A permease encoded by *STL1* is required for active glycerol uptake by *Candida albicans*

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Candida albicans accumulates large amounts of the polyols glycerol and D-arabitol when the cells are exposed to physiological conditions relevant to stress and virulence in animals. Intracellular concentrations of glycerol are determined by rates of glycerol production and catabolism and of glycerol uptake and efflux through the plasma membrane. We and others have studied glycerol production in *C. albicans*, but glycerol uptake by *C. albicans* has not been studied. In the present study, we found that [¹⁴C]glycerol uptake by *C. albicans* SC5314 was (i) accumulative; (ii) dependent on proton-motive force; (iii) unaffected by carbon source; and (iv) unaffected by large molar excesses of D-arabitol or other polyols. The respective K_m and V_{max} values were 2.1 mM and 460 $\mu\text{mol h}^{-1}$ (g dry wt)⁻¹ in glucose medium and 2.6 mM and 268 $\mu\text{mol h}^{-1}$ (g dry wt)⁻¹ in glycerol medium. To identify the *C. albicans* glycerol uptake protein(s), we cloned the *C. albicans* homologues of the *Saccharomyces cerevisiae* genes *GUP1* and *STL1*, both of which are known to be involved in glycerol transport. When multicopy plasmids encoding *C. albicans* *STL1*, *C. albicans* *STL2* and *C. albicans* *GUP1* were introduced into the corresponding *S. cerevisiae* null mutants, the transformants all acquired the ability to grow on minimal glycerol medium; however, only *S. cerevisiae* *stl1* null mutants transformed with *C. albicans* *STL1* actively took up extracellular [¹⁴C]glycerol. When both chromosomal alleles of *C. albicans* *STL1* were deleted from *C. albicans* BWP17, the resulting *stl1* null mutants grew poorly on minimal glycerol medium, and their ability to transport [¹⁴C]glycerol into the cell was markedly reduced. In contrast, deletion of both chromosomal alleles of *C. albicans* *STL2* or of *C. albicans* *GUP1* had no significant effects on [¹⁴C]glycerol uptake or the ability to grow on minimal glycerol medium. Northern blot analysis indicated that *C. albicans* *STL1* was expressed in both glucose and glycerol media, conditions under which we detected wild-type active glycerol uptake. Furthermore, *STL1* was highly expressed in salt-stressed cells; however, the *stl1* null mutant was no more sensitive to salt stress than wild-type controls. We also detected high levels of *STL2* expression in glycerol-grown cells, even though deletion of this gene did not influence glycerol uptake activity in glycerol-grown cells. We conclude from the results above that a plasma-membrane H⁺ symporter encoded by *C. albicans* *STL1* actively transports glycerol into *C. albicans* cells.

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INTRODUCTION

The three-carbon polyol glycerol plays a protective role when yeast or fungal cells encounter environmental stresses, and formation of glycerol is also essential for redox balance in some yeast species (Ansell *et al.*, 1997). Yeast and fungal

cells have consequently developed adaptive mechanisms to control glycerol levels within limits suitable for growth. These mechanisms include modulation of glycerol production and catabolism, retention of intracellular glycerol or its release into the extracellular environment, and transport of extracellular glycerol across the plasma membrane and into the cell (Kayingo *et al.*, 2001a).

Glycerol transport is best understood for the budding yeast *Saccharomyces cerevisiae*. *S. cerevisiae* controls glycerol

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

A supplementary table of primers is available with the online version of this paper.

retention and efflux during osmoregulation via the plasma-membrane glycerol channel protein Fps1p (Tamás *et al.*, 1999, 2000). The Fps1p channel closes in response to osmotic up-shock, which causes glycerol to be retained within the cell (Luyten *et al.*, 1995). In contrast, the Fps1p channel opens in response to osmotic down-shock, which results in rapid and massive glycerol efflux (Tamás *et al.*, 1999). Glycerol is also actively transported into *S. cerevisiae* cells by an H⁺ symporter (Lages & Lucas, 1997) encoded by *STL1* (Ferreira *et al.*, 2005). The *STL1* protein product (Stl1p) belongs to the HXT family of sugar transporters (Nelissen *et al.*, 1997), and *STL1* expression is regulated in a complex manner by carbon source (Lages & Lucas, 1997; Rep *et al.*, 2000; Haurie *et al.*, 2001; Ferreira *et al.*, 2005), during growth on nonfermentable carbon sources (Lages & Lucas, 1997), and in a Cat8p-dependent manner (Rep *et al.*, 2000; Haurie *et al.*, 2001) upon entry into stationary phase (Lages & Lucas, 1997). Furthermore, Stl1p is subject to glucose inactivation during endocytosis (Lages & Lucas, 1997; Ferreira *et al.*, 2005). Expression of *STL1* is also induced by salt and osmotic shock in a Hog1p- and Hot1p-dependent manner (Rep *et al.*, 2000; Gasch *et al.*, 2000). Moreover, the transporter has been suggested to be involved in the oxidative stress response (Ferea *et al.*, 1999) and high temperature response (Ferea *et al.*, 1999; Ferreira & Lucas, 2007), as well as being under regulation by Ca²⁺/calcineurin (Yoshimoto *et al.*, 2002).

In *S. cerevisiae*, glycerol transport is also strongly influenced by the deletion of the gene *GUP1* (Holst *et al.*, 2000). Although *S. cerevisiae* Δ *gup1* mutants retain the ability to transport glycerol into the cell (Neves *et al.*, 2004), these mutants do not grow efficiently on glycerol and have a severely reduced transport $V_{\max}$ (Holst *et al.*, 2000). Deletion of *GUP1* also causes many pleiotropic effects that are associated with cell structure organization and biogenesis, including alteration of lipid composition (Oelkers *et al.*, 2000; Ferreira & Lucas, 2008), budding pattern (Ni & Snyder, 2001), vacuole morphology (Bonangelino *et al.*, 2002), and cell wall composition, assembly, stability and morphology (Ferreira *et al.*, 2006). Gup1p has been shown to belong to the MBOAT family of membrane-bound O-acyltransferases (Hofmann, 2000; Neves *et al.*, 2004), acting as a GPI anchor remodelase (Bosson *et al.*, 2006). Although the exact nature of the influence of *GUP1* on glycerol transport remains unclear, there is a clear phenotypic connection between glycerol transport and cell wall biogenesis relating first and foremost with the *GUP1* gene, and secondly with the PKC (protein kinase C) and HOG (high osmolarity glycerol) MAP kinase pathways (Wojda *et al.*, 2003; Gomes *et al.*, 2005).

Active glycerol transport systems have also been reported in several other fungi. For example, an active glycerol transport system has been characterized biochemically in *Fusarium oxysporum* and was shown to be involved in glycerol utilization and repressed by glucose (Castro & Loureiro-Dias, 1991). Active glycerol uptake systems also

occur in the osmotolerant yeasts *Debaryomyces hansenii* (Lucas *et al.*, 1990) and *Zygosaccharomyces rouxii* (van Zyl *et al.*, 1990) and the extremely salt-tolerant yeasts *Pichia sorbitophila* (Lages & Lucas, 1995) and *Candida versatilis* (syn. *Candida halophila*) (Silva-Graça & Lucas, 2003). Moreover, the constitutiveness of glycerol transport was suggested to serve an evolutionary advantage, observed in yeasts able to tolerate more than 2 M NaCl (Lages *et al.*, 1999). Finally, channel-like proteins or genes similar to *S. cerevisiae* Fps1p or *FPS1*, respectively, have been demonstrated in several other yeasts (Kayingo *et al.*, 2001b; Kayingo *et al.*, 2004; Neves *et al.*, 2004).

The human pathogen *Candida albicans* synthesizes and accumulates large amounts of glycerol and of the five-carbon polyol D-arabitol in culture and in the tissues of infected animals (Kiehn *et al.*, 1979; Bernard *et al.*, 1981; Wong *et al.*, 1982; San José *et al.*, 1996). *C. albicans* mutants that underproduce glycerol are hypersusceptible to environmental stresses and are hypovirulent in mice (San José *et al.*, 1996; Alonso-Monge *et al.*, 1999, 2003). Because glycerol appears to be the main protective solute with which cells maintain osmotic homeostasis (San José *et al.*, 1996; Kayingo & Wong 2005), having efficient mechanisms for regulating glycerol accumulation may be important for pathogenic fungi to colonize and survive in mammalian hosts in which environmental stresses such as nutrient deprivation, high osmolarity, high temperatures, low oxygen levels and oxidative killing by host phagocytes are frequently encountered. To date, the mechanisms by which *C. albicans* regulates glycerol accumulation are not well understood. There have been some studies on glycerol production (San José *et al.*, 1996; Kayingo & Wong 2005), but the means by which glycerol is transported into and out of *C. albicans* cells and the role of glycerol transport systems in cellular homeostasis have not been studied. Therefore, in the present study, we used both biochemical and molecular genetic approaches to investigate the nature of glycerol transport systems in *C. albicans* and to assess the roles these systems play in glycerol utilization and environmental stress tolerance.

METHODS

Yeast strains, media and growth conditions. Strains used in this work are shown in Table 1. Growth on glycerol as the main carbon and energy source was analysed on yeast nitrogen base (YNB) medium containing 0.4% glycerol and 0.05% peptone (Rønnow & Kielland-Brandt, 1993) at 30 °C for 10 days. Growth in stress conditions and on alternative polyols (D-arabitol, L-arabitol, mannitol, xylitol and ribitol) was assessed similarly. The effect of pH on growth of various yeast strains was tested on YNB containing 2% glucose and 0.4% glycerol at pH 5.4 (unbuffered YNB), pH 3.5 (containing 200 mM glycine/HCl), pH 5.5 (containing 10 mM MES) and pH 7.0 (containing 150 mM HEPES).

To analyse glycerol uptake, batch cultures were grown to exponential phase in liquid YNB (2% glucose or 2% glycerol) with shaking at 30 °C. For growth curves, exponentially growing cells were harvested from YNB-glucose liquid medium, washed three times in water, and

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
<i>C. albicans</i>		
SC5314	Wild-type	Gillum <i>et al.</i> (1984)
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	Fonzi & Irwin (1993)
BWP17	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>	Wilson <i>et al.</i> (1999)
DAY185	<i>ura3Δ::λimm434/ura3Δ::λimm434 HIS1::his1::hisG/his1::hisG ARG4::URA3::arg4::hisG/arg4::hisG</i>	Davis <i>et al.</i> (2000)
GK-Y003-a	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG gup1Δ::URA3-dpl200/GUP1</i>	This study
GK-Y003	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG gup1Δ::URA3-dpl200/gup1Δ::ARG4</i>	This study
GK-Y004-a	<i>ura3Δ::λimm434/ura3Δ::λimm434, his1::hisG/his1::hisG arg4::hisG/arg4::hisG stl1Δ::URA3-dpl200/STL1</i>	This study
GK-Y004	<i>ura3Δ::λimm434/ura3Δ::λimm434, his1::hisG/his1::hisG arg4::hisG/arg4::hisG stl1Δ::URA3-dpl200/stl1Δ::ARG4</i>	This study
GK-Y004-b	<i>ura3Δ::λimm434/ura3Δ::λimm434,his1::hisG/his1::hisG arg4::hisG/arg4::hisG stl1Δ::ARG4/STL1::HIS1</i>	This study
GK-Y005-a	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG stl2Δ::URA3-dpl200/STL2</i>	This study
GK-Y005	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG stl2Δ::URA3-dpl200/stl2Δ::ARG4</i>	This study
<i>S. cerevisiae</i>		
W303-1A	<i>MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0</i>	Thomas & Rothstein (1989)
BHY61-1A	<i>MATα ura3 leu4 trp1 his3 ade2 ygl084c::his5⁺ fps1::HIS3</i>	Holst <i>et al.</i> (2000)
<i>stl1</i> Y15831	BY4742; <i>MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YDR536w::kanMX4</i>	EUROSCARF

inoculated into fresh YNB-glucose (2 %) medium with or without NaCl (5 %) or fresh YNB-glycerol (0.4 % or 2 %) medium, followed by incubation at 30 °C and measurement of OD₆₀₀ at suitable intervals.

Glycerol uptake and accumulation. The methods of Lages & Lucas (1995, 1997) and Lages *et al.* (1999) were used to measure initial rates of [¹⁴C]glycerol uptake, concomitant external alkalization, and the ratios of intracellular-to-extracellular glycerol concentrations (in/out accumulation ratios). To measure [¹⁴C]glycerol uptake, *C. albicans* cells were washed twice and resuspended in ice-cold water to a final concentration of approximately 30 mg dry wt ml⁻¹; 10 μl of the cell suspension was mixed with 10 μl 100 mM Tris/citrate buffer (pH 5.0) and incubated for 2 min at 25 °C, and the reaction was started by adding 5 μl 20 mM [¹⁴C]glycerol (Amersham, 2000 d.p.m. nmol⁻¹). The reaction was stopped by adding 5 ml ice-cold water, after which the cells were harvested on Whatman GF/C filters, washed twice with 5 ml water and added to vials containing 5 ml scintillation fluid (LKB Scintillation Products). [¹⁴C]Glycerol was quantified with a liquid scintillation counter (Packard Instruments). Initial uptake rates and kinetic constants were calculated using a regression-analysis program (Graph Pad Software).

To determine if proton fluxes into or out of cells accompanied glycerol uptake, cell suspensions (0.5 ml, 30 mg dry wt ml⁻¹) in 4.5 ml water were incubated until the extracellular pH stabilized, 100 μl 1 M glycerol (pH 5.0) was added, and extracellular pH was measured with an electrode (PHM82, Radiometer) immersed in a water-jacketed chamber of 10 ml capacity with a magnetic stirrer and a flat-bed recorder (Kipp & Zonen).

To measure transport-driven ratios of intracellular to extracellular [¹⁴C]glycerol (in/out accumulation ratios), cell suspensions (80 μl) and 110 μl 100 mM Tris/citrate (pH 5.0) were incubated for 2 min at 30 °C with magnetic stirring, and 10 μl (200 mM) [¹⁴C]glycerol

(300 d.p.m. nmol⁻¹) was added. At suitable intervals thereafter, 10 μl aliquots were filtered through Whatman GF/C filters, and the filters were washed twice with 5 ml ice-cold water and transferred to vials of scintillation fluid. For biomass measurements, cells were harvested on pre-weighed filters and washed, and the filters were dried at 80 °C overnight and weighed. Intracellular [¹⁴C]glycerol concentrations were calculated using estimated intracellular volumes of 2 μl (mg dry wt)⁻¹ (Lages *et al.* 1999).

Molecular methods. Potential *C. albicans* glycerol uptake proteins were identified by searching the *C. albicans* genome database (<http://www-sequence.stanford.edu/group/candida/index.html>) for homologues of the *S. cerevisiae* genes *GUP1* and *STL1*. The genes of interest were cloned by PCR with the oligonucleotide primers listed in Supplementary Table S1, available with the online version of this paper. Standard methods were used for restriction analysis, PCR amplification, subcloning of selected restriction fragments and PCR products into convenient plasmid vectors, and introduction of the resulting plasmids into *Escherichia coli* or *S. cerevisiae*. Multicopy plasmids encoding *C. albicans* *GUP1*, *C. albicans* *STL1* or *C. albicans* *STL2* were introduced into the corresponding *S. cerevisiae* null mutants by the lithium acetate method. The transformants were initially selected on minimal medium without uracil, after which they were replica plated and screened for the ability to grow on YNB supplemented with 0.4 % glycerol plus 0.05 % peptone. The *C. albicans* genes *STL1*, *STL2* and *GUP1* were expressed in *S. cerevisiae* under their native promoters and terminator regions, and they were not codon-optimized for expression in *S. cerevisiae*.

Construction of *C. albicans* *gup1Δ/gup1Δ* (strain GK-Y003), *stl1Δ/stl1Δ* (strain GK-Y004) and *stl2Δ/stl2Δ* (strain GK-Y005) mutants was done by the short flanking homology PCR method in *C. albicans* BWP17 (Wilson *et al.*, 1999). The targeting DNA fragments were amplified by PCR from plasmid pRS-Arg4Δ*SpeI* (Wilson *et al.*, 1999)

and plasmid pDDB57, which contains the *URA3-dpl200* cassette (Wilson *et al.*, 2000). Synthetic oligonucleotides *GUP1*-DEL-5 and *GUP1*-DEL-3 (Table S1) were used to amplify the targeting DNA fragments for *GUP1*, oligonucleotides *STL1*-DEL-5 and *STL1*-DEL-3 were used to amplify the targeting fragments for *STL1*, and oligonucleotides *STL2*-DEL-5 and *STL2*-DEL-3 were used to amplify the targeting fragments for *STL2* (Table S1).

In all targeted gene deletion experiments, one chromosomal allele of the gene of interest was replaced by *URA3*, and *Ura*⁺ prototrophs were selected and tested for homologous integration of the *URA3* cassette within the target locus. The resultant mutants (e.g. *GUP1/gup1Δ::URA3-dpl200*, strain GK-Y003-a) were transformed with an *ARG4*-containing cassette, thereby creating the desired homozygous mutant (e.g. *gup1Δ::ARG4/gup1Δ::URA3-dpl200*, strain GK-Y003). Homologous chromosomal integrations were verified by gene-specific PCR and by Southern hybridization, as previously described for other *C. albicans* mutants (Kayingo & Wong, 2005).

STL1 and *STL2* expression was examined by Northern hybridization (Sambrook & Russell, 2001). *ACT1* expression served as a loading control. Exponential-phase cultures of *C. albicans* SC5314 were divided, inoculated into YNB+2% glucose, YNB+2% glycerol and YNB+2% glucose+1 M NaCl, and then cultured at 30 °C. After incubation for 0, 0.5, 1, 3 and 14 h, RNA was extracted by the method of Amberg *et al.* (2005), and then separated by electrophoresis in denaturing agarose gels and transferred to nylon membranes. The membranes were probed with either an *STL1* fragment amplified by PCR with the primers STL1F and STL1R (Table S1), an *STL2* fragment amplified by PCR with the primers STL2F and STL2R (Table S1) or an *ACT1* fragment amplified by PCR with primers ACT1F and ACT1R (Table S1; Enjalbert *et al.* 2006). For each treatment, two independent sets of RNA samples were analysed by Northern analysis.

RESULTS

[¹⁴C]Glycerol uptake by *C. albicans*

Glucose-grown *C. albicans* cells accumulated [¹⁴C]glycerol against a concentration gradient (in/out accumulation ratios >10), and this process was blocked by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Fig. 1). Glycerol uptake by *C. albicans* conformed to Michaelis–Menten kinetics, with *V*_{max} and *K*_m values as shown in Table 2. Also, first-order kinetics were measured at higher [¹⁴C]glycerol concentrations, with an estimated *K*_d value of 0.09 l h⁻¹ (g dry wt)⁻¹. The specificity of glycerol transport was examined by determining if the polyols D-arabitol, L-arabitol, ribitol, xylitol and mannitol inhibited glycerol uptake. At concentrations between 0.1 mM and 100 mM (at least 50-fold higher than the *K*_m for glycerol uptake), none of the tested polyols significantly reduced [¹⁴C]glycerol uptake or accumulation (data not shown).

Addition of unlabelled glycerol or CCCP to cells that had previously accumulated [¹⁴C]glycerol resulted in efflux of [¹⁴C]glycerol (Fig. 1). Because inhibition by CCCP suggested that glycerol uptake was dependent on proton-motive force, we measured extracellular pH when glycerol was added to an unbuffered suspension of *C. albicans* cells. Rapid alkalinization of the extracellular medium was observed (Table 2), as would be expected if glycerol was

Fig. 1. Transport-driven accumulation of [¹⁴C]glycerol by *C. albicans*. Wild-type cells accumulated [¹⁴C]glycerol against a concentration gradient (●), and this process was blocked by the protonophore CCCP (■). When 85 mM unlabelled glycerol (○) or 50 μM CCCP (□) was added to cells that had previously accumulated glycerol for 40 min (arrow), there was an efflux of [¹⁴C]glycerol out of the cells. Accumulation ratios were determined at pH 5 and 30 °C for cells that were grown in YNB with 2% (w/v) glucose medium and were harvested at mid-exponential growth phase.

transported across the plasma membrane by an H⁺ symporter.

Because carbon source and NaCl stress have been shown to influence glycerol uptake by some yeast species (Lages *et al.*, 1999), we compared the initial glycerol uptake rates and glycerol accumulation ratios in *C. albicans* cells grown in glucose (fermentable) or glycerol (non-fermentable). Whereas glycerol uptake in *S. cerevisiae* is subject to glucose inhibition (Lages & Lucas, 1997), there were no significant differences in the rates of glycerol uptake by glucose- or glycerol-grown *C. albicans* cells (Table 2). Furthermore, to determine if NaCl stress would increase active uptake of glycerol in *C. albicans* [as it does in the halotolerant yeast *Zygosaccharomyces rouxii* (van Zyl *et al.*, 1990)], *C. albicans* cells were incubated in 1 M NaCl for 1 h before starting the accumulation assay. There were no significant differences in the in/out accumulation ratios between cells grown in the presence or absence of 1 M NaCl at any time tested (data not shown), indicating that glycerol uptake is not affected by salt stress.

Functions of *C. albicans* *STL1* and *STL2*

C. albicans orf19.5753 and orf19.7093 were identified by searching the *C. albicans* genome database for homologues of *S. cerevisiae* *STL1*, the known gene encoding a glycerol-uptake protein. *C. albicans* orf19.5753 encodes a deduced protein of 547 amino acids with multiple membrane-spanning domains that is 60% identical to *S. cerevisiae* Stl1p; this gene has been designated *C. albicans* *STL1*. *C. albicans* orf19.7093 encodes a deduced protein of 553 amino acids with multiple membrane-spanning domains that is 34% identical to *C. albicans* Stl1p and 36% identical

Table 2. Glycerol transport by wild-type *C. albicans* and the *gup1* null mutant

Kinetic parameters, accumulation ratios and external alkalization were determined in cells grown on minimal medium with glucose or glycerol and collected during the exponential growth phase.

Strain	Carbon source	[¹⁴ C]Glycerol uptake*		[¹⁴ C] Glycerol accumulation†	External alkalization upon glycerol addition
		<i>K_m</i> (mM)	<i>V_{max}</i> [μmol h ⁻¹ (g dry wt) ⁻¹]		
WT	Glucose	2.1 ± 0.9 (4)	460 ± 110.6 (4)	+	+
	Glycerol	2.6 ± 0.7 (3)	268 ± 26.3 (3)	+	ND
<i>gup1Δ</i>	Glucose	1.0 ± 0.7 (4)	382 ± 87.6 (4)	+	+

*Mean ± SE; the number of independent experiments is shown in parentheses. ND, Not determined.

†Positive results mean in/out ratios higher than 1.

to *S. cerevisiae* Stl1p; this gene has been designated *C. albicans* STL2.

To assess the functions of *C. albicans* STL1 and STL2, the two ORFs of interest and their promoter and terminator regions were amplified from *C. albicans* genomic DNA by PCR and ligated into the yeast multicopy shuttle plasmid YEplac195 in their non-codon-optimized form. The resulting plasmids were then introduced into a *S. cerevisiae* *stl1* null mutant. *S. cerevisiae* *stl1* null mutants transformed with *C. albicans* STL1 grew better on YNB with 0.4 % glycerol and 0.05 % peptone (Fig. 2a) and accumulated 42-fold more [¹⁴C]glycerol intracellularly than did the empty-vector control (Fig. 2b). *S. cerevisiae* *stl1* null mutants transformed with *C. albicans* STL2 also grew slightly better on glycerol medium than did empty-vector controls, but these mutant strains accumulated no more [¹⁴C]glycerol than did empty-vector controls (data not shown). These data suggested that *C. albicans* STL1 encodes the active glycerol uptake protein in *C. albicans*; however, the inability of *C. albicans* STL2 to cause increased glycerol transport uptake by *S. cerevisiae* could be due to Stl2p's inability to transport glycerol across the plasma membrane, or to problems with heterologous expression, such as an inefficient or inactive native promoter or mistranslation of STL2 due to the presence of CTG codons.

To determine more directly if *C. albicans* STL1 and/or STL2 encode glycerol transport proteins, we constructed and analysed *C. albicans* *stl1* and *stl2* null mutants. The *C. albicans* *stl1* null mutants grew poorly on solid and liquid minimal glycerol media, and this growth defect was reversed by reintroduction of a single wild-type copy of STL1 (*stl1Δ* + STL1) (Fig. 3a, b). Furthermore, growth of the *C. albicans* *stl1* null mutants was still impaired when the glycerol concentration was increased to 2 % (data not shown), suggesting that a low-affinity glycerol transport system does not compensate in the absence of STL1. Moreover, the ability to transport glycerol into the cell was reduced by 95 % for glucose- or glycerol-grown *C. albicans* *stl1* null mutants, and reintroduction of a single wild-type copy of STL1 restored glycerol uptake almost to wild-type

levels (Fig. 3c). In contrast, *C. albicans* *stl2* null mutants did not differ significantly from wild-type controls in their abilities either to grow on either 0.4 % glycerol (Fig. 3a, b) or 2 % glycerol (data not shown), or to transport glycerol into the cell (Fig. 3c).

Because the ability to transport glycerol into the cell contributes to salt tolerance in other fungi (Lages *et al.*, 1999), we investigated the effects of the *stl1* null mutation on the ability of *C. albicans* to tolerate NaCl and other environmental stresses. Despite impaired growth on glycerol as a sole carbon source and a corresponding reduction in glycerol uptake activity, *C. albicans* *stl1* null mutants grew as well on solid media containing 1 M NaCl as did wild-type controls and the *stl2* null mutant (which has wild-type glycerol uptake) (Fig. 4a). To detect growth differences between the *stl1* mutant and wild-type at earlier time points, we also conducted growth curves in liquid media. Growth of the *stl1* null mutant was similar to growth of wild-type controls in YNB, YNB + 1 M NaCl, or YNB + 1 M NaCl supplemented with 30 mM glycerol (Fig. 4b), mirroring the results on solid media. Moreover, the *stl1* null mutants and wild-type controls did not differ significantly in their abilities to grow in the presence of 1 mM H₂O₂, at 25 °C, 37 °C, and 42 °C (Fig. 4c), or in YNB that was buffered at pH values of 3.5, 5.5 or 7.0 (data not shown). Lastly, there were no significant differences in growth between these mutants and the wild-type on any of the polyols used in the competition experiments, including mannitol, D-arabitol and L-arabitol (Fig. 4c), or ribitol and xylitol, which gave results similar to mannitol (data not shown). That the mutants grew like wild-type controls on all of the polyols tested provides additional evidence that the *C. albicans* H⁺ symporter is highly specific for glycerol.

The level of STL1 and STL2 mRNA expression in cells grown in medium with glucose (control), glycerol (mimicking diauxic transition), or glucose with 1 M NaCl (salt shock) was assessed by Northern analysis to: (i) determine if STL1 expression can account for glycerol uptake by *C. albicans* when grown on glucose, which is in

Fig. 2. Heterologous expression of *C. albicans* *STL1* in the *S. cerevisiae* *stl1* null mutant. (a) When multicopy plasmids encoding *C. albicans* *STL1* were introduced into the *S. cerevisiae* *stl1* null mutant, the transformants all acquired the ability to grow on minimal glycerol. Growth phenotypes on minimal glycerol were scored by tenfold dilutions from top to bottom and plates were incubated at 30 °C for 10 days. (b) *S. cerevisiae* *stl1* null mutants transformed with *C. albicans* *STL1* actively took up extracellular [¹⁴C]glycerol, and accumulated 42-fold more [¹⁴C]glycerol intracellularly than did empty-vector controls. Transport and accumulation of [¹⁴C]glycerol were measured in cells harvested at the diauxic shift.

direct contrast to *S. cerevisiae*, where *STL1* expression and Stl1p activity are under glucose repression (Ferreira *et al.*, 2005); and (ii) eliminate the possibility that *STL2* actually does encode a glycerol uptake protein but that we observed no function because the gene is not expressed under the conditions tested. Results of our analysis (Fig. 5) revealed that *STL1* is weakly expressed upon transfer to both glucose and glycerol, and it is expressed at transiently high levels upon salt shock. Thus, *STL1* expression can account for glycerol uptake activity in glucose-grown cells of *C. albicans* and is not under glucose repression as is observed

Fig. 3. Effect of *stl1* and *stl2* null mutations on growth and glycerol uptake by *C. albicans*. (a, b) Deletion of *C. albicans* *STL1* (*stl1Δ* / *stl1Δ*) and *STL2* (*stl2Δ* / *stl2Δ*) had no effect on growth of *C. albicans* on minimal glucose medium; however, deletion of *STL1* (but not *STL2*) resulted in reduced growth on glycerol as a sole carbon and energy source, and reintroduction of a single copy of wild-type *STL1* (*stl1Δ* + *STL1*) restored the ability of the *stl1* null mutants to grow on minimal glycerol, whether on solid (a) or liquid (b) medium. (c) Deletion of *STL1* (*stl1Δ* / *stl1Δ*) reduced glycerol uptake by approximately 95% on glucose-grown (white bars) or glycerol-grown (dark bars) cells as compared to wild-type strains, and reintroduction of a single copy of wild-type *STL1* (*stl1Δ* + *STL1*) restored glycerol uptake to almost wild-type levels. In contrast, deletion of *STL2* (*stl2Δ* / *stl2Δ*) did not significantly alter glycerol uptake in glucose-grown or glycerol-grown cells.

for *S. cerevisiae*. *STL1* can also account for uptake in the presence of glycerol and salt stress. In contrast, *STL2* was transiently expressed at high levels upon transition from glucose to glycerol medium (mimicking diauxic shift), but it was not expressed upon transfer to glucose or salt stress. Because *STL2* was strongly expressed in glycerol-grown cells (Fig. 5), where the *stl1* null mutant is highly reduced

Fig. 4. Effect of *stl1* and *stl2* null mutations on growth of *C. albicans* under environmental stress conditions. (a) Deletion of *C. albicans* *STL1* (*stl1Δ/stl1Δ*) and *STL2* (*stl2Δ/stl2Δ*) had no effect on growth on minimal glucose medium supplemented with NaCl. (b) Growth curves of wild-type *C. albicans* SC5314 (black symbols) and *stl1Δstl1Δ* (white symbols) on YPD alone (Δ , $\blacktriangle$), and on YPD supplemented with 1.5 M NaCl ($\square$, $\blacksquare$) or with 1.5 M NaCl and 30 mM glycerol ($\circ$, $\bullet$). (c) Growth assays under other environmental stress conditions, including growth on minimal glucose medium (i); minimal glucose medium + 1 M NaCl (ii); minimal glucose medium + 1 mM H_2O_2 (iii); minimal glucose medium at various temperatures (25 °C, 37 °C or 42 °C) (iv); or minimal medium containing various polyols as sole carbon sources (v).

in glycerol uptake, lack of *STL2* expression cannot account for the lack of activity by *STL2*. Therefore, *STL2* does not appear to encode a glycerol uptake protein, at least under the conditions tested.

Effect of *C. albicans* *GUP1*

In *S. cerevisiae*, deletion of *GUP1* (which encodes a membrane-bound *O*-acyltransferase) results in changes in glycerol uptake kinetics including a decrease in glycerol transport V_{max} and a reduction in growth rate on glycerol

as a sole carbon and energy source (Holst *et al.*, 2000). *C. albicans* orf19.4985 encodes a deduced protein of 584 amino acids that is 53 % identical to *S. cerevisiae* Gup1p (encoded by *GUP1*). When *C. albicans* *GUP1* was introduced into the *S. cerevisiae* *gup1* null mutant it restored the ability of this mutant to grow on glycerol (data not shown). However, deletion of *GUP1* from the genome of *C. albicans* did not alter the ability of *C. albicans* to (i) take up or accumulate radiolabelled glycerol as compared to wild-type controls (Table 2); (ii) grow on glycerol as the sole carbon source within the first 5 days (Fig. 6a),

Fig. 5. Expression of *STL1* and *STL2* by wild-type *C. albicans* SC5314. Northern blot analysis with *C. albicans* *STL1* and *STL2* as probes revealed that patterns of *STL1* and *STL2* expression are transient according to growth phase and vary among treatments. A single culture was grown in minimal glucose medium to an OD₆₀₀ of 1.5, a sample taken ($t=0$), followed by equal division of the remaining culture between the following three treatments: minimal glucose medium (Gluc), minimal glycerol medium (Glyc), and minimal glycerol medium amended with 1 M NaCl (NaCl). Treated cultures were then incubated at 30 °C, 200 r.p.m. for 0.5, 1, 3 and 14 h, the time points at which cells were harvested for RNA extraction. *ACT1* served as an RNA loading control.

although the wild-type controls grew slightly better than the *gup1* mutants after longer periods of incubation (data not shown); (iii) grow in the presence of 1 mM H₂O₂ (data not shown) or at various temperatures, including 25 °C and 37 °C (data not shown); or (iv) conserve glycerol intracellularly when subjected to salt stress (Fig. 6b). These results suggest that *GUP1* in *C. albicans* does not influence H⁺/glycerol symporter activity; however, it should be

noted that the *gup1Δ* null mutant was slightly sensitive to higher temperatures (i.e. 42 °C, Fig. 6a)

DISCUSSION

Yeast cells can regulate intracellular glycerol concentrations by controlling glycerol production and catabolism, by retaining glycerol inside the cell, and by actively acquiring it from the environment. We and others have previously studied glycerol production and accumulation in *C. albicans* (San José *et al.*, 1996; Alonso-Monge *et al.*, 1999, 2003; Kayingo & Wong, 2005), but glycerol uptake by *C. albicans* has not previously been studied. In this study, we found that *C. albicans* takes up glycerol actively through an H⁺ symporter similar to the glycerol transporters previously described in *S. cerevisiae* and other yeasts (Lages & Lucas 1995, 1997; Silva-Graça & Lucas, 2003). Several lines of evidence support this conclusion. First, initial rates of [¹⁴C]glycerol uptake followed Michaelis–Menten-type kinetics with high affinity, commensurate with *K_m* values obtained for other yeasts. Second, entry of glycerol into the cell was accompanied by transient and fast alkalinization of the extracellular medium, indicating co-transport with protons. Third, transport was accumulative and against a glycerol concentration gradient. Fourth, both uptake and accumulation of glycerol were sensitive to the action of a protonophore (uncoupler), CCCP, suggesting that transporter activity is dependent on proton-motive force. And lastly, the transporter appeared to be very highly specific, since addition of other polyols such as D-arabitol did not inhibit uptake or provoke efflux of accumulated [¹⁴C]glycerol.

Furthermore, we found that the *C. albicans* H⁺/glycerol symporter was encoded by *STL1*, a homologue of the gene encoding the active glycerol transporter in *S. cerevisiae*. Of the two *C. albicans* homologues of *S. cerevisiae* *STL1*, *C. albicans* *STL1* (orf.5753) and *STL2* (orf.7093), only

Fig. 6. Effects of the *gup1* null mutation on growth and glycerol accumulation by *C. albicans*. (a) No significant differences were observed in the abilities of wild-type strain DAY185, a heterozygous *gup1Δ/GUP1* mutant or a homozygous *gup1Δ/gup1Δ* mutant to grow on minimal glucose or glycerol medium at 30 °C, or on glucose medium supplemented with salt; however, growth of the *gup1* null mutant was slightly impaired at 42 °C. (b) There was also no significant difference in the amount of glycerol accumulated intracellularly when the *C. albicans* *gup1* null mutant (dark bars) and wild-type strain DAY185 (white bars) were exposed to osmotic stress (minimal glucose medium + 1 M NaCl) at 30 °C for 0–300 min.

deletion of *C. albicans STL1* resulted in a significant reduction in glycerol transport as compared to wild-type. Additionally, reintroduction of wild-type *STL1* into the *C. albicans stl1* null mutant restored wild-type glycerol uptake. Thus, *C. albicans STL1*, not *C. albicans STL2*, encodes the glycerol transporter in *C. albicans*, at least under the conditions tested. We cannot rule out that Stl2p could be a glycerol transporter under other conditions or as a subordinate of Stl1p with a phenotype seen only in the absence of *STL1*; however, that the *C. albicans stl1* mutant growth impairment is not rescued in the presence of high levels of glycerol argues against Stl2p functioning as a low-affinity glycerol importer.

Although our results suggest that *C. albicans STL2* does not function in glycerol uptake, we expect that *STL2* still provides a function for *C. albicans* related to glycerol homeostasis. This is based on the conclusion of Palma *et al.* (2007) that the nature of the sugar compounds available in a particular yeast species' habitat provides the driving force for the amplification of its different sugar transporters, and correspondingly, that the observed gene amplifications reflect important sugars in the environment. Indeed, *STL2* transcription is strongly upregulated by *C. albicans* upon transfer of exponentially growing cells from glucose to glycerol (mimicking diauxic transition). Furthermore, expression of *C. albicans STL2* complemented the mutant growth phenotype of the *S. cerevisiae stl1Δ* mutant on glycerol-based medium. Together, these results suggest that *C. albicans STL2* encodes a functional protein that positively affects glycerol uptake and/or utilization, although its exact function will require further investigation.

Despite the two species sharing a homologous glycerol transporter, regulation of glycerol transport in *C. albicans* differed from that in *S. cerevisiae*. That glycerol transport activity by wild-type *C. albicans* was similar in glucose- and glycerol-grown cells indicated that this transport is not regulated by carbon source, as it is in *S. cerevisiae*, where glycerol transport is under glucose repression and inactivation (Lages & Lucas, 1997) and is subject to glucose-derived inactivation by endocytosis (Ferreira *et al.*, 2005). Although these results suggested that transcription of *C. albicans STL1* might be constitutive, we found that *C. albicans STL1* mRNA is not constitutively expressed. Instead, *C. albicans STL1* was expressed upon transfer of cells into media with either glucose or glycerol as the carbon source and mRNA levels quickly declined despite the observed constitutive glycerol transport activity. This was most likely due to a much longer life-span of the *C. albicans* Stl1p protein than of the corresponding mRNA.

Similar to what is observed for *S. cerevisiae STL1* (Rep *et al.*, 2000) and Stl1p (Ferreira *et al.*, 2005), *C. albicans STL1* was strongly upregulated in response to salt stress, which suggests a role for glycerol uptake in response to osmotic shock. Even so, the *stl1* null mutant was no more sensitive to salt stress than wild-type controls when they were grown in the presence of 1 M NaCl, whether or not glycerol was

available in the medium. Thus, it appears that either upregulation of glycerol production or retention of glycerol within the cell by the closing of a putative efflux channel may be more important for survival under salt stress than the recovery of extracellular glycerol by active uptake. Indeed, transcription of the genes for glycerol production is upregulated in the presence of salt stress (Enjalbert *et al.*, 2006). Furthermore, impairment of the glycerol production pathway by deletion of *GPPI*, encoding the terminal phosphatase (Fan *et al.*, 2005), or of the combination of *GPD1* and *GPD2*, encoding the two glycerol-3-phosphate dehydrogenases, results in an increased sensitivity to osmotic stress as compared to wild-type (R. Andrie and others, unpublished data). However, despite our efforts to investigate the relative importance of glycerol efflux and glycerol uptake for the survival of *C. albicans* in the presence of osmotic stress, we have yet to identify the gene(s) and protein(s) responsible for *C. albicans* glycerol efflux. Just as was the case in *Schizosaccharomyces pombe* (Kayingo *et al.*, 2004), deletion of the only aqua- or aquaglyceroporin in the *C. albicans* genome, *CaAQY1* (Carbrey *et al.*, 2001; Tang *et al.*, 2005), did not alter glycerol efflux as compared to wild-type (data not shown). In our effort for a full understanding of glycerol homeostasis and its role in the stress response and virulence of *C. albicans*, characterization of glycerol efflux by *C. albicans* continues to be a focus of our research.

Finally, in contrast to the requirement of *GUP1* for *S. cerevisiae* glycerol transport and growth on glycerol as a sole carbon source (Holst *et al.*, 2000), deletion of the only *C. albicans* *GUP* homologue (*GUP1*) had no effect on growth on glycerol (except for a slight sensitivity at 42 °C) or on glycerol uptake or accumulation by *C. albicans*. This clearly indicates a different role in glycerol homeostasis for *GUP1* in *C. albicans* from that in *S. cerevisiae*. Because *S. cerevisiae* Gup1p has recently been associated with a large number of wall-related phenotypes (Ferreira *et al.*, 2006; Ferreira & Lucas, 2008) and has been shown to belong to the MBOAT family of membrane-bound O-acyltransferases (Hofmann, 2000; Neves *et al.*, 2004) acting as a GPI anchor remodelase (Bosson *et al.* 2006), it is possible that *C. albicans* Gup1p also plays a role in cell wall biosynthesis and composition. That both *S. cerevisiae* Gup1p (Holst *et al.*, 2000; Blevé *et al.* 2005) and *C. albicans* Gup1p (G. Kayingo & B. Wong, unpublished results) localize to the plasma membrane and the endoplasmic reticulum provides support for this possibility; however, further experimentation will be necessary to elucidate the functions of *GUP1* in *C. albicans*.

In conclusion, although *C. albicans* shares some similarities with *S. cerevisiae* with regard to glycerol homeostasis, we have identified several differences that potentially could be specific to the pathogenic lifestyle of *C. albicans*. In particular, the constitutive nature of glycerol symport observed in *C. albicans* differs from that previously reported in *S. cerevisiae*, where glycerol is actively transported only in cells grown on non-fermentable carbon

sources and not in cells grown on glucose. Having a constitutive glycerol uptake system may be one of the ways that *C. albicans* has evolved to adapt to stressful environments in the colonization of its host.

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