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Glutathione S-transferases and oxidative stress in *Saccharomyces cerevisiae*

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Abbreviations used

GSH – glutathione

GSTs – glutathione S-transferases

CDNB – 1-chloro-2,4-dinitrobenzene

MAPEG – membrane associated proteins involved in eicosanoid and glutathione metabolism

G-site – glutathione-binding site

H-site – hydrophobic substrate binding site

eEF – eukaryotic elongation factor

Grx – glutaredoxin

ROS – reactive oxygen species

SOD – superoxide dismutase

γ-GC – γ -glutamyl cysteine

Gsh2 – glutathione synthetase

Glr – glutathione reductase

Trx – thioredoxin

GPx – glutathione peroxidase

PHGPx – phospholipid hydroperoxide glutathione peroxidase

PUFA – polyunsaturated fatty acid

4-HNE – 4-hydroxynonenal

MMA(V) – monomethylarsonic acid

DMA(V) – dimethylarsinic acid

MMA(III) – monomethylarsonous acid

DMA(III) – dimethylarsinous acid

MIP – major intrinsic protein

NCR – nitrogen catabolite repression

Acr2p – arsenate reductase

Ycf1 – yeast cadmium factor

MAPKs – mitogen-activated protein kinases

JNK – c-Jun NH_2-terminal kinase

PKC – protein kinase C

MAPKKs – mitogen-activated protein kinase kinases

MAPKKKs – mitogen-activated protein kinase kinase kinases

bZIP – basic region leucine zipper

TOR – target of rapamycin

Gdh1 – NADPH-dependent glutamate dehydrogenase

Gdh2 – NAD^+-dependent glutamate dehydrogenase

Gln1 – glutamine synthetase

CONTENTS

Introduction

Chapter 1

The glutathione S-transferase family of enzymes: essential catalysts of cellular detoxification

1. General characteristics and functions of glutathione S-transferases

1.1. Overview of cell detoxification

Living organisms are continuously exposed to exogenous and endogenous toxic chemical species, which may cause adverse and sometimes lethal effects. The ability of living organisms to survive the risk posed by such compounds represents a fundamental biological adaptation for survival. Different strategies have been adopted by cells to counter the effect of toxic compounds and their metabolites. Defense mechanisms are usually general, rather than specific for a given chemical or organism. Among the defense mechanisms, such as sequestration and binding, catalytic biotransformation was evolved as a crucial mode of protection against toxic chemical species (Sheehan *et al*., 2001).

Cells possess a broad ensemble of enzymes capable of transforming a wide range of different chemical structures and functionalities. The enzymatic detoxification of xenobiotics has been classified into three distinct phases, which act in a tightly integrated manner. Phases I and II enzymes catalyze the conversion of a lipophilic, non-polar xenobiotic into a more water-soluble and therefore less toxic metabolite, which can then be eliminated more easily from the cell. Phase I of detoxification is mainly the result of action by the cytochrome P450 system and mixed function oxidases (Ishikawa *et al.*, 1997). These proteins are responsible for a wide range of reactions, of which oxidations appear to be the most important (Guengerich, 2001).

Phase II enzymes catalyze the conjugation of activated xenobiotics to an endogenous water-soluble substrate, such as reduced glutathione (GSH) or uridine diphosphate (UDP)-glucuronic acid. Quantitatively, conjugation to GSH, which is catalyzed by glutathione S-transferases (GSTs) is the major phase II reaction in many species (Sheehan *et al*., 2001).

The resulting more soluble compounds are eliminated from cells in phase III of the detoxification process. In eukaryotic organisms, they are actively excreted or compartmentalized in the vacuole by ATP-dependent GS-X pumps (Ishikawa *et al.*, 1997; Ernst *et al*., 2005). Indeed, as the glutathionylated moiety is hydrophilic, the conjugate cannot usually simply re-diffuse back into the cell.

1.2. Detoxification function of glutathione S-transferases

Glutathione S-transferases promote the inactivation and degradation of a wide range of compounds by the formation of glutathione conjugates. Enzymes of this family are generally characterized by broad substrate specificity and low affinity (high Km values). A low catalytic

efficiency (Vmax/Km) is a hallmark of the functional flexibility of the family, and has probably been integral to the evolution of GSTs as detoxifiers of a broad spectrum of endogenous and environmental chemicals (Tew & Ronai, 1999).

The reaction of glutathione (GSH) with electrophilic substrates can be represented by the following general scheme:

$$GSH + R\text{-}X \rightarrow GSR + HX$$

The function of GSTs is to bring the substrate into close proximity with GSH by binding both the electrophilic substrate and GSH to the active site and to activate the sulfhydryl group of GSH, thereby allowing nucleophilic attack of glutathione on the substrate.

GSTs can catalyze nucleophilic aromatic substitutions, Michael additions to α,β-unsaturated ketones, and epoxide ring-opening reactions, all of which result in the formation of GSH conjugates and the reduction of hydroperoxides, resulting in the formation of oxidized glutathione (GSSG) (Armstrong, 1996).

The variety of electrophilic compounds (presented as R-X in the above equation) that can be conjugated to glutathione by GSTs includes halogenated aromatic and aliphatic compounds, peroxides and epoxides, α,β-unsaturated carbonyl compounds, isothiocyanates and low molecular weight or protein disulfides. The most commonly used substrates for determination of GST enzymatic activity, usually by UV-VIS spectroscopy, are presented in Fig. 1.

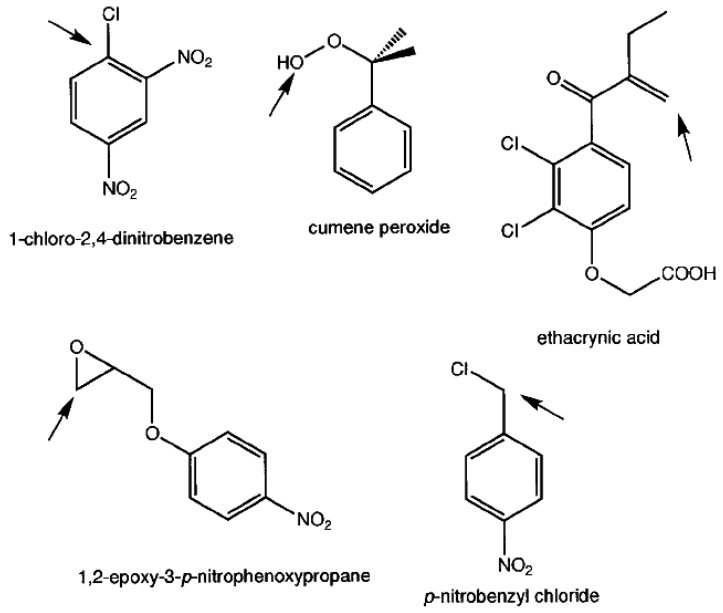


Fig.1. Standard substrates for measure of glutathione S-transferase activity. Arrows indicate the site for GSH addition (Vuilleumier, 1997).

In addition to GSH conjugation of electrophiles and peroxide reduction, GSTs have regularly been reported to be associated with other biologically important processes. Some GST proteins are involved in ion channel modulation (Dulhunty *et al*., 2001), in the synthesis of eicosanoids, leukotrienes and prostaglandins, and in GSH dependent transferase or isomerase reactions (Bartling *et al.*, 1993; Kanaoka *et al*., 1997; Fernandez-Canon & Penalva, 1998). A prominent example for a GST with a non-detoxification function is the human GSTO1–1, which modulates ryanodine receptors (RyR), a group of calcium channels in the endoplasmic reticulum of muscular cells (Dulhunty *et al*., 2001). Human omega class GSTs also possess dehydroascorbate reductase and thioltransferase activity (Board *et al*., 2000), and prostaglandin D synthase activity was demonstrated for human sigma GST (Kanaoka *et al*., 1997). In maize, a GST encoded by the *Bz2* gene was found to conjugate GSH to cyanidin 3-glucoside, the final step in the biosynthesis of the anthocyanin pigments (Marrs *et al*., 1995). Some GSTs are involved in catabolism of phenylalanine/tyrosine, catalyzing the isomerization of maleylacetoacetate to fumarylacetoacetate (Fernandez-Canon & Penalva, 1998).

Interestingly, some GSTs also possess a noncatalytic ligand-binding function, whereby they sequester hydrophobic planar aromatic ligands, such as dianthraquinones, at a poorly defined binding site or sites. In mammals, binding of steroids, hemes, and other ligands at this site(s) may alter GST tissue distribution (Lu & Atkins, 2004). This GST site has been referred to as the "ligandin" site. No detoxification role for the ligandin function has yet been demonstrated.

Finally and worthy of note, other glutathione S-transferases act non-enzymatically as signal transduction modulators (this function will be discussed in more detail below, Chapter 3).

1.3. Classification of enzymes within the glutathione S-transferase family

The GST superfamily has been subdivided into an ever-increasing number of classes basing on a variety of criteria, including amino acid/nucleotide sequence, physical structure of the genes (i.e. intron number and position), and immunological, kinetic and tertiary/quaternary structural properties of the corresponding proteins (Sheehan *et al*., 2001; Frova, 2006).

GST enzymes were first discovered in mammalian tissues during early 60s and even now they are best studied in higher eukaryotes, especially in rat, human and mouse. The characterization of mammalian GSTs has been facilitated by their activity with the model substrates, as 1-chloro-2,4-dinitrobenzene (CDNB) (Fig. 1) and their ability to bind to glutathione affinity matrices.

These GSTs were initially divided into three classes, alpha, mu, and pi, on the basis of their distinct but broad and overlapping specificity for model substrates.

The discovery of GST enzymes which do not bind to glutathione agarose columns and have poor activity towards 1-chloro-2,4-dinitrobenzene, the model "universal" GST substrate, provoke the determination of a new class of GST enzymes, theta (Meyer *et al*., 1991). For a long time, such atypical GSTs, including fungal and bacterial GSTs, were placed into this class (Vuilleumier, 1997).

With the development of bioinformatic analysis and the sequencing of entire genomes, many new glutathione S-transferases have been identified on the basis of characteristic motifs and generalized GST profiles (Vuilleumier & Pagni, 2002). Computational analysis led the definition of new classes whose number and nomenclature still remains somewhat arbitrary. To allow a better characterization of such enzymes, generalized profiles for GSTs, designed at the basis of pairwise and multiple alignments of the corresponding sequences can be used (Vuilleumier & Pagni, 2002). Two distinct profiles are available in InterPro (PFAM database) (http://www.ebi.ac.uk/interpro/), the N-terminal (accession PF02798.GST_N) and C-terminal (accession PF00043.GST_C) GST domains, which can be use for the classification of GST protein sequences and for inferring their function. A search with this two domains revealed that there are more than 1800 proteins sequences with PF02798.GST_N and more than 1700 with PF00043.GST_C. Using the predictors described in Vuilleumier & Pagni, 2002, there are more than 2400 protein sequences which share the N-terminal GST domain (described with pre:GST_N) and more than 2400 sequences sharing the C-terminal GST domain (described with pre:GST_C, respectively). More than 2600 sequences share both PF02798.GST_N and PF00043.GST_C or pre:GST_N and pre:GST_C domains (Table 1).

Table 1. Number of protein sequences in different taxonomical groups matching N-terminal or C-terminal GST domain according to InterPro databases (Suisprot & TrEMBL, April 2007)

Taxonomy group	Protein sequences with GST_N domain		Protein sequences with GST_C domain		Protein sequences with both domains
	pre:GST_N	PF02798.GST_N	pre:GST_C	PF00043.GST_C	
S. cerevisiae	9	8	9	9	6
Fungi	231	162	232	157	249
Archaea	0	0	4	4	0
Bacteria	2238	1724	2187	1542	2415
Total	2478	1894	2432	1712	2670

While there are no clearly established criteria concerning the extent of sequence similarity required for placing a GST in a particular class, it is generally accepted that GSTs share greater than 60% identity within a class, and those with less than 30% identity are assigned to separate classes (Sheehan *et al*., 2001). Much emphasis tends to be placed on the primary structure at the N-terminus because, within the classes, this region tends to be better conserved than others, as it often includes an important part of the active site.

According to the most recent review dedicated to GSTs (Frova, 2006), three main subfamilies of GSTs are generally recognized, each encoded by distinct multigene families:

1. soluble or cytosolic GSTs (canonical);
2. microsomal GSTs (MAPEG – membrane associated proteins involved in eicosanoid and glutathione metabolism);
3. plasmid-encoded bacterial fosfomycin-resistance GSTs.

Presently, the cytosolic GSTs are classified as follows:

- Seven classes of cytosolic GSTs are recognized in mammals, namely the specific Alpha, Mu, Pi and the common Sigma, Theta, Zeta and Omega.
- Plants have six classes, Lambda, Phi, Tau, DHAR (dehydroascorbate reductases) (specific), Theta and Zeta.
- Five classes have been recognized in insects, the Delta (specific), Sigma, Theta, Zeta and Omega.
- In unicellular organisms the picture is less clear. Bacteria possess a specific class named Beta, in addition to other enzymes more related to the common Theta and possibly other classes.

Microsomal GSTs, now designated MAPEG, are also ubiquitous, with a well documented presence in a wide spectrum of organisms occupying all positions in the evolutionary scale (Bresell *et al.*, 2005). Previously, MAPEG family included 13 proteins, 6 mammalian and 7 from plants, fungi and bacteria. According to multiple sequence alignments, four groups were recognized, termed I, II, III and IV (Jakobsson *et al*., 2000). Groups I and IV include human GSTs, while group III is exclusively bacterial (one *E. coli* and one *V. cholerae* sequence).

Finally, group II is the most heterogeneous as to the origin of the sequences, including human as well as members from plants and fungi (Frova, 2006).

The original set of 13 MAPEG members has meanwhile substantially expanded thanks to bioinformatics. A total of 131 distinct MAPEG proteins were found, 52 from prokaryotes and 79 from a wide spectrum of eukaryotes (Bresell *et al*., 2005), and an additional 24 members were detected in plants. This illustrates quite well the complicated and dynamic classification of MAPEG enzymes, as well as this of whole GST family.

The plasmid-encoded bacterial fosfomycin-resistance GST family includes the metallothiol bacterial fosfomycin resistance proteins, which are related to glyoxylases, lactoglutathione lyases, and extradiol dioxygenases (Rigsby *et al*., 2005).

In addition to cytosolic, microsomal and fosfomycin-resistance enzymes, an important number of known GSTs is presently not classified. Others form minor, separated groups, as this of Kappa GSTs, which structural and evolutionary position is very distinct from the existing classes.

1.4. Structural characteristics of GST enzymes

1.4.1. Cytosolic GSTs

To date, the crystal structures of over 200 soluble GSTs, belonging to the main plant, animal and bacterial classes, have been resolved. The common structural framework of the main cytosolic classes is presented in Fig. 2. Their analysis clearly demonstrates that, despite the sequence divergence, all soluble GST proteins show structural conservation, displaying a common 3D-fold and a dimeric organization.

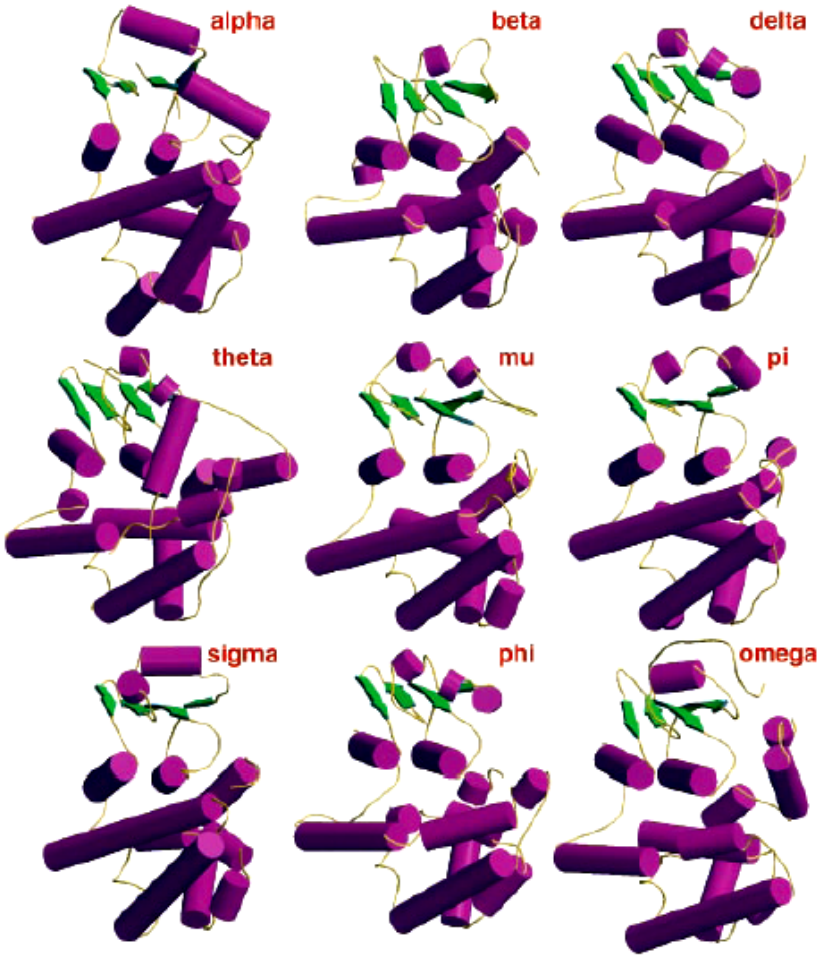


Fig. 2. The common chain fold of the soluble GST family. Helices are represented as purple cylinders, and β-strands are represented as green arrows (Board *et al*., 2000).

Soluble (cytosolic) GSTs are, as a general rule, biologically active as homo- or hetero-dimers of subunits of 23–30 kDa and an average length of 200–250 aminoacids. Each subunit is composed of two spatially distinct domains: an N-terminal domain (domain I), consisting of β strands and α helices as secondary elements, and an all helical C-terminal domain (domain II) (for detailed review see Armstrong, 1997).

Domain I adopts a thioredoxin-like fold (βαβαββα) (Fig. 3), which consists of two typical structural motifs, the N-ter $\beta_1\alpha_1\beta_2$ and the C-ter $\beta_3\beta_4\alpha_3$, linked together by a long loop containing an α-helix (α_2). Together the two regions form a β-sheet of three parallel ($\beta_1\beta_2\beta_4$) and one antiparallel (β_3) strands, sandwiched between the α_2 helix on one side and helices α_1 and α_3 on the other side. Helix α_2 and strand β_3 are connected by a loop containing a cis-Proline which is highly conserved in all GSTs. The cis-Pro loop, though not directly involved in catalysis, is important in maintaining the protein in a catalytically competent structure (Allocati *et al*., 1999).

Domain II consists of a variable number (4–7) of α–helices connected to domain I by a short linker sequence.

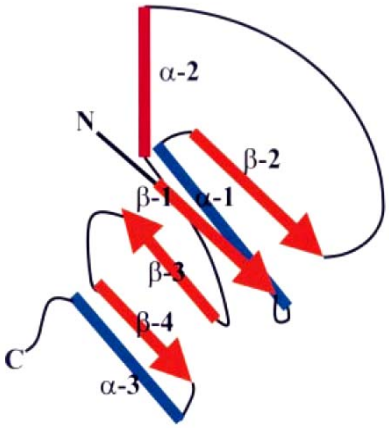


Fig. 3. The thioredoxin-like fold of Domain I. α-helices are shown as cylinders, while β-sheets are shown as red arrows. The four β-sheets are essentially co-planar, with one helix (α_2) shown in red above this plane and the other two α-helices (α_1 and α_3) shown in blue below the plane. The *cis*-Pro loop links α_2 to β_3 (Sheehan *et al*., 2001).

There are two ligand-binding sites per subunit. A specific glutathione-binding site (G-site) constructed mainly from residues of domain I, and the hydrophobic substrate binding site (H-site), which is formed primarily by residues with non-polar side chains lying in domain II. The two sites together constitute the catalytically active site. The N-terminal domain is quite conserved, and contains specific residues critical for GSH binding and catalytic activity. In particular, the highly conserved Tyr7 of the mammalian Alpha/Mu/Pi classes, and Ser17 of the ubiquitous Theta and Zeta, of the plant specific Phi and Tau and of insect Delta classes, have a crucial role in the catalytic activation of GSH. The Tyr/Ser hydroxyl group acts as hydrogen bond donor to the thiol group of GSH, promoting the formation and stabilization of the highly reactive thiolate anion which is the target for nucleophilic attack of an electrophilic substrate

Some GST classes have, instead of a Ser or Tyr, a cysteine at the usual active site position, a residue that promotes the formation of mixed disulphides with glutathione rather than the formation of the thiolate anion (Frova, 2006). All these enzymes have poor, when not null, conjugation activity to GSH, and are implicated in redox reactions instead.

Unlike domain I, domain II is quite variable both in sequence and topology, and this diversity determines the ample and distinct hydrophobic substrate specificities observed for the different enzymes.

1.4.2. Microsomal GSTs

Most MAPEG proteins are involved in the synthesis of eicosanoids, leukotrienes and prostglandins, catalyzing GSH-dependent transferase or isomerase reactions (Frova, 2006). Microsomal GSTs have a sequence identity with the soluble GSTs less than 10% (Frova, 2006). Usually, their subunits are shorter, with an average length of 150 aminoacids. Crystallization experiments have been reported for three members of the MAPEG family: microsomal glutathione S-transferase 1 (MGST1), microsomal prostaglandin E synthase 1 (MPGES1) and leukotriene C4 synthase (LTC4S) (reviewed in Hebert *et al*., 2005).

MAPEG proteins characterized so far have four transmembrane domains, the amino and carboxyl termini of the protein protruding into the luminal side of the membrane, while putative sites for GSH and substrate binding are located in loops facing the cytosol (Bresell *et al.*, 2005).

The 3D map of MGST1 and the projection structures of LTC4S and PGES1 indicate the enzymes as homotrimers (Hebert *et al*., 2005). Three-dimensional maps of MGST1 also demonstrate that a large proportion of the protein monomer forms a left-handed four α-helix bundle (Holm *et al*., 2002). Because the projection maps of all known MAPEG proteins are similar, it is very likely that the protein fold determined for MGST1 shows a common property among most, if not all, MAPEG proteins.

1.4.3. Plasmid-encoded bacterial fosfomycin-resistance GSTs

Three similar but mechanistically distinct fosfomycin resistance proteins that catalyze the opening of the oxirane ring of fosfomycin are known: FosA is a Mn^{2+} and K^{+}-dependent glutathione transferase. FosB is a Mn^{2+}-dependent L-cysteine thiol transferase. FosX is a Mn^{2+}-dependent fosfomycin-specific epoxide hydrolase (Rigsby *et al*., 2005).

Structural and mechanistic analysis of FosA established that it catalyzes the Mn^{2+} and K^{+}-dependent addition of GSH to fosfomycin (Bernat *et al.*, 1997). The crystal structures of this enzyme have been determined, showing a unique repeated domain structure that is distinct from either the cytoplasmic or MAPEG glutathione S-transferase families (Rife *et al*., 2002). The apoenzyme is a dimer of 16 kDa subunits. Each subunit contains a mononuclear Mn^{2+} center that interacts strongly with the substrate fosfomycin and very weakly or not at all with GSH (Bernat *et al.*, 1997).

1.4.4. Kappa class GSTs

In eukaryotes, these enzymes are typically found in mitochondria and not in the cytosol. In addition, a human kappa enzyme was detected also in peroxisomes (Morel *et al.*, 2004). All mammalian Kappa enzymes are encoded by single copy genes, highly similar in mouse, rat and human both in sequence (approximately 70% aminoacid identity) and in organization: they all consist of eight exons and seven introns at conserved positions (Jowsey *et al.*, 2003; Morel *et al.*, 2004). Like the soluble GST proteins, it forms a dimer, but the secondary structural elements of its subunit differ significantly from the common model (Ladner *et al.*, 2004). Instead of the canonical N-terminal α,β domain followed by an all α-helical domain, the structure has an α-helical domain inserted between helix α_2 and strand β_3 of the thioredoxin-like α,β domain.

1.5. Regulation of GST enzymes

GST enzymes can either be expressed constitutively or be induced by a wide range of compounds and factors of both natural and xenobiotic origin, suggesting that their promoters contain either a set of different specific motifs or general regulatory sequences activated by different molecules.

In eukaryotes, induction of GSTs by xenobiotics is mediated via several different transcriptional mechanisms (Eaton & Bammler, 1999). The rat *GSTA2* gene promoter region contains a glucocorticoid-response element (GRE), a xenobiotic response element (XRE) and an antioxidant-response element (ARE). The GRE mediates induction via glucocorticoids, and the XRE mediates induction by planar aromatic hydrocarbons. Induction of transcription of *rGSTA*2 in response to phenolic antioxidants was shown to be mediated by ARE.

In mammals, high expression levels of another GST enzyme – GSTP1, are characteristic for both physiological cellular proliferation, e.g. embryogenesis and pathological proliferation such as in tumorigenesis. During chemically induced hepatocarcinogenesis in the rat, rGSTP1 is over-expressed, not evenly distributed throughout the liver, but in foci. This was shown to be controlled largely by an element, in the *rGSTP* upstream called GPE1 (part of this element has sequence resembling ARE) (Muramatsu & Sakai, 2001).

In yeast, the promoter region of *GTT1* gene contains a xenobiotic response element and an antioxidant response element (Choi *et al.*, 1998). It also contains several copies of a stress response element (STRE), a post-diauxic shift element (PDS), and a sequence recognized by

yeast yAP1 transcriptional factor. The transcription of *GTT1* is induced by osmotic stress and xenobiotics, and, most significantly, after diauxic shift.

1.6. Evolution of the GST enzyme family

GSTs constitute a very ancient protein superfamily, which is thought to have evolved from a thioredoxin-like ancestor in response to the development of oxidative stress (Martin, 1995). Other GSH- and cysteine-binding proteins also share the thioredoxin-like fold, and it is increasingly becoming clear that GSTs share sequence and structural similiarities with several stress-related proteins in a wide range of organisms. It is thought that the multiple GST classes arose by a process of gene amplification followed by divergence, perhaps involving a mechanism similar to DNA shuffling, resulting in novel catalytic activities (Armstrong, 1998). Domain shuffling facilitated GSTs becoming involved in a wide range of other tasks at an early stage in the evolution of eukaryotes (McGoldrick *et al.*, 2005).

2. Glutathione S-transferase-like proteins in yeast

2.1. The diversity of microbial GSTs

In contrast to higher eukaryotes, both bacteria and fungi have a relatively small number of known GSTs with very diverse and often unusual functions. For a long time, GST enzymes from microbial sources were neglected and were not systematically studied. One of the reasons for this was the poor activity of microbial GSTs with CDNB as a model substrate for GST activity, which led to the conclusion that these enzymes are rare in unicellular organisms (Vuilleumier, 1997). The accidentally characterized enzymes were placed in the theta class, the most heterogeneous and diversified group which also included enzymes from plants, mammals, fish, birds and insects. With the increasing publication of numerous bacterial and fungal genomes, however it became clear that GST genes exist in a wide range of bacteria and fungi.

Microbial GSTs differ functionally and structurally from the mainstream GST classes of plants and mammals. In 2002, an exploratory analysis of existing genome databases revealed the presence of more than 103 putative GST genes in bacteria (Vuilleumier & Pagni, 2002). This number has since continuously increased. To date, more than 2500 microbial proteins were found to contain both GST_N and GST_C profile (see Table 1).

At the protein level, bacterial GSTs of known function catalyze specific reactions in degradation pathways of recalcitrant chemicals such as dichloromethane, 1,2-dichloroepoxyethane and lignin

compounds, which can be used for growth by bacteria (Vuilleumier, 1997). In contrast to eukaryotic GSTs, these bacterial enzymes are very specific for each substrate and have no activity toward CDNB or other typical GST substrates. Dichloromethane (DCM) dehalogenase, 1,2-dichloroepoxyethane epoxidase and tetrachlorohydroquinone reductase are the best characterized bacterial GSTs at the sequence and functional level (Vuilleumier, 1997).

Besides the catabolic GSTs with strict substrate specificity, those that have activity toward typical substrates such as CDNB have been identified from *Proteus mirabilis* (Mignogna *et al.*, 1993) and *Escherichia coli* (Nishida *et al*., 1994). They are 53% identical in primary structure, but have low homology with those from eukaryotes. These bacterial enzymes have not only GSH-conjugating activity toward typical substrates such as CDNB and ethacrynic acid but also GSH-dependent peroxidase activity toward cumene hydroperoxide (Nishida *et al*., 1994; Perito *et al*., 1996). In total, there are seven genes that code for GST-like proteins with divergent sequences in the *E. coli* genome (Rife *et al*., 2003). Two of them, yfcF and yfcG gene products, exhibit GST activity toward CDNB and GSH-dependent peroxidase activity toward cumene hydroperoxide (Kanai *et al*., 2006).

2.2. GSTs of fungi and yeasts

As bacterial GSTs, most fungal glutathione S-transferases do not fit easily into any of the previously characterized classes of the family. They display a wide diversity of primary structures, which also often strongly differ from mammalian GST sequences.

In a recent study, sixty-seven known GST-like proteins from 21 fungal species were grouped in five clusters – clusters 1, 2, EF1Bγ, Ure2p and MAK16 (McGoldrick *et al.,* 2005). These clusters are quite different from previously defined classes and are considered as separate groups with an uncertain phylogenic relation to the mainstream eukaryotic GSTs.

According to these authors, clusters 1 and 2 group 13 GST sequences, including the best characterized GST genes in fungi – *S. cerevisiae GTT1, S. pombe GST3* and *Aspergillus nidulans GSTA*. Surprisingly, the second "classic" GST gene in *S. cerevisiae, GTT2,* was not part of any major cluster of fungal GST sequences, despite detectable identity to *GTT1* (11% identity and additional 32% similarity (Choi *et al.*, 1998)). The sequence alignments at the basis of the reported phylogenetic relationships were not presented, so the rationale behind such observations remains unclear.

Twenty-three of the known 67 fungal GST sequences were placed in the cluster of elongation factors EF1Bγ. This cluster could be regarded as a distinct GST class, widespread in yeasts and fungi, which was probably recruited into the elongation machinery of eukaryotic cells at an early stage of evolution (McGoldrick *et al.,* 2005). Sequence alignment, motif searching and homology modeling had initially revealed that the N-terminal domain of fungal EF1Bγ is closely related to GSTs (Koonin *et al*., 1994). While all fungal EF1Bγ proteins seem to contain this N-terminal domain, the C-terminal domain is of variable length in various fungal species. It is absent in *A. nidulans*, where the EF1Bγ protein consists of the N-terminal GST-like domain only (Koonin *et al*., 1994). This suggests that the functionality of the EF1Bγ may reside in the GST-like thioredoxin-fold domain. Despite this sequence homology, it is unclear whether fungal EF1Bγ proteins retain the GST-like catalytic activities, predicted from sequence analysis. Indeed, only the recombinant EF1Bγ from *Oryza sativa* has been shown to catalyze GSH conjugation to 1-chloro-2, 4-dinitrobenzene (Kobayashi *et al*., 2001).

Other GST sequences have been clustered together in a group of *URE2*-like genes. The protein Ure2 of *S. cerevisiae* has been characterized in detail at the structural level. It is a dimer of subunits of approx. 50 kDa. The C-terminal domain (residues 94–354) shows a low level (11–20%) of sequence identity to GST, but its crystal structure shows unmistakable similarity to the typical GST fold (Bousset *et al*., 2001). Interestingly, residues implicated in substrate binding and catalysis in GSTs seem to have mutated to non-catalytic residues in Ure2p, which might explain why it does not seem to catalyze GSH dependent conjugation or bind GSH.

Finally, the MAK16 cluster consists of several fungal GST genes coded for a group of nuclear proteins that play a role in both cell cycle progression and biogenesis of 60S ribosomal subunits – the MAK16 proteins (McGoldrick *et al.,* 2005). This cluster is not well characterized and its precise functional significance remains unclear, but it is clearly distinct from the previous four groupings of GST-like proteins and may be related to Kappa and MAPEG GSTs.

2.3. GSTs of known function in Saccharomyces cerevisiae

2.3.1. Gtt1 and Gtt2

The first *S. cerevisiae* genes coding for functional GST enzymes which were identified were *GTT1* and *GTT2* (Choi *et al.*, 1998). Blast search of GenBank identified GST from maize form IV as most similar known GST enzyme to Gtt1 and Gtt2 (35% identity with Gtt1p and 27% with

Gtt2p). Despite this limited identity, recombinant Gtt1p and Gtt2p exhibit GST activity with 1-chloro-2,4-dinitrobenzene as a substrate (Choi *et al.*, 1998).

Both Gtt1p and Gtt2p are able to form homodimers. Subcellular fractionation demonstrated that Gtt1p associates with the endoplasmic reticulum (Choi *et al.*, 1998).

Transcription from *GTT1* promoter is induced by osmotic stress and xenobiotics, and, after diauxic shift. Expression of *GTT1* remains high throughout the stationary phase. *GTT1* and *GTT2* are not essential to the yeast cell, but *gtt1Δ*, *gtt2Δ*, and *gtt1Δgtt2Δ* mutant strains exhibit significantly reduced thermotolerance in the stationary phase, and limited ability to grow at 39°C. Therefore, it is likely that Gtt1p and Gtt2p participate, via their GST activity, in the elimination of toxic metabolites that accumulate during the stationary phase as well as under other stress conditions (Choi *et al.*, 1998).

2.3.2. Tef3p and Tef4p

At least three proteins form eukaryotic elongation factor 1 (eEF1) responsible for the elongation step of protein synthesis in yeast (Jeppesen *et al*., 2003). EF1A binds aminoacyl-tRNAs to the 80S ribosome while EF1B which contains two distinct subunits EF1Bα and EF1Bγ facilitates the guanine nucleotide exchange (see Chapter 2 of Results for details and figures). EF1Bγ is a dimer of approximately 50 kDa subunits which consist of distinct N- and C-terminal domains. The N-terminal domain possesses a GST similarity (Koonin *et al*., 1994).

The *S. cerevisiae* eEFB1γ is encoded by two homologue genes *TEF3* and *TEF4* (Kinzy *et al*., 1994). These genes are both not essential for growth. The crystal structure of the recombinant 219 residue N-terminal domain (domain 1) of Tef3p has been determined (Jeppesen *et al*., 2003), showing unambiguously that this protein is a member of the GST structural superfamily (Fig. 4). However the GST domain of Tef3 recombinant protein displayed no GST-like catalytic activity, and GSH was absent from the putative catalytic site. In addition, the *TEF3*-encoded form of eEF1Bγ has no obvious catalytic residue, while the form of eEF1Bγ encoded by the *TEF4* gene contains serine 11, which may act catalytically.

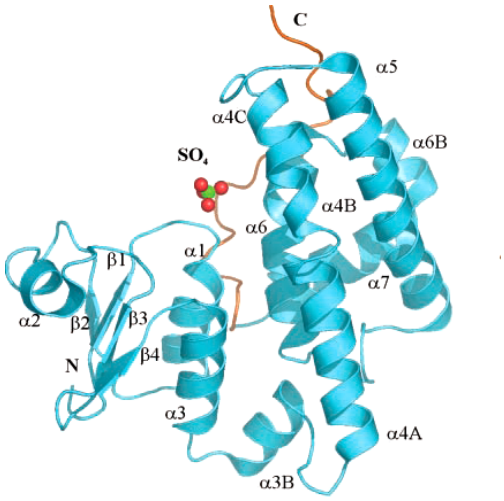


Fig. 4. The eEF1Bγ domain 1 monomer viewed from the dimer interface side. The N-terminal subdomain 1N containing the sulfate ion is shown on the left and subdomain 1C on the right. The N and C termini are labeled along with the secondary structural elements and the sulfate ion. The last 20 amino acids constituting the C terminus of subdomain 1C are colored orange (Jeppesen *et al*., 2003).

2.3.3. Ure2p

Ure2p negatively regulates GATA factor-mediated transcription in *S. cerevisiae,* and plays a key role in nitrogen catabolite repression. This protein possesses prion-like characteristics and has properties similar to mammalian prions (Wickner, 1994). The N-terminus encodes a functional prion region (residues 1–93) rich in Asn and Gln while the C-terminal region (residues 94–354) is responsible for suppression of GATA-factor-mediated transcription. The crystal structure of the truncated C-region in both the apo- form and with GSH bound has confirmed the GST classification of Ure2p. The structure of Ure2p 95–354 region (Fig. 5) shows a two-domain protein forming a globular dimer. The N-terminal domain is composed of a central 4 strand β-sheet, flanked by four α-helices, two on each region side. In contrast, the C-terminal domain is entirely α-helical. The fold of Ure2p 95–354 resembles that of the beta class GSTs (Bousset *et al*., 2001). However there is a significant structural difference – an insertion of 32 residues (residues 267–298) located in the C-terminal domain of Ure2p and called cap region.

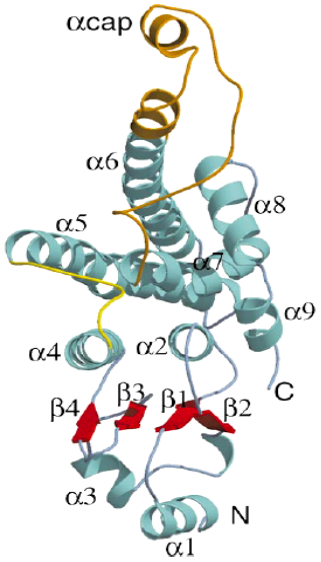


Fig. 5. Ribbon representation of the three-dimensional structure of the functional region of Ure2p (residues 94–354) in its monomeric form. A short linker region, shown in yellow, separates the two domains. The cap region (residues 267–298) is shown in orange. The N-terminal domain (residues 95–196) consists of a central four-stranded β sheet (red) flanked on both sides by α helices (blue). The larger C-terminal domain (residues 206–354) is all α-helical (Bousset *et al*., 2001).

Ure2p does not show a detectable level of GST activity with typical substrates such as CDNB (Choi *et al.*, 1998). However, *ure2Δ* mutants have been found to show greater sensitivity than isogenic wild type strains to a range of GST substrates and compounds generating oxidative stress (Rai *et al.*, 2003). Most specifically, an N-terminal residue that participates in catalysis in GSTs of known function – Tyr, Ser, Cys or His is not present in Ure2p. This residue is critical for destabilization of the cysteine S–H bond, thereby facilitating formation of the active thiolate anion. The region of Ure2p that binds glutathione contains an asparagine, Asn124 which is situated at a location and distance that are consistent with permitting it to function in destabilization of this critical S–H bond (Bousset *et al*., 2001).

2.3.4. Omega class GSTs

The *S. cerevisiae* genome encodes three proteins that display similarities with human omega class glutathione S-transferases – hGSTO1-1 and hGSTO2-2. It is remarkable that, while most bacterial and fungal GSTs are structurally separated from mammalian GSTs, those with the basic structure of the Omega class are present across all domains of life.

The three *S. cerevisiae* proteins have been named Gto1, Gto2 and Gto3 (Garcera *et al*., 2006). Their purified recombinant forms are active as thiol transferases (glutaredoxins) against *β*-

hydroxyethyl disulphide, as dehydroascorbate reductases and as dimethylarsinic acid reductases, while they are not active against the standard GST substrate CDNB (1-chloro-2,4-dinitrobenzene). Their glutaredoxin activity is also detectable in cell free extracts.

Analysis of point mutants derived from wild-type Gto2 indicates that, among the three cysteine residues of the molecule, only the residue at position 46 is required for the glutaredoxin activity (Garcera *et al*., 2006). This indicates that the thiol transferase acts through a monothiol mechanism. Replacing the active site of the yeast monothiol glutaredoxin Grx5 with the proposed Gto2 active site containing Cys46 allows Grx5 to retain some activity against *β*-hydroxyethyl disulphide. Therefore the residues adjacent to the respective active cysteine residues in Gto2 and Grx5 are important determinants for the thiol transferase activity against small disulphide-containing molecules (Garcera *et al*., 2006).

Gto1 is located in the peroxisomes, whereas Gto2 and Gto3 are in the cytosol (Barreto *et al*., 2006). Among the *GTO* genes, *GTO2* shows strongest induction of expression after exposure to agents such as diamide, 1-chloro-2,4-dinitrobenzene, *tert*-butyl hydroperoxide or cadmium, in a manner that is dependent on transcriptional factors Yap1 and/or Msn2/4 (Barreto *et al*., 2006). Phenotypic analyses with single and multiple mutants in the *S. cerevisiae* glutathione S-transferase genes show that, in the absence of Gto1 and the two Gtt proteins, cells display increased sensitivity to cadmium (Barreto *et al*., 2006). A *gto1*-null mutant also shows growth defects on oleic acid-based medium, which is indicative of abnormal peroxisomal functions, and altered expression of genes related to sulfur amino acid metabolism. As a consequence, growth of the *gto1Δ* mutant is delayed in growth medium without lysine, serine, or threonine, and the mutant cells have low levels of reduced glutathione (Barreto *et al*., 2006).

Chapter 2

Antioxidant roles of glutathione S-transferases

1. Oxidative stress defense in yeast

1.1. Oxygen metabolism in aerobic organisms

All aerobic organisms use molecular oxygen (O_2) for respiration and/or oxidation of nutrients in order to obtain energy in an efficient way. The oxygen molecule is therefore indispensable for their life, as electron acceptor in oxidation processes. While oxidation is the most efficient biological way to produce energy, organismal tolerance for oxygen and oxidative by-products or reactive oxygen species (ROS), is limited (Vichnevetskaia & Roy, 1999; Scandalios, 2005). Thus, a narrow optimum for oxygen tolerance exists in living organisms with an aerobic lifestyle.

Molecular oxygen is relatively unreactive due to its electron configuration (two unpaired electrons with parallel spins). In the cellular electron transport chain, oxygen is reduced by four electrons to water. Activation of oxygen (i.e. first univalent reduction step) is energy dependent and requires an electron donation [eq. 1]. The subsequent one-electron reduction steps are not energy dependent and can occur spontaneously or require appropriate electron and proton donors. The superoxide anion ($O_2^{-\cdot}$), produced in the first step, can be protonated at a low pH to yield the perhydroperoxyl radical $^{\cdot}HO_2$ [eq. 2]. Both $O_2^{-\cdot}$ and $^{\cdot}HO_2$ undergo spontaneous dismutation to produce hydrogen peroxide (H_2O_2) [eq. 3 & 4].

$$O_2 + e^- \rightarrow O_2^{-\cdot} \text{ [1]}$$

$$H^+ + O_2^{-\cdot} \leftrightarrow {}^{\cdot}HO_2 \text{ [2]}$$

$${}^{\cdot}HO_2 + {}^{\cdot}HO_2 \rightarrow H_2O_2 + O_2 \text{ [3]}$$

$${}^{\cdot}HO_2 + O_2^{-\cdot} + H_2O \rightarrow H_2O_2 + O_2 + OH^{\cdot} \text{ [4]}$$

Although H_2O_2 is less reactive than $O_2^{-\cdot}$, in the presence of reduced transition metals such as Fe^{2+} in a chelated form (which is the case in biological systems), the formation of hydroxyl radicals ($OH^{\cdot}$) can occur in the Fenton reaction (Vichnevetskaia & Roy, 1999; Scandalios, 2005):

$$Fe^{2+}\text{complex} + H_2O_2 \rightarrow Fe^{3+}\text{complex} + OH^{\cdot} + OH^- \text{ [5]}$$

Fe^{3+}complex can be efficiently reduced by $O_2^{-\cdot}$ [eq. 6], the product of one electron oxygen reduction, thus closing the cycle of Fenton reaction:

$$O_2^{-\cdot} + Fe^{3+}complex \rightarrow O_2 + Fe^{2+}complex \quad [6]$$

In total: $$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^{-} \quad [7]$$

All above reactions (eq. 1 – 7) occur during normal metabolic processes in aerobic cells, as the four-electron reduction is always accompanied with a partial one- to three-electron reduction, yielding the formation of intermediate products: hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\cdot}$) and hydroxyl radical ($OH^{\cdot}$). These compounds, often named ROS (reactive oxygen species), are highly reactive and can directly oxidize cellular macromolecules, including DNA, protein, and lipids. The interaction of ROS with lipids is particularly damaging to cells because a single ROS molecule can generate a secondary chain reaction with autocatalytic propagation of lipid peroxidation reactions, thus forming a number of toxicants such as hydroperoxides (ROOH), peroxyradicals ($ROO^{\cdot}$), alkoxy radicals ($RO^{\cdot}$), and α,β-unsaturated aldehydes.

In addition to being generated in the physiological processes, ROS can be produced as primary products under a variety of stress conditions (Scandalios, 2005). For example, numerous toxic environmental chemicals such as xenobiotics, pesticides, herbicides, fungicides, ozone, and radiation cause their harmful environmental effects via generation of free radicals and other ROS. The consequences of ROS formation depend on the intensity of the stress and on the physicochemical conditions in the cell (antioxidant status, redox state and pH).

1.2. Main antioxidant defense systems in yeast

Cells possess a range of enzymatic and non-enzymatic defense systems to counter oxidative stress and to protect cellular constituents. Non-enzymatic defense systems typically consist of small molecules which are soluble in an aqueous environment or, in some instances, a lipid environment. They act in general as radical scavengers, being oxidized by ROS and thereby removing oxidants from solution. Non-enzymatic defense systems include glutathione, ascorbate, threhalose, tocopherols, phytochelatins, polyamines and metallothioneins. Enzymatic defenses include glutaredoxins, thioredoxins, superoxide dismutase (SOD), glutathione S-transferases (GST) and peroxidases such as catalase and glutathione peroxidase.

These different systems are reviewed in detail in the following section, with special emphasis on *S. cerevisiae.*

1.2.1. Non-enzymatic defense system

Among non-enzymatic defenses, the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine; GSH) plays a pivotal role. Its biological significance is mainly related to the free sulphydryl moiety of the cysteine residue, which confers unique redox (E'_o=-0.24 V for thioldisulfide exchange) and nucleophilic properties (Pocsi *et al.*, 2004). GSH is found in cells as reduced GSH, oxidized disulfide form GS-SG, and mixed disulfides, GS-S-CoA and GS-S-Cys. Under normal conditions, glutathione is primarily present in its reduced form (GSH), with only a small proportion being present in the fully oxidized state (GSSG) (Pocsi *et al.*, 2004).

Glutathione has multifunctional roles in both regular cellular metabolism and in defense against ROS (Fig. 6). It reacts nonenzymatically with a series of ROS including hydroxyl radical ($OH^{\bullet}$), hypochlorous acid (HOCl), alkoxy radicals ($RO^{\bullet}$), peroxyradicals ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), as well as with many nitrogen and carbon containing radicals through the formation of thiyl ($GS^{\bullet}$) radicals (Halliwell & Gutteridge, 1999).

In addition to its role as a free-radical scavenger in cells, glutathione serves as a co-factor for several enzymes involved in the overall antioxidant defense, and in heavy metal and xenobiotic stresses. As discussed below, these enzymes include glutathione peroxidases, glutathione reductase and glutathione S-transferases.

Glutathione can also serve as a sulfur and nitrogen source for yeast growth, and to participate in reactions involving the synthesis of proteins and nucleic acids (Penninckx, 2002).

A brief overview of GSH role in fungal cell in context of the overall metabolism is presented in Fig. 6.

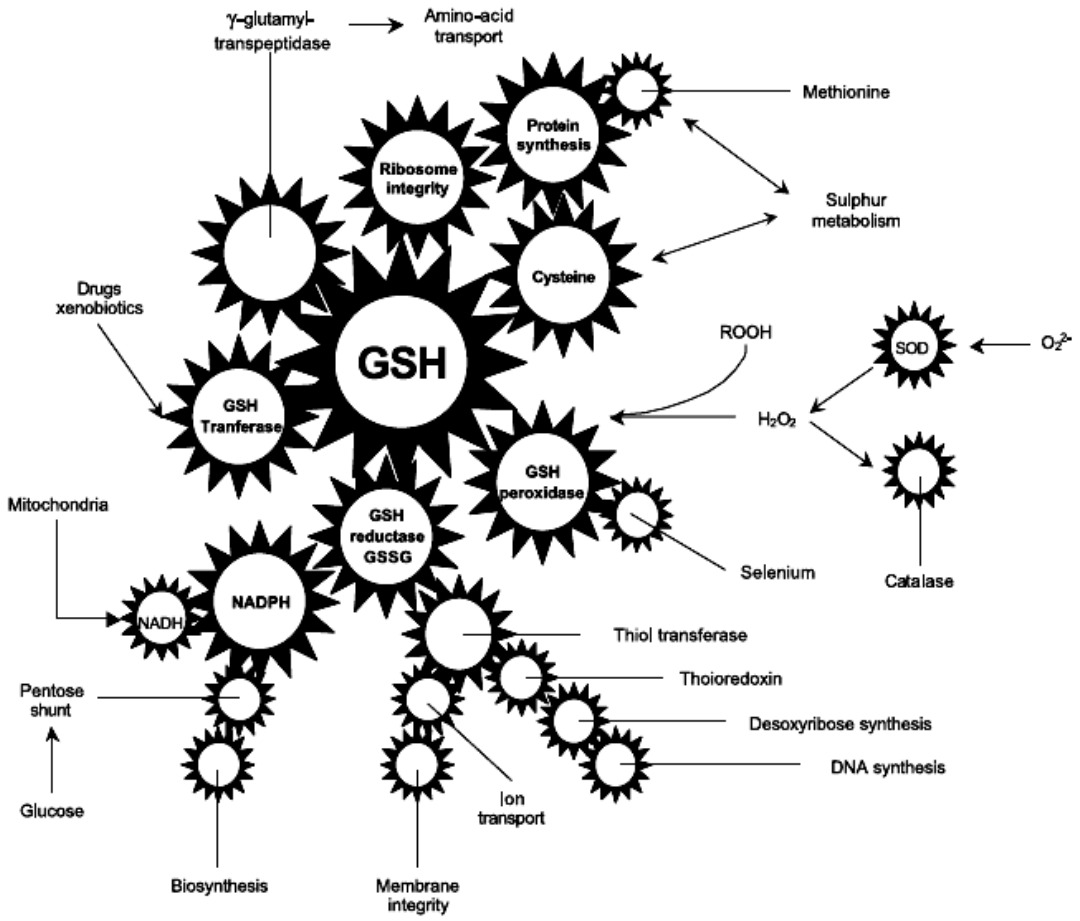


Fig. 6. GSH production and consumption machines in the metabolic network of fungi (Penninckx, 2002).

In yeast, GSH is synthesized via a two-step, ATP-dependent process. Glutamate and cysteine are conjugated by the enzyme γ-glutamyl cysteine synthetase, encoded by *GSH1* gene in *S. cerevisiae* (Lisowsky, 1993). The product, γ -glutamyl cysteine (γ-GC) is combined with glycine to form GSH in a reaction catalyzed by glutathione synthetase, encoded by *GSH2* (Grant *et al*., 1997). Yeast mutants lacking *GSH1* are unable to grow in the absence of GSH, and are sensitive to oxidative stress caused by H_2O_2 and various alkyl hydroperoxides (Grant *et al*., 1996). Conversely, *gsh2Δ* mutants are relatively insensitive to oxidative stress, presumably due to the high concentrations of the antioxidant dipeptide γ-GC which accumulates in these mutants (Grant *et al*., 1997). However, γ-GC cannot fully substitute for GSH, as the *gsh2Δ* mutant grows poorly on GSH-deficient media and is more sensitive to heat shock (Grant *et al.*, 1997).

The breakdown of GSH is initiated by γ-glutamyl transpeptidase, which catalyzes transfer of the γ-glutamyl group of GSH (and of other γ-glutamyl compounds) to acceptors (amino acids, dipeptides, GSH, H_2O) (Pocsi *et al.*, 2004).

Utilisation of GSH in both enzymatic and non-enzymatic defense mechanisms results in its conversion to the oxidized form (GSSG) which must be recycled to GSH to maintain the high intracellular ratio of GSH to GSSG, required for balancing redox potential in cell (Pocsi *et al.*, 2004). Glutathione reductase (Glr) is a flavoprotein which catalyzes reduction of GSSG to GSH using the reducing power of NADPH, thus regenerating reduced GSH (Fig. 7).

1.2.2. Enzymatic antioxidant defense system

Glutaredoxins and thioredoxins

Glutaredoxins and thioredoxins are ubiquitous small heat-stable oxidoreductases that have proposed functions in many cellular processes, including deoxyribonucleotide synthesis, repair of oxidatively damaged proteins, protein folding and sulphur metabolism (Wheeler & Grant, 2004). Glutaredoxins and thioredoxins are structurally similar and have been conserved throughout evolution, particularly in the region of their active site which contains two conserved cysteine residues.

Glutaredoxins form part of the glutaredoxin system, comprising NADPH, GSH and glutathione reductase (Glr) (Fig. 7), which transfers electrons from NADPH to glutaredoxins via GSH (Michelet *et al*., 2006).

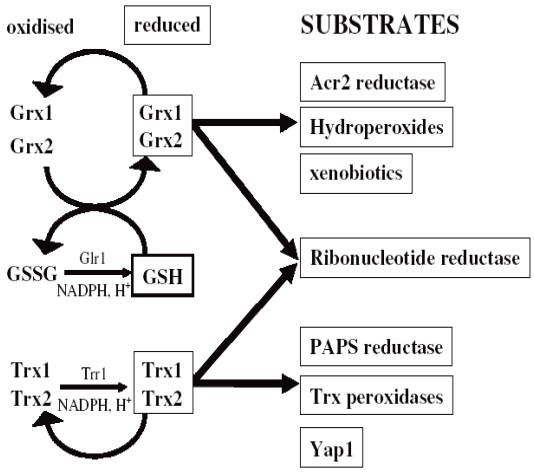


Fig. 7. The GSH/glutaredoxin and thioredoxin systems in *S. cerevisiae*. Yeast contains two gene pairs encoding cytoplasmic glutaredoxins (*GRX1*, *GRX2*) and thioredoxins (*TRX1*, *TRX2*). The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase (Trr1). In contrast, oxidized glutaredoxin is reduced by glutathione (GSH), and oxidized GSSG is in turn reduced in an NADPH-dependent reaction catalyzed by glutathione reductase (Glr1). Glutaredoxins and thioredoxins are believed to act as hydrogen donors for a range of enzymes: ribonucleotide reductase, 3'-phosphoadenosine 5'-phosphosulphate reductase (PAPS reductase), thioredoxin peroxidases, Yap1, arsenate reductase (Acr2) and can directly reduce hydroperoxides and xenobiotics (Wheeler & Grant, 2004).

Yeast contains two glutaredoxin genes, designated *GRX1* and *GRX2* (Grant, 2001). Strains deleted for both *GRX1* and *GRX2* are viable but lack heat-stable oxidoreductase activity, using β-hydroxyethyl disulphide as a model disulphide substrate (Luikenhuis *et al*., 1997). In addition, the *grx1Δ* mutant is sensitive to the superoxide anion ($O_2^{\cdot-}$) induced oxidative stress, whereas a strain that lacks *GRX2* is sensitive to hydrogen peroxide (H_2O_2).

Both Grx1p and Grx2p of *S. cerevisiae* were shown to be active as glutathione S-transferases and glutathione peroxidases, and their activity with model substrates such as 1-chloro-2,4-dinitrobenzene, hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide is similar to that of glutathione peroxidase GPx2 and glutathione S-transferases Gtt1 and Gtt2 (Collinson *et al*., 2002; Collinson & Grant, 2003).

A new family of glutaredoxin-related proteins has been recently identified in yeast (Grx3-5) (Rodriguez-Manzaneque *et al*., 1999). These glutaredoxin-like proteins differ from classical glutaredoxins in that they contain a single cysteine residue at their putative active sites. Grx3, 4 and 5 may be required to reduce protein-mixed disulphides, which are formed during exposure to ROS, a reaction that proceeds via a monothiol mechanism (Rodriguez-Manzaneque *et al.*, 1999). Loss of *GRX3, GRX4* or *GRX5* results in decreased oxidoreductase activity, and the triple mutant lacking all three isoforms is unviable. In addition, the *grx5Δ* mutant is particularly sensitive to oxidative stress induced by H_2O_2 and the superoxide anion, shows elevated levels of oxidative protein damage and is slow growing.

Yeast contains two genes encoding cytoplasmic thioredoxins (*TRX1* and *TRX2*) (Fig. 7) that are dispensable under normal growth conditions (Gan, 1991). Deletion of both *TRX1* and *TRX2* affects the cell cycle, resulting in a prolonged S phase and a shortened G1 interval (Muller, 1991). Like glutaredoxins, Trx2 functions in protection against ROS, as *trx2Δ* mutants are sensitive to H_2O_2 (Kuge & Jones, 1994).

Glutathione peroxidases

Glutathione peroxidases (GPxs) represent the major enzymatic defense against oxidative stress caused by hydroperoxides. They reduce hydrogen peroxide and organic hydroperoxides, such as fatty acid hydroperoxides, to the corresponding alcohols, using reducing power provided by GSH (Herbette *et al*., 2007).

Two principal forms of glutathione peroxidase have been characterized: classical GPx (cGPx or GPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) (Arthur, 2000). PHGPx

is monomeric and partly membrane-associated, often evident in both soluble and membrane fractions, whereas GPx is tetrameric and soluble. Moreover, PHGPx is functionally distinct from GPx, as they reduce lipid hydroperoxides esterified to biomembranes in addition to the peroxides that are substrates of both Gpx and PHGPx. Phospholipid hydroperoxides are central intermediates in the lipid peroxidation chain reaction and lipid peroxidation being one of the major types of oxidative damage in cells is associated with membrane perturbation, inactivation of membrane proteins, and cell lysis. Thus, PHGPx is the principal cellular enzyme capable of membrane lipid peroxidation repair, and is generally considered to represent the main line of enzymatic defense against oxidative membrane damage (Avery & Avery, 2001).

Notably, *S. cerevisiae* does not contain any classical GPx, but expresses three PHGPx, which have activity with phospholipid hydroperoxides as well as non-phospholipid hydroperoxides (Inoue *et al*., 1999). The expression of three PHGPxs, combined with the absence of a conventional GPx, is a unique feature of *S. cerevisiae*. Avery & Avery (2001) suggested that *S. cerevisiae* has been under a particular selective pressure to evolve and sustain a high capacity for enzymatic repair of membrane lipid peroxidation, specifically, in response to various stresses. Paradoxically, *S. cerevisiae* is unusual among eukaryotes in that its membrane phospholipids are normally devoid of polyunsaturated fatty acid (PUFAs) as a result of the absence of the corresponding desaturases (Avery *et al*., 1996). Thus, it can be expected that *S. cerevisiae* is less susceptible than many other organisms to potential damage from lipid peroxidation. Nonetheless, lipid peroxidation does occur in *S. cerevisiae,* and has been shown to account for membrane damage and killing, for example during metal-induced oxidative stress (Howlett & Avery, 1997). Therefore, one possible explanation for the absence of PUFA synthesis together with the expression of three PHGPx enzymes could be that *S. cerevisiae* is prone to particularly high fluxes of reactive oxygen species generated in or directed toward membranes than other organisms (Avery & Avery, 2001). In such a model, both PUFA-lacking membranes and high PHGPx activity (possibly along with the capacity to ferment) might be considered evolutionary adaptations to help *S. cerevisiae* combat such fluxes.

Superoxide dismutases

Superoxide dismutase (SOD) activity is present in all aerobic and in some anaerobic organisms as well as in all subcellular compartments susceptible to oxidative stress. This enzyme was discovered in 1969 by McCord and Fridovich in bovine blood, and catalyzes the following reaction:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

There are three types of this enzyme, which can be discriminated by their metal cofactor (Fridovich, 1986): the structurally similar Fe SOD (procaryotic organisms, chloroplast stroma) and Mn SOD (procaryotic organisms and the mitochondrial matrix of eukaryotes); and structurally unrelated Cu/Zn SOD (cytosolic and mitochondrial intermembrane space enzyme). Apart from their localisation, these isoenzymes differ in their sensitivity to inhibitors: Cu/Zn SOD is sensitive to KCN, whereas H_2O_2 inhibits both the Cu/Zn SOD and Fe SOD.

S. cerevisiae, like most other eukaryotes, contains two SOD enzymes: Cu/Zn SOD (the product of the *SOD1* gene) in the cytosol, nucleus, intermembrane space of mitochondria and lysosomes and Mn SOD (the product of the *SOD2* gene) in the mitochondrial matrix (Gralla & Kosman, 1992). Mn SOD is believed to represent the major means of protection against mitochondrial superoxide. The sources of ROS relevant to Cu/Zn SOD are less understood, because of the location of this enzyme within the cytosol.

Catalases

The final step in the detoxification of ROS (the elimination of excess H_2O_2) is carried out by catalases, which are ubiquitous family of isoenzymes with an iron containing active site that catalyze the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

S. cerevisiae has two such enzymes, the peroxisosomal catalase A encoded by *CTA1* and the cytosolic catalase T encoded by *CTT1*. Limited data is available regarding the role(s) of Ctt1 and Cta1 in the yeast cell. The coordinated regulation of *CTA1* with peroxisomal structures and fatty acid metabolism has led to the suggestion that Cta1 functions in the detoxification of H_2O_2 generated from fatty acid β-oxidation (Jamieson, 1998). Ctt1 is thought to play a more general role as an antioxidant during exposure to oxidative stress, since *CTT1* expression is induced by various stress conditions including heat, osmotic, starvation and hydrogen peroxide stress (Belazzi *et al*., 1991 and references therein).

2. The role of GSTs in the response against oxidative stress

GSTs show broad substrate specificity and detoxify a variety of electrophiles by conjugation to reduced GSH. As xenobiotics are a primary source of oxidative stress, it is not surprising that

GSTs also play an important although indirect role in antioxidant defense, by eliminating toxic substances and preventing their subsequent deleterious effect. An example for such an oxidative stress limiting role is the arsenate reduction by mammalian GST Omega (Aposhian & Aposhian, 2006). This GST detoxifies As(V) species and in this way efficiently decreases arsenate-stimulated generation of ROS. In addition to this general defensive role, some GSTs are directly implicated in the oxidative stress reponse by their GPx function (Awasthi *et al.*, 2004). These GSTs can efficiently reduce inorganic and organic exogenous peroxides or eliminate endogenous lipid and fatty acid peroxides (Zhao *et al*., 1999; Hubatsch *et al.*, 1998; Veal *et al*., 2002; Garcera *et al*., 2006; Bai *et al*., 2004). Thus, GSTs can provide protection against electrophilic xenobiotics, toxic metals and drugs not only via conjugation to GSH, but also by alleviating the oxidative stress and subsequent lipid peroxidation that is often associated with exposure to xenobiotics.

2.1. The glutathione peroxidase function of GSTs

2.1.1. Protection role of GSTs against lipid peroxidation in mammals

Enzymes such as catalase, superoxide dismutases, and glutathione peroxidases (GPxs) and the nonenzymatic defense such as glutathione provide the first line of oxidant defense, by inactivating ROS and scavenging free radicals generated in the electron transport chain. However, even the small amounts of ROS escaping this first line of defense can initiate an autocatalytic increase in lipid peroxidation, resulting in the formation of a variety of toxic electrophilic species. A second line of defense against ROS involves specific mechanisms dedicated to the protection against lipid peroxidation.

The most important lipid peroxidation products are alkoxyradicals ($RO^{\bullet}$), peroxyradicals ($ROO^{\bullet}$), hydroperoxides (ROOH) such as fatty acid hydroperoxides (FA-OOH) and phospholipid hydroperoxides (PL-OOH), and relatively stable toxic and reactive end products such as 4-hydroxyalkenals (4-hydroxynonenal (4-HNE)), malondialdehyde, and acrolein (Awasthi *et al.*, 2004 and references therein). These compounds exert various cytotoxic effects, including genotoxicity, cytoskeletal modifications, alteration of membrane fluidity, inactivation of enzymes and inhibition of DNA synthesis.

In mammals, the protection against lipid peroxidation is mainly provided by GPxs and Alpha class GSTs (Awasthi *et al.*, 2004). GSTs attenuate the effect of lipid peroxidation via two

separate functions: (1) by their selenium-independent GPx activity towards FA-OOH and PL-OOH and (2) by GSH conjugation of oxidation end products such as 4-HNE.

In humans, some of the Alpha GST isozymes (hGSTA1-1 and hGSTA2-2) can efficiently reduce FA-OOH, PL-OOH (Fig. 8) as well as cumene hydroperoxide, and can also interrupt the autocatalytic chain of lipid peroxidation by reducing these hydroperoxides (Zhao *et al*., 1999). In addition, these two enzymes provide 55-75% of the total GPx activity in human liver (Yang *et al.*, 2001). This suggests that in humans, the Alpha class GSTs perhaps play a more important role than the classical GPxs in defense mechanisms against lipid peroxidation.

Other Alpha class GST isozymes, in particular mGSTA4-4 (mice), rGSTA4-4 (rats), hGST5.8, and hGSTA4-4 (humans) have high GSH conjugation activity toward 4-HNE and other α,β-unsaturated aldehydes (Fig. 8) (Hubatsch *et al.*, 1998; Singhal *et al*., 1994). In addition, the subcellular localization of GSTA4 – approximately 25% of the cellular mGSTA4-4 is localized in the mitochondrial matrix, the cellular compartment with highest ROS production, (Raza *et al*., 2002) and the presence of antioxidant response elements in the gene promoter (Hayes *et al.*, 2005) – are consistent with a possible antioxidant role of this enzyme.

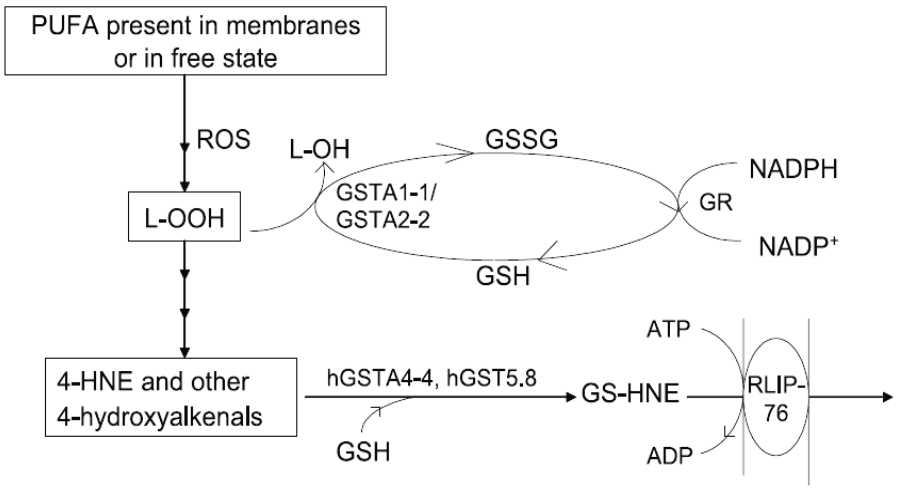


Fig. 8. Role of GSTs in attenuation of lipid peroxidation in human cells. Lipid peroxidation of PUFA is initiated by ROS/free radicals to generate L-OOH, which can autocatalytically propagate lipid peroxidation by continually generating free radicals. GSTA1-1/A2-2 reduce L-OOH to corresponding alcohols, break the chain of lipid peroxidation, and limit HNE production. hGSTA4-4 and hGST5.8 conjugate HNE to GSH-forming GS-HNE, which is transported out of cells (Awasthi *et al.*, 2004).

In addition, recent studies suggest that these enzymes can also affect cell cycle signaling by regulating the intracellular concentrations of 4-HNE, which is also an important signaling molecule. Signaling functions mediated by GSTs are an expanded area of interest which is specially discussed below (Chapter 3).

2.1.2. Role of yeast GSTs in oxidative stress response

In yeast species however, the role of GST enzymes in the response to oxidative stress is not clear, in particular because the functions of GSTs have yet to be fully characterized.

Three genes encoding glutathione S-transferases in *S. pombe* are involved in the oxidative stress response (Veal *et al*., 2002). All three of the proteins have GST activity with CDNB, and the expression of the corresponding genes is induced by peroxide and required for peroxide resistance. The *GST1*, *GST2* and *GST3* genes were found to be required for normal resistance to *tert*-butyl hydroperoxide and H_2O_2. Only one of these proteins, Gst3, has peroxidase activity and displays substantial sequence divergence to the other two proteins (Veal *et al*., 2002).

In *S. cerevisiae,* only Gtt1p and Ure2p have glutathione peroxidase activity *in vitro* when expressed in recombinant form. Ure2p is active with cumene hydroperoxide, hydrogen peroxide and *tert*-butyl hydroperoxide as substrates (Bai *et al*., 2004). An Ure2p variant, lacking the unstructured N-terminal prion domain showed kinetic parameters identical to the wild type protein and the fibrillar aggregates of the prion [URE3] form showed the same level of effectiveness towards peroxides as the native protein.

Recombinant Gtt1p has glutathione peroxidase activity against cumene hydroperoxide, hydrogen peroxide and *tert*-butyl hydroperoxide, but is not active towards 4-HNE (Garcera *et al*., 2006), suggesting that in *S.cerevisiae* GSTs, do not have overlapping roles with PHGPx enzymes.

Finally, three omega class GSTs in *S. cerevisiae* have poor GPx activity and possess activity as glutaredoxins via a monothiol mechanism (Garcera *et al*., 2006). This indicates a possible `housekeeping' function of Omega-class GSTs, which may act as GSH-dependent thiol transferases, removing S-thiolated adducts which some proteins form with GSH and cysteine in response to oxidative stress.

2.2. Role of GSTs in oxidative stress caused by arsenic species

2.2.1. Modes of arsenic action

Arsenic is a highly toxic metalloid which is naturally present in a number of minerals and is released into the environment by industrial and agricultural activities. It has two biologically important oxidation states, arsenate (As(V)) and arsenite (As(III)). As(V) occurs meanly as

arsenic acid (H_3AsO_4) while As(III) is present as arsenous acid, also called arsenic trioxide (As_2O_3). In solution at neutral pH, arsenic acid exists as the arsenate oxyanion (AsO_4^{3-}). The pKa of arsenous acid (As_2O_3) is 9.2, so that, at physiological pH, it is primarily present in solution as neutral $As(OH)_3$ (Rosen, 2002).

Inorganic arsenic can be methylated under microbial processes to form organic complexes such as monomethylarsonic acid (MMA(V)), dimethylarsinic acid (DMA(V)) monomethylarsonous acid (MMA(III)) and dimethylarsinous acid (DMA(III)) (Aposhian, 1997). These species are also formed as intermediate products in cellular detoxification of arsenic in higher eukaryotes.

The specific toxicity of arsenate is generally attributed to its chemical similarity to phosphate (Tamas & Wisocki, 2001). Arsenate is capable of mimicking the role of phosphate in cellular transport and enzymatic reactions. Thus, arsenate may replace an essential phosphate in various metabolic processes. Arsenate uncouples oxidative phosphorylation by the formation of unstable arsenate esters, which substitute for phosphate esters in ATP formation, thus contributing to decreased ATP cellular levels. The in vivo reduction of As(V) into the more toxic As(III) may further explain the acute toxicity of As(V).

In mammals, arsenite-stimulated generation of ROS, known to damage proteins, lipids and DNA, is probably the direct cause of the carcinogenicity of arsenite (Liu *et al.*, 2001). For many years, arsenite has been thought to react with -SH groups of cysteine residues, which constitute an integral part of the active site of many enzymes, thereby inhibiting their catalytic activity. The major effect of acute As(III) poisoning in mammals is carbohydrate depletion, which results in reduced coenzyme A production and in an inhibition of the citric acid cycle and pyruvate metabolism (Tamas & Wisocki, 2001). One of the most studied enzyme models has been the pyruvate dehydrogenase (PDH) complex. It has been considered to be the most sensitive target site to inhibition by arsenite. More specifically, the site has been believed to be the lipoamide dehydrogenase subunit of the large PDH multienzyme complex (Adamson & Stevenson, 1981).

Recently, it was shown that arsenic trioxide does not bind to thiols of the enzyme complex, but rather that it stimulates ROS production that causes PDH inactivation by oxidation (Samikkannu *et al*., 2003). Indeed, these results have led to a reassessment of the mechanism of inorganic arsenic inhibition of enzymes and other metabolic processes.

Further, concerning the nature of generated ROS, clear evidence has been provided that arsenite increases the levels of superoxide-driven hydroxyl radicals in human–hamster hybrid (A_L) cells (Liu *et al.*, 2001). It has been also shown to induce lipid peroxidation of membranes and the formation of lipid peroxide (Maiti & Chatterjee, 2000).

On the basis of these findings, Hei & Filipic (2004) have proposed a model describing the induction of free radical network in mammalian cells after exposure to arsenic which results in organelle damage and mutagenesis (Fig. 9).

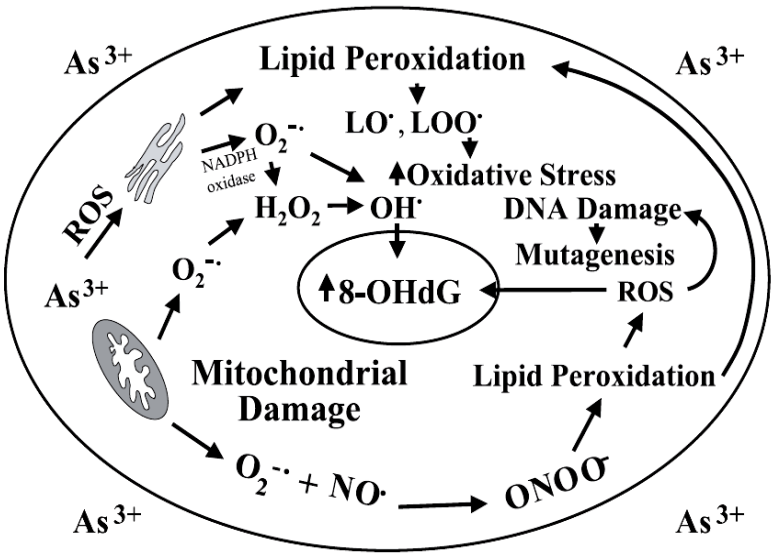


Fig. 9. Working model on the induction and pathways of ROS- (reactive oxygen species) and RNS- (reactive nitrogen species) mediated genotoxicity of arsenic in mammalian cells. Trivalent sodium arsenite induces ROS formation within minutes after entering cells. ROS and RNS induce an increase in intracellular oxidative stress that results in the induction of oxidative DNA lesions (an example is 8-OHdG 8-hydroxy-2'-deoxyguanosine) and mutagenesis. Both mitochondrial membrane damage and the induction of lipid peroxidation contribute to the genotoxicity of arsenic. Stimulation of membrane-bound NADPH oxidase by arsenic enhances ROS production. Damage to the mitochondrial membrane may result in the leakage of superoxide anions into the cytosol, and the subsequent production of peroxynitrite anions may provoke the increase in the intracellular oxidative stress level (Hei & Filipic, 2004).

2.2.2. Arsenic detoxification in mammals: specific role of GSTs

Arsenate and arsenite have different pathways to enter the cell. First of all, when organisms are exposed to arsenate, some of it is reduced to arsenite. This conversion of an arsenic species with an oxidation state of +5 to one of +3 is catalyzed by arsenate reductase (Radabaugh & Aposhian, 2000). In mammals, this activity can be supplied by either the arsenate reductase/purine nucleoside phosphorylase (PNP) protein or by the MMA(V) reductase/GST omega class protein. These two enzymes catalyze conversion of either inorganic or organic arsenate species (Fig. 10).

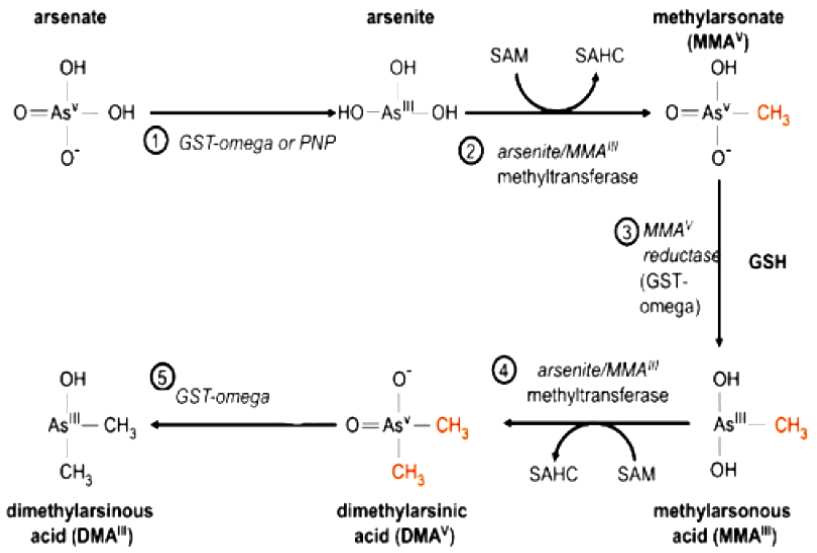


Fig. 10. Biotransformation of arsenate in mammalian systems. SAM, S-adenosylmethionine; SAHC, S-adenosylhomocysteine (Aposhian & Aposhian, 2006).

As shown in Fig. 10, human glutathione-S-transferase omega (hGSTO1) can reduce arsenate, MMA(V), and DMA(V) to arsenite, MMA(III), and DMA(III), respectively, and changes the oxidation state of the As^{+5} to As^{+3}. MMA(V) reductase/GST omega is the rate-limiting enzyme for the inorganic arsenic methylation pathway of As(V) metabolites (Zakharyan & Aposhian, 1999) and has an absolute requirement for GSH. As indicated by mRNA transcription levels, GSTO1 is expressed in many human tissues (Yin *et al*., 2001), and has a cysteine (Cys32) in its active site (Aposhian & Aposhian, 2006).

2.2.3. Mechanism of arsenic detoxification in yeast

Pathways of arsenic entry in Saccharomyces cerevisiae

Two pathways of cellular entry of arsenic have been identified in *S. cerevisiae*. As(V) enters cells via phosphate transporters, while glycerol channels belonging to the major intrinsic protein (MIP) family were shown to mediate As(III) uptake (Fig. 11).

Pentavalent arsenate is analogous to inorganic phosphate (Pi), and both anions utilise the same pathway to enter cells. Two systems are suggested to operate in active transport of Pi in *Saccharomyces cerevisiae* (Tamai *et al*., 1985). One has high affinity for external Pi, and the other has low affinity. *PH084* has been shown to encode a major component of the Pi transporter for the high affinity system (Bun-ya *et al*., 1991). Mutations in the *PHO84* or in the *PHO87* and *PHO88* genes, encoding proteins that are believed to be associated with Pho84p in

the plasma membrane, result in increased As(V) tolerance (Bun-ya *et al*., 1996; Yompakdee *et al*., 1996). Furthermore, cells with a mutation in the *PHO86* gene, encoding a protein that is required for transport of Pho84p from the endoplasmic reticulum to the Golgi apparatus, also exhibit enhanced As(V) tolerance (Bun-ya *et al*.,1996; Yompakdee *et al*., 1996).

For a long time As(III) was thought to enter cells by passive diffusion. GlpF, a glycerol facilitator in *E. coli,* was first identified as a trivalent metalloid transporter (Sanders *et al*., 1997). The *S. cerevisiae* glycerol channel Fps1p is homologous to GlpF, and both proteins belong to the ubiquitous MIP family of water and solute transporters. The transport activity of Fps1p is regulated by changes in osmolarity, and the physiological role of the protein is to control the cellular content of glycerol under conditions of osmotic stress (Tamás *et al*., 1999). In addition, Fps1p also constitutes the main entrance route of arsenite and antimonite in *S. cerevisiae* (Wysocki *et al*., 2001), but it does not exhibit a significant transport rate for methylated As(III) (Liu *et al*., 2006). Inactivation of this system improves cellular tolerance to trivalent As(III) salts. Furthermore, yeast cells expressing a constitutively active and unregulated form of the Fps1p channel are hypersensitive to As(III). Direct transport assays have confirmed the ability of Fps1p to recognise As(III) as a substrate. Cells mediating a high level of uptake are more sensitive to metalloids than cells with a low level of uptake (Wysocki *et al*., 2001).

Importantly, Fps1p-mediated influx of toxic metalloids, appears to be highly regulated in yeast (Wysocki *et al*., 2001). The presence of arsenite or antimonite in the growth medium results in a rapid repression of the *FPS1* gene. Thus, reduction of metalloid influx by inactivation of the uptake system may be a general mechanism in acquisition of arsenic tolerance, nevertheless the exact regulation pathway is unknown.

While both physiological and transport data support the role of Fps1p in metalloid uptake, it is clear that alternative entrance pathways for arsenic exist in yeast. In glucose-free medium, hexose permeases appear to be responsible for the majority of $As(OH)_3$ accumulation in *Saccharomyces cerevisiae* (Liu *et al.,* 2004).

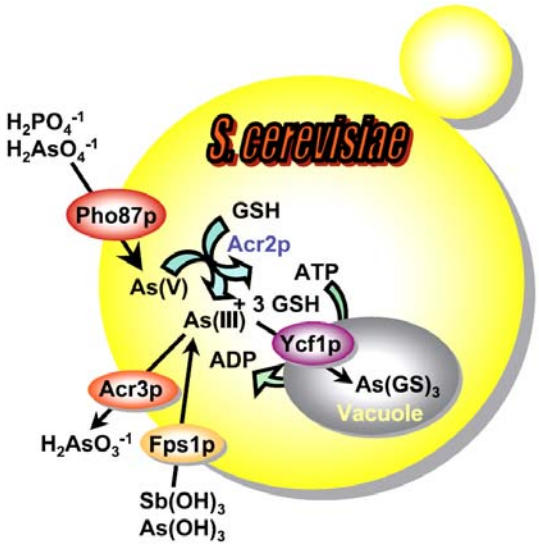


Fig. 11. Arsenic detoxification in *S. cerevisiae.* Arsenate is taken up by phosphate transporters, and As(III) is taken up by Fps1p. Arsenate is reduced to arsenite by the Acr2p enzyme. Arsenite is extruded from the cells by Acr3p (a plasma membrane arsenite efflux protein), and Ycf1p, which transports $As(GS)_3$ into the vacuole (Rosen, 2002).

Arsenate reduction in Saccharomyces cerevisiae

When arsenate is taken up, it is reduced to As(III) prior to extrusion or sequestration (Fig. 11). While arsenate reduction can however take place in a non-enzymatic glutathione-dependent way, this process may be too slow to be biologically significant (Delnomdedieu *et al.*, 1994), necessitating enzymatic mechanisms for reduction. The *S. cerevisiae ACR2* gene confers enhanced arsenate tolerance when present in multiple copies in yeast cells, whereas its disruption results in arsenate sensitivity. Since the *acr2Δ* mutant is not As(III) sensitive, *ACR2* is thought to encode a yeast arsenate reductase (Bobrowicz *et al.*, 1997). Indeed, purified Acr2p catalyzes the reduction of arsenate to arsenite with glutathione and glutaredoxin as sources of reducing equivalents (Mukhopadhyay *et al*., 2000). Arsenate reduction involves non-covalent binding of the As(V) oxyanion and subsequent reduction to As(III), with one electron transferred from a cysteine thiolate from the Acr2 protein and a second electron from the thiolate of glutathione (Mukhopadhyay *et al*., 2000). The mixed disulfide between glutathione and the protein is then reduced, with glutaredoxin acting as electron donor to regenerate the active arsenate reductase. Incidentally, this is reminiscent of the mechanism observed in certain prokaryotic GSTs involved in the dehalogenation of aromatic compounds (PcpC protein from *Sphingomonas chlorophenolica* and LinD from *Sphingobium japonicum* UT26) (Anandarajah *et al*., 2000; Nagata *et al.*, 1999). However, the Acr2 protein shows no sequence similarity to these enzymes.

Arsenite efflux in Saccharomyces cerevisiae

In *S. cerevisiae*, metalloids that enter the cytosol are removed via two independent transport systems: the plasma membrane transporter Acr3p and the vacuolar ABC transporter Ycf1p (Rosen, 2002) (Fig. 11).

The *S. cerevisiae* plasma membrane transporter Acr3p mediates the efflux of As(III), probably as an oxyanion. Expression of *ACR3* from a multicopy vector improves cellular As(III) tolerance (Bobrowicz *et al*., 1997), while deletion of the gene results in sensitivity to both As(V) and As(III) (Ghosh *et al*., 1999). Consistently, cells expressing *ACR3* have low cytosolic arsenite content, while the *acr3Δ* mutant accumulates high amounts of As(III).

The *YCF1* (yeast cadmium factor) gene was isolated for its ability to confer high level of cadmium tolerance to yeast cells, when present on a multicopy plasmid. Ycf1p is a member of the ABC transporter superfamily and is associated with the vacuolar membrane. It transports a wide range of glutathione-conjugated substrates into the vacuole, including $As(GS)_3$ (Ghosh *et al.*, 1999). In agreement with its role in metalloid detoxification, mutants lacking *YCF1* are highly sensitive to As(III).

Genetic data further support the notion that *ACR3* and *YCF1* encode independent detoxification systems. The hypersensitivity of yeast cells to metalloids is additive in a mutant lacking both transporters, compared to the two single mutants (Ghosh *et al*., 1999).

In spite of these studies, little is known about the detailed mechanisms of arsenic tolerance in unicellular eukaryotes. Comparing the resistance pathways in yeasts and mammals several questions can be addressed. In particular, is there a detoxification system involving methylation of arsenic species in yeast? And, if there is such a system which is the enzyme reducing arsenate methylated forms and can Acr2p reduce methylated arsenate or is there a functional homologue of hGSTO1 in yeast, which can reduce arsenate, MMA(V), and DMA(V) to arsenite, MMA(III), and DMA(III)? These questions still need further investigative efforts. Another crucial point of interest is the mechanism of arsenate reduction by Acr2p. This mechanism is (1) glutathione-dependent and (2) similar to the mechanism of dehalogenation of aromatic compounds by some bacterial GSTs. These two observations suggest a possible GST role in yeast arsenic detoxification which still remains to be investigated.

Chapter 3

Regulatory roles of GSTs: entering new territory

1. Introduction

A striking feature of glutathione S-transferases is their multifunctionality. This has initially referred to their broad substrate specificity, because GSTs have been shown to react with a wide range of structurally different toxic compounds, including polycyclic aromatic hydrocarbons, α,β unsaturated aldehydes, epoxides, chemotherapeutic agents, quinones, prostaglandins, toxic metals, lipid peroxidation products and other reactive oxygen species. This large diversity of detoxification reactions occurs by two distinct mechanisms – nucleophilic addition of glutathione to electrophilic chemicals and glutathione dependent reduction (for a review see Armstrong, 1996).

More recently, it has become apparent that the role of GSTs is not restricted to detoxification metabolism. For example, some glutathione S-transferases act as isomerases or thiol transferases catalyzing specific reactions in eicosanoid synthesis, steroid hormone metabolism, and amino acid degradation (Board *et al*., 2000; Fernandez-Canon & Penalva, 1998; Kanaoka *et al*., 1997). Although often different in their catalytic mechanism, these functions are also carried out in a glutathione dependent manner via the enzymatic active site.

More importantly, GST enzymes have been shown to participate in processes which, in contrast to those described above, do not require GSH-dependent activity. Some GSTs are able to bind non-catalytically, hydrophobic compounds such as porphyrins, bile acid, bilirubin, drugs, pigments, steroid and thyroid hormones (Lu & Atkins, 2004). Therefore, they act as "ligandins" realizing non-covalent or covalent interactions with molecules that are not substrates for GST enzymatic activity. The binding of non-substrate ligands does not require reduced glutathione and is independent of the glutathione-binding site (G-site) and the hydrophobic substrate binding site (H-site), which constitute the catalytically active site of GST enzymes. The exact ligand-binding function of GSTs remains unclear, but sequestering molecules may prevent the interaction of cytotoxic ligands with their targets or may modulate their physicochemical properties.

In addition to such rather unexpected roles for GSTs, it has increasingly become clear that at least some GSTs can modulate the activity of several enzymes and transcription factors by way of direct non-catalytic protein-protein interactions. This intriguing function may result in the regulation of a broad series of cellular processes, since the targets for GST protein-protein binding are often central signaling molecules (Adler *et al*., 1999; Bertram *et al*., 2000). The two best studied examples so far – the inhibition of MAP kinase pathway by mammalian GST P1-1 and the inhibition of GATA signal cascade by yeast GST Ure2p, described in more detail below

– provide evidence that such regulatory roles of glutathione S-transferases may represent a conserved feature of these proteins during the course of evolution.

2. Indirect regulatory role of GSTs

However, when discussing such possible regulatory roles for GSTs, it is essential to be able to discriminate between direct regulation as a result of non-catalytic binding with signal transducers, and indirect regulation as consequence of the detoxification enzyme activity of GSTs. The latter phenomenon is presumably unspecific, and based on the elimination of chemical species such as ROS, and compounds generating ROS or other molecules which may act as second messengers.

Perhaps the best studied example for a regulation role of GSTs as a result of detoxification activity is the elimination of 4-HNE by human class Alpha GSTs (for detailed reviews see Awasthi *et al.*, 2004; Sharma *et al*., 2004, see also Chapter 2 of Introduction). This function arises from the fact that 4-HNE is also a signal molecule which can affect cell cycle, growth and apoptosis (Uchida *et al.*, 1999). Therefore, the detoxification by GSTs has an important regulatory effect at two levels: first, reduction of fatty acid and phospholipid peroxides by GSTs interrupts the peroxidation reaction and limits production of 4-HNE, and second, the glutathione conjugation activity eliminates 4-HNE that has been produced.

4-Hydroxynonenal is the most abundant end product of lipid peroxidation. In addition to its deleterious effect as toxicant, it can modulate cell growth and affect signal transduction pathways. The moderately high concentrations of 4-HNE in human cell cultures can induce cell apoptosis and differentiation, and affect signaling pathways including activation of adenylate cyclase, caspase 3 and kinases as c-Jun NH_2-terminal kinase (JNK) and protein kinase C (PKC) (Awasthi *et al.*, 2004 and reference therein). On the other hand, low levels of 4-HNE have been shown to promote cell proliferation, suggesting that it also affects cell cycle events in a concentration-dependent manner. For example, submicromolar concentrations of 4-HNE activate rat hepatocyte protein kinase C-bII, a serine/threonine kinase that is activated intracellularly by various phospholipases, whereas micromolar concentrations inhibit its activity (Chiarpotto *et al.*, 1999).

4-HNE is mainly generated during lipid peroxidation, and its homeostasis seems to be regulated by both production and metabolism. Alpha GSTs (hGSTA1-1 and hGSTA2-2) can reduce the 4-HNE precursors FA-OOH, PL-OOH in order to interrupt the autocatalytic chain of lipid

peroxidation and to decrease the 4-HNE production (Fig. 12). Human cells overexpressing hGSTA1-1 or hGSTA2-2 have reduced 4-HNE levels and during oxidative stress, 4-HNE formation is decreased in these cells (Yang *et al.*, 2001; Yang *et al.*, 2002). Moreover, hGSTA2-transfected K562 cells do not have oxidant-mediated activation of the JNK pathway, which is involved in apoptosis caused by exogenous stimuli such as UV radiation, H_2O_2, ceramide and TNF-α (Yang *et al.*, 2001). Likewise, H_2O_2-induced activation of caspase 3, which is believed to be involved in the execution phase of apoptosis, is blocked in hGSTA1 or hGSTA2 transfected human lens epithelial (HLE B-3) cells (Yang *et al.*, 2002).

Other GSTs (GSTA4-4 and hGST5.8) can also regulate HNE concentrations in mammalian cells by conjugating it to GSH (Fig. 12), as the majority of 4-HNE is metabolized via its conjugation to glutathione (Awasthi *et al.*, 2000). mGSTA4 transfected cells have significantly lower steady-state levels of 4-HNE due to its accelerated metabolism, and show neither apoptosis nor activation of JNK or caspase 3. They have lower (<50%) steady-state levels of 4-HNE, and proliferate at a significantly higher rate compared to corresponding parental cells (Cheng *et al.*, 1999). In contrast, mGSTA4 null mice tissue has steady-state concentrations of 4-HNE about 3-fold higher than the corresponding tissue of wild type mice (Engle *et al.*, 2004).

In contrast to mammals, little is known about such a regulation in yeast and bacteria. The possible GST regulation role via 4-HNE detoxification still remains to be investigated in these organisms.

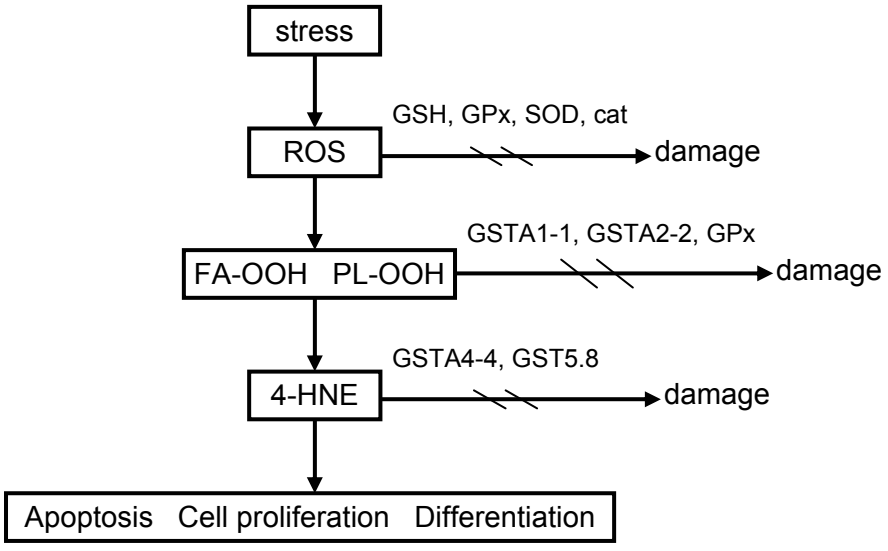


Fig. 12. Role of GSTs in regulation of 4-HNE signaling in mammals. Primary antioxidant defenses such as GSH, GPxs, SOD and catalases eliminate ROS generated during stress exposure. However, ROS can escape the first line of defense and initiate autocatalytic lipid peroxidation. GSTs regulate the intracellular concentration of both intermediate and end lipid peroxidation products, and modulate oxidant-dependent signaling (modified from Sharma *et al*., 2004).

3. GSTs as direct regulators of cell signalling

In addition to the indirect regulatory role of some GSTs, others can directly bind to signal molecules in regulatory pathways and modulate cellular metabolism. This GST function is discussed in detail below.

3.1. GSTs modulate the JNK signaling cascade

Mitogen-activated protein kinases (MAPKs) are an enzyme family involved in various and often opposing cellular responses to external or endogenous signals (Ip & Davis, 1998). They play a predominant role in both the stress response and in many normal physiological processes. The MAPK signaling pathway regulates cellular proliferation and differentiation, tissue morphogenesis, cell migration, apoptosis, inflammatory responses and malignant transformation. It is composed of three separated groups of protein kinases (Fig. 13): MAPKKKs (mitogen-activated protein kinase kinase kinases), which phosphorylate and thereby activate MAPKKs (mitogen-activated protein kinase kinases), which phosphorylate MAPKs (mitogen-activated protein kinases). In turn, MAPKs regulate several transcription factors from the family of AP-1 proteins, via phosphorylaton (Fig. 13). The AP-1 family consists of several groups of bZIP-domain (bZIP=basic region leucine zipper) proteins: the Jun, the Fos, and the ATF-2 subfamilies. Like all bZIP transcription factors, AP-1 proteins have to dimerize before they can bind to their DNA target sites TGACTCA and TGACGTCA (Leppa & Bohmann, 1999). Different homo- and heterodimeric combinations with different regulatory properties can be formed to assure the fine regulation of cell stress response and development. In mammals, Jun proteins are presented by c-Jun, JunB, and JunD. The first one is best characterized and is the main transcription factor to regulate a variety of processes such as cell growth, stress response and apoptosis.

The transcriptional activity of the c-Jun protein is greatly enhanced by phosphorylation of two serine residues at positions 63 and 73 in its activation domain (Whitmarsh & Davis, 1996). Phosphorylation of the N-terminus of c-Jun is catalyzed by a group of MAPKs, c-Jun NH_2-terminal kinases (JNKs) (Fig. 13). JNKs are activated by dual phosphorylation of Thr and Tyr residues within a Thr-Pro-Tyr motif located in kinase subdomain VIII (Whitmarsh & Davis, 1996) and can be inactivated by Ser/Thr and Tyr protein phosphatases. JNK activation is mediated by two MAPKKs: MKK4 and MKK7. The presence of two MAP kinase activators upstream of JNK in mammals indicates that signals that lead to JNK activation may be mediated

by either MKK4 or MKK7 (Ip & Davis, 1998). Alternatively, MKK4 and MKK7 might respond to distinct stimuli.

Numerous protein kinases that function as MAPKKKs in the JNK pathway have been also described, including MEKK1, ASK1, MEKK2, MEKK3, MEKK4, MEKK5, MST/MLK2, MUK/DLK, MLK3/SPRK, TAK1, and Tpl-1/2 (Ip & Davis, 1998). They phosphorylate and activate MKK4 and MKK7.

All these kinases function in order to provide the correct phosphorylation of c-Jun transcription factor by JNK. The c-Jun transcription factor has a short half-life because it is rapidly degraded by an ubiquitin-mediated proteasome pathway (Whitmarsh & Davis, 1996). Activation of JNK causes phosphorylation of c-Jun which leads to the inhibition of c-Jun ubiquitination and degradation (Fuchs *et al.*, 1996). Thus, JNK activation increases the half-life of c-Jun and causes an accumulation of the c-Jun protein. This increased level of c-Jun contributes to JNK-stimulated AP-1 transcription of genes responsible for the response to stimuli such as UV, H_2O_2, or TNF-α.

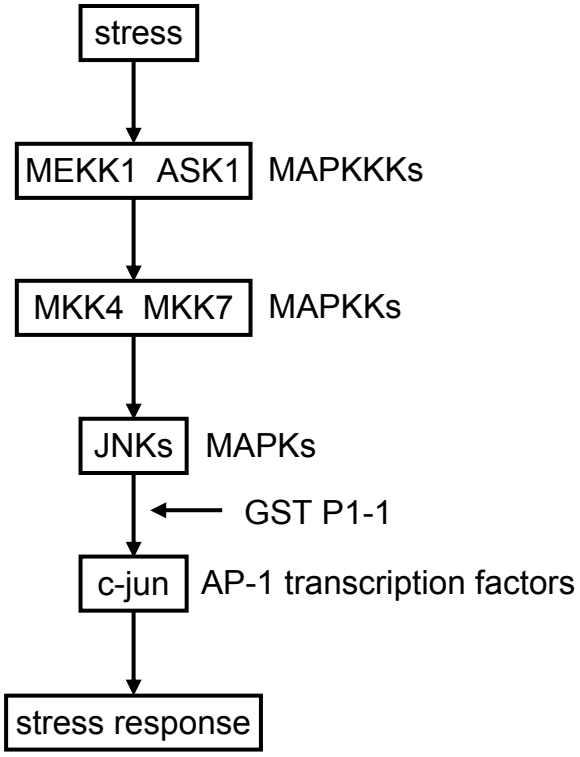


Fig. 13. General structure of the mammalian JNK signal transduction pathway. Under stress signals the MAPKKKs, MEKK1 and ASK1 are activated to phosphorylate the downstream MAPKKs, which in turn initiate phosphorylation of JNKs. Once activated, JNKs phosphorylate transcription factor target c-Jun, which activates the transcription of stress response genes.

Despite the evidence that the JNK kinase is constitutively phosphorylated, the JNK activity is relatively low under normal conditions (Minden *et al*., 1994). In addition, stress conditions do not affect the transcript or protein levels of JNK. Together, these observations lead to the speculation that an inhibitor of JNK may exist to regulate upstream kinases-independent JNK activation when switching to stress conditions. This putative inhibitor was successfully isolated from mouse fibroblast cells, purified, and surprisingly characterized as GST P1-1 (Adler *et al*., 1999). GST P1-1 inhibits JNK by forming a tripartite complex with JNK and c-Jun. This direct interaction decreases JNK phosphorylation of c-Jun in a dose-dependent manner, but GST P1-1 is not able to remove phosphate groups from already prephosphorylated c-Jun and JNK (Adler *et al*., 1999).

Other GST isozymes such as Alpha, Mu, bacterially expressed and mutated in essential residues are also capable although to a lesser degree to associate with and to inhibit the Jun–JNK complex *in vitro* (Adler *et al*., 1999). In addition, a human GST P1-1 which can efficiently modulate JNK activity has been recently isolated from human erythrocytes and gastric cancer cells (Ranganathan *et al*., 2005).

The inhibition of JNK phosphorylation activity by GST P1-1 is found in non-stressed cells, while UV or H_2O_2 exposure reduces this inhibition and increases JNK activity in a dose dependent manner. Furthermore, it was shown that only the monomeric form of GST P1-1 regulates JNK, while di- and multimers are not able to inhibit JNK signaling (Adler *et al*., 1999). UV irradiation reduces GST–JNK association, probably as a result of the formation of GST–GST dimers and multimers. ROS scavengers, such as N-acetyl cysteine and ethyl-ester of GSH, which inhibit the formation of GST P1-1 multimers, maintain the low basal activity of JNK (Adler *et al*., 1999). While the precise mechanism for the disruption of the ligand-binding interaction between GST P1-1 and JNK is not yet defined, aggregation of GST monomers following oxidative stress suggests intermolecular disulfide bridge formation between available cysteine residues. In particular, it has been shown that cysteine residues at positions 14, 47, 117, and 169 of GSTp are particularly susceptible to oxidation (Shen *et al*., 1993). Although it was determined that residues 47 and 101 were critical to the maintenance of catalytic activity, the protein:protein interactions between GST P1-1 and JNK appear to be distal from the catalytic site of GST P1-1.

Moreover, under physiological conditions, GST P1-1 inhibition of JNK takes place in spite of the presence of active MKK4 and MEKK1, possibly through direct inhibition of JNK-mediated

Jun phosphorylation (Adler *et al*., 1999). This suggests that GST–JNK association impairs JNK kinase activity.

Additional support for the importance of GST regulation in signal transduction is provided by the observations that a glutathione S-transferase of the Mu class can regulate JNK upstream kinases in the activation of the c-Jun pathway.

ASK1 (apoptosis signal-regulating kinase) and MEKK1, two MAPKKKs that activate the JNK pathway, are inhibited via protein:protein interactions by mouse GSTM1 (Cho *et al.*, 2001; Ryoo *et al.*, 2004). Overexpression of mGSTM1 blocked ASK1 oligomerization and repressed ASK1-dependent apoptotic cell death (Cho *et al.*, 2001). Transfection of human embryonic kidney cells with GSTM1 resulted in inhibition of H_2O_2-induced activation of MEKK1 (Ryoo *et al.*, 2004).

Interestingly, GSTM1 appears to interact with these two kinases in different ways. GSTM1 binds the C-terminal region of MEKK1, which contains the catalytic domain and inhibits the interaction between MEKK1 and its substrate, the downstream kinase MKK4/SEK1 (Ryoo *et al.*, 2004). In contrast, it binds the N-terminal domain of ASK1 and inhibits its oligomerization (Cho *et al.*, 2001).

The JNK signaling cascade is, thus, a prominent example of regulatory function performed by GSTs, as several members of the same enzyme family modulate one transduction cascade at different levels (Fig. 14). GSTs regulate mammalian JNK pathway at least at five well defined points. First, GSTA1-1 and GSTA2-2, by their GPx activity, attenuate the production of 4-HNE, which is an activator of JNK (Uchida *et al.*, 1999; Yang *et al.*, 2001). Second, GSTA4-4 and hGST5.8 detoxify residual 4-HNE by conjugation to glutathione, inhibiting 4-HNE activation of JNK signaling (Awasthi *et al.*, 2000). Third, GSTM1 binds to ASK1, inhibiting its oligomerization and thus repressing the JNK-dependent phosphorylation of c-Jun (Cho *et al.*, 2001). Further, the same enzyme binds non- catalytically to another MAPKKK in the JNK control network, MEKK1 (Ryoo *et al.*, 2004). In this way, GSTM1 inhibits the interaction of MEKK1 with the next kinase in the JNK cascade, interrupting the phosphorylation chain. And finally, GST P1-1 directly blocks JNK and represses the phosphorylation and therefore the activation of c-Jun transcription factor (Adler *et al*., 1999).

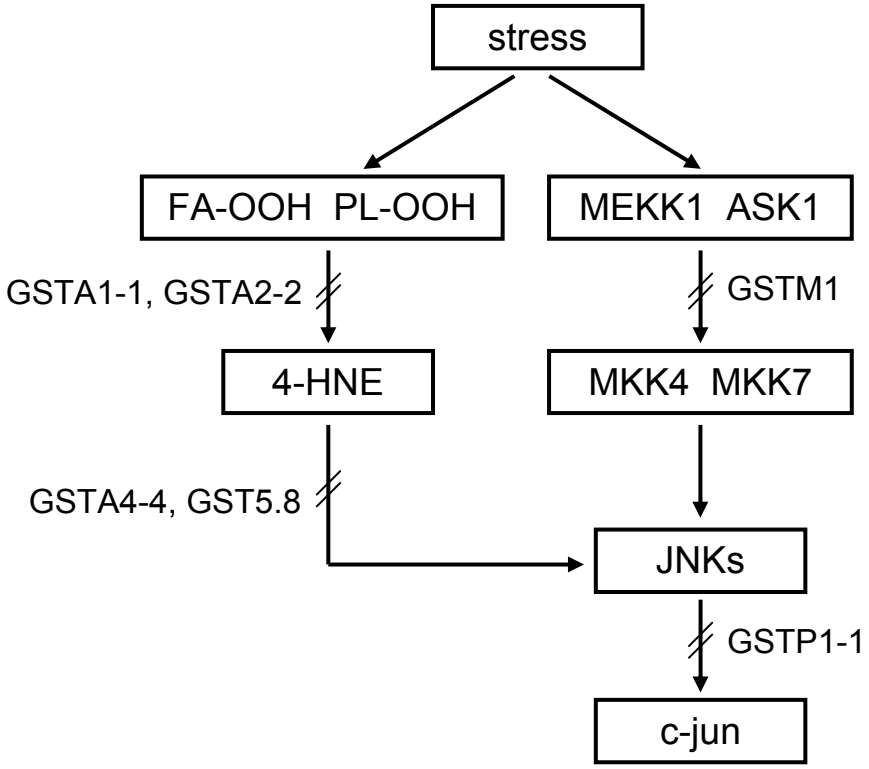


Fig. 14. GSTs regulate c-Jun signaling at different levels. Environmental stress can provoke both activation of JNK signal cascade and 4-HNE formation. As described above, alpha class GSTs may efficiently detoxify lipid peroxidation products and attenuate 4-HNE activation of JNK, while Mu and Pi GSTs interact directly with kinases to modulate c-Jun phosphorylation.

3.2. Ure2p: an essential regulator of GATA signaling pathway in yeast

3.2.1. GATA factor family

At least four proteins, the activators Gln3p and Gat1/Nil1p, as well as the repressors Dal80p and Deh1/Gzf3p, define the GATA family of transcription factors in *S. cerevisiae.* These proteins were originally discovered to regulate nitrogen utilisation in yeast (for review see Magasanik & Kaiser, 2002). Nitrogen regulation (nitrogen catabolite repression, NCR) is a mechanism that prevents unnecessary production of permeases and other proteins for the utilisation of compounds that are non-preferred nitrogen sources, when a preferred nitrogen source is available.

All GATA factors have a homologous Zn^{2+}-chelating zinc-finger motif (C-X_2-C-N-C-X_2-C) with a loop size (N) ranging from 17 to 20 residues (Cooper, 2002). The target of GATA family proteins is a DNA sequence located in the gene promoter, which contains five consecutive core nucleotides GATAA which can be found on both strands, as GATAA or TTATC respectively. In the genome of *S. cerevisiae*, GATA sequences are relatively well represented (3,24 GATAA/TTATC sequences per 1 kb), and the presence of such a sequence in a gene promoter

does not necessarily imply GATA factor regulation of its expression (Cooper, 2002). It seems that a significant number of GATA motifs are not active and are not recognized by transcription factors. In addition, each of the GATA factors has a preferred target site. For example, Gln3p binds preferably to GATAAGA, and Gat1p to AGATAAG sequences (Harbison *et al,* 2004). Dal80p and Deh1p, which both form dimers through leucine zipper motifs, require two GATAA elements for binding. For Dal80p these GATAA elements must be 15-35 bp apart, in either orientation in the promoter, and oriented head-to-tail or tail-to-tail, but not head-to-head (Cunningham & Cooper, 1993). However it is possible for different factors to bind in competition for the same targets, as is the case of Gln3p and Dal80p, which bind the same GATAA motifs upstream of the genes *DAL3* (ureidoglycolate hydrolase) and *UGA4* (gamma-aminobutyrate permease) (Cunningham *et al,* 1994). As Gln3p and Dal80p act antagonistically, the relative levels of these proteins in the nucleus will determine the activation or repression of expression of *DAL3* and *UGA4* genes. Therefore, the ability of GATA factors to enter the nucleus in response to external stimuli is likely a central determinant of GATA regulated gene transcription.

3.2.2. The GST Ure2p inhibits gene expression by GATA factors

The protein Ure2 of *S. cerevisiae* was discovered in 1969 by François Lacroute, who isolated mutants able to counter the effect of NCR. Further experiments illuminated the exact role of Ure2p and defined it as the major GATA negative regulator in the yeast cell (for review see Cooper, 2002). Ure2p binds to Gln3p and Gat1p preventing their nucleus localization and therefore inhibiting the transcription of GATA regulated genes. In response to external stimuli (for example, nitrogen limitation), Gln3p and Gat1p are concentrated in the nucleus where they bind GATA sequences upstream of GATA-sensitive genes and activate their transcription.

Ure2p is a cytoplasmic homodimeric protein. Determination of its gene sequence and 3D structure showed a homology with GSTs (Coshigano & Magasanik, 1991). In addition, the same protein was reported to possess characteristics of prion, e.g. to form insoluble self-propagating amyloids which are in the base of an inheritable prion-like phenotype [URE3] (Wickner, 1994).

Each Ure2p subunit is composed of two distinct regions. The N-terminal region (residues 1-93) is rich in asparagine and glutamine residues (43 of 93 amino acids, ~ 45%) and in serine and threonine residues (~ 20%) (Bousset *et al,* 2001). This region is sufficient to induce [URE3] phenotype, while the C-terminal region (residues 94-354) is the functional GATA repressor part, and shares the GST profile.

3.2.3. Mechanism of GATA signal transduction

Ure2p regulates Gln3p and Gat1p localization by physically sequestering them in the cytoplasm. In this way, expression of regulated genes is totally (as for NCR-regulated genes) or partially abolished (as it is the case for genes implicated in glutathione degradation and salt stress response, in which other regulatory networks are also implicated). In response to changes in the environment of the cells, Ure2p releases Gln3p and/or Gat1p which enter the nucleus and increase the expression of corresponding genes (Cunningham *et al.*, 2000; Kulkarni *et al.*, 2001)). The second level of repression occurs at this transcriptional level as a result of reciprocal regulation of GATA factors. In addition to target gene, Gln3p and Gat1p also activate the transcription of the GATA repressors which in their turn may repress the expression of the gene as well as this of activator genes (Fig. 15) (Cunningham *et al*., 2000). Expression of all GATA factor encoding genes, except *GLN3*, is regulated by all of other GATA factors. This includes autogenous regulation of *GAT1*, *DAL80* and *DEH1* expression. Furthermore, the fine regulation of activation/repression of a given gene may require the presence of all of the factors or only some of them. The resulting model is quite complex and the molecular basis of signaling still requires to be clarified, but it is evident that such a regulation network can modulate the expression of many genes in parallel, in response to various environmental signals and stimuli.

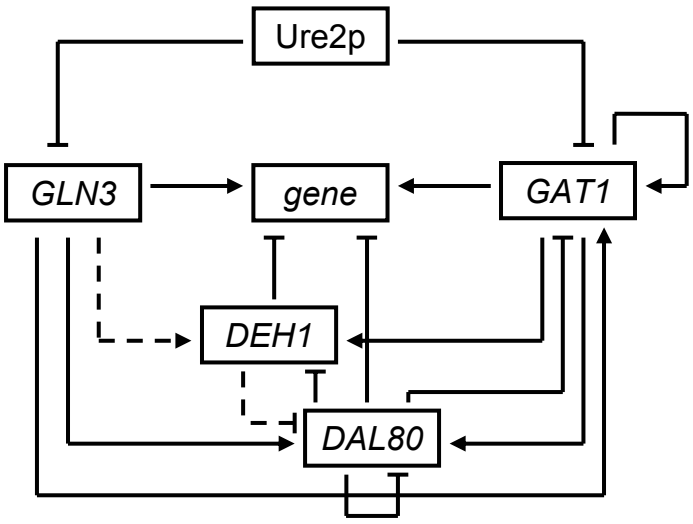


Fig. 15. Model for GATA regulation. Arrows and bars designate positive and negative regulation, respectively. Dashed areas designate weak regulation (according to Cooper, 2002).

The picture has become even more complicated by the discovery of a second cytoplasm repressor of Gln3p, the TOR (target of rapamycin) kinase proteins (Cardenas *et al*., 1999; Hardwick *et al*., 1999; Beck & Hall, 1999), which participates in an evolutionary conserved signaling pathway used by all eukaryotic cells. This pathway was discovered through the action

of the antibiotic rapamycin, a potent inhibitor of T-cell proliferation, which combines with the small immunophilin FKBP and targets the TOR kinase (for review see Schmelzle & Hall, 2000). The TOR kinases are members of the phosphatidylinositol 3-kinase (PI-3K) superfamily of enzymes, which regulate cell growth and differentiation in response to nutrient availability.

Treating yeast cells with rapamycin and therefore inhibiting the TOR kinase pathways has several distinctive effects, such as inhibition of protein synthesis, cell cycle arrest at the G1/S boundary, onset of autophagy, and inhibition of ribosomal biogenesis (Schmelzle & Hall, 2000). TOR kinase activity is essential for the transcription of ribosomal RNA and ribosomal protein genes, for the operation of glycolytic pathway, the TCA cycle, and in nitrogen metabolism.

It was shown, that TOR, Ure2p and Gln3p form stable complexes in the cytoplasm (Bertram *et al*., 2000) and that tripartite complex cannot enter the nucleus (Fig. 16, left). Two-hybrid analysis has suggested that TOR binds to all other GATA factors, while the ability of Ure2p to bind Gat1p, Dal80p and Deh1p has not been studied (Bertram *et al*., 2000). Indeed, the efforts to understand the mechanism of GATA regulation have always focused on Gln3p, since it is thought to be the "master" GATA factor.

Treatment of *S. cerevisiae* with rapamycin, which inhibits TOR kinase activity, results in rapid and strong induction of Gln3-dependent gene transcription (Beck & Hall, 1999). The same effect was reported in mutants lacking a functional copy of *URE2* gene. These observations indicate that TOR and Ure2p are equally important for Gln3p cytoplasm sequestration. However, there are several possible interpretations for the identity of the direct causative event preventing Gln3p from entering the nucleus. It is clear that an intact kinase domain or the kinase activity of TOR is required for repressing Gln3p-dependent genes (Bertram *et al*., 2000), and that phosphorylation is indispensable for cytoplasm localization of Gln3p. But there is a disagreement as to whether Ure2p facilitates phosphorylation of Gln3p by TOR or whether phosphorylation facilitates Ure2p-Gln3p complex formation (Cooper, 2002).

TOR interacts with Gln3p in the absence of Ure2p, but TOR interacts with Ure2p only when Gln3p is present, indicating that Ure2p binds to TOR by the mediation of Gln3p (Bertram *et al*., 2000). In addition, Ure2p binds to both hyperphosphorylated and unphosphorylated Gln3p, thus the phosphorylation status of Gln3p does not appear to affect the binding to Ure2p. Finally, Ure2p-bound Gln3p is more resistant than free Gln3p to phosphatases, which leads to the conclusion that Ure2p stabilizes the phosphorylated form of Gln3p (Bertram *et al*., 2000).

Finally, the exact mechanism of tripartite Ure2p-Gln3p-TOR complex dissociation is not clear. It seems that in response to extracellular stimuli, several cell phosphatases, such as Tap42:Sit4

and Pph3, dephosphorylate Gln3p (Bertram *et al*., 2000) in parallel with its release from TOR and Ure2p. Again there is consensus about neither the primary event nor the final result. In other words, it is not clear whether Ure2p removal leads to dephosphorylation of Gln3p or whether phosphatase activity provokes complex dissociation (Cooper, 2002).

3.2.4. GATA regulation beyond nitrogen catabolite repression

Although GATA regulation was originally discovered to function in nitrogen catabolite repression, its role has now been expanded to an implication in the cell response to salt (Crespo *et al.*, 2001) and to oxidative stress (Springael & Penninckx, 2003). In particular, the transcription of *ENA1,* a gene encoding a lithium and sodium ion transporter essential for salt tolerance in yeast, is controlled by the TOR/GATA signaling pathway (Crespo *et al.*, 2001). Ena1 is a P-type ATPase, involved in the extrusion of redundant Li^+ and Na^+ ions from the cytoplasm under high salt concentrations. Mutants lacking *ENA1* show increased sensitivity to both high concentrations of Li^+ and Na^+. *ENA1* expression is strongly induced under TOR-inactivating conditions, such as rapamycin treatment, while the absence of Gln3p and Gat1p results in reduced basal and salt-induced expression of *ENA1* (Crespo *et al.*, 2001). The double mutant *gln3Δgat1Δ* is sensitive to high concentrations of lithium and sodium. In contrast and as it can be expected the mutant *ure2Δ* is more resistant relatively to the parental strain in salt stress conditions (Crespo *et al.*, 2001). In this mutant, both Gln3p and Gat1p are constantly free to enter the nucleus and to overexpress the transcription of *ENA1*, therefore the cells extrude more efficiently toxic concentrations of these ions.

The expression of *CIS2* gene is also regulated by the nature of the nitrogen source and is GATA factor dependent (Springael & Penninckx, 2003). In *S. cerevisiae*, the *CIS2* gene encodes γ-glutamyl transpeptidase, the main GSH-degrading enzyme. The transcription of *CIS2* is regulated according to the nature of the nitrogen source. Expression is higher in cells grown on a poor nitrogen source. Under these conditions, the GATA factors Gln3p and Gat1p are both required for *CIS2* expression, the second one appearing as the more important factor. As in the above case, disruption in *URE2* gene has a contrary effect – the expression of *CIS2* is increased in the *ure2Δ* mutant. Thus the higher level of *CIS2* transcripts may result in rapid glutathione degradation and may be in the basis of *ure2Δ* oxidant sensitivity.

These two examples show the pleiotropic function of GATA signal transduction in *S. cerevisiae*. Previously, thought to be implicated only in nitrogen regulation, this signaling network is now extended far beyond nitrogen catabolism. Genes as diverse as those encoding a membrane

ATPase (*PMA1*), a high-affinity zinc-permease (*ZRT1*) or unknown open reading frames (*YHI9* and YGR125w) have been shown to be GATA-regulated in a genome-wide transcriptional analysis by mini-array membrane hybridization (Cox *et al.*, 1999). However, these genes have not been studied in detail and the exact regulation mechanisms still remain to be clarified. It must be point out that until now the identification of new GATA-regulated genes beyond the NCR is more or less hazardous and further efforts are need to understand the role of GATA signal transduction in the overall metabolism of *S. cerevisiae*.

3.3. GST regulation in mammals and yeast: common threads?

Despite the limited information available for GST regulation role, the two known examples, mammalian GST P1-1 and yeast Ure2p show some common functional features, which may indicate a common regulatory mechanism.

GST P1-1 is a cytosolic mammalian class Pi glutathione S-transferase, while Ure2p is a yeast GST homologue. Their primary structures are not related, but the tertiary structures of these proteins possess the characteristic profile and general architecture of GSTs. GST P1-1 and Ure2p are both homodimers and act as glutathione peroxidases. In addition, the two enzymes share another common function, they are repressors in major signal pathways in mammals and yeast, respectively.

The model for GST regulation in mammals (Fig. 16, right side) suggests that through its association with the Jun-JNK complex, GSTp inhibits JNK phosphorylation and activity under non-stressed conditions (Adler *et al*., 1999). Changes in cell environment result in a dissociation of the GST–JNK complex. The liberated JNK regains its functional capacity to be phosphorylated and to phosphorylate and activate c-Jun.

A similar tripartite model has been proposed for Ure2p regulation of yeast GATA signal pathway (Bertram *et al*., 2000). In this model, Ure2p associates with the Gln3p-TOR complex under nitrogen rich conditions and probably inhibits Gln3p dephosphorylation or at least stabilizes the phosphorylated form of Gln3p (Fig. 16, left side). When the preferred nitrogen is exhausted or replaced with non-preferred source, Ure2p dissociates from Gln3p, enabling its dephosphorylation and nucleus entry.

However, there are some features that distinguish the Ure2p-Gln3p-TOR model. First, in contrast to JNK signaling, where GST P1-1 regulates the kinase and not the transcription factor, Ure2p inhibits directly the transcription activator Gln3p. And second, the causative activation

event for the accumulation of GATA factors in the nucleus is the dephosphorylation of Gln3p, while phosphorylation results in its inactivation and in cytoplasm sequestration of GATA factors.

Under non-stressed conditions, GSTs inhibit the nucleus localization by physical sequestration of the corresponding transcription factors, thereby preventing unnecessary gene expression. The exact stimuli and sensing mechanisms for their activation under stress conditions, as well as the events leading the release of Gln3p or c-Jun are not known. Establishing the cause-effect relationships associated with GST control of transcription factor localization and activation will require a more detailed understanding of the molecular interactions prevailing in the formed complexes.

The binding interactions in the complex GST P1-Jun-JNK were identified to occur between the C-terminus of JNK (Wang *et al*., 2001) and the 194-201 residues region of GST P1-1 (Monaco *et al.*, 1999). The potential JNK interaction site of GST P1-1 is distal from the GST subunit dimerization domain (involving Cys-47 and Cys-101) and the active site, indicating that the essential activity residues are not implicated in the regulatory function of the enzyme. Moreover, the 194-201 sequence of GST P1-1 contains residues that are positively charged or contain polar groups in their side chains, while the sequence analysis of the C terminus of JNK1 shows both a loop region and an α-helix rich in negatively charged residues, particularly glutamic and aspartic acids (Wang *et al*., 2001). These observations lead the hypothesis, that JNK1 C terminus region can form a negatively charged binding face capable of interacting with GST P1-1.

The Gln3p region required for complex with Ure2p are residues 1–103, while N and C termini of the Ure2p region required for interact with Gln3p are situated between residues 101–151 and 330–346, respectively (Kulkarni *et al*., 2001). These regions are neither overlapped with Ure2p prion induction region (Maddelein & Wickner, 1999) and G- and H-binding sites (Bousset *et al.*, 2001). Neither the residues, implicated in Ure2p dimer formation Phe-295, Asp-296, Tyr-297 and Arg-267 to Met-272 (Umland *et al.*, 2001) are required for Ure2p-Gln3p interaction.

It is evident that in both mammalian and yeast GST regulation, enzymatic activity and active G- and H-sites are not required. Interestingly, the prion form of Ure2p, [URE3] has the same level of GPx activity *in vitro* as the native protein (Bai *et al*., 2004). In contrast, the conversion of Ure2p to [URE3] results in total loss of regulatory function (Lacroute, 1971). This suggests that the two functions are independent, and that the loss of regulatory function is attributable to a steric blocking mechanism, rather than to conformational changes. In addition, while GST activity is conferred by the physiologically occurred dimeric forms of the proteins, inhibition of

signal transduction may depend on monomeric protein, at least in the case of GST P1-1. Whether the monomer-dimer ratio is critical for GST regulatory role of Ure2p remains to be clarified. Adler *et al*. (1999) proposed an attractive model in which GST P1-1 is thought to serve as a sensor of intracellular changes in redox potential. UV or H_2O_2 treatment increases GST dimerization and decreases the amount of monomeric GST P1-1, resulting in reduction of GST-JNK-Jun complex formation.

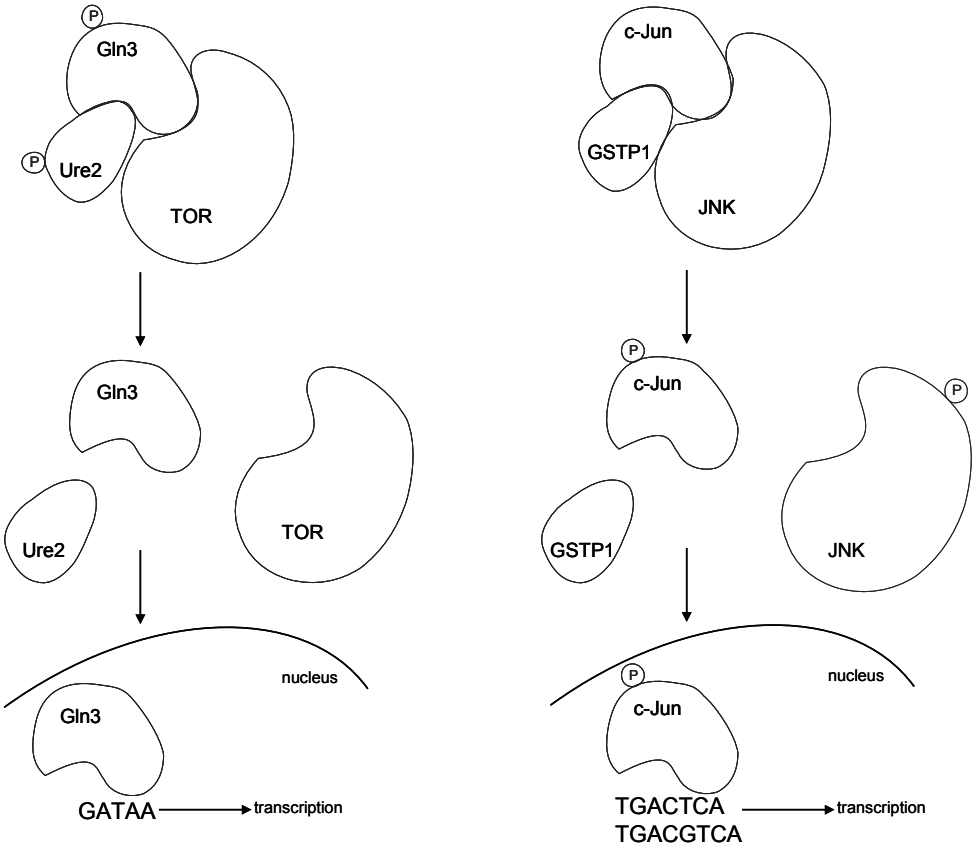


Fig. 16. Tentative model of Ure2p inhibition of GATA regulation and GST P1-1 inhibition of JNK signaling.

It remains to be clarified whether such a phenomenon is restricted to the above examples or is a more general feature of other members of the GST family. The sequence divergence of GST family and the relatively limited information about regulatory active GSTs restrict the application of bioinformatic analysis. Therefore, a promising area for future research would be to look for GST-signal transducer couples in mammalian GATA signal cascade and in the yeast MAPK pathway, respectively. Whether similar regulatory mechanisms also operate in the bacterial domain of life also requires further investigations. Approaches such as two-hybrid analysis (Bertram *et al*., 2000) and micro-array techniques (Cox *et al.*, 1999), although laborious, may allow the identification of such new GST protein-protein interactions.

Objectives and outline of the thesis

Glutathione S-transferases (GSTs, EC 2.5.1.18) define an enzyme family of multifunctional proteins with important roles in cellular detoxification of exogenous and endogenous compounds. As described in the first part of this thesis, GSTs are important enzymes in metabolic detoxification processes, and catalyze the conjugation of the tripeptide glutathione to electrophilic compounds to form more soluble and usually less toxic derivatives. In addition, GSTs can serve as peroxidases, isomerases and thiol transferases, and have non-catalytic functions, among which binding of non-substrate ligands and modulation of signaling processes.

Saccharomyces cerevisiae was the first eukaryote whose genome was completely sequenced in 1996. The yeast genome databases have been annotated in great detail and represent a very important tool for developing basic knowledge about eukaryotic physiology. The availability of the complete genome sequence and disruption mutants for all genes/ORFs of *S. cerevisiae* permits the use of genome-wide experimental approaches for the detailed understanding of eukaryotic metabolic pathways. *S. cerevisiae* has thus become an important model system for studying the role of genes of interest in complex physiological processes and cellular functions.

The major interest of our research laboratories lies in a better understanding of the metabolic processes involved in microbial resistance and adaptation to toxic polluants. Among these, detoxification pathways involving GST enzymes are still poorly studied in yeast. Yeast GSTs are structurally and functionally quite distinct from the already defined classes of the GST family. The main research objective of this work was to study the role of glutathione metabolism and in particular the specific requirement for GSTs in response to different toxic compounds in *S. cerevisiae*. A systematic approach, using defined GST mutants in the same genetic background, has been taken to investigate connections between yeast GSTs and oxidative stress.

Specifically, screening for arsenic and oxidant sensitivity in *S. cerevisiae* GST mutants has revealed that two yeast GSTs (Ure2p and Tef4p) may play a role in arsenic and oxidant detoxification (Chapter 1 of the Results).

The GST-like protein Tef4 is a homolog of yeast elongation factor eEF1Bγ. The physiological function and the possible detoxification role of this protein beyond its similarity to GST proteins have yet to be understood. Our preliminary results prompted us to investigate the toxic phenotype of *tef4Δ* mutant by studying its sensitivity to other GST substrates. Another important objective of this work was to clone the gene *TEF4* in order (i) to complement the observed mutant phenotype and (ii) to express and characterize the corresponding protein Tef4p.

The second chapter of the Results section is devoted to a detailed study of the *tef4Δ* mutant. The phenotypic characterization of the mutant as well as our attempts to isolate the protein are described. The main results concerning the specific sensitivity phenotypes of *tef4Δ* mutant were already presented at the Eleventh Congress of the Bulgarian Microbiologists with International Participation in St. Constantine, Varna, October 5-7, 2006, as a poster presentation ("Sensitivity of *Saccharomyces cerevisiae* GST mutants to arsenate").

The third chapter of the results section deals with the role of Ure2p in arsenic metabolism in yeast. In contrast to Tef4p, Ure2p is a well-studied protein. It is a yeast prion homolog, and acts as a central repressor of the GATA signalling pathway. The sensitivity to arsenic and oxidants has already been addressed by others, but the exact role of the Ure2 protein is not yet known. The main focus of our work has been to determine which of its two major functions – GATA factor repression or glutathione conjugation – is responsible for arsenic and oxidant detoxification in yeast.

We also present new evidence that the GATA regulatory pathway is at the origin of the observed regulation of arsenite transport in *S. cerevisiae*, that will be submitted for publication (working title: "Regulation of arsenite uptake – a novel non-enzymatic role for the glutathione S-transferase Ure2 of *Saccharomyces cerevisiae"*.

The final chapter of the Results section concerns the response of *ure2Δ* mutant to oxidative stress. A manuscript describing the results obtained on this topic will also be submitted (working title: "Oxidant response in *ure2Δ* mutant of *Saccharomyces cerevisiae*").

Results

Chapter 1

Screening of GST mutant bank

Résumé en français

Criblage d'une banque de mutants GST

Les glutathion S-transférases (GSTs) représentent une famille d'enzymes qui jouent un rôle important dans la détoxification de composés électrophiles très variés chez les mammifères et chez les plantes, mais dont le rôle chez les organismes unicellulaires demande encore à être étudié en détail. Dans un contexte environnemental, la fonction des GSTs la plus connue est leur activité de catalyse des réactions de conjugaison entre le glutathion et des molécules réactives comportant des sites électrophiles. Cette activité catalytique des GSTs représente la première étape de détoxification de tels composés, avec la formation de produits moins toxiques et plus hydrosolubles que les molécules de départ. Chez les levures, les membres de cette famille d'enzymes sont étonnamment peu nombreux, en comparaison tant avec les protéobactéries qu'avec les eucaryotes supérieurs.

Au commencement de ce travail, 2 gènes codant pour des GSTs (*GTT1* & *GTT2*), 5 gènes homologues des GSTs (*URE2, TEF3, TEF4, EFB1*, *GUS1*) et 4 cadres ouverts de lecture présentant une similarité partielle de séquence avec les GSTs ont été identifiés et partiellement caractérisés chez *S. cerevisiae* (http://www.yeastgenome.org/; http://cbi.labri.fr/Genolevures/). L'objectif majeur de ce travail a été de contribuer à la caractérisation des rôles physiologiques de ces enzymes chez *Saccharomyces cerevisiae* pour la résistance au stress induit par différents oxydants comme les peroxydes et aux polluants inorganiques comme l'arsenic. La résistance à l'arsenic a été choisie comme un des principaux thèmes de cette étude du fait de l'existence chez les levures de mécanismes de résistance à l'arsenic glutathion-dépendants encore imparfaitement caractérisés. A noter par ailleurs que la détoxification de l'arsenic chez l'être humain, en particulier l'étape de réduction des formes pentavalentes (l'arséniate, l'acide monométhylarsonique – MMA^V et l'acide diméthylarsonique – DMA^V) dépend de l'enzyme hGSTO-1, une GST de classe Omega.

Les premières étapes du travail de thèse ont été consacrées à la caractérisation phénotypique, par rapport au stress oxydatif et à la résistance à l'arsenic, d'un éventail de 23 souches de mutants de *Saccharomyces cerevisiae* obtenues de la collection EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) et inactivés sélectivement et de la même manière dans un seul gène de leur génome. Ces mutants couvrent les 11 gènes codant une protéine présentant une homologie aux glutathion S-transférases (5 protéines homologues sur les deux domaines N et C-

terminaux des GSTs, et 6 autres sur un seul de ces deux domaines), ainsi qu'un panel représentatif de gènes impliqués dans le métabolisme du glutathion (glutathion synthétase et réductase, glutaredoxines, glutathion peroxydases et canaux protéiques de transport de conjugués du glutathion). Dans un premier temps, la sensibilité à l'arséniate As(V) (0-4 mM), à l'arsénite As(III) (0-2 mM) et au peroxyde d'hydrogène (0-7 mM) des mutants sélectionnés a été évaluée en milieu solide et liquide, par une caractérisation détaillée du taux de croissance des différents mutants en présence de ces composés, et de leur viabilité après exposition à ces différents traitements. Pour résumer brièvement l'ensemble des données obtenues, les résultats les plus marquants sont la démonstration:

- d'une sensibilité du mutant GST *ure2Δ* à l'As(III) et au H_2O_2
- d'une sensibilité du mutant GST *tef4Δ* à l'As(V) et au H_2O_2
- d'une sensibilité du mutant *gpx3Δ* au H_2O_2
- d'une sensibilité du mutant *glr1Δ* à l'As(V) et au H_2O_2
- d'une sensibilité du mutant *ycf1Δ* à l'As(V) et à l'As(III)
- d'une sensibilité du mutant *grx5Δ* à l'As(V), à l'As(III) et au H_2O_2

La sensibilité des mutants *gpx3Δ, glr1Δ, grx5Δ* et *ycf1Δ* avait été notée précédemment. Dans ce contexte, le travail a été continué et approfondi sur les deux mutants *ure2Δ* et *tef4Δ*.

1. Introduction

Compared with GST enzymes from other organisms, those of fungi, especially *S. cerevisiae* are both functionally and structurally more diverse (McGoldrick *et al*., 2005). When this work was initiated only two GST genes had been cloned and sequenced from *S. cerevisiae,* and the corresponding recombinant proteins studied (Choi *et al*, 1998). These two enzymes – Gtt1p and Gtt2p show little sequence similarity (approx. 30%) to the standard GST classes and have poor CDNB activity.

Several other yeast proteins, namely Ure2p, Tef3p and Tef4p had been noted to display similarity in sequence and structure to GSTs, but their GST-like properties had not been studied in detail, under a GST-specific point of view.

Despite this ignorance concerning GST enzymes of *S. cerevisiae* two general observations could be made:

- In contrast with mammals and plants, and even with other fungi, a relatively small number of GSTs is present in *S. cerevisiae*.
- The evolutionary and structural relationship between the GSTs of *S. cerevisiae* and those of known classes of GSTs remains unclear.

2. Results

2.1. Identification of S. cerevisiae *GST mutants sensitive to arsenic and to hydrogen peroxide*

A preliminary objective of the present thesis was to obtain an overview of all sequences with similarity to GSTs in *S. cerevisiae* by exploring the available databases. The InterPro (PROSITE, PRINTS, Pfam, ProDom, SMART, TIGRFAMs, PIRSF, SUPERFAMILY, Gene3D and PANTHER) databases of multiple alignments of conserved protein regions and domains (http://www.ebi.ac.uk/interpro/; Mulder *et al.*, 2005; Mulder *et al.*, 2007) were searched for sequences containing matches to N- and C-GST generalized domains (see also Chapter 1 of Introduction). This revealed the presence of 11 genes and ORFs in the genome of *S. cerevisiae* with homology to known GST sequences, which possess N-, and/or C-GST profile according to one or more of the HMM databases (Table 1). As GSTs share the thioredoxin-like fold (Sheehan *et al*., 2001), most of the obtained sequences have also been noted to belong to the thioredoxin superfamily.

At the beginning of this study, a considerable number of these ORFs were of unknown function and there was no knowledge of their physiological role. With the progress of this work, some of the listed genes were cloned and their products were defined as functional GSTs – such as the three omega class GSTs Gto1p, Gto2p and Gto3p (Garcera *et al*, 2006). For others, such as *URE2,* several physiological functions were described, and an important gain of knowledge was acquired (Rai & Cooper, 2005). Finally, a third group of yeast GST homologues (YGR201c, *EFB1* and *GUS1*) is still uncharacterized to this day.

The main purpose of this work was to characterize the physiological roles of yeast GSTs in resistance to oxidant compounds, such as peroxides and arsenic, because of the long-standby interest of both host laboratories of the thesis project in oxidative stress in yeast (Nedeva *et al.*, 2004; Petrova *et al.*, 2004) and microbial glutathione S-transferases (Vuilleumier, 1997; Vuilleumier & Pagni, 2002). The first experimental aim of the work was to study the sensitivity to arsenic and hydrogen peroxide of available single disruption GST mutants of *S. cerevisiae*. The corresponding mutants in GST-like genes which were investigated in our experiments are listed in Table 2. A further step of our study was to initiate a detailed characterization of the mutants that had been shown to display sensitivity to one or more of the studied toxicants.

GSTs are known to have overlapping roles in the protection against stress conditions with the antioxidant enzymes glutathione peroxidases and glutaredoxins (e.g. Bai *et al.*, 2004; Collinson & Grant, 2003). In addition to the common cofactor glutathione, they often act on similar, often electrophilic toxic substrates, such as inorganic and organic peroxides, CDNB and DCNB (Collinson & Grant, 2003). To demonstrate the role of genes encoding components of the glutathione metabolic pathway in mediating arsenic and oxidant toxicity/resistance in yeast, another 11 genes whose protein products participate in glutathione metabolism were also investigated for their possible participation in arsenic and oxidant detoxification. These genes code for proteins such as glutathione peroxidases, glutaredoxins, glutathione synthetase, glutathione reductase and glutathione S-conjugate transporter (listed in the second part of Table 2).

Table 1. Protein sequences of *S. cerevisiae* found to share N- or C- GST profiles according to different databases (PHAM, Superfamily, Gene3D and Panther). The expectation values (E-values) representing the possibility that a hit to the corresponding motif occurs by chance are presented for each protein.

HMM domains		Protein Matches (E-values)										
		Tef3p	Tef4p	Gtt1p	Gtt2p	Ure2p	YG4D	Tef5p	Gto1p	Gto2p	Gto3p	Gus1p
PFAM	PF02798 glutathione S-transferase N-domain	$1{,}7e^{-15}$	$2{,}3e^{-15}$	$3{,}6e^{-18}$	$2{,}3e^{-12}$	$8{,}5e^{-21}$						
	PF00043 glutathione S-transferase C-domain	$1{,}6e^{-19}$	$1{,}3e^{-18}$	$1{,}1e^{-16}$	$2{,}2e^{-12}$	$4{,}6e^{-16}$	$2{,}4e^{-13}$					
Superfamily	SSF47616 glutathione S-transferase C-domain	$5{,}4e^{-22}$	$1{,}5e^{-21}$	$1{,}4e^{-14}$	$5e^{-24}$	$2{,}4e^{-26}$	$1{,}9e^{-18}$	0,00024	$3{,}1e^{-20}$	$5{,}6e^{-24}$	$2{,}4e^{-25}$	$1{,}3e^{-06}$
	SSF52833 thioredoxin-like fold	$3{,}1e^{-09}$	$1{,}6e^{-07}$	$1{,}1e^{-12}$	$4{,}2e^{-19}$	$4{,}4e^{-16}$	0,0013		$1{,}7e^{-05}$	$3{,}9e^{-07}$	0,0011	
Gene3D	G3DSA_3.40.30.10 thioredoxin fold	$7{,}5e^{-28}$	$3{,}3e^{-23}$	$1{,}8e^{-08}$	$5{,}5e^{-19}$	$4e^{-27}$	$2{,}5e^{-19}$					
	G3DSA_1.20.1050.10 glutathione S-transferase	$2e^{-40}$	$4{,}3e^{-34}$		$7{,}9e^{-08}$	$1{,}3e^{-49}$	$4e^{-31}$					
Panther	PTHR11260 glutathione S-transferase	$3{,}7e^{-74}$	$1{,}8e^{-58}$	$2e^{-98}$	$4{,}4e^{-136}$	$1{,}1e^{-59}$	$2{,}8e^{-62}$					

Table 2. GST homologues and genes encoding components of the glutathione metabolic pathway in *Saccharomyces cerevisiae.*

Gene	ORF	Description (according to SGD http://www.yeastgenome.org/)	GST profile	Genotype of corresponding EUROSCARF mutant used in this work
TEF3/CAM1	YPL048w	Homolog of translation elongation factor eEF1Bγ, participates in the regulation of GTP-binding protein eEF1A	N+C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPL048w::kanMX4
TEF4/EFC1	YKL081w	Homolog of translation elongation factor eEF1Bγ	N+C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YKL081w::kanMX4
GTT1	YIR038c	Endoplasmic reticulum associated glutathione S-transferase	N+C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YIR038c::kanMX4
GTT2	YLL060c	Glutathione S-transferase	N+C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLL060c::kanMX4
URE2	YNL229c	Glutathione S-transferase; nitrogen catabolite repression regulator; altered form of Ure2p creates [URE3] prion	N+C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL229c::kanMX4
YG4D (Swiss-Prot entry name)	YGR201c	Putative protein of unknown function	C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGR201c::kanMX4
TEF5/EFB1	YAL003w	Translation elongation factor eEF1Bα; stimulates nucleotide exchange to regenerate eEF1A-GTP; facilitates binding of aminoacyl-tRNA to the ribosomal A site	C-profile	BY4743; Mat a/α; his3Δ1/his3Δ1; leu2Δ0 /leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0 /ura3Δ0; YAL003w::kanMX4/YAL003w
GTO1	YGR154c	Putative peroxisomal omega-class glutathione S-transferase	C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGR154c::kanMX4
GTO2/ECM4	YKR076w	Putative omega class glutathione S-transferase; participates in cell wall biogenesis	C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YKR076w::kanMX4
GTO3	YMR251w	Putative cytosolic omega class glutathione S-transferase	C-profile	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR251w::kanMX4 BY4742; Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YMR251w::kanMX4
GUS1/GSN1	YGL245w	Glutamyl-tRNA synthetase, forms a complex with methionyl-tRNA synthetase; increases the catalytic	C-profile	BY4743; Mat a/α; his3Δ1/his3Δ1; leu2Δ0 /leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0;

		efficiency of both tRNA synthetases and ensures their correct localization in the cytoplasm		ura3Δ0 /ura3Δ0; YGL245w::kanMX4/YGL245w
YCF1	YDR135c	Vacuolar glutathione S-conjugate transporter of the ATP-binding cassette family, has a role in detoxifying metals such as cadmium, mercury, and arsenite; also transports unconjugated bilirubin;		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR135c::kanMX4
GPX1	YKL026c	Phospholipid hydroperoxide glutathione peroxidase; protects cells from phospholipid hydroperoxides and nonphospholipid peroxides during oxidative stress		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YKL026c::kanMX4
GPX2/AMI1	YBR244w	Phospholipid hydroperoxide glutathione peroxidase; protects cells from phospholipid hydroperoxides and nonphospholipid peroxides during oxidative stress		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YBR244w::kanMX4
GPX3/HYR1/ORP1	YIR037w	Thiol peroxidase, functions as a hydroperoxide receptor to sense intracellular hydroperoxide levels and transduces a redox signal to the Yap1p transcription factor		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YIR037w::kanMX4
GLR1/LPG17	YPL091w	Cytosolic and mitochondrial glutathione oxidoreductase, converts oxidized glutathione to reduced glutathione		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPL091w::kanMX4
GSH2	YOL049w	Glutathione synthetase, catalyzes the ATP-dependent synthesis of glutathione (GSH) from γ-glutamylcysteine and glycine		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOL049w::kanMX4
GRX1	YCL035c	Hydroperoxide and superoxide-radical responsive heat-stable glutathione-dependent disulfide oxidoreductase, shows glutathione peroxidase activity		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YCL035c::kanMX4
GRX2/TTR1	YDR513w	Cytoplasmic glutaredoxin, thioltransferase, glutathione-dependent disulfide oxidoreductase involved in maintaining redox state of target proteins, shows glutathione peroxidase activity		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR513w::kanMX4

GRX3	YDR098c	Monothiol glutaredoxin subfamily member; protects cells from oxidative damage		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR098c::kanMX4
GRX4	YER174c	Monothiol glutaredoxin subfamily member; protects cells from oxidative damage		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER174c::kanMX4
GRX5	YPL059w	Monothiol glutaredoxin subfamily member; protects cells from oxidative damage; mitochondrial matrix protein involved in the synthesis/assembly of iron-sulfur centers		BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPL059w::kanMX4

To determine whether the investigated genes are involved in resistance to arsenic and oxidative stress, all single disruption mutants, in which the corresponding ORFs were replaced with the kanamycin resistance cassette, were tested for hypersensitivity to arsenite, arsenate and H_2O_2 by spotting onto YPD plates with different concentrations of substances (for details on growth conditions see Annex 1). All used mutants were isogenic to the same wild-type background strain BY4741 (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). The ability to grow of each mutant was determined after 3 days of incubation in relation to the growth of the isogenic parental strain. Disruption mutants defective in genes for known glutathione S-transferases (*gtt1Δ* and *gtt2Δ*) did not show hypersensitivity to arsenic and hydrogen peroxide relatively to the parental strain BY4741. In contrast, six other mutants were sensitive to one or more of tested toxic compounds:

- The GST mutant *tef4Δ* is sensitive to As(V) and H_2O_2;
- The GST mutant *ure2Δ* is sensitive to As(III) and H_2O_2;
- The mutant *ycf1Δ* is sensitive to As(III) and As(V);
- The mutant *glr1Δ* is sensitive to As(V) and H_2O_2;
- The mutant *gpx3Δ* is sensitive to H_2O_2;
- The mutant *grx5Δ* is sensitive to As(III), As(V) and H_2O_2.

The results for the arsenic and peroxide sensitivity of studied mutants are summarized in Fig. 1, and the detailed pattern of the observed phenotypes is presented in Table 3.

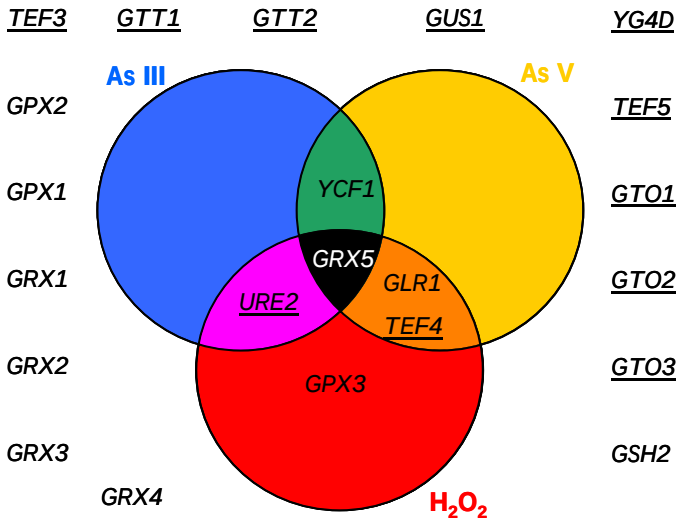
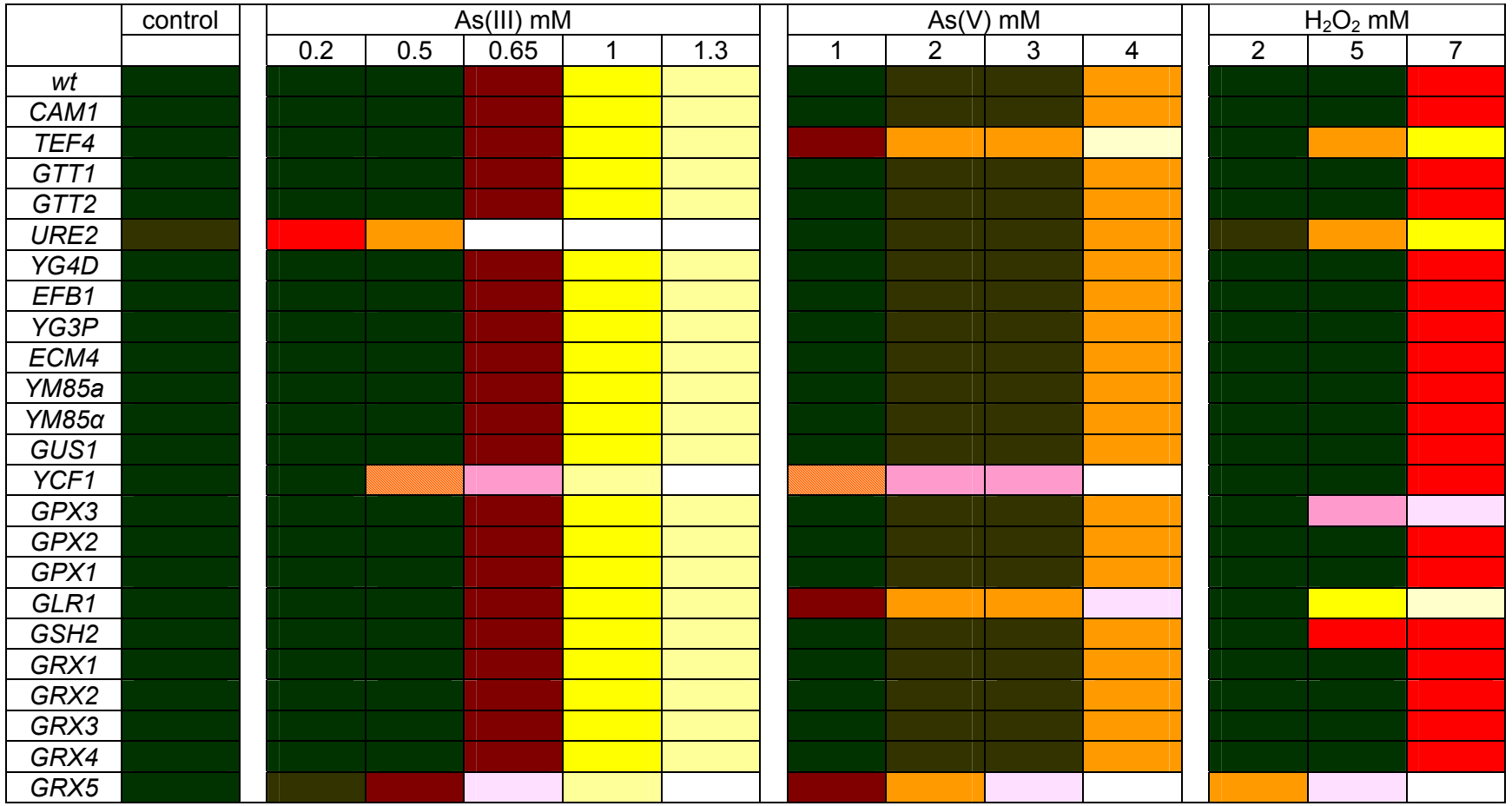


Fig.1 Sensitivity of studied mutants to arsenic and H_2O_2 (in red – genes implicated in H_2O_2 resistance, in yellow – in As(V) resistance, and in blue – in As(III) resistance, around the circles – genes which single disruption mutants did not show any growth inhibition in presence of arsenic or/and H_2O_2.

Table 3. Sensitivity of tested mutants to arsenic and H_2O_2 on solid YPD media. Growth is expressed as the last decimal dilution at which growth was observed: 10^{-5}; $10^{-4.5}$; 10^{-4}; $10^{-3.5}$; at 10^{-3}; $10^{-2.5}$; 10^{-2}; $10^{-1.5}$; 10^{-1}; $10^{-0.5}$; □ no growth.



The sensitivity of mutants defective in genes coding for components of glutathione metabolism (i.e. *YCF1*, *GLR1*, *GPX3* and *GRX5)*, have already been well characterized (Ghosh *et al*, 1999; Inoue *et al*, 1999; Rodrıguez-Manzaneque *et al*, 1999). However, we decided to validate our experimental strategy by comparing the phenotypes obtained in our study with results previously obtained by others. We further evaluated mutant sensitivity by monitoring growth kinetics of investigated strains in the presence or absence of As and H_2O_2. Growth was determined in liquid rich medium supplemented with various concentrations of the toxicants, and the corresponding specific growth rates were determined. A brief description of each class of phenotype observed is presented below.

2.2. The ycf1Δ *mutant is sensitive to As(V) and As(III)*

Ycf1p (yeast cadmium factor), a member of the ABC transporter superfamily, is a vacuolar GSH conjugates transporter which catalyzes the ATP-driven uptake of As(III) into the vacuole (Ghosh *et al*, 1999). Direct comparison between the *S. cerevisiae* strain harbouring a deletion of the *YCF1* gene and the isogenic wild type strain demonstrated that Ycf1p is required for increased resistance to the toxic effects of arsenic, as well as to other toxic metals, cadmium and mercury (Li *et al*, 1996; Gueldry *et al*, 2003).

The Ycf1p catalyzes the vacuolar sequestration of As(III), which may originate both as a product of As(V) reduction or from direct As(III) uptake. It was not, thus surprising that the corresponding *ycf1Δ* mutant showed hypersensitivity to both forms of arsenic. The observed growth defect of this mutant occurred at relatively low concentrations of arsenic, at which the parental BY4741 strain grew without any detectable inhibition (Fig. 2).

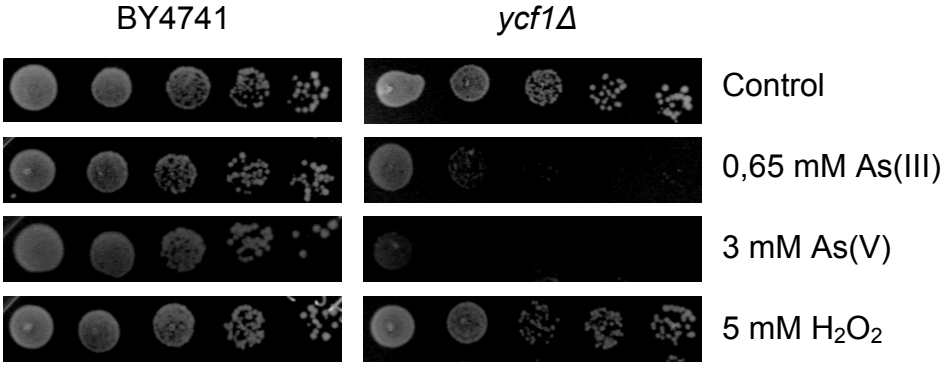


Fig. 2. Sensitivity of *ycf1Δ* mutant to arsenite, arsenate and hydrogen peroxide. Parental strain BY4741 and mutant *ycf1Δ* were cultivated overnight in liquid YPD medium, serially diluted, and 5 µL of each dilution were spotted on YPD plates with the indicated concentrations of As(III), As(V) and H_2O_2.

In liquid medium, the mutant *ycf1Δ* showed approximately 80% decrease of growth at 0,5 mM As(III) and 0,2 mM As(V), whereas this decrease was only 10-15% for the wild type (Fig. 3). In contrast, the mutant was not more sensitive to H_2O_2 than the wild-type on both solid and liquid media, confirming that Ycf1p has no role in peroxide metabolism.

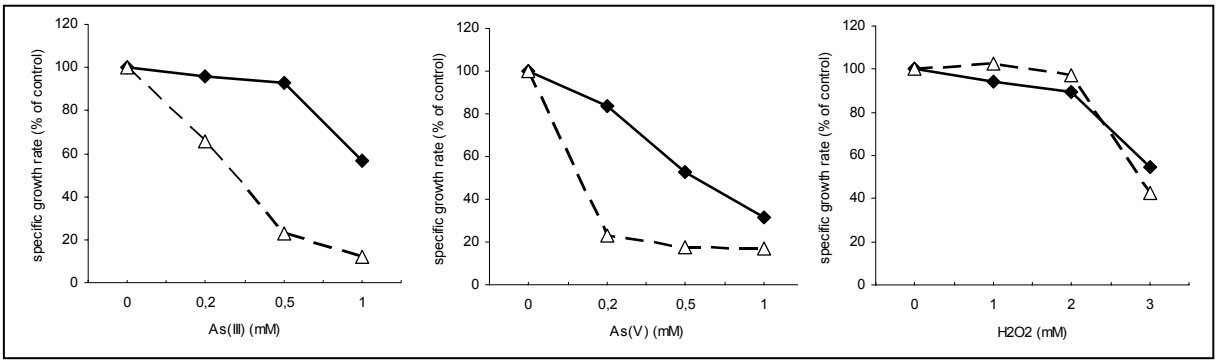


Fig.3. Sensitivity of *ycf1Δ* mutant to arsenic and H_2O_2 in liquid YPD medium. The specific growth rate of BY4741 (■) and *ycf1Δ* (Δ) in medium with various concentrations of As(III), As(V) and H_2O_2 was calculated from the growth curves and is presented as a percentage of the specific exponential growth rate (h^{-1})of each strain in YPD medium without any toxicant. Presented values are mean of two independent experiments.

A similar pattern for arsenic sensitivity of a mutant with disruption in *YCF1* gene had already been noted in the study of Ghosh *et al*, 1999. These authors were the firsts to demonstrate the presence of two parallel and independent pathways for removal of toxic arsenite from yeast cytosol – the plasma membrane efflux by Acr3p, and the ATP-driven uptake of As(III) into the vacuole catalyzed by Ycf1p (see Introduction, Chapter 2, Fig. 11).

2.3. The gpx3Δ *mutant is sensitive to* H_2O_2

Three glutathione peroxidase enzymes are expressed in *S. cerevisiae* – Gpx1p, Gpx2p and Gpx3p (Inoue *et al*, 1999). The *gpx3Δ* mutant is hypersensitive to peroxides, whereas null mutants of the *GPX1* and *GPX2* genes do not show any obvious oxidant phenotype. The glutathione peroxidase activity decreased approximately by 50% in the *gpx3Δ* mutant compared to wild type (Inoue *et al*, 1999). The demonstrated peroxide sensitive phenotype of *gpx3Δ* mutant was confirmed in our experiments – its growth was almost fully abolished in the presence of H_2O_2 at concentrations greater than 5 mM (Fig. 4).

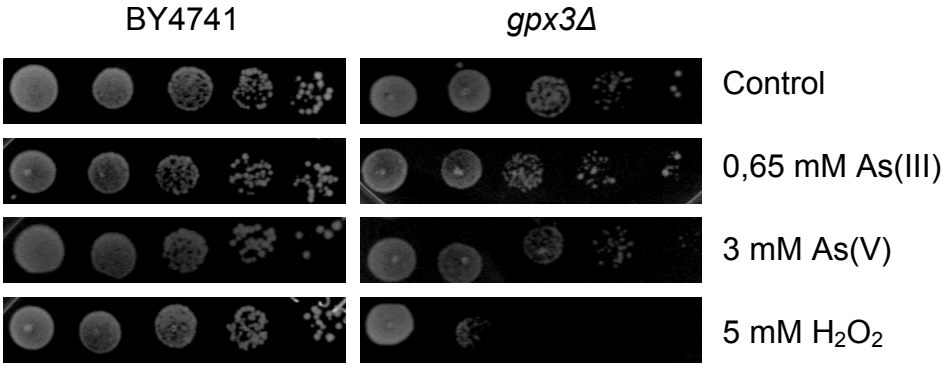


Fig. 4. Sensitivity of *gpx3Δ* mutant to arsenite, arsenate and hydrogen peroxide. For details see legend of Fig. 2. The data on the parental strain (Fig. 2) are repeated here for convenience.

2.4. The glr1Δ *mutant is sensitive to H_2O_2 and As(V)*

The reduced state of glutathione (GSH) is maintained by NADPH-dependent glutathione reductase, which catalyzes the regeneration of oxidized GS-SG to reduced GSH. The gene encoding glutathione reductase in *S. cerevisiae* (*GLR1*) has been identified and null mutants, although viable, accumulate an excess of oxidized glutathione and are hypersensitive to oxidants (Grant *et al*, 1996). This sensitivity to the oxidative effect of hydrogen peroxide was demonstrated here when we spotted *glr1Δ* mutant onto plates with H_2O_2 (Fig. 5) – according to our experiments, the concentration limit at which the mutant was able to grow was 5 mM, while the wild type grew quite well at concentrations of 7 mM H_2O_2 (see Table 3).

The *glr1Δ* mutant was also sensitive to As(V) (Fig. 5). Thus, the tripeptide GSH plays critical roles in both the enzymatic and nonenzymatic reduction of pentavalent arsenicals to arsenic(III). Since the detoxification of arsenate requires reduced GSH the enhanced sensitivity of the mutant is probably a consequence of decreased intracellular levels of reduced glutathione.

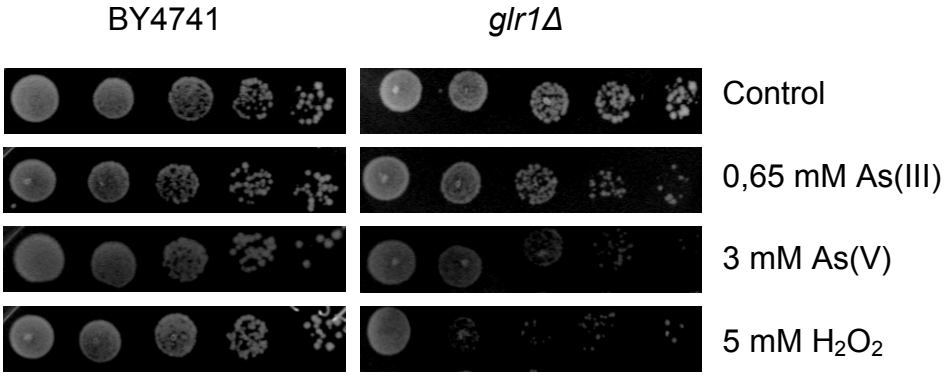


Fig. 5. Sensitivity of *glr1Δ* mutant to arsenite, arsenate and hydrogen peroxide. For details see legend of Fig. 2. The data on the parental strain (Fig. 2) are repeated here for convenience.

We next examined the growth kinetics in liquid medium of the *glr1Δ* mutant in the presence or absence of arsenic salts and H_2O_2. At concentrations of 2-3 mM H_2O_2, the disruption *glr1Δ* mutant showed approximately 10-15% stronger inhibition of the growth than the parental strain (Fig. 6). This growth defect became more manifest at higher peroxide concentrations, as shown by the experiments on solid YPD medium performed with 5-7 mM H_2O_2 (Fig. 5, Table 3).

In liquid rich medium supplemented with arsenate, growth of the *glr1Δ* mutant was inhibited by 70% at concentration of 0,5 mM As(V), while that of the parental strain BY4741 was inhibited by 50%. In contrast, the growth of both strains in presence of As(III) was similar.

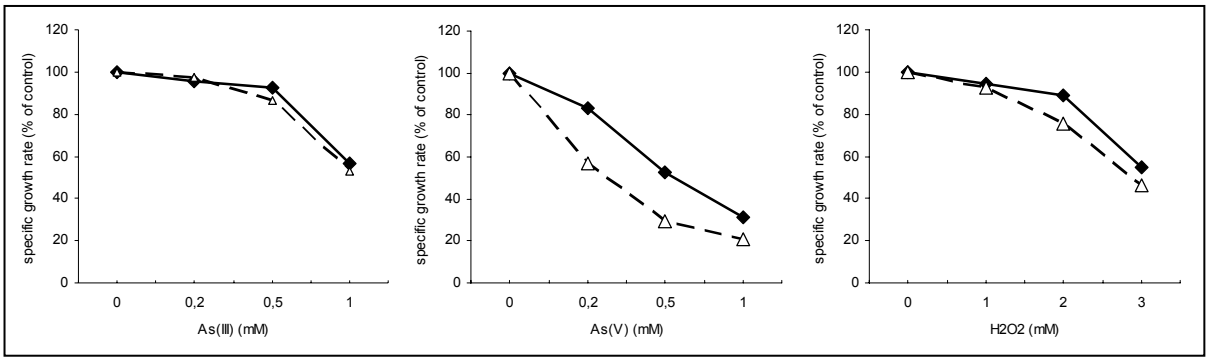


Fig.6. Sensitivity of *glr1Δ* mutant (Δ) to arsenic and H_2O_2 in liquid YPD medium, compared to that of parental BY4741 strain (■). Growth details are presented in the legend of Fig. 3.

The strong glutathione requirement of arsenate reduction (Mukhopadhyay *et al.*, 2000), and the need for reduced GSH to counter free-radical production after arsenic exposure may have additive

effects in defining arsenate resistance in *S. cerevisiae,* which may explain the observed phenotype of mutant *glr1Δ*. Indeed, deficiency in GSH-dependent enzymes such as glutathione reductase can have deleterious effects not only on direct oxidant elimination, but also on the overall detoxification process, more generally.

2.5. The grx5Δ mutant is hypersensitive to H_2O_2, As(V) and As(III)

Glutaredoxin Grx5p is known to be an essential mitochondrial protein (Rodrıguez-Manzaneque *et al*, 1999). Grx5p plays an important role in protection against oxidative stress both during ordinary growth conditions and after externally induced damages. Mutants lacking Grx5p are growth deficient in both rich and minimal media, and are also highly sensitive to oxidative damage (Rodrıguez-Manzaneque *et al*, 1999). A significant increase in total carbonylated protein content is observed in *grx5Δ* cells, and a number of specific proteins, including transketolase, appears to be highly oxidized in this mutant (Rodrıguez-Manzaneque *et al*, 1999).

In our study, the *grx5Δ* mutant showed poor growth and sensitivity to all conditions tested (Fig.7 & 8). This probably results from the general growth defect of the mutant. Mitochondria are known target sites for damage action of arsenic – one of the hypotheses for genotoxicity of arsenic includes apoptosis by direct effect on mitochondrial transmembrane potential (Larochette *et al*, 1999). Arsenic also inhibits citric acid metabolism and blocks respiration (Tamas & Wisocki, 2001). Since hydrogen peroxide as well as arsenic both cause indirect and direct damages to mitochondria, the observed sensitivity of the *grx5Δ* mutant was expected. However, our experiments show two additional phenotypes of the *grx5Δ* mutant – sensitivity to As(III) and to As(V).

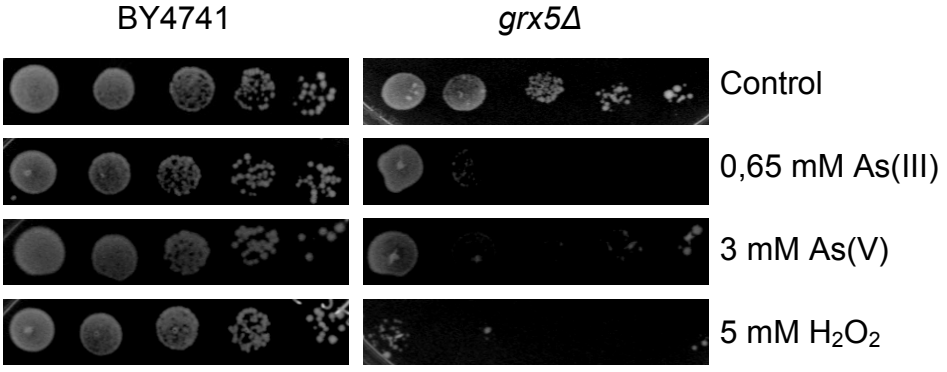


Fig. 7. Sensitivity of *grx5Δ* mutant to arsenite, arsenate and hydrogen peroxide. For details see legend of Fig. 2. The data on the parental strain (Fig. 2) are repeated here for convenience.

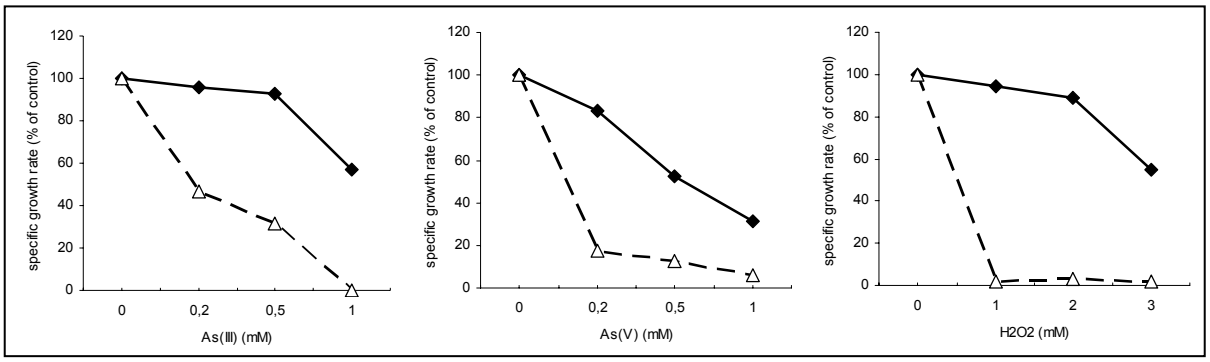


Fig.8. Sensitivity of *grx5Δ* mutant (Δ) to arsenic and H_2O_2 in liquid YPD medium, compared to that of parental BY4741 strain (■). Growth details are presented in the legend of Fig. 3.

3. Conclusion and perspectives

The presented results confirm that glutathione has important roles in defense against reactive oxygen species as well as in overall cellular detoxification metabolism and validate the chosen approach for the detection of sensitivity of selected mutant strains to the exposure to toxic oxidants, such as H_2O_2 and arsenic species. Indeed, glutathione participates as a general free-radical scavenger and as a cofactor of antioxidant enzymes, readily explaining the observed hydrogen peroxide sensitivity of *gpx3Δ, glr1Δ* and *grx5Δ* mutants. In addition to its antioxidative role, glutathione also serves as a cofactor for enzymes implicated in heavy metal protection. A large variety of mutant phenotypes testifying the specific protective role of glutathione in arsenic detoxification, were obtained. In particular, this role is underlined by the sensitivity to As(III) and As(V) of the *ycf1Δ* mutant, which is unable to carry out glutathione conjugation and vacuolar sequestration of arsenic (Ghosh *et al*, 1999). Likewise, the sensitivity to As(V) of the *glr1Δ* mutant, deficient in reduced glutathione results from the GSH requirement in the process of As(V) reduction.

Most importantly, however, the sensitivity of *ure2Δ* and *tef4Δ* mutants to arsenic and peroxide were new findings which form the basis for the main part of the work performed in the present thesis. These results are presented in the following chapters.

Annex 1

Materials and methods

Strains and media

The *S. cerevisiae* strains used in this study are listed in Table 2 and are all isogenic to BY4741 (*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*). They were obtained from *EUROSCARF* (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/).

Strains were grown on commercial available YPD rich medium – 1% yeast extract, 2% peptone, 2% glucose (Q-BioGene).

Arsenic and oxidant sensitivity assays

All strains were grown overnight in YPD liquid medium (30ºC/140rpm). Grown cultures (OD_{600}~1-1,5) were pelleted, washed with fresh medium and then diluted to OD_{600} ~ 1; 0,1; 0,01; 0,001; 0,0001 with fresh YPD medium. Samples (5 µl) of each diluted culture were spotted onto agar plates of the same medium containing arsenic or H_2O_2 at different concentrations, and incubated at 30ºC for 3 days. As(III), as 0,13 M stock solution of $NaAsO_2$ (Sigma) was used at final plate concentrations of 0,2 mM; 0,5 mM; 0,65 mM; 1,0 mM and 1,3 mM. As(V), as 0,26 M stock solution of $Na_2HAsO_4.7H_2O$ (Sigma) was at final concentrations of 1 mM; 2 mM; 3 mM and 4 mM, respectively. H_2O_2 (30% stock solution, Fluka) was used at 2 mM, 5 mM and 7 mM final concentration.

Determination of growth rate in liquid cultures

Two hundred µL of YPD with 0,2 mM; 0,5 mM; 1,0 mM As(III) ($NaAsO_2$), 0,2 mM; 0,5 mM; 1,0 mM As(V) ($NaHAsO_4.7H_2O$) or 1 mM; 2 mM; 3 mM H_2O_2 were inoculated with overnight cultures to an initial OD_{600}~0,05. Cultures were incubated in a *96 MicroWell*™ flat-bottom plate (Nunclon) for 24 hours at 25°C/160 rpm, and the OD_{600} was measured at regular intervals with a Bio-Tek KC4 plate reader. Specific growth rates were determined from exponential phase of growth (usually between 6 and 14 hour after inoculation).

Chapter 2

Characterisation of the arsenic and peroxide sensitivity of the *tef4Δ* mutant of *S. cerevisiae*

Résumé en français

Caractérisation de la sensibilité du mutant *tef4* à l'arsenic et au H_2O_2

Chez les eucaryotes, la traduction des protéines est divisée en trois phases principales : l'initiation, l'élongation et la terminaison. Pendant l'initiation, le ribosome se fixe sur l'extrémité de l'ARNm, accompagné de l'ARNt qui porte la méthionine. L'élongation comprend le déplacement du ribosome sur l'ARNm et l'association de chaque codon à un ARNt correspondant. L'élongation de la traduction nécessite un nombre important de facteurs, dont le facteur eEF1 composé des deux éléments eEF1A et eEF1B. La protéine eEF1A est responsable de la liaison de l'aminoacyl-ARNt aux ribosomes et le complexe multimérique eEF1B recharge eEF1A en GTP à chaque cycle de l'élongation. eEF1B est constitué de deux sous-unités α et γ. Chez *Saccharomyces cerevisiae,* la sous-unité γ est codée par deux gènes *TEF3* et *TEF4*, dont les protéines sont identiques à 64 %.

Le rôle physiologique de Tef3p et Tef4p est encore inconnu. Il existe des données concernant leur structure (Jeppesen *et al*., 2003) mais notre travail est le premier à notre connaissance à suggérer un rôle fonctionnel de la protéine Tef4p. Nous avons démontré pour le mutant *tef4Δ* plusieurs phénotypes nouveaux – sensibilité à l'As(V), au H_2O_2 et au CDNB, un composé aromatique chloré et substrat modèle des GSTs. Une différence de sensibilité au H_2O_2 selon la phase de croissance a été observée de manière reproductible. En contraste, le mutant *tef3Δ* présente une sensibilité à l'As(V), au H_2O_2 et au CDNB comparable à celle du type sauvage.

Notre travail a concerné le clonage du gène *TEF4* dans le plasmide-navette p413-GALS permettant l'expression chez les levures, et dans le plasmide pET24b permettant sa surexpression dans *E. coli*. Etant donné que le gène contient une région non-codante dans sa structure, les expériences ont été éffectuées en parallèle à partir d'ADN génomique et à partir d'ARN total cellulaire. Cependant, la complémentation du phénotype du mutant et la surexpression de la protéine à partir des vecteurs utilisés n'ont pas pu être obtenues. L'étude au niveau protéique de Tef4p et la caractérisation phénotypique du mutant représentent une perspective ouverte pour la poursuite de ce travail.

1. Introduction

The process of protein biosynthesis is divided into three distinct phases – initiation, elongation, and termination (Kapp & Lorsch, 2004). In eukaryotes, during initiation the complex Met-tRNA is bound to the ribosomal P-site and base-paired with an initiator AUG codon on the mRNA. In the elongation cycle, the aminoacylated tRNA is brought to the ribosomal A-site by elongation factor eEF1A, a 50-kDa G-protein (Fig. 1). The ribosome acts as a GTPase activator for eEF1A in the presence of a correct codon-anticodon match between the aminoacylated tRNA and the A-site codon of mRNA. To ensure the energy supply of the process, eEF1A hydrolyzes GTP, and the resulted eEF1A-GDP leaves the ribosome. The eukaryotic elongation factor eEF1A acts in complex with another elongation factor 1B (eEF1B), which is the guanine nucleotide exchange factor for eEF1A. In *S. cerevisiae*, translation elongation factor eEF1B contains two distinct subunits, eEF1Bα and eEF1Bγ. The observed ratio of eEF1A:eEF1Bγ:eEF1Bα subunits in *S. cerevisiae* is 2:2:2, and the estimated molecular mass of the complex is 240 kDa (Jeppesen *et al*., 2003). Subunit eEF1Bα is an essential protein and is coded by the *TEF5* gene. The protein eEF1Bγ appears to stimulate GTP/GDP nucleotide exchange of eEF1Bα, and also to physically attach this complex to membranes of the endoplasmic reticulum or to tubulin.

Yeast eEF1Bγ is present as two isoforms able to bind the eEF1Bα subunit (Olarewaju *et al.*, 2004) and encoded by the *TEF3* and *TEF4* genes respectively. Both genes are not essential for growth or for proper translation (Olarewaju *et al.*, 2004). The *TEF3* gene was isolated as a gene encoding a calcium-dependent membrane binding protein (Kambouris *et al*., 1993). This gene was identified independently as a gene dosage extragenic suppressor of the cold-sensitive mutant *drs2,* which is deficient in the assembly of 40S ribosomal subunits (Ripmaster *et al*., 1993). In contrast, the product of *TEF4*, the second homologue gene for eEFB1γ in *S. cerevisiae*, displays high identity to Tef3p (64,5%), but does not suppress the cold-sensitive 40S ribosomal subunit deficiency of *drs2* mutant strain (Kinzy *et al*., 1994). These findings indicate that Tef3p and Tef4p have different functions in the cell.

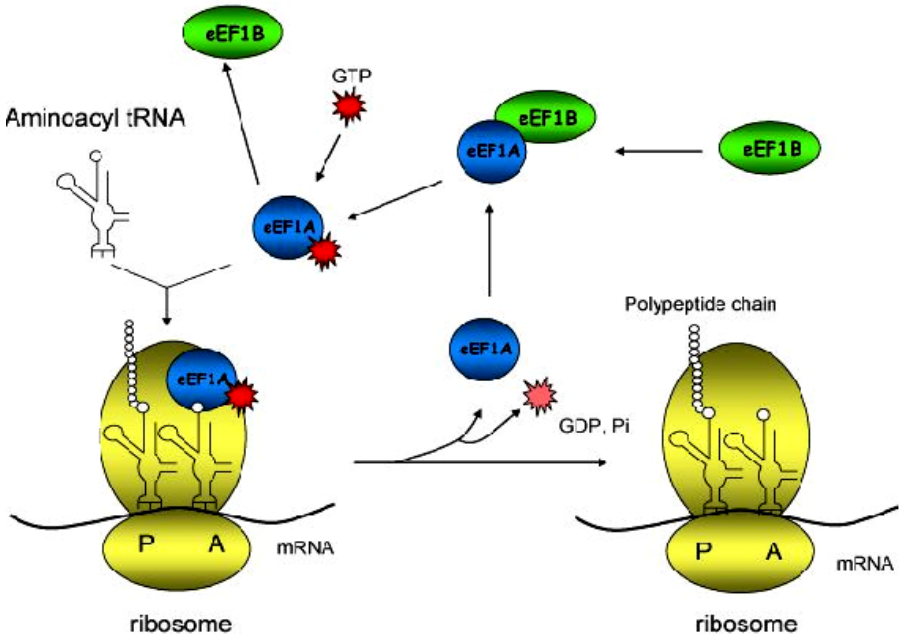


Fig. 1. A general scheme of translation elongation in eukaryotes (for details see text; image source http://www.genetics.med.ed.ac.uk/transelong/)

The two homologous genes *TEF3* and *TEF4* are located on chromosome XVI and XI of *S. cerevisiae,* respectively. The intron-less gene *TEF3* encodes a protein of 415 amino acids with molecular weight of 47 kDa, while the product of *TEF4* is 412 amino acids long (46,5 kDa). The structure of *TEF4* gene contains a 326 bp intron, coding for a small nucleolar RNA (snoRNA) (Fig. 2). WU-BLAST2 alignment reveals an identity of 64% between the two protein sequences with the highest sequence conservation in the C terminal part of the protein and 47% identity between the GST domains of Tef3p and Tef4p (Fig. 3).

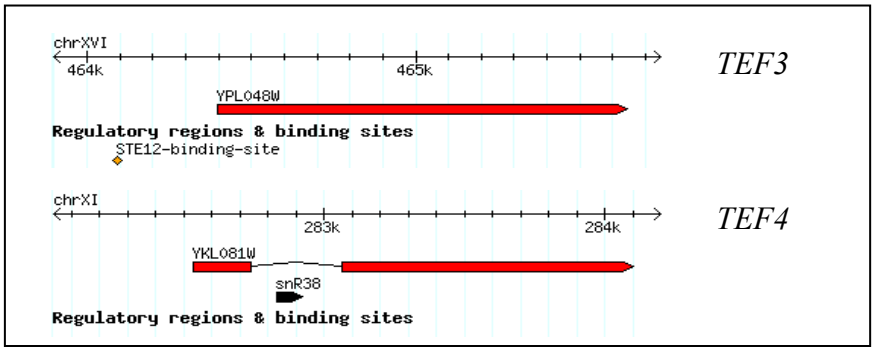


Fig. 2. Schematic view of *TEF3* and *TEF4* genes (image source – SGD, http://www.yeastgenome.org/). Genes are represented in red, relatively to the chromosome coordinates. Putative regulatory sequences and binding sites in the gene up-stream regions (chromosome XVI) are also presented in yellow.

Sequence alignment, motif searching and homology modeling had revealed that both forms of yeast EF1Bγ are closely related to GSTs (also see Table 1 in Chapter 1 of Results). The N-terminal domain has sequence motifs characteristic of the Theta class glutathione S-transferases (GST) and, accordingly has been suggested to form homodimers and to be enzymatically active (Koonin *et al*., 1994). Indeed, GST activity was observed toward the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) with the recombinant eEF1Bγ subunit from *Oryza sativa* expressed in *Escherichia coli,* and for the full native eEF1B complex (Kobayashi *et al*., 2001). Limited proteolysis demonstrates that yeast and human eEF1Bγ consist of two structural domains connected by a flexible linker (Jeppesen *et al*., 2003). The so called domain 1 (residues 1 – 219 in the yeast Tef3p, see Fig. 3) is the GST domain. Importantly, structural details of the catalytic site are conserved in the yeast eEF1Bγ domain 1, but it is not able to bind to a GSH affinity matrix and does not show any activity toward the GST model substrate CDNB (Jeppesen *et al*., 2003). The *TEF3*-encoded form of eEF1Bγ has no obvious catalytic residue. However, the form of eEF1Bγ encoded by the *TEF4* gene contains serine 11 (Fig. 3), which may act catalytically as aligns to active site residues in other GST proteins. In addition, an alignment of 20 eEF1Bγ sequences resulted in the identification of 12 conserved residues in the GST domain of these proteins (Jeppesen *et al*., 2003). Two of them, *cis*-Pro-50 and Glu-62, are conserved in most GST proteins, and are involved in the positioning and binding of glutathione in the active site (Armstrong, 1996). This suggests that eEF1Bγ in yeast has GST-like functions, as the evolutionary maintenance of such a specific structure seems very unlikely in the absence of some specific purpose. A novel GST activity with a specific substrate of still unknown identity is therefore a strong possibility. Alternatively, the GST fold may be explained with the regulatory role of the EF1Bγ protein, as the monomer interface can participate in protein-protein interaction.

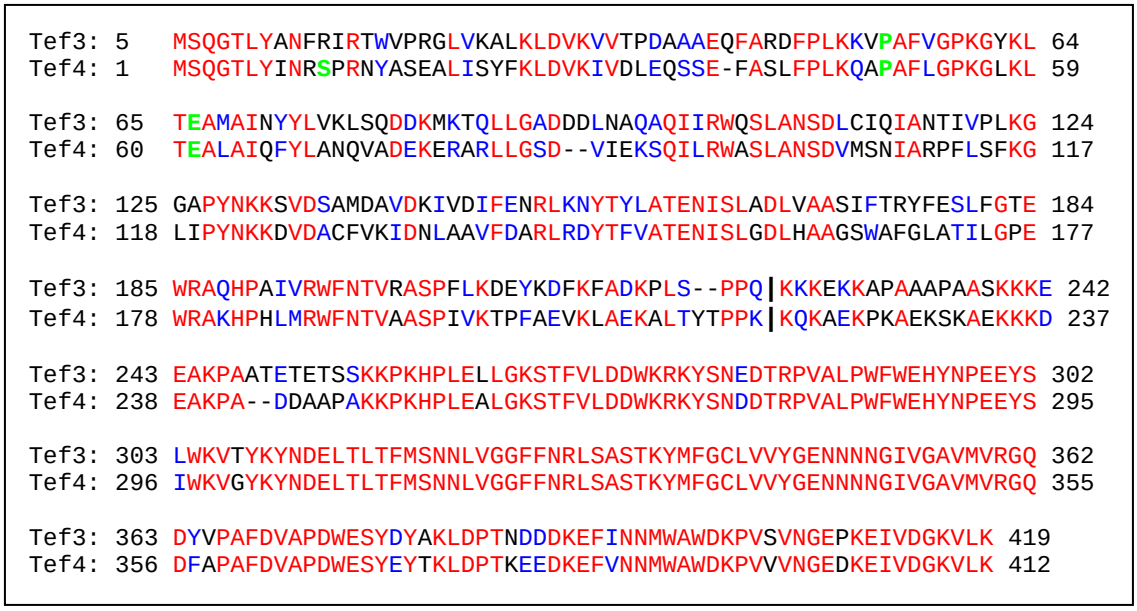


Fig. 3. Alignment of *S. cerevisiae* Tef3p and Tef4p. Amino acid sequences were aligned using ClustalW. Numbers indicate the position of the adjacent amino acid in the protein sequence. Amino acids that are identical are shown in red, and conserved substitutions are shown in blue. The putative catalytic Ser-11 of Tef4p and the conserved *cis*-Pro and Glu are shown in green. The limit of the GST domain is shown as a black dash.

2. Results

2.1. Phenotypic characterization of the tef4Δ *mutant*

The preliminary screening of *S. cerevisiae* GST mutants revealed that disruption of the *TEF4* gene conferred sensitivity to As(V) and to H_2O_2 (see Chapter 1 of Results). The *tef4Δ* showed significant growth inhibition even at relatively low concentrations of As(V), at which the parental strain BY4741 did not show any detectable growth perturbation. The *tef4Δ* mutant did not grow at concentrations higher than 6 mM As(V) (Fig. 4 and data not shown). In contrast, the wild type strain is still able to grow at 8 mM As(V). This suggested a possible role of the Tef4 protein in arsenate and oxidant detoxification. Despite the high identity observed between two eEF1Bγ homologues Tef3p and Tef4p, the mutant *tef3Δ* was not sensitive to these compounds (Fig. 4 and 7, see also Table 3 in Chapter 1 of Results).

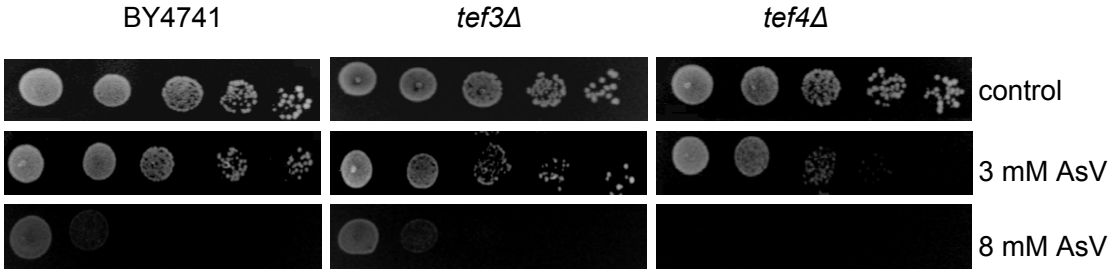


Fig.4. Sensitivity of mutants *tef3Δ* and *tef4Δ* to $NaHAsO_4.7H_2O$ (As(V)) on YPD medium. Parental strain BY4741 and mutants *tef3Δ* and *tef4Δ* were cultivated overnight at 30°C/140 rpm in liquid YPD medium, serially diluted, and 5 µL of each dilution were spotted on YPD plates at the indicated concentration of As(V). Colonies were photographed after 5 days incubation.

Because the spot analysis reports only on the end point of growth and colony formation, we decided to investigate in more details the kinetics of mutant growth under stress conditions. A growth analysis was performed for BY4741, *tef3Δ* and *tef4Δ* over a period of 24 hours. In medium supplemented with As(III), the three strains showed similar growth rates (Fig. 5). In contrast, in presence of As(V), the *tef4Δ* mutant grew slower, with a maximal specific growth rate 20-40% lower than that of parental strain BY4741 and of the mutant *tef3Δ*.

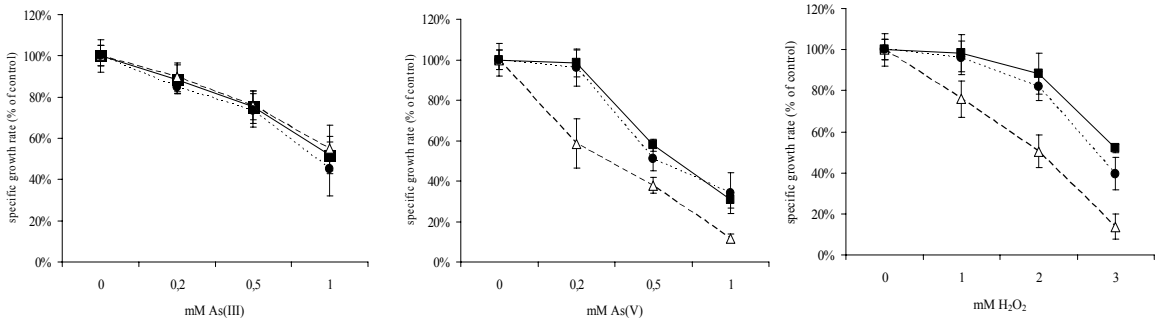


Fig.5. Sensitivity of *tef4Δ* mutant to arsenic and H_2O_2 in liquid YPD medium. Cultures of BY4741, *tef3Δ* and *tef4Δ* mutants, with initial OD_{600}~0,05 were cultivated for 24 hours. The specific growth rate of BY4741 (■), *tef3Δ* (●) and *tef4Δ* (Δ) in medium with various concentrations of As(III) (A), As(V) (B) and H_2O_2 (C) was calculated from the growth curves, and presented as the percentage of the specific growth rate (h^{-1}) of each strain on YPD without addition of arsenic or hydrogen peroxide. Vertical bars represent the standard error of three independent experiments.

As discussed in Chapter 2 of the Introduction, the initial steps of detoxification for As(III) and As(V) differ in *S. cerevisiae*. As(V) is taken up via phosphate transporters, and As(III) by the aquaglyceroporin Fps1p. As(V) is reduced to As(III) which is then extruded or sequestered in the vacuole. In yeast, the reduction of As(V) requires GSH as a donor of reducing equivalents in the

action of arsenate reductase (Mukhopadhyay *et al*., 2000). In humans, arsenate reduction is carried out by an omega class GST, hGSTO-1 (Aposhian *et al*., 2004), for which As(V), MMA(V) (monomethylarsonic acid) and DMA(V) (dimethylarsonic acid) can act as substrates. An attractive hypothesis for the arsenate sensitivity phenotype of *tef4Δ* mutant, therefore would be that Tef4p acts as an active GST catalyzing an alternative detoxification reaction with an As(V) species as the substrate. Testing As(V) transformations requires methods based on high-performance liquid chromatography (HPLC) separation coupled with inductively coupled plasma atomic emission spectrometry (ICP-AES) (Chausseau *et al*., 2000). Unfortunately, these methods are not yet widely available.

Nevertheless, in order to investigate the hypothesis for GST function of Tef4p, we tested the viability of the disruption mutant to the most frequently used GST model substrate –CDNB (1-chloro-2,4-dinitrobenzene). The strain lacking *TEF4* gene was more sensitive than the wild-type strain and *tef3Δ* mutant, and presented a significant growth defect when spotted onto plates containing CDNB (Fig. 6).

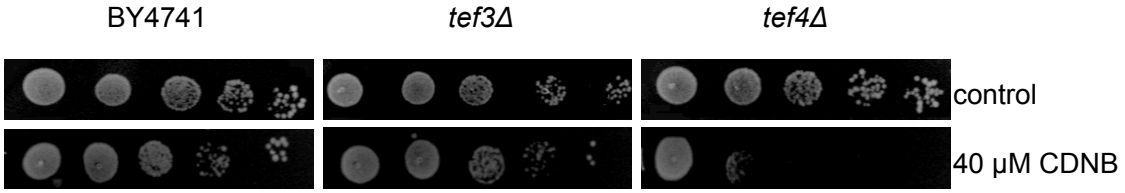


Fig.6. Sensitivity of mutants *tef3Δ* and *tef4Δ* to CDNB (1-chloro-2,4-dinitrobenzene). See legend of Fig. 4. for growth condition details.

In addition to its sensitivity to arsenate and CDNB, the mutant *tef4Δ* also showed decreased viability upon H_2O_2 exposure in both solid and liquid rich media (Fig. 5 and 7). As CDNB, hydrogen peroxide is a GST substrate, with many GSTs exhibiting glutathione peroxidase activity. However, GPx activity was not observed for rice recombinant EF1Bγ (Kobayashi *et al*., 2001), which is the only such enzyme to have been investigated experimentally.

As presented in the right panel of Fig. 5, the specific growth rate of *tef4Δ* at concentrations of 1-3 mM H_2O_2 was 30-40% lower that the specific growth rates of BY4741 or *tef3Δ*. Interestingly, growth of the mutant in presence of H_2O_2 depended on the growth phase of the preculture used (Fig. 7). The mutant was more sensitive to peroxide when the preculture used was in exponential phase of growth (OD_{600}~0,3) than when it was in stationary phase (OD_{600}~1,5). This was not

entirely surprising, as in general *S. cerevisiae* is more resistant to H_2O_2 in stationary growth phase, when the membrane permeability for H_2O_2 is decreased (Sousa-Lopes *et al*., 2004).

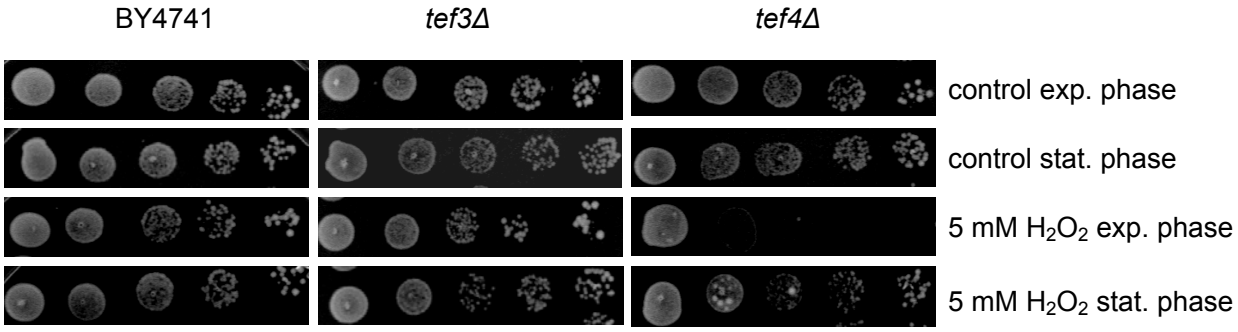


Fig.7. Sensitivity of mutants *tef3Δ* and *tef4Δ* to H_2O_2. Strains were cultivated in liquid YPD medium until exponential (OD_{600}~0,3) or stationary growth phase (OD_{600}~1,5). Each culture was serially diluted and then spotted on solid YPD medium with or without addition of 5 mM H_2O_2.

The phenotype presented by *tef4Δ* mutant was quite similar to that of the glutathione reductase deletion mutant, *glr1Δ* (described in Chapter 1 of Results), which is a result of decreased glutathione content in mutant cells. Glutathione is the primary mean of detoxifying H_2O_2 in *S. cerevisiae* (Grant *et al*., 1998), and is also required for protection against arsenate and electrophilic compounds as CDNB. Therefore, alternative explanation for arsenate, peroxide and CDNB sensitivity of *tef4Δ* would be impaired glutathione metabolism. Indeed, the mutant showed a decrease in the level of both reduced and oxidized glutathione in cell free extracts (Table 1). The content and redox state of GSH was analysed in wild-type and mutant strains over a range of stress conditions, respectively exposure to As(III), As(V) and H_2O_2. Oxidized glutathione (GSSG) was detected at low levels in unstressed cells (YPD cultivation), approximately 5-fold lower than that of reduced GSH. There was a significant increase in both GSH and GSSG after arsenic and peroxide exposure, approximately 2-fold for the reduced GSH and 4-fold for oxidized glutathione. An important parameter of GSH metabolism is the redox ratio (GSSG/GSH), which gives a measure of the proportion of GSH present in the oxidized state relative to the reduced form. The GSH redox ratio was substantially increased in all tested conditions, indicating that arsenic and H_2O_2 shift the redox balance of the cell to a more oxidized state, as expected. The main difference between the wild type and *tef4Δ* mutant was that the glutathione levels in the *tef4Δ* mutant strain were decreased by approximately 2-fold, whereas the ratio between the reduced and oxidized GSH remained unaffected (Table 1).

The overall phenotypic characterization of *tef4Δ* mutant evokes two possible hypotheses for a role of Tef4 protein in overall detoxification process of *S. cerevisiae*. It is possible that Tef4p is an active GST playing a critical role in arsenate, CDNB and peroxide detoxification. Another possibility is that Tef4p functions in glutathione metabolism through an unknown pleiotropic mechanism, and that the sensitivity of the disruption mutant to these toxic compounds is a result of the decreased GSH and GSSG content. To address this question, we attempted to isolate and purify the corresponding Tef4 protein.

Table 1. Glutathione content of *tef4Δ* mutant after arsenic and peroxide exposure.

strain	condition	GSH*		GSSG*		GSSG/GSH ratio
		mmol mL mg protein^{-1}	% of GSH content on YPD	mmol mL mg protein^{-1}	% of GSSG content on YPD	
BY4741	YPD	0.14	100	0.03	100	0,21
BY4741	YPD+As(III) 0,2 mM	0.30	214	0.11	367	0,37
BY4741	YPD+As(V) 0,5 mM	0.25	179	0.14	467	0,56
BY4741	YPD+H_2O_2 2 mM	0.29	207	0.10	333	0,34
tef4Δ	YPD	0.09	100	0.02	100	0,22
tef4Δ	YPD+As(III) 0,2 mM	0.16	178	0.07	350	0,44
tef4Δ	YPD+As(V) 0,5 mM	0.13	144	0.07	350	0,54
tef4Δ	YPD+H_2O_2 2 mM	0.14	156	0.08	400	0,57

* presented values are mean of two independent experiments

2.2. Cloning and expression of TEF4 *gene*

The first aim of cloning *TEF3* and *TEF4* genes was to confirm by complementation that the observed mutant phenotypes were the result of disrupted genes and not a secondary effect. In order to characterize the products of *TEF3* and *TEF4* genes, expression of the proteins in both homologous and heterologous overexpression systems was attempted.

Gene specific PCR primers were designed based upon the DNA sequences of *TEF3* and *TEF4*, and used to amplify a 2300 or a 2600 bp fragment respectively (for details on primer design and PCR conditions see Annex 2). The primers were designed to amplify the genes as well as ~1000 bp

upstream sequence of the coding regions which could be involved in regulation of gene expression. Since *TEF4* features an intron in its structure, the coding sequence from total mRNA was also amplified by RT-PCR reaction.

The PCR products were digested with *Xba*I/*Xho*I (*TEF3*) or *BamH*I/*Xho*I (*TEF4*), and the isolated DNA fragments were ligated into the yeast shuttle vector p413 (Funk *et al*., 2002) previously digested with the appropriate enzymes. Resulting plasmids were pME8510 (p413-$TEF4_{DNA}$), which contains the promoter region of *TEF4*, as well as the genomic DNA copy of the gene with the intron; pME8511 (p413-$TEF4_{RNA}$), which has *BamH*I/*Xho*I inserted *TEF4,* amplified from mRNA without intron and any upstream sequences and pME8520 (p413-*TEF3*), which contains the genomic DNA copy of *TEF3* gene with ~1000 bp upstream sequence of the gene. The correct gene insertion, orientation and sequences were confirmed by DNA sequencing.

Each of the three plasmids was transformed into mutant and parental strains to complement the phenotype of *tef4Δ* mutant. Transformants were selected on minimal medium without histidine. Expression from the GALS promoter was induced with galactose as described in Annex 2, and transformed strains were spotted on minimal galactose medium with previously used concentrations of arsenate, H_2O_2 and CDNB. No complementation of the *tef4Δ* mutant phenotype was observed with any of the investigated plasmids. As shown in Fig. 8, there was no detectable overexpression in cell free extracts from wild type cells transformed with pME8511.

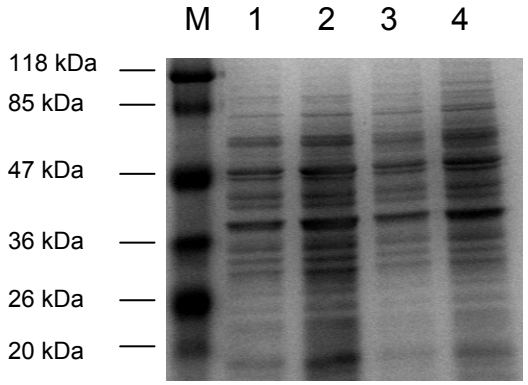


Fig. 8. Analysis of overexpressed Tef4p in *S. cerevisiae* by SDS–PAGE (12%). Protein (approx. 10 µg on each lane, determinated by the method of Bradford) was visualized by staining with Coomassie brilliant blue. Lane M, Protein standards; lane 1, crude extract from uninduced culture BY4741 + pME8511, grown in glucose medium; lane 2, crude extract from pre-induced culture, transferred after 16 hours of growth in glucose in medium containing 1% glucose + 1% galactose; lanes 3 & 4, crude extract from induced cultures, transferred after 6 hours of growth in pre-induction medium into galactose induction medium, and grown for additional 16 or 20 hours respectively.

It was then attempted to express the recombinant forms of Tef3p and Tef4p heterologously in *E. coli* using the pET overexpression system (http://www.merckbiosciences.co.uk/docs/NDIS/C183-002.pdf). The two genes (*TEF3* and *TEF4* without intron) were digested from plasmids pME8511 and pME8520 and ligated to pET24b vector to obtain the *E. coli* expression plasmids pME8512 (pET24b-$TEF4_{RNA}$) and pME8521 (pET24b-*TEF3*). *BL21(DE3)* cells were transformed and induced as described in Annex 2. Upon addition of IPTG, a visible band corresponding to the molecular weight of Tef3p and Tef4p (47 kDa) was observed in SDS-PAGE gels (Fig. 9). However, no detectable CDNB activity was measured in cell free extracts of *BL21(DE3)* transformed with either pME8512 or pME8521 (data not shown).

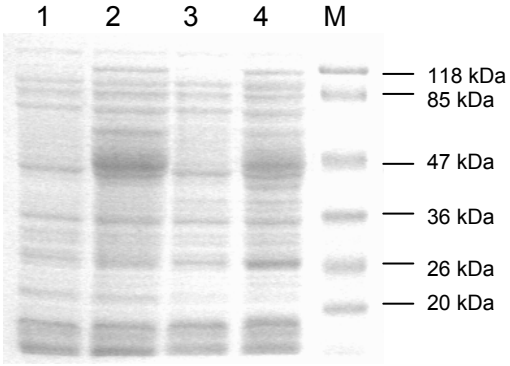


Fig. 9. Analysis of overexpressed Tef4p and Tef3p in *E.coli BL21(DE3)* cells by SDS–PAGE (12%). Protein was visualized by staining with Coomassie brilliant blue. Lane 1, crude extract from uninduced *BL21(DE3)*, transformed with pME8521 (pET24b-*TEF3*); lane 2, crude extract from induced with IPTG, *BL21(DE3)* + pME8521 (pET24b-*TEF3*); lane 3, crude extract from uninduced *BL21(DE3)*, transformed with pME8512 (pET24b-$TEF4_{RNA}$) and lane 4, crude extract from induced with IPTG, *BL21(DE3)*, transformed with pME8512(pET24b-$TEF4_{RNA}$). Total protein loaded in each lane was approx. 15 µg.

Despite this encouraging result, the question why a functional copy of the *TEF4* gene could not complement the mutant phenotype has remained unclear. In addition, it must be pointed out that some authors have reported exactly the opposite phenotype for *tef4Δ* mutant – namely resistance to oxidative stress (Olarewaju *et al.*, 2004). Was the determined phenotype a non specific and secondary effect as a result of gene disruption? Or there was a not determined problem with the correct expression of the protein? All these questions need to be clarified in future experiments. One possibility for future work would be to test other vectors, promoters and expression conditions. However, the most promising and immediate perspective of this work is the isolation and

purification of Tef4p and Tef3p from heterologous system which may permit functional characterization of the recombinant proteins. Further utilisation of the Tef3/Tef4 GST couple may contribute to the determination of functionally important residues for *tef4Δ* mutant phenotypes. Approaches such as random and/or directed mutagenesis and DNA shuffling would be another interesting possibility to uncover a catalytic and/or regulatory function for either of the *TEF* homologues investigated here.

Annex 2

Materials and methods

Strains, media and growth conditions

The strains, media and growth conditions used are the same as in Chapter 1 of Results (described in Annex 1).

Determination of glutathione level

BY4741 and *tef4Δ* strains were cultivated in YPD medium until OD_{600} reached approximately 0,8. The cultures were then treated with 0,2 mM $NaAsO_2$ (Sigma), 0,5 mM $NaHAsO_4.7H_2O$ (Sigma) or 2 mM H_2O_2 (Fluka) for 30 min. Cells were collected, washed, resuspended in equal volume of 0,05 M potassium phosphate buffer pH 7,8 and disrupted with glass beads (diameter – 0,5 mm, Sigma) (4x10 min) on ice. The resulting homogenates were centrifuged 30 min at 14000 rpm at 4°C resulting in cell free extracts.

Cellular GSH was determined by the method of Ellman (Zhang, 2000). One mL of 5% TCA was added to 2 mL sample to precipitate the proteins and the extract was centrifuged for 5 min at 5000 rpm. Fifty µL of the obtained supernatant was added to 400 µL 0,1 M potassium phosphate buffer pH 7,5. The reaction was started with 50 µL 10mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; dissolved in methanol, Fluka) and the OD at 412 nm was measured after 2 min of incubation at room temperature.

GS-SG was quantified after conversion of cellular GS-SG to GSH by glutathione reductase and NADPH. One hundred µL of the sample was incubated with 900 µL reaction mixture (0,1 M potassium phosphate buffer pH 7,5, 0,5 EU/mL glutathione reductase (Fluka), 0,3 mM NADPH) at 25°C for 30 min. Total GSH was quantified as above, and the amount of GSH measured before reduction was subtracted to obtain GS-SG content of each sample.

Concentration of GSH was calculated by dividing the OD_{412} by 13600 M^{-1} cm^{-1} (the extinction coefficient of DTNB reagent), and by multiplying by the total volume/sample volume ratio of the solution. All results were normalized for protein concentration in each sample, determined by the

method of Bradford with bovine serum albumin as a standard (BioRad Protein Assay; Bradford, 1976).

DNA and RNA isolation

S. cerevisiae was cultivated in YPD medium at 30°C until OD_{600}~1. Cells from 10 mL were collected by centrifugation (2500 rpm, 10 min). Pellets were washed with water and resuspended in breaking buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA, 100 mM NaCl, 1% SDS and 2% Triton X-100). Glass beads (0,5 mm) and 250 µL phenol/chloroform solution were added, and cells were disrupted by vortexing (3 min). After centrifugation (12000 rpm, 5 min), DNA was ethanol-precipitated from supernatant (2x), air dried and collected in 50 µL TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8).

Total cellular RNA was isolated by using TRIzol Reagent (Invitrogen). Cells from 30 mL cultures were harvested and homogenized with 1 mL TRIzol and 0,2 mL chloroform, vortexed with glass beads and the RNA presented in the aqueous phase was precipitated with isopropyl alcohol. After 10 min of incubation, RNA samples were centrifuged, washed with ethanol, air dried and dissolved in RNase-free water.

Isolated RNA was DNAse1 treated (RQ1, Promega) with one unit of enzyme per 1 µg of RNA and conserved in aliquots at -80°C until further use. The purity of prepared RNAs was determined spectrophotometrically. Only RNAs with A_{260}/A_{280}>1,8 were used.

Amplification of TEF3 and TEF4 genes

A *Taq* DNA polymerase (New England BioLabs) and standard PCR protocol (30 cycles of denaturation at 95°C for 1 min, annealing step for 90 sec and polymerization at 72°C for 2 min.) were used. The specific annealing temperatures were 45°C for *TEF3* amplification and 48°C for *TEF4*. Primers for amplification of *TEF3* were tt5 (‘5CGTCTAGATTAGCAACAGGAGGAA3’) – forward primer, amplifying 980 bp upstream from ATG codon and introducing *Xba*I restriction site, and the reverse primer tt6 (‘5CTACTCGAGCATACTTTATAATAAA3’), amplifying 20 bp downstream from the stop codon and introducing *Xho*I site. *TEF4* was amplified from genomic wild-type DNA with forward primer tt7 (‘5TAGGATCCTAATGGTATTTCATTTC3’) recognizing

1000 bp upstream from ATG codon and introducing *BamH*I restriction site and tt8 (‘5TGACTCGAGTGTATATTGGGAAGAAAA3’) amplifying 20 bp downstream from gene stop and introducing *Xho*I restriction site. In parallel, *TEF4* was amplified from total cellular RNA with QIAGEN OneStep RT-PCR Kit and oligos tt13 forward (‘5TTTGGATCCCTCGATAGATTCAAGA3’), recognizing 20 bp upstream from ATG and introducing *BamH*I site and the reverse primer tt8. The annealing temperature was 51°C and the standard kit protocol was used.

Cloning of TEF3 *and* TEF4 *genes*

Amplified products were gel extracted (QIAquick Gel Extraction Kit, QIAgen) and digested with the appropriate restriction enzymes. They were ligated with Quick Ligation Kit (New England BioLabs) to *Xba*I/*Xho*I or *BamH*I/*Xho*I digested shuttle vector p413 (a 5611 bp yeast expression vector with GALS-promotor, a kind gift from Ivan Tarassov) or *E. coli* expression vector pET24b (Novagen). The ligation mixtures were transformed into competent *E. coli* DH5alpha, and the resulting plasmids pME8510 (p413-$TEF4_{DNA}$), pME8511 (p413-$TEF4_{RNA}$), pME8512 (pET24b-$TEF4_{RNA}$), pME8520 (p413-*TEF3*) and pME8521 (pET24b-*TEF3*), were isolated and sequenced.

Expression of TEF3 *and* TEF4 *in yeast*

Plasmids pME8510, pME8511, pME8520 and vector p413 were transformed into wild type, *tef3Δ* and *tef4Δ* strains by electroporation (BioRad Micropulser, standard manufacturer’s protocol). Transformants were selected on minimal YNB medium without histidine.

Transformant strains were cultivated until OD_{600}~1 in minimal medium with 2% glucose as carbon source for induction of GALS promoter. The glucose was then removed by repeated washing and the cells were transferred to induction medium containing galactose at concentration of 2%. After 24 hours of induction, cells were harvested and were either spotted on YNB plates with galactose as the carbon source and various concentrations of arsenate, H_2O_2 and CDNB or disrupted to obtain cell free extracts as described above.

Expression of TEF3 *and* TEF4 *in E.coli*

Plasmids pME8512 (pET24b-$TEF4_{RNA}$) and pME8521 (pET24b-*TEF3*) were transformed into the *E. coli* expression strain *BL21*(*DE3*). Bacteria were grown at 37°C in LB medium containing 50 $mg.L^{-1}$ kanamycin. Target protein expression was induced by addition of 0.25 mM IPTG to mid-log phase cultures (OD_{600}~0.6). After 4 h of additional growth, bacteria were harvested by centrifugation. The cell pellet was resuspended in a buffer containing 20 mM potassium sodium phosphate buffer pH 7, 1 mM GSH and 10% glycerol. Cells were disrupted on ice by sonication (altering cycles of 2 sec sonication pulses with 9 sec pause for a total of 5 min). Cell debris were spun down by centrifugation at 15000 g, 4°C for 45 min. Supernatant aliquots with equal protein content were subsequently loaded to 12% SDS-PAGE and electrophoretically separated (approx. 100 mV for 1 hour).

SENSITIVITY OF *SACCHAROMYCES CEREVISIAE* GST MUTANTS TO ARSENATE

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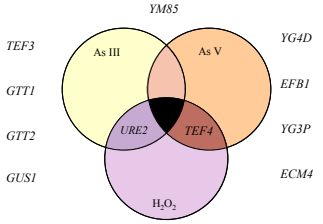
Arsenic is a toxic metalloid present in natural and polluted industrial environments. It is a human carcinogen, but is also used in treatment of acute promyelocytic leukemia and protozoan parasitic diseases. When mammals are exposed to arsenate, it is reduced to arsenite either by PNP arsenate reductase or by MMA(V) reductase, a member of the omega class of glutathione S-transferases (GST). This family of enzymes catalyzes the conjugation of electrophilic toxic compounds to the thiol group of glutathione (GSH), and therefore plays a critical role in the detoxication of xenobiotics. GSTs have been grouped into eight classes basing on their sequence, their substrate specificity, and on structural and immunological properties. In contrast to GST representatives from mammals, plants and even bacteria, little is known about the GSTs of yeasts and fungi, although they appear to be particularly diverse both structurally and functionally.

We have taken a systematic approach to study the potential role of GSTs in arsenic toxicity in *Saccharomyces cerevisiae*. A search of annotations in the *Saccharomyces* genome database suggests the presence of 11 genes and ORFs with homology to GSTs in *S. cerevisiae*. Mutants of *S. cerevisiae* in which a single *GST* gene was disrupted were tested for their sensitivity to As(III) and As(V). A mutant disrupted in the gene *TEF4* encoding a translation elongation factor EF1γ and GST homolog displayed hypersensitivity to As(V), indicating a possible participation of the Tef4 protein in arsenate detoxification.

Screening of GST mutant bank

Mammalian GSTs are implicated in arsenic detoxification → Sensitivity to arsenic



GSTs have glutathione peroxidase activity → Sensitivity to a typical oxidant substrate - H_2O_2

Sensitivity of a *TEF4* disruption mutant to arsenate and GST model substrates

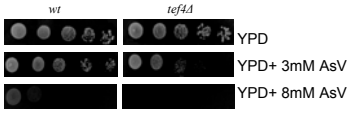


Fig.1. Sensitivity of mutant *tef4Δ* to $NaHAsO_4.7H_2O$ on YPD medium. Parental strain BY4741 (wt) and mutant *tef4Δ* were cultivated overnight at 30°C/140 rpm in liquid YPD medium, serially diluted, and 5 µL of each dilution were spotted on YPD plates at the indicated concentration of $NaHAsO_4.7H_2O$ (AsV). Colonies were photographed after 5 days incubation.

Fig.2. Sensitivity of mutant *tef4Δ* to H_2O_2. Parental strain BY4741 (wt) and mutant *tef4Δ* were cultivated in liquid medium until exponential or stationary growth phase. Each culture was serially diluted and then spotted on solid YPD medium containing 5mM H_2O_2.

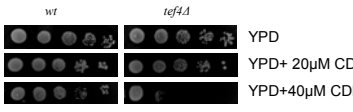


Fig.3. Sensitivity of mutant *tef4Δ* to CDNB (1-chloro-2,4-dinitrobenzene). Parental strain BY4741 (wt) and mutant *tef4Δ* were cultivated overnight in liquid YPD medium. Each strain culture was serially diluted and then spotted on solid medium containing different concentrations of CDNB.

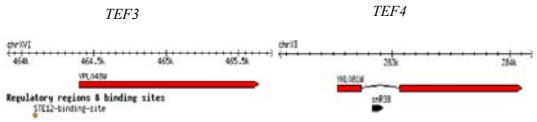


Fig. 4. Schematic view of *TEF3* and *TEF4* genes. Tef3p and Tef4p are translation elongation factors which share 64.5 % identity and 77.5 % similarity in sequence at the protein level.

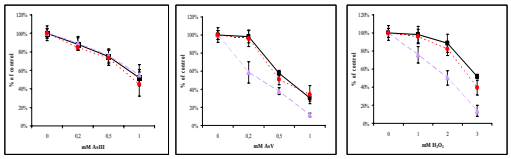


Fig.5. Sensitivity of *tef4Δ* mutant to arsenic and to H_2O_2 in liquid YPD medium. Cultures of BY4741, *tef3Δ* and *tef4Δ* with initial OD_{600}~0,05 were cultivated for 24 hours. The specific growth rate of BY4741 (■), *tef3Δ* (●) and *tef4Δ* (▲) in medium with different concentrations of AsIII (A), AsV (B) and H_2O_2 (C) was calculated from the growth curves, and is presented here as the percentage of the specific growth rate of each strain on YPD without addition of arsenic or hydrogen peroxide. Vertical bars represent the standard error of three independent experiments.

strain	condition	GSH (µmol/ml/mg protein)	GS-SG (µmol/ml/mg protein)
wild type	YPD	0.14	0.03
wild type	YPD+AsIII 0.2mM	0.30	0.11
wild type	YPD+AsV 0.5mM	0.25	0.14
wild type	YPD+H_2O_2 2mM	0.29	0.10
tef4Δ	YPD	0.09	0.07
tef4Δ	YPD+AsIII 0.2mM	0.16	0.07
tef4Δ	YPD+AsV 0.5mM	0.13	0.07
tef4Δ	YPD+H_2O_2 2mM	0.14	0.08

Table 1. Glutathione content of mutant *tef4Δ*. Concentrations of reduced and oxidized GSH were measured in cell-free extracts of wild type and mutant cultures induced for 30 min with arsenic or hydrogen peroxide.

Conclusions

The screening of mutants in all GST-like genes of *S. cerevisiae* showed that the mutant *tef4Δ* is sensitive to arsenate and hydrogen peroxide. The gene *TEF4* together with *TEF3* code for translation elongation factors EF1γ. Despite their high similarity in sequence, the two factors Tef3p and Tef4p probably have different functions. Only the product of *TEF4* gene is required for arsenate and oxidant detoxification in *S. cerevisiae*. In addition, the disruption mutant is sensitive to the model GST substrate CDNB, and has a lower level of reduced and oxidized intracellular glutathione. These findings suggest that Tef4p is a GST with a possible role in arsenate reduction and in the oxidative stress response of *S. cerevisiae*.

References: 1. S. Vuilleumier & M. Pagni (2002) Appl Microbiol Biotechnol 58:138–146; 2. H. V. Aposhian et al. (2004) Toxicol Appl Pharmacol 198: 327–335; 3. T. G. Kinzy, T. L. Ripmaster & J. L. Woolford Jr (1994) Nucleic Acids Res 22: 2703-2707; 4. http://www.yeastgenome.org

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Chapter 3

Regulation of arsenite uptake – a novel non-enzymatic role for the glutathione S-transferase Ure2 of *Saccharomyces cerevisiae*

Résumé en français

Régulation du transport de l'arsénite : un rôle inédit de la glutathion S-transférase Ure2p chez *Saccharomyces cerevisiae*

La protéine Ure2p est un "prion" faisant l'objet de nombreuses études au niveau international. Les prions sont des agents infectieux de nature protéique impliqués dans nombreuses maladies neurodégénératives comme la maladie de Creutzfeldt Jacob (MCJ) chez l'homme ou la maladie de «la vache folle» chez les animaux. Les hypothèses avancées pour expliquer la propagation de ces pathogénies reposent sur la capacité de la protéine prion à changer de conformation : passant d'une forme soluble, active et sensible à la protéolyse, à une forme agrégée, insoluble et résistante à la protéolyse. Les événements moléculaires à l'origine de la propagation des prions sont encore mal connus et font l'objet d'une forte mobilisation scientifique. La découverte chez la levure et les champignons filamenteux, de protéines non pathogènes, présentant des caractéristiques similaires à celles du prion de mammifère a ouvert la voie à des recherches fondamentales et appliquées sur des modèles d'abord plus simples que les prions des mammifères.

Chez la levure *S. cerevisiae*, la protéine Ure2 est à l'origine de l'apparition du phénotype [URE3] qui est dominant, invasif et transmissible. Les rôles de cette protéine, déjà bien caractérisée au niveau structurel, sont multiples et complexes : régulation de type GATA du métabolisme de l'azote, de la dégradation du glutathion, de la signalisation du glucose et participation à la résistance au stress osmotique. Par ailleurs, différentes études récentes ont révélé un rôle de cette protéine dans la résistance à différents métaux.

Dans le cadre de notre travail, des expériences complémentaires visant à éclairer le rôle du gène *URE2* dans le stress à l'arsenic ont été entreprises. Le mutant *ure2Δ* a fait l'objet d'une étude plus détaillée, concernant en particulier la détoxification de l'arsénite. L'activité glutathion péroxydase, qui est la seule activité connue de la protéine Ure2, n'est pas altérée en extrait cellulaire après exposition à l'arsenic. En revanche, une dépendance de la réponse à l'arsenic de la source d'azote utilisée a été démontrée pour *S. cerevisiae*, indiquant une interaction possible entre la détoxification de l'arsenic et la régulation de type GATA. Une des énigmes les plus persistantes sur le rôle des GSTs concerne l'implication avérée de plusieurs de ces protéines dans la régulation de différents processus, rôle dont nous suspections de ce fait l'existence dans le cas de Ure2p.

Dans notre travail, nous avons montré que des motifs caractéristiques GATA sont présents dans le promoteur du gène *FPS1* (codant pour le transporteur majeur de l'arsénite), et que l'expression de ce gène est légèrement augmentée au niveau de la transcription chez le mutant *ure2Δ*. Par ailleurs, en conditions excluant le transport de l'arsénite par Fps1p (milieu sans hexoses), le mutant *ure2Δ* n'est plus sensible à l'arsénite.

Au regard des résultats obtenus et des études publiées, nous proposons une hypothèse impliquant une régulation de type GATA par Ure2p pour la détoxification de l'arsenic chez la levure, agissant en particulier au niveau du transport de l'arsénite.

1. Introduction

The protein Ure2 of *Saccharomyces cerevisiae* has been extensively studied in three main different aspects: as a major negative regulator of GATA dependent transcription (Cooper, 2002); as a precursor of protease resistant altered form, [URE3], a prion-like protein (Wickner *et al.*, 2000); and as a glutathione S-transferase (GST) (Choi *et al*., 1998; Bousset *et al*., 2001).

The *URE2* gene was characterized in detail, as a consequence of the classical work of Lacroute in the 1960s (Lacroute, 1971), who isolated recessive (*ure2*) and dominant (*URE3*) mutations both determining the ability to overcome the effect of nitrogen catabolite repression (NCR), a regulatory mechanism involved in the cell response to different nitrogen sources. When *S. cerevisiae* is cultivated on a preferred nitrogen source such as ammonia, the expression of genes required for the utilisation of alternative nitrogen sources is strongly repressed (for reviews see Cooper, 2002; Magasanik & Kaiser, 2002). In the absence of a preferred nitrogen source, these genes are induced and expressed at high levels to ensure the assimilation of the non-preferred nitrogen source. Under N-rich conditions, the *URE2* gene product forms cytoplasm-localized complexes with two proteins Gln3p and Gat1p, which belong to the GATA family of transcription regulators and recognize specific GATAA containing core sequences in the promoter regions of genes for the assimilation of non-preferred nitrogen sources (Cooper, 2002). Accordingly, in *ure2Δ* mutants or in strains expressing the [URE3] prion form, Gln3p and Gat1p are free to enter the nucleus and to activate transcription of GATA regulated genes. The GATA regulation system was originally discovered in the context of nitrogen utilisation, but it has since become implicated in the cell response to salt stress (Crespo *et al.*, 2001) and in the degradation of glutathione (Springael & Penninckx, 2003).

The Ure2p molecule is composed of two distinct sequence regions. The N-terminal part, from residue 1 to residue 93 represents the prion-inducing domain, responsible for self-propagation in [URE3] aggregates (Masison & Wickner, 1995). The C-terminal part (residues 94-354) is the part of the protein whose sequence and three-dimensional structure are homologous to that of glutathione S-transferases (Coschigano & Magasanik, 1991; Bousset *et al*., 2001), a family of enzymes catalyzing the conjugation of toxic electrophilic compounds to glutathione (Vuilleumier & Pagni, 2002). Despite its similarity to GSTs and the finding that Ure2p binds glutathione and has glutathione peroxidase activity with inorganic and organic peroxides (Bai *et al.*, 2004), attempts to demonstrate activity of Ure2p with canonical GST model substrates such as 1-chloro-2,4-dinitrobenzene (CDNB), have been unsuccessful so far (Choi *et al*., 1998). Nevertheless, recent

studies with other electrophilic compounds, such as transition metals and oxidants, have demonstrated a particular sensitivity of *URE2* disruption mutants to a broad series of toxic agents (Al, As, Cr, Se, Cd, Ni, Co, Cu, Fe, Ag, Hg, H_2O_2, diamide, cumene hydroperoxide and *t*-butyl hydroperoxide) (Rai *et al*., 2003; Basu *et al*., 2004; Rai & Cooper, 2005). Among those, arsenic has been the focus of much recent interest because of its presence as a toxic contaminant of drinking water in many parts of the world (Aposhian *et al*., 2004). Arsenic exists in two major oxidation states – As(III) (arsenite) and As(V) (arsenate). In *S. cerevisiae*, detoxification of As(III) and As(V) differs by the transport mechanisms associated by these processes. Arsenite is transported by aquaglyceroporins, whereas arsenate is transported by inorganic phosphate careers (Tamas & Wysocki, 2001). Somewhat paradoxically given the greater toxicity of As(III), As(V) is reduced to the more toxic form As(III) when entering the cell (Rosen, 2002). In humans, arsenate reduction is catalyzed by a glutathione-S-transferase, hGSTO1 (Zakharyan & Aposhian, 1999), but such a mechanism has not been described in microorganisms. However, glutathione-dependent reduction of arsenate indeed occurs in *S. cerevisiae* (Mukhopadhyay & Rosen, 1998). This reduction requires glutathione as a donor of reduction equivalents and the action of arsenate reductase. In yeast, As(III) can be conjugated to glutathione and sequestered in the vacuole by a protein pump, Ycf1p, or extruded by the membrane protein Acr3p (Rosen, 2002). The vacuole-associated transporter Ycf1p transports As(III) in the form of $As(GS)_3$, whose formation requires glutathione conjugation, but the identity of the enzyme catalyzing the conjugation reaction is not yet known. Both glutathione-dependent detoxification steps, i.e. arsenate reduction and $As(GS)_3$ formation, have not yet been fully studied and their mechanism still remains to be clarified. These observations, and the fact that the exact role of Ure2p in heavy metal resistance has not yet been elucidated, prompted us to address the implication of the GST Ure2p in arsenic detoxification in more detail.

In the present study, we show that Ure2p, and more specifically its GST domain, is required for arsenite resistance in *S. cerevisiae,* but has no effect on arsenate resistance. A dependence of arsenic sensitivity on the nitrogen source used for growth is clearly demonstrated. We also present experimental data supporting the existence of GATA-type regulation of arsenic resistance in yeast, and propose a mechanism for regulation of arsenite uptake in *S. cerevisiae.*

2. Materials and methods

2.1. Strains and media

The *S. cerevisiae* strains used in this study were BY4741 (*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), and the isogenic disruption mutants *ure2Δ* (BY4741 *YNL229c::kanMX4*), *gln3Δ* (BY4741 *YER040w::kanMX4*), and *gat1Δ* (BY4741 *YFL021w::kanMX4*). All strains were obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/).

Strains were grown on rich YPD medium – 1% yeast extract, 2% peptone, 2% glucose (Q-BioGene) and on minimal medium – 0,17% YNB without amino acids and ammonia (Q-BioGene). Minimal medium was supplemented with a source of nitrogen (0,1% glutamate, 0,1% proline or 0,5% $(NH_4)_2SO_4$) and carbon (2% glucose, 2% maltose or 2% glycerol) as indicated. Auxotrophic supplements were added as required (Ura, His and Met at 20 mg L^{-1} and Leu at 60 mg L^{-1} final concentration).

2.2. Arsenic sensitivity assays

Yeast strains were grown overnight at 30°C/140 rpm in YPD or YNB liquid medium. Cells were pelleted, washed twice with fresh medium and then resuspended to OD_{600}~1. Ten-fold serial dilutions with OD_{600}~1; 0,1; 0,01; 0,001; 0,0001 were prepared. Samples (5 µL) of each diluted strain culture were spotted onto YPD or YNB plates containing $NaAsO_2$ (As(III)) or $NaHAsO_4.7H_2O$ (As(V)) (Sigma) at different concentrations. Plates were incubated at 30°C for 3 and 5 days for YPD and YNB medium, respectively.

2.3. Growth experiments

Cultures of *S. cerevisiae* were grown overnight in YPD liquid medium, collected and washed twice with fresh medium. Two-hundred µL of YPD with or without $NaAsO_2$ (As(III)) or $NaHAsO_4.7H_2O$ (As(V)) at the indicated concentrations were inoculated with overnight cultures to an initial OD_{600} of 0,05. Cultures were then incubated in 96 *MicroWell*™ flat-bottom plates (Nunclon) for 24 hours at 25°C/160 rpm, and OD_{600} was measured at regular intervals using a Bio-Tek KC4 plate reader.

2.4. Complementation of ure2Δ mutant phenotype

For complementation, genes *URE2* and *URE2ΔN* (a truncated *URE2* gene with a 279 bp 5' end deletion) were cloned from plasmids pET23b-URE2 and pET23b-URE2(ΔN) (Choi *et al*., 1998) by digestion with *Xba*I and *Xho*I. The resulting 1,0 kb and 0,7 kb fragments were cloned into

*Xba*I/*Xho*I digested yeast/*E. coli* shuttle vector p413 (Funk *et al*., 2002), under the control of the *GALS* promoter. After transformation into *E. coli* DH5α, the resulting plasmids pME8530 (p413-*URE2*) and pME8531 (p413-*URE2ΔN*) were transformed in the *ure2Δ* mutant strain by electroporation (BioRad Micropulser), using the standard protocol provided by the manufacturer. Transformants were selected on minimal medium without histidine. Both the wild-type and mutant strains were transformed with the p413 vector as a control. Transformant strains were cultivated in minimal medium with glucose as the carbon source to an OD_{600}~1. Cells were then transferred to induction medium with galactose at a concentration of 2%. After 24 hours of induction, when OD_{600} of cultures had reached approximately 1, cultures were spotted on YNB plates containing As(III) at various concentrations using galactose as carbon source, as described above.

2.5. RNA isolation and RT-PCR

Total cellular RNA was isolated using TRIzol Reagent (Invitrogen). Briefly, strains were cultivated to an OD_{600}~0,8 at 30°C/140 rpm in YPD, $NaAsO_2$ was added to a final concentration of 1 mM and cultures were further cultivated for 30 min. Cultures (30 mL), either untreated or exposed to arsenite, were harvested and homogenized with 1 mL TRIzol and 0,2 mL chloroform, vortexed with glass beads, and RNA in the aqueous phase was precipitated with isopropyl alcohol. After 10 min of incubation, RNA samples were centrifuged, washed with ethanol, air-dried and dissolved in RNase-free water (50 µL). Isolated total RNA was DNAse1 treated (RQ1, Promega; one unit of enzyme per 1 µg of RNA) and conserved in aliquots at -80°C until use. The purity of prepared RNAs was determined spectrophotometrically, and only preparations with A_{260}/A_{280}>1,8 were used.

Template mRNA (10 ng) was amplified in 10 µL final volume using the OneStep RT-PCR Kit (Qiagen) with gene specific primers at a final concentration of 0,6 µM. Primers of similar length and annealing temperature were designed to amplify a 191 bp fragment of the genes of interest using Primer3 (http://frodo.wi.mit.edu/). Primer sequences were: *FPS1* gene, 5'-AGGAATTTTTAGCCGAGTTT-3' and 5'-GGATGAAACCAAGGATGTTA-3'; *PHO84* gene, 5'-TACTGGGTATCCGTCTTCAC-3' and 5'-ATAAAGGTGGTTGTGTTTGG-3'; *PUT1* gene, 5'-GTTACTAAATCGCGCATACC-3' and 5'-AGTTCCTTCTTGGGAAGTGT-3'; 18S rRNA gene, 5'-TTAACGAGGAACAATTGGAG-3' and 5'-CACAAGGACTCAAGGTTAGC-3'. The reaction was initiated at 50°C for 30 min to synthesize cDNA, and reverse transcriptase was then inactivated at 95°C for 15 min. Amplification of cDNA by PCR was then performed by 30 cycles of 30 sec at 95°C; 1 min at 50°C and 1 min at 70°C. These conditions were chosen so that none of the

analyzed PCR products reached a plateau at the end of the amplification protocol, i.e. PCR reactions were in the exponential phase of amplification. Amplified products were visualized on 1% agarose gels and analyzed by Quantity One Software (BioRad Gel Documentation System). The signal intensity of PCR products under different conditions was normalized relative to 18S rRNA as an external constitutive gene reference. Control PCR reactions omitting the reverse transcriptase step demonstrated the absence of contaminating DNA in the initial RNA preparation.

2.6. Biochemical assays

BY4741 and *ure2Δ* strains were cultivated in YPD medium up to an OD_{600} of approximately 0,8. Cultures were then induced for 30 min with 1 mM of $NaAsO_2$ or $NaHAsO_4.7H_2O$. Cells were collected, washed, resuspended in an equal volume of 0,05 M potassium phosphate buffer pH 7,8, and disrupted with glass beads (0,5 mm, Sigma) in a vibration homogenizer VHG1 (Biotech International Melsungen) on ice (4x10min). The resulting suspensions were centrifuged for 30 min at 14000 rpm at 4°C to yield crude cell-free extracts.

Glutathione peroxidase (GPx) was measured as described (Veal *et al.*, 2002), with H_2O_2 as substrate. One unit of enzyme activity represents 1 μmol NADPH oxidized in 1 min. Initial rates were determined from the linear slope of progress curves after subtracting the non-enzymatic velocities due to the auto-oxidation of GSH by the hydroperoxide determined from the corresponding blank, and using an extinction coefficient for NADPH of 6220 M^{-1} cm^{-1}.

NADPH dependent glutamate dehydrogenase (Gdh1) and NAD-dependent glutamate dehydrogenase (Gdh2) were assayed as described (Doherty, 1970), with the following modifications. To measure the activity of Gdh1, 50 μL of ten-fold diluted cell-free extract were added to 450 μL reaction mixture (50 mM potassium phosphate buffer pH 7,75; 50 mM NH_4Cl; 10 mM α-ketoglutarate and 2 mM NADPH). The reaction mixture for NAD-dependent glutamate dehydrogenase (Gdh2) was 50 mM potassium phosphate buffer pH 8; 0,1 mM β mercaptoethanol; 0,5 mM EDTA; 50 mM NH_4Cl; 10 mM α-ketoglutarate and 2 mM NADH. The oxidation of NADPH or NADH was monitored by recording the absorbance at 340 nm, as described above.

Glutamine synthetase (Gln1) activity was determined as follows: 50 μL of cell-free extract were incubated for 10 min at 45°C with 50 mM imidazole-HCl buffer pH 7,15; 100 mM glutamate; 50 mM NH_4Cl; 50 mM $MgCl_2$ and 1 mM ATP in a final volume of 0,5 mL. The reaction was stopped with 1 mL 10% TCA. After centrifugation, the inorganic phosphate produced from ATP

degradation was measured in the supernatant. One unit of enzyme activity represents 1 μmol inorganic phosphate produced per mg protein.

Cellular GSH content was determined by the method of Ellman (Zhang, 2000). One mL of 5% TCA was added to 2 mL cell-free extract to precipitate the proteins, and the resulting suspension was centrifuged for 5 min at 5000 rpm. Fifty μL of the resulting supernatant was added to 400 μL 0,1 M potassium phosphate buffer pH 7,5. The reaction was initiated by addition of 50 μL 10mM 5,5'-dithio-bis(2-nitro)benzoic acid (dissolved in methanol, final concentration 1 mM). Concentration of GSH was determined spectrophotometrically at 412 nm, using an extinction coefficient of 13600 M^{-1} cm^{-1}.

GS-SG was quantified after conversion of cellular GS-SG to GSH by glutathione reductase (Sigma) and NADPH. One hundred μL of the sample was incubated with 900 μL reaction mixture (0,1 M potassium phosphate buffer pH 7,5, 0,5 EU/mL glutathione reductase, 0,3 mM NADPH) at 25°C for 30 min. The total GSH was quantified as above, and the amount of GSH measured before reduction was subtracted to obtain the GS-SG content of each sample.

All results were normalized for protein concentration in each sample, determined by the method of Lowry with bovine serum albumin as a standard (Lowry *et al.*, 1951).

3. Results

3.1. The GST domain of Ure2p confers arsenite resistance to Saccharomyces cerevisiae

The specific requirement for Ure2p in arsenic detoxification in *S. cerevisiae* was investigated with the wild-type strain BY4741 and ure2Δ mutant. Growth response to arsenite (As(III)) and arsenate (As(V)) was determined by spotting strains onto YPD plates containing arsenic (Fig. 1). After 3 days of incubation, a strong inhibition of ure2Δ ability to grow on medium containing arsenite was observed. The mutant was sensitive even at the relatively low concentrations of 0,2 mM arsenite, while the wild type was able to grow up to 1,3 mM As(III) (Fig. 1 and data not shown). A similar pattern of arsenite sensitivity of a *S. cerevisiae* mutant with a disruption in the *URE2* gene was observed previously in the study of Rai & Cooper, 2005, but in a different strain background.

We characterized strain growth in the presence of arsenite in liquid medium in more detail (Fig. 2A). The wild-type strain BY4741 showed approximately 50% growth inhibition at a concentration of 1 mM As(III), while the viability of ure2Δ mutant was strongly reduced and its specific growth rate was inhibited (approx. 90% reduction of the growth rate, Fig. 2A).

Somewhat surprisingly, however, disruption of the *URE2* gene had no effect on viability of *S. cerevisiae* when cells were spotted on YPD+As(V) plates (Fig. 1). Indeed, although the growth rate of ure2Δ was only ~ 60% of wild type growth rate in medium without any arsenic addition, it is evident that it grew at least at the same rate as BY4741 when cultivated in presence of As(V) (Fig. 2B). In contrast, the study of Rai & Cooper (2005) mentioned above suggested a similar sensitivity of S. cerevisiae to As(V) and As(III) in the background of TCY5 strain.

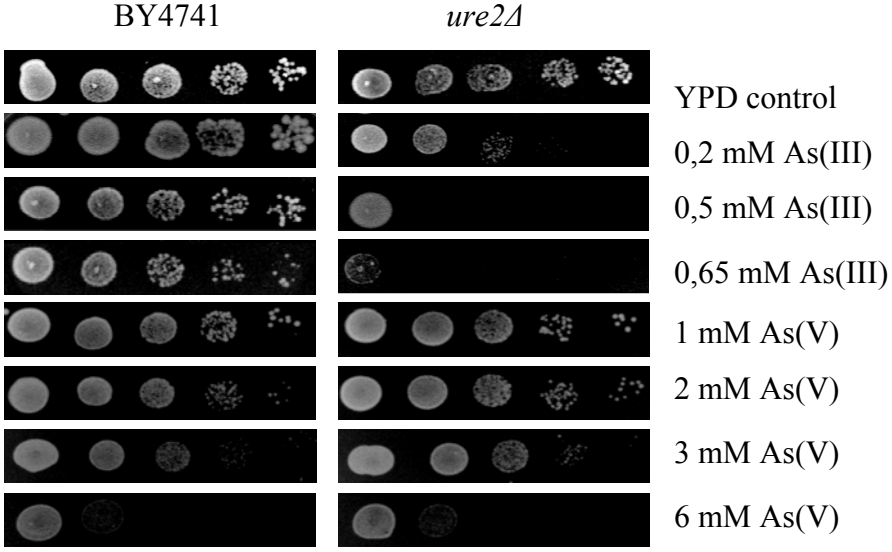

Fig. 1. Sensitivity of *ure2Δ* mutant to arsenite (As(III)) and arsenate (As(V)). Wild-type strain BY4741 and mutant *ure2Δ* strains were cultivated overnight in liquid YPD medium, serially diluted in decimal increments, and 5 µL of each dilution were spotted on YPD plates containing the indicated concentrations of sodium arsenite or sodium arsenate. Plates were photographed after 3 days incubation.

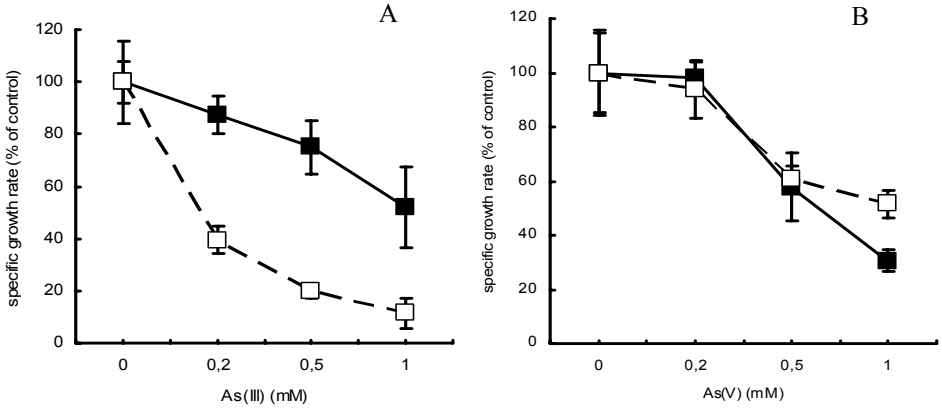

Fig.2. Sensitivity of *ure2Δ* mutant to arsenic in liquid YPD medium. Cultures of BY4741 and *ure2Δ* inoculated from liquid pre-cultures at an initial OD_{600}~0,05 were cultivated for 24 hours, 25°C, 160 rpm. The maximal growth rate in the exponential phase of BY4741 (■) and *ure2Δ* (□) in medium with various concentrations of As(III) (A) or As(V) (B) is presented as the percentage of the specific growth rate of each strain without arsenic (0,173 ± 0,014 h^{-1} for the wild type BY4741 and 0,110 ± 0,017 h^{-1} for the *ure2Δ* mutant). Vertical bars represent the standard error of three independent experiments.

To confirm that arsenite sensitivity of *ure2Δ* was a result of *URE2* gene disruption in strain BY4741, the mutant strain was transformed with plasmid pME8530, a centromeric low copy plasmid bearing the gene *URE2* under the control of the weak inducible promoter *GALS*. After galactose induction, the plasmid completely restored the growth of *ure2Δ* mutant in presence of As(III) (Fig. 3).

The conversion of Ure2p to its amyloidal form is dependent on N-terminal first 93 residues of the sequence, whereas the GST-like C-terminal part of the protein regulates GATA-mediated gene expression (Masison & Wickner, 1995) and has a GPx activity (Bai *et al.*, 2004). To determine which of the two domains of Ure2p confers resistance to arsenite, mutant *ure2Δ* was transformed with plasmid pME8531, bearing a copy of the GST domain of *URE2* without the prion propagation region (279 bp truncation at its 5'-end) (Choi *et al*., 1998). The GST part of *URE2* also complemented the sensitivity of *ure2Δ* mutant to arsenite, while the negative control (*ure2Δ*+p413) remained sensitive to the same degree as the untransformed strain (Fig. 3). Expression of the two forms of Ure2p was confirmed by Western blot analysis using anti-Ure2p antiserum (data not shown).

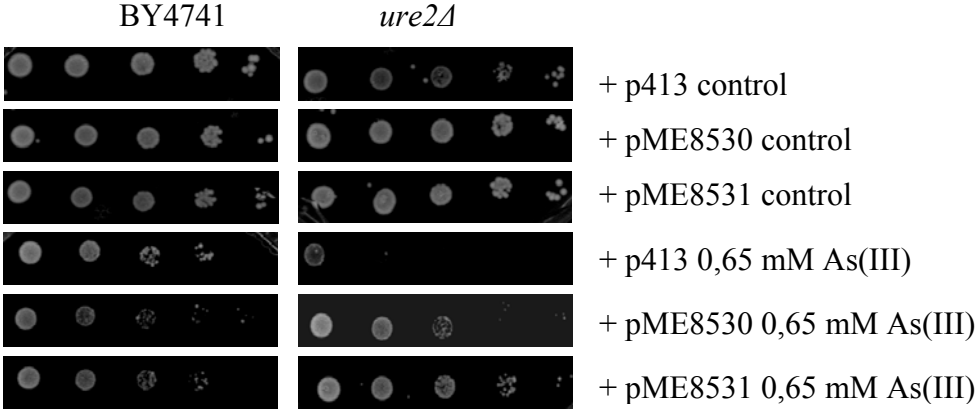

Fig.3. Complementation of *ure2Δ* mutant phenotype. Both wild-type BY4741 and *ure2Δ* mutant strains were transformed with plasmids p413 (vector), pME8530 (p413-*URE2*) and pME8531 (p413-*URE2ΔN*). Resulting transformants were cultivated in minimal medium containing 2% glucose. The cells were then induced with 2% galactose for 24 hours, and 10-fold serial dilutions were spotted (5 µL) on galactose plates with or without 0,65 mM sodium arsenite (As(III)).

3.2. Effects of arsenic exposure on glutathione peroxidase activity, NCR regulation and glutathione content in the ure2Δ *mutant*

Purified recombinant Ure2p shows *in vitro* glutathione peroxidase activity whit both H_2O_2 and organic peroxides (Bai *et al.*, 2004). In order to clarify whether the observed hypersensitivity of

ure2Δ mutant to arsenite is related to the enzymatic properties of the protein, we measured the GPx activity with H_2O_2 in cell-free extracts of BY4741 and *ure2Δ* mutant after 30 min arsenic exposure. As shown in Table 1, the mutant did not show any difference in GPx activity: in both strains, treatment with arsenic resulted in an overall increase of GPx activity in cell-free extracts (by approx. 15% for As(V) and 20% for As(III)).

Table 1. Enzyme activities and glutathione content in cell-free extracts of wild-type (BY4741) and *ure2Δ* cells mutant after arsenic exposure.

Strain/condition	Enzyme activities (U mg protein^{-1})				GSH content (mmol mg protein^{-1})		
	GPx[a*]	Gdh1[b]	Gdh2[c]	Gln1[d]	reduced	oxidized	ratio ox/red
BY4741	23,5±2,9	1,26±0,07	0,11±0,02	0,35±0,07	0,14±0,03	0,02±0,04	0,1
ure2Δ	23,8±0,1	2,25±0,41	4,00±0,12	0,38±0,08	0,03±0,01	0,03±0,01	1,0
BY4741+1mM As(III)	28,6±1,7	1,40±0,12	0,09±0,01	0,36±0,11	0,33±0,01	0,10±0,02	0,3
ure2Δ+1mM As(III)	28,4±3,3	2,50±0,18	4,72±0,18	0,42±0,17	0,04±0,01	0,10±0,02	2,5
BY4741+1mM As(V)	27,5±1,3	1,43±0,23	0,10±0,01	0,40±0,02	0,28±0,01	0,13±0,01	0,5
ure2Δ+1mM As(V)	26,5±4,1	2,19±0,13	4,10±0,25	0,41±0,07	0,03±0,01	0,09±0,02	3,0

[a*]Glutathione peroxidase (in mU mg protein^{-1})
[b]NADPH dependent glutamate dehydrogenase
[c]NAD dependent glutamate dehydrogenase
[d]Glutamine synthetase

The negative regulation of genes for assimilation of poor nitrogen sources is lost in the *ure2Δ* mutant, in which nitrogen catabolic genes are expressed at high levels, irrespectively of the nature of nitrogen source in the medium. When *S. cerevisiae* wild-type is cultivated with ammonia as nitrogen source, two enzymes are notably induced – NADPH-dependent glutamate dehydrogenase (Gdh1), which catalyzes the reaction of glutamate synthesis from α-ketoglutarate and NH_4^+ ions, and glutamine synthetase (Gln1) which adds a second ammonium ion to glutamate to form glutamine (Schure *et al.*, 2000). During growth on glutamate as sole nitrogen source, in contrast, glutamate is converted to ammonium by NAD^+-dependent glutamate dehydrogenase (Gdh2). The expression of Gdh2 is strongly modulated by Ure2p and is induced by non-preferred nitrogen sources, especially glutamate. *GDH1* and *GLN1* genes are also GATA regulated, albeit to a lesser degree (Schure *et al.*, 2000). Moreover, the expression of *GDH2* and *GLN1* genes is strongly derepressed in the *ure2Δ* mutant (Coschigano & Magasanik, 1991). We therefore tested whether

glutamate dehydrogenase and glutamine synthetase activities were affected under conditions of arsenic stress. The data presented in Table 1 reveal that the activities of Gdh1p, Gdh2p and Gln1p in cell-free extracts of BY4741 and *ure2Δ* mutant are not significantly altered after exposure to As(III) or As(V), and therefore cannot be associated with the observed arsenic sensitivity of the *ure2Δ* mutant.

Ure2p also regulates the expression of the *CIS2* gene encoding γ-glutamyl transpeptidase, the first enzyme implicated in glutathione degradation, and the *ure2Δ* mutant shows increased activity of γ-glutamyl transpeptidase, resulting in faster glutathione degradation (Springael & Penninckx, 2003). As glutathione (GSH) may detoxify heavy metals by chelation, or protect against associated oxidation stresses, the sensitivity of *ure2Δ* mutant to As(III) could be explained by a decreased intracellular GSH level in the *ure2Δ* mutant, as previously noted in the *ure2Δ* mutant in the absence of arsenic (Perrone *et al.*, 2005). We thus measured the intracellular level of reduced and oxidized forms of glutathione after As(III) and As(V) exposure, in both wild-type BY4741 and *ure2Δ* mutant strains. Indeed, disruption of the *URE2* gene resulted in a significant decrease in GSH level, and in a strongly altered ratio of oxidized to reduced glutathione (Table 1). The GSH concentration in BY4741 increased 2-folds after exposure to As(III) or As(V), while it remained the same in the *ure2Δ* mutant. Most notably, both As(III) and As(V) exposure caused a similar increase of GS-SG levels in both strains.

3.3. GATA-mediated regulation of arsenic sensitivity in S. cerevisiae

The above findings indicated that neither glutathione peroxidase activity, decreased level of reduced glutathione nor the inhibitory function of Ure2p on the nitrogen assimilation enzymes were associated with arsenite sensitivity in the *ure2Δ* disruption mutant. We therefore investigated whether a direct Ure2p-mediating GATA repression role was at the origin of the observed *ure2Δ* As(III) phenotype. We thus asked whether As(III) sensitivity of *S. cerevisiae* would be affected by the nitrogen source used for growth. Accordingly, wild-type and *ure2Δ* mutant strains were spotted on solid minimal medium, supplemented either with ammonium or with glutamate as sole nitrogen sources, and with addition of 0,65 mM arsenite or 6 mM arsenate. Greater sensitivity to As(III) was observed for both strains growing on glutamate as the nitrogen source (Fig. 4). In contrast, the sensitivity to As(V) was more pronounced when strains were cultivated on ammonium supplemented YNB medium. Similar results were observed on minimal medium with proline as a nitrogen source (data not shown). Taken together, these results confirm and extend the preliminary

observations of Rai & Cooper, 2005, but put the observation of the arsenic sensitivity in *S. cerevisiae* in a new perspective. More specifically, the role of the Ure2p in arsenic resistance may not be enzymatic, but rather regulatory, by specifically affecting the fate of one the arsenic forms.

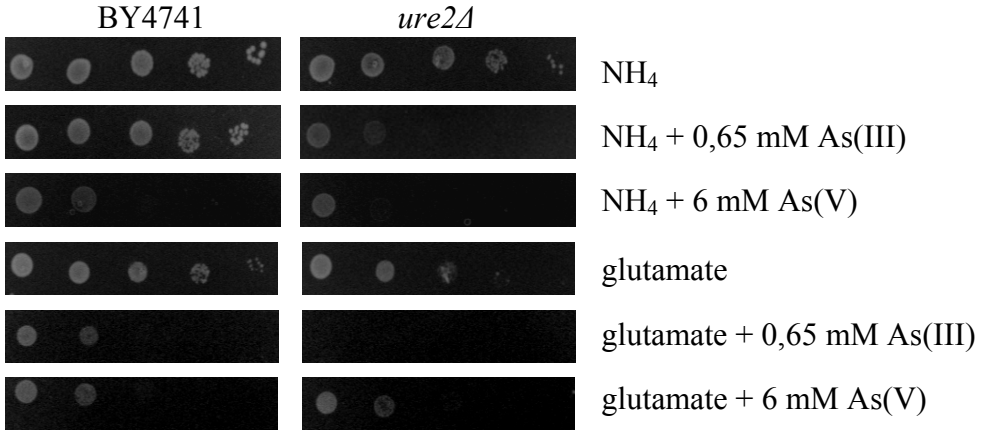

Fig. 4. Sensitivity of *ure2Δ* to arsenite and arsenate in minimal medium, supplemented with ammonium or glutamate as a nitrogen source. After overnight cultivation in minimal medium with glucose as a carbon source and the indicated nitrogen source, 5 µL of serial dilutions of each strain were spotted on the corresponding plates with 0,65 mM sodium arsenite (As(III)) or 6 mM sodium arsenate (As(V)).

Since the *ure2Δ* disruption mutant is sensitive to arsenite but not to arsenate (Fig. 1), this suggested to us that Ure2p may play a role in an initial step of arsenite detoxification, e.g. in As(III) influx, mediated by *FPS1*. In *S. cerevisiae*, arsenite is transported by the membrane-associated protein Fps1p (encoded by the *FPS1* gene), a member of the MIP (major intrinsic protein) family (Wysocki *et al.*, 2001). Arsenate, in contrast, due to its resemblance to inorganic phosphate, is taken up by yeast using phosphate transporters (Bun-ya *et al.*, 1996), and is efficiently reduced to arsenite in the cell (Mukhopadhyay & Rosen, 1998). To investigate whether *FPS1* gene could be GATA regulated, the *S. cerevisiae* genome sequence was searched for the occurrence of potential binding sites for GATA-type regulators (Table 2). Six GATAA/TTATC motifs in the upstream (-1kb) region of *FPS1* were identified (Fig. 5). This number corresponds well with that previously found for known GATA regulated genes, such as general nitrogen assimilation genes, as well as the *ENA1* and *CIS2* genes (Table 2). In contrast, GATAA/TTATC motifs are not found in the promoter region of the *URE2* gene. Additionally, two GATAA sequences were present as a tandem AGATAAGATAAAG site in the *FPS1* upstream region, and two other TTGGT and TTGTT sequences, suggested to define possible binding sites for auxiliary regulators (Magasanik & Kaiser, 2002), were also found.

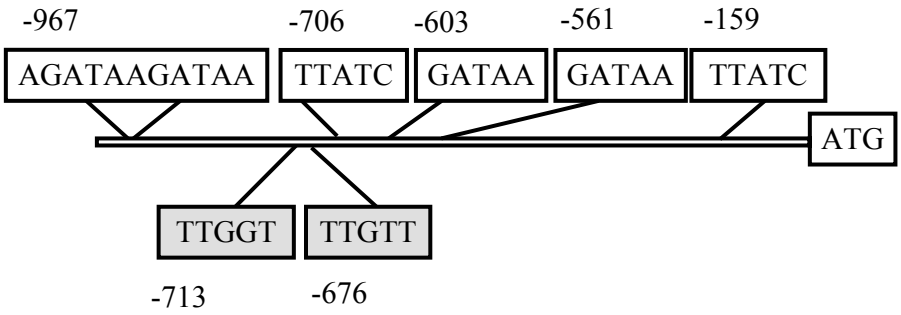

Fig. 5. Scheme of *FPS1* upstream regions (-1kb). Open boxes represent the GATAA/TTATC motifs, shaded boxes are the TTGGT/TTGTT auxiliary motifs. The numbers shows the nucleotide position relative to the ATG start codon of the gene.

Table 2. Comparative analysis of GATAA/TTATC motifs in *S. cerevisiae* genome. The bioinformatics study was done in SGD (*Saccharomyces* Genome Database) with Pattern Matching tool. The number of GATAA/TTATC in *FPS1* upstream (-1 kb) region is compared to those of known nitrogen regulated genes, *ENA1* (encoding a P-type Na+-ATPase essential for sodium detoxification), *CIS2* (γ-glutamyl transpeptidase, a glutathione degradation enzyme) and *PHO84* (arsenate transporter).

Gene	Description	Hits GATAA	Hits TTATC	Total
DUR3	Urea transporter	3	10	13
DAL5	Allantoin and ureidosuccinate permease	8	4	12
UGA4	Gamma-aminobutyrate permease	4	5	9
CAN1	Arginine permease	4	4	8
DAL4	Allantoin permease	5	3	8
DUR1,2	Urea amidolyase	7	1	8
DAL80	Negative regulator of nitrogen-regulated genes	1	6	7
GAP1	General amino acid permease	5	2	7
GAT1	Transcriptional activator of nitrogen-regulated genes	7	0	7
DAL2	Allantoicase	4	3	7
DAL7	Malate synthase	2	5	7
FPS1	Glycerol and arsenite channel	4	2	6
CIS2/ECM38	Gamma-glutamyltranspeptidase	1	5	6
PUT1	Proline oxidase	3	3	6
PHO84	Phosphate, manganese and arsenate transporter	2	4	6
ENA1	P-type ATPase sodium pump	3	3	6
DAL1	Allantoinase	2	4	6
DAL3	Ureidoglycolate hydrolase	5	1	6
GDH1	NADPH-dependent glutamate dehydrogenase	2	3	5
GLN1	Glutamine synthetase	4	1	5
UGA1	Gamma-aminobutyrate transaminase	3	2	5
GZF3/DEH1	GATA zinc finger protein	1	3	4
GDH2	NAD-dependent glutamate dehydrogenase	3	0	3
MKS1	Regulator in Ras-CAMP and lysine biosyntheses and nitrogen regulation	1	1	2
GLN3	Transcriptional activator of nitrogen-regulated genes	1	0	1
URE2	Nitrogen catabolite repression regulator	0	0	0
total genome		19831	19564	39395

Therefore, the expression pattern of *FPS1* gene in wild-type and mutant strains was investigated by RT-PCR to assess whether *FPS1* is regulated by Ure2p at the transcriptional level (Fig. 6). The level of *FPS1* transcripts was 57% higher in the *ure2Δ* mutant than in the wild-type strain BY4741. Similarly, for *PUT1*, a gene previously shown to be overexpressed in an *ure2Δ* mutant (Coffman *et al.*, 1994), transcript levels were 73% higher under our experimental conditions. After treatment of a late-exponential phase cultures (OD_{600} ~ 0,8) with As(III) for 30 min, a decrease by 20% in *FPS1* transcript levels was observed in the case of the wild type only. In contrast, the transcript abundance of *PHO84*, a gene implicated in As(V) uptake (Bun-ya *et al.*, 1996), was not significantly altered in the *ure2Δ* mutant under the tested conditions.

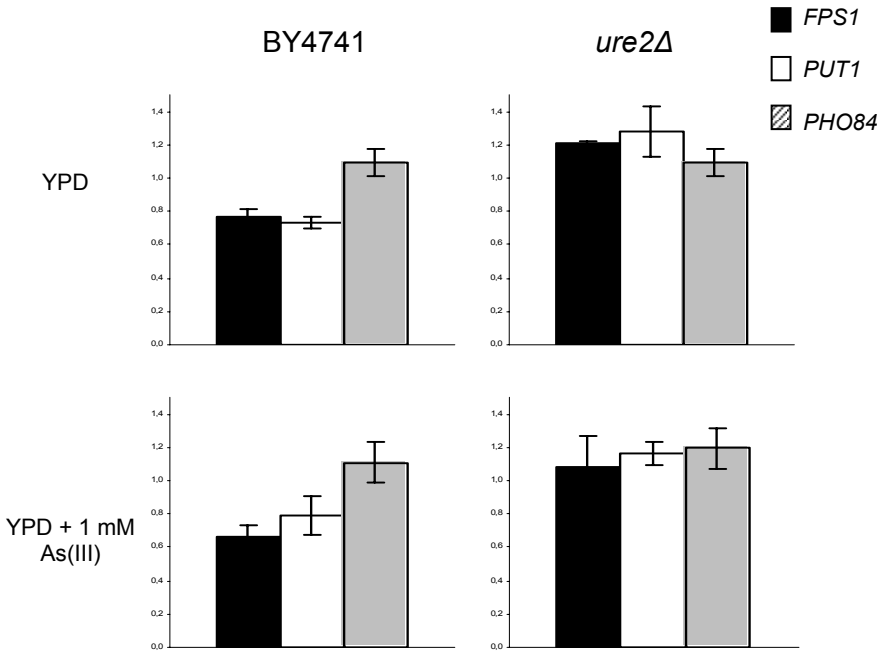

Fig. 6. Increased expression of the *FPS1* gene in the *ure2Δ* mutant. Wild type (BY4741) and mutant strain (*ure2Δ*) grown in YPD to late exponential phase (OD_{600}~0,8) were shifted to YPD + 1 mM As(III) ($NaAsO_2$) for 30 min, and the extracted RNA was amplified by RT-PCR. The intensity of each cDNA band was normalized against the intensity of cDNA obtained from 18S rRNA of the corresponding sample. The intensity of 18S rRNA was set to 1. *PUT1*, a gene coding for proline oxidase (Coffman *et al.*, 1994), was used as a positive GATA- and Ure2p-regulated control. The expression of *PHO84* encoding for an arsenate transporter was also measured and used as a control for a gene not regulated by Ure2p.

Further, it is also known that Fps1p is a major transporter of As(III) (in the form of $As(OH)_3$) in cells grown in high glucose medium, while As(III) is mainly transported by membrane hexose

permeases in the absence of glucose (hexoses) (Liu *et al.*, 2004). If indeed Ure2p specifically regulates the As(III) transporter Fps1, no sensitivity of the *ure2Δ* mutant to As(III) would be expected under conditions limiting Fps1p-medited arsenite uptake, e.g. growth on maltose or glycerol as the sole carbon source. Indeed, cultivation of the *ure2Δ* mutant in the absence of glucose and using the disaccharide maltose as the sole C-source (Fig. 7) demonstrated that the *ure2Δ* mutant did not show higher sensitivity to As(III) relative to the wild type (similar results were obtained by using glycerol as the sole carbon source, data not shown).

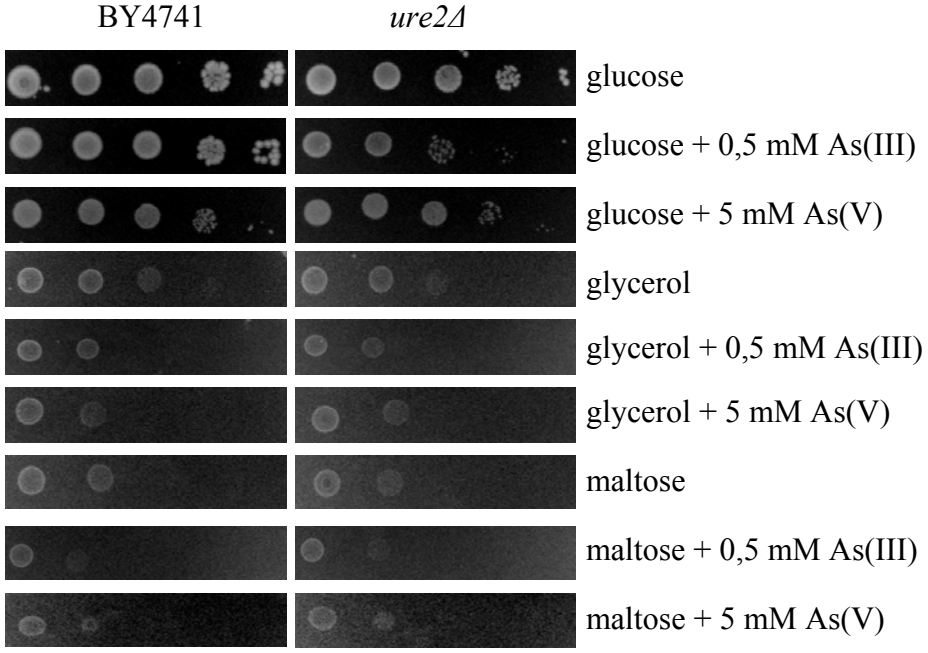

Fig. 7. Sensitivity of *ure2Δ* to arsenite and arsenate in minimal medium, supplemented with 2% of glucose or maltose as a carbon source. Five µL of ten-fold serial dilutions of overnight cultures were spotted on YNB plates with the indicated carbon source and arsenic concentrations. In all cases, the nitrogen source was NH_4^+ and the incubation time was 5 days.

Two other disruption mutants of *S. cerevisiae* were investigated to further evaluate an implication of Ure2p by way of the GATA pathway as a regulatory mechanism for As(III) transport in *S. cerevisiae.* As mentioned above, Gln3p and Gat1p are two transcription activators which participate in GATA mediated gene expression. Gln3p and Gat1p operate as activators, enhancing the expression of regulated genes in response to environmental signals (Cooper, 2002). Thus, the modulation of arsenic toxicity in *S. cerevisiae* by the GATA regulatory pathway predicts an increased resistance towards arsenite of GATA activator mutants as the outcome. This arsenite sensitivity of *gln3Δ* and *gat1Δ* mutants was investigated by growth in liquid rich medium at various

concentration of As(III). As presented in Fig. 8, the two disruption mutants *gln3Δ* and *gat1Δ* were indeed slightly more resistant to As(III) relatively to wild type, this resistance being more pronounced for the *gat1Δ* mutant.

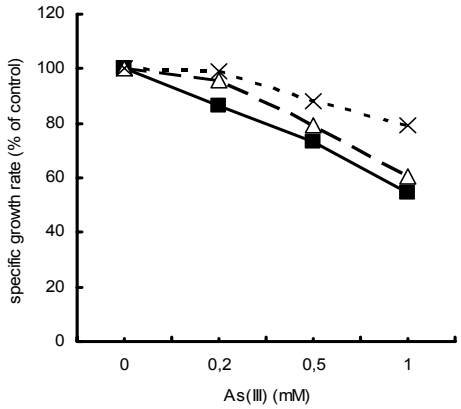

Fig.8. Resistance of *gln3Δ* and *gat1Δ* mutants to As(III) in liquid YPD medium. The specific growth rate of BY4741 (■), *gln3Δ* (Δ) and *gat1Δ* (x) in medium with various concentrations of As(III) was calculated from the growth curves and is presented as the percentage of the specific growth rate of each strain without arsenic (mean of two independent experiments).

4. Discussion

While an enzymatic function of physiological importance for Ure2p has yet to be demonstrated, the results presented here suggest a specific regulatory role of Ure2p in the tolerance of *S. cerevisiae* to arsenite toxicity. The *URE2* disruption mutant is hypersensitive to arsenite, but is resistant to arsenate (Fig.1 & 2). In addition, expression of the GST domain of Ure2p completely restores arsenite tolerance in the *ure2Δ* mutant to wild-type levels (Fig. 3).

The well-known pleiotropic functions of Ure2p (Wickner *et al.*, 2001; Cooper, 2002; Bai *et al.*, 2004) suggest three possible explanations for the observed sensitivity of the *ure2Δ* mutant toward As(III): (1) Direct enzymatic participation of Ure2p in the detoxification of arsenite; (2) Sensitivity as an indirect, non-specific consequence of a GATA-regulatory role of Ure2p; and (3) Sensitivity as a direct consequence of Ure2p-mediated GATA regulation.

An enzymatic activity of Ure2p in arsenic detoxification is an attractive hypothesis. However, the differential sensitivity of the *ure2Δ* mutant to reduced and oxidized forms of arsenic cannot be explained readily by such a hypothesis, since loss of the enzymatic activity with As(III) of Ure2p will also affect resistance towards As(V), which is reduced intracellulary to As(III) by Acr2p (Mukhopadhyay & Rosen, 1998). In such a case, *ure2Δ* mutant would be sensitive to both As(III)

and As(V) forms. This phenotype is indeed also observed for the *ycf1Δ* mutant (Ghosh *et al*., 1999, and our data not shown) lacking Ycf1p, a protein involved in intracellular As(III) transport to the vacuole. In this study, moreover, glutathione peroxidase activity previously reported for the recombinant Ure2 protein was similar in both wild-type and *ure2Δ* mutant cell-free extracts after exposure to As(III) and As(V) (Table 1). Taken together, these data suggest that Ure2p peroxidase activity is unlikely to be associated with arsenite sensitivity of the mutant.

An alternative explication for the observed As(III) sensitivity of the *ure2Δ* mutant and for a role of Ure2p in arsenic detoxification is an inhibitory role of Ure2p on GATA-type regulation of gene expression. Several genes, such as *GDH1, GDH2* and *GLN1* are overexpressed in an *ure2Δ* mutant (Coschigano & Magasanik, 1991). The activities of the corresponding enzymes, however, were not altered by arsenic exposure (Table 1). This indicates that the observed phenotype is unlikely to be due to uncontrolled overproduction of enzymes for nitrogen assimilation. Moreover, the *CIS2* gene encoding γ-glutamyl transpeptidase, a glutathione degradation enzyme, is also regulated by Ure2p. This is of particular interest, as glutathione is known to play an important role in the reduction of arsenate to arsenite. Indeed, the *ure2Δ* mutant displays strongly decreased intracellular content of reduced GSH. Nevertheless, this decreased reduced glutathione of *ure2Δ* was not affected by arsenite exposure and the mutant was resistant to arsenate, which requires reduced glutathione for its detoxification. Moreover, it is known that exogenous addition of GSH to the medium does not change the sensitivity of *ure2Δ* mutant to As(III) (Rai & Cooper, 2005).

The third possible explanation, i. e. that arsenite detoxification occurs by way of direct GATA regulation of *FPS1* implies that arsenite sensitivity of *S. cerevisiae* depends on the nitrogen source used for growth. Indeed, when wild type and *ure2Δ* mutant were cultivated on medium with glutamate as a nitrogen source, both strains were more sensitive to As(III) (Fig.4). This could adequately be explained by the fact that under these conditions, GATA activators localize to the nucleus and activate the transcription of genes whose products increase arsenite sensitivity in yeast.

Should an explication along these lines hold true, the full ensemble of genes regulated by GATA factors remains to be identified. We suggest that the *FPS1* gene, coding for the main arsenite transporter (Wysocki *et al*., 2001), could be such a GATA-regulated gene. Indeed, arsenic resistance in yeast appears to be determined both by the presence of influx systems (Wysocki *et al.*, 2001) and by active efflux of toxic arsenic compounds (Ghosh *et al*., 1999). In such a situation, increased sensitivity could be caused either by overexpression of an uptake protein, or by

deletion/repression of gene(s) coding for detoxification and/or efflux system component(s). In the case of arsenic, however, the latter alternative would cause sensitivity to both As(III) and As(V) forms (Rosen, 2002). Therefore, among the genes known to be involved in arsenic detoxification, only *FPS1* seems to be a likely target for Ure2p regulation, as it participates only in detoxification of the As(III) form of arsenic, and since its overexpression should result in increased arsenite sensitivity. In particular, GATA-regulated expression of *FPS1* may explain differential As(III) sensitivity of both wild-type and *ure2Δ* mutant strains on preferred and non-preferred nitrogen sources (Fig. 4 & Fig. 9). According to such a model, when wild-type strain is cultivated on a preferred nitrogen source (such as ammonia), GATA-mediated transcription is repressed and the *FPS1* gene is expressed at a normal level. In the *ure2Δ* mutant, the *FPS1* gene is overexpressed as a result of nuclear localization of Gln3p/Gat1p, and the mutant is more sensitive to As(III) as a consequence. Similarly, cultivation in the presence of a non-preferred nitrogen source (such as glutamate) induces nucleus accumulation of Gln3p/Gat1p in both strains, which activates the expression of GATA-regulated genes including *FPS1*. This then leads to increased arsenite sensitivity in both wild-type and *ure2Δ* mutant, due to an increased level of As(III) transport in the cell. This is exactly what was observed in our experiments (Fig. 9).

The potentially crucial role of Fps1p in arsenite tolerance, as mediated by Ure2p, is further confirmed by the other experiments of growth of *S. cerevisiae* with different carbon sources. Accordingly, when *S. cerevisiae* is cultivated on hexose-containing medium in millimolar concentrations, hexose transporters (Hxts) are occupied by sugars, and arsenite (presented in maximal concentration of 500 μM) is mainly transported by Fps1p (Liu *et al*., 2004). In medium with maltose or glycerol as the sole carbon source, in contrast, the carbon source is acquired through maltose transporters (Novak *et al.*, 2004) or Fps1p (Sutherland *et al.*, 1997), respectively. In this situation, hexose permeases are therefore free to transport arsenite into the cell (Liu *et al*., 2004). If Ure2p participates in arsenic metabolism by negatively regulating transcription of the *FPS1* gene, as is indeed suggested by our RT-PCR experiments (Fig. 6), then the sensitivity of the *ure2Δ* mutant to arsenite would be comparable to that of wild type, if cultivated on medium not supplied with hexoses as a carbon source. This is indeed what was observed in our experiments (Fig. 10).

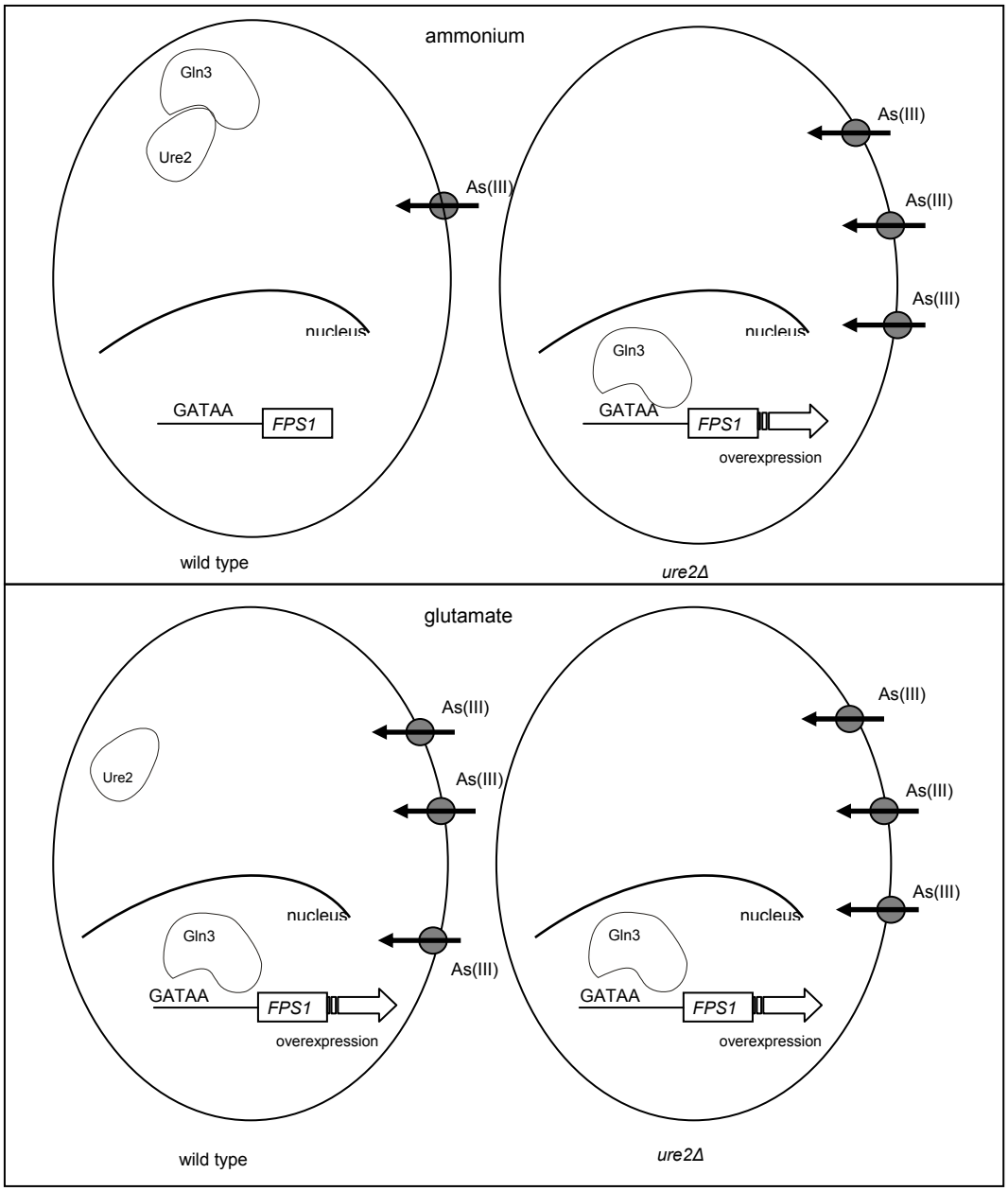

Fig. 9. A model for nitrogen source-dependent regulation of arsenite sensitivity in S. *cerevisiae* (see text for details). Black arrows indicate As(III) transport and grey circles represent Fps1p.

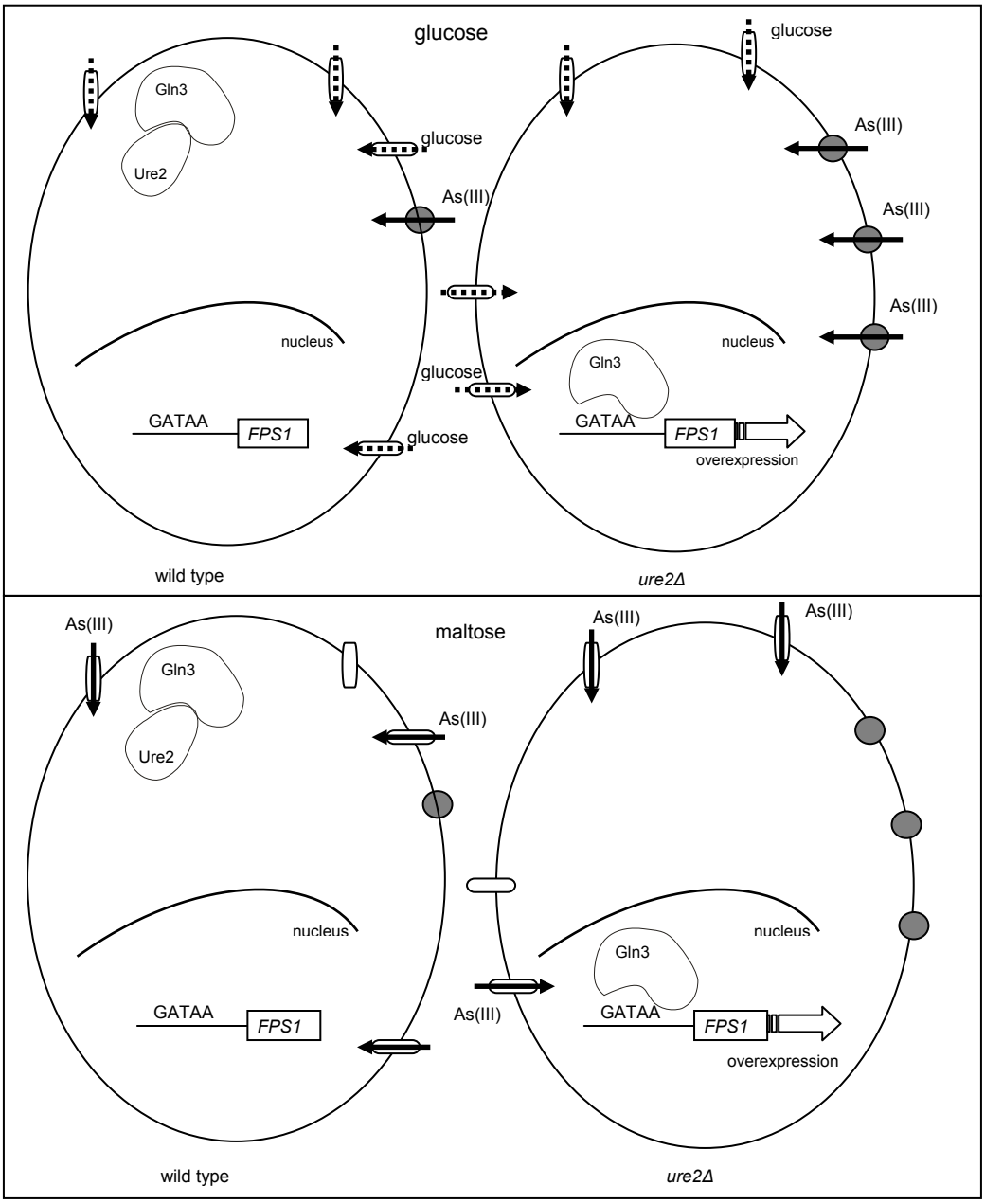

Fig. 10. A model for carbon source-dependent arsenite sensitivity of *ure2Δ* mutant. Black arrows indicate As(III) transport, dashed arrows indicate glucose transport. Grey circles represent Fps1p and white rectangles indicate hexose transporters.

Summing up, the increased level of *FPS1* transcripts in the *ure2Δ* mutant (Fig. 6), together with the observed arsenite resistance of GATA activator mutants *gln3Δ* and *gat1Δ* (Fig. 8) lead us to propose a simple general model for arsenite regulation in *S. cerevisiae* (Fig. 11). When the protein Ure2p is expressed, it forms cytoplasmic complexes with Gln3p and Gat1p that prevent nuclear entry of these regulatory proteins, and in consequence transcription of *FPS1*. In the *ure2Δ* mutant, the two

activators Gln3p and Gat1p are free to enter the nucleus, leading to overexpression of *FPS1* and thus greater sensitivity of *S. cerevisiae* to As(III). The higher resistance to As(III) of the *gat1Δ* mutant (Fig. 8) suggests that the main regulation occurs via Gat1p rather than via Gln3p. This is in line with the observation that the promoter region of *FPS1* contains an AGATAAG site, recently found to be the preferred site for DNA binding of Gat1p (Harbison *et al,* 2004). Interestingly, the degradation of glutathione, which is another nitrogen-independent GATA-regulated process, also appears to depend primary on Gat1p and not on Gln3p (Springael & Penninckx, 2003).

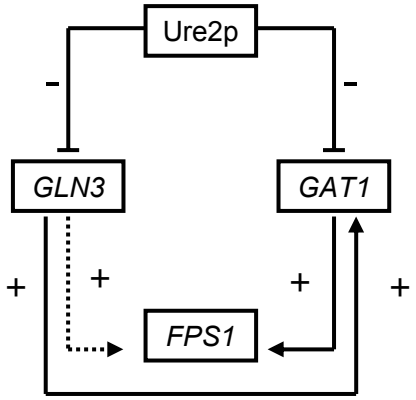

Fig.11. GATA regulation of *FPS1* gene. Each line represents a binding event. The bars and arrows designate negative and positive control modes, respectively. The dashed line represents a presumably weaker interaction (see also Fig. 8).

In conclusion, our work further emphasizes the importance of GATA control as one of the central modes of metabolic regulation in *S. cerevisiae*. This features the central regulatory role of a GST-like protein, Ure2p, of still uncertain enzymatic function. In our view, the involvement of this GST in arsenic resistance represents a good example of the complex and intriguing relationships between central metabolism and detoxification of environmental pollutants.

We are grateful to Ivan Tarassov for providing the p413 vector and to Ales Vancura for pET23b-URE2 and pET23b-URE2(ΔN) plasmids.

Chapter 4

Oxidant response in *ure2Δ* mutant of *Saccharomyces cerevisiae*

Résumé en français

La réponse au stress oxydatif chez le mutant *ure2Δ* de *Saccharomyces cerevisiae*

Pour se protéger contre les effets toxiques de l'oxygène, les organismes disposent de systèmes de défense qui permettent de réguler la production d'espèces oxygénées réactives. Ces systèmes sont composés d'enzymes spécifiques et de molécules antioxydantes non-spécifiques (vitamines, oligo-éléments, peptides, caroténoïdes). Le système de défense enzymatique comprend des catalases, des superoxyde dismutases et des peroxydases.

Les catalases sont des enzymes extrêmement actives localisées chez les levures dans les peroxisomes, les mitochondries et le cytosol (encodées par les gènes *CTA* et *CTT*, respectivement).

Les superoxyde dismutases de *Saccharomyces cerevisiae* (au Cu/Zn et au Mn, codées respectivement par les gènes *SOD1* et *SOD2*) catalysent l'élimination de l'anion superoxyde, première espèce toxique formée à partir de l'oxygène. Elles assurent de ce fait la première ligne de défense contre le stress oxydatif.

Les peroxydases (cytochrome *c* peroxydase, glutathion peroxidase, etc.) sont capables d'extraire l'hydrogène d'un substrat pour former deux molécules d'eau à partir d'une molécule de peroxyde.

Par ailleurs et de manière générale comme les autres eucaryotes et les bactéries Gram-négatives aérobies, la levure *Saccharomyces cerevisiae* dispose d'un antioxydant non-enzymatique, le glutathion (γ-L-glutamyl-L-cystinylglycine), qui peut d'une part participer dans la défense antioxydante comme un source d'équivalents réducteurs, et d'autre part servir de cofacteur à certaines enzymes impliquées dans la protection contre le stress oxydatif.

Après la démonstration d'une sensibilité du mutant *ure2Δ* au H_2O_2, le travail a été complété par l'étude de la sensibilité aux autres oxydants comme le ménadione et le paraquat. En contraste avec le H_2O_2, qui consomme les équivalents réduits de la cellule (GSH), ces composés endommagent les structures cellulaires par la formation de l'anion radical superoxyde ($O_2^{\bullet -}$). Le mutant *ure2Δ* est moins sensible à ce type d'oxydants, indiquant que la sensibilité au stress oxidatif de ce mutant est limitée aux péroxides.

Notre travail a été complété par l'étude biochimique des systèmes antioxydants de *S. cerevisiae* (détermination biochimique des activités enzymatiques et caractérisation électrophorétique des

isoenzymes antioxydants). Dans ce contexte, les systèmes de défense enzymatique et non-enzymatique au stress oxydatif chez le mutant *ure2Δ* ont été étudiés. En particulier, le contenu en glutathion et l'activité glutathion peroxidase, catalase et superoxyde dismutase ont été mesurés pour différentes phases de croissance en milieu riche (YPD). Le mutant *ure2Δ* présente une activité plus élevée de catalase et de superoxyde dismutase, avec l'apparition d'une forme isoenzymatique de catalase absente chez le type sauvage correspondant.

1. Introduction

Glutathione-S-transferases (GSTs) are an enzyme family playing an important role in antioxidant defense of both prokaryotes and eukaryotes (for a review see Sheehan *et al.*, 2001 and Frova, 2006). These enzymes catalyze a wide variety of reactions, mainly in the detoxification of electrophilic chemicals and lipid peroxidation products (Hubatsch *et al*., 1998), but also include isomerisation reactions (Fernandez-Canon & Penalva, 1998), and glutathione peroxidase activities (Zhao *et al*., 1999; Garcera *et al.*, 2006), by conjugation to the tripeptide glutathione (GSH), the major non-enzymatic radical scavenger protecting cells from reactive oxygen species (ROS) (Posci *et al.*, 2004).

Saccharomyces cerevisiae has two GST genes – *GTT1* and *GTT2*, whose products are known to catalyze typical GST reactions (Choi *et al*., 1998). A third gene, *URE2,* codes for a protein that exhibits sequence and structural homology to GSTs, but the attempts to demonstrate a GST activity of Ure2p beyond its GPx activity (Bai *et al.*, 2004) have been unsuccessful (Choi *et al*., 1998). The disruption in *URE2* gene confers a sensitivity of *S. cerevisiae* to toxic and oxidant compounds such as Al, Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Se, diamide, hydrogen peroxide, cumene and *t*-butyl hydroperoxides (Basu *et al*., 2004; Rai *et al.,* 2003; Rai & Cooper, 2005).

Ure2p has been originally discovered as a major negative regulator in the physiological process of nitrogen catabolite repression (NCR), a sensing mechanism mediated by the GATA signal transduction pathway (Lacroute, 1971). In brief, when a preferred nitrogen is provided in the medium, Ure2p forms cytoplasmic complexes with two transcription activators Gln3p and Gat1p to prevent the expression of the genes required for assimilation of non-preferred nitrogen sources such as proline and glutamate (for a review see Cooper, 2002). When the preferred nitrogen source (typically ammonia) is exhausted, Gln3p and Gat1p are liberated from the complexes and enter the nucleus to increase the expression of target genes for assimilation of alternative nitrogen sources.

The pleiotropic functions of Ure2p raise the question of whether its requirement in the detoxification of heavy metals and peroxides is a result of enzymatic Ure2p participation (as a GST enzyme) or alternatively, is an effect of direct or indirect GATA regulation of one or more genes whose products are involved in oxidative and metal stress. As toxic metals and peroxides are known GST substrates, it is possible that Ure2p acts enzymatically on such compounds in ways that remain to be elucidated. On the other hand, the sensitivity of *ure2Δ* mutant to heavy metals is controversial. Deletion of *URE2* gene has been described to confer resistance to Zn(II) and Mo(VI) (Rai &

Cooper, 2005) and to As(V) (Chapter 3 of the Results), a fact which can be explained if the detoxification pathway of each toxic compound is regulated in opposite ways by the GATA transduction pathway and by the protein Ure2p, respectively.

In the present work we tested the sensitivity of the GST mutant *ure2Δ* towards a number of oxidant substrates during the growth on different nitrogen sources. Having detected the hypersensitivity to hydrogen peroxide and resistance to paraquat and menadione, we measured the levels of the main antioxidant defense systems in *S. cerevisiae* – on the one hand, glutathione and glutathione peroxidase which protect the cell from peroxides, and on the other hand, superoxide dismutase and catalase which promote the superoxide anion detoxification. Our findings suggest that the sensitivity of mutant *ure2Δ* to oxidants is specific for glutathione oxidizing agents such as peroxides, and is a result of strongly decreased cellular glutathione levels in this mutant.

2. Materials and methods

Strains, media and plasmids

Saccharomyces cerevisiae strains: BY4741 (*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and its isogenic disruption mutant *ure2Δ* (*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL229c::kanMX4*) were obtained from *EUROSCARF* (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html).

Rich YPD medium (Q-BioGene) and minimal YCB medium (1.17% yeast carbon base) without amino acids and NH_4^+ (Difco) were used. Minimal medium was supplemented with the indicated nitrogen sources (0.1% proline, 0.1% glutamate or 0.5% $(NH_4)_2SO_4$) and the required amino acids.

The plasmids used were pME8530 (bearing the entire gene *URE2*) and pME8531 (bearing only the GST part of the gene *URE2*), both constructed on the base of the yeast expression vector p413 (Funk *et al.*, 2002; kindly provided by Ivan Tarassov) and under the control of *GALS* promoter, as described elsewhere (Chapter 3 of the Results). The *ure2Δ* mutant was transformed with either pME8530 or pME8531 and the expression of the genes was achieved as described. As controls, both parental and mutant strains were transformed with the vector p413.

Oxidant sensitivity assays

Yeast strains were grown overnight at 30°C/140 rpm in 10 mL of YPD medium. Cells were pelleted and washed twice with fresh medium. Ten-fold serial dilutions with OD_{600}~1; 0.1; 0.01; 0.001;

0.0001 were prepared. Samples (5 μL) of each diluted culture were spotted onto plates containing the indicated in each figure, concentration of H_2O_2, paraquat (paraquat dichloride; 1,1'-Dimethyl-4,4'-bipyridinium dichloride hydrate, Sigma) or menadione (2-Methyl-1,4-naphthoquinone, Sigma). Where specified, 2 mM reduced glutathione (Fluka) was added to the medium. The plates were incubated at 28°C for 5 days.

Alternatively, for assaying sensitivity in liquid medium, strains were pre-grown in YPD medium until log phase (OD_{600}~0.3). Five mL aliquots of YPD containing various concentrations of H_2O_2, paraquat or menadione were inoculated from the precultures to an initial OD_{600}~0.05. Cultures were incubated for 15 hours at 30°C/140 rpm and the final OD_{600} was measured.

Biochemical assays

Aliquots of 95 mL YPD medium were inoculated with 5 mL preculture in the same medium to an initial OD_{600} ~ 0.05 and cultivated in 500 mL Erlenmeyer flaks on rotary shaker (140 rpm/min) at 30°C for 72 hours. Growth was monitored every 3 hours by measuring the optical density at 600 nm (OD_{600}).

Strain biomass was collected at 12 h, 24 h, 48 h and 72 h of cultivation. Cells were disrupted with 4 cycles of agitation (10 min each) in the presence of glass beads (0.5 mm, Sigma) and 0.05 M potassium phosphate buffer on a vortex mixer with 1 min of pause between the cycles, followed by centrifugation (20 min/15000 rpm). The resulting cell free extracts were subsequently used for enzymatic analyses.

The cellular GSH and GS-SG were determined by the method of Ellman as described previously (Zhang, 2000).

Glutathione peroxidase (GPx) was measured as described by Veal *et al.* (2002). One unit of enzyme activity represents the μmoles oxidized NADPH for 1 min.

The activity of catalase was measured spectrophotometrically (Aebi, 1981). Enzyme activity was expressed as rate of change of absorbance at 240 nm resulting from H_2O_2 decomposition (ΔE_{240} min^{-1} mg $protein^{-1}$).

SOD assay was performed by the inhibition method of Beauchamp & Fridovich (1971). One unit of activity was expressed as the amount of cell-free protein that caused 50% inhibition of the reduction

of *p*-Nitro-Blue tetrazolium chloride (NBT) to blue formazan under the test conditions (25°C, pH 7.8).

All results were normalised for protein concentration in each sample, determined by the method of Lowry with bovine serum albumin as a standard (Lowry *et al.*, 1951).

Western blot

Twenty µg of proteins from extracts of yeast strains BY4741, BY4741 + pME8530, BY4741 + pME8531, *ure2Δ, ure2Δ* + pME8530, *ure2Δ* + pME8531 were separated on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Amersham). The transfer was performed on Mini Trans-Blot Cell (BioRad) according to the standard manufacturer protocol. The primary anti-Ure2 bodies (kindly provided by Michel Aigle) were obtained in rabbit and were used in dilution 1:1000. The enhanced chemiluminescence western blotting detection and analysis system (Amersham) was used to detect the proteins as instructed by the manufacturer (secondary anti-rabbit antibody dilution was 1:10000).

Native PAGE

Native polyacrylamide gel electrophoresis (PAGE) was performed on 10% vertical gel using Mighty Small II vertical electrophoresis (Hoefer Scientific Instruments).

SOD activity was visualized by soaking the gels in 10 mM potassium phosphate buffer, pH 7.2 containing 0.1 mM riboflavin and 2 mM *o*-dianisidine dihydrochloride for 1 h at room temperature in dark, followed by brief rinsing with distilled water and illumination for 15 min (Misra & Fridovich, 1977). The type of SOD enzymes was detected by treatment with 2 mM KCN of gels before staining.

Catalase profiles were obtained in 10% polyacrylamide gels containing 1% starch and visualized by soaking the gels in solution of 1.5% Na_2SO_3 and 1% H_2O_2, followed by coloration with iodine (0.3% I_2 and 2% KI).

Rhodamine 123 staining

Free intracellular radicals were detected with dihydrorhodamine 123 (Sigma) as described (Madeo *et al*., 1999). Cultures of parental strain BY4741, mutant strain *ure2Δ*, and the mutant strain, transformed either with pME8530 (*ure2Δ*+pME8530) or pME8531 (*ure2Δ*+pME8531) were grown overnight in liquid minimal medium with 2% galactose and appropriate supplements.

Dihydrorhodamine 123 was added at 5 μg per mL to BY4714 and *ure2Δ* cultures at OD_{600} ~ 1. After 2 hours of incubation at 25°C, the cells were harvested, washed twice with fresh medium and viewed without further processing through a rhodamine optical filter using a Leica DM 4000B microscope. At least 400 cells in three independent experiments were examined to estimate the frequency of oxidant phenotype.

3. Results and discussion

3.1. Sensitivity of URE2 *disruption mutant to oxidants*

The *ure2Δ* mutant is sensitive to hydrogen peroxide, cumene and *t*-butyl hydroperoxide, and to the oxidant compound diamide (Rai *et al.,* 2003; Rai & Cooper, 2005). To further define the oxidant phenotype of *ure2Δ* mutant beyond peroxide and diamide sensitivity, growth of the mutant in the presence of other oxidants such as paraquat and menadione was investigated. In contrast to peroxides and diamide which use up reduced cell equivalents (e.g. GSH) by oxidation, menadione and paraquat divert electrons from the mitochondrial transport chain and undergo redox cycling (Bolton *et al.*, 2000). Thus, they are reduced by one electron and form radical species, which react rapidly with O_2 to generate superoxide anions *in situ*. Consistent with previous studies, the *ure2Δ* mutant exhibited a reduced ability to grow in rich medium supplemented with H_2O_2 (at concentration of 2 mM H_2O_2, it showed 7% survival versus 79% of the wild type) (Fig. 1). Surprisingly, the disruption of *URE2* had no effect on cell growth in the presence of paraquat and menadione (Fig. 1).

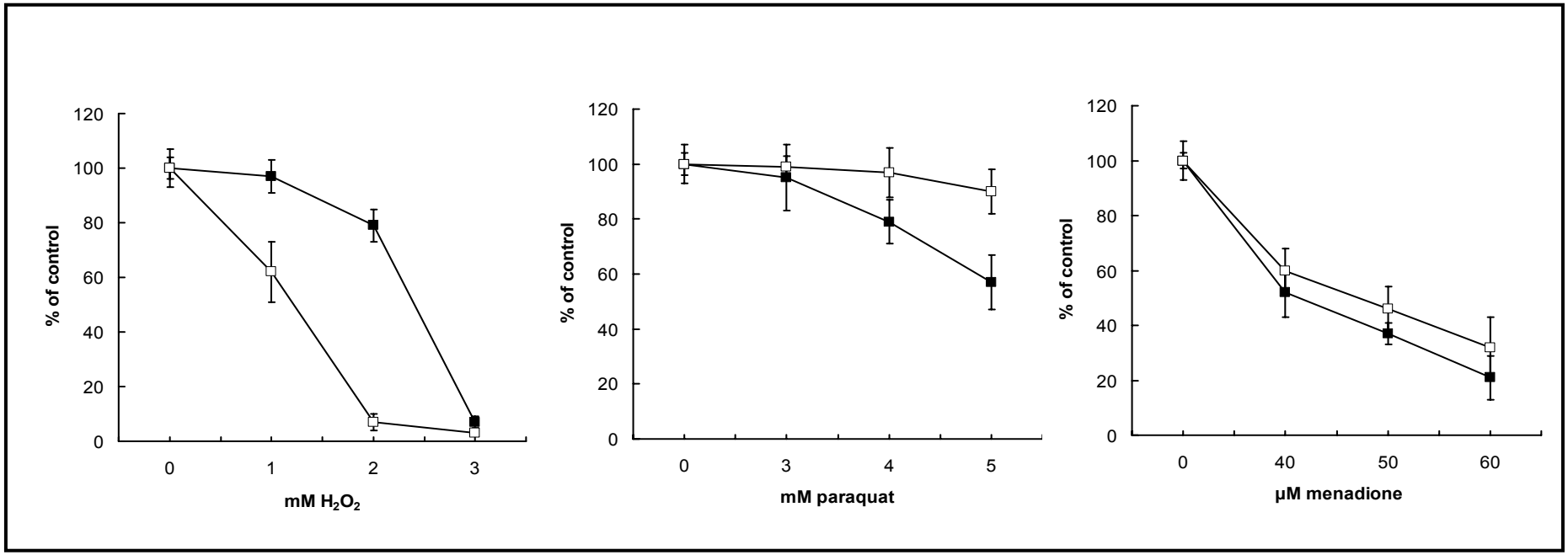

Fig.1. Sensitivity of *ure2Δ* mutant to H_2O_2, paraquat and menadione in liquid YPD medium. Cultures of BY4741 (■) and *ure2Δ* (□) with initial OD_{600} ~ 0.05 were cultivated at 28˚C with the indicated concentrations of oxidants. Fifteen hours after inoculation, the final OD_{600} of cultures was measured. The growth is presented as a percentage of control strain growth (final OD_{600}) in YPD medium without any addition of oxidants (BY4741 final OD_{600} ~ 1,2; *ure2Δ* mutant OD_{600} ~ 0,8). The vertical bars represent standard error of three independent experiments.

Many genes regulated by Ure2p are subject of nitrogen catabolite repression, therefore their expression rate is easily altered by growth conditions, such as the addition of preferred or non-preferred nitrogen sources to the medium. To analyse whether the observed oxidant phenotype of the disrupted mutant was related to the type of nitrogen source, its sensitivity on minimal medium containing various concentrations of H_2O_2, paraquat or menadione and supplemented either with ammonia (Fig. 2A) or proline (Fig. 2B) as the sole nitrogen source, was investigated. Proline is a non-preferred nitrogen source used in many studies dealing with yeast nitrogen catabolite repression (Cooper, 2002). Ammonia is reported to repress the expression of proline utilisation genes *PUT1* and *PUT2* in *S. cerevisiae* (Xu *et al*., 1995), and therefore acts as a preferred nitrogen source.

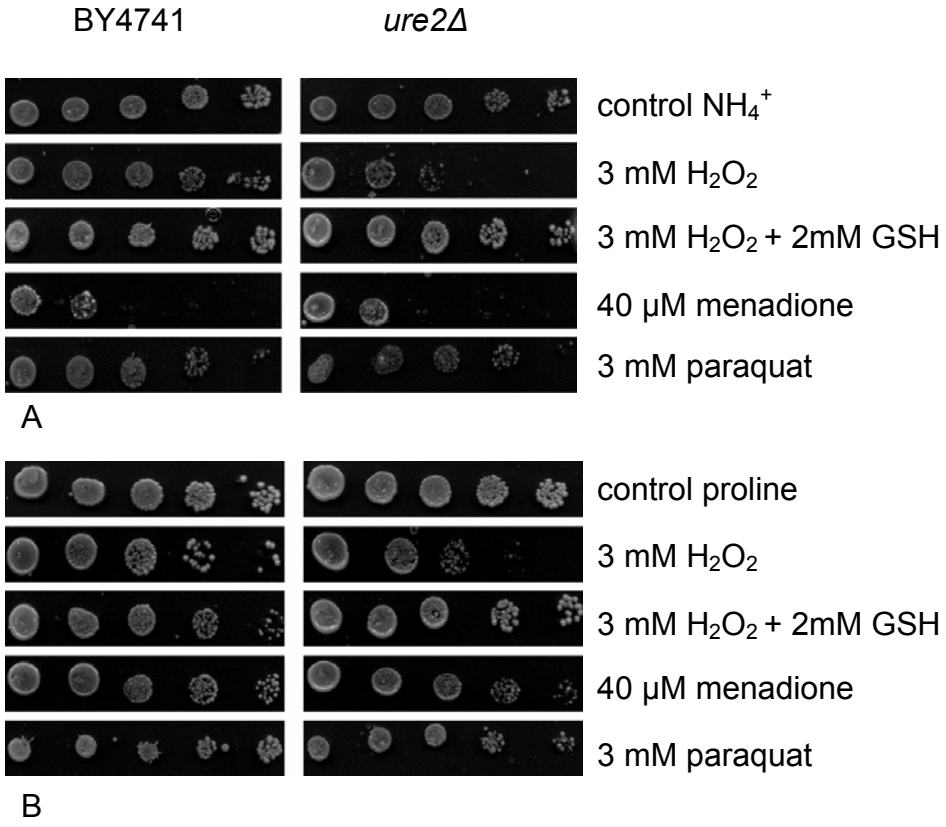

Fig. 2. Sensitivity of *ure2Δ* to oxidant compounds on preferred and non-preferred nitrogen source. Parental strain BY4741 and *ure2Δ* mutant were cultivated overnight in liquid YPD medium, serial diluted and then 5 µL of each dilution were spotted on minimal medium with ammonium (A) or proline (B) as nitrogen source containing the indicated concentrations of H_2O_2, menadione or paraquat and supplemented or not with 2 mM glutathione (GSH). The plates were incubated for 5 days at 28˚C.

As shown in Fig. 2, growth of *ure2Δ* mutant in the presence of H_2O_2 was inhibited to the same degree regardless of whether ammonia or proline was provided in the medium, indicating that peroxide sensitivity did not depend on the type of nitrogen source. In contrast to H_2O_2, paraquat and menadione sensitivity of *S. cerevisiae* differed depending on the nitrogen source, with the preferred nitrogen source apparently being determinant for the oxidant sensitivity. It could be speculated that the growth on preferred nitrogen source confers higher metabolic activity of the cells, active oxidation of substrates and mitochondrial proliferation, which makes cells more vulnerable to toxic action of redox active compounds causing "uncoupling" of oxidative phosphorilation. On the other hand, the decreased sensitivity of *S. cerevisiae* to superoxide-producing compounds on proline could be due to the protective role of proline as a general antioxidant (Terao *et al*., 2003). Should this be the case, however, the similar sensitivity of *S. cerevisiae* to H_2O_2 during growth on ammonia and proline cannot be explained. In addition, similar results were obtained with glutamate as nitrogen source (data not shown). The interpretation that these different phenotypes are a result of nitrogen source dependent regulation of superoxide detoxification therefore seems more attractive.

3.2. Effect of glutathione on the sensitivity of ure2Δ *mutant to* H_2O_2

There are two possible pathways for detoxification of H_2O_2 in *Saccharomyces cerevisiae* – by reduction with glutathione or through catalase action. A yeast strain which lacks a functional copy of the *GSH1* gene, encoding the enzyme for the first step in the biosynthesis of GSH, was found to be sensitive to exogenous H_2O_2 and *t*-butyl hydroperoxide, whereas strains deficient in catalase genes are resistant to H_2O_2, suggesting that GSH based reactions are the primary means of detoxifying this oxidant, rather than catalase-mediated removal (Grant *et al*., 1998).

Ure2p inhibits the expression of *CIS2* gene (encodes γ-glutamyl transpeptidase, the first enzyme in glutathione degradation) (Springael & Penninckx, 2003). In this context, the sensitivity of *ure2Δ* mutant to hydrogen peroxide can result from increased GSH degradation in response of deregulated localization of Gln3/Gat1 factors that activate the transcription of γ-glutamyl transpeptidase. The growth of wild-type and *ure2Δ* cells in the presence of 3 mM H_2O_2 in minimal medium supplemented with 2 mM GSH was therefore investigated. Glutathione addition completely restored growth of the mutant on both ammonia and proline (Fig. 2). This finding strongly supports the hypothesis of the glutathione importance in the detoxification of peroxides in yeast, and provides an explanation for the sensitivity to H_2O_2 in the *ure2Δ* mutant.

3.3. Glutathione redox ratio and glutathione peroxidase activity in ure2Δ *mutant*

To determine the time points for biomass collection and antioxidant measurement, a detailed growth analysis for BY4741 and *ure2Δ* mutant was performed over a period of 72 h in YPD medium (Fig. 3). During cultivation in rich medium, *S. cerevisiae* shows a typical diauxic growth maintained first by the glucose and then by the complex carbon sources. As stationary phase increases yeast resistance towards oxidants such as H_2O_2 (Sousa-Lopes *et al*., 2004), the antioxidant levels were monitored in parental BY4741 strain and in *ure2Δ* mutant at four time points – at 12 h, at 24 h, at 48 h and at 72 h.

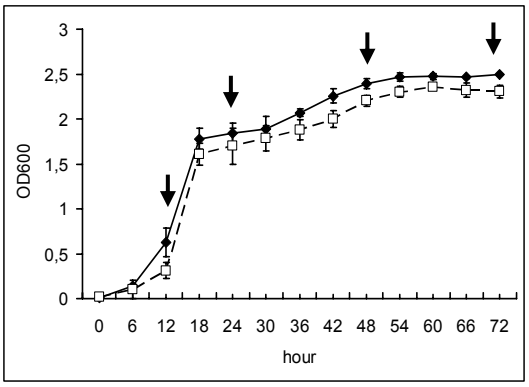

Fig.3. Growth of BY4741 (closed symbols) and *ure2Δ* mutant (open symbols) in YPD medium. Optical density at 600 nm was measured at 3 hours intervals between 0 and 72 h of postinoculation. The vertical bars represent the standard error of two independent experiments and arrows indicate the time points for biomass collection.

Intracellular levels of reduced and oxidized glutathione (GSH/GS-SG) were measured. As expected, the disruption of *URE2* gene resulted in nearly 5-fold decreased GSH level (Table 1). Since the level of the oxidized form was not changed, the mutant displayed a strongly altered redox state. The glutathione level of *ure2Δ* correlated well with the observed increased sensitivity to peroxides and to diamide, which are agents that deplete the cellular pool of reduced glutathione by oxidation.

Second, as the protein Ure2 shows *in vitro* glutathione peroxidase activity with both H_2O_2 and organic peroxides (Bai *et al.*, 2004), the GPx activity with H_2O_2 as substrate was tested in cell-free extracts of parental and mutant strain at the indicated time points (Table 1). The measured activities did not differ in cell free extracts of both strains, and increased with the time. These results are not surprising keeping in mind that only one of the three glutathione peroxidase disruption mutants of *S. cerevisiae, gpx3Δ* has a decreased GPx activity *in vivo* (Inoue *et al*., 1999), while both GPx2p and GPx3p are active *in vitro* (GPx1p has not been successfully expressed in soluble form, Avery & Avery, 2001).

Table 1. Levels of main antioxidants in cell-free extracts of BY4741 and *ure2Δ* at different time points of long-term YPD cultures.

	GSH[a] (mmol mg protein^{-1})		GS-SG[b] (mmol mg protein^{-1})		GS-SG/GSH ratio		GPx[c] activity (mU mg protein^{-1})		Catalase activity (ΔE min^{-1} mg protein^{-1})		SOD[d] activity (U mg protein^{-1})	
	BY4741	*ure2Δ*	BY4741	*ure2Δ*	BY4741	*ure2Δ*	BY4741	*ure2Δ*	BY4741	*ure2Δ*	BY4741	*ure2Δ*
Time (h)												
12 hours	0,12±0,02	0,03±0,02	0,03±0,01	0,03±0,02	0,3	1,0	24,6±2,5	24,1±4,2	0,05±0,01	0,06±0,01	8,5±2,2	9,4±0,8
24 hours	0,16±0,03	0,03±0,01	0,03±0,02	0,04±0,01	0,2	1,3	26,9±0,9	27,5±2,3	0,36±0,06	0,66±0,04	10,3±1,6	11,8±2,0
48 hours	0,48±0,12	0,10±0,09	0,12±0,03	0,09±0,04	0,3	0,9	32,4±3,0	30,1±0,8	0,78±0,03	1,40±0,11	12,8±2,4	16,7±1,3
72 hours	0,51±0,10	0,12±0,01	0,16±0,01	0,11±0,05	0,3	0,9	36,3±1,2	34,5±0,2	0,73±0,02	1,05±0,15	14,6±1,8	18,6±1,9

[a]Reduced glutathione
[b]Oxidized glutathione
[c]Glutathione peroxidase
[d]Superoxide dismutase

The absence of significant difference between the GPx activity of the wild type and the *ure2Δ* cell free extracts prompted us to investigate the effect of *URE2* overexpression on GPx activity in *ure2Δ*. Mutant cells were transformed with plasmids pME8530 (yeast expression plasmid p413 + *URE2* gene) and pME8531 (p413 + *URE2ΔN,* the GST homologous part of the gene). After 24 hours of galactose induction, the GPx activity with hydrogen, cumene and *t*-butyl hydroperoxides as substrates was measured in cell-free extracts of the transformed strain. No increase in total GPx activity was observed in cells overexpressing *URE2* gene (data not shown). Protein overexpression of proteins was not apparent on SDS-PAGE gels (Fig. 4, upper panel), but a western blot with Ure2p antibodies showed clear overexpression of Ure2 and Ure2ΔN proteins in both parental and mutant strains (Fig. 4, bottom panel). This indicates that GPx activity of Ure2p, if present, is unlikely to be associated with the increased oxidative stress in the *ure2Δ* mutant.

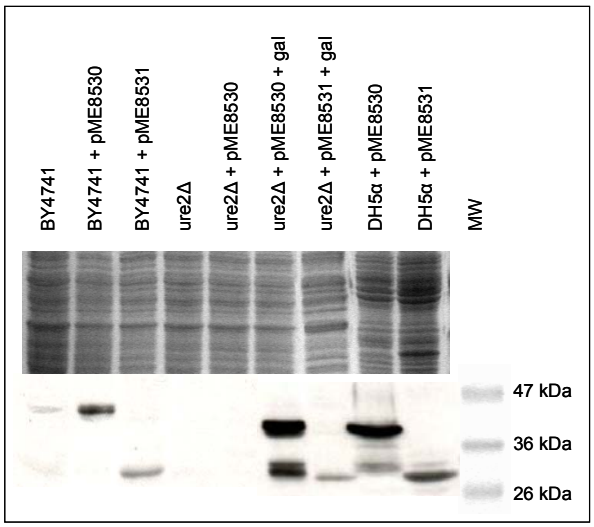

Fig. 4. SDS-PAGE (upper panel) and Western blot (bottom panel) analysis of cell extracts from wild-type and *ure2Δ* mutant strains. Each lane was loaded with 20 μg of total protein. Immunoblots were probed with rabbit antiserum against Ure2p. *E. coli* DH5α cells transformed with pME8530 and pME8531 were used as positive controls for Ure2p expression.

3.4. Catalase and superoxide dismutase activities in the ure2Δ *mutant*

In contrast to H_2O_2, exposure of wild-type yeast to menadione results in only modest depletion of total glutathione levels (Stephen & Jamieson, 1996), indicating that in this case GSH-dependent detoxification is a secondary defense mechanism. In fact, the destruction of superoxide formed through paraquat and menadione exposure requires the cooperative action of superoxide dismutase and catalase. Accordingly, yeast overexpressing human Cu/Zn-SOD is more resistant to paraquat

and menadione (Yoo *et al*., 1999). Furthermore, mitochondrial overexpression of catalase provides protection against menadione toxicity in insulin-producing RINm5F cells (Gurgul *et al*., 2004).

Since the *ure2Δ* mutant is resistant to superoxide producing agents whose detoxification requires superoxide dismutase and catalase, these enzymatic activities were measured in the parental and mutant strain. As can be seen in Table 1, total catalase activity in cell-free extracts reached the highest value at 48 hours of cultivation during the second exponential phase. In addition, nearly two-fold increased activity was observed in *ure2Δ* mutant (1,40±0,11 units at 48 hours of cultivation) compared to the parental BY4741 strain (0,78±0,03 units). The maximal values of SOD activity were determined in the second stationary phase. Similarly, SOD levels were increased in the mutant (18,56±1,9 units versus 14,64±1,8 units of the parental strain, at 72 hours of cultivation).

Native PAGE allowed direct visualization of catalase and SOD activity (Fig. 5). Cell-free extracts from both strains were isolated at the hours of highest enzyme activity. This revealed the presence in both strains of three clearly observed SOD isoforms, two of which seemed to be cyanide insensitive. In contrast to the SOD isoforms which were undistinguishable, one supplementary band correlating with the highest catalase activity (Fig. 5, first panel) was observed in the *ure2Δ* mutant. Both biochemical and eletrophoretical data suggest that *ure2Δ* mutant has an increased level of catalase and SOD enzymes, which thus can explain the observed *ure2Δ* resistance to paraquat and menadione.

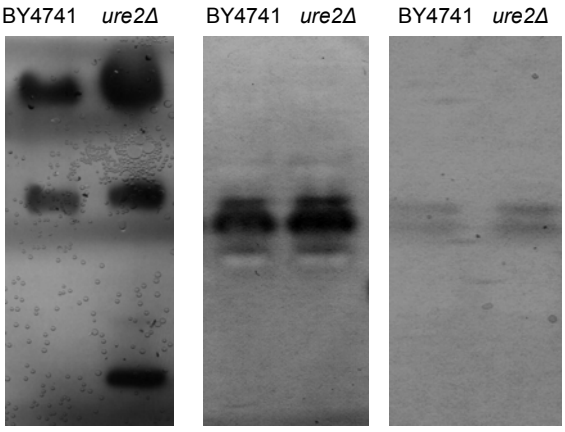

Fig. 5. Visualization of catalase (first panel), total SOD (second panel) and Mn SOD (third panel) isoenzymes in BY4741 (left lanes) and *ure2Δ* mutant (right lanes). Cell-free extracts were isolated at 48 hours of cultivation for the catalase determination and at 72 hours for SOD gels; 150 μg of total protein were loaded on each lane and subjected to 10% native PAGE. The gels were stained for specific catalase activity with iodine solution and for SOD activity – with riboflavin and dianizidine dihydrochloride. Mn SOD isoforms were detected after inhibition with 2 mM KCN.

3.5. Accumulation of reactive oxygen species in ure2Δ *mutant*

The "paradoxical" oxidant response capacity of *ure2Δ* mutant – significantly decreased glutathione levels in the context of unaltered GPx activity and increased catalase and SOD activities – prompted us to estimate the total oxidant levels in *ure2Δ* mutant cells by measuring the free intracellular radicals under normal non-stressed culture conditions. These were determined with the oxidant-sensitive compound dihydrorhodamine 123 which crosses the cell wall by passive diffusion, and converts to the red fluorescent rhodamine 123 in the presence of intracellular ROS. The mutant showed a 3-fold higher number of rhodamine-stained cells in comparison with the parental strain BY4741 (Fig. 6). When the *ure2Δ* mutant was complemented with the expression vectors containing the entire *URE2* gene (plasmid pME8530) or the functional GST part of the gene alone (pME8531) after overnight induction on galactose medium, the strain showed similar intracellular free radical levels as the parental strain BY4741.

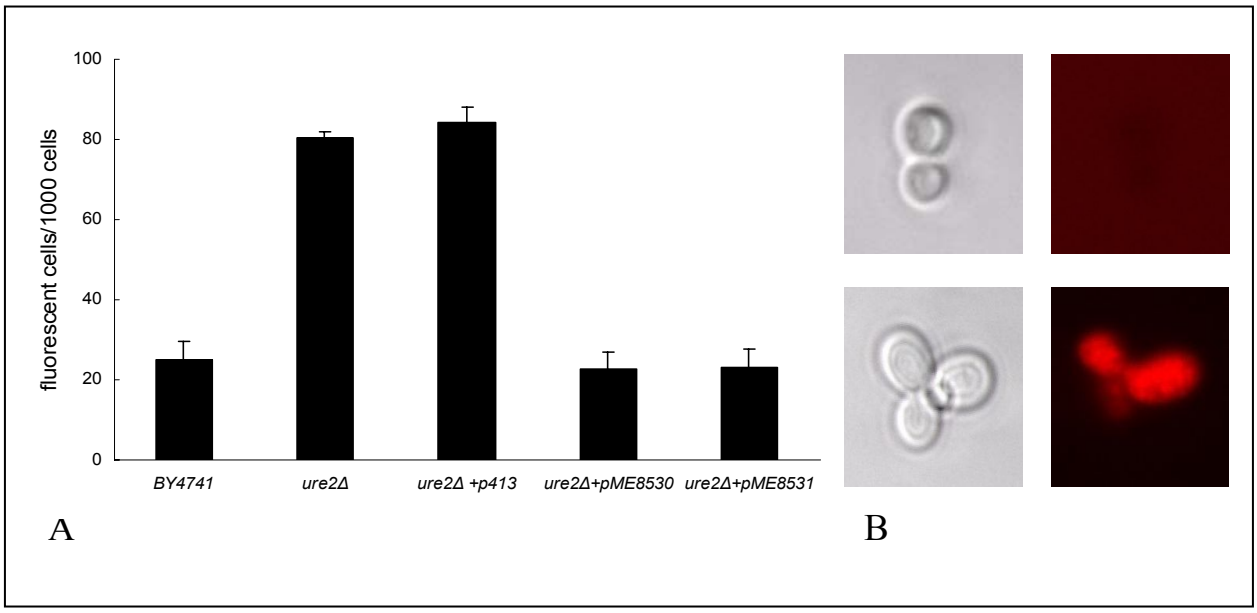

Fig. 6. Accumulation of ROS in *ure2Δ* mutant. Cultures of BY4741, *ure2Δ*, *ure2Δ*+pME8530 and *ure2Δ*+pME8531 were grown overnight in liquid induction medium with 2% galactose and appropriate supplements. The strains were incubated for 2 hours with dihydrorhodamine 123 and the number of fluorescent cells per 1000 of total cells was counted (A). (B) represents non-fluorescent wild-type cells (first line, right panel) and a typical view of rhodamine 123 fluorescence in *ure2Δ* mutant cells (second line, right panel). In left are the corresponding phase contrast displays.

Previous reports suggested a requirement for a functional *URE2* gene for oxidant protection in *S. cerevisiae* (Basu *et al*., 2004; Rai *et al.,* 2003; Rai & Cooper, 2005). Here, we elucidate this

requirement and link it with the glutathione oxidizing agents. GATA transduction cascade controls the process of glutathione degradation by regulating the expression of *CIS2* gene (encoding γ-glutamyl transpeptidase) (Springael & Penninckx, 2003). Since Ure2p is the major repressor of the expression of GATA-regulated genes, the *ure2Δ* mutant has increased *CIS2* transcript levels (Springael & Penninckx, 2003). The deletion mutant shows also increased excretion of glutathione in the medium (Perrone *et al.*, 2005). Indeed, our experiments confirm that the *ure2Δ* mutant has decreased intracellular glutathione levels. Despite the presumed compensatory role of catalase and SOD, whose activities were increased in *ure2Δ*, the mutant fails to cope with the lower glutathione content, and shows increased level of intracellular oxidants. Thus, our results underline the primary antioxidant role of glutathione in yeast, and confirm the hypothesis that glutathione deficiency is at the origin of peroxide sensitivity of *ure2Δ* mutant.

We would like to thank Michel Aigle for Ure2 antibodies, Ivan Tarassov for p413 expression plasmid and Ales Vancura for pET23b-URE2 and pET23b-URE2(ΔN) plasmids.

General summary and perspectives

Glutathione S-transferases (GSTs) represent a family of detoxification enzymes that play an important role in toxicant resistance in both prokaryotic and eukaryotic organisms. Their action is a crucial component of cellular responses to environmental pollutants, carcinogens, drugs, and products of oxidative stress. GSTs have the general function of conjugating glutathione to electrophilic substances to reduce their toxicity. Members of the family can act as peroxidases, isomerases or thiol transferases. In addition, some GSTs plays a role in the modulation of cellular signal transduction, most notably inhibiting two of the major regulatory cascades in the eukaryotic cells, GATA regulation in yeast (Cooper, 2002) and JNK signaling in mammals (Adler *et al.*, 1999).

The first yeast GST on which the present thesis has focused, Tef4p, is a homologue of elongation factor EF1Bγ. Yeast elongation factor 1 comprises subunits EF1A, EF1Bα, and EF1Bγ. Unlike EF1A and EF1Bα, neither homologue of EF1Bγ (encoded by *TEF3* and *TEF4*) is essential for yeast. In this work, sensitivity to As(V), H_2O_2 and CDNB, as well as decreased glutathione levels were observed for the *tef4Δ* mutant, indicating a possible role of Tef4p in arsenate, oxidant and organohalogens detoxification. In contrast, the *tef3Δ* mutant was not sensitive to these toxic compounds. These phenotypes of *tef4Δ* mutant could be explained by two alternative hypotheses: (1) Tef4p is a GST with enzymatic activity, important for arsenate, CDNB and peroxide detoxification in *S. cerevisiae,* or (2), Tef4p plays a yet unknown role in glutathione metabolism.

However, attempts to complement the phenotypes of the *tef4Δ* mutant with an expression vector containing a copy of the *TEF4* gene were unsuccessful and, we were unable to confirm that the observed phenotype was a direct result of *TEF4* disruption. Future work should therefore involve the isolation and purification of Tef4p and Tef3p as recombinant proteins, in order to assess the pertinence of these two hypotheses, and to study in more detail the physiological relative roles and specificities of these GST proteins.

The other yeast GST, investigated in this work, the protein Ure2 of *Saccharomyces cerevisiae* is a GST homologue with multisubstrate glutathione-dependent peroxidase activity (Bai *et al.*, 2004). It is also a precursor of the yeast prion [URE3] (Wickner, 1994), and is a major negative regulator of the GATA transcription factors (Cooper, 2002). In this work, we have presented evidence that Ure2p is also required for the detoxification of trivalent arsenic in *S. cerevisiae*. The protective role of Ure2p against arsenite is determined by the C-terminal domain of the protein, which displays GST similarity and determines GATA factor regulation role. This raises the question as to whether

this effect derives from enzymatic Ure2p action (as GST) or, alternatively, from the direct or indirect GATA-type regulation of one or more genes whose products are implicated in the arsenic stress response. The data presented in this thesis suggest that Ure2p is implicated in arsenite transport by regulating the expression of *FPS1* gene (coding for the main arsenite transporter) in yeast. The previously reported glutathione peroxidase activity of Ure2p, the decreased level of reduced glutathione in the *ure2Δ* mutant, as well as the inhibitory function of Ure2p in the expression of enzyme for non-preferred nitrogen assimilation do not seem to have an effect on arsenic sensitivity of the disruption mutant.

GATA transduction was originally discovered to regulate nitrogen utilisation in *S. cerevisiae*. Further, it has also been implicated in glutathione degradation since GATA factors and Ure2p regulate the expression of the *CIS2* gene which encodes γ-glutamyl transpeptidase, the first enzyme in glutathione degradation (Springael & Penninckx, 2003). The resulting decreased glutathione content in the *ure2Δ* mutant thus probably determines the observed sensitivity of the *ure2Δ* disruption mutant to hydrogen peroxide. The finding that glutathione deficiency is at the origin of H_2O_2 sensitivity in the *ure2Δ* mutant is supported by two observations: (1) glutathione addition to the growth medium completely restores tolerance to H_2O_2 in the *ure2Δ* mutant and (2) the *ure2Δ* mutant is not sensitive to other oxidants such as paraquat and menadione, which do not affect GSH content of the yeast cell.

The results presented here raise intriguing questions regarding the GST-dependent regulation of cellular signaling. Previously, *URE2* disruption mutants have been shown by others to be sensitive to a broad series of toxic agents (Al, As, Cr, Se, Cd, Ni, Co, Cu, Fe, Ag, Hg, H_2O_2, diamide, cumene hydroperoxide and t-butyl hydroperoxide) (Basu *et al*., 2004; Rai *et al*., 2003; Rai & Cooper, 2005). These toxicants are GST substrates, so that the sensitivity of *ure2Δ* mutant argues in favor of Ure2 being a glutathione S-transferase. Nevertheless, studies so far have not unequivocally distinguished whether the *ure2Δ* mutant phenotype is a direct or indirect effect of the absence of expression of *URE2*. Our data demonstrate that the effect of *URE2* disruption to arsenic and peroxide resistance is a result of the loss of the negative Ure2p regulation of Gat1p and/or Gln3p transcription factors, which are pleiotropic regulators and possibly involved in arsenic and peroxide detoxification. The exact GATA regulation mechanism of arsenite uptake in yeast remains, however, to be clarified. Which of the known GATA factors are implicated in this regulation? Another interesting question is the nature of the primary signal event. While the mechanism of

Ure2/GATA factor interaction has been clarified, the mechanism of signal transmission upstream of formation of the complex Ure2p-Gln3 is still unknown. Importantly, arsenite transport is a newcomer in GATA regulated processes in *S. cerevisiae*. This not only expands the scope of GATA regulated processes far beyond nitrogen assimilation, but also raises the question "How such different physiological processes can be fine-regulated using one mechanism?". It is clear that unknown members of GATA regulation cascade exist, and it would be of great interest to determine their nature and their exact role in each of the processes regulated by the GATA system and Ure2p, respectively. As the *ure2Δ* mutant shows a number of sensitive phenotypes, it will be also interesting to study its role in the metabolism of other toxic metals, and to extend the present study to other species to gain a more detailed understanding of GST regulatory roles in lower eukaryotes.

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Title in English: Glutathione S-transferases and oxidative stress in *Saccharomyces cerevisiae*

Glutathione S-transferases are an enzyme family of multifunctional proteins playing an important role in cellular detoxification of exogenous and endogenous toxic compounds. In addition, GSTs can serve as peroxidases, isomerases and thiol transferases or have non-catalytic functions, among which binding of non-substrate ligands and the modulation of signaling processes.
A systematic approach, using defined GST mutants in the same genetic background, has been taken to demonstrate the connection between GSTs and the oxidative stress caused by peroxide and arsenic in *S. cerevisiae*. This screening revealed that two yeast GSTs (Ure2p and Tef4p) may play a role in arsenic and oxidant detoxification. Tef4p is a homologue of yeast elongation factor EF1Bγ and our studies showed that the corresponding single disruption mutant is sensitive to H_2O_2 and As(V).
Regarding the role of Ure2p, we present evidence that Ure2p is required for the detoxification of trivalent arsenic in *S. cerevisiae*. The protection role of Ure2p towards arsenite is determined by the GST domain of the molecule and is a result of the GATA repression role of Ure2p. GATA regulation is also in the origin of oxidant sensitivity of the mutant *ure2Δ*.

Keys words: glutathione S-transferases, arsenic, oxidative stress, regulation

Titre en français: Glutathion S-transférases et stress oxydatif chez *Saccharomyces cerevisiae*

Les glutathion S-transférases répresentent une famille d'enzymes qui joue un rôle important dans la détoxification de composés toxiques d'origine éxogène et endogène. Les GSTs peuvent avoir des activités péroxidase, isomérase ou thiol transférase et également d'exprimer fonctions non-catalytiques, comme l'interaction avec differents ligands ou la modulation de la signalisation cellulaire.
Un éventail des mutants de *S. cerevisiae* inactivés sélectivement et de la même manière dans un seul gène de leur génome a été choisi pour étudier le rôle des glutathion S-transférases dans la réponse au stress oxydatif et à l'arsenic. Notre criblage a montré l'existance de deux protéines (Ure2p et Tef4p) qui peuvent avoir un rôle dans la détoxification de l'arsenic et du peroxide. Tef4p représente un des homologues du facteur d'élongation EF1Bγ chez la levure et nous avons démontré pour le mutant *tef4Δ* une sensibilité à l'As(V) et au H_2O_2.
Concernant Ure2p, un rôle de la protéine dans la détoxification de l'arsenic trivalent chez *S. cerevisiae* a été proposée. Le rôle protecteur de Ure2p déterminé par la partie GST de la molécule est résultat de la fonction de la protéine dans la régulation du type GATA. Le meme rôle est dans l'origine également de la sensibilité aux oxydants du mutant *ure2Δ*.

Mots clés : glutathion S-transférases, arsenic, stress oxydatif, régulation

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