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Protection of yeast lacking the Ure2 protein against the toxicity of heavy metals and hydroperoxides by antioxidants

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Abstract
The aim of this study was to examine the protection of the yeast lacking the "antioxidant-like" prion precursor protein (Ure2p), by antioxidants and to elucidate how modification of redox homeostasis affects toxicity of agents inducing oxidative stress in the *Δure2* cells. We found a diverse ability of a range of antioxidants to ameliorate the hypersensitivity of the *Δure2* disruptant to oxidants and heavy metal ions. Glutathione and then ascorbate were the most effective antioxidants; Tempol, Trolox and melatonin were much less effective or even hampered the growth of the *Δure2* cells exposed to tested agents. The intracellular level of ROS was augmented in the *Δure2* mutant under normal growth conditions (1.7-fold), and after treatment with H_2O_2 (2.3-fold) and Cd(II) (2.8-fold), with respect to its wild-type counterpart. Glutathione was unable to prevent the increase in ROS production caused by $CdCl_2$. The *Δure2* disruptant was also hypersensitive to heat shock, like mutants lacking glutathione *S*-transferases.

Keywords: *Yeast, Saccharomyces cerevisiae, Ure2p, heavy metals, antioxidants, glutathione S-transferase*

Abbreviations: *CDNB, 1-chloro-2,4-dinitrobenzene; CumOOH, cumene hydroperoxide; DCF, 2′,7′-dichlorofluorescein; GST, glutathione S-transferase; H_2DCF, 2′,7′-dichlorodihydrofluorescein; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; NAC, N-acetylcysteine; NCR, nitrogen catabolite repression; ROS, reactive oxygen species; tBOOH, t-butyl hydroperoxide*

Introduction

The *Saccharomyces cerevisiae* Ure2p is a protein interesting in several respects. Firstly, it plays a role as a transcriptional regulator of genes involved in nitrogen catabolite repression (NCR) [1,2]. Under nitrogen-rich conditions (the presence of glutamine or ammonia), Ure2p forms complexes with GATA-factor transcription activators such as Gln3 and Gat1/Nil1 which turns off transcription of genes responsible for the use of poor nitrogen sources [3,4]. To the contrary, in the presence of non-preferred nitrogen sources (e.g. proline) these genes are highly expressed as a consequence of dephosphorylation of Gln3 and its translocation to the nucleus.

Secondly, Ure2p is a precursor of yeast [URE3] prion. This property of Ure2p is ascribed to its N-terminus rich in asparagine and glutamine [5]. Such amino-acid repeats are found in proteins connected with a range of neurodegenerative diseases, especially amyloid- fiber based diseases [6,7]. Hence, the yeast prion system is a promising model to study biochemical and molecular facets of mammalian prions occurrence, propagation, transmission and clearance [8–10].

Thirdly, Ure2p seems to be involved in the defense against heavy metals and oxidants. The protein shares

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homology with glutathione *S*-transferases (theta class), according to protein sequence alignment and 3D similarity [3,11–13]. Nevertheless, it fails to show activity with the common GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) [3,14–16]. However, no detectable GST activity towards CDNB characterizes also other members of the most diverse theta class of GSTs (identified mainly by structural similarities) [17]. It has been reported repeatedly that deletion of the *URE2* gene enhances the toxicity of a range of metal ions, especially Cd(II), As(III), As(V), Cr(III), Cr(VI), Se(IV) and prooxidative agents, such as H_2O_2, *t*BOOH and CumOOH, to yeast cells [16,18,19]. *In vitro* studies have revealed that Ure2p shows a GSH-dependent peroxidase activity towards hydroperoxides (H_2O_2 and organic peroxides) [20]. Additionally, a saprophytic ascomycete *Aspergillus nidulans* lacking *gstA* gene, a homolog of the *S. cerevisiae URE2* gene, is also more sensitive to heavy metals (selenium, silver, nickel), xenobiotics and a systemic fungicide carboxin [21].

The aim of this study to check whether disruption of the *URE2* gene imposes oxidative stress on the yeast, affecting the level of production of reactive oxygen species. Here, we show that toxicity of hydrogen peroxide and Cd (II) to the *Δure2* cells may be associated with increased intracellular ROS production. Previously, we found that exogenous antioxidants can protect yeast deficient in antioxidant proteins [22–24]. In this study, we examined also if antioxidants can affect the growth of the *Δure2* disruptant subjected to oxidative stress. Since Ure2p can be considered as a GST analog, we checked also whether Ure2p may contribute to heat shock resistance which has been found for "classical" yeast glutathione *S*-transferase 1, Gtt1, and glutathione *S*-transferase 2, Gtt2, [14].

Materials and methods

Chemicals

Cumene hydroperoxide was from Fluka and Trolox was from Aldrich. All other reagents were purchased from Sigma (Poznan, Poland) and were of analytical grade.

Yeast strains and growth conditions

The following yeast strains were used: wild-type CC30 *MATa trp1-1*, *ade2-1*, *leu2-3,112*, *his3-11,15*, *ura2::HIS3*; CC32 a *URE2* disruptant in CC30 YNL229CΔ KanR; CC34 [URE3] isogenic to CC30 [25] which were obtained from Dr Christophe Cullin (Bordeaux University, France), wild type CY4 *MATa ura3-52*, *leu2-3,112*, *trp1-1*, *ade2-1*, *his3-11*, *can1-100*; and disruptants Y117 *grx1::LEU2 grx2::HIS3*; Y836 *gtt1::TRP1 gtt2::URA2*; Y781 *grx1::LEU2 grx2::HIS3 gtt1::TRP1 gtt2::URA2* which were kindly provided by Dr Chris Grant (University of Manchester, UK) [26]. Yeast was grown either on liquid YPD medium (1% Difco Yeast Extract, 1% Difco Yeast Bacto-Peptone, 2% glucose, or on solid YPD or minimal YNB medium (0.67% Yeast Nitrogen Base, 0.5% glucose) with appropriate additives, containing 2% agar, at 28°C.

The deletion mutants were checked on YPD Petri dishes containing 200 μg/ml G418 sulphate or on YNB plates ± Ura, Ade, Leu, His, Trp, respectively.

Tests for assessment of heavy metal and oxidant toxicity and assay of growth restoration by antioxidants

Several dilutions (1×10^7, 1×10^6, 1×10^5, 1×10^4 cells/ml) of a yeast exponential phase culture in a volume of 5 μl were inoculated on YPD or YNB agar supplemented with the appropriate amino acids or nucleic acid bases containing the tested agents ± antioxidants, incubated at 28°C and inspected after 48 h. Stock solutions of antioxidants were added to sterile media which were cooled to just above the solidification point (temperature of ~50°C). All stock solutions of antioxidants were freshly prepared before adding to the media.

For spotting experiments involving $AlCl_3$ was used a modified low-pH, low-phosphate (LPP) medium [18] to avoid troubles with solubility and to augment toxicity of Al. LPP medium is a YNB minimal medium (0.67% Yeast Nitrogen Base, 2% glucose) with 78 μM K_2HPO_4, 1.1 mM KH_2PO_4 and 3.2 mM KCl, pH 3.5.

Modification of heavy metal and oxidant resistance by pre-treatment with D-arabinose and NAC

Yeast cells from the logarithmic phase at a concentration of 1×10^7 cells/ml were incubated in 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.1% glucose with an antioxidant precursor (NAC or D-arabinose) for 2 h or were grown in YNB medium with NAC or D-arabinose for 18 h and then harvested, washed and diluted (1×10^7, 1×10^6, 1×10^5, 1×10^4 cells/ml) and inoculated on appropriate plates at 28°C for 48 h.

Intracellular ROS production

Cells were sedimented by centrifugation, washed twice and suspended in 0.1 M phosphate buffer, pH 7.0, containing 0.1% glucose and 1 mM EDTA ± $CdCl_2$ or H_2O_2, to a density of 10^8 cells/ml. Generation of reactive oxygen species was estimated with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, 27 μM). Fluorescence intensity due to oxidation of 2′,7′-dichlorodihydrofluorescein (H_2DCF) to 2′7′-dichlorofluorescein (DCF) was monitored in a Hitachi F2500 fluorescence spectrophotometer. Measurement conditions were: $\lambda_{ex} = 495$ nm, $\lambda_{em} = 525$ nm; temperature, 28°C.

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Heat shock treatment

Cells were grown to logarithmic or stationary phase and then subjected to heat shock at 50°C for 1, 2, 3 and 5 min. After exposure cell suspensions were diluted and plated onto solid YPD medium. Cultures were left to grow at 28°C for 48 h and their survival was estimated by colony counting.

The results represent means ± SD from at least three independent experiments.

Results

Sensitivity to the Δure2 disruptant to heavy metal ions and oxidants

Since Ure2p may take part in the detoxification of heavy metals and hydroperoxides [16,18,19] we decided to get a closer insight into the phenotype of the *Δure2* mutant. Results of assessment of heavy metals and oxidants toxicity to the *Δure2* cells is summarized in Table I.

Hydroperoxides hampered the growth of the *Δure2* mutant both on YPD and on YNB media. The most toxic was cumene hydroperoxide which affected the growth of the *Δure2* disruptant at a concentration of 10 μM on YNB medium (Table I). Similar effects were seen for *t*-butyl hydroperoxide at a concentration of 70 μM and for 300 μM hydrogen peroxide. Harmful effects of hydroperoxides were observed at much higher concentrations, of 300 μM and 1.5 mM for organic peroxides and H_2O_2, respectively, on the rich (YPD) medium. We checked also whether redox cycling agents may cause inhibition (delay) of growth of the *Δure2* cells. However, no hypersensitivity to menadione, a superoxide-generating agent, was detected in cells lacking *URE2* gene (Table I).

The *Δure2* mutant grew slower than the wild-type yeast on YNB plates when subjected to Cd(II), As(III), Cr(VI), Ni(II), Se(IV) and Co(II). Also As(V), Cr(III) and Al(III) affected more strongly the growth of the *Δure2* cells than of its wild-type counterpart, but at much higher concentrations (Table I). On the YPD medium, toxicity of some metals, especially Cr(III), Cr(VI) and Ni(II) was masked. Some metals (Hg(II), Ag(I) and Mo(VI)) had limited inhibitory effect on the growth of the *Δure2* disruptant, as compared with the wild-type strain, both on rich and minimal media. The *Δure2* disruptant spotted onto YNB plate was even slightly more resistant to Mo(VI) than the wild-type strain (Table I).

In parallel, yeast in the [URE3] state, containing aggregated and altered Ure2p, was subjected to spotting experiments. We were unable to observe any increased sensitivity of the yeast strain of the [URE3] prion phenotype towards the agents tested when compared to its wild-type counterpart (data not shown).

Since structural homology of Ure2p with glutathione *S*-transferases Gtt1 and Gtt2 proteins has been demonstrated [14], we compared the sensitivity of disruptants in genes coding for these proteins and also in genes coding for Grx1p (glutaredoxin 1) and Grx2p (glutaredoxin 2) to heavy metals and oxidants. Hydroperoxides hampered the growth of the *Δgrx1Δgrx2* and *Δgrx1Δgrx2Δgtt1Δgtt2* disruptants [26,27] but they had no effect on the kinetics of growth of the *Δgtt1Δgtt2* mutant (data not shown). Generally (with few exceptions), the *Δgrx1Δgrx2*, *Δgtt1Δgtt2* and *Δgrx1Δgrx2Δgtt1Δgtt2* mutants were no more sensitive to a range of heavy metals tested than their parental strains (data not shown). A mutant lacking *GTT1* and *GTT2* genes showed a more severe growth impairment than its wild-type counterpart on YPD medium in the presence of 500 μM As(III) (Figure 1), although this hypersensitivity was lower than that of the *Δure2* cells which were unable to grow in the presence of 300 μM As(III) (Table I). The *Δgrx1Δgrx2* disruptant was more sensitive to selenite (especially 2 mM) than its isogenic wild-type counterpart (Figure 1).

Protection by antioxidants

Antioxidants, including ascorbate, GSH, Tempol, Trolox and melatonin, were checked for their ability to ameliorate growth restriction of the *Δure2* cells caused by some heavy metals and oxidants (Table II).

Glutathione at a concentration of 5 mM completely restored growth of the *Δure2* mutant in every system tested. At lower concentrations, 1–2 mM, it also improved the ability of colony formation in almost all cases. No protective effect of glutathione against Cd(II) toxicity has been reported previously [19]. Differences in the concentrations of Cd(II) and GSH used and in the duration of incubation may be responsible for the contradictory results obtained by Rai and Cooper [19] and found in the present study. Ascorbate also conferred protection to the *Δure2* strain, especially with respect to hydroperoxides. It was less protective against heavy metal toxicity (cobalt and arsenic exposure in YNB medium) (Table II). However, an opposite effect (better protection by ascorbate than by glutathione) was seen on YPD medium (Figure 2).

To the contrary, protection against Cd(II) by ascorbate was seen on YNB medium but not on the rich YPD medium (Table II).

Three other antioxidants tested were much less effective (Table II). Tempol partially restored growth of the *Δure2* disruptant treated with Cd(II), As(III) and hydrogen peroxide. Trolox conferred intermediate protection to the *Δure2* mutant exposed to As(III) and modest protection in the cases of exposure to Cr(VI) and hydrogen peroxide. Melatonin exerted only a very weak protection to the *Δure2* cells

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Table I. Sensitivity of the *Δure2* disruptant to metals and oxidants.

Medium		YPD		YNB	
Metal/oxidant	Concentration	WT	*Δure2*	WT	*Δure2*
$CdCl_2$	5 μM	+++	+++	+++	++
	7–10 μM	+++	++	+++	+
	20 μM	+++	–	++	–
$NaAsO_2$	50 μM			+++	+++
	100 μM	+++	+++	+++	++
	200 μM	+++	++	++	–
	300 μM	+	–	–	–
	1 mM	–	–		
Na_2HAsO_4	50–500 μM	+++	+++	+++	+++
	1 mM	+++	++	+++	++
	2 mM			++	+
	5 mM			–	–
$CoCl_2$	100 μM			+++	+++
	200–400 μM			+++	++
	500 μM	+++	+++	+	–
	1 mM	+++	++	–	–
	2 mM	–	–		
$Cr(NO_3)_3$	100–500 μM			+++	+++
	1–2 mM			+++	++
	5 mM	+++	+++	–	–
K_2CrO_4	100 μM			+++	++
	200 μM	+++	+++	++	–
	500 μM	++	++	–	–
	1 mM	–	–		
Na_2SeO_3	100–200 μM	+++	+++	+++	+++
	500 μM–2 mM	+++	++	+++	++
	3 mM	+++	++	++	+
$NiCl_2$	50 μM			+++	+++
	100 μM			+++	++
	200 μM			++	+
	500 μM			–	–
	1 mM	+++	+++		
	1.5 mM	++	++		
	2 mM	–	–		
$HgCl_2$	5–20 μM			+++	+++
	30 μM			+++	++
	40 μM			+	+
	50 μM			–	–
	500 μM	+++	+++		
	1 mM	++	++		
	2 mM	–	–		
$AgNO_3$	1 μM			+++	+++
	2–5 μM			+	+
	10–50 μM			–	–
	200 μM	+++	+++		
	500 μM	–	–		
Na_2MoO_4	100–200 μM			+++	+++
	500μM–1 mM			++	+++
	2 mM	+++	+++	+	++
$AlCl_3$*	50–500 μM	+++	+++		
	1–3 mM	+++	++		
	5 mM	+	–		
H_2O_2	200 μM			+++	+++
	300 μM			+++	++
	500 μM			–	–
	1 mM	+++	+++		
	1.5 mM	+++	++		
	2 mM	++	–		
*t*BOOH	20 μM			+++	+++
	70 μM			+++	++
	100 μM			++	+
	200 μM	+++	+++	–	–
	300 μM	+++	++		

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Table I – *continued*

Medium		YPD		YNB	
Metal/oxidant	Concentration	WT	*Δure2*	WT	*Δure2*
	400 μM	++	+		
	500 μM	+	–		
CumOOH	10 μM			+++	++
	20 μM			++	+
	50 μM			+	–
	100 μM			–	–
	200 μM	+++	+++		
	300 μM	+++	++		
	400 μM	++	–		
	500 μM	–	–		
Menadione	1–10 μM			+++	+++
	20 μM			++	++
	30 μM			+	+
	50 μM	+++	+++	–	–
	70 μM	++	++		
	90 μM	+	+		

+++ growth unaltered (growth at all dilutions tested, as of non-treated cells), ++ partial inhibition (no growth at concentrations ranging from 1×10^4 to 1×10^5 cells/ml), + strong inhibition (no growth at a concentration of 1×10^6 cells/ml),—complete inhibition of growth (no growth at a concentration of 1×10^7 cells/ml), * test with Al(III) was performed on LPP medium (see Materials and methods).

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subjected to hydrogen peroxide and As(III). Additionally, higher concentrations of Tempol, Trolox and melatonin, viz. 1, 1 and 0.5 mM, respectively, were themselves toxic to yeast cells (data not shown). In some cases these antioxidants even augmented the toxicity of the agents tested, e.g. melatonin, 0.5 mM, aggravated the growth inhibition caused by heavy metal ions and oxidants in every system studied (Table II).

The data presented above are based on the growth of yeast in the presence of antioxidants and oxidative agents, so we cannot exclude some direct oxidant-antioxidant interactions in the tested media. In order to ascertain that the protective effect was due to intracellular action of the antioxidants, we increased intracellular level of two yeast antioxidants, erythroascorbic acid (a five-carbon ascorbic acid analog synthesized in the yeast) and glutathione by D-arabinose and N-acetylcysteine pre-treatment, respectively. The antioxidant precursors were washed off, and then cells of the *Δure2* mutant were subject to oxidative challenge. Treatments with both antioxidant precursors resulted in an increase of the resistance of the cells to heavy metals, the effect of NAC being more pronounced (Table III), especially in a case of short term pre-incubation in buffer (Table IIIA), most probably because of overshoot of synthesis of antioxidants in the initial period of feeding with their precursors.

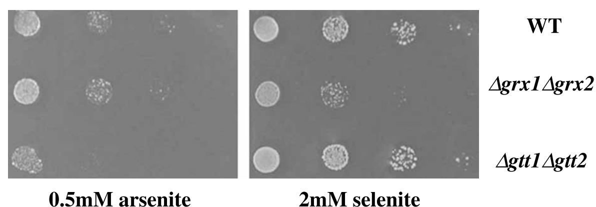

Figure 1. As(III) toxicity to the *Δgtt1Δgtt2* mutant and Se(IV) toxicity to the *Δgrx1Δgrx2* mutant. Cells from logarithmic phase were spotted onto YPD medium at decreasing concentration (from left to right: 1×10^7, 1×10^6, 1×10^5, 1×10^4 cells/ml). Growth was inspected after 48 h.

Intracellular ROS production

Under normal growth conditions the rate of H_2DCF oxidation was 1.7-fold higher in the *Δure2* disruptant compared to its wild type counterpart (Figure 3).

We also checked the influence of two agents, hydrogen peroxide and Cd(II), on the intracellular level of ROS in the *Δure2* mutant. Both agents caused an additional increase of the intensity of DCF fluorescence, 2.3 and 2.8-fold, respectively (Figure 3). However, assuming the basal level of DCF fluorescence in wild-type strain as 100% and expressing results obtained in other systems as % of this value, one obtains augmentation of the rate of H_2DCF oxidation by hydrogen peroxide and Cd(II) of about 7 and 40-fold, respectively. Since ascorbate can decrease the steady-state level of ROS in mutant lacking *SOD1* gene [28] we checked if glutathione can alter intracellular ROS production in the *Δure2* strain subjected to 1 mM H_2O_2 or 10 μM Cd(II). We were unable to observe any decrease of the rate of H_2DCF oxidation (data not shown).

Sensitivity to the Δure2 cells to heat shock treatment

Gtt1p and Gtt2p, two proteins homologous to Ure2p, confer resistance to heat shock in the stationary phase and mutants lacking *GTT1* and/or *GTT2* gene lose

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Table II. Protection of the *Δure2* yeast from toxicity of metal ions and oxidative agents by antioxidants

Antioxidant		Metal/oxidant									
		YPD			YNB						
Compound	Concentration	A	B	C	D	E	F	G	H	I	J
Ascorbate	1 mM	0	*	***	*	0	***	0	***	***	***
	5 mM	0	**	***	**	0	***	0	***	***	***
	10 mM	0	***	***	***	0	***	0	***	***	***
	20 mM	0	***	***	***	*	***	0	***	***	***
	30 mM	0	***	***	***	*	***	0	***	***	***
GSH	50 μM	**	**	**	0	0	0	*	**	0	0
	100 μM	**	**	**	0	0	0	*	**	*	*
	1 mM	***	**	***	***	*	***	*	***	***	***
	2 mM	***	**	***	***	**	***	*	***	***	***
	5 mM	***	***	***	***	***	***	***	***	***	***
Tempol	50 μM	*	**	***	0	*	0	0	0	0	0
	100 μM	0	**	*	0	0	0	0	0	0	0
	1 mM	–	0	0	0	0	–	0	0	–	0
Trolox	100 μM	0	0	*	0	0	*	0	0	0	**
	500 μM	0	–	0	–	0	0	–	0	0	**
	1 mM	–	–	0	–	–	0	–	0	0	*
Melatonin	50μM	0	*	*	0	0	–	0	0	0	0
	100μM	0	0	0	0	0	–	0	0	0	0
	500μM	–	–	–	–	–	–	–	–	–	–

A: 10 μM Cd(II), B: 200 μM As(III), C: 1.5 mM H_2O_2, D: 7 μM Cd(II), E: 100 μM As(III), F: 100 μM Cr(VI), G: 400 μM Co(II), H: 0.3 mM H_2O_2, I: 70 μM *t*BOOH, J: 10 μM CumOOH.
0 no effect of an antioxidant (growth the same as of the yeast treated with a tested metal ion or oxidant, in the absence of any protective agent), *some protective effect (restoration of growth at a concentration of 1×10^6 cells/ml), ** medium protective effect (restoration of growth at a concentration of 1×10^5 cells/ml), *** restoration of growth to the level observed in the absence of the metal/oxidant (growth at all dilutions tested),—inhibition of growth by the antioxidant.

ability to grow at +39°C [14]. These findings prompted us to examine the survival of the *Δure2* strain after exposure to +50°C for 1, 2, 3 and 5 min, by the colony formation assay. In the logarithmic phase, survival of the *Δure2* mutant was lower when compared with the wild-type strain: 74%/95%, 66%/91%, 45%/78% and 1%/8% for the *Δure2* mutant and wild-type strain after 1, 2, 3 and 5 min of heat treatment, respectively (Figure 4).

A similar dependence was observed for the *Δure2* cells from stationary phase of growth (data not shown).

Discussion

The role of Ure2p in the defense of the yeast cell against exogenous oxidants and metal ions [16,19] is puzzling. The present results (Table I) confirm a role of Ure2p in the protection against heavy metals and hydroperoxides, demonstrated by other authors [16,18,19]. Additionally, we found that Ure3p, an aggregated prion form of Ure2p, can confer resistance to metal ions and oxidants tested as efficiently as the native protein (data not shown). This preservation of function by the protein transformed to the prion form is intriguing. It may be linked to the fact that the glutathione peroxidase activity of Ure2p is maintained upon structural conversion to the aggregated prion form [20], which suggests that the loss of regulatory function is attributable to a steric blocking mechanism rather than to conformational changes *per se* [29].

Despite the structural similarity to glutathione *S*-transferases, Ure2p lacks detectable GST activity towards CDNB [3,14–16]. However, CDNB, a classical GST substrate, was found to be slightly more toxic to the *Δure2* strain than to its wild type counterpart; hence Ure2p may marginally take part in CDNB detoxification [16]. The view that the high toxicity of heavy metals and hydroperoxides to the *Δure2* cells is a consequence of the function of Ure2p as a negative regulator of GATA-factor transcription activators Gln3 and Gat1/Nil1 has been rebutted [16]. The question whether Ure2p acts directly as glutathione *S*-transferase remains still unresolved.

Four proteins with some overlapping functions present in *S. cerevisiae*, Gtt1, Gtt2, Grx1, Grx2, are known to possess the ability to detoxify CDNB. Interestingly, Gtt1 and Gtt2 are homologous to Ure2p [14,26,30]. These findings prompted us to check the sensitivity to the *Δgrx1Δgrx2*, *Δgtt1Δgtt2* and *Δgrx1Δgrx2Δgtt1Δgtt2* disruptants to a range of heavy metals ions and oxidants. Mutants lacking *GRX1* and/or *GRX2* genes are hypersensitive to oxidative stress conditions [27] while Gtt1p and Gtt2p do not

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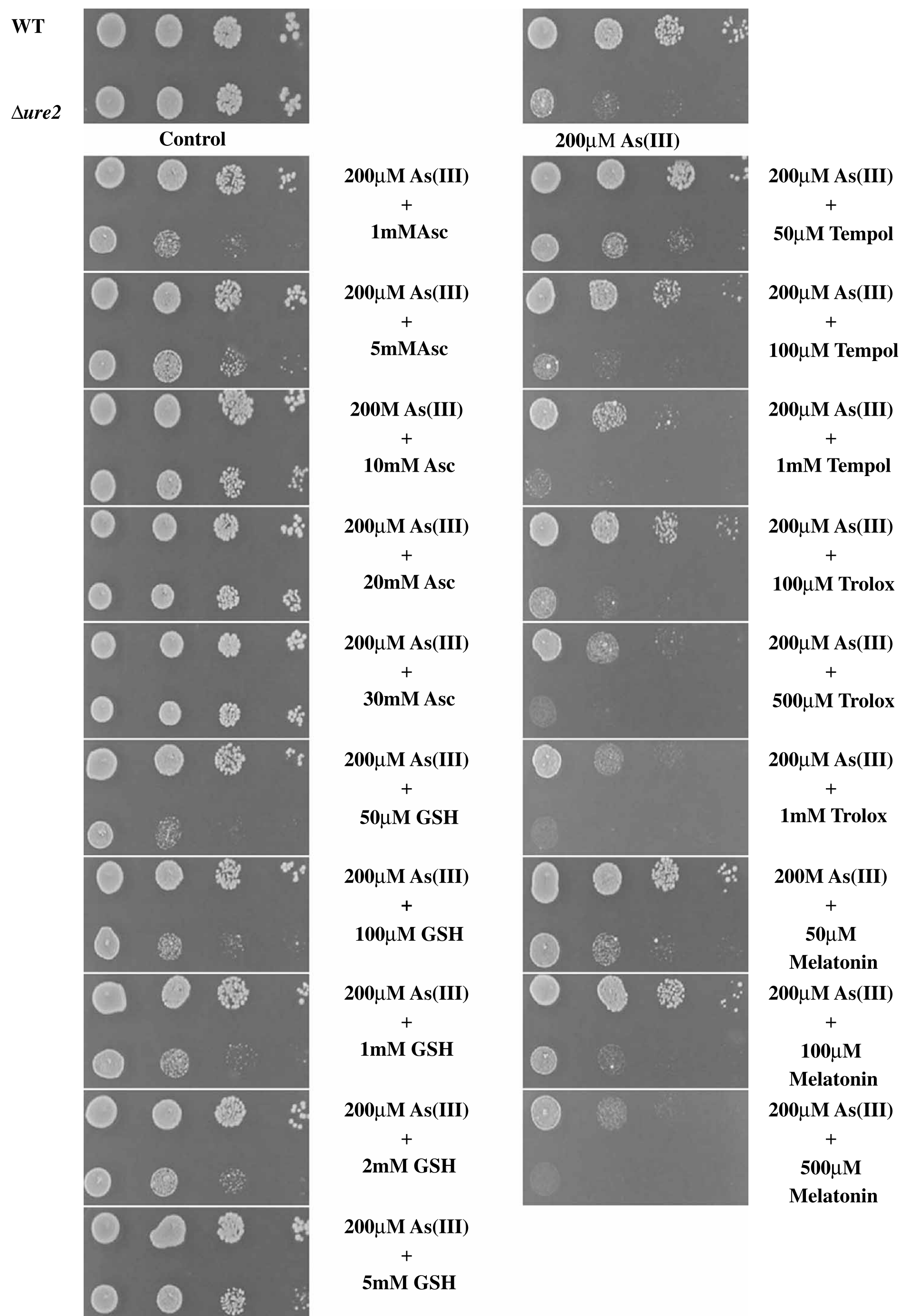

Figure 2. Suppression of growth impairment of the *Δure2* mutant subjected to 200 μM As(III) by selected antioxidants in YPD medium. Increasing dilutions of exponential phase cells (1×10^7, 1×10^6, 1×10^5 and 1×10^4 cells/ml, from left to right) were plated in a volume of 5 μl. Growth was assessed after 48 h.

contribute to the protection against oxidants and xenobiotics [14].We observed growth impairment of the *Δgrx1Δgrx2* and the *Δgrx1Δgrx2Δgtt1Δgtt2* mutants exposed to hydroperoxides (data not shown). In principle, we were unable to notice any differences in growth rates of the *Δgrx1Δgrx2*, *Δgtt1Δgtt2* and *Δgrx1Δgrx2Δgtt1Δgtt2* disruptants exposed to heavy metals in comparison with their isogenic wild-type strain (data not shown). Only the disruptant in *GTT1* and *GTT2* genes exhibited hypersensitivity to 500 μM As(III) when grown on YPD medium (Figure 1). This sensitivity was lower than that observed for the *Δure2* cells which failed to grow in rich medium supplemented with 300 μM As(III) (Table I). The *Δgrx1Δgrx2* mutant subjected to selenite showed also a limited ability to grow (Figure 1). This result may be due to the fact that this disruptant is hypersensitive to conditions that promote oxidative stress [27] and selenite toxicity in yeast is closely related to its prooxidant action [31].

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Table III. Effect of pre-treatment with NAC or D-arabinose on the sensitivity of the *Δure2* mutant to metal ions and oxidants.

		Metal/oxidant									
		YPD			YNB						
Compound	Concentration	A	B	C	D	E	F	G	H	I	J
(A) 2 h incubation in 0.1M phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.1% glucose with NAC or D-arabinose											
NAC	1 mM	0	*	*	0	***	0	0	***	**	0
	2 mM	0	*	*	0	***	0	0	***	**	0
	5 mM	0	**	**	*	***	**	0	***	**	**
	10 mM	0	**	***	*	***	**	*	***	**	**
D-arabinose	1 mM	0	0	0	0	0	0	0	0	0	0
	2 mM	*	0	0	0	0	0	0	0	*	0
	5 mM	*	0	*	0	0	0	0	0	*	0
(B) 18 h growth in YNB medium with NAC or D-arabinose											
NAC	1 mM	0	0	0	0	0	0	0	*	0	0
	2 mM	0	0	0	0	0	0	0	*	0	0
	5 mM	*	0	*	0	*	*	0	**	0	0
	10 mM	*	0	**	*	*	*	*	**	0	0
D-arabinose	1 mM	0	0	0	0	0	0	0	0	0	0
	2 mM	0	0	0	0	*	*	*	0	0	0
	5 mM	*	0	0	0	*	*	*	0	0	0

A: 10 μM Cd(II), B: 200 μM As(III), C: 1.5 mM H_2O_2, D: 7 μM Cd(II), E: 100 μM As(III), F: 100 μM Cr(VI), G: 400 μM Co(II), H: 0.3 mM H_2O_2, I: 70 μM *t*BOOH, J: 10 μM CumOOH.
0 no effect of an antioxidant precursor (growth the same as of the yeast treated with a tested metal ion or oxidant, in the absence of any protective agent), *some protective effect (restoration of growth at a concentration of 1×10^6 cells/ml), ** medium protective effect (restoration of growth at a concentration of 1×10^5 cells/ml), *** restoration of growth to the level observed in the absence of the metal/oxidant (growth at all dilutions tested).

Se(IV) behaving as an oxidant can induce oxidative stress response genes such as *GLR1* (encoding for glutathione reductase) and *TRR1* (encoding for thioredoxin reductase) in a *YAP1*-dependent manner [31]. Overexpression of glutathione reductase also confers resistance to selenite [31]. Taken together, among the proteins considered, only Ure2p can serve as a protector against broad spectrum of heavy metals.

Toxic effects of metals may involve inactivation of enzymes, blocking functional groups of other biomolecules [32] as well as generation of ROS in the Fenton reaction and subsequent lipid peroxidation and

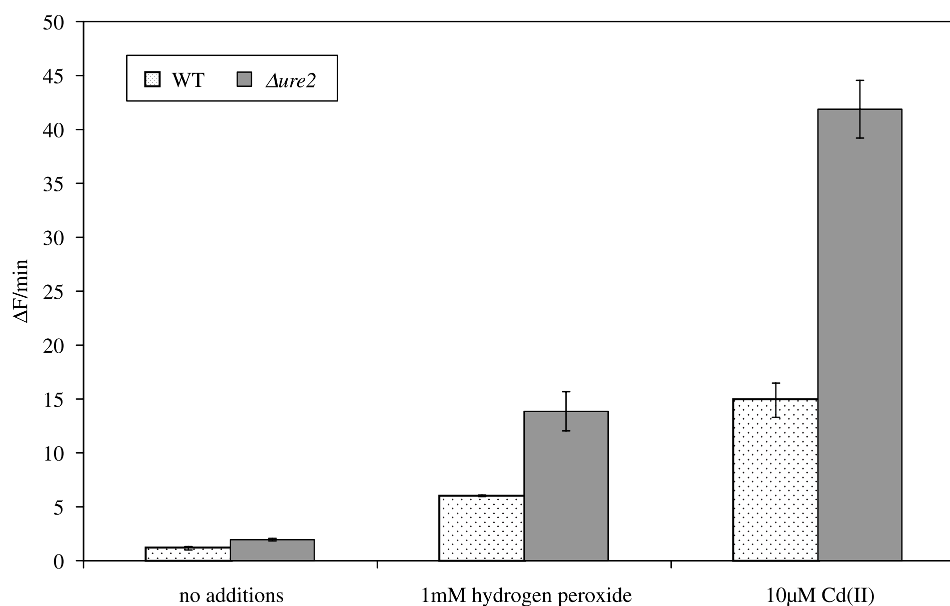

Figure 3. Effect of hydrogen peroxide and Cd(II) on intracellular ROS production estimated by the rate of increase of DCF fluorescence. Yeast cells were incubated with the fluorogenic probe, H_2DCF-DA, in 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1% glucose at 28°C for 30 min. Bars indicate SD, $n = 3$.

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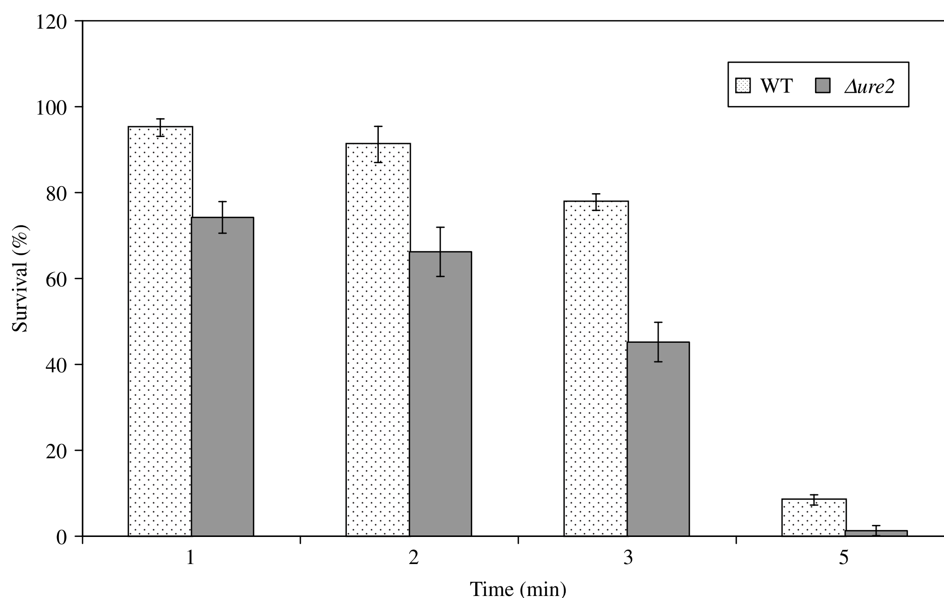

Figure 4. Sensitivity of the *Δure2* disruptant to the heat shock. Cells from logarithmic phase at a concentration of 1×10^7 cells/ml were subjected to heat shock (+50°C for 1, 2, 3 and 5 min) and then spread onto YPD agar plates. Survival was determined by colony counting. Mean values ± SD, $n = 3$.

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damage to other biomolecules [33,34]. Fenton reaction, characteristic for transition metals (e.g. iron, copper, cobalt, chromium(VI)) leads to generation of the very reactive hydroxyl radical [33,34]. Non-transition metals such as cadmium, arsenic, mercury and nickel can also induce oxidative stress by depleting GSH and binding to protein sulfhydryl groups [33,35].

It has been reported that yeast cells lacking antioxidant proteins exhibit elevated sensitivity to heavy metals [36–38]. Additionally, antioxidants may provide protection against metal-mediated free radical attack [34]. We have previously demonstrated that antioxidants may alleviate growth defects of the yeast caused by lack of antioxidant proteins such as the Cu,Zn- superoxide dismutase [22,24,28] or glutaredoxin 5 [23]. It seemed therefore worthwhile to check whether the augmented sensitivity of the *Δure2* mutant to heavy metals and oxidative agents can be ameliorated by antioxidants. The present data (Table II) demonstrate that antioxidants can indeed protect the *Δure2* mutant not only against oxidants such as peroxides but also to heavy metals, and confirm previous findings that only some antioxidants are protective for the yeast [22–24,39]. Glutathione and ascorbate were most effective in restoring growth of the *Δure2* cells in the presence of heavy metals. These results may indicate, although indirectly, that growth impairment of the *Δure2* mutant caused by heavy metals is mediated by oxidative stress. In the case of Co(II), ascorbate did not affect the growth inhibition. It has been reported that co-incubation of Co derivatives with ascorbate causes generation of hydrogen peroxide and exposure of the cells to a Co(II)/ascorbate system leads to apoptosis [40]. Others antioxidants, especially thiols, have been reported to augment the toxicity of cobalt, especially in cell-free systems [41]. However, in our hands, glutathione (5 mM) successfully restored growth of the *Δure2* mutant subjected to cobalt treatment (Table II) which may be attributed to formation of metal complexes with glutathione, inhibiting the reactivity of metal ions. Tempol, Trolox and melatonin showed only a minor ability to ameliorate the growth restriction by heavy metals and oxidants (Table II, Figure 2). Moreover, these compounds alone may behave as prooxidants (data not shown) and augment toxicity of the chemicals tested (Table II, Figure 2). Such a mode of action may be due to high redox potential of the secondary radicals of these antioxidants formed in their reactions with oxidative agents [22]. Prooxidant effects of nitroxides [42,43], Trolox [44] and melatonin [45] were also reported previously.

Tests involving co-incubaton of oxidants or heavy metals with antioxidants in the environment of the cells, although used routinely, rise problems with interpretation of the mechanism of action of antioxidants. It is not obvious whether the antioxidants examined protect the cells intracellularly or act outside the cells. Therefore, we have used another approach to assess the intracellular action of antioxidants, increasing GSH and erythroascorbic acid pool in the cells by NAC [46] and D-arabinose pre-treatment [47,48], respectively (Table III). N-acetylcysteine, a stimulator of endogenous glutathione biosynthesis, was able to revert the toxic effect of heavy metals. D-arabinose, a substrate for the

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pathway of erythroascorbic acid biosynthesis, exerted a similar, though less pronounced effect (Table III). This difference in effects may be due to the fact that N-acetylcysteine is an antioxidant itself [49], and even if its excess is not deacetylated and incorporated into GSH, it may contribute to the antioxidant capacity of the cells. Similar experiments, consisting in increasing intracellular levels of GSH [46] and erythroascorbic acid [48] were used to demonstrate the role of these antioxidants in the resistance of yeast cells to diphenyl diselenide and hydrogen peroxide, respectively.

Our results confirm directly altered redox equilibrium in the *Δure2* mutant by showing increased rate of H_2DCF oxidation both under normal growth conditions and during treatment with exogenous stressors (Figure 3). Although it is assumed usually that the increase of intracellular DCF fluorescence reflects mainly the rate of formation of hydrogen peroxide [50,51], this probe is known to react with a range of oxidants [52]. It seems therefore more appropriate to interpret H_2DCF oxidation as a general index of the ROS production. The rate of oxidation of H_2DCF was elevated in the *Δure2* mutant (Figure 3) which strongly suggests that the lack of *URE2* gene is associated with redox imbalance. Exposure the *Δure2* cells to hydrogen peroxide and cadmium resulted in a further increase of DCF fluorescence intensity, which indicates that Cd(II) toxicity may be, at least in part, mediated by generation of ROS. Co-incubation the *Δure2* cells with cadmium and glutathione (1 mM) did not result in any decrease of the rate of H_2DCF oxidation (data not shown). Therefore, the role of GSH in this system cannot consist in scavenging of free radicals formed in excessive amounts in the presence of cadmium. According to the current knowledge, the main mechanism for cadmium detoxification in *S. cerevisiae* is the formation of the glutathione complex with cadmium [53], spontaneous or assisted by GSTs, with subsequent export of the adduct to the vacuole mediated by the Ycf1p transporter [54,55].

Mutant lacking *URE2* gene exhibited increased sensitivity to heat shock treatment (Figure 4), a phenotype characteristic also for the *Δgtt1*, *Δgtt2* and *Δgtt1Δgtt2* disruptants. Although such phenomenon was seen only in the stationary phase of growth of mutants lacking *GTT1*, *GTT2* or *GTT1* and *GTT2* genes [14], it may be another link between Ure2p and the family of glutathione *S*-transferases.

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