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CLONING AND FUNCTIONAL ANALYSIS OF THE *GSH1/MET1* GENE COMPLEMENTING CYSTEINE AND GLUTATHIONE AUXOTROPHY OF THE METHYLOTROPHIC YEAST *Hansenula polymorpha*

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The Hansenula polymorpha GSH1/MET1 gene was cloned by complementation of glutathione-dependent growth of H. polymorpha gsh1 mutant isolated previously as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) resistant and cadmium ion sensitive clone. The H. polymorpha GSH1 gene was capable of restoring cadmium ion resistance, MNNG sensitivity, normal glutathione level and cell proliferation on minimal media without addition of cysteine or glutathione, when introduced into the gsh1 mutant cells. It was shown that the H. polymorpha GSH1 gene has homology to the Saccharomyces cerevisiae MET1 gene encoding S-adenosyl-L-methionine uroporphyrinogen III transmethylase, responsible for the biosynthesis of sulfite reductase cofactor, sirohaem. The H. polymorpha GSH1/MET1 gene deletion cassette (Hpgsh1/met1::ScLEU2) was constructed and corresponding null mutants were isolated. Crossing data of the point gsh1 and null gsh1/met1 mutants demonstrated that both alleles were located to the same gene. The null gsh1/met1 mutant showed total growth restoration on minimal media supplemented with cysteine or glutathione as a sole sulfur source, but not with inorganic (sulfate, sulfite) or organic (methionine, S-adenosylmethionine) sources of sulfur. Moreover, both the point gsh1 and null gsh1/met1 mutants displayed increased sensitivity to the toxic carbon substrate methanol, formaldehyde, organic peroxide and cadmium ions.

Key words: methylotrophic yeast, Hansenula polymorpha, glutathione, sulfate assimilation, MET1.

Regulation of the glutathione (GSH) metabolism, the most abundant tripeptide thiol in aerobic prokaryotic and eukaryotic cells, is under thorough investigation in aspects of oxidative stress response, heavy metal detoxification, sulfur and nitrogen nutrition etc. [1]. Study of the role that GSH plays in defence of unicellular eukaryotes, yeasts, is important for biotechnology and environmental protection. In methylotrophic yeast, this compound is especially important due to its role in detoxification of the intermediates of methanol metabolism, such as formaldehyde and hydrogen peroxide, as well as organic peroxides [2]. GSH could detoxify their excess in formaldehyde dehydrogenase and glutathione peroxidase reactions. Previously we described isolation and characterization of *Hansenula polymorpha* GSH-deficient mutants of two genetic groups, *gsh1* and *gsh2*, which are *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) resistant and simultaneously sensitive to cadmium ions and methanol [3]. The *H. polymorpha GSH2* gene cloned from *H. polymorpha* gene library by complementation of the *gsh2* mutation, was shown to be a homologue of the *Saccharomyces cerevisiae GSH1* gene (*ScGSH1*) coding for the first enzyme of glutathione biosynthesis, gamma-glutamylcysteine synthetase (GCS; EC 6.3.2.2.) [4, 5].

The aim of this work was to clone and characterize the gene responsible for the *gsh1* mutation. In this paper we report the isolation and identification of the *H. polymorpha GSH1* gene, which restored the glutathione level, MNNG sensitivity, cadmium resistance and growth on minimal GSH-deficient medium of the *H. polymorpha gsh1* mutant. It was shown that the *H. polymorpha GSH1* gene displayed homology to the *S. cerevisiae MET1* gene encoding S-adenosyl-L-methionine uroporphyrinogen III transmethylase (EC 2.1.1.107) responsible for the biosynthesis of sulfite reductase cofactor, sirohaem. Finally, we discussed the possible involvement of the *H. polymorpha GSH1/MET1* gene (*HpGSH1/MET1*) in sirohaem-dependent sulfate assimilation and cysteine supply for GSH biosynthesis, cadmium and chromate tolerance.

Materials and Methods

Yeast strains and media. Strains constructed and used in this study are listed in Table 1. Yeast cells were cultivated in rich YPD medium (1% glucose, 1% peptone, and 1% yeast extract) or minimal synthetic medium, which contained 10 g/l glucose and was supplemented with vitamins and trace elements [6]. Sulfur-deficient medium used for the sulfur source-dependent growth phenotype analysis of *H. polymorpha gsh1* and Δ*gsh1/met1* mutants contained the following compounds (per 1 litre): 10 g glucose, 1 g KH_2PO_4, 0.4 g $MgCl_2 \times 6H_2O$, 2.8 g NH_4Cl, 0.1 g $CaCl_2 \times 6H_2O$, and was supplemented with 2.6 mM $(NH_4)_2SO_4$, 0.1 mM Na_2SO_3, 0.1 mM S-adenosylmethionine (SAM), 0.1 mM S-adenosylhomocysteine, 0.1 mM methionine, 0.1 mM homocysteine, 0.1 mM cysteine or 0.1 mM GSH. For GCS activity assay the yeast cells were cultivated in sulfur-free medium B [7], which contained 2% glucose and was supplemented with 0.1 mM cysteine, 0.1 mM GSH or 38 mM $(NH_4)_2SO_4$, or in standard synthetic medium (2% glucose, 0.17% yeast nitrogen base without amino acids, and 0.5% $(NH_4)_2SO_4$). The medium for hybridization of yeast strains contained 2% malt extract. According to the auxotrophic requirements of strains appropriate amounts of amino acids and nucleic bases were added to all synthetic media. *Escherichia coli* DH5α strain used for plasmid propagation was cultured in LB medium (1%

Table 1. H. polymorpha strains constructed and used in this study

Designation	Genotype or relevant features	Reference or source
Wild type strains (WT)		
NCYC495 *leu1-1*		National Collection of Cultures, Food Research Institute, Norwich UK
NCYC495 *leu1-1 ade11*		Collection of A. Sibirny, Lviv, Ukraine
DL-1 *leu2*		Collection of H. Kang, Daejeon, Korea
CBS4732 *leu2-2 ura3-20*		K. Lahtchev, Sofia, Bulgaria
CBS4732 *leu2-2 met2-2*		K. Lahtchev, Sofia, Bulgaria
CBS4732 *leu2-2*		K. Lahtchev, Sofia, Bulgaria
Transformants of NCYC495 genetic line		
pG1	*gsh1 leu1-1* +(pYT3+ORF1+ORF2+ORF3+ORF4)	[4]; this study
pG1-23	*gsh1 leu1-1* +(pYT3+ORF2+ORF3)	This study
pG1-36	*gsh1 leu1-1* +(pYT3+ORF4)	This study
pG1-47	*gsh1 leu1-1* +(pYT1+ORF4)	This study
Mutant strains		
gsh1 point	*gsh1 leu1-1* (NCYC495)	[3]
gsh2 point	*gsh2 leu1-1* (NCYC495)	[3]
Δgsh1/met1 ade11	*leu1-1, ade11, Δgsh1/met1::ScLEU2* (NCYC495)	This study
Δgsh1/met1 ura3-20	*leu2-2, ura3-20, Δgsh1/met1::ScLEU2* (CBS4732)	This study
Δgsh1/met1 met2-2	*leu2-2, met2-2, Δgsh1/met1::ScLEU2* (CBS4732)	This study
Δgsh2 ade11	*leu1-1, ade11, Δgsh2::ScLEU2* (NCYC495)	This study
Δgsh2 met2-2	*leu2-2, met2-2, Δgsh2::ScLEU2* (CBS4732)	This study

NaCl, 1.5% peptone, and 0.5% yeast extract) with ampicillin (100 μg/ml) at 37 °C.

Construction of H. polymorpha GSH1/MET1 deletion cassette and null gsh1/met1 strain. To construct the *Hpgsh1/met1::ScLEU2* mutant allele, in which the coding sequence of the amino acid residues from 1 to 178 was replaced by a DNA fragment containing the *S. cerevisiae LEU2* gene, the 5′-DNA fragment (557 bp) corresponding to the *HpGSH1/MET1* promoter was amplified from the genomic DNA of *H. polymorpha* CBS4732 *leu2* by polymerase chain reaction (PCR) using primers VU5F/VU6R (Table 2). The 3′-DNA fragment of the *HpGSH1/MET1* coding and terminator sequence (1509 bp) cloned into the 3′-flanking region of the *ScLEU2* gene fragment was obtained by elimination of *Bam*HI/*Bam*HI fragment and subsequent self-ligation of pYT1+5.3 kb plasmid (Table 3; Results and Discussion). The resultant plasmid was digested with endonucleases *Hind*III and *Pst*I and used for cloning of *Hind*III/*Pst*I digested promoter of the *HpGSH1/MET1* gene (Fig. 1, *A*, *B*). The *Hpgsh1/met1::ScLEU2* deletion cassette was released as 4.29 kb fragment with *Hind*III and *Sac*I and transformed into *H. polymorpha* NCYC495 *leu1-1 ade11*, CBS4732 *leu2-2 met2-2*, and CBS4732 *leu2-2 ura3-20* wild type strains by electroporation [5]. Leu^+ transformants were selected on glucose-containing medium without leucine and subsequently analysed for Gsh- phenotype on minimal glucose-containing medium without exogenous GSH. Total genomic DNA was isolated from several Leu^+ Gsh^- transformants. Correct chromosomal replacement of the wild type *HpGSH1/MET1* gene with the *Hpgsh1/met1::ScLEU2* null mutant allele was confirmed by PCR analysis using two sets of primers: VU11F/VU12R for detection of the wild type *HpGSH1/MET1* allele and VU13F/VU12R for the null *gsh1/met1* allele (Table 2; Fig. 2, *A*, *B*).

Construction of H. polymorpha GSH2 deletion cassette and isolation of null gsh2 mutant. To create

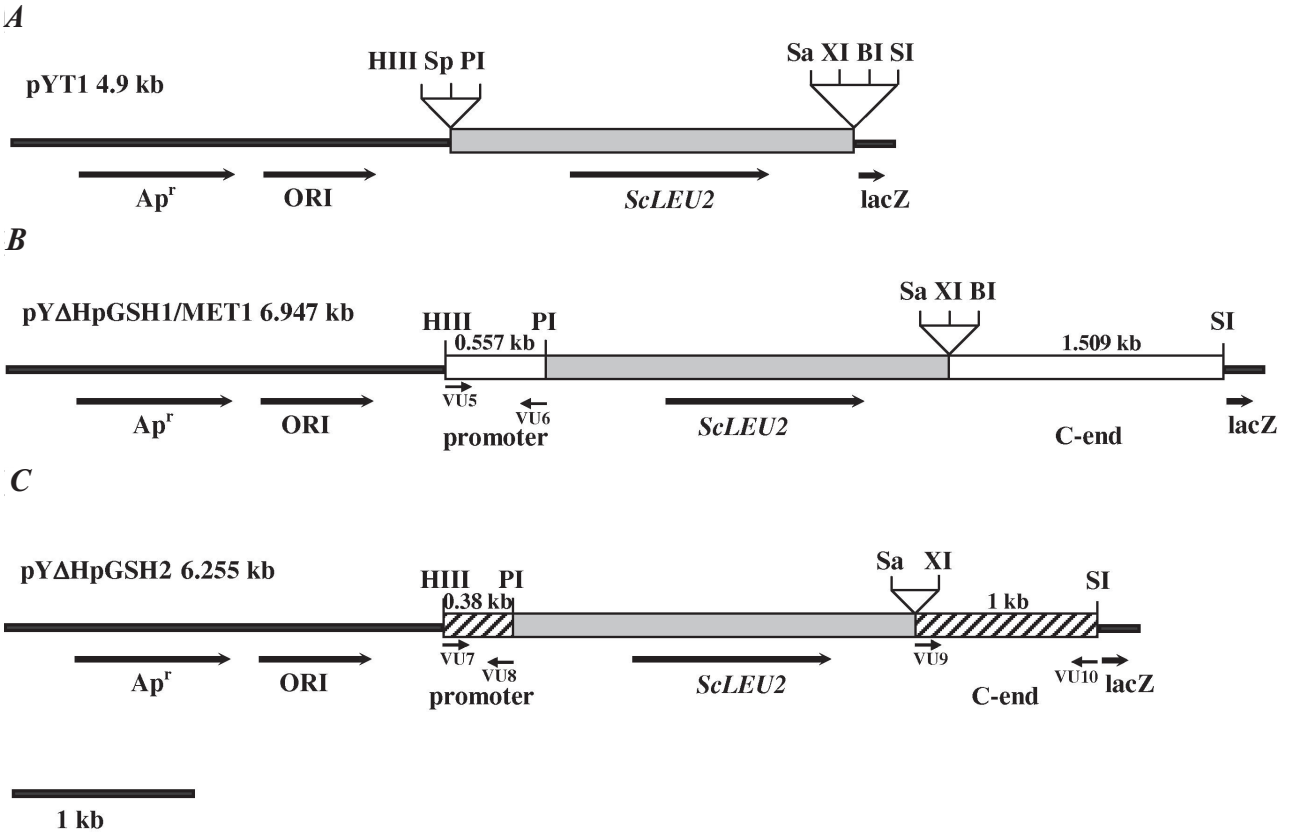

Fig. 1. Linear schemes of the plasmids. A: pYT1 (4.9 kb); B: pYΔHpGSH1/MET1 (6.947 kb); C: pYΔHpGSH2 (6.255 kb). Denotation: DNA fragment harboring the S. cerevisiae LEU2 gene (thick grey line), promoter and C-end regions of the H. polymorpha GSH1/MET1 gene (colourless fragments), promoter and C-end regions of the H. polymorpha GSH2 gene (thick striped line), pUC19 sequence (thin black line). Restriction sites: HIII, HindIII; Sp, SphI; PI, PstI; Sa, SalI; XI, XbaI; BI, BamHI; SI, SacI

Table 2. Oligonucleotides used as primers for PCR amplification in this study

Name	Sequence	Site
Primers for construction of Hpgsh1/met1::ScLEU2 deletion cassette		
primers for isolation of 5′-flanking region of *HpGSH1/MET1* gene:		
VU5F	5′-GGAAGCTTGGCACTCCAGAATGA-3′	(*Hind*III)
VU6R	5′-AACTGCAGTGCAAGGAGAACGTTT-3′	(*Pst*I)
Primers for confirmation of null HpGSH1/MET1 mutation		
primers for identification of wild type *HpGSH1/MET1* gene:		
VU11F	5′-TCTGTGTGCTCACGAATGCT-3′	
VU12R	5′-AGTATCCGGTCACCAGCAAT-3′	
Primers for identification of null *Hpgsh1/met1* gene:		
VU13F	5′-AAGAAGATCGTCGTTTTGCC-3′	
VU12R	5′-AGTATCCGGTCACCAGCAAT-3′	
Primers for construction of Hpgsh2::ScLEU2 deletion cassette		
primers for isolation of 5′-flanking region of *HpGSH2* gene:		
VU7F	5′-GGAAGCTTTCGGGCTGGCAGTGTTA-3′	(*Hind*III)
VU8R	5′-GAACTGCAGGGTCGATAAGGTTTTTC-3′	(*Pst*I)
Primers for isolation of 3′-flanking region of *HpGSH2* gene:		
VU9F	5′-GCTCTAGAGTACCTCAAGCTGGTGA-3′	(*Xba*I)
VU10R	5′-GTGAGCTCTAGCGTGCAATTTTTCC-3′	(*Sac*I)
Primers for confirmation of null HpGSH2 mutation		
primers for identification of wild type *HpGSH2* gene:		
VU15F	5′-GTCAACATCGCTCCATTGAT-3′	
VU10R	5′-GTGAGCTCTAGCGTGCAATTTTTCC-3′	(SacI)
Primers for identification of null *Hpgsh2* gene:		
VU30F	5′-TCTAGAATCAGCCTCCACATAAGCC-3′	
VU31R	5′-CACCTGGCAAAACGACGATCTTCTT-3′	

Underlined nucleotides mark the restriction sites indicated on the right

the *Hpgsh2::ScLEU2* mutant allele, in which the coding sequence of the amino acid residues from 1 to 574 was replaced by a DNA fragment harboring the *ScLEU2* gene, the 5′-DNA fragment (380 bp) of the *HpGSH2* promoter and the 3′-DNA fragment (1000 bp) containing a part of the *HpGSH2* coding and terminator sequence were amplified from the genomic DNA of *H. polymorpha* CBS4732 *leu2* by PCR using two sets of primers: VU7F/VU8R for the 5′-DNA fragment and VU9F/VU10R for the 3′-DNA fragment of *HpGSH2* (Table 2). The 5′-DNA fragment of the *HpGSH2* gene was cloned as a *Hind*III/*Pst*I fragment into the *Hind*III/*Pst*I digested pYT1 plasmid, which was harboring the *ScLEU2* gene. The resultant plasmid was digested by endonucleases *Xba*I and *Sac*I and subsequently ligated with the *Xba*I/*Sac*I digested 3′-DNA fragment of the *HpGSH2* gene (Fig. 1, *A*, *C*). The *Hpgsh2::ScLEU2* deletion cassette was released as 3.604 kb fragment with *Hind*III and *Sac*I and transformed into *H. polymorpha* NCYC495 *leu1-1 ade11* and CBS4732 *leu2-2 met2-2* wild type strains by electroporation [5]. Several Leu^+ Gsh^- transformants unable to grow on minimal glucose-containing medium without exogenous GSH were picked for total genomic DNA isolation and further PCR analysis. Correct replacement of the wild type *HpGSH2* gene with the *Hpgsh2::ScLEU2* null

Table 3. Plasmids constructed and used in this study

Plasmid name	Description	Reference or source
pYT1	Yeast-bacterium shuttle vector, originated from pUC19, harboring *ScLEU2* gene	Kindly provided by J. M. Cregg
pYT3	Yeast-bacterium shuttle vector, originated from pUC19, harboring *ScLEU2* gene and ARS element of *H. polymorpha*	'GenBank: AF347016.1
pG1 (pYT3+8.6 kb)	Plasmid harboring *H. polymorpha* ORF1, ORF2, ORF3, ORF4	[4]; this study
(pYT3+4.4 kb)	Plasmid harboring *H. polymorpha* ORF2 and ORF3	This study
(pYT1+5.3 kb)	Plasmid containing *H. polymorpha* ORF4	This study
(pYT3+5.3 kb)	Plasmid containing *H. polymorpha* ORF4	This study
pYΔHpGSH1/MET1	Plasmid containing *Hpgsh1/met1::ScLEU2* deletion cassette	This study
pYΔHpGSH2	Plasmid containing *Hpgsh2::ScLEU2* deletion cassette	This study

mutant allele was confirmed using the following sets of primers: VU15F/VU10R for detection of the wild type *HpGSH2* allele and VU30F/VU31R for the null *Hpgsh2* allele (Table 2; Fig. 2, *C*, *D*).

Genetic analysis of point and null gsh1 and gsh2 mutant strains. Hybridization of the auxotrophic *H. polymorpha* strains was carried out as previously described [3]. A point *gsh1 leu1-1* mutant was crossed with the null mutant strains *Δgsh1/met1 ade11*, *Δgsh1/met1 met2-2*, and *Δgsh1/met1 ura3-20*, while a point *gsh2 leu1-1* mutant was mated with the null mutant strains, *Δgsh2 ade11* and *Δgsh2 met2-2*. Diploid cells obtained on rich medium (2% malt extract) were examined for prototrophy and Gsh^- phenotype by replica plating them on minimal medium, which contains 1% glucose without appropriate amino acids and nucleic bases, in the presence or absence of 100 μM GSH.

Molecular techniques. General DNA manipulations were performed as previously described [8]. Plasmids constructed and used in this study are listed in Table 3. Yeast transformants were analyzed for vector stability, as previously described for *Pichia pastoris* [9]. 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 next corporations: Sigma (USA), Reanal (Hungary), Fermentas (Lithuania), New England Bio Labs (USA) and Promega (USA). DNA sequencing was performed at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). For DNA sequence analysis the ORF Finder, graphical analysis tool of the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA), was used. Protein homology search was performed using the BLAST algorithm of the NCBI (Bethesda, MD, USA), http://www.ncbi.nlm.nih.gov/BLAST/. Multiple sequence alignments were constructed using Multalin algorithm, version 5.4.1, http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html and shaded using Boxshade 3.21, http://www.ch.embnet.org/software/BOX_form.html.

Analytical assays. Total reduced and oxidized glutathione (GSH+GSSG) content was analysed in cell-free extracts as previously described [6, 3] using the standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase and NADPH. The protein concentration was determined by Lowry method [10] using bovine serum albumin as the standard.

Activity of GCS was assayed in yeast cells, which were pregrown in 3 ml of sulfur-free medium B in the presence of 38 mM $(NH_4)_2SO_4$, 0.1 mM cysteine (*gsh1* and *Δgsh1/met1*) or 0.1 mM GSH (*Δgsh2*) for 1 day and then transferred into 125 ml of the same medium with an initial $OD_{600\ nm}$ ~ 0.04 and grown till the late logarithmic phase. Cells of *Δgsh2* mutant, grown in the presence of GSH, were washed twice with water and then additionally transferred into medium B with 0.1 mM cysteine for 6 hours. *H. polymorpha* wild type strains of different genetic lines were precultivated in 20 ml of standard synthetic medium for 2 days, transferred into 250 ml of the same medium with initial $OD_{600\ nm}$ ~ 0.01 and grown till the early logarithmic or stationary phase. Harvested cells were washed twice with water and frozen for homogenization. Activity of GCS was measured as previously described [11], but with some modifications. Cells were resuspended in 1 volume of 100 mM Tris-HCl buffer pH 8.0 containing 2 mM EDTA,

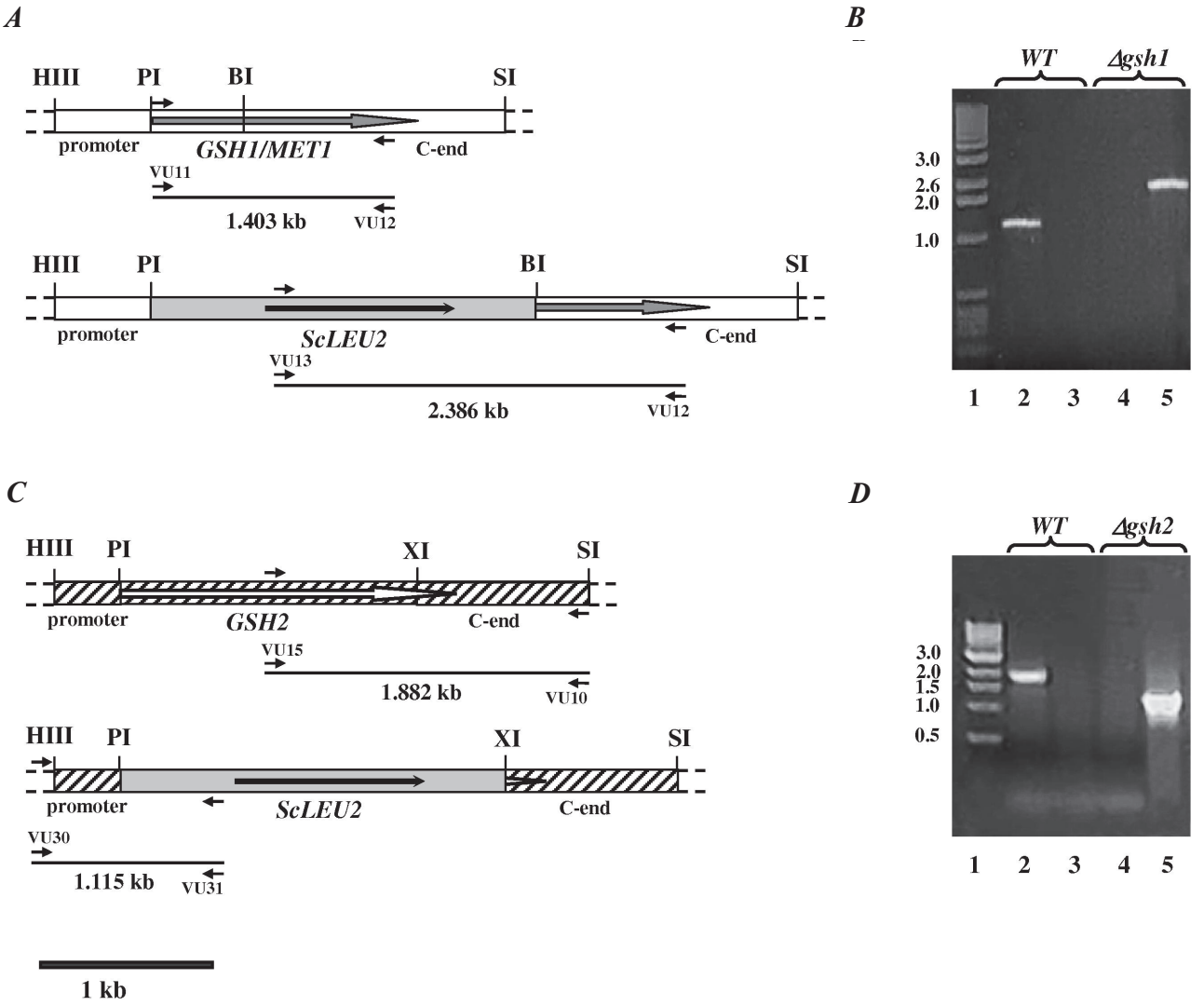

Fig. 2. Schematic representation and PCR analysis of the H. polymorpha GSH1/MET1 (A, B) and GSH2 (C, D) genes in the wild type strain and correspondent null mutant strains. PCR primers are indicated as small arrows. Lanes 2 and 4 represent wild type GSH1/MET1-specific primers (B) or wild type GSH2-specific primers (D); lanes 3 and 5 – Δgsh1/met1-specific primers (B) or Δgsh2-specific primers (D). As template for PCR amplification were used genomic DNAs of wild type strain (WT) (B, D; lanes 2 and 3) and Δgsh1/met1 (B; lanes 4 and 5) or Δgsh2 (D; lanes 4 and 5) mutant strains. Restriction sites: HIII, HindIII; PI, PstI; BI, BamHI; SI, SacI; XI, XbaI

10 mM $MgCl_2$, 150 mM KCl, 5 mM glutamate, 2 mM phenylmethanesulfonyl fluoride (PMSF) and 1 volume of acid washed glass beads (diameter 425-600 μm; Sigma) was added. Homogenization was performed with cell mill (20 min, regimen 90, 4 °C). After centrifugation (15000 × g, 20 min, 4 °C), 250-300 μl of supernatant was desalted on Sephadex G-25 column (0.76 × 5.5 cm) calibrated with the same Tris-HCl buffer, but without PMSF. Aliquots of 200 μl of desalted supernatants were collected and taken for the protein determination. The protein curve in dependence on absorption of aliquots at 280 nm was built. The first part of the peak (Fig. 3), which probably contained low molecular mass compounds, was cut off and fractions with the highest protein concentration were combined and taken for the GCS activity measurement.

The incubation mixture for determination of GCS activity contained 135 μl of the desalted extract (2-3 mg/ml protein), 25 μl 100 mM glutamate, 25 μl 100 mM cysteine/1.2 M dithiothreitol (Sigma, USA), 12.5 μl 100 mM ATP, 25 μl 100 mM phospho(enol)pyruvate, 10 μl 1 M Tris-HCl pH

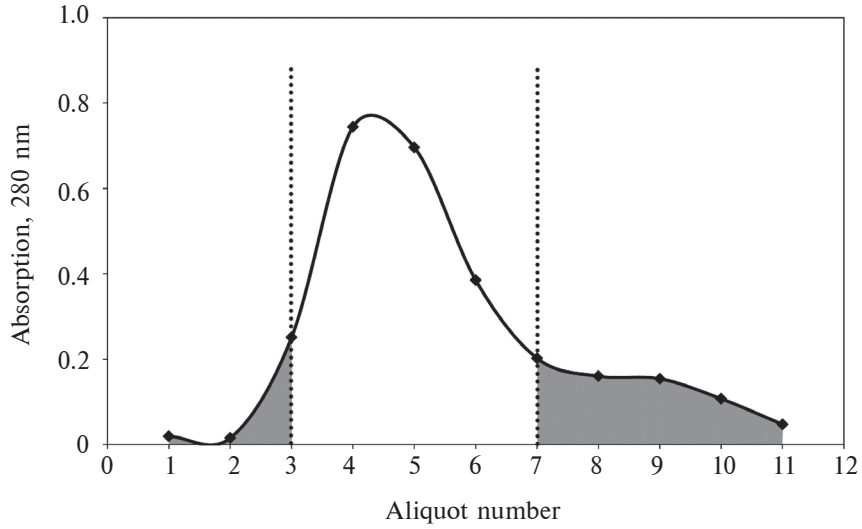

Fig. 3. The protein curve in the dependence to absorption of aliquots of desalted cell-free extracts of H. polymorpha wild type strain NCYC495 leu1-1 at 280 nm. Yeast cells were grown in medium B with 0.1 M cysteine and 2% glucose. Aliquots 1-3 and 7-11 were discarded; aliquots 4-6 were combined and used for GCS activity measurement

8.0, 2.5 µl 0.5 M $MgCl_2$, and 3.5 U pyruvate kinase (Sigma, USA). The same incubation mixture but only without cysteine was used as a control reaction. The reaction was performed at 37 °C during 1 h. Afterwards the incubation mixture in total volume of 250 µl was treated with 37.5 µl of sodiumborohydride (1 mg/ml of sodiumborohydride in 2.5 M NaOH) during 5 min at room temperature. Then proteins were precipitated by treatment with 50 µl 3.5 M HCl, incubated 10 min on ice and centrifuged (12 000 × g, 10 min). The supernatant was filtered and injected into the HPLC column. The separation was carried out on the RP 18 column with solvent A (0.05% phosphoric acid) and solvent B (acetonitrile). It was started at 100% A, and then applied a gradient up to 20% solvent B within 20 min. The detection of thiol dipeptide was performed by post-column derivatisation using Ellman's reagent (100 mg/l in 0.05 M potassium-phosphate buffer pH 8.0) and measurement of the absorbance at 410 nm. The product of GCS reaction, γ-glutamylcysteine, was identified using authentic compound from Sigma (USA) for calibration. Specific enzyme activity of GCS was expressed in $nmol \cdot h^{-1} \cdot mg\ protein^{-1}$.

Statistic analysis was done in program SigmaPlot 11.0 using Student's *t*-test.

Results and Discussion

Molecular cloning and sequence analysis of the H. polymorpha GSH1 gene functionally complementing gsh1 mutation. In the previous study we described cloning of a chromosomal DNA fragment more than 7 kb from the *H. polymorpha* CBS4732 genomic DNA library, which functionally complemented the glutathione-deficient phenotype of *gsh1* mutant [4]. The obtained Gsh^+ transformants restored their ability to grow on GSH-deficient synthetic media with methanol or multicarbon substrates and acquired resistance to Cd (II) and Cr (VI) ions and sensitivity to MNNG at the levels similar to those of the wild type strain (data non shown). In the present study it was shown that pG1 plasmid isolated from Gsh^+ transformants of *gsh1* mutant contained the 8.6 kb *H. polymorpha* genomic DNA fragment inserted in *Bam*HI restriction site of pYT3 plasmid. Sequence analysis revealed that the 8.6 kb fragment contained at least 4 open reading frames (ORFs) (Fig. 4). ORF1 showed homology to *S. cerevisiae* CDC5 gene involved in the regulation of cytokinesis; ORF2 appeared to be homological to a hypothetical protein; ORF3 displayed homology to *S. cerevisiae* gene of CDP-alcohol phosphatidyl transferase, which participates in metabolism of phospholipids; ORF4 was found to be a homolog of the *S. cerevisiae MET1* gene, coding for S-adenosyl-L-methionine uroporphyrinogen III transmethylase responsible for sirohaem biosynthesis (Fig. 4). To identify which of four ORFs could complement the Gsh^- phenotype of point *gsh1* mutant, we have constructed a set of plasmids harboring the subfragments of 4.4 kb (containing ORF2 and ORF3) cloned into *Sac*I site of pYT3 vector and 5.3 kb (containing ORF4) into *Sac*I site of pYT3 and pYT1 vectors. DNA subfragments of 4.4 kb and

5.3 kb were obtained from pG1 plasmid digested by *Sac*I restriction enzyme after fractionation by agarose electrophoresis and subsequent elution from gel. Transformation of *H. polymorpha gsh1* mutant with the constructed plasmids demonstrated that the wild type phenotypes in respects of growth on synthetic medium, glutathione level, MNNG sensitivity and cadmium ion resistance were restored only in the transformants (pG1-36 and pG1-47) harboring the ORF4-containing plasmids (Fig. 4). Vector stability study of these transformants in rich YPD medium showed that both ORF4-containing plasmids supported autonomously in the cells of yeast transformants and were responsible for restoration of the Gsh^+ phenotype. For the next study the transformant pG1-47 containing ORF4 on the base of pYT1 plasmid was chosen. The cellular total glutathione (GSH+GSSG) level of pG1-47 and pG1 transformants was found to be 2.3 and 3.1 times higher than in point *gsh1* mutant under conditions of normal growth and 10.4 and 11.3 times higher under cadmium ion induction. However, under both conditions these values were a little bit lower than that of wild type strain (Table 4). The extent of restoration of resistance to cadmium ions in the transformants is shown in Table 4.

The ORF4 of 1.539 kb, designated as the *H. polymorpha GSH1/MET1* gene, was predicted to encode a polypeptide of 513 amino acids with appropriate molecular mass of 58 kDa. A search of the protein databases revealed significant sequence similarity between the deduced C-terminus amino acid sequence of *H. polymorpha* Gsh1p/Met1p (246-472 a.a.) and those of a number of proteins possessing uroporphyrinogen III transmethylase domain. The protein with the highest similarity was a potential uroporphyrin-3 C-methyltransferase from *Candida albicans* (50% identity, 70% similarity, Accession No. EAK96258.1). *H. polymorpha* Met1p was also found to share 49% identity and 69% similarity with S-adenosyl-L-methionine uroporphyrinogen III transmethylase from *P. pastoris* (Accession No. CAY67134.1). Other proteins with high similarity included: S-adenosyl-L-methionine uroporphyrinogen III transmethylase from *S. cerevisiae*, ScMet1p, (43% identity and 59% similarity, Accession No NP_012995), a putative uroporphyrinogen-III C-methyltransferase from *Schizosaccharomyces pombe* (42% identity, 60% similarity, Accession No O74468), uroporphyrin-III C-methyltransferase from *Neurospora crassa* (38% identity, 54% similarity, Accession

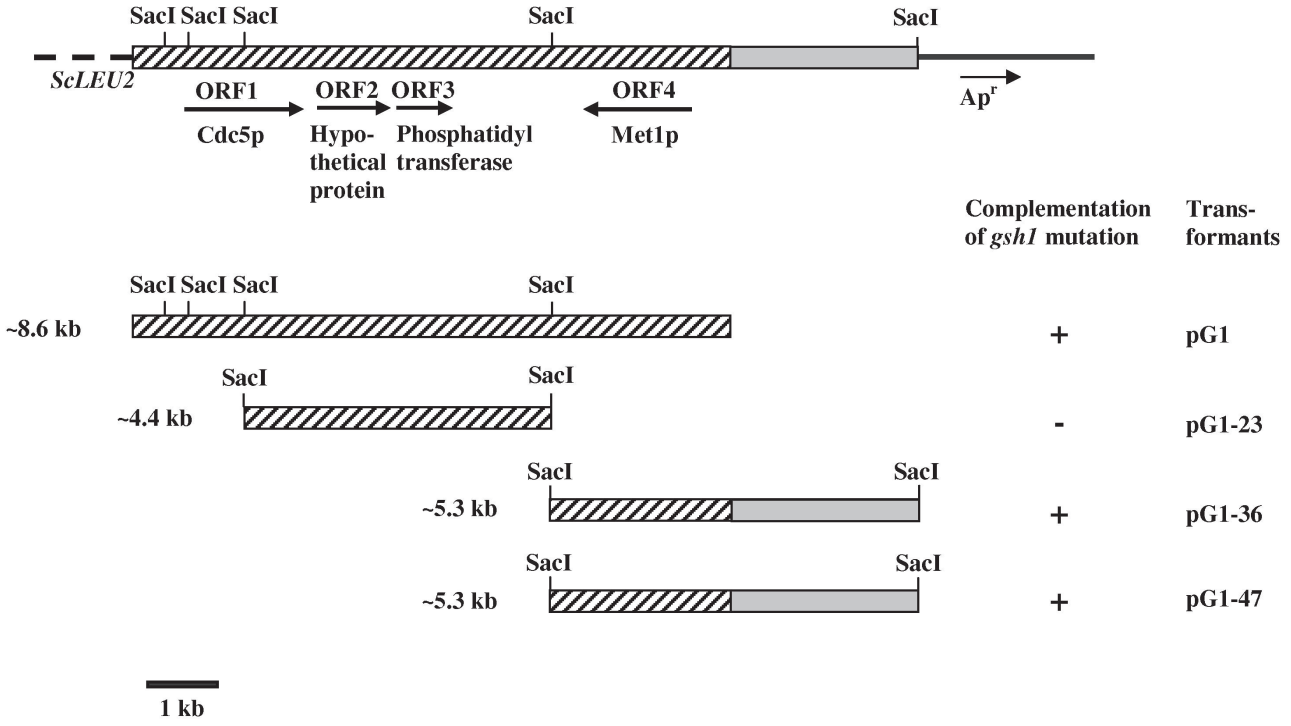

Fig. 4. Scheme of the linear pG1 plasmid and subcloning of 8.6 kb chromosomal DNA fragment, harboring the H. polymorpha GSH1/MET1 gene. Denotation: S. cerevisiae LEU2 gene, ScLEU2 (black dotted line), H. polymorpha 8.6 kb chromosomal DNA fragment (striped line), H. polymorpha ARS-element (thick grey line), and bacterial part of the plasmid pUC19 (thin black line)

Table 4. Growth and intracellular GSH+GSSG content of H. polymorpha wild type strain NCYC495 leu1-1, point gsh1 mutant and transformants (pG1 and pG1-47), depending on the presence of Cd^{2+} ions (M ± m, n = 4)

Strain	GSH+GSSG, nmol·mg protein^{-1} *		Growth on minimal synthetic medium**			
	Control	$CdSO_4$, 0.1 mM	Control	$CdSO_4$, mM		
				0.1	0.4	1.0
NCYC495	128.0 ± 6.1	209.8 ± 8.4$^{\#}$	++	+±	±	−
gsh1	29.2 ± 0.8$^{\#\#}$	11.0 ± 0.3$^{\#,\#\#}$	+±	±	−	−
pG1	90.1 ± 2.6$^{\#\#}$	124.0 ± 5.7$^{\#,\#\#}$	+++	+++	++	+±
pG1-47	68.0 ± 1.9$^{\#\#}$	114.3 ± 5.2$^{\#,\#\#}$	+++	+++	++	+

* Yeast strains were pregrown in the minimal synthetic medium containing 1% glucose till the logarithmic phase and then transferred for incubation into the same medium with or without Cd^{2+} ions for 13 hours. Initial growth medium for *gsh1* mutant was additionally supplied with 0.05% yeast extract; ** Yeast growth on replica plates after 3 days of incubation at 37 °C: (+++) − very intensive growth; (++) − intensive growth; (+±) − moderate growth; (+) − weak growth; (±) - very weak growth; (−) − absence of growth. Growth media for *gsh1* mutant were additionally supplied with 100 μM GSH; $^{\#}$ $P < 0.05$ as compared to correspondent control values; $^{\#\#}$ $P < 0.05$ as compared to correspondent wild type values

No CAE76594). Significantly lower homology was also detected to the multifunctional CysG protein, sirohaem synthase from *E. coli* (28% identity, 48% similarity, Accession No CBG36455). Alignment of the deduced amino acid sequence of the putative *H. polymorpha* Gsh1p/Met1p with the homologous proteins from *C. albicans*, *P. pastoris*, *S. cerevisiae*, *S. pombe*, *N. crassa* and *E. coli* is presented in Fig. 5.

To confirm that the *HpGSH1/MET1* gene was the wild type allele complementing directly the point *gsh1* mutant phenotypes, the null *gsh1/met1* mutant strains (*Δgsh1/met1::ScLEU2*) were constructed by the gene replacement method as described in Materials and Methods (Fig. 1, *A*, *B*; 2, *A*, *B*). Data of crossing of the point *gsh1* mutant with the null *gsh1/met1* mutant strains showed that all obtained prototrophic diploid cells could grow on minimal medium only in the presence of exogenous GSH, demonstrating that the hybridized mutants belonged to the same genetic group. One of the null *gsh1/met1* mutants, particularly *Δgsh1/met1 ade11*, was chosen for more detailed study.

Growth, GCS activity and total GSH+GSSG level of the wild type strain, point gsh1 and Δgsh1/met1 mutants of H. polymorpha depending on sulfur source. The cloned *HpGSH1/MET1* gene could be involved in sirohaem-dependent sulfite reduction reaction of sulfate assimilation, and consequently in sulfur amino acid and GSH biosynthesis pathways. Therefore, we studied the effect of different sulfur sources on the glutathione-dependent phenotypes of point *gsh1* and null *gsh1/met1* mutants. It is known that the cell requirement for sulfur can be fulfilled by the uptake of sulfur-containing amino acids, glutathione, or by assimilation of sulfate (through consequent reduction to sulfite and sulfide) into organic compounds such as cysteine and/or homocysteine [12, 13]. Similar to the *S. cerevisiae met1* mutant, the *H. polymorpha Δgsh1/met1* mutant did not grow in minimal medium with sulfate and sulfite as a sole sulfur source. However, in contrast to the *S. cerevisiae met1* mutant that satisfied nutritional needs in sulfur with S-amino acids and their derivatives [12], the null *H. polymorpha gsh1/met1* mutant strain displayed negligible ability to assimilate methionine and SAM, and reduced growth activity on S-adenosylhomocysteine and homocysteine as a sole sulfur source (Fig. 6, *A*, *B*). In sulfur-free medium supplemented with cysteine or GSH *H. polymorpha* null *gsh1/met1* mutant showed total growth restoration. At the same time, *H. polymorpha* point *gsh1* mutant displayed the absence of growth on inorganic sulfur sources (sulfate, sulfite), negligible growth activity on SAM and less pronounced growth reduction on methionine, S-adenosylhomocysteine and homocysteine (data not shown). Discrepancy in growth phenotype of *met1* mutants from *S. cerevisiae* and *H. polymorpha* could be explained by distinctions in sulfate assimilation pathways (on the step of H_2S incorporation in cysteine or/and homocysteine) and transsulfuration reactions between cysteine and homocysteine (be- or unidirectional) [12]. It might be possible that similar to *S. pombe*, the methylotrophic yeast *H. polymorpha*, might lack reverse transsulfuration pathway, which is necessary for

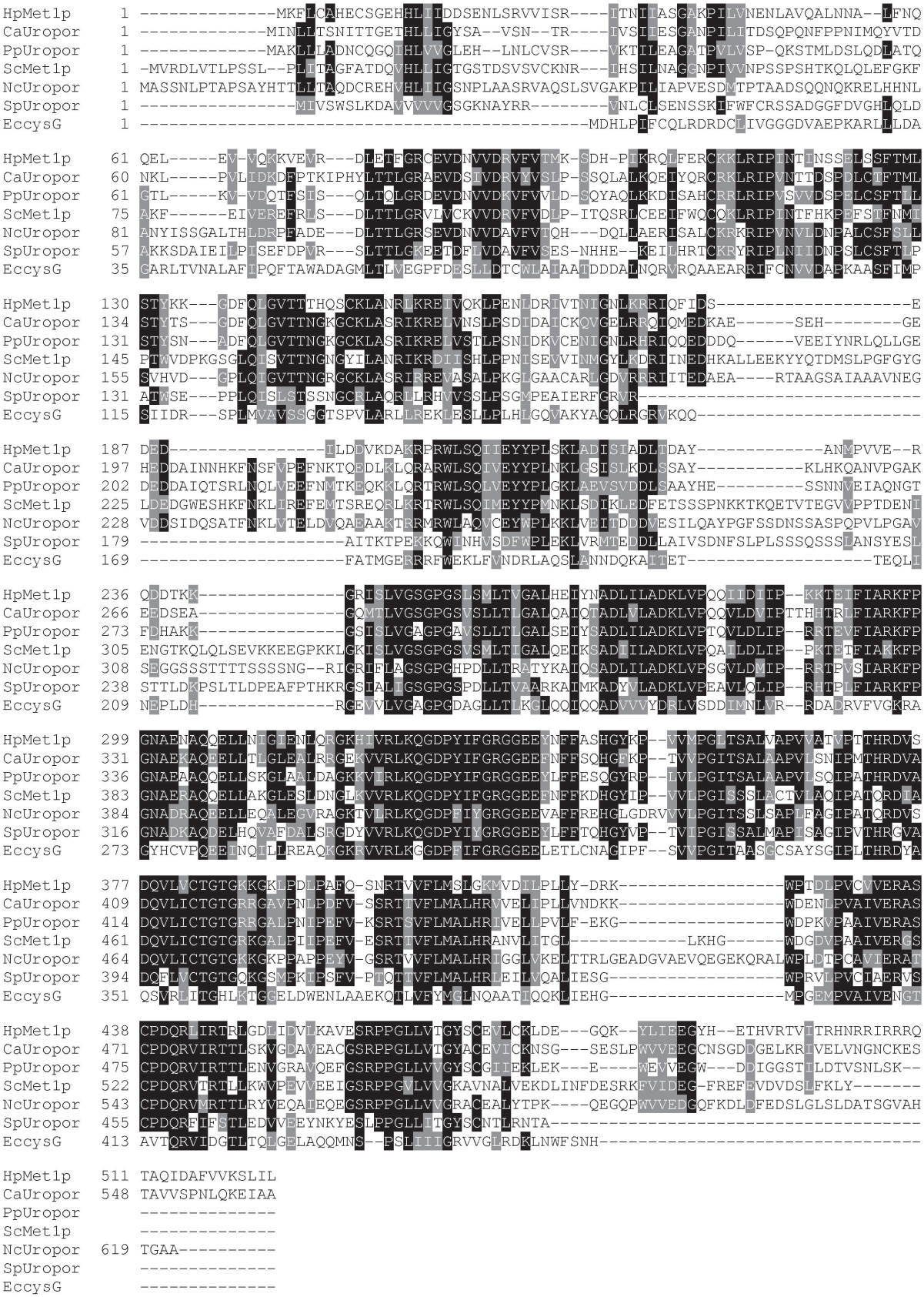

Fig. 5. Alignment of the deduced amino acid sequence of the putative Gsh1p/Met1p protein from H. polymorpha with the homologous proteins from C. albicans (CaUropor), P. pastoris (PpUropor), S. cerevisiae (ScMet1p), N. crassa (NcUropor), S. pombe (SpUropor), and E. coli (EccysG)

the transformation of methionine through homocysteine to cysteine. However, *H. polymorpha* wild type strain could effectively utilize methionine and SAM, as a sole sulfur source (Fig. 6, *A*). Therefore, it could be assumed that yeast *H. polymorpha*, similar to *S. pombe*, might convert methionine to sulfate followed by reduction and incorporation into homocysteine or/and cysteine [14], or similar to some bacteria, protozoa and plants possess methanethiol degradation pathway [15, 16] or other alternative pathway of methionine transformation into cysteine. Cellular glutathione level was partially restored in the null *H. polymorpha gsh1/met1* mutant in sulfur-free medium supplemented with homocysteine, cysteine or GSH, compared to that of the wild type strain (Fig. 6, *A*, *B*). It was also shown that the activity of GCS was not impaired in both point and null *gsh1/met1* mutants grown on cysteine as a sole sulfur source (Table 5). As a negative control, the *H. polymorpha Δgsh2 ade11* mutant strain with the deleted gene of GCS, constructed by the gene replacement method as described in Materials and Methods (Fig. 1, *A*, *C*; 2, *C*, *D*), was used. The *Δgsh2* mutant, grown in the sulfur-free medium supplemented with GSH, displayed only residual level of GCS activity (Table 5). It was also demonstrated that GCS activity was not regulated by the sulfur source (sulfate ions, cysteine or GSH) in *H. polymorpha* NCYC495 *leu1-1* wild type strain (Table 5). Besides, it was shown that GCS activity did not correlate with the increased cellular GSH+GSSG levels in *H. polymorpha* wild type strains of different genetic lines (Table 6), indicating that the cellular level of glutathione is balanced by complex processes of transport, degradation, as well as of biosynthesis of GSH precursor, cysteine. On the other hand, cellular glutathione level of the wild type strains

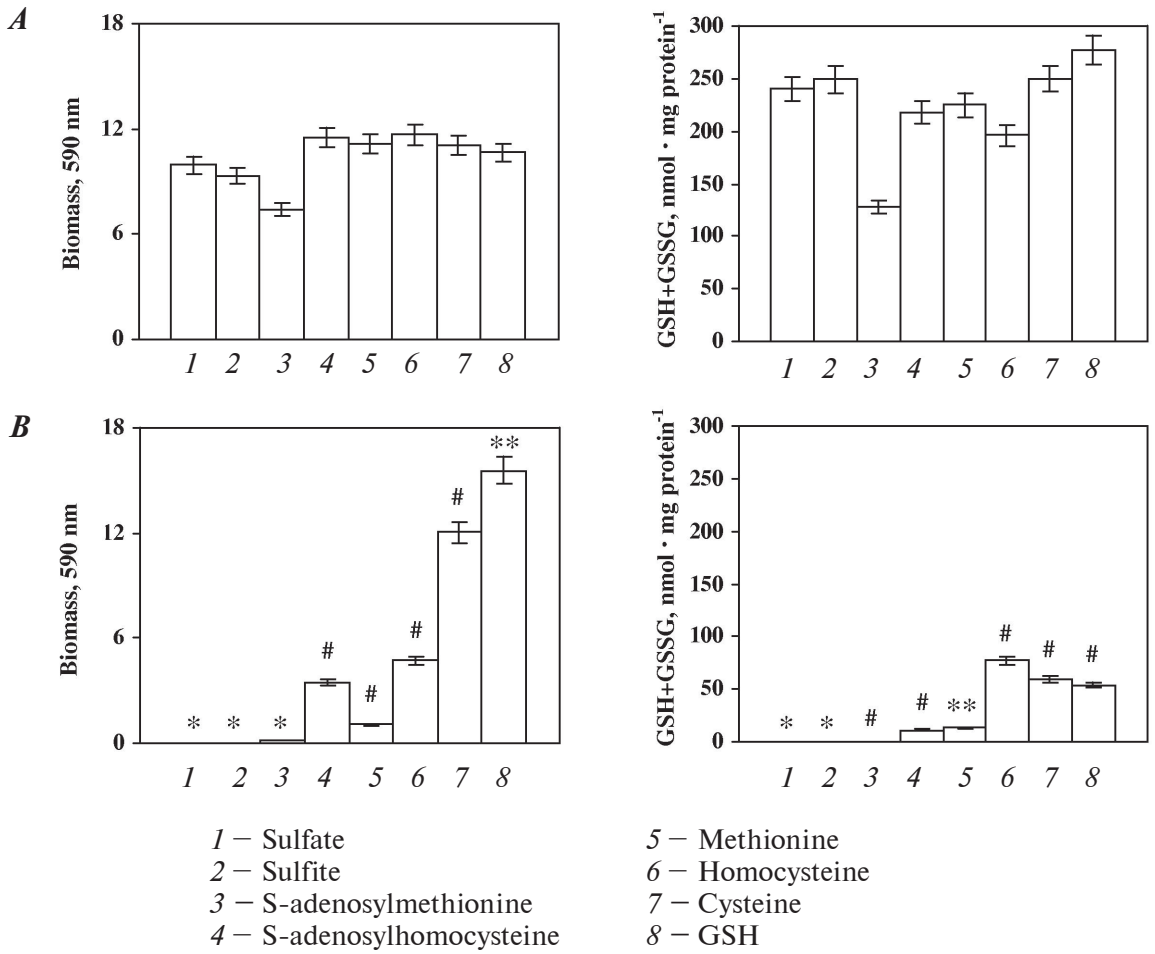

*Fig. 6. Growth and intracellular glutathione level of H. polymorpha wild type strain (A) and Δgsh1/met1 (B) mutant depending on sulfur source. Yeast cells were inoculated with start OD_{590} ~ 0.001. Growth data are presented on the 5-th day; n = 4; * P < 0.001, ** P < 0.01, # P < 0.05 as compared to correspondent wild type values*

Table 5. GCS activity (nmol·mg protein^{-1}·h^{-1}) of H. polymorpha NCYC495 leu1-1 wild type strain, point gsh1 and null gsh1/met1 and gsh2 mutants depending on sulfur source (M ± m, n = 3)

Strain	Sulfur source		
	SO_4^{2-}	GSH	Cys
NCYC495	281.0 ± 12.6#	290.8 ± 11.2#	261.7 ± 9.2#
gsh1	–	–	255.6 ± 9.5#
Δgsh1/met1	–	–	341.8 ± 13.7#
Δgsh2	–	43.6 ± 0.4	–

# $P < 0.05$ as compared to inactive protein

Table 6. GCS activity, cellular GSH+GSSG level and cadmium ions sensitivity of H. polymorpha NCYC495 leu1-1, CBS4732 leu2-2 and DL-1 leu2 wild type strains (M ± m, n = 3)

Strain	GCS activity,* nmol·mg protein^{-1}·hour^{-1}		GSH+GSSG,* nmol·mg protein^{-1}		Growth on YPD medium**	
	logarithmic	stationary	logarithmic	stationary	$CdSO_4$, mM	
					0	1
NCYC495	235.4 ± 11.5#	276.0 ± 12.4#	100.2 ± 3.4	180.0 ± 6.9##	+++	–
CBS4732	351.5 ± 16.3#	144.6 ± 6.1#,##	140.0 ± 4.2	405.1 ± 14.3##	+++	+
DL-1	329.0 ± 14.8#	251.9 ± 10.6#,##	160.4 ± 3.9	512.3 ± 16.2##	+++	++

* Yeast cells were grown in liquid standard synthetic medium with 2% glucose till indicated phase; ** Yeast growth on plates after 2 days of incubation at 37 °C: (+++) – very intensive growth; (++) – intensive growth; (+) – weak growth; (–) – absence of growth; # $P < 0.05$ as compared to inactive protein; ## $P < 0.05$ as compared to correspondent values in logarithmic phase

appeared to be correlated well with cadmium ions sensitivity (Table 6).

Growth of point and null gsh1 and gsh2 mutants in the media with stress-inducing agents. The effects of the *gsh1* mutation on the glutathione-dependent growth of *H. polymorpha* on different carbon sources and on the resistance to different stress factors were estimated in the drop test in comparison with the wild type strain, point *gsh2* and *Δgsh2* mutants. Both point and null *gsh1* and *gsh2* mutants were able to restore their growth on glucose, methanol, ethanol and glycerol as a sole carbon source in the presence of exogenous GSH (Fig. 7). It was also demonstrated that point and null *gsh1* and *gsh2* mutants were sensitive to the toxic carbon substrate methanol, formaldehyde, organic peroxide and ions of heavy metal cadmium, compared to the wild type strain (Fig. 7). Since GSH plays a pivotal role in oxidative stress response, it could be assumed, that sensitive phenotypes of *gsh1/met1* mutants to the tested factors are related with reduced cysteine biosynthesis and consequently reduced GSH production, and in the case of *gsh2* mutants – with impaired GSH biosynthesis. We previously reported that *H. polymorpha* was unable to synthesize phytochelatins in response to cadmium ion treatment [17]. Thus, GSH is the main molecule involved in detoxification of intracellular cadmium ions in this yeast. Incorporation of sulfide into $Cd(GSH)_2$ complexes in some yeasts significantly increased its Cd-binding capacity and increased detoxification effect [18]. Therefore, the possible lack of sulfide production in the *gsh1/met1* mutant could contribute to increased cadmium sensitivity. Besides, it was shown that the *MET1* gene of *S. cerevisiae* belongs to the genes, which expression was specifically induced in response to cadmium ion treatment [19]. It is interesting to note that tight connection between reduction in cadmium tolerance, glutathione, haem (sirohaem) biosynthesis and sirohaem-dependent activity of sulfite reductase (EC 1.8.1.2) was also observed for *Candida glabrata hem2* mutant with defected *HEM2* gene encoding porphobilinogen synthase (EC 4.2.1.24) [20]. The addition of cysteine, but not methionine increased glutathione levels and tolerance to cadmium ions of both the wild type and *hem2* mutant strains. Both point and null *gsh1/met1* mutants of *H. polymorpha* also manifested increased sensitivity to chromium ions, compared to

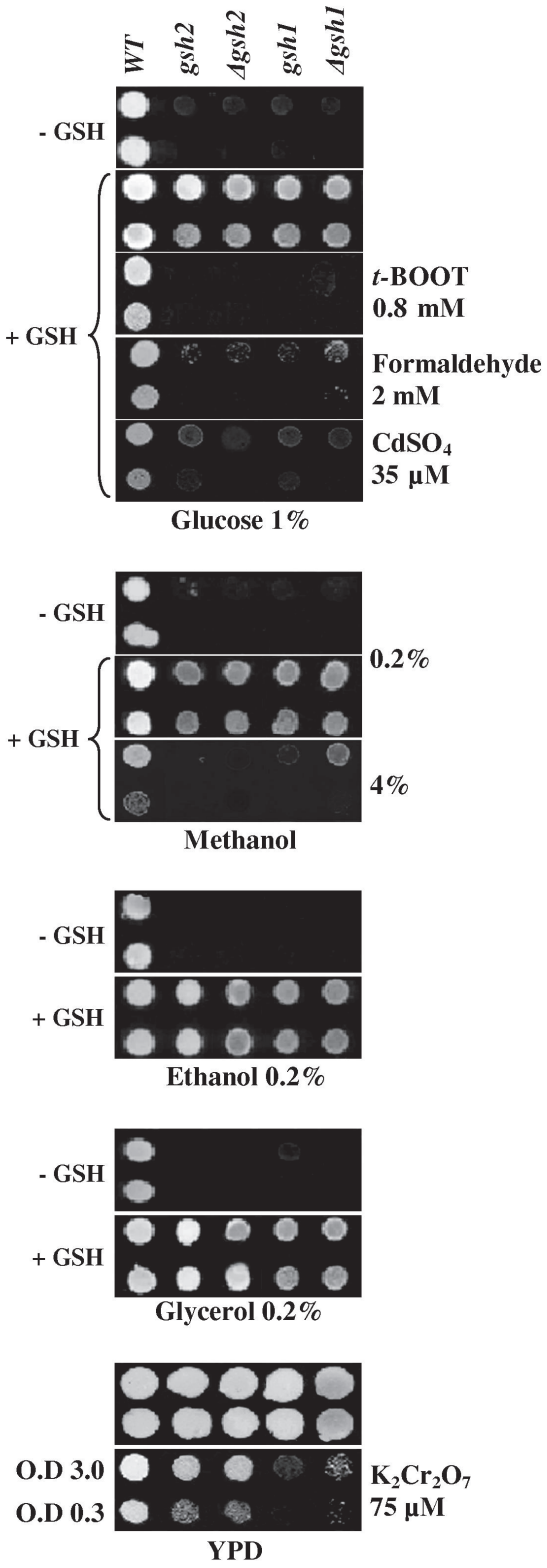

Fig. 7. Growth sensitivity of H. polymorpha wild type strain NCYC495 leu1-1 (WT), point and null gsh1 and gsh2 mutants on different carbon sources depending on the presence of 20 μM GSH or different stress induced factors: chromate ions ($K_2Cr_2O_7$), methanol, tert-butyl hydroperoxide (t-BOOH), formaldehyde, and cadmium ions ($CdSO_4$). Minimal media with stress induced factors additionally contained 20 μM GSH. Cultures of each strain were grown till stationary phase and spotted in 4μl suspensions on the plates with OD_{590} 3.0 (upper row) and 0.3 (bottom row). Growth was estimated after 3 days of incubation at 37 °C

gsh2 mutants and wild type strain. It is known that sulfate and chromate anions share the common assimilation system for their transport and subsequent reduction in the cell [21]. It could be speculated that the increased sensitivity of the *H. polymorpha gsh1/met1* mutant might be related with overaccumulation of the most toxic intermediate compound Cr^{5+} instead of a less toxic Cr^{3+} form due to impairment in sirohaem-dependent sulfite reductase reaction.

In the present study we cloned the *HpGSH1/MET1* gene, which displayed homology to *S. cerevisiae MET1* gene encoding S-adenosyl-L-methionine uroporphyrinogen III transmethylase by functional complementation of glutathione-deficient phenotype in the *H. polymorpha gsh1* mutant. We also discussed the possible involvement of the *HpGSH1/MET1* gene in sirohaem-dependent sulfate assimilation and cysteine supply for GSH biosynthesis, cadmium and chromate tolerance.

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КЛОНУВАННЯ І ФУНКЦІОНАЛЬНИЙ АНАЛІЗ *GSH1/MET1* ГЕНУ, ЩО КОМПЛЕМЕНТУЄ АУКСОТРОФНІСТЬ ЗА ЦИСТЕЇНОМ ТА ГЛУТАТІОНОМ У МЕТИЛОТРОФНИХ ДРІЖДЖІВ *Hansenula polymorpha*

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Ген *GSH1/MET1 Hansenula polymorpha* клонували шляхом комплементації глутатіонзалежного росту мутанта *gsh1 H. polymorpha*, попередньо виділеного як клон, що виявляв резистентність до *N*-метил-*N'*-нітро-*N*-нітрозогуанідину (MNNG) і чутливість до іонів кадмію. Ген *GSH1 H. polymorpha* відновлював резистентність до іонів кадмію, чутливість до MNNG, нормальний рівень глутатіону та проліферацію клітин на мінімальному середовищі без додавання цистеїну або глутатіону за введення у клітини мутанта *gsh1*. Показано, що ген *GSH1 H. polymorpha* є гомологічним гену *MET1 Saccharomyces cerevisiae*, що кодує S-аденозил-L-метіонін уропорфіриноген III трансметилазу, яка відповідає за біосинтез кофактора сульфітредуктази, сірогему. Нами була сконструйована касета з делецією гену *GSH1/MET1 H. polymorpha* (*Hpgsh1/met1::ScLEU2*) та одержані відповідні нуль-мутанти (із делецією значної частини структурної ділянки цього гену). Дані зі схрещування точкового *gsh1* мутанта та нуль-мутантів за *gsh1/met1* продемонстрували, що обидві алелі розміщені в одному й тому ж гені. Нуль-мутант за *gsh1/met1* повністю відновлював ріст на мінімальному середовищі із цистеїном або глутатіоном як єдиному джерелі сірки, але не з неорганічними (сульфат, сульфіт) чи органічними (метіонін, S-аденозилметіонін) джерелами сірки. Окрім того, обидва мутанти – точковий *gsh1* і нуль-мутант за *gsh1/met1* – виявляли підвищену чутливість до токсичного вуглецевого субстрату метанолу, до формальдегіду, органічного пероксиду та іонів кадмію.

Ключові слова: метилотрофні дріжджі, *Hansenula polymorpha*, глутатіон, асиміляція сульфату, *MET1*.

КЛОНИРОВАНИЕ И ФУНКЦИОНАЛЬНЫЙ АНАЛИЗ *GSH1/MET1* ГЕНА, КОТОРЫЙ КОМПЛЕМЕНТИРУЕТ АУКСОТРОФНОСТЬ ПО ЦИСТЕИНУ И ГЛУТАТИОНУ У МЕТИЛОТРОФНЫХ ДРОЖЖЕЙ *Hansenula polymorpha*

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Ген *GSH1/MET1 Hansenula polymorpha* клонировали путем комплементации глутатионзависимого роста мутанта *gsh1 H. polymorpha*, предварительно изолированного как клон,

который проявлял резистентность к *N*-метил-*N'*-нитро-*N*-нитрозогуанидину (MNNG) и чувствительность к ионам кадмия. Ген *GSH1 H. polymorpha* восстанавливал резистентность к ионам кадмия, чувствительность к MNNG, нормальный уровень глутатиона и пролиферацию клеток на минимальной среде без добавления цистеина или глутатиона при введении в клетки мутанта *gsh1*. Показано, что ген *GSH1 H. polymorpha* выявляет гомологию к гену *MET1 Saccharomyces cerevisiae*, кодирующему S-аденозил-L-метионин уропорфириноген III трансметилазу, которая отвечает за биосинтез кофактора сульфитредуктазы, серогема. Нами была сконструирована кассета с делецией гена *GSH1/MET1 H. polymorpha* (*Hpgsh1/met1::ScLEU2*) и получены соответственные нуль-мутанты (с делецией значительной части структурного участка этого гена). Данные по скрещиванию точечного *gsh1* мутанта и нуль-мутантов при *gsh1/met1* продемонстрировали, что обе аллели расположены в одном и том же гене. Нуль-мутант *gsh1/met1* проявлял полное восстановление роста на минимальной среде с цистеином или глутатионом в качестве единственного источника серы, но не с неорганическими (сульфат, сульфит) или органическими (метионин, S-аденозилметионин) источниками серы. Кроме того, оба мутанты – точечный *gsh1* и нуль-мутант при *gsh1/met1* проявляли повышенную чувствительность к токсическому углеродному субстрату метанолу, к формальдегиду, органическому пероксиду и ионам кадмия.

Ключевые слова: метилотрофные дрожжи, *Hansenula polymorpha*, глутатион, ассимиляция сульфата, *MET1*.

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