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Archaeal promoter-directed expression of the *Halobacterium salinarum* catalase-peroxidase gene

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Abstract The *Halobacterium salinarum* catalase-peroxidase gene was subcloned into shuttle vectors pWL102 and pWL202 and expressed under the control of different archaeal promoters. When *Hbt. salinarum* was transformed with the catalase-peroxidase gene under the control of its own promoter, catalase-peroxidase activity increased twofold. Catalase-peroxidase activity increased threefold when *Hbt. salinarum* was transformed with the catalase-peroxidase gene under the control of a tRNA promoter. This bifunctional enzyme in *Hbt. salinarum* was not induced by environmental stresses such as H_2O_2, intense light, darkness, high temperature, low temperature, redox inhibitors, heavy metals, or ions.

Key words Bifunctional enzyme · Induction · Oxidative stress · Polymerase chain reaction

Introduction

Catalase-peroxidase, a member of the hydroperoxidase family, is one of three distinct groups of related enzymes: catalases, peroxidases, and bifunctional catalase-peroxidases. These proteins serve to limit H_2O_2 exposure in cells by removing peroxide in an enzymatic redox reaction. Catalases employ H_2O_2 as the reductant, whereas peroxidases utilize organic peroxides in this function.

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Catalase: $H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$
Peroxidase: $H_2O_2 + R(OH)_2 \rightarrow 2H_2O + RO_2$

Catalase-peroxidases are unique bifunctional proteins that exhibit both catalase and peroxidase activities while sharing characteristics in common with both enzymes. Catalase-peroxidase was first purified from *Escherichia coli* (Claiborne and Fridovich 1979). Since then, similar bifunctional enzymes have been isolated from a variety of different microorganisms (Nagy et al. 1995; Hilar and Loewen 1995). Most catalase-peroxidases are dimers or tetramers possessing equally sized subunits with molecular weights about 80 kDa and are readily reduced by dithionite, a characteristic commonly found in peroxidases. In addition, these bifunctional enzymes show properties distinct from either catalase or peroxidase. They have narrow pH ranges for maximal activity, are not inhibited by 3-amino-1,2,4-triazole, are readily inactivated at temperatures above 50°C, are inactivated by H_2O_2, exhibit a low K_m for H_2O_2, and have a relatively low content of heme per molecule (Goldberg and Hochman 1989; Hochman and Goldberg 1991; Hochman et al. 1992).

Catalases and peroxidases have recently taken on an industrial importance (Potera 1998). Industrial processes frequently require enzymes to function in extreme environments such as high salt, acidic or basic pH, and high temperatures. The industrial potential of extremophilic enzymes such as those from halophiles and thermophiles has encouraged us to purify these enzymes, and to clone and attempt to overexpress the proteins. We have purified the catalase-peroxidase from *Hbt. salinarum* (Brown-Peterson and Salin 1993), and the gene encoding the enzyme has been cloned (Long and Salin, 2000).

In this article, we describe the expression of the catalase-peroxidase gene under the control of different promoters. In addition, we report on the inability of this enzyme to be induced under stress conditions normally associated with catalase or peroxidase induction in other organisms.

Materials and methods

Vectors, organisms, and culture conditions

The shuttle vector pWL102 was purchased from ATCC, (Rockville, MD, USA) whereas pWL202 was a gift from Dr. Charles Daniels of the Department of Microbiology, Ohio State University. *Halobacterium salinarum* (strain NRL) was cultured in complex medium (250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g trisodium citrate $\cdot 2H_2O$, 2 g KCl, 10 g peptone in 1 l H_2O, pH 7.0) under constant shaking (200 rpm) at 40°C. *Escherichia coli* XL1-Blue was grown in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 l H_2O, pH 7.0) at 37°C with constant shaking (200 rpm). Ampicillin (Sigma, St. Louis, MO, USA) was used at a final concentration of 100 μg/ml.

Expression of the catalase-peroxidase gene

The PCR-amplified *Hbt. salinarum* catalase-peroxidase gene along with its own promoter (Hcat) (Long and Salin, 2000), HCP, an rRNA promoter from *Halobacterium Cutirubrum* (Jolley et al. 1996), and HSP, an rRNA promoter from *Hbt. salinarum* (Brown et al. 1989), were cloned into pWL102. The gene was also cloned into pWL202, which contains a $tRNA^{Lys}$ promoter from *Haloferax volcanii* (Nieuwlandt and Daniels 1990). This construct is referred to as HTP. Recombinant plasmids were transformed into *Hbt. salinarum* for subsequent expression.

The HCP and HSP promoter regions were first amplified by PCR. The PCR reaction mixture, in a volume of 50 μl, contained: 1 × cloned *Pfu* buffer (20 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 100 μg/ml nuclease-free BSA), 0.2 mM dNTP, 5 U *Pfu*Turbo DNA polymerase (Stratagene, La Jolla, CA, USA), 0.2 μM of HCP5′ primer (5′-CCAGGTAC CGATGCCCTTAAGTACAACAGGGTACT-3′) and HCP3′ primer (5′-CAACATATGCGTTCGCATTCCA CCGAAGTACCCTG-3′) for HCP promoter construction, or 0.2 μM of HSP5′ primer (5′-CATGGTACCGACGGT GTTTTATGTACCCCACCACTC-3′) and HSP3′ primer (5′-CAACATATGACGTCGTTCGCATCTCATCCGAGTG GTG-3′) for HSP promoter construction. The PCR reaction was performed as follows: 28 cycles at 96°C for 30 s, 65°C for 30 s, and 72°C for 1 min, and finally an extra 5 min of extension at 72°C. The catalase-peroxidase gene was also amplified by PCR using the previously cloned gene (Long and Salin, 2000) as template. Primers CE5′ (5′-CAACATATGGAGAACGAAGACCACA-3′) and HCXBN1 (5′-CGGTCTAGAAGTGGCGAGCACT ACGT-3′) were used for amplification of the catalase-peroxidase gene from the translational start codon to the end of the 3′-flanking region. For amplification of the catalase-peroxidase gene from the transcriptional start site to the end of the 3′-flanking region, primers HPINDEL (5′-GGAATTCCATATGGGGAGATCCGTACCA-3′) and HCXBN1 were used. The PCR reaction was performed at 96°C for 2 min, followed by 28 cycles at 96°C for 1 min, 65°C for 1 min, and 72°C for 6 min, and finally an extra 10 min of extension at 72°C. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Santa Clarita, CA, USA).

The HCP and HSP promoters along with the catalase-peroxidase gene were digested with *Nde*I and purified by agarose gel electrophoresis and a QIAquick Gel Extraction Kit (Qiagen). The promoters were then ligated with the catalase-peroxidase gene, diluted, and used as a template for PCR. Primers HCP5′ and HCXBN1 were used for amplification of the HCP promoter-linked catalase-peroxidase gene, whereas the primers HSP5′ and HCXBN1 were used for amplification of the HSP promoter-linked catalase-peroxidase gene. Primers HCKPN1 (5′-CCAGG TACCGGATCCGATGGTCGTGGCA-3′) and HCXBN1 were used to amplify the catalase-peroxidase gene under control of its own promoter.

Primers HTP5 (5′-GCTTCTAGAATGGAGAACGA AGACCACA-3′) and HTP3 (5′-TGGGGTACCGAAGT GGCGAGCACTACGT-3′) were used to amplify the HTP promoter-controlled catalase-peroxidase gene from the translational start codon to the end of the 3′-flanking region. Alternately, primers HTXB (5′-GCTCTAGAGA GATCCGTACCATTGCCA-3′) and HTP3 were used for amplification of the HTP promoter-controlled catalase-peroxidase gene from the transcriptional start site to the end of the 3′-flanking region. The PCR reaction was performed at 96°C for 2 min, followed by 28 cycles at 96°C for 1 min, 65°C for 1 min, and 72°C for 6 min, and finally an extra 10 min of extension at 72°C. The PCR products were purified and digested with *Kpn*I and then *Xba*I. Shuttle vectors pWL102 and pWL202 were also digested with *Kpn*I and then *Xba*I. The linearized vector and PCR products were purified and ligated by T4 DNA ligase and then transformed into *E. coli* XL1-Blue via electroporation according to the manufacturer's protocol (E-C Apparatus, St. Petersburg, FL, USA). The recombinant plasmid DNAs were purified with a QIAprep Spin Miniprep Kit (Qiagen) and transformed into *Hbt. salinarum* according to the method of Cline et al. (1989). The PCR method was used to determine if the catalase-peroxidase gene under the control of different promoters had been transformed into *Hbt. salinarum.* The *Hbt. salinarum* transformants with the catalase-peroxidase gene under the control of different promoters were grown in 50 ml complex medium with 20 μM mevinolin (Merck, Whitehouse Station, NJ, USA) until the cultures reached an OD_{600} of 0.4–0.6. Cell cultures were harvested by centrifugation at 7,500 × g for 10 min at 4°C and then washed twice with 50 mM potassium phosphate buffer (pH 7.0) containing 2 M NaCl. Cells were resuspended in 10 ml wash buffer and then frozen (–70°C, 15 min) and thawed (37°C, 3 min) five times. Next, cells were sonicated at full power on a Fisher sonifier Fisher sonifier (Atlanta, GA, USA) for 20 s at 4°C followed by 2 min of cooling on ice to prevent excessive heating. After a total of 3 min sonication, 0.2 mg of DNase I was added to the homogenate and the suspension was stirred at room temperature for 1.5 h. The extract was centrifuged for 15 min at 10000 *g* at 4°C, and the supernatant was assayed for catalase-peroxidase activity.

Induction of catalase-peroxidase

Halobacterium salinarum was grown in 500 ml complex medium as described previously. When cultures reached early to mid-log phase of growth (OD_{600}, 0.3–0.5), they were separated into different flasks, and different concentrations of heavy metals, redox inhibitors, H_2O_2, and ions were added or the cells were subjected to the alterations in light and temperature conditions as noted. At various time points, 40 ml of cell cultures was harvested, lysed, and assayed for catalase-peroxidase activity.

Enzyme assays

All enzyme activities were assayed in triplicate using a UV-VIS spectrophotometer (Cary 3 C; Varian, Sugar Land, TX, USA) at 25°C in a buffer containing 50 mM potassium phosphate and 2 M NaCl. The pH of the buffer was adjusted to 6.5 for the catalase assay and to 7.5 for the peroxidase assay. Catalase activity was determined spectrophotometrically at 240 nm with 20 mM H_2O_2 (Beers and Sizer 1952). Peroxidase activity was determined spectrophotometrically at 510 nm with 0.06 mM 4-aminoantipyrine in 40 mM phenol and 2 mM H_2O_2 as substrates (Worthington 1988). One unit of activity for both catalase and peroxidase was defined as the decomposition of 1 μmol H_2O_2 per minute at 25°C at the specified pH. Protein concentration was determined by the Bio-Rad Protein Assay Kit (Hercules, CA, USA). The average of three replicates was taken, with no statistically significant differences noted in the three replicate determinations.

Results

Expression of catalase-peroxidase gene

Figure 1 diagrammatically depicts the constructs used in our expression studies. Along with the gene's own promoter (Hcat), two archaeal rRNA promoters (HSP and HCP) were employed as was an archaeal $tRNA^{Lys}$ promoter (HTP). In addition, a series of constructs were employed in which these promoters were linked to the gene in a region upstream of the open reading frame (ORF) to include the transcriptional start site "G" at position –183. These constructs were termed HGSP, HGCP, and HGTP, respectively.

The catalase-peroxidase activities of all transformed *Hbt. salinarum* clones are shown in Table 1. Cells transformed with catalase-peroxidase containing its own pro-

Table 1. Catalase-peroxidase activity of transformed *Halobacterium salinarum*

Promoter constructs	Catalase SA (U/mg protein)	Fold increase	Peroxidase SA (U/mg protein)	Fold increase
HS	12.0	1.0	0.10	1.0
Hcatp	26.4	2.2	0.19	1.9
HCP1	16.6	1.4	0.12	1.1
HCP2	12.4	1.0	0.11	1.1
HSP1	13.6	1.1	0.12	1.2
HSP2	11.3	0.9	0.12	1.1
HS	16.8	1.0	0.10	1.0
HTP1	46.4	2.8	0.24	2.5
HTP2	48.4	2.9	0.23	2.3
HS	12.8	1.0	0.12	1.0
HGCP1	12.1	0.9	0.12	1.1
HGCP2	14.0	1.1	0.12	1.0
HGSP1	13.2	1.0	0.10	0.8
HGSP2	14.1	1.1	0.14	1.3
HGTP1	27.6	2.2	0.19	1.7
HGTP2	23.5	1.8	0.18	1.6

The number after the constructs refers to the fact that in several transformants two clones were chosen for analysis
HS, nontransformed *Halobacterium salinarum*; Hcatp, transformed *Hbt. salinarum* containing the catalase-peroxidase gene with its own promoter; HCP, HSP, and HTP, transformed *Hbt. salinarum* containing HCP, HSP, or HTP promoter-controlled catalase-peroxidase gene; HGCP, HGSP, and HGTP, transformed *Hbt. salinarum* containing HCP, HSP, or HTP promoter-controlled catalase-peroxidase gene starting from the transcriptional start site; SA, enzyme-specific activity

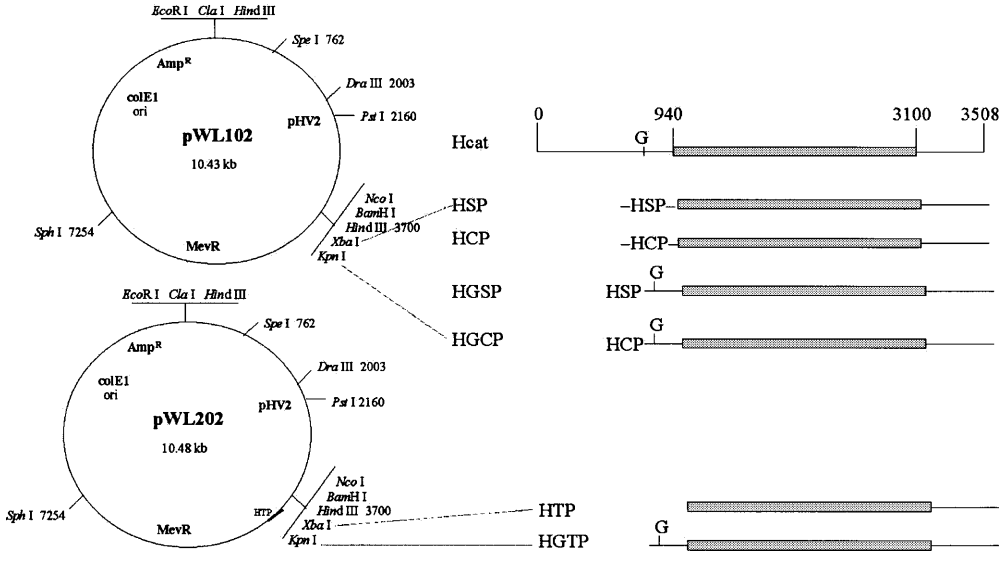

Fig. 1. Constructs used for expression of the catalaseperoxidase gene in *Halobacterium salinarum*. Constructs were incorporated into shuttle vector pWL102 at the designated site. *Gray blocks* represent open reading frame; *G* denotes the transcriptional start site; *HSP*, *HCP*, and *HTP* represent the HSP, HCP, and HTP promoter regions. *Numbers* represent nucleotide positions

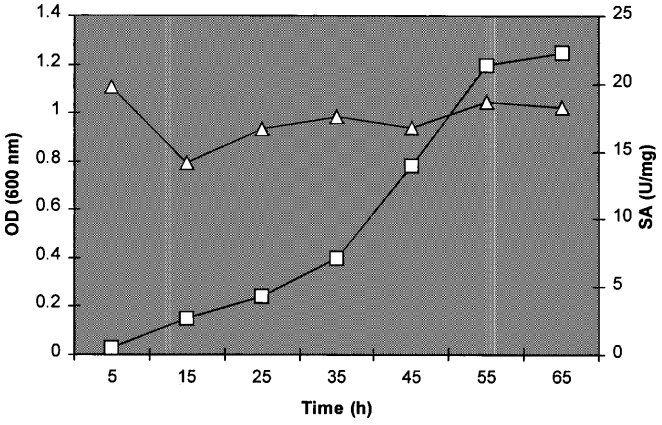

Fig. 2. Catalase activity of *Hbt. salinarum* at different growth phases. *Open squares*, growth measured as OD_{600}; *open triangles*, specific activity of catalase; *SA*, specific activity (U/mg protein)

moter or the HTP promoter had a level of catalase activity 2.2-fold or 2.9-fold and peroxidase activity 1.9-fold or 2.5-fold higher than that of the wild-type *Hbt. salinarum*, respectively. Those transformants with catalase-peroxidase containing the HCP or HSP promoter showed no increase in catalase and peroxidase activity compared with wild-type *Hbt. salinarum*. In addition, inclusion of the region upstream of the ORF to encompass the transcriptional start site "G" along with the promoters did not result in any enhancement of catalase-peroxidase activity in the recombinants.

Induction of catalase-peroxidase under stress conditions

The activity of catalase-peroxidase in wild-type *Hbt. salinarum* during growth was monitored in cell extracts at different growth phases (Fig. 2). A constant, constitutive level of catalase activity was found at all growth phases, thereby indicating that catalase-peroxidase is growth-phase independent in *Hbt. salinarum*. Treatment of *Hbt. salinarum* with various concentrations of H_2O_2 for different times during the exponential phase did not yield a significant induction of catalase activity, a point that was supported by Northern blot analysis (data not shown).

Treatment of *Hbt. salinarum* with redox inhibitors for various lengths of times did not result in any significant increase in catalase activity (Table 2). Moreover, *Hbt. salinarum* cells were killed at higher methyl viologen (MV) and N_3^- concentrations (represented by 0 catalase activity). When *Hbt. salinarum* was treated with 0.1 mM heavy metals or ions for various times during the exponential phase, only Cd^{2+} treatment for 18 h resulted in a twofold increase in catalase activity. In all other treatments, no increases were apparent (Table 3).

When *Hbt. salinarum* was grown in the dark or under intense light conditions (1400 foot-candles provided by a quartz iodide lamp), catalase activities were the same as in cells grown under normal overhead light conditions (data not shown). Hence, extremes in light conditions did not induce the bifunctional enzyme in *Hbt. salinarum*. Finally, growth of the organism at 10° or 50°C did not increase catalase activity compared with those cells grown at the optimal growth temperature (37°C) (Fig. 3). In fact, as expected, high temperature resulted in loss of activity and cell death.

Table 2. Effect of redox inhibitors on catalase-peroxidase induction

Inhibitor (mM)	Time (h)					
	2	8	18	24	32	42
MV						
0	14.0	12.2	12.3	13.6	9.8	9.5
0.1	10.4	5.3	2.6	2.4	0	0
1.0	4.3	0	0	0	0	0
10	0	0	0	0	0	0
CN^-						
0	13.2	13.0	12.2	18.7	7.0	8.9
0.1	13.1	13.3	13.2	15.5	14.0	8.8
1.0	13.3	11.3	14.3	16.3	13.4	12.7
10	16.6	14.0	11.9	12.1	13.3	13.2
N_3^-						
0	12.1	9.0	13.5	14.2	12.7	11.1
0.1	15.1	6.6	11.3	11.1	10.6	11.5
1.0	9.2	5.8	3.5	5.2	4.6	6.3
10	5.0	0.7	0	0	0	0

Values denote specific activity of catalase (U/mg protein); 0 values indicate complete loss of activity on cell death
MV, methyl viologen

Table 3. Effect of heavy metals and ions on catalase-peroxidase induction

Metal or ion	Time (h)					
	2	8	18	24	32	42
0	12.1	10.5	9.9	10.9	10.6	8.1
Zn^{2+}	14.3	11.9	14.5	18.9	14.9	12.3
Hg^{2+}	11.2	10.4	14.1	9.6	9.6	11.0
Pb^{2+}	14.8	13.2	6.7	18.0	14.4	13.5
0	14.3	14.0	12.8	14.0	18.1	16.82
Cd^{2+}	16.2	20.3	26.5	25.8	24.0	23.62
Fe^{3+}	13.7	15.2	15.7	17.7	12.7	13.93
Al^{3+}	15.9	10.5	13.0	12.1	9.8	12.77
0	8.8	8.7	16.8	15.6	9.3	14.2
F^-	8.9	8.6	17.4	17.8	10.0	13.6
I^-	10.0	9.8	17.2	18.9	9.3	8.8

Values denote specific activity of catalase (U/mg protein)
All metals and ions were at 0.1 mM concentration, with the exception of the "0" value in which no metal or ion was present

Discussion

Expression of the catalase-peroxidase gene under control of its own promoter showed a twofold increase in enzyme activity compared with wild-type cells, and thus we placed the gene under control of three different strong archaeal promoters that have been shown previously to result in significant increases in gene expression. The rRNA promot-

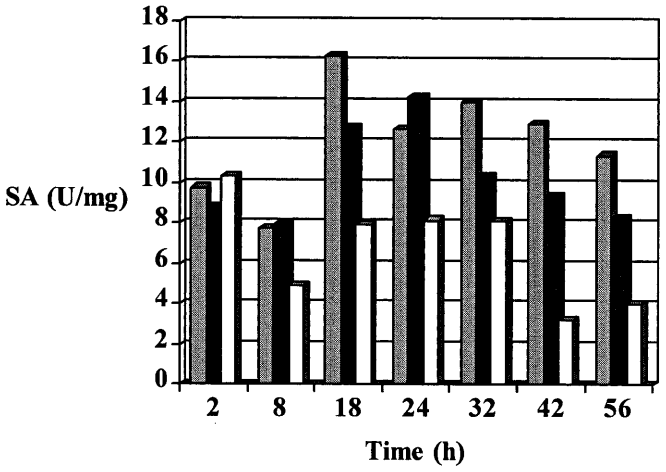

Fig. 3. Effect of temperature on induction of catalase-peroxidase in *Hbt. salinarum*. *Gray column*, 37°C treatment; *black column*, 10°C treatment; *white column*, 50°C treatment. Time values represent the duration of treatment. *SA*, specific activity (U/mg protein)

ers of *Hbt. cutirubrum* (HCP) (Jolley et al. 1996) and *Hbt. salinarum* (HSP) (Brown et al. 1989), and the $tRNA^{Lys}$ promoter of *Haloferax volcanii* (HTP) (Nieuwlandt and Daniels 1990), were utilized as replacements for the 5′-flanking region of the catalase-peroxidase gene. Dihydrolipoamide dehydrogenase in *Haloferax volcanii* transformed with the HCP promoter resulted in a 15-fold-higher activity than in wild-type cells (Jolley et al. 1996). However, in our study, enzyme activity increased only threefold in *Hbt. salinarum* transformed with the HTP-controlled catalase-peroxidase gene. No increased enzyme activity was detected in the other two transformants. Employing three additional transformants in which the HCP-, HSP-, and HTP-linked catalase-peroxidase gene incorporated the 183-bp leading untranslated sequence also did not result in further increases in gene expression. Therefore, apparently the leading sequence does not play a critical role in catalase-peroxidase expression, a conclusion that is supported by our finding of a Shine–Dalgarno sequence within the open reading frame of the gene (Long and Salin, 2000).

Bacteria have developed defensive systems against oxidative stress that could damage DNA, protein, and membranes. Several mechanisms for oxidative stress responses have been identified in Eubacteria. In *E. coli*, there are two hydroperoxidases, HPI and HPII. The former is a bifunctional catalase-peroxidase induced by H_2O_2 and is encoded by a *KatG* gene (Claiborne and Fridovich 1979; Triggs-Raine et al. 1988). The latter is a monofunctional catalase and is encoded by a *KatE* gene (Loewen et al. 1985; Mulvey et al. 1990). The oxidative stress responses in *Hbt. salinarum* have not been studied as extensively as those in Eubacteria. Superoxide dismutase activity increased two- to threefold when *Hbt. salinarum* was heat stressed or treated with redox inhibitors and increased nearly tenfold when subjected to a hyposaline stress (Begonia and Salin 1991; Brown-Peterson and Salin 1994a, b). In the work presented here, catalase-peroxidase was not induced by H_2O_2 or by any other stress conditions employed.

The upstream region of the *Hbt. salinarum* catalase-peroxidase gene does not contain sequences similar to the *KatG* of *E. coli*, which can bind the regulatory protein OxyR (Long and Salin, 2000). Moreover, the upstream region of the *Hbt. salinarum* catalase-peroxidase gene does not contain sequences similar to *Fur* (ferric uptake regulator), which has been identified in upstream regions of catalase-peroxidase genes in *E. coli*, *Mycobacterium tuberculosis*, and *Streptomyces reticuli* (Bagg and Neilands 1987; Deretic et al. 1995; Zou et al. 1999). These *Fur* genes encode Fur proteins, which regulate the expression of catalase-peroxidase. Therefore, *Hbt. salinarum* catalase-peroxidase is probably not involved in a transitory stress response and hence other defensive mechanisms must be employed. When *Hbt. salinarum* was subjected to a hyposaline stress, catalase activity increased nearly two orders of magnitude (Brown-Peterson and Salin 1994b). However, this increased activity emanated from an induced monofunctional "mesohalic" catalase rather than an enhanced synthesis of the bifunctional catalase-peroxidase (Brown-Peterson and Salin 1995).

In summary, we have expressed the catalase-peroxidase gene in the halophilic archeon *Hbt. salinarum*. The greatest enzymatic activity (threefold increase) was found in *Hbt. salinarum* transformed with a tRNA promoter-controlled catalase-peroxidase gene. Moreover, we have determined that this enzyme in *Hbt. salinarum,* unlike that of similar enzymes in other microorganisms, is not induced by environmental stresses such as H_2O_2, intense light, darkness, high temperature, low temperature, redox inhibitors, heavy metals, or ions.

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