

## Multilevel Control of GSH Accumulation in Mutant and Wild-type Strains of *S. cerevisiae* Under Conditions of Smooth Cysteine Addition

Nisamedtinov, I.<sup>1,2</sup>, Orumets, K.<sup>1,3</sup>, Kevvai, K.<sup>1</sup>, Viiard, E.<sup>1,3</sup>, Sarand, I.<sup>1</sup>, Paalme, T.<sup>1,3</sup>

<sup>1</sup> Competence Centre of Food and Fermentation Technologies, Tallinn, Estonia

<sup>2</sup> Lallemand Inc., Montreal, Canada

<sup>3</sup> Tallinn University of Technology, Chair of Food Processing, Tallinn, Estonia

Corresponding e-mail address: inisamedtinov@lallemand.com

We studied the molecular mechanisms leading to glutathione over-accumulation in a *S. cerevisiae* strain selected via random mutagenesis. Comparative sequencing of *GSH1* in the mutant and its wild-type parent strain and the fed-batch experiments with continuous cysteine addition demonstrated that glutathione over-accumulation in the mutant were not caused by resistance to feedback inhibition of Gsh1p activity. Transcript analyses revealed that the expression levels of the genes leading to cysteine biosynthesis (*CYS3* and *CYS4*), but also *GSH1* and its transcription factor *YAP1* were several fold higher than those of the wild-type strain. The latter, together with an increased intracellular cysteine concentration, are most likely the reasons for glutathione over-accumulation in the mutant.

### 1. Introduction

Glutathione (GSH) is the most abundant non-protein thiol in eukaryotic cells. Due to a wide range of cellular physiological functions, in particular antioxidative functions, it has become an important compound for the treatment of a host of diseases and malfunctions, including cancer and neurodegenerative diseases (Martin and Teissman, 2009; Galant et al., 2009). Because of the increasing number of practical applications of GSH, there is an increasing need to reduce its production cost. Even though a number of technologies exist for the commercial production of GSH (e.g. chemical and enzymatic synthesis), microbial synthesis in yeast, in particular *S. cerevisiae*, has remained the current choice (Bacchawat et al., 2009). The biosynthesis of GSH occurs over two consecutive ATP-dependent reactions, catalyzed by  $\gamma$ -glutamylcysteine synthetase (Gsh1p) and GSH synthetase (Gsh2p). Generally, the first reaction has been considered the rate-limiting step in GSH biosynthesis, controlled via the feedback inhibition by GSH at both the transcriptional and post-translational levels to avoid its over-accumulation (Pócsi et al., 2004). The *GSH1* gene is regulated by the coupling of two transcriptional activators – redox sensitive Yap1p and Met4p, a principal factor mediating regulation of sulfur assimilatory pathways. The down-regulation of *GSH1* was suggested to be caused via ubiquitination of Met4p, which allows the repressor

Cbf1p to bind to the promoter of *GSH1* and inhibit Yap1p induced expression (Wheeler et al., 2003). Post-translational regulation is a non-allosteric feedback inhibition of Gsh1p activity (Soltaninassab et al., 2000). In addition to these two control mechanisms, cysteine has been shown to be the major limiting precursor for GSH accumulation (Alfajara et al., 1992) and thus, the regulation of sulfur assimilatory pathways also becomes important from the point of view of cysteine biosynthesis.

Among different strategies to improve GSH accumulation, the GSH biosynthetic enzymes Gsh1p and Gsh2p have been over-expressed in *S. cerevisiae* (Grant et al., 1997). These strains, however, failed to accumulate GSH concentrations higher than two fold compared to the initial physiological level, which can be explained by the feedback regulation of the Gsh1p reaction. To overcome nutrient limitation, overproduction of a few key enzymes in sulfur assimilatory pathways leading to cysteine biosynthesis have been suggested (Bacchawat et al., 2009), however the effect of such genetic modifications has not been experimentally demonstrated.

In addition to recombinant strains, several mutation strategies have been described in the patent literature for the isolation of GSH over-accumulating strains. The over-accumulation in those strains has been related to the feedback resistance of Gsh1p (Li et al., 2004). In the present work we investigated possible molecular mechanisms allowing GSH over-accumulation in a *S. cerevisiae* strain selected via random mutagenesis.

## 2. Materials and Methods

### 2.1. Yeast strain and cultivation process

A commercial baker's yeast *S. cerevisiae* (LYCC6267) and its GSH over-accumulating mutant (LYCC562671) were provided by Lallemand, Inc. (Montréal, Canada). A 7 L "BioBench" fermenter (Applikon, The Netherlands), equipped with two variable speed feeding pumps, a fixed speed titration pump and a pH and EtOH sensor (Mine Safety Appliances, USA) was used in the cultivation experiments. Cultivations were started in the batch mode using culture medium described in van Hoek et al., 2000. After the consumption of ethanol a fed-batch cultivation on mineral feeding medium without cysteine was started in ethanol-stat mode ( $\text{EtOH}_{\text{setpoint}}=0.2 \text{ g L}^{-1}$ ). When the biomass concentration reached  $\sim 40 \text{ g dwt L}^{-1}$  the shift from the basic feeding medium to the one containing cysteine [ $56$  and  $112 \mu\text{mol (g sucrose)}^{-1}$  in Exp. 1 and Exp. 2, respectively] was carried out and cultivation was continued as described above.

### 2.2. Analytical methods

Culture samples (6-7 mL) were quickly collected into 15 mL tubes on ice in an interval of 20 minutes, distributed into 6 Eppendorf microcentrifuge tubes (1 mL into each one) and washed with ice cold RNase free [dimethyl pyrocarbonate (DMPC)-treated] water, frozen in liquid nitrogen and stored at  $-80 \text{ }^{\circ}\text{C}$  until analysis. All measurements were carried out in duplicate. Intracellular thiols were quantified after extraction in 0.1 N formic acid ( $70 \text{ }^{\circ}\text{C}$ , 1 hour) by derivatization with 5,5'-dithiobis(2-nitrobenzoic acid) and determination using ACQUITY UPLC system (Waters, USA) using a  $\text{C}_{18}$  column (BEH C18,  $100 \times 2.1 \text{ mm}$ ,  $1.7 \mu\text{m}$ , Waters, USA) and a photo diode array detector. The specific rate of Gsh1p reaction ( $Q_{\text{Gsh1}}$ ,  $\mu\text{mol g}^{-1} \text{ h}^{-1}$ ) was expressed as follows:

$$Q_{Gsh1} = \frac{d(GSH_X \cdot X \cdot V)}{dt(X \cdot V)} + \frac{d(\gamma GCS_X \cdot X \cdot V)}{dt(X \cdot V)} \quad (1)$$

where  $GSH_X$  and  $\gamma GCS_X$  – concentration of glutathione and  $\gamma$ -glutamylcysteine in the biomass ( $\mu\text{mol g}^{-1}$ );  $X \cdot V$  – amount of biomass in the fermenter (g).

Expression of *GSH1*, *GSH2*, *ECM38*, *SUL1*, *MET17*, *CYS3*, *CYS4*, *STR2*, *STR3*, *YAP1* and *MET4* (Fig. 1) was monitored by a method termed transcript analysis with the aid of affinity capture (TRAC, Plexpress OY, Finland) using a pool of oligonucleotide probes designed for the respective target genes, separable and quantifiable by capillary electrophoresis. For sequencing *GSH1* the gene was amplified by PCR in two fragments (approx. 1200 bp) using genome DNA as a template. The amplified fragments were cloned into a pJET1.2 vector as described by the manufacturer (Fermentas, Lithuania). Four clones *per* fragment were sequenced using plasmid specific primers flanking the cloning site. The sequencing reactions were set up using a BigDye kit as described by manufacturer (Applied Biosystems, USA). The sequences obtained were analyzed using BLAST.

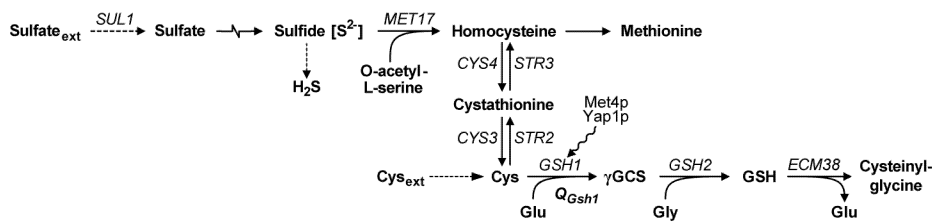


Figure 1 Schematic representation of the main biosynthetic pathways involved in the metabolism of cysteine and GSH.  $Q_{Gsh1}$  – specific rate of *Gsh1p* reaction ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ ); *SUL1* – high affinity sulfate permease; *MET17* – homocysteine synthase; *CYS4* – cystathionine beta-synthase; *CYS3* – cystathionine gamma-lyase; *STR3* – cystathionine beta-lyase; *STR2* – cystathionine gamma-synthase; *GSH1* – gamma-glutamylcysteine synthetase; *GSH2* – glutathione synthetase; *ECM38* – gamma-glutamyltranspeptidase; *Met4p* – leucine zipper transcriptional activator; *Yap1p* – redox sensitive transcriptional activator.

### 3. Results and discussion

The concentration of GSH in the mutant during fed-batch cultivation at near maximum respiratory growth rate  $\mu \approx \mu_{crit}$  (ethanol-stat) without the addition of cysteine was 40-45  $\mu\text{mol g}^{-1}$ . This was over three fold higher than the concentration observed in the parent strain under the same growth conditions (Fig. 2, Table 1). The comparative sequencing of *GSH1* did not reveal any mutations in the ORFs, which could lead to a disruption or release of the feedback inhibition of GSH on *Gsh1p* in the mutant.

The shift to cysteine containing feeding medium caused a rapid but short-term increase in the specific reaction rates of *Gsh1p* (and *Gsh2p*) reactions and a two fold increase in GSH concentration in the wild-type strain but not in the mutant (Fig. 2). This can be explained by the lower initial GSH concentration and the resulting weaker feedback inhibition of *Gsh1p* in the parent strain. However, with increasing GSH concentration the *Gsh1p* reaction rate decreased to a level comparable with the mutant. No down-

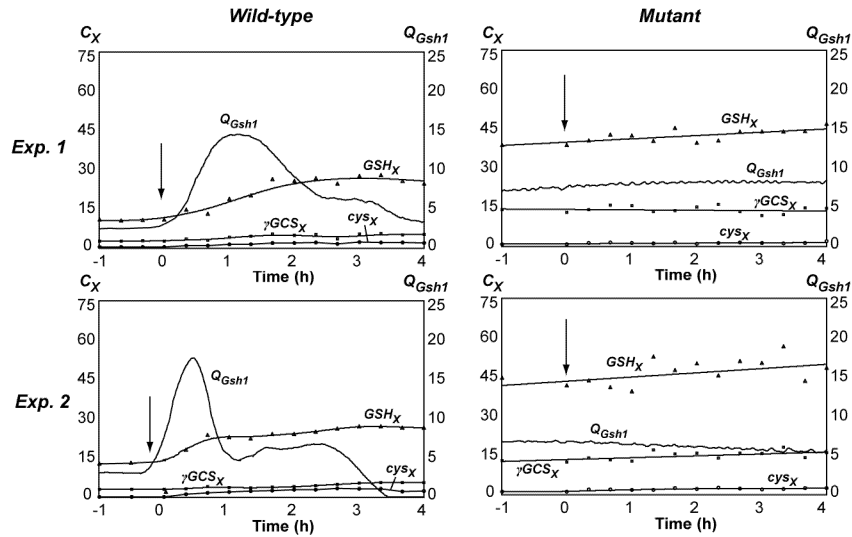


Figure 2 Accumulation of cysteine,  $\gamma$ GCS and GSH ( $\mu\text{mol g}^{-1}$ ) and the calculated Gsh1p reaction rates ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ ) in the mutant and wild-type strains during the continuous addition of cysteine:  $56 \mu\text{mol g}^{-1}$  sucrose in Exp. 1;  $112 \mu\text{mol g}^{-1}$  sucrose in Exp. 2. The arrows denote the start of cysteine addition.

regulation in GSH1 expression was observed in either strain at higher GSH concentrations (Fig. 4). These results indicate that the feedback inhibition of Gsh1p activity by GSH was functional in the mutant and the mutation(s) must be in other locations instead of GSH1 to cause GSH over-accumulation. Notably, the mRNA levels of both ECM38 and YAP1 increased in Exp. 2. Although the concentration of cysteinylglycine was not determined in the sample extracts, the increased expression of ECM38 suggests a possible increase in GSH turnover.

The results of gene expression analysis revealed that the major difference distinguishing the mutant from the parent strain was that the mRNA levels of CYS3, CYS4, GSH1,

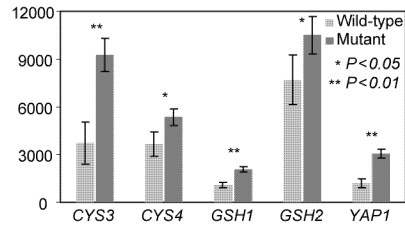


Figure 3 Initial expression levels of the key genes of cysteine and GSH biosynthesis in wild-type and mutant strains. The averages of two independent experiments are shown  $\pm$  SD. The means were compared using two-sample Student's *t*-test.

Table 1 Concentration of cysteine,  $\gamma$ GCS and GSH ( $\mu\text{mol g}^{-1}$ ) in mutant and parent strains and specific growth rates ( $\text{h}^{-1}$ ) before cysteine addition; maximum Gsh1p reaction rates observed during the experiments ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ ). <sup>(1)</sup>, <sup>(2)</sup> – Exp. 1 and Exp. 2, respectively.

	Wild-type	Mutant
Cys	0.41 <sup>(1)</sup> , N/A <sup>(2)</sup>	0.74 <sup>(1)</sup> , 0.99 <sup>(2)</sup>
$\gamma$ GCS	2.64 <sup>(1)</sup> , 2.88 <sup>(2)</sup>	12.95 <sup>(1)</sup> , 13.9 <sup>(2)</sup>
GSH	10.9 <sup>(1)</sup> , 14.5 <sup>(2)</sup>	38.7 <sup>(1)</sup> , 44.5 <sup>(2)</sup>
$\mu_{crit}$	0.18	0.13
$Q_{Gsh1}^{max}$	15 <sup>(1)</sup> , 16 <sup>(2)</sup>	8 <sup>(1)</sup> , 9 <sup>(2)</sup>

*GSH2* and *YAP1*, relative to those of the maintenance genes *ACT1* and *SHR3*, were several fold higher in the mutant (Fig. 3). No statistically significant differences between the initial expression levels were observed in the other genes studied (*SUL1*, *MET17*, *STR2*, *STR3*, *ECM38* and *MET4*) (data not shown). These results suggest that the higher GSH accumulation in the mutant might be caused by either i) higher cysteine accumulation and/or ii) higher Gsh1p and Gsh2p levels in the cells. Indeed, comparing the initial intracellular levels of thiols in the mutant and parent strains, we observed that, in addition to GSH, the concentration of intracellular cysteine as well as  $\gamma$ GCS were also remarkably higher in the mutant (Table 1). The  $K_m$  value for *S. cerevisiae* Gsh1p has not been quantified, however, for *E. coli* and mammalian cells it has been reported to be 0.09 and 0.15 mM, respectively (Murata, 1994; Griffith, 1999). Considering these  $K_m$  values and knowing that the intracellular water content is between 65-69% in yeast (Alcázar et al., 2000), the initial cysteine concentration in the wild-type strain was close to  $K_m$ . Thus, when the Gsh1p reaction rate is cysteine limited, a higher intracellular cysteine concentration may result in an increased flux and lead to a further increase in the GSH concentration, as was observed shortly after the switch of the feeding media in the experiments with the wild-type strain (Fig. 2).

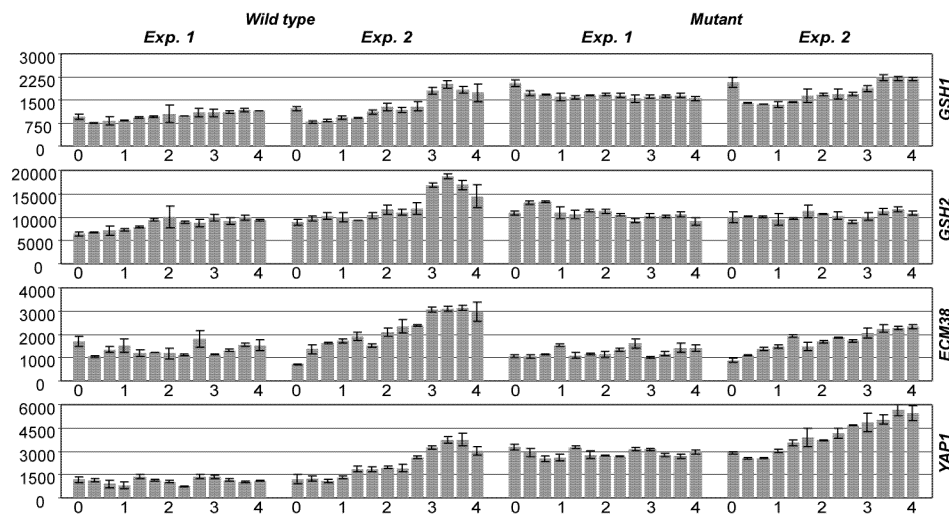


Figure 4 Expression of the target genes, relative to internal hybridization control and the expression of reference genes (*ACT1* and *SHR3*) during the continuous addition of cysteine:  $56 \mu\text{mol g}^{-1}$  sucrose in Exp. 1;  $112 \mu\text{mol g}^{-1}$  sucrose in Exp. 2. The means of duplicates are shown  $\pm$  average absolute deviation. The numbers under the columns represent the cultivation time (in h from the switch of the feeding media).

#### 4. Conclusion

GSH over-accumulation in the mutant used in the present study is caused by higher expression of *CYS3* and *CYS4* resulting in higher concentration of cysteine – the major limiting precursor for GSH synthesis, and by higher Gsh1p and Gsh2p levels in the cells. The molecular mechanisms (mutations) leading to the higher expression of *CYS3*, *CYS4*, *GSH1*, *GSH2* and *YAP1* in the mutant strain remain to be elucidated.

## Acknowledgements

The financial support for this research was provided by the Enterprise Estonia project EU22704, Estonian Ministry of Education and Research grant SF0140090s08 and by Estonian Science Foundation grant G7323. We would like to thank Mr David Schryer for critical revision of the manuscript.

## References

- Alcázar E.B., Rocha-Leão M.H.M. and Dweck J., 2000. Yeast Intracellular Water Determination by Thermogravimetry. *J Thermal Anal Cal* 59 (3), 643-648.
- Alfajara C., Miura K., Shimizu H., Shioya S. and Suga K., 1992. Cysteine addition strategy for maximum glutathione production in fed-batch culture of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 37, 141-146.
- Bachhawat A.K., Ganguli D., Kaur J., Kasturia N., Thakur A., Kaur H., Kumar A. and Yadav A., 2009. Glutathione Production in Yeast. *In: Yeast Biotechnology: Diversity and Applications*. Eds. Satyanarayana, T. and Kunze G. Springer, Dordrecht.
- Galant N.J., Wang H., Lee D.R., Mucsi Z., Setiadi D.H., Viskolcz B. and Csizmadia I.G., 2009. Thermodynamic role of glutathione oxidation by peroxide and peroxybicarbonate in the prevention of Alzheimer's disease and cancer. *J Phys Chem A* 113 (32), 9138-9149.
- Grant C.M., MacIver F.H. and Dawes I.W., 1997. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gamma-glutamylcysteine. *Mol Biol Cell* 8, 1699-1707.
- Griffith O.W., 1999. Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med* 27, 992-935.
- Li Y., Wei G. and Chen J., 2004. Glutathione: a review on biotechnological production. *Appl Microbiol Biotechnol* 66, 233-242.
- Martin H.L. and Teismann P., 2009. Glutathione – a review on its role and significance in Parkinson's disease. *FASEB J*. E-pub ahead of print.
- Murata K., 1994. Glutathione and Its Derivatives: Produced by Recombinant *Escherichia coli* and *Saccharomyces cerevisiae*. *Bioprocess Technol* 19, 159-83.
- Pócsi I., Prade R.A. and Penninckx M.J., 2004. Glutathione, altruistic metabolite in fungi. *Adv Microb Physiol* 49, 1-76.
- Soltaninassab S.R., Sekhar K.R., Meredith M.J. and Freeman M.L., 2000. Multi-faceted regulation of gamma-glutamylcysteine synthetase. *J Cell Physiol* 182, 163-170.
- van Hoek P., de Hulster E., van Dijken J.P. and Pronk J.T., 2000. Fermentative capacity in high-cell-density fed-batch cultures of baker's yeast. *Biotechnol Bioeng* 68, 517-523.
- Wheeler G.L., Trotter E.W., Dawes I.W. and Grant C.M., 2003. Coupling of the transcriptional regulation of glutathione biosynthesis to the availability of glutathione and methionine via the Met4 and Yap1 transcription factors. *J Biol Chem* 278, 49920-49928.