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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 *sod1*Δ and *sod2*Δ mutant strains are different. Compared with the parent strain and the *sod1*Δ mutant, the *sod2*Δ 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 *sod2*Δ showing the highest sensitivity, especially when cultured in air. Addition of 1 mmol/L glutathione to the medium restores aerobic growth of the *sod1*Δ mutant but has only a minor effect on the growth of the *sod2*Δ strain at 37 ºC. The *sod2*Δ 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

PDS	post-diauxic-shift (element)	STRE	stress response (element)
ROS	reactive oxygen species		
SOD	superoxide dismutase (EC 1.15.1.1)	Cu,Zn-SOD (SOD1)	cytosolic superoxide dismutase
		Mn-SOD (SOD2)	mitochondrial superoxide dismutase

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; *sod1*Δ, but not *sod2*Δ, grows very poorly even under atmospheric oxygen (Gralla and Kosman 1992). In addition, the *sod1*Δ mutant (not *sod2*Δ) shows lysine and methionine auxotrophy when grown in air and is more sensitive than *sod2*Δ 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 *sod2*Δ 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 *sod2*Δ mutant of *S. 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 ºC, demonstrating that Mn-SOD in *S. 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 H_2O_2, possibly due to the presence of the putative Yap1p-binding site in the gene's promoter sequence (Jamieson 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 *sod1*Δ and *sod2*Δ 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 (MATα *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 ºC. 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 ºC.

For spot test analyses the strains were cultured in liquid medium for 20 h, brought to the same absorbance (A_{600}) 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 As^{III} ($NaAsO_2$) and As^{V} ($Na_2HAsO_4{\cdot}7H_2O$), 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 *sod1*Δ (JS-C50) and *sod2*Δ (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 *sod2*Δ, but not the *sod1*Δ 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 *sod1*Δ mutant were also sensitive to elevated salt concentrations, showing growth inhibition at both 0.25 and 0.5 mol/L NaCl, the *sod2*Δ mutant was by far the most sensitive to the lower NaCl concentration (0.25 mol/L) (Table I). This large growth defect of the *sod2*Δ mutant was rescued to the level shown by the parent and *sod1*Δ 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 *sod1*Δ 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, %)[a] of the strains at different concentrations of NaCl

Treatment		US50-18c (parent)	JS-C50 (*sod1*Δ)	JS-C55 (*sod2*Δ)
NaCl, mol/L	GSH, mmol/L			
0.25	0	62.9 ± 3.84	76.2 ± 2.95	38.0 ± 8.24
0.25	2	70.3 ± 0.18	81.7 ± 0.96	81.4 ± 0.44
0.5	0	16.6 ± 1.64	24.2 ± 1.46	8.6 ± 0.24
0.5	2	46.5 ± 3.32	48.6 ± 6.65	35.6 ± 0.78

[a]Strains were incubated in YPD medium for 1 d under hypoxic conditions at 30 ºC. 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 ºC, with *sod2*Δ showing the greatest sensitivity especially when the cells were cultured in air (Fig. 1). The addition of 1mmol/L glutathione to the medium partially restored the growth of the *sod1*Δ mutant under normoxic conditions at both 30 and 37 °C, but had only a minor effect on the growth of the *sod2*Δ mutant at 37 ºC. Exposure to heat shock (1 h at 41 ºC) induces the expression of *GSH1* and *GSH2*

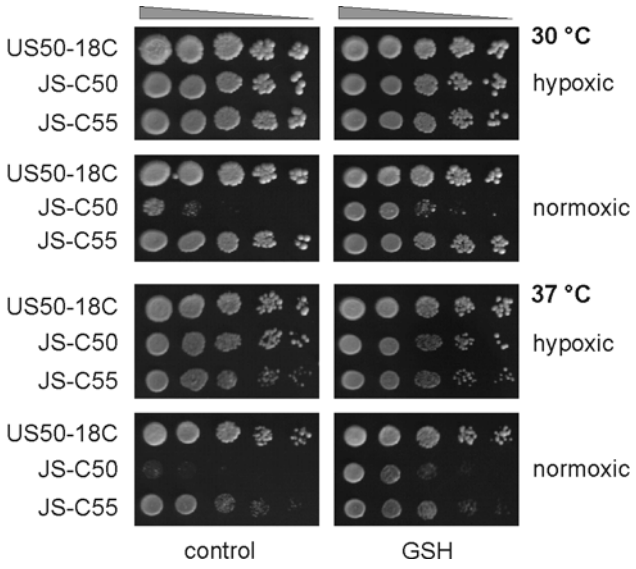

Fig. 1. Effect of the addition of 1 mmol/L glutathione (GSH) to the medium on hypoxic and normoxic growth of the strains at 30 and 37 ºC; US50-18C – parental strain, JS-C50 – *sod1*Δ mutant strain, JS-C55 – *sod2*Δ mutant strain; *triangles on the top* show the decreasing number of plated cells.

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 *sod2*Δ mutant cultured at 37 ºC 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

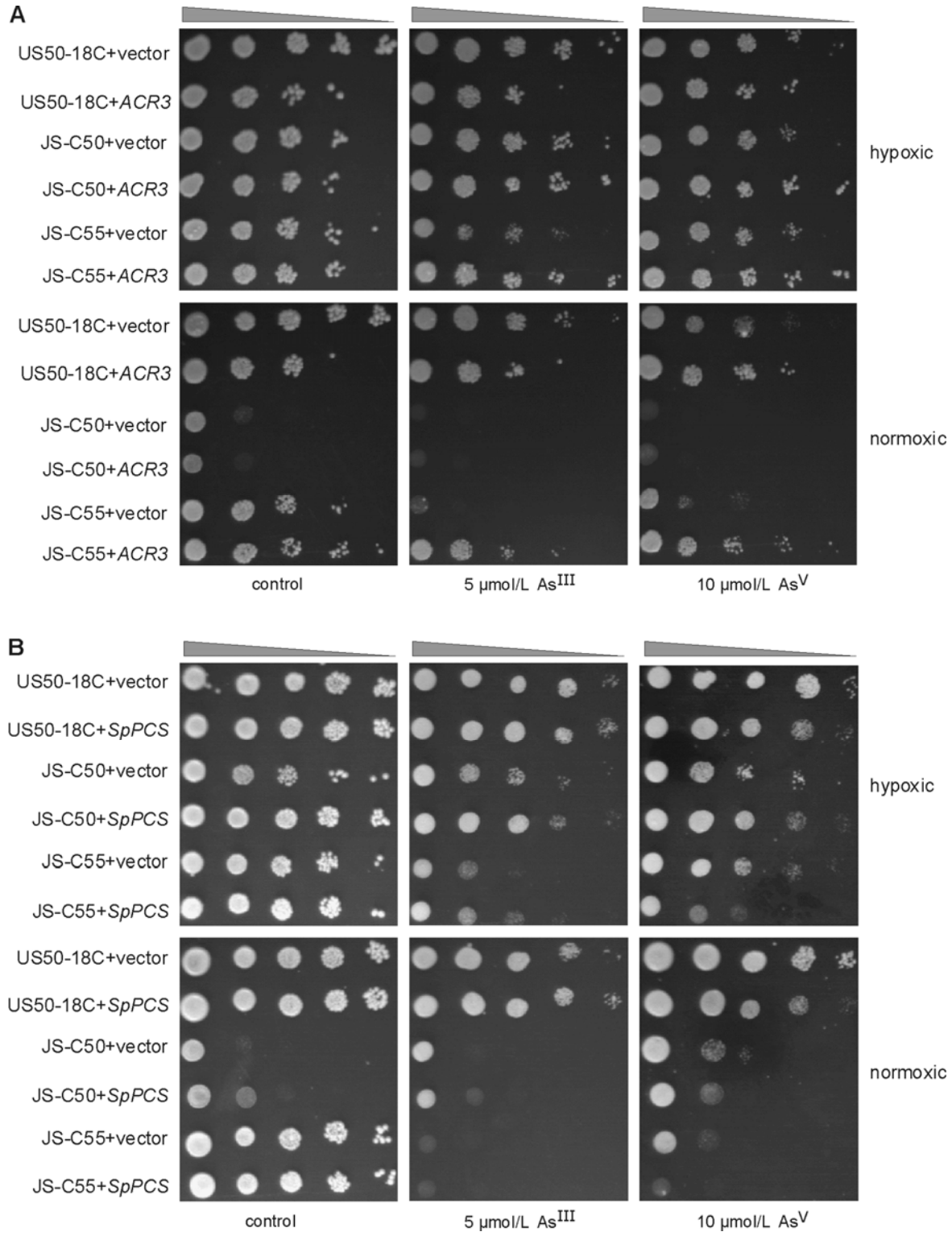

Fig. 2. Effect of ACR3p (**A**; minimal SD medium with 2 % glucose) and SpPCSp (**B**; minimal SD medium with 2 % galactose) on the growth of the strains on media with metalloids; As^{III} or As^{V} was added to a final concentration of 5 and 10 µmol/L, respectively. The plates were incubated either in air or under hypoxic conditions at 30 ºC (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 *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 *and references therein*). A recent study also implicated As^{III} and As^{V} in the induction of oxidative stress response through Yap1p in *S. cerevisiae* (Wysocki *et al*. 2004). However, it has also been reported that in *S. pombe* responses to arsenite are different from those generated by ROS, as revealed by exposure to H_2O_2 (Rodriguez-Gabriel and Russell 2005).

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

Strain *sod2*Δ was found to be sensitive to both As^{III} and As^{V} and its sensitivity was much more pronounced under normoxic growth conditions. Overexpression of ACR3p complemented the *sod2*Δ sensitivity, although only partially for As^{III} when cells were grown in air (Fig. 2A). On the other hand, expression of the

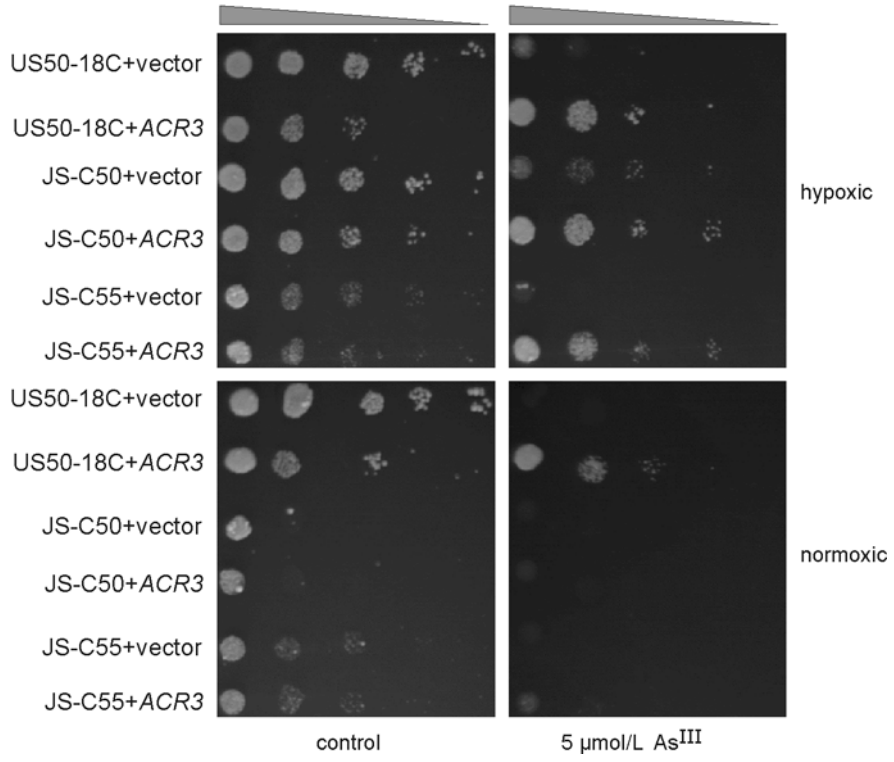

Fig. 3. Effect of growth at an elevated temperature (37 ºC) on arsenite sensitivity; As^{III} was added to the minimal SD medium with 2 % glucose to a final concentration of 5 µmol/L; the plates were incubated either in air or under hypoxic conditions (note that incubation in minimal medium further increases the sensitivity of *sod2*Δ strain to elevated temperature); for further explanation *see* Figs 1 and 2.

phytochelatin synthase gene only led to a partial complementation of the JS-C55 sensitivity to As^{III} under hypoxic conditions. It should be noted that *SpPCSp* overexpression decreased the tolerance of *sod2*Δ and also parent cells, albeit to a lower extent, to As^{V} (Fig. 2B). A similar sensitizing effect was previously observed for parent W303-1A and *ycf1* but not for *acr3* strains (Wysocki *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 ºC 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 *sod1*Δ *sod2*Δ double mutant has been previously shown to be sensitive to cadmium (Brennen and Schiestl 1996). In our experiments, both *sod1*Δ and *sod2*Δ strains were sensitive to this metal, with *sod2*Δ again showing the most severe growth defect (*data not shown*). The relative resistance of *sod1*Δ 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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