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Glutathione Deficiency Leads to Riboflavin Oversynthesis in the Yeast *Pichia guilliermondii*

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Abstract The *Pichia guilliermondii GSH1* and *GSH2* genes encoding *Saccharomyces cerevisiae* homologues of glutathione (GSH) biosynthesis enzymes, γ-glutamylcysteine synthetase and glutathione synthetase, respectively, were cloned and deleted. Constructed *P. guilliermondii Δgsh1* and *Δgsh2* mutants were GSH auxotrophs, displayed significantly decreased cellular GSH+GSSG levels and sensitivity to *tert*-butyl hydroperoxide, hydrogen peroxide, and cadmium ions. In GSH-deficient synthetic medium, growths of *Δgsh1* and *Δgsh2* mutants were limited to 3–4 and 5–6 cell divisions, respectively. Under these conditions *Δgsh1* and *Δgsh2* mutants possessed 365 and 148 times elevated riboflavin production, 10.7 and 2.3 times increased cellular iron content, as well as 6.8 and 1.4 fold increased ferrireductase activity, respectively, compared to the wild-type strain. Glutathione addition to the growth medium completely restored the growth of both mutants and decreased riboflavin production, cellular iron content, and ferrireductase activity to the level of the parental strain. Cysteine also partially restored the growth of the *Δgsh2* mutants, while methionine or dithiothreitol could not restore the growth neither of the *Δgsh1*, nor of the *Δgsh2* mutants. Besides, it was shown that in GSH presence riboflavin production by both *Δgsh1* and *Δgsh2* mutants, similarly to that of the wild-type strain, depended on iron concentration in the growth medium. Furthermore, in GSH-deficient synthetic medium *P. guilliermondii Δgsh2* mutant cells, despite iron overload, behaved like iron-deprived wild-type cells. Thus, in *P. guilliermondii* yeast, glutathione is required for proper regulation of both riboflavin and iron metabolism.

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Introduction

The yeast *Pichia guilliermondii* belongs to a group of yeast species that produce significant amounts of riboflavin (vitamin B_2) in response to iron limitation [1]. A large collection of *P. guilliermondii* mutants defective in the regulation of vitamin B_2 biosynthesis and iron transport were described during the last decade. Riboflavin overproducing *rib80*, *hit1,* and *red6* mutants exhibit a considerably increased rate of iron transport, cellular iron accumulation, ferrireductase activity and possess inhibited activity of Fe/S cluster proteins [11, 12, 33, 37]. Further studies reported that the deletion of the yeast frataxin homologue *YFH1* in *P. guilliermondii* and *Candida albicans* leads to misregulation of riboflavin biosynthesis and iron acquisition, as well as defects in Fe/S cluster proteins and hypersensitivity to oxidative stress [25, 29]. In addition, *yfh1* mutation impairs sulfate assimilation in *P. guilliermondii* [25]. It was assumed that Fe/S clusters, rather than free iron, are involved in the regulation of iron acquisition and riboflavin biosynthesis in *P. guilliermondii* [11]. Meanwhile, it was shown that *Saccharomyces cerevisiae Δyfh1* mutant cells are characterized by defects in the biosynthesis of Fe/S clusters, iron and glutathione hyperaccumulation into mitochondria, severe mitochondrial glutathione-dependent oxidative stress, and thiol oxidation of key mitochondrial enzymes [3, 9]. Besides, it was established that mutations *rib80, rib81,* and *hit1*, affecting the regulation of riboflavin biosynthesis and iron acquisition, cause oxidative stress in *P. guilliermondii.* Induction of oxidative stress by superoxide generating

agents increased flavinogenic activity and iron accumulation in the cells [6, 23]. Thus, it was hypothesized that the increased riboflavin production is an element of antioxidant defense in *P. guilliermondii*.

It is generally known that antioxidant thiol buffers—glutathione (GSH) and thioredoxin—are responsible for the maintenance of cellular reduction–oxidation (redox) status. Due to high concentration in the cell (1–10 mM), low redox potential (−240 mV), and stability that results from the unusual γ-glutamyl-cysteine bond, GSH is regarded as a major cellular redox buffer [5]. GSH fulfills a variety of biological functions: it participates in nutritional and oxidative stress response and in detoxification of electrophilic xenobiotics and heavy metals, serves as a reservoir of redox labile sulfur amino acid cysteine, and influences—through redox—several essential processes such as gene expression, cell proliferation, and apoptosis [2, 10, 22]. In addition, GSH performs a specific function in the maturation of cytosolic Fe/S proteins [16, 35]. It is believed that the status of Fe/S clusters serves as the main sensor mechanism in iron homeostasis in the yeast *S. cerevisiae* and probably in regulation of riboflavin biosynthesis in the yeast *P. guilliermondii.* Since the genome of *P. guilliermondii* has been sequenced, and efficient methods of gene manipulation have been developed [6], this yeast species is a convenient model organism for studying interrelationships between riboflavin biosynthesis, iron metabolism, and cellular GSH status.

The genes encoding enzymes of GSH biosynthesis were cloned from a number of organisms including yeasts *S. cerevisiae*, *Schizosaccharomyces pombe*, as well as *Escherichia coli*, plants, mouse, rat, and human [15, 22]. In the yeast *S. cerevisiae* GSH is synthesized nonribosomally by the consecutive action of two cytosolic enzymes γ-glutamylcysteine synthetase (γGCS) and glutathione synthetase, encoded by the *GSH1* and *GSH2* genes, respectively. Mutant strains of *S. cerevisiae* lacking *GSH1* gene are unable to grow in the absence of exogenous GSH and are sensitive to oxidative stress caused by hydrogen peroxide, *tert*-butyl hydroperoxide [13], and cadmium ions. In contrast, strains lacking *GSH2* gene grow poorly, since the dipeptide intermediate, γ-glutamylcysteine, could partially substitute for GSH [13, 14].

The aim of this work was to estimate the likely involvement of GSH in the regulation of riboflavin biosynthesis and iron accumulation in the flavinogenic yeast *P. guilliermondii*. For that purpose, *P. guilliermondii GSH1* and *GSH2* genes were cloned, and the correspondent null mutant strains were constructed and characterized. This study shows that the blocking of glutathione biosynthesis leads to riboflavin oversynthesis and increased cellular iron accumulation in the yeast *P. guilliermondii*.

Materials and Methods

Yeast Strains and Media

The used *P. guilliermondii* strains were ATCC 6260 and R-66 (*MAT*$^{-}$*hisX ura3*) [25] wild-type strains and null *gsh1* (*Δgsh1::pPGK-ScURA3 ura3 hisX)* and *gsh2* (*Δgsh2::pPGK-ScURA3 ura3 hisX*) mutants, obtained in this study. Yeast cells were cultivated on saccharose-containing (2 %) synthetic Burkholder medium at 30 °C and supplemented with uridine (50–100 mg L^{-1}), histidine (40 mg L^{-1}), and GSH (0.001–0.5 mM), if required [32]. Iron-supplemented media contained 3.6 μM of iron, added as ammonium ferrous sulfate hexahydrate. Iron-deficient media contained approximately 0.18 μM of iron. Iron was removed from the medium with 8-hydroxyquinoline as described earlier [34]. *P. guilliermondii* null *gsh1* and *gsh2* mutants were picked from a medium containing 3 % saccharose, 0.67 % yeast nitrogen base (YNB), 0.4 % vitamin-free casamino acids, 0.2 mM GSH, and 2 % agar. The selected strains were subsequently analyzed for Gsh^{-} phenotype on the same medium without vitamin-free casamino acids and GSH, or on solid or liquid synthetic Burkholder medium. *E. coli* DH5α strain used for plasmid propagation was cultured in LB medium (1 % NaCl, 1.5 % peptone, and 0.5 % yeast extract) with ampicillin (100 μg/ml) at 37 °C.

Cloning of *P. guilliermondii* GSH1 Gene, Construction of *gsh1*::*pPGK-ScURA3* Deletion Cassette and Null *gsh1* Strain

To clone the *P. guilliermondii GSH1* gene, a 4.547 kb DNA fragment, bearing *PgGSH1* gene, was amplified from the genomic DNA of *P. guilliermondii* wild-type strain ATCC 6260 by polymerase chain reaction (PCR), using primers JB13F and JB14R (Table 1), subsequently digested with *Kpn*I restriction enzyme and cloned into the *Kpn*I site of the pUC57 vector. The obtained plasmid was named pUC57+PgGSH1. To create the *gsh1::pPGK-ScURA3* deletion cassette, the coding sequence of the amino acid residues from 57 to 567 was replaced by a modified *S. cerevisiae URA3* gene under the control of the *P. guilliermondii* phosphoglycerate kinase (*PGK1*) gene promoter. For that purpose, the 5′-DNA fragment (1389 bp) and the 3′-DNA fragment (1624 bp) of the *PgGSH1* gene were amplified from pUC57 + PgGSH1 plasmid by PCR using primers JB13F/JB32R and JB33F/JB14R, respectively (Table 1). The 5′-DNA fragment corresponded to the promoter and part of the *PgGSH1* coding sequence, while the 3′-DNA fragment contained part of the *PgGSH1* coding and terminator sequence. Both PCR products were purified, digested with *Kpn*I and *Bam*HI restriction endonucleases,

Table 1 Oligonucleotides used as primers for PCR amplification in this study

Name	Sequence (5′–3′)	Site
Primers for cloning of *P. guilliermondii GSH1* gene		
JB13F	ATGGTACCATTGGAGGACACTCTTTTG	*Kpn*I
JB14R	AAGGTACCTTTCCTTTCCTGAGC	*Kpn*I
Primers for construction of *gsh1::pPGK-ScURA3* deletion cassette		
Primers for isolation of 5′-DNA fragment of *PgGSH1* gene		
JB13F	ATGGTACCATTGGAGGACACTCTTTTG	*Kpn*I
JB32R	AAGGATCCGACAAGCATGTATTCAAC	*Bam*HI
Primers for isolation of 3′-DNA fragment of *PgGSH1* gene		
JB33F	AAGGATCCTTGATCTCAGCGAATTTG	*Bam*HI
JB14R	AAGGTACCTTTCCTTTCCTGAGC	*Kpn*I
Primers for confirmation of null *PgGSH1* mutation		
JB29F	GGTGTTAGTGTTGATAACGGAAAC	
JB4R	CCCTGCAGGGTAATTCTAGCAATCGATC	
Primers for cloning of *P. guilliermondii GSH2* gene		
JB17F	TAAGGTACCTTATCGTTGAACAACAG	*Kpn*I
JB18R	GAGGTACCTCATTGGCTTCTTTGAG	*Kpn*I
Primers for construction of *gsh2::pPGK-ScURA3* deletion cassette		
JB20F	TAAGATCTTATCGTATCCCTTCAAAG	*Bgl*II
JB19R	GTAGATCTACATATTAGCTCACGTG	*Bgl*II
Primers for confirmation of null *PgGSH2* mutation		
JB30F	CGACTCTTTGACTATCTTGAAGAC	
JB4R	CCCTGCAGGGTAATTCTAGCAATCGATC	

Underlined nucleotides mark the restriction sites indicated on the right

and simultaneously cloned into the *Kpn*I site of a pUC57-BHI vector, which was obtained from the pUC57 vector by elimination of the *Bam*HI restriction site and subsequent self-ligation. The resultant plasmid was linearized with *Bam*HI restriction enzyme and ligated with PGK-URA3/*Bam*HI fragment. The latter fragment was gained from pPGKURA3 plasmid, digested with endonuclease *Bam*HI after fractionation by agarose electrophoresis and subsequent elution from gel. The *gsh1::pPGK-ScURA3* deletion cassette was released as a 4.509 kb fragment with *Kpn*I and transformed into *P. guilliermondii MAT*$^{-}$*hisX ura3* wild-type strain by the lithium-acetate method. Ura^{+} transformants were selected on a solid saccharose-containing medium without uridine. Several Ura^{+} Gsh^{-} transformants unable to grow in liquid synthetic saccharose-containing medium without exogenous GSH were picked for total genomic DNA isolation and further PCR analysis. The *gsh1::pPGK-ScURA3* deletion cassette appeared to be integrated into the genome of selected transformants by homologous recombination that led to a knock-out of the *PgGSH1* structural gene as confirmed by PCR analysis, using primers JB29F and JB4R (Table 1). Single integration of the deletion cassette into the genome of selected transformants was verified by Southern blot analysis, using open reading frame (ORF) of *S. cerevisiae URA3* gene as a probe (data not shown).

Cloning of *P. guilliermondii GSH2* Gene, Construction of *gsh2::pPGK-ScURA3* Deletion Cassette and Null *gsh2* Strain

To clone the *P. guilliermondii GSH2* gene, a 3.732 kb DNA fragment, carrying *PgGSH2* gene, was amplified from the genomic DNA of *P. guilliermondii* wild-type strain ATCC 6260 by PCR, using primers JB17F and JB18R (Table 1), subsequently digested with *Kpn*I restriction enzyme, and cloned into the *Kpn*I site of the pUC57 vector. The obtained plasmid, pUC57+PgGSH2, was used for construction of the *gsh2::pPGK-ScURA3* deletion cassette, in which the modified *S. cerevisiae URA3* gene (*pPGK-ScURA3*) replaced the coding sequence of the *PgGSH2* gene. A vector harboring promoter (1077 bp) and terminal (970 bp) regions of the *PgGSH2* gene was obtained by amplification of pUC57+PgGSH2 plasmid by PCR, using primers JB20F and JB19R (Table 1), subsequent treatment with T4DNA polymerase, and self-ligation. The resultant plasmid was linearized with *Bgl*II restriction enzyme and ligated with PGK-URA3/*Bam*HI fragment, which was obtained as described above. The *gsh2::pPGK-ScURA3* deletion cassette was released as a 3.543 kb fragment with *Kpn*I and transformed into *P. guilliermondii MAT*$^{-}$*hisX ura3* wild-type strain by the lithium-acetate method. Ura^{+} transformants were selected on a saccharose-containing medium without uridine and subsequently analyzed for Gsh^{-} phenotype by replica plating on a synthetic saccharose-containing medium with and without exogenous GSH. Several Ura^{+} Gsh^{-} transformants were picked for total genomic DNA isolation and further PCR and Southern blot analysis. Correct chromosomal replacement of the wild-type *PgGSH2* gene with the *gsh2::pPGK-ScURA3* null mutant allele was confirmed by PCR analysis, using primers JB30F and JB4R (Table 1). Integration of the single copy of the deletion cassette into the genome of selected transformants was proven by Southern blot analysis, using ORF of *S. cerevisiae URA3* gene as a probe (data not shown).

Molecular Techniques

General DNA manipulations were performed as previously described [28]. Yeast transformation was carried out according to [7]. Plasmids constructed and used in this study are listed in Table 2. Synthetic oligonucleotide primers, produced by "IDT Technologies" (USA), were used for the amplification of DNA fragments by PCR. Reagents and restriction enzymes were purchased from

Table 2 Plasmids constructed and used in this study

Plasmid name	Description	Reference or source
pUC57	Yeast-bacterium shuttle vector	GI:2440162
pUC57-BHI	Yeast-bacterium shuttle vector lacking *Bam*HI restriction site	This study
pPGKURA3	Yeast-bacterium shuttle vector, originated from pUC19, harboring ORF of *ScURA3* gene under the PGK1 gene promoter of *P. guilliermondii*	[25]
pUC57+PgGSH1	Plasmid harboring cloned *P. guilliermondii GSH1* gene	This study
pUC57+PgGSH2	Plasmid containing cloned *P. guilliermondii GSH2* gene	This study
pUC57-BHI+N_C_PgGSH1	Plasmid harboring 5′- and 3′-flanking regions of *P. guilliermondii GSH1* gene	This study
pUC57+N_C_PgGSH2	Plasmid harboring promoter and terminal regions of *P. guilliermondii GSH2* gene	This study
pΔPgGSH1	Plasmid containing *gsh1::pPGK-ScURA3* deletion cassette	This study
pΔPgGSH2	Plasmid containing *gsh2::pPGK-ScURA3* deletion cassette	This study

following corporations: "Sigma" (USA), "Reanal" (Hungary), "Fermentas" (Lithuania), "New England Bio Labs" (USA), and "Promega" (USA). Protein homology search and multiple sequence alignments were performed using the BLAST algorithm of the National Center for Biotechnology Information (Bethesda, MD, USA) and Multalin algorithm, version 5.4.1, available at http://www.ncbi.nlm.nih.gov/BLAST/ and http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html respectively.

Analytical Assays

Total reduced and oxidized glutathione (GSH+GSSG) content was analyzed in cell-free extracts as previously described [39] using the standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) in the presence of glutathione reductase and NADPH [8]. The protein concentration was determined by the Lowry method using bovine serum albumin as a standard [19]. Riboflavin was measured in culture medium fluorometrically with a Turner Quantech FM 109510–33 fluorometer using a solution of synthetic riboflavin as a standard. To avoid errors during the assay of riboflavin content, the samples, as well as a standard riboflavin solution were additionally treated with sodium hydrosulfite (10 % $Na_2S_2O_4$/5 % $NaHCO_3$). Hydrosulfite completely extinguishes riboflavin fluorescence. The cellular iron content was determined with 2,2-dipiridyl as described earlier [12]. Thin-layer chromatography was carried out on Silufol plates with a system of 5 % solution of KH_2PO_4 in water. The ferrireductase activity of washed cells was assayed spectrophotometrically (Helios Gamma UVG-100105 spectrophotometer) with ferric citrate (0.2 mM) as a substrate [12].

Cell Preparation for the Assay of Ferrireductase Activity and Cellular Iron Content

Yeast strains were precultivated in synthetic glutathione-supplemented (50 μM) Burkholder medium. To obtain sufficient cell yield (about 45–50 mg), null *gsh1* mutant cells were inoculated parallely in two flasks containing 150 ml of GSH-lacking synthetic Burkholder medium with initial $OD_{600nm} \sim 0.1$, cultured for 3–4 divisions, and combined before harvesting. Simultaneously, one flask containing 150 ml of the same medium was used for the cultivation of each yeast strain in GSH presence, as well as for the wild-type strain and null *gsh2* mutant—in GSH absence. Cells were washed with water and processed for the indicated assays.

Results and Discussion

Sequence Analysis of the *P. guilliermondii* GSH1 and GSH2 Genes

The genes that encode putative γ-glutamylcysteine synthetase and glutathione synthetase were identified in the *P. guilliermondii* genome based on their primary sequence homology to *S. cerevisiae GSH1/YJL101C* and *GSH2/YOL049W* genes respectively. The *P. guilliermondii* gene designated as *PgGSH1* was predicted to encode a polypeptide of 767 amino acids with a theoretical molecular mass of 88.3 kDa. Alignment of the putative PgGsh1p with the homologous proteins from other organisms revealed that PgGsh1p shares the strongest homology with glutamate–cysteine ligase from *Pichia stipitis* (66 % identity and 78 % similarity, Accession No. XP_001385377.2). Other proteins with high similarity included: glutamate-cysteine ligase from *C. albicans* (53 % identity, 70 % similarity, Accession No. gb|EEQ42421.1|), γGCS from *Hansenula polymorpha* (48 % identity, 63 % similarity, Accession No. AF435121_1), γGCS from *Pichia pastoris* (45 % identity, 62 % similarity, Accession No. XP_002489831.1), Gsh1p from *S. cerevisiae* (44 % identity, 57 % similarity, Accession No.

NP_012434.1), and γGCS from *S. pombe* (41 % identity, 55 % similarity, Accession No. emb|CAA59379.1|).

The *P. guilliermondii* gene designated as *PgGSH2* encodes a protein of 481 amino acids with a predicted molecular mass of 55.2 kDa. Protein sequence analysis revealed that PgGsh2p possesses the highest similarity to glutathione synthetase from *P. stipitis* (62 % identity, 79 % similarity, Accession No. XP_001383934.1). *P. guilliermondii* Gsh2p also shares high similarity with putative glutathione synthetase from *C. albicans* (60 % identity and 75 % similarity, Accession No. XP_716243.1), as well as with glutathione synthetase from *P. pastoris* (57 % identity and 71 % similarity, Accession No. XP_002490629.1), *H. polymorpha* (53 % identity and 69 % similarity, Accession No. AF397211_1), *S. cerevisiae* (49 % identity and 68 % similarity, Accession No. NP_014593.1), and *Dekkera bruxellensis* (50 % identity and 65 % similarity, Accession No. gb|EIF45717.1|).

Phenotypic Analysis of *P. guilliermondii* Null *gsh1* and *gsh2* Mutant Strains

To elucidate the putative role of glutathione in the regulation of flavin production, GSH-deficient strains of the yeast *P. guilliermondii* were constructed by the gene replacement method as described in "Materials and Methods" section. Phenotypic analysis showed that the growth of *P. guilliermondii Δgsh1* and *Δgsh2* mutants in a liquid synthetic medium without exogenous GSH was limited to 3–4 and 5–6 cell divisions, respectively. Under these conditions both *Δgsh1* and *Δgsh2* mutants of *P. guilliermondii* accumulated significant amounts of a fluorescent substance in cultural medium, which was identified as riboflavin, using absorption spectra analysis and thin-layer chromatography (data not shown). Riboflavin production by the *Δgsh1* and *Δgsh2* mutants was, respectively, 365 and 148 times higher when compared to the parental strain (Table 3). Addition of 10 μM GSH to the growth medium completely restored the growth of both mutants and decreased riboflavin production to the level of the parental strain. GSH in concentration of 0.5 mM led to growth inhibition (Table 3). Cysteine also partially restored the growth of *P. guilliermondii Δgsh2* mutants, while methionine or dithiothreitol (DTT) did not restore it (Fig. 1). At the same time, cysteine, methionine, or DTT could not substitute for GSH for cell growth in *P. guilliermondii Δgsh1* mutants (Fig. 1). On the contrary, beta-mercaptoethanol and cysteine partially restored the growth of the *H. polymorpha* point *gsh2* mutant strain, with impaired γGCS [38]. Besides, a previous study had reported that reducing compounds containing a free sulphydryl group, such as beta-mercaptoethanol, DTT, and

Table 3 Growth and riboflavin productivity of *P. guilliermondii* wild-type strain R-66 (*WT*), *Δgsh1* and *Δgsh2* mutants in liquid synthetic iron-sufficient medium depending on GSH concentration

GSH (mM)	Biomass (mg dry cells* mL^{-1})			Riboflavin productivity (μg*mg dry $cells^{-1}$)		
	WT	*Δgsh1*	*Δgsh2*	*WT*	*Δgsh1*	*Δgsh2*
0	4.62	0.04	0.29	0.05	18.23	7.4
0.001	4.47	2.16	5.43	0.06	0.28	0.06
0.01	4.66	3.09	6.13	0.05	0.09	0.05
0.05	4.54	3.14	5.76	0.06	0.08	0.06
0.1	4.56	3.09	5.85	0.06	0.07	0.06
0.2	4.34	2.97	5.3	0.06	0.08	0.08
0.5	1.59	2.68	2.64	0.09	0.11	0.13

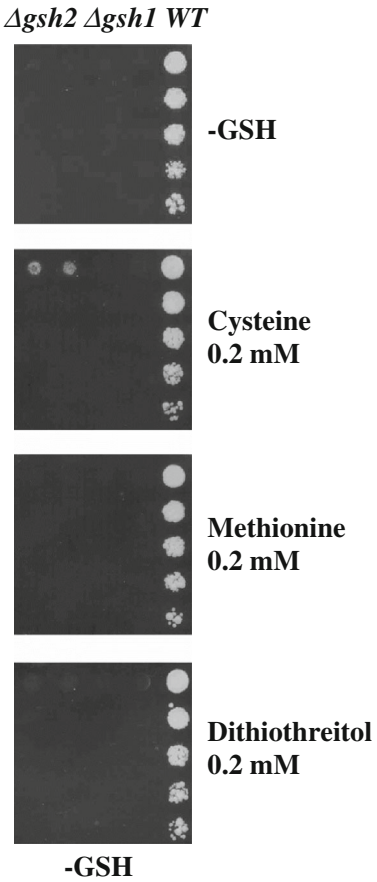

Fig. 1 Growth of *P. guilliermondii* wild-type strain R-66 (*WT*) and null *gsh1* and *gsh2* mutants on solid synthetic YNB medium depending on different sulfur sources. Cultures of each strain were grown in liquid synthetic medium supplemented with 100 μM glutathione and 100 mg/L of uridine for 24 h and diluted to an optical density $A_{600} = 0.2$. Serial five times dilutions were made. Four microliter suspensions of each dilution were spotted on the plates. Growth was estimated after 3–5 days of incubation at 30 °C

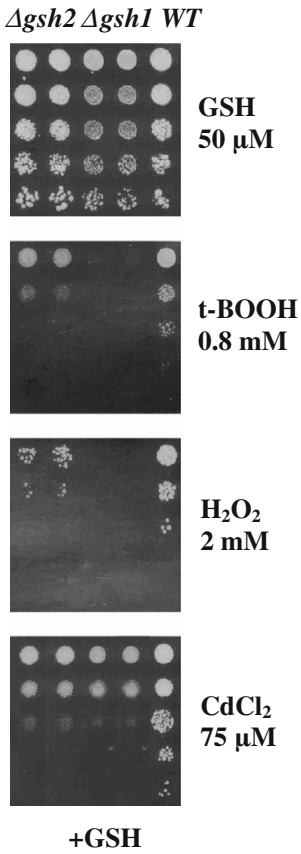

Fig. 2 Sensitivity of *P. guilliermondii* wild-type strain R-66 (*WT*) and null *gsh1* and *gsh2* mutants to the different stress-induced factors: *tert*-butyl hydroperoxide (t-BOOH), hydrogen peroxide, and cadmium ions ($CdCl_2$). Solid synthetic YNB medium was supplemented with 50 μM GSH. Cell preparation and incubation were performed as described in Fig. 1

cysteine could relieve GSH auxotrophy of the *S. cerevisiae Δgsh1* mutant, lacking functional γGCS [13]. However, contradictory results obtained later demonstrated that only exogenous glutathione can restore the growth of the *S. cerevisiae Δgsh1* mutant, while high DTT concentrations only delay the time necessary for exhaustion of the cellular GSH pool [4, 30, 36]. Thus, glutathione in the yeast *P. guilliermondii*, similarly to *S. cerevisiae*, is essential for growth during non-stress conditions. Besides, *P. guilliermondii* wild-type strain and constructed *Δgsh1* and *Δgsh2* mutants were tested for growth in the presence of stress-generating agents. It was shown that null *gsh1* and *gsh2* mutants of *P. guilliermondii*, similarly to GSH-deficient mutants of *S. cerevisiae* [13] and *H. polymorpha* [40], were more sensitive to *tert*-butyl hydroperoxide, hydrogen peroxide, and cadmium ions, than the wild-type strain (Fig. 2).

Growth and cellular GSH+GSSG content of the wild-type strain and of *Δgsh1* and *Δgsh2* mutants of the yeast *P. guilliermondii*, as well as their riboflavin production, were estimated in liquid synthetic medium supplemented with 50 μM GSH depending on iron concentration. In iron-deprived medium, both *Δgsh1* and *Δgsh2* mutants, similar to the wild-type strain, displayed an affected growth rate compared to the iron-replete media (Table 4). Besides, it was shown that *Δgsh1* as well as *Δgsh2* mutants exhibited significantly decreased cellular GSH+GSSG levels as compared to that of the wild-type strain, independent from iron concentration in the growth medium (0.18, 3.6, 8.9 μM; Table 4). Under condition of GSH supplementation, riboflavin production by both *Δgsh1* and *Δgsh2* mutants, similar to that of the wild-type strain, depended on iron concentration in the growth medium. It was high in iron-deprived medium and consequently low in iron-replete media (Table 4). Besides, it was observed that high concentration of iron in GSH-deficient growth medium did not suppress riboflavin production by the *P. guilliermondii Δgsh2* mutant in contrast to the wild-type strain (Table 5). Furthermore, the addition of the reducing agent dithiothreitol (0.5 mM) to the growth medium reduced, but did not abolish riboflavin production by the *P. guilliermondii Δgsh2* mutant (Table 5). In addition, cellular iron content and ferrireductase activity in the wild-type strain and in *Δgsh1* and *Δgsh2* mutants of *P. guilliermondii* grown in GSH-deficient and GSH-supplemented (50 μM) synthetic media were estimated in this study. It was established that under GSH deficiency the cellular iron content in *Δgsh1* and *Δgsh2* mutants was, respectively, 10.7 and 2.3 times increased compared to the parental strain (Fig. 3a). Moreover, under these conditions, the *Δgsh1* mutant manifested 6.8 times increased ferrireductase activity, while the *Δgsh2* mutant showed only a 1.4 times increase as compared to the wild-type strain (Fig. 3b). Glutathione addition decreased cellular iron content and ferrireductase activity in both mutants to the level of the parental strain.

The relation between riboflavin biosynthesis, iron accumulation, and cellular GSH status in the yeast *P. guilliermondii* was explored in the present study. It was shown that the constructed *P. guilliermondii* null *gsh1* and *gsh2* mutants share properties of GSH-deficient mutants (i.e., GSH auxotrophy and significantly decreased cellular GSH+GSSG levels) and of mutants defective in the regulation of vitamin B_2 biosynthesis (i.e., riboflavin overproduction, increased cellular iron accumulation, and ferrireductase activity). Furthermore, the *Δgsh1* and *Δgsh2* mutants display sensitivity to the compounds that cause oxidative stress, which is common for both mutant groups. It is known that *P. guilliermondii,* similar to other flavinogenic yeasts, exhibits a coordinated regulation of riboflavin biosynthesis and iron accumulation [1, 12, 31]. The results presented in this study do not contradict that such regulation could also occur under conditions of GSH deficiency. Besides, it was established that, similarly to *S. cerevisiae*, GSH is indispensable for the growth of the

Table 4 Growth, riboflavin productivity, and cellular GSH+GSSG content of *P. guilliermondii* wild-type strain R-66 (*WT*), *Δgsh1* and *Δgsh2* mutants in liquid synthetic glutathione-supplemented (50 μM) medium depending on iron concentration

Strain	Biomass (mg dry cells* mL^{-1})			Riboflavin productivity (μg*mg dry $cells^{-1}$)			GSH+GSSG (nmol*mg $protein^{-1}$)		
	Iron (μM)								
	0.18	3.6	8.9	0.18	3.6	8.9	0.18	3.6	8.9
WT	1.41	7.06	6.47	10.3	0.12	0.15	110	173.1	137.8
Δgsh1	1.37	3.21	2.65	5.11	0.34	0.26	0	1.29	1.29
Δgsh2	1.7	6.53	6.93	13	0.16	0.13	20.7	5.03	4.23

Table 5 Growth and riboflavin productivity of *P. guilliermondii* wild-type strain R-66 (*WT*) and *Δgsh2* mutant depending on iron concentration or sulfur source

Strain	Biomass (mg dry cells* mL^{-1})[a]			Riboflavin productivity (μg*mg dry $cells^{-1}$)[a]			Biomass (mg dry cells* mL^{-1})[b]			Riboflavin productivity (μg*mg dry $cells^{-1}$)[b]		
	Iron (μM)						SO_4^{2-}	DTT	GSH	SO_4^{2-}	DTT	GSH
	0.18	3.6	8.9	0.18	3.6	8.9						
WT	1.14	8.53	6.69	15.4	0.12	0.12	4.29	4.25	4.39	0.05	0.08	0.06
Δgsh2	0.25	0.25	0.23	5.99	6.47	7.76	0.46	0.64	4.86	7.23	4.32	0.08

[a] Yeast strains were grown in liquid synthetic glutathione-deficient medium with varying iron concentration

[b] Yeast strains were grown in liquid synthetic iron-supplemented medium with different sulfur sources

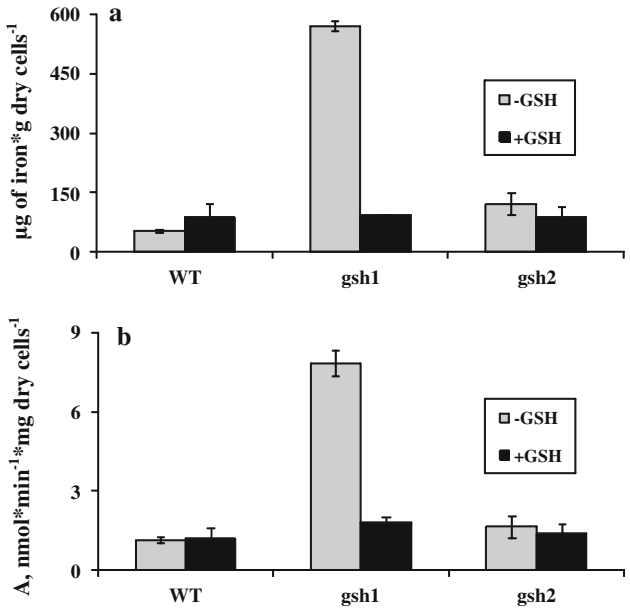

Fig. 3 Iron content (**a**) and ferrireductase activity (**b**) in wild-type strain R-66 (*WT*), null *gsh1* and *gsh2* mutants of the yeast *P. guilliermondii* in the absence or presence of 50 μM GSH

P. guilliermondii Δgsh1 mutant under non-stress conditions. Earlier, glutathione was shown to be necessary for maturation of cytosolic Fe/S proteins and for maintaining of cellular redox status in yeast *S. cerevisiae* [35]. Moreover, it was demonstrated that thiol-redox maintenance duties demand high GSH concentration, while iron metabolism function could be satisfied with a trace amount of GSH [16]. Recent studies suggested that GSH plays an ancillary role in cellular thiol-redox control in *S. cerevisiae*, while its vital function is associated with iron metabolism [16, 17]. The current study demonstrates that even such a low concentration of glutathione as 1 μM is sufficient for a considerable growth restoration of both *P. guilliermondii* GSH-deficient mutants and causes a drastic decrease in riboflavin production (Table 3). This could indicate that, similarly to *S. cerevisiae*, very small amounts of glutathione could satisfy the functions important for the viability of the yeast *P. guilliermondii*. The sufficient GSH concentration for the vitality of the *S. cerevisiae Δgsh1* mutant is 0.5 μM [16]. Besides, the observed iron-deprived phenotype of *P. guilliermondii* null *gsh1* and *gsh2* mutant cells, in GSH-deficient iron-supplemented synthetic medium, correlates well with the recently shown iron starvation-like response, triggered by GSH depletion in the yeast *S. cerevisiae*, which was associated with cytoplasmic Fe/S enzymes inactivation [16]. Obtained data also display that addition of DTT to the GSH-deficient growth medium of the *P. guilliermondii Δgsh2* mutant reduced, but did not abolish riboflavin production by the mutant (Table 5). It was shown previously that iron, but not DTT, could partially rescue maturation of cytosolic Fe/S proteins in *S. cerevisiae* GSH depleted cells [16, 35]. Nevertheless, DTT apparently could substitute for some GSH redox functions, thereby decreasing its consumption [4, 30, 36] and consequently partially sparing its vital iron function. Thus, one could assume that the essential function of glutathione in

the yeast *P. guilliermondii* might also be associated with its participation in iron metabolism, rather than with its role in controlling of cellular redox status.

It is worth to note that in *S. cerevisiae*, glutathione, as well as the monothiol glutaredoxins Grx3 and Grx4, take part in iron sensing by the Aft1 transcriptional regulator of iron homeostasis, which in turn indirectly senses iron through the cellular Fe/S biogenesis status [21, 24, 27]. Furthermore, existence of unusual GSH–FeS–Grx3/4 complexes has been proved in vitro [18, 26] and in vivo, and their participation in iron delivery to all iron-containing proteins and to mitochondria has been suggested [20]. In addition, it was supposed that Fe/S clusters, rather than free iron, are involved in the regulation of iron acquisition and riboflavin biosynthesis in *P. guilliermondii* [11]. Thus, one may assume that a functional role of GSH in the regulation of riboflavin biosynthesis and iron accumulation in the yeast *P. guilliermondii* might be realized through its probable involvement in biogenesis and/or maintenance of Fe/S cluster status. However, to support this notion and to shed light on the molecular mechanisms underlying the function of GSH in the regulation of riboflavin biosynthesis and iron homeostasis in the yeast *P. guilliermondii* further comprehensive studies and probably identification of the other critical components involved in these processes are required.

In conclusion, it has been found for the first time that blocking of glutathione biosynthesis leads to riboflavin oversynthesis and increased cellular iron accumulation in the yeast *P. guilliermondii*. Obtained results indicate the presence of common elements regulating these processes.

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Conflict of interest None.

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