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Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*

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ABSTRACT **The cause for death after lethal heat shock is not well understood. A shift from low to intermediate temperature causes the induction of heat-shock proteins in most organisms. However, except for HSP104, a convincing involvement of heat-shock proteins in the development of stress resistance has not been established in *Saccharomyces cerevisiae*. This paper shows that oxidative stress and antioxidant enzymes play a major role in heat-induced cell death in yeast. Mutants deleted for the antioxidant genes catalase, superoxide dismutase, and cytochrome *c* peroxidase were more sensitive to the lethal effect of heat than isogenic wild-type cells. Overexpression of catalase and superoxide dismutase genes caused an increase in thermotolerance. Anaerobic conditions caused a 500- to 20,000-fold increase in thermotolerance. The thermotolerance of cells in anaerobic conditions was immediately abolished upon oxygen exposure. HSP104 is not responsible for the increased resistance of anaerobically grown cells. The thermotolerance of anaerobically grown cells is not due to expression of heat-shock proteins. By using an oxidation-dependent fluorescent molecular probe a 2- to 3-fold increase in fluorescence was found upon heating. Thus, we conclude that oxidative stress is involved in heat-induced cell death.**

Most living cells are sensitive to sudden heat exposure. A shift in temperature from a low to an intermediate temperature induces the stress response or heat-shock response (1–3), which is considered to be an evolutionarily conserved genetic system advantageous to living organisms. After a temperature shift from 23 to 37°C in cells of the yeast *Saccharomyces cerevisiae*, 80 proteins were transiently induced; 20 of these proteins are now classified as major heat-shock proteins (HSPs) (2). Some of these HSPs have been characterized, but the function of many of them is still unclear (4).

Initial studies suggested that HSPs play an essential role in the acquisition of stress tolerance. On the other hand, a convincing involvement of HSPs in the development of stress resistance has not been established in yeast. Except for *HSP104*, none of the other HSP disruption mutants show any block in the acquisition of stress resistance in yeast (5). Furthermore, a yeast strain with a temperature-sensitive mutation in the heat-shock factor (*hsfl-m3*) that leads to a general block in heat-shock-induced protein synthesis was not affected in the acquisition of thermotolerance (6). Therefore, HSPs may not be important for stress tolerance acquisition but rather for a rapid recovery after heat shock (4).

The main factors causing death after heat exposure are still unknown. Thus, the heat-shock response may not elucidate why cells die in response to heat exposure but rather how they repair the damage afterwards. To investigate why cells die in response to heat exposure, we completely avoided the induction of the heat-shock response by exposing cells immediately to lethal heat. In particular, we investigated the possible involvement of oxidative stress in the lethal effect of heat exposure.

Aerobic organisms must deal with reactive oxygen species that are generated during the normal course of aerobic metabolism to avoid oxidative damage. Thus, cellular antioxidants and enzymes capable of rapidly detoxifying active oxygen have evolved. Active oxygen species, including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•), are noted for their high reactivity and resultant damage to proteins, lipid membranes, and DNA (7), and some circumstantial evidence implicates active oxygen species with hyperthermia (8). The enzymatic reduction of superoxide anions to hydrogen peroxide and its subsequent removal is facilitated in yeast by superoxide dismutase, catalases, and cytochrome *c* peroxidase (9–11).

We show that mutants deficient in the key antioxidant enzymes catalase, superoxide dismutase (SOD), and cytochrome *c* peroxidase were sensitized to a 50°C heat exposure. Overexpression of catalase and SOD led to a protection from lethal heat shock. Anaerobically grown cells were several orders of magnitude more resistant to lethal heat shock, and this protection was immediately abolished upon air exposure. Anaerobic cultures of antioxidant gene-deletion mutants or antioxidant gene overexpression strains did not differ significantly in their sensitivity. Intracellular conditions became pro-oxidant during lethal heating when measured by an oxidant-sensitive fluorescent probe, and overexpression of antioxidant enzymes considerably reduced probe oxidation.

MATERIALS AND METHODS

Strains. Yeast strains used in this study are described in Table 1. *Escherichia coli* strain DH5α [*supE44ΔlacU169*-(*ϕ80laczΔM15*)*hsdR17recAendA1gyrA96thi-1relA1*] was used for all DNA propagations.

Media. Growth and minimal media were prepared as described (15).

Molecular Biology Procedures. Large- and small-scale plasmid isolations from *E. coli* were prepared using DNA isolation kits (Promega); *E. coli* transformation and electrophoresis of DNA were performed as described (16). Transformation of yeast was carried out by treating intact cells with lithium acetate to promote DNA uptake (17, 18).

Plasmid Construction. DNA handling and manipulations, including restriction enzyme digests, DNA ligations, and other cloning procedures, were carried out as described (16). Plasmid p7308 containing the *S. cerevisiae* catalase T gene (*CTT1*) was obtained from W. Spevak (19) as a *Hin*dIII fragment in plasmid YEp13. The 195CTT1 plasmid was created from p7308 by subcloning the *Hin*dIII fragment containing the

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Abbreviations: HSP, heat-shock protein; SOD, superoxide dismutase.

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Table 1. Strains used

Strain	Genotype	Ref.
RS112	*MATa ura3-52 leu2-3,112 trp5-27 arg4-3 ade2-40 ilv1-92 HIS3::pRS6 LYS2* *MATα ura3-52 leu2-Δ98 TRP5 ARG4 ade2-101 ilv1-92 his3Δ200 lys2-801*	12
DBY 747	*MATa leu2 his3 trp1 ura3*	13
GA74T-1A	*MATa leu2 ura3::CTT1-lacZ his3 trp1 ade8 cta1-2 can1 ctt1::URA3*	13
W303-1a	*MATa ade2-1 can1-110 trp1-1 his3-11,15 leu2-3,112 ura3-1*	11
ccp1::HIS3	*MATa ade2-1 can1-100 trp1-1 his3-11,15 leu2-3,112 ura3-1 ccp1::HIS3*	11
ccp1::URA3	*MATa ade2-1 can1-100 trp1-1 his3-11,15 leu2-3,112 ura3-1 ccp1::URA3*	11
W303-1a Δhsp104	*MATa ade2-1 can1-100 trp1-1 his3-11,15 leu2-3,112 ura3-1 hsp104::LEU2*	5
EG103	*MATa leu2-3,11 his3 trp1-289 ura3-52*	14
EG118	*MATa leu2-3,11 his3 trp1-289 ura3-52 sod2ΔA::TRP1*	14
EG110	*MATa leu2-3,11 his3 trp1-289 ura3-52 sod1ΔA::URA3*	14

CTT1 gene into the unique *Hin*dIII site of the plasmid YEplac195.

The *S. cerevisiae* peroxisomal catalase gene (*CTA1*) was also obtained from W. Spevak (19), in the *Eco*RI site of the multicopy plasmid YEp352. The centromere-containing plasmid was created by releasing the 2.7-kb *Eco*RI fragment containing the entire gene sequence and cloning it into the *Eco*RI site of the centromere-containing plasmid pRS413 (Stratagene) to create pRS413-CTA1. The 195CTA1 plasmid was constructed in the same way by cloning the 2.7-kb *CTA1*-containing fragment into the unique *Eco*RI cloning site of the multicopy plasmid YEplac195.

The *S. cerevisiae* copper-zinc SOD gene (*SOD1*) was kindly donated by E. Gralla (14). A 2.05-kb *Sph* I fragment containing the complete gene was initially cloned into the *Sph* I cloning site of the multicopy plasmid YEp352E. It was recloned into the *Sph* I site of the multicopy plasmid YEplac195 to form the plasmid 195SOD1.

Plasmid 195SOD1CTT1 was constructed by cloning the *Hin*dIII/*Bam*HI *CTT1* fragment obtained from the 195CTT1 plasmid into the yeast shuttle vector pRS406 (Stratagene) at the unique *Hin*dIII/*Bam*HI sites. The *CTT1* gene was released by restriction digest with the enzymes *Sal* I/*Spe* I. This was to enable plasmid p195SOD1 to be opened at the multiple cloning site by restriction with *Xba* I/*Sal* I, enabling insertion of the *CTT1* gene into the 195SOD1 plasmid without disturbing the *SOD1* gene.

195SOD1CTA1 was created by digesting the 195CTA1 vector with *Sph* I and inserting the *SOD1* 2.05-kb *Eco*RI fragment.

Mutations in Antioxidant Genes. EG118 (*sod1*) mutant was made by one-step gene deletion/replacement in EG103 using the *URA3* marker as described (14). EG110 (*sod2*) was made in a similar manner using the *TRP1 Eco*RI/*Bgl* II fragment (end filled) to replace the *Spe* I/*Nru* I fragment of the *SOD2* gene as described (20). Catalase mutants GA74-1A described in legend to Fig. 1. Cytochrome *c* peroxidase mutants were made by one-step gene deletion/replacement in W303-1a using both *HIS3* marker or the *URA3* markers as described (11).

Catalase and SOD Assay. Catalase activity was measured spectrophotometrically by the disappearance of H_2O_2 at 240 nM (21). SOD activity was measured as described (22). For all assays, yeast strain RS112 (Table 1) was grown overnight at 30°C in 300 ml of YPAD medium to a cell density of 2×10^7 cells per ml. Cells were washed in 0.87% saline and concentrated to 1×10^9 cells per ml. Aliquots (150 μl) were prepared in 1.5-ml Eppendorf tubes, and cell lysis with glass beads was achieved by six 30-sec periods of vigorous Vortex mixing. Tubes were maintained on ice between each Vortex mixing. Cellular debris was removed by two 10-min centrifugation steps at $3000 \times g$. The supernatant was collected after each spin, and the resultant crude lysate was assayed. Protein levels were determined by the method of Bradford (23) using reagents purchased from Bio-Rad.

Lethal Heat Survival Assay. Strains were grown to mid-logarithmic phase (5×10^6 cells per ml) in YPAD medium, washed in 0.87% saline, and concentrated to 2×10^8 cells per ml. Aliquots of 100 μl were prepared in PCR Eppendorf tubes for each time point and placed on ice. Unless otherwise stated, lethal heating was performed in a PCR thermal cycler set with maximum temperature ramping to 50°C and taking ≈80 sec to reach 50°C from room temperature. Time points were taken with respect to the time that the samples reached 50°C. Tubes were removed at set time points and immediately placed on ice. The aliquots were then diluted accordingly and plated onto solid YPAD. Colonies were counted after 3 days of incubation at 30°C, and the percent viability was calculated with respect to cells not heated.

Anaerobic Lethal Heat Survival Assay. Anaerobic media were prepared by autoclaving in flasks stopped with rubber bungs and crimped metal caps (Baxter Scientific Products, McGaw Park, IL); the flasks were flushed with nitrogen. All cultures were grown in media containing 0.01% resazurin (Sigma) as an oxygen indicator at 30°C, which turns red upon exposure to oxygen and becomes reduced during autoclaving (24). Flushing with nitrogen and inoculation were achieved via needle injection through the rubber bung. Cultures were maintained anaerobically for growth and heat-shock manipulations in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). The lethal heating survival assay was done as described in Fig. 1.

Oxygen Exposure Reoxygenation Experiment. A lethal heating survival assay with mid-logarithmic phase anaerobic cultures (5×10^6 cells per ml) was performed with the exception that all manipulations were performed within an Atmosbag (Aldrich) previously flushed with nitrogen gas. Cultures were then exposed to the atmosphere, shaken vigorously, and immediately exposed to 50°C in a thermal cycler as described above.

Measurement of *in Vivo* Molecular Oxidation. Direct molecular evidence of *in vivo* intracellular oxidation using the oxidant-sensitive probe 2′,7′-dichlorofluoroscin diacetate was used to measure the levels of oxidation developed during lethal heating at 50°C. Fluorescence was measured in strain RS112 (Table 1) transformed with plasmids YEplac195, 195SOD1, 195CTT1, and 195SOD1CTT1 (see Table 2) using a Spex Industries (Edison, NJ) spectrofluorimeter set at an excitation wavelength of 504 nm and an emission wavelength of 524 nm (25). As a control, the fluorescence was recorded over 10 min at 50°C without any cells and with cells previously killed at 50°C (data not shown). Cells were grown overnight at 30°C with shaking in 50 ml of liquid SC medium minus uracil medium to 1×10^7 cells per ml. The next day, the cells were counted and diluted in 10 ml of fresh YPAD liquid medium to a final concentration of 0.5×10^7 cells per ml and allowed to double at 30°C with shaking. A 5-mM stock solution of

dichlorofluoroscin diacetate dissolved in ethanol (20 μl) was added to each culture 15 min before the assay and allowed to incubate at 30°C. One-milliliter volumes of culture were concentrated to 100 μl and heated for 0, 2, 4, 6, 8, and 10 min in a thermal cycler at 50°C. Immediately after lethal heating, the cells were cooled on ice. They were washed twice in ice-cold distilled water, resuspended in 200 μl of water, and kept on ice. Cells were lysed by agitation with a Vortex mixer at maximum speed for 5 min. The supernatant was obtained after centrifugation in a microcentrifuge for 10 min. Crude extract (70 μl) was suspended in 2.5 ml of water, and fluorescence was recorded at an excitation wavelength of 504 nm and an emission wavelength of 524 nm.

RESULTS

We investigated whether oxidative stress contributed to the lethality of lethal heat shock in *S. cerevisiae* cells. To do this, we avoided the adaptive response in our experiments by a rapid shift of cells grown in liquid medium at 30°C to a lethal heat shock of 50°C for various periods of time. This procedure did not leave time for the expression of HSPs.

Mutations in Antioxidant Genes Sensitize Cells to Lethal Heat shock. The effect of mutations in the genes coding for cytoplasmic and mitochondrial SOD (*SOD1, SOD2*) (10), peroxisomal catalase A (*CTA1*), cytosolic catalase T (*CTT1*) (26), and mitochondrial cytochrome *c* peroxidase (*CCP1*) (11) on viability after lethal heat shock was determined. A SOD^+ wild-type strain, the isogenic *sod1* deletion strain, and the isogenic *sod2* deletion strain were subjected to lethal heating conditions. Wild-type cells showed a survival of 20×10^{-6} after 60 min compared with the *sod1* deletion, which showed a survival of 5×10^{-6}, and the *sod2* deletion, which showed a survival of 10×10^{-6} (Fig. 1*C*). Isogenic yeast mutants *cta1* or *ctt1* as well as double mutant *cta1, ctt1* were used (Fig. 1*A*). The *ctt1* mutant survival levels were 5×10^{-4} and the *cta1* mutant survival levels were 0.5×10^{-4} compared with the $CTT1^+CTA1^+$ strain survival of 1.5×10^{-4} after 60 min at 50°C. The double mutant *cta1, ctt1* showed an enhancement of heat sensitivity and had 0.11×10^{-4} surviving cells after 60 min at 50°C. A *CCP1* wild-type strain and two different isogenic *ccp1* disruption mutants were used. Both *ccp1* disruption mutants were more sensitive after 60-min heat shock, with survivals of 3×10^{-5} and 4×10^{-5} for the mutants compared with 93×10^{-5} for the wild-type strain (Fig. 1*B*).

Overexpression of Antioxidant Genes Protects Cells Against Lethal Heating. The effect of expression of both genes *CTT1* and *SOD1* from a multicopy plasmid on viability after lethal heat shock was determined. The specific activities of catalase (21) and SOD (27) were measured in extracts from cells carrying the antioxidant genes on multicopy plasmids. Cells with the multicopy plasmid containing the *CTT1* gene showed a 3-fold higher specific activity of catalase, and cells containing the multicopy plasmid with the *SOD1* gene showed about a 3-fold higher SOD activity than the cells with only the vector (Table 2).

Cells overexpressing *CTT1* and both *CTT1* and *SOD1* were considerably protected after 60 min at 50°C. Cells overexpressing *CTT1* showed 90×10^{-6} survival and cells overexpressing *CTT1* and *SOD1* showed 110×10^{-6} survival compared with 4×10^{-6} survival of cells containing the YEplac195 vector alone.

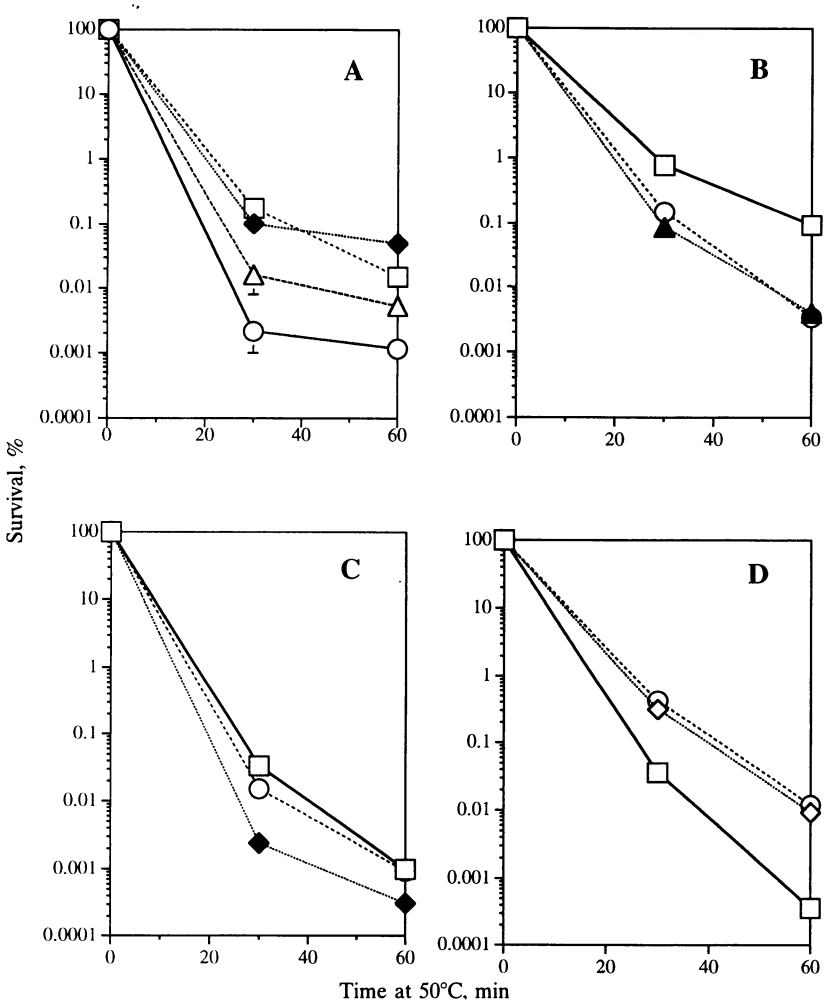

FIG. 1. Lethal heat survival assay. Strains were grown to late logarithm (2×10^7 cells per ml) in SC medium, washed in 0.87% saline, and concentrated to 2×10^8 cells per ml. Aliquots of 1 ml were prepared in Eppendorf tubes for each time point and placed on ice. Lethal heating was performed in a water bath set at 50°C for times 0, 30, and 60 min. All other manipulations were as described. All results are shown as the mean ± SEs of four experiments. In some cases, error bars are smaller than symbols. (*A*) Thermosensitivity of catalase mutants. Isogenic strains GA74-1A *MATa leu2 ura3::CTT1-lacZ his3 trp1 ade8 cta1–2 can1* (*cta1*) (△) and GA74T-1A [same as GA74-1A with *ctt1::URA3* (*cta1, ctt1*)] (13) (○). Plasmid pRS413-CTA1 was used to complement the *cta1* mutation in strains GA74-1A and GA74T-1A to give rise to the isogenic wild-type (□) and *ctt1* mutant (◆) strains. (*B*) Thermosensitivity of cytochrome *c* peroxidase mutants. W303-1a (□) and deletion mutants W303-1a ccp1::HIS3 (▲) and W303-1a ccp1::URA3 (○) were used (11) (Table 1). (*C*) Thermosensitivity of superoxide dismutase mutants. Isogenic strains EG103 (SOD^+) (□), EG118 (*sod1*Δ) (◆), and EG110 (*sod2*Δ) (○) were used (14, 20) (Table 1). (*D*) Thermotolerance of strains with antioxidant genes on multicopy plasmids. Yeast strain RS112 (Table 1) was transformed with plasmids YEplac195 (□), 195CTT1 (◇), and 195SOD1CTT1 (○). (Error bars ± 1 SE.)

Table 2. Catalase and SOD enzyme activity

Plasmid	Catalase specific activity, units/mg of protein	SOD specific activity, units/mg of protein
YEplac195	0.75	118
195CTT1	2.42	ND
195SOD1	0.98	437
195SOD1CTT1	2.27	ND

Strain DBY747 (for genotype, see Table 1) was grown in YPD to 5 × 10^6 cells per ml. A crude cell extract was prepared, and enzyme assays were done as described. Protein was measured by the Bradford assay using bovine serum albumin as a protein standard. Results are the means of two experiments. ND, not determined.

To further investigate whether oxidative stress is involved in the early events in heat lethality, cells were exposed for 1 to 5 min to 50°C. Cells overexpressing both *CTT1* and *SOD1* showed a resistant shoulder after 1 min of heat exposure with no death seen, whereas <40% survival was observed in wild-type cells. An LD_{50} difference of 2- to 3-fold can be estimated. (Fig. 2 *A–C*).

Two more yeast strains were used. A protective effect of *SOD1* or the human manganese SOD (expressed under the control of a yeast promoter) alone was seen in two out of three strains; however, protection was always observed when both SOD and catalase were expressed simultaneously.

It was determined whether acquiring thermotolerance after overexpression of the antioxidant genes was indirectly due to expression of HSPs. Proteins labeled metabolically with [^{35}S]methionine were separated using SDS/PAGE with extract from strains containing control vectors or the overexpression constructs incubated at 25°C and after a shift to 39°C. The major HSPs were induced after the shift to 39°C, but there was no difference between lanes from strains containing the overexpression constructs vs. the vector alone (data not shown).

Effect of Anaerobiosis on Heat Lethality. The effect of anaerobic conditions on viability after lethal heating was determined. Wild-type cells as well as strains containing the *sod* and *ccp1* mutations and the *SOD1* and *CTT1* multicopy plasmids were grown anaerobically and aerobically to mid-logarithmic phase and exposed to 50°C for 30 min. Remarkably, all strains showed 20–40% viability (Table 3). Compared with the aerobically grown cells, this result means an increase in heat resistance of 500-fold for the wild-type cells to >20,000-fold for the *sod1* deletion strain. Under these anaerobic conditions, no obvious difference in heat resistance was found for strains containing deletions or overexpression constructs of the antioxidant genes.

Table 3. Resistance of anaerobic cells to a 50°C heat shock for 30 min

Strain	Anaerobic, % viability	Aerobic, % viability
DBY747	24.23 ± 1.23	0.035 ± 0.004
DBY747 195CTT1	14.50 ± 2.65	0.315 ± 0.051
DBY747 195SOD1	24.64 ± 0.54	0.211 ± 0.005
DBY747195SODCTT1	9.68 ± 6.08	0.421 ± 0.022
EG103	31.25 ± 14.67	0.034 ± 0.005
EG110	41.72 ± 19.76	0.002 ± 0.003
EG118	22.90 ± 11.12	0.015 ± 0.010
W3031A	32.37 ± 12.84	0.770 ± 0.078
W3031A ccp1::HIS3	36.35 ± 24.62	0.090 ± 0.016
W3031A ccp1::URA3	28.66 ± 20.54	0.140 ± 0.030

Cultures were maintained anaerobically for all growth and heat-shock manipulations. The lethal heat survival assay was performed as described above. Results are the means of two experiments (errors ± 1 SD).

The thermotolerance associated with anaerobiosis may involve a number of factors involved with the shift in metabolism. Efforts to disseminate the influence of oxygen from the metabolic shift to fermentation have involved growing cells aerobically in 20% glucose to produce anaerobic-like conditions of metabolism. Under these conditions, cells were actually more sensitive to a 50°C heat shock (data not shown). To investigate more directly whether metabolic conditions under anaerobiosis or the presence of oxygen caused the sensitivity of aerobic cells, we did reoxygenation experiments. Anaerobic cultures exposed to the atmosphere were immediately (at least within 5 sec) sensitized to lethal heat. With the conditions employed for these reoxygenation experiments, survival levels after 10 min at 50°C were 0.93% ± 0.78% for anaerobic cells compared with 0.006% ± 0.0013% for the same culture exposed briefly to the atmospheric oxygen. These data strongly suggest that oxygen is the major causative agent for the lethal effect of heat.

If HSPs were induced under anaerobic conditions, the above result would be less surprising. An involvement in acquisition of thermotolerance has so far been shown only for HSP104 (5). However, the *hsp104* deletion mutant was also >1000 times more resistant to a lethal heat shock at 50°C for 10 min when

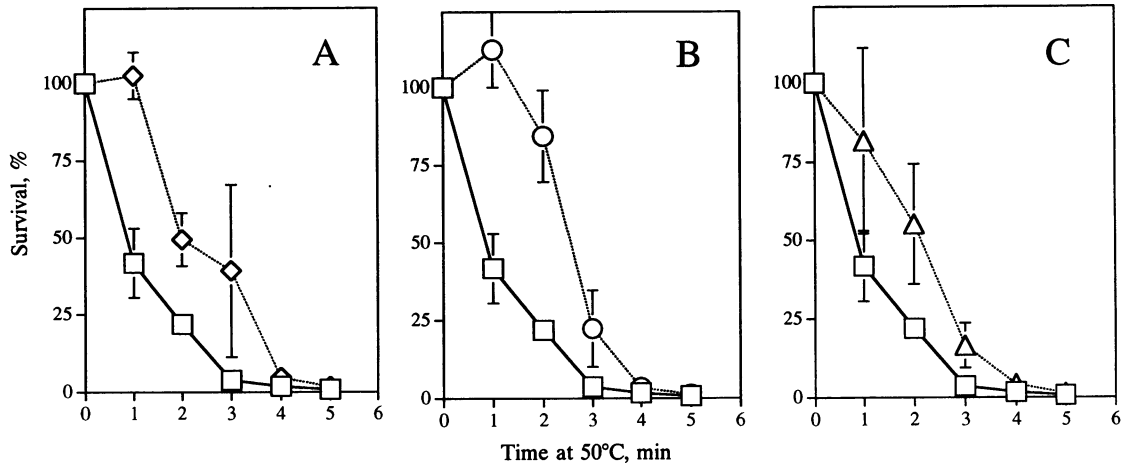

FIG. 2. Antioxidant overexpression LD_{50}. (*A–C*) Strain RS112 (12) (Table 1) was transformed with plasmids YEplac195 (□), and (*A*) 195SOD1CTT1 (◇), (*B*) 195CTT1 (○), and (*C*) 195SOD1 (△) (Table 2) were grown in SC medium lacking uracil to 5 × 10^6 cells per ml. Cells were washed as described and heated for times 0, 1, 2, 3, 4, and 5 min in a PCR thermal cycler. After heating, tubes were immediately cooled on ice and plated on YPAD plates. Because of the linear scale, differences in survival levels at <1% have overlapping symbols and are better shown in Fig. 1*D*.

grown in the absence of oxygen than when grown aerobically. Therefore, *HSP104* is not involved in the acquisition of thermotolerance under anaerobic conditions (data not shown). In addition, proteins labeled metabolically with [^{35}S]methionine were separated using SDS/PAGE with extract from strains grown aerobically and anaerobically. The major HSPs were not induced under anaerobic conditions at 23°C (data not shown).

Oxidation of an Intracellular Fluorescent Probe During Lethal Heating. Direct molecular evidence of *in vivo* intracellular oxidation using the oxidant-sensitive probe 2′,7′-dichlorofluoroscin diacetate was used to measure the levels of oxidation developed during lethal heating at 50°C. Deacetylation by esterases to dichlorofluoroscin occurs within the cell, and subsequent oxidation to dichlorofluoroscein can be measured spectrofluorimetrically (25). A 2- to 3-fold increase in fluorescence evolved over 10 min in cells containing the control plasmid compared with samples containing no cells (Fig. 3). Cells previously killed by exposure to 50°C for 4 hr did not increase in fluorescence (data not shown). Furthermore, this increase in fluorescence was reduced by 2-fold in cells containing the antioxidant genes *CTT1* and *SOD1* (Fig. 3). The same experiment was performed with cells overexpressing *CTT1* or *SOD1* alone, revealing that *CTT1* overexpression may be responsible for most of the decrease in fluorescence (Fig. 3).

DISCUSSION

Much of the focus of investigations into the effect of stress conditions on living organisms has centered on the investigations of the acquisition of thermotolerance in yeast. These investigations have tremendously enhanced our understanding of the stress response and how the cell reacts to heat shock; however, the initial cause for the lethal effect of heat is still unknown. Therefore, in our experiments we avoided the adaptive response (expression of heat-shock genes), and cells incubated in liquid medium at 30°C were immediately exposed to 50°C.

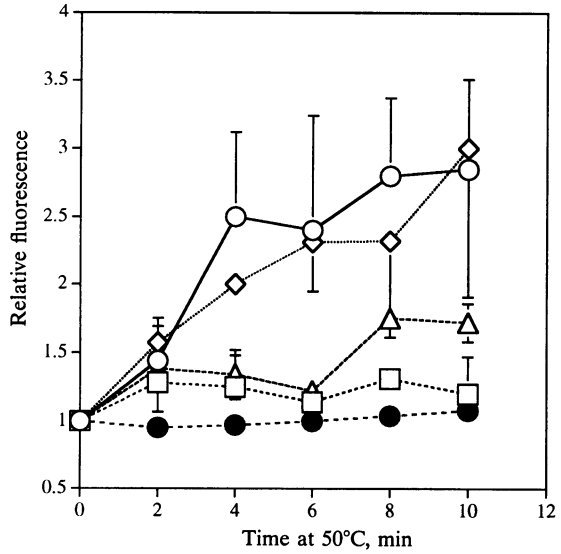

FIG. 3. Measurement of *in vivo* molecular oxidation. Fluorescence was measured in crude cell extracts from strain RS112 (Table 1) transformed with plasmids YEplac195 (○), 195SOD1CTT1 (△), 195SOD1 (◇), and 195CTT1 (□) (see Table 2) as described. As a control, the fluorescence was recorded over 10 min at 50°C without any cells (●) and with cells previously killed by 50°C for 4 hr (data not shown). Fluorescence was recorded at an excitation wavelength of 504 nm and an emission wavelength of 524 nm. The result is the mean of two experiments. (Error bars ± 1 SE.)

All our results support the possibility that oxidative stress plays a major role in the lethal effect of heat in eukaryotes. Most important, viability was improved 500- to 20,000-fold under anaerobic conditions, and surprisingly this protection was completely and immediately removed upon exposure to air. Anaerobic cultures of mutants in the major antioxidant enzymes still showed ≈30% viability after 30 min at 50°C. This percentage is higher than the gain in thermotolerance reported by pretreatment of cells at 37°C and induction of HSPs. This result, in fact, may argue that most of the lethal effect of heat at this temperature is caused by oxidative stress.

Other results support this interpretation. Cells deleted for oxidant genes were more sensitive to lethal heat shock, and cells overexpressing antioxidant genes were more resistant to lethal heat shock. However, under anaerobic conditions, no effect on the expression level of the antioxidant genes was found. In addition to these results, we have demonstrated heat-dependent intracellular oxidation by following the fluorescence of an oxidation-sensitive probe during lethal heat exposure. Oxidative fluorescence induced by heat is quenched in cells overexpressing antioxidant enzymes.

While this manuscript was under review, it was shown that the aerobic heat sensitivity is greatly enhanced in *sodA* and *sodB* mutants in *E. coli* (27) and anaerobic conditions alleviate this lethality. Also, the addition of a manganic porphyrin SOD mimic has been shown to protect *sodA* and *sodB* mutants in *E. coli* cells from O_2^- lethality during stationary phase and lethal heating at 42°C (28). In these experiments, oxidative stress (in particular O_2^-) is implicated in prokaryote cell killing during a lethal heat shock.

Several signals within the cell, such as AppppA (29), at least five genes (30), and potassium channels (31), are induced by heat as well as by oxidative stress. Furthermore, antioxidant genes such as *CTT1* in yeast (13) and *SOD* in *E. coli* (28, 32) are induced after heat shock. There may be many different reasons explaining the heat-induced oxidative stress. Upon heating, several molecules within the cell, such as iron (33), sulfhydrol compounds (34), or polyamine oxidation (35), may induce oxidative stress. It has been shown for instance that concentrations of iron, which were not toxic at 37°C, became toxic in a dose-dependent fashion during hyperthermia treatment in Chinese hamster ovary cells (33). In addition, lethal heat shock stimulates polyamine oxidation that generates hydrogen peroxide in mammalian cells (35). Furthermore, it has been shown that the reactivity of superoxide increases upon heating (36). Each of these examples depends upon the presence of oxygen. Alternatively, heat-induced protein denaturation may lower the normal level of cellular antioxidant enzymes with the consequence of oxidative stress.

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