Received: 24 November 2021 | Revised: 18 February 2022 | Accepted: 7 March 2022

DOI: 10.1002/yea.3702

RESEARCH ARTICLE

Yeast WILEY

Insights into the glycerol transport of *Yarrowia lipolytica*

Anna M. Erian[1,2] | Michael Egermeier[1,2] | Hans Marx[1,2] | Michael Sauer[1,2]

[1]CD-Laboratory for Biotechnology of Glycerol, Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

[2]Department of Biotechnology, Institute of Microbiology and Microbial Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

Correspondence
Michael Sauer, CD-Laboratory for Biotechnology of Glycerol, Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria.
Email: michael.sauer@boku.ac.at

Present address
Michael Egermeier, Department of Wood Chemistry and Biotechnology, Wood K plus-Kompetenzzentrum Holz GmbH, Altenberger Strasse 69, Linz, 4040, Austria.

Funding information
Christian Doppler Forschungsgesellschaft; OMV Refining & Marketing GmbH; Vogelbusch GmbH

Abstract

Cellular membranes separate cells from the environment and hence, from molecules essential for their survival. To overcome this hurdle, cells developed specialized transport proteins for the transfer of metabolites across these membranes. Crucial metabolites that need to cross the membrane of each living organism, are the carbon sources. While many organisms prefer glucose as a carbon source, the yeast *Yarrowia lipolytica* seems to favor glycerol over glucose. The fast growth of *Y. lipolytica* on glycerol and its flexible metabolism renders this yeast a fascinating organism to study the glycerol metabolism. Based on sequence similarities to the known fungal glycerol transporter *Sc*Stl1p and glycerol channel *Sc*Fps1p, ten proteins of *Y. lipolytica* were found that are potentially involved in glycerol uptake. To evaluate, which of these proteins is able to transport glycerol in vivo, a complementation assay with a glycerol transport-deficient strain of *Saccharomyces cerevisiae* was performed. Six of the ten putative transporters enabled the growth of *S. cerevisiae stl1Δ* on glycerol and thus, were confirmed as glycerol transporting proteins. Disruption of the transporters in *Y. lipolytica* abolished its growth on 25 g/L glycerol, but the individual expression of five of the identified glycerol transporters restored growth. Surprisingly, the transporter-disrupted *Y. lipolytica* strain retained its ability to grow on high glycerol concentrations. This study provides insight into the glycerol uptake of *Y. lipolytica* at low glycerol concentrations through the characterization of six glycerol transporters and indicates the existence of further mechanisms active at high glycerol concentrations.

KEYWORDS

aquaglyceroporin, glycerol facilitator, glycerol uptake, MFS transporter, plasma membrane transport

1 | INTRODUCTION

Plasma membranes build the interface between the cell and its environment, separating the intracellular metabolism from the extracellular space. To transfer nutrients and end products of the metabolism, to maintain the cellular homeostasis, and to enable intercellular communication, cells have developed specialized transport proteins that mediate the exchange of ions and molecules across this barrier. These plasma membrane transport proteins, together with transporters of intracellular membranes, account for

Anna M. Erian and Michael Egermeier are joint first authors.

approximately 10% of all cellular proteins (Saier et al., 2020). Currently, the Transporter Classification Database (tcdb.org) lists over 21,000 transport proteins and classifies them into more than 1500 families composed of proteins that share sequence, structural or functional attributes. Although the advent of metagenome sequencing and progress in bioinformatics aided the detection of novel transporters, only a fraction of these proteins has been functionally characterized so far. The transport proteins, that have already been described, feature a vast range of substrate specificities, such as the transport of diverse drugs (The International Transporter Consortium, 2010), carboxylic acids (Casal et al., 2008) or ions (Prakash et al., 2003), to name but a few. Even transport proteins that facilitate the transfer of water or urea through the plasma membrane are known, although these molecules are able to diffuse through lipid bilayers (Engel et al., 1994; You et al., 1993).

Essential molecules that need to cross the membrane of each living organism are the carbon sources. Diverse transport proteins for the import of carbon sources were discovered, whereupon especially transporters for glucose, the most abundant monosaccharide in nature, have been extensively studied in all kingdoms of life (Albers et al., 1999; Buhr & Erni, 1993; Huang & Czech, 2007; Özcan & Johnston, 1999; Sauer et al., 1990). As most organisms prefer glucose as a primary energy source, research mainly focused on the characterization of glucose and other hexose transporters. However, some organisms prefer other substrates over glucose and came to the fore because of their ability to efficiently utilize alternative carbon sources. One of these organisms is the yeast *Yarrowia lipolytica*.

Y. lipolytica is a dimorphic yeast that utilizes an array of carbon sources, including hydrophobic substrates as well as sugars, to accumulate lipids or to produce sugar alcohols and organic acids (Barth & Gaillardin, 1996). Due to its peculiar metabolism, *Y. lipolytica* became a yeast model for various processes, such as lipid production and protein secretion, or for studies on dimorphism (Nicaud, 2012). The flexible metabolism of *Y. lipolytica*, which allows it to adapt to various environmental conditions, and its preference for glycerol over glucose render *Y. lipolytica* a very interesting yeast for studies on the glycerol metabolism (Egermeier et al., 2017; Lubuta et al., 2019; Papanikolaou et al., 2002). Although many enzymes involved in the glycerol metabolism have already been identified, information on the proteins essential for glycerol uptake in *Y. lipolytica* is scarce (Rywińska et al., 2013).

The molecular mechanism of glycerol uptake in yeasts has been best studied in *Saccharomyces cerevisiae*, whereas details about the glycerol transport in other yeasts and fungi are limited. In *S. cerevisiae*, several proteins were proposed to be involved in the import of glycerol, including *Sc*Gup1p, *Sc*Gup2p (Holst et al., 2000), and the channel protein *Sc*Fps1p (Luyten et al., 1995; Sutherland et al., 1997). The proteins *Sc*Gup1p and *Sc*Gup2p were later identified as *O*-acyltransferases that might influence the uptake of glycerol indirectly, instead of being glycerol uptake proteins themselves (Bosson et al., 2006). The initial assumption that *Sc*Fps1p mediates the import, as well as the export of glycerol by facilitated diffusion, was revised too. *Sc*Fps1p turned out to control the glycerol export under osmotic stress, rather than the import of glycerol (Oliveira et al., 2003; Tamás et al., 1999). Instead, the glycerol import of *S. cerevisiae* was shown to rely on the active transport by one single protein, the glycerol/H^+-symporter *Sc*Stl1p (Ferreira et al., 2005).

Take Away

- Six proteins of *Yarrowia lipolytica* were identified as glycerol transporters.
- Two channel proteins and four active transporters facilitated glycerol uptake.
- Identified transporters are involved in glycerol uptake <25 g/L glycerol.
- Indication of further glycerol transporters in *Y. lipolytica* was obtained.

According to the Transporter Classification Database, *Sc*Stl1p is a member of the major facilitator superfamily (MFS), which is the largest family of secondary carriers (Pao et al., 1998). The MFS comprises transport proteins for a variety of molecules, including transporters for hexoses and carboxylic acids, some of which have already been characterized in *Y. lipolytica* (Erian et al., 2020; Guo et al., 2015; Lazar et al., 2017; Y.-K. Park & Nicaud, 2020). Transporters of the MFS mediate the transport of solutes in response to chemiosmotic ion gradients (Pao et al., 1998), whereupon *Sc*Stl1p was shown to be an active symporter of glycerol and H^+ (Ferreira et al., 2005). Based on sequence similarities to *Sc*Stl1p, glycerol transporters were identified and functionally characterized in several other yeasts, such as *Candida albicans* (Kayingo et al., 2009), *Zygosaccharomyces rouxii* (Dušková et al., 2015), *Candida glycerinogenes* (Ji et al., 2018), *Wickerhamomyces anomalus* (da Cunha et al., 2019) and *Kluyveromyces marxianus* (Zhang et al., 2020).

In comparison to the active transport mechanism of *Sc*Stl1p, *Sc*Fps1p is a glycerol channel of the family of major intrinsic proteins (MIPs) that transports glycerol passively. The MIP family diverts into three subfamilies, the aquaporins, the aquaglyceroporins, and the superaquaporins (J. H. Park & Saier, 1996). These channel proteins span the plasma membrane typically with six transmembrane helices and are selective for small uncharged molecules such as water (aquaporins) or glycerol (aquaglyceroporins). Additional to the export of glycerol, *Sc*Fps1p of *S. cerevisiae* facilitates the uptake of arsenite, antimonite, and the undissociated form of acetic acid (Mollapour & Piper, 2007; Wysocki et al., 2001). Several orthologues to *Sc*Fps1p were identified in other yeasts, including Fps1p of *Z. rouxii* (Dušková et al., 2015), *K. marxianus*, and *Kluyveromyces lactis* (Neves et al., 2004). While these *Sc*Fps1p orthologues have a function similar to *Sc*Fps1p concerning glycerol export and osmotic stress response, several proteins were found that have a similar sequence to *Sc*Fps1p but the reverse transport function. The proteins Fps2p of *Pachysolen tannophilus* and Fps1p of *Cyberlindnera jadinii*, for example, facilitate the glycerol uptake

in *S. cerevisiae stl1Δ* and enhance the growth of wild-type *S. cerevisiae* on glycerol (Klein et al., 2016).

The loss of a single protein (i.e.Stl1p) renders *S. cerevisiae* unable to grow on glycerol, but it requires the removal of at least seven transport proteins to stop its growth on glucose (Reifenberger et al., 1995; Wieczorke et al., 1999). Likewise, some yeasts that achieve higher growth rates on glycerol than *S. cerevisiae* possess more than only one glycerol transporter. For instance, two glycerol transporters were identified in *C. glycerinogenes* (Ji et al., 2018) and four potential glycerol transporters were found in the genome of *Komagataella phaffii* (Mattanovich et al., 2009). Remarkably, *Y. lipolytica* has even seven putative active glycerol transporters (Lazar et al., 2017) and two putative glycerol channels (Pettersson et al., 2005). A similarly high level of genetic redundancy is present in *Y. lipolytica* for the transport of hexoses. Six hexose transporters can be found in *Y. lipolytica*, three of which are broad-range hexose transporters mediating the transport of glucose, galactose, mannose, and fructose (Lazar et al., 2017). The loss of two of these broad-range transporters (YALI0C06424p and YALI0E23287p) drastically reduces growth on fructose, glucose, and mannose.

The expression of the putative glycerol transporters of *Y. lipolytica* has already been analyzed in several transcriptome and proteome studies. In particular, one orthologous protein to *Sc*Fps1p, that is, YALI0F00462p, was shown to be strongly induced by glycerol (Lubuta et al., 2019), whereas some of the *Sc*Stl1p-like proteins were found to be upregulated on xylose (Ryu et al., 2016; Walker et al., 2021) or at low nitrogen concentrations (Hapeta et al., 2020). Although several expression studies are available, only one experimental study with one of the nine proteins has been conducted so far to support the expected glycerol transport function. In the study of Klein et al. (2016), one of the putative glycerol channels (YALI0E05665p) was expressed in a wild-type strain of *S. cerevisiae*, which significantly increased its growth rate on glycerol and therefore suggests a role of the channel protein in glycerol import. However, the final proof that this protein is a transport protein is still lacking. Moreover, the role of the other eight putative transport proteins in glycerol uptake is still unclear.

In this study, we provide further evidence for the glycerol transport activity of YALI0E05665p and evaluated the transport function of nine other proteins of *Y. lipolytica*. To this end, we performed a complementation assay with glycerol-transporter deficient strains of *S. cerevisiae* and *Y. lipolytica*, the latter of which was obtained during this study. In total, six proteins of *Y. lipolytica*, including YALI0E05665p, were experimentally verified as having glycerol import activity. Moreover, an indication was obtained that further transport proteins or transport mechanisms exist in *Y. lipolytica* which allow glycerol uptake at high glycerol concentrations.

2 | MATERIALS AND METHODS

2.1 | Strains and media

Y. lipolytica strain DSM 3286 was obtained from the Leibnitz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Precultures were grown in a rich liquid medium containing per liter deionized water: 9 g yeast extract, 18 g soy peptone, and 10 g glycerol (YPG) or 10 g glucose (YPD), adjusted to pH 7.5. For solid media, 20 g/L agar–agar were added to the medium. Shake flask cultivations were performed in media (YNB) containing per liter deionized water: 1.7 g yeast nitrogen base without amino acids (BD Difco, Thermo Fisher Scientific), 6.25 g $(NH_4)_2SO_4$, 1.99 g K_2HPO_4, 1.85 g KH_2PO_4, and either 25 g glycerol (YNBG) or glucose (YNBD). A defined medium was used for all bioreactor cultivations containing per liter deionized water: 100 g glycerol, 3.1 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 1.3 g $Na_2HPO_4 \times 2H_2O$, 1.0 g $MgSO_4 \times 7H_2O$, 0.2 g $CaCl_2 \times 2H_2O$, 0.5 g citric acid, 21 mg $FeCl_3$, 1 mg Thiamine-HCl, 0.5 mg H_3BO_3, 0.06 mg $CuSO_4 \times 5H_2O$, 0.1 mg KI, 0.45 mg $MnSO_4 \times H_2O$, 0.71 mg $ZnSO_4 \times 7H_2O$ and 0.23 mg $Na_2MoO_4 \times 2H_2O$. For selection purposes and to maintain plasmids during cultivation, 400 mg/L nourseothricin were added. *S. cerevisiae* BY4741 *stl1Δ* was obtained from the EUROSCARF collection under accession number Y05831 and was maintained on YP agar with 20 g/L glucose and 300 mg/L hygromycin B if applicable. Precultures of *S. cerevisiae* were grown in YPD medium with 300 mg/L hygromycin B. Plates for spot test assays of *S. cerevisiae* contained per liter deionized water: 20 g agar, 1.7 g YNB, 50 g $(NH_4)_2SO_4$, 10 g glycerol, 100 mg L-leucine, 20 mg L-methionine, and 20 mg L-histidine.

2.2 | Plasmid construction

Expression plasmids were created by Golden Gate cloning in *Escherichia coli* DH10B as described in detail by Egermeier et al. (2019). Plasmid maps of standard vectors used in this study are available at www.addgene.com and can be accessed with their corresponding plasmid number. All coding sequences (CDS) were cured in silico from *Bsa*I and *Bpi*I restriction sites before cloning. CDS of YALI0D05665g (*FPS1*), YALI0F00462g (*FPS2*), YALI0D01111g (*STL1*), YALI0C04730g (*STL2*), YALI0C16522g (*STL3*), YALI0B17138g (*STL6*), YALI0F25553g (*STL7*), and YALI2C00079g (*STL8*) were ordered as plasmids or gBlocks from Integrated DNA Technologies (www.idtdna.com) with suitable fusion sites for Golden Gate cloning. The CDS of YALI0F06776g (*STL4*) and YALI0A08998g (*STL5*) were amplified by polymerase chain reaction (PCR) from genomic DNA of *Y. lipolytica* DSM 3286 simultaneously removing potential *Bsa*I/*Bpi*I restriction sites. The plasmids were directly used for cloning and CDS were assembled with the native *TEF1* or *GPD1* promoter and *CYC1* transcription terminator of either *Y. lipolytica* or *S. cerevisiae* to expression cassettes. The PCR amplified fragments were first cloned into a plasmid (BB1_L_23_syn_BsaI; Plasmid #89915) without a promoter or terminator, were sequenced (Microsynth Austria GmbH), and subsequently assembled with the appropriate promoters and terminators (BB2_L_AB_syn_BbsI; Plasmid #89917). The expression cassettes were further cloned into expression vectors for *Y. lipolytica* or *S. cerevisiae*. The expression vector pMEG_BB3_YL68N_AC (Plasmid #117830) for *Y. lipolytica* contained an autonomous replication sequence (CEN/ARS68) and the resistance marker for

nourseothricin (*natMX*). The vector BB3_arscen_URA3_Hygro (Plasmid #118077) for *S. cerevisiae* carried *URA3*, an *hph* expression cassettes and an autonomous replication sequence (CEN/ARS6). The final plasmids and empty vectors were used for yeast transformations according to the lithium-acetate method established by Barth and Gaillardin (1996).

For the generation of sGFP-fusion proteins, the CDS of *FPS1* and *sGFP* were PCR amplified. Fusion sites for the assembly with a vector were added 5′ to the CDS of *FPS1* and 3′ to the CDS of *sGFP*. At the 3′ end of *FPS1*, the first part of a glycine-serine linker (amino acids GGG) and a unique fusion site were added, simultaneously removing the stop codon. The same fusion site and the second part of a glycine-serine linker (amino acids GS) were added at the 5′ end of *sGFP*. Both PCR fragments were assembled in a vector BB1_L_23_syn_BsaI (Plasmid #89915) and verified by Sanger Sequencing (Microsynth Austria GmbH). Further cloning was performed as described above with promoter *TEF1* and terminator *CYC1* of *Y. lipolytica*.

2.3 | Genome editing with CRISPR/Cas9

A CRISPR/Cas9 based system was used to disrupt genes by insertion/deletion mutations in the 5′ region of the CDS as previously described by Egermeier et al. (2019). Briefly, a plasmid was created by Golden Gate cloning which expressed a humanized Cas9 and a single guide RNA (gRNA) flanked by a Hammerhead type ribozyme and a hepatitis delta virus ribozyme. *Y. lipolytica* DSM 3286 was transformed with this plasmid and after an outgrowth phase of 4 days at 30°C, the cells were plated on selection plates. Cells carrying a frameshift mutation were detected by Sanger sequencing (Microsynth Austria GmbH) and were cured from the plasmid by restreaking them 2–3 times on YPG plates. The entire CDS of *STL8* was knocked out using the same CRISPR/Cas9 based system with a single vector containing two gRNAs. Protospacers were selected that are directly up- and downstream of the CDS. The successful knockout was confirmed by colony PCR and Sanger sequencing, and the strains were cured as described before.

2.4 | Bioinformatics tools

Standard in silico analysis, for example, Sanger sequencing analysis and DNA and protein sequence alignments, were performed with CLC Main Workbench (Version 8.1). The *Y. lipolytica* DSM 3286 genome assembly ASM1449061v1 was used as a reference genome. BLAST searches were done with the online protein–protein blast tool of NCBI using the predefined parameters, that is, search in the nonredundant protein sequences database with a BLOSUM62 scoring matrix, restricted to *Y. lipolytica* (tax id: 4952). Phylogenetic trees were generated with the Constraint-based Multiple Alignment Tool (COBALT) available at NCBI. Prediction of protein localizations was done with the online tool ProtComp 9.0 (Softberry Inc.). Prediction of transmembrane helices was performed with the online tool TMHMM 2.0 (Krogh et al., 2001). Gene clusters of *FPS1*-like genes were identified with the Sequence Similarity DataBase (SSDB, available on kegg.jp) using YALI0F00462g as query (Kanehisa et al., 2004).

2.5 | Spot test assay

A total of 10 ml YPD was inoculated with a single colony of *S. cerevisiae* and cells were grown overnight at 30°C and 180 rpm. Cells were harvested, washed once with deionized water and a 10-fold serial dilution from OD_{600} 1 to 10^{-4} was prepared. Four microliters of each dilution were spotted on YNBD and YNBG agar and plates were incubated for 7 days at 30°C.

2.6 | Shake flask cultivations

A total of 10 ml YPD was inoculated with a single colony and incubated overnight on a shaker at 180 rpm and 30°C. Cells were harvested and washed once with sterile deionized water. The cell density was determined with a photometer (Biochrom WPA CO8000 Cell Density Meter) at 600 nm and the cells were used for inoculation of 10 ml YNBG or YNBD medium (in 100 ml shake flasks) with an OD_{600} of 1. The shake flasks were incubated at 30°C on a rotary shaker at 180 rpm. All cultivations were performed in triplicates and an unpaired *t*-test was performed for statistical analysis.

2.7 | Bioreactor cultivations

All cultivations were carried out in a DASGIP Parallel Bioreactor System (Eppendorf AG) with four parallel bioreactors and a maximum working volume of 1.2 L. pH was monitored with a pH probe (Mettler-Toledo) and adjusted to pH 5.5 by the automated addition of 5 M NaOH or 1 M H_2PO_4. The dissolved oxygen concentration was monitored by a VisiFerm DO 120 probe (Hamilton Company) and was controlled at 50% throughout the cultivation by adjusting the gassing rate of pressurized air and the stirrer speed. To reduce foam formation, 5% (wt/vol) Struktol (SB 2121; Schiller+Seilacher GmbH) were added dropwise whenever necessary. All cultivations were performed at 30°C.

For preculture, 100 ml YPD was inoculated with a single colony and incubated overnight on a shaker at 180 rpm and 30°C. Cells were harvested and washed once with sterile deionized water. The cell density was determined with a photometer (Biochrom WPA CO8000 Cell Density Meter) at 600 nm and the cells were used for inoculation of 500 ml cultivation medium with an OD_{600} of 1. All cultivations were performed in triplicates.

2.8 | Metabolite and biomass determination

Glycerol concentrations were determined by high-performance liquid chromatography analysis (Shimadzu) with an Aminex HPX-87H

column (300 × 7.8 mm; Bio-Rad Laboratories). The column was operated at 60°C with 8 mM H_2SO_4 as mobile phase and a flow rate of 0.6 ml/min for 25 min. Peaks were detected and quantified with a refraction index detector (RID-10A; Shimadzu) or with a photodiode array detector at 254 nm (SPD-M20A; Shimadzu). To determine cell dry mass (CDM) of *Y. lipolytica*, 2 ml culture broth were centrifuged for 5 min at 10,000 g, the pellet was washed once with deionized water and dried for 48 h at 100°C. CDM was determined in duplicates.

2.9 | Gene expression analysis

A comprehensive data set of previous bioreactor cultivations of *Y. lipolytica* DSM 3286 was used for differential gene expression analysis. In these experiments, a 72-h batch cultivation was performed in bioreactors with 100 g/L glycerol or glucose either at pH 5.5 or pH 3.5. Samples for RNA-Seq were taken in the early exponential growth phase at pH 5.5 (after 8 h, approx. 2 g/L CDM and 95 g/L residual glycerol) and in the nitrogen-limited stationary phase at both pH conditions (after 28 h, approx. 14.5 g/L CDM and 18 g/L residual glycerol). A total of 18 samples for strain DSM 3286 was analyzed by Illumina sequencing (Eurofins Austria GmbH). Expression data are provided as $\log_2$-FPKM values for biological triplicates. The full data set, including details on sample treatment and data processing, is available in the GEO database with entry number GSE151659.

2.10 | Protein localization analysis

Single colonies of strains expressing the sGFP-fusion proteins were inoculated in 10 ml YPG and were incubated overnight at 30°C and 180 rpm. Cells were harvested, washed once with deionized water and used for inoculation of fresh medium at OD_{600} of 2. After incubation at 30°C and 180 rpm for approx. 3 h, the cells were analyzed with an Axio Observer Z1 (Carl Zeiss Microscopy GmbH) using an HXP 120 V lightning unit (Carl Zeiss Jena GmbH), filter set 38 HE (Carl Zeiss Microscopy GmbH), and Software ZEN 2.3 pro. Pictures of representative cells were taken in the brightfield, and after excitation/emission at 488/509 nm.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of glycerol transporters in a loss-of-function mutant of *S. cerevisiae*

In *S. cerevisiae*, the import of glycerol relies on the glycerol/H^+-symporter Stl1p. A BLAST search for proteins in *Y. lipolytica* similar to *Sc*Stl1p yielded a list of several proteins with *e*-values ranging from 10^{-131} to 10^{-23}. All of these proteins can be classified as transporters of the Sugar Porter Family and have been phylogenetically analyzed by Lazar et al. (2017). The phylogenetic analysis showed that eight of these proteins form a cluster with *Sc*Stl1p (Figure 1), which suggests a potential role in glycerol transport (Table 1). Therefore, these eight proteins were selected for analysis of their function. Two further proteins were considered as putative glycerol transporters that have amino acid sequences similar to the glycerol channel Fps2p of *P. tannophilus* (Table 1). The expression of one of these proteins, termed *Yl*Fps1p, enhanced the glycerol uptake of *S. cerevisiae* in a previous study (Klein et al., 2016). The other gene, termed *FPS2*, is located directly upstream of the glycerol kinase *GUT1*, forming a cluster. The same cluster of (experimentally characterized) glycerol facilitators with glycerol kinases can also be found for instance in the genomes of *K. marxianus*, *K. lactis*, and *Z. rouxii*.

A high gene expression of important glycerol transporters was expected to occur in *Y. lipolytica* when glycerol is present in the medium. Therefore, we compared the transcriptome of *Y. lipolytica* DSM 3286 cultivated in either glycerol- or glucose-containing

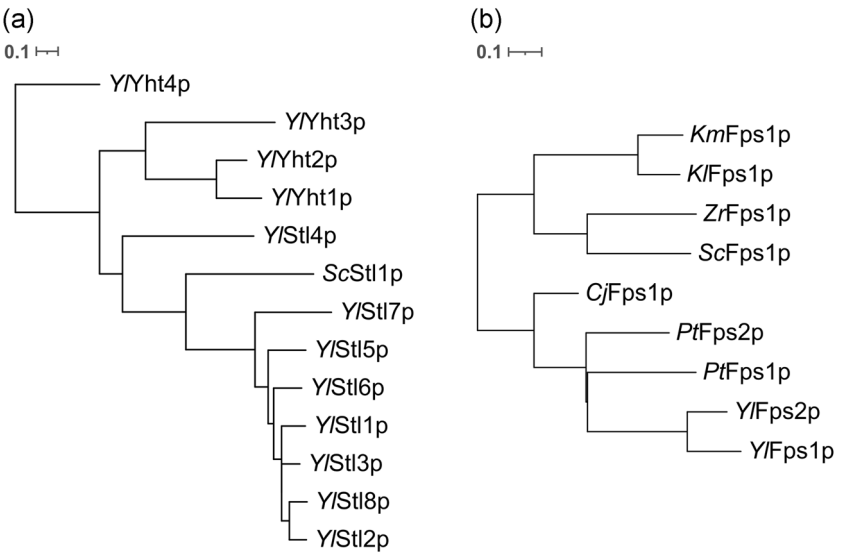


FIGURE 1 Unrooted phylogenetic tree of glycerol transporters. (a) Putative active glycerol transporters of *Yarrowia lipolytica* are compared to *Sc*Stl1p (YDR536W) and known hexose transporters of *Y. lipolytica* (Yht1p (YALI0C06424p), Yht2p (YALI0C08943p)), Yht3p (YALI0F19184p), Yht4p (YALI0E23287p). (b) Putative glycerol channels of *Y. lipolytica* are compared to the known glycerol channels *Cj*Fps1p (ODV72620.1), *Kl*Fps1p (AAQ01788.1), *Km*Fps1p (AAS47031.1), *Sc*Fps1p (CAA38096.1), *Pt*Fps1p (AFN43530.1), *Pt*Fps2p (AFN43531.1), and *Zr*Fps1p (AAQ16650.1)

TABLE 1 Selected proteins of *Yarrowialipolytica* from a BLAST search with *Pt*Fps2p and *Sc*Stl1p as reference proteins

Locus tag			BLAST analysis			Gene expression analysis	
DSM 3286	CLIB122	Name	Query	Query cover	*E*-value	log_2-FPKM Growth	log_2-FPKM N-Limit
YALI2F00006	YALI0F00462	*Yl*Fps2p	*Pt*Fps2p	87%	4E−107	6.91 ± 0.02	3.23 ± 0.24
YALI2E00127	YALI0E05665	*Yl*Fps1p	*Pt*Fps2p	86%	5E−105	6.99 ± 0.05	7.13 ± 0.06
YALI2D01102	YALI0D01111	*Yl*Stl1p	*Sc*Stl1p	95%	4E−131	5.90 ± 0.17	0.43 ± 0.07
YALI2C00080	YALI0C04730	*Yl*Stl2p	*Sc*Stl1p	86%	2E−130	0.04 ± 0.06	0.81 ± 0.19
YALI2C00079	YALI0C04686	*Yl*Stl8p	*Sc*Stl1p	95%	2E−129	n.a.	n.a.
YALI2B00179	YALI0C16522	*Yl*Stl3p	*Sc*Stl1p	93%	2E−129	0.09 ± 0.08	4.89 ± 0.01
YALI2E00997	YALI0F25553	*Yl*Stl7p	*Sc*Stl1p	95%	2E−126	1.44 ± 0.21	3.09 ± 0.40
YALI2C00742	YALI0B17138	*Yl*Stl6p	*Sc*Stl1p	88%	6E−120	0.24 ± 0.05	2.5 ± 0.19
YALI2A00136	YALI0A08998	*Yl*Stl5p	*Sc*Stl1p	90%	2E−120	0.19 ± 0.07	3.57 ± 0.07
YALI2F00135	YALI0F06776	*Yl*Stl4p	*Sc*Stl1p	82%	5E−78	5.29 ± 0.02	7.34 ± 0.06

Note: Log_2-FPKM expression values of a transcriptome analysis from previous cultivations of *Y. lipolytica* DSM 3286 in the growth phase at pH 5.5 and the nitrogen-limited production phase at pH 3.5 with glycerol as substrate are given as mean values and standard deviations of biological triplicates. *STL4* and *FPS1* are the only genes showing high expression profiles in both cultivation phases. *STL8* is not represented in the analyzed transcriptome data set and therefore, no gene expression data is available. All query cover and *e*-values refer to the proteins of *Y. lipolytica* DSM 3286. The corresponding locus tags in *Y. lipolytica* CLIB122 are additionally indicated. Additional information on differential gene expression on glycerol and glucose can be found in Table S1.

Abbreviation: n.a., not available.

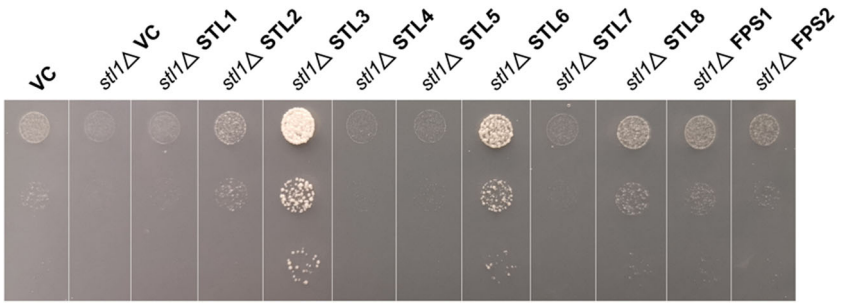


FIGURE 2 Characterization of putative glycerol transporters of *Yarrowia lipolytica* in *Saccharomyces cerevisiae*. Growth of *S. cerevisiae* BY4741 carrying an empty vector (VC) and *S. cerevisiae* BY4741 *stl1Δ* expressing putative glycerol transporters of *Y. lipolytica* or an empty vector (VC) was determined on plates containing 1% (wt/vol) glycerol. To verify the growth of all strains, a control with 1% (wt/vol) glucose was performed and can be found in Figure S2

medium. However, contrary to the expected upregulation of transporters on glycerol, the expression levels were similar at either condition or even higher on glucose than glycerol. The only gene being upregulated on glycerol compared to glucose is *FPS1* during the growth phase of the culture (Table S1).

To evaluate if the putative transporters facilitate import of glycerol, a complementation assay was performed with a knock-out strain of *S. cerevisiae* that lacks its native glycerol transporter Stl1p. *S. cerevisiae* BY4741 *stl1Δ* was transformed with plasmids carrying an expression cassette of 1 of the 10 putative transporters of *Y. lipolytica* under control of the endogenous *TEF1* promoter and *CYC1* terminator or with an empty vector (VC) as control. All strains were spotted on agar plates with 10 g/L glycerol and growth phenotypes were determined.

In comparison to the control strain *S. cerevisiae* BY4741 *stl1Δ*, which grew poorly (Figure 2), growth was enhanced by the expression of YALI0C04730g (*STL2*), YALI0C16522g (*STL3*), YALI0B17138g (*STL6*), YALI2C00079g (*STL8*), YALI0E05665g (*FPS1*), or YALI0F00462g (*FPS2*). The restored growth of these strains confirmed a glycerol transport function of *Yl*Stl2p, *Yl*Stl3p, *Yl*Stl6p, *Yl*Stl8p, *Yl*Fps1p, and *Yl*Fps2p. The expression of *Yl*Stl3p and *Yl*Stl6p improved growth of *S. cerevisiae stl1Δ* even substantially in comparison to the wild-type strain expressing an empty vector (Figure 2).

The ability to import glycerol via *Yl*Fps1p and *Yl*Fps2p contrasts with the function of *Sc*Fps1p which facilitates predominantly the export of glycerol from cells (Tamás et al., 1999). However, similar to *Yl*Fps1p and *Yl*Fps2p, proteins Fps1p of *C. jadinii* (Klein et al., 2016) and Fps2p of *P. tannophilus* (Liu et al., 2013) were demonstrated to

enable glycerol import in *S. cerevisiae stl1Δ*. In a phylogenetic analysis, *Cj*Fps1, *Pt*Fps1, *Pt*Fps2, *Yl*Fps1, and *Yl*Fps2 grouped in a branch separate to the nonglycerol importers *Kl*Fps1, *Km*Fps1, *Zr*Fps1, and *Sc*Fps1 (Figure 1). Comparison of the amino acid sequences of all aforementioned Fps1p-homologs shows significant differences in the length of the proteins. All known glycerol facilitators that enable import of glycerol in *S. cerevisiae stl1Δ* (i.e., *Cj*Fps1p, *Pt*Fps2p, *Yl*Fps1p, *Yl*Fps2p) consist of 322–385 amino acids, whereas proteins complementing a loss of *Sc*Fps1p in *S. cerevisiae* (i.e., *Kl*Fps1p, *Km*Fps1p, *Zr*Fps1p) have 564–658 amino acids. The core of all these proteins is rather conserved, but the glycerol importers lack the long N-terminal domain present in *Sc*Fps1p, which is the target for MAP kinase *Sc*Hog1p involved in the closure of the channel in *S. cerevisiae* (Lee et al., 2013).

More than 70% of the amino acid sequence of *Yl*Stl2p, *Yl*Stl3p, *Yl*Stl6p, and *Yl*Stl8p are identical, differing mainly in the N- and C-terminal regions (Figure S2). All four proteins are predicted by the stand-alone software TMHMM 2.0 to form 12 transmembrane helices, which is typical for the Sugar Porter Family of transporters (Saier, 2000). Although *Yl*Stl1p and *Yl*Stl5p are very similar to *Yl*Stl2p, *Yl*Stl3p, and *Yl*Stl6p (~70% identities; Table S2), they did not complement the loss-of-function in *S. cerevisiae stl1Δ* at the tested conditions (Figure 2).

To further characterize the six identified glycerol transporters, their impact on glycerol uptake in *Y. lipolytica* was determined. Additional to these proteins, we also considered YALI0F06776p (*Yl*Stl4p) as an interesting transport protein of *Y. lipolytica* although it did not complement the loss-of-function in *S. cerevisiae stl1Δ* (Figure 2). The preceding transcription analysis of *Y. lipolytica* DSM 3286 showed that *STL4* is highly transcribed during the growth as well as the nitrogen-limited production phase (Table 1). In contrast, all other *Sc*Stl1p-like proteins were either weakly expressed (*STL7*) or downregulated during growth (*STL2*, *STL3*, *STL5*, *STL6*) or production (*STL1*). Due to the interesting expression profile of *STL4*, which is similar to the expression pattern observed for the previously reported glycerol transporter *FPS1* by Klein et al. (2016), the encoded protein *Yl*Stl4p was also considered as putative glycerol transporter of *Y. lipolytica*, although the complementation assay in *S. cerevisiae* did not show such a result for the assumed phenotype. It may be noted at this point that the expression data for *STL8* is not depicted in Table 1 because the respective gene is not represented in the available data set used for expression analysis as it is a pseudogene in the reference genome of *Y. lipolytica* CLIB122.

3.2 | Disruption of the identified glycerol transporters in *Y. lipolytica* abolishes growth

Apart from detecting the ability to transport glycerol, it was of great interest to investigate which of the glycerol transporters are essential for the import of glycerol into *Y. lipolytica* cells. Hence, we disrupted the putative glycerol transporters in *Y. lipolytica* DSM 3286 and determined the glycerol uptake of the generated strains.

While *Sc*Fps1p is a channel protein that enables active diffusion, *Sc*Stl1p is actively transporting H^+ and glycerol into the cells. Thus, both proteins have fundamentally different transport properties, and we chose to examine both types of proteins separately in *Y. lipolytica*. To that end, two *Y. lipolytica* strains were generated that had a disruption of either *FPS1* and *FPS2*, yielding strain YldFPS, or a disruption of *STL2*, *STL3*, *STL4*, *STL6*, and *STL8*, yielding strain YldSTL. Both strains and a wild-type strain were cultivated in shake flasks with 25 g/L glycerol and their growth was determined. Additionally, all strains were cultivated with 25 g/L glucose to exclude a growth defect that is not related to glycerol uptake. Growth and substrate uptake of both strains were comparable to the wild-type strain in glycerol (Figure 3) and glucose media (data not shown). Thus, loss of these transporters resulted in no phenotypic change of *Y. lipolytica* under the tested conditions and no vital glycerol import function could be attributed to them.

The deletion of transporters may not result in any detectable change of growth as long as at least one other transporter is present that can compensate for the loss. Therefore, we generated a strain with a disruption of all seven genes (*FPS1*, *FPS2*, *STL2*, *STL3*, *STL4*, *STL6*, and *STL8)*, yielding strain YldSF, and determined growth in shake flasks with 25 g/L glycerol or glucose, respectively. Growth of YldSF in the glycerol-containing medium was not only slower than the wild-type but was stopped and no glycerol was taken up within 96 h (Figure 3). Growth of YldSF in the glucose-containing medium was unaffected (data not shown), thus, the growth defect of YldSF on glycerol can be assumed to be the consequence of lacking essential glycerol transporters.

The disruption of either *Sc*Stl1p-like proteins (strain YldSTL) or *Sc*Fps1p-like proteins (strain YldFPS) had no influence on the growth of *Y. lipolytica* DSM 3286, whereas the disruption of *Sc*Stl1p-like and *Sc*Fps1p-like proteins (strain YldSF) diminished growth. Although both types of proteins rely on different mechanisms for the transport of glycerol across the plasma membrane, *Y. lipolytica* seemed to be able to compensate the loss of active diffusion with active, H^+-coupled transport and vice versa at the tested conditions. Hence, an effect on growth and glycerol uptake was only detectable upon the simultaneous disruption of both transporter types.

3.3 | Individual expression of each glycerol transporter restores the growth of YldSF

Disruption of *STL2*, *STL3*, *STL4*, *STL6*, *STL8*, *FPS1*, and *FPS2* in *Y. lipolytica* DSM 3286 resulted in a strain that showed a growth deficiency when using glycerol as the sole carbon source. Hence, this strain was an excellent host to investigate the transport ability of each individual transporter. To this end, fourteen expression vectors were generated, each carrying one transporter-coding gene under the control of either the endogenous *GPD1* or *TEF1* promoter of *Y. lipolytica*, whereof *TEF1* has a higher relative expression strength (Blazeck et al., 2011). These constitutive promoters were used for the expression of all genes to ensure comparable results by avoiding unanticipated transcriptional regulations. All expression vectors were used for the transformation of YldSF. Additionally, YldSF and

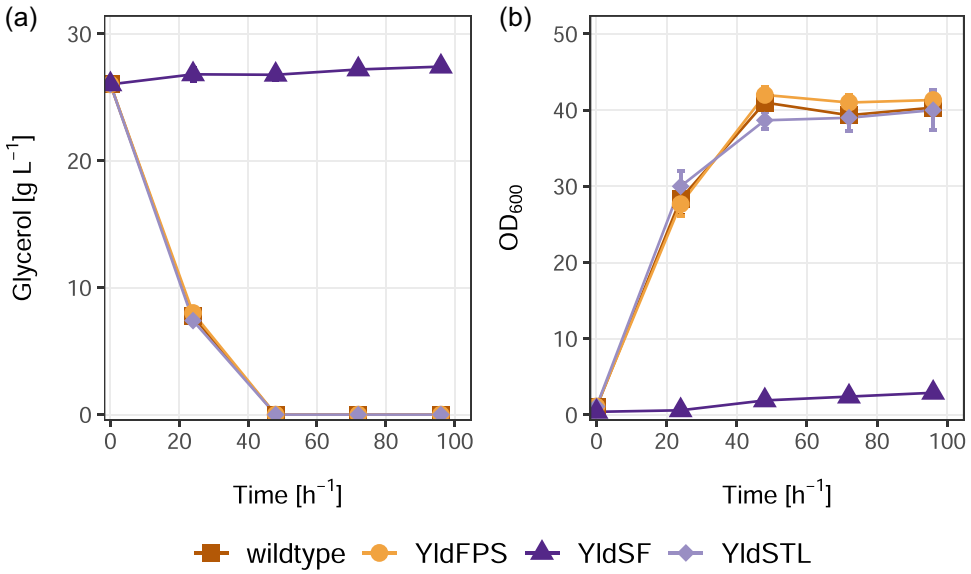


FIGURE 3 Shake flask cultivation of *Yarrowia lipolytica* DSM 3286 and mutant strains with a disruption of *FPS1* and *FPS2* (YldFPS), or a disruption of *STL2*, *STL3*, *STL4*, *STL6*, and *STL8* (YldSTL) or all of the aforementioned seven genes (YldSF). The strains were cultivated in shake flasks at a starting pH of 6.5 with 25 g/L glycerol and the time-course of (a) glycerol consumption and (b) growth was monitored. Mean values of triplicate cultivations are shown with standard deviations as error bars

wild-type *Y. lipolytica* DSM 3286 were transformed with a vector control, generating strains YldSF_VC and 3286_VC, respectively. The obtained strains were cultivated in shake flasks with 25 g/L glycerol and growth and substrate uptake were determined after 48 h.

The wild-type derivative strain 3286_VC grew to an OD_{600} of 38 and consumed the entire glycerol within 48 h, whereas the deletion strain derivative YldSF_VC neither grew nor consumed any glycerol (Figure 4). Strains expressing *STL2*, *STL3*, *STL4*, *STL6*, or *STL8* from the weak *GPD1* promoter did not significantly grow either. However, growth and glycerol uptake were restored by the expression of *FPS1* or *FPS2*. Expressing the protein *Yl*Fps1p even facilitated the growth of YldSF to OD_{600} values similar to the wild-type strain and enabled the complete uptake of glycerol. When the transporters were expressed with a stronger promoter (*TEF1*), all of them reconstituted the growth of YldSF except for *Yl*Stl4p and *Yl*Stl8p (Figure 4). *Yl*Stl2p, *Yl*Stl3p, *Yl*Fps1p, and *Yl*Fps2p allowed wild-type-like growth, whereas the final OD_{600} of *Yl*Stl6p was significantly lower ($p < 0.01$). To exclude a detrimental effect of the constitutive gene expression on the growth of the recombinant strains, all strains were additionally cultivated in a medium with 25 g/L glucose. The strains grew synchronously and reached an OD_{600} of ~39–43, showing no growth defect.

Concluding, even a weak expression of either *Yl*Fps1p or *Yl*Fps2p compensated the lack of five other glycerol transporters, whereas *Sc*Stl1-like proteins required a stronger expression to restore the growth of YldSF. *Yl*Stl4p, which was selected due to its interesting expression profile, was not verified as a glycerol transporter and may have another, not yet determined function. A recent proteome study emphasizes a role of *Yl*Stl4p in the transport of xylose (Walker et al., 2021). Surprisingly, *Yl*Stl8p did not enable the growth of YldSF on glycerol although it restored growth of *S. cerevisiae stl1Δ* in the preceding complementation assay (Figure 2). This observation might hint to a posttranscriptional regulation or posttranslational modification, which obstructs the glycerol uptake function in *Y. lipolytica*.

3.4 | *Yl*Fps1p is localized in the plasma membrane

Proteins that import substrates into the cell are components of the plasma membrane, linking the cytoplasm to the extracellular environment. Hence, the identified glycerol transporters were expected to be localized in the plasma membrane, which was supported by bioinformatical predictions with very high scores of 9.9–10 using the stand-alone software ProtComp.

Especially the transporter *Yl*Fps1p appeared in the shake flask experiments as a potent glycerol facilitator which enabled wild-type-like growth and glycerol uptake already upon weak expression. Thus, *Yl*Fps1p was chosen for a more detailed characterization and the experimental verification of its subcellular localization. For this purpose, *Yl*Fps1p was fused with a C-terminal superfolder GFP (sGFP) tag and was expressed in *Y. lipolytica* DSM 3286 from the constitutive *TEF1*-promoter. As a control, *Y. lipolytica* DSM 3286 was transformed with a plasmid carrying an untargeted sGFP. Both strains were cultivated in glycerol-containing medium and were analyzed under a fluorescence microscope.

Fluorescence microscopy of *Y. lipolytica* DSM 3286 expressing the fusion protein showed that YlFps1p is localized in the plasma membrane of *Y. lipolytica* (Figure 5b), whereas sGFP was not visibly targeted to the plasma membrane (Figure 5a). A faint fluorescence signal inside the cells expressing the fusion protein was detected as well, which might be an artifact of strong expression of *Yl*Fps1p-sGFP or due to a distorted

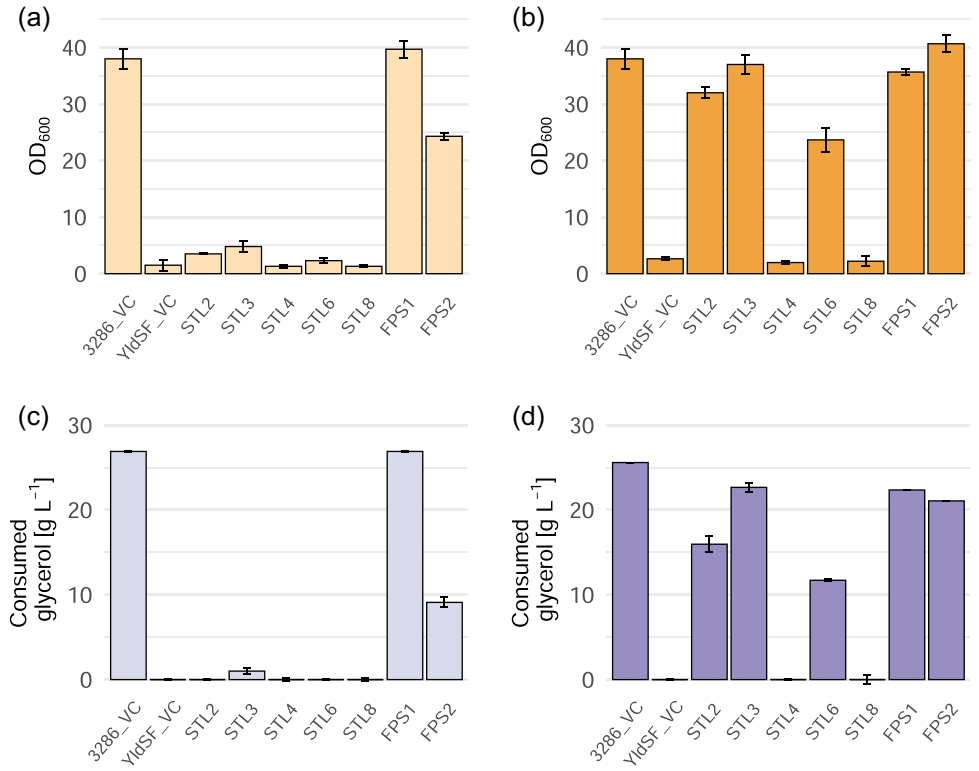
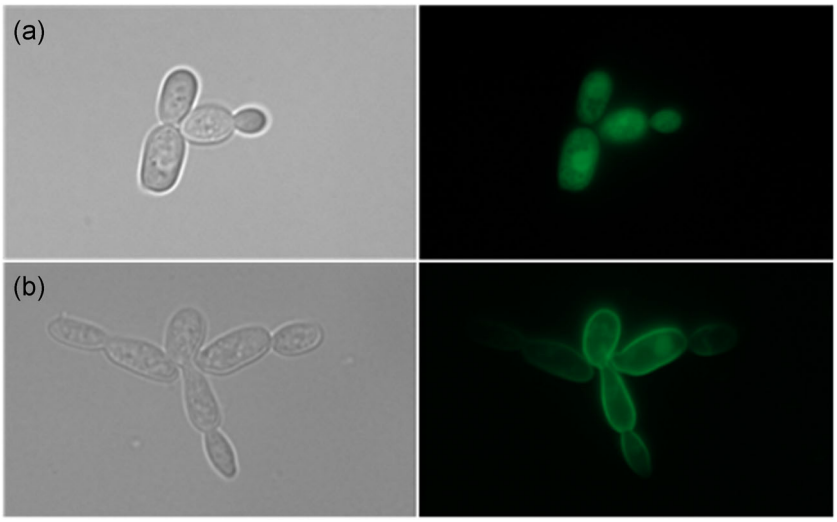


FIGURE 4 Shake flask cultivation of YldSF expressing putative glycerol transporters from different promoters. *Yarrowia lipolytica* DSM 3286 with a disruption of *STL2*, *STL3*, *STL4*, *STL6*, *STL8*, *FPS1*, and *FPS2* (YldSF) expressing transporter genes from either *GPD1* (Panels A and C) or *TEF1* promoter (Panels B and D) were cultivated with 25 g/L glycerol for 48 h. *Y. lipolytica* DSM 3286 and YldSF carrying empty vectors (3286_VC, YldSF_VC) were cultivated as control strains. OD_{600} values (Panels A and B) and consumed glycerol concentrations (Panels C and D) are displayed as mean values of triplicate cultivations with standard deviations as error bars. Asterisks indicate a significant difference ($p < 0.01$) to YldSF_VC

FIGURE 5 Subcellular localization of *Yl*Fps1p. Exponentially growing cells of (a) *Yarrowia lipolytica* DSM 3286 expressing untargeted sGFP and (b) *Y. lipolytica* expressing *Yl*Fps1p with a C-terminal sGFP-tag were analyzed with a fluorescence microscope. Pictures of representative cells were taken from the brightfield (left) and after excitation/emission at 488/509 nm (right)



structure of the fusion protein. However, the strong fluorescence of the plasma membrane clearly supports the bioinformatic prediction that *Yl*Fps1p is integrated into the plasma membrane of *Y. lipolytica*. We verified that the fusion protein is functional, as it complements the deletion phenotype of YldSF (not shown). The absence or presence of glycerol had no impact on localization (not shown).

3.5 | Transport mechanism of glycerol at high concentrations remains unknown

Although *Yl*Stl2p, *Yl*Stl3p, *Yl*Stl6p, *Yl*Fps1p, and *Yl*Fps2p were confirmed to transport glycerol, an influence on the uptake of glycerol had not been observed before the gene disruption of all five

proteins (plus *Yl*Stl4p and *Yl*Stl8p). So far, all experiments were carried out with relatively low glycerol concentrations of 25 g/L. Therefore, it was of interest to analyze if the strains YldSTL and YldFPS were still able to compensate the loss of two or three transporters, respectively, at higher glycerol concentrations. Both strains, as well as YldSF and wild-type *Y. lipolytica* DSM 3286, were cultivated in medium containing 100 g/L glycerol. The cultivations were performed under controlled conditions in bioreactors to ensure sufficient oxygen supply, which is essential at high glycerol concentrations.

In accordance with the results obtained from shake flask cultivations with 25 g/L glycerol (Figure 3), the glycerol uptake and growth of strain YldSTL was wild-type-like (Figure 6). Although growth of YldFPS was also wild-type-like, the uptake of glycerol differed. Glycerol concentrations, and hence, the glycerol uptake, of both strains remained similar for 26 h but started to diverge afterward (Figure 6). While glycerol was already depleted after 48 h by the wild-type, 10.0 g/L glycerol were still present in the cultivations of YldFPS at the same time point. The cells required another 20 h to consume the residual glycerol. The difference in glycerol uptake of YldFPS between the cultivation in shake flasks and bioreactors might be due to the inherently different cultivation conditions. While the oxygen supply in shake flasks is limited and the pH fluctuates, both parameters were controlled in the bioreactor to ensure the best growth conditions. Hence, the regulation of gene expression might have changed due to the different conditions and altered glycerol uptake of YldFPS. Moreover, the better oxygenation in bioreactors allows high glycerol conversion rates, which might only be sufficiently supported by the glycerol uptake rates of *Sc*Fps1p-like transporters but not *Sc*Stl1p-like transporters at low concentrations. The slower uptake of strain YldFPS but not YldSTL re-emphasizes the good transport capability of *Yl*Fps1p and *Yl*Fps2p, which became evident even at a weak expression in YldSF in the preceding shake flask experiment (Figure 4).

Remarkably, strain YldSF was able to grow in the bioreactor cultivation (Figure 6), which is in stark contrast to the previous shake flask cultivations (Figure 3). After a long lag phase, the cells started to grow and reached the same maximum biomass concentration as the wild-type. However, YldSF was not able to completely metabolize glycerol within 97 h, which is double the time the wild-type required to take up the entire amount. The glycerol uptake rates of the wild-type peaked between glycerol concentrations of 40–70 g/L with an average rate of 0.32 g/g_{CDM}/h. In comparison, the uptake rate of YldSF in the same glycerol range was only 0.13 g/g_{CDM}/h and decreased to less than 0.03 g/g_{CDM}/h below 36 g/L glycerol. This very low uptake rate at low glycerol concentrations corresponds to the results of the shake flask experiments, where no uptake was detected at a concentration of 25 g/L and an OD_{600} 1 (equal to approximately 0.25 g_{CDM}/L).

During the bioreactor cultivation, wild-type *Y. lipolytica* DSM 3286 secreted citrate, ketoglutarate, mannitol, arabitol, and erythritol. The same metabolites were also produced by YldSF, but the accumulation of erythritol was reduced significantly compared to the wild-type (Figures 7 and S3). Erythritol concentrations of the wild-type, YldFPS, and YldSTL peaked between 3.6 and 5.4 g/L but did not exceed 0.28 g/L during the cultivation of YldSF (Figure 7). Erythritol is typically accumulated by *Y. lipolytica* DSM 3286 until glycerol is depleted and is consumed afterward. In

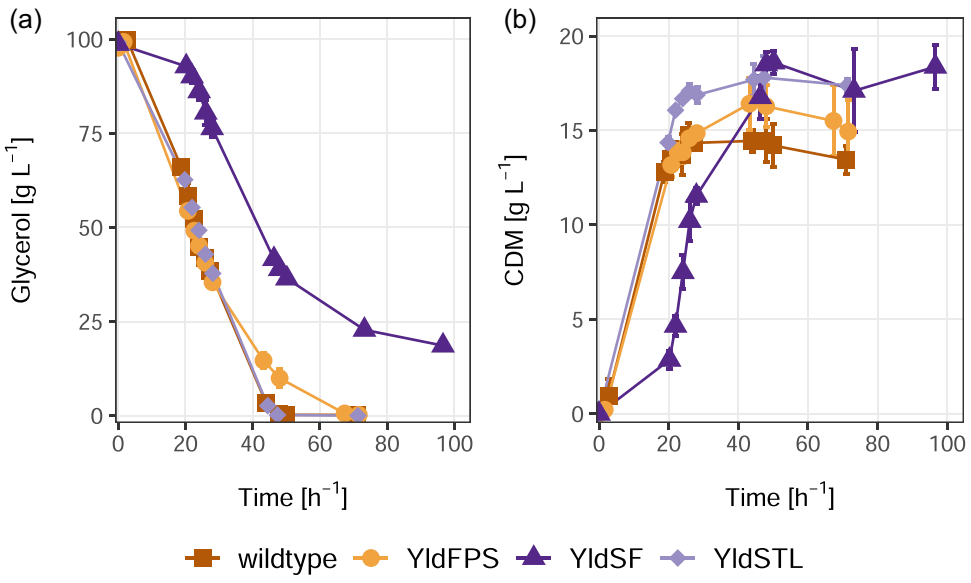


FIGURE 6 Bioreactor cultivation of *Yarrowia lipolytica* DSM 3286 wild-type and mutant strains with 100 g/L glycerol. *Y. lipolytica* DSM 3286 with a disruption of *FPS1* and *FPS2* (YldFPS), *STL2*, *STL3*, *STL4*, *STL6*, and *STL8* (YldSTL) or *STL2*, *STL3*, *STL4*, *STL6*, *STL8*, *FPS1*, and *FPS2* (YldSF) were cultivated in minimal medium with 100 g/L glycerol at pH 5.5. The time course of (a) glycerol uptake and (b) biomass production are shown of cultivations in triplicate cultivations with standard deviations as error bars. The carbon balances close at 101 ± 3% and can be found in detail in Table S3. CDM, cell dry mass

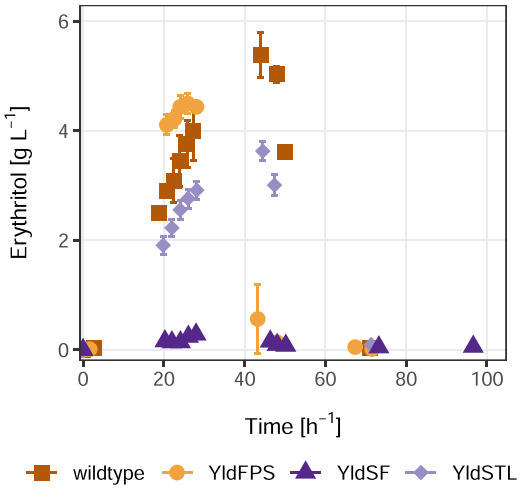


FIGURE 7 Erythritol production of *Yarrowia lipolytica* DSM 3286 and mutant strains. *Y. lipolytica* DSM 3286 with a disruption of *FPS1* and *FPS2* (YldFPS), *STL2*, *STL3*, *STL4*, *STL6*, and *STL8* (YldSTL) or *STL2*, *STL3*, *STL4*, *STL6*, *STL8*, *FPS1*, and *FPS2* (YldSF) were cultivated in bioreactors with 100 g/L glycerol at pH 5.5. The time course of erythritol production is shown of cultivations in triplicate cultivations with standard deviations as error bars

strain YldSF erythritol accumulated only slightly until the glycerol concentration was between 70 and 80 g/L and was subsequently taken up. An early reconsumption of erythritol was also detected in strain YldFPS, which started to consume erythritol after 26 h despite the availability of 40.8 g/L glycerol (Figures 6a and 7). To evaluate, if the identified glycerol transporters of *Y. lipolytica* are also involved in the transport of erythritol, *Y. lipolytica* DSM 3286 and YldSF were cultivated in shake flasks with glucose- or erythritol-containing medium. When grown on 100 g/L glucose, both strains produced similar amounts of erythritol (approx. 1.25 g/L) within 96 h. When grown on 12.5 g/L erythritol, both strains grew to an OD_{600} of 25 and consumed the entire erythritol within 24 h. Concluding, erythritol appears to be better accessible than glycerol at high glycerol concentrations upon the loss of important glycerol importers, and none of the transporters seems to be crucial for erythritol import or export.

The ability of YldSF to import glycerol indicates the presence of other proteins that can transport glycerol into the cells additional to the five identified transporters. These additional transporters might facilitate glycerol import only at high concentrations due to a low affinity for glycerol or due to transcriptional repression or post-translational regulation at low concentrations. The uptake of substrates is an intricately regulated process and is best described in yeasts for the hexose uptake of *S. cerevisiae* (Bisson et al., 2016; Özcan & Johnston, 1999). Several sensor proteins and transcription factors coordinate more than 20 hexose transporters to regulate hexose uptake of *S. cerevisiae* at different substrate concentrations. For instance, one of the regulated hexose transporters, *Sc*Hxt1p, is a low-affinity glucose transporter whose expression increases linearly with glucose concentration and reaches maximal expression not before a glucose concentration of 40 g/L (Özcan & Johnston, 1995). Similar regulatory mechanisms might be present in *Y. lipolytica* that influence yet unidentified transporters and enable the glycerol uptake at high concentrations. The proteins *Yl*Stl1p, *Yl*Stl5p, and *Yl*Stl7p might also be reconsidered as glycerol transporters although they did not complement the loss of function in *S. cerevisiae stl1Δ* (Figure 2). Eventually, glycerol transport did not occur at the tested conditions in the heterologous host but might occur in the native host *Y. lipolytica*.

Moreover, passive diffusion of glycerol into the cells might play a role at high glycerol concentrations. Glycerol is generally recognized as a molecule that can pass the lipid bilayer of the plasma membrane by passive diffusion along a concentration gradient (Gancedo et al., 1968; Heredia et al., 1968; Romano, 1986). This concept of passive diffusion of glycerol into yeast cells was however questioned by Oliveira et al. (2003), who hypothesized that any detectable passive diffusion of glycerol into *S. cerevisiae* is in fact facilitated by *Sc*Fps1p, eventually together with other less specific proteins. Nevertheless, the same authors referred to the dynamic nature of plasma membranes and stated that passive diffusion of glycerol into cells cannot be completely excluded. The composition of plasma membranes is known to be highly dynamic and flexible and is influenced by environmental stresses (Beney & Gervais, 2001). It has been shown that the deletion of membrane proteins, such as glycerol channels GlpF of *E. coli* or Fps1p of *S. cerevisiae*, influences the lipid composition of the plasma membrane and thereby alters its permeability (Toh et al., 2001; Truniger & Boos, 1993). Hence, passive diffusion of glycerol into YldSF might occur at high glycerol concentrations and might be accompanied by changes in the lipid bilayer composition of the plasma membrane, reflected in the long lag phase of YldSF.

In conclusion, out of 10 putative glycerol transporters, five were experimentally confirmed to have glycerol transport activity in *Y. lipolytica* (YALI0B17138p, YALI0C04730p, YALI0C16522p, YALI0E05665p, YALI0F00462p). Each of these five proteins and YALI2C00079p complemented a loss of the sole glycerol importer Stl1p in *S. cerevisiae* and reconstituted the yeast's ability to take up glycerol. Although the disruption of the identified transporters in *Y. lipolytica* abolished growth in medium containing 25 g/L glycerol, it did not impair growth at higher concentrations. These results provide an excellent starting point for further studies on the intriguing glycerol transport mechanism and glycerol metabolism of efficiently growing yeast strains.

ACKNOWLEDGMENTS

The financial support by the Christian Doppler Research Association, Vogelbusch GmbH and OMV Refining & Marketing GmbH, Vienna, Austria is gratefully acknowledged.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Anna M. Erian https://orcid.org/0000-0003-2411-2413
Michael Egermeier https://orcid.org/0000-0001-7872-1287
Hans Marx https://orcid.org/0000-0002-0451-1719
Michael Sauer https://orcid.org/0000-0003-0510-2447

REFERENCES

Albers, S.-V., Elferink, M. G. L., Charlebois, R. L., Sensen, C. W., Driessen, A. J. M., & Konings, W. N. (1999). Glucose transport in the extremely thermoacidophilic *Sulfolobus solfataricus* involves a high-affinity membrane-integrated binding protein. *Journal of Bacteriology*, *181*(14), 4285–4291. https://doi.org/10.1128/JB.181.14.4285-4291.1999

Barth, G., & Gaillardin, C. (1996). Yarrowia lipolytica. In K. Wolf (Ed.), *Nonconventional yeasts in biotechnology: A handbook* (pp. 313–388). Springer. https://doi.org/10.1007/978-3-642-79856-6_10

Beney, L., & Gervais, P. (2001). Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. *Applied Microbiology and Biotechnology*, *57*(1), 34–42. https://doi.org/10.1007/s002530100754

Bisson, L. F., Fan, Q., & Walker, G. A. (2016). Sugar and glycerol transport in *Saccharomyces cerevisiae*. In J. Ramos, H. Sychrová, & M. Kschischo (Eds.), *Yeast membrane transport* (pp. 125–168). Springer International Publishing. https://doi.org/10.1007/978-3-319-25304-6_6

Blazeck, J., Liu, L., Redden, H., & Alper, H. (2011). Tuning gene expression in *Yarrowia lipolytica* by a hybrid promoter approach. *Applied and Environmental Microbiology*, *77*(22), 7905–7914. https://doi.org/10.1128/AEM.05763-11

Bosson, R., Jaquenoud, M., & Conzelmann, A. (2006). *GUP1* of *Saccharomyces cerevisiae* encodes an *O*-acyltransferase involved in remodeling of the GPI anchor. *Molecular Biology of the Cell*, *17*(6), 2636–2645. https://doi.org/10.1091/mbc.E06-02-0104

Buhr, A., & Erni, B. (1993). Membrane topology of the glucose transporter of *Escherichia coli*. *Journal of Biological Chemistry*, *268*(16), 11599–11603. https://doi.org/10.1016/S0021-9258(19)50243-5

Casal, M., Paiva, S., Queirós, O., & Soares-Silva, I. (2008). Transport of carboxylic acids in yeasts. *FEMS Microbiology Reviews*, *32*(6), 974–994. https://doi.org/10.1111/j.1574-6976.2008.00128.x

da Cunha, A. C., Gomes, L. S., Godoy-Santos, F., Faria-Oliveira, F., Teixeira, J. A., Sampaio, G. M. S., Trópia, M. J. M., Castro, I. M., Lucas, C., & Brandão, R. L. (2019). High-affinity transport, cyanide-resistant respiration, and ethanol production under aerobiosis underlying efficient high glycerol consumption by *Wickerhamomyces anomalus*. *Journal of Industrial Microbiology and Biotechnology*, *46*(5), 709–723. https://doi.org/10.1007/s10295-018-02119-5

Dušková, M., Ferreira, C., Lucas, C., & Sychrová, H. (2015). Two glycerol uptake systems contribute to the high osmotolerance of *Zygosaccharomyces rouxii*. *Molecular Microbiology*, *97*(3), 541–559. https://doi.org/10.1111/mmi.13048

Egermeier, M., Russmayer, H., Sauer, M., & Marx, H. (2017). Metabolic flexibility of *Yarrowia lipolytica* growing on glycerol. *Frontiers in Microbiology*, *8*, 49. https://doi.org/10.3389/fmicb.2017.00049

Egermeier, M., Sauer, M., & Marx, H. (2019). Golden Gate-based metabolic engineering strategy for wild-type strains of *Yarrowia lipolytica*. *FEMS Microbiology Letters*, *366*(4), fnz022. https://doi.org/10.1093/femsle/fnz022

Engel, A., Walz, T., & Agre, P. (1994). The aquaporin family of membrane water channels. *Current Opinion in Structural Biology*, *4*(4), 545–553. https://doi.org/10.1016/S0959-440X(94)90217-8

Erian, A. M., Egermeier, M., Rassinger, A., Marx, H., & Sauer, M. (2020). Identification of the citrate exporter Cex1 of *Yarrowia lipolytica*. *FEMS Yeast Research*, *20*(7), foaa055. https://doi.org/10.1093/femsyr/foaa055

Ferreira, C., van Voorst, F., Martins, A., Neves, L., Oliveira, R., Kielland-Brandt, M. C., Lucas, C., & Brandt, A. (2005). A member of the sugar transporter family, Stl1p is the glycerol/H^+ symporter in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*, *16*(4), 2068–2076. https://doi.org/10.1091/mbc.e04-10-0884

Gancedo, C., Gancedo, J. M., & Sols, A. (1968). Glycerol metabolism in yeasts. *European Journal of Biochemistry*, *5*(2), 165–172. https://doi.org/10.1111/j.1432-1033.1968.tb00353.x

Guo, H., Liu, P., Madzak, C., Du, G., Zhou, J., & Chen, J. (2015). Identification and application of keto acids transporters in *Yarrowia lipolytica*. *Scientific Reports*, *5*(1), 1–10. https://doi.org/10.1038/srep08138

Hapeta, P., Kerkhoven, E. J., & Lazar, Z. (2020). Nitrogen as the major factor influencing gene expression in *Yarrowia lipolytica*. *Biotechnology Reports*, *27*, e00521. https://doi.org/10.1016/j.btre.2020.e00521

Heredia, C. F., Sols, A., & Fuente, G. D. (1968). Specificity of the constitutive hexose transport in yeast. *European Journal of Biochemistry*, *5*(3), 321–329. https://doi.org/10.1111/j.1432-1033.1968.tb00373.x

Holst, B., Lunde, C., Lages, F., Oliveira, R., Lucas, C., & Kielland-Brandt, M. C. (2000). *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. *Molecular Microbiology*, *37*(1), 108–124. https://doi.org/10.1046/j.1365-2958.2000.01968.x

Huang, S., & Czech, M. P. (2007). The *GLUT4* glucose transporter. *Cell Metabolism*, *5*(4), 237–252. https://doi.org/10.1016/j.cmet.2007.03.006

Ji, H., Lu, X., Zong, H., & Zhuge, B. (2018). Functional and expression studies of two novel *STL1* genes of the osmotolerant and glycerol utilization yeast *Candida glycerinogenes*. *The Journal of General and Applied Microbiology*, *64*(3), 121–126. https://doi.org/10.2323/jgam.2017.10.001

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., & Hattori, M. (2004). The KEGG resource for deciphering the genome. *Nucleic Acids Resources*, *32*, D277–D280. https://doi.org/10.1093/nar/gkh063

Kayingo, G., Martins, A., Andrie, R., Neves, L., Lucas, C., & Wong, B. (2009). A permease encoded by *STL1* is required for active glycerol uptake by *Candida albicans*. *Microbiology*, *155*(Pt 5), 1547–1557. https://doi.org/10.1099/mic.0.023457-0

Klein, M., Islam, Z., Knudsen, P. B., Carrillo, M., Swinnen, S., Workman, M., & Nevoigt, E. (2016). The expression of glycerol facilitators from various yeast species improves growth on glycerol of *Saccharomyces cerevisiae*. *Metabolic Engineering Communications*, *3*, 252–257. https://doi.org/10.1016/j.meteno.2016.09.001

Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *Journal of Molecular Biology*, *305*(3), 567–580. https://doi.org/10.1006/jmbi.2000.4315

Lazar, Z., Neuvéglise, C., Rossignol, T., Devillers, H., Morin, N., Robak, M., Nicaud, J.-M., & Crutz-Le Coq, A.-M. (2017). Characterization of hexose transporters in *Yarrowia lipolytica* reveals new groups of Sugar Porters involved in yeast growth. *Fungal Genetics and Biology*, *100*, 1–12. https://doi.org/10.1016/j.fgb.2017.01.001

Lee, J., Reiter, W., Dohnal, I., Gregori, C., Beese-Sims, S., Kuchler, K., Ammerer, G., & Levin, D. E. (2013). MAPK Hog1 closes the *S. cerevisiae* glycerol channel Fps1 by phosphorylating and displacing its positive regulators. *Genes & Development*, *27*(23), 2590–2601. https://doi.org/10.1101/gad.229310.113

Liu, X., Mortensen, U. H., & Workman, M. (2013). Expression and functional studies of genes involved in transport and metabolism of

glycerol in *Pachysolen tannophilus*. *Microbial Cell Factories*, *12*(1), 27. https://doi.org/10.1186/1475-2859-12-27

Lubuta, P., Workman, M., Kerkhoven, E. J., & Workman, C. T. (2019). Investigating the influence of glycerol on the utilization of glucose in *Yarrowia lipolytica* using RNA-seq-based transcriptomics. *G3: Genes|Genomes|Genetics*, *9*(12), 4059–4071. https://doi.org/10.1534/g3.119.400469

Luyten, K., Albertyn, J., Skibbe, W. F., Prior, B. A., Ramos, J., Thevelein, J. M., & 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. *The EMBO Journal*, *14*(7), 1360–1371.

Mattanovich, D., Graf, A., Stadlmann, J., Dragosits, M., Redl, A., Maurer, M., Kleinheinz, M., Sauer, M., Altmann, F., & Gasser, B. (2009). Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris*. *Microbial Cell Factories*, *8*(1), 29. https://doi.org/10.1186/1475-2859-8-29

Mollapour, M., & Piper, P. W. (2007). Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Molecular and Cellular Biology*, *27*(18), 6446–6456. https://doi.org/10.1128/MCB.02205-06

Neves, L., Oliveira, R., & Lucas, C. (2004). Yeast orthologues associated with glycerol transport and metabolism. *FEMS Yeast Research*, *5*(1), 51–62. https://doi.org/10.1016/j.femsyr.2004.06.012

Nicaud, J.-M. (2012). Yarrowia lipolytica. *Yeast*, *29*(10), 409–418. https://doi.org/10.1002/yea.2921

Oliveira, R., Lages, F., Silva-Graça, M., & Lucas, C. (2003). Fps1p channel is the mediator of the major part of glycerol passive diffusion in *Saccharomyces cerevisiae*: Artefacts and re-definitions. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1613*(1), 57–71. https://doi.org/10.1016/S0005-2736(03)00138-X

Özcan, S., & Johnston, M. (1995). Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. *Molecular and Cellular Biology*, *15*(3), 1564–1572. https://doi.org/10.1128/mcb.15.3.1564

Özcan, S., & Johnston, M. (1999). Function and regulation of yeast hexose transporters. *Microbiology and Molecular Biology Reviews*, *63*(3), 554–569. https://doi.org/10.1128/MMBR.63.3.554-569.1999

Pao, S. S., Paulsen, I. T., & Saier, M. H. (1998). Major facilitator superfamily. *Microbiology and Molecular Biology Reviews*, *62*(1), 1–34. https://doi.org/10.1128/MMBR.62.1.1-34.1998

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., & Marc, I. (2002). *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. *Journal of Applied Microbiology*, *92*(4), 737–744. https://doi.org/10.1046/j.1365-2672.2002.01577.x

Park, J. H., Saier, Jr., M. H. (1996). Phylogenetic characterization of the MIP family of transmembrane channel proteins. *The Journal of Membrane Biology*, *153*(3), 171–180. https://doi.org/10.1007/s002329900120

Park, Y.-K., & Nicaud, J.-M. (2020). Screening a genomic library for genes involved in propionate tolerance in *Yarrowia lipolytica*. *Yeast*, *37*(1), 131–140. https://doi.org/10.1002/yea.3431

Pettersson, N., Filipsson, C., Becit, E., Brive, L., & Hohmann, S. (2005). Aquaporins in yeasts and filamentous fungi. *Biology of the Cell*, *97*(7), 487–500. https://doi.org/10.1042/BC20040144

Prakash, S., Cooper, G., Singhi, S., & Saier, M. H. (2003). The ion transporter superfamily. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1618*(1), 79–92. https://doi.org/10.1016/j.bbamem.2003.10.010

Reifenberger, E., Freidel, K., & Ciriacy, M. (1995). Identification of novel HXT genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on qlycolytic flux. *Molecular Microbiology*, *16*(1), 157–167. https://doi.org/10.1111/j.1365-2958.1995.tb02400.x

Romano, A. H. (1986). Microbial sugar transport systems and their importance in biotechnology. *Trends in Biotechnology*, *4*(8), 207–213. https://doi.org/10.1016/0167-7799(86)90263-5

Ryu, S., Hipp, J., & Trinh, C. T. (2016). Activating and elucidating metabolism of complex sugars in *Yarrowia lipolytica*. *Applied and Environmental Microbiology*, *82*(4), 1334–1345. https://doi.org/10.1128/AEM.03582-15

Rywińska, A., Juszczyk, P., Wojtatowicz, M., Robak, M., Lazar, Z., Tomaszewska, L., & Rymowicz, W. (2013). Glycerol as a promising substrate for *Yarrowia lipolytica* biotechnological applications. *Biomass and Bioenergy*, *48*, 148–166. https://doi.org/10.1016/j.biombioe.2012.11.021

Saier, M. H. (2000). Families of transmembrane sugar transport proteins. *Molecular Microbiology*, *35*(4), 699–710. https://doi.org/10.1046/j.1365-2958.2000.01759.x

Saier, M. H., Reddy, V. S., Moreno-Hagelsieb, G., Hendargo, K. J., Zhang, Y., Iddamsetty, V., Lam, K. J. K., Tian, N., Russum, S., Wang, J., & Medrano-Soto, A. (2020). The Transporter Classification Database (TCDB): 2021 update. *Nucleic Acids Research*, *49*(D1), D461–D467. https://doi.org/10.1093/nar/gkaa1004

Sauer, N., Friedländer, K., & Gräml-Wicke, U. (1990). Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. *The EMBO Journal*, *9*(10), 3045–3050. https://doi.org/10.1002/j.1460-2075.1990.tb07500.x

Sutherland, F. C., Lages, F., Lucas, C., Luyten, K., Albertyn, J., Hohmann, S., Prior, B. A., & Kilian, S. G. (1997). Characteristics of Fps1-dependent and -independent glycerol transport in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, *179*(24), 7790–7795. https://doi.org/10.1128/jb.179.24.7790-7795.1997

Tamás, M. J., Luyten, K., Sutherland, F. C. W., Hernandez, A., Albertyn, J., Valadi, H., Li, H., Prior, B. A., Kilian, S. G., Ramos, J., Gustafsson, L., Thevelein, J. M., & Hohmann, S. (1999). Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Molecular Microbiology*, *31*(4), 1087–1104. https://doi.org/10.1046/j.1365-2958.1999.01248.x

The International Transporter Consortium. (2010). Membrane transporters in drug development. *Nature Reviews Drug Discovery*, *9*(3), 215–236. https://doi.org/10.1038/nrd3028

Toh, T.-H., Kayingo, G., van der Merwe, M. J., Kilian, S. G., Hallsworth, J. E., Hohmann, S., & Prior, B. A. (2001). Implications of *FPS1* deletion and membrane ergosterol content for glycerol efflux from *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *1*(3), 205–211. https://doi.org/10.1111/j.1567-1364.2001.tb00035.x

Truniger, V., & Boos, W. (1993). Glycerol uptake in *Escherichia coli* is sensitive to membrane lipid composition. *Research in Microbiology*, *144*(7), 565–574. https://doi.org/10.1016/0923-2508(93)90006-N

Walker, C., Dien, B., Giannone, R. J., Slininger, P., Thompson, S. R., & Trinh, C. T. (2021). Exploring proteomes of robust *Yarrowia lipolytica* isolates cultivated in biomass hydrolysate reveal key processes impacting mixed sugar utilization, lipid accumulation, and degradation. *BioRxiv*, *6*, 0044321. https://doi.org/10.1101/2021.04.12.439577

Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C. P., & Boles, E. (1999). Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Letters*, *464*(3), 123–128. https://doi.org/10.1016/S0014-5793(99)01698-1

Wysocki, R., Chéry, C. C., Wawrzycka, D., Hulle, M. V., Cornelis, R., Thevelein, J. M., & Tamás, M. J. (2001). The glycerol channel Fps1p

mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Molecular Microbiology*, *40*(6), 1391–1401. https://doi.org/10.1046/j.1365-2958.2001.02485.x

You, G., Smith, C. P., Kanai, Y., Lee, W.-S., Stelzner, M., & Hediger, M. A. (1993). Cloning and characterization of the vasopressin-regulated urea transporter. *Nature*, *365*(6449), 844–847. https://doi.org/10.1038/365844a0

Zhang, B., Ren, L., Wang, H., Xu, D., Zeng, X., & Li, F. (2020). Glycerol uptake and synthesis systems contribute to the osmotic tolerance of *Kluyveromyces marxianus*. *Enzyme and Microbial Technology*, *140*, 109641. https://doi.org/10.1016/j.enzmictec.2020.109641

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Erian, A. M., Egermeier, M., Marx, H., & Sauer, M. (2022). Insights into the glycerol transport of *Yarrowia lipolytica*. *Yeast*, 39, 323–336. https://doi.org/10.1002/yea.3702