Isolation and characterization of the *LGT1* gene encoding a low-affinity glucose transporter from *Torulaspora delbrueckii*

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Running Title: A low-affinity glucose transporter of *Torulaspora delbrueckii*

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Abstract

Torulaspora delbrueckii PYCC 5321 displayed a mediated glucose transport activity best fitted assuming a biphasic Michaelis-Menten kinetics with a low- and a high-affinity component. A genomic library of this yeast strain was used to transform a mutant of *Saccharomyces cerevisiae* deficient in glucose transport. Sequence analysis of a DNA fragment cloned, revealed the presence of a 1,704 bp-length ORF. This ORF, named *LGT1*, displayed a high homology to yeast glucose transporter genes. Functional characterization of the *LGT1* gene product in *S. cerevisiae* revealed that it encodes a low-affinity transporter, able to mediate the uptake of glucose and fructose. In consonance with this, 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. The GeneBank Accession No. for *LGT1* is AY598344.

Keywords: *Torulaspora delbrueckii*; Yeast; *LGT1*; *HXT*; Hexose transporter; Low-affinity glucose transporter

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 (Diderich *et al.*, 1999; Goffrini *et al.*, 2002; Rossell *et al.*, 2002). Consequently, a great effort has been traditionally devoted to decipher the biochemical and genetic mechanisms that control the expression, function and regulation of hexose transporters (for a review see Boles and Hollenberg, 1997; Özcan and Johnston, 1999). The best physiologically and molecularly characterized yeast is by far *Saccharomyces cerevisiae*. In this organism, 34 genes encoding established or putative sugar permeases, the largest family of the major facilitator superfamily (MFS), have been identified (Nelissen *et al.*, 1997).

In the past few years, the characterization of non-conventional yeasts, from both fundamental and technological aspects, has received special attention. In the yeast-based baking industry, efforts have been centred in the study and potential application of non-*Saccharomyces* baker's yeasts with improved commercially important traits, as osmotolerance and cryoresistance (Randez-Gil *et al.*, 2003). *Torulaspora delbrueckii*, one of the most abundant yeast species present in home-made corn and rye bread dough (Almeida and Pais, 1996a) have been recognized as the most promising alternative to industrial strains of *S. cerevisiae* (Randez-Gil *et al.*, 2003). Indeed, *T. delbrueckii* strains display freeze/thaw tolerance (Almeida and Pais, 1996b) and an exceptional resistance to osmotic and Na⁺ injury (Nakata *et al.*, 1995; Ok and Hashinaga, 1997; Hernandez-Lopez *et al.*, 2003). Nevertheless, the regular use of this yeast in the bread making industry has not been established due to some drawbacks. *T. delbrueckii* strains show considerable variation in their abilities to ferment and to assimilate carbon

compounds, as galactose or maltose (Kurtzman, 1998), poor maltase activity and low fermentative capacity (Hernandez-Lopez *et al.*, 2003). On top of this, there is a lack of knowledge on the physiology and molecular biology of this organism. In spite of the phylogenetic proximity of this yeast to *S. cerevisiae*, the differences observed between the two species, demonstrate that the behaviour of *T. delbrueckii* cannot be directly inferred from that of *S. cerevisiae*. Furthermore, and specifically in what regards sugar transport, results from previous work have shown that other yeast species, also phylogenetically close to *S. cerevisiae*, show relevant differences at this level (Gonçalves *et al.*, 2000; Milkowski *et al.*, 2001). Therefore, an in-depth investigation is required to gain insight into the function and regulation of *T. delbrueckii* sugar transporters.

This paper describes, for the first time, the isolation and characterisation of a low-affinity glucose transporter, *LGT1*, in the non-conventional yeast *T. delbrueckii*. A kinetic analysis of the sugar transport driven by Lgt1p in a hexose transport-null mutant strain of *S. cerevisiae* and its transcriptional regulation by Rgt1p, Mig1p and Mig2p are also reported.

Methods

Strains and culture media

T. delbrueckii PYCC 5321, *S. cerevisiae* CEN.PK2-1C (*MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289 SUC2 MAL2-8^C MAL3*) and a glucose transport-null mutant of *S. cerevisiae*, EBY.VW4000 (Wieczorke *et al.*, 1999) were used throughout this work. Yeast cells were cultured at 30 °C in YPD media (1% yeast extract, 2% peptone and 2% glucose) or SD (0.67% yeast nitrogen base without amino acids, DIFCO) plus 2%

glucose or maltose and supplemented with the appropriate auxotrophic requirements (Sherman *et al.*, 1986). Yeast transformants containing the nourseothricin resistance module (natMX4), were selected on YPD-agar plates supplemented with 50 µg/ml of nourseothricin (cloNAT, WERNER Bioagents, Germany). *Escherichia coli* DH5α host strain was grown in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract and 0.5% NaCl) supplemented with ampicillin (50 µg/ml). All amino acids, sugars and antibiotics were filter-sterilized and added to autoclaved medium. Solid media contained 2% agar. Yeast cells were transformed by the lithium acetate method and *E. coli* was transformed by electroporation following standard protocols.

Glucose uptake assays

For glucose uptake assays, [U-¹⁴C]glucose with a specific activity of 310 mCi/mmol (Amersham) was used. *S. cerevisiae* mutants were grown on SD with 2% maltose and transferred to SD with 2% glucose for 4 hours. For *T. delbrueckii* SD was substituted by a mineral medium with vitamins (van Uden, 1967). The cells were harvested at exponential phase (OD₆₄₀, 0.5-0.6) by centrifugation, washed twice with cold water, and suspended in water to a cellular density of 35-45 mg (biomass dry weigh) of yeast cells ml⁻¹. Zero-trans influx of glucose was determined essentially as previously described for maltose uptake (Alves-Araújo *et al.*, 2004) and monitored with a liquid scintillation counter (Packard Instrument Co., Inc.). Glucose uptake was determined with glucose concentrations from 0.1 mM to 100 mM. To determine the inhibiting effect of other sugars on the glucose transport, competition experiments were carried out by measuring the uptake of glucose at each concentration in the presence of an excess of other unlabeled sugar. The two sugars were added simultaneously to the reaction mixture.

Concentrations of the inhibitor were 50 and 100 mM for low and high range of glucose concentration, respectively. Triplicate determinations were performed at each glucose concentrations. Kinetic parameters were derived using computer-assisted non-linear regression by using GraphPad Prism software (Microsoft Corp.), however, when the regression was best fitted to two equations, values were obtained from Eadie-Hofstee plots.

DNA manipulations and sequencing

Standard DNA manipulations were carried out as described by Sambrook *et al.* (1989). Genomic DNA from *T. delbrueckii* cells was prepared as described by Sherman *et al.* (1986). DNA sequencing was performed in both strands by the dideoxy-chain termination procedure using the primers TD1 to TD11 described in Table 1. Analysis of sequence data was carried out using DNAMAN sequence analysis software (Lynnon Biosoft). Similarity searches were performed at the Swiss Institute of Bioinformatics (Appel *et al.*, 1994) and the National Center for Biotechnology Information, using BLAST software (Altschul *et al.*, 1997). Multiple sequence alignment was done using the clustalW (Thompson *et al.*, 1994) and Multalin (Corpet, 1988) programs. Search of Lgt1p transmembrane regions was carried out by scanning the sequence against protein profile databases using TMHMM server v. 2.0, CBS (Krogh, *et al.*, 2001) at the Center for Biological Sequence Analysis (Technical University of Denmark).

Construction of plasmids and disruption strains

Disruption of the *RGT1* gene of *S. cerevisiae* was carried out by the PCR-short flanking homology technique (Wach *et al.*, 1994). The disruption cassette containing the

nourseothricin-resistance *natMX4* module was generated by PCR using the pAG25 plasmid (Goldstein and McCusker, 1999) as template and two specific synthetic oligonucleotides, TD12 and TD13 (Table 1) containing each 16 bases homologous to the pFA6-kanMX4 MCS (Wach *et al.*, 1994) flanked by short homology regions to the *RGT1* gene. Detection of the correct gene disruption was done by diagnostic PCR. The reaction was performed with whole yeast cells (Huxley *et al.*, 1990) from isolated colonies, using a set of oligonucleotides, TD14-TD15 (Table 1), designed to bind outside of the replaced *RGT1* sequence. Because of the high GC content of the nourseothricin acetyltransferase-encoding gene *nat1* from *Streptomyces noursei* (Krugel *et al.*, 1993), the PCR-reaction mixture contained 5% DMSO as previously described (Goldstein and McCusker, 1999). The plasmid YEpLGT1 was generated by cloning a 2,911-bp *EcoRI-SpeI* fragment from plasmid YEpT-6 into the *EcoRI-XbaI* sites of the YEp lac181 vector (Gietz and Sugino, 1988). *LGT1* promoter fusion to *lacZ*, *LGT1::lacZ*, was constructed by cloning the PCR product (+30 to -1219, primers TD16 and TD17, Table 1) with a *Bam*HI site and a *Hind*III site into the vector YEp357 (Myers *et al.*, 1986).

β -Galactosidase assays

β -Galactosidase activity was assayed in cell free extracts as described previously (Guarante, 1983). Activities are given in units (U) per mg of protein and are the averages of three to seven independent assays. Cell mass (10-20 mg wet weight) was taken from the cultures at different times after transference to the induction medium, sedimented by centrifugation and crude extracts were prepared with glass beads according to Ciriacy (1975). Total protein content in cell free extracts was determined

using the commercial BioRad protein assay kit (Bio-Rad Laboratories, USA) based on Bradford method (1976), and bovine serum albumin (BSA) as standard.

Results

Cloning of *LGT1* gene involved on glucose transport

A genomic library of *T. delbrueckii* PYCC 5321 (Hernandez-Lopez *et al.*, 2002) constructed into the vector YEplac181 (Gietz and Sugino, 1988) 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. Transformants were selected on a leucine-free medium with glucose as a sole carbon source. Six of these transformants, able to grow on glucose, were further analysed by plasmid isolation and compared on the basis of their restriction map, which revealed four different patterns (data not shown). A representative of each group (referred as YEplT-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 (Figure 1A). Furthermore, we analysed the ability of the transformants to transport glucose. Cells were grown on SD-maltose and transferred to SD-glucose for 4 h before harvesting. YEplac181 (empty plasmid) transformants were used as a control. As is depicted in Figure 1B, all the transformants showed mediated glucose transport, with K_m values in the range of 12-25 mM. One plasmid, YEplT-6, was further analysed. DNA sequencing at the middle of the insert 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. Hence, we named this gene

LGT1, for low-affinity glucose transporter. (The GeneBank Accession No. for *LGT1* is AY598344).

Characterisation of the *LGT1* gene

Analysis of the *LGT1* upstream sequence (985 bp upstream from the ATG) showed the presence of several Mig1p- and Rgt1p-binding sequences. The transcriptional factors *MIG1* and *RGT1* have been reported as required for the transcriptional regulation of hexose transporters in *S. cerevisiae*. *MIG1* mediates the repression at high concentrations of glucose of *HXT2* and *HXT4* expression, whereas *RGT1* acts as a bifunctional regulator of *HXT1-HXT4* (Özcan *et al.*, 1996, Kim *et al.*, 2003). We found three sequences, -410/-415, -421/-416 and -680/-685 containing a perfect Mig1p-binding GC box, (G/C)(C/T)GGGG (Nehlin and Ronne, 1990). Nevertheless, only the later contains a 5' AT-rich region adjacent to the GC box, ATTTA (positions -2 to -6 from the GC box). This sequence appears to determine, at least in part, the specificity of Mig1p, and is found in all experimentally confirmed Mig1-binding sites (Lundin *et al.*, 1994). We also mapped in the promoter of *LGT1* 12 sequences in both orientations matching the Rgt1p consensus binding site 5'-CGGANNA-3' (Kim *et al.*, 2003), all but one arrayed in two clusters, -164 to -325 (6 sites) and -699 to -798 (5 sites). The existence of multiple Rgt1p binding sites distributed in clusters has also been reported in the *S. cerevisiae* *HXT* promoters (Kim *et al.*, 2003). In particular, the low-affinity hexose transporters *HXT1* and *HXT3* contain 15 and 13 sites, respectively, which determine the efficient *in vivo* repression by Rgt1p (Kim *et al.*, 2003). Sequence analysis of the 5'-region upstream of *LGT1* also showed the presence of four potential TATA boxes at positions -117, -264, -400 and -575 from the ATG codon. These

elements are often found in yeast promoters, playing a critical role in transcription (Struhl, 1982). Another relevant feature from the 5'-flanking sequence of *LGT1*, was the presence of "A" at the positions -1 and -3 with respect to the initiation codon. This feature has been associated with highly expressed genes and was previously observed in *T. delbrueckii* (Hernandez-Lopez *et al.*, 2002, Alves-Araújo *et al.*, 2004) and in general for eukaryotes (Kozak, 1987). Some consensus A-rich sequences known to be positioning elements (Guo and Sherman, 1995) 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.

Sequence characterisation of Lgt1p

As expected, significant similarities were found between the amino-acid sequence predicted from *LGT1* and other yeast hexose transporters (Figure 2), with identity values of about 32-36% for the *Schizosaccharomyces pombe* Ght5-6p, 56-57% for the *Pichia stipitis* Sut1-3p, 64-72% for the *Kluyveromyces lactis* Kht2p and Rag1p, and 61-78% for the Hxtp family of *S. cerevisiae*. For the later, the highest similarity was observed for Hxt9p/Hxt11p (78%), two genes not regulated by glucose, which play a role in drug resistance (Nourani *et al.*, 1997), for Hxt12p, which is almost identical to Hxt11p, and for Hxt6p/Hxt7p (77%) and Hxt4p (76%) encoding high- and intermediate-affinity transporters, respectively. *LGT1* encoded a potential 567-amino acid protein showing the typical structure of a membrane protein transporter, which has been predicted to contain 11 hydrophobic membrane-spanning domains, a larger cytoplasmatic loop between the sixth and seventh transmembrane helix and less well conserved cytoplasmic amino- and carboxy-terminal extensions. When compared with

sequences of transporter proteins that are predicted to have 12 transmembrane domains, the 7th domain is missing in this sequence. Indeed, the region corresponding to the 7th domain contains a few more hydrophilic residues not reaching the threshold hydrophobicity required to specify it as a transmembrane domain. Nevertheless, it has been speculated for other transporter proteins, that such a region could span the lipid bilayer if it is largely in contact with other more hydrophobic membrane-spanning segment (Lodish, 1988; Kruckeberg, 1996). Additionally, Lgt1p sequence showed a leucine-zipper motif (L-Xaa6-L-Xaa6-I-Xaa6-I) at the position 106 to 127, starting five amino acids before the N-terminal of membrane-spanning domain 2 and extending through that domain. These motifs have been shown to mediate protein-protein interactions by formation of a coiled-coil structure (Abel and Maniatis, 1989; Ahn *et al.*, 2003), involved in dimer formation (Wang *et al.*, 2001). The leucine-zipper motif is highly conserved in this position in *S. cerevisiae* Hxt/Gal2, *K. lactis* Kht2/Rag1, and *P. stipitis* Sut proteins. Two putative asparaginyl glycosylation sites (consensus sequence N-X-S/T), located at the positions N87 and N226, were also found in Lgt1p. The first one occurs between transmembrane domains 1 and 2, and the second one is located between domains 5 and 6, both on the extracellular face of the plasma membrane. The position of the asparaginyl glycosylation motif in these two topological regions is also highly conserved in yeast hexose transporters (Bisson *et al.*, 1993). Lgt1p also contained the two sugar transporter signature sequences as well as a PESP motif (Bairoch, 1993; Kruckeberg, 1996) starting at amino acid positions 172, 373 and 253, respectively (Falquet *et al.*, 2002). These highly conserved motives occur in all members of the sugar transporter family from prokaryotes to eukaryotes (Weierstall *et al.*, 1999). Finally, no PEST-like sequences were apparent in the Lgt1 protein sequence.

These sequences have been reported to be involved in the glucose-induced degradation of some sugar permeases (Medintz *et al.*, 2000). The lack of PEST motifs in Lgt1p is consistent with its apparent function as glucose transporter.

Kinetic characterisation of glucose transport mediated by Lgt1p and by wild type *T. delbrueckii*

Kinetic characterisation of glucose transport was performed with the strain *S. cerevisiae* EBY.VW4000 transformed with the *LGT1* gene and for comparison, with the wild type *Torulaspora delbrueckii* strain.

Glucose-grown cells of *T. delbrueckii* PYCC 5321 showed kinetics of glucose transport best fitted assuming a biphasic Michaelis-Menten kinetics with a low- and a high-affinity component (Figure 3). This behaviour is similar to what has been described for other yeasts (Özcan and Johnston, 1999). Although, presenting some variations in the kinetic parameters, a biphasic kinetics of glucose transport was also observed for fructose and maltose-grown cells (Table 2). Both the low- and high-affinity components of the glucose transport were competitively inhibited by fructose and maltose (Figure 4), suggesting that both these sugars shared the glucose transporters. However, in a previous study, no measurable transport of radiolabelled maltose was detected in glucose-grown cells of *T. delbrueckii* (Alves-Araújo *et al.*, 2004). These observations could be interpreted as the result 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* (Diderich *et al.*, 1999).

Plasmid YEpLGT1, which contains the whole *LGT1* gene, 751-bp of the promoter and 456 bp of the terminator, was used to transform the *hxt* null mutant strain of *S. cerevisiae*. Glucose and fructose uptake was measured in the transformed cells and, as a control, in cells harbouring the empty vector YEplac181. Cells were grown in SD-maltose (OD_{640} 0.2-0.3) and transferred to SD-glucose for 4 hours. Under these conditions the control strain did not exhibit any measurable glucose uptake. As expected, cells transformed with the *LGT1* gene exhibited glucose uptake with a K_m value of $36.5 (\pm 3.1)$ mM, in the range of the low-affinity component, and a V_{max} of $1.1 (\pm 0.04)$ $\text{nmol}^{-1} \text{s}^{-1} \text{mg}^{-1}$ dry wt. Lgt1p was also able to mediate significant fructose uptake in the *hxt* mutant, although with a lower affinity than for glucose, K_m value of $51.4 (\pm 3.0)$ mM and V_{max} of $1.4 (\pm 0.04)$ $\text{nmol}^{-1} \text{s}^{-1} \text{mg}^{-1}$ dry wt. In agreement with this, the presence of 100 mM fructose inhibited the zero *trans*-influx of glucose with features of competitive inhibition (Figure 5). Thus, V_{max} remained constant, whilst the apparent K_m increased to $77.6 (\pm 5.3)$ mM, confirming that fructose was transported by Lgt1p. As for the *T. delbrueckii* wild type strain, maltose inhibited competitively the glucose transport (Figure 5) suggesting that this permease allows the binding of one of the glucose residues of maltose as was interpreted above. However, maltose inhibition of glucose uptake in the *hxt* mutant overexpressing *LGT1* was weaker than that observed in the parental *T. delbrueckii* PYCC 5321 strain (Figure 4), pointing to the presence of other hexose transporters in *T. delbrueckii* able to bind maltose. The presence of 100 mM mannose inhibited the glucose transport in a non-competitive manner, the K_m value remaining constant and the V_{max} value lowering to $0.8 \text{ nmol}^{-1} \text{s}^{-1} \text{mg}^{-1}$ dry wt (Figure 5). This result suggested that Lgt1p did not transport mannose, contrary to what has been described for the major hexose transporters Hxt1-4, Hxt6 and Hxt7 from *S. cerevisiae*.

(Reinfsenberger *et al.*, 1997; Wiczorke *et al.*, 1999). However, this observation deserves further investigation since the *hxt* null mutant strain of *S. cerevisiae* transformed with the plasmid YEpLGT1 presented some residual growth on mannose, which was not evident in cells transformed with the empty vector (results not shown).

Induction of *LGT1* expression is dependent of glucose concentration and requires the inactivation of the repressor Rgt1p

To study the transcriptional regulation of *LGT1* by glucose, we used a fusion of its promoter to the reporter gene *lacZ*. Since glucose transport mediated by several Hxt proteins of *S. cerevisiae* has been proposed to be required for regulation of other *HXT* genes (Özcan and Johnston, 1999), the *LGT1::lacZ* fusion was not expressed in the *hxt* null mutant, but in the parental CEN.PK.2-1C strain. Cells were grown on SD-2% galactose, transferred to the same medium or to SD containing low (0.2%) or high (4%) glucose concentrations, and the levels of β -galactosidase activity were measured. As can be seen in Table 3, the *LGT1::lacZ* fusion was expressed at very low basal level in galactose and induced about 16-fold by 0.2% glucose. Higher glucose concentrations resulted in a further increase in the reporter gene expression, which reached values in the same range of those reported for *HXT1* and *HXT3* in multicopy plasmids (Özcan and Johnston, 1996). Thus, *LGT1* appears to be a highly expressed functional hexose transporter. However, regulation of *LGT1* was apparently different from that of *HXT1* and *HXT3*. The former is only induced by high glucose, whereas induction of *HXT3* expression by glucose is independent of sugar concentration. Regulation of *LGT1* resembles more that of low-affinity glucose transporters of *K. lactis*, *KHT1* and *RAG1*,

which show an enhanced expression with the external glucose concentration (Chen *et al.*, 1992; Milkowski *et al.*, 2001).

We also analysed if the Rgt1p and Mig1p consensus binding sites found in the promoter region of *LGT1* were functional. For this, we measured the *LGT1::lacZ* expression in *rgt1* and *mig1* single mutants of the parental CEN.PK2-1C strain. As shown in Table 3, deletion of *RGT1* caused *LGT1* to be highly expressed in the absence of glucose. Moreover, lack of this regulatory protein resulted in a higher induction at low glucose levels, but a significant increase, about 3-fold, was also evident at 4% glucose. Thus, the *S. cerevisiae* Rgt1p appears to act exclusively as a repressor of *LGT1* expression, and not as a bifunctional regulator. Conversely, deletion of *MIG1* had no effect on *LGT1* expression, suggesting that this pathway does not have a relevant role in the regulation of the transporter, or that *MIG1* and *MIG2* could have redundant roles in its regulation, as has been described for some glucose repressed genes in *S. cerevisiae* (Lutfiyya *et al.*, 1998). For this reason we tested *LGT1::lacZ* expression in the double mutant $\Delta mig1 \Delta mig2$ of the same parental strain (Table 3). Additional disruption of *MIG2* in a *mig1* background resulted in a higher induction of *LGT1::lacZ*, particularly at high glucose levels, indicating that Mig2p or both Mig1p and Mig2p in a redundant way, act as repressors of *LGT1* expression at high glucose concentrations. This is consistent with the role of these regulatory proteins in *S. cerevisiae*. Nevertheless, these results could not necessarily reflect the *in vivo* function of the putative homologues in *T. delbrueckii*.

Concluding remarks

This study has shown the presence in *T. delbrueckii* of a low-affinity hexose transporter, Lgt1p, able to mediate the uptake of glucose and fructose. Comparison of sequencing

data and regulatory studies of *LGT1* expression in *S. cerevisiae* suggest that this permease is closely related to the previously reported low-affinity transporters *KHT1/RAG1* of *K. lactis*. This organism, a respirofermentative yeast closely related to *T. delbrueckii*, has been shown to contain an “ancestral” gene cluster formed by a low- and a high-affinity glucose transporter, *KHT1-KHT2*, which is the only metabolically relevant in *K. lactis* (Belloch *et al.*, 2000; Hernandez-Lopez *et al.*, 2002; Aller-Arranz *et al.*, 2003). Thus, we can speculate that a similar situation occurs in *T. delbrueckii*. In consonance with this, preliminary sequencing data of the plasmids YEpT-1/-2/-4 appears to indicate that all of them contain different fragments of the same chromosomal locus reported here for YEpT-6. This could explain the lower fermentative capacity of *Torulaspora* strains in comparison with that exhibited by *S. cerevisiae*, which contains at least two of these functionally relevant gene clusters located in chromosomes IV (*HXT3-HXT6/HXT7*) and VIII (*HXT1-HXT4*). Results of our laboratory also indicate that *T. delbrueckii* has a higher rate of respiration on glucose and maltose than *S. cerevisiae* (unpublished results). Overall, these evidences suggest that the sugar uptake step could be a bottleneck in the fermentative performance of *T. delbrueckii*. Comparison of the kinetics of glucose transport in the wild type *T. delbrueckii* and in *S. cerevisiae* *hxt* null mutant expressing *LGT1*, indicates that other glucose transporters must be functional in *T. delbrueckii*. A further characterisation of putative additional transporters linked to *LGT1* will help to confirm the role of sugar permeases in the fermentative capacity of this biotechnological important yeast and will provide new clues on the mechanisms that have driven the evolution of hexose transporters in yeasts.

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Table 1. Oligonucleotides used in this study.

Primer	Sequence 5' to 3'	Comments
TD1	ATGACCATGATTACGCCAA	Sequencing
TD2	GTAAAACGACGGCCAGT	Sequencing
TD3	CGTGATATCCTCCATTTC	Sequencing
TD4	GCTACTCCTTGATTACTATCT	Sequencing
TD5	GTCTACTACAGAGCATACAC	Sequencing
TD6	GAATAATACCACCGATAGC	Sequencing
TD7	TTCGGCTTCCACACCAG	Sequencing
TD8	CGTAACACCGACAGATGC	Sequencing
TD9	GTGTTATTCGATATTTATC	Sequencing
TD10	GGAAAGCTCCTGAGTGATG	Sequencing
TD11	TCACCGTATCTGTCGAAACC	Sequencing
TD12	TGACTACAAAGATGAAAAGGGGGTCTGCT CAAAC TGCCAA <u>CGTACGCTGCAGGTCG</u>	PCR, <i>RGT1</i> disruption cassette construction (bases homologous to the pFA6-kanMX4 MCS underlined)
TD13	GATAGAGGTTGAGTTTCCACATATTCCGA TAAGAGTGAAGAT <u>TCGATGAATTCGAGC</u>	PCR, <i>RGT1</i> disruption cassette construction (bases homologous to the pFA6-kanMX4 MCS underlined)
TD14	ATGAACGAGCTGAACACTGT	Diagnostic PCR, <i>RGT1</i> disruption
TD15	GTCAAATCTGAATAGACTCCTG	Diagnostic PCR, <i>RGT1</i> disruption
TD16	TTTCACCTAAGGATCCTTTCTATT	<i>LGT1::lacZ</i> fusion
TD17	GTCAACTGGTGTAAGCTTTGTAGT	<i>LGT1::lacZ</i> fusion

Table 2. Kinetic parameters of glucose uptake in cells of *T. delbrueckii* PYCC 5321 grown in medium with glucose, fructose or maltose (1%, w/v).

Kinetic parameters	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	13.67±0.96	1.32±0.19	13.91±0.77

Table 3 – *LGT1* gene expression in cells of *mig1*, *mig1mig2* and *rgt1* mutants of *S. cerevisiae* CEN.PK2-1C^a

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

^a 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.

Figure legends

Figure 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 (Wieczorke *et al.*, 1999). 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 (OD₆₄₀, 0.2-0.3) to SD medium containing 2% glucose. After 4 h the zero trans-influx of [U-¹⁴C]glucose was measured. For more details see the Materials and Methods section.

Figure 2. Relationships between Lgt1p and other yeast hexose transporters. The dendrogram 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) and *Saccharomyces carlsbergensis* (Scar).

Figure 3. Eadie-Hofstee plots of glucose initial uptake rates in cells of *T. delbrueckii* PYCC 5321. Cells were grown in 1% glucose and harvested during exponential growth (OD₆₄₀, 0.5-0.6). Radiolabelled glucose uptake was measured as described in Materials and Methods.

Figure 4. Eadie-Hofstee plots of glucose initial uptake rates in the presence of other sugars in cells of *T. delbrueckii* PYCC 5321. Cells were grown in 0.5 % (open symbols) or 4% (close symbols) glucose for measurements in the low and high range of glucose concentration, respectively. This was done in order to try to decrease the influence of the low and high affinity components of glucose transport on each other. The cells were harvested during exponential growth (OD₆₄₀, 0.5-0.6), and the zero *trans*-influx of [U-¹⁴C]glucose was measured in the absence (■, □) or in the presence of maltose (▲, △) or fructose (●, ○). Concentrations of inhibitors were: 50 mM for low glucose concentrations and 100 mM for high glucose concentrations. Radiolabelled glucose uptake was measured as described in Materials and Methods.

Figure 5. Eadie-Hofstee plots of glucose initial uptake rates in YEpLGT1 transformant cells of the *S. cerevisiae* glucose transport-null mutant EBY.VW4000. SD-maltose-grown cells were washed with chilled-water, and transferred (OD₆₄₀, 0.2-0.3) to SD medium containing 2% glucose. After 4 h the zero *trans*-influx of [U-¹⁴C]glucose was measured in the absence (△) and presence of 100 mM maltose (■), 100 mM mannose (○) or 100 mM fructose (▼). Radiolabelled glucose uptake was measured as described in the Materials and Methods section.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5