Advanced Journal of Microbiology Research Vol. 2010
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Molecular cloning and characterization of *STL1* gene of *Debaryomyces hansenii*

Juan Carlos González-Hernández

Laboratorio de Bioquímica del Departamento de Ingeniería Bioquímica, Instituto Tecnológico de Morelia; Avenida Tecnológico 1500. C. P. 58120. Morelia, Michoacán, México. E-mail: jcgh1974@yahoo.com. Tel: (+52-433) 3121570 Ext. 240. Fax: (+52-433) 3121570 Ext. 211.

Accepted 26 April, 2010

Debaryomyces hansenii **is often found in salty environments. This yeast species is not only halotolerant, but also halophilic. Its genome sequence is known completely, but the mechanisms behind its halotolerance are poorly understood. It was compared to the STL1 protein sequence of *Saccharomyces cerevisiae* against the translated sequence from the *D. hansenii* genome sequence database released by Génolevure. An ORF (DEHA0E01122g) was found with 54% homology and 39% identity with Stl1p from *S. cerevisiae*. *DhSTL1* was heterologously expressed successfully in a *S. cerevisiae* (BY4741) wild type and in another strain lacking its own system for the glycerol transport (*STL1*) gene. The *DhSTL1* gene in transformed *S. cerevisiae* strains showed a slight but significant difference in the doubling times in growth curves obtained in liquid YNB-ura medium, with glycerol as carbon source. *DhSTL1* gene in transformed *stl1* yeast strain showed phenotype growth at pH 7.5 under salt stress conditions (glucose as carbon source). The kinetic parameters of transport and glycerol accumulation conferred by *DhSTL1* in the *S. cerevisiae* transformant strains did not show significant differences. An increase in the transcript level of *DhSTL1* gene in the presence of saline stress at pH 5.6; whereas, at 7.5 pH, it was expressed in all evaluated conditions.**

Key words: *Debaryomyces hansenii*, glycerol, transport, salt tolerance.

INTRODUCTION

In yeasts, defense responses to salt stress are based on osmotic adjustment by osmolyte synthesis and cation transport systems for sodium exclusion. Polyols, and especially glycerol, are the major osmolytes produced by yeasts (Blomberg and Adler, 1992). In *Saccharomyces cerevisiae*, physiological and molecular studies have previously shown the presence of an active uptake system driven by proton motive force (Holst et al., 2000), only operative when glycerol is the carbon and energy source, this was the first report on a gene product involved in active transport of glycerol in yeasts. According to previous studies in other yeast species, more halotolerant than *S. cerevisiae*, like *Pichia sorbitophila* (Lages and Lucas, 1995), *Zygosaccharomyces rouxi* (Zyl et al., 1990) or *Debaryomyces hansenii* (Lucas et al., 1990), glycerol was found to be actively transported, establishing and maintaining a glycerol gradient in the presence of high salt concentrations and counterbalancing glycerol's natural leakage.

Ferreira et al. (2005) identified genes involved in active glycerol uptake in *S. cerevisiae* by screening a deletion mutant collection comprising 321 strains. They found that deletion of *STL1*, which encodes a member of the sugar transporter family, eliminates active glycerol transport. Stl1p is present in the plasma membrane of *S. cerevisiae* during conditions in which glycerol symport is functional. Both the Stl1p and the active glycerol transport are subject to glucose-induced inactivation. These last authors concluded that, the glycerol proton symporter in *S. cerevisiae* is encoded by *STL1*.

When *D. hansenii* was subjected to increased NaCl stress, there was a decrease of intracellular K^+ and an increase of intracellular Na^+ (Norkrans, 1968; Norkrans

and Kylin, 1969). However, the total salt level in the cells was not sufficient to balance the water potential of the medium. For this reason, additional osmotically active solutes must be accumulated (Chen and Wadso, 1982). Many eukaryotic microorganisms accumulate polyols when exposed to osmotic stress (Adler et al., 1985; Brown, 1978), and, in *D. hansenii*, a positive correlation was demonstrated between the intracellular glycerol level and the salinity of the surrounding medium (Adler et al., 1985; Andre et al., 1988; González-Hernández et al., 2005).

In addition, tolerance to a sudden osmotic dehydration shock is higher in cells carrying an increased amount of intracellular polyols (Adler and Gustafsson, 1980). The two polyols produced and accumulated by *D. hansenii* under saline stress are glycerol, which is the major internal solute in exponentially growing cells and arabinitol, which predominates in stationary-phase cells (Adler and Gustafsson, 1980; González-Hernández et al., 2005).

In *D. hansenii* a striking relationship between the intracellular glycerol concentration and the metabolic activity, measured as heat production rate, has been reported when cells were grown in presence of 2.7 M NaCl (Gustaffson, 1979), suggesting a specific role for glycerol when *D. hansenii* is exposed to these conditions. Determinations of intracellular glycerol levels with respect to the osmotic volumes revealed that increases in intracellular glycerol may counterbalance up to 95% of the external osmotic pressure due to added NaCl (Reed, 1987).

Lucas et al. (1990) reported that, NaCl concentration and glycerol accumulation are linked through a putative sodium-glycerol symport that uses the sodium gradient as a driving force for maintaining the glycerol gradient.

This transporter would also accept potassium as co-substrate. Furthermore, it has been observed that, the glycerol uptake is accompanied by proton uptake when extracellular NaCl is present and that the protonophore (CCCP) induces collapse of the glycerol gradient, supporting earlier proposals that the intracellular Na^+ concentration is kept lower by an active Na^+-H^+ exchange mechanism (Luyten et al., 1995).

It is also interesting that, this halotolerant-halophilic yeast has the natural capacity to adapt to high salt concentrations, Na^+ being accumulated without producing any apparent toxicity. On the contrary, cells show some better functional characteristics in the presence of NaCl (González-Hernández et al., 2004).

Yeasts have revealed along the years that, the information contained in their genes may be successfully transferred to other microorganisms, plants and other higher eukaryotes, resulting in the improvement of resistance capabilities (Domínguez, 1998). Thus, heterologous expression of genes associated with salt stress resistance remains one of the most important goals in salt stress research. However, the mechanisms underlying long-term resistance to high salt concentrations are yet poorly understood. Considering the important role of glycerol in osmoregulation, described for *D. hansenii,* it is of great importance to characterize the glycerol transport from both molecular and physiological points of view, in order to understand the mechanisms underlying salt resistance in this yeast. Here, describing with the available tools, the molecular and physiological characterization of a glycerol transporter from this halophilic and alkaline tolerant yeast.

MATERIALS AND METHODS

Yeasts strains and bacteria

The type strains *D. hansenii* PYC2968 (CBS767) and *S. cerevisiae* BY4741 (MAT a; his3 1; leu2 0; met15 0; ura3 0) were used as genetic background strains; *S. cerevisiae* Y05831 (BY4741Mat a; his3 1; leu2 0; met15 0; ura3 0; YDR536w::kanMX4) was obtained from EUROSCARF consortium (Table 1). Competent cells of *Escherichia coli* XL1-Blue (Invitrogen) were also used for plasmid (Yep352) selection and propagation (Hanahan, 1985). JCGHZERO is the wild type with the empty plasmid, JCGHSTL1 is the wild type with the plasmid plus *DhSTL1*, JCGH*zero* is the *STL1* mutant with the empty plasmid, and JCGH*stl1* is also the mutant with the plasmid plus the *DhSTL1* gene.

Growth media

D. hansenii strain was routinely maintained in YPD medium (10 g yeast extract, 10 g peptone, 20 g glucose and 20 g agar per liter). The transformant strains were routinely maintained on solid YNB-ura (w/o amino acids) medium with 2% glucose, supplemented, when required, with the adequate requirements for prototrophic growth (Pronk, 2002). *E. coli* XL1-Blue strain was routinely maintained in Luria-Bertani medium (LB) at 37°C; ampicillin (100 µg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal, 4 µg ml-1) were used as supplements (Sambrook, 1989) when required.

Cloning strategy and DNA manipulations

Sequence data for *D. hansenii* were obtained from Génolevures Consortium website (Génolevures, 2001), by performing tblastn search with the Stl1p sequence from *S. cerevisiae* against the *D. hansenii* genomic sequence. Using the BLASTP 2.2.14 program (NCBI, Bethesda, MD, USA) (Altschul et al., 1997), ORF revealing homology to *S. cerevisiae* Stl1p protein were identified. Based on the nucleotide sequences of this ORF together with the contiguous upstream and downstream regions, primers were designed to amplify a region comprised between approximately -1000 bp from the ATG start codon and +200 bp after the TAA stop codon of the gene. Specific primers designed and modified to incorporate a restriction site (underlined) for *Bam* HI forward primer (5´-CGGGATCCCGTTTTGTCTTTGCTGACTCCC-3´), and for reverse primer, *Pst* 1; (5´-AACTGCAGAACCAATGCATTGGTCACGGTTCAAGTGTCTTAAA-3´), to amplify STL1 ORF and the flanking regions (-1000 bp, +200 bp). PCR amplification was carried out in an Eppendorf thermocycler with DNA polymerase from BIOTOOLS, for 30 cycles, at 64°C (annealing temperature chosen according to the primer characteristics). Using this approach, one fragment of 2.6 kb was

Table 1. List of bacteria, yeasts strains, and plasmids used in the present work

Plasmids and strains	Characteristics	Source
Bacteria		
Escherichia coli DH5α		(Hanahan, 1985)
Strains		
D. hansenii (CBS767)	Type strain	Portuguese yeast culture collection
S. cerevisiae (BY4741) (wild type)	MAT a; his3 1; leu2 0; met15 0; ura3 0	EUROSCARF
S. cerevisiae (BY4741) (*stl1*)	BY4741Mat a; his3 1; leu2 0; met15 0; ura3 0; YDR536w:: kanMX4	EUROSCARF
JCGHZERO	BY4741 (YEp352 empty)	This work
JCGHSTL1	BY4741 (JCGHpSTL1)	This work
JCGH*zero*	BY4741 *stl1* (Yep352 empty)	This work
JCGH*stl1*	BY4741 *stl1* (JCGHpSTL1)	This work
Plasmid		
YEp352	Yeast episomal vector, 2 µm, URA3 yeast marker and Amp^R	(Hill et al., 1986)
JCGHpSTL1	YEp352 derivative containing *DhSTL1* gene	This work

obtained, using *D. hansenii* CBS 767 genomic DNA as a template. The amplified products were digested with *ECoR* I and *Hind* III, purified using the purification kit "GFX PCR DNA and Gel Band Purification" (GE Healthcare). The fragment was cloned into the XL1-Blue *E. coli* strain (YEp352, see Table 1), characterized at the molecular level, and used to transform a wild type *S. cerevisiae* strain and *stl1 S. cerevisiae* strain by the lithium acetate method (Geitz and Schiestl, 1995). Current plasmid isolation was performed by alkaline extraction as described in Birnboim and Doly (1979), modified as in Sambrook et al. (1989). For plasmid isolation from yeasts, the procedure described by Hoffman and Winston (1987) was followed. Agarose gel electrophoresis and restriction site mapping were performed according to standard methods (Sambrook, 1989). Yeast genomic DNA from *D. hansenii* was isolated (Cryer, 1975), after a previous treatment with liticase. Yeast genomic pDNA was isolated using QIAprep Spin Miniprep Kit Protocol according to manufacturer's directions. Constructs were named as: JCGHZERO for plasmids without *DhSTL1*; JCGHSTL1, for plasmids containing the *DhSTL1* gene (Table 1).

Transformants were selected on minimal medium with methionine, leucine, and histidine; 40 transformants (JCGHZERO), 147 transformants (JCGHSTL1), 48 transformants (JCGH*zero*), and 119 transformants (JCGH*stl1*) were obtained. One representative clone from each transformant was used for heterologous expression studies.

Isolation of RNA and northern blot analysis

Total RNA was extracted from exponential phase yeast cells after 6 h of incubation under saline stress conditions. Cells were then collected by centrifugation, frozen using liquid nitrogen and kept at -80°C until RNA extraction was performed. Total RNA was extracted by the hot phenol extraction protocol (Schmitt et al., 1990), modified by Daniela Castro (personal communication), as described below. Frozen cells were re-suspended in 470 µl of 100 mM sodium acetate, pH 5.0, 5 mM $MgCl_2$ plus 1/10 volume of 10% SDS (w/v), 5 µl DEPC, and 500 µl glass beads, and vortexed for 1 min. After vortexing, cells were subjected to three hot phenol extractions (5 min at 65°C) with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), pH 5.0, and one extraction with 1 volume of chloroform : isoamyl alcohol at room temperature. RNA precipitation was performed as described by Schmitt et al. (1990). Total RNA was fractionated through formaldehyde-agarose gels and transferred to N^+-Hybond membranes (GE Healthcare). Hybridization was performed with a digoxygenin (DIG)-labelled probe prepared from an internal fragment of *DhSTL1* labelled using the DIG system (Roche) by random priming, according to manufacturer's instructions. Hybridizations were performed in DIG Easy Hyb (Roche), at 50ºC (16 h). Membranes were then washed under high-stringency conditions and exposed to X-ray films for a maximum of 48 h.

Growth assays

Specific growth rates were determined in liquid YNB-ura medium (2% (w/v) of glucose or glycerol) starting from a 50 ml pre-inoculum of cells grown in the same medium. The pre-inoculum was grown at 28ºC for 24 h in an orbital shaker at 180 rpm and used to inoculate 250 ml flasks containing 100 ml of liquid medium at 0.2 of the initial optical density. Growth was followed by measuring the absorbance at 640 nm, with a Shimadzu spectrophotometer, model UV-160A. The ability of transformant strains to grow in the presence of different NaCl and KCl concentrations (0.6, 1.5 and 3.0 M) at two different pH (5.6 and 7.5, adjusted with Trizma base), and two carbon sources (2% (w/v) of glucose or glycerol) was assessed on solid YNB-ura media with KCl and NaCl to the desired final concentrations. Transformants were grown for 24 h in 25 ml of YNB-ura liquid medium (2% glucose) to a final density of approximately 3×10^7 ml^{-1}. Plates were inoculated with serial 10-fold dilutions of these cultures and incubated at 28ºC. Growth was recorded after 1 or 2 weeks (in the case of the 3 M NaCl or KCl medium).

Glycerol transport measurements

Initial uptake rates and accumulation ratios of radiolabeled glycerol

were measured using the method described by Lucas et al. (1990) with modifications. Cells were grown to exponential phase (OD_{640} nm 0.4-1.0), harvested by centrifugation, washed twice, and resuspended in ice cold water to a final concentration of 0.25 g/ml and kept on ice. The cell suspension (25 µl) was mixed with 100 mM MES or MOPS buffer (pH 5.6 or 7.5, 50 µl; adjusted with Trizma base) in 10 ml conical centrifuge tubes. After 2 min at 28°C in a water bath, the reaction was started by adding 25 mM aqueous solution of D-[U-^{14}C]-glycerol (specific activity of ≈ 700 cpm/nmol) at appropriate concentrations. After 10 s, the incorporation was stopped by diluting with 5 ml of ice cold water. Cells were immediately collected on Whatman GF/C filters (Whatman, Maidstone, England) at reduced pressure, washed with 5 ml cold de-mineralized water, and immersed in vials containing 5 ml of scintillation fluid (Optiphase `Hisafe´ 2, Perkin Elmer; Life sciences manufactured by Fisher Chemical Products, England). The radiolabeled glycerol taken up by cells was measured in a scintillation counter (Beckman LS3801). All determinations were performed in triplicate and referenced to a blank made by inverting the sequence of addition of glycerol and water.

The initial uptake rates were also measured in the presence of 0.6 M of NaCl or KCl. In order to determine the transport-driven in/out accumulation ratios of ^{14}C-glycerol, 25 µl of cell suspension (0.25 g/ml) was mixed with 5 µl glucose [1 M] and 30 µl of 100 mM MES or MOPS (adjusted at pH 5.6 or 7.5 with TRIZMA base) in 10 ml conical centrifuge tubes and incubated at 28°C. After 2 min of incubation, the reaction was started by adding 40 µl (25 mM) of ^{14}C-glycerol (specific activity ≈ 700 cpm/nmol). At appropriate time intervals, 10 µl aliquots were taken and filtered through Whatman GF/C filters at reduced pressure, washed twice with 5 ml ice-cold water, and transferred to vials containing 5 ml scintillation fluid. The accumulation ratio assays were also performed in the presence of 0.6 M of NaCl or KCl. Efflux of the radiolabel upon the addition of nonradioactive glycerol at a final concentration of 1 M or of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) at a final concentration of 50 µM was tested. The *D. hansenii* and *S. cerevisiae* intracellular volumes, used to calculate the intracellular glycerol concentrations were determined previously (González-Hernández et al., 2004).

Reproducibility

All assays were repeated at least three times, and the data reported are means or representative values.

RESULTS

The release of the complete genome sequence of *D. hansenii* by the Génolevure consortium, led me to search for *D. hansenii* sequences with homology to genes involved in glycerol transport in order to clone and characterize the putative transporter (s). By performing a tBlastn search, It was compared the STL1 protein sequence of *S. cerevisiae* against the translated sequence from the *D. hansenii* genome sequence database released by Génolevure. An ORF (DEHA0E01122g) was found with 54% homology and 39% identity with Stl1p from *S. cerevisiae*; located in chromosome E (anti-sense strand) and previously annotated as a gene of the sugar permease family STL1 uptake transporter protein of 510 amino acids (aa). The chromosomal regions corresponding to *DhSTL1* were amplified together with it own putative promoter and termination regions. The amplified fragments were cloned and used to transform a wild type and a *S. cerevisiae* mutant (*stl1*). Table 1 lists plasmids and strains used for cloning this transporter.

In order to assess the possible contribution of the DhStl1p protein under study to the glycerol transport, growth of *D. hansenii* and the *S. cerevisiae* transformants was evaluated in YNB-ura liquid mineral medium without stress conditions with glucose or glycerol as carbon sources. Figure 1 illustrates the growth results obtained with the transformants. It is very clear that the presence of *STL1* gene disturbs growth on glucose in both types of transformants (Figure 1A) whereas growth on glycerol is improved (Figure 1B). Although Figure 1 only represents one set of results, they were consistent in three sets of experiments. This effect was more evident in the transformant obtained with the *stl1* mutant of *S. cerevisiae*. In this case the doubling time decreased from 55 to 35 h. Table 2 shows the doubling times of the growth curves of Figure 1; *S. cerevisiae* transformant cell grown in glucose showed similar or lower doubling time values; in the glycerol curves of *S. cerevisiae* transformants, the doubling times showed clearly the contribution of *DhSTL1* in the *S. cerevisiae* (*stl1*) strain.

Figure 2 shows the ability of *D. hansenii* and transformants strains to grow on solid YNB-ura media without salt, and the presence of NaCl and KCl (0.6 M, of each salt), at pH 5.6 (A), and 7.5 (B), and glucose as carbon source. The phenotype conferred by *DhSTl1* was evaluated, inserted into the YEp352 plasmid, and cloned into a wild type *S. cerevisiae* strain and into another lacking the *STL1* gene, obtaining JCGHZERO, JCGHSTL1, JCGH*zero*, and JCGH*stl1*. Growth of these transformants was assessed in plates with 0.6 M, 1.5 M, and 3.0 M of NaCl or KCl. The *S. cerevisiae* transformant strains and *D. hansenii* grown in presence of glucose without salt were able to grow in plates in presence of the salts (0.6 and 1.5 M). It is worth mentioning that the phenotype of Na^+ or K^+ tolerance in the *S. cerevisiae* (*stl1*) transformant was conferred by *DhSTL1* gene; slightly at pH 5.6, and clearly at pH 7.5, growth was recorded after 1 week. The plates growth in the presence of 1.5 and 3.0 M of the salts (data not shown); in the presence of 1.5 M of salts the results were similar to those obtained in the plates with 0.6 M of salts; the plates with 3.0 M of salts did not show growth of *S. cerevisiae* transformants, only *D. hansenii* had the capacity to grow after 2 weeks in presence of KCl or NaCl (3 M). In the presence of glycerol as carbon source, *D. hansenii* grew in all experimental conditions evaluated in the presence of 3.0 M of the salts, showing a slight growth after two weeks (data not shown); the *S. cerevisiae* transformant cells showed growth more clearly in YNB-ura medium without salt at both pHs; the phenotype conferred by *DhSTL1* in JCGHSTL1 transformant strain was observed in the presence of 0.6 M of salts; the other *S. cerevisiae* transformant cells did not show a clear phenotype; at 1.5 M of salt a slight growth was observed, whereas at 3.0 M

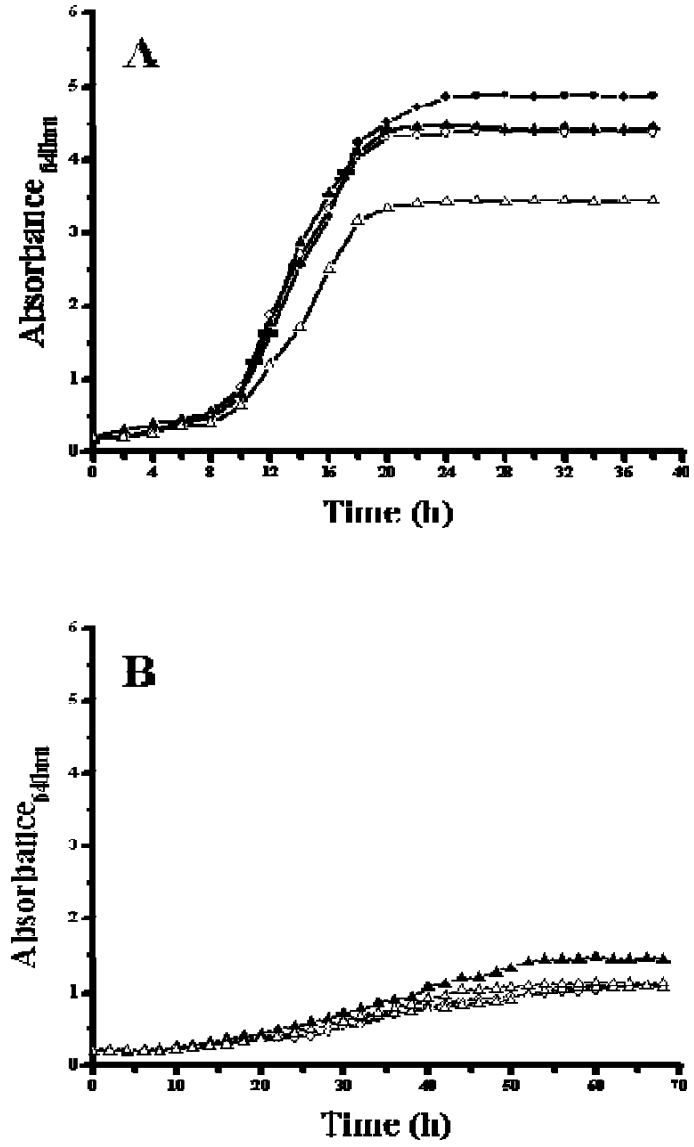

Figure 1. (Gonzalez-Hernández JC) growth of *S. cerevisiae* transformants cultivated in YNB-ura medium (2% (w/v) glucose [**A**]

Table 2. Doubling time of D. hansenii and S. cerevisiae transformants cultivated in YNB-ura medium (2% (w/v) glucose and 2% (w/v) glycerol).

	Doubling time (h)	
	Glucose	**Glycerol**
D. hansenii	9.41	9.38
JCGHZERO	2.52	32.3
JCGHSTL1	2.55	31.35
JCGH *zero*	2.72	55.42
JCGH *stl1*	3.13	35.51

Doubling time was determined from the exponential phase of the curves. Cells were grown as described under Materials and Methods. The results are the means of three experiments.

no growth was observed (data not shown).

The kinetic properties of the glycerol transport system in *D. hansenii* and *S. cerevisiae* transformants is shown in Table 3, at pH 5.6 and 7.5. The results of glycerol kinetic parameters can be summarized as follows: a) in *D. hansenii* (pH 5.6) the Vmax values are similar, the presence of NaCl or KCl increased the affinity for ^{14}C-glycerol uptake; b) the Vmax values for all *S. cerevisiae* transformants (pH 5.6) are around 6-fold lower as compared with *D. hansenii* strain; c) the Km values (pH 5.6) for *S. cerevisiae* transformant yeasts did not show significant differences between the cells incubated with or without salt; d) in *D. hansenii* (pH 7.5), the Vmax values are similar too, but the presence of NaCl or KCl decreased the affinity for ^{14}C-glycerol uptake; e) the Vmax values for all *S. cerevisiae* yeast strains (pH 7.5) are around 6 or 10-fold lower as compared with *D. hansenii* strain; f) the Km values (pH 7.5) for *S. cerevisiae* transformants decreased the affinity for ^{14}C-glycerol uptake in the cells incubated with salt; g) at both pHs, in the JCGH*zero* strain, a simple diffusion (D) of ^{14}C-glycerol transport was observed; h) JCGH*stl1* conferred glycerol transport phenotype at both pHs, it was saturable and adjustable to one component in *stl1* strain.

The influence of extracellular pH on maximum glycerol accumulation ratio in *D. hansenii* was studied at 5.6 and 7.5 pH values Cells grown in YNB-ura medium with glycerol as carbon source showed accumulation of labeled glycerol (Figure 3) against gradient. CCCP prevented slightly accumulation and elicited a significant efflux of labeled glycerol when added after 20 min of incubation. A similar behavior caused the addition, at the same time of 1 M of cold glycerol after 20 min incubation time. Figure 3A shows that, the presence of KCl increased the in/out accumulation rate more than NaCl at pH 5.6; whereas, at pH 7.5, the salts decreased the in/out accumulation rate. In/out ratios presented significant variations in comparison with the value previously obtained at pH 5.6, and CCCP prevented glycerol accumulation at the pH value tested. In Figure 3B, incubation in the presence of a salt diminished the accumulation rate of labeled glycerol, the results obtained in the Figure 3A showed that maximum accumulation ratio did not exceed 20 times in *D. hansenii* without salt, whereas the accumulation rate of *D. hansenii* without salt at pH 7.5 increased 1-fold as compared with the experiments at pH 5.6.

The accumulation rates of labeled glycerol was evaluated in the *S. cerevisiae* transformant strains (Figure 4), these were also incubated in the presence of CCCP, or cold glycerol (data not shown); in all conditions, the incubation decreased the glycerol accumulation rate, the CCCP elicited efflux of labeled glycerol when it was added at the start of the incubation reaction or after 20 min of incubation. Another point to describe about this experiment are the very low ratios of accumulated exposed to high NaCl concentrations (Adler and Gustafsson, 1980). Some authors (Serrano, 1996) have considered the

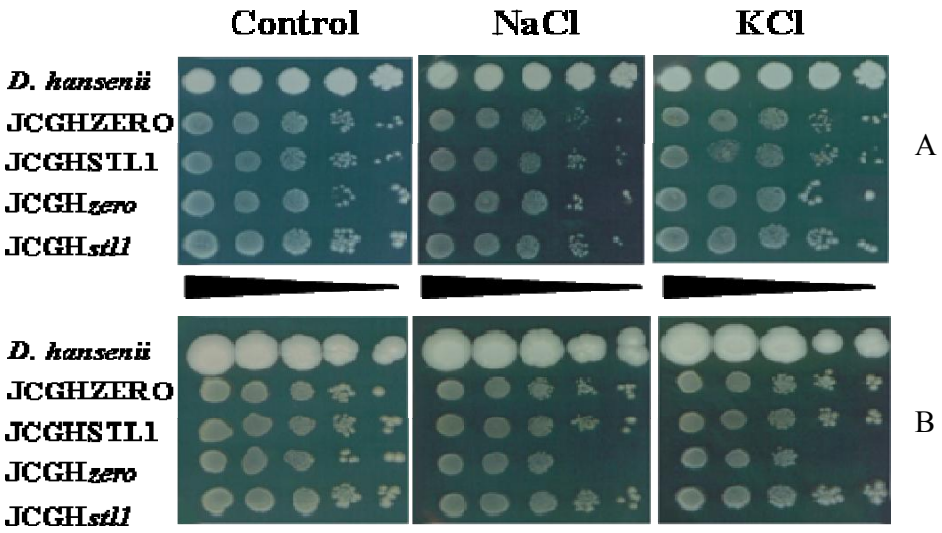

Figure 2. (Gonzalez-Hernández JC) Growth of *D. hansenii* and transformants of *S. cerevisiae* containing YEp352 with or without fragment of *STL1* genomic DNA from *D. hansenii*. Cells were grown for 24 h in 5 ml of YNB-ura liquid medium up to final density approximately 3×10^7 ml^{-1}. Plates were inoculated with serial 10-fold dilutions (the black arrow indicates the dilution direction) of these cultures onto YNB-ura medium with glucose as carbon source (pH 5.6 [A], and pH 7.5 [B]), and different growth conditions (YNB-ura medium, 0.6 M of NaCl, and KCl, respectively), and incubated at 28ºC. *D. hansenii*, and the *S. cerevisiae* transformants (JCGHZERO, JCGHSTL1, JCGH*zero*, JCGH*stl1*). Growth was recorded after 1 week. Data are representative of three experiments.

Table 3. Kinetic parameters of ^{14}C-glycerol transport (pH 5.6 and 7.5) of *D. hansenii* and *S. cerevisiae* transformants cultivated in YNB-ura medium, and incubated in the absence or the presence of 0.6 M NaCl or KCl.

	pH 5.6		pH 7.5	
	Vmax	**Km**	**Vmax**	**Km**
D. hansenii	0.77	0.68	0.56	0.30
0.6 M KCl	0.78	0.49	0.52	0.60
0.6 M NaCl	0.79	0.49	0.40	0.43
JCGHZERO	0.13	0.90	0.051	0.38
0.6 M KCl	0.19	1.05	0.08	0.38
0.6 M NaCl	0.12	0.77	0.15	0.73
JCGHSTL1	0.086	0.69	0.034	0.10
0.6 M KCl	0.13	0.48	0.024	0.13
0.6 M NaCl	0.12	0.60	0.034	0.12
JCGH *stl1*	0.14	0.56	0.14	0.71
0.6 M KCl	0.12	1.12	0.17	1.12
0.6 M NaCl	0.20	0.92	0.11	0.57

	D = mmol g^{-1} d. wt.	
	pH 5.6	**pH 7.5**
JCGH *zero*	0.14	0.56
0.6 M KCl	0.12	1.12
0.6 M NaCl	0.20	0.92

The transport of labeled cations was measured as described under Methods. (Vmax = mmol h^{-1} g^{-1} d. wt; Km = [mM]; D = diffusion coefficient). Results represent the means ± SEM (n = 3).

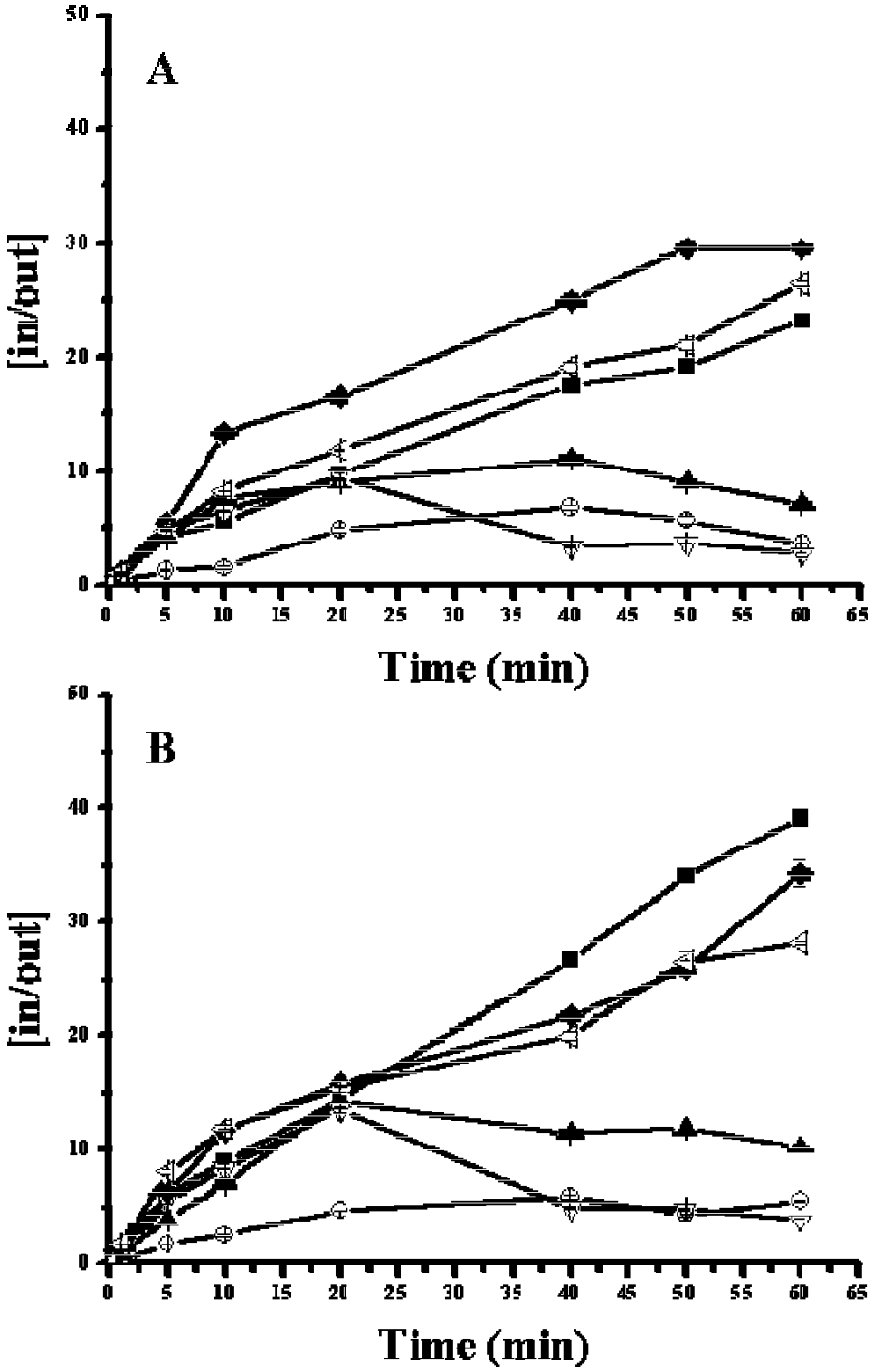

Figure 3. Accumulation of 14C- glycerol at pH 5.6 (A) or 7.5 (B) by D. hansenii grown in YNB-ura medium (2% glycerol), 25 µl cell suspension (0.25 g/ml), 5 µl glucose [1 M], and 30 µl 100 mM MES or MOPS (accumulation transport was also assessed in the presence of 0.6 M of NaCl or KCl). The experiment was started by adding 14C-glycerol. Aliquots of 10 µl cell suspension were taken at the indicated times and handled as described under Methods. Results represent the means ± SEM (n = 3). *D. hansenii* (), Accumulation of radiolabel was prevented by adding 50 µM of CCCP ( ), Efflux of radiolabel after the addition of 50 µM of CCCP at 20 min of incubation ( ), Efflux of radiolabel after the addition of nonradioactive glycerol at 20 min of incubation (, D. hansenii incubated with 0.6 M of KCl (), D. hansenii incubated with 0.6 M of NaCl ( ).

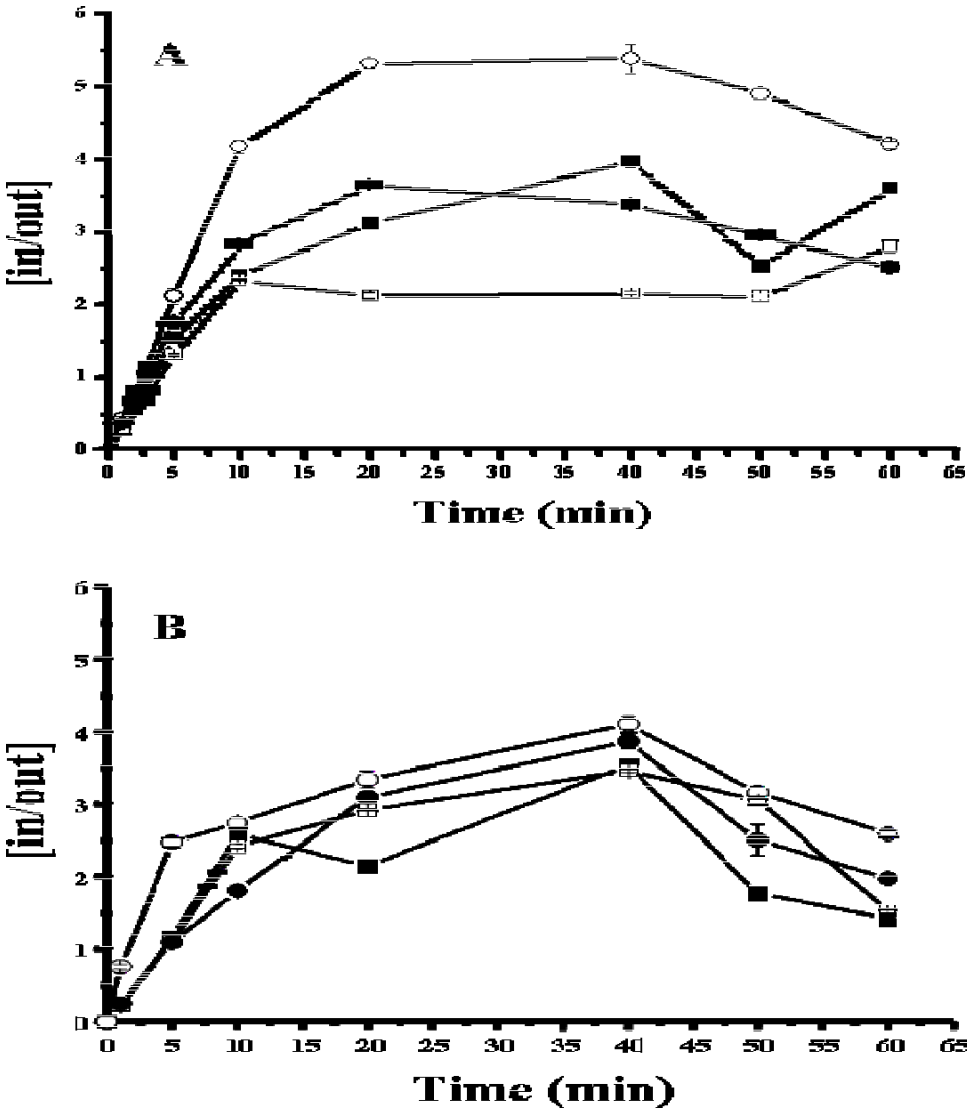

Figure 4. Accumulation of ^{14}C-glycerol at pH 5.6 (A) or 7.5 (B) by *S. cerevisiae* transformants grown in YNB-ura medium (2% glycerol), 25 µl of cell suspension (0.25 g/ml), 5 µl glucose 1 M, and 30 µl 100 mM MES or MOPS. The experiment was started by adding ^{14}C-glycerol. Aliquots of 10 µl cell suspension were taken at the indicated times and handled as described under Methods. Results represent the means ± SEM (*n* = 3). JCGHZERO (), JCGHSTL1 (), JCGH*zero* (), JCGH*stl1* ( ).

possibility of obtaining genes from *S. cerevisiae* involved in salt resistance, with the objective of eventually transferring these genes to plants. It would be worthwhile to consider at least the identification of one or several key genes from yeasts, such as *D. hansenii*, glycerol, which were almost 10-fold lower as compared with the ratios of accumulated glycerol in *D. hansenii*. Figure 4 shows the in/out accumulation rate obtained in the *S. cerevisiae* transformants strains grown in YBN-ura medium without salt in the presence of glycerol as carbon source at pH 5.6 or 7.5; at both pHs. The phenotype conferred by *DhSTL1* to the JCGHSTL1 transformant was observed, which increased the in/out accumulation rate of labeled glycerol, more accumulated glycerol at pH 5.6 than 7.5 was observed.

The northern blot analysis was performed for *DhSTL1* transcripts in *D. hansenii* using total RNA prepared from *D. hansenii* cells that had been grown in YPD medium (Figure 5); cells were grown to exponential phase on YPD glucose medium and shifted to the same medium with or without 0.6 M of KCl or NaCl. *DhSTL1* gene expression was observed slightly in absence of salts (pH 5.6), whereas, in the presence of salts, it was observed an increase in the transcript level of *DhSTL1* at

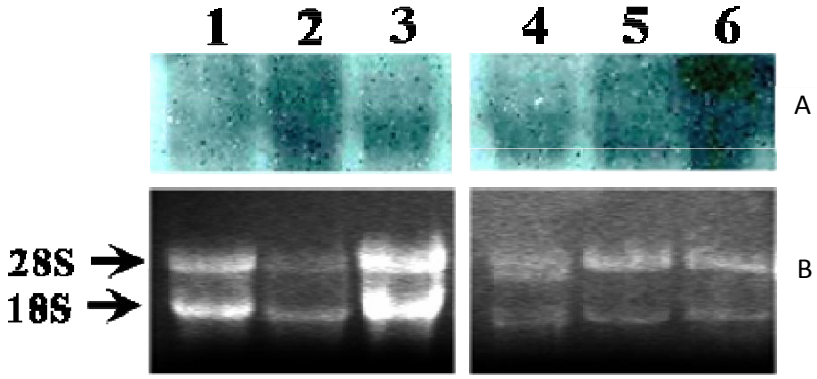

Figure 5. (Gonzalez-Hernández JC) Northern blot analyses. Total RNA (30 µg) was extracted and loaded onto 1.0 % agarose gel, transferred onto a membrane, and hybridized with DIG-labeled *STL1* Easy Hyb (Roche). Total RNA extracted from cells grown in YNB medium and incubated for 6 h in YPD medium without salt (lanes 1,4); with 0.6 M of KCl (lanes 2, 5) or 0.6 M of NaCl (lanes 3, 6). For lanes 1-3, the cells were incubated at pH 5.6; and for lanes 4-6, at pH 7.5 (A). Ethidium bromide-stained (0.01 µg ml^{-1}) pattern (B). Arrowheads indicate the positions of ribosomal RNA. Data are representative of three experiments.

pH 5.6; at 7.5 pH (Figure 5).

DISCUSSION

High NaCl concentrations cause loss of cellular water, which leads to cell shrinkage and growth arrest (Blomberg and Adler, 1992). As part of the adaptation to high NaCl concentrations, compatible solutes are produced and high levels of these solutes are obtained inside the cell, resulting in re-entry of lost water, regaining cell volume (Brown, 1978; Yancey, 1982). Both *D. hansenii* and *S. cerevisiae*, produce high intracellular levels of glycerol as the main compatible solute when which are naturally halotolerant or halophilic to the same purpose. *STL1* was identified during a screen for genes encoding membrane proteins involved in glycerol utilization, and it has been previously assigned as an active glycerol/H^+ symporter in *S. cerevisiae* (Ferreira et al., 2005).

The molecular biology of *D. hansenii* is poorly established, and recently the genome has been explored in the Génolevures project (Génolevures, 2001). In this project, the genomes of *S. cerevisiae* and other yeast species of the Hemiascomycetes class are compared. In that sense, the available genetic tools were used (*D. hansenii* genome database) to search for sequences of genes involved in glycerol transport in yeasts. This procedure allow to detect sequences and design primers to amplify and clone successfully the complete Stl1 protein sequence, as well as the upstream and downstream regions eventually involved in their regulation.

One of the phenotypes of the *S. cerevisiae* transformant strains is a slow, and low growth in YNB-ura medium when glycerol is the carbon and energy source, this experiments are similar to those reported by Lages and Lucas (1995). Ferreira et al. (2005), reported that deletion of the *STL1* gene in both BY4742 and W303-1A genetic backgrounds resulted in cells that grew poorly on glycerol, the present experiments were made on BY4741 genetic background, obtained from EUROSCARF consortium. The genomic clones carrying the *DhSTL1* gene (from which the probe was derived) includes upstream and downstream non-coding sequences, therefore the expression of the *DhSTL1* gene was under the control of its own promoter.

To identify if Stl1 protein is involved in active uptake of glycerol in *D. hansenii*, the ability of *S. cerevisiae* transformant strains to complement *D. hansenii* growth phenotypes were evaluated (Figure 2); this was verified by cloning *DhSTL1* gene and inserting in YEp352 plasmid. The JCGH*stl1* transformant strain showed a slight but significant difference in the doubling times in growth curves made in liquid YNB-ura medium, with glycerol as carbon source. In an experiment performed in solid YNB-ura medium, In *STL1* gene-deleted transformant strains, the, *DhSTL1* depicts a slow growth on glycerol as a sole carbon source in the absence or presence of salts, but a phenotype was observed in the experiments; whereas, in glucose-grown cells, the phenotype of *DhSTL1* in *stl1* transformant strain is clearer.

S. cerevisiae has earlier been found to possess the ability to take up glycerol from the surroundings against a concentration gradient (Lages and Lucas, 1997). The uptake was shown to be driven by electrogenic proton symport. Recently, it has been reported (Ferreira et al.,

2005), that the cloning glycerol proton symporter in *S. cerevisiae* is encoded by the *STL1* gene. The successful of the *DhSTL1* gene in *S. cerevisiae* wild type, and in the *STL1* gene-deleted strain to characterize and identify if this protein is responsible for the glycerol transport in *D. hansenii*. All the transport and accumulation assays were evaluated in cells grown in YNB-ura medium using glycerol as carbon source, considering that in the *S. cerevisiae* transport system was under glucose repression and inactivation, glucose-grown cells presented, instead, a lower affinity permease for glycerol, probably a facilitated diffusion (Lages and Lucas, 1997).

Earlier reports, in which glycerol transport was evaluated in cells grown in mineral medium with vitamins and 2% of glucose as carbon source at 25ºC, confirmed that *D. hansenii* showed a constitutive active glycerol transport system that is not subject to glucose repression and mediates glycerol accumulation as a function of extracellular NaCl concentration (Lucas et al., 1990). The results were evaluated too at pH 7.5, because *D. hansenii* is considered an alkaline-tolerant yeast. In this sense, the performed experiments showing that glycerol transport was saturable and adjustable to one component at both pHs, 5.6 and 7.5. The kinetic parameter (pH 5.0) of glycerol transport reported in *S. cerevisiae* show an affinity uptake system with Km of 1.7 mM and Vmax of 441 µmol h^{-1} g^{-1} dry weight, the values obtained in the present experiments and the values reported in the presence of glucose as carbon source (Lucas et al., 1990) for *D. hansenii* showed a higher affinity uptake and velocity rate compared with the values reported for *S. cerevisiae* (Lages and Lucas, 1997). The *S. cerevisiae* transformant strains used in this work revealed that the *DhSTL1* gene conferred phenotype for JCGH*stl1* transformant strain.

As has been published for *S. cerevisiae* (Lages and Lucas, 1997), most of the strains able to grow on glycerol as the single carbon and energy source show evidence of inductive active transport. The existence of an H^+-glycerol symport induced by growth on glycerol is consistent with a role of such a carrier in glycerol catabolism. In contrast, a constitutive transporter can more easily be associated with both the salt stress response and glycerol assimilation.

Another way to analyze the components of glycerol uptake is to measure glycerol accumulation against a concentration gradient. The accumulation of compatible solutes, such as glycerol, in the yeast *S. cerevisiae*, is a ubiquitous mechanism in cellular osmoregulation. The rate of glycerol uptake is strongly reduced during growth at high osmolarity, indicating that yeast cells possess mechanisms that control the transport rate (Luyten, 1995; Sutherland, 1997). The action of a protonophore, eliminating ΔpH and lowering intracellular pH (Serrano, 1991), can affect active uptake and prevent the consequent accumulation, but the possibility cannot be disregarded that it also affects enzymes from the first steps of catabolism, creating artifacts.

Rep et al. (2000) reported that expression of this *STL1* gene is undetectable under normal conditions but strongly induced after osmotic shock (in presence of NaCl and/or sorbitol), as confirmed by Northern blot analysis. The slight expression observed of the *DhSTL1* gene without salt (pH 5.6), but, in the presence of salts, It was observe an increase resulting from Stl1p expression (Figure 5). The obtained results at more alkaline pH showed similar Stl1p expression pattern in absence or presence of salts. Importantly, the levels of Stl1p and glycerol accumulation activity were directly correlated, when the experiments were evaluated at pH 5.6. The increased levels of Stl1p appearing after shifting to KCl or NaCl salts, and the accumulation rate in *D. hansenii* wild type strain was higher (pH 5.6) in the presence of salts (Figure 5). The slight expression of this transport and a higher level of salt-stress resistance suggest that this could be an evolutionary advantage for growth under such conditions. Correspondingly, glycerol symport has previously been shown to be repressed by glucose, induced by growth on nonfermentable carbon sources, and transiently detectable during diauxic shift upon growth on glucose (Lages and Lucas, 1997). *STL1* gene is also highly and transiently induced by osmotic shock during exponential growth on glucose-based media (Posas, 2000; Rep, 2000). The rapid appearance of Stl1p under these conditions suggests a role for the glycerol symporter during the immediate response to osmotic shock. This might be important in nature, considering the extreme, diverse, and rapid changes in environmental conditions yeasts may experience. Because yeast cells leak a substantial amount of the produced glycerol into the medium (Shen, 1999), this induction of Stl1p is not surprising.

D. hansenii yeast appears to respond to salinity stress in a similar manner to *S. cerevisiae* during early exponential phase, increasing the production of glycerol in response to salinity stress, whereas cells in late exponential phase show conservation of glycerol intracellularly, as in *Z. rouxii* (Zyl, 1990); the molecular basis for these differences in glycerol permeability is unknown.

None of the upstream regulatory proteins involved in glycerol transport had been studied and identified in *D. hansenii*. This paper allows suggesting that probably *DhSTL1* is not the principal gene involved in the glycerol transport in *D. hansenii*. Further investigation is being developed to know the actual role of *DhGUP1* and *DhAQPY1* in this solute transport; thus, the information is still scarce to present any regulatory mechanism implicated in the glycerol response functioning in *D. hansenii*. Due to the main role in osmoregulation played by glycerol, understanding its regulation in *D. hansenii* may allow us to comprehend the basis for the different halotolerance that characterizes these two yeasts. *D. hansenii* is not a friendly microorganism to work with.

The development of molecular tools for the manipulation of *D. hansenii* genes is a necessity an urgent task; the laboratory and others involved in this task have recently initiated the study of auxotrophic mutants or resistance molecular markers for this yeast, without any success.

ACKNOWLEDGMENTS

This work was supported by grant SFRH/BPD/19913/2004 to Juan Carlos González-Hernández, granted by FCT, Portugal.

REFERENCES

Adler L, Blomberg A, Nilsson A (1985). Glycerol metabolism and osmoregulation in the salt-tolerant yeast *Debaryomyces hansenii*. J. Bacteriol. 162: 300-306.

Adler L, Gustafsson L (1980). Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. Arch. Microbiol. 124: 123-130.

Andre L, Nilsson A, Adler L (1988). The role of glycerol in osmotolerance of the yeast *Debaryomyces hansenii*. J. Gen. Microbiol. 134: 669-677.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Blomberg A, Adler L (1992). Physiology of osmotolerance in fungi. Adv. Microb. Physiol. 33: 145-212.

Brown AD (1978). Compatible solutes and extreme water stress in eukaryotic micro-organisms. Adv. Microb. Physiol. 17: 181-242.

Chen A, Wadso I (1982). A test and calibration process for microcalorimeters used as thermal power meters. J. Biochem. Biophys. Methods. 6: 297-306.

Cryer DR, Ecclesmall R, Marmur J (1975). Academic Press, New York. Isolation of yeast DNA. In: Methods in Cell Biology (Prescott, D.M., Ed.) 12: 39.

Domínguez JM (1998). Xylitol production by free and immobilized *Debaryomyces hansenii.* Biotechnol. Lett. 20: 53-56.

Ferreira C, van Voorst F, Martins A, Neves L, Oliveira R, Kielland-Brandt MC, Lucas C, Brandt A (2005). A member of the sugar transporter family, Stl1p is the glycerol/H+ symporter in *Saccharomyces cerevisiae*. Mol. Biol. Cell. 16: 2068-2076.

Geitz RD, Schiestl RH (1995). Transforming yeast with DNA. Methods Mol. Cell. Biol. 5: 255-269.

Génolevures (2001). Genomic Exploration of the Hemiascomycete yeasts. http://cbi.labri.fr/Genolevures/. Center for Bioinformatics, Bordeaux.

González-Hernández JC, Cárdenas-Monroy CA, Peña A (2004). Sodium and potassium transport in the halophilic yeast *Debaryomyces hansenii*. Yeast 21: 403-412.

González-Hernández JC, Jiménez-Estrada M, Peña A (2005). Comparative analysis of trehalose production by *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under saline stress. Extremophiles 9: 7-16.

Gustaffson L (1979). The ATP pool in relation to the production of glycerol and heat during growth of the halotolerant yeast *Debaryomyces hansenii*. Arch. Microbiol. 120: 15-23.

Hanahan D (1985). Techniques for transformation of *E. coli*. In: DNA Cloning: A Practical Approach (Glover, D.M., Ed.) 109-135.

Hill JE, Myers AM, Koerner TJ, Tzagoloff A (1986). Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast 2: 163-167.

Hoffman CS, Winston F (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *E. coli*. Gene. 57: 267-272.

Holst B, Lunde C, Lages F, Oliveira R, Lucas C, Kielland-Brandt MC (2000). *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. Mol. Microbio. 37: 108-124.

Lages F, Lucas C (1995). Characterization of a glycerol/H^+ symport in the halotolerant yeast *Pichia sorbitophila*. Yeast 11: 111-119.

Lages F, Lucas C (1997). Contribution to the physiological characterization of glycerol active uptake in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. 1322: 8-18.

Lucas C, Da Costa M, Van Uden N (1990). Osmoregulatory active sodium-glycerol co-transport in the halotolerant yeast *Debaryomyces hansenii*. Yeast 6: 187-191.

Luyten K, Albertyn J, Skibbe WF, Prior BA, Ramos J, Thevelein JM, Hohmann S (1995). Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. EMBO J. 14: 1360-1371.

Norkrans B (1968). Studies on marine ocurring yeasts: respiration, fermentation and salt tolerance. Archiv. Fur. Mikrobiologie 62: 358-372.

Norkrans B, Kylin A (1969). Regulation of the potassium to sodium ratio and of the osmotic potential in relation to salt tolerance in yeasts. J. Bacteriol. 100: 836-845.

Posas F, Chambers JR, Heyman JA, Hoeffler JP, de Nadal E, Arino J (2000). The transcriptional response of yeast to saline stress. J. Biol. Chem. 275: 17249-17255.

Pronk JT (2002). Auxotrophic yeast strains in fundamental and applied research. Appl. Environ. Microbiol. 68: 2095-2100.

Reed RH, Chudek JA, Foster R, Gadd GM (1987). Osmotic significance of glycerol accumulation in exponentially growing yeasts. Appl. Environ. Microbiol. 53: 2119-2123.

Rep M, Krantz M, Thevelein JM, Hohmann S (2000). The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J. Biol. Chem. 275: 8290-8300.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schmitt ME, Brown TA, Trumpower BL (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. 18: 3091-3092.

Serrano R (1991). Transport across yeast vacuolar and plasma membranes. In The Molecular and Cell Biology of the Yeast *Saccharomyces cerevisiae*. Genome Dynamics, Protein Synthesis pp. 523-585.

Serrano R (1996). Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int. Rev. Cytol. 165: 1-52.

Shen B, Hohmann S, Jensen RG, Bohnert H (1999). Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol. 121: 45-52.

Sutherland FC, Lages F, Lucas C, Luyten K, Albertyn J, Hohmann S, Prior BA, Kilian SG (1997). Characteristics of Fps1-dependent and-independent glycerol transport in *Saccharomyces cerevisiae*. J. Bacteriol. 179: 7790-7795.

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982). Living with water stress: evolution of osmolyte systems. Science 217: 1214-1222.

Zyl PJ, Kilian SG, Prior BA (1990). The role of an active transport mechanism in glycerol accumulation during osmoregulation by *Zygosaccharomyces rouxii*. Appl. Microbiol. Biotechnol. 34: 231-235.