New insights on glycerol transport in *Saccharomyces cerevisiae*

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Abstract Previous studies evidenced in *Saccharomyces cerevisiae* the activity of a H^+/glycerol symport, derepressed by growth on non-fermentable carbon sources, later associated with *GUP1* and *GUP2* genes. It was also demonstrated that only the combined deletion of *GUP1, GUP2* together with *GUT1* (glycerol kinase) abolished active transport in ethanol-induced cells. In this work, we show that a glycerol H^+/symport, with identical characteristics to the previously described, was found in *gup1gup2gut1* grown under salt-stress, particularly high in cells collected during diauxic-shift. These results suggest different roles for Gup1/2p than glycerol transport. The gene encoding for glycerol active uptake is thus yet unknown.
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1. Introduction

Glycerol, the main compatible solute used by yeasts under low a_w in high osmolarity environments [1], can permeate *Saccharomyces cerevisiae* plasma membrane through a constitutively expressed channel, Fps1p [2–4], and a H^+/symport, active in cells derepressed by growth on non-fermentable carbon sources [5]. This transport system was associated with the expression of the genes *GUP1* and *GUP2* [6]. *GUP1* was found in a two step screening: one corresponding to a glycerol-mediated salt phenotype recovery in a *gpd1gpd2* background, i.e., a strain impaired in glycerol synthesis, and another corresponding to a gut-diagnostic medium phenotype [6]. *GUP2* was obtained searching *S. cerevisiae* genome database, according to its high degree of similarity to *GUP1*. Even so, its disruption did not present similar phenotypes and did not complement *gup1* mutation [6]. Furthermore, mutants characterization as to glycerol active transport has shown that this could still be measured in cells deleted in both genes, provided they were cultivated under derepression conditions. This was attributed to transport measurements artefacts, created by glycerol kinase (Gut1p), the first enzyme from glycerol catabolism. Consistently, no glycerol active transport could be measured on a *gup1gup2gut1* mutant grown on YPE [6]. On the other hand, as expected [4,6], Fps1p-mediated glycerol entry by first order kinetics was always present.

Still a contradiction remained, the fact that *GUP1* allows the recovery of salt-stress phenotype in spite of cells being cultivated on glucose, which has been shown to repress glycerol active transport [6]. Further studies on *GUP1* and *GUP2* expression have shown these genes to be very low and constitutive, and no correlation was found between *GUP1* and *GUP2* expression, glycerol intracellular levels and glycerol active transport detection [7]. Moreover, the high similarity between *GUP1*-like sequences from different yeast species [8] is not consistent with the detection of glycerol active transport in the same species [9]. Also, and despite that these genes were attributed an exclusive family in the Transport Protein database (TC-DB2.A.50.1.1), they were preferentially included in a superfamily of membrane-bound *O*-acyl transferases (MBOAT/DltB/KOG3860) [10].

Considering that genes may be of completely different value for the cells under different physiological conditions, to complement previous studies and to try to clarify *GUP1* and *GUP2* roles in relation to glycerol active transport, this was studied in strains combining mutations on *GUP1*, *GUP2* and *GUT1* genes cultivated under salt stress.

2. Materials and methods

2.1. Yeast strains, media and growth conditions

Saccharomyces cerevisiae strains used in this work were W303-1A [11], CLy3, isogenic to W303-1A but *ygl084c::His5+ ypl189w::KanMX* and BHY67, isogenic to W303-1A but *gut1 ygl084c::His5+ ypl189w::KanMX* [6]. Cells were grown in rich media [YP: 1% (w/v) yeast extract; 2% (w/v) peptone] with 2% (w/v) glucose (YPD). Salt-stress media were based on YPD with 1 M NaCl, with or without 15 mM glycerol. Batch cultures were grown in Erlenmeyer flasks with air/culture proportion of 2/1, at 30 °C, 160 r.p.m. and growth was monitored spectrophotometrically, measuring $A_{640\,nm}$.

2.2. Quantification of extracellular solutes by high performance liquid chromatography

Carbon source consumption and metabolites present in the growth media were followed by high performance liquid chromatography according to Lages and Lucas [5].

2.3. Glycerol transport studies

Initial rates of glycerol uptake, external alkalinization upon glycerol addition, as well as in/out accumulation ratios, were determined as previously described [5,9,12,13]. The intracellular volume values used to determine intracellular glycerol molarity have been determined by Lages and Lucas [5].

3. Results

W303-1A, *gup1gup2* and *gup1gup2gut1* were grown on YPD and YPD supplemented with 1 M NaCl, with or without 15 mM glycerol. Specific growth rates during exponential growth phase, as well as the pattern of compound variation in the

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growth medium, were identical in the three strains for each medium (example in Fig. 1). Lag phase duration was slightly shortened by the addition of the mentioned small amount of glycerol to the growth media.

Glycerol uptake and accumulation experiments were performed in cells collected in: (1) mid-exponential growth phase, while fermenting glucose; (2) during diauxic shift, when glucose was exhausted from the media and cells were using ethanol as carbon source; and (3) closer to the stationary phase (arrows in Fig. 1).

Either strain cells, cultivated on YPD and collected during exponential growth phase, presented only a first order kinetics, indicative of channel-mediated entry [4,6]. This is in accordance with previously published results that show the active transport system to be under glucose repression [5,6]. When cells were collected during diauxic shift, W303-1A and *gup1gup2* strains presented accumulation of radiolabelled glycerol against gradient, not measurable in the *gup1gup2gut1* strain (Fig. 2). These results are also consistent with published ones, from ethanol-grown cells, which showed that transport was only missing when *GUT1* gene, besides *GUP1* and *GUP2*, was deleted [6].

In cells grown in YPD with 1 M NaCl, with or without 15 mM glycerol, and collected in any growth phase, all the three strains presented glycerol accumulation against gradient. This is shown in Fig. 3 for cells grown in the presence of salt and glycerol. In cells collected in mid-exponential growth phase, according to published results, the uptake V_{max} values were
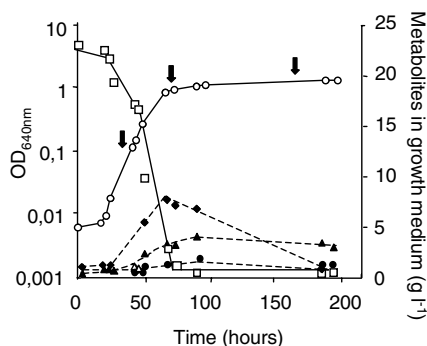

Fig. 1. *gup1gup2gut1* growth curve in YPD + 1 M NaCl + 15 mM glycerol at 30 °C (○) and variation of metabolites in growth media (glucose (□); ethanol (◆); glycerol (▲); acetate (●)). Arrows show sampling O.D. chosen for transport assays.
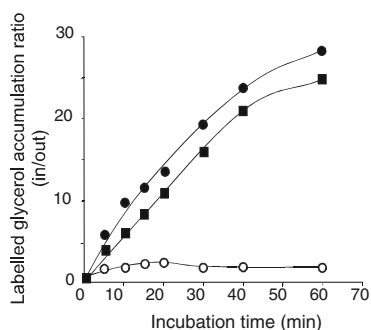

Fig. 2. Accumulation ratios of [^{14}C]glycerol at pH 5 and 30 °C, of cells cultivated in YPD and harvested during diauxic shift. W303-1A (●); *gup1gup2* (■); *gup1gup2gut1* (○).
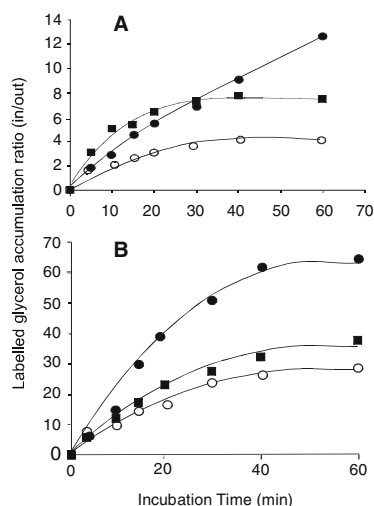

Fig. 3. Accumulation ratios of [^{14}C]glycerol at 30 °C of cells cultivated in YPD + 1 M NaCl + 15 mM glycerol and harvested during mid-exponential growth phase (A) and diauxic shift (B). W303-1A (●); *gup1gup2* (■); *gup1gup2gut1* (○).

lower than 50 μmol h^{-1} g^{-1} dry weight and K_m, consequently, could not be determined [6]. This was the *criterium* used in previously published work to consider as absence of transport [6]. During diauxic shift instead, as exemplified in Fig. 4, transport presented much higher V_{max} and for this reason K_m could be determined. The values calculated are presented in Table 1. At first hand, they appear to be identical to the ones previously determined in W303-1A for ethanol-grown cells [5,6]. To sustain this statement, ANOVA test was applied. All the K_m values available from strains with W303-1A background from previous works [6,14] were used together with the ones here obtained. Values were not significantly different: $p > 0.05$ in all cases. The presence of 15 mM glycerol in media did not change uptake parameters significantly.

Glycerol uptake in *gup1gup2gut1* proved to be sensitive to the action of the ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), V_{max} being decreased in about 80%. Incubation in the presence of CCCP prevented glycerol accumulation against gradient, and radiolabelled glycerol efflux was obtained by the addition of either cold glycerol or CCCP, consistently with the fact that this strain cannot metabolize glycerol (Fig. 5). Maximum accumulation ratios did
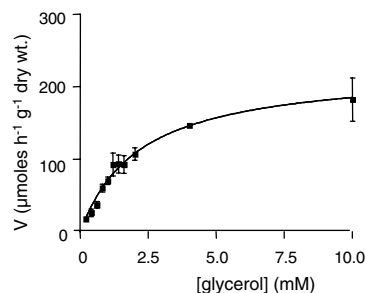

Fig. 4. *gup1gup2gut1* [^{14}C]glycerol initial uptake rates at pH 5 and 30 °C, in the presence of 1 M NaCl. Cells were harvested during diauxic shift on YEPD + 1 M NaCl + 15 mM glycerol. Kinetic parameters were determined and evaluated by computer regression analysis (GraphPad PrismR, 1994–97 Copyright GraphPad Software, Inc.).

Table 1
Kinetic parameters of glycerol uptake and accumulation of glycerol against gradient in cells grown on glucose in the presence of salt and 15 mM glycerol and collected during diauxic shift

Strains	[^{14}C]Glycerol uptake		[^{14}C]Glycerol accumulation	External alkalinization upon glycerol addition
	K_m (mM)	V_{max} (µmol h^{-1} g^1 dw)		
W303-1A	4.1 ± 1.17 (3)	494 ± 91.5 (3)	+	+
gup1gup2	3.9 ± 2.15 (3)	309 ± 118 (3)	+	+
gup1gup2gut1	2.2 ± 0.31 (3)	226 ± 15.7 (3)	+	+

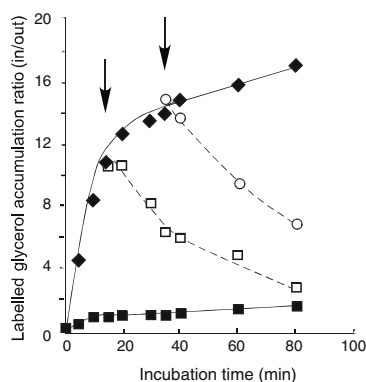*n*, Number of independent experiments given in parentheses.
± S.E.

Fig. 5. *gup1gup2gut1* [^{14}C]glycerol accumulation ratios at pH 5 and 30 °C, in the presence of 1 M NaCl (◆). Cells were harvested during diauxic shift on YEPD + 1 M NaCl + 15 mM glycerol. Efflux of radioactivity observed after the addition (arrow) of 85 mM "cold" glycerol (○); efflux of radioactivity observed after the addition (arrow) of 50 µM CCCP (□); accumulation prevented by the addition of 50 mM CCCP (■).

not vary with extracellular pH. This is considered as an indirect consequence of the active transport responsible for accumulation against chemical gradient being dependent on proton-motive force [5,9,13]. Also, variation of NaCl concentration in assay buffer did not affect accumulation rates significantly. Furthermore, external alkalinization upon addition of glycerol was observed. Though proton/glycerol stoichiometry was not determined, these results indicate the presence of a transport system similar to the one previously described in *S. cerevisiae*, a H$^+$/glycerol symport [5].

Regardless to growth media, cells collected in pre-stationary phase presented the same profile of glycerol uptake and accumulation against gradient as found during diauxic shift, although values obtained were more erratic.

4. Discussion

Glycerol transport in W303-1A cells, derepressed by growth on non-fermentable carbon sources [5], has been previously associated with *GUP1* gene [6]. On the other hand, transport in cells grown on glucose with salt-stress was measured, but exclusively in a *gpd1gpd2* background. This has been associated with *GUP2* gene [6]. Previously, Luyten and collaborators [3] had shown that glycerol could be taken up by *S. cerevisiae* cells in a salt-stress based medium. They also showed that *fps1* mutant strain, which took glycerol very poorly in YPD, increased substantially in glycerol uptake once cultivated under stress. Therefore, glycerol active transport, although not detected

before, was beforehand expected to be present in salt-stress-grown cells [3] in spite of being under glucose repression [5].

Results hereby presented show that, regardless of the deletions in *GUP1*, *GUP2* and *GUT1* genes, which abolished active transport in ethanol growing cells [6], cells cultured under salt-stress and collected during diauxic shift, i.e., under derepression conditions, presented glycerol active uptake. Thus considering, neither of these three genes, though associated with glycerol transport [6], cannot, in themselves, be considered as structural gene(s) for the symporter activity described here. This could correspond to a different system from the one previously described 2functioning in different physiological conditions. Yet, the transport characteristics now observed are identical to the ones published before [5,6], from which we stress the same K_m value. This points to the possibility of Gup1p and Gup2p not being glycerol carriers, but, instead, regulatory proteins, since they do interfere with transport activity [6]. This hypothesis is consistent with the *GUP1* and *GUP2* low and constitutive expression [7] and with the observation that the presence of *GUP1* homologues in several yeasts [8] does not correlate to the detection of H$^+$/glycerol symport activity [8,9]. We thus suggest for Gup1p and Gup2p roles other than glycerol active transport, being the gene encoding for glycerol active transport yet unknown.

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