INTRODUCTION

Studies to characterize hexose transporters in yeasts usually refer to glucose as the substrate, but most transporters characterized so far at the molecular level do not discriminate between glucose and fructose. Hexose transport has been investigated in great detail in *Saccharomyces cerevisiae*, in which a family of more than 20 genes mediate the facilitated diffusion of glucose, fructose and mannose. This genetic complexity seems also to be present in other ascomycetous yeasts, such as *Candida glabrata* (http://cbl.labri.fr/Genolevures) and *Candida albicans* (Fan et al., 2002). Classic studies have reported that, in *S. cerevisiae*, the affinity for glucose is five (Cirillo, 1968) or ten (Kotyk, 1967) times higher than for fructose. The kinetic characterization of individual transporters of *S. cerevisiae* indicates that, for all transporters tested, *Km* is higher for fructose than for glucose (Reifenberger et al., 1997), confirming the classic data. The only exceptions to this, in yeasts, were the Fsy1p-specific fructose-H+ symporter from *Saccharomyces pastorianus* (Gonçalves et al., 2000) and its close homologue, Frt1, from *Kluyveromyces lactis* (Diezemann & Boles, 2003).

*Zygosaccharomyces bailii* is a food-spoilage yeast that has evolved the ability to grow under rather inhospitable conditions, such as those present in preserved food and beverages: low water activity, low pH and the presence of weak acid preservatives, in particular (Fleet, 1992). In contrast with most yeasts, and in particular with *S. cerevisiae*, *Z. bailii* consumes fructose faster than glucose, deserving the designation of a fructophilic yeast. This behaviour was explained by Sousa-Dias et al. (1996) by taking into account the kinetics of hexose uptake observed in this yeast. Fructose is taken up by a high-capacity, low-affinity transporter, specific for fructose (no other sugar inhibits fructose uptake); a second transporter takes up glucose, fructose and 2-deoxyglucose in a low-capacity and high-affinity manner. Both involve facilitated diffusion mechanisms. Moreover, fructose promotes the inactivation of the glucose transporter, preventing the utilization of this sugar when fructose is also available (Sousa-Dias et al., 1996).

This work deals with the cloning and characterization of the fructose-specific transporter, Ffz1, of *Z. bailii*. Our strategy involved functional complementation of a strain of *S. cerevisiae* incapable of growth on hexoses.

METHODS

Micro-organisms and culture conditions. *S. cerevisiae* strain EBY.VW4000 was used as host strain for the *Z. bailii* genomic

Ffz1, a new transporter specific for fructose from *Zygosaccharomyces bailii*

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The basis of fructophily in the yeast *Zygosaccharomyces bailii* has been shown to reside in the performance of transport systems for hexoses. In this study, a gene encoding a fructose-specific transporter was characterized. The strategy involved the functional complementation of a *Saccharomyces cerevisiae* strain that does not take up hexoses (*hxt-null* strain). This strain was transformed with a genomic library of *Z. bailii*. One transformant capable of growing on fructose, but not on glucose, was obtained. This transformant did not transport D-[14C]glucose, and the kinetic parameters for D-[14C]fructose were *V*ₘₐₓ = 3·3 mmol h⁻¹ g⁻¹ and *Kₘ = 80·4 mM. As in the original strain of *Z. bailii*, fructose uptake was not inhibited by the presence of other hexoses or uracyl. The plasmid responsible for the observed phenotype was found to carry an ORF encoding a 616 amino acid protein with the characteristics of a membrane transporter, which was designated *FFZ1* (fructose facilitator *Zygosaccharomyces*). The impairment in function observed in an *S. cerevisiae* transformant expressing a truncated Ffz1 protein lacking 67 amino acids at the C-terminus suggests an important role for this terminal part in the proper structure of the transporter.
library. This *S. cerevisiae* strain lacks the ability to transport hexoses (Wieczorke et al., 1999). Strain EBY.VW4000 was routinely kept on solid yeast nitrogen base (YNB) medium with 20 g maltose l\(^{-1}\), supplemented with uracil, leucine, tryptophan and histidine. Growth in the same liquid medium was monitored by measuring OD\(_{640}\) with a Spectronic 20D spectrophotometer (Milton Roy). *Escherichia coli* XL-1 Blue (Stratagene) cells were grown in Luria–Bertani (LB) medium and, when required, ampicillin (100 μg ml\(^{-1}\)), X-Gal (4 μg ml\(^{-1}\)) and IPTG (4 μg ml\(^{-1}\)) were used as supplements.

**Screening of the library.** The genomic library of *Z. bailii* ISA1307 constructed by Rodrigues et al. (2001) was used in this work. For library plasmid DNA isolation, 45 000 clones, representing four times the total number of independent clones of the genomic library, were grown on LB medium supplemented with ampicillin. Strain EBY.VW4000 was transformed with library plasmid DNA (about 4 μg DNA yielded approximately 2 × 10\(^6\) transformants), using the lithium acetate method (Agatep et al., 1998). The transformation mixture was first plated onto solid YNB medium with 20 g maltose l\(^{-1}\) as the sole carbon and energy source, supplemented with leucine, tryptophan and histidine. After 7 days at 28 °C, colonies were replica plated onto the same medium containing 10 g l\(^{-1}\) of either fructose or glucose, instead of maltose. The growth phenotype on both media was evaluated after 7 days at 28 °C. The transformants were cured of the library plasmids by selection in 5-fluoroorotic acid (5-FOA)-containing medium, according to the procedure described by Boeke et al. (1987).

**Initial uptake measurements.** Cells were harvested at an OD\(_{640}\) of 0.5–0.8 by centrifugation (5000 g for 5 min), washed twice with cold distilled water and resuspended in distilled water to a final concentration of approximately 50 mg dry weight ml\(^{-1}\). The cell suspension (20 μl) was mixed with 20 μl 100 mM Tris/citrate buffer, pH 5.0, in 10 ml conical centrifuge tubes. After 2 min incubation in a water bath at 22 °C, uptake was initiated by the addition of 20 μM uranyl nitrate, in the same buffer, were added to the D-\([\text{U-}^{14}\text{C}]\)fructose solutions. In this case, the incorporation was started by the addition of the cell suspension.

For competition assays, 250 mM of either glucose, 2-deoxyglucose, mannose or sorbose, or 2.5 mM uranyl nitrate, in the same buffer, were added to the D-\([\text{U-}^{14}\text{C}]\)fructose solutions. In this case, the incorporation was started by the addition of the cell suspension.

The filters were placed in scintillation liquid (OptiPhase HiSafe II; Amersham). The radioactivity remaining on the filters was measured with a Beckman LS6000LL scintillation counter.

**DNA manipulations.** The procedure followed for plasmid rescue from yeast transformants was as described by Hoffman & Winston (1987). DNA manipulations were performed essentially as described by Sambrook et al. (1989). The complete DNA sequence of both strands of the *FFZ1*-coding region was obtained by primer walking, using an ABI PRISM 310 Genetic Analyser (Perkin-Elmer).

**RESULTS**

**Selection of transformants**

*S. cerevisiae* strain EBY.VW4000 is unable to use hexoses as sole carbon and energy source, because it lacks the ability to transport these sugars. This strain was transformed with a genomic library of *Z. bailii* based on centromeric plasmid pRS316 (URA3) (Sikorski & Hieter, 1989). URA\(^{-}\) transformants (approximately 2 × 10\(^6\)) were selected on maltose-containing medium, and were subsequently replica plated onto YNB fructose and YNB glucose media. One transformant (TF1) was identified that was able to grow only on fructose as sole carbon and energy source, while a second transformant (TFG1) grew both on fructose and on glucose (Fig. 1).

To ascertain whether the observed ability of the two selected transformants to utilize hexoses was conferred by a library plasmid, the transformants were plated on a medium containing 5-FOA. The Ura\(^{-}\) strains were unable to grow on either glucose or fructose, indicating that the restoration of hexose utilization was dependent in both cases on the presence of the library plasmid. The plasmids present in transformants TF1 and TFG1 were rescued in *E. coli*. Both transformants were found to contain only one library plasmid each (named pTF1 and pTFG1), as assessed by the restriction pattern of the purified plasmids. pTF1 and pTFG1 were subsequently used to retransform host strain EBY.VW4000. The phenotypes of the resulting transformants with respect to growth on glucose and on fructose were similar to those observed for the original transformants, TF1 and TFG1. This result suggested that TF1 carried a plasmid that encoded the *Z. bailii* fructose-specific transporter. We also anticipated that TFG1 might carry the low-capacity transporter of *Z. bailii*, since the strain could grow both on fructose and glucose.

![Fig. 1. Growth of *S. cerevisiae* strain EBY.VW4000 transformed with an empty vector (plasmid pRS316) and transformants carrying *Z. bailii* genomic library plasmids pTF1 and pTFG1. The medium was YNB supplemented with 1% maltose, fructose or glucose, as indicated.](image-url)
Kinetics of sugar transport

Transformant TF1 grew in liquid YNB medium containing 1% fructose as sole carbon and energy source with a duplication time of 3 h. TF1 was able to take up D-[U-14C]fructose, but no D-[U-14C]glucose uptake could be measured (Fig. 2). The kinetic parameters calculated for fructose uptake were $V_{\text{max}} = 3.3 \text{ mmol h}^{-1} \text{g}^{-1}$ and $K_m = 80-4 \text{ mM}$, at 22°C and pH 5. The specificity of the carrier was determined by testing the effect of other sugars as possible competitive inhibitors of initial [14C]fructose uptake. The uptake of fructose was not affected by the presence of 100 mM glucose, mannose, sorbose or 2-deoxyglucose (results not shown). Uranyl (1 mM), which functions as a competitive inhibitor of glucose uptake in several biological systems (Fuhrmann et al., 1992), also did not interfere with fructose uptake.

Transformant TFG1 failed to grow in liquid medium, both when fructose and when glucose was used as carbon and energy source.

The FFZ1 gene

Strain EBY.VW4000 transformed with plasmid pTF1 displayed fructose-uptake characteristics that resembled those found for the specific fructose facilitator of Z. bailii, indicating that pTF1 encodes this transporter.

The plasmid was found to carry a Z. bailii genomic DNA insert of approximately 4.5 kb. The insert was sequenced in its entire length, revealing the presence of an ORF of 616 amino acids that exhibited the characteristics of an integral membrane protein with 12 membrane-spanning regions (as predicted by the HMMTOP server, version 1.1). The gene was named FFZ1 (fructose facilitator of Zygosaccharomyces) (see footnote for EMBL accession number). A similarity search in public databases, using the predicted amino-acid sequence of Ffz1p, disclosed a low degree of homology with fungal membrane proteins, among which were sugar transporters and multidrug resistance proteins (Fig. 3). The closest relative of Ffz1p appeared to be a hitherto uncharacterized membrane transporter from Schizosaccharomyces pombe (Yao5p).

The 4.3 kb Z. bailii genomic DNA insert present in pTFG1 was also sequenced. Analysis of this sequence uncovered an incomplete FFZ1 gene, lacking the region that encodes the C-terminal 67 amino acids of the predicted Ffz1 protein. This suggests an important role of the C-terminus of Ffz1p for proper function and specificity of the carrier, since TFG1 grows slowly on both glucose- and fructose-based solid media.

DISCUSSION

In this work, what is believed to be the first isolation by functional complementation in S. cerevisiae of a permease-encoding gene from Z. bailii is described. This permease (Ffz1p) is, as previously shown, a facilitated diffusion system specific for fructose. The phenotype conferred by the presence of an FFZ1-containing plasmid on an S. cerevisiae host strain unable to transport hexoses reproduces the peculiarities of fructose utilization by Z. bailii. As predicted, the S. cerevisiae transformant TF1 could grow on fructose, but was incapable of growth on glucose and, in line with this, initial uptake assays showed that fructose, but not glucose, was efficiently transported. It is remarkable that neither other hexoses nor uranyl inhibited fructose uptake, as previously observed in the original strain of Z. bailii (Sousa-Dias et al., 1996). Fuhrmann et al. (1992) observed that 10 μM uranyl was enough to affect glucose transport in S. cerevisiae. In contrast, fructose transport through Ffz1p was not affected by 1 mM uranyl, supporting the view that the molecular mechanism involved in Ffz1p-mediated transport

![Fig. 2. Initial uptake of fructose (■) and glucose (○) measured in cells of transformant TF1 grown on YNB with 1% fructose. Cells were incubated for 5 s with [14C]fructose or [14C]glucose.](http://mic.sgmjournals.org)
is different from the mechanism involved in hexose transport in \textit{S. cerevisiae}.

Two closely related genes encoding specific \(H^+\)-fructose symporters were previously cloned from yeasts: \textit{FSY1} from \textit{S. pastorianus} (Gonçalves et al., 2000) and \textit{FRT1} from \textit{K. lactis} (Diezemann & Boles, 2003). \textit{Ffz1p}, a facilitated diffusion system, does not resemble these proteins (Fig. 3), suggesting that fructose specificity does not have a common phylogenetic background in these cases. The homology with other facilitated diffusion systems that transport glucose and fructose, like the Hxt family from \textit{S. cerevisiae}, is also poor. The putative protein was found to be most similar to \textit{Yao5p} (probable membrane transporter) of \textit{Schizosaccharomyces pombe} (28 \% identity).

The values previously reported for the kinetic parameters of fructose uptake in \textit{Z. bailii} are quite similar to those measured in \textit{S. cerevisiae} transformant TF1 carrying the \textit{FFZ1} gene in a centromeric plasmid (low copy number). The \(K_m\) values measured in the original strain and in transformant TF1 were, respectively, 65 mM and 80 mM. Transport via \textit{Ffz1p} can, therefore, be classified as low affinity. The \(H^+\)-fructose symporters displayed a much higher affinity (\(K_m\) values of 0-16 mM in both cases) (Gonçalves et al., 2000; Diezemann & Boles, 2003). In general, the \(H^+\)-glucose symporters that have been characterized so far in yeasts also present higher affinities than facilitated diffusion systems (Spencer-Martins & van Uden, 1985; Loureiro-Dias, 1987; Peinado et al., 1989). We can speculate that yeasts invest proton motive force in sugar transport only when the sugar is present in low concentrations in the environment; energy is then necessary to provide an adequate intracellular concentration of the sugar in order for metabolism to proceed. In environments where sugars are present at high levels, the concentration gradient across the plasma membrane is enough to maintain an active catabolism; in this situation it is favourable that sugars cross the membrane by facilitated diffusion without energy dissipation. The presence of a low-affinity, fructose-specific carrier in \textit{Z. bailii} is, therefore, in keeping with the fact that this yeast is frequently isolated from high-sugar-content foods and beverages.

The maximum velocity of fructose uptake measured in the transformant expressing only \textit{Ffz1p} (TF1) was 3-3 mmol h\(^{-1}\) g\(^{-1}\). This value is comparable to that previously reported for the original strain of \textit{Z. bailii} (5 mmol h\(^{-1}\) g\(^{-1}\)) (Sousa-Dias et al., 1996), and is much higher than the values found for the \(H^+\)-fructose symporters of \textit{S. pastorianus} and \textit{K. lactis}, which were of the order of 0-1 mmol h\(^{-1}\) g\(^{-1}\) (Cason et al., 1986; Diezemann & Boles, 2003). Also, \(H^+\)-glucose symporters characterized in yeasts display, in general, much lower maximum velocities than facilitated diffusion systems (Spencer-Martins & van Uden, 1985; Loureiro-Dias, 1987; Peinado et al., 1989).

Many questions remain to be answered. An intriguing issue concerns the specificity of the novel permease. What are the key structural differences between \textit{Ffz1p} and the many multi-substrate facilitated-diffusion hexose carriers, like those belonging to the Hxt family? A first clue may reside in the observation that the absence of the 67 amino acids at the C-terminus clearly affects \textit{Ffz1p} function, causing not only reduced growth of the respective \textit{S. cerevisiae} transformant (TFG1) on fructose, but also affecting the specificity of the transporter, since growth of TFG1 on glucose could be detected (Fig. 1). This suggests that the C-terminus has a prominent structural-functional role.

Although the fructophilic behaviour of \textit{Z. bailii} is not yet fully elucidated, a first step has been accomplished: since \textit{Ffz1p} is specific for fructose, when this sugar is present in high concentrations in the environment it crosses the plasma membrane at a higher rate than glucose and, consequently, can be metabolized faster.

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**REFERENCES**


