Protective Role of Mitochondrial Superoxide Dismutase against High Osmolarity, Heat and Metalloid Stress in *Saccharomyces cerevisiae*

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**ABSTRACT.** Superoxide dismutases, both cytosolic Cu,Zn-SOD encoded by *SOD1* and mitochondrial Mn-SOD encoded by *SOD2*, serve *Saccharomyces cerevisiae* cells for defense against the superoxide radical but the phenotypes of *sod1Δ* and *sod2Δ* mutant strains are different. Compared with the parent strain and the *sod1Δ* mutant, the *sod2Δ* mutant shows a much more severe growth defect at elevated salt concentrations, which is partially rescued by 2 mmol/L glutathione. The growth of all three strains is reduced at 37 ºC, the *sod2Δ* showing the highest sensitivity, especially when cultured in air. Addition of 1 mmol/L glutathione to the medium restores aerobic growth of the *sod1Δ* mutant but has only a minor effect on the growth of the *sod2Δ* strain at 37 ºC. The *sod2Δ* strain is also sensitive to As(III) and As(V) and its sensitivity is much more pronounced under aerobic conditions. These results suggest that, unlike the Sod1p protein, whose major role is oxidative stress defense, Sod2p also plays a role in protecting *S. cerevisiae* cells against other stresses – high osmolarity, heat and metalloid stress.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PDS</td>
<td>post-diauxic-shift (element)</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase (EC 1.15.1.1)</td>
</tr>
<tr>
<td>Cu,Zn-SOD</td>
<td>cytosolic superoxide dismutase</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>mitochondrial superoxide dismutase</td>
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<tr>
<td>STRE</td>
<td>stress response (element)</td>
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</table>

Aerobic organisms are continuously exposed to various ROS produced as a result of their own metabolism or exposure to environmental stress. Such ROS as the superoxide radical anion, hydrogen peroxide and hydroxyl radical can cause oxidative damage to proteins, lipids and DNA leading to impairment of cell growth and even to cell death (Sigler et al. 1999; Sturtz and Culotta 2002). Cells of *S. cerevisiae* contain two SOD, cytosolic Cu,Zn-SOD encoded by *SOD1* and mitochondrial Mn-SOD, encoded by *SOD2*, that play a role in the protection against the superoxide radical, whose main source is thought to be mitochondrial respiration (Longo et al. 1996; Jamieson 1998). Mutants in both of these genes show high sensitivity to hyperoxic conditions; *sod1Δ*, but not *sod2Δ*, grows very poorly even under atmospheric oxygen (Gralla and Kosman 1992). In addition, the *sod1Δ* mutant (not *sod2Δ*) shows lysine and methionine auxotrophy when grown in air and is more sensitive than *sod2Δ* to redox cycling agents such as paraquat (Sturtz and Culotta 2002). Strains devoid of SOD2 grow slowly on nonfermentable carbon sources and show high sensitivity to ethanol (Longo et al. 1996; Costa et al. 1997). Like other ROS-induced adverse effects, these growth defects can be rescued by externally added antioxidants (Krasowska et al. 1999, 2000, 2006). Both SOD are essential for stationary phase survival in baker’s yeast, with Mn-SOD being more important under low aeration conditions (Longo et al. 1996). Overexpression of both SOD extends the survival of yeast cells by ≈30 % (Fabrizio et al. 2003). It thus appears that Mn-SOD protects the mitochondria from ROS generated during respiration and Cu,Zn-SOD, acting in the cytoplasm, is the major enzyme protecting cells from exogenous oxidant agents. The Cu,Zn-SOD is also present in the intermembrane space of yeast mitochondria, and protects mitochondrial proteins, especially those with 4Fe–4S clusters, from oxidative damage (Sturtz et al. 2001; Wallace et al. 2004).

Cytoplasmic and mitochondrial SOD have also been characterized in other fungi. In *Candida albicans* Cu,Zn-SOD is essential for filamentous growth and virulence and also for protection against oxidative stress (Hwang et al. 2002). In contrast, strains devoid of Mn-SOD show no auxotrophy, changes in cell or colony morphology or diminished virulence but are sensitive to hyperoxic conditions, ethanol and superox-
ide-generating agents (Hwang et al. 2003). The sod2Δ mutant was also sensitive to high osmolarity and heat. Taken together, these data point to different physiological roles of the two SOD enzymes in C. albicans (Hwang et al. 2003). The sod2Δ mutant of P. pombe, like its counterpart in C. albicans, is sensitive to menadione but also to heat and high osmolarity. The sod2′ gene was induced by KCl, menadione and 42 °C, demonstrating that Mn-SOD in P. pombe is a general stress-regulated protein (Jeong et al. 2001). The regulation of expression of S. cerevisiae SOD genes was also studied. Yeast cytoplasmic SOD1 expression is primarily induced by oxygen and ROS-generating agents, such as menadione and H2O2, possibly due to the presence of the putative Yap1p-binding site in the gene’s promoter sequence (Janieson 1998; Cyrne et al. 2003) and also by copper levels in the medium, through an Ace1 transcription factor (Gralla et al. 1991). Mitochondrial SOD2 expression is induced by growth on nonfermentable carbon sources through the HAPB motif in its promoter sequence and by decreased cAMP levels through STRE and PDS elements. All those elements are involved in the induction of SOD2 expression when cells enter the stationary phase (Flattery-O’Brien et al. 1997). The expression of SOD2 gene is also upregulated by heat treatment as well as oxidative and osmotic stress (Saccharomyces Genome Database; Piper 1995). We investigated whether sod1Δ and sod2Δ mutants of S. cerevisiae were sensitive to conditions other than oxidative stress.

MATERIALS AND METHODS

Strains and plasmids. Null mutant strains with deleted SOD1 (JS-C50) and SOD2 (JS-C55) genes in the genetic background of S. cerevisiae strain US50-18C (MATa PDR1-3 ura3 his1) (Balzi et al. 1987) were obtained using loxP–kanMX–loxP replacement marker (Güldener et al. 1996) as described by Krasowska et al. (2003). Plasmids PA103, bearing ACR3 gene on pFL44S (Bonneaud et al. 1991; Bobrowicz et al. 1997) and pSpPCS, with SpPCS gene on pYES2 (Clemens et al. 1999) (Invitrogen), kindly provided by R. Wysocki and S. Clemens, respectively, were used in the yeast transformation experiments performed by the lithium acetate method (Ito et al. 1983).

Culture conditions and growth assays. The strains were grown on YPD medium (in %: glucose 2, peptone 2, yeast extract 1) at 30 °C. Transformants carrying plasmids were cultured on a minimal medium (2 % glucose for pFL44S or 2 % galactose for pYES2, and 0.67 % yeast nitrogen base without amino acids) supplemented according to the auxotrophic requirements. Solid media were prepared by adding 2 % agar (Difco).

Liquid cultures were performed in test tubes in 5 mL media maintained for 1 d without shaking to ensure low-oxygen growth conditions. For phenotypic analyses the YPD medium was supplemented with appropriate concentrations of NaCl and/or glutathione and the test tubes were incubated at 30 °C.

For spot test analyses the strains were cultured in liquid medium for 20 h, brought to the same absorbance (A600) and then serially 10-fold diluted in sterile water. Three-μL aliquots of respective dilutions were spotted on plates supplemented with glutathione or various concentrations of AsIII (NaAsO2) and AsV (Na2HAsO4·7H2O), and incubated for 3 (rich media) or 5 d (minimal media) at a desired temperature either in air (normoxic conditions) or in airtight jars filled with nitrogen gas (< 7 % oxygen, hypoxic conditions) (Sturtz and Culotta 2002; Krasowska et al. 2003).

Representative results from at least three independent experiments are shown.

RESULTS AND DISCUSSION

The general phenotypes of mutant sod1Δ (JS-C50) and sod2Δ (JS-C55) strains were consistent with the literature data on these mutants in different genetic backgrounds. Strain JS-C50, but not JS-C55, grew poorly under atmospheric oxygen pressure and showed lysine and methionine auxotrophy when grown in air. Both strains were sensitive to superoxide-generating agents such as paraquat, with JS-C55 being more resistant. On the other hand, the sod2Δ, but not the sod1Δ mutant, showed growth deficiency when cultured on nonfermentable carbon sources such as glycerol. These results suggest that the Sod1 protein is the major oxidative-stress-defense agent in S. cerevisiae, with Sod2p gaining importance when the respiration chain is activated. We investigated other possible physiological functions of Sod1 and Sod2 proteins in the yeast cell.

Protection against heat and osmotic stress. Since it was found that the SOD2 gene was necessary for the induction of adaptive response to stress produced not only by menadione but also by heat and ethanol and that this induction was more pronounced in strains lacking the SOD1 gene (Pereira et al. 2003), we inves-
tigated whether Cu,Zn-SOD and/or Mn-SOD play a protective role against heat and osmotic stress in *S. cerevisiae*.

**Growth of the strains at different concentrations of NaCl.** Even though both the parent strain US50-18C and the sod1Δ mutant were also sensitive to elevated salt concentrations, showing growth inhibition at both 0.25 and 0.5 mol/L NaCl, the sod2Δ mutant was by far the most sensitive to the lower NaCl concentration (0.25 mol/L) (Table I). This large growth defect of the sod2Δ mutant was rescued to the level shown by the parent and sod1Δ strains by the addition of 2 mmol/L glutathione to the culture medium, suggesting that both glutathione and Sod2p, known to be involved in protection against oxidative stress, are also necessary for the survival of cells confronted with a high osmolarity environment. Glutathione had a slight effect on the growth of parent US50-18C and the sod1Δ strain, which showed a 25–37 % growth decrease when cultured with 0.25 mol/L NaCl. At 0.5 mol/L NaCl the addition of glutathione partially rescued the growth defect of all studied strains, implying that at this salt concentration the growth inhibition is due to the sensitivity inherent to the parent strain.

**Table I.** Effect of glutathione (GSH) on the growth (relative growth rate, %) of the strains at different concentrations of NaCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>US50-18c (parent)</th>
<th>JS-C50 (sod1Δ)</th>
<th>JS-C55 (sod2Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl, mol/L</td>
<td>GSH, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>62.9 ± 3.84</td>
<td>76.2 ± 2.95</td>
</tr>
<tr>
<td>0.25</td>
<td>2</td>
<td>70.3 ± 0.18</td>
<td>81.7 ± 0.96</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>16.6 ± 1.64</td>
<td>24.2 ± 1.46</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>46.5 ± 3.32</td>
<td>48.6 ± 6.65</td>
</tr>
</tbody>
</table>

*Strains were incubated in YPD medium for 1 d under hypoxic conditions at 30 °C. Culture growth, evaluated by measuring *A*600, was related to untreated control (i.e. culture in YPD only or YPD with 2 mmol/L GSH but no NaCl); means ± SD (n = 3).

**Effect of elevated temperature on the growth of mutant strains.** The growth of all strains was inhibited at 37 °C, with sod2Δ showing the greatest sensitivity especially when the cells were cultured in air (Fig. 1). The addition of 1 mmol/L glutathione to the medium partially restored the growth of the sod1Δ mutant under normoxic conditions at both 30 and 37 °C, but had only a minor effect on the growth of the sod2Δ mutant at 37 °C. Exposure to heat shock (1 h at 41 °C) induces the expression of *GSH1* and *GSH2* genes and intracellular glutathione content also increases but only when the cells are cultured in air (Sugiyama *et al.* 2000). According to the authors this could imply that elevated temperature enhances the rate of respiration and ROS production in the mitochondria, leading to a heat-shock response of *GSH1* and *GSH2*. Our results indicate that the growth defect of the sod2Δ mutant cultured at 37 °C is only partially dependent...
on air and that glutathione, at least at the concentration tested, cannot help the cells in dealing with this stress. Together with the data from another group showing that heat and ethanol shock do not increase glutathione levels in the cell (Piper 1995) this could suggest that at least under certain circumstances the yeast response

**Fig. 2.** Effect of ACR3p (A; minimal SD medium with 2 % glucose) and SpPCS (B; minimal SD medium with 2 % galactose) on the growth of the strains on media with metalloids; AsIII or AsV was added to a final concentration of 5 and 10 μmol/L, respectively. The plates were incubated either in air or under hypoxic conditions at 30 °C (for further details see Materials and Methods); for further explanation see Fig. 1; ACR3, SpPCS – genes.
to these stressful conditions does not require elevated glutathione concentrations. The above results indicate that mitochondrial SOD provides protection in \textit{S. cerevisiae} against high osmolarity and heat, and suggest that different mechanisms, both dependent on and independent of glutathione, are likely to be involved in these two cases.

**Protection against metalloids.** It has been documented that exposure to metal ions can lead to oxidative stress in the cell either through direct free radical generation (Cu, Fe) or indirect effects such as glutathione depletion (Cd); also metalloids, such as arsenite, were shown to induce the oxidative response (Brennen and Schiestl 1996; Avery 2001 \textit{and references therein}). A recent study also implicated As\textsuperscript{III} and As\textsuperscript{V} in the induction of oxidative stress response through Yap1p in \textit{S. cerevisiae} (Wysocki \textit{et al.} 2004). However, it has also been reported that in \textit{S. pombe} responses to arsenite are different from those generated by ROS, as revealed by exposure to H\textsubscript{2}O\textsubscript{2} (Rodriguez-Gabriel and Russell 2005).

**Effect of As\textsuperscript{III} and As\textsuperscript{V} on the growth of mutant strains.** The strains were transformed with plasmids containing either the \textit{ACR3} gene responsible for detoxication of arsenic compounds in \textit{S. cerevisiae} (Fig. 2A) or \textit{SpPCS} gene encoding a phytochelatin synthase involved in metal tolerance in \textit{S. pombe} (Fig. 2B).

Strain \textit{sod2}\textsuperscript{Δ} was found to be sensitive to both As\textsuperscript{III} and As\textsuperscript{V} and its sensitivity was much more pronounced under normoxic growth conditions. Overexpression of ACR3p complemented the \textit{sod2}\textsuperscript{Δ} sensitivity, although only partially for As\textsuperscript{III} when cells were grown in air (Fig. 2A). On the other hand, expression of the phytochelatin synthase gene only led to a partial complementation of the JS-C55 sensitivity to As\textsuperscript{III} under hypoxic conditions. It should be noted that \textit{SpPCS}\textsubscript{Sp} overexpression decreased the tolerance of \textit{sod2}\textsuperscript{Δ} and also parent cells, albeit to a lower extent, to As\textsuperscript{V} (Fig. 2B). A similar sensitizing effect was previously observed for parent W303-1A and \textit{ycf1} but not for \textit{acr3} strains (Wysocki \textit{et al.} 2003) suggesting that Sod2p may have an indirect role in arsenate tolerance. Additional heat stress applied by raising the temperature of incubation of the plates to 37 °C leads to a dramatic increase of arsenite sensitivity of all studied strains beyond the capacity for complementation by Acr3p (Fig. 3), pointing to a synergistic effect of cell damage induced by oxidative, heat and metalloid stress.
The sod1Δ sod2Δ double mutant has been previously shown to be sensitive to cadmium (Brennen and Schiestl 1996). In our experiments, both sod1Δ and sod2Δ strains were sensitive to this metal, with sod2Δ again showing the most severe growth defect (data not shown). The relative resistance of sod1Δ strain could be explained by increased expression of Ycf1 and Cup1 proteins in this mutant (Adamis et al. 2004), both of which are involved in cadmium detoxication.

In S. pombe and C. albicans, Mn-SOD is required not only for protection of mitochondria against the superoxide radical but also for defending the cells against osmotic and heat stress (Jeong et al. 2001; Hwang et al. 2003). S. cerevisiae Mn-SOD was shown to be necessary for the induction of adaptive response to stress produced by oxidizing agents but also by heat and ethanol (Pereira et al. 2003). Our results suggest that Sod2p in S. cerevisiae, like mitochondrial SOD of other fungi (Jeong et al. 2001; Hwang et al. 2003), is a general stress response protein. On the other hand, Sod1p appears to play a key role in controlling the redox status of the cell, keeping the concentration of ROS generated by the changing environment at a low level that minimizes the damage to the cell components but is sufficient to induce necessary adaptive changes in the gene expression (Pereira et al. 2003).

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