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EXPERIMENTAL ARTICLES

Cloning of the *GSH1* and *GSH2* Genes Complementing the Defective Biosynthesis of Glutathione in the Methylotrophic Yeast *Hansenula polymorpha*

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Abstract—The cloning of 7.2- and 9.6-kbp fragments of the methylotrophic yeast *Hansenula polymorpha* DNA restored the wild-type phenotype Gsh^+ in the glutathione-dependent *gsh1* and *gsh2* mutants of this yeast defective in glutathione (GSH) synthesis because of a failure of the γ-glutamylcysteine synthetase reaction. The 9.6-kbp DNA fragment was found to contain a 4.3-kbp subfragment, which complemented the Gsh^- phenotype of the *gsh2* mutant. The Gsh^+ transformants of the *gsh1* and *gsh2* mutants, which bear plasmids pG1 and pG24, having the 7.2- and 4.3-kbp DNA fragments, respectively, had a completely restored wild-type phenotype with the ability to synthesize GSH and to grow in GSH-deficient synthetic media on various carbon sources, including methanol, and with acquired tolerance to cadmium ions. In addition, the 4.3-kbp DNA fragment borne by plasmid pG24 eliminated pleiotropic changes in the *gsh2* mutants associated with methylotrophic growth in a semisynthetic (GSH-supplemented) medium (poor growth and alterations in the activity of the GSH-catabolizing enzyme γ-glutamyltransferase and the methanol-oxidizing enzyme alcohol oxidase).

Key words: methylotrophic yeasts, glutathione biosynthesis, gene cloning.

Genes controlling glutathione (GSH) biosynthesis have been well studied in many organisms. The *GSH1* (or *GCS*) gene, encoding γ-glutamylcysteine synthetase (γGCS), the first enzyme of GSH synthesis, has been cloned from *Escherichia coli*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, rat, human, and other organisms [1–3]. The molecular structure of the *GSH2* gene, coding for glutathione synthetase, the second enzyme of GSH biosynthesis, has been studied in *S. cerevisiae* and *S. pombe* [4, 5]. Investigations along this line led to the establishment of the regulatory zones of the GSH biosynthesis gene promoters, as well as the proteins (Yap1 and Skn7) and low-molecular-weight factors that control the expression of these genes in *S. cerevisiae* and *S. pombe* exposed to various kinds of stress [5–8]. Of particular importance is the role of GSH in methylotrophic yeasts, which produce two highly cytotoxic compounds, hydrogen peroxide and formaldehyde, in the first reaction of methanol catabolism. These compounds are potential inducers of oxidative and electrophilic stresses [9], a fact which greatly enhances the role of GSH as an antistress factor. For instance, GSH is directly involved in the oxidation of formaldehyde in the formaldehyde dehydrogenase reaction [10]. In the presence of methanol in the medium, the intracellular content of GSH in methylotrophic yeasts considerably rises [11], especially in their formaldehyde dehydrogenase–defective mutants, which accumulate great amounts of toxic formaldehyde in the culture medium [12]. For this reason, the genetic control of GSH biosynthesis in methylotrophic yeasts may include, in addition to the known mechanisms, some specific regulatory pathways.

In a previous work, we obtained *gsh1* and *gsh2* mutants of the methylotrophic yeast *Hansenula polymorpha* defective in GSH biosynthesis because of the complete or partial loss of γGCS activity and suggested that these mutants have lesions in the structural and/or regulatory genes responsible for the first step of GSH synthesis [13]. These mutants were not able to grow on a number of carbon sources, including methanol, in a synthetic medium lacking GSH, but did grow in a GSH-supplemented synthetic or complex medium [13]. Unlike the *gsh2* mutants, which could not grow on methanol irrespective of whether or not GSH was present in the culture medium, the *gsh1* mutants were able to grow on methanol in the presence of GSH.

The aim of the present work was to reveal the origin of the *gsh1* and *gsh2* mutations in the methylotrophic yeast *H. polymorpha* by cloning DNA fragments able to complement the mutant phenotypes to the wild-type phenotype.

MATERIALS AND METHODS

Strains and cultivation conditions. Experiments were carried out with the wild-type methylotrophic

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yeast *Hansenula polymorpha* CBS 4732 (*leu2*) and its glutathione-dependent mutants *gsh1-2 leu2*, *gsh2-1 leu2* [13], *hsh1-2 leu1-1*, and *gsh2-1 leu1-1*. The auxotrophic marker *leu1-1* was introduced into the yeast genotype by means of hybridization of the mutants *gsh1-2 ura3* and *gsh2-2 ura3* [13] with the strain *H. polymorpha* NCYC 496 (*leu1-1*), which was derived from the wild-type strain obtained from the National Collection of Yeast Cultures, Food Research Institute, Colney Lane, Norwich NR4 7UA, United Kingdom. Plasmids were amplified using the *Escherichia coli* strain DH5α.

The *H. polymorpha* strains were grown at 37°C either in a complex YPD medium containing 1% of each yeast extract, bacto peptone, and glucose, or in synthetic YNB medium (0.67% Difco YNB), which was supplemented with a carbon source (1% glucose, 1% ethanol, or 0.5% methanol) and, when required, 20 mg/l leucine and/or 30 mg/l GSH (Sigma). The *E. coli* strain was grown at 37°C in an LB medium containing (%) bacto peptone, 1.5; NaCl, 1; and yeast extract, 0.5.

DNA manipulations. The isolation and cleavage of plasmid DNA by restriction endonucleases and the separation of DNA fragments by electrophoresis in agarose gel were carried out by standard methods [14, 15]. *H. polymorpha* and *E. coli* cells were transformed with DNA by electroporation [16]. The *H. polymorpha* gene bank was constructed using the *H. polymorpha–E. coli* shuttle vector pYT3 [17]. The 9.6-kbp DNA fragment complementing the Gsh^- phenotype was subcloned using the same shuttle vector, pYT3. Recombinant plasmids were constructed using the DNA subfragments obtained by cleaving plasmid pG2 (plasmid pYT3 + the 9.6-kbp DNA fragment) with restriction endonuclease *Bam*HI or *Xba*I. The DNA subfragments were fractionated by electrophoresis in agarose gel, followed by electroelution in dialysis sacks. DNA was hydrolyzed, dephosphorylated, and ligated using enzymes purchased from MBI Fermentas (Lithuania), Promega, and New England Biolabs.

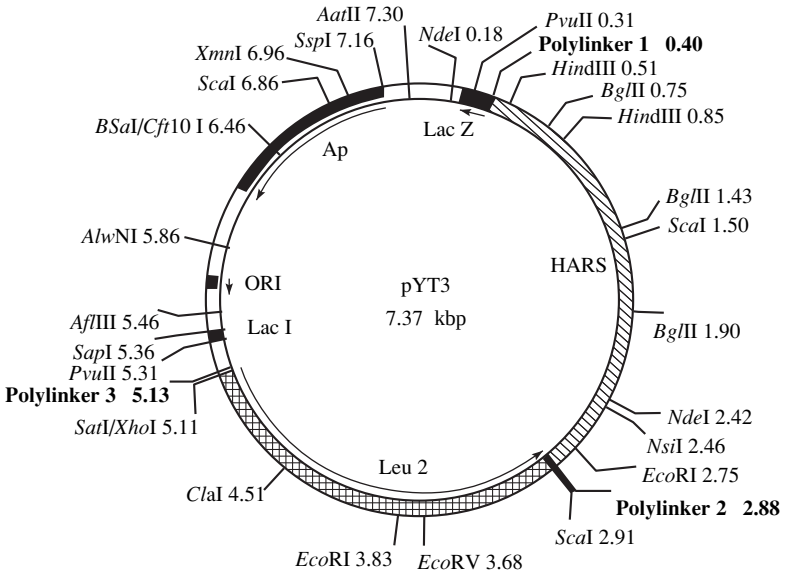
Biochemical methods. An aliquot (0.5 ml) of a cell suspension was diluted with 50 mM Tris buffer (pH 10.5) containing 0.25 M sucrose, 1 mM $MgCl_2$, and 1 mM PMSF, mixed with an equal volume of glass beads, and cells were broken by treatment in a Vortex homogenizer for 10 min in the cold. The pH of the cell homogenate was adjusted to 7.5, and the homogenate was centrifuged first at 1475 *g* for 5 min and then at 4340 *g* for 5 min. The second supernatant (cell-free extract) was used to assay alcohol oxidase and to determine the total content of glutathione (GSH + GSSG), as described earlier [13]. The γ-glutamyltransferase (γGT) activity of the cell extract was determined by a modified method of Payne and Payne [18]: 0.5 ml of the reaction mixture, containing 0.1 M Tris–HCl buffer (pH 8.0), 2.5 mM γ-L-glutamyl-*p*-nitroanilide, and 1 mg protein of the cell extract was incubated for 1–2 h at 37°C. The reaction was stopped by adding 0.35 ml of 2.5 M acetic acid, and the mixture was centrifuged at 10000 *g* for 5 min. *p*-Nitroaniline present in the supernatant was determined spectrophotometrically at 410 nm using the coefficient of molar extinction for *p*-nitroanilide, equal to 8800 M^{-1} cm^{-1}.

The protein concentration was determined by the method of Lowry *et al.* [19], using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The wild-type phenotype of the *gsh1* and *gsh2* mutants of the methylotrophic yeast *H. polymorpha* was restored by transforming these mutants with the *H. polymorpha* CBS 4732 gene bank constructed with the aid of plasmid pYT3 (Fig. 1). Gsh^+ transformants were selected based on their ability to grow in a GSH-deficient synthetic medium with glucose as the carbon source. The selective medium for the recipient mutant strain with the auxotrophic marker *leu2* was supplemented with leucine, since the *LEU2* gene of *S. cerevisiae* marking the shuttle vector pYT3 complements the *leu1-1* but not the *leu2* mutation in the *H. polymorpha* genome. We succeeded in obtaining two Gsh^+Leu^+ transformants of the *gsh1* mutants and one Gsh^+ transformant of the *gsh2* mutants, which exhibited the wild-type phenotype when subcultured on the respective selective media. The transformation of *E. coli* cells with the total DNA isolated from each of the transformants grown in the GSH-deficient synthetic medium showed that one of the two Gsh^+Leu^+ transformants did not contain plasmid DNA, which is indicative of plasmid integration in this transformant. Analysis of the plasmid DNA isolated from the other Gsh^+Leu^+ transformant (12 ampicillin-resistant clones) and from the Gsh^+ transformant (4 ampicillin-resistant clones) showed that 11 of the 12 clones contained plasmid pYT3 bearing a DNA fragment about 7.2 kbp in size, which corresponded to the plasmid DNA of the Gsh^+Leu^+ transformant of the *gsh1* mutants. Furthermore, all 4 ampicillin-resistant clones exhibited the presence of plasmid pYT3 with a DNA fragment about 9.6 kbp in size, which corresponded to the plasmid DNA of the Gsh^+ transformant of the *hsh2* mutants. The isolated plasmids carrying the 7.2- and 9.6-kbp DNA fragments were designated pG1 and pG2, respectively.

Plasmids pG1 and pG2 were used to retransform the mutants *gsh1-1 leu1-1* and *gsh2-1 leu1-1*, respectively. The resultant Gsh^+ retransformants were able to grow on methanol in the GSH-deficient synthetic medium, had normal sensitivity to cadmium ions (Table 1), and exhibited positive mitotic instability under nonselective conditions (after 15 generations in the presence of GSH, only 72–87% of the Gsh^+Leu^+ transformants and 21–57% of the Gsh^+ transformants retained the ability to grow in the absence of GSH). The identicalness of the plasmid DNA isolated from the respective transfor-



Polylinker 1: 0.40/*Eco*RI, *Sac*I, *Kpn*I.
Polylinker 2: 2.88/*Bam*HI, *Xba*I.
Polylinker 3: 5.13/*Bsp*MI, *Pst*I, *Sph*I, *Hin*dIII.

Fig. 1. The restriction map of the *H. polymorpha–E. coli* shuttle vector pYT3, on which the *H. polymorpha* gene bank was constructed. The plasmids of this bank contain 5- to 10-kbp fragments of the total DNA of *H. polymorpha* CBS 4732, which were inserted at polylinker 2 of plasmid pYT3 [17].

mants to plasmids pG1 and pG2 was confirmed by analysis with the restriction endonuclease *Eco*RI.

The 7.2- and 9.6-kbp DNA fragments of plasmids pG1 and pG2, respectively, were mapped using restriction endonucleases (Figs. 2, 3). Figure 3 shows the DNA subfragments of plasmid pG2 (A, B, C, and D) that were used for subcloning on the basis of plasmid pYT3.

The recombinant plasmids pG21, pG22, pG23, and pG24, which bore, respectively, the subfragments A, B, C, and D of plasmid pG2, were used to transform the mutant *H. polymorpha gsh2-1 leu1-1* strain. Transformants were selected based on their ability to grow in the medium lacking leucine. The Leu^+ transformants carrying plasmids pG21, pG22, and pG23 turned out to be unable to grow in the minimal synthetic medium containing no GSH. At the same time, the subfragment D cloned by plasmid pG24 complemented the Gsh^-Leu^- phenotype to the wild-type Gsh^+Leu^+ phenotype. Three of the Gsh^+Leu^+ transformants were found to be able to

Table 1. Growth characteristics of the wild-type *H. polymorpha* strains, their *gsh* mutants, and the Gsh^+ transformants of these mutants

Strain	Growth substrate						
	glucose		ethanol		methanol		Glucose + 0.05 mM Cd^{2+} + 0.02 mM GSH
	–GSH	+GSH	–GSH	+GSH	–GSH	+GSH	
4732 *leu 2* (wild type)	++	+++	++	+++	+	++	++
gsh1-2 leu2 mutant	–	+++	–	+++	–	++	–
gsh2-1 leu2 mutant	–	+++	–	+++	–	±	–
495 *leu1-1* (wild type)	++	+++	++	+++	+	++	++
gsh1-2 leu1-1 mutant	–	++	–	++	–	+	–
gsh2-1 leu1-1 mutant	–	++	–	++	–	–	–
Gsh^+Leu^+ transformant (pG1)	++	++	++	++	+	+	+++
Gsh^+ transformant (PG2)	++	++	++	++	++	++	+
Gsh^+Leu^+ transformant (PG24)	++	++	++	++	+	+	+++

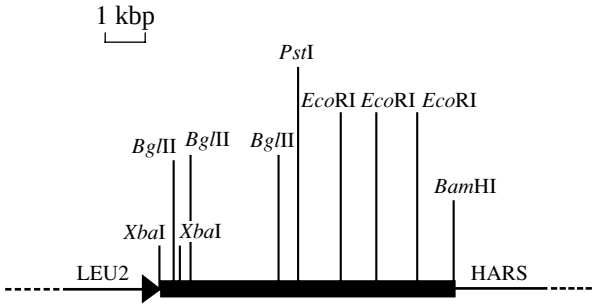


Fig. 2. The restriction map of the plasmid pG1–borne 7.2-kbp fragment of the chromosomal DNA of *H. polymorpha.* The flanking regions of the shuttle vector pYT3 are shown by thin lines.

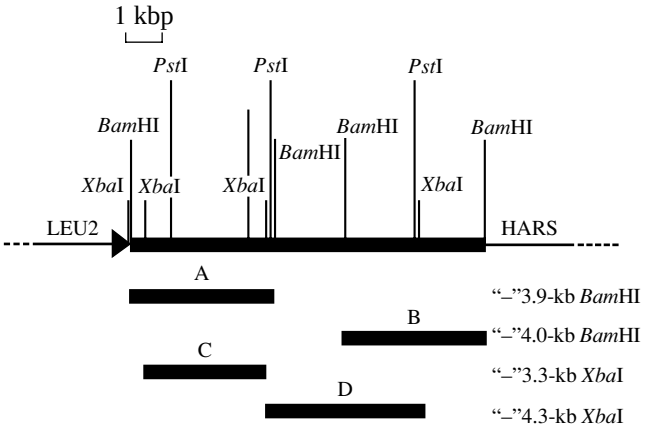


Fig. 3. The restriction map of the plasmid pG2–borne 9.6-kbp fragment of the chromosomal DNA of *H. polymorpha.* The flanking regions of the shuttle vector pYT3 are shown by thin lines. The subfragments A, B, C, and D of the 9.6-kbp fragment are shown below the map. "+" and "–" alongside of the subfragments indicate, respectively, the ability or inability of the particular subfragment to restore the wild-type phenotype in the *gsh2-1 leu1-1* mutant.

grow on methanol in the GSH-deficient synthetic medium, had normal sensitivity to cadmium ions (Table 1), exhibited positive mitotic instability under nonselective conditions, and contained a plasmid identical to pG24, i.e., the plasmid which was used to retransform the recipient *gsh2-1 leu1-1* strain.

When grown in the GSH-deficient synthetic medium with various carbon sources, the Gsh^+Leu^+ transformants of the *gsh1* and *gsh2* mutants (containing plasmids pG1 and pG24, respectively) exhibited a considerably higher level of intracellular GSH than the respective recipient mutant strains (Table 2). In this case, the methanol-grown Gsh^+Leu^+ transformants of the *gsh2* mutants exhibited no pleiotropic changes typical of these mutants (Fig. 4), namely, the poor growth of the *gsh2-1 leu2* mutant and the inability of the *gsh2-1 leu1-1* mutant to grow on methanol in the GSH-supplemented synthetic medium (Table 1) and the increased (by 3.5–5 times) activity of γGT in both mutants as compared with their growth in the complex YPD medium and with the wild-type strain and *gsh1* mutants grown in the semisynthetic medium (Fig. 4). The increase in the γGT activity correlated with a decrease in the level of GSH consumed from the medium and in the activity of alcohol oxidase (Fig. 4). The very low level of intracellular GSH in the methanol-grown *gsh2* mutants may result from the increased activity of γGT or may be due to alterations in metabolism, transport, or mechanisms maintaining the necessary level of GSH in the vacuoles [9]. The low level of GSH in the *gsh2* mutants may be responsible for the inability of these mutants to synthesize active alcohol oxidase because of a failure at the stages of apoenzyme synthesis or holoenzyme formation in the peroxisomes and, in the final analysis, may limit the growth of these mutants on methanol. In addition, the very high activity of γGT may reduce the efficiency of methanol catabo-

Table 2. Intracellular levels of the total glutathione (GSH + GSSG, nmol/mg protein) in the wild-type *H. polymorpha* strain, its *gsh* mutants, and the Gsh^+Leu^+ transformants of the *gsh1* and *gsh2* mutants, bearing plasmids pG1 and pG24, respectively

Strain	Growth substrate*					
	glucose		ethanol		methanol	
	–GSH	+GSH	–GSH	+GSH	–GSH	+GSH
495 *leu1-1* (wild type)	202	128	62	104	106	163
gsh1-2 leu1-1 mutant	<0.1**	81	<0.1**	33	<0.1**	75
gsh2-1 leu1-1 mutant	<0.1**	7	<0.1**	8	<0.1**	13
Gsh^+Leu^+ transformant (pG1)	83	ND	58	ND	101	ND
Gsh^+Leu^+ transformant (pG24)	87	ND	63	ND	151	ND

* Yeast cells were grown in the synthetic (–GSH) or semisynthetic (+ GSH) medium with different carbon sources to the midexponential growth phase.
** The *gsh1-2 leu1-1* and *gsh2-1 leu1-1* mutants were grown in the semisynthetic (+ GSH) medium with different carbon sources to the midexponential growth phase and then incubated for 16 h in the synthetic (–GSH) medium with the respective carbon source. ND stands for "not determined."

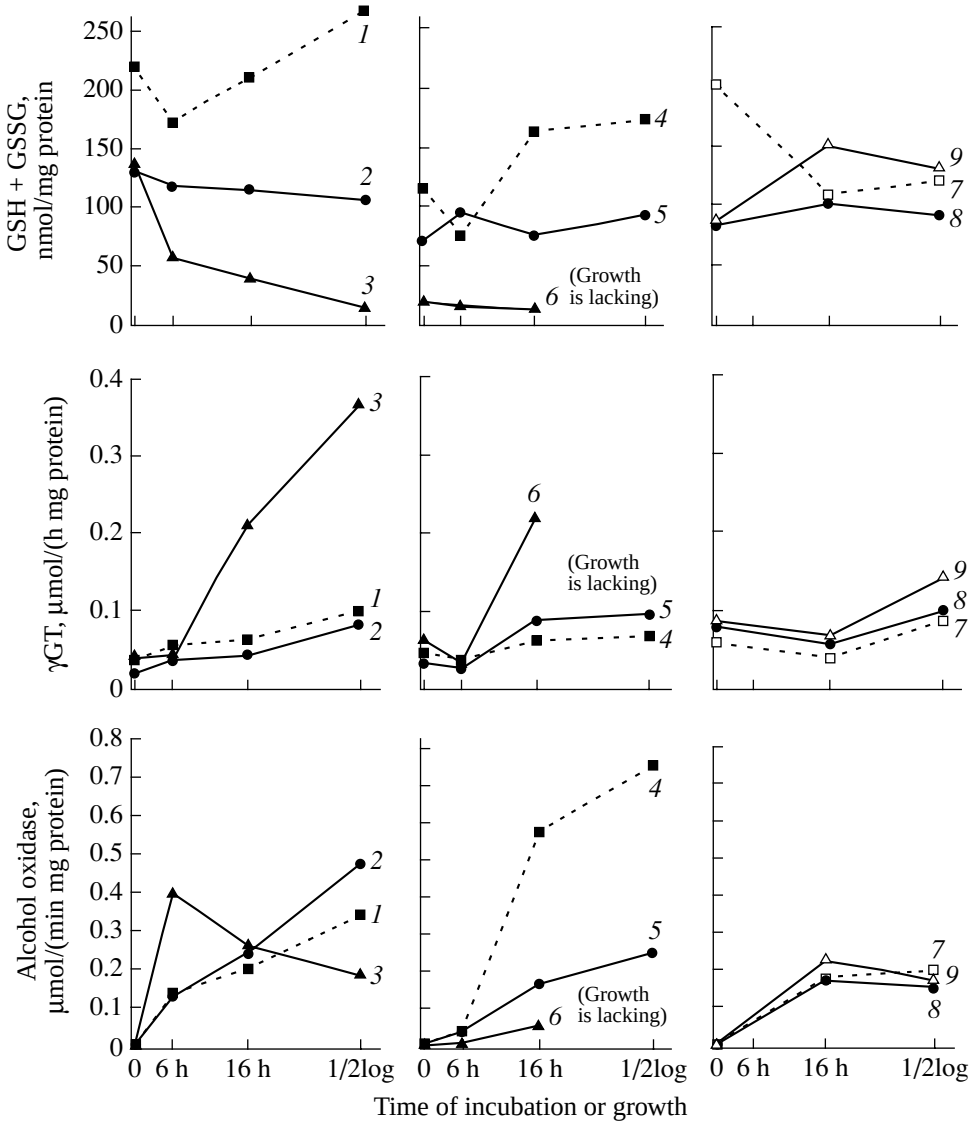


Fig. 4. The intracellular total content of glutathione (GSH + GSSG) and the activities of γ-glutamyltransferase and alcohol oxidase in the cell-free extract of (*1*) the wild-type *H. polymorpha* CBS 4732 (*leu2*), (*4, 7*) the wild-type NCYC 495 (*leu1-1*), the recipient mutants (*2*) *gsh1-2 leu2*, (*3*) *gsh2-1 leu2*, (*5*) *gsh1-2 leu1-1*, (*6*) *gsh2-1 leu1-1*, and (*8, 9*) the Gsh^+Leu^+ transformants of, respectively, the *gsh1* and *gsh2* mutants after 6 and 16 h of incubation and in the logarithmic phase of growth in the GSH-supplemented synthetic medium with methanol. The incubation medium was inoculated with yeast cells grown in (*1–6*) complex YPD medium or (*7–9*) the GSH-deficient synthetic medium with glucose and leucine. (*7–9*) The wild-type NCYC 495 (*leu1-1*) cells and the Gsh^+Leu^+ transformants were grown and incubated in the GSH-deficient synthetic medium with methanol.

lism in the *gsh2-1 leu2* mutant (data not shown) by diverting formaldehyde to the so-called mercapturic acid pathway, which assimilates GSH-conjugated electrophilic compounds, such as GSH-conjugated formaldehyde, in higher eukaryotes [20].

To conclude, the cloned DNA fragments complement defects in the biosynthesis of GSH in the *H. polymorpha gsh1* and *gsh2* mutants and likely represent genes involved in GSH synthesis and/or its regulation. These genes are arbitrarily referred to as *GSH1* and *GSH2*. The latter gene is presumably responsible not only for the synthesis but also for the catabolism of GSH, which makes the role of this gene in the methylotrophic growth of yeasts particularly important. *GSH2* may represent a regulatory gene that coordinates the biosynthesis and catabolism of GSH.

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