

Cloning novel sugar transporters from *Scheffersomyces (Pichia) stipitis* allowing D-xylose fermentation by recombinant *Saccharomyces cerevisiae*

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Abstract

Objectives Since uptake of xylose limits its fermentation, we aimed to identify novel sugar transporters from *Scheffersomyces stipitis* that allow xylose uptake and fermentation by engineered *Saccharomyces cerevisiae*.

Results An *hxt*-null *S. cerevisiae* strain, lacking the major hexose transporters (*hxt1Δ-hxt7Δ* and *gal2Δ*) but having high xylose reductase, xylitol dehydrogenase and xylulokinase activities, was transformed with a genomic DNA library from *S. stipitis*. Four plasmids allowing growth on xylose contained three genes

encoding sugar transporters: the previously characterized *XUT1* permease, and two new genes (*HXT2.6* and *QUP2*) not previously identified as xylose transporters. High cell density fermentations with the recombinant strains showed that the *XUT1* gene allowed ethanol production from xylose or xylose plus glucose as carbon sources, while the *HXT2.6* permease produced both ethanol and xylitol, and the strain expressing the *QUP2* gene produced mainly xylitol during xylose consumption.

Conclusions Cloning novel sugar transporters not previously identified in the *S. stipitis* genome using an *hxt*-null *S. cerevisiae* strain with a high xylose-utilizing pathway provides novel promising target genes for improved lignocellulosic ethanol production by yeasts.

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Sugar transporter · Xylose

Introduction

Lignocellulosic biomass, an abundant and renewable feedstock, is an attractive raw material for bioethanol production since it does not compete with food and feed production (Sarkar et al. 2011; Caspeta et al. 2013; Nogueira et al. 2013). The major fermentable sugars from hydrolysis of this lignocellulosic biomass

are D-glucose and D-xylose, and to obtain an economically feasible industrial process for bioethanol production it is necessary to efficiently ferment both sugars into ethanol (Lin and Tanaka 2006; Stambuk et al. 2008; Kim et al. 2012). The development of robust microorganisms for xylose fermentation is required for efficient bioethanol production, and abundant research has been devoted to improve xylose utilization by recombinant strains of *Saccharomyces cerevisiae*, an industrial yeast that it is not able to ferment xylose (Matsushika et al. 2009; Young et al. 2010; Kim et al. 2012; Laluece et al. 2012; Cai et al. 2012; Nielsen et al. 2013; Sánchez Nogué and Karhumaa 2015).

A major focus in metabolic engineering for xylose fermentation has been to establish and improve an intracellular xylose catabolic pathway in *S. cerevisiae*. Two strategies are currently being used: overexpression of xylose isomerase (XI), or overexpression of xylose reductase (XR) and xylitol dehydrogenase (XDH). Since both pathways transform xylose into xylulose, it is also required to overexpress xylulokinase (XK) that will enhance the entrance of xylulose into the pentose-phosphate pathway. However, independently of the xylose utilizing pathway used, the uptake of xylose across the *S. cerevisiae* plasma membrane occurs through a large family of hexose transporters, encoded by the *HXT1-HXT17* and *GAL2* genes, that has been reported to have substantial metabolic flux control, especially when the intracellular pathway is optimized (Kötter and Ciriacy 1993; Hamacher et al. 2002; Lee et al. 2002; Gárdonyi et al. 2003; Sedlak and Ho 2004; Saloheimo et al. 2007; Bertilsson et al. 2008; Parachin et al. 2011; Gonçalves et al. 2014).

A strategy to overcome this problem has focused on the isolation of heterologous sugar transporters with better xylose-transporting properties for functional expression in xylose fermenting *S. cerevisiae* cells (Saloheimo et al. 2007; Hector et al. 2008; Katahira et al. 2008; Madhavan et al. 2009; Runquist et al. 2009, 2010; Young et al. 2011, 2014; Tanino et al. 2012; Diao et al. 2013). However, up to now very few heterologous xylose transporters have been characterized in *S. cerevisiae*, and recent surveys with over 34 heterologous known and putative sugar transporters from seven different organisms revealed that only half of the expressed permeases allowed significant utilization of xylose by *S. cerevisiae* cells (Young et al.

2011, 2014). Furthermore, most of these studies have just analyzed the capacity of the transformed cells to grow on xylose, and very few have verified the contribution of the permeases for xylose fermentation by the recombinant strains.

The yeast *Scheffersomyces stipitis* (formerly *Pichia stipitis*) is capable of fermenting a variety of sugars present in the lignocellulosic biomass, is one of the best xylose fermenting species found in nature, but unfortunately has several drawbacks for industrial fuel ethanol production mainly due to its low tolerance to the fermentation product (Toivola et al. 1984; Nigam 2001; Jeffries et al. 2007; Farias et al. 2014; Parambil and Sarkar 2014). Nevertheless, this yeast has been a major source of xylose-utilizing genes for genetic engineering of *S. cerevisiae*, including not only the necessary activities of the XR and XDH enzymes (encoded by the *XYL1* and *XYL2* genes), and other enzymes of the pentose-phosphate pathway (Kötter and Ciriacy 1993; Jin et al. 2005; Matsushika et al. 2009), but also many heterologous sugar transporters. The analysis of xylose transport by this yeast revealed complex kinetics, indicating the presence of several permeases with differing properties, a situation also found for hexose transport in *S. cerevisiae* (Kilian and Van Uden 1988; Does and Bisson 1989).

Up to now 15 monosaccharide transporters from *S. stipitis* have been identified and functionally analyzed in *S. cerevisiae*. These include the hexose transporters *SUT1-SUT4*, some of them able to mediate xylose transport in yeast cells (Weierstall et al. 1999; Katahira et al. 2008; Runquist et al. 2010; Moon et al. 2013), but also several other putative transporters encoded by the *XUT1-XUT7*, *AUT1*, *HGT2*, *RGT2* and *STL1* genes, of which few have been shown to allow significant xylose transport, fermentation and/or growth when expressed in *S. cerevisiae* recombinant strains (Du et al. 2010; Young et al. 2011, 2012; Moon et al. 2013).

We have recently developed a *hxt*-null *S. cerevisiae* strain, lacking the major hexose transporters (*hxt1Δ-hxt7Δ* and *gal2Δ*), with a high activity of the XR, XDH and XK enzymes due to overexpression (through an integrative plasmid) of the *XYL1*, *XYL2* and *XKS1* genes, and analyzed the impact that several *S. cerevisiae* *HXT* permeases have in anaerobic batch fermentations using xylose, glucose, or xylose plus glucose as carbon sources (Gonçalves et al. 2014). In the present report, a genomic library of *S. stipitis* was

screened with this *hxt*-null strain to identify novel sugar transporters that allow xylose uptake and fermentation when expressed in *S. cerevisiae*, a required metabolic engineering approach to increase catabolic rates in recombinant yeasts for biofuel applications.

Materials and methods

Strains and media

The yeast strains used in this study are listed in Table 1. *Escherichia coli* strain DH5 α was grown in lysogeny broth (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl) supplemented with ampicillin (at 100 mg l⁻¹). Yeasts were grown in rich YP medium (1 % yeast extract, 2 % Bacto-peptone) or synthetic complete (SC) medium lacking uracil (0.67 % yeast nitrogen base without amino acids, supplemented with adequate auxotrophic requirements), containing 2 % maltose, glucose, fructose or xylose. The pH of the medium was adjusted to pH 5 with HCl. When required, 2 % Bacto-agar and 0.5 mg aureobasidin A l⁻¹ (Takara Bio, Japan) were added to the medium.

Molecular genetic techniques

Standard methods for bacterial transformation, DNA manipulation and analysis were employed (Ausubel et al. 1995). The yeast *S. stipitis* (strain NBRC1687, Watanabe et al. 2011) genomic DNA library was constructed in the pPGK plasmid (Table 1). Genomic DNA was extracted using the MasterPure Yeast DNA purification kit (Epicenter, USA), digested with *Bam*HI, and 3–5 kb fragments were isolated by gel electrophoresis. These DNA fragments were cloned into the *Bam*HI site of plasmid pPGK. The genomic library was transformed into the *S. cerevisiae* *hxt*-null strain DLG-K1 (Table 1), which has high XR, XDH, and XK activities (Gonçalves et al. 2014), using the Yeastmaker yeast transformation kit (Clontech Laboratories, USA). The *hxt*-null DLG-K1 strain is available to the scientific community for research non-commercial purposes from the Collection of Microorganisms, DNA and Cells of Universidade Federal de Minas Gerais (member of the World Federation of Culture Collections, see http://www.wfcc.info/ccinfo/index.php/collection/by_id/1029/) under accession number UFMG-CM-Y5856. Strains transformed with the pPGK-derived plasmids were selected in SC

Table 1 Yeast strains, plasmids, and primers used in this study

	Relevant genotype or description	Source or reference
Yeast strains		
DLG-K1	<i>MATa hxt1Δ::HIS3::Δhxt4 hxt2Δ::HIS3 hxt3Δ::LEU2::hxt6 hxt5::LEU2 hxt7::HIS3 gal2Δ ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 AUR1::pAUR-XKXDHXR</i>	Gonçalves et al. (2014)
BBY-6	DLG-K1, pPGK-6	This work
BBY-24	DLG-K1, pPGK-24	This work
BBY-37	DLG-K1, pPGK-37	This work
BBY-90	DLG-K1, pPGK-90	This work
Plasmids		
pPGK	<i>URA3 PGK1p-PGK1t</i>	Kang et al. (1990)
pPGK-6	<i>URA3 PGK1p-HXT2.6-PGK1t</i>	This work
pPGK-24	<i>URA3 PGK1p-XUT1-PGK1t</i>	This work
pPGK-37	<i>URA3 PGK1p-QUP2-PGK1t</i>	This work
pPGK-90	<i>URA3 PGK1p-QUP2-PGK1t</i>	This work
Primers		
pPGK_seq_F	CAGATCATCAAGGAAGTAATTATCT	–
pPGK_seq_R	TCTATCGATTTC AATTCAATTCAAT	–

medium lacking uracil and supplied with aureobasidin A and 2 % maltose or xylose. Plasmids were extracted from the recombinant yeast strains using the Zymo-prep Yeast Plasmid Miniprep II kit (Zymo Research, USA). Plasmids recovered from yeast transformants were analyzed by restriction digestion and/or diagnostic PCR using primers pPGK_seq_F and pPGK_seq_R (Table 1). These same primers were used to sequence the 5' and 3' ends of the DNA fragments present in the recombinant pPGK vectors (ACTGene Análises Moleculares Ltda, Brazil), and the sequences were used to identify the cloned genes by comparison with the known genome of *S. stipitis* (Jeffries et al. 2007).

Phylogenetic analysis

Amino acid sequences of *S. stipitis* sugar transporters were obtained from NCBI (www.ncbi.nlm.nih.gov/protein/) with the following accession numbers: *AUT1* (GI:4836720); *HGT2* (GI:4851832); *HXT2.4* (GI:4850978); *HXT2.6* (GI:4838414); *QUP2* (GI:4840652); *RGT2* (GI:4840859); *STL1* (GI:4838168); *SUT1* (GI:4851252); *SUT2* (GI:4838413); *SUT3* (GI:4839762); *SUT4* (GI:4850775); *XUT1* (GI:4839826); *XUT2* (GI:4852047); *XUT3* (GI:4851844); *XUT4* (GI:4840896); *XUT5* (GI:4840252); *XUT6* (GI:4841106); and *XUT7* (GI:4851701). Sequences were first aligned (see Supplementary Fig. 1) using Clustal Omega (Sievers et al. 2011), and then the phylogenetic tree was calculated and constructed using the neighbor joining method with Mega 5 (Tamura et al. 2011).

Growth and fermentations conditions

Yeast strains were pre-grown in SC medium containing 2 % (w/v) maltose for 36 h at 28 °C. The cells were collected by centrifugation at 6000×g for 5 min at 4 °C, washed twice with sterile water, and inoculated (0.1 ± 0.02 g of dry yeast l⁻¹) into SC medium containing 2 % (w/v) maltose, xylose, glucose or fructose. Cells were grown aerobically at 28 °C with shaking (160 rpm) in Erlenmeyer flasks filled to 1/5 of the volume with medium. Cellular growth was followed from OD₆₀₀ values and culture samples were harvested regularly, centrifuged (5000×g, 1 min), and their supernatants used for the determination of substrates and fermentation products as described below. For batch fermentations, cells were pre-grown

as described above and inoculated at a high cell density (5.5 ± 0.5 g of dry yeast l⁻¹) into 25 ml synthetic SC medium containing 2 % glucose or/and xylose. Anaerobic batch fermentations were performed at 30 °C in closed 50-ml bottles with a magnetic stir bar to allow mild agitation (100 rpm). Samples were collected regularly and processed as described above.

Determination of substrates and fermentation products

Glucose, xylose, ethanol, xylitol, and glycerol were determined by HPLC equipped with a refractive index detector using an Aminex HPX-87H column (Bio-Rad). HPLC was run at 40 °C using 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.1 ml min⁻¹ and 0.01 ml injection volume.

Results

The genomic library of *S. stipitis* DNA was transformed into the *S. cerevisiae* *hxt*-null strain DLG-K1, which is deleted for its hexose transporter genes *HXT1* to *HXT7* and *GAL2*, and although it overexpresses the *XYL1*, *XYL2* and *XKS1* genes for efficient xylose fermentation, this *hxt*-null strain is unable to take up and grow on xylose, glucose or other monosaccharides as the sole carbon source (Gonçalves et al. 2014). Transformants were selected on a synthetic SC medium lacking uracil, and containing 2 % (w/v) xylose as carbon source and aureobasidin A. More than 90 transformant colonies grew up within 5 days at 28 °C, and after further inoculation into new SC-2 % xylose plates, eight transformants were selected by their capacity to grow in both xylose and glucose. Indeed, all these eight transformants were able to grow (to different extents) in the presence of several monosaccharides, including glucose, fructose, mannose, galactose and xylose (data not shown), indicating that the genes present on the pPGK plasmids encode for general monosaccharide transporters. These eight transformants able to grow on xylose were further analyzed by plasmid isolation and retransformation into the *hxt*-null strain DLG-K1. Four plasmids (pPGK-6, pPGK-24, pPGK-37 and pPGK-90, Table 1) were able to restore growth on SC medium containing xylose as a carbon source, and

restriction enzyme analysis and partial sequencing showed that these four plasmids (Fig. 1a) contained three genes present in the known genome of *S. stipitis* (Jeffries et al. 2007).

Plasmid pPGK-6 has an insert of ~3 kb containing the *HXT2.6* gene, present in chromosome 4 of *S. stipitis*, encoding for a transporter with 534 amino acid residues. Plasmid pPGK-24 contained a ~2.3 kb insert with the *XUT1* gene, present in chromosome 6 of *S. stipitis*, encoding for a transporter with 561 amino acid residues. Plasmids pPGK-37 and pPGK-90 had two different inserts (they differ by the 5' *Bam*HI cleavage site that generated the DNA fragments), containing the same *QUP2* gene (present in chromosome 7 of *S. stipitis*) that encodes for a transporter with 521 amino acid residues. Since the yeast strain transformed with these two plasmids containing the *QUP2* had similar behavior, the results shown for them are the mean values of the two strains containing the pPGK-37 or pPGK-90 plasmids. All the three transporters mediate the uptake of glucose, fructose or xylose, allowing growth and fermentation of these sugars by strain DLG-K1 (Supplementary Table 1), although the specific growth rate, ethanol yield, and amount of sugar consumed by these strains with xylose as carbon source were lower, when compared to cell growth on glucose or fructose.

Figure 2 shows the kinetics of glucose or/and xylose consumption and fermentation by the recombinant yeast strains containing the cloned *S. stipitis* sugar transporters using high cell density fermentation conditions. The three genes expressed in the *hxt*-null

DLG-K1 strain allowed glucose consumption in less than 3 h, showing high volumetric glucose consumption and volumetric ethanol production rates, as well as (with the exception of the *QUP2* gene) high ethanol yields (Table 2). During glucose fermentations the major co-product found was glycerol, which accumulated up to 2.5 g l⁻¹ when the *hxt*-null yeast strain contained the *HXT2.6* gene, up to 1.5 g l⁻¹ in the case of the *XUT1* permease, and 2.6 g l⁻¹ with the *QUP2* gene (data not shown). Regarding xylose fermentation, the *HXT2.6* gene allowed the consumption of this sugar, which during the first 10 h was fermented into ethanol but from that point on the cells stopped producing ethanol and the consumed xylose was reduced into xylitol, which accumulated in the media into the same levels as ethanol (Fig. 2). In contrast, the transporter that allowed the best xylose fermentation yields by the cells was *XUT1* (Table 2), due to the production of very low levels of xylitol (Fig. 2). The permease encoded by the *QUP2* gene, although allowed the uptake of xylose with considerable rates (Table 2), the volumetric ethanol production rates and yields were low as these cells converted the sugar mainly into xylitol, and not ethanol (Fig. 2).

During xylose and glucose co-fermentations the rates of glucose consumption were practically not affected (Table 3; Fig. 2) in the strains expressing the *HXT2.6*, *XUT1*, or *QUP2* genes. From the data shown in Fig. 2 and Tables 2 and 3, it is evident that there is roughly a 40–50 % decrease in the rates of xylose consumption by these strains with equal amounts of both sugars present in the medium, when compared

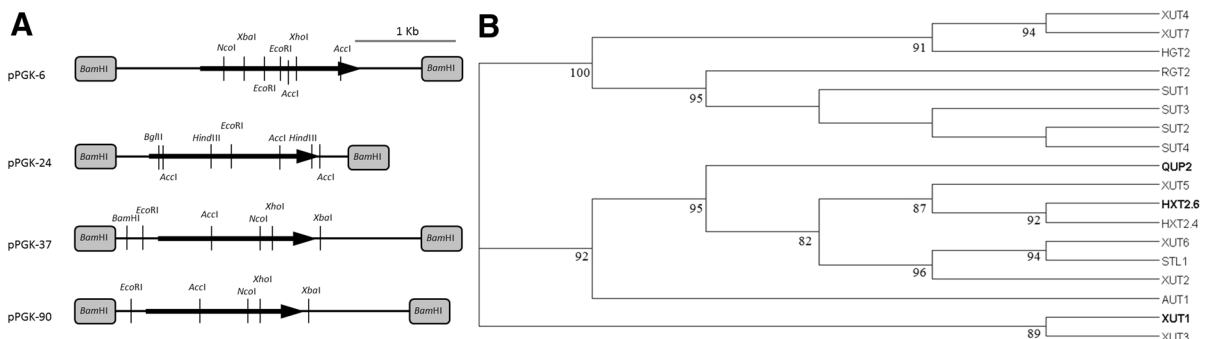


Fig. 1 **a** Structure and restriction maps of the DNA fragments present in plasmids pPGK-6, pPGK-24, pPGK-37 and pPGK-90. The grey boxes indicate pPGK vector sequences, the lines indicate *S. stipitis* DNA sequences, and the black arrows indicate the identified open reading frames. **b** Maximum

likelihood phylogeny with bootstrap values from alignment of sugar transporter-related proteins present in the *S. stipitis* genome, already characterized in *S. cerevisiae* (including the cellobiose transporter *HXT2.4*), with the three genes cloned in the present work in bold characters

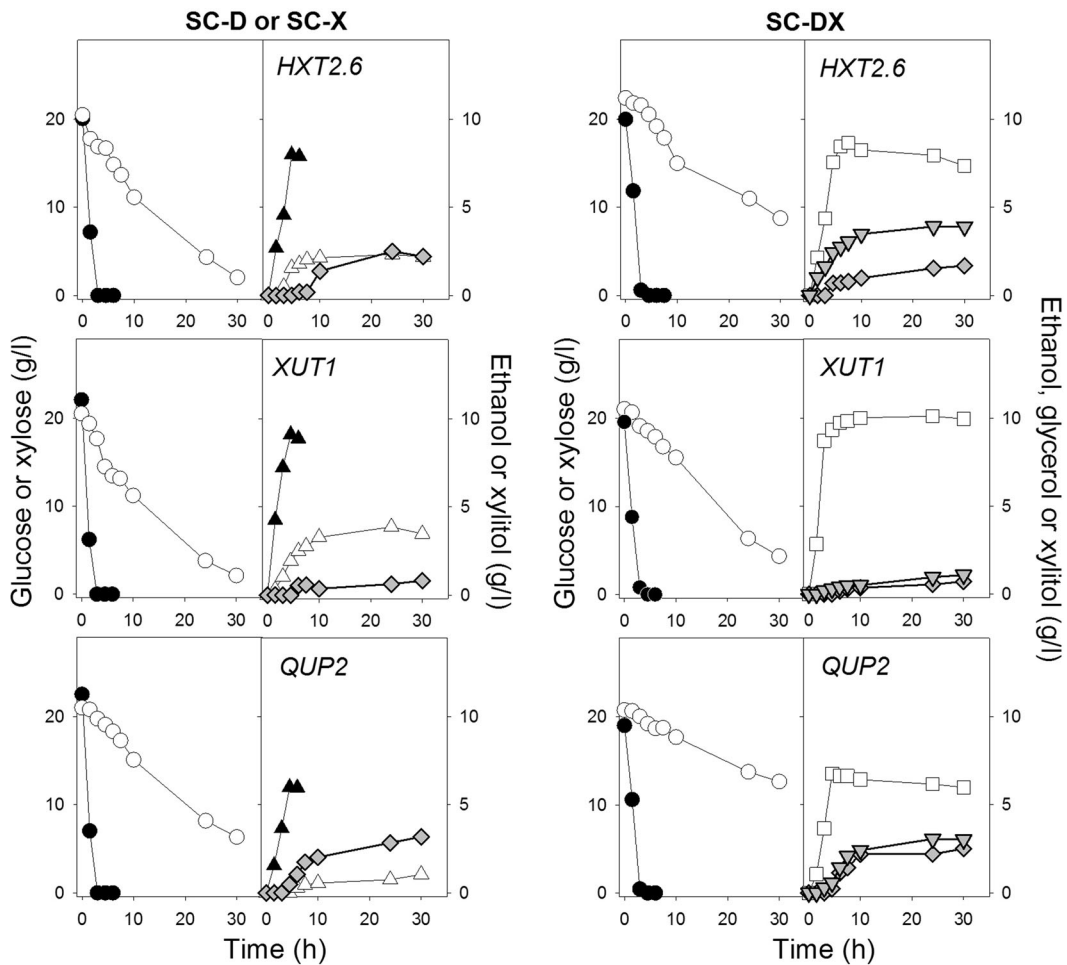


Fig. 2 Time course of glucose (SC-D), xylose (SC-X) or glucose plus xylose (SC-DX) fermentations by the *hxt*-null strain DLG-K1 expressing the *HXT2.6* gene (strain BBY-6), *XUT1* gene (strain BBY-24), or *QUP2* gene (strains BBY-37 and BBY-90). The kinetics of glucose (black circles) or xylose (white circles) consumption, ethanol production during glucose (black triangles), xylose (white triangles) or glucose plus xylose

(white squares) fermentations, and the production of xylitol (grey diamonds) or glycerol (inverted grey triangles) during xylose or glucose plus xylose fermentation, respectively, were determined as described in “Materials and Methods” section. Data are averages from two independent experiments, or the average of strains BBY-37 and BBY-90 for the *QUP2* gene

with the fermentations carried out with each sugar individually. In the case of the cells expressing the *QUP2* permease, all the ethanol produced seems to come from glucose fermentation (Table 2, 3), and again high levels of xylitol (and glycerol) were produced during xylose consumption (Fig. 2). With the cells expressing the *HXT2.6* and *XUT1* permeases xylose consumption seemed to contribute to the ethanol yields (Fig. 2, Tables 2 and 3), but the amount of ethanol produced by the strain expressing the *XUT1* gene was higher due to a very low production of xylitol and glycerol (Fig. 2). Finally, during glucose/xylose

co-fermentations by the strain expressing the *HXT2.6* permease, high quantities of glycerol accumulated in the media (Fig. 2).

Discussion

Scheffersomyces stipitis is of biotechnological interest as it ferments several sugars present in lignocellulosic hydrolysates and thus its sugar transporters have been the subject of extensive research. As mentioned in the Introduction, 15 monosaccharide transporter genes

Table 2 Rates and yields for anaerobic high cell density batch fermentation of glucose or xylose by recombinant yeast strains

Sugar ^a and transporter ^b	Sugar consumption (%)	V_S (g l ⁻¹ h ⁻¹) ^c	V_E (g l ⁻¹ h ⁻¹) ^d	$Y_{E/S}$ (g g ⁻¹) ^e
Glucose				
<i>HXT2.6</i>	100	6.7 ± 0.1	1.76 ± 0.01	0.40 ± 0.01
<i>XUT1</i>	100	7.36 ± 0.1	1.62 ± 0.02	0.41 ± 0.01
<i>QUP2</i>	100	7.51 ± 0.7	1.48 ± 0.05	0.27 ± 0.03
Xylose				
<i>HXT2.6</i>	90	0.94 ± 0.1	0.60 ± 0.02	0.14 ± 0.01
<i>XUT1</i>	90	1.17 ± 0.2	0.55 ± 0.03	0.23 ± 0.02
<i>QUP2</i>	70	0.55 ± 0.04	0.15 ± 0.05	0.07 ± 0.02

^a Fermentations were performed on complete synthetic SC medium containing 2 % of the indicated sugars during 30 h (Fig. 2)

^b The data for *HXT2.6* was obtained with strain BBY-6, for *XUT1* with strain BBY-24, and for *QUP2* with strains BBY-37 and BBY-90

^c Maximum volumetric sugar consumption rate

^d Maximum volumetric ethanol production rate

^e Yield based on g of ethanol produced per g of sugar consumed. Values are averages of two independent experiments, or the average of strains BBY-37 and BBY-90 for the *QUP2* gene

Table 3 Rates and yields for anaerobic high cell density batch fermentation of glucose plus xylose by recombinant yeast strains

Transporter ^a	Glucose consumption (%)	V_G (g l ⁻¹ h ⁻¹) ^b	Xylose consumption (%)	V_X (g l ⁻¹ h ⁻¹) ^c	V_E (g l ⁻¹ h ⁻¹) ^d	$Y_{E/S}$ (g g ⁻¹) ^e
<i>HXT2.6</i>	100	6.46 ± 0.3	61	0.59 ± 0.01	1.8 ± 0.02	0.28 ± 0.02
<i>XUT1</i>	100	6.26 ± 0.1	80	0.62 ± 0.02	1.53 ± 0	0.3 ± 0.01
<i>QUP2</i>	100	6.19 ± 0.2	39	0.34 ± 0.03	1.25 ± 0.04	0.26 ± 0.03

^a The data for *HXT2.6* was obtained with strain BBY-6, for *XUT1* with strain BBY-24, and for *QUP2* with strains BBY-37 and BBY-90. Fermentations were performed on complete synthetic SC medium containing equal amounts (2 %) of glucose and xylose during 30 h (Fig. 2)

^b Maximum volumetric glucose consumption rate

^c Maximum volumetric xylose consumption rate

^d Maximum volumetric ethanol production rate

^e Yield based on g of ethanol produced per g of sugar consumed. Values are averages of two independent experiments, or the average of strains BBY-37 and BBY-90 for the *QUP2* gene

from this yeast (see Fig. 1b) have been analyzed in recombinant *S. cerevisiae* cells, and our cloning and screening approach with a *hxt*-null strain with high XR, XDH and XK activities allowed us to identify three genes that allow growth on and fermentation of xylose, of which two of them (*HXT2.6* and *QUP2*) have not been identified before as capable of transporting xylose into yeast cells. [It should be noted that we were not expecting to clone many gene members of the *S. stipitis* sugar transporter family (e.g. *SUT1*, *SUT2*, *XUT5*, *XUT6* and *STL1*), because they contain

*Bam*HI-cleaving sites in their ORFs that would impair their functional cloning from a genomic library made with DNA cleaved with this restriction enzyme.]

Based on amino acid sequence comparisons of these *S. stipitis* sugar transporter proteins studied in *S. cerevisiae* (Fig. 1b), many transporters (including *SUT1-SUT4*, *RGT2*, *HGT2*, *XUT4* and *XUT7*) cluster together and are not closely related to the permeases cloned in this work. The *XUT1* permease is closely related to *XUT3*, but distant to the *HXT2.6* and *QUP2* transporters that cluster with a distinct set of sugar

transporters including *XUT2*, *XUT5*, and *XUT6*, a permease homolog to the *STL1* glycerol- H^+ symporter of *S. cerevisiae*, a putative arabinose- H^+ symporter (*AUT1*), and the cellobiose transporter encoded by the *HXT2.4* gene.

The *XUT1* and *XUT3* transporters have been characterized as permeases with moderate transport efficiency but higher xylose preference (Young et al. 2011, 2012). Both contain a conserved motive (G-G/F-x-x-x-G) at the end of the first transmembrane span that confers better xylose transport properties (Young et al. 2014). While this motif is absent in the *HXT2.6* permease, the new *QUP2* transporter identified in this work has a partial overlapping sequence (A-G-F-V-G-G) in this region of the protein. The three sugar transporters cloned in the present work also have the conserved PESPR motif right after the sixth transmembrane span, a common motif of sugar transporters that is required for efficient transport activity (Sun et al. 2012).

It is surprising that the transporter encoded by the *HXT2.6* gene allows monosaccharide transport and fermentation by the *hxt*-null DLG-K1 strain, as this transporter has been included into another family of *S. stipitis* closely related sugar transporters (*HXT2.1*–*HXT2.6*) postulated to encode cellobiose permeases (Jeffries and van Vleet 2009; Ha et al. 2013). These genes are closely located with genes encoding endo-glucanases, α -glucosidases, or even other sugar permeases. While the recently characterized cellobiose transporter *HXT2.4* (Ha et al. 2013) is found in a gene cluster with an endo-glucanase (*EGC2*) and a α -glucosidase (*BGL5*) in chromosome 1 of *S. stipitis*, the *HXT2.6* permease is in close proximity with a α -glucosidase (*BGL1*) and the monosaccharide transporter *SUT2* in chromosome 4. This *HXT2.6* gene has been reported to be induced (together with *BGL1*) only after aerobic growth with cellobiose as the unique carbon source (Jeffries and van Vleet 2009). Thus, our results indicate that although this permease in *S. stipitis* is induced by cellobiose, its function is to transport the released monosaccharides after cellobiose (and maybe other oligosaccharides) cleavage by the α -glucosidase encoded by *BGL1*.

While we have not yet characterize the kinetic properties or sugar transport mechanism (facilitated diffusion or H^+ -symport) of the new cloned transporters, our results show that all three permeases allow not only growth on several monosaccharides, but also

efficient xylose consumption and fermentation by the *hxt*-null DLG-K1 strain. It would also be nice to analyze the performance of these new transporters in a *S. cerevisiae* wild-type strain with all its native hexose transporters. Nevertheless, the *hxt*-null DLG-K1 strain with high XR, XDH and XK activities (Gonçalves et al. 2014) used in our genomic library screen to clone new *S. stipitis* xylose transporters is a promising strain platform to identify and characterize other new xylose permease genes from, for example, xylose-fermenting yeast species recently described (Cadete et al. 2009, 2013; Wohlbach et al. 2011; Lobo et al. 2014). The characterization of new xylose transporters can expand our understanding of permease function as well as suggest potentially useful classes of transport proteins or molecular engineering approaches for improving xylose utilization in recombinant *S. cerevisiae* for biofuel applications.

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Supporting information Supplementary Table 1—Growth rates and ethanol yields of the recombinant yeast strains grown on synthetic SC medium with the indicated sugars.

Supplementary Fig. 1—Amino acid sequence alignment of *S. stipites* sugar transporters.

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