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 (Hp*sh1/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 Δgsh1/mef1 mutants contained the following compounds (per 1 litre): 10 g glucose, 1 g KH₂PO₄, 0.4 g MgCl₂ × 6H₂O, 2.8 g NH₄Cl, 0.1 g CaCl₂ × 6H₂O, and was supplemented with 2.6 mM (NH₄)₂SO₄, 0.1 mM Na₂SO₃, 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 (NH₄)₂SO₄, or in standard synthetic medium (2% glucose, 0.17% yeast nitrogen base without amino acids, and 0.5% (NH₄)₂SO₄). 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>
<thead>
<tr>
<th>Table 1. H. polymorpha strains constructed and used in this study</th>
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<tbody>
<tr>
<td>Designation</td>
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<tr>
<td>Wild type strains (WT)</td>
</tr>
<tr>
<td>NCYC495 leu1-1</td>
</tr>
<tr>
<td>NCYC495 leu1-1 ade11</td>
</tr>
<tr>
<td>DL-1 leu2</td>
</tr>
<tr>
<td>CBS4732 leu2-2 ura3-20</td>
</tr>
<tr>
<td>CBS4732 leu2-2 met2-2</td>
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<table>
<thead>
<tr>
<th>Transformants of NCYC495 genetic line</th>
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<tbody>
<tr>
<td>pG1</td>
</tr>
<tr>
<td>pG1-23</td>
</tr>
<tr>
<td>pG1-36</td>
</tr>
<tr>
<td>pG1-47</td>
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</table>

<table>
<thead>
<tr>
<th>Mutant strains</th>
</tr>
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<tbody>
<tr>
<td>gsh1 point</td>
</tr>
<tr>
<td>gsh2 point</td>
</tr>
<tr>
<td>Δgsh1/mef1 ade11</td>
</tr>
<tr>
<td>Δgsh1/mef1 ura3-20</td>
</tr>
<tr>
<td>Δgsh1/mef1 met2-2</td>
</tr>
<tr>
<td>Δgsh2 ade11</td>
</tr>
<tr>
<td>Δgsh2 met2-2</td>
</tr>
</tbody>
</table>
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 HpgS1/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 HpgS1/Met1 coding and terminator sequence (1509 bp) cloned into the 3'-flanking region of the ScLEU2 gene fragment was obtained by elimination of BamHI/BamHI fragment and subsequent self-ligation of pYT1+5.3 kb plasmid (Table 3; Results and Discussion). The resultant plasmid was digested with endonucleases HindIII and PstI and used for cloning of HindIII/PstI 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 HindIII and SacI 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 HpgS1/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

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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: HindIII, Sp, SphI, PI, PstI, Sa, SalI, XbaI, Bl, BamHI, SI, SacI.
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 HindIII/PstI fragment into the HindIII/PstI digested pYT1 plasmid, which was harboring the ScLEU2 gene. The resultant plasmid was digested by endonucleases XbaI and SacI and subsequently ligated with the XbaI/Saci 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 HindIII and SacI and transformed into H. polymorphaNCYC495 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 2. Oligonucleotides used as primers for PCR amplification in this study |
|-----------------------------|-----------------------------|-----------------------------|
| Name                        | Sequence                    | Site                        |
| **Primers for construction of Hpgsh1/met1::ScLEU2 deletion cassette** |                            |                             |
| VU5F                        | 5'-GGAAAGCTTTGCCGAGCTCTGTTTA-3' | (HindIII)                  |
| VU6R                        | 5'-AACTGCAGTGGCAAGGACACGTTT-3' | (PstI)                     |
| **Primers for confirmation of null Hpgsh1/MET1 mutation** |                            |                             |
| VU11F                       | 5'-TCTGTGTGCTCCAGAATGCT-3'   |                             |
| VU12R                       | 5'-AGTATCCGGTACCCAGCAAT-3'   |                             |
| **Primers for identification of null Hpgsh1/met1 gene** |                            |                             |
| VU13F                       | 5'-AAGAAGATCGTGTTTTGCCC-3'   |                             |
| VU12R                       | 5'-AGTATCGGGTCACCAGCAAT-3'   |                             |
| **Primers for construction of Hpgsh2::ScLEU2 deletion cassette** |                            |                             |
| VU7F                        | 5'-GGAAAGCTTTGCCGAGCTCTGTTTA-3' | (HindIII)                  |
| VU8R                        | 5'-AACTGCAGTGGCAAGGACACGTTT-3' | (PstI)                     |
| **Primers for isolation of 3′-flanking region of Hpgsh2 gene** |                            |                             |
| VU9F                        | 5'-GCTCTAGAAGTACCTCAAGGCTGGTGA-3' | (XbaI)                  |
| VU10R                       | 5'-GTGAGCTCTAGCGTGCAATTTTTCC-3' | (SacI)                     |
| **Primers for confirmation of null Hpgsh2 mutation** |                            |                             |
| VU15F                       | 5'-GTCAACATCGTGCCTCCATTGAT-3' |                             |
| VU10R                       | 5'-GTGAGCTCTAGCGTGCAATTTTTCC-3' | (SacI)                     |
| **Primers for identification of null Hpgsh2 gene** |                            |                             |
| VU30F                       | 5'-TCTAGATCGGCCCTCCACATAAGCC-3' |                             |
| VU31R                       | 5'-CACCTGGCAAACGACGATTTTC-3' |                             |

Underlined nucleotides mark the restriction sites indicated on the right.
null mutant strains, while a point mutant was mated with gsh2 leu1-1 ade11 Δgsh1
Hybridization of the auxotrophic mutant strains. described [3]. A point mutant was gsh1 leu1-1
DNA sequencing was performed at the Korea Re-
England Bio Labs (USA) and Promega (USA).
Reanal (Hungary), Fermentas (Lithuania), New
by PCR. Reagents and restriction enzymes were
were used for the amplification of DNA fragments
primers, produced by IDT Technologies (USA),
for the null gsh2 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 manipu-
lations 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 ITD Technologies (USA), were used for the amplification of DNA fragments by PCR. Reagents and restriction enzymes were purchased from next corp: Sigma (USA), Recanal (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.
cb.nlm.nih.gov/BLAST/. Multiple sequence alignments were constructed using Multalin algorithm, version 5.4.1, http://bioinfo.genopole-

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 se-
rum 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 (NH₄)₂SO₄, 0.1 mM cysteine (gsh1 and Δgsh1/met1) or 0.1 mM GSH (gsh2) for 1 day and then transferred into 125 ml of the same medium with an initial OD₆₀₀ 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 dif-
ferent genetic lines were precultivated in 20 ml of standard synthetic medium for 2 days, transferred into 250 ml of the same medium with initial OD₆₀₀ nm ≈ 0.01 and grown till the early loga-
rithmic or stationary phase. Harvested cells were washed twice with water and frozen for homogeniza-
tion. 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,
10 mM MgCl₂, 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
8.0, 2.5 μl 0.5 M MgCl₂, 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·h⁻¹·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 BamHI 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 SacI site of pYT3 plasmid and 5.3 kb (containing ORF4) into SacI site of pYT3 and pYT1 vectors. DNA subfragments of 4.4 kb and
5.3 kb were obtained from pG1 plasmid digested by SacI 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

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**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 \( \textit{H. polymorpha} \) wild type strain \( \text{NCYC495 leu1-1, point gsh1 mutant and transformants (pG1 and pG1-47), depending on the presence of Cd}^{2+} \) ions (\( M \) \( \pm \) \( m \), \( n = 4 \))

<table>
<thead>
<tr>
<th>Strain</th>
<th>GSH+GSSG, nmol/mg protein(^{1, *})</th>
<th>Growth on minimal synthetic medium**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CdSO(_{4}), 0.1 mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCYC495</td>
<td>128.0 ± 6.1</td>
<td>209.8 ± 8.4(^{a})</td>
</tr>
<tr>
<td>gsh1</td>
<td>29.2 ± 0.8(^{a})</td>
<td>11.0 ± 0.3(^{a, xx})</td>
</tr>
<tr>
<td>pG1</td>
<td>90.1 ± 2.6(^{a, xx})</td>
<td>124.0 ± 5.7(^{a, xx})</td>
</tr>
<tr>
<td>pG1-47</td>
<td>68.0 ± 1.9(^{a, xx})</td>
<td>114.3 ± 5.2(^{a, xx})</td>
</tr>
</tbody>
</table>

* 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 \( \mu \)M GSH; \(^{a} P < 0.05\) as compared to correspondent control values; \(^{xx} P < 0.05\) as compared to correspondent wild type values.

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

To confirm that the \( \text{HpGSH1/ME1} \) gene was the wild type allele complementing directly the point gsh1 mutant phenotypes, the null gsh1/\( \text{met1} \) mutant strains (\( \Delta \)gsh1/\( \text{met1} \)::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/\( \text{met1} \) 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/\( \text{met1} \) mutants, particularly \( \Delta \)gsh1/\( \text{met1 ade1} \), was chosen for more detailed study.

Growth, GCS activity and total GSH+GSSG level of the wild type strain, point gsh1 and \( \Delta \)gsh1/\( \text{met1} \) mutants of \( \text{H. polymorpha} \) depending on sulfur source. The cloned \( \text{HpGSH1/ME1} \) 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/\( \text{met1} \) 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 \( \text{S. cerevisiae met1} \) mutant, the \( \text{H. polymorpha} \) \( \Delta \)gsh1/\( \text{met1} \) mutant did not grow in minimal medium with sulfate and sulfite as a sole sulfur source. However, in contrast to the \( \text{S. cerevisiae} \) \( \text{met1} \) mutant that satisfied nutritional needs in sulfur with S-amino acids and their derivatives [12], the null \( \text{H. polymorpha} \) gsh1/\( \text{met1} \) mutant strain displayed negligible ability to assimilate methionine and SAM, and reduced growth activity on \( \text{S-adenosylhomocysteine} \) and homocysteine as a sole sulfur source. In sulfur-free medium supplemented with cysteine or GSH \( \text{H. polymorpha} \) null gsh1/\( \text{met1} \) mutant showed total growth restoration. At the same time, \( \text{H. polymorpha} \) 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, \( \text{S-adenosylhomocysteine} \) and homocysteine (data not shown). Discrepancy in growth phenotype of \( \text{met1} \) mutants from \( \text{S. cerevisiae} \) and \( \text{H. polymorpha} \) could be explained by distinctions in sulfate assimilation pathways (on the step of \( \text{H}_{2}\text{~S} \) incorporation in cysteine or/and homocysteine) and transsulfuration reactions between cysteine and homocysteine (be- or unidirectional) [12]. It might be possible that similar to \( \text{S. pombe} \), the methylotrophic yeast \( \text{H. polymorpha} \), 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 \textit{H. polymorpha} with the homologous proteins from \textit{C. albicans (CaUropor)}, \textit{P. pastoris (PpUropor)}, \textit{S. cerevisiae (ScMet1p)}, \textit{N. crassa (NcUropor)}, \textit{S. pombe (SpUropor)}, and \textit{E. coli (EcCyysG)}
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/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 OD590 ~ 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](image-url)
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 Δgsh1 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 decreased 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sulfur source</th>
<th>GSH</th>
<th>Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SO$_4^{2-}$</td>
<td>GSH</td>
<td>Cys</td>
</tr>
<tr>
<td>NCYC495</td>
<td>281.0 ± 12.6</td>
<td>290.8 ± 11.2</td>
<td>261.7 ± 9.2</td>
</tr>
<tr>
<td>gsh1</td>
<td>–</td>
<td>–</td>
<td>255.6 ± 9.5</td>
</tr>
<tr>
<td>Δgsh1/met1</td>
<td>–</td>
<td>–</td>
<td>341.8 ± 13.7</td>
</tr>
<tr>
<td>Δgsh2</td>
<td>–</td>
<td>43.6 ± 0.4</td>
<td>–</td>
</tr>
</tbody>
</table>

* P < 0.05 as compared to inactive protein

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)

<table>
<thead>
<tr>
<th>Strain</th>
<th>GCS activity,* nmol mg protein$^{-1}$ hour$^{-1}$</th>
<th>GSH+GSSG,* nmol mg protein$^{-1}$</th>
<th>Growth on YPD medium**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>logarithmic</td>
<td>stationary</td>
<td>logarithmic</td>
</tr>
<tr>
<td>NCYC495</td>
<td>235.4 ± 11.5</td>
<td>276.0 ± 12.4</td>
<td>100.2 ± 3.4</td>
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<tr>
<td>CBS4732</td>
<td>351.5 ± 16.3</td>
<td>144.6 ± 6.1</td>
<td>140.0 ± 4.2</td>
</tr>
<tr>
<td>DL-1</td>
<td>329.0 ± 14.8</td>
<td>251.9 ± 10.6</td>
<td>160.4 ± 3.9</td>
</tr>
</tbody>
</table>

* 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; (+) – weak growth; (–) – absence of growth; * P < 0.05 as compared to inactive protein; ** P < 0.05 as compared to correspondent values in logarithmic phase
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 (K₂Cr₂O₇), methanol, tert-butyl hydroperoxide (t-BOOH), formaldehyde, and cadmium ions (CdSO₄). 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 OD₅₉₀ 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(VI) instead of a less toxic Cr(III) form due to impairment in sirohaem-dependent sulfate 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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КЛЮЧЕВІ СЛОВА: метилотрофні дріжджі, Hansenula polymorpha, глутатіон, асіміляція сульфату, MET1.

Ген GSH1/MET1 Hansenula polymorpha клонували шляхом комбінації глутатіонзалежного росту мутанта gsh1 H. polymorpha, попередньо виділеного як клон, що виявляв резистентність до N-метил-N'-нітро-N'-нітроазоганідину (MNNG) і чутливість до іонів кадмію. Ген GSH1 H. polymorpha відновлював резистентність до іонів кадмію, чутливість до MNNG, нормальний рівень глутатіону та продіференцію 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-метіоніну уroporphyrinogen III трансмісапазу, яка відповідає за біосинтез кофактора сульфідредуктази, сіроугру. Нами була сконструйована касета з делецією гену GSH1/ MET1 H. polymorpha (Hpgsh1/met1::ScLEU2) та одержані відповідні нуль-муантів (із делецією значної частини структурної ділянки цього гена). Дані зі схрещування точкового gsh1 мутанта та нуль-муантів за gsh1/met1 продемонстрували, що обидві алелі розміщені в одному й тому ж гені. Нуль-муант gsh1/met1 повністю відновлював ріст на мінімальному середовищі із цистеїном і глутатіоном, але не з неорганічними (сульфат, сульфіт) чи органічними (метіонін, глутатіон) джерелами глутатіону. Окрім того, обидва мутанти – точковий gsh1 і нуль-муант gsh1/met1 – виявили підвищену чутливість до токсичного вуглецевого субстра-ту метанолу, до формальдегіду, органічного пероксиду та іонів кадмію.

Ген GSH1/MET1 Hansenula polymorpha клонували шляхом комбінації глутатіонзалежного росту мутанта gsh1 H. polymorpha, попередньо виділеного як клон, що виявляв резистентність до N-метил-N'-нітро-N'-нітроазоганідину (MNNG) і чутливість до іонів кадмію. Ген GSH1 H. polymorpha відновлював резистентність до іонів кадмію, чутливість до MNNG, нормальний рівень глутатіону та продіференцію 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-метіоніну уroporphyrinogen III трансмісапазу, яка відповідає за біосинтез кофактора сульфідредуктази, сіроугру. Нами була сконструйована касета з делецією гену GSH1/ MET1 H. polymorpha (Hpgsh1/met1::ScLEU2) та одержані відповідні нуль-муантів (із делецією значної частини структурної ділянки цього гена). Дані зі схрещування точкового gsh1 мутанта та нуль-муантів за gsh1/met1 продемонстрували, що обидві алелі розміщені в одному й тому ж гені. Нуль-муант за gsh1/met1 – виявили підвищену чутливість до токсичного вуглецевого субстра-ту метанолу, до формальдегіду, органічного пероксиду та іонів кадмію.

Ключові слова: метилотрофні дріжджі, Hansenula polymorpha, глутатіон, асіміляція сульфату, MET1.
который проявлял резистентность к N-метил-N'-нитро-N'-нитрозогуанидину (MNNNG) и чувствительность к ионам кадмия. Ген GSH1 H. polymorpha восстанавливал резистентность к ионам кадмия, чувствительность к МННГ, 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 трансметодазу, которая отвечает за биосинтез кофактора сульфитредуктазы, серогема. Нами была сконструирована кассета с делецией гена GSH1/MET1 H. polymorpha (HpGSH1/mel1::ScLEU2) и получены соответствующие 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 проявили повышенную чувствительность к токсиническому углеродному субстрату метанолу, к формальдегиду, органическому пероксиду и ионам кадмия.

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


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