A Member of the Sugar Transporter Family, Stl1p Is the Glycerol/H⁺ Symporter in *Saccharomyces cerevisiae*

Célia Ferreira,^{*,†} Frank van Voorst,^{*} António Martins,[†] Luisa Neves,[†]
Rui Oliveira,[†] Morten C. Kielland-Brandt,^{*} Cândida Lucas,[†] and Anders Brandt^{*}

^{*}Carlsberg Laboratory, DK-2500 Copenhagen Valby, Denmark; and [†]Centro de Biologia (CB-UM)/Departamento de Biologia da Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Submitted October 8, 2004; Revised January 13, 2005; Accepted January 21, 2005
Monitoring Editor: Benjamin Glick

Glycerol and other polyols are used as osmoprotectants by many organisms. Several yeasts and other fungi can take up glycerol by proton symport. To identify genes involved in active glycerol uptake in *Saccharomyces cerevisiae* we screened a deletion mutant collection comprising 321 genes encoding proteins with 6 or more predicted transmembrane domains for impaired growth on glycerol medium. Deletion of *STL1*, which encodes a member of the sugar transporter family, eliminates active glycerol transport. Stl1p is present in the plasma membrane in *S. cerevisiae* during conditions where glycerol symport is functional. Both the Stl1 protein and the active glycerol transport are subject to glucose-induced inactivation, following identical patterns. Furthermore, the Stl1 protein and the glycerol symporter activity are strongly but transiently induced when cells are subjected to osmotic shock. *STL1* was heterologously expressed in *Schizosaccharomyces pombe*, a yeast that does not contain its own active glycerol transport system. In *S. pombe*, *STL1* conferred the ability to take up glycerol against a concentration gradient in a proton motive force-dependent manner. We conclude that the glycerol proton symporter in *S. cerevisiae* is encoded by *STL1*.

INTRODUCTION

Glycerol, a C₃ polyalcohol, is an important intermediate in energy metabolism in both prokaryotes and eukaryotes. It has long been used for therapeutic and industrial processes. Aspects of glycerol metabolism are also important in biotechnology, e.g., for bio-alcohol production yields or wine smoothness. Essential roles of glycerol in basic biochemical aspects have been extensively studied in several yeasts and fungi. These include biosynthesis of glycerophospholipid and triacylglycerol from glycerol 3-phosphate and dihydroxyacetone phosphate (Kohlwein *et al.*, 1996; Müllner and Daum, 2004), redox balance (Ansell *et al.*, 1997; Valadi *et al.*, 2004), osmoadaptation (reviewed by Hohmann, 2002), oxidative stress protection (Påhlman *et al.*, 2001), and response to heat shock (Siderius *et al.*, 2000). Responses to elevated temperatures and high osmolarity involve several signaling pathways including the protein kinase C pathway and the HOG pathway, which regulates intracellular levels of glycerol (Hohmann, 2002; Wojda *et al.*, 2003).

In cells ranging from mammals (Lang *et al.*, 1998) to archaea (Kempf and Bremer, 1998), osmolytes play an important role in the response to osmotic stress caused by low water availability in environments as diverse as poorly irrigated soils or high-sugar musts. In eukaryotic microorganisms like algae or yeasts, polyols, primarily glycerol, act as osmolytes (reviewed by Brown, 1977 and Wang *et al.*, 2001). Their production, consumption and retention are consequently tightly

regulated and dynamic processes (reviewed by Hohmann, 2002). *Magnaporthe grisea* (rice blast) a phytopathogenic fungus with a strong impact on world economy, accumulates glycerol, which allows the penetration of the appressorium into the plant host cell (Thines *et al.*, 2000). Glycerol has also been shown to accumulate in the human pathogenic yeast *Candida albicans* in response to several types of osmotic stress in a HOG pathway-dependent manner (San José *et al.*, 1996). This pathway, besides controlling osmotic sensitivity in *C. albicans* as in other yeasts, also plays a role in dimorphic differentiation and virulence (Alonso-Monge *et al.*, 1999). Moreover, Ochiai *et al.* (2002) showed that glycerol accumulates in *C. albicans* as a consequence of treatment with azole fungicides, suggesting a role for stress-signaling pathways in the control of hypha formation. In human physiology, plasma glycerol levels are correlated with obesity (reviewed by Large *et al.*, 2004). Glycerol has also been found to stimulate glucose-responsive neurons (Yang *et al.*, 1999). Furthermore, fasting glycerolemia is a predictor of impaired glucose tolerance in certain families (Gaudet *et al.*, 2000; see also review by Brisson *et al.*, 2001).

Glycerol can permeate the cell membrane of many organisms by specific members of the aquaporin family, the aquaglyceroporin channel proteins (Engel and Stahlberg, 2002). In the yeast *Saccharomyces cerevisiae*, the gene *FPS1* encodes a channel-type protein belonging to the major intrinsic protein (MIP) family (Luyten *et al.*, 1995). Fps1p allows regulated glycerol release under osmotic down-shift (Tamás *et al.*, 1999, 2000). Furthermore, the channel has been shown to close upon osmotic up-shock, thus contributing to fast glycerol retention (Tamás *et al.*, 1999). Fps1p has recently been shown to mediate the first-order kinetics of glycerol uptake (Oliveira *et al.*, 2003), formerly considered a passive diffusion process (Lages *et al.*, 1999).

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-10-0884>) on February 9, 2005.

Address correspondence to: Cândida Lucas (clucas@bio.uminho.pt).

Table 1. *S. cerevisiae* strains used in the present work

Strain	Genotype	Reference
W303-1A	<i>MATa leu2-3 leu2-112 ura3-1 trp1-1 his3-11 his3-15 ade2-1 can1-100</i>	Thomas and Rothstein (1989)
YSH481	Isogenic to W303-1A but <i>gpd1::LEU2</i>	Hohmann, S.
YSH642	Isogenic to W303-1A but <i>gpd1::TRP1 gpd2::URA3</i>	Hohmann, S.
RH266-1D	<i>MATa end3 ura3 his4 leu2 bar1-1</i>	Raths <i>et al.</i> (1993)
BHY52	Isogenic to W303-1A but <i>fps1::HIS3</i>	Holst <i>et al.</i> (2000)
BHY63	Isogenic to W303-1A but <i>fps1::HIS3 gup1::his+ gup2::KanMX</i>	Holst, B. (unpublished)
CLY1	Isogenic to W303-1A but <i>gut1</i>	Holst <i>et al.</i> (2000)
FVVY21	Isogenic to W303-1A but <i>gpd1::TRP1 gpd2::URA3 stl1::KanMX</i>	This study
FVVY22	Isogenic to W303-1A but <i>fps1::HIS3 stl1::KanMX</i>	This study
FVVY23	Isogenic to W303-1A but <i>gut1 stl1::KanMX</i>	This study
FVVY24	Isogenic to W303-1A but <i>stl1::KanMX</i>	This study
FVVY28	Isogenic to W303-1A but <i>STL1-ZZ KanMX</i>	This study
FVVY38	Isogenic to YSH481 but <i>STL1-ZZ KanMX</i>	This study
FVVY39	Isogenic to YSH642 but <i>STL1-ZZ KanMX</i>	This study
FVVY40	Isogenic to RH266-1D but <i>STL1-ZZ KanMX</i>	This study

In addition, an active glycerol transport system of the proton symport type is present in *S. cerevisiae* when grown on nonfermentable carbon sources like glycerol or ethanol (Lages and Lucas, 1997). This transport system operates independently of Fps1p-mediated diffusion (Sutherland *et al.*, 1997).

GUP1 and its homologue *GUP2*, encoding proteins with multiple membrane spans, have been suggested to be involved in active glycerol transport in *S. cerevisiae* (Holst *et al.*, 2000). *GUP1* is essential for efficient growth on glycerol as sole carbon and energy source. *GUP1* was also found to be important for glycerol-dependent survival upon salt stress in a mutant strain defective in glycerol synthesis. The strain was deleted for both isogenes of glycerol 3P-dehydrogenase, *GPD1* and *GPD2*, and was therefore unable to produce sufficient amounts of glycerol to counteract osmotic stress (Albertyn *et al.*, 1994; Ansell *et al.*, 1997; Oliveira and Lucas, 2004). Such mutants can be rescued by inclusion of small amounts of glycerol in the medium. Additional deletion of *GUP1* makes these strains unable to grow on high-osmotic media with glycerol, suggesting a role of *GUP1* in glycerol uptake (Holst *et al.*, 2000). *GUP2* is a paralogue of *GUP1*. Its disruption did not yield similar phenotypes (Holst *et al.*, 2000). The deletion of *GUP1* yielded a decrease in glycerol transport $V_{\max}$. Additional deletion of *GUP2* further decreased the $V_{\max}$ but was not enough to abolish transport. A remaining saturable component of glycerol uptake kinetics in these strains was attributed to an artifact created by the glycerol kinase (Gut1p), the first enzyme of glycerol catabolism, because the triple mutant *gup1 gup2 gut1* did not show active uptake of glycerol when grown in ethanol-based medium (Holst *et al.*, 2000). However, it was later found that this mutant has the ability to take up glycerol actively during the diauxic shift if grown on glucose-based media containing high amounts of salt (Neves *et al.*, 2004a). Therefore, the roles of *GUP1* and *GUP2* in active glycerol uptake remain unclear. In addition, both *GUP1* and *GUP2* are expressed constitutively, an expression pattern that is distinct from glycerol transport activity (Oliveira and Lucas, 2004). Furthermore, Gup1p and Gup2p are, by sequence homology, members of a superfamily of membrane-bound O-acyl transferases (Hofmann, 2000; Neves *et al.*, 2004b). *gup1* deletion mutants exhibit a number of additional phenotypes apparently unrelated to glycerol uptake, like defects in bipolar bud site selection (Ni and Snyder, 2001), defects in sorting of carboxypeptidase Y, proteinase A, and alkaline

phosphatase (Bonangelino *et al.*, 2002) and changes in telomere length (Askree *et al.*, 2004). Taking these different data into account, it is unlikely that *GUP1* and *GUP2* encode glycerol transporters, although they are involved in glycerol uptake under some circumstances. We presently have no explanation for this involvement.

A deeper understanding of the ways glycerol is produced, transported, and retained in model organisms like yeasts will contribute to an improved understanding of glycerol-related aspects ranging from human health to biotechnology. With the view that the active glycerol transporter in *S. cerevisiae* still remained to be identified, we designed a screen to find it in the EUROSCARF yeast deletion collection. We identified a mutant lacking a membrane protein in the sugar permease family (Nelissen *et al.*, 1997) that was unable to transport glycerol. The present work presents consistent evidence that the gene *STL1*, sugar transporter like (Zhao *et al.*, 1994), encodes the *S. cerevisiae* glycerol/H⁺ symporter.

MATERIALS AND METHODS

Strains

S. cerevisiae strains used in this study comprise two different genetic backgrounds: BY4742 (Brachmann *et al.*, 1998) and W303-1A (Thomas and Rothstein, 1989). The collection of single deletion mutants in BY4742 was obtained from EUROSCARF. The strains with W303-1A genetic background are listed in Table 1. All *stl1* deletion mutants were made by transformation with a PCR-generated fragment containing the *KanMX* cassette (Wach *et al.*, 1994) and a 200-base pair flanking region amplified from the corresponding BY4742 mutant obtained from EUROSCARF. Deletions were verified by PCR. The construct used to complement the *stl1* mutation was made by amplification of the gene and flanking regions from genomic DNA using primers 5' CCG GCT CGA GTT GCA GAT TAC GAA AGA ATC 3' and 5' GGC CTC TAG AGA ATA TCA YGG CAA GAC CGC 3'. The resulting PCR product was cloned between the *XhoI* and *XbaI* of pRS316. The construct was verified by DNA sequencing. *Schizosaccharomyces pombe* EG573 (*mat1-M mat2/3-del::leu- ura4-D18 ade6*) was used for heterologous expression of *STL1*. *Escherichia coli* strains XL1-Blue and DH5 α were used for plasmid selection and propagation.

Yeast Media and Growth Conditions

Batch cultures of yeast were grown aerobically in complex medium (YP: 1% [wt/vol] yeast extract; 2% [wt/vol] peptone) supplemented with 2% (wt/vol) glucose (YPD), ethanol (YPE), or glycerol (YPG) as carbon and energy sources. Incubation was performed at 30°C, 180 rpm, orbital shaking with air/liquid ratio 2.5/1. The inability to utilize glycerol as a carbon and energy source was recognized on glycerol-based defined media supplemented with 0.05% peptone (Rønnow and Kielland-Brandt, 1993). Synthetic complete (SC) and SC drop-out media were made according to Burke and collaborators (2000). Drop tests were performed from cellular suspensions containing $\sim 1 \times 10^6$ cells/ml.

Tenfold serial dilutions were made, and 5 μ l of each suspension was applied. *S. pombe* EG573 was cultivated on SC with 2% (wt/vol) glucose in identical physiological conditions as *S. cerevisiae*.

Transformation and DNA Manipulation

S. cerevisiae cells were transformed with the lithium acetate method (Ausubel *et al.*, 1996). Transformants were selected on selective synthetic medium (SC without uracil) or YPD containing 300 μ g/ml G418. *E. coli* strains were transformed with a CaCl_2 /heat shock-based protocol (Ausubel *et al.*, 1996). Transformants were selected on LB agar supplemented with 100 μ g/ml ampicillin. *S. pombe* EG573 was transformed using the method of Bähler *et al.* (1998). Transformants were selected on SC without uracil, supplemented with 2% (wt/vol) agar and 15 μ M thiamine.

DNA isolation and manipulation was performed by standard procedures (Sambrook *et al.*, 1989). DNA fragments obtained by PCR were purified using the QIAquick PCR product Purification Kit (Qiagen, Hilden, Germany). The concentration and purity of DNA was checked spectrophotometrically (Sambrook *et al.*, 1989).

Glycerol Transport Studies

The methods of Lages and Lucas (1995, 1997) and Lages *et al.* (1999) were used to measure initial rates of radiolabeled glycerol uptake and the ratios of intracellular-to-extracellular glycerol concentrations (in/out accumulation ratios). To measure initial uptake rates of [^{14}C]glycerol (Amersham, Freiburg, Germany), cells were washed twice and resuspended in ice-cold water to a final concentration of ~ 30 mg/ml (dry weight). The yeast suspension (10 μ l) was mixed with 100 mM Tris/citrate buffer, pH 5.0 (10 μ l), in a conical centrifuge tube and incubated for 2 min at 30°C in a water bath. The reaction was started by addition of 5 μ l of a 20 mM aqueous solution of [^{14}C]glycerol with a specific activity of 2000 dpm/nmol, corresponding to a final concentration of 4 mM. Uptake at this concentration is close to V_{max} (Lages and Lucas, 1997; Sutherland *et al.*, 1997). After 10 s, the reaction was stopped by dilution with 5 ml of ice-cold water. Cells were immediately collected on Whatman GF/C filters (Whatman, Maidstone, England), washed twice with 5 ml water, and immersed in vials containing 5 ml of scintillation fluid (Optiphase Hisafe2, Wallac Manufactured Products, Turku, Finland). The radio-labeled glycerol taken up by cells was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter (Canberra Packard International, Zurich, Switzerland). The assays were made in triplicate and referenced to a blank made by inverting the sequence of addition of glycerol and water.

To determine the transport-driven ratios of [^{14}C]glycerol inside versus outside the cells (in/out accumulation ratios), the cell suspension (80 μ l) and 110 μ l 100 mM Tris/citrate (pH 5.0) were transferred to conical glass centrifuge tubes and incubated at 30°C with magnetic stirring. After 2 min of incubation the experiment was started by addition of 10 μ l (200 mM) of [^{14}C]glycerol (specific activity 300 dpm/nmol). At appropriate time intervals, 10- μ l aliquots were taken and filtered through Whatman GF/C filters. The filters were then washed twice with 5 ml ice-cold water and transferred to vials containing 5 ml scintillation fluid.

The protonophores/ionophores carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) were used to prevent accumulation of glycerol through glycerol/proton symport activity, as well as to induce efflux of intracellular radiolabeled glycerol, in assay concentration of 50 μ M. The *S. pombe* and *S. cerevisiae* intracellular volumes used to calculate the intracellular glycerol concentrations were determined previously (Lages *et al.*, 1999).

Subcellular Localization Using Green Fluorescent Protein as Reporter

For expression of the *STL1*-GFP fusion protein we made use of pUG35 (U. Güldener and J. Hegemann, unpublished results), a centromere-based vector containing the *MET25* promoter, followed by a polylinker and a sequence encoding yeast-enhanced green fluorescent protein (GFP). *STL1* was amplified by PCR using primers 5' GGC CTC TAG AAT GAA GGA TTT AAA ATT ATC G 3' and 5' GGC CGA ATT CAC CCT CAA AAT TTG CTT TAT C 3'. The resulting PCR product was cloned between the *Xba*I and *Eco*RI sites of pUG35, in frame with GFP, yielding pUG35-*STL1*. The construct was verified by DNA sequencing. Strains were transformed with pUG35-*STL1*. Cells were grown overnight in SC medium without uracil, at 30°C, with glycerol as carbon source. Cells were then diluted in the same medium to an $\text{OD}_{600} \approx 0.25$ and allowed to reach $\text{OD}_{600} = 0.6$. Subsequently the cells were shifted to medium without uracil and methionine to induce the *MET25* promoter. After 1–2 h of induction, cells were visualized by fluorescence microscopy. The remaining culture was then shifted to medium containing 2% glucose and methionine to switch off the promoter. Cells were visualized after 1 h of growth in glucose. A Leica TCS SP2 confocal scanning microscope (Heidelberg, Germany) was used to obtain the images. GFP was excited at 488 nm and emitted light between 500 and 570 nm.

Western Blotting

A PCR-generated fragment encoding a duplicated IgG binding domain (Z) of protein A, and containing a *KanMX* cassette, was amplified from plasmid pFZ

according to Whyte and Munro (2001) using primers 5' CAA ACA TCA AAA ATG AAG ATA CAG TGA ACG ATA AAG CAA ATT TTG AGG GTG GAG CAG GGG CGG GTG 3' and 5' AAT GCT TTC TTA AGT AAA TTA CAA AAT ATG ATT TGT GAG TTG TGT GTG AAG GTC GAC GGT ATC GAT AAG 3'. This was used to transform yeast to construct the epitope-tagged strains. Correct integration was verified by PCR. For Western blots, cells (0.2 mg dry weight) were collected by centrifugation, and protein extracts were prepared according to Burke *et al.* (2000), separated by SDS-PAGE (10%), and transferred to nitrocellulose filters (Invitrogen, Carlsbad, CA) according to Kyhse-Andersen (1994). The filters were incubated with a commercial mixture of horseradish peroxidase and antiperoxidase rabbit antibody (PAP, cat. no. Z0113, Dako-Cytomation A/S, Copenhagen, Denmark), and reacting polypeptides were visualized using ECL Plus Western Blotting Detection system (Amersham Biosciences) and a Storm 860 Scanner (Molecular Dynamics, Sunnyvale, CA). GFP-containing proteins were detected using rabbit antibodies raised against rx-YFP (Østergaard *et al.*, 2001) by Pineda Antikörper (Berlin, Germany; a kind gift of Drs. Jakob Winther and Henrik Østergaard).

Expression of *STL1* in *S. pombe*

A chromosomal copy of *STL1* was obtained by PCR using the primers 5'GGC CTC GAG ATG AAG GAT TTA AAA TTA TCG 3' and 5'GGC CCC GGG TCA ACC CTC AAA ATT TGC 3', including restriction sequences. It was inserted into *E. coli*/*S. pombe* shuttle multicopy expression vectors pREP82X and pREP4X between the *Xho*I and *Xma*I sites. These plasmids contain a thiamine-repressible promoter, *mtt1*, and *ura4*⁺, and Amp^r as selective markers. They differ in promoter strength, weak and strong, respectively, because of increased expression-modulating mutations (Maundrell, 1993). The integrity of the constructs was verified by sequencing. *S. pombe* EG573 was transformed with pREP4X-*STL1* or with pREP82X-*STL1*. Transformants were selected and cultivated in liquid SC medium without uracil and supplemented with 2 nM thiamine for the lower expression plasmid pREP82X and 15 μ M thiamine for the higher promoter strength plasmid pREP4X. Cells were harvested by centrifugation at $\text{OD}_{600} = 1.2$ (exponential growth phase), resuspended in the same medium without added thiamine (Sieiro *et al.*, 2003), and grown for 6 h for cultures from medium with 2 nM thiamine or for 30 h for cultures from medium with 15 μ M thiamine. Cells were recollected by centrifugation and resuspended in water to a cell density of 30 mg/ml (dry weight) and kept on ice before glycerol transport assays.

RESULTS

Identification of Mutants Impaired in Glycerol Utilization

We began our screen with the assumptions that a mutant lacking the glycerol transporter would grow less efficiently on glycerol than the parent and that the transporter would be a multispansing membrane protein. On the basis of these assumptions, we screened a subset of the EUROSCARF single-deletion mutant collection, comprising 321 open reading frames with six or more predicted membrane spans, for glycerol utilization. Ten deletion mutants with a growth defect on a defined medium containing glycerol as the sole carbon source were found. Five did not grow (*PER1*, *PCP1*, *DRS2*, *PMT5*, and *RHK1*), whereas the remaining five grew poorly (*PMR1*, *GEF1*, *ERG4*, *GUP1*, and *STL1*). The strains deleted for *PER1* (protein processing in the ER), *PCP1* (processing of cytochrome c peroxidase), *PMR1* ($\text{Ca}^{2+}/\text{Mn}^{2+}$ P-type ATPase, Golgi), and *GEF1* (chloride channel localized to Golgi involved in intracellular iron metabolism) are listed at the SGD database (<http://www.yeastgenome.org/>) as having growth defects on respiratory substrates. Deletions of *DRS2* (integral membrane Ca^{2+} -ATPase) and *PMT5* (protein O-mannosyl transferase of the ER) are listed as having a growth defect on lactate as a carbon source. Thus deletion of six of the genes identified in the present screen are known to be generally compromised for growth on nonfermentable carbon sources and thus probably not directly involved in glycerol uptake. In addition, *RHK1* (involved in synthesis of the dolichol-linked oligosaccharide donor for N-linked glycosylation of proteins) and *ERG4* (C24-sterol reductase) are both predicted to have 9 transmembrane domains and to be located in the ER. Interestingly, ergosterol is required for targeting of the tryptophan permease to the membrane (Umebayashi and Nakano, 2003); this may also apply to the

Figure 1. Growth phenotypes on glycerol-based medium (2% wt/vol). Tenfold serial dilutions from left to right. Plates were incubated at 30°C for 5 d.

glycerol transporter. *STL1* (Zhao *et al.*, 1994) and *GUP1* (Holst *et al.*, 2000) are the only genes from this group encoding proteins with 12 putative transmembrane domains, and *STL1* is the only one with an unknown function. Stl1p belongs to the sugar transporter family in *S. cerevisiae* (Nelissen *et al.*, 1997).

Most studies on active glycerol uptake have been performed in the W303-1A genetic background. Therefore *STL1* was disrupted with the *KanMX* cassette (Wach *et al.*, 1994) in this strain. Deletion of *STL1* in both BY4742 and W303-1A genetic backgrounds resulted in cells that grew poorly on glycerol (Figure 1). A copy of the *STL1* gene comprising 2 kbp of the promoter region and 200 base pairs of the terminator region was introduced into the *stl1*-deleted strain using a centromere-based plasmid. The resulting strain grew like the wild-type strain, demonstrating functional complementation of the *stl1* growth defect (Figure 1).

Stl1p Is Essential for Active Glycerol Uptake

The glycerol utilization phenotype prompted us to analyze glycerol transport in the *stl1* mutant. Wild-type cells and *stl1* cells were grown in glucose-based complex medium containing 1 M NaCl and 15 mM glycerol. Cells were harvested at the diauxic shift, and glycerol transport was measured. These conditions were chosen because they are known to allow detection of glycerol accumulation even in a strain lacking *GUP1*, *GUP2*, and *GUT1* (Neves *et al.*, 2004a). Deletion of *STL1* eliminated active transport-driven glycerol accumulation (Figure 2A). The same occurred in the mutant strains *stl1 fps1* and *stl1 gut1* (data not in the figure). Interestingly, the *gpd1 gpd2 stl1* strain, unlike the *gpd1 gpd2* strain, did not grow on salt even in the presence of small amounts of glycerol. Additionally, cells deleted for all three genes previously known to be directly or indirectly involved in glycerol transport in *S. cerevisiae*, *GUP1*, *GUP2*, and *FPS1* (Holst *et al.*, 2000; Tamás *et al.*, 1999), accumulated glycerol against a chemical gradient (Figure 2B). This accumulation was sensitive to the depletion of proton motive force by CCCP. Moreover, glycerol symport activity has previously been observed on nonfermentable carbon sources like ethanol (Lages and Lucas, 1997). To avoid interference of metabolism with measurements of transport kinetics (Holst *et al.*,

Figure 2. (A) Accumulation ratios of [14 C]glycerol in W303-1A (●) and W303-1A *stl1* cells (▲) at the diauxic shift after growth in glucose-based complex medium (YPD) containing 1 M NaCl and 15 mM glycerol. Efflux of radiolabel after the addition of nonradioactive glycerol to 1 M (Δ) or the ionophore CCCP to 50 μ M (○). (B) Accumulation ratios of [14 C]glycerol in the mutant W303-1A *gup1 gup2 fps1* (●). Efflux of radiolabel after the addition of nonradioactive glycerol to 1 M (Δ) or the ionophore CCCP to 50 μ M (○). Cells were assayed at the diauxic shift after growth in YPD containing 1 M NaCl and 15 mM glycerol. (C) Accumulation ratios of [14 C]glycerol in W303-1A *gut1* (●) and W303-1A *stl1 gut1* (▲). Cells were collected during exponential growth in ethanol-based complex medium (YPE). Efflux of radiolabel after the addition of nonradioactive glycerol to 1 M (Δ) or of the ionophore CCCP to 50 μ M (○). The arrows indicate the time point of the addition of either CCCP or nonradioactive glycerol.

2000; Oliveira *et al.*, 2003), experiments were also performed in a *gut1* background. The deletion of *STL1* in this background eliminated the active transport-driven glycerol accumulation (Figure 2C). This was also observed in the mutant strains *stl1 gpd1 gpd2* and *stl1 fps1* grown on ethanol (data not in the figure). Thus, under all growth conditions where *S. cerevisiae* is known to take up glycerol by active transport, deletion of *STL1* abolished this capacity.

Stl1p and Active Glycerol Transport Are Induced in Response to Osmotic Shock

Transcription of *STL1* in glucose-grown cells is induced upon a shift to high salt conditions. The expression is transient and dependent on Hog1p and Hot1p (Rep *et al.*, 2000). Glycerol/ H^+ symport activity is absent in wild-type cells during exponential growth in glucose-based complex me-

Figure 3. (A) Transient induction of active glycerol transport by salt shock in W303-1A (*GPD1 GPD2*) cells grown in YPD. NaCl to 0.7 M was added at $t = 0$ during the exponential growth phase. [^{14}C]Glycerol initial uptake rates (at 4 mM; gray bars) and maximum accumulation ratios (white bars). (B) Transient induction of Stl1p by salt shock. W303-1A (*GPD1 GPD2*), YSH481 (*gpd1 GPD2*), or YSH642 (*gpd1 gpd2*) cells containing *STL1-ZZ* were grown and incubated as in A. Samples were taken every 30 min after the addition of NaCl and analyzed by Western blot.

dium irrespective of the presence of NaCl (Holst *et al.*, 2000). It becomes measurable during the diauxic shift when glucose is exhausted (Lages and Lucas 1997; Neves *et al.*, 2004a).

To analyze the expression of *STL1* in more detail, we integrated DNA encoding a duplicated IgG-binding domain (Z) of the *Staphylococcus aureus* protein A to the chromosomal copy of *STL1*. The strain expressing the fusion protein (Stl1p-ZZ) grew on glycerol medium like the wild-type, indicating that the fusion protein is functional. Subsequently, we analyzed the Stl1p level and measured uptake of glycerol under osmotic shock conditions. Cells were grown to exponential phase on glucose and shifted to medium containing 0.7 M NaCl. Samples were taken at time intervals and the $V_{\max}$ of active transport using labeled glycerol at a single concentration was estimated. The same cells were used to determine the maximum in/out concentration ratio, reflecting active transport-driven accumulation (Figure 3A). Osmotic shock led to a sharp increase in glycerol accumulation capability. After 1.5 h of incubation at 30°C, significant amounts of Stl1p were produced in cells experiencing osmotic shock (Figure 3B). Stl1p was not produced in cells that were not osmotically shocked. Importantly, the levels of Stl1p and the glycerol uptake activity directly correlated. The high levels of Stl1p appearing after 1.5 h shift to NaCl gradually disappeared, and the protein was no longer detectable after 4 h (Figure 3B, *GPD1 GPD2*). These results are in agreement with the transient appearance

Figure 4. Induction of Stl1p in W303-1A cells containing *STL1-ZZ* during growth in YPD in the continuous presence of 0.7 M NaCl. Samples were taken at the points included in the growth curve shown in A and analyzed by Western blot (B). The arrow indicates the time point with the highest expression level. (C) W303-1A (*GPD1 GPD2*), YSH481 (*gpd1 GPD2*), or YSH642 (*gpd1 gpd2*) cells containing *STL1-ZZ* were grown in YPD and 0.7 M NaCl. Samples were taken during the exponential growth phase every 4 h and analyzed by Western blot.

of the *STL1* transcript after salt shock, reported by Rep *et al.* (2000). They found that the transcript appeared 0.5 h after the shock, but was undetectable after 1.5 h. Synthesis and accumulation of glycerol are major responses of yeast to sudden increases in the osmolarity (Hohmann, 2002). The glycerol synthesis is accomplished by induction of the genes *GPD1* and *GPD2*, encoding a glycerol 3-phosphate dehydrogenase and a glycerol 3-phosphate-specific phosphatase, respectively (Ansell *et al.*, 1997). The closure of the Fps1p channel ensures that glycerol does not exit the cell during osmotic shock (Tamás *et al.*, 1999). Interestingly, in cells that are deleted for the two isogenes *GPD1* and *GPD2*, Stl1p appeared during the osmotic shock, but the down-regulation/degradation was prevented (Figure 3B, *gpd1 gpd2*), presumably because the osmotic imbalance persisted in the absence of glycerol synthesis. The *gpd1 gpd2* strain exhibits a significant lag phase and somewhat slower growth after salt shock compared with the parent, so we also analyzed a strain that is deleted only for *GPD1* and that is not affected in growth rate after salt shock. Under these conditions, the presence of Stl1p was also stabilized (Figure 3B, *gpd1 GPD2*).

If cells were grown continuously in glucose-based medium with a high concentration of salt, Stl1p was detectable only when the cells entered the diauxic shift (Figure 4A, arrow, and B), consistent with active glycerol transport (Holst *et al.*, 2000; Neves *et al.*, 2004a). However, *gpd1* and *gpd1 gpd2* cells, which produce only residual amounts of glycerol (Oliveira and Lucas, 2004), made significant amounts of Stl1p during exponential growth in 0.7 M NaCl (Figure 4C). This is consistent with the fact that *gpd1 gpd2* cells display glycerol uptake during exponential growth in glucose-based medium with NaCl (Holst *et al.*, 2000). This is not surprising, considering the growth defect of the *gpd1 gpd2 stl1* strain on media containing 1 M NaCl and 15 mM

Figure 5. (A) Localization in vivo of fluorescent Stl1-GFP in W303-1A cells containing a centromere-based vector with the *STL1*-GFP fusion. Cells were grown in glycerol-based synthetic medium (left panels) and shifted to glucose and visualized after 1 h (right panels). (B) Western blot analysis of the fate of the Stl1-GFP protein. Full-length Stl1p-GFP as well as the 38-kDa degradation product (Stl1p-GFP*) are indicated. (C) Western blot analysis of Stl1-ZZ protein level in ethanol (YPE)-grown W303-1A cells and RH266-1D (*end3*) containing *STL1*-ZZ at the indicated times (hours) after the addition of glucose.

glycerol. Thus, the absence of glycerol synthesis apparently prevented the down-regulation and degradation of Stl1p in this mutant.

Stl1p Is Localized to the Plasma Membrane and Is Subject to Glucose Inactivation

The subcellular localization of Stl1p was analyzed using the GFP fused to the carboxy-terminus of Stl1p, with the gene under the control of the methionine-repressible *MET25* promoter. The fusion protein was functional in uptake of [14 C]glycerol. The majority of the fluorescence was detected at the plasma membrane in cells growing exponentially on glycerol-based medium (Figure 5A). This is consistent with a function of Stl1p as a plasma membrane-localized glycerol transporter. The symporter activity (V_{max}) decreases progressively after transfer from ethanol- to glucose-containing medium. The activity is reduced to 10% within 30 min after transfer (Lages and

Lucas, 1997). In full accordance, when cells were shifted from glycerol to glucose, and the promoter driving the expression of the fusion protein was turned off by the addition of methionine, the fluorescence signal disappeared from the plasma membrane within 30 min. Most fluorescence was detected in the vacuolar lumen 1 h after the shift to glucose (Figure 5A). Western blot analysis using anti-GFP antibodies showed a 90-kDa band, presumably reflecting the full-length fusion protein. In addition, a 38-kDa degradation product was detected. On shift to glucose-containing medium the full-length protein disappeared, and the degradation product accumulated and appeared to be stable for at least 2.5 h (Figure 5B). These observations indicate that the protein is internalized into the vacuole, and that the GFP moiety is detached from Stl1p, releasing it from the membrane. Inactivation of Stl1p-ZZ by growth in glucose was also followed by Western blot analysis. When glucose was added to cells growing exponentially in ethanol-based complex medium, Stl1p progressively disappeared (Figure 5C, WT) and was no longer detected 1.5 h after the addition of glucose. This is consistent with the behavior of the GFP fusion protein. If, however, the fate of Stl1p was followed in an *end3* mutant, which is defective in endocytosis (Raths *et al.*, 1993), Stl1p was not degraded within 4 h, and a larger molecular species appeared (Figure 5C, *end3*). This larger form of Stl1p may represent one or more ubiquitinated species of Stl1p. We conclude that Stl1p is inactivated by growth in glucose, internalized to the vacuole by endocytosis in an *END3*-dependent manner, where it is subsequently degraded.

The S. cerevisiae STL1 Gene Provides Active Uptake of Glycerol When Expressed in S. pombe

Fission yeast, *S. pombe*, does not take up glycerol by active transport (Lages *et al.*, 1999). This yeast does not have any genes with high sequence homology to *STL1*. Therefore, we chose to introduce the *S. cerevisiae STL1* gene into *S. pombe* to test if this gene indeed codes for the glycerol/ H^+ symporter. *S. pombe* transformants expressing *STL1* from two promoters of different strength were obtained. The time needed for optimal expression of *STL1* was initially determined by glycerol uptake experiments using a single concentration of radiolabeled glycerol in the transport V_{max} range. For each construct, a short time interval was found in which glycerol accumulation could be measured after the promoter was turned on. This was 6 and 30 h for the weaker and the stronger promoter, respectively.

We determined glycerol accumulation ratios in a single transformant for each construct (Figure 6A) and compared it with untransformed cells as well as with control transformants harboring plasmids without *STL1* insert (Figure 6B). Accumulation exceeded equilibrium and was higher for the transformants expressing the gene with the stronger promoter. The accumulation was sensitive to the dissipation of proton motive force through the action of the protonophores/ionophores CCCP and FCCP (Figure 6A). Furthermore, these drugs elicited glycerol efflux (Figure 6C). We conclude that the expression of the *S. cerevisiae STL1* gene in *S. pombe* introduces uptake of glycerol in this yeast that is dependent on the proton motive force.

DISCUSSION

STL1 was identified in a screen for genes encoding membrane proteins involved in glycerol utilization. It has not previously been assigned a function. We propose that Stl1p is the active glycerol/ H^+ symporter in *S. cerevisiae* based on the following arguments. First, *stl1* mutants are not able to efficiently utilize glycerol as a sole carbon and energy source.

Figure 6. (A) Accumulation ratios of $[^{14}\text{C}]$ glycerol in *Schizosaccharomyces pombe* transformants harboring the plasmids pREP82X-STL1 (■) ($n = 5$) and pREP4X-STL1 (○) ($n = 8$) and the transformant harboring pREP82X-STL1 in the presence of CCCP (▲) and FCCP (△). (B) Accumulation ratios of $[^{14}\text{C}]$ glycerol in untransformed *S. pombe* cells (□) ($n = 3$) and in *S. pombe* cells transformed with the empty vector pREP82X (●) ($n = 3$). (C) Accumulation of $[^{14}\text{C}]$ glycerol in *S. pombe* transformants harboring pREP82X-STL1 (■) and subsequent efflux of radiolabel after addition of CCCP (▲) or FCCP (△). The arrow indicates the addition of the ionophore. n , number of independent assays with each strain.

Second, active uptake of glycerol is absent in *stl1* mutants. Third, the expression data from the literature as well as protein data presented here show that *STL1* expression and Stl1 protein correlate directly with glycerol uptake activity. Fourth, the localization of Stl1p in the plasma membrane and its glucose inactivation are fully consistent with the function as a transporter. Fifth, heterologous expression of *STL1* in *S. pombe* enables this yeast to take up glycerol via an active mechanism. Notably, the glycerol utilization phenotype of the *stl1* mutant proved to be a direct consequence of its involvement in the glycerol transport, as the deletion of *STL1* abolished glycerol accumulation.

Yeast utilizes glucose by fermentation and produces ethanol and small amounts of glycerol. The production of glycerol under fermentative conditions provides the cell with an electron sink that enables it to maintain redox balance (Ansell *et al.*, 1997). The produced glycerol exits the cell *via* the Fps1p channel. When all glucose is consumed, the yeast culture enters the diauxic shift, during which major changes in gene expression alter the fermentative to oxidative metabolism, allowing the yeast to utilize the produced ethanol and glycerol before entry into the stationary phase. Cat8p is a transcription factor that regulates a subset of gluconeogenic genes when cells are grown on nonfermentable carbon sources such as ethanol (Hedges *et al.*, 1995). *STL1* is induced, in concert with 36 other genes, in a Cat8p-dependent manner during the diauxic shift (Haurie *et al.*, 2001). Most of these Cat8p-induced genes are related to ethanol utilization but in addition to *STL1*, the gene encoding the lactate permease, *JEN1* is also induced (Makuc *et al.*, 2001). Correspondingly, glycerol symport has previously been shown to be repressed by glucose, induced by growth on nonfermentable carbon sources and transiently detectable during diauxic shift upon growth on glucose (Lages and Lucas, 1997). *STL1* is also highly and transiently induced by osmotic shock during exponential growth on glucose-based media (Posas *et al.*, 2000; Rep *et al.*, 2000; Yale and Bohnert 2001; this work). The rapid appearance of Stl1p under these conditions suggests a role for the glycerol symporter during the imme-

diolate response to osmotic shock. This might be important in nature, considering the extreme, diverse and rapid changes in environmental conditions yeasts may experience. Because yeast cells leak a substantial amount of the produced glycerol into the medium (Shen *et al.*, 1999), this induction of Stl1p is not surprising. Degradation of Stl1p involves *END3*-mediated endocytosis. Several plasma membrane proteins with transport functions are subject to down-regulation via ubiquitination and subsequent endocytosis (Katzmann *et al.*, 2002; Hicke and Dunn, 2003). Among other examples in yeast are the glucose inactivation of the lactate permease Jen1p (Andrade and Casal, 2001; Paiva *et al.*, 2002) and the galactose transporter Gal2p (Horak and Wolf, 2001). The occurrence of higher-molecular-weight Stl1-ZZ species, in particular in the *end3* mutant (Figure 5), is consistent with the idea that Stl1p degradation is also signaled by ubiquitination.

The *STL1* gene product is one of the 34 members of the sugar permease family in *S. cerevisiae* (Nelissen *et al.*, 1997), which is a part of the major facilitator superfamily (MFS). However, Stl1p is distinct from the hexose transporter subfamily, the maltose transporter subfamily and the inositol permease subfamily of the sugar permeases in *S. cerevisiae*. Hence the sugar permease family in yeast includes members that transport hexoses and inositol by facilitated diffusion and members that transport maltose and now glycerol by proton symport. Stl1p is predicted to contain 12 membrane-spanning helices. The elucidation of the structure of two members of the MFS, the glycerol 3-phosphate transporter and the lactose/ H^+ symporter, both from *E. coli*, revealed that they have very similar structures despite a low degree of sequence similarity (Abramson *et al.*, 2003; Huang *et al.*, 2003). Differences in residues that are involved in substrate binding and translocation are expected to generate the different transporter functions within the MFS family. It is thus not unexpected that a member of the sugar permease family in *S. cerevisiae* transports glycerol, given the structural resemblance between sugars and glycerol.

Polyols accumulate in plants and fungi in response to salt, low temperature, and drought. Polyols increase the osmotic potential of the cytoplasm and protect cellular structures and metabolism. In a number of plant species, mannitol and sorbitol are, in addition to sucrose, primary products of photosynthesis (Cheng *et al.*, 2004). As these plants experience salt or water stress, they synthesize polyols that are transported from the leaves, where they are synthesized, to nonphotosynthetic organs. *Apium graveolans* (celery) synthesizes and transports mannitol from leaves to other organs during water or salt stress. Mannitol is transported by a proton symport mechanism across the plasma membrane of the phloem strands. The transporter is a member of the sugar permease family of the MFS (Noiraud *et al.*, 2001). In addition, a sorbitol proton symporter has been identified in *Prunus cerasus* (sour cherry; Gao *et al.*, 2003) and in *Malus domestica* (apple; Ramsperger-Gleixner *et al.*, 2004; Watari *et al.*, 2004). Recently a polyol proton symporter in *Arabidopsis thaliana* has also been identified (Reinders *et al.*, 2004). These plant polyol proton symporters all belong to the sugar permease family of the MFS.

Debaryomyces hansenii (Lucas *et al.*, 1990) and *Candida albicans* (G. Kayingo, personal communication) also exhibit active glycerol transport. Stl1p homologues in *D. hansenii* (Accession no. CAG87598.1) and *C. albicans* (Accession no. CA0472) share 63 and 59% identity with Stl1p, respectively. Other yeasts for which genomic sequences are partially available via the Génolévures Project (Souciet *et al.*, 2000) that display active glycerol transport (Lages *et al.*, 1999) also

contain sequences encoding putative proteins with a significant degree of identity to *Stl1p*, e.g., *P. angusta*, *Zygosaccharomyces rouxii*, and *Kluyveromyces marxianus*. *K. lactis* contains a gene (Accession no. XP_456249) encoding a protein with 71% identity to *Stl1p*. However, the type of glycerol uptake mechanism in this yeast is presently not known. Furthermore, several phytopathogenic fungi contain genes encoding putative proteins with a considerable degree of similarity, in particular *Eremothecium gossypii* (Accession no. NP_984235.1), *Aspergillus nidulans* (Accession no. XP_413305.1), and *Magnaporthe grisea* (Accession no. EAA53889.1), which are 70, 53, and 44% identical to *Stl1p*, respectively. *Eremothecium* and *Magnaporthe* depend on glycerol for appressorial turgor, allowing invasiveness (de Jong *et al.*, 1997). *Aspergillus* is able to take up glycerol, although it is not known whether this operates via an active mechanism (Visser *et al.*, 1988). These interspecies homologies are high considering that the closest relatives in *S. cerevisiae*, members of the hexose transporter family, only share 27% or less sequence identity with *Stl1p*. This suggests that active glycerol uptake is a mechanism that operates in many different yeasts and fungi.

ACKNOWLEDGMENTS

We thank Dr. J. H. Hegemann from Heinrich-Heine-Universität, Institut für Mikrobiologie, Düsseldorf; Dr. H. Riezman from University of Geneva, Biochemistry Institute; and Dr. R. Egel, from the University of Copenhagen, for kindly providing the pUG35 GFP expression vector, *S. cerevisiae* RH266-1D, and *S. pombe* EG573, respectively. We thank J. Greisvold for skilful technical assistance, Dr. J. Winther for help with the bio-informatical analysis, and Dr. C. Tachibana for critical reading of the manuscript. C.F. is a PhD student from FCT (Fundação para a Ciência e Tecnologia), Ministério da Ciência e do Ensino Superior, Portugal, Ref. SFRH/BD/4714/2001. L.N. is PRODEP (Portugal) PhD student no. 53/258.002/00.

REFERENCES

- Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003). Structure and mechanism of the lactose permease of *Escherichia coli*. *Science* 301, 610–615.
- Albertyn, J., Hohmann, S., Thevelein, J. M., and Prior, B. A. (1994). GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol. Cell. Biol.* 14, 4135–4144.
- Alonso-Monge, R., Navarro-García, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., Sánchez, M., and Nombela, C. (1999). Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J. Bacteriol.* 181, 3058–3068.
- Andrade, R. P., and Casal, M. (2001). Expression of the lactate permease gene *JEN1* from the yeast *Saccharomyces cerevisiae*. *Fungal Genet. Biol.* 32, 105–111.
- Ansell, R., Granath, K., Hohmann, S., Thevelein, J. M., and Adler, L. (1997). The two isoenzymes for yeast NAD⁺-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. *EMBO J.* 16, 2179–2187.
- Askree, S. H., Yehuda, T., Smolikov, S., Gurevich, R., Hawk, J., Coker, C., Krauskopf, A., Kupiec, M., and McEachern, M. J. (2004). A genome-wide screen for *Saccharomyces cerevisiae* deletion mutants that affect telomere length. *Proc. Natl. Acad. Sci. USA* 101, 8658–8663.
- Ausubel, F. M., Struhl, K., Smith, J. A., Seidman, J. G., Moore, D. D., Kingston, R. E., and Brent, R. (1996). *Current Protocols in Molecular Biology*, New York: John Wiley & Sons.
- Bähler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., III, Steever, A. B., Wach, A., Philippsen, P., and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based targeting in *Schizosaccharomyces pombe*. *Yeast* 14, 943–951.
- Bonangelino, C. J., Chavez, E. M., and Bonifacio, J. S. (2002). Genomic screen for vacuolar protein sorting genes in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 13, 2486–2501.
- Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14, 115–132.
- Brisson, D., Vohl, M. C., St-Pierre, J., Hudson, T. J., and Gaudet, D. (2001). Glycerol: a neglected variable in metabolic processes? *Bioessays* 23, 534–542.
- Brown, A. D. (1977). Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Adv. Microbiol. Physiol.* 17, 181–242.
- Burke, D., Dawson, D., and Stearns, T. (2000). *Methods in Yeast Genetics*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Cheng, L., Zhou, R., Reidel, E. J., Sharkey, T. D., and Dandekar, A. M. (2004). Antisense inhibition of sorbitol synthesis leads to up-regulation of starch synthesis without altering CO₂ assimilation in apple leaves. *Planta (in press)*.
- de Jong, J. C., McCormack, B. J., Smirnov, N., and Talbot, N. J. (1997). Glycerol generates turgor in rice blast. *Nature* 389, 244–245.
- Engel, A., and Stahlberg, H. (2002). Aquaglyceroporins: channel proteins with a conserved core, multiple functions, and variable surfaces. *Int. Rev. Cytol.* 215, 75–104.
- Gao, Z., Maurousset, L., Lemoine, R., Yoo, S. D., van Nocker, S., and Loescher, W. (2003). Cloning, expression, and characterization of sorbitol transporters from developing sour cherry fruit and leaf sink tissues. *Plant Physiol.* 131, 1566–1575.
- Gaudet, D. *et al.* (2000). Glycerol as a correlate of impaired glucose tolerance: dissection of a complex system by use of a simple genetic trait. *Am. J. Hum. Genet.* 66, 1558–1568.
- Haurie, V., Perrot, M., Mini, T., Jenö, P., Sagliocco, F., and Boucherie, H. (2001). The transcriptional activator Cat8p provides a major contribution to the reprogramming of carbon metabolism during the diauxic shift in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 276, 76–85.
- Hedges, D., Proft, M., and Entian, K. D. (1995). CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 15, 1915–1922.
- Hicke, L., and Dunn, R. (2003). Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* 19, 141–172.
- Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling. *Trends Biochem. Sci.* 25, 111–112.
- Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* 66, 300–372.
- Holst, B., Lunde, C., Lages, F., Oliveira, R., Lucas, C., and Kielland-Brandt, M. C. (2000). GUP1 and its close homologue GUP2, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 37, 108–124.
- Horak, J., and Wolf, D. H. (2001). Glucose-induced monoubiquitination of the *Saccharomyces cerevisiae* galactose transporter is sufficient to signal its internalization. *J. Bacteriol.* 183, 3083–3088.
- Huang, Y., Lemieux, M. J., Song, J., Auer, M., and Wang, D. N. (2003). Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. *Science* 301, 616–620.
- Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002). Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell. Biol.* 3, 893–905.
- Kempf, B., and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* 170, 319–330.
- Kohlwein, S. D., Daum, G., Schneider, R., and Paltauf, F. (1996). Phospholipids: synthesis, sorting, subcellular traffic—the yeast approach. *Trends Cell Biol.* 6, 260–266.
- Kyhse-Andersen, J. (1994). The semi-dry electroblotter. *J. Biophys. Biochem. Methods* 10, 203–209.
- Lages, F., and Lucas, C. (1995). Characterization of a glycerol/H⁺ symport in the halotolerant yeast *Pichia sorbitophila*. *Yeast* 11, 111–119.
- Lages, F., and Lucas, C. (1997). Contribution to the physiological characterization of glycerol active uptake in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1322, 8–18.
- Lages, F., Silva-Graca, M., and Lucas, C. (1999). Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. *Microbiology* 145, 2577–2585.
- Lang, F., Busch, G. L., Ritter, M., Völkl, H., Waldegger, S., Gulbins, E., and Häussinger, D. (1998). Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* 78, 247–306.
- Large, V., Peroni, O., Letexier, D., and Beylot, M. (2004). Metabolism of lipids in human white adipocyte. *Diabetes Metab.* 30, 294–309.

- Lucas, C., da Costa, M., and van Uden, N. (1990). Osmoregulatory active sodium-glycerol co-transport in the halotolerant yeast *Debaromyces hansenii*. *Yeast* 6, 187–191.
- Luyten, K., Albertyn, J., Skibbe, W. F., Prior, B. A., Ramos, J., Thevelein, J. M., and Hohmann, S. (1995). Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J.* 14, 1360–1371.
- Makuc, J., Paiva, S., Schauen, M., Krämer, R., André, B., Casal, M., Leão, C., and Boles, E. (2001). The putative monocarboxylate permeases of the yeast *Saccharomyces cerevisiae* do not transport monocarboxylic acids across the plasma membrane. *Yeast* 18, 1131–1143.
- Maundrell, K. (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* 123, 127–130.
- Müllner, H., and Daum, G. (2004). Dynamics of neutral lipid storage in yeast. *Acta Biochim. Pol.* 51, 323–347.
- Nelissen, B., de Wachter, R., and Goffeau, A. (1997). Classification of all putative permeases and other membrane plurispansers of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 21, 113–134.
- Neves, L., Lages, F., and Lucas, C. (2004a). New insights on glycerol transport in *Saccharomyces cerevisiae*. *FEBS Lett.* 565, 160–162.
- Neves, L., Oliveira, R., and Lucas, C. (2004b). Yeast orthologues associated with glycerol transport and metabolism. *FEMS Yeast Res.* 5, 51–62.
- Ni, L., and Snyder, M. (2001). A genomic study of the bipolar bud site selection pattern in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 12, 2147–2170.
- Noiraud, N., Maurousset, L., and Lemoine, R. (2001). Identification of a mannitol transporter, AgMat1, in celery phloem. *Plant Cell* 13, 695–705.
- Ochiai, N., Fujimura, M., Oshima, M., Motoyama, T., Ichiishi, A., Yamada-Okabe, H., and Yamaguchi, I. (2002). Effects of iprodione and fludioxonil on glycerol synthesis and hyphal development in *Candida albicans*. *Biosci. Biotechnol. Biochem.* 66, 2209–2215.
- Oliveira, R., and Lucas, C. (2004). Expression studies of *GUP1* and *GUP2*, genes involved in glycerol active transport in *Saccharomyces cerevisiae*, using semi-quantitative RT-PCR. *Curr. Genet.* 46, 123–139.
- Oliveira, R., Lages, F., Silva-Graca, M., and Lucas, C. (2003). Fps1p channel is the mediator of the major part of glycerol passive diffusion in *Saccharomyces cerevisiae*: artefacts and re-definitions. *Biochim. Biophys. Acta* 1613, 57–71.
- Østergaard, H., Henriksen, A., Hansen, F. G., and Winther, J. R. (2001). Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein. *EMBO J.* 20, 5853–5862.
- Påhlman, A. K., Granath, K., Ansell, R., Hohmann, S., and Adler, L. (2001). The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. *J. Biol. Chem.* 276, 3555–3563.
- Paiva, S., Kruckeberg, A. L., and Casal, M. (2002). Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of *Saccharomyces cerevisiae*. *Biochem. J.* 363, 737–744.
- Posas, F., Chambers, J. R., Heyman, J. A., Hoefler, J. P., de Nadal, E., and Arino, J. (2000). The transcriptional response of yeast to saline stress. *J. Biol. Chem.* 275, 17249–17255.
- Ramsperger-Gleixner, M., Geiger, D., Hedrich, R., and Sauer, N. (2004). Differential expression of sucrose transporter and polyol transporter genes during maturation of common plantain companion cells. *Plant Physiol.* 134, 147–160.
- Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. (1993). *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* 120, 55–65.
- Reinders, A., Panshyshyn, J. A., and Ward, J. M. (2004). Analysis of transport activity of *Arabidopsis* sugar alcohol permease homolog AtPLT5. *J. Biol. Chem.* 280, 1594–1602.
- Rep, M., Krantz, M., Thevelein, J. M., and Hohmann, S. (2000). The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. *Hot1p* and *Msn2p/Msn4p* are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J. Biol. Chem.* 275, 8290–8300.
- Rønnow, B., and Kielland-Brandt, M. C. (1993). GUT2, a gene for mitochondrial glycerol 3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. *Yeast* 9, 1121–1130.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- San José, C., Alonso-Monge, R., Pérez-Díaz, R. M. Pla, J., and Nombela, C. (1996). The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. *J. Bacteriol.* 178, 5850–5852.
- Shen, B., Hohmann, S., Jensen, R. G., and Bohnert, H. J. (1999). Roles of sugar Alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. *Plant Physiol.* 121, 45–52.
- Siderius, M., van Wuytswinkel, O., Reijenga, K. A., Kelders, M., and Mager, W. H. (2000). The control of intracellular glycerol in *Saccharomyces cerevisiae* influences osmotic stress response and resistance to increased temperature. *Mol. Microbiol.* 36, 1381–1390.
- Sieiro, C., Poza, M., Vilanova, M., and Villa, T. G. (2003). Heterologous expression of the *Saccharomyces cerevisiae* *PGU1* gene in *Schizosaccharomyces pombe* yields an enzyme with more desirable properties for the food industry. *Appl. Environ. Microbiol.* 69, 1861–1865.
- Souciet, J. L. *et al.* (2000). Genomic exploration of the hemiascomycetous yeasts: 1. A set of yeast species for molecular evolution studies. *FEBS Lett.* 487, 3–12.
- Sutherland, F.C.W., Lages, F., Lucas, C., Luyten, K., Albertyn, J., Hohmann, S., Prior, B. A., and Killian, S. G. (1997). Characteristics of Fps1-dependent and -independent glycerol transport in *Saccharomyces cerevisiae*. *J. Bacteriol.* 179, 7790–7795.
- Tamás, M. J. *et al.* (1999). Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* 31, 1087–1104.
- Tamás, M. J., Rep, M., Thevelein, J. M., and Hohmann, S. (2000). Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress. *FEBS Lett.* 472, 159–165.
- Thines, E., Roland, W., Weber, S., and Talbot, N. J. (2000). MAP kinase and protein kinase A-dependent mobilization of triacylglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea*. *Plant Cell* 12, 1703–1718.
- Thomas, B. J., and Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell* 56, 619–630.
- Umebayashi, K., and Nakano, A. (2003). Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. *J. Cell Biol.* 161, 1117–1131.
- Valadi, A., Granath, K., Gustafsson, L., and Adler, L. (2004). Distinct intracellular localization of Gpd1p and Gpd2p, the two yeast isoforms of NAD⁺-dependent glycerol-3-phosphate dehydrogenase, explains their different contributions to redox-driven glycerol production. *J. Biol. Chem.* 279, 39677–39685.
- Visser, J., van Rooijen, R., Dijkema, C., Swart, K., and Sealy-Lewis, H. M. (1988). Glycerol uptake mutants of the hyphal fungus *Aspergillus nidulans*. *J. Gen. Microbiol.* 134, 655–659.
- Wach, A., Brachat, A., Pöhlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 10, 1793–1808.
- Wang, Z.-X., Zhuge, J., Fang, H., and Prior, B. A. (2001). Glycerol production by microbial fermentation: a review. *Biothec. Adv.* 19, 201–223.
- Watari, J., Kobae, Y., Yamaki, S., Yamada, K., Toyofuku, K., Tabuchi, T., and Shiratake, K. (2004). Identification of sorbitol transporters expressed in the phloem of apple source leaves. *Plant Cell Physiol.* 45, 1032–1041.
- Whyte, J. R., and Munro, S. (2001). The Sec34/35 Golgi transport complex is related to the exocyst, defining a family of complexes involved in multiple steps of membrane traffic. *Dev. Cell* 1, 527–537.
- Wojda, I., Alonso-Monge, R., Bebelman, J. P., Mager, W. H., and Siderius, M. (2003). Response to high osmotic conditions and elevated temperature in *Saccharomyces cerevisiae* is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways. *Microbiology* 149, 1193–1204.
- Yale, J., and Bohnert, H. J. (2001). Transcript expression in *Saccharomyces cerevisiae* at high salinity. *J. Biol. Chem.* 276, 15996–16007.
- Yang, X. J., Kow, L. M., Funabashi, T., and Mobbs, C. V. (1999). Hypothalamic glucose sensor: similarities to and differences from pancreatic beta-cell mechanisms. *Diabetes* 48, 1763–1772.
- Zhao, S., Douglas, N. W., Heine, M.J.S., Williams, G. M., Winther-Larsen, H. C., and Meaden, P. G. (1994). The *STL1* gene of *Saccharomyces cerevisiae* is predicted to encode a sugar transporter-like protein. *Gene* 146, 215–219.