Mitochondrial superoxide dismutase is essential for ethanol tolerance of Saccharomyces cerevisiae in the post-diauxic phase

V. Costa, M. A. Amorim, E. Reis, A. Quintanilha and P. Moradas-Ferreira

This work reports the role of both superoxide dismutases - CuZnSOD (encoded by SOD1) and MnSOD (encoded by SOD2) - in the build-up of tolerance to ethanol during growth of Saccharomyces cerevisiae from exponential to post-diauxic phase. Both enzyme activities increase from the exponential phase to the diauxic shift and from the diauxic shift to the post-diauxic phase. The levels of mRNA-SOD1 and mRNA-SOD2 increase from the exponential phase to the diauxic shift; however, during the post-diauxic phase mRNA-SOD1 levels decrease while mRNA-SOD2 levels remain unchanged. These data indicate the existence of two regulatory mechanisms involved in the induction of SOD activity during growth: synthesis de novo of the proteins (until the diauxic shift), and post-transcriptional or post-translational regulation (during the post-diauxic phase). Ethanol does not alter the activities of either enzyme in cells from the diauxic shift or post-diauxic phases, although the respective mRNA levels decrease in post-diauxic-phase cells treated with ethanol (14% or 20%). Results of experiments with sod1 and sod2 mutants show that MnSOD, but not CuZnSOD, is essential for ethanol tolerance of diauxic-shift and post-diauxic-phase cells. Evidence that ethanol toxicity is correlated with the production of reactive oxygen species in the mitochondria is obtained from results with respiration-deficient mutants. In these cells, the induction of superoxide dismutase activity by ethanol is low; also, the respiratory deficiency restores the capacity of sod2 cells to acquire ethanol tolerance.

Keywords: yeast, ethanol tolerance, superoxide dismutase, post-diauxic phase

INTRODUCTION

The increasing ethanol concentration during batch fermentation affects the growth, viability and fermentation rate of Saccharomyces cerevisiae cells (van Uden, 1984); this toxicity has been associated with protein denaturation and membrane fluidity, leading to membrane leakage (Casey & Ingledeew, 1986; Piper, 1995). Ethanol has also been considered to be responsible for promoting mitochondrial DNA mutagenesis (Bandas & Zakharov, 1980) and, indeed, mitochondria have been suggested as a target for ethanol damage (Aguilera & Benitez, 1985; Sá-Correia & van Uden, 1986). As for other stress conditions, S. cerevisiae cells seem to be more ethanol tolerant when they reach the stationary phase (Werner-Washburne et al., 1993; Piper, 1995). Exponential-phase cells can also become tolerant to lethal ethanol concentrations if they undergo a previous sublethal heat or ethanol stress (Watson & Cavicchioli, 1983; Costa et al., 1993). During both these stress adaptations, the expression of a subset of proteins is highly induced (Plesset et al., 1982; Werner-Washburne et al., 1993), including the stress proteins Hsp26, Hsp30 (Panaretou & Piper, 1992), Hsp70, Hsp104 (Sanchez et al., 1992), catalase T (Belazzi et al., 1991; Wieser et al., 1991) and MnSOD (Costa et al., 1993). However, only Hsp104 and MnSOD were shown to be required for the acquisition of ethanol tolerance. Plasma membrane
ATPase activity (Panaretou & Piper, 1990; Rosa & Sá-Correa, 1996), as well as the accumulation of trehalose (Odumuru et al., 1993) and the increase in membrane concentrations of unsaturated fatty acids and ergosterol (Beaven et al., 1982; Del Castillo Agudo, 1992), were also correlated with ethanol tolerance (Piper, 1995).

We have previously reported that the mitochondrial superoxide dismutase (MnSOD) plays a key role in the acquisition of ethanol tolerance in exponential-phase yeast cells (Costa et al., 1993). In addition to MnSOD, the system of primary antioxidant defences (enzymatic and non-enzymatic) includes CuZnSOD, cytochrome-c-peroxidase, catalases A and T, metallothionein, thio-redoxin, thioredoxin-peroxidase and glutathione (Moradas-Ferreira et al., 1996). It has been found that in exponential yeast cells growing mainly by fermentation, the synthesis of many antioxidant defences is repressed, and that derepression only occurs at the diauxic shift phase, before the onset of respiratory growth (Werner-Washburne et al., 1993; Moradas-Ferreira et al., 1996). Therefore, the induction of antioxidant defences during this respiratory adaptation may contribute to the increased oxidative stress tolerance observed in these cells (Jamieson, 1992). Ethanol toxicity was correlated with the production of reactive oxygen species (ROS), since MnSOD and catalase T are induced by ethanol stress, leading to a higher ethanol tolerance (Costa et al., 1993; Wieser et al., 1991). Cells with increased levels of MnSOD and catalase T are able to avoid the damaging effects of ROS, such as superoxide (O_2^-) and hydroxyl (•OH) radicals, and hydrogen peroxide (H_2O_2) (Halliwell & Gutteridge, 1989).

With the aim of understanding the role of both CuZnSOD (encoded by SOD1) and MnSOD (encoded by SOD2) in the tolerance of S. cerevisiae to ethanol stress, we analysed mRNA-SOD levels and SOD activities during growth from the exponential to the post-diauxic phase. In addition, ethanol tolerance of mutant cells deficient in either CuZnSOD (sod1 cells) or MnSOD (sod2 cells) was studied. The role of ROS in the induction of SODs under ethanol stress conditions was addressed using respiration-deficient mutants.

**METHODS**

**Yeast strains and growth conditions.** The strains of Saccharomyces cerevisiae used in this study are listed in Table 1. Respiration-deficient mutants were prepared by prolonged exposure to ethidium bromide and selected as cells unable to form colonies on YPG plates (1% yeast extract, 2% bactopeptone, 3%, v/v, glycerol). Cells were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) to early exponential phase (OD_{600} 0.6), diauxic shift phase (OD_{600} 30 ± 0.1 for the aBR10 strain; OD_{600} 39 ± 0.1 for DL1, DL1sod2 and Dscd2-2C strains) or post-diauxic phase (OD_{600} 57 ± 0.1 for the aBR10 and DL1sod2 strains; OD_{600} 70 ± 0.1 for the DL1 strain; OD_{600} 90 ± 0.1 for the Dscd2-2C strain) (Fig. 1), in an orbital shaker, at 26 °C, and 120 r.p.m., with a ratio of flask volume/medium volume of 5:1. Growth of aBR10p and DL1sod2p cells (respiration-deficient mutants) in the exponential phase was similar to that observed in the aBR10 and DL1sod2 cells (data not shown).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td>aBR10</td>
<td>MATa gal1 trp1 his4 ade</td>
<td>Rymond et al. (1983)</td>
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<tr>
<td></td>
<td>cyc1</td>
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<tr>
<td>aBR10p*</td>
<td>[aBR10] rho^-</td>
<td>This work</td>
</tr>
<tr>
<td>Dscd2-2C</td>
<td>MATa ura3 arg4 sod1</td>
<td>Bilinski et al. (1985)</td>
</tr>
<tr>
<td>DL1</td>
<td>MATa his3-11,3-15 ura3-&gt;251,3-372,3-328</td>
<td>van Loon et al. (1986)</td>
</tr>
<tr>
<td>DL1sod2</td>
<td>[DL1] sod2</td>
<td>van Loon et al. (1986)</td>
</tr>
<tr>
<td>DL1sod2p*</td>
<td>[DL1] sod2 rho^-</td>
<td>This work</td>
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* Respiration-deficient mutants.

**Ethanol tolerance.** Yeast cells were grown to the diauxic shift or post-diauxic phase. Aliquots of these cultures were treated with 14% (v/v) or 20% (v/v) ethanol for 30 min. Cultures of the sod2p strain, growing in early exponential phase (OD_{600} 0.6) at 26 °C, or pre-exposed to a sublethal ethanol stress (8%, v/v) for 30 min, were subsequently treated with 14% ethanol for 30 or 60 min.

Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26 °C for 3 d.

**SOD (EC 1.15.1.1) activity.** Yeast extracts were prepared in 0.05 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, by vigorous shaking of the cell suspension, in the presence of glass beads, for 3 min. Short pulses of 30 s were used, with 30 s intervals on ice. Proteins were assayed by the Lowry method, using bovine serum albumin as a standard. One hundred micrograms of total protein was used for the enzyme assay. Total SOD activity was determined spectrophotometrically at 550 nm, in the presence of cytochrome c, using the xanthine-xanthine oxidase system (Flohe & Ottig, 1984). MnSOD activity was assayed in the presence of 2 mM KCN. SOD activity of extracts was determined by reference to a standard curve prepared with known amounts of bovine SOD (Sigma), and expressed as U (mg protein)^-1. One unit of SOD is the amount of enzyme which inhibits the rate of cytochrome c reduction by 50%.

**Preparation and analysis of RNA.** RNA was isolated as described by Brown (1994). Total RNA (30 µg) was denatured with glyoxal and dimethyl sulfoxide, blotted onto Hybond N membranes and probed as described by Sambrook et al. (1989). The following gene probes were used: a 0.5 kb HindIII fragment of the SOD1 gene (Birmingham-McDonogh et al., 1988); a 2 kb BamHI fragment of the SOD2 gene (Marres et al., 1985); a 1.1 kb EcoRI fragment of the CTT1 gene, encoding catalase T (Spevak et al., 1983); and a 1 kb HindIII-EcoRI fragment of the ACT1 gene, encoding actin (Gallwitz & Sures, 1980). Band intensities were evaluated using an Ultra Scan XL Enhancer laser densitometer.

**Statistical analysis.** Data are expressed as mean values ± SD of at least three independent experiments. Values were compared by Student's t-test. The 0.05 probability level was chosen as the point of statistical significance throughout.
MnSOD and ethanol tolerance in post-diauxic phase

RESULTS

Ethanol does not induce SOD activities in cells growing from the diauxic shift to the post-diauxic phase

As previously shown for exponentially growing cells, ethanol induces MnSOD activity, which could be correlated with the acquisition of ethanol tolerance (Costa et al., 1993). As the post-diauxic-phase yeast cells are resistant to high concentrations of ethanol (Piper, 1995), we analysed the build-up of this tolerance by determining the SOD activities and levels of mRNA-SOD1 and mRNA-SOD2 in S. cerevisiae aBR10 cells during the different growth phases (Fig. 1). CuZnSOD and MnSOD activities increased 100% and 200%, respectively, from the exponential phase to the diauxic shift, and 35% and 170%, respectively, from the diauxic shift to the post-diauxic phase (Fig. 2a). Exposure of diauxic shift or post-diauxic phase cells to ethanol (14% or 20%, v/v) did not significantly affect either CuZn-SOD or MnSOD activity (Fig. 2b). To assess whether the increased SOD activities were due to the synthesis de novo of the proteins, we analysed the respective mRNA levels. Our results (not shown) confirmed the data previously obtained by Galiazzo & Labbe-Bois (1993): both mRNA-SOD1 and mRNA-SOD2 levels increased threefold during growth from the exponential to the diauxic shift phase. However, during growth to the post-diauxic phase, mRNA-SOD1 levels decreased 40%, while mRNA-SOD2 levels remained identical (Fig. 3). When post-diauxic cells were stressed with ethanol, both mRNA levels were further reduced 30–40%. A similar depletion of mRNA-CTT1 and mRNA-ACT1 was observed (Fig. 3a). This effect on mRNA-SOD levels suggests that, besides a general decrease for mRNA levels as cells enter the post-diauxic phase, ethanol stimulates mRNA degradation or inhibits transcription. In contrast, ethanol did not affect mRNA-SOD levels of cells from the diauxic shift (Fig. 3) or exponential phase (Fig. 4). Notably, in exponentially growing cells, heat shock induced a 2-fold increase for mRNA-SOD1 and a 2.5-fold increase for mRNA-SOD2 (Fig. 4).

MnSOD deficiency renders post-diauxic phase yeast cells hypersensitive to ethanol

The results in Fig. 2 suggested that ethanol tolerance at the post-diauxic phase is associated with a higher activity of CuZnSOD and MnSOD. Considering the role of SODs in ethanol tolerance, it was decided to assess the relevance of each enzyme during growth using sod1 and sod2 null mutants.

![Fig. 1. Growth of S. cerevisiae aBR10 (△), DL1 (□), sod1 (▲) and sod2 (■) cells in YPD medium. Arrows indicate the growth phases at which the experiments were performed: the exponential phase (Exp), the diauxic shift phase (DS) and the post-diauxic phase (PD).](image)

![Fig. 2. Analysis of CuZnSOD and MnSOD activities in S. cerevisiae aBR10 cells. (a) Both CuZnSOD and MnSOD activities increase during growth from the exponential (Exp) to the diauxic shift (DS) and post-diauxic (PD) phases [results for exponential-phase cells are from Costa et al. (1993)]. Values are means±SD of five independent experiments. **P<0.05 (DS compared to Exp, and PD to DS). (b) Exposure of diauxic shift (DS) and post-diauxic (PD) cells to 14% (v/v) or 20% (v/v) ethanol for 30 min does not affect CuZnSOD or MnSOD activity. Values are means±SD of five independent experiments.](image)
In *S. cerevisiae* aBR10 cells, 80–90% of cells from either the diauxic shift phase or the post-diauxic phase remained viable when exposed to 14% (v/v) ethanol stress (Table 2). During the transition from the diauxic shift to the post-diauxic phase, cells became tolerant to higher ethanol concentrations, up to 20% (v/v): 55% of cells remained viable at the diauxic shift phase, while more than 80% of post-diauxic phase cells survived. The *sod1* mutation did not impair the acquisition of ethanol tolerance during the transition to the diauxic shift and post-diauxic phase (Table 2). In contrast, the *sod2* mutation rendered yeast cells very sensitive to ethanol (Table 2). In this mutant, 100% of diauxic-shift cells and 90% of post-diauxic-phase cells became unviable after 30 min in the presence of 20% ethanol, whereas in aBR10 cells, as well as in DL1 cells (the isogenic, wild-type strain of the *sod2* mutants; data not shown) only 45% cell death occurred in diauxic-shift cells and 20% in post-diauxic-phase cells. Despite the high sensitivity of *sod2* mutants to ethanol, their tolerance slightly increased in the post-diauxic phase, compared to the diauxic shift phase.

**Ethanol induces CuZnSOD activity of sod2 cells in the diauxic shift and post-diauxic phase**

As *sod2* cells are very sensitive to ethanol, but can still acquire a low degree of tolerance when they reach the post-diauxic phase, we analysed the contribution of CuZnSOD activity to the observed tolerance. CuZnSOD activity of *sod2* cells increased 40% during growth from the exponential to the diauxic shift phase (data not
The high sensitivity of sod2 cells to ethanol supports the correlation between ethanol toxicity and production of ROS in the mitochondria. Therefore, we addressed the question of whether a respiratory deficiency would increase ethanol tolerance of sod2 cells, since the generation of reactive species in the mitochondria is impaired. Indeed, the tolerance to 14% ethanol of respiration-deficient sod2 cells (S. cerevisiae sod2p) growing exponentially was significantly enhanced when cells were pre-exposed to 8% ethanol for 30 min: 15% of exponential-phase cells were able to form colonies after exposure to 14% ethanol for 60 min, while 80% of ethanol-pretreated cells remained viable. This acquisition of ethanol tolerance by sod2Δ cells was similar to that observed in the wild-type strain, S. cerevisiae DL1 (data not shown).

To investigate if ROS are involved in the induction of MnSOD, the activity was analysed in respiration-deficient mutants of the aBR10 strain (S. cerevisiae aBR10p). The constitutive SOD activities of aBR10p mutants (Fig. 5a) were identical to those of wild-type cells (Fig. 2a); however, when cells were exposed to 8% ethanol, only a small increase of MnSOD activity was observed (Fig. 5a). A comparative analysis with heat shock showed no significant effect on the activity of either enzyme (Fig. 5a). The analysis of mRNA levels showed that ethanol treatment caused decreased mRNA-SOD1 levels in the aBR10Δ strain, similar to the effect observed in wild-type cells, but caused an increase in mRNA-SOD2 (90% after 60 min; Fig. 5b). Heat shock did not affect mRNA-SOD1 levels and increased the levels of mRNA-SOD2 (45% and 95% after 30 and 60 min, respectively); however the induction was lower than that determined in aBR10 cells (150% and 130%, respectively).

**DISCUSSION**

When exponential-phase cells of S. cerevisiae growing on glucose are exposed to a sublethal thermal or ethanol stress, antioxidant defences, such as MnSOD and catalase T, are induced and ethanol tolerance increases (Watson & Cavicchioli, 1983; Wieser et al., 1991; Costa et al., 1993; Schuller et al., 1994). The yeast cells can also become more tolerant to ethanol and other stress agents when they shift from fermentative to respiratory growth (Piper, 1995). Indeed, when fermentation comes to an end, a number of genes downregulated by glucose are activated, including genes encoding antioxidant defences (Werner-Washburne et al., 1993; Moradas-Ferreira et al., 1996). As previously reported, the acquisition of ethanol tolerance is dependent on the activity of MnSOD (Costa et al., 1993). The present work was aimed at analysing the correlation between the activity of CuZnSOD and MnSOD and ethanol tolerance during different phases of growth.

When cells enter the diauxic shift phase, the activity of both SODs increased. This increase in activity was correlated with higher levels of mRNA, and thus with an increased translation of the apoproteins. However, measurements of the dismutase activity of cells in the post-diauxic phase revealed that the activity of CuZnSOD was only moderately induced while the activity of MnSOD...
MnSOD was much more significantly induced, and this occurred while mRNA-SOD1 levels decreased and mRNA-SOD2 remained constant, compared to diauxic-shift cells (Figs 2 and 3). These results suggest that the induction of CuZnSOD and MnSOD during growth from exponential to diauxic shift phase is due to a de novo synthesis of the proteins. In contrast, their induction during growth into the post-diauxic phase involves post-transcriptional regulatory mechanisms or post-translational activation of the apoproteins. These post-transcriptional regulatory mechanisms have been shown to occur in yeast cells. The CuZnSOD apoprotein is post-translationally activated by copper during aeratation of hypoxic cells both in yeast and mammalian cells (Galliczio et al., 1991; Rossi et al., 1994). An increased translatability of mRNA-RAS2, -ENO1, -RPB4 and -BCY1, and post-translational modifications of Bcy1p have been observed as cells grow into the post-diauxic phase (Brevario et al., 1988; Jigami et al., 1986; Werner-Washburne et al., 1991).

When post-diauxic phase cells were stressed with ethanol, the dismutase activity did not change; however, the levels of both mRNA-SODs decreased. A similar depletion of mRNA-CTT1 and mRNA-ACT1 indicates that ethanol either stimulates mRNA degradation in general or represses gene transcription.

The high SOD activities and ethanol tolerance observed in post-diauxic yeast cells suggest a possible correlation between these two phenotypes. The role of MnSOD in ethanol tolerance is supported by the high sensitivity of sod2 mutant cells to ethanol during growth. However, sod2 cells are still more tolerant in the post-diauxic phase than in the diauxic shift phase, adding support to the idea that other factors might be involved that are induced at the post-diauxic phase, such as Hsp26, Hsp104 and catalase T, together with changes in membrane lipids (Piper, 1995).

It is clear that ethanol tolerance is independent of CuZnSOD activity, as sod1 mutant cells display a tolerance similar to that of wild-type cells during all growth phases. Furthermore, despite the higher CuZnSOD activity found in sod2 mutants in the post-diauxic phase [8.5 vs 6.9 U (mg protein)⁻¹], these cells were highly sensitive to ethanol (Tables 2 and 3). However, CuZnSOD may play a minor role in ethanol tolerance of
cells deficient in MnSOD. In fact, CuZnSOD activity increased in post-diauxic sod2 cells exposed to ethanol, and these cells have a higher ethanol tolerance than diauxic shift cells. This minor role of CuZnSOD in ethanol tolerance was also suggested in exponential-phase cells (Costa et al., 1993). These results, however, cannot rule out an important role of CuZnSOD in other stress tolerances associated with post-diauxic-phase cells (Werner-Washburne et al., 1993). The localization of MnSOD in the mitochondrial matrix, where most of the ROS are produced during respiration, seems to be important for ethanol tolerance. By trapping \( \cdot \text{O}_2^- \) produced in excess within mitochondria of yeast cells under ethanol stress conditions, MnSOD would prevent its diffusion to the cytosol, thereby protecting lipids, proteins and nucleic acids from oxidative damage (Halliwell & Gutteridge, 1989). The \( \text{H}_2\text{O}_2 \) produced by dismutation of \( \cdot \text{O}_2^- \) catalysed by MnSOD can be decomposed by catalase T. It has been shown that the CTT1 gene is derepressed in the post-diauxic phase, as well as upon heat or ethanol stress of exponential-phase cells (Wieser et al., 1991; Schuller et al., 1994). In fact the highest ethanol tolerance is achieved by the coordinated action of MnSOD and catalase T. The induction of CTT1 is higher in exponential-phase cells exposed to ethanol than in those exposed to heat shock, and sublethal ethanol pretreatment confers higher ethanol tolerance than a sublethal thermal stress (Costa et al., 1993). In addition, CTT1 is only derepressed in the post-diauxic phase, and these cells are more tolerant to ethanol than diauxic-shift cells (Piper, 1995).

If ethanol induces the generation of \( \cdot \text{O}_2^- \) in mitochondria, it would be expected that a respiration-deficient strain would be more tolerant to ethanol. Indeed, in contrast to sod2 cells, exponentially growing sod2p cells are able to acquire ethanol tolerance. Besides, the data also indicate that \( \cdot \text{O}_2^- \) may regulate SOD expression upon thermal or ethanol stress. In fact, the levels of mRNA-SOD1 and mRNA-SOD2 increased upon heat shock and mRNA-SOD2 increased upon ethanol stress; however the induction was lower than that observed in wild-type cells (Figs 4 and 5). The induction of MnSOD activity by ethanol was rather low in petite (respiration-deficient) cells and no longer occurred upon heat shock, compared with wild-type cells, giving further evidence of a post-translational regulation of MnSOD, which may involve \( \cdot \text{O}_2^- \) under these stress conditions.

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**REFERENCES**


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