Full Paper

Oxidative stress tolerance of a spore clone isolated from Shirakami kodama yeast depends on altered regulation of Msn2 leading to enhanced expression of ROS-degrading enzymes

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We analyzed the stress response in a spore clone from Shirakami kodama yeast, *Saccharomyces cerevisiae*, with an exceptional high tolerance to oxidative stress. The levels of reactive oxygen species (ROS) in this clone were very low, whereas the genes for superoxide dismutase (*SOD2*) and catalase (*CTT1*) were highly expressed and those enzymes also had high activities even under non-stress conditions. Both genes are regulated by general stress-responsive transcription factors Msn2 and Msn4, and Yap1, a transcription factor required for oxidative stress tolerance, and the removal of Msn2 or Yap1 caused a significant decrease in *CTT1*-expression. Under non-stress conditions, Msn2 was ~3.6-fold more abundant in the nucleus of the spore clone compared with a laboratory strain, whereas the nuclear abundance of Yap1 remained unchanged. Thus, a high tolerance to oxidative stress in this spore clone results from a high expression of ROS-degrading enzymes by the abundant accumulation of Msn2 in the nucleus. We found that oxidative stress caused by the presence of furfural did not impair fermentation by this strain, which could make it attractive for ethanol production from lignocellulosic biomass.

Key Words: furfural; Msn2; oxidative stress; ROS; Shirakami kodama yeast

Introduction

Reactive oxygen species (ROS) are produced under several environmental conditions. ROS are highly reactive molecules that can oxidize proteins, DNA, and lipids, and can lead to extensive damage of cellular components and cell death. Oxidative stress means an imbalance in the redox status of cells towards an oxidized state. In *Saccharomyces cerevisiae*, which is a model species for basic studies in cell biology, enzymatic strategies are in place to minimize the effect of ROS (Herrero et al., 2007). ROS are directly detoxified with enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase, encoded by *SOD2* and localized at the mitochondrial matrix, catalyzes the reduction of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (Culotta et al., 2006). Catalase T, encoded by *CTT1*, converts H_2O_2 to water and oxygen in the cytosol (Izawa et al., 1996). Glutathione peroxidase catalyzes the reduction of H_2O_2 and lipid hydroperoxides to water and corresponding alcohols (Carmel-Harel and Storz, 2000; Inoue et al., 1999). *S. cerevisiae* contains three glutathione peroxidase homologs, *GPX1*, *GPX2* and *GPX3*, of which mainly *GPX2* is used in the oxidative stress response (Inoue et al., 1999).

S. cerevisiae cells respond to oxidative stress by altering their transcriptional program (Herrero et al., 2007). One of the target genes is *CTT1*, which is regulated by the oxidative stress transcription factors Yap1 and Skn7 and by the general stress response complex Msn2/4 (Toone and Jones, 1998). Its promoter region contains the following regulatory elements: The general stress-responsive

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element (STRE, 5'-AGGGG-3' or 5'-CCCCT-3'), which is bound by the Msn2 and Msn4 activators (Martinez-Pastor et al., 1996) and the AP-1 element, which is triggered by the Yap family of activators (Fernandes et al., 1997). Skn7 cooperates with Yap1 in activating many oxidative stress response genes, such as *GPX2* (Inoue et al., 1999; Lee et al., 1999).

The transcriptional activity of Yap1 is modulated by its regulated import into the nucleus upon oxidative stress (Gulshan et al., 2005), whereas the transcription factor Skn7 constitutively localizes in the nucleus (He et al., 2009). Under normal growth conditions, Msn2 and Msn4 are largely sequestered in the cytosol with the help of the TOR (target of rapamycin) signaling pathway (Beck and Hall, 1999). Upon onset of stress conditions, Msn2 and Msn4 are relocalized to the nucleus in a process controlled by the cyclic AMP (cAMP)-dependent protein kinase (PKA) (Görner et al., 1998; Petkova et al., 2010). The cAMP signal is produced by adenylate cyclase via the stimulation of the Ras protein and its main target is the cAMP-dependent protein kinase A (PKA). Msn2 and Msn4 share a conserved nuclear localization signal (NLS) which is negatively regulated by PKA-dependent phosphorylation, but not by the target of rapamycin complex 1 (TORC1) (Görner et al., 1998, 2002). PKA is composed of catalytic subunits, Tpk1, Tpk2 and Tpk3 (Toda et al., 1987b), and a regulatory subunit, Bcy1, which is a negative regulator of PKA (Toda et al., 1987a). Inactivation of cAMP is caused by degrading it through cAMP phosphodiesterases, Pde1 and Pde2 (Nikawa et al., 1987). Deletion of *PDE2* results in a highly activated cAMP-PKA pathway (Park et al., 2005).

H₂O₂ can act as a second message and is then transduced by peroxiredoxin, which relays the signal to thioredoxin (Trx). Oxidized thioredoxins inhibit PKA-dependent phosphorylation, allowing Msn2 and Msn4 to concentrate in the nucleus (Bodvard et al., 2017). Kinases Rim15 and Yak1 can positively regulate Msn2/4 dependent transcription, for example of starvation-specific genes (Zhang et al., 2013). Rim15 integrates nutrient, redox or oxidative stress signals to induce an appropriate transcriptional program through Msn2 and Msn4 (Cameroni et al., 2004).

Yeast cells used in the baking, brewing and bioethanol industries are exposed to oxidative stresses during freezing, drying, and fermentation (Morano et al., 2012); for example, in the production of bioethanol from polysaccharides derived from lignocellulose. In order to increase the saccharification of cellulose and hemicellulose and their release from the surrounding lignin, the lignocellulosic materials must be pretreated with weak acid, steam explosion and liquid hot water (Margeot et al., 2009). During this process, byproducts containing aldehydes such as furfural and phenolic compounds are produced. Because furfural causes ROS in cells (Allen et al., 2010), it inhibits fermentation by yeast cells (Palmqvist and Hahn-Hägerdal, 2000; Taherzadeh et al., 1999).

In 1997, Shirakami kodama yeast, a strain of *S. cerevisiae*, was isolated from leaf mold from the Shirakami Mountains, and is used as a commercial baker's yeast (Takahashi, 2007). This yeast is very tolerant to frost and droughts because of its exceptionally high level of intrac-

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype ^{a)}
Shirakami kodama yeast	<i>MATa/MATα</i>
MCY3605	<i>MATa ura3-52 leu2-3,112 his3-200 ho</i>
IB287	<i>MATa/MATα</i> diploid of MCY3605
IB1306	<i>MATα ho</i>
IB1670	IB1306 except <i>ura3::natMX</i>
IB1707	IB1306 except <i>ura3::loxP trp1::natMX</i>
IB1714	IB1707 except <i>ctt1::CgTRP1</i>
IB1716	IB1707 except <i>gpx2::CgTRP1</i>
IB1717	IB1707 except <i>sod2::CgTRP1</i>
IB1737	IB1707 except <i>msn2::CgTRP1</i>
IB1738	IB1707 except <i>msn4::CgTRP1</i>
IB1741	IB1707 except <i>yap1::CgTRP1</i>
IB1752	<i>MATa/MATα</i> diploid of IB1707 ^{b)}
IB1761	IB1707 expect <i>rim15::CgTRP1</i>
IB1762	IB1707 expect <i>yak1::CgTRP1</i>
IB1812	IB1707 except <i>skn71::CgTRP1</i>
IB1817	IB1707 expect <i>bcy1::CgTRP1</i>
IB1818	IB1707 expect <i>pde2::CgTRP1</i>

^{a)} *CgTRP1* is *TRP1* from *Candida glabrata*.

^{b)} IB1707 was diploidized using the homothallic gene *HO*.

ellular trehalose (Takahashi, 2007). We isolated a spore clone form of Shirakami kodama yeast with an even higher tolerance to oxidative stress than the parental strain. Here, we show that this spore clone is very capable of reducing ROS due to a high constitutive expression of *SOD2* and *CTT1*, which are caused by an increased abundance of Msn2 in the nucleus, even under non-stress conditions. This spore clone could make an attractive possibility for the production of ethanol from lignocellulosic biomass, as indicated by its fermentation characteristics in the presence of furfural.

Materials and Methods

Microorganisms and plasmids. The *S. cerevisiae* strains used are listed in Table 1. The oligonucleotides used are shown in Table 2. *Escherichia coli* DH5α was used for the manipulation of plasmid DNAs. MCY3605 was a gift from Dr. M. Carlson. Commercial dry yeast (Shirakami kodama yeast dry, Akita Jujyo Kasei Co., Ltd.) was used as Shirakami kodama yeast. All *S. cerevisiae* strains were heterothallic, as they were of the *ho* genotype. IB1306, a spore clone isolated from Shirakami kodama yeast, was used for the construction of strain IB1670 and its derivatives by gene-disruption or -replacement using the Cre/*loxP* system (Güldener et al., 1996). Target genes were replaced by homologous recombination using a PCR-fragment carrying a marker flanked by *loxP* sites. Table 3 shows the strains, primers, and templates used. In the case of a gene-disruption, the marker genes were removed by recombination between the *loxP* sites upon expression of the Cre recombinase.

To construct the *CTT1p-CYC1-lacZ* reporter genes, the promoter region of *CTT1* was amplified from MCY3605 genomic DNAs with primers OLI690 and OLI691, and cloned in plasmid pΔ5'728 (a gift from Dr. D. Stuart), yielding plasmid pIB650. Plasmid pRS314 cup1 cup-GFP-Yap1 and pAdh1-Msn2-GFP (based on pRS316), used for

Table 2. Oligonucleotides used in this study.

Name	Sequence 5'-3'	Purpose
OLI521	TTTCCAGAATAGCCAACCAC	to test <i>RIM15</i> disruption
OLI522	TTGCGCCGAAGATTAGC	to test <i>RIM15</i> disruption
OLI536	ACGGAAACCTTGAGATAAC	to test <i>MSN2</i> disruption
OLI537	CTTGCCAGAAGTGGATGTGC	to test <i>MSN2</i> disruption
OLI538	GTTTTTCGCAACTGGTCAAC	to test <i>MSN4</i> disruption
OLI539	CACCAAAAGCATCGTCTTCA	to test <i>MSN4</i> disruption
OLI690	GCATGCCAGAGATGATGAGGAACCGATGAC	cloning <i>CTT1</i> -promoter
OLI691	CTCGAGGTAGCAGTAGGGGATACGATAAATGCAC	cloning <i>CTT1</i> -promoter
OLI713	CAGCTCAGCTTCACAAATGA	to test <i>CTT1</i> disruption, <i>CTT1</i> probe
OLI714	CAGCCTTATCTCCAGTCAAG	to test <i>CTT1</i> disruption, <i>CTT1</i> probe
OLI721	GAAGGGTGGTTTGTGATTGC	to test <i>SOD2</i> disruption, <i>SOD2</i> probe
OLI722	GCTTCTTTCCAGTTGACCAC	to test <i>SOD2</i> disruption, <i>SOD2</i> probe
OLI725	GAATGCAAGGACAAGAAAGG	to test <i>GPX2</i> disruption, <i>GPX2</i> probe
OLI726	TGTTCCGGATAATTGCTTGA	to test <i>GPX2</i> disruption, <i>GPX2</i> probe
OLI739	TGAGCCCCTGGTTGACTTGT	to test <i>YAP1</i> disruption
OLI740	TGTCGTTGTGCTTATCAAGC	to test <i>YAP1</i> disruption
OLI743	CAATGCCCCCTCCTCAGATT	to test <i>YAK1</i> disruption
OLI744	CTGGTGTAAGCGGGATAACT	to test <i>YAK1</i> disruption
OLI747	AGGGACACTTTACGCTTTCC	to test <i>BCY1</i> disruption
OLI748	GCTGCTCGGACTTTTCTTA	to test <i>BCY1</i> disruption
OLI765	TCCACTATGAGCTTTTCCAC	to test <i>SKN7</i> disruption
OLI766	CAACAGCTCTGTGGTGGATT	to test <i>SKN7</i> disruption
OLI769	GATAAAGTGGGATCTTCCTG	to test <i>PDE2</i> disruption
OLI770	GATGGTGATACATCCAGTGG	to test <i>PDE2</i> disruption

Table 3. PCR templates and primers used in gene-disruption/replacement.

Strain	Parent	Template	Primer (F)	Primer (R)	PCR Fragment ^{a)}	Cre ^{b)}
IB1707	IB1306	pUG6-natMX ^{c)}	OLI475	OLI476	<i>ura3::natMX</i>	+
IB1707	IB1306	pUG6-natMX ^{c)}	OLI479	OLI480	<i>trp1::natMX</i>	
IB1714	IB1707	p3010 ^{d)}	OLI711	OLI712	<i>ctt1::CgTRP1</i>	
IB1716	IB1707	p3010 ^{d)}	OLI723	OLI724	<i>gpx2::CgTRP1</i>	
IB1717	IB1707	p3010 ^{d)}	OLI719	OLI720	<i>sod2::CgTRP1</i>	
IB1737	IB1707	p3010 ^{d)}	OLI530	OLI531	<i>msn2::CgTRP1</i>	
IB1738	IB1707	p3010 ^{d)}	OLI532	OLI533	<i>msn4::CgTRP1</i>	
IB1741	IB1707	p3010 ^{d)}	OLI737	OLI738	<i>yap1::CgTRP1</i>	
IB1761	IB1707	p3008 ^{d)}	OLI517	OLI518	<i>rim15::CgLEU2</i>	
IB1762	IB1707	p3010 ^{d)}	OLI741	OLI742	<i>yak1::CgTRP1</i>	
IB1812	IB1707	p3010 ^{d)}	OLI763	OLI764	<i>skn7::CgTRP1</i>	
IB1817	IB1707	p3010 ^{d)}	OLI745	OLI746	<i>bcy1::CgTRP1</i>	
IB1818	IB1707	p3010 ^{d)}	OLI767	OLI768	<i>pde2::Cg TRP1</i>	

^{a)} *CgTRP1* and *CgLEU2* are *TRP1* and *LEU2* from *Candida glabrata*, respectively.

^{b)} Removal of marker by induction of Cre-recombinase.

^{c)} A gift from Dr. Kodama.

^{d)} Sugiyama et al. (2005).

the observation of Yap1- and Msn2-localization, were gifted by Dr. Kuge and Dr. Schüller, respectively (Görner et al., 1998; Kuge et al., 1997).

Media and genetic methods. The media and genetic methods used for the manipulation of *S. cerevisiae* and *E. coli* cells were as described by Amberg et al. (2005) and Sambrook and Russell (2001), respectively. Yeast cells were grown on a rich medium (YPD, 1% yeast extract, 2% polypeptone, 2% glucose) or a supplemented synthetic medium (SD, 0.67% yeast nitrogen base without amino acids, 2% glucose, and a supplement with amino acids). For the diploidization of IB1707, the *HO* gene was expressed from a YRp type plasmid containing *TRP1* in IB1707. Diploid cells were confirmed by a mating type-test.

Stress survival test. Twenty-eight spore clones of Shirakami kodama yeast were isolated by means of a micromanipulator, and their resistance to oxidative stress was determined. For this, cells were grown overnight at 30°C in 5 ml YPD medium, harvested by centrifugation, diluted to an OD₆₀₀ of 0.5 in 10 ml of fresh YPD medium and cultured at 30°C to an OD₆₀₀ of about 2. For the oxidative stress test, 1 ml of growing cells were harvested by centrifugation, resuspended in 1 ml of sterile water containing 30 mM H₂O₂, and further incubated at 30°C for 30 min. The resistance of *sod2Δ*, *ctt1Δ*, and *gpx2Δ* cells to oxidative stress was determined as above with cells grown in an SD medium lacking tryptophan to an OD₆₀₀ of 1 and resuspended in 5 ml of SD medium lacking tryptophan containing 3 mM H₂O₂. The control cells were not exposed to these stress conditions. The cultures were appro-

proportionately diluted with sterile water, plated on a solid YPD medium, and then incubated at 30°C for 2 days to measure the colony forming units (CFU). The percentage of survival was normalized to the CFU of the control samples (set at 100%).

Measurement of the intracellular oxidation level. The level of the intracellular reactive oxygen species (ROS) induced in a cell during oxidative stress was measured with the oxidant-sensitive probe 2',7'-dichlorofluorescein diacetate (H₂DCFDA) (Molecular Probes, Eugene, OR). Cells were grown overnight at 30°C in an SD medium with uracil, leucine, and histidine (SD+ULH) to an OD₆₀₀ of about 2, were diluted to an OD₆₀₀ of 0.2 in 50 ml of fresh SD+ULH, and cultured at 30°C to an OD₆₀₀ of about 0.5. Then 0.3 mM H₂O₂ was added to the culture and cells were grown for 0.5, 1, or 2 h at 30°C. Cells were harvested, washed with sterile water, and resuspended in 5 ml SD+ULH containing 0.1 mM H₂DCFDA and incubated for 20 min at 30°C with shaking. Cells were harvested, washed with distilled water, resuspended in 250 µl of distilled water and then disrupted with glass beads in a Delta Mixer (Se-08, Taitec, Saitama, Japan) for 20 min at 4°C. After adding 250 µl of distilled water, cell extracts were harvested, and then the fluorescence was measured with λ_{EX} = 490 nm and λ_{EM} = 524 nm using a fluorescence spectrophotometer (SpectroMax M3, Molecular Devices, CA, USA). The cell protein weight was determined following the manufacturer's instructions using a Bio-Rad protein Assay (Bio-Rad, CA, USA). The value of λ_{EM} = 524 nm was normalized to the amount of protein in the mixture.

Enzyme assay. Cells were grown overnight at 30°C in a selective SD medium to an OD₆₀₀ of about 2, harvested by centrifugation, diluted to an OD₆₀₀ of 0.2 in 25 ml of fresh medium, and cultured at 30°C to an OD₆₀₀ of about 0.5. To test the effect of oxidative stress, 0.3 mM H₂O₂, or sterile water, was added to the cultures and cells were grown for the indicated times at 30°C, after which the cells were harvested and their enzymatic activities were measured. The cell protein weight was determined following the manufacturer's instructions using a Bio-Rad protein Assay (Bio-Rad, CA, USA). The β-galactosidase (EC 3.2.1.23) activities from the *CTT1p-CYC1-lacZ* reporter gene in strains MCY3605 and IB1306 were determined after transformation with plasmid pIB650. After a 1 h exposure to 0.3 mM H₂O₂, β-galactosidase activities in the cells were determined with ortho-nitrophenyl-β-D-galactose (ONPG) as the substrate, as described by Amberg et al. (2005). For the measurement of intracellular superoxide dismutase and catalase activities, cells were exposed to 0.3 mM H₂O₂ for 30 min and tested with the SOD assay kit-AST (Dojindo Molecular Technologies, Gaithersburg, MD, USA) and the Colorimetric activity kit (Arbor Assays, Ann Arbor, MN, USA) according to the manufacturer's instructions. Superoxide dismutase and catalase activities were expressed as % per mg protein.

Northern blot hybridization. RNA isolation and Northern hybridization were carried out as described by Sambrook and Russell (2001). To isolate RNA, cells were pregrown overnight at 30°C in 5 ml of a selective SD

medium, harvested by centrifugation, diluted to an OD₆₀₀ of 0.2 in 50 ml of a fresh SD medium and cultured at 30°C to an OD₆₀₀ of 0.5. Cells were treated with 0.3 mM H₂O₂ for 30 min at 30°C (the same conditions as for measuring superoxide dismutase and catalase activities). The total RNA (10 µg) was separated on 1.0% formaldehyde agarose and blotted to a nylon membrane (Biodyne B PALL Corp. N.Y., USA) and hybridized to *CTT1*-, *SOD2*- and *GPX2*-specific ³²P-labeled probes. As probes, ~1 kbp PCR fragments amplified from MCY3605 genomic DNA with primer sets OLI713/OLI714 (*CTT1*), OLI721/OLI722 (*SOD2*) and OLI725/OLI726 (*GPX2*) were labeled using the BcaBEST Labeling kit (Takara). A radioisotope signal was detected on a FLA-3000 (Fujifilm) phosphor imager.

Microscopy. Cells expressing GFP-Yap1 or Msn2-GFP were cultured as described for enzymatic assays and exposed to H₂O₂ for 15 min at 30°C. Nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI). Green fluorescent protein (GFP) localization was monitored directly with a BX51 fluorescent microscope (Olympus). Quantification of the nuclear accumulation of GFP-Yap1 or Msn2-GFP was carried out by microscopic examination of at least 300 cells per sample. The percentages of nuclear accumulation were determined by dividing the number of cells, in which GFP-Yap1 or Msn2-GFP fluorescence colocalized with DAPI stained nuclei, by the number of all DAPI stained cells.

Fermentation test. For the fermentation test, cells were pregrown at 30°C in 5 ml of SD medium with appropriate amino acids and bases, washed once with distilled water, inoculated into 100 ml of the 20% glucose-containing the same medium with 18 mM furfural or sterile water at a final OD₆₀₀ of 0.1, and then further incubated at 30°C for 5 days with shaking. Ethanol concentrations were analyzed by ALCHOMEITO AL-2 (Riken Keiki Co., Tokyo, Japan) following the manufacturer instructions.

Results

Isolation of spore clones from *Shirakami kodama* yeast

Shirakami kodama, a strain of *S. cerevisiae* known for its stress-tolerance, is used as a commercial baker's yeast (Takahashi, 2007). We set out to examine how this yeast copes with oxidative stress so that it can be applied in other industrial processes. For this, we isolated twenty-eight spore clones from *Shirakami kodama* yeast and measured their tolerance to oxidative stress. A spore clone, IB1306, revealed higher viability after exposure to 30 mM H₂O₂ than that of the *Shirakami kodama* yeast (Table 4). To gain insight in the mechanisms underlying the oxidative stress response, we set out to elucidate how IB1306 cells acquired such a high tolerance to oxidative stress.

We first measured the intracellular ROS levels of IB1306 cells with the oxidant-sensitive probe H₂DCFDA during H₂O₂ treatment (Fig. 1). The fluorescence intensity in a laboratory strain gradually increased over time and was about 1.6-fold higher after a 2 h exposure to H₂O₂, indicating that intracellular ROS generation was induced by H₂O₂ treatment. In IB1306 cells, however, fluorescence intensity increased about 2-fold after 0.5 h exposure to

Table 4. Viability of Shirakami kodama yeast and IB1306 cells under 30 mM H₂O₂ stress conditions.

Strain	Viability (%)
Shirakami kodama yeast	0.003 ± 0.001
IB1306	0.1 ± 0.01

Viability is the average of three independent experiments.

Fig. 1. Intracellular ROS levels in MCY3605 and IB1306 cells.

The intracellular oxidation level was measured after exposure to 0.3 mM H₂O₂ at 30°C for the indicated time. The mean and standard deviation for three independent experiments is indicated. White and gray bars represent values of MCY3605 and IB1306 cells, respectively.

H₂O₂, which was sustained over 1 h, but, after 2 h in the presence of H₂O₂, the fluorescence had dropped back to the starting levels. Fluorescence intensity in IB1306 cells decreased about 1.5-fold at the start and was at the same level after 0.5 and 1 h exposure to H₂O₂, compared with that in a laboratory strain. These results indicate that IB1306 cells have a high ability to reduce ROS.

High activities of superoxide dismutase and catalase

Antioxidant defenses include a number of protective enzymes that are present in different subcellular components and can be upregulated in response to ROS exposure (Morano et al., 2012). We therefore measured the activities of superoxide dismutase and catalase in IB1306 cells (Table 5). Interestingly, upon 0.3 mM H₂O₂ stress conditions, IB1306 cells revealed at least 1.3-fold increased superoxide dismutase activity compared with the laboratory strain. The superoxide dismutase activity in IB1306 cells under non-stress conditions was almost 2.3-fold higher than that in the laboratory strain. Similarly, the catalase activity in IB1306 cells was almost 1.5-fold greater under H₂O₂ stress conditions. Like the superoxide dismutase activity, the catalase activity in IB1306 cells under non-stress conditions also indicated an almost 1.6-fold increase compared with that in the laboratory strain. Thus, high activities of superoxide dismutase and catalase contributed to reduce elevated ROS by H₂O₂ exposure and, even under non-stress conditions, superoxide dismutase and catalase in IB1306 cells are relatively more active.

Next, we investigated, by a means of Northern hybridization, whether the expression of *SOD2*, *CTT1*, and *GPX2* in IB1306 cells, which encode superoxide dismutase, cata-

Table 5. Superoxide dismutase and catalase activities (U/mg protein).

Strain	Superoxide dismutase		Catalase	
	–	+	–	+
MCY3605	505.7 ± 131.7	984.0 ± 76.5	4.3 ± 0.6	7.4 ± 0.0
IB1306	1142.7 ± 152.1	1575.5 ± 217.0	6.8 ± 0.7	11.1 ± 1.9

+ and – indicate the treatment with and without 0.3 mM H₂O₂ for 30 min, respectively. These activities are the average of three independent cultures.

lase, and glutathione peroxidase, respectively, are induced under oxidative stress conditions. Transcription of the three genes in MCY3605 cells increased upon exposure to H₂O₂ (Fig. 2, lanes 1 and 2, 5 and 6, 9 and 10). Interestingly, in IB1306 cells, *SOD2* and *CTT1* seem to be highly expressed irrespective of exposure to H₂O₂ (Fig. 2, lanes 3 and 4, 7 and 8), whereas the transcription of *GPX2* in IB1306 cells was at the same level as that in MCY3605 cells (Fig. 2, lanes 9 to 12). These results indicate that the observed differences in superoxide dismutase and catalase activities relate to increased expression levels of *SOD2* and *CTT1* in IB1306 cells, while the expression of *GPX2* does not change.

Msn2 is required for high levels of *CTT1* expression in IB1306 cells

In order to gain more insight into the contribution of *SOD2*, *CTT1* or *GPX2* to IB1306 tolerance to oxidative stress, the genes were disrupted by replacement with *TRP1* from *Candida glabrata* (Sugiyama et al., 2005; see Materials and Methods, Table 3). We examined the effect of these genes on the survival rate of IB1306 cells after exposure to oxidative stress (Fig. 3). The cell viability of gene-disrupted cells after exposure to 3 mM H₂O₂ was significantly lower than that of wild-type cells. *ctt1Δ* cells were more sensitive to 3 mM H₂O₂ stress than *sod2Δ* or *gpx2Δ* cells. Taken together, our results indicate that the catalase is relatively more active in IB1306 cells under 3 mM H₂O₂ stress conditions. Therefore, we focused on the expression of *CTT1* in the following experiments.

CTT1 is regulated by the oxidative-stress transcription factors Yap1 and Skn7, and by the general stress response complex Msn2/4 (Toone and Jones, 1998). To examine how these proteins regulate *CTT1* expression, the genes for *MSN2*, *MSN4*, *YAP1*, or *SKN7* were disrupted in IB1306 (Table 1) and the levels of *CTT1* transcription were determined under conditions with or without oxidative stress (Fig. 4). Under non-stress conditions, in *msn2Δ*, *msn4Δ*, and *yap1Δ* cells *CTT1* transcription was not observed (Fig. 4, lanes 3, 5 and 7). Upon oxidative stress conditions, only in *msn4Δ* cells was *CTT1* highly expressed (Fig. 4, lane 6) indicating that Msn4 is not required for the *CTT1* transcription under oxidative stress conditions, and suggesting that Msn2 and Msn4 have different roles as a transcription factor. Under both conditions, in *skn7Δ* cells *CTT1* transcription decreased to about half compared with that in wild-type cells (Fig. 4, lanes 1, 2, 9 and 10). Thus, in IB1306 cells, under non-stress conditions Msn2, Msn4, and Yap1 are mainly required for the *CTT1* expression and,

Fig. 2. *SOD2*, *CTT1*, and *GPX2* transcription under H_2O_2 stress conditions in MCY3605 and IB1306 cells. The Northern blot was prepared with total RNA isolated from MCY3605 (lanes 1, 2, 5, 6, 9 and 10) and IB1306 (lanes 3, 4, 7, 8, 11 and 12) cells grown in an SD medium with (+) or without (–) 0.3 mM H_2O_2 for 30 min.

Fig. 3. Effect of *SOD2*, *CTT1*, and *GPX2* deletions on tolerance to H_2O_2 stress.

Survival percentages of wild-type (IB1707) and derived strains carrying deletions *sod2Δ* (IB1717), *ctt1Δ* (IB1714) and *gpx2Δ* (IB1716) were determined after exposure to 3 mM H_2O_2 for 30 min. Results indicate the mean and standard deviation for three independent experiments.

when exposed to oxidative stress, Msn2 and Yap1 primarily contribute to that. We focused on the function of Msn2 to explore in more detail the mechanism for tolerance to oxidative stress in IB1306 cells, because Msn2 is required for *CTT1* transcription under both non- and oxidative stress conditions (Fig. 4, lanes 3 and 4), and no change was observed in the expression of *GPX2* (Fig. 2, lanes 9 to 12) which is regulated by Yap1 and Skn7, but not Msn2/4 (Inoue et al., 1999).

Msn2 localizes to the nucleus under non-stress conditions in IB1306

It was previously shown that in the absence of stress, Msn2 is mainly localized in the cytoplasm. Following exposure to stress, Msn2 and Msn4 are rapidly translocated to the nucleus, where it facilitates the expression of stress-induced genes (Görner et al., 1998). We examined whether subcellular localization of Msn2 in IB1306 cells follows a similar pattern by means of a GFP fusion protein expressed from a single copy plasmid, pAdh1-Msn2-GFP (Fig. 5A). Surprisingly, even under non-stress conditions, the proportion of Msn2-GFP that accumulated in the nucleus of IB1306 cells (39%) was much higher than in case of the laboratory strain (11%). This was also the case upon exposure to H_2O_2 , when, in IB1306 cells, 2-fold more Msn2-GFP existed in the nucleus compared with the laboratory strain. Therefore, it seems likely that Msn2 accu-

Fig. 4. *CTT1* transcription under H_2O_2 stress conditions in *msn2Δ* (IB1737), *msn4Δ* (IB1738), *yap1Δ* (IB1741) and *skn7Δ* (IB1812) cells.

The Northern blot was prepared with total RNA isolated from wild-type, IB1670, (lanes 1 and 2), IB1737 (lanes 3 and 4), IB1738 (lanes 5 and 6), IB1741 (lanes 7 and 8) and IB1812 (lanes 9 and 10) cells grown in an SD medium containing uracil with (+) or without (–) 0.3 mM H_2O_2 for 30 min. Values at the bottom show the quantification of the Northern-blot-signals for *CTT1* normalized to the rRNA loading control using Quantity One assay software (Bio Rad CA, USA).

Table 6. β -galactosidase activity from the *CTT1p-CYC1-lacZ* reporter gene (units/mg protein).

Strain	Characteristic	0.3 mM H_2O_2	
		–*	+*
IB1670	Wild-type	112.3 $\pm$ 6.7	170.2 $\pm$ 14.2
IB1761	<i>rim15Δ</i>	54.4 $\pm$ 4.1	82.5 $\pm$ 2.9
IB1762	<i>yak1Δ</i>	100.3 $\pm$ 4.1	96.5 $\pm$ 8.5
IB1817	<i>bcy1Δ</i>	25.4 $\pm$ 3.3	22.1 $\pm$ 1.9
IB1818	<i>pde2Δ</i>	8.0 $\pm$ 0.3	8.8 $\pm$ 0.6

* β -galactosidase activities were determined after 1 h incubation in selective SD medium with (+) or without (–) 0.3 mM H_2O_2 . The β -galactosidase activity is the average of three independent cultures.

mulation in IB1306 and MCY3605 cells is differently regulated. The subcellular localization of Yap1, which we also tested by means of a GFP fusion protein, did not differ significantly between IB1306 and the laboratory strain (Fig. 5B). Because Yap1 activity is mainly regulated by its intracellular localization (Kuge et al., 1997), these results indicate that the accumulation of Msn2, but not Yap1, in the nucleus causes a high expression of *CTT1* under no-stress conditions in IB1306.

Fig. 5. Nuclear accumulation of Msn2-GFP and GFP-Yap1 in response to H₂O₂.

Msn2-GFP (A), and GFP-Yap1 (B) subcellular localization, and the position of the cell nucleus (DAPI) were assessed by fluorescence microscopy. The numbers indicate the percentage of fluorescence in the nucleus. Exposure to 0.3 mM H₂O₂ is indicated by +, the absence of stress by –.

Fig. 6. Growth curves (A) and ethanol production (B) of IB287 (circle) and IB1752 (square) cells in the presence (closed symbol) or absence (open) of 18 mM furfural.

The values are the mean from three independent experiments; standard deviations are indicated.

cAMP-dependent protein kinase A might have a low activity in IB1306 cells

Gene-expression that relies on transcription factors Msn2 and Msn4 can be positively regulated by kinases Rim15 and Yak1 (Cameroni et al., 2004; Zhang et al., 2013). To investigate whether Rim15 or Yak1 are involved in *CTT1* expression, their genes were disrupted in IB1306 cells and the levels of *CTT1* expression were determined in the mutant strains by a means of a *lacZ* reporter assay after 1 h incubation in selective SD medium with or without 0.3 mM H₂O₂ (Table 6). Upon oxidative stress, *rim15Δ*, as well as *yak1Δ*, cells revealed about a 2-fold decreased β -galactosidase activity from the *CTT1p-CYC1-lacZ* reporter as compared with wild-type IB1306 cells. Under non-stress conditions, there was a difference between *yak1Δ* and *rim15Δ* cells: the β -galactosidase activity in *yak1Δ* cells was like the wild-type, whereas in *rim15Δ* cells this was ~2-fold reduced compared with the wild-type. These observations suggest that under conditions without

oxidative stress, the high expression of *CTT1* is maintained by Rim15 rather than by Yak1 in IB1306 cells.

To investigate the role of the cAMP/PKA cascade in the expression of *CTT1*, we disrupted the PKA regulatory subunit *BCY1* or cAMP phosphodiesterase *PDE2* in IB1306 cells. Under conditions with or without H₂O₂, induced PKA activity in either the *bcy1Δ* or *pde2Δ* disruptant resulted in a 4- or 14-fold reduced β -galactosidase activity from the *CTT1p-CYC1-lacZ* reporter, respectively, as compared with the wild-type cells (Table 6). These results suggest that transcriptional activity of Msn2 in the nucleus, which is counteracted by an activated cAMP/PKA pathway, contributes to the expression of *CTT1*.

IB1306 cells are attractive for bioethanol production from lignocellulosic biomass

In view of the enhanced oxidative stress tolerance of IB1306, we wondered how the strain would cope with the conditions which cells would encounter during bioethanol

production from lignocellulosic materials. For this we made a diploid, uracil and tryptophan auxotroph strain, IB1752, which would be more robust than the haploid IB1306 (Table 1). The fermentation potential of IB1752, in comparison with a diploid laboratory strain, IB287, was evaluated by measuring the growth rate and the ethanol production (Fig. 6). In the absence of furfural, IB1752 cells reached a stationary phase faster and at a higher cell density, and produced more ethanol in the first three days than the control strain. When exposed to 18 mM furfural stress, IB1752 had a shorter lag-phase, and a higher growth rate and ethanol production than the control strain. Interestingly, the final level of ethanol production by IB1752 was ~2%, irrespective of the presence of furfural, whereas ethanol production in the control strain was very sensitive to stress. These results indicate that IB1752 cells can adapt very well to the presence of furfural.

Discussion

A haploid spore clone, IB1306, isolated from diploid Shirakami kodama yeast showed a higher tolerance to oxidative stress than Shirakami kodama yeast. We have sought to gain more insight into the mechanism for this increased tolerance to oxidative stress. We found that this strain efficiently countered elevated levels of ROS induced by H₂O₂ exposure due to high activities of superoxide dismutase and catalase, and also maintained this in the absence of stress (Table 5). This correlated with a high expression of *SOD2* and *CTT1*, which was caused by Msn2 and Msn4, irrespective of exposure to H₂O₂ (Fig. 2). This can be explained by the constitutive nuclear accumulation of Msn2: even under non-stress conditions, about 40% of the Msn2 pool accumulated in the nucleus, which increased to 90% after exposure to H₂O₂ stress (Fig. 5). Another oxidative-stress response factor, Yap1, was found not to show such a constitutive nuclear localization in IB1306.

Interestingly, it has been found that Msn2 overexpression activates STRE-regulated genes including *SOD2* and *CTT1* in the absence of stress (Schmitt and McEntee, 1996; Watanabe et al., 2009), and results in tolerance to ethanol stress in a sake yeast, to oxidative stress in bioethanol producing strains and to freeze-thaw stress in baker's yeast (Sasano et al., 2012a, b; Watanabe et al., 2009). Overall, it appears that reduced regulation of Msn2 export can lead to enhanced stress tolerance. In the case of IB1306, in which ROS-degrading enzymes become highly expressed and are continuously active, this has led to a high tolerance to oxidative stress.

Yak1 and Rim15 kinases positively regulate starvation-specific gene expression mediated by the Msn2/4 transcription factors (Zhang et al., 2013). In IB1306 cells, Rim15, which can phosphorylate Msn2 *in vitro* (Lee et al., 2013), might be involved in sustaining the high expression of *CTT1* under non-stress conditions (Table 6). Under nutritional or non-stress conditions, Rim15 activity is inhibited by phosphorylation mediated by PKA and Sch9, a major substrate of the target of rapamycin complex 1 (TORC1) (Wanke et al., 2008). Upregulation of the cAMP/PKA activity by disruption of *BCY1* or *PDE2* reduced the

expression of *CTT1* under non-stress conditions (Table 6). Therefore, it is presumed that, in IB1306 cells, high *CTT1* expression under non-stress conditions is a result of reduced inhibition of Rim15 by PKA.

Stresses to which yeast strains are exposed in various industrial processes could be countered by overexpression of Msn2 (Sasano et al., 2012a, b; Watanabe et al., 2009). Being related to increased Msn2 activity, the enhanced oxidative stress-tolerance of IB1306 could make this strain suitable for bioethanol production from lignocellulosic biomass, in which aldehydes, such as furfural, are present which cause oxidative stress in yeast. In growth experiments in which ethanol production was recorded, we found that a diploid strain derived from IB1306 performed much better than a standard strain when exposed to furfural (Fig. 6). Future tests will have to show whether this strain can accommodate the full variety of compounds released during preparation of lignocellulosic biomass that repress fermentation in other yeast strains.

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