Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasaemic *Saccharomyces cerevisiae*

Shingo IZAWA, Yoshiharu INOUE* and Akira KIMURA

Laboratory of Molecular Breeding of Microorganisms, Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

Controversy about the importance of catalase in the detoxification of H_2O_2 in human erythrocytes continues. It has been suggested that catalase has no role in the clearance of H_2O_2 in erythrocytes. In the present study we investigated the role of catalase in the defence mechanism against oxidative stress using *Saccharomyces cerevisiae*. *S. cerevisiae* has two catalases, catalase A and catalase T. We constructed a double mutant (acatalasaemic mutant) unable to produce either catalase A or catalase T, and compared it with wild-type and single-mutant cells. The acatalasaemic mutant cells showed a similar growth rate to wild-type cells under non-oxidative stress conditions, and showed a similar susceptibility to H_2O_2 stress in the exponential growth phase. The acatalasaemic mutant cells at stationary phase were, however, much more sensitive to H_2O_2 stress than wild-type and single-mutant cells. Moreover, the ability of acatalasaemic and single-mutant cells to show adaptation to 2 mM H_2O_2 was distinctly inferior to that of wild-type cells. These results suggest that catalase is not essential for yeast cells under normal conditions, but plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of these cells.

INTRODUCTION

All aerobic organisms use molecular oxygen ($^3O_2$) for respiration or oxidation of nutrients to obtain energy efficiently. During the reduction of molecular oxygen to water via the acceptance of four electrons, reactive oxygen species such as the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$) are generated. These species are capable of damaging DNA, protein and lipid membranes, and are known to be causative factors in degenerative diseases such as cancer. For defence against reactive oxygen species, cells contain antioxidative enzymes such as superoxide dismutase, catalase and several peroxidases, as well as antioxidants such as ascorbate, tocopherol and glutathione.

H_2O_2 is enzymically catabolized in aerobic organisms by catalase and several peroxidases. In animals, H_2O_2 is enzymically detoxified by catalase and glutathione peroxidase (GPx). In animal cells, and especially in human erythrocytes, the principal antioxidant enzyme for the detoxification of H_2O_2 has for a long time been considered to be GPx, as catalase has much lower affinity for H_2O_2 than does GPx. Furthermore, it is reported that, in humans lacking erythrocyte catalase activity, these cells are not susceptible to haemolysis induced by oxidative stress [1]. Therefore it has been suggested and widely accepted that almost all H_2O_2 is detoxified by GPx and that catalase has no role in the clearance of H_2O_2.

More recently, however, mammalian and yeast catalases were found to contain tightly bound NADPH, and to require NADPH to prevent the formation of catalase compound II (inactive form) by H_2O_2 [2–4]. This finding means that both GPx and catalase are dependent on NADPH, and raises the possibility that catalase, as well as GPx, is important in the detoxification of H_2O_2. Subsequently, several studies using acatalasaemic (catalase-deficient) cells and glucose-6-phosphate dehydrogenase (G6PDH)-deficient cells have reported that catalase plays an important role, in addition to that of GPx, in protection against H_2O_2 stress [5–9]. Controversy about the significance of catalase in the detoxification of H_2O_2 in animal cells continues to this day.

On the other hand, in bacterial cells it has been reported that catalase may not defend individual bacterial cells against H_2O_2 stress. Ma and Eaton [10] reported that individual catalase-deficient *Escherichia coli* cells showed an identical sensitivity to H_2O_2 to that of wild-type cells, but high-density or colonial catalase-deficient cells showed greater susceptibility than wild-type cells under the same conditions. Similar results were reported in *Salmonella typhimurium* [11]. These findings suggested that catalase may function to protect groups of bacteria rather than discrete, isolated, cells.

In the yeast *Saccharomyces cerevisiae*, H_2O_2 is enzymically catabolized by catalase and cytochrome *c* peroxidase (CCP). It is still controversial as to whether *S. cerevisiae* contains GPx or not. *S. cerevisiae* has two catalases, peroxisomal catalase (catalase A) and cytosolic catalase (catalase T), encoded by the *CTA1* and *CTT1* genes respectively [12,13]. Much work has been done by Ruis and co-workers [14–19] on the regulation of these genes and their transcriptional factors. Despite the progress in elucidating the regulation of catalase genes, the real function of catalase in the oxidative stress response in *S. cerevisiae* is still in question.

In the present paper we investigate the role of catalase in the tolerance to and the adaptive response to H_2O_2 stress in *S. cerevisiae* using disruption mutants of the genes encoding the two catalases. We have carefully tested the susceptibility of catalase-deficient mutants to H_2O_2, and we discuss the differences in the role of catalase between yeast and other organisms.

EXPERIMENTAL

Yeast strains and medium

The yeast strains used in this study were as follows: YPH250 (*MAT***a** *trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*),

Abbreviations used: GPx, glutathione peroxidase; CCP, cytochrome *c* peroxidase; G6PDH, glucose-6-phosphate dehydrogenase.

* To whom correspondence should be addressed.

YTT7 (*MAT***a** *trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ctt1::URA3*), YIT2 (*MAT***a** *his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 cta1::TRP1*), YWT1 (*MAT***a** *his3-Δ200 lys2-801 leu2-Δ1 ade2-101 cta1::TRP1 ctt1::URA3*). YPH250 was obtained from the Yeast Genetic Stock Center (University of California at Berkeley, CA, U.S.A.). Yeast cells were cultured in 50 ml of YPD medium (2% glucose, 2% peptone, 1% yeast extract, pH 5.5) at 28 °C with reciprocal shaking (350 rev./min) in 200 ml Sakaguchi flasks. Exponential-phase cells were harvested at an attenuance of the culture at 610 nm (A_{610}) of 0.1–0.2. Stationary-phase cells were harvested after cultivation for 72 h.

Disruption of the *CTT1* and *CTA1* genes and plasmid construction

The *ctt1* deletion mutant was constructed by transforming YPH250 with the plasmid *ctt1::URA3* digested with *Eco*RI/*Eco*RI and selecting for Ura$^+$ transformants, producing strain YTT7 (Δ*ctt1*). To delete the *CTA1* gene the following oligonucleotide primers were used in a PCR to amplify a 1.6 kb fragment containing the open reading frame of the *CTA1* coding sequence from genomic DNA: 5′-ATGTCGAAATTGGGAC-AAGA-3′ and 5′-AAAATTTGGAGTTACTCGAAAGC-3′. PCR was carried out using the following conditions: 94 °C for 1 min (denaturation), 55 °C for 2 min (annealing), 72 °C for 2 min (extension), for 31 cycles. An *Ssp*I/*Hin*cII *CTA1* fragment was cloned into pUC19. The 0.8 kb *Eco*RI/*Pst*I fragment from plasmid YRpG1, containing the *TRP1* gene, was inserted between the *Bam*HI site internal to the *CTA1* gene, giving plasmid pCT721. Plasmid pCT721 was digested with *Eco*RI/*Eco*47III to linearize the *cta1::TRP1* fragment, prior to the transformation of strain YPH250 to construct strain YIT2 (Δ*cta1*). A *ctt1* and *cta1* double-deletion mutation was also constructed, producing strain YWT1 (Δ*cta1* and Δ*ctt1*). Replacement of the wild-type *CTT1* and *CTA1* alleles by the *ctt1::URA3* and *cta1::TRP1* disruption mutations was verified by PCR and catalase-activity staining [20]. Transformations were performed by the method of electropolation (Bio-Rad; Gene Pulser II).

Enzymic assays

Cells were disrupted by vortexing with glass beads in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM PMSF and 1 μg/ml pepstatin A. Catalase activity was measured by the method of Roggenkamp et al. [21]. The rate of disappearance of H_2O_2 was measured spectrophotometrically at 240 nm. One unit of catalase activity is defined as the amount of enzyme catalysing the degradation of 1 μmol of H_2O_2/min at 25 °C. CCP activity was assayed by the method of Yonetani [22]. One unit of this activity is defined as the amount of enzyme oxidizing 1 μmol of cytochrome *c*/min at 25 °C. G6PDH activity was measured by the method of Kornberg and Horecker [23]. One unit of G6PDH activity is defined as the amount of enzyme oxidizing 1.0 μmol of D-glucose 6-phosphate/min at 25 °C.

Catalase-activity staining

The method of catalase-activity staining was essentially that described by Clare et al. [20]. Cells in stationary phase were disrupted and cell-free extracts were applied to native electrophoresis, which was performed on 15% polyacrylamide gels. The gel was soaked in horseradish peroxidase (50 mg/ml) in 100 mM potassium phosphate buffer (pH 7.0) for 45 min. H_2O_2 was then added to a concentration of 5.0 mM and soaking was continued for 10 min. The gel was then rapidly rinsed twice with distilled water and soaked in 0.5 mg/ml diaminobenzidine in potassium phosphate buffer until staining was complete.

H_2O_2 treatment

Conditions for the treatment of cells were essentially as described by Flattery-O'Brien et al. [24]. Cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial $A_{610} = 0.1$. This represented approx. 2×10^6 cells/ml. To observe the susceptibility of yeast to H_2O_2, various concentrations of H_2O_2 were added to 5 ml samples, and cell survival was monitored by taking samples at 20 min intervals, diluting in the same buffer and plating aliquots on YPD plates. For adaptation experiments, cells were pretreated by resuspension in fresh YPD medium containing a sublethal concentration of H_2O_2 (0.2 mM) and incubated with shaking at 28 °C for 1 h. Pretreated cells were then harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4), and challenged with the lethal concentration of H_2O_2 (2 mM).

RESULTS

Construction of catalase-deficient mutants

The catalase-deficient phenotypes of mutant strains were confirmed by catalase-activity staining. As showing in Figure 1, YTT7 (Δ*ctt1*) expressed only catalase A (lane 2), YIT2 (Δ*cta1*)

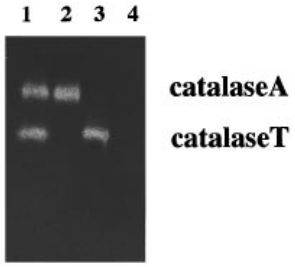

Figure 1 Catalase-activity staining

Cell-free extracts of stationary-phase cells of wild-type and catalase-deficient mutants were applied to native PAGE and then the gel was stained. The upper band is catalase A activity and lower band is catalase T. Lanes: 1, wild-type; 2, YTT7 (Δ*ctt1*); 3, YIT2 (Δ*cta1*); 4, YWT1 (Δ*cta1*/Δ*ctt1*).

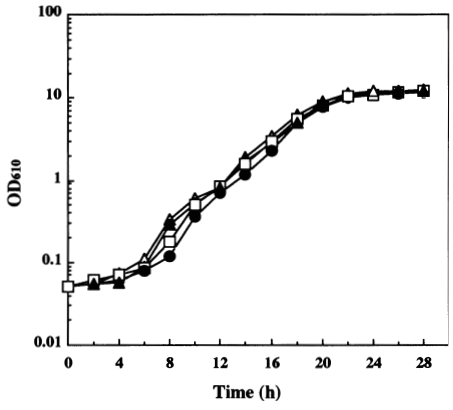

Figure 2 Comparison of growth of wild-type cells and catalase-deficient mutants

Cells were cultured in YPD medium at 28 °C, and growth was monitored by measuring the absorbance at 610 nm (OD_{610}). Strains: ●, YPH250; △, YIT2; ▲, YTT7; □, YWT1.

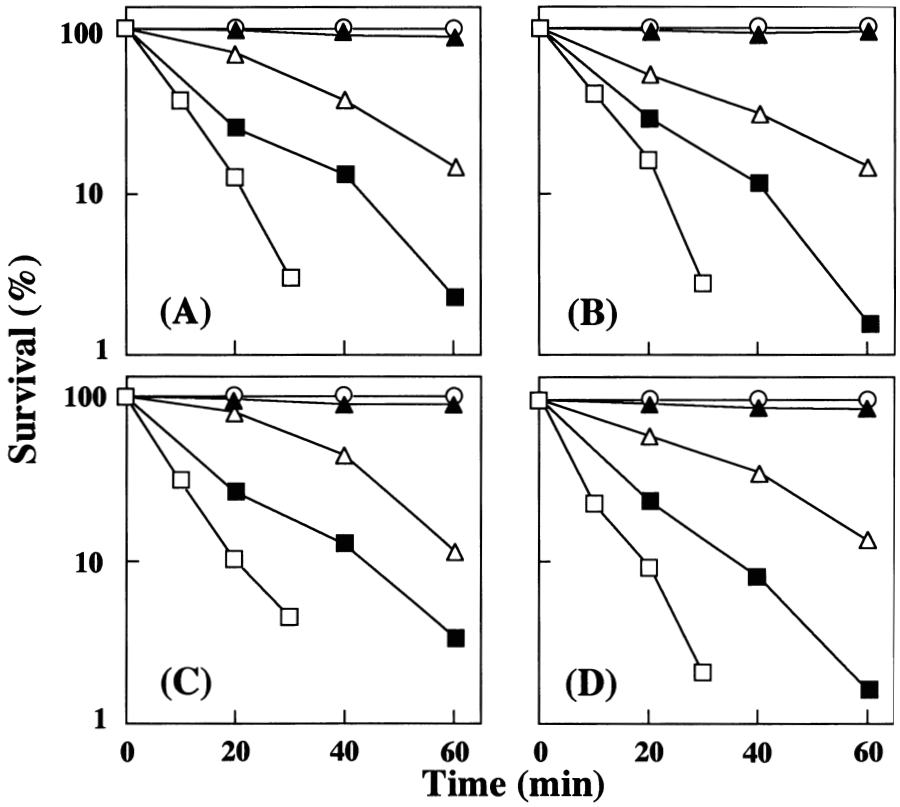

Figure 3 Susceptibility of exponential-phase cells to H_2O_2 stress

Cells in exponential phase were harvested and resuspended in 100 mM potasium phosphate buffer (pH 7.4) to obtain the initial $A_{610} = 0.1$ (2×10^6 cells/ml), and then treated with various concentrations of H_2O_2. Samples were diluted and plated on YPD agar plates to monitor cell viability. H_2O_2 concentrations (mM): 0 (○), 0.2 (▲), 1 (△), 2 (■) and 5 (□). Strains: (**A**) YPH250, (**B**) YIT2, (**C**) YTT7 and (**D**) YWT1. Results represent the means of four independent experiments.

expressed only catalase T (lane 3), and YWT1 (Δ*cta1*/Δ*ctt1*) expressed neither catalase (lane 4).

Effect of catalase on growth in batch culture

We investigated the effect of catalase deficiency on cell growth in batch culture. Figure 2 shows the growth curves of wild-type and catalase-deficient mutant cells in YPD medium. All four strains showed similar growth rates. The presence or absence of catalase had little or no effect on growth rate under conditions without H_2O_2 stress.

Susceptibility to H_2O_2 stress of exponential-phase cells

The susceptibility to H_2O_2 stress of wild-type and catalase-deficient mutant cells in exponential phase was monitored (Figure 3). In exponential phase, all mutants showed an almost identical susceptibility to H_2O_2 stress. Only a small number of cells of all strains were capable of surviving under 2 mM H_2O_2 stress for 60 min. In the presence of 0–5 mM H_2O_2, there was no marked difference between wild-type and mutant cells. These results suggest that, in the exponential growth phase, catalase may not act to defend individual *S. cerevisiae* cells against H_2O_2 stress, in analogy with bacterial cells [10,11].

Induction of adaptation to H_2O_2 stress

It is well known that cells pre-exposed to comparatively mild and sublethal stress conditions show induction of resistance to subsequent lethal stress. This phenomenon was observed not only in bacterial cells but also in eukaryotic organisms, including *S. cerevisiae*, and was termed adaptation [25,26]. We investigated the role of catalase in the induction of adaptation to H_2O_2 stress. Adaptation was induced by the pretreatment of exponential-phase cells with 0.2 mM H_2O_2 for 60 min in YPD medium. The pretreated cells were harvested and then challenged to 2 mM H_2O_2 stress in potassium phosphate buffer, and cell survival was monitored.

With a 2 mM H_2O_2 challenge, a difference in susceptibility was noted between wild-type cells and catalase-deficient mutants (Figure 4). Very little increase in tolerance was induced by the pretreatment in catalase-deficient mutant cells, especially in acatalasaemic cells. The single-mutant catalase-deficient cells showed a slight increase in tolerance to 2 mM H_2O_2 stress (Figures 4B and 4C), whereas there was almost no increase in tolerance in acatalasaemic cells (Figure 4D). These results suggest that catalase plays an important role in the induction of adaptation to H_2O_2 stress.

Susceptibility to H_2O_2 stress of stationary-phase cells

It has been reported that, if microbial cells enter the stationary growth phase, they acquire resistance to several environmental stresses such as heat, oxidative damage and osmotic stress. It has been previously reported that *S. cerevisiae* in stationary phase acquires resistance to higher concentrations of H_2O_2, in the order of 10–20 mM, when compared with cells in exponential phase

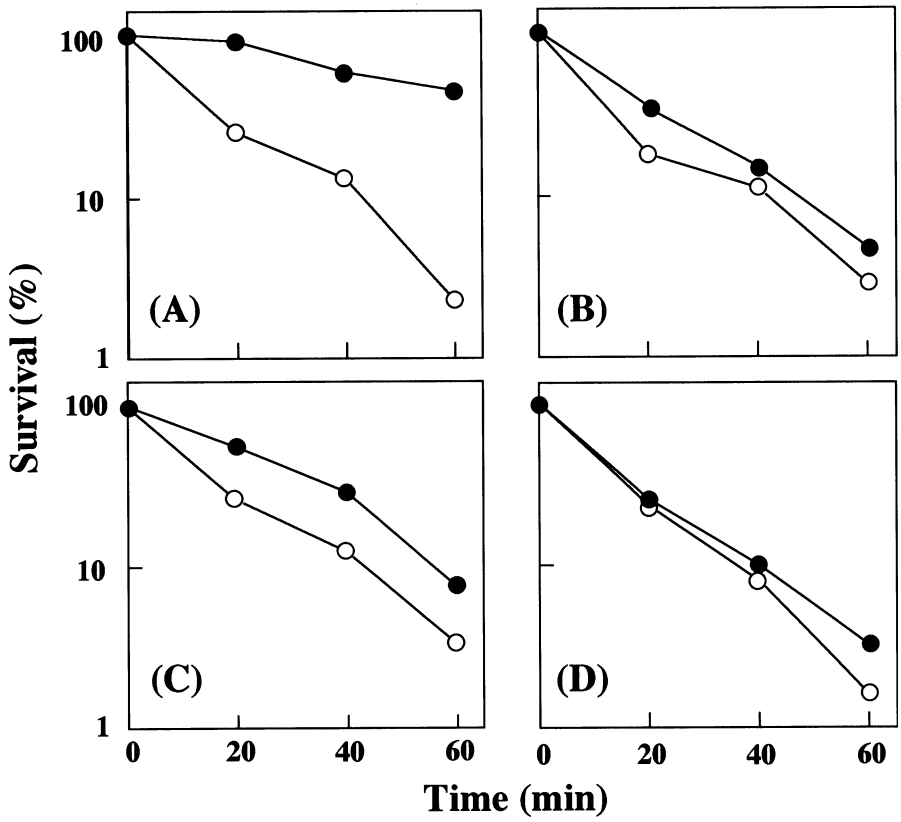

Figure 4 Effect of catalase deficiency on adaptation to 2 mM H_2O_2

Cells in exponential phase were pretreated with 0.2 mM H_2O_2 (●) for 1 h in fresh YPD medium, and then resuspended in 100 mM potasium phosphate buffer (pH 7.4) to obtain the initial A_{610} = 0.1 and challenged with 2 mM H_2O_2. Cells in exponential phase were also directly challenged with 2 mM H_2O_2 without pretreatment (○). Strains: (**A**) YPH250, (**B**) YIT2, (**C**) YTT7 and (**D**) YWT1. Results represent the means of four independent experiments.

[26–28]. The susceptibilities of wild-type and catalase-deficient mutant cells in stationary phase were thus investigated (Figure 5). The wild-type and the single-mutant catalase-deficient cells acquired a distinct resistance to H_2O_2 stress on shifting from exponential to stationary phase. All of these three strains showed a similar susceptibility to H_2O_2 stress. In the presence of 2 mM H_2O_2, almost all cells survived at 60 min, and approx. 50% of total cells survived under 20 mM H_2O_2 stress for 60 min (Figures 5A–5C). In contrast, the acatalasaemic cells displayed a greater susceptibility to high concentrations (5 mM or more) of H_2O_2. No viable cells were detected after treatment with 20 mM H_2O_2 for 60 min, and approx. 90% of the acatalasaemic mutant cells were killed in only 5 mM H_2O_2 by 60 min (Figure 5D).

Although the acatalasaemic cells were much more sensitive to H_2O_2 stress than the wild-type and the single-mutant catalase-deficient cells in stationary phase, the acatalasaemic cells did acquire resistance to H_2O_2 stress on the shift from exponential phase to stationary phase. More than 80% of the acatalasaemic cells in stationary phase survived, whereas approx. 90% of the acatalasaemic cells in exponential phase were killed, by 2 mM H_2O_2 stress for 60 min (Figures 3D and 5D). Apparently the acatalasaemic mutant cells in stationary phase were much more resistant to H_2O_2 stress than the cells in exponential phase.

Changes in enzyme activities

The activities of catalase, CCP and G6PDH were measured under three different conditions as follows: exponential-phase cells without H_2O_2 treatment, exponential-phase cells pretreated with 0.2 mM H_2O_2 for 60 min, and stationary-phase cells.

Catalase

Table 1 shows the different catalase activities in each strain under the various conditions, and relative activities are shown in Figure 6. In wild-type cells in exponential phase, the activity of catalase was increased 1.7-fold by treatment with 0.2 mM H_2O_2 for 60 min (the pretreatment condition of the adaptation experiment). It has already been reported that expression of catalase is induced by many kinds of stresses, including oxidative stress, and by entering stationary phase [16,29,30]. Jamieson et al. [28] reported that transcription of the *CTT1* gene was induced 2-fold, and transcription of the *CTA1* gene was slightly induced, by H_2O_2. Each catalase-deficient single mutant, Δ*cta1* and Δ*ctt1*, shows approximately half of the catalase activity of the wild-type in exponential phase. On pretreatment, the catalase activities of these two mutants increased approx. 2-fold; however, each activity was at most equal to or less than that of non-pretreated wild-type cells (Figure 6).

The activity of catalase increased dramatically (more than 20-fold) on transition from the exponential to the stationary phase in wild-type cells. The catalase activities of Δ*cta1* cells and Δ*ctt1* cells in the stationary phase were also increased 6–8-fold in comparison with those in the exponential phase. No activity was detected in acatalasaemic cells under any conditions.

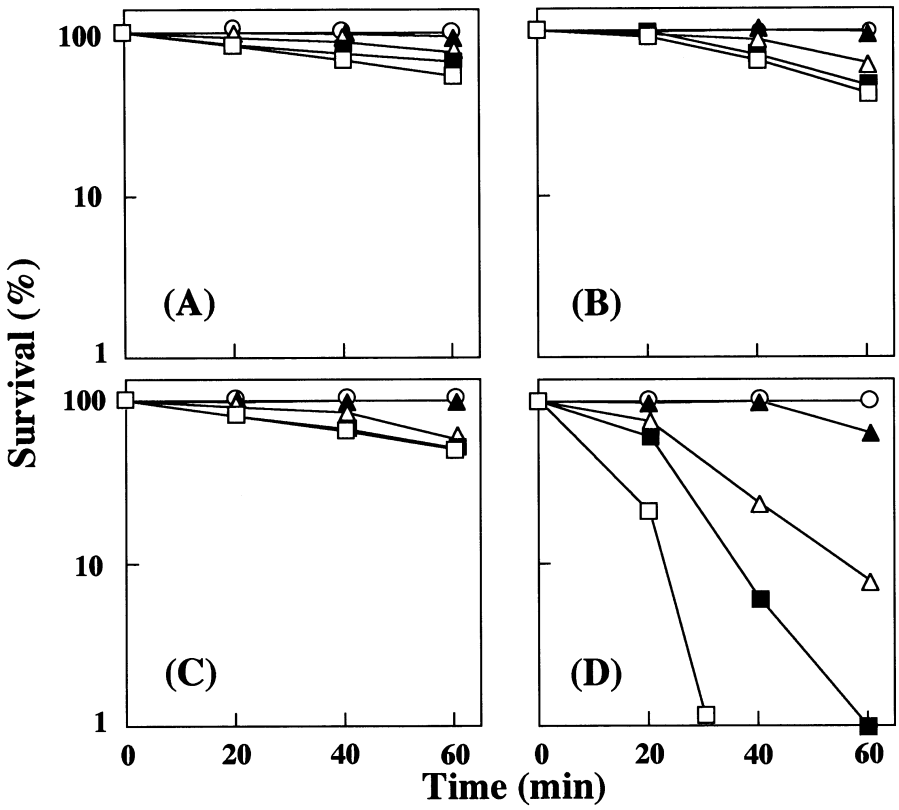

Figure 5 Susceptibility of stationary-phase cells to H_2O_2 stress

Cells in stationary phase were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial $A_{610} = 0.1$ and then treated with various concentrations of H_2O_2. Samples were diluted and plated on YPD agar plates to monitor cell viability. H_2O_2 concentrations (mM): 0 (○), 2 (▲), 5 (△), 10 (■) and 20 (□). Strains: (**A**) YPH250, (**B**) YIT2, (**C**) YTT7 and (**D**) YWT1. Results represent the means of four independent experiments.

Table 1 Effect of conditions on catalase activity in the various *S. cerevisiae* strains

Where applicable, H_2O_2 was added at 0.2 mM for 60 min. One unit of catalase activity is the amount that catalyses the degradation of 1 μmol of H_2O_2/min at 25 °C. Values are means ± S.E.M. of four independent experiments. N.D., not detected.

	Activity (units/mg of protein)		
	Exponential-phase cells		
Strain	$-H_2O_2$	$+H_2O_2$	Stationary-phase cells
YPH250 (wild-type)	1.32 ± 0.28	2.28 ± 0.59	32.21 ± 4.20
YIT2 (Δ*cta1*)	0.61 ± 0.15	1.24 ± 0.41	5.10 ± 1.24
YTT7 (Δ*ctt1*)	0.77 ± 0.33	1.30 ± 0.49	5.23 ± 1.18
YWT1 (Δ*cta1*/Δ*ctt1*)	N.D.	N.D.	N.D.

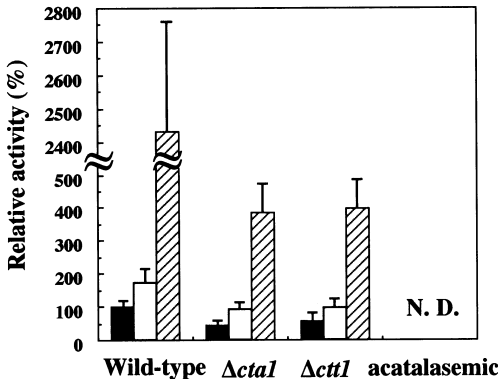

Figure 6 Changes in the relative activities of catalase

The catalase activity of non-treated wild-type cells in exponential phase (1.32 units/mg of protein) was taken as 100%. Cell conditions were as follows: filled bar, exponential-phase cells without pretreatment; empty bar, exponential-phase cells pretreated with 0.2 mM H_2O_2 for 1 h in fresh YPD; hatched bar, stationary-phase cells. N. D., not detected.

CCP

CCP also detoxifies H_2O_2, in addition to catalase. The activities of CCP are shown in Table 2. There was no significant difference in CCP activity between the wild-type and mutants. The CCP activity of each strain was increased 2.4–2.7-fold by pretreatment with 0.2 mM H_2O_2 in the exponential phase, and was increased 4–5-fold by the shift to the stationary phase.

G6PDH

Yeast catalases contain tightly bound NADPH. NADPH prevents compound II accumulation [2]. G6PDH is the key enzyme for the generation of NADPH via the pentose phosphate cycle.

Table 2 Effect of conditions on CCP activity in the various *S. cerevisiae* strains

Where applicable, H_2O_2 was added at 0.2 mM for 60 min. One unit of CCP activity is defined as the amount that oxidizes 1 μmol of cytochrome *c*/min at 25 °C. Values are means ± S.E.M. of four independent experiments.

	Activity (m-units/mg of protein)		
	Exponential-phase cells		
Strain	$-H_2O_2$	$+H_2O_2$	Stationary-phase cells
YPH250 (wild-type)	4.1 ± 0.6	11.2 ± 0.9	18.3 ± 3.4
YIT2 (Δ*cta1*)	4.2 ± 0.1	10.0 ± 0.5	19.8 ± 2.2
YTT7 (Δ*ctt1*)	3.6 ± 0.2	9.7 ± 0.2	18.7 ± 4.3
YWT1 (Δ*cta1*/Δ*ctt1*)	3.5 ± 0.1	9.3 ± 0.7	14.7 ± 2.2

Table 3 Effect of conditions on G6PDH activity in the various *S. cerevisiae* strains

Where applicable, H_2O_2 was added at 0.2 mM for 60 min. One unit of G6PDH activity is defined as the amount that oxidizes 1 μmol of D-glucose 6-phosphate/min at 25 °C. Values are means ± S.E.M. of four independent experiments.

	Activity (m-units/mg of protein)		
	Exponential-phase cells		
Strain	$-H_2O_2$	$+H_2O_2$	Stationary-phase cells
YPH250 (wild-type)	447 ± 7	686 ± 66	320 ± 13
YIT2 (Δ*cta1*)	425 ± 11	793 ± 9	361 ± 15
YTT7 (Δ*ctt1*)	431 ± 51	842 ± 25	349 ± 21
YWT1 (Δ*cta1*/Δ*ctt1*)	545 ± 13	874 ± 50	338 ± 16

The presence of G6PDH and glucose 6-phosphate serves to keep the catalase-bound NADPH fully reduced and to keep catalase fully active. The activities of G6PDH under the various conditions are shown in Table 3. All strains showed similar patterns of G6PDH activity. The G6PDH activity of each strain was increased approx. 1.5-fold by pretreatment with 0.2 mM H_2O_2 in the exponential phase, and was decreased 20–30 % by the shift to the stationary phase.

DISCUSSION

The focus of our study was to clarify the role of catalase in the response to oxidative stress in *S. cerevisiae*. To accomplish this objective, we used and analysed catalase-deficient mutants. In the absence of H_2O_2 stress, acatalasaemic yeast cells showed an identical growth rate to that of wild-type cells (Figure 2). This result indicates that catalase may not function in scavenging endogenous H_2O_2 generated in metabolic reactions such as respiration or β-oxidation in growing yeast cells. It is likely that in *S. cerevisiae* H_2O_2 is practically scavenged in the absence of oxidative stress conditions by CCP or other mechanisms, but not by catalase. It has been suggested that in human erythrocytes all H_2O_2 is practically scavenged by GPx, and not by catalase, under physiological conditions. However, some studies have indicated that catalase is equally important as GPx for the detoxification of H_2O_2 in human erythrocytes [5,7–9]. In *S. cerevisiae* we did not obtain any results supporting the idea that catalase plays a similarly important role to peroxidase under normal conditions. Our results may give a clue to solving the controversy about the role of catalase in erythrocytes.

In the exponential growth phase, there was no difference in susceptibility to H_2O_2 stress between wild-type and mutant yeast cells (Figure 3). Catalase activities in Δ*cta1* and Δ*ctt1* cells were approximately half of that in wild-type cells, and in acatalasaemic cells catalase activity was not detected at all (Table 1, Figure 6). These results indicate that catalase may not be necessary for scavenging for H_2O_2 in actively growing cells. It is well known that the expression of the catalase-encoding genes of *S. cerevisiae* is regulated by glucose and cAMP [15,31]; thus catalase activity in exponential-phase cells is lower than that in stationary-phase cells. Furthermore, there was no significant difference between wild-type and mutant cells in CCP activity (Table 2). These facts further support the idea that catalase may not defend growing cells against H_2O_2 stress. It seems that exponential-phase cells accommodate and respond to H_2O_2 stress using mainly CCP or other systems, but not catalase.

We investigated the role of catalase in the adaptive response to H_2O_2 stress. The acquisition of tolerance by mutant cells on pretreatment with 0.2 mM H_2O_2 was imperfect in comparison with that by wild-type cells (Figure 4). With a 2 mM H_2O_2 challenge (a relatively high concentration of H_2O_2), very little increase in tolerance was observed in mutant cells, especially acatalasaemic cells (Figure 4). Catalase activity was increased 1.5–2.0-fold by the pretreatment in wild-type and single-mutant catalase-deficient cells (Table 1, Figure 6). However, the catalase activities of pretreated single mutants were the same as or lower than that of non-pretreated wild-type cells (Δ*cta1*, 93.9 %; Δ*ctt1*, 98.5 % of the activity of non-pretreated wild-type), and approximately half that of pretreated wild-type cells (Table 1, Figure 6). In acatalasaemic cells catalase activity was not detected at all.

The *de novo* synthesis of at least 21 proteins is increased on adaptation to H_2O_2 in *S. cerevisiae* [32], and the expression of catalase is also inducible by oxidative stress [16,18]. Our experiments confirmed the induction of not only catalase but also CCP activity on adaptation of yeast cells (Table 2). There was no distinct difference in CCP activity among all four strains under any conditions. In each strain, CCP activity was increased 2–3-fold by pretreatment with 0.2 mM H_2O_2 (Table 2). However, a difference was observed in cell survival on subsequent treatment with 2 mM H_2O_2 between wild-type and catalase-deficient mutant cells (Figure 4). Without an increase in catalase activity, i.e. with only the increase in CCP and other defensive activities, it would be almost impossible for the cells to acquire a sufficient increase in tolerance in the adaptive response of *S. cerevisiae*. These results indicate that the induction of catalase is as important as that of CCP in the adaptation response to H_2O_2. Especially when challenged with higher concentrations of H_2O_2, an appropriate increase in catalase activity seems to be necessary. From these results we conclude that not only the increase in CCP activity but also appropriate levels of catalase activity are essential for the cells to acquire a sufficient adaptation response.

On shifting from the exponential to the stationary phase, an increase in tolerance to H_2O_2 stress was observed in all four strains. Even in acatalasaemic yeast cells a remarkable increase in tolerance to H_2O_2 was observed on the shift in growth phase (Figures 3D and 5D). A significant increase in CCP activity was observed in stationary-phase acatalasaemic cells and wild-type cells (Table 2). Thus the increase in tolerance to H_2O_2 in acatalasaemic cells is dependent on the induction of CCP and other defence systems. A difference in susceptibility between wild-type and acatalasaemic cells is reflected by the absence of catalase in the latter, which indicates that catalase is essential in order to acquire maximal tolerance on shifting to the stationary

phase. On the other hand, both of single-mutant cells, Δ*cta1* and Δ*ctt1*, showed a similar susceptibility to H_2O_2 to that of wild-type cells in the stationary phase. There were no significant differences in the CCP and G6PDH activities of these cells, and the catalase activities of Δ*cta1* cells and Δ*ctt1* cells in the stationary phase were increased 6–8-fold in comparison with those in the exponential phase. These results suggest that a catalase activity of approx. 5.00 units/mg of protein is enough in order to acquire maximal tolerance to H_2O_2 on shifting to the stationary phase. Furthermore, the localization of catalase, in peroxisomes or in the cytosol, apparently has little or no effect on susceptibility to H_2O_2, and catalase A and catalase T seem to be able to compensate for the absence of each other in the defence of yeast cells against H_2O_2 stress.

G6PDH activity was also increased approx. 1.5-fold on pretreatment with 0.2 mM H_2O_2, but in contrast it was decreased in the stationary phase in all strains (Table 3). We previously reported that intracellular glutathione plays an important role in the adaptive response in *S. cerevisiae*, and G6PDH is induced to recycle glutathione [33]. In addition, G6PDH is essential for defence against oxidative stress in the mouse [34]. As shown in Table 3, there was no significant difference in G6PDH activity between wild-type and mutant cells, and an increase in activity on H_2O_2 pretreatment was observed even in catalase-deficient mutant cells. In the stationary phase a decrease in G6PDH activity was observed, in contrast with the increase in catalase activity (Tables 1 and 3). An effect of catalase deficiency on the change in G6PDH activity was not observed in this study.

In *E. coli*, expression of the catalase genes (*katE* and *katG*) is induced in the stationary phase and by H_2O_2 stress under the control of RpoS and OxyR [35,36]. On the other hand, it has been reported that the protective effect of bacterial catalase is cell-density-dependent. Thus under low-cell-density conditions catalase deficiency has no effect on the susceptibility to H_2O_2 of bacterial cells [10,11,37]. It is thought that catalase is able to decrease H_2O_2 by a mass effect at high cell density. At low cell density, catalase is incapable of maintaining an internal/external concentration gradient of H_2O_2, since the rate of H_2O_2 permeation into each individual cell appears to exceed the rate of degradation by catalase. In the present study, all experiments on susceptibility and adaptation were carried out at low cell density (2×10^6 cells/ml). Surprisingly, in yeast cells in stationary phase, the effect of catalase on H_2O_2 stress was observed even under conditions of low cell density. Acatalasaemic yeast cells in stationary phase were distinctly more sensitive to H_2O_2 stress than wild-type and single-mutant cells at low cell density (Figure 5). Moreover, catalase played an important role in the acquisition of tolerance in the adaptation of exponential-phase cells (Figure 4). Thus in *S. cerevisiae* catalase may protect cells even at low cell density in the adaptative response to H_2O_2 and in stationary-phase cells. It seems that there is a difference in the role of catalase between yeast and bacterial cells. A difference in the role of glutathione in the response to oxidative stress has also been observed between *E. coli* and *S. cerevisiae* [33,38]. The defence mechanism of yeast seems to be more complicated than that of bacterial cells [39]. The differences in the stress-response systems may have an interesting relationship with the evolution of the organisms, as suggested by Ma and Eaton [10].

In conclusion, in *S. cerevisiae* catalase does not play an important role in the detoxification of H_2O_2 under physiological conditions. However, under conditions of stress, in the stationary phase or in the adaptive response, catalase is necessary and plays an important role in the acquisition of a sufficient increase in tolerance to H_2O_2. Thus catalase seems to be necessary in an 'emergency'. Further studies on role of catalase in the response to other kinds of stress are under way in order to clarify the function of catalase in *S. cerevisiae*.

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