TARGETING GENES OF Cd INDUCED OXIDATIVE STRESS RESPONSE IN YEASTS

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ABSTRACT
The microbial biosynthesis of nanoparticles has become one of the most studied fields of nanobiotechnology. However, exposure of cells to heavy metals and metalloids profoundly affects biological systems as it generally leads to intracellular oxidative damage and strongly depends on the cellular metabolic status. In this respect, the cadmium resistance of 7 yeast species differing in their type of glucose oxidation and energy generation (Saccharomyces cerevisiae, Candida glabrata, Schizosaccharomyces pombe, Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis and Rhodotorula graminis) was studied. It was shown that the cellular growth of S. pombe and C. glabrata was not significantly impaired when high Cd concentration was applied. Unusually elevated survival levels were also detected in H. polymorpha and R. graminis yeasts. To further investigate the cellular resistance to Cd ions, a comprehensive in silico analysis of key antioxidant enzymes were performed. Applying a computational approach, it was shown that Crabtree positive Schizosaccharomyces pombe and Candida glabrata, as well as the oxidative yeast Rhodotorula graminis possess genetically determined advantages for surviving when higher concentrations of toxic Cd ions are present in the environment: existence of duplicated copies of genes encoded key antioxidant enzymes (catalases, glutathione peroxidases and glutathione S-transferases). Moreover, the proteins involved in cellular antioxidant defence in those yeast species possess numerous targeting signals allowing their localization plasticity in different subcellular structures. In spite of the lack of antioxidant genetic advantages, Hansenula polymorpha also revealed high Cd resistance.


Keywords: yeasts, cadmium, metal toxicity, in silico analysis, antioxidant enzymes

Introduction
In the last decades, the bioproduction of nanocrystalline materials has become one of the most studied fields of nanobiotechnology (2). The biosynthesis of nanoparticles, using microorganisms, attracted a lot of attention as this route has the potential to lead to production of industrially important monodispersed systems (39). It has been well documented that numerous microorganisms could synthesize a great variety of inorganic nanocrystals, e.g. Ag0, Au0, ZnS, cdS and AgS (24). Of all microbial cells, yeasts are shown to possess the highest biosynthetic potential to produce these substances (10, 24, 37, 39, 42). Exposure of Candida glabrata (10, 23) to cadmium ions leads to intracellular formation of cadmium nanoparticles. Torulopsis species is well-known for synthesis of lead sulphide nanocrystals intracellularly when challenged with this metal (21, 22). Schizosaccharomyces pombe is capable of producing cadmium sulphide within the yeast cells (21, 22, 23). Pichia capsulata exhibited the most efficient production of silver nanoparticles (39). Rhodosporidium diobovatum produced intracellular stable lead sulfide nanoparticles (37).

The biosynthesis of nanoparticles by yeasts strongly depends on the cellular metal ion homeostasis, which is maintained through highly regulated processes of uptake, storage and secretion. Exposure to metals is associated with the activation of a variety of intracellular signal transduction pathways, including those regulated by mitogen activated protein kinases (MAPKs) (40). However, when present in excessive amounts, metals and metalloids could profoundly affect biological systems because they are toxic and harmful. The toxicity of a given metal is governed by its mechanisms of uptake, oxidation state, and intracellular distribution. It also depends on interactions with various macromolecules, as well as on its physicochemical properties and ligand creation preferences. Generally, the exposure to metals is associated with increased levels of intracellular oxidative damage, including lipid peroxidation, protein denaturation, and DNA strand breaks (40). Cadmium (Cd) is from the so-called “soft” transition metals and generally causes oxidative stress, lipid peroxidation, and mutagenesis; but the molecular mechanisms leading to these cellular effects remain elusive (4).

To understand Cd tolerance of eukaryotic microorganisms a suitable yeast model was used – Saccharomyces cerevisiae. It possesses well characterised expression profiles of proteins involved in oxidative stress defence (superoxide dismutase, thioredoxin, glutathione and thioredoxin reductases) and Yap1, the basic leucine-zipper (bZIP) transcriptional activator, elements having important role in heavy metal detoxification (43). Moreover, it was found that resistance to cadmium is mediated by genes involved in sulphate assimilation,
Fig. 1. Sensitivity of *S. cerevisiae, Candida glabrata, Schizosaccharomyces pombe, Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis* and *Rhodotorula graminis* to different concentrations of Cd(NO₃)₂.
glutathione biosynthesis pathways, maintenance of cell wall integrity and those needed for bud neck formation (19, 43).

Thus many of the genes and cognate regulatory pathways responsive to cadmium toxicity in \textit{S. cerevisiae} have been identified and this knowledge can be used to study the consequence of metal toxicity. To address this gap and to assess the potential of different yeast microorganisms to produce metal nanoparticles, a Cd sensitivity assay was performed. Yeast strains differing in their mode of sugar utilization (\textit{Saccharomyces cerevisiae}, \textit{Schizosaccharomyces pombe}, \textit{Pichia pastoris}, \textit{Hansenula polymorpha}, \textit{Kluyveromyces lactis}, \textit{Hansenula polymorpha} – crabtree positive, \textit{Rhodotorula graminis} – crabtree negative, and \textit{Rhodotorula graminis} – obligate oxidative) were analysed (41). In this way the influence of the yeast metabolic type on Cd toxicity was also anticipated. The obtained \textit{in vivo} results were, furthermore, confirmed by comprehensive \textit{in silico} analysis of key genes involved in the maintenance of redox and metal homeostasis.

**Materials and Methods**

**Strains and media**

Yeast microorganisms used in this investigation were: \textit{Saccharomyces cerevisiae} NBIMCC 583, \textit{Kluveromyces lactis} NBIMMCC 1446 and \textit{Rhodotorula graminis} NBIMCC 1686, \textit{Pichia pastoris} X-33 and \textit{Hansenula polymorpha} CBS 4732. The strains were grown in standard liquid YPD medium prior to cadmium sensitivity assay.
Selection of gene sets
Target genes related to the antioxidant defence system were selected through the SGD Pathway tools (http://pathway.yeastgenome.org) and related publication data (11, 12).

Identification of orthologous genes
Protein sequences of selected genes were downloaded from the site of the Genolevures project (http://www.genolevures.org) for Kluyveromyces lactis and Candida glabrata, BOGAS genome annotation system (http://bioinformatics.psb.ugent.be/webtools/bogas/) for Pichia pastoris, DOE Joint Genome Institute (http://www.jgi.doe.gov) for Hansenula polymorpha and Rhodotorula graminis (13, 14). Candidates for orthologous genes of the S. cerevisiae set were selected as the best identity matches with $E < 10^{-4}$ and then verified with comparison of the annotation data of each gene. Putative paralogous genes were identified as described by Gu et al. (15) ($E < 10^{-10}$).

Intracellular localisation
The intracellular localisation of the proteins encoded by the orthologous genes in K. lactis, C. glabrata, P. pastoris, H. polymorpha and R. graminis were analysed through WoLF PSORT (http://wolfsort.org/) and PSORT II Prediction software (http://psort.hgc.jp) (29). The cellular targeting data for S. cerevisiae were downloaded from SGD (http://www.yeastgenome.org).

Cadmium sensitivity assay
Strains were grown overnight at 30 °C/204 rpm in 10 ml YPD medium. Cultures were pelleted and washed twice with fresh medium. Ten-fold serial dilutions (OD$_{40}$ ~ 1; 0.1; 0.01; 0.001; 0.0001) were prepared, and samples (5 µL) of each dilution were spotted onto plates with different concentrations of Cd(NO$_3$)$_2$ (0.5 mmol/L, 0.75 mmol/L, 1.0 mmol/L, 1.25 mmol/L and 1.5 mmol/L). Plates were incubated at 28 °C for 5 days.

Results and Discussion
Oxygen metabolism affects growth on Cd
In vivo study of the toxic effects of different Cd concentrations was performed by using spot analysis. The growth inhibition, proceeding from nonessential to highly toxic, was measured and the survival level of 7 yeast species differing in their type of glucose oxidation and energy generation (Crabtree negative: Candida glabrata and Schizosaccharomyces pombe, and Crabtree positive: Pichia pastoris, Hansenula polymorpha, Kluyveromyces maxianus and Rhodotorula graminis) was assessed (41) (Fig. 1). When exposing S. cerevisiae, K. lactis, and P. pastoris to low concentrations of Cd(NO$_3$)$_2$ (0.5 mmol/L and 0.75 mmol/L) a significant decrease in cellular colony growth was observed. The obtained results agree with the findings of Pasternakiewicz (32), who established that the growth of S. cerevisiae is slowed by concentrations of cadmium higher than 10 µmol/L, and entirely stops at 1 mmol/L. It was shown that a higher level of cadmium in the medium impairs the synthesis of proteins. Cadmium can also cause structural damages in the plasma membrane of these yeasts and, more importantly, the absorption of cadmium is accompanied by release of intracellular potassium (30). On the contrary, an elevated resistance to high cadmium concentrations was detected in S. pombe and C. glabrata (Fig. 1). Both yeast strains were viable even when 1.5 mmol/L Cd(NO$_3$)$_2$ was added to the growth medium. This could be attributed to the fact that in S. pombe and C. glabrata, similarly to plants, Cd detoxification involves synthesis of phytochelatins (27). Surprisingly, the growth of H. polymorpha and R. graminis was not significantly impaired even when very high cadmium concentrations were used (Fig. 1). As regards R. graminis, capsule formation, as well as the presence of carotenoid pigments, probably contributes to the resistance of these microorganisms to heavy metals. This finding also corresponds to the investigations of Li et al. (25), who showed that Rhodotorula sp. Y11 could grow in a considerable concentration of cadmium, and can be of great use in in situ bioremediation of contaminated industrial wastewaters. Another interesting observation was the higher resistance of H. polymorpha to Cd. Until recently, it was considered that the sequestration of this heavy metal in H. polymorpha occurs similarly to that found in Saccharomyces cerevisiae (with glutathione being the main Cd intracellular chelator), but different from Schizosaccharomyces pombe and Candida glabrata (27). However, several papers report identification of cadmium-ion tolerant Hansenula species (31, 44), suggesting that much profounder investigations should be made on the cadmium tolerance of these yeast microorganisms.

Antioxidant enzymes impact fitness effect against Cd toxicity
To better understand the observed toxic effect and the mechanisms of Cd resistance in the different metabolic types of yeasts, a study of the fitness effect of antioxidant enzymes against metal toxicity was performed, using computational biology approach. In order to explain the detected levels of resistance to different Cd concentrations, the antioxidant genes and their sequences were explored. The copy number of the key genes encoding proteins responsible for the detoxification of reactive oxygen species was identified and the homology between these genes and those of the model S. cerevisiae was assessed. Studies were conducted on the genes encoding the enzymes SOD, catalase, glutathione peroxidases and glutathione S-transferases, as well as on enzymes involved in glutathione synthesis: γ-glutamylcystein and glutathione S-transferases (Table 1). The results showed that, although in general these genes were conservative in all tested yeast strains, some differences could be observed (Table 2). The diversity of antioxidant genetic markers and its relation to the different types of glucose metabolism was also speculated on.

The first enzymatic safeguard against Cd metal toxicity is superoxide dismutase (43). It is known that the yeast S. cerevisiae express two forms of superoxide dismutase enzymes: Mn SOD, encoded by the gene SOD2 and localized mainly in the mitochondrial matrix, and CuZn SOD, encoded by the gene SOD1 and acting in the cytosol and mitochondrial intermembrane space (35). The two genes have no similarity...
in the protein sequence of the encoded enzymes and are not regarded as orthologs. SODs catalyse the breakdown of the superoxide radical, O$_2^-$, to an oxygen molecule (dioxygen) and hydrogen peroxide and have an important role in heavy metal stress tolerance (5, 38). Analysis of the genome of C. glabrata, S. pombe, P. pastoris, H. polymorpha, K. lactis and R. graminis showed that these two enzymes have relevant orthologs and their degree of homology with S. cerevisiae was 48 % to 68 % and 36 % to 55 %, respectively for CuZn SoD and Mn SoD (Table 2 and Fig. 2A). Interestingly, in the genome of R. graminis, a gene encoding Cu/Zn SOD was not identified. It was considered that in pigmented basidiomycetous yeasts, the availability of SoD enzymes was scarce to none and the crucial role in preventing oxidative damages was awarded to the endogenous carotinoids and their antioxidant properties (28). Recent genetic and biochemical experiments revealed that in Rhodotorula mucilaginosa and Rhodotorula graminis changes in the structure of the SOD1 gene generate stop codons at different positions, which correlates with the absence of peptides or active proteins in the cells. However, for other basidiomycetous yeasts, such as R. glutinis and U. puniceus, the cloned nucleotide sequence contains all necessary information to produce a functional protein (16, 20). Moreover, studies with S. cerevisiae cells lacking the cytosolic Sod1, showed that cadmium was removed twice as effectively as in the control strain, while those deficient in the mitochondrial Sod2 exhibited poor metal absorption (1). It was observed that the deficiency of Sod1 increases the expression of both cup1 (a metallothionein) and Ycf1 (a vacuolar glutathione S-conjugate pump), proteins involved in protection against cadmium, indicating that Mn SoD plays a more important role in protecting yeast cells from heavy metal toxicity. In this respect, the lack of Cu/Zn SOD in some Rhodotorula species probably is not of critical importance for the regulation of Cd homeostasis in the cell.

Another enzyme involved in the protection against metal Cd ion toxicity is catalase (43). It was found that in the cells of S. cerevisiae two catalase enzymes are available which catalyse the decomposition of hydrogen peroxide to water and oxygen. Both isoforms, catalase A and catalase T, are encoded

### Table 1

<table>
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<th>Gene name</th>
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<td>SOD1</td>
<td>1.15.1.1</td>
<td>Cu/Zn superoxide dismutase</td>
</tr>
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<td>SOD2</td>
<td>1.15.1.1</td>
<td>Mn superoxide dismutase</td>
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<tr>
<td>CTA1</td>
<td>1.11.1.6</td>
<td>Catalase A</td>
</tr>
<tr>
<td>CTT1</td>
<td>1.11.1.6</td>
<td>Catalase T</td>
</tr>
<tr>
<td>GPX1</td>
<td>1.11.1.9</td>
<td>Glutathione peroxidase</td>
</tr>
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<td>GPX2</td>
<td>1.11.1.9</td>
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<td>GPX3</td>
<td>1.11.1.9</td>
<td>Glutathione peroxidase</td>
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<td>6.3.2.2</td>
<td>γ-Glutamylcysteine synthetase</td>
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<td>6.3.2.3</td>
<td>Glutathione synthetase</td>
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<td>GTT3</td>
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### Table 2

Number of paralogous genes encoding key proteins involved in the antioxidant defense system of S. cerevisiae and their orthologs in C. glabrata, S. pombe, P. pastoris, H. polymorpha, K. lactis and R. graminis

<table>
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<th>CTT1</th>
<th>GPX1</th>
<th>GPX2</th>
<th>GPX3</th>
<th>GTT1</th>
<th>GTT2</th>
<th>GTT3</th>
<th>GSH1</th>
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<tr>
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Comparison of the cellular localization of \textit{S. cerevisiae} antioxidant gene products and these of their \textit{C. glabrata}, \textit{S. pombe}, \textit{P. pastoris}, \textit{H. polymorpha}, \textit{K. lactis} and \textit{R. graminis} best orthologs. (A) Localization of SODs, catalases and glutathione peroxidases; (B) Localization of glutathione S-transferases and enzymes involved in glutathione synthesis. The intracellular distribution of \textit{C. glabrata}, \textit{S. pombe}, \textit{P. pastoris}, \textit{H. polymorpha}, \textit{K. lactis} and \textit{R. graminis} proteins was predicted by \textit{in silico} analysis. The percentage of probability of the prediction is indicated when more than one possible localizations were returned. The localization for \textit{S. cerevisiae} proteins are displayed according to the annotation on the SGD website. When several paralogs exist, an inner border divides the localization data for each gene product.

(A)

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{S. cerevisiae} & \textbf{C. glabrata} & \textbf{S. pombe} & \textbf{P. pastoris} & \textbf{H. polymorpha} & \textbf{K. lactis} & \textbf{R. graminis} \\
\hline
\textbf{Sod1} & cytoplasmic mitochondrial IMS & 39 \% cytoplasmic 13 \% mitochondrial IMS & 41 \% cytoplasmic 23 \% mitochondrial IMS & 43 \% cytoplasmic 16 \% mitochondrial IMS & 47 \% cytoplasmic 17 \% mitochondrial IMS & cytoplasmic & - \\
\hline
\textbf{Sod2} & mitochondria & 30 \% mitochondria 17 \% peroxisomal & mitochondrial & mitochondrial & mitochondrial & mitochondrial & 52 \% mitochondrial 22 \% cytoplasmic \\
\hline
\textbf{Cta1} & peroxisomal mitochondrial & peroxisomal & - & peroxisomal & peroxisomal & peroxisomal & - \\
\hline
\textbf{Ctt1} & cytoplasmic & - & 52 \% cytoplasmic 15 \% mitochondrial & - & - & cytoplasmic & 48 \% cytoplasmic 17 \% mitochondrial cytoplasmic \\
\hline
\textbf{Gpx1} & unknown & - & cytoplasmic & - & - & - & - \\
\hline
\textbf{Gpx2} & cytoplasmic mitochondrial nuclear & cytoplasmic & - & - & - & cytoplasmic & cytoplasmic \\
\hline
\textbf{Gpx3} & cytoplasmic & 48 \% cytoplasmic 22 \% mitochondrial cytoplasmic & - & cytoplasmic & cytoplasmic & cytoplasmic & - \\
\hline
\end{tabular}
\caption{Comparison of the cellular localization of \textit{S. cerevisiae} antioxidant gene products and these of their \textit{C. glabrata}, \textit{S. pombe}, \textit{P. pastoris}, \textit{H. polymorpha}, \textit{K. lactis} and \textit{R. graminis} best orthologs.}
\end{table}

(B)

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{S. cerevisiae} & \textbf{C. glabrata} & \textbf{S. pombe} & \textbf{P. pastoris} & \textbf{H. polymorpha} & \textbf{K. lactis} & \textbf{R. graminis} \\
\hline
\textbf{Gtt1} & mitochondrial endoplasmic reticulum & - & cytoplasmic (glutamate-cysteine ligase) & 39 \% cytoplasmic 39 \% mitochondrial & - & mitochondrial & cytoplasmic cytoplasmic \\
\hline
\textbf{Gtt2} & mitochondrial & - & cytoplasmic & - & - & - & - \\
\hline
\textbf{Gtt3} & unknown & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic & 33 \% endoplasmic reticulum 22 \% vacuolar 22 \% plasma membrane & - \\
\hline
\textbf{Gsh1} & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic (glutamate-cysteine ligase) & - \\
\hline
\textbf{Gsh2} & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic & cytoplasmic \\
\hline
\end{tabular}
\caption{Comparison of the cellular localization of \textit{S. cerevisiae} antioxidant gene products and these of their \textit{C. glabrata}, \textit{S. pombe}, \textit{P. pastoris}, \textit{H. polymorpha}, \textit{K. lactis} and \textit{R. graminis} best orthologs.}
\end{table}

by the genes \textit{CTA1} and \textit{CTT1} and have different subcellular localization. Catalase A is localized in peroxisomes and mitochondria (35), while catalase T is found in the cytosol (18). Comparative analysis of the amino acid sequence of the two catalases shows 39 \% similarity, which indicates that both enzymes in \textit{S. cerevisiae} could be considered as paralogs.

On the other hand, the \textit{in silico} analysis of the genome of \textit{C. glabrata}, \textit{P. pastoris}, \textit{H. polymorpha} and \textit{S. pombe} revealed that all four yeast species have only one catalase enzyme (Table 2 and Fig. 1B). In \textit{C. glabrata}, \textit{H. polymorpha} and \textit{P. pastoris} the enzyme is ortolog to CTA1, with 81 \%, 57 \% and 59 \% homology in amino acid sequences, respectively; and in
S. pombe, to CTT1 with 50 % homology (Fig. 1B). Analysis of the K. lactis genome revealed presence of two catalase enzymes, similarly to S. cerevisiae: one which is orthologous to CTA1 with 80 % identity, and the other, orthologous to CTT1 with 67 % identity. The identity between both K. lactis catalases was found to be 43 %, which indicates that they are paralogous copies (Fig. 2B). Surprisingly, in R. graminis three catalase paralogs were detected with 36 % average identity between them. All the three catalase genes are orthologs to S. cerevisiae CTT1.

The role of catalase enzymes in acquiring heavy metal stress tolerance is still elusive. When studying the effects of copper and cadmium on the growth and catalase activity in different yeast strains, Romandini et al. (36) found that the catalase level remains almost unchanged in the tested conditions. On the other hand, Chen et al. (6) observed about a 2.6-fold increase in catalase activity of the C. albicans cells treated with 1 mmol/L Cd²⁺. Likewise, Cho et al. (7) showed that, in S. pombe, catalase activity was greatly induced after treatment with cadmium chloride and even a low concentration (0.01 mmol/L) of cadmium also exerted a stimulating effect on the catalase activity. In this respect R. graminis seems to have the greatest potential to cope with H₂O₂ generated during heavy metal exposure.

The role of glutathione peroxidase enzymes in heavy metal toxicity is not clearly revealed but some recent investigations showed that in Saccharomyces cerevisiae yeasts exposed to CdCl₂, increased activities of GPx were detected (26). In this respect, search of S. cerevisiae glutathione peroxidase orthologs was performed in the corresponding databases. Three GPx homologs were found in the Saccharomyces cerevisiae genome: GPX1, GPX2, and GPX3, which are induced by glucose starvation and are generally involved in cellular protection against phospholipid hydroperoxides and nonphospholipid peroxides formed during oxidative stress (3, 17). These three genes encode proteins which show 65 % identity between each other. The analysis of the other 6 yeast genomes revealed that in the most analysed yeast species only one copy of glutathione peroxidase enzyme is preserved and the average identity with S. cerevisiae genes was around 62 %. However, the K. lactis and C. glabrata strains were an exception. In K. lactis two glutathione peroxidases were detected, orthologous to GPX2 and GPX3, respectively; and in C. glabrata the GPx orthologs were 3 with about 55 % identity to those of S. cerevisiae (Fig. 2C).

The main role of glutathione S-transferases (GST) is the detoxification of various electrophilic xenobiotic compounds such as Cd and other heavy metals and products of oxidative stress. The reaction proceeds with the participation of GSH, which in the process of catalysis forms a soluble conjugate which is excreted from the cell. Structurally, glutathione S-transferases belong to the family of thioredoxin enzymes. It is known that the yeast S. cerevisiae express two standard forms of the enzyme (Gtt1 and Gtt2). In recent years, a third open reading frame was identified, coding the protein Gtt3, which has been shown to have an essential role in glutathione metabolism and protection against oxidative stress. It is also believed that Gtt3 is a glutathione transferase, although this has not been fully proven. Moreover, one general mechanism for cadmium detoxification, both in S. cerevisiae and mammals, is the chelation of the metal by glutathione and the subsequent compartmentalization of the GSH–metal complex (33). Many xenobiocins can react either spontaneously with the thiol moiety of GSH to form GSH-conjugates, or via glutathione S-transferases (9, 18). Analysis of the genome of the studied yeast strains revealed that C. glabrata, P. pastoris and H. polymorpha have only one glutathione S-transferase orthologous either to CTT1, or to GTT3 (Table 2 and Fig. 2D). The average identity with S. cerevisiae genes was calculated to be 41 %. In the genome of K. lactis and R. graminis two glutathione S-transferases were detected. In K. lactis they are with higher similarity to GTT1 and GTT3 (64 % and 27 %, respectively) and in R. graminis both are orthologous to GTT1 with 32.5 % identity. However, in the genome of S. pombe, four orthologs of the three glutathione S-transferases of S. cerevisiae were found. Two are with higher homology to GTT2 and the other two are orthologs to GTT1 and GTT3, respectively. Taking into account that Saccharomyces cerevisiae cells mutated in the GTT1 or GTT2 genes showed twice as high cadmium sensitivity as the wild type strain (1), it could be presumed that yeast strains having one or more copies of GTT1 and GTT2 would be less susceptible to Cd toxicity and would form metal nanoparticles in greater amounts. In this respect R. graminis and especially S. pombe possess obvious advantages in comparison to other yeast strains. However, research carried out by Collinson and Grant (9) showed that in S. cerevisiae two other proteins, namely Grx1p and Grx2p, have an overlapping function with that of glutathione S-transferases and are primarily responsible for intracellular glutathione S-transferase activity. Thus, cells that lack glutathione S-transferases can probably also carry out the disposal of various xenobiocins and heavy metals by alternative expression of multifunctional glutaredoxins.

Glutathione (gamma-glu-cys-gly; GSH) is usually present at high concentrations in most living cells, being the major reservoir of non-protein reduced sulfur. Because of its unique redox and nucleophilic properties, GSH serves in bio-reductive reactions as an important line of defense against reactive oxygen species, xenobiocins and heavy metals, including Cd. GSH is synthesized from its constituent amino acids by two ATP-dependent reactions catalysed by gamma-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2). Both enzymes are localized in the cytosol. Analysis of the genome of the all 7 yeast species showed that gamma-glutamylcysteine synthetase and glutathione synthetase have their relevant orthologs (Table 2 and Fig. 1E). The degree of homology between the amino acid sequences of gamma-glutamylcysteine synthetase of S. cerevisiae and those of the tested yeast microorganisms ranged from 47 % in P. pastoris and R. graminis to 68 % in C. glabrata. Similar data
were obtained for glutathione synthetase, whose degree of identity varies between 35% in *R. graminis* to 55% in *C. glabrata* (Fig. 2E). The observed similarity in the number of gene copies and protein sequence alignments showed the high evolutionary conservativeness of the enzymes involved in glutathione biosynthesis. The latter is probably due to the central role of glutathione in maintaining the intracellular redox homeostasis and cell resistance against reactive oxygen species, organic and inorganic oxidants, as well as heavy metals (8).

### Intracellular localization of antioxidant enzymes influences Cd resistance

As the possibility for targeting proteins to different cellular compartments enables a rapid and coordinated response to various exogenous and endogenous stresses, the intracellular localization of the antioxidant enzymes was also analysed. The presence of different signal sequences was allocated through PSORT II Prediction software (http://psort.ims.u-tokyo.ac.jp/form2.html) and the obtained *in silico* data were presented in Table 3 (A and B). Additionally, the available data about the intracellular targeting of the key antioxidant enzymes in *S. cerevisiae* was also summarized (according to the SGD website). After reviewing the obtained results it was shown that in most cases an overlapping between the known targeting sites and the results from the *in silico* analysis was observed. However, in some yeast species, the bioinformatics search showed the presence of additional possible subcellular locations. For example, the Sod2 enzyme in *S. cerevisiae* was known to be localized in yeast mitochondria (34), but a possibility for targeting of this protein to peroxisomes was detected in *C. glabrata* yeasts due to the presence of the second type of peroxisomal targeting signal (PTS2). Probability for residing in the cytosol was discovered also for the Mn SOD in *R. graminis*. The *in silico* analysis further revealed that catalase A orthologs are generally targeted to yeast peroxisomes. However, the catalase T enzyme orthologs, besides their typical localization in the cytosol, could also be targeted to the mitochondria in *S. pombe* and *R. graminis*. As regards the glutathione peroxidase orthologs, it was found that in all the studied yeast strains they are generally localized in the cytosol, similarly to *S. cerevisiae* proteins. The only exception is the *C. glabrata* Gpx3 protein, which possesses a cluster of positive amino acids and a Gavel cleaving site determining its possible import into cellular mitochondria. On the contrary, for some of the analysed enzymes the typical localization for *S. cerevisiae* did not coincide with the available targeting signals in the orthologs. Such a relative discrepancy was shown for glutathione S-transferases. Although for most of them a cytosolic location has been shown, for the *P. pastoris* Gtt1 ortholog possible mitochondrial targeting was revealed and for the *K. lactis* Gtt3 ortholog signals for the endoplasmic reticulum, vacuole and plasma membrane were identified. After analysing the amino acid sequences of Gsh1 and Gsh2 enzymes, it was found that in all the studied yeast species these proteins were located in the cytosol. All this data suggests that

### Conclusions

The investigation of 7 yeast species differing in their glucose and energy metabolism, revealed that four of them possess elevated resistance levels to higher Cd concentrations: namely, *Schizosaccharomyces pombe*, *Candida glabrata*, *Hansenula polymorpha* and *Rhodotorula graminis*. Further, by applying *in silico* approach, distinct genetically determined advantages were described in the Crabtree positive *S. pombe* and *C. glabrata*, as well as in the oxidative yeast *Rhodotorula graminis*. In these yeasts the better genetic potential includes the presence of duplicated copies of key antioxidant enzymes, such as catalase, glutathione peroxidase and glutathione S-transferases. These genetic characteristics allow the expression of several isoenzymes functioning in the organism in different environmental and physiological conditions, thus contributing to the maintenance of metal ion homeostasis. Additionally in these yeast species, enzymes involved in the cellular antioxidant defence possess numerous targeting signals, allowing plasticity in localization in different subcellular structures. Consequently, besides the well-known producers of Cd nanoparticles – *S. pombe* and *C. glabrata*, *R. graminis* also exhibited higher genetic and biochemical potential for formation of Cd microcrystallites. Moreover, the yeast *Hansenula polymorpha* also displayed elevated resistance to Cd, although without any observable genetic advantage in terms of the studied antioxidant genes. Contrary, although Crabtree negative with high antioxidant capacity, *K. lactis* and *P. pastoris* did not show either biochemical or genetic ability to cope with the harmful effects of Cd. All this data suggests that the type of metabolism in yeast species is not directly related to the Cd resistance and, apart from a genetic prerequisite for cellular antioxidant capacity, other mechanisms are also involved in Cd detoxification. In this respect, further detailed investigations are needed to make a more conclusive hypothesis about the role and relative significance of both the genetic and the physiological capacity for the resistance to toxic metals and the ability to produce metal nanoparticles.

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### REFERENCES