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New strategies for efficient expression of heterologous sugar transporters in *Saccharomyces cerevisiae*

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Abstract: In our previous work we had developed an *hxt*-null *Saccharomyces cerevisiae* strain displaying high xylose reductase, xylitol dehydrogenase and xylulokinase activities that proved to be useful as a chassis strain to study new xylose transporters, as *SsXUT1* from *Scheffersomyces stipitis*. *Spathaspora passalidarum* and *Spathaspora arborariae* have in their genomes genes with high sequence similarity (78-80%) to *SsXUT1*. To characterize these putative transporter genes (*SpXUT1* and *SaXUT1*, respectively) they were expressed in the same chassis strain as *SsXUT1*. Surprisingly, the cloned genes could not restore the ability to grow in several monosaccharides tested, although the strains expressing the *SsXUT1* and *SpXUT1* permeases, after growth on maltose, showed the presence of ^{14}C-glucose and ^{14}C-xylose transport activity. An important feature of these permeases is that *SsXUT1* lacks lysine residues in its N-terminal domain with high-confidence ubiquitinylation potential, and has only one at the C-terminal domain, while the *SpXUT1* transporter had several of such residues at its C-terminal domain. When the *SpXUT1* gene was cloned in a truncated version lacking such lysine residues, the permease allowed grow on glucose or xylose, and even promoted xylose fermentation by the *hxt*-null strain. In another approach, we deleted two arrestins known to be involved in sugar transporter ubiquitinylation and endocytosis (*ROD1* and *ROG3*), but only the *rog3Δ* strain allowed modest growth on these sugars. Taken together, these results suggest that to allow efficient sugar transporter expression in *S. cerevisiae* the lysines involved in transporter endocytosis should be removed from the sequence of the permease.

Keywords: xylose; *hxt*-null; ubiquitinylation; lysine; truncated permease; endocytosis; *XUT1*; *ROD1, ROG3*

1. Introduction

The development and use of sustainable alternatives to fossil fuels has been ongoing for the last few decades, and the use of ethanol as a renewable fuel has intensify specially in the USA and Brazil, which account for over 80% of the global ethanol production [1-4]. Presently, three generations of biofuels are being implemented: first generation (1G) biofuels from food based plant materials; second generation (2G) biofuels from lignocellulosic biomasses; and third generation (3G) biofuels from algae-based biomasses [5]. Bioethanol is produced by fermentation of the sugars present in the above biomasses, particularly those present in lignocellulosic plant materials obtained from agricultural and forestry residues, which exists in large quantities and prevails on Earth, and does not contribute to the food vs. fuel controversy [4,6]. These sugars can be used to produce not

only ethanol, but also other valuable chemical products. Biochemical conversion of lignocellulosic biomass into ethanol involves several steps including pre-treatment, enzymatic hydrolysis and fermentation, being this last step a major bottleneck to economically feasible 2G ethanol production [7,8].

Lignocellulosic biomass is composed of cellulose (a linear polymer of glucose molecules linked by β1-4 glycosidic bounds), hemicellulose (a branched and highly heterogeneous polymer containing both hexoses and pentoses), and lignin. Thus, the resulting biomass hydrolysates have various hexoses and pentoses available, and after glucose, xylose and arabinose are the second and third most abundant sugars in plant biomass hydrolysates [9,10]. While glucose can be easily fermented by industrial *Saccharomyces cerevisiae* strains [11], this yeast is not naturally able to ferment the pentoses xylose or arabinose, unless it is genetically modified to express the assimilation routes for these sugars [12-14]. In the case of xylose, genes encoding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Scheffersomyces stipitis* or *Spathaspora passalidarum*, or xylose isomerase (XI) from bacteria and fungi have been extensively used [14-18]. Since both pathways transform xylose into xylulose, it is also required to overexpress the endogenous xylulokinase (XK) gene that will enhance the entrance of this sugar into the pentose-phosphate pathway. While the XI pathway can provide higher ethanol yields, the XR/XDH route allows higher ethanol productivities and higher xylose consumption rates by engineered *S. cerevisiae* yeasts [19,20].

When the metabolic pathway for xylose is established intracellularly, the transport of the sugar across the plasma membrane becomes the limiting step in the assimilation and fermentation of this pentose. The uptake of xylose in *S. cerevisiae* is mediated by a large family of hexose transporters, encoded by the *HXT1-HXT17* and *GAL2* genes, that have significant lower affinities for this pentose than for their main substrate glucose [12,21]. Thus, the discovery and heterologous expression of pentose transporters in *S. cerevisiae* has also being one of the alternatives used to improve 2G ethanol production [22-27]. Consequently, the engineering of pentose transporters in recombinant *S. cerevisiae* yeasts is the subject of several recent reviews in the scientific literature [28-31]. A suitable platform to clone and characterize monosaccharide transporters was created in 1999 by deleting all known hexose transporters in *S. cerevisiae*, as this *hxt⁰*-null EBY.VW4000 strain is not able to grow on media with glucose, fructose or mannose as the sole carbon source (although it still grows very slowly on galactose), but grows normally on maltose as this sugar is transported by different maltose-specific permeases encoded by *MALx1* genes [32,33]. This *hxt⁰*-null strain has being used to characterize hundreds of hexose transporters from other yeasts, fungi, plants, and other eukaryotes including human GLUT transporters, as well as xylose transporters, after the introduction of a xylose utilization pathway in this *hxt⁰*-null strain [24,34]. One drawback of this *hxt⁰*-null EBY.VW4000 strain is that it required 16 successive deletion rounds with the LoxP/Cre system, which resulted in gene losses and several chromosomal rearrangements, affecting, for example, sporulation and spore germination by this *hxt⁰*-null strain [35]. Nevertheless, a recent publication shows that using CRISPR-SpCas9 technology is possible to construct an *hxt⁰*-null strain without these drawbacks [36].

Some years ago our group used an *hxt*-null strain, deleted in the principal hexose transporters (*hxt1Δ* to *hxt7Δ* and *gal2Δ*) and thus unable to grow on glucose, fructose and other monosaccharides, as a platform to study xylose transport because it had high xylose reductase, xylitol dehydrogenase and xylulokinase activities. This DLG-K1 strain was used to characterize the xylose fermentation capacity of yeast strains expressing individual hexose transporters (e.g. *HXT1*, *HXT2*, *HXT5* and *HXT7*) from *S. cerevisiae* [21], and allowed the cloning and characterization of three novel xylose transporters (*XUT1*, *QUP2* and *HXT2.6*) from *Scheffersomyces stipitis* [27]. Results showed that the *SsXUT1* gene allowed ethanol production from xylose or xylose plus glucose as carbon sources, while the *SsHXT2.6* permease produced both ethanol and xylitol, and the strain expressing the *SsQUP2* gene produced mainly xylitol during xylose consumption. Since the

genome of other new xylose-fermenting yeast species (e.g. *Spathaspora passalidarum* [37,38] or *Sp. arborariae* [39,40]) have genes encoding transporters with high sequence similarity (78-80%) to *SsXUT1*, we decided to clone and characterize these putative permeases with our *hxt*-null DLG-K1 strain. In the present work we show that the cloned genes could not restore the ability of the yeast strain to grow in several monosaccharides tested, although the strains expressing the *SsXUT1* and *SpXUT1* permeases, after growth on maltose, showed ^{14}C-glucose and ^{14}C-xylose transport activity. Thus, we analyzed the presence of lysine residues in the N- or C-terminal domain of these transporters with ubiquitinylation potential, as well as the involvement of two α-arrestins known to be involved in sugar transporter ubiquitinylation and endocytosis in *S. cerevisiae*. Our results indicate that to allow efficient sugar transporter expression in *S. cerevisiae* the lysines involved in transporter endocytosis should be ideally removed from the sequence of the permease.

2. Materials and Methods

2.1. Strains, media and growth conditions

The yeast strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strain DH5α was used for cloning, and was grown in Luria broth (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) supplemented with 100 mg/L ampicillin. Yeasts were grown on rich YP medium (1% yeast extract, 2% Bacto peptone, Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil), synthetic complete (YNB) medium (0.67% yeast nitrogen base without amino acids, supplemented with adequate auxotrophic requirements, Sigma-Aldrich), or optimized [41] YNB medium (o-YNB, containing 1.34% yeast nitrogen base without amino acids, supplemented with 2x adequate auxotrophic requirements and 1.5% casaminoacids, Sigma-Aldrich), with 2% maltose, glucose or xylose as carbon source. When required, 2% Bacto agar, 0.5 g/L zeocin (Invivogen, San Diego, CA, USA) or 200 mg/L geneticin sulfate (G-418, Sigma-Aldrich) were added to the medium. The pH of the medium was adjusted to pH 5.0 with HCl, or to pH 8.0 (adjusted with NaOH) when zeocin was used. Cells were pre-grown in YNB-2% maltose and used to inoculate new medium containing the carbon sources tested with an initial cell density of 0.1 A_{600nm} (Cary 60 UV-VIS spectrophotometer, Agilent Technologies, Santa Clara, CA, USA). Growth was performed aerobically in cotton plugged Erlenmeyer flasks filled to 1/5 of the volume with medium at 28ºC with 160 rpm orbital shaking. Cellular growth was followed by absorbance measurements at 600 nm (A_{600nm}), and culture samples were harvested regularly, centrifuged (5,000 g, 1 min at 4ºC), and supernatants used for the quantification of substrates and fermentation products as described below. Alternatively, yeast strains were grown in 100 µl of o-YNB medium (lacking uracil) containing 2% maltose, glucose or xylose at 30ºC in 96-well plates in a Tecan INFINITY M200 PRO microplate reader (Tecan Austria GMBH, Grodig, Austria). All wells in the plate were tightly sealed with AccuClear Sealing Film for qPCR (E & K Scientific, Santa Clara, CA, USA), and growth of each culture was monitored by measuring the A_{600nm} every 10 h, with high intensity orbital shaking between measurements. After ~190 h of growth the plates were centrifuged (3,500 g, 10 min at 4ºC), and the supernatants used for ethanol determination as described below. For batch fermentations, cells were pre-grown in synthetic complete YNB medium containing 2% maltose for 20 hours at 28°C, the cells were collected by centrifugation at 6,000 g for 5 min at 4°C and washed twice with sterile water, and inoculated at a high cell density (10.0 ± 0.5 g dry cell weight (DCW)/L) into 25 mL of synthetic YNB medium containing 2% glucose or xylose. 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.

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

Strains, plasmids and primers	Relevant features, genotype or sequence	Source

Yeast strains:		
Sp. arborariae UFMG-HM19.1A^T	Isolated from rotting wood in Minas Gerais, Brazil	[39]
Sp. passalidarum UFMG-CM-Y474	Isolated from rotting wood in Roraima, Brazil	[17]
S. cerevisiae DLG-K1	*MATa gal2Δ ura3-52 his3-11,15 leu2-3,112 hxt2Δ::HIS3 hxt5::LEU2 hxt7::HIS3 hxt3Δ::LEU2::hxt6 hxt1Δ::HIS3::Δhxt4 MAL2 SUC2 AUR1*::pAUR-XKXDHXR	[21]
S. cerevisiae DLG-K1ΔR1	Isogenic to DLG-K1, but *rod1Δ*::LoxP-*KanMX6*-LoxP	This work
S. cerevisiae DLG-K1ΔR3	Isogenic to DLG-K1, but *rog3Δ*::LoxP-*Ble^R*-LoxP	"
S. cerevisiae DLG-K1ΔR1ΔR3	Isogenic to DLG-K1, but *rog3Δ*::LoxP-*Ble^R*-LoxP *rod1Δ*::LoxP-*KanMX6*-LoxP	"
Plasmids:		
pUG6	LoxP-P$_{TEF}$-*KanMX6*-T$_{TEF}$-LoxP	[42]
pUG66	LoxP-P$_{TEF}$-*Ble^R*-T$_{TEF}$-LoxP	"
pPGK	2μ *URA3* P$_{PGK1}$-T$_{PGK1}$	[43]
pGPD-426	2μ *URA3* P$_{TDH3}$-T$_{CYC1}$	[44]
pPGK-*SsXUT1*	2μ *URA3* P$_{PGK1}$-*SsXUT1*-T$_{PGK1}$	[27]
pPGK-*SpXUT1*	2μ *URA3* P$_{PGK1}$-*SpXUT1*-T$_{PGK1}$	This work
pPGK-*SpXUT1*ΔC	2μ *URA3* P$_{PGK1}$-*SpXUT1*ΔC-T$_{PGK1}$	"
pGPD-*SaXUT1*	2μ *URA3* P$_{TDH3}$-*SaXUT1*-T$_{CYC1}$	"
pGPD-*SaXUT1*ΔNC	2μ *URA3* P$_{TDH3}$-*SaXUT1*ΔNC-T$_{CYC1}$	"
Primers: [1]		
pPGK-SpXUT1-F	AGATCG**GAATTC**AAGCTT<u>ATGCACGGAGGTTCAGACG</u>	"
pPGK-SpXUT1-R	GCC**GGATCC**GGC<u>TTAAGCACTGTCAGCATCAGC</u>	"
pPGK-SpXUT1ΔC-R	GGC**GGATCC**AAATTA<u>GTCAGAGTCTAATTCTTCTCCGCC</u>	"
pGPD-SaXUT1-F	AGATCGGAATTCAAGCTT**GGATCC**<u>ATGCACGGAGGTTCAGATAGTAA</u>	"
pGPD-SaXUT1-R	GCC**CTCGAG**GTCGACCCCGGGGGC<u>TTAATCAGCATCAGCAACCTTTTC</u>	"
pGPD-SaXUT1ΔNC-F	GCC**GGATCC**AAAATG<u>CGTTTAGAAATCGCCGGTAAACC</u>	"
pGPD-SaXUT1ΔNC-R	GCC**CTCGAG**GTCGACTTA<u>ATCAGAATCTAAGTCTTCTAATCC</u>	"
ROD1Δ-F	*ATGTTTTCATCATCATCTCGACCTTCAAAAGAGCCATTAC*<u>CCAGCTGAAGCTTCGTACGC</u>	"
ROD1Δ-R	*CTATGAGCGATCCCGTTTTGTGAACATCTCCATTAAATTA*<u>GCATAGGCCACTAGTGGATC</u>	"
ROG3Δ-F	*GGCGTTGATAAAGAGCCAATATCTATTGTTGCTACATAGA*<u>CCAGCTGAAGCTTCGTACGC</u>	"
ROG3Δ-R	*CGACTATCGTTTGTTACCCTTTGATAGAAAACCTCCCATA*<u>GCATAGGCCACTAGTGGATC</u>	"
V-ROD1-F	AGTCGAGTCCCTTGGTACAT	"
V-ROD1-INT-F	CTGCCGTCACTTATGCTCTG	"
V-ROD1-R	CGAATGATGTCTGTGGGATC	"
V-ROG3-F	GCAAGTACAGAGTCCTACCA	"
V-ROG3-INT-F	CTGTGTGCAAGATTGTGATG	"
V-ROG3-R	GCCAGTTAGAGTGCGTAAAT	"
V-Kan^R-F	CCGGTTGCATTCGATTCC	"
V-Ble^R-F	CCTTCTATGAAAGGTTGGGC	"

[1] Bold sequences indicate restriction enzyme sites (*Bam*HI, *Eco*RI or *Xho*1) used for cloning, underlined sequences allow amplification of genes or the transformation modules present in plasmids pUG6 and pUG66, and italicized sequences are homologous to the upstream and downstream region of the target genes that were deleted.

2.2. Molecular biology techniques

Standard methods for DNA manipulation and analysis, as well as bacterial and yeast transformation, were employed [45,46]. The genomic DNA from the *Sp. passalidarum* and *Sp. arborariae* strains was purified using a YeaStar Genomic DNA kit (Zymo Research, Irvine, CA, USA). Based on the genome sequence of *Sp. arborariae* and *Sp. passalidarum* [38,40], primers were designed (Table 1) to amplify genes with high sequence homology to the *XUT1* gene from *Sc. stipitis* (*SsXUT1* [27]), introducing restriction sites for cloning into multicopy shuttle vectors containing strong and constitutive promoters and terminators (pPGK and p423-GPD, Table 1) as well as the *URA3* gene used as selective marker. Alternatively, the *SpXUT1* gene was amplified using a reverse primer (pPGK-SpXUT1ΔC-R) that introduces a premature stop codon and removes the last 22 amino acid residues of the protein. In the case of the *SaXUT1* gene the permease was truncated both in the N- and C-terminal domains, removing the first 17 amino acid residues (primer pGPD-SaXUT1ΔNC-F, which introduces a methionine after the 17 residues deleted), and removing the last 18 amino acid residues (primer pGPD-SaXUT1ΔNC-R) as described for the *SpXUT1* permease.

In another approach, we deleted the *ROD1* and/or *ROG3* genes in strain DLG-K1 using the PCR-based gene replacement procedure [42]. Briefly, the LoxP-*KanMX6*-LoxP knockout cassette from plasmid pUG6 (Table 1) was amplified with primers ROD1Δ-F and ROD1Δ-R (Table 1), and the resulting PCR product of 1,615-bp (flanked by ~40 bp of homology to the upstream and downstream regions of the *ROD1* gene) containing the *KanMX6* gene was used to transform competent yeast cells. After 2-hour cultivation on YP-2% glucose, the cells were plated on the same medium containing G-418 and incubated at 28°C. G-418-resistant isolates were tested for proper genomic integration of the LoxP-*KanMX6*-LoxP cassette at the *ROD1* locus by diagnostic colony PCR using 4 primers (V-ROD1-F, V-ROD1-INT-F, V-ROD1-R and V-Kan^R-F; Table 1). This set of primers amplified a 3,556-bp fragment (primers V-ROD1-F and V-ROD1-R) or a 2,633-bp fragment (primers V-ROD1-INT-F and V-ROD1-R) from a normal *ROD1* locus, or yielded a 2,720-bp fragment (primers V-ROD1-F and V-ROD1-R) and a 1,500-bp fragment (primers V-Kan^R-F and V-ROD1-R) if the LoxP-*KanMX6*-LoxP cassette was correctly integrated at the *ROD1* locus, producing strain DLG-K1ΔR1 (*rod1*Δ::LoxP-*KanMX6*-LoxP, Table 1). A similar approach was used to delete the *ROG3* gene from strains DLG-K1 or DLG-K1ΔR1. The LoxP-*Ble^R*-LoxP knockout cassette from plasmid pUG66 (Table 1) was amplified with primers ROG3Δ-F and ROG3Δ-R (Table 1), and the resulting PCR product of 1,265-bp (flanked by ~40 bp of homology to the upstream and downstream regions of the *ROG3* locus) containing the *Ble^R* gene was used to transform competent cells. After 2-hour cultivation on YP-2% glucose, the transformed cells were plated on the same medium containing zeocin and incubated at 28°C. Zeocin-resistant isolates were tested for proper genomic integration of the LoxP-*Ble^R*-LoxP cassette at the *ROG3* locus by diagnostic colony PCR using 4 primers (V-ROG3-F, V-ROG3-INT-F, V-ROG3-R and V-Ble^R-F; Table 1). This set of primers amplified a 2,967-bp fragment (primers V-ROG3-F and V-ROG3-R) or a 1,249-bp fragment (primers V-ROG3-INT-F and V-ROG3-R) from a normal *ROG3* locus, or yielded a 1,757-bp fragment (primers V-ROG3-F and V-ROG3-R) and a 750–bp fragment (primers V-Ble^R-F and V-PHO13-R) if the *LoxP-Ble^R-LoxP* module replaced and deleted the *ROG3* gene, producing strain DLG-K1ΔR3 (*rog3*Δ::LoxP-*Ble^R*-LoxP, Table 1) and strain DLG-K1ΔR1ΔR3 (*rod1*Δ::LoxP-*KanMX6*-LoxP and *rog3*Δ::LoxP-*Ble^R*-LoxP, Table 1).

2.3. Transport assays

The DLG-K1 yeast strain transformed with plasmids pPGK-*SsXUT1* or pPGK-*SpXUT1* were grown in YNB-2% maltose to mid-log phase (A_{600nm} of 0.6-1.0), centrifuged, washed twice with cold distilled water, and suspended in water to a cell density of 20 g DCW/L. The uptake of D-[U-^{14}C]glucose or D-[U-^{14}C]xylose (both from Amersham, Little Chalfont, UK) was determined by placing 20 μL of the yeast suspension with 20 μL of 100 mM Tris-citrate buffer, pH 5.0, in the bottom of 8-mL Röhren tubes (Sarstedt AG & Co. KG, Numbrecht, Germany). The tube was incubated at 25 °C for 5 min, and the

reaction started by adding 10 μL of the radiolabelled substrate (102-104 cpm/nmol) at the desired final sugar concentration with vigorous shaking. After 5 s the reaction was stopped with 5 mL of ice-cold distilled water and vigorous shaking, immediately filtered in Whatman glass microfiber GF/C membranes (2.4 cm diameter), and the filters washed twice with 10 mL of ice-cold distilled water. The filters were placed into scintillation vials containing 6 ml of liquid scintillation cocktail (OptiPhase 'HiSafe' 2, Wallac, Turku, Finland), and the radioactivity retained on filters was counted using a liquid scintillation counter (Tri-Carb™ 1600 CA, Packard, Downers Grove, IL, USA). The kinetic parameters (K_m and V_{max}) of glucose and xylose transport by the cloned permeases were determined by fitting the experimental data to the Michaelis-Menten equation, using SigmaPlot v. 11.0 (Systat Software Inc., San Jose, CA, USA).

2.4. Analytical Methods

Glucose, xylose, ethanol, xylitol, and glycerol were determined by high performance liquid chromatography (Prominence HPLC system) equipped with a RID-20A refractive index detector (Shimadzu Co., Tokyo, Japan) using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The HPLC apparatus was operated at 50°C using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min, and 10 μL injection volume.

3. Results

The *SsXUT1* permease from *Ss. stipitis* has been studied previously in *S. cerevisiae*, showing the highest preference for xylose over glucose among several other transporters analyzed [24,27]. Using the sequence of this transporter with 566 amino acids allowed the identification of two other putative permeases with high sequence identity from the xylose-fermenting yeasts *Sp. passalidarum* and *Sp. arborariae*. The open-reading frame (ORF) from the *Sp. passalidarum* genome encodes for a transporter with 576 amino acids and 78.8% sequence identity with *SsXUT1*, while the ORF present in the genome of *Sp. arborariae* encodes for a transporter with 572 amino acids and 79.51% sequence identity with *SsXUT1*. These genes were cloned into multicopy plasmids as described in the Materials and Methods section, and named *SpXUT1* and *SaXUT1*, respectively. To characterize these putative transporters the DLG-K1 (*hxt*-null) strain transformed with plasmids containing these genes were pre-grown on YNB-2% maltose, and inoculated (initial A_{600nm} of 0.1) into YNB medium containing 2% glucose or xylose. However, no significant growth or sugar consumption was observed during 120 h of incubation for both permeases when expressed in strain DLG-K1. Figure 1 shows as example the data for strain DLG-K1 transformed with plasmid pPGK-SpXUT1. Absence of growth was also observed when the cells were inoculated in media containing 2% fructose or galactose (data not shown).

These were quite surprising results since the *SpXUT1* permease expressed in strain DLG-K1, after growth on 2% maltose, showed a clear ^{14}C-glucose and ^{14}C-xylose transport activity similar to the transport activity present in the same *hxt*-null strain expressing the *SsXUT1* permease (Figure 2). The *SsXUT1* permease allows ^{14}C-glucose transport with a K_m of 24.5 mM, but with a low V_{max}, while ^{14}C-xylose transport occurs with a much lower affinity, although with higher capacity (Figure 2, Table 2). In the case of the *SpXUT1* permease ^{14}C-glucose transport is mediated with a similar affinity of 26.1 mM, and also a low V_{max}, and ^{14}C-xylose transport is also mediated with an even lower affinity but high capacity, when compared to glucose uptake by this permease expressed in strain DLG-K1 (see Table 2).
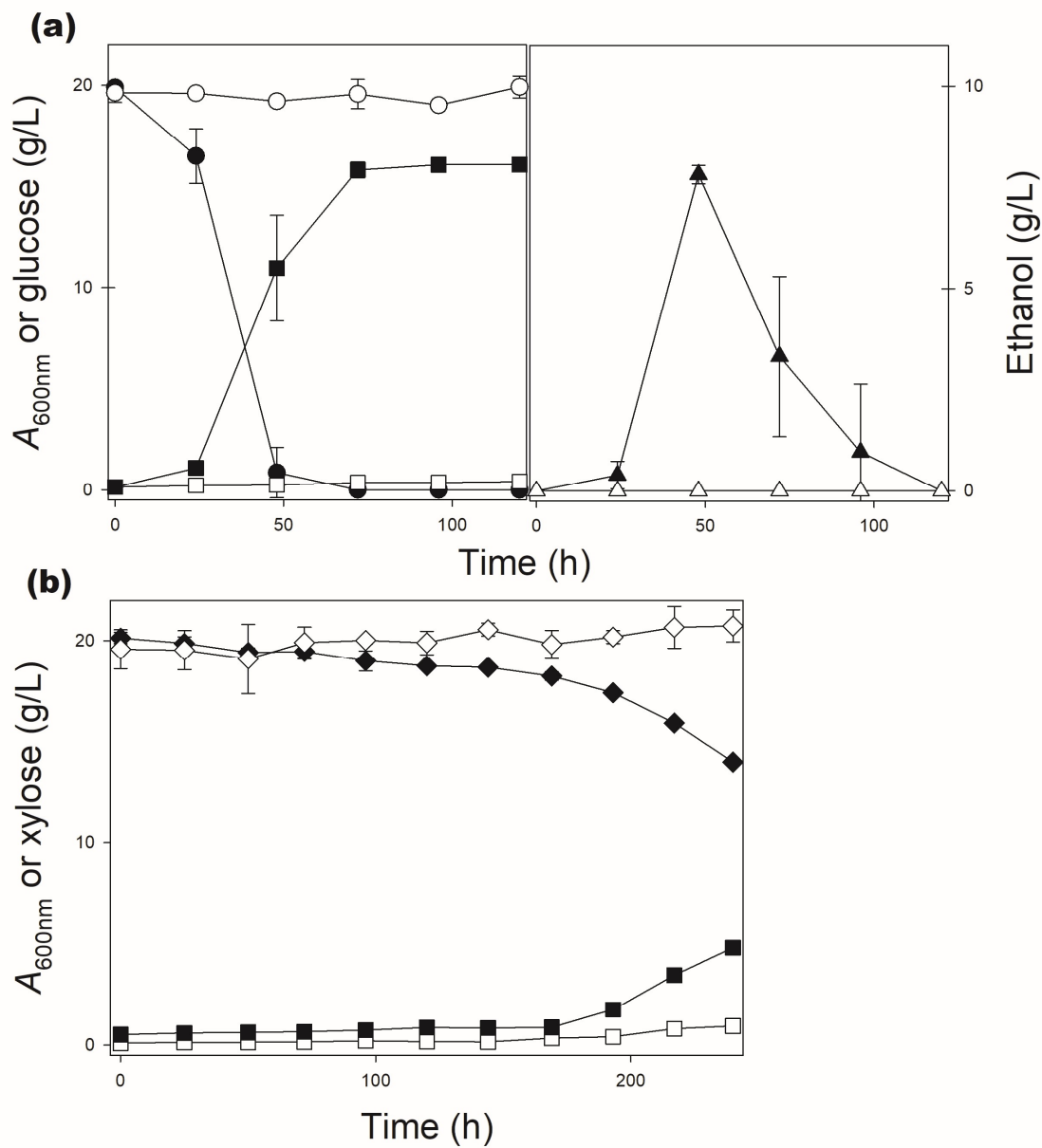
Figure 1. Growth of strain DLG-K1 transformed with plasmid pPGK-SpXUT1 (open symbols) or plasmid pPGK-SpXUT1ΔC (black symbols) on YNB medium (lacking uracil) containing 2% glucose (**a**) or xylose (**b**) as carbon source. At the indicated time points the cell growth (squares), glucose (circles), xylose (diamonds) and ethanol (triangles) concentrations were determined. No ethanol was produced during xylose consumption.

Table 2. Kinetic parameters of ^{14}C-glucose and ^{14}C-xylose transport by the *SsXUT1* and *SpXUT1* permeases.

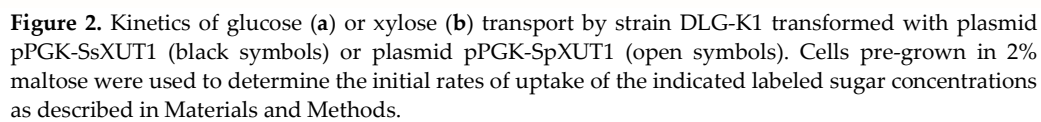| Strain:	Transport of ^{14}C-Glucose		Transport of ^{14}C-Xylose	
	K_m (mM)	V_{max} (mmol h^{-1} gDCW^{-1})	K_m (mM)	V_{max} (mmol h^{-1} gDCW^{-1})
DLG-K1 + pPGK-SsXUT1	24.5 ± 4.1	10.8 ± 1.8	417.7 ± 176	72.4 ± 27
DLG-K1 + pPGK-SpXUT1	26.1 ± 11.5	4.3 ± 1.9	711 ± 550	72.3 ± 7.5

Figure 2. Kinetics of glucose (**a**) or xylose (**b**) transport by strain DLG-K1 transformed with plasmid pPGK-SsXUT1 (black symbols) or plasmid pPGK-SpXUT1 (open symbols). Cells pre-grown in 2% maltose were used to determine the initial rates of uptake of the indicated labeled sugar concentrations as described in Materials and Methods.

Despite the high sequence identity between these three transporters, a significant difference was observed in their N- and C-terminal cytoplasmic domains regarding the presence of lysine residues with high-confidence ubiquitinylation potential (Figure 3). In the case of the *SsXUT1* permease it does not have any lysine in its N-terminal domain, and has a single one with high-confidence ubiquitinylation potential at the end of the C-terminal domain (residue K-558). The permease that had the higher number of lysine residues at their cytoplasmic domains was the *SpXUT1* transporter, since this permease had a medium-confidence possible ubiquitinylated lysine at its N-terminal domain (residue K-17), and also had 3 lysine residues at its C-terminal cytoplasmic domain with high-confidence ubiquitinylation potential (residues K-555, K-557 and K-567, see Figure 3). In the case of the *SaXUT1* permease the lysine residues with ubiquitinylation potential were the same as the *SpXUT1* permease, although residues K-555 and K-567 were considered with medium-confidence ubiquitinylation potential by the UbPred program used to identify such residues [48].

Figure 3. Sequence alignment of the N-terminal (**A**) and C-terminal (**B**) cytoplasmic domains of the *SsXUT1*, *SpXUT1* and *SaXUT1* transporters. The protein sequences were aligned using Clustal Omega [47] and the lysine residues with medium- (blue) or high-confidence (red) ubiquitinylation potential were determined with the UbPred program [48].

Considering that such lysine residues with ubiquitinylation potential could be involved in removing the transporters from the plasma membrane through endocytosis [49], we decided to remove these terminal lysine residues by simply truncating the permeases. In the case of the *SpXUT1* permease we removed the last 22 amino acid residues of the protein by introducing a premature stop codon during cloning as described in Materials and Methods, and this modified transporter was denominated *SpXUT1*ΔC. For the *SaXUT1* permease we not only removed the last 18 amino acid residues, but also the first 17 amino acid residues, producing the *SaXUT1*ΔNΔC permease that lacks all lysine residues with ubiquitinylation potential from their N- and C-terminal domains.

As can be seen in Figure 1, when the DLG-K1 strain was transformed with the pPGK-SpXUT1ΔC plasmid the cells were able to consume, growth on and ferment efficiently glucose (Figure 1a), and consumption of xylose also occurred, although was delayed and incomplete (Figure 1b). Nevertheless, this strain expressing the *SpXUT1*ΔC permease was able to ferment xylose efficiently, producing both ethanol and xylitol, while the strain expressing the full-length transporter (*SpXUT1*) was unable to consume the pentose under fermentative conditions (Figure 4). In the case of the *SaXUT1* permease truncated in both the N- and C-terminal domains (*SaXUT1*ΔNΔC) we also observed clear improvements in growth on glucose or xylose, but not as impressive as with the results obtained with the *SpXUT1* and *SpXUT1*ΔC permeases shown above. For example, under the fermentative conditions shown in Figure 4, after 48 h the DLG-K1 strain transformed with plasmid pGPD-SaXUT1ΔNΔC consumed only ~5 g/L of xylose, producing ~1.5 g/L of ethanol, while the strain expressing the full length *SaXUT1* permease was unable to consume the pentose (data not shown).
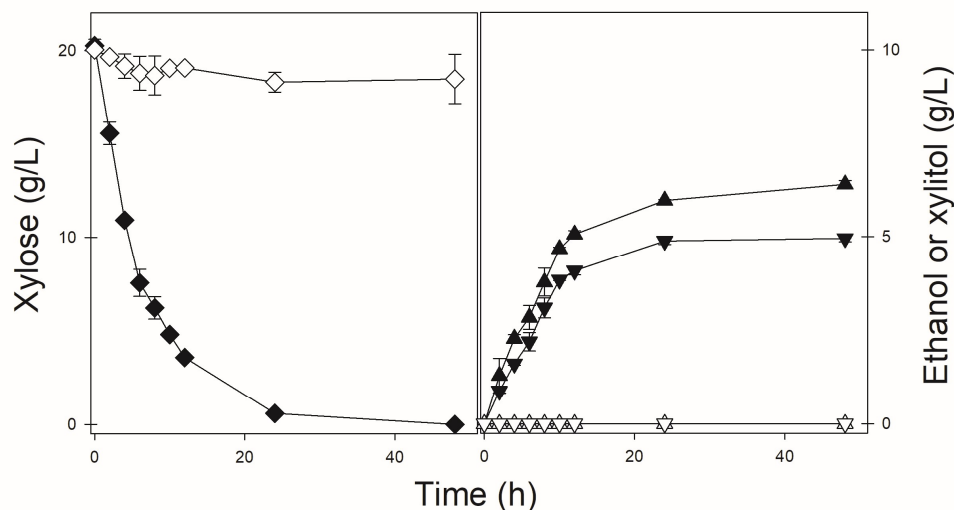

Figure 4. Xylose fermentation by the DLG-K1 strain transformed with the pPGK-*SpXUT1* (open symbols) or pPGK-*SpXUT1*ΔC (black symbols) plasmids. The batch fermentation was performed with high cell concentrations (10 g DCW/L) and the amount of xylose (diamonds), ethanol (tringles) and xylitol (inverted triangles) in the medium were determined at the indicated time points as described in Materials and Methods

Since our results suggested that the ubiquitinylation of heterologous sugar permeases impair their successful expression in *S. cerevisiae*, and since this post-translational modification that triggers the endocytosis of the plasma membrane transporters depends on the activity of α–arrestins that function as adaptors for the E3 ubiquitin ligase encoded by the essential gene *RSP5* [50,51], we tested if deleting α–arrestins involved in sugar transporter endocytosis could be another strategy to improve the functional expression of the cloned transporters in *S. cerevisiae*. Although this yeast has 14 known α–arrestins, we

focused in two α–arrestins known to be involved in sugar transporter endocytosis: *ROD1* (also known as *ART4* for *a*rrestin-*r*elated *t*rafficking adaptors) which has been shown to mediate the ubiquitinylation and endocytosis of the high-affinity *HXT6* and *HXT4* glucose transporters [52-54], the low-affinity *HXT1* and *HXT3* glucose permeases [55], as well as the *GAL2* galactose permease [56]; and its paralog *ROG3* (also known as *ART7*) that has been shown to be implicated in the ubiquitinylation and endocytosis of the low-affinity *HXT1* and *HXT3* glucose permeases [54,55].

To verify the influence of these two α–arrestins on the functionality of the *SpXUT1* and *SaXUT1* permeases in *S. cerevisiae* we constructed in our *hxt*-null DLG-K1 genetic background (Table 1) strains deleted in *ROD1* (DLG-K1ΔR1), *ROG3* (DLG-K1ΔR3), or both genes (DLG-K1ΔR1ΔR3). Figure 5 shows the growth patterns of these 3 strains, transformed with plasmids pGPD-SaXUT1 or pPGK-SpXUT1, in three carbon sources: maltose, glucose and xylose. Analyzing the data it is evident that the deletion of both α-arrestins (*rod1*Δ, *rog3*Δ) has a negative impact on growth even with the control carbon source (2% maltose). While all strains produced 8.7 ± 0.7 g ethanol/L at the end of growth on this carbon source, strain DLG-K1ΔR1ΔR3 transformed with both plasmids produced less ethanol (7.2 ± 1.1 g/L) from 2% maltose. Deletion of both genes had also a negative impact when the cells were grown on the two other carbon sources.
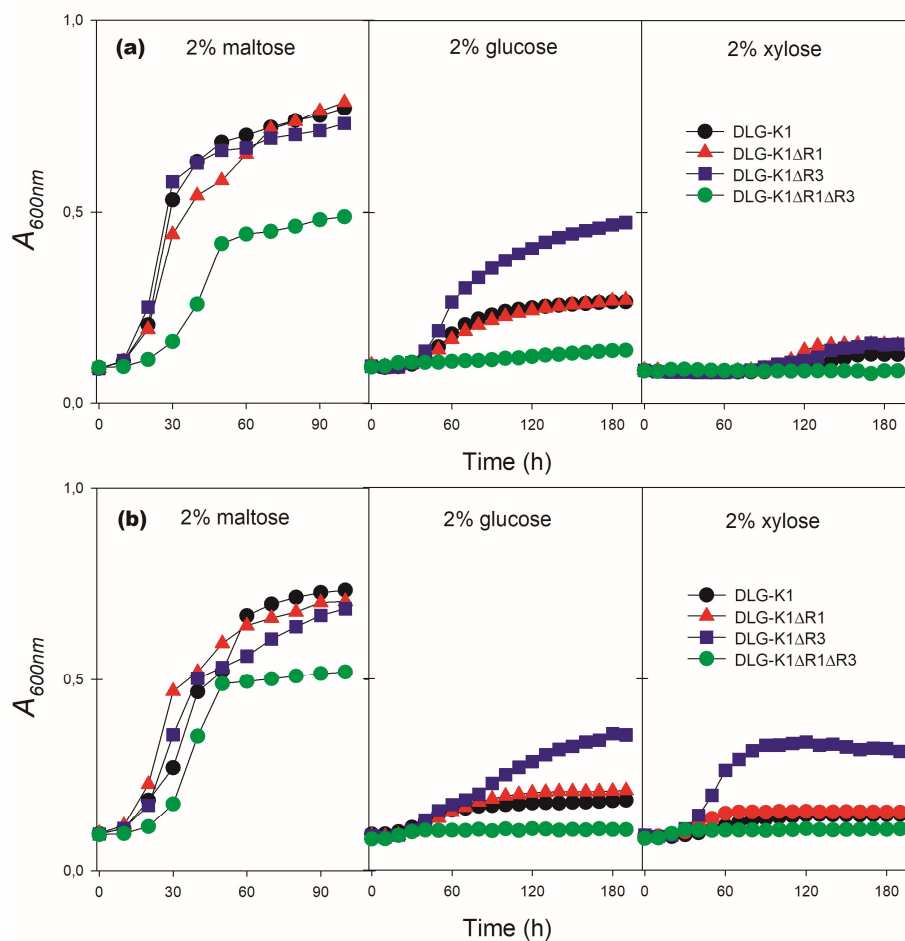

Figure 5. Growth of the indicated *hxt*-null strains containing the pPGK-SpXUT1 (**a**) or pGPD-SaXUT1 (**b**) plasmids on o-YNB medium containing 2% of the indicated carbon sources in a microplate reader as described in Materials and Methods.

Growth on glucose was clearly improved in the *rog3Δ* strain DLG-K1ΔR3 expressing both *XUT1* permeases (Figure 5). The *rog3Δ* cells expressing the *SpXUT1* transporter produced 7.1 ± 0.2 g ethanol/L at the end of growth on 2% glucose, while these same cells expressing the *SaXUT1* permease produced only 1.1 ± 0.1 g ethanol/L, a lower performance as already mentioned for cells expressing this permease. Regarding growth on xylose, for the cells expressing the *SpXUT1* permease none of the strains deleted in *ROD1* and/or *ROG3* improved the utilization of this carbon source by the *hxt*-null strains, indicating that probably other α–arrestins might be involved in ubiquitinylation of this transporter in the presence of the pentose. A different pattern was obtained with the strains expressing the *SaXUT1* permease, as the DLG-K1ΔR3 strain showed some growth on xylose (Figure 5), but without ethanol production. The improvements observed with the strains deleted in the two α–arrestins shown in Figure 5 (performed in a microplate reader with 96-well plates) were confirmed when these strains DLG-K1ΔR3 expressing both transporters were grown aerobically in cotton plugged Erlenmeyer flasks (data not shown).

4. Discussion

Our group had previously used the *hxt*-null strain DLG-K1 to clone and characterize three sugar transporters from *Sc. stipitis* that allowed growth and xylose fermentation by *S. cerevisiae* [27]. One of the transporters (*SsXUT1*) was already known to allow growth of this yeast on xylose [24], but the two others (*SsHXT2.6* and *SsQUP2*) were completely new and even annotated for other functions in the published genome sequence of *Sc. stipitis* [27]. An interesting characteristic of these three permeases is that, as shown here for the *SsXUT1* permease (see Figure 3), they had only one lysine residue with high-confidence ubiquitinylation potential at their C-terminal cytoplasmic domain (*SsXUT1* and *SsQUP2*), or at its N-terminal domain (*SsHXT2.6*). In contrast, the two putative sugar transporters cloned in this work from the xylose-fermenting yeasts *Sp. passalidarum* and *Sp. arborariae*, having high sequence identity with *SsXUT1*, had several lysine residues with high-confidence ubiquitinylation potential at their C-terminal domain (e.g. *SpXUT1*), as well as lysine residues with medium-confidence ubiquitinylation potential also at their N-terminal cytoplasmic domain (see Figure 3).

In *S. cerevisiae* sugar transporters can be ubiquitinylated and removed from the plasma membrane through endocytosis depending not only on the type (and quantity) of the sugar present in the medium, but also in response to other environmental perturbations [49-51]. For example, low affinity and high capacity glucose transporters (e.g. *HXT1* and *HXT3*) are endocytated in the absence of glucose (or presence of other carbon sources, like galactose, ethanol or lactate), while high affinity glucose transporters (e.g. *HXT2*, *HXT6* and *HXT7*) are removed from the plasma membrane in the presence of excess glucose, but also in the absence of glucose (or presence of other carbon sources) [53,55,57-59]. The influence of ubiquitinylation and endocytosis in the expression of heterologous sugar transporters in *S. cerevisiae* is only starting to be understood [31].

Indeed, several efforts to express heterologous sugar transporters in this yeast have been hampered by the low success of such approaches. For example, Young and coworkers [24] cloned 23 transporters from different organisms (bacteria, plant and several yeasts) and expressed them in a *hxt*-null strain with a xylose utilization pathway. Of these 23 heterologous transporters, only 7 (including *SsXUT1*) conferred significant growth phenotypes on one or more of six different carbon sources tested. Several other recent manuscripts reporting the identification and cloning of sugar transporters from yeasts and fungi, with rates of successful expression of less than 1/3 of the putative permeases in a *hxt*-null strain, are common [60-62]. Although there are many possible reasons for failure to efficiently express a putative sugar transporter in *S. cerevisiae*, including misfolding of the protein, problems with correct traffic of the permease to the plasma membrane, or even the lipid environment of the host, the stability at the cell surface is

also of concern, and transporter ubiquitinylation and endocytosis is certainly an issue that could influence de heterologous expression of sugar transporters in this yeast.

Ubiquitinylation of lysine residues at the N- or C-terminal cytoplasmic domains are part of the molecular signals used for endocytic removal of sugar permeases, and several studies have shown that by mutating or deleting such residues the transporters are stabilized at the plasma membrane. For example, the *S. cerevisiae HXT1* transporter has 4 lysine residues at its N-terminal domain involved in its ubiquitinylation and endocytosis, and truncating the whole N-terminal domain (deleting 56 residues to remove all 4 lysine residues) gives a functional transporter that is stable at the plasma membrane [58]. We have used recently this strategy to enhance xylose consumption and fermentation by an industrial *S. cerevisiae* strain [63], while site-directed mutation of these 4 residues in the *HXT1* transporter was also shown to improve cell growth on this carbon source [64]. Another example involves one of the few yeast active xylose transporters characterized in *S. cerevisiae*, the *Candida intermedia CiGXS1* gene encoding for a glucose/xylose H+-symporter [65]. This transporter in a *hxt*-null strain was submitted to several rounds of mutagenesis aiming to remove glucose inhibition, especially during glucose-xylose co-fermentations, and among several interesting mutations found there were some that truncated the C-terminal domain of the permease [66]. A systematic analysis of this C-terminal domain revealed that the best results in terms of growth on glucose plus xylose medium was when 27 amino acids were deleted from the end of the *CiGXS1* permease, which included two lysine residues [66]. Finally, C-terminal truncations that enhance the stabilization and transport activity of two heterologous cellobiose transporters expressed in *S. cerevisiae* have been also reported, eliminating 1-2 lysine residues present at their C-terminal domains probably involved in ubiquitinylation and endocytosis [41]. Thus, our approach of truncating the *SpXUT1* or *SaXUT1* permeases, removing the potentially ubiquitinylation lysine residues at their N- and/or C-terminal domains, is certainly an interesting strategy to increase the repertory of heterologous sugar (especially pentose) transporters for molecular engineering approaches to improve biomass sugar utilization in recombinant *S. cerevisiae* strains for biofuel and chemical production.

Besides eliminating lysine residues from the cytoplasmic domains of the transporters by truncating their cytoplasmic terminal domains, we also tested the involvement of two α–arrestins (*ROD1* and *ROG3*) known to be involved in sugar transporters ubiquitinylation and endocytosis in *S. cerevisiae*. While deletion of *ROD1* or both α–arrestins in our *hxt*-null strain had no effect on the absence of growth of the strains expressing the two *XUT1* permeases, deletion of *ROG3* had a positive effect with both permeases during growth on glucose, but during growth on xylose only the *SaXUT1*-expressing strain DLG-K1ΔR3 had its growth enhanced (Figure 5). This more complex situation might reflect the fact that most α–arrestins have overlapping functions when promoting the ubiquitinylation of the permeases by the *RSP5* ubiquitin ligase, and many transporters are regulated by two or even up to four α–arrestins, reflecting the diverse signaling pathways that mediate transporter ubiquitinylation and endocytosis triggered by changing environmental conditions [41,50,51,54-56]. Nevertheless, our results highlight the involvement of the *ROG3* α–arrestin in down-regulation of the heterologous *SpXUT1* and *SaXUT1* permeases expressed in *S. cerevisiae* in the presence of glucose, while a more complex situation is evident when xylose is the carbon source for growth.

5. Conclusions

In the present work we have cloned two *XUT1* permeases from the xylose-fermenting yeasts *Sp. passalidarum* and *Sp. arborariae*. While these two transporters seemed to be not functional in *S. cerevisiae*, the truncation of the N- and/or C-terminal domains, eliminating lysine residues with ubiquitinylation potential, allowed the functional expression of the transporters in an *hxt*-null strain. Thus, this might be an interesting strategy to increase the repertory of heterologous sugar transporters for molecular engineering approaches to improve biomass sugar utilization by *S. cerevisiae*. This work

therefore highlights the importance of post-translational modifications in the correct expression of novel sugar transporters in recombinant *S. cerevisiae* strains, aiming at an efficient 2G bioethanol production.

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