Expression studies of *GUP1* and *GUP2,* genes involved in glycerol active transport in *Saccharomyces cerevisiae,* using semi-quantitative RT-PCR

Rui Oliveira and Cândida Lucas

Centro de Biologia da Universidade do Minho (CB-UM) / Departamento de Biologia

Campus de Gualtar, 4710-057 Braga, Portugal

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Corresponding author.

Phone: +351-253 604313/10; **Fax**: +351-253678980; **E-Mail**: clucas@bio.uminho.pt

Abstract

Glycerol active uptake in *Saccharomyces cerevisiae*, characterised physiologically as a H^+-symport, was previously described as repressed by glucose, induced by growth on non-fermentable carbon sources and unresponsive to growth under salt stress. *GUP1* and *GUP2* were identified and characterised as genes involved in glycerol active uptake. Using SQ-RT-PCR, *GUP1* and *GUP2* transcription was measured. Unlike active transport activity determined previously, this was shown to be constitutive and not affected by either glucose repression or growth under salt stress. Furthermore, transcription of *GUP1* and *GUP2* was still not affected in the *gpd1gpd2* mutant strain grown under salt stress in the presence of small amounts of glycerol, in which case a very high Vmax of glycerol uptake has been reported. Intracellular compounds were determined. Glycerol, acetate and trehalose were found to be the major compounds accumulated. Surprisingly, *gpd1gpd2* mutant was found to produce significant amounts of glycerol. Yet, results do not evidence a correlation between the amount of each compound and glycerol transport activity in any of the strains.

Introduction

Glycerol active uptake systems have been described in many yeast species (Lages et al. 1999). From these, some were physiologically characterised in detail (Lucas et al. 1990; van Zyl et al. 1991, 1993; Lages and Lucas 1995, 1997; Silva-Graça et al. 2003). The predicted biological function of glycerol transport systems, can be at first hand, connected with glycerol utilisation as carbon and energy source. Yeasts presenting ~~such~~ active uptake grow well on glycerol-based media (Lages and Lucas 1999). On the other hand, glycerol is the main compatible solute yeasts accumulate under low a_w stress. The activity of glycerol active transport has been shown to be indispensable for salt-stress survival (Oliveira et al. 1996). Furthermore, it has been suggested that it could contribute to glycerol/osmolite intracellular retention and accumulation (van Aelst et al. 1991). Accordingly, the more salt stress-resistant yeasts have been shown to present constitutively expressed glycerol active transport (Lages et al. 1999). Yet, the main mechanism involved in salt stress response in *S. cerevisiae*, as in other yeasts, has been reported to be the increase in glycerol biosynthesis and consequent intracellular accumulation (Brown 1978), through the upregulation of *GPD1* (glycerol 3P dehydrogenase) (Albertyn et al. 1994; Ansell et al., 1997) and *GPP2* (glycerol 3P phosphatase) (Påhlman et al. 2001). Efficient glycerol retention has been shown to be due to the closing of the FPS1p channel (Tamás et al. 1999, 2000).

In *S. cerevisiae*, a glycerol active transport of the H^+/symport type has been physiologically characterised (Lages and Lucas 1997). It has been shown to be under glucose repression, induced by growth on non-fermentable carbon sources and unresponsive to growth under salt stress (Lages and Lucas 1997). It has been later associated with two genes: *GUP1* and *GUP2* (Holst et al. 2000).

GUP1 identification was done using two independent and parallel screenings. The first was based on searching for glycerol-based growth phenotype on a *gut*-diagnostic medium (Rønnow and Kielland-Brandt 1993), and the second, on searching for salt stress rescue by extracellular glycerol on a strain impaired in glycerol synthesis by deletion of both *GPD1* and its isogene *GPD2* (Holst et al. 2000). *GUP2* was found in databases, according to its high degree of similarity to *GUP1* (Holst et al. 2000).

Some modulation of transport activity was found in multiple mutant combinations of *GUP1*, *GUP2*, *GUT1*, *GPD1*, *GPD2* and other genes from glycerol metabolic pathway, suggesting subtle regulation mechanisms difficult to define (Holst et al. 2000). Transport was abolished in ethanol growing cells (derepression conditions for glycerol H^+/symport), by the combination of deletions of *GUP1* together with *GUT1* (glycerol kinase, first enzyme from glycerol consumption pathway) (Holst et al. 2000). Furthermore, transport activity regulation studies revealed that *gpd1gpd2* mutants presented an extremely high transport Vmax when cultivated on glucose (repression conditions for glycerol/H^+ symport), provided the medium was supplemented with NaCl and a small amount of external glycerol, the same conditions of one of the surveys leading to *GUP1* cloning above mentioned. Under these circumstances, transport activity was abolished by both *GUP1* and *GUP2* deletions (Holst et al. 2000).

The more striking differences in active transport Vmax came from comparing (1) W303-1A cells grown on either glucose or ethanol (Lages and Lucas 1997; Holst et al. 2000) and (2) *gpd1gpd2* cells grown on either glucose or glucose with salt and a small amount of glycerol (Holst et al. 2000). To assign Gup1p and Gup2p as glycerol symporter(s), these remarkable differences found in glycerol transport Vmax were expected to have some counterpart on gene expression regulation.

Thus considering, semi-quantitative reverse transcriptase-polymerase chain reaction (SQ-RT-PCR) was used to study *GUP1* and *GUP2* transcription in the mutant strains and growth conditions used previously (Holst et al. 2000). The reason underneath the choice of this methodology resides in the fact that it allows the study of very poorly expressed genes, which is the case of *GUP1* and *GUP2*, according to preliminary data. Results obtained suggest that the transcription of these genes is constitutive and not significantly affected by salt stress.

Furthermore, due to the complexity of the transport activity modulation found in the various mutant combinations involving genes from glycerol production and consumption pathways (Holst et al. 2000), it seems plausible that a fine-tuning of glycerol intracellular levels can eventually be achieved, not only by metabolism and channel-mediated retention, but presumably also by active transport. Intracellular concentrations of glycerol, as well as trehalose and acetate measured in this work do not

correlate, either negatively or positively, with transport activity determined previously under the same circumstances.

Materials and methods

Yeast strains and growth conditions

The following *S. cerevisiae* strains were used: W303-1A (Thomas and Rothstein 1989), YSH642 (kindly provided by S. Hohmann.) and BHY22 (Holst et al. 2000). YSH642 and BHY22 are both isogenic to W303-1A, but respectively *gpd1::TRP1gpd2::URA3* and MATα *gpd1::TRP1gpd2::URA3gup1::Tn-LEU2.* They will be referred throughout the text by their main mutation combinations. Maintenance was performed at 4°C in YNB w/o amino acids (Difco) supplemented with 2% (w/v) glucose and auxotrophic requirements, according to the strains demands, or YEPD at –70°C with glycerol 30% (w/v). Batch cultures were performed using complete medium (YEP) supplemented with 2% (w/v) glucose, ethanol or glycerol as carbon and energy sources. Incubation conditions were standardised at 30°C and 180 rpm orbital shaking, in 500 ml Erlenmeyer flasks containing 200 ml of growth medium. Growth was followed spectrophotometrically at A_{600nm}. Cells grown on YEPD were harvested at early- to mid-exponential growth phase (A_{600nm} 0.3-0.4), at the diauxic shift (A_{600nm} 1.2-1.4), which corresponds to the exhaustion of glucose in the medium and entering stationary phase (A_{600nm} 2.0) (Oliveira et al. 2003).

RNA preparation

RNA extraction was performed using the hot acidic phenol method and integrity and purity were determined with standard methodologies (Ausubel et al. 1996). Because small amounts of DNA can be co-purified with RNA and that could act as target in subsequent PCR reactions, a DNase I incubation step was included. Total RNA (1 µg) was incubated with RNase-free DNase I (3 U) in DNase buffer (20 mM tris·HCl, pH

8.3; 50 mM potassium chloride; 20 mM magnesium chloride), in a total volume of 10 µl. Incubation was performed at room temperature for 15 min and inactivation of DNase was done with 2.5 mM EDTA and incubation at 65ºC for 10 min.

Semi-quantitative reverse transcriptase - polymerase chain reaction (SQ-RT-PCR)

Reverse transcription conditions were followed strictly according to manufacturer's suggestion. First strand cDNA was synthesized using Thermoscript™ RT-PCR System (Life Technologies™), a modified Avian Reverse Transcriptase lacking RNase H activity and presenting high thermal stability. After confirmation of purity and integrity by visualisation after separation by electrophoresis under denaturing conditions, 100 ng of total RNA was denatured by incubation at 65ºC for 5 min with 50 ng random hexamers and kept on ice until use. The reaction mixture of 20 µl was transferred to cDNA synthesis buffer (50 mM tris acetate, pH 8.4; 75 mM potassium acetate; 8 mM magnesium acetate), 5 mM DTT, and 15 U of the reverse transcriptase Thermoscript RT. Reverse transcription reaction was done in a pre-heated thermal cycler ~~at 25ºC~~ programmed to 25ºC, 10 min for random hexamers annealing, 50ºC, 50 min for reverse transcription in order to avoid secondary structures of mRNAs, and 85ºC, 5 min for denaturation of Thermoscript RT. Complementary RNA was degraded by incubation of cDNA with 2U of RNase H, at 37ºC, 20 min, in order to improve sensitivity. cDNA samples were stored at -20ºC. Multiplex PCR reactions were performed with 2 µl of cDNA as template, with 50 mM $MgCl_2$, 10 mM dNTP mix, 10 µM of each primer, 4 µl 18S primer/Competimer™ mix (for *GUP1*: 2 µl of 5 µM 18S rRNA primer pair/8 µl Competimers™; for *GUP2*: 1 µl 5 µM 18S rRNA primer pair/9 µl Competimers™), 2 U Platinum *Taq* DNA polymerase (which enables a "hot-start" method to improve specificity of annealing), 20 mM tris-HCl (pH 8.4) and 50 mM KCl in a total volume of 50 µl. Sequences of primers used in this work are for RT*GUP1*f: CGTGGTTCCCTTGATGTTCT; for RT*GUP1*r: TATGGGGTGTGCAGCAGTTA; for RT*GUP2*f: TTCAAAAGGCGACAAAGGAT; for RT*GUP2*r; for 18S rRNAf: AGGAATTGACGGAAGGGCAC; and for 18S rRNAr: GCACATCTAAGGGCATCACA. Primers for internal standard 18S rRNA were obtained from commercial source (Quantu<u>mRNA</u>™ 18S Internal Standards, Ambion),

which included Competimers™ consisting in 3'-end-modified 18S rRNA primers that cannot be extended by DNA polymerase. The Competimers™ were used to modulate the amount of amplicon of the internal standard to obtain comparable signals between samples and internal standard. PCR program for *GUP1* was as follows: 94ºC, 30 sec; 55ºC, 30 sec; 72ºC, 1 min in a total of 29 cycles, being the first denaturation step of 2 min and the last extension step of 5 min. For *GUP2*, the program was similar except for the annealing temperature, which was 57ºC and the number of cycles which were 34.

Linear range of amplification was determined for each target (*GUP1* and *GUP2*) together with the internal standard 18S rRNA. Reverse transcription was made in a RNA sample from cells of strain W303-1A harvested at mid-exponential growth phase using ethanol as carbon and energy source, ensuring derepression of glycerol active uptake (Lages and Lucas 1997). *GUP1* target was co-amplified with a segment of 18S rRNA in a multiplex PCR, and 10 µl aliquots of reaction mixes were removed at different convenient cycle numbers and analysed. Plots of PCR product fluorescence *versus* cycle number revealed linearity of amplification up to cycle number 30 for both *GUP1* and 18S rRNA, and up to cycle number 36 for *GUP2* and 18S rRNA (not shown), with correlation values 0.990. These results allowed to select the cycle number at which reactions should stop for analysis of PCR products in order to obtain data proportional to the initial levels of target and internal standard, respectively, cycle 29 for *GUP1* mRNA and cycle 34 for *GUP2* mRNA.

To determine the limits of detection of variations of mRNA and the upper and lower amounts of total RNA input allowing quantification in RT-PCR experiments, known as dynamic range, different amounts of input total RNA were assayed. The same samples and reaction conditions used for linear range determinations were used for dynamic range studies. Linear correlation between input RNA (from 1µl to 4 µl, corresponding to 5 ng to 20 ng input RNA) and amplicons were found (not shown).

For each sample, the ratio between the fluorescence of the target gene and the internal standard was used to overcome variability between samples caused by RNA quality, RNA quantification errors, and random tube-to-tube variation in PCR and reverse transcription reactions. Results presented are mean values of, at least, three independent experiments.

PCR reactions were performed to detect genomic DNA contamination in all DNase-treated total RNA samples with RT*GUP1*f and RT*GUP1*r primers and conditions mentioned for detection of *GUP1* mRNA in RT-PCR reactions. In addition, detection of contaminating DNA in all samples was performed in RT-PCR reaction mixtures in which thermoscript RT has been omitted. The absence of amplification, under these conditions, meant absence of enough genomic DNA for successful amplification by PCR and subsequent detection with ethidium bromide staining.

Measurements of intracellular compounds

Identification and quantification of solutes were performed by chromatography (HPLC), using the same methodology applied before (Lages and Lucas 1995). Intracellular volume values, used to calculate intracellular compounds concentration, were determined previously (Lages et al. 1999; Silva-Graça et al. 2003).

Results

Results for GUP1 *and* GUP2 *transcription*

The glycerol active transport system, characterised physiologically in *S. cerevisiae* as a H^+-symport, has been shown to be repressed by glucose and induced by growth on non-fermentable carbon sources (Lages and Lucas 1997). *GUP1* and *GUP2* mRNA levels were measured in glucose growing cells collected in early- to mid-exponential growth phase, during diauxic shift and entering stationary phase (Table I). These did not seem to differ from each other, except, from the lower level of *GUP1* expression found during the diauxic shift in glucose-based medium. Furthermore, cells growing exponentially on glucose were compared with cells growing exponentially on ethanol or glycerol (Table I). No significant differences were observed in *GUP1* and *GUP2* transcripts according to carbon source.

The glycerol active transport system has been shown to be unresponsive to growth under salt stress in W303-1A cells (Lages and Lucas 1997). In the *gpd1gpd2* mutant,

this transport system was highly induced by growth under salt stress (Holst et al. 2000). For this reason, the influence of salt stress on *GUP1* and *GUP2* expression was investigated as well. Cells growing in media with glucose supplemented with 1 M NaCl alone or with further supplementation with 15 mM glycerol were collected in early- to mid-exponential growth and analysed for *GUP1* and *GUP2* mRNA's relative content (Table II). No significant differences in expression for both genes were observed. The same assays were repeated for *GUP1* and *GUP2* mRNAs using the *gpd1gpd2* strain and *GUP2* mRNA using the *gpd1gpd2gup1* strain (Table II). *GUP1* expression did not vary significantly in *gpd1gpd2* genetic background as compared with W303-1A. The only result found to be apparently different, was the higher *GUP2* expression observed in the *gpd1gpd2* strain in salt stress medium with 15 mM glycerol compared to W303-1A cells (Table II). Applying the ANOVA test to all these results (Tables I and II), it became clear that the small differences observed were not significant, being $p>0.05$ in all combinations.

Detection and quantification of intracellular compounds

The unexpected invariant and constitutive transcription of *GUP1* and *GUP2* suggested the possibility of a post-transcriptional regulation step of transport activity. Theoretically, the substrate can affect its own transport activity through a biochemical feed-back inhibition effect. *S. cerevisiae* cells growing on glucose are known to produce glycerol (Blomberg and Adler 1989), from which a considerable amount is excreted to the medium (Blomberg and Adler 1989; Oliveira et al., 2003). Under osmotic/salt stress glycerol production increases substantially (Blomberg and Adler 1989) and the Fps1p channel closes avoiding excretion to the medium (Tamás et al. 1999, 2000). A feed-back inhibition of glycerol on its own active transport system could explain results of Vmax obtained in uptake experiments reported by Holst and co-workers (2000). In particular, the observation of the high glycerol active transport Vmax detected in *gpd1gpd2* mutant cells supposed to be impaired in glycerol synthesis. The amounts of intracellular compounds were measured by HPLC in W303-1A and *gpd1gpd2* strains, cultivated in the same growth conditions and collected in the same growth phases as tested for RT-PCR and are presented in Table III.

Intracellular glycerol in the W303-1A strain under salt stress reached, as predicted, a very high level (± 400 mM). When cells were exposed to salt stress together with 15 mM glycerol, intracellular glycerol was about half that value. On the other hand, glucose growing cells in the absence of salt, presented, as expected, very low levels of intracellular glycerol (Table III).

In the *gpd1gpd2* strain, in spite of the deletions in the genes encoding the key enzymes responsible for glycerol production, glycerol could still be measured in glucose-grown cells being the amounts not significantly different from the ones in W303-1A cells (Table III). In opposition to the parental strain grown under salt stress, in this mutant, more intracellular glycerol was measured when the salt stress-medium was further supplemented with 15mM glycerol. No detectable growth could be obtained in YEPD plates supplemented with 1M NaCl (not shown). Nevertheless, growth was still possible in liquid YEPD with 1M NaCl, regardless of further glycerol supplementation. Specific growth rates were $0.112h^{-1}$ and $0.162h^{-1}$ respectively with and without glycerol supplementation. Lag phase lasted 3 and 6 days for the same growth conditions. The salt-sensitive phenotype detected on plate for the *gpd1gpd2* strain may derive from the extended lag phase duration.

Two other compounds were found to accumulate intracellularly, trehalose and acetic acid (Table III). Though the amount of intracellular trehalose was higher in cells cultivated under salt stress, it was not higher in *gpd1gpd2* mutants compared to wild type, as it would be expected if trehalose would replace glycerol as osmolite. On the other hand, intracellular concentration of acetic acid increased in cells cultivated under salt stress together with a small amount of glycerol.

Discussion

GUP1 *and* GUP*2 are transcribed constitutively*

Expression analysis by SQ-RT-PCR of *GUP1* and *GUP2* revealed constitutive and invariant expression of both genes in all conditions tested. These included exponential and post-diauxic phases of growth on glucose, exponential growth on ethanol, glycerol

and glucose under salt stress, in W303-1A and *gpd1gpd2* strains. Being so, the substantial differences in glycerol uptake Vmax previously reported between W303-1A and *gpd1gpd2* strains (Holst et al. 2000), as well as the fact of the glycerol active uptake be under glucose repression and induced by growth on non-fermentable carbon sources (Lages and Lucas 1997), do not have a correspondence on *GUP1* and *GUP2* transcription levels.

Genome-wide expression analyses by microarrays have been used to study expression reprogramming of yeast cells when facing an osmotic shock (Rep et al. 2000; Posas et al. 2000; Gasch et al. 2000; Causton et al. 2001; Yale and Bohnert, 2001).

Transcription induction of *GUP1* by osmotic shock of 1 M NaCl (Causton et al. 2001; Yale and Bohnert 2001) and absence of induction or repression by 0.4 to 0.8M NaCl, as well as for sorbitol shock (Rep et al. 2000; Posas et al. 2000) was reported. In this work we observed constant levels of *GUP1* mRNA in cells cultivated either in the presence or in the absence of salt. However, the experimental design for RT-PCR experiments was different since, instead of determining transcript levels in salt-shocked cells, steady-state transcript levels of adapted cells to salt stress were measured. This distinction of transcriptional activity of *GUP1* as function of osmotic shock or osmotic stress might constitute a major difference. In fact, dramatic changes in transcript profile follow a stress shock, but after adaptation of cells to the new environmental conditions imposed by stress during growth, gene expression can undergo different changes. The cell reaches a new survival steady-state which may be only slightly different from the initial non-stress conditions (Gasch et al. 2000). Hence, according to a model proposed by these authors, the induction of transcriptional activity of *GUP1* by salt-shock, which could provide a rapid and presumably small increase in Gup1p levels (Gasch et al. 2000), can be temporary, until the cell reaches a new equilibrium state under the osmotic stress constraint.

In what concerns *GUP2* expression, determined by microarrays, transcript levels upon salt shock have been, like in this work, reported to be approximately constant (Rep et al. 2000; Posas et al. 2000; Gasch et al. 2000; Causton et al. 2001; Yale and Bohnert 2001).

S. cerevisiae *cells accumulate glycerol, trehalose and acetate*

Transport regulation by direct binding of the substrate as a feed-back regulator is theoretically plausible, and has been proposed in yeasts, for uracil and inositol transport in *S. cerevisiae* (Robinson et al. 1996; Séron et al. 1999). Both substrates are found in the extracellular medium, suggesting a tight control of the correspondent intracellular levels, presumably through transport activity regulation (Séron et al. 1999). Similarly, in *S. cerevisiae*, glycerol is excreted (Oliveira et al. 2003) and intracellular levels are most probably also very tightly regulated (Blomberg, 2000). This regulation involves changes in transcription rates of several genes from the metabolic pathway as well as the activity of the Fps1p glycerol channel (Tamás et al. 1999, 2000). Different amounts of intracellular concentration of glycerol were detected in W303-1A cells grown on glucose alone or under salt stress with and without external supplementation of glycerol. Nevertheless, active uptake of glycerol was not detected in this strain growing exponentially under any of these conditions (Holst et al. 2000). Therefore, these differences in internal levels of glycerol are unlikely to be related to a feed-back regulation effect on uptake.

Significant amounts of glycerol were found inside *gpd1gpd2* mutant cells grown in the presence of 1M NaCl, either with or without glycerol supplementation. The origin of this intracellular glycerol is not clear. However, considering that the culture medium is devoid of this compound, one possibility could be biosynthesis through the dihydroxyacetone pathway, previously described in *Schizosaccharomyces pombe* (May and Sloan, 1981; May et al. 1982). Although this pathway has been reported to be present in *S. cerevisiae* (Norbeck and Blomberg 1997; Molin et al. 2003), further work is required to confirm its activity under the conditions tested in this work.

Trehalose, according to Mager and Varela (1993), might help the cell to survive stress though it does not substitute for glycerol as an osmolite. Consistently, the activity as osmolite seems to be unlikely, since intracellular levels are nearly constant regardless of glycerol content, as observed in both W303-1A and *gpd1gpd2* strains. Blomberg (2000) proposed that both glycerol and trehalose would undergo salt-stress stimulated turnover of ATP-consuming futile cycles to avoid substrate accelerated cell death through increased ATP net production. The higher trehalose content under salt stress observed in this work is apparently consistent with this hypothesis. The similar trehalose levels observed in W303-1A and *gpd1gpd2* suggest that this turnover is apparently not

activated by the impairment of the glycerol biosynthesis. Therefore, the lack of ATP consumption created by deficient glycerol turnover does not seem to be compensated by trehalose metabolism.

Intracellular acetic acid increased in cells growing under salt stress when a small amount of glycerol was present in the medium. According to the literature, expression of *ALD3*, encoding NAD^+-dependent acetaldehyde dehydrogenase, increases under salt stress (Blomberg and Adler 1989; Navarro-Aviño et al. 1999; Norbeck and Blomberg 2000). The increase in NADH needed for the reduction of dihydroxyacetone phosphate by Gpd1/2p towards glycerol production could be partially compensated through Ald3p function. Nevertheless, this is eventually not the case since the amounts of intracellular acetate found in wt and in *gpd1gpd2* mutant cells grown in the absence and in the presence of salt were not substantially different, unless excretion has been increased. This is in accordance with the absence of salt-sensitive phenotype of *ald2,3* mutants reported by Navarro-Aviño and co-workers (1999). Acetate concentrations increased considerably when cells were grown with small amounts of glycerol but *gpd1gpd2* mutant showed identical amounts of acetate as wt cells. This way, the metabolic adjustment underlying acetate increase for the time being remains undetermined.

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Table I

Expression of *GUP1* and *GUP2* genes in W303-1A cultivated in different media and collected during different growth phases (SQ-RT-PCR arbitrary units)

	YEPD			YEPE	YEPG
	Exponential growth (Fermentation)	**Diauxic shift**	**Pre-stationary phase (Respiration)**	**Exponential growth**	**Exponential growth**
GUP1	1.76 ± 0.25 (4)	0.43 ± 0.12 (3)	1.29 ± 0.43 (3)	1.16 ± 0.12 (4)	0.97 ± 0.26 (3)
GUP2	0.91 ± 0.12 (3)	0,69 ± 0,41 (3)	0.66 ± 0.21 (3)	1.20 ± 0.33 (3)	1.06 (1)

(n) number of independent assays
± Standard error

Table II

Expression of *GUP1* and *GUP2* genes in different strains cultivated in YEPD, under salt stress and supplemented or not with a small amount of glycerol (SQ-RT-PCR arbitrary units)

Media	YEPD	YEPD 1M NaCl	YEPD 1M NaCl 15mM Glycerol	YEPD	YEPD 1M NaCl	YEPD 1M NaCl 15mM Glycerol
Genes / Strains		*GUP1*			*GUP2*	
W303-1A	1.76 ± 0.25 (4)	1.27 ± 0.10 (4)	1.20 ± 0.26 (3)	0.91 ± 0.12 (3)	1.39 (1)	1.21 ± 0.14 (3)
gpd1gpd2	1.31 ± 0.13 (3)	1.38 ± 0.38 (3)	1.61 ± 0.15 (3)	1.57 ± 0.24 (3)	1.96 ± 0.40 (3)	2.04 ± 0.44 (3)
gpd1gpd2gup1	0 (1)	0 (1)	0 (1)	1.12 (1)	2.37 ± 0.33 (3)	1.60 ± 0.16 (3)

(n) number of independent assays
± Standard error

Table III Intracellular concentration of substrates (mM) measured in exponentially growing cells of W303-1A and the correspondent *gpd1gpd2* mutant in different growth media

Strain	W303-1A			*gpd1gpd2*		
Growth medium	YEPD	YEPD + 1M NaCl	YEPD + 1M NaCl +15mM Glycerol	YEPD	YEPD + 1M NaCl	YEPD + 1M NaCl +15mM Glycerol
Glycerol	0.75 ± 0.35 (4)	383.4 ± 140.0 (6)	238.9 ± 19.3 (3)	1.1 (2)	34.4 ± 5.8 (3)	138.4 ± 24.3 (3)
Trehalose	70.3 ± 18.7 (5)	108.7 ± 55.6 (6)	106.4 ± 14.1 (3)	28.7 ± 7.1 (3)	117.9 ± 84.9 (3)	114.0 ±36.3 (6)
Acetate	6.3 ± 3.3 (4)	5.4 ± 4.5 (5)	39.4 ± 8.1 (3)	2.3 ± 1.2 (3)	12.4 (2)	26.8 ± 12.1 (4)

(n) number of independent assays
± Standard error