of intramolecular disulfide linkage in AhpC\_H1 from *B. subtilis*.** A, B: Soluble fraction of *B. subtilis* overnight cultures was precipitated with 30%, 35%, 40%, 45%, 50%, 55%, and 60% ammonium sulfate. After dialysis of solubilized precipitates, 20  $\mu$ g of each fraction was separated in 14% reducing SDS-PAGE gel, and stained with Coomassie brilliant blue (A) or processed by western blotting using anti-AhpC\_H1 antibody (for western blotting, 1  $\mu$ g protein was separated). Correlation of band intensities in (A) and (B) indicates that the major band marked as a is an AhpC\_H1 monomer; b marks an AhpC\_H1 dimer; C: Proteins (20  $\mu$ g) of the 40% fraction were incubated in the presence or absence of 2 mM TCEP, reacted with 5 mmol/L of AMS for 30 min at 30  $^{\circ}$ C, separated by non-reducing SDS-PAGE, and stained with Coomassie Brilliant Blue; D: Similarly treated proteins (100 ng) were subjected to western blotting. Triple bands (1, 2, and 3) around the position of the monomer indicate AMS modification of Cys residues after the reduction by TCEP. MT, AhpC\_H1-deficient *B. subtilis*; the absence of the AhpC\_H1 band indicates antibody specificity. SM, molecular weight markers (15, 20, 25, 37, 50, 75, 100, 150, 250 kDa).

inhibited AhpC\_H2 activity (Figure 7C), indicating that AhpC\_H1 is a much more robust enzyme compared to AhpC\_H2.

#### Trx-dependent peroxidase activity of AhpC\_H1 mutants

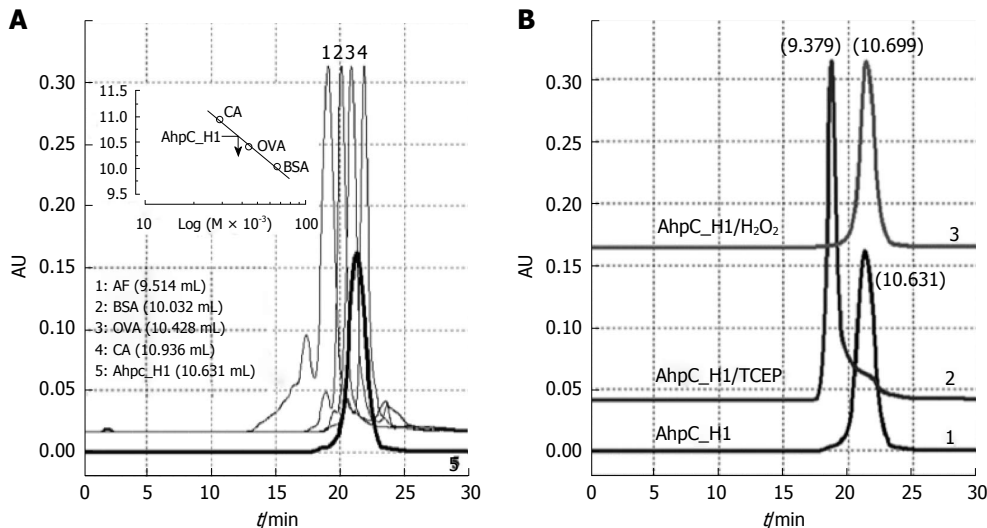
Figure 8 shows Trx-dependent peroxidase activity of the wild-type AhpC\_H1 and its C37S, C47S, C166S mutants toward *t*-butyl hydroperoxide substrate. Compared to almost inactive C47S and C166S mutants, the C37S protein showed a substantial enzymatic activity (approximately 19.6% of that of the wild-type protein), indicating that, in addition to Cys47 and Cys166, Cys37 is also required for the peroxidation activity of AhpC\_H1.

#### AhpC\_H1 expression in *B. subtilis*

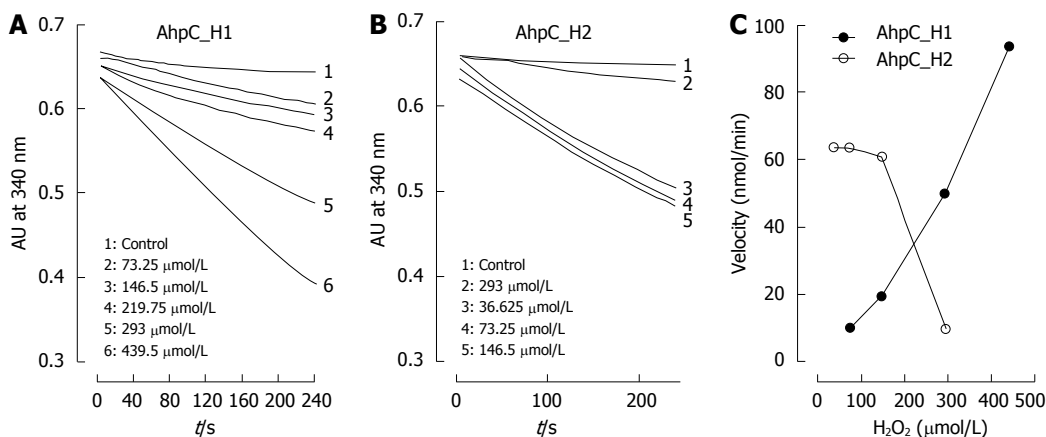
*B. subtilis* genome encodes four Prxs (B\_BCP, B\_TPx, B\_AhpC\_H1, and B\_AhpC\_H2) (Figure 1), and their expression as antioxidant proteins should be regulated

by growth conditions in a gene-specific manner. SDS-PAGE analysis of *B. subtilis* crude extracts indicates high expression of AhpC\_H1 (Figure 5). To further evaluate the regulation of Prx enzymes in *B. subtilis*, mutant strains deficient in B\_BCP ( $\Delta$ BCP), B\_TPx ( $\Delta$ TPx), B\_AhpC\_H1 ( $\Delta$ AhpC\_H1), or B\_AhpC\_H2 ( $\Delta$ AhpC\_H2) were constructed and analyzed by 2D electrophoresis. Protein patterns visualized by Coomassie Brilliant Blue staining indicate that one of the predominant spots represented the AhpC\_H1 monomer (Figure 9A, a), whereas the other spot detected in the cumene hydroperoxide (CMOOH)-stressed wild-type strain (Figure 9A, W + CMOOH, b) is suggested to be the AhpC\_H1 dimer.

Except for AhpC\_H1, other Prxs were not detected by 2D electrophoresis (Figure 9A). As expected, AhpC\_H1 (Figure 9A, a) was seen in all mutants but  $\Delta$ AhpC\_H1. It is worth noting that another spot corresponding to the AhpC\_H1 dimer was detected



**Figure 6** High-performance liquid chromatography gel-filtration chromatograms of AhpC\_H1 purified from *B. subtilis*. AhpC\_H1 was loaded onto a ZORBAX® Pro 10/300 GF450 column and eluted with 50 mmol/L phosphate buffer, pH 7.4, containing 150 mmol/L KCl. A: AhpC\_H1 elution profile (bold line, 5); protein molecular mass standards: 1, apoferritin (AF, 443 kDa); 2, bovine serum albumin (BSA, 66 kDa); 3, ovalbumin (OVA, 44 kDa); 4, carbonic anhydrase (CA, 29 kDa). Inset shows a calibration curve used to determine AhpC\_H1 molecular mass; B: The profiles of TCEP-reduced AhpC\_H1 (AhpC\_H1/TCEP) and AhpC\_H1 treated with 2 mmol/L H<sub>2</sub>O<sub>2</sub> for 30 min at 30 °C (AhpC\_H1/H<sub>2</sub>O<sub>2</sub>) were compared with that of untreated AhpC\_H1. Elution volume (mL) is in parentheses. Oligomeric AhpC\_H1 was eluted at 9.379 mL.



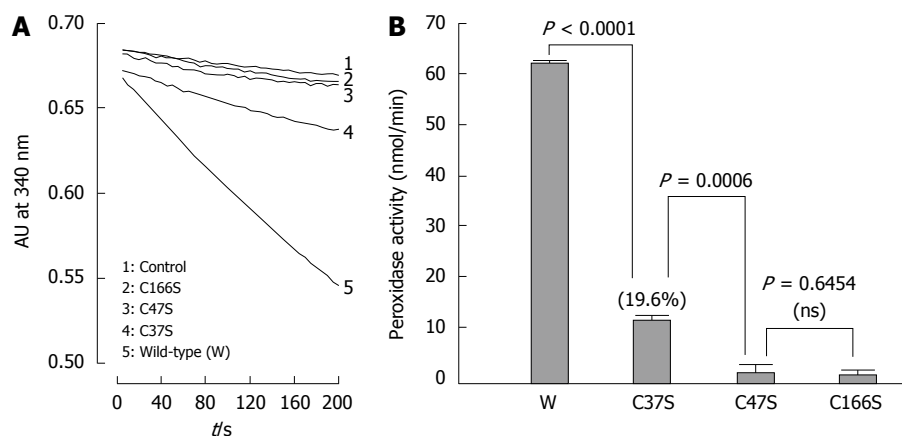
**Figure 7** Susceptibility of AhpC\_H1 with AhpC\_H2 to inactivation by hydrogen peroxide. Peroxidase activity of AhpC\_H1 (A) and AhpC\_H2 (B) was continuously monitored by the decrease in 340-nm absorbance due to NADPH oxidation at 25 °C in the presence of 50 mmol/L of HEPES-NaOH buffer, pH 7.4, containing 1 mmol/L EDTA, 100 nmol/L of *E. coli* Trx reductase, 0.14 mmol/L of NADPH, 5 μmol/L of *E. coli* Trx, 2 μmol/L of AhpC\_H1 and AhpC\_H2 and varying concentrations of H<sub>2</sub>O<sub>2</sub>. Control, absorbance without H<sub>2</sub>O<sub>2</sub>; C: Changes in initial peroxidase activity rate depending on H<sub>2</sub>O<sub>2</sub> concentration. AhpC\_H1 was resistant, while AhpC\_H2 was sensitive to inactivation by H<sub>2</sub>O<sub>2</sub>.

by western blotting analysis using anti-AhpC\_H1 antibodies (Figure 9B, W/2D Western, b). Taken together, these results suggest that the expression of AhpC\_H1 in *B. subtilis* is much higher than that of B\_BCP, B\_TPx, and AhpC\_H2.

### Hyperoxidation of AhpC\_H1

In oxidative stress-exposed *B. subtilis*, the position of the AhpC\_H1-corresponding spot at a more acidic area (Figure 9A, W+CMOOH, b) suggests that Cys residue(s) could be hyperoxidized to Cys-SO<sub>2</sub>H or Cys-SO<sub>3</sub>H<sup>[34]</sup>. To further investigate AhpC\_H1 response to hyperoxidation, exponentially growing *B. subtilis* was treated with increasing concentrations

of CMOOH, and protein migration was analyzed by 2D electrophoresis. Figure 9C shows that CMOOH treatment stimulated, at a concentration-dependent manner, the appearance of two different protein spots (b and c) at more acidic positions; at the same time, the original protein spot (a) gradually disappeared, while spot c increased. Considering that sulfinic acid (Cys-SO<sub>2</sub>H) is more negatively charged than sulfenic acid (Cys-SO<sub>2</sub>H), spots b and c seem to correspond to the sulfenic and sulfinic forms of AhpC\_H1. The predominant appearance of the more acidic AhpC\_H1 form in the oxidative stress-exposed *B. subtilis* might be considered as evidence that Cys166 residue is the primary site for hyperoxidation, provided that only



**Figure 8** Trx system-supported alkyl hydroperoxide peroxidase activity of the wild-type and mutant AhpC\_H1 proteins. A: Peroxidase reaction was initiated by adding 2  $\mu\text{mol/L}$  of the wild-type (W) or C37S, C47S, and C166S mutated AhpC\_H1 enzymes to the reaction mixture containing 50 mmol/L Hepes-NaOH, pH 7.4, 1 mmol/L EDTA, 100 nmol/L of *E. coli* Trx reductase, 5  $\mu\text{mol/L}$  of *E. coli* Trx, and 0.14 mM NADPH; the activity was continuously monitored by the decrease in 340-nm absorbance at 25  $^{\circ}\text{C}$ ; B: Initial peroxidase activity rate of the wild-type and mutant AhpC\_H1. The data are expressed as the mean  $\pm$  standard error of three independent measurements.

Cys166 is reduced among the three Cys residues of AhpC\_H1.

#### AhpC\_H1 induction in *B. subtilis* exposed to alkyl hydroperoxides

AhpC\_H1 expression in *B. subtilis* in response to oxidative stress was analyzed in exponentially growing cultures treated with different concentrations of  $\text{H}_2\text{O}_2$ , *t*-butyl hydroperoxide (tBOOH), CMOOH, paraquat (PQ), and diamide for 45 min. Western blotting analysis (Figure 10) indicates that AhpC\_H1 levels were not significantly affected by  $\text{H}_2\text{O}_2$ , paraquat, and diamide; however, organic peroxides, especially CMOOH, markedly increased AhpC\_H1 expression in *B. subtilis*, suggesting the function of AhpC\_H1 as an antioxidant scavenger of lipid hydroperoxides.

#### Phenotype of the AhpC\_H1-deficient mutant strain

To determine functional significance of four *Bacillus* Prxs, we compared the growth of the  $\Delta\text{AhpC\_H1}$ ,  $\Delta\text{AhpC\_H2}$ ,  $\Delta\text{B\_BCP}$ , and  $\Delta\text{B\_TPx}$  deletion mutants with that of the parental wild-type strain. All of the mutants exhibited gradual reduction of cell density in the stationary phase (Figure 11A), which could be caused by a decrease of cell viability. Figure 11B shows that among the four null mutants,  $\Delta\text{AhpC\_H1}$  exhibited better survival in the stationary phase, despite much higher levels of AhpC\_H1 expression in the wild-type *B. subtilis* compared to other Prxs.

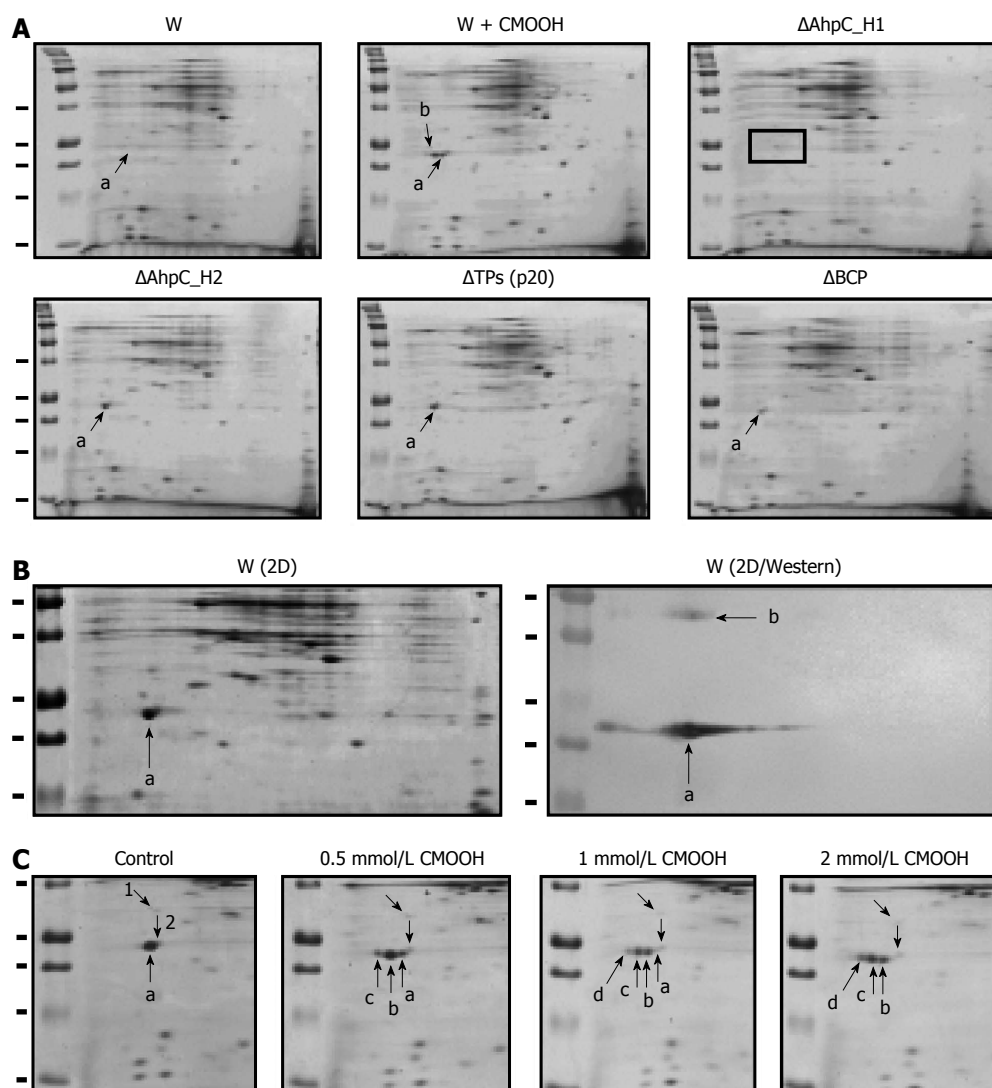
## DISCUSSION

Sequence homology and phylogenetic analysis performed in this study reveal that AhpC\_H1 is closely related to *E. coli* AhpC (E\_AhpC), a typical representative of bacterial AhpC, suggesting that AhpC\_H1 may be an E\_AhpC ortholog. AhpC\_H1 has the highest sequence similarity to E\_AhpC among the four analyzed *B. subtilis*

Prxs (65.24% identity in a 187-amino acid region), although it possesses an extra Cys37 in addition to two conserved residues, Cys47 and Cys166. AhpC\_H2, another AhpC enzyme in *B. subtilis*, showed 38.4% identity with E\_AhpC in a 159-amino acid sequence. Our results suggest that unlike AhpC\_H1, AhpC\_H2 is a member of the typical 2-Cys AhpC/Prx1 subfamily, which includes *E. coli* alkyl hydroperoxide reductase. Comparative structural analysis and evaluation of peroxidase activity, resistance to inactivation by peroxide substrates, and phenotypes of null mutants have demonstrated that AhpC\_H1 is a new type of bacterial AhpC, with the structure and functional activity distinct from those of AhpC\_H2. Thus, our data indicate that AhpC\_H2, not AhpC\_H1, is an ortholog of *E. coli* AhpC. AhpC\_H1 (GenBank accession number BAA11268) was first suggested to be an AhpC subunit of the *B. subtilis* alkyl hydroperoxide reductase induced in response to stressful conditions such as heat and high salinity or growth inhibition at the stationary phase<sup>[35-37]</sup>. We found a gradual decrease in the viability of Prx-deficient mutant strains in the stationary phase, which is consistent with previous reports, suggesting a functional role of these enzymes in *B. subtilis* stress response during the stationary growth phase. Together with AhpC\_H1, other three *B. subtilis* Prxs are likely to be involved in bacterial defense system against toxic conditions created by increased oxidative stress<sup>[38]</sup>.

Bacterial AhpC enzymes are known as electron acceptors from the Trx system, although they show much higher reactivity with their specialized flavoprotein reductase AhpF<sup>[9]</sup>. AhpC proteins from *Mycobacterium tuberculosis*<sup>[39]</sup> and *Helicobacter pylori*<sup>[40]</sup> have been identified as Trx-dependent alkyl hydroperoxide reductases. In this study, we observed that, regardless of the nature of disulfide linkages (intra- or intermolecular), all four *B. subtilis* Prxs could be easily reduced by the Trx system consisting of Trx1, Trx reductase, and NADPH,



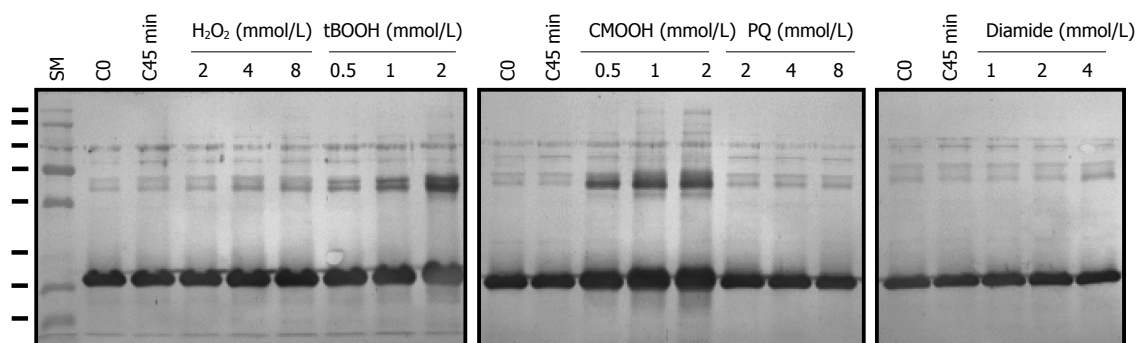


**Figure 9** Expression of peroxiredoxins in the wild-type and Prx-deficient *B. subtilis* strains. A: Two-dimensional (2D) electrophoresis of soluble proteins from untreated (W) and cumene hydroperoxide (CMOOH)-treated (W + CMOOH) wild-type *B. subtilis*, and Prx-null mutant strains. Soluble protein fraction (20  $\mu\text{g}$ ) obtained from exponential *B. subtilis* cultures treated with or without 1 mM CMOOH for 30 min was separated in 2D gels and analyzed for AhpC\_H1 expression after staining with Coomassie Brilliant Blue. a indicates AhpC\_H1; b indicates AhpC\_H1 shift to acidic area; in AhpC\_H1 deletion mutant ( $\Delta\text{AhpC\_H1}$ ), AhpC\_H1-specific signal is absent; B: *B. subtilis* was grown and treated as in (A), and soluble proteins (1  $\mu\text{g}$ ) were separated by 2D electrophoresis and stained with Coomassie Brilliant Blue (W/2D) or analyzed by western blotting using polyclonal antibodies against AhpC\_H1 (2D/Western); a and b indicate the position of AhpC\_H1 monomeric and dimeric forms, respectively; C: *B. subtilis* cultures were grown as in (A), treated with the indicated concentrations of CMOOH for 30 min, and soluble proteins were separated by 2D electrophoresis followed by Coomassie Brilliant Blue staining. a, indicates original and b, c, and d indicate oxidized AhpC\_H1 proteins; 1 and 2 denote internal standards. 2D electrophoresis was performed using 4–7 pH gradients (left to right); molecular weight markers (50, 37, 25, 20, and 15 kDa) are shown in the left lines.

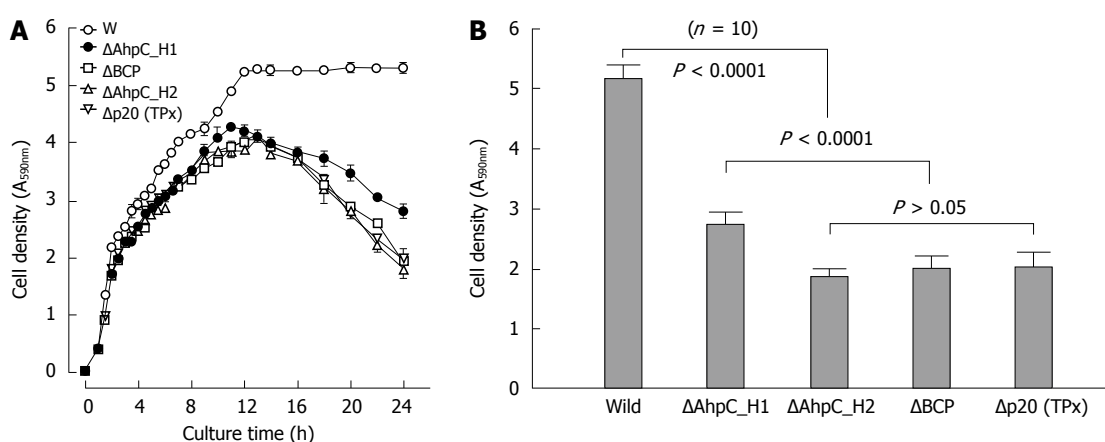
although in the oxidized AhpC\_H1, the intramolecular disulfide bond was formed between Cys37 and Cys47 and not between the conserved Cys47 and Cys166 residues. Conversion of the reduced AhpC\_H1 from a dimeric form to a high-molecular mass oligomer raises a possibility that the disulfide bond in AhpC\_H1 has a more complex role as a functional motif, which promotes conformational transitions unrelated to disulfide linkage. In the case of typical 2-Cys Prx members belonging to the AhpC/Prx1 subfamily, hyperoxidation of the conserved Cys residue(s) has been known as a covalent modification, which stabilizes higher order structures<sup>[33,41]</sup>. We have also demonstrated the existence of the hyperoxidized AhpC\_H1 as a predominant form in oxidative-stress-exposed *B. subtilis*. Based on the

observation of oxidation-induced dimerization (Figure 6B), we could speculate that the reduction of the disulfide bond between Cys37 and Cys47 may be the first step in the oligomerization process. Covalent modification of Cys residues could stabilize higher order structures as is the case with typical 2-Cys Prx members that lack the intramolecular disulfide bond.

Similar to AhpC\_H1, *M. tuberculosis* AhpC has been reported to possess three Cys residues, two at positions 174 and 176 in the C-terminal region, and the conserved Cys61 at the N-terminus. Both Cys174 and Cys176 can form an intermolecular disulfide bond with the conserved N-terminal Cys61 during cyclic catalysis, and all three Cys residues are shown to be important for enzymatic activity<sup>[42]</sup>. In AhpC\_H1, the



**Figure 10** Effects of various oxidants on AhpC\_H1 expression in *B. subtilis*. Exponentially grown bacterial cultures were treated with indicated concentrations of H<sub>2</sub>O<sub>2</sub>, t-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CMOOH), paraquat (PQ), and diamide for 45 min at 30 °C. Soluble proteins (5 µg) were separated in 14% reducing SDS-PAGE gels, transferred to nitrocellulose membrane, and reacted with the polyclonal AhpC\_H1 antibody. C0 and C45min indicate untreated *B. subtilis* at time 0 and after 45-min growth, respectively. SM, molecular weight markers (15, 20, 25, 37, 50, 75, 100, and 150 kDa).



**Figure 11** Growth of the wild-type and Prx-deficient  $\Delta$ AhpC\_H1,  $\Delta$ AhpC\_H2,  $\Delta$ BBCP, and  $\Delta$ BTPx *B. subtilis* strains. A: Bacterial growth was monitored by the optical density at 600 nm (OD<sub>600</sub>) for up to 25 h after addition of the same cell density of exponentially grown culture in aerobic condition; the data are expressed as the mean  $\pm$  standard error of five independent measurements; B: The growth of the wild-type and Prx-deficient *B. subtilis* strains after 1 d of culture. The data are expressed as the mean  $\pm$  standard deviation of 10 independent measurements.

additional Cys37 was also observed to be involved in peroxidation catalysis. Distinct catalytic activities of AhpC\_H1 and AhpC\_H2 toward H<sub>2</sub>O<sub>2</sub> (Figure 7C) may provide a rationale for the existence of two different AhpC enzymes in *B. subtilis*, which may function at different concentrations of H<sub>2</sub>O<sub>2</sub>.

Peroxiredoxins, a class of ubiquitously expressed cysteine-dependent peroxidases, serve not only as ROS detoxifiers but also as regulators of signal transduction pathways and peroxide responses. Thus, an intriguing feature of AhpC\_H1 is redox-dependent oligomerization, which regulates enzyme activity. Other functional consequences of this dimer-oligomer conversion remain to be solved, but we hypothesize that the reduction of the intra-subunit disulfide linkage between Cys37 and Cys47 by the Trx system or other electron donors serves to stabilize the oligomeric structure, and further regulates some physiological processes. Given that AhpC\_H1 is the most abundant protein among the four types of *B. subtilis* Prxs, the detailed *in vivo* function of AhpC\_H1 is worth further investigation.

In summary, structural and functional differences

between AhpC\_H1 and AhpC\_H2 revealed in this study clearly demonstrate that AhpC\_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC. The present data also suggest that AhpC\_H2, as a typical 2-Cys enzyme, is a true member of the AhpC/Prx1 subfamily, although AhpC\_H1 has the highest sequence similarity to *E. coli* AhpC among the four *B. subtilis* Prxs.

## COMMENTS

### Background

Peroxiredoxins (Prxs) are versatile and predominantly thiol-dependent peroxidases that detoxify hydrogen peroxide and various hydroperoxides. They are ubiquitously expressed, with multiple isoforms present in most organisms. Prxs exhibit high reactivity with hydrogen peroxide and organic hydroperoxides and are involved in peroxide detoxification and signal transduction. The present comparative study was designed to characterize two alkyl hydroperoxide reductase (AhpC) homologs and reveal the biological significance of the existence of two homologous AhpC enzymes in *B. subtilis*.

### Research frontiers

This paper is the first report of a detailed structure-functional analysis of the bacillus 2-Cys AhpC homologs AhpC\_H1 and AhpC\_H2.

## Innovations and breakthroughs

AhpC\_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC, and AhpC\_H2, as a typical 2-Cys enzyme, is a true member of the AhpC/Prx1 subfamily, although AhpC\_H1 has the highest sequence similarity to *E. coli* AhpC among the four *B. subtilis* Prxs.

## Applications

Peroxiredoxins, a class of ubiquitously expressed cysteine-dependent peroxidases, serve not only as ROS detoxifiers but also as regulators of signal transduction pathways and peroxide responses. Thus, an intriguing feature of AhpC\_H1 is redox-dependent oligomerization, which regulates enzyme activity. The reduction of the intra-subunit disulfide linkage between Cys37 and Cys47 by the Trx system or other electron donors serves to stabilize the oligomeric structure, and further regulates some physiological processes. Given that AhpC\_H1 is the most abundant protein among the four types of *B. subtilis* Prxs, the detailed *in vivo* function of AhpC\_H1 is worth further investigation.

## Terminology

Peroxiredoxins (Prxs): a ubiquitous family of antioxidant enzymes that convert a harmful peroxide to water and oxygen. Peroxiredoxins are frequently referred to as alkyl hydroperoxide reductase (AhpC) in bacteria. Other names include thiol specific antioxidant (TSA). This family contains AhpC and TSA, as well as related proteins; Phylogenetic analysis: An analysis of relationships between collection of genes and proteins that are derived from a common ancestor; Orthologous proteins: any proteins found in two or more species that can be traced to a common ancestor; specif. one of two homologous genes that is descended from a common ancestor, but which has evolved in a different way; Homologous proteins: proteins having a very similar primary, secondary, and tertiary structure.

## Peer-review

The paper examines the two proteins in an attempt to discover the function of these two homologous enzymes in the same organism, both of which are postulated to protect against oxidative damage in cells. These two proteins were initially identified by sequence homologies, cloned, expressed, and purified. The authors have also generated a number of site-directed mutants of the proteins and examined the role of Cys residues in the proteins. Overall, the work in the manuscript carefully examines both the biochemistry and genetics of the AhpC H1 and H2 proteins. AhpC H1 levels increase in response to alkyl peroxides. The authors have indicated that at least AhpC H1 protects against oxidative stress. It appears that there is a significant drop in cell density. Using the mutant proteins and complementation with the AhpC H1 or H2 would have provided further proof of the role of these proteins. Also, showing actual survival of the cells in response to alkyl peroxides or hydrogen peroxide would have been useful.

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