Molecular and biochemical characterization of glucose transport in *Torulaspora delbrueckii*

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Introduction

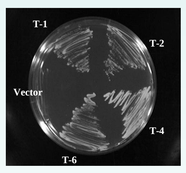
Most of yeast biotechnological applications rely on their ability to efficiently ferment a great variety of sugars. This property is closely related to their sugar transport capacity, which has been widely considered a rate-limiting step of sugar metabolism. In *Saccharomyces cerevisiae* 34 genes encoding established or putative sugar permeases, the largest family of the major facilitator superfamily (MFS), have been identified (Nelissen *et al.*, 1997).

Torulaspora delbrueckii, one of the yeast species most frequently found in home-made corn and rye bread dough (Almeida and Pais, 1996a) has been recognized as the most promising alternative to industrial strains of *S. cerevisiae*. Indeed, *T. delbrueckii* strains display freeze/thaw tolerance (Almeida and Pais, 1996b) and an exceptional resistance to osmotic and Na^+ injury (Hernandez-Lopez *et al.*, 2003). Nevertheless, there is a lack of knowledge on the physiology and molecular biology of this organism, an in-depth investigation being required to gain insight into the function and regulation of *T. delbrueckii* sugar transporters.

Cloning of genes involved on glucose transport

A genomic library of *T. delbrueckii* PYCC 5321 (Hernandez-Lopez *et al.*, 2002) constructed into the vector YEplac181 was transformed into the *S. cerevisiae* strain EBY.VW4000 (Wieczorke *et al.*, 1999), which is deleted for 22 genes, *HXT1-17*, *GAL2*, *AGT1*, *YDL247w*, *YJR160c*, *STL1*, and cannot transport glucose. The plasmids recovered from the selected transformants presented 4 different restriction patterns. A representative of each group (referred as YEpT-1/-2/-4/-6) was used to retransform the *S. cerevisiae* mutant strain, confirming that all of them were able to confer the ability to grow on glucose as sole carbon source and mediated glucose transport, with K_m values in the range of 12-25 mM (Fig. 1).

A.



B.

Transformants	K_m (mM)	V_{max} (nmol s^{-1} mg^{-1} dry wt)
YEpT-6	25.30 ± 2.65	1.11 ± 0.04
YEpT-2	11.90 ± 0.61	2.34 ± 0.04
YEpT-1	25.68 ± 2.56	0.60 ± 0.02
YEpT-4	15.36 ± 1.72	0.85 ± 0.03

Fig. 1. Analysis of Hxt^+ transformants of a *S. cerevisiae* glucose transport-null mutant. A: Growth on glucose plates of YEpT-1, YEpT-2, YEpT-4 and YEpT-6 transformants of the *hxt* null *S. cerevisiae* mutant strain EBY.VW4000. Transformant cells harbouring an empty plasmid (YEplac181) were used as control. Cells were pre-grown on liquid SD-maltose and streaked on solid SD media containing glucose. The plates were incubated at 30 ° C for 3 days. B: Kinetic parameters of glucose transport determined in the same transformants. Cells were grown on SD-maltose, washed with chilled-water, and transferred (OD_{640}, 0.2-0.3) to SD medium containing 2% glucose. After 4 h the zero trans-influx of [U-^{14}C]glucose was measured.

Characterization of *LGT1* gene and of putative LGT1p

LGT1 displayeds a high homology to other yeast glucose transporter genes (Fig. 3).

Analysis of the *LGT1* upstream sequence (985 bp upstream from the ATG) showed the presence of several Mig1p- and Rgt1p-binding sequences and of 4 potential TATA boxes at positions -117, -264, -400 and -575 from the ATG codon.

Some consensus A-rich sequences were found in the *LGT1* 3'-untranslational region (positions 1827 to 1832, and 1953 to 1958), and some in-frame stop codons were present after the TGA codon.

The ORF codifies a potential 567-amino acid protein with the typical structure of a transport protein which has been predicted to contain 11 transmembrane domains. Lgt1p sequence also showed the presence of 1 PESP motif, 2 glucose transport sygnatures, 1 "Leucine-zipper" and 2 potential glycosilation sites.

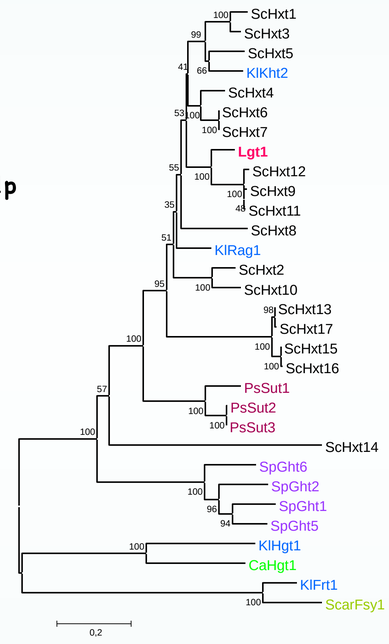


Fig. 3. Relationships between Lgt1p and other yeast hexose transporters. The dendogram was obtained using CLUSTALX (Clustal method) and includes proteins from *Saccharomyces cerevisiae* (Sc), *Kluyveromyces lactis* (Kl), *Pichia stipitis* (Ps), *Schizosaccharomyces pombe* (Sp), *Candida albicans* (Ca) to *Saccharomyces carlsbergensis* (Scar). Numbers given at nodes are the percentage of frequencies with which a given branch appeared in 1000 bootstrap replications.

Induction of *LGT1* expression

Expression of *LGT1* in *S. cerevisiae* was high in media containing 4% of glucose and almost undetectable in galactose as sole carbon source.

In the absence of glucose, repression of *LGT1* expression required the transcription factor Rgt1p. However, a functional Rgt1p does not appear to be required for a full induction of *LGT1* at high glucose levels.

Deletion of the gene coding for the general repressor Mig1p had no effect on *LGT1* expression, but additional disruption of *MIG2* in a *mig1* background indicated that Mig2p or both Mig1p and Mig2p in a redundant way, act as repressors of *LGT1* expression at high glucose concentrations.

Table I. *LGT1* gene expression was measured in cells of *mig1*, *mig1mig2* and *rgt1* mutants of *S. cerevisiae* CEN.PK2-1C. Precultures were grown on SD medium lacking uracil with 2% galactose and transferred to SD medium without uracil containing the indicated sugars and grown for more 4 hours before assay.

Relevant genotype	β-galactosidase activity (Miller units) ± SD		
	2% Galactose	0.2% Glucose	4% Glucose
wt *LGT1::lacZ*	1.3 ± 0.3	21.1 ± 0.8	265 ± 30
mig1 LGT1::lacZ	1.5 ± 0.4	5.7 ± 1.4	189 ± 21
Δmig1 Δmig2 LGT1::lacZ	10.4 ± 0.6	19.9 ± 2.5	683 ± 91
Δrgt1 LGT1::lacZ	1284 ± 132	893 ± 65	722 ± 72

Southern blot analysis

Southern blot analysis revealed the presence of several genes with high homology to *LGT1* in *T. delbrueckii* genome (Fig. 7). These results are according to the kinetics of glucose transport showed by *T. delbrueckii*. These evidences suggest that like has been described for other yeasts *T. delbrueckii* contains several hexoses transporters.

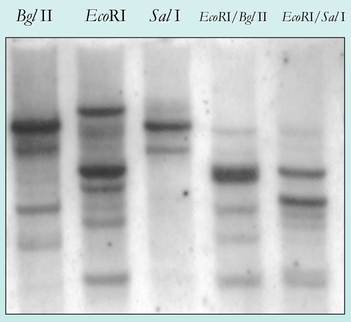


Fig. 7. Southern blot analysis of *T. delbrueckii* wild type using a DNA probe with 300bp, homologous to 18 *S.cerevisiae* hexoses transporters and to *T. delbrueckii LGT1*.

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Identification of *LGT1* gene

DNA sequencing of the insert of plasmid YEpT-6 revealed the presence of a 1,704 bp-length uninterrupted open reading frame (ORF) showing a high similarity (70% to 80% of total identity) to previously reported yeast hexose transporters. This gene was named *LGT1*, for low-affinity glucose transporter. (The GeneBank Accession No. for *LGT1* is AY598344). For further studies the insert from plasmid YEpT-6 was cut with the enzymes *Eco*RI and *Spe*I and the fragment obtained which contained the whole *LGT1* gene, 751-bp of the promoter and 456 bp of the terminator, was used to construct YEpLGT1 (Fig. 2).

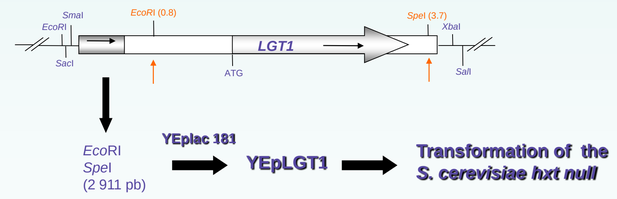

Fig. 2. Schematic representation of the DNA contained in plasmid YEpT-6. A fragment of this DNA was used to construct the plasmid YEpLGT1.

Glucose transport in the *LGT1* transformant of *S. cerevisiae hxt* null strain *versus* *T. delbrueckii* PYCC 5321

Glucose-grown cells of *T. delbrueckii* PYCC 5321 showed kinetics of glucose transport best fitted assuming a biphasic kinetics with a low- and a high-affinity component (Fig. 4). A biphasic kinetics of glucose transport was also observed for fructose and maltose-grown cells (Table II).

Cells of *S. cerevisiae hxt* null strain transformed with the *LGT1* gene exhibited glucose uptake in the range of the low-affinity component. Lgt1p was also able to mediate significant fructose uptake in the *hxt* null mutant (Fig. 5).

In *T. delbrueckii* both the low and high-affinity components of the glucose transport were competitively inhibited by fructose and maltose. In *S. cerevisiae* cells transformed with the *LGT1* gene the presence of fructose or maltose also inhibited the zero *trans*-influx of glucose with features of competitive inhibition (Fig. 6). However, previous results showed that no measurable transport of radiolabelled maltose was detected in glucose-grown cells of *T. delbrueckii* (Alves-Araújo *et al.*, 2004). The results obtained could be interpreted as the consequence of the binding of one glucose residue of maltose to the extracellular binding site of the glucose transporters, impairing glucose transport, as previously suggested for *S. cerevisiae* and *S. bayanus*.

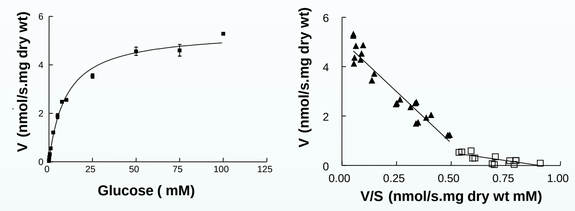

Fig. 4. Direct representation and Eadie-Hofstee plot of glucose initial uptake rates in cells of *T. delbrueckii* PYCC 5321. Cells were grown in 1% glucose (w/v).

Table II. Kinetic parameters of glucose uptake in cells of *T. delbrueckii* PYCC 5321 grown in medium with glucose, fructose or maltose (1%, w/v).

Kinetic parameter	Glucose		Fructose		Maltose	
	High-affinity component	Low-affinity component	High-affinity component	Low-affinity component	High-affinity component	Low-affinity component
V_{max} (nmol s^{-1} mg^{-1}dry wt)	1.17±0.24	5.07±0.15	1.12±0.25	5.07±0.20	1.14±0.13	7.26±0.23
K_m (mM)	1.30±0.34	8.32±0.55	1.77±0.54	13.6±0.96	1.32±0.19	13.9±0.77

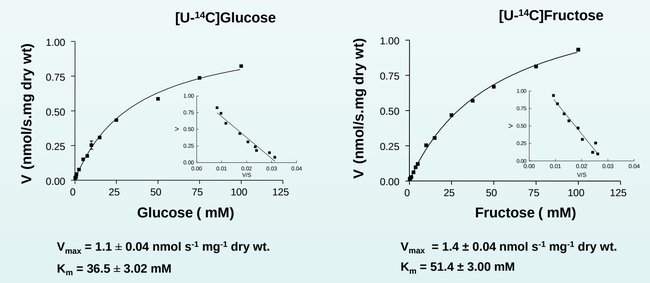

Fig. 5. Direct representation and Eadie-Hofstee plots (insert) of glucose and fructose initial uptake rates in *LGT*1 transformant cells of the *S. cerevisiae hxt* null mutant. Cells were grown in YNB 2% maltose (w/v) , tranfered to and incubated YNB 2% glucose (w/v) and incubated for additional 4 hours.

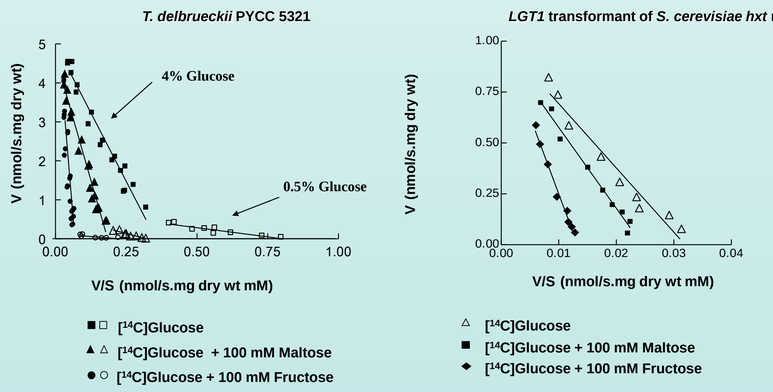

Fig. 6. Eadie-Hofstee plots of glucose initial uptake rates in the presence of other sugars in cells of *T. delbrueckii,* grown either in 0.5 % or 4 % glucose (w/v) and LGT1 transformant of *S. cerevisiae hxt* null strain, prepared as described in Fig. 5.

References
Almeida MJ, Pais CS. 1996a. *Lett Appl Microbiol* **23:** 154-158; Almeida MJ, Pais CS. 1996b. *Appl Environ Microbiol* **62:** 4401-4401; Hernandez-Lopez MJ, Prieto JA, Randez-Gil F. 2002. *Yeast* **19:** 1431-1435; Hernandez-Lopez MJ, Prieto JA, Randez-Gil F. 2003. *Antonie van Leeuwenhoek* **84:** 125-134; Nelissen B, De Wachter R, Goffeau A. 1997. *FEMS Microbiol Rev* **21:** 113-134; Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E. 1999. *FEBS Lett* **464:** 123-128.