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ORIGINAL PAPER

Roles of Mn-catalase and a possible heme peroxidase homologue in protection from oxidative stress in *Thermus thermophilus*

Akio Ebihara[1] · Miho Manzoku[2] · Kenji Fukui[2,3] · Atsuhiro Shimada[2,4] · Rihito Morita[2,5] · Ryoji Masui[2,6] · Seiki Kuramitsu[2]

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Abstract Hydrogen peroxide (H_2O_2) produces hydroxyl radicals that directly attack a variety of biomolecules and cause severe cellular dysfunction. An extremely thermophilic bacterium, *Thermus thermophilus* HB8, possesses at least three enzymes that can scavenge H_2O_2: manganese-containing catalase (TTHA0122, MnCAT), a possible peroxiredoxin homologue (TTHA1300), and a possible heme peroxidase (HPX) homologue (TTHA1714). To investigate the roles of these proteins, we attempted to disrupt each of these genes in *T. thermophilus* HB8. Although we were able to completely disrupt *ttha1300,* we were unable to completely delete *ttha0122* and *ttha1714* because of polyploidy. Quantitative real-time PCR showed that, compared to the wild type, 31 % of *ttha0122* and 11 % of *ttha1714* remained in the Δ*ttha0122* and Δ*ttha1714* disruption mutants, respectively. Mutants with reduced levels of *ttha0122* or *ttha1714* exhibited a significant increase in spontaneous mutation frequency. Δ*ttha1714* grew slower than the wild type under normal conditions. Δ*ttha0122* grew very poorly after exposure to H_2O_2. Moreover, Δ*ttha0122* did not show H_2O_2-scavenging activity, whereas Δ*ttha1300* and Δ*ttha1714* scavenged H_2O_2, a property similar to that exhibited by the wild type. MnCAT purified from *T. thermophilus* HB8 cells scavenged H_2O_2 in vitro. The recombinant form of the possible HPX homologue, reconstituted with hemin, showed peroxidase activity with H_2O_2 as an oxidant substrate. Based on these results, we propose that not only MnCAT but also the possible HPX homologue is involved in protecting the cell from oxidative stress in *T. thermophilus*.

Keywords *Thermus thermophilus* · Hydrogen peroxide · Oxidative stress · Manganese-containing catalase · Peroxiredoxin · Heme peroxidase

Abbreviations

ROS	Reactive oxygen species
MnCAT	Manganese-containing catalase
HPX	Heme peroxidase

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✉ Seiki Kuramitsu
kuramitu@bio.sci.osaka-u.ac.jp

Akio Ebihara
aebihara@gifu-u.ac.jp

[1] Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

[2] Department of Biological Sciences, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

[3] Department of Biochemistry, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan

[4] Picobiology Institute, Graduate School of Life Science, University of Hyogo, 3-2-1 Kouto, Kamigouri, Akou-gun, Hyogo 678-1297, Japan

[5] Department of Biological and Chemical Systems Engineering, Kumamoto College, National Institute of Technology, 2627 Hirayamashinmachi, Yatsushiro, Kumamoto 866-8501, Japan

[6] Department of Biology, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka-shi 558-8585, Japan

HTK	Thermostable kanamycin-resistance gene
HRP	Horseradish peroxidase
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
MES	2-morpholinoethanesulfonic acid
MOPS	3-(*N*-morpholino)propanesulfonic acid
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Introduction

Molecular oxygen gives rise to reactive oxygen species (ROS) as inevitable byproducts of aerobic metabolism. The initial products in ROS formation are superoxide anions, which strongly oxidize iron-sulfur clusters in proteins (Imlay 2003, 2008). In an aerobic organism, superoxide dismutase eliminates superoxide but produces another ROS, hydrogen peroxide (H_2O_2). H_2O_2 has the lowest reactivity and the highest stability among the biologically relevant ROS (Giorgio et al. 2007); however, when ferrous iron transfers an electron to H_2O_2 (the Fenton-reaction), it produces a hydroxyl radical. This radical is the only ROS that can directly damage most biomolecules (Imlay 2003, 2008). For example, it attacks DNA to generate oxidatively damaged bases such as 8-oxoguanine (Imlay 2008; Morita et al. 2010). These DNA lesions are toxic and mutagenic. To protect itself from oxidative stress due to ROS, an aerobic organism possesses fundamental enzymes for detoxifying ROS (Mishra and Imlay 2012) and repairing oxidized DNA (Imlay 2008; Morita et al. 2010).

There are multiple H_2O_2-scavenging enzymes in an aerobic organism (Mishra and Imlay 2012). Seaver and Imlay analyzed the phenotypes of *E. coli* mutants lacking scavenging enzymes and proposed that alkyl hydroperoxide reductase scavenges low levels of H_2O_2, whereas catalases scavenge high levels (Mishra and Imlay 2012; Seaver and Imlay 2001a). As is the case in *E. coli*, scavenging enzymes in each organism are thought to play different roles in coping with the oxidative stress caused by H_2O_2.

The extremely thermophilic bacterium, *Thermus thermophilus* HB8, is an aerobic bacterium (Oshima and Imahori 1974). This organism has a relatively small genome size (2.2 Mbp), and its biological system is thought to consist of minimum essential proteins (Yokoyama et al. 2000). *T. thermophilus* HB8 is thought to have at least three enzymes that can scavenge H_2O_2. The first protein, TTHA0122, is a manganese-containing catalase (MnCAT). MnCAT converts H_2O_2 into water and oxygen and is different from other heme-containing catalases in that it has a di-manganese center in the active site (Chelikani et al. 2004; Whittaker 2012). The second protein, TTHA1300, is a possible peroxiredoxin homologue. Peroxiredoxin reduces H_2O_2 into water through its active site cysteine (Wood et al. 2003). The resulting sulfenic acid is recycled to a thiol by a cellular reducing system such as the NADPH-dependent thioredoxin system (Wood et al. 2003). The third protein, TTHA1714, is a possible heme peroxidase (HPX) homologue (Ebihara et al. 2005). TTHA1714 was originally annotated as a conserved hypothetical protein. Based on its crystal structure (PDB ID: 1VDH), we showed that TTHA1714 has a heme-binding site with an Fe-His-Asp triad. This is a common feature of HPX proteins and, therefore, we proposed it as a possible HPX homologue (Ebihara et al. 2005). This set of homologous proteins has a highly conserved tertiary structure containing a heme-binding site and is classified in the dye-decolorizing peroxidase family (Zubieta et al. 2007a, b). Although the aforementioned three proteins in *T. thermophilus* HB8 are thought to be involved in protection from H_2O_2-induced oxidative stress, their contribution to the cellular protection system is poorly understood.

In this study, we attempted to disrupt *ttha0122*, *ttha1300*, or *ttha1714* in *T. thermophilus* HB8 to investigate the effect on spontaneous mutation frequency and sensitivity to oxidative stress caused by H_2O_2. We showed that not only MnCAT but also the possible HPX homologue, TTHA1714, has a protective role against oxidative stress in *T. thermophilus*.

Materials and methods

Disruption of the *ttha0122*, *ttha1300*, and *ttha1714* genes

The *ttha0122*, *ttha1300*, and *ttha1714* genes were disrupted in *T. thermophilus* HB8 by substituting the target gene with the thermostable kanamycin resistance gene, *HTK* (Hoseki et al. 1999), through homologous recombination, as previously described (Hashimoto et al. 2001). The plasmids for homologous recombination were constructed by inserting *HTK,* flanked by approximately 500 bp upstream and downstream of each gene, into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The 500-bp DNA fragments from upstream of each gene were amplified by PCR using the primer sets, 5′-CCGGGGTGAGCTCCTCCCGCACCGAGAGGG-3′ and 5′-CGCCGTCAACGGGTACCGCGGTCTATCCTC-3′, 5′-GCCACTACACCCCCATGCTCAAGTTCGCCC-3′ and 5′-CGCCGTCAACGTCTAGAGTGCCCACTTCCA-3′, and 5′-GTTGAGGCCGAGGTTGGAGAGGAAGAGGAG-3′ and 5′-CGCCGTCAACGGGTACCTTCGGGAACGTGC-3′ (BEX, Tokyo, Japan) for *ttha0122*, *ttha1300*, and *ttha1714*,

respectively. The 500-bp DNA fragments from downstream of each gene were amplified by PCR using primer sets, 5′-TGTTGGTTACGCTGCATCTCTACGAGAAGG-3′ and 5′-ACGGATGGACCTCCTCTCCGAGTTCCCCTT-3′, 5′-TGTTGGTTACGCTGCAGTGAAGGCGCTCAG-3′ and 5′-GTCCCCGTCCATGCGGAGCTCGGGCTCCCC-3′, and 5′-CATGTTGGTTACGCTGCAGCTGCGGGCCTT-3′ and 5′-GCCAAAGAAAAGGACCAGCCAGCGCACCAG-3′ (BEX) for *ttha0122*, *ttha1300*, and *ttha1714*, respectively. To produce the *ttha0122*, *ttha1300,* and *ttha1714* mutants (Δ*ttha0122,* Δ*ttha1300,* and Δ*ttha1714*), *T. thermophilus* HB8 cells were transformed with the above plasmids, as previously described (Hashimoto et al. 2001). Gene disruptions were confirmed by PCR amplification, using isolated genomic DNA as the template.

Quantitative real-time PCR

Approximately 25 μL of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was mixed with an equal volume of a solution containing 5, 2.5, 0.25, 0.05, or 0.01 ng/μL template of genomic DNA and 200 nM forward and reverse primers. It was then subjected to real-time PCR using a 7300 Real-Time PCR system (Applied Biosystems). The forward and reverse primer sets used for *ttha0122* and *ttha1714* amplification were 5′-ATGTTCCTGAGGATAGACCGCC-3′ and 5′-ATCTCCACCCCGGTGAGGC-3′, and 5′-ATGGAGCGGCACGTTCCC-3′ and 5′-GGGAACGTGCCGCTCCAT-3′ (BEX), respectively. Melting curve analysis confirmed specific amplification from the genomic DNA with each primer set (data not shown). Data were analyzed as previously described (Cao et al. 2010; Pfaffl 2001). The *ttha1934* gene, which is present in equal amounts in the wild type and mutants, was used as a reference gene. The efficiency (E) is given by:

$$10^{(-1/\text{slope})},$$

where slope is the slope of the plot of log(dilution) vs. threshold cycle number (C_t). The determined efficiencies were $E_{(ttha0122)} = 1.59$, $E_{(ttha1714)} = 1.16$, and $E_{(ttha1934)} = 1.98$. The relative copy number of the gene (*ttha0122* or *ttha1714*) in the mutant cell compared to the wild type is given by

$$\left(E_{(gene)}\right)^{\Delta Ct(gene)} / \left(E_{(ttha1934)}\right)^{\Delta Ct(ttha1934)},$$

where $\Delta C_{t(gene)}$ is [$C_{t(gene)}$ for wild type − $C_{t(gene)}$ for mutant].

Estimation of spontaneous mutation frequency

The mutation frequency of *T. thermophilus* HB8 was estimated based on how frequently streptomycin-resistant mutants occurred, as calculated from the means of the modified Luria-Delbrück fluctuation test (Luria and Delbruck 1943). Pre-cultured wild type, Δ*ttha0122*, Δ*ttha1300*, and Δ*ttha1714* strains of *T. thermophilus* HB8 were diluted 1:60 with 3 mL of TR medium (0.8 % polypeptone, 0.4 % yeast extract, 0.2 % NaCl; pH 7.2) and shaken at 70 °C for 2 h. After addition of 30 μL of 0 or 250 mM H_2O_2, cells were cultured at 70 °C for 5 h. One milliliter of each culture was spread on a plate containing 50 μg/mL streptomycin. Subsequently, the same cultures were diluted 1:10^5 with TR medium, and 100 μL of each diluted culture was spread on a drug-free plate. The plates were incubated at 70 °C for 20 h. The frequency of streptomycin-resistant mutants per 10^8 cells was calculated from the numbers of colonies formed on the streptomycin-containing and drug-free plates. The Mann–Whitney test was performed to statistically evaluate the results.

Growth of the mutants and measurement of sensitivity to oxidative stress

The wild type, Δ*ttha0122,* Δ*ttha1300,* and Δ*ttha1714* strains of *T. thermophilus* HB8 were pre-cultured aerobically overnight at 70 °C in 4 mL of TT medium [0.8 % polypeptone, 0.4 % yeast extract, 0.2 % NaCl, 0.4 mM $CaCl_2$, 0.4 mM $MgCl_2$; pH 7.2 (Hashimoto et al. 2001)]. Then, 255 mL of fresh TT medium was inoculated with 0.5–1 mL of the pre-culture, and the cells were cultivated aerobically at 70 °C. The growth of the cells was monitored by measuring the absorbance at 600 nm (OD600) at various times during the culture.

To examine the sensitivity to H_2O_2, H_2O_2 was added to the culture medium at a final concentration of 10 mM at the mid-log phase, and OD600 was measured again to monitor the growth of *T. thermophilus* HB8.

Estimation of scavenging capability of the mutants

At the mid-log phase, H_2O_2 was added to a culture medium at a final concentration of 10 mM. A small volume of the culture medium was taken at 0, 1, 3, 5, 7, and 15 min after the addition, and the cells were immediately removed from the culture medium by filtration with a 0.22-μm cutoff filter and an aspirator. The concentration of residual H_2O_2 in the filtrate was measured by a horseradish peroxidase (HRP) enzyme assay with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate. Approximately 100 μL of the diluted cell filtrate was added to 10 μL of 10 mM ABTS, 50 μL of 200 mM potassium phosphate (pH 7.0), and 20 μL of water. Subsequently, 20 μL of 2.5 μg/mL HRP was added to the solution. The absorbance at 414 nm was measured using a SpectraMax 190 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

The absorbance was converted to H_2O_2 concentration using a standard curve of H_2O_2 concentration (0–75 μM).

Purification of MnCAT from *T. thermophilus* HB8

T. thermophilus HB8 cells were grown in a culture medium containing polypeptone, yeast extract, and NaCl. Approximately 100 g of frozen *T. thermophilus* cells was suspended and disrupted by sonication in 500 mL of Buffer A (20 mM Tris–HCl, 100 mM NaCl, 10 mM EDTA, and 10 mM beta-mercaptoethanol; pH 8.0). The cell lysate was ultracentrifuged (200,000×*g*) for 30 min at 4 °C. Ammonium sulfate was added to the clear supernatant in a stepwise manner to produce 30, 45, 60, and 75 % saturation. After stirring for an hour, the precipitate was recovered by centrifugation at each stage. The supernatant containing 75 % ammonium sulfate was left overnight and the resulting precipitate was collected by centrifugation. Each precipitate was dissolved in Buffer B (20 mM Tris–HCl, 1 mM EDTA, and 10 mM beta-mercaptoethanol; pH 8.0). The five protein fractions were separately dialyzed overnight against Buffer B. To find the MnCAT-containing fraction, H_2O_2-scavenging activity was measured using a ferric-xylenol orange method (Gay et al. 1999). A 1:1000 diluted solution of each fraction was incubated at room temperature for 5 min in 50 mM potassium phosphate (pH 7.0) containing 41 μM H_2O_2. The reaction was stopped by adding H_2SO_4. The resulting solution was mixed at a 1:1 ratio with a solution containing 0.25 mM xylenol orange, 0.1 mM ferrous ammonium sulfate, and 25 mM H_2SO_4. After 30 min, the absorbance at 560 nm was measured to determine the H_2O_2-scavenging activity. Three fractions with activity (45–60, 60–75, and 75 % overnight) were separately loaded onto a TOYOPEARL SuperQ-650 M column (Tosoh Bioscience, Tokyo, Japan) equilibrated with Buffer B and eluted with a linear gradient of 0–1 M NaCl. H_2O_2-scavenging activity was measured again to find the MnCAT-containing fractions. All the fractions with activity were pooled, desalted into Buffer B, and loaded to a Resource Q column (GE Healthcare, Buckinghamshire, England) equilibrated with Buffer B. Proteins were eluted with a linear gradient of 0–0.25 M NaCl. The MnCAT-containing fractions were pooled, desalted into 10 mM sodium phosphate buffer, 150 mM NaCl, 1 mM EDTA, and 10 mM beta-mercaptoethanol (pH 7.0), and loaded onto a Bio-Scale CHT10-I column (Bio-Rad) equilibrated with the same buffer. The proteins were eluted with a linear gradient of 10–250 mM sodium phosphate. The MnCAT-containing fractions were pooled, desalted against 20 mM 2-morpholinoethanesulfonic acid (MES), 1 mM EDTA, and 10 mM beta-mercaptoethanol (pH 6.0), and loaded onto a RESOURCE S column (GE Healthcare) equilibrated with the same buffer. Proteins were eluted with a linear gradient of 0–0.25 M NaCl. The MnCAT-containing fractions were pooled and subjected to gel filtration on a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) equilibrated with 20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, and 10 mM beta-mercaptoethanol (pH 8.0). The MnCAT-containing preparation was polished on a Bio-Scale CHT5-I column (Bio-Rad Laboratories, Hercules, CA) with a linear gradient of 10–250 mM sodium phosphate, followed by desalting against 20 mM MOPS, 1 mM EDTA, and 10 mM beta-mercaptoethanol (pH 7.0) using a HiPrep 26/10 Desalting column (GE Healthcare). The protein concentration of MnCAT was determined with a molecular extinction coefficient at 280 nm (30,337 M^{-1} cm^{-1}) that is calculated according to the formula provided by Kuramitsu et al. (1990). The final concentration was 33 mg/mL. The proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and identified by peptide mass fingerprinting using MALDI-TOF MS (Ultraflex, Bruker Daltonics, Bremen, Germany).

Characterization of MnCAT

The metal content of MnCAT was determined by CIROS-160EOP inductively coupled plasma spectrometry (Rigaku, Tokyo, Japan). The H_2O_2-scavenging activity was measured using a SpectraMax 190 Microplate Spectrophotometer (Molecular Devices). MnCAT (50 nM) was incubated with various concentrations of H_2O_2 in 50 mM potassium phosphate (pH 7.0) at 25 °C. The decomposition of H_2O_2 was monitored at 240 nm ($\varepsilon_{240} = 43.6$ M^{-1} cm^{-1}) (Beers and Sizer 1952), and the steady-state kinetics of MnCAT were analyzed. The K_m and k_{cat} values were determined using the Lineweaver–Burk plot.

Preparation and characterization of possible HPX homologue

The recombinant form of the possible HPX homologue (TTHA1714) was expressed in *E. coli* BL21(DE3) and was produced as the apo-form (Ebihara et al. 2005). The holo-form of the possible HPX homologue was prepared in vitro by reconstitution of the intact preparation with hemin (Ebihara et al. 2005). Total protein concentration was determined using the bicinchoninic acid method (Smith et al. 1985). Heme concentration was determined by pyridine hemochrome analysis (Berry and Trumpower 1987). Peroxidase activity was measured at 25 °C using a U-3010 spectrophotometer (Hitachi, Tokyo, Japan) and a 1-cm quartz cuvette. Reaction mixtures contained 1 μM reconstituted possible HPX homologue, 2 mM reductant substrate (ABTS or guaiacol), 2 mM H_2O_2, and 50 mM potassium phosphate (pH 7.0). Oxidations of ABTS and guaiacol were determined from the increase in absorbance

at 730 nm ($\varepsilon_{730} = 1.4 \times 10^4$ M^{-1} cm^{-1}) and at 470 nm ($\varepsilon_{470} = 3.8 \times 10^3$ M^{-1} cm^{-1}), respectively (Matsui et al. 1999). The reaction was initiated by the addition of H_2O_2. Only the linear rate was used to calculate the activity. Intact possible HPX homologue (apo-form), hemin, myoglobin, and HRP were used as controls of peroxidase activity.

Results

Disruptions of the *ttha0122*, *ttha1300*, and *ttha1714* genes of *T. thermophilus* HB8

In order to test their H_2O_2-scavenging abilities, we tried to disrupt the *ttha0122*, *ttha1300,* and *ttha1714* genes of *T. thermophilus* HB8. Gene disruption was carried out by substituting the target gene with the thermostable kanamycin-resistant gene, *HTK* (Fig. 1a). Three cycles of gene disruption were carried out to obtain the disruption mutants. Ohtani et al. (2010) showed that *T. thermophilus* HB8 is a polyploid organism. Therefore, we examined the extent to which the gene was disrupted in the mutants obtained. Using standard PCR methods, a DNA fragment of *HTK* was amplified from the isolated genomic DNA of all mutants (Fig. 1b, lanes 3, 7, and 11), suggesting that the relevant gene was disrupted by homologous recombination, as expected. On the other hand, DNA fragments of *ttha0122* and *ttha1714* were amplified from the genomic DNA of the Δ*ttha0122* and Δ*ttha1714* strains, respectively (Fig. 1b, lanes 9 and 13). A DNA fragment of *ttha1300* was not amplified from the genomic DNA of Δ*ttha1300* strain (Fig. 1b, lane 5).

Characterization by PCR revealed that the genomes of the kanamycin-resistant mutants obtained by *ttha0122*- and *ttha1714*-gene disruption retained a certain number of wild type copies of *ttha0122* and *ttha1714*, respectively. In order to estimate the number of *ttha0122* and *ttha1714* genes remaining in the mutants, quantitative real-time PCR was performed. The *ttha1934* gene was used as a reference gene. Compared with the wild type, 31 % of *ttha0122* and 11 % of *ttha1714* were present in Δ*ttha0122* and Δ*ttha1714*, respectively (Table 1). Our results indicate that some, but not all, copies of *mncat* and *hpx* were disrupted in Δ*ttha0122* and Δ*ttha1714*, while all copies of *ttha1300* were disrupted in Δ*ttha1300*.

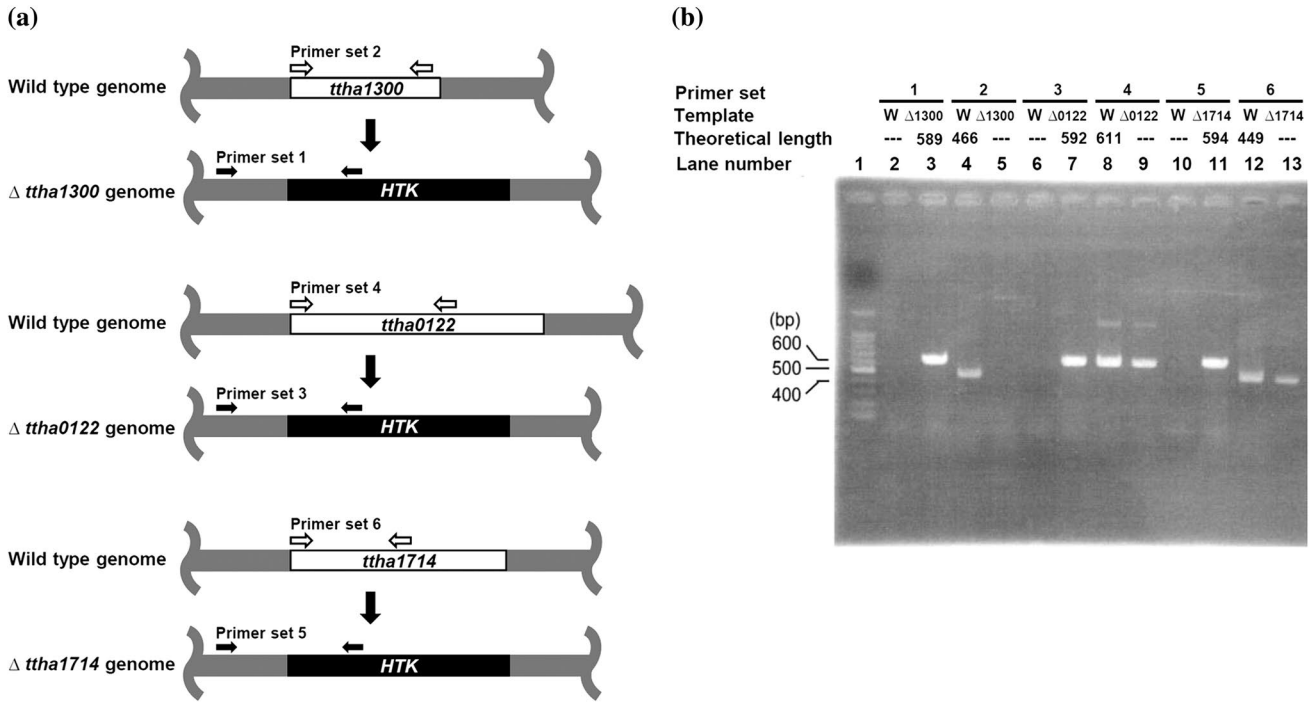

Fig. 1 Disruptions of *ttha1300*, *ttha0122*, and *ttha1714* examined by PCR. **a** A schematic representation of the amplified regions in wild type and mutants. *Arrows* represent primers used for PCR. Primer sets 1 and 2, 3 and 4, and 5 and 6 were used for examination of the *ttha1300*, *ttha0122*, and *ttha1714* disruptions, respectively. **b** Amplified DNA fragments were analyzed by agarose gel electrophoresis. PCR reactions contained genomic DNA isolated from the wild type (W), Δ*ttha1300* (Δ*1300*), Δ*ttha0122* (Δ*0122*), or Δ*ttha1714* (Δ*1714*) strains, as well as primer set 1 (*lanes 2* and *3*), 2 (*lanes 4* and *5*), 3 (*lanes 6* and *7*), 4 (*lanes 8* and *9*), 5 (*lanes 10* and *11*), and 6 (*lanes 12* and *13*). *Lane 1* DNA size marker. The lengths of the amplified fragments showed good concordance with the theoretical lengths of the targeted regions

Table 1 Analysis of Δ*ttha0122* and Δ*ttha1714* mutants by quantitative real-time PCR

Experiment #	Strain	$C^{a}_{t\,(gene)}$	$C^{a}_{t\,(ttha1934)}$	Relative copy number
1	Wild type	22.77	32.82	1.0
	Δ*ttha0122*	22.97	31.27	0.306
2	Wild type	30.26	32.82	1.0
	Δ*ttha1714*	33.61	30.76	0.110

[a] PCR cycle number at which fluorescence intensity reached a threshold value, using the disruption gene-specific or *ttha1934*-specific primers. Results are averages of triplicate determinations

Phenotypes of the mutants

To investigate whether each protein contributes to protection from oxidative stress, we measured the spontaneous mutation frequencies of Δ*ttha1300*, Δ*ttha0122*, and Δ*ttha1714* by measuring the spontaneous generation of streptomycin-resistant mutants. Streptomycin resistance can be acquired by single-base substitutions including AT-CG transversion and AT-GC transition mutations (Bonny et al. 1991). These mutations can be generated by oxidative DNA damage, such as the formation of 8-oxoguanine and 5-formyluracil (Wallace 2002). The wild type strain showed a similar mutation frequency under normal and oxidative stress conditions (Fig. 2). This result indicates that the H_2O_2-induced oxidative stress protection mechanism works wells in the wild type. Compared to the wild type, Δ*ttha1300* showed no significant increase in mutation frequency under either condition. On the other hand, Δ*ttha0122* and Δ*ttha1714* exhibited statistically significant increases in the mutation frequencies (approximately 8- and 6-fold higher, respectively) under normal conditions when compared to the wild type (Fig. 2). Moreover, these increases in mutation frequencies were more significant under oxidative stress conditions (Fig. 2). These results indicate that under normal conditions, MnCAT (TTHA1012) and a possible HPX homologue (TTHA1714) contribute to protection from oxidative DNA damage. Furthermore, the fact that Δ*ttha1300* did not show a significant increase in spontaneous mutation frequency (Fig. 2) indicates that this possible peroxiredoxin homologue (TTHA1300) does not significantly contribute to oxidative stress protection under our experimental conditions.

Next, we compared the growth curves of the wild type, Δ*ttha1300*, Δ*ttha0122,* and Δ*ttha1714* strains (Fig. 3). We observed that Δ*ttha1300* and Δ*ttha0122* grew at a rate similar to that of the wild type (Fig. 3b, c), while Δ*ttha1714* grew slower than the wild type, throughout the culture (Fig. 3d, filled circles). Notably, after a long culture period, the culture medium of Δ*ttha1714* turned red, compared to the wild type (Fig. 4).

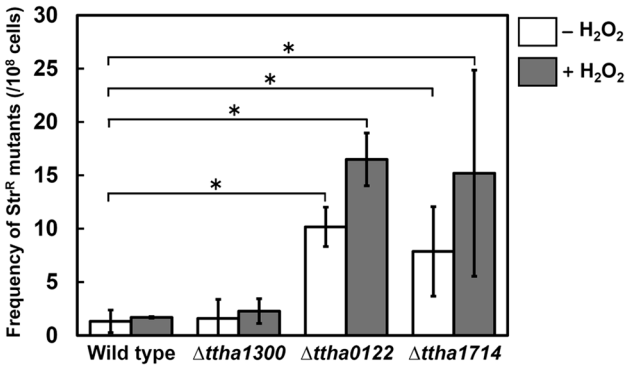

Fig. 2 Spontaneous mutation frequencies of the wild type, Δ*ttha1300*, Δ*ttha0122,* and Δ*ttha1714* strains. The spontaneous mutation frequencies were evaluated by measuring the frequency of streptomycin-resistant (Str^R) mutants, as described in the "Materials and methods". Normal conditions (without H_2O_2), *white bar*; oxidative stress conditions (with H_2O_2), *gray bar*. *Error bars* indicate the standard deviation of the results ($n = 3$ wild type, Δ*ttha1300*, Δ*ttha0122* strains analyzed; $n = 4$ Δ*ttha1714* strain analyzed). *$P = 0.05$ by Mann–Whitney test

To examine the sensitivity to H_2O_2-induced oxidative stress, we added H_2O_2 to the culture medium at the mid-log phase and monitored the cell growth. H_2O_2 rapidly diffuses across the cell membrane (Imlay 2008; Seaver and Imlay 2001b). Thus, when H_2O_2 is added to the culture medium, it can enter the cells and cause oxidative stress (Imlay 2008). When the wild type was treated with 5 mM H_2O_2, it grew at a rate similar to that of the untreated control (Supplemental Fig. S1). When the wild type was treated with 10 mM H_2O_2, it showed slight growth retardation and was not able to recover by the end of the culture period (Fig. 3a). These results indicate that the oxidative stress imposed by 10 mM H_2O_2 is large enough to cause a significant change in cell growth. After exposure to H_2O_2, Δ*ttha1300* and Δ*ttha1714* showed a growth defect similar to that shown by the wild type (Fig. 3b, d). On the other hand, Δ*ttha0122* grew very poorly under oxidative stress and exhibited the most significant growth defect of all the mutants (Fig. 3c). These results indicate that mutants with reduced levels of MnCAT are highly sensitive to H_2O_2-induced oxidative stress.

Since the cell membrane is permeable to H_2O_2 (Imlay 2008; Seaver and Imlay 2001b), H_2O_2 must be detoxified by enzymes within the cells. To extrapolate the H_2O_2-scavenging capability of the wild type and of each mutant, we measured the H_2O_2 concentration in the culture medium over time after addition of H_2O_2. Both Δ*ttha1300* and Δ*ttha1714* scavenged H_2O_2 as well as the wild type (Fig. 5). On the other hand, Δ*ttha0122* exhibited virtually no scavenging activity (Fig. 5, filled circles). H_2O_2 was not degraded in the culture medium that was not inoculated with *T. thermophilus* cells (Fig. 5, open squares), or in the filtrate where *T. thermophilus* cells were removed from the

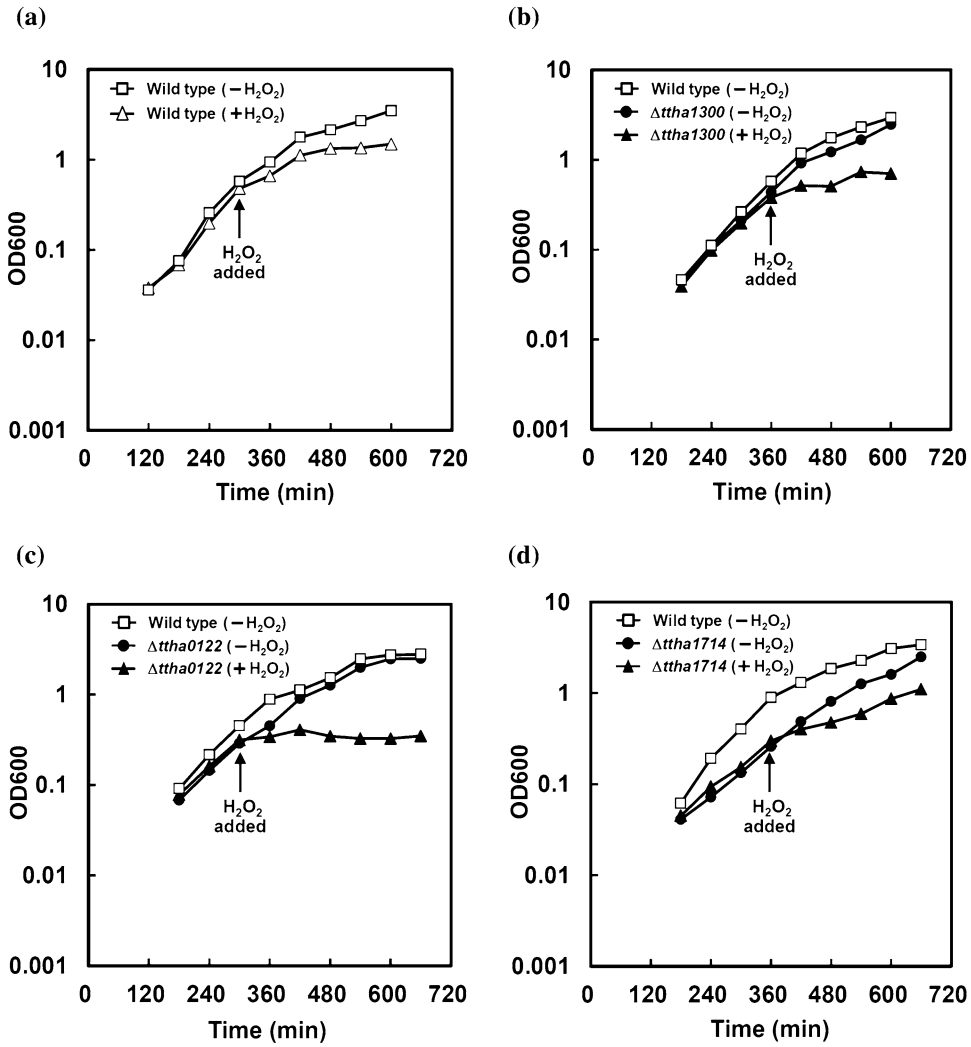

Fig. 3 Effects of gene disruption on cell growth and sensitivity to H_2O_2-induced oxidative stress. Growth curves of the wild type (**a**), Δ*ttha1300* (**b**), Δ*ttha0122* (**c**), and Δ*ttha1714* (**d**) strains were obtained by OD600 measurement. The wild type was cultured in parallel with each gene mutant. The wild type, grown under normal conditions (*open squares*); the wild type, grown with H_2O_2 treatment (*open triangles*), the gene mutant, grown under normal conditions (*filled circles*); the gene mutant, grown with H_2O_2 treatment (*filled triangles*). To impose oxidative stress, H_2O_2 was added at a final concentration of 10 mM at the mid-log phase (the time indicated by the *arrow*, 300 min for the wild type and Δ*ttha0122*; 360 min for Δ*ttha1300* and Δ*ttha1714*)

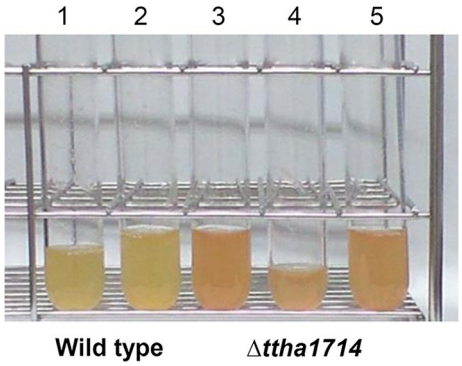

Fig. 4 Cultures of the wild type and Δ*ttha1714*. The wild type and Δ*ttha1714* were cultured aerobically overnight (12.5 h) at 70 °C in 4 mL TT medium. Cultures 1 and 2 are the wild type (duplicate). Cultures 3–5, Δ*ttha1714* (triplicate). A large amount of the culture medium (255 mL) of Δ*ttha1714* also became red

culture medium harvested at the mid-log phase (data not shown). These results indicate that MnCAT (TTHA0122) is a strong scavenger of H_2O_2 in *T. thermophilus* HB8. The presence of MnCAT in Δ*ttha1300* and Δ*ttha1714* is likely responsible for their scavenging capability.

Characterization of MnCAT purified from *T. thermophilus* HB8 cells

To confirm its H_2O_2-scavenging capability in vitro, we purified MnCAT from the *T. thermophilus* HB8 cells using the successive steps of ammonium sulfate fractionation, anion-exchange column chromatography, hydroxyapatite column chromatography, cation-exchange column chromatography, and gel filtration. A protein with H_2O_2-scavenging activity was purified to near homogeneity on SDS-PAGE (Fig. 6). The apparent molecular weight was 37×10^3. The major band was identified as MnCAT by peptide mass fingerprinting using MALDI-TOF MS. A gel filtration analysis gave an apparent molecular weight of 140×10^3 for the purified MnCAT. Inductively coupled plasma spectrometry of MnCAT preparation showed a Mn ion/MnCAT ratio of 0.52. The steady-state kinetics of this preparation was

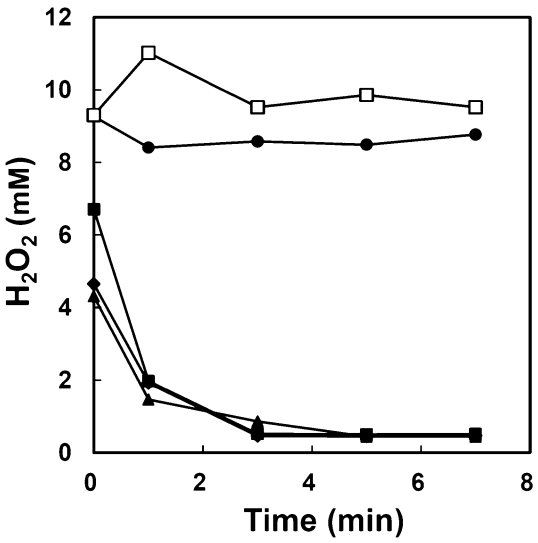

Fig. 5 H_2O_2-scavenging capability of the wild type and the mutants. A small volume of culture medium was taken at various time points after addition of H_2O_2 (final concentration, 10 mM), and the concentration of residual H_2O_2 in the culture filtrate was measured by an enzyme assay described in the "Materials and methods". Controls without inoculation (*open squares*); the wild type (*filled squares*); Δ*ttha1300* (*filled triangles*), Δ*ttha0122* (*filled circles*), and Δ*ttha1714* (*filled diamonds*)

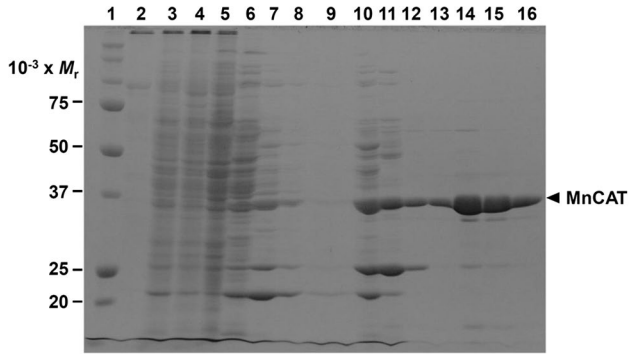

Fig. 6 SDS-PAGE analysis of MnCAT preparations from *T. thermophilus* HB8. The pooled fractions after each purification step were subjected to 12 % SDS-PAGE followed by Coomassie Brilliant Blue staining. *Lane 1* molecular weight marker, *lane 2* pellet of cell lysate, *lane 3* supernatant of cell lysate, *lane 4* 30 % ammonium sulfate fraction, *lane 5* 30–45 % ammonium sulfate fraction, *lane 6* 45–60 % ammonium sulfate fraction, *lane 7* 60–75 % ammonium sulfate fraction, *lane 8* 60–75 % ammonium sulfate fraction (left overnight), *lane 9* supernatant of the 75 % ammonium sulfate fraction, *lane 10* pooled fractions after TOYOPEARL SuperQ-650 M column chromatography, *lane 11* after Resource Q column chromatography, *lane 12* after Bio-Scale CHT10-I column chromatography, *lane 13* after Resource S column chromatography, *lane 14* after HiLoad 16/60 Superdex 200 pg column chromatography, *lane 15* after Bio-Scale CHT5-I column chromatography, and *lane 16* final preparation. Molecular weights of the marker proteins are shown on the *left*. *Arrowhead* shown on the *right* represents the size of MnCAT

performed using H_2O_2 as a substrate (Table 2). The K_m value of this preparation was similar to that of MnCAT in a previous study although the k_{cat} value was much lower (Shank et al. 1994). The k_{cat}/K_m of this preparation was 6.3×10^4 M^{-1} s^{-1} (Table 2). These results indicate that *T. thermophilus* possesses an intracellular protein, MnCAT, which scavenges H_2O_2 in vitro.

Characterization of the possible HPX homologue

To examine if the possible HPX homologue functions as a peroxidase, we expressed the recombinant form of this protein using an *E. coli* expression system. Since this protein was produced in an apo-form, the intact preparation (apo-form) was reconstituted with hemin. The peroxidase activity of the reconstituted preparation was measured using ABTS or guaiacol as a reductant substrate and H_2O_2 as an oxidant substrate (Supplemental Fig. S2). For both reductant substrates, the reconstituted preparation showed a larger increase in absorbance than the intact preparation and hemin. The absorbance increase detected with myoglobin is consistent with a previous report in which myoglobin showed peroxidase activity with ABTS and guaiacol (Matsui et al. 1999).

Table 3 shows a comparison of peroxidase activities measured using reconstituted and intact preparations, hemin, myoglobin, and HRP. The heme content of the reconstituted possible HPX homologue was estimated to be 0.59 mol mol^{-1} protein (data not shown). Peroxidase activities of reconstituted preparation were higher than those of hemin and myoglobin, although much smaller than those of HRP, a typical peroxidase (Table 3). These results indicate that the possible HPX homologue (TTHA1714) functions as a peroxidase with H_2O_2 as an oxidant substrate.

Discussion

T. thermophilus HB8 possesses at least three enzymes that can scavenge H_2O_2: MnCAT (TTHA0122), a possible peroxiredoxin homologue (TTHA1300), and a possible HPX homologue (TTHA1714). To investigate the roles of these proteins, we attempted to delete the *ttha0122*, *ttha1300,* and *ttha1714* genes from the *T. thermophilus* HB8 chromosome by homologous recombination. This method has been widely used to disrupt genes of interest in *T. thermophilus* HB8 (Agari et al. 2008, 2011; Fukui et al. 2011; Nakane et al. 2011; Shimada et al. 2010). In this study, we completely disrupted the *ttha1300* gene, but were unable to delete every copy of *ttha0122* or *ttha1714* (Fig. 1b; Table 1). Our inability to completely delete *ttha0122* is consistent with a previous study, which reported unsuccessful attempts to knock out the *mncat* gene in *T. thermophilus*

Table 2 Kinetic parameters of MnCAT from *T. thermophilus*

MnCAT	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
As isolated[a]	4.5×10^3	71	6.3×10^4
As isolated[b]	2.6×10^5	83	3.1×10^6

[a] This work

[b] Shank et al. (1994)

Table 3 Peroxidase activity of the possible HPX homologue from *T. thermophilus*

Substrate	Reconstituted HPX	Intact HPX	Hemin	Myoglobin	HRP
ABTS	58 (98)	ND	(7.2)	49	7.1×10^3
Guaiacol	52 (88)	ND	(11)	16	6.0×10^4

Reconstituted possible HPX homologue (1 μM), intact possible HPX homologue (1 μM), hemin (1 μM), myoglobin (1 μM), and HRP (1 nM) were assayed for peroxidase activity as described in "Materials and methods". Activity is expressed as μM product per min per μM protein

ND not detected

Activity values in parentheses are expressed as μM product per min per μM heme

HB27 (Moreno et al. 2004). Partial disruption of a gene has been previously reported with the deletion of *recJ* in *Deinococcus radiodurans* (Cao et al. 2010), an extremely radioresistant and polyploid bacterium (Hansen 1978) closely related to the genus *Thermus* (Omelchenko et al. 2005). Our inability to produce *ttha0122* and *ttha1714* null mutants suggests that their gene products exert essential functions in *T. thermophilus* HB8.

The Δ*ttha0122* and Δ*ttha1714* strains showed conspicuous phenotypes despite containing residual wild type genes. The partial disruption of each gene caused a significant increase in the spontaneous mutation frequency: eightfold higher for Δ*ttha0122* and sixfold higher for Δ*ttha1714* under normal conditions (Fig. 2). Nakane et al. (2011) measured the spontaneous mutation rate for the Δ*mutM* strain of *T. thermophilus* HB8. MutM removes an oxidatively damaged base, 8-oxoguanine, from DNA (Morita et al. 2010). While Δ*mutM* shows no significant increase in its spontaneous mutation frequency under the normal conditions, it exhibits a roughly threefold increase in the rate of spontaneous mutations under oxidative conditions (Nakane et al. 2011). Notably, this increase is less than that of Δ*ttha0122* and Δ*ttha1714* under normal conditions (Fig. 2). These findings indicate that the oxidative stress occurring in Δ*ttha0122* and Δ*ttha1714* under normal conditions is high enough to cause mutagenic effects and suggest an important role for MnCAT and the possible HPX homologue in scavenging ROS.

Moreno et al. (2004) used an antisense RNA to inhibit the function of MnCAT in *T. thermophilus* HB27 and showed that this causes the cells to be more sensitive to H_2O_2. Consistent with this previous report, Δ*ttha0122* was highly sensitive to H_2O_2-induced oxidative stress in our study (Fig. 3c). MnCAT (TTHA0122) is annotated as an intracellular enzyme that degrades H_2O_2. To confirm its presence, we purified it from *T. thermophilus* HB8 cells (Fig. 6). Enzymatic analysis showed that it scavenges H_2O_2 with substantial catalytic efficiency (Table 2). The virtual absence of scavenging activity in Δ*ttha0122* (Fig. 5) is probably due to the decreased amount of MnCAT in the mutant strain. Collectively, our results indicate that MnCAT is the primary scavenger for intracellular H_2O_2 in *T. thermophilus*.

Figure 5 shows that the HPX mutant, Δ*ttha1714*, scavenges H_2O_2 as well as the wild type. This finding indicates that this possible HPX homologue is not the primary H_2O_2 scavenger. However, it does help *T. thermophilus* cells cope with oxidative stress, since Δ*ttha1714* showed a significant increase in spontaneous mutation frequency (Fig. 2). Based on the similarities in amino acid sequence and three-dimensional structure, TTHA1714 is considered as a possible HPX homologue, and is classified as a dye-decolorizing peroxidase that uses H_2O_2 to degrade various compounds such as an anthraquinone-type dye (Sugano 2009). Enzymatic analysis suggests that TTHA1714 acts as a peroxidase with H_2O_2 as an oxidant substrate (Table 3). The culture medium of Δ*ttha1714* turned red after a long culture period (Fig. 4). Decreased levels of the possible HPX homologue in Δ*ttha1714* may cause the accumulation of a red-colored dye and H_2O_2, leading to hydroxyl radical production and oxidative damage.

Agari et al. (2010) used DNA microarray analysis to identify a host of genes under the control of the oxidative stress-responsible transcriptional activator, SdrP, from *T. thermophilus* HB8. Since the *mncat* and *hpx* genes are not included in the list, another transcriptional regulator probably controls the expression of these two genes.

In aerobically growing cells, various types of ROS scavengers contribute to diminish oxidative damage and maintain aerobic metabolism. In accordance with a previous study by Moreno et al. (2004), our results provide further evidence that MnCAT is the primary scavenger for H_2O_2 and serves essential functions in *T. thermophilus*. It is noteworthy in this study that both MnCAT and the possible HPX homologue exhibited a similar and significant increase in spontaneous mutation frequency even under normal conditions (Fig. 2). This finding indicates that the protective role of the possible HPX homologue against oxidative stress is comparable to that of MnCAT. Although present in the HPX mutant (Δ*ttha1714*), MnCAT may not be able to complement the deficiency of the possible HPX

homologue in the mutant. Based on these findings, we propose that not only MnCAT but also the possible HPX homologue, a member of dye-decolorizing peroxidase, acts as ROS scavengers to protect *T. thermophilus* cells from oxidative stress. This is the first study on the involvement of the possible HPX homologue TTHA1714 in protecting cells from oxidative stress in this thermophile. Further biochemical and gene expression analyses are warranted to understand the discrete roles of these scavenging enzymes.

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