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High-affinity transport, cyanide-resistant respiration, and ethanol production under aerobiosis underlying efficient high glycerol consumption by *Wickerhamomyces anomalus*

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Abstract
Wickerhamomyces anomalus strain LBCM1105 was originally isolated from the wort of *cachaça* (the Brazilian fermented sugarcane juice-derived Brazilian spirit) and has been shown to grow exceptionally well at high amounts of glycerol. This paramount residue from the biodiesel industry is a promising cheap carbon source for yeast biotechnology. The assessment of the physiological traits underlying the *W. anomalus* glycerol consumption ability in opposition to *Saccharomyces cerevisiae* is presented. A new *Wa*Stl1 concentrative glycerol-H^+ symporter with twice the affinity of *S. cerevisiae* was identified. As in this yeast, *WaSTL1* is repressed by glucose and derepressed/induced by glycerol but much more highly expressed. Moreover, LBCM1105 aerobically growing on glycerol was found to produce ethanol, providing a redox escape to compensate the redox imbalance at the level of cyanide-resistant respiration (CRR) and glycerol 3P shuttle. This work is critical for understanding the utilization of glycerol by non-*Saccharomyces* yeasts being indispensable to consider their industrial application feeding on biodiesel residue.

Keywords *Wickerhamomyces anomalus* · Glycerol metabolism · *Cachaça* · Biotechnological applications · Glycerol transport · *STL1*

Introduction

Glycerol is aby-product of biodiesel production processes. The transesterification reaction, using vegetable oils or animal fats and alcohol, produces stoichiometrically 10% of crude glycerol per weight of the oils consumed [37]. Crude glycerol contains mostly glycerol (35–50%), in addition to water (10–30%), methanol (20–50%), and sulfuric acid (5–10%), as well as small amounts of NaCl and several types of fatty acids and impurities. Actually, its composition differs depending on the alcohol/oil ratio and the catalytic agent employed [17]. In view of the global biodiesel market boost [23, 46], the worldwide production of crude glycerol is currently growing enormously. For example, in Brazil, the consumption of biodiesel was predicted to increase up to 4 billion liters per year in 2022, 40% more than in 2013 [5], which could be highly underestimated, because the percentage of biodiesel added to common diesel is also predicted to double in the same period [6]. Therefore, Brazil alone is predicted to produce by then 400 million liters of crude glycerol per year. Although glycerol is a compound that is widely used in numerous industrial processes and products and the price of glycerine (a commercial term for a sample containing more than 95% glycerol) has fallen dramatically [75], the heterogeneous and mixed nature of crude glycerol precludes its direct use in industry [1]. As a result, crude

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glycerol faces problems of proper disposal [47] and environmental impact [33] and demands acceptable solutions [17].

Using glycerol for microbial growth and the subsequent production of value-added products by single-cell factories would be ideal to improve the ecological sustainability of the biofuel industry while contributing to its economic viability [75]. Crude glycerol has been shown to be usable as a substrate by bacteria, fungi, and yeasts producing diverse chemicals and polymers (reviewed in [25]). In particular, lipolytic yeast strains from the genera of *Lipomyces*, *Rhodotorula*, and *Yarrowia* have been shown to produce single-cell fine oils [32, 34, 63], whereas the xylose consumer *Pachysolen tannophilus* produced 28.1 g L^{-1} of ethanol from crude glycerol. Therefore, the identification of new wild strains that are able to grow and/or to produce different substances of biotechnological interest from this substrate is highly justifiable.

The microflora from the spontaneous fermentation underlying the production of *cachaça* (the Brazilian sugarcane-derived spirit) is highly diverse [9, 19]. In a previous survey on the physiological traits of more than 100 different yeasts isolated from the wort of *cachaça*, a robust isolate of *Wickerhamomyces anomalus* LBCM1105 (previously LBCM105 [9]) was identified, which could grow on 25% glycerol (reagent-grade). *W. anomalus* was formerly designated as *Pichia anomala*, *Hansenula anomala*, or *Candida pelliculosa*. It is mostly known for being a wine spoilage yeast [44, 49] and has been isolated from several habitats frequently associated with spoilage or processing of food and grain products [50]. Nevertheless, under its several aliases, *W. anomalus* has been suggested as an interesting cell factory for the production of aromas for alcoholic beverages [27], biosurfactants [62], lipids [2, 61], enzymes [12, 21], and bread [41, 71]. Importantly, it has been shown to produce ethanol from glucose, sucrose, or xylose [9]. In line with this metabolic versatility, *W. anomalus* can grow on very diverse carbon or nitrogen sources, at both high and low pH, under osmotic stress/low a_w and anaerobic conditions [16].

The efficient use of a substrate is the first step to consider any microorganism as a putatively economic viable biotechnological tool [66]. This depends not only on fast and tightly regulated metabolism, but also on an efficient transport system that can generously supply progressing metabolic fluxes without *drowning* the cell. This work was aimed at a first assessment of the *W. anomalus* LBCM1105 glycerol consumption ability. A new *STL1* gene was identified, *WaSTL1*. *Wa*Stl1 was shown to work as a glycerol-H^+ symporter with more than twice the affinity of the *S. cerevisiae* counterpart, and *WaSTL1* was shown to be identically repressed by glucose and derepressed/induced by glycerol, albeit in considerably larger amounts. Moreover, the growth of *W. anomalus* on glycerol was accompanied by the aerobic production of ethanol, most probably providing redox compensation for cyanide-resistant respiration (CRR). More efficient glycerol utilization, therefore, appears to derive not only from the high-affinity character of *Wa*Stl1 permease combined with a higher expression of *WaSTL1*, but also from displaying aerobic ethanol production. The present work paves the way for a better understanding of the metabolism of *W. anomalus*, as well as of the possibility of optimizing its growth on glycerol wastes for industrial applications.

Materials and methods

Microbial strains and cultivation conditions

The microbial strains used in this study are listed in Table 1. Yeasts were grown at 30 °C on YP (1% yeast extract, 2% peptone) or YNB without amino acids (HiMedia, Mumbai, India). Amino acids were added as previously described [58]. The YNB media were prepared in a citrate/phosphate buffer (for 1 L: 614.5 mL of 0.1 M citric acid + 385.5 mL of 0.2 M Na_2HPO_4), adjusted to a pH value of 4.0 for liquid and 6.5 for solid media. Batch cultures were preinoculated

Table 1 Biological materials used in this study

Strain	Genotype	Origin
Wickerhamomyces anomalus:		
LBCM1105 wt	Isolate from *cachaça* wort	Conceição et al. [9]
Saccharomyces cerevisiae:		
BY4741 wt	*MATa*; *ura3Δ0*; *leu2Δ0*; *his3Δ1*; *met15Δ0*	Euroscarf
BY4741 *Δstl1*	BY4741; *MATa*; *ura3Δ0*; *leu2Δ0*; *his3Δ1*; *met15Δ0*; YDR536w::kanMX4	Euroscarf
BY4741 *Δstl1* pCevhph	BY4741; *MATa*; *ura3Δ0*; *leu2Δ0*; *his3Δ1*; *met15Δ0*; YDR536w::kanMX4; pCev-G2-Km-hph	This study
BY4741 *Δstl1* pCev*WaSTL1*	BY4741; *MATa*; *ura3Δ0*; *leu2Δ0*; *his3Δ1*; *met15Δ0*; YDR536w::kanMX4; pCev-G2-Km-hph-*WaSTL1*	This study
E. coli:		
TOP10	*F-mcrAΔ(mrr-hsdRMS-mcrBC)φ80lacZΔM15 ΔlacX74 nupG recA1araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-*)	Invitrogen (NL)

into 20 mL of YNB or YP and 0.3% glucose for 12 h, centrifuged, and then washed in a citrate/phosphate buffer and resuspended in 5 mL. This was used to set OD 0.1 inoculum in a 50 mL medium with 3, 5, 10, or 20% glycerol or 2% glucose, at an air/liquid ratio of 5:1. Incubation took place at 30 °C with 200 rpm orbital shaking. Growth was quantified by measuring OD_{600nm}. *Escherichia coli* TOP10 was maintained in a Luria–Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) and grown at 37 °C with 200 rpm orbital shaking. Solid media had 2% agar. All concentrations mentioned in % correspond to w/v.

High-performance liquid chromatography (HPLC) analysis

Glycerol and ethanol in the growth media were quantified using HPLC (Shimadzu, Kyoto, Japan), equipped with an autosampler (SIL-20ACHT, Shimadzu), a binary pump (LC-20AD, Shimadzu), a column oven (CTO-20A, Shimadzu), a refractive index detector (RID-10A, Shimadzu), and a 300 × 7.8 mm, 9 µm particle-size column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA). A mobile phase of 2.5 mM H_2SO_4 was used. Runs were performed at 55 °C for 30 min at a flow rate of 0.6 mL min^{-1}. The injection volume for all samples was 30 µL. Samples of yeast culture (1.5 mL) were collected and centrifuged (13,000 rpm, 3 min), and then, the supernatant was stored at − 20 °C. Prior to injection, the samples were filtered through a 0.22 µm filter and diluted appropriately in ultrapure water.

General molecular biology procedures

Yeast genomic DNA was extracted as previously described [35]. Polymerase chain reaction (PCR) was performed using a T100 thermocycler (Bio-Rad, CA, USA) and GoTaq® DNA Polymerase (Promega, Madison, WI, USA) according to the manufacturer's protocol. Primers (Table 2) were synthesized by Integrated DNA Technologies (IDT Corporation, Newark, NJ, USA). Yeast strains were transformed using the lithium acetate–polyethylene glycol method as previously described [10] and *E. coli* by $CaCl_2$/heat-shock-based protocol [3]. PCR products were purified and sequenced using capillary electrophoresis (Sanger's method) using the ABI3130 platform from Life Technologies (Myleus Biotecnologia, Belo Horizonte, MG, Brazil). Plasmids from *E. coli* were purified using a Wizard®Plus SV Minipreps DNA Purification System (Promega, WI, USA).

Amplification of the *STL1* Gene in *W. anomalus*

Degenerate primers for *STL1* (Table 2) were designed according to the conserved amino acid sequences of the Stl1 predicted proteins from the following NCBI accession numbers: *S. cerevisiae* P39932, *Millerozyma farinosa* XP_004195829.1, *Candida albicans* EEQ 46634, and *Scheffersomyces stipitis* ABN65745. The amplified fragment was purified, sequenced, and compared in silico against the genome of *W. anomalus* LBCM1105 available at CTBE (Brazilian Bioethanol Science and Technology Laboratory, Campinas, SP, Brazil) [53, 57]. The obtained chromosomal alignment of *WaSTL1* was used to design specific primers (Fw_*WaSTL1* and Rv_*WaSTL1* in Table 2), which allowed the amplification of the full open-reading frame (ORF).

In silico analysis of *Wa*Stl1p

The comparative analysis of amino acid sequences from *Wa*Stl1p and *Sc*Stl1p was performed using SnapGene®

Table 2 Primer sequences used in this study

Primers	Sequence (5′–3′)
Fw_*WaSTL1*_degen	GGTTATGATCAAGGT
Rv_*WaST1*_degen	ACCAGCAGTTTCTGG
Fw_*WaSTL1*	ATGGGTTTTGATTGGAAAT
Rv_*WaSTL1*	TTAAACATTATCATCACCTTTTTTAAC
Fw_hph_ho_pCev	AACTTCGTATAATGTATGCTATACGAAGTTATTAGGTGATGTGGTCGGCTGGAGATCGG
Rv_hph_ho_pCev	TTCTCAGGTATAGCATGAGGTCGCTCCTAGTGGATCTGATCAGTATAGCGACCAGCATTC
Fw_*WaSTL1*_ho_pCev	AGGATCCGTAATACGACTCACTATAGGGCCCGGGCGTCGAATGGGTTTTGATTGGAAATC
Rv_*WaSTL1*_ho_pCev	TCGAGGTCTTCTTCGGAAATCAACTTCTGTTCCATGTCGATTAAACATTATCATCACCTT
Fw_*ACT1*_qPCR	CGTCTGGATTGGTGGTTCT
Rv_*ACT1*_qPCR	GTGGTGAACGATAGATGGAC
Fw_*ScSTL1*_qPCR	CAAGGGTTGATGGCAAGTCT
Rv_*ScSTL1*_qPCR	CCTGCACTACAGTTGCGTGT
Fw_*WaSTL1*_qPCR	ACCGCCACTATTCCAGTTTG
Rv_*WaSTL1*_qPCR	TGAACCTTCCAAGTTGACCA

software Version 2.3.2 (Chicago, IL, USA) software. The "alternative yeast nuclear" codon usage was assumed for *W. anomalus*. The amino acid sequence alignment was performed by Clustal (using default parameters). Several servers were used to predict the hydropathy profile and therein the topology of *Wa*Stl1p putative transmembrane nature: HMMTOP 2.0 (Hidden Markov Model for Topology Prediction) [68], available at http://www.enzim.hu/hmmtop/; TMHMM v.2.0 (membrane protein topology prediction method, based on a hidden Markov model) [26], available at http://www.cbs.dtu.dk/services/TMHMM/; PredictProtein (online prediction of proteins' structural and functional features) [74], available at http://www.predictprotein.org/; HHpred (a server for protein remote homology detection and 3D structure prediction) [76], available at https://toolkit.tuebingen.mpg.de/#/tools/hhpred; PSIPRED (Protein Sequence Analysis Workbench) [7], available at http://bioinf.cs.ucl.ac.uk/psipred/?memsatsvm=1. Ortholog prediction was performed using egg NOG 4.5 [22], available at http://eggnogdb.embl.de/#/app/home.

Vector construction and yeast transformation

The pCEV-G2-Km plasmid was a gift from Lars Nielsen and Claudia Vickers (Addgene plasmid #46815) [70]. This plasmid has *TEF1* and *PGK1* promoter-controlled expression cassettes associated with geneticin (G418) resistance. To add hygromycin (hph) resistance, the corresponding cassette hphMX6 was amplified from the pJET1,2-attB-hph-attP plasmid [72] using Fw_hph_ho_pCev and Rv_hph_ho_pCev primers (Table 2). An amplicon was used to transform *S. cerevisiae* BY4741 by homologous recombination together with *Eco*RV digested pCEV-G2-Km [10], and pCevhph was obtained. The same strategy was used to generate pCev*WaSTL1*, but pCEV-G2-Km was digested with *Eco*RV and *Sal*I instead. *WaSTL1* was amplified with specific primers (primers Fw_*WaSTL1*_ho_pCev and Rv_*WaSTL1*_ho_pCev in Table 2). Transformants were selected for resistance to 500 µg mL^{-1} hygromycin (Cayman Chemical, Ann Arbor, MI, USA). Figure 1 illustrates the strategy used for plasmid construction. Plasmids were rescued from yeasts as described by Singh and Weil [60] and then amplified in *E. coli* according to the standard procedures [3]. Transformants were selected in 100 µg mL^{-1} ampicillin (MIDSCI, Valley Park, MO, USA). The correctness of the constructions was checked by PCR; restriction analysis using *KpnI*, *BamHI*, and *SpeI* enzymes; sequencing.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mL of a yeast culture in the middle log growth phase. The cells were centrifuged and the total RNA was extracted using the phenol/chloroform method. The concentration of each RNA sample was measured using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of RNA and cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 0.1 µg of each total RNA sample was reverse-transcribed using random primers and MultiScribe™ Reverse Transcriptase according to the manufacturer's instructions and then stored at −20 °C. Prior to the cDNA synthesis, a DNase treatment was performed using RQ1 RNase-free DNase according to the manufacturer's instructions (Promega).

RT-qPCR procedures

RT-qPCR was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA) using the SYBR Green Master Mix Kit (Applied Biosystems, CA, USA). For each reaction of a 20 µL final volume, 10 µL of SYBR Green Master Mix 2X, 1 µL of both oligonucleotides (10 µM Fw/Rv), 6 µL of DEPC water, and 2 µL of cDNA were used. The plate was sealed and centrifuged. The reaction proceeded as follows: one cycle of 2 min at 50 °C, one cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, and one cycle of 1 min at 60 °C. The primers used are listed in Table 2. The tests for amplification efficiency and validation of the experiments were performed as described previously [52]. The expression level of the *STL1* target gene was normalized according to the constitutive *ACT1* gene. For each sample (in triplicate); the value of the quantitative relative expression in 5% glycerol was determined as $2^{-\Delta\Delta C_t}$ regarding the *STL1* gene with reference to BY4741 or LBCM1105 expression in 2% glucose.

Measurement of glycerol initial uptake rates

Glycerol transport was characterized as previously described [28, 29, 36] with some adaptations. Radiolabeled [^{14}C]glycerol (Amersham, Freiburg, Germany) was used. Cells were harvested at the exponential growth phase, double-washed with ultrapure water, washed again with 100 mM Tris/citrate buffer (pH = 5.0), and then resuspended in the same buffer at a final concentration of ~ 10 mg mL^{-1} (dry weight). The glycerol initial uptake rates of 10 s were determined in the same buffer. The glycerol concentrations ranged from 0.05 to 5 mM, and the radiolabel-specific activities (SAs) ranged from 30 to 60 GBq·mol^{-1}. All assays were carried out in duplicate. To test the active nature of *Wa*Stl1-mediated glycerol transport and its dependence on the proton motive force (p.m.f.), cells were incubated for 10 s on a 4 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) ionophore prior to the transport assay. Under these conditions, transport was assayed using 0.2, 2, and 5 mM [^{14}C]glycerol. The

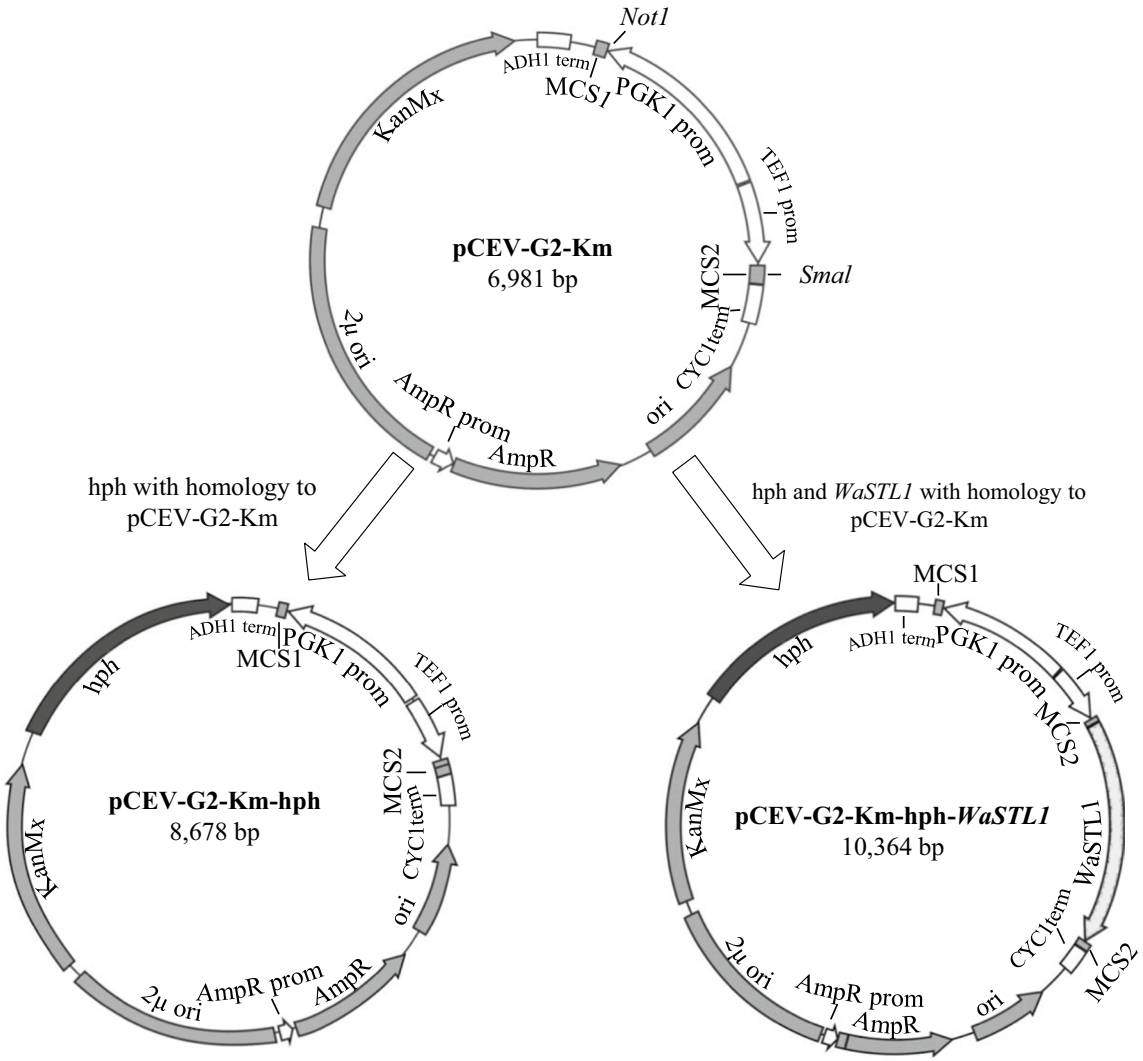

Fig. 1 A diagram illustrating the plasmid construction. hph stands for hygromycin-B-phosphotransferase protein conferring resistance to hygromycin, whereas KanMx stands for kanamycin resistance

concentrative nature of the transporter was confirmed by measuring the radiolabeled glycerol accumulation ratio as previously described [29]. A single concentration of 500 mM [^{14}C]glycerol with an SA of 2000 GBq·mol^{-1} was used. The generally accepted value for intracellular volumes in yeasts [2 µL·mg^{-1} dry weight] was assumed to estimate the therein glycerol intracellular molarity. The same concentration of CCCP as above was used to counteract the Stl1-driven glycerol accumulation, causing the efflux of nonmetabolized glycerol from the cell. Radioactivity was measured in a Beckman LS6000 scintillation counter.

Statistical analysis and reproducibility

An analysis of variance (ANOVA), Tukey's test, *t* test, linear regression, and nonlinear fit of Michaelis–Menten kinetic curves were performed using Prism 6.01 software (GraphPad Software, La Jolla, CA, USA). Results were considered statistically significant when $p < 0.05$. The results of glycerol transport and consumption, growth in liquid media, and RT-qPCR derived from at least two independent assays, each one performed in duplicate.

Results and discussion

Assessment of *W. anomalus* as a glycerol-metabolizing strain

Wickerhamomyces anomalus strain LCBM1105 (formerly *Pichiaanomala, Hansenula anomala* and *Candida pelliculosa*) was isolated from the Brazilian *cachaça* wort and

identified in a previous survey as an excellent glycerol consumer that is able to grow on solid YP with up to 25% glycerol [9]. In this study, growth on liquid YP or YNB with increasing glycerol concentrations up to 10% was assessed. Specific growth rates were statistically invariant ($0.2\,h^{-1}$) on YNB and YP up to 5% glycerol. The highest μ_g of $0.28\,h^{-1}$ was obtained in YP with 10% glycerol (Fig. 2a). This is compatible with the supposedly osmophilic nature of *W. anomalus* [16]. *S. cerevisiae* wild-type (WT) BY4741 under the same conditions grew much slower: $\mu_g = \pm 0.1\,h^{-1}$ in either YNB or YP. *S. cerevisiae* is known to be an extremely poor

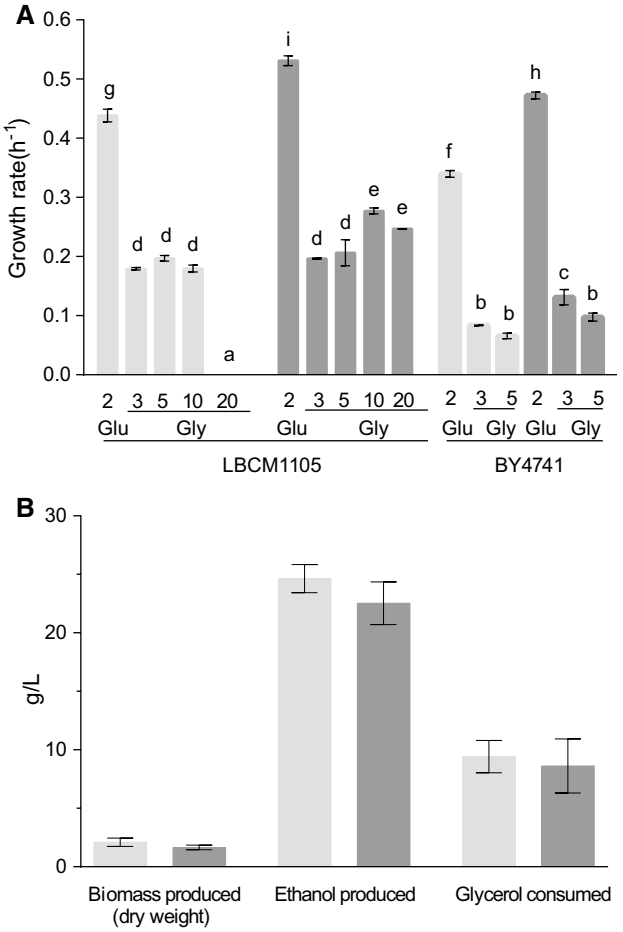

Fig. 2 Growth of *W. anomalus* LBCM1105 and *S. cerevisiae* BY4741 on glycerol. **a** Batch cultures with an air/liquid ratio of 5:1 and different concentrations of glycerol (% w/v) were used to estimate the specific growth rate during the log phase. Controls in 2% glucose are also presented. Cells were grown in YNB (light-gray bars) or YP (dark-gray bars).The results represent the average and standard deviation of at least two independent assays. Different letters represent a statistically significant difference with $p < 0.05$ (Tukey's test). BY4741 was not able to grow in 10% glycerol. (B) Biomass and ethanol production and glycerol consumption after 72 h of culture in YP with 3% glycerol (light-gray bars) or YP with 5% glycerol (dark-gray bars). The results represent the average and standard deviation of at least two independent assays. There was no statistical difference between the results obtained in each medium

grower on YNB glycerol, demanding nitrogen-rich supplements like peptone or glutamic acid [39]. Some strains do not grow on glycerol at all. The most recurrent reason pointed out in the literature for this inability is NAD(H) redox imbalance, caused at the level of glycerol 3P shuttle between the cytosol and the mitochondria ($Gpd1/Gut2_{mit}$) [31]. In *S. cerevisiae*, the operational glycerol assimilation pathway involves the glycerol permease Stl1, the glycerol kinase Gut1, and the glycerol 3P shuttle inner mitochondrial membrane glycerol 3P dehydrogenase $Gut2_{mit}$ [43]. Nevertheless, there is no consensus regarding the causes underlying the difference between *S. cerevisiae* and other yeast species that, otherwise, can grow well on glycerol. Attempts to randomly evolve nonuser strains into users were accomplished, but they were often unstable and easily reverted [39, 45, 67]. Reciprocal hemizygosity analysis between a bad and a good glycerol consumer suggested that only Gut1 activity governs the glycerol growth ability [65]. Surprisingly, in another study using the same method, two apparently glycerol-unrelated *loci* were found to improve the yeast's ability to grow on glycerol, the *SSK1* encoding an osmoregulation signaling effector, and *UBR2* encoding a component of the ubiquitin ligase complex [64]. Why and how these proteins are able to promote growth on glycerol are presently unknown, and neither *SSK1*- or *UBR2*-like genomic sequences could be found in the *W. anomalus* genome [53, 57]. These authors further expressed in *S. cerevisiae* an allele from the glycerol channel *FPS1* originating from a good glycerol-consuming yeast [64], obtaining a maximum μ_g on glycerol in a minimal medium of $\pm 0.1\,h^{-1}$ μ_g of the best yeast glycerol growers, assessed in YNB 2% glycerol [30], was generally at least 2.5 × higher, and specifically that of *W. anomalus* (PYCC4121) was $0.29\ h^{-1}$, identical to μ_g obtained in the present work in YP 10% glycerol.

In the present work, yeasts were cultivated at 30 °C. This temperature was chosen in view of the environmental conditions of the origin of the *W. anomalus* strain LBMC1105 [9]. Nevertheless, *W. anomalus* apparently prefers lower temperatures [30]. Survival for 4 weeks on a malt extract medium of one strain has been reported to be best at 10 °C [38]. Noticeably, glycerol is used by yeast cells for many purposes, including high-temperature stress resistance [59, 73]. It is, therefore, not uncalled for that glycerol utilization at higher temperatures might yield less biomass and a slower μ_g, because the cell needs to maintain proper intracellular glycerol levels [59, 73]. In *S. cerevisiae*, this response concomitantly promotes the alleviation of glucose repression over the glycerol permease gene *STL1* [14].

The production of biomass (dry weight) and the corresponding amount of glycerol consumed were quantified in *W. anomalus* cultured on YP with 3 and 5% glycerol (Fig. 2b). The values were statistically identical ($p < 0.05$) according to the *t* test under both conditions; at 5% glycerol,

a large amount of substrate was left unconsumed (2.8%). This suggests that, although da Conceição et al. [9]. showed that *W. anomalus* LCBM1105 is able to grow well on solid media with different glycerol concentrations ranging from 2 to 25%, on a liquid medium, the efficiency of glycerol utilization is mostly lost above 3%. In addition, the chromatographic analysis of growth media revealed the presence of ethanol (Fig. 2b). Identically to the biomass produced and glycerol consumed, the ethanol produced was no different in 3 or 5% glycerol (*t* test, $p < 0.05$). Considering the theoretical conversion of glycerol to ethanol ($C_3H_6O_3 \rightarrow C_2H_5OH + CO_2$), only a very small fraction of glycerol was probably used to produce ethanol, around 8.5% of the total glycerol consumed. Therefore, glycerol fermentation occurs but appears to be residual.

Glycerol can be anaerobically fermented by several bacterial species, producing ethanol, other alcohols, formate, or polyols [8]. Yeasts are, otherwise, able to utilize glycerol as a carbon source only aerobically [4]. Glycerol consumption in *S. cerevisiae* occurs through the intermediate glycerol 3-phosphate and supplies mitochondria with reduced $FADH_2$ derived from the activity of $Gut2_{mit}$, part of the abovementioned shuttle that couples the cytosolic and mitochondrial redox pools [31]. *W. anomalus*, unlike *S. cerevisiae*, is a Crabtree-negative yeast; therefore, it is incapable of aerobic fermentation of glucose [69]. It shares with other yeasts of the cyanide-resistant respiration (CRR) chain that does not have complexes III and IV and is, therefore, resistant to antimycin-A and cyanide [69]. An alternative cyanide-insensitive ubiquinol oxidase, Aox, already identified in *W. anomalus* [56] performs O_2 fixation [55]. The operation of this alternative respiratory chain has been suggested to generate a weaker p.m.f. and therein lower ATP production. In *W. anomalus*, glucose represses both the metabolic flux through this alternative pathway [40] and the expression of *AOX* [56]. Accordingly, growth on glycerol should allows the alleviation of repression over *AOX*, promoting the functioning of the CRR. There is no information available as to whether *W. anomalus* shares with *S. cerevisiae* the same steps of glycerol assimilation. That being said, the function of Gut2 would be important to supply mitochondria with $FADH_2$ needed for the function of complex II, this way contributing to increasing the efficiency of respiration in comparison to glucose utilization. Moreover, although, in plants, it has been suggested that CRR promotes the dissipation of unchanneled redox potential as heat [42], in yeasts, there is no evidence supporting that hypothesis. Instead, the present work's finding of ethanol in *W. anomalus* glycerol cultures suggests that the NADH surplus generated by CRR could be reoxidized by producing ethanol. The residual nature of the putative glycerol/ethanol conversion suggested above is consistent with this possibility. Moreover, the expression of an alcohol dehydrogenase [11] could be rate-limiting the amount of glycerol that can be consumed, causing a halt of glycerol consumption at ≥ 3% as described above. Any attempt to improve *W. anomalus* glycerol consumption for biotechnological purposes will need to take this into consideration.

Identification and sequencing of *WaSTL1*

The good performance of *W. anomalus* on glycerol could, at least in part, derive from the efficiency of the systems ensuring glycerol supply to the cell. An active permease operating as a glycerol/H^+ symporter was primordially described in *S. cerevisiae* [29, 48] and the gene identified as *STL1* [15], a member of the HXT superfamily. Subsequently, this permease was also characterized in *C. albicans* [24], *Debaryomyces hansenii* [51], and Zygosaccharomyces rouxii [13]. Other yeast species whose genome has been sequenced display *STL1*-like sequences (NCBI Database), although the corresponding protein function has not been confirmed. Stl1 proteins share a high degree of identity, with large parts of the amino acid sequence being conserved. The alignment of Stl1 from *S. cerevisiae* S288c (AJV19167), *C. albicans* WO-1 (EEQ 46634), *Millerozyma M. farinosa* CBS7064 (CCE85634), and *Scheffer somyces S. stipitis* CBS6054 (XP_001383774) was used to design degenerate primers covering, on the basis of *S. cerevisiae*, the region between the 43rd and the 478th amino acid (Fig. 3). The resulting *W. anomalus* amplicon of 1287 bp was sequenced and observed to align with the genome available at CTBE (Bioethanol Science and Technology National Lab., Campinas SP, Brazil) [53, 57], allowing the identification of a putative ORF with 1686 bp. Nucleotide BLAST analysis of this DNA sequence revealed 99% identity with a hypothetical ORF XM_019185120 from *W. anomalus* strain NRRL Y-366-8, and 87% identity with the *STL1* from *W. ciferrii* (CCH43320). Both genes putatively encode proteins that are considered members of the sugar transporters superfamily like *S. cerevisiae* Stl1. In silico translation of the *W. anomalus* LCBM1105 amplicon yielded a sequence of 561 amino acid residues with the highest identity and similarity to *C. albicans* Stl1 [72% identity (372/520) and 84% similarity (441/520)], followed by *S. stipitis* [71% identity (371/524) and 83% similarity (438/524)] and *S. cerevisiae* [61% identity (317/521) and 77% similarity (403/521)]. The alignment of these four Stl1 proteins clearly shows a high degree of conservation (Fig. 3). The Stl1 referred to above from *Z. rouxii* (CAR30743), *D. hansenii* CBS767 (CAG85514), and *M. farinosa* shares with *Wa*Stl1 an increasingly less identity, 58, 40, and 39%, respectively.

*Wa*Stl1 was subsequently analyzed in silico. In general, transporters like Stl1 display 10–12 hydrophobic α-helix domains that should correspond in vivo to transmembrane domains (TMDs) of the protein [54]. Several servers were

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ScSTL1      MKDLKLSNFKGKFISRTSHWGLTGKKLRYFITIASMTGFSLFGYDQGLMASLITGKQFNY
WaSTL1      ------MG-FDW-KSRTNTSGLSGRALRVSVTLTATLGFSLFGYDQGLMSGLITGHEFNY
CaSTL1      -----MGGFIDNIFKRTTTAGLTGRKLRAAVTITATLGFSLFGYDQGLMAGLISAEQFNW
SsSTL1      ------MAYLDWLTARTNTFGLRGKKLRAFITVVAVTGFSLFGYDQGLMSGIITADQFNS
                      .    **.  ** *: **  :*:.:  ************:.:*:..:**

ScSTL1      EFPATKENGDHDRHATVVQGATTSCYELGCFAGSLFVMFCGERIGRKPLILMGSVITIIG
WaSTL1      EFPATKGN-------SVNQGAVTACYEIGCFFGALFAMFRGERIGRRPLIILGAFIIIIG
CaSTL1      EFPATKDN-------SVIQGAVTASYELGCFFGAIFALLRGDALGRKPIIFFGATIIILG
SsSTL1      EFPATRNN-------STIQGAVTSCYELGCFFGAVFALLRGERIGRRPLVLCGSLIIILG
            *****: *       :. ***.*:.**:*** *::*.:: *: :**:*::: *: * *:*

ScSTL1      AVISTCAFRGYWALGQFIIGRVVTGVGTGLNTSTIPVWQSEMSKAENRGLLVNLEGSTIA
WaSTL1      TLISVTAYRDDWGTGQFVVGRVITGIGNGMNTATIPVWQSEMSKPEKRGMLVNLEGSVVA
CaSTL1      TIISVTPFRPHWPLGQFVVGRVITGIGNGMNTATIPVWQSEMSKPENRGKLVNLEGAVVA
SsSTL1      TVISVTAFHPHWSLGQFVIGRVITGIGNGMNTATIPVWQSEMSRAENRGRLVNLEGSVVA
            ::**.  ::  *  ***:***:**:*.*:**:**********: *:** ******:.:*

ScSTL1      FGTMIAYWIDFGFSYTNSSVQWRFPVSMQIVFALFLLAFMIKLPESPRWLISQSRTEEAR
WaSTL1      IGTFVAYWVDFGMSYVNNSSQWRFPVAFQIFFAFFVMIGALQLPESPRWLLSKGRREEAV
CaSTL1      FGTFIAYWLDFGLSYVDSSVSWRFPVAFQIFFALWVIFGIIQLPESPRWLISKDRKPEAF
SsSTL1      VGTCIAYWLDFGLSYVDNSVSWRFPVAFQIVFASVLFVGMLQLPDSPRWLVANHRRAEAL
            .** :***:***:**.:.* .*****::**.**  ::   ::**:*****::: *  **

ScSTL1      YLVGTLDDADPNDEEVITEVAMLHDAVNRTKHEKHSLSSLFSRGRSQNLQRALIAASTQF
WaSTL1      FVLAALNDVPIDDEGVDEEATAIQDAVEKVSKKQISIKEVFTGGKTQHFRRMLIGSSTQF
CaSTL1      EVLAALNDTTPDDDAIVAEASVIIDAVRRNAKVQAGFKDLFTGGKTAHFQRMLIGSSTQF
SsSTL1      QVLSALKDLPEDDEEILNEAEVIQESVDKFA-GHASVKEVFTGGKTQHWQRMVIGSSTQF
             ::.:*.*   :*: :  *.  : ::* :    : ....:*: *:: : :* :*.:****

ScSTL1      FQQFTGCNAAIYYSTVLFNKTIKL--DYRLSMIIGGVFATIYALSTIGSFFLIEKLGRRK
WaSTL1      FQQFTGCNAAIYYSTVLFEQTIGL--TGKLPLILGGVFATIYALSTIPSFFLIEKLGRRN
CaSTL1      FQQFTGCNAAIYYSTLLFYETVFHHTKYRLSMILGGVFATIYALATLPSFFLIDTLGRRN
SsSTL1      FQQFTGCNAAIYYSTVLFQDTIGL--ERRMALIIGGVFATVYAIFTIPSFFLVDTLGRRN
            ***************:** .*:      :: :*:******:**: *: ****::.****:

ScSTL1      LFLLGATGQAVSFTITFACLVKENKENARGAAVGLFLFITFFGLSLLSLPWIYPPEIASM
WaSTL1      LFLIGATGQGCSFIITFGCLVKDTTQNAKGAAVGLFLFIVFFAFTILPLPWVYPPEINPL
CaSTL1      LFLIGAIGQGISFLISFACLINPTEQNAKGAAVGIYLFIVFFAFTILPLPWIYPPEINPL
SsSTL1      LFLIGAMGQGIAFTITFACLIDDTENNAKGAAVGLFLFICFFAFTILPLPWVYPPEINPL
            ***:** **. :* *:*.**:. . :**:*****::*** **.:::* ***:*****  :

ScSTL1      KVRASTNAFSTCTNWLCNFAVVMFTPIFIGQSGWGCYLFFAVMNYLYIPVIFFFYPETAG
WaSTL1      RTRTVASAISTCTNWLTNFAVVMFTPVFIANARWGCYLFFAVMNFIFVPIIFFFYPETAG
CaSTL1      RTRTTASAVSTCTNWLTNFAVVMFTPPFISASGWGCYLYFAVMNFLFVPIIFFFYPETAG
SsSTL1      RTRTIASAISTCTNWICNFAVVMFTPVFVTNTRWGAYLFFAVMNFLFVPIIFFFYPETAG
            :.*: :.*.******: ********* *:  : **.**:*****:::*:**********

ScSTL1      RSLEEIDIIFAKAYEDGTQPWRVANHLPKLSLQEVEDHANALGSYDDEM-EKEDFGEDRV
WaSTL1      RSLEEMDIMFAKAHVEKKPVWRVAQTMPKMNHREVERAAVELGLFDEIEFQNDGFDDKAS
CaSTL1      RSLEEIDIIFAKAYVEKRQPWRVAATLPKLSLQEVEDESKSLGLYDDD-FEKDNFETKED
SsSTL1      RSLEEIDIIFAKAFVDKRQPWRVAATMPKLSNHEIEDEANRLGLFDDGTFDKEAFETKEN
            *****:**:****. :    ****  :**:. :*:*  :  ** :*:   ::: *  .

ScSTL1      EDTYNQINGDNSSS-SSNIKNEDTVNDKANFEG----------
WaSTL1      A-----SIPDAEKEGTSGTDTPDSQENNNNHSGLFVKKGDDNV
CaSTL1      I-----SDGTASNS----------------NGVFEKPENV--
SsSTL1      A-----SSSS--------------------------------
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◂**Fig. 3** *Wa*Stl1 amino acid sequence alignment. *W. anomalus* LBCM1105-*WaSTL1* (AKU75155.2) was reverse-translated in silico and aligned against with the Stl1 proteins from *S. cerevisiae* S288C-*ScSTL1* (AJV19167.1), *C. albicans* WO-1-*CaSTL1* (EEQ 46634.1), and *S. stipitis* CBS6054-*SsSTL1* (XP_001383774). Alignment was performed at https://pir.georgetown.edu/pirwww/search/multialn.shtml using ClustalWat its default settings. The underlined amino acids are the positions corresponding to the Fw- and Rv-degenerated primers

used (see "Materials and methods"), all of which predicted, identically to *Sc*Stl1, that the protein locates at the plasma membrane and that it has 11 or 12 TMDs. Hence, the sequence identity, the hydropathy profile analysis, and the predicted subcellular location strongly suggest that the *W. anomalus* amplicon corresponds to an *S. cerevisiae* Stl1 ortholog. The probability of orthology was estimated using eggNOG 4.5 [22], and it was very high for the pairs *Wa*Stl1/*Sc*Stl1 (*e* value = 1.18×10^{-260}), *Wa*Stl1/*S. stipitis* Stl1 (*e* value = 1.25×10^{-293}), and *Wa*Stl1/*Yarrowia lipolytica* Stl1p (*e* value = *e value* 1.9×10^{-245}). The *WaSTL1* genomic sequence was recorded at GenBank under the accession number KP856970.2, as well as in the Transporter Classification Database (TCDB) under the number 2.A.1.1.137. The latter included *WaSTL1* in the Major Facilitator Superfamily (2.A.1); that is, in the group of electrochemical potential-driven symporters. The following results confirm the appropriateness of this inclusion.

Complementing *S. cerevisiae Δstl1* with *WaSTL1*

STL1 from *W. anomalus* was expressed in *S. cerevisiae* (BY4741 *Δstl1*). This should provide evidence on the function of permease in a heterologous environment, while simultaneously improving *S. cerevisiae*'s glycerol consumption ability through improved uptake in the recipient yeast. To accomplish this, specific primers designed to amplify the *WaSTL1* complete ORF and hph cassette (Table 2) were used to obtain two amplicons that were inserted in the pCev-G2-Km (Table 1, Fig. 1), and *WaSTL1* was inserted in frame with the *TEF1* strong promoter. The construction (pCev*WaSTL1*) was verified by PCR amplification, digestion, and sequencing. After confirmation, the plasmid was used to transform *S. cerevisiaeΔstl1* (Table 1). Growth on glycerol should provide a rough indirect assessment of the heterologous expression of the glycerol transporter. Therefore, transformants and controls (untransformed *S. cerevisiae* and *W. anomalus* WT strains) were grown on YNB with 5% glycerol (reagent-grade) as the sole carbon and energy source (Fig. 4). Antibiotic resistance was kept to ensure plasmid maintenance. Results show, as expected [9], that *W. anomalus* grows better than *S. cerevisiae* [15], although the strain used in the present work, BY4741, is different from the ones assessed before in that regard. On the other hand, the *S. cerevisiae Δstl1* mutant, alone or harboring an empty plasmid, did not grow on glycerol, whereas the transformant recovered the ability to grow on glycerol, although it took longer to resume growth. This suggests that the construction ensures the appropriate expression of the gene. To verify this, all these strains/conditions were used to quantify *STL1* expression by RT-qPCR using *ACT1* as a reference gene. In all cases, cells were collected in the log phase. The results (Fig. 5) show that, in *W. anomalus*, as reported for *S. cerevisiae* [15], glucose exerts a repressive effect over the expression of *STL1*, whereas glycerol acts as an inducer. Moreover, according to the results from growth on glycerol observed in liquid media (Fig. 4), the expression of *STL1* in *W. anomalus* is almost twice that in *S. cerevisiae*, suggesting a crucial role of the transporter in supplying a metabolic flux. The expression of *WaSTL1* in *S. cerevisiae* Δ*stl1* appears to be slightly lower than in identically cultivated WT *S. cerevisiae*, but these results were actually not statistically different. The higher affinity of the *Wa*Stl1 compared to *Sc*Stl1 was, therefore, not enough per se to ensure a significantly better growth of the recipient *S. cerevisiae* on glycerol.

*Wa*Stl1 glycerol transport

To characterize the mechanism of *Wa*Stl1-mediated glycerol transport, radiolabeled glycerol uptake was quantified using the well-established methodologies [29, 48]. In all yeasts where Stl1 was studied in detail, this transporter was shown to operate as an H^+ symporter, a saturable active permease. Simultaneously, glycerol can permeate through the Fps1 channel. Therefore, glycerol uptake kinetics present two branches: one corresponding to the saturable, enzyme-like active transport and the other to the nonsaturable passive diffusion of glycerol through the open channel, following a full equation corresponding to $V = V_{max} \cdot [S]/(K_m + [S]) + K_d \cdot [S]$ [48]. As shown above, the *S. cerevisiae* WT and transformed strains were grown on YNB with 5% glycerol. To promote the growth of the deleted strain, 0.3% glucose was added. Cultures were collected in the log phase and used to assay the initial uptake rates of radiolabeled glycerol (Fig. 6). The data from the WT and transformant strains followed the full equation, whereas the *S. cerevisiae* Δ*stl1* mutant strain presented only the second component of linear diffusion as expected. The estimated value of the corresponding K_d constant was $0.0043 \pm 0.0001\ L \cdot h^{-1} \cdot g^{-1}$ ($p < 0.0001$), a value similar to the previously reported one with the other genetic backgrounds [15, 29]. This was used to iterate the data from the strains displaying the full equation, allowing the calculation of the kinetic constants of the active transport component. The *S. cerevisiae* WT strain presented K_m of 2.26 ± 0.22 mM, slightly higher than the previously determined value in different *S. cerevisiae* genetic backgrounds [15, 29], and V_{max} of $527.4 \pm 25.91\ \mu mol \cdot h^{-1} \cdot g^{-1}$

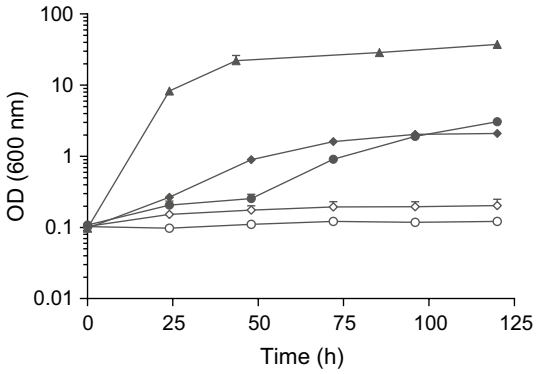

Fig. 4 Ability of the recombinant yeasts to grow on glycerol. Cultures of *S. cerevisiae* BY4741 WT (filled circle), Δ*stl1* (open circle), Δ*stl1* pCevhph (open diamond), Δ*stl1* pCev*WaSTL1* (filled diamond), and *W. anomalus* LBCM1105 (filled triangle) in YNB with 5% glycerol. The results represent the average and standard deviation of two independent experiments performed in duplicate. The bars represent the standard deviation of the mean

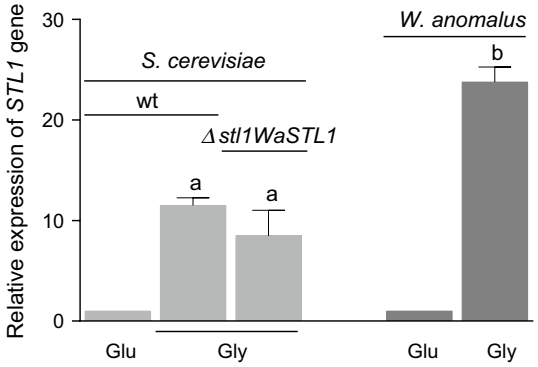

Fig. 5 Quantification of *STL1* mRNA by RT-qPCR. Results of the expression of *STL1* on *S. cerevisiae* BY4741 WT cultivated on 2% glucose or 5% glycerol, as well as Δ*stl1* harboring pCev*WaSTL1* cultivated on 5% glycerol (gray bars), and on *W. anomalus* LBCM1105 (dark bars), grown on 2% glucose or 5% glycerol. Cells were collected in the mid-log phase. RT-qPCR was performed using *ACT1* as an expression reference. The results represent the average and standard deviation of at least two independent assays. Different letters represent a statistically significant difference with $p < 0.05$ (Tukey's test)

($r^2 = 0.9801$). *Wa*Stl1-mediated transport in the recombinant strain presented more than twice the affinity, with K_m of 0.96 ± 0.15 mM, but a much lower V_{max} of 148.3 ± 10.16 μmol·h^{-1}·g^{-1} ($r^2 = 0.9224$). This is consistent with using a plasmid expression, even under a *TEF1* promoter, which was recently found to be able to induce a high expression in glycerol synthetic media [20]. The lower V_{max} from the transformant is consistent with the lower *STL1* mRNA (Fig. 5), although the corresponding values could not be considered statistically different.

CCCP is a well-known ionophore that acts as an uncoupler, affecting the activity of the secondary transporters

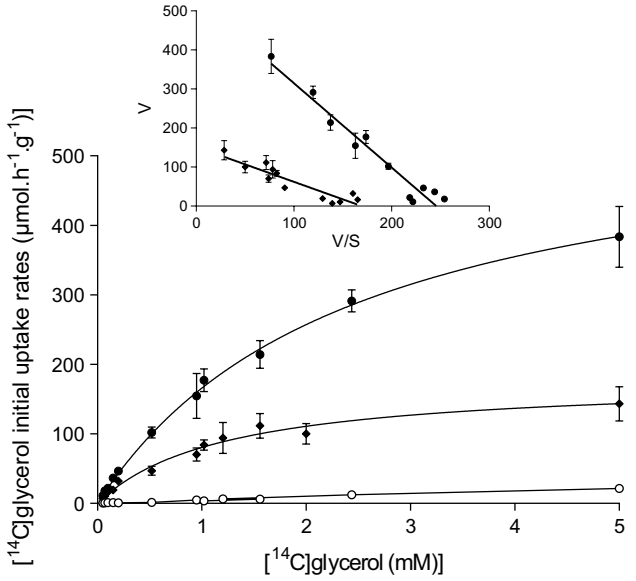

Fig. 6 Kinetics of glycerol transport. A Michaelis–Menten plot of [^{14}C]glycerol's initial uptake rates by BY4741 WT (filled circle) and Δ*stl1* pCev*WaSTL1* (filled diamond) cultivated in YNB with 5% glycerol and Δ*stl1* (open circle) cultivated in YNB with 5% glycerol, with 0.3% glucose added. The results represent the mean of transport obtained from two independent experiments performed in duplicate. They also represent the average and standard deviation of at least two independent assays. Inset: Eadie–Hofstee plot of the same results, except for the BY4741 Δ*stl1*

depending on ΔpH and/or ΔΨ, as is the case of H^+ symporters like *Sc*Stl1. Bearing in mind that this drug previously proved effective in eliminating the uptake of glycerol by *Sc*Stl1 [15], CCCP was used to test *Wa*Stl1-mediated glycerol transport. A single concentration of CCCP was used [15, 18] against three radiolabeled glycerol concentrations around the K_m values mentioned above: 0.2, 2, and 5 mM. The initial uptake rates were determined in the absence and in the presence of CCCP (Fig. 7). As expected, the drug had no effect on the uptake of glycerol by the Δ*stl1* mutant. Since this strain does not express the transporter, the remaining glycerol entry should occur through the open Fps1 glycerol channel, which should not be affected by the action of uncouplers. In the other two strains, CCCP significantly decreased glycerol uptake, leaving an almost identical residual glycerol entry, again possibly occurring through Fps1 [48]. This confirms that *Wa*Stl1 operates in the dependence of the p.m.f. most probably as an H^+ symporter like *Sc*Stl1 [15, 30, 48]. Accordingly, the accumulative nature of *Wa*Stl1 was assessed by quantifying the Stl1-driven glycerol in/out accumulation ratio. This was performed in glycerol and glucose-grown cells under identical physiological conditions (Fig. 8). The maximum accumulation ratio of glycerol on glycerol-grown *W. anomalus* was approximately 15×, well above the chemical equilibrium, indicating concentrative

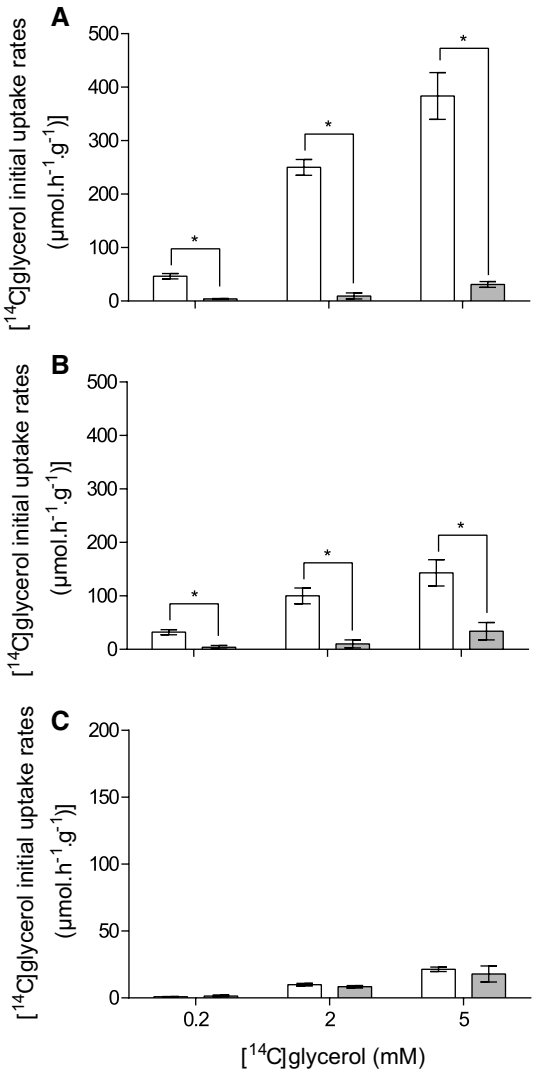

Fig. 7 Prevention of glycerol transport by the ionophore CCCP. Transport of [^{14}C]glycerol (0.2, 2, and 5 mM) in the absence (blank bars) and in the presence (gray bars) of CCCP, by BY4741 WT (**a**) and Δ*stl1* pCev*WaSTL1* (**b**) cultivated in YNB with 5% glycerol and Δ*stl1* (**c**) cultivated in YNB with 5% glycerol and 0.3% glucose. *Statistically significant difference with $p < 0.05$ between transport with (w/) and without (w/o) CCCP

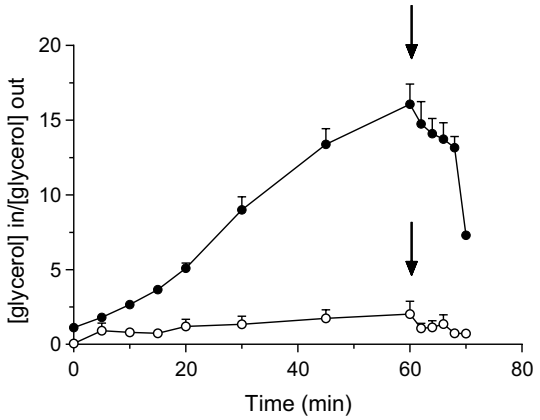

Fig. 8 Stl1-mediated glycerol accumulation. Radiolabeled glycerol int/out ratio on *W. anomalus* grown on YP with 2% glycerol (filled circle) or glucose (open circle). The arrow indicates the time point of addition of the uncoupler ionophore CCCP

uptake. In WT *S. cerevisiae*, the in/out glycerol ratio (as V_{max}) varied significantly depending on the strain in use [29, 48], indicating large differences in the regulation of expression. As expected, CCCP induced an efflux of nonmetabolized intracellular radiolabeled glycerol consistent with the dissipation of the accumulation ratio by the elimination of the driving p.m.f. On the other hand, the same strain cultivated on glucose presented an in/out glycerol accumulation ratio very close to equilibrium, compatible with the transport visible on glycerol-grown cells being repressed by glucose. These results suggest that, in *W. anomalus*, as in *S. cerevisiae*, not only does the *STL1* gene encode a concentrative secondary transporter of glycerol dependent on p.m.f., but also it is under glucose repression and is induced by growth on glycerol, as also shown by RT-qPCR quantification of *STL1* expression (Fig. 5). Importantly, the affinity of *W. anomalus* Stl1 permease is higher than the one determined in *S. cerevisiae* in both strains used previously [15, 29] and the BY4741 used now. All of these features confirm the better growth on glycerol observed in *W. anomalus*.

Conclusions

The *W. anomalus* strain LBCM1105 that was used in this work was previously noticed to have a remarkable ability to grow on glycerol and to use it as a sole carbon and energy source [9]. Among other possibilities, this should be achieved with the contribution of an efficient glycerol transport, able to fuel fast metabolism. The present work was aimed at identifying and characterizing the associated glycerol transporter. The genome of *W. anomalus* was primordially only partially sequenced and no *STL1*-like sequence was available; therefore, we used a degenerate primer-based strategy that allowed the retrieval of the full ORF. This was found to be quite similar to DNA sequences from other yeast species presumably encoding Stl1 proteins. Moreover, the putative *Wa*Stl1 protein has 561 amino acids and 11 or 12 putative transmembrane helices, and probably locates at the plasma membrane, all of which consistent with a transporter. The aminoacid sequences showed a high degree of conservation with the numerous Stl1-like proteins available in databases, most of which named exclusively on the basis of amino acid sequence homology. The Stl1 proteins that were actually studied, from *S. cerevisiae* [15], *C. albicans* [24], *Z.*

rouxii [13], and *D. hansenii* [18, 51], all function as high-affinity, concentrative glycerol-specific permeases of the H^+ symport type, consequently in the dependence of a functional p.m.f. The present results show that this is also the case for *W. anomalus* Stl1. The gene was successfully cloned and expressed in *S. cerevisiae*, complementing the Δ*stl1* mutant phenotype on glycerol. Moreover, *Wa*Stl1 displayed saturation kinetics with twice the affinity of *Sc*Stl1, as well as sustained glycerol intracellular accumulation against the chemical gradient in the dependence of p.m.f. As reported for *S. cerevisiae*, the results of *STL1* mRNA quantification by RT-qPCR, as well as by radiolabeled glycerol kinetics and accumulation, showed that *WaSTL1* is under glucose repression and is highly induced by growth on glycerol. Importantly, *STL1* mRNA was also much higher in *W. anomalus* than in WT *S. cerevisiae*. The better growth of *W. anomalus* on glycerol compared to *S. cerevisiae* could, therefore, derive at least in part from a combination of Stl1 higher affinity with more proteins. Importantly, another cause for the better performance of *W. anomalus* is apparently related to the production of residual amounts of ethanol and the derepression of *AOX* from the CRR. Channeling glycerol through the glycerol 3P shuttle fuels mitochondria with $FADH_2$, whereas producing ethanol by a glycerol-derepressed ethanol dehydrogenase could compensate for the CRR-generated NADH excess. The present work paved the way for a better understanding of the metabolism of *W. anomalus*, as an important non-*Saccharomyces* biotechnological tool, as well as the possibility of optimizing its growth on glycerol wastes for industrial applications.

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Compliance with ethical standards

Conflict of interest Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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