RESEARCH ARTICLE

D-glucose overflow metabolism in an evolutionary engineered high-performance D-xylose consuming *Saccharomyces cerevisiae* strain

Jeroen G. Nijland[1], Hyun Yong Shin[1], Eleonora Dore[1], Donny Rudinatha[1], Paul P. de Waal[2] and Arnold J. M. Driessen[1,*,†]

[1]Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology, University of Groningen, Zernike Institute for Advanced Materials and Kluyver Centre for Genomics of Industrial Fermentation, Groningen, The Netherlands and [2]DSM Biotechnology Center, Alexander Fleminglaan 1, 2613 AX, Delft, The Netherlands

*Corresponding author: Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747AG Groningen, The Netherlands. E-mail: a.j.m.driessen@rug.nl

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†Arnold J.M. Driessen, http://orcid.org/0000-0001-9258-9104

ABSTRACT

Co-consumption of D-xylose and D-glucose by *Saccharomyces cerevisiae* is essential for cost-efficient cellulosic bioethanol production. There is a need for improved sugar conversion rates to minimize fermentation times. Previously, we have employed evolutionary engineering to enhance D-xylose transport and metabolism in the presence of D-glucose in a xylose-fermenting *S. cerevisiae* strain devoid of hexokinases. Re-introduction of Hxk2 in the high performance xylose-consuming strains restored D-glucose utilization during D-xylose/D-glucose co-metabolism, but at rates lower than the non-evolved strain. In the absence of D-xylose, D-glucose consumption was similar to the parental strain. The evolved strains accumulated trehalose-6-phosphate during sugar co-metabolism, and showed an increased expression of trehalose pathway genes. Upon the deletion of *TSL1*, trehalose-6-phosphate levels were decreased and D-glucose consumption and growth on mixed sugars was improved. The data suggest that D-glucose/D-xylose co-consumption in high-performance D-xylose consuming strains causes the glycolytic flux to saturate. Excess D-glucose is phosphorylated enters the trehalose pathway resulting in glucose recycling and energy dissipation, accumulation of trehalose-6-phosphate which inhibits the hexokinase activity, and release of trehalose into the medium.

Keywords: sugar transport; D-xylose transporter; trehalose-6-phosphate; bioethanol; yeast; glycolysis

ABBREVIATIONS

bp:	base pair	FC:	fold change
DW:	dry-weight	Hxk:	hexokinase
		Hxt:	hexose transporter
		LC-MS:	Liquid chromatography–mass spectrometry

MM: minimal medium
OD: optical density
XKS: xylulose kinase
XI: xylose isomerase

INTRODUCTION

Increasing energy demand and concerns of obtaining this energy from fossil fuels have stimulated the development of liquid fuels from renewable feedstock. Bioethanol, mostly used as a fuel additive, is produced from readily fermentable agricultural feedstock's like sugar cane and corn. However, this is less desired because the production of these feedstock's requires large amounts of arable land while competing with food supply (Solomon 2010). A more sustainable source of feedstock is lignocellulosic biomass from hardwood, softwood and agricultural residues (Zaldivar, Nielsen and Olsson 2001). A major drawback of lignocellulosic feedstock's is the inability of the most commonly used yeast in industry, *Saccharomyces cerevisiae*, to ferment the substantial fraction (up to ~30%, (Gírio *et al.* 2010)) of pentose sugars, such as D-xylose, that besides the hexose sugars are released upon conversion of lignocellulose (Carroll and Somerville 2009). In recent years, two main strategies have been developed to equip *S. cerevisiae* with the ability to convert D-xylose into bioethanol: (i) the XR-XDH pathway, a two-step redox pathway in which xylose reductase (XR) first catalyzes the reduction of xylose to xylitol, which is subsequently oxidized via xylitol dehydrogenase (XDH) to form xylulose (Kotter and Ciriacy 1993; Tantirungkij, Seki and Yoshida 1994; Jeffries and Jin 2004; Bera *et al.* 2011) and (ii) the XI pathway, a one-step conversion from xylose into xylulose using either a bacterial or fungal xylose isomerase (Kuyper et al. 2003, 2004, 2005). The latter pathway, overexpressing the fungal xylose isomerase of *Piromyces sp.* E2 is used in this study. D-xylulose is subsequently phosphorylated by the xylulose kinase Xks1, which has been overexpressed in engineered strains (Van Maris *et al.* 2007; Peng *et al.* 2011; Zha *et al.* 2014). The resulting D-xylulose-5-phosphate enters the pentose phosphate pathway (PPP) and, via glyceraldehyde-3-phosphate and fructose-6-phosphate, D-xylose catabolism is connected to glycolysis and subsequent ethanol fermentation. Various other mutations like e.g. the deletion of Gre3 (Traff *et al.* 2001; Shao *et al.* 2009; Wisselink *et al.* 2009) and the deletion of Pmr1 (Verhoeven *et al.* 2017) have improved D-xylose consumption on solely D-xylose. However, the transport of D-xylose into the cell, in the presence of D-glucose, remained a major hurdle in order to obtain co-consumption of D-glucose and D-xylose (Hamacher *et al.* 2002b; Sedlak and Ho 2004; Saloheimo *et al.* 2007). In an industrial setting, it is preferred that both sugars are fermented simultaneously and at high rates (von Sivers *et al.* 1994) to generate an economically feasible and robust process. However, because of competition with D-glucose, impaired D-xylose transport in xylose-fermenting *S. cerevisiae* strains prevents simultaneous consumption of D-glucose and D-xylose (Hamacher *et al.* 2002b). D-xylose and D-glucose are both transported into the cell via the hexose transporters (Hxt) (Reifenberger, Freidel and Ciriacy 1995; Reifenberger, Boles and Ciriacy 1997; Hamacher *et al.* 2002a). However, the preferred D-glucose consumption of *S. cerevisiae* is the direct result of the sugar specificities of these hexose transporters. All Hxt transporters are specific for D-glucose and their affinity for this sugar is significantly (on average a 100-fold) higher compared to D-xylose (Kotter and Ciriacy 1993; Hamacher *et al.* 2002b). This prevents

efficient D-xylose transport in the presence of high concentrations of D-glucose (Young *et al.* 2012). Different approaches have been followed to improve D-xylose transport including the introduction of specific D-xylose transporters derived from other organisms, but, in general, the achieved D-xylose transport rates are insufficient to allow for maximal growth (Saloheimo *et al.* 2007; Du, Li and Zhao 2010; Runquist, Hahn-Hagerdal and Radstrom 2010; Young *et al.* 2011; Wang *et al.* 2015; Nijland and Driessen 2020). In recent studies, D-xylose transport in the presence of D-glucose, has improved dramatically based on the mutagenesis of endogenous Hxt transporters. This specifically concerns a conserved asparagine (at position 366, 376, 370 and 376, in Hxt11 (Shin *et al.* 2015), Hxt36 (Nijland *et al.* 2014), Hxt7 (Farwick *et al.* 2014) and Gal2 (Farwick *et al.* 2014; Verhoeven *et al.* 2018b), respectively) which, when mutated, results in a reduced D-glucose affinity with a mostly unaffected or even improved affinity for D-xylose. In a further evolutionary engineering strategy employing a D-glucose metabolism deficient strain (lacking all four hexokinases) (Nijland *et al.* 2019a) that was selected for improved growth rates on D-xylose in the presence of inhibitory concentrations of D-glucose, high-performance D-xylose consumption and growth was achieved. In the evolved strain (DS71054-evo6), now carrying the aforementioned N367I mutation in Hxt37, overall D-xylose transport was optimized via the downregulation of Hxt1, Hxt2 and the deletion of Hxt7. Furthermore, morphological analysis of the evolved DS71054-Evo6 strain showed an increased cell size, which can be attributed to a change in ploidy. The increase in cell surface area together with the decreased expression of the (re)main(ing) Hxt transporters, allowing D-glucose insensitive uptake of D-xylose via Hxt37 N367I, appear major factors in the improved growth on D-xylose.

Here, we used the aforementioned evolved lineage of xylose fermenting *S. cerevisiae* strains (Nijland *et al.* 2019a) that were optimized for high performance D-xylose metabolism in the presence of high concentrations of D-glucose, and reintroduced the hexokinase Hxk2 to restore D-glucose metabolism. These strains also support the co-consumption of D-xylose and D-glucose but the increasing rates of D-xylose metabolism are paralleled by a progressively reduced rate of D-glucose metabolism. In these strains, upregulation of the trehalose pathway enzymes occurs resulting in increasing levels of trehalose-6-phosphate, an inhibitor of hexokinase. We hypothesize that during mixed sugar fermentation and a high rate of D-xylose entry into the cell, the glycolytic flux becomes saturated and excess sugar is channeled into the trehalose pathway resulting in a futile cycle of energy dissipation.

MATERIALS AND METHODS

Yeast stains, media and culture conditions

Xylose-fermenting *S. cerevisiae* strains used in this study were provided by DSM Bio-based Products & Services and described elsewhere (Table S1, Supporting Information). They are made available for academic research under a strict Material Transfer Agreement with DSM (contact: johan.doesum-van@dsm.com). Aerobic shake flask experiments were done at 200 rpm in minimal medium (MM) supplemented with vitamin solution, urea, trace elements and D-xylose and/or D-glucose (Luttik *et al.* 2000). In the fermentation experiments under micro-aerobic conditions, on 7% D-glucose and 3% D-xylose or solely 7% D-glucose or 3% D-xylose, a starting OD_{600} of 2.0–2.5 was used. Cell growth

was monitored by optical density (OD) at 600 nm using an UV-visible spectrophotometer (Novaspec PLUS).

Cloning of hexokinases

The hexokinase genes were amplified using the primers listed in Table S2 (Supporting Information) using the Phusion® High-Fidelity PCR Master Mix with HF buffer (Thermo Fisher Scientific, CA, USA). Genomic DNA of DS71054 was used as a template for Hxk2, Hxk1 and Glk1. To amplify *Sp*Hxk2 a *S. cerevisiae* codon optimized gene block was ordered (IDT, Leuven, Belgium). Hxk2-Y, with one mutation as compared to Hxk2, was made using overlap PCR (primers listed in Table S2, Supporting Information). The PCR fragments of Hxk2, Hxk1, Hxk2-Y and *Sp*Hxk2 were cut with restriction enzymes XbaI and Cfr9I (Thermo Fisher Scientific, CA, USA). Glk1 was cut with BcuI and BamHI. The vector pRS313-P7T7 was used for the expression of Hxt transporters under control of the HXT7 promotor and was derived from pRS313 (kindly supplies by DSM Biotechnology Center, The Netherlands) as backbone containing the histidine selection marker and the Cen/ARS low copy origin for cloning in yeast. pRS313 was digested with the restriction enzymes XbaI and Cfr9I or BcuI and BamHI. The subsequent ligation was performed using T4 DNA ligase (Thermo Fisher Scientific, CA, USA).

Intracellular metabolite extraction

Cell free extracts of all strains complemented with Hxk2 and grown in minimal medium containing 7% D-glucose and 3% D-xylose were isolated after 2, 4 or 12 hours using an ethanol boiling extracted method (Canelas *et al.* 2008) with minor adjustments. Cells were collected and quenched by adding 60% methanol of −40°C and snap-frozen at −80°C. Each tube containing 1.5 mg dry weight cells was taken from the −80°C freezer and 1 ml 75% (v/v) boiling ethanol was added. Each tube was immediately vortexed and placed in a thermomixer (Eppendorf, Hamburg, Germany) at 95°C. After 5 min each tube was stored in the −80°C freezer. Further processing was done via evaporation and re-suspending the intracellular content in water and filtering through a 0.2 μm PTFE 13 mm syringe filter (VWR, Amsterdam–The Netherlands).

Analytical methods

High performance liquid chromatography (Shimadzu, Kyoto, Japan) on culture supernatants was performed using an Aminex HPX-87H column at 65°C (Bio-RAD) and a refractive index detector (Shimadzu, Kyoto, Japan) was used to measure the concentrations of D-glucose, D-xylose, acetic acid and ethanol. The mobile phase was 0.005 N H_2SO_4 at a flow rate of 0.55 ml/min. The analysis of intracellular metabolites was performed with an Accella1250 HPLC system using an Aminex HPX-87H column at 60°C (Bio-RAD) coupled with the ES-MS Orbitrap Exactive (Thermo Fisher Scientific, CA, USA). The intracellular concentrations of glucose-6-phosphate, ATP/ADP and NAD+/NADH were measured using the Glucose-6-phosphate Assay Kit, the ATP Assay Kit and the NAD/NADH Quantitation Kit (all from Sigma-Aldrich, Zwijndrecht, The Netherlands), respectively and performed as described by manufacturer.

Gene deletion

Strains were transformed with plasmid p414-KanMX-TEF1p-Cas9-CYCt (Nijland *et al.* 2017) to express Cas9. Target and repair

fragments were designed using www.yeastriction.com and the CRISPR/Cas9 protocol described was used (Mans *et al.* 2015). Oligonucleotides used for the gene deletions are listed in Table S3 (Supporting Information).

Transport assays

To determine the kinetic parameters of sugar transport, cells were grown for 16 hours in shake flasks in minimal medium containing 2% D-xylose or 2% D-glucose and standard uptake procedure was followed as shown before (Nijland *et al.* 2014). Uptakes were performed with [^{14}C] D-xylose and [^{14}C] D-glucose (ARC, USA) at 50 and 380 mmol l^{-1}, respectively, with various inhibiting sugar concentrations. The uptake incubation times were 10 and 60 s for D-glucose and D-xylose, respectively.

RNA extraction and cDNA synthesis

Total RNA was isolated from *S. cerevisiae* cells by a glass-bead disruption Trizol extraction procedure and performed as described by manufacturer (Life Technologies, Bleiswijk, The Netherlands). Yeast pellets from 2 ml of exponential phase cell culture (OD$_{600}$ of ∼ 4) were mixed with 0.2 ml of glass beads (diameter 0.45 mm) and 900 μl of Trizol with 125 μl chloroform, and disrupted in a Fastprep FP120 (Thermo Savant) for 45 s at speed 6. The extracted total RNA (500 ng) was used to synthesize cDNA using the iScript cDNA synthesis Kit (Bio-rad, CA, USA).

RNAseq and analysis

Total RNA of all strains complemented with Hxk2, grown in minimal medium containing 7% D-glucose and 3% D-xylose, was isolated in duplicates after 7 h. The RNA was prepared for sequencing using the QuantSeq 3' mRNA-Seq Library Prep (FWD for Illumina) Kit (Lexogen, Vienna, Austria) and run on an Illumina HiSeq 2500 with single-read 100 bp read mode and V4 chemistry. The average number of reads per sample was 4064011 and was consistent in all samples. The FastQ files were run through a BowTie2-TopHat-SamTools pipeline and the resulting BAM files were analysed using SeqMonk V0.27.0. The CEN.PK113–7D strain was used as a reference genome. All genes were quantified in CPM (count per million) with a cut-off of 15 and run in an intensity difference statistical test in which a statistical difference of below 0.05 was used ($P < 0.05$).

Glycolytic protein levels

Cell lysates were prepared using a glass bead lysis method in which yeast cells (OD$_{600}$ of ∼ 4) were mixed with 0.2 ml of glass beads (diameter 0.45 mm) and 500 μl 50 mM Tris-HCl pH 8.0, complemented with 1 mM PMSF, and disrupted in a Fastprep FP120 (Thermo Savant) for 45 seconds at speed 6. Cell debris was subsequently removed by ultracentrifugation at 4°C for 30 min at 45 000 rpm using a 45Ti rotor (Beckman). Relative protein concentrations of the proteins part of the glycolytic pathways were quantified using targeted proteomics. Briefly, in-gel digestion was performed on 15 μl yeast homogenates using trypsin (sequencing grade modified trypsin V5111; Promega) after reduction with 10 mmol/L dithiothreitol and alkylation with 55 mmol/l iodoacetamide proteins, followed by solid-phase extraction (SPE C18-Aq 50 mg/1 ml, Gracepure) for sample clean-up. Liquid chromatography (LC) on a nano-ultra high-performance liquid chromatography system (Ultimate

UHPLC focused; Dionex) was performed to separate the peptides. The endogenous target peptides were analyzed by a triple quadrupole mass spectrometer (MS) equipped with a nano-electrospray ion source (TSQ Vantage; Thermo Scientific, CA, USA). For the LC-MS measurements, an amount of the digested peptides equivalent to 1 μl yeast lysate starting material was injected together with 1 μg total protein from a ^{15}N-isotopically labelled yeast standards peptide digest mix. For the isotopically labeled standards, used in the mass spectrometry measurements, protein extracts from isotopically labeled ^{15}N YSBN6 yeast cells were used. For the preparation of the ^{15}N standards YSBN6 was cultivated in a 2.5 l fermenter on minimal medium, supplemented with 10 g/L glucose and using ^{15}N-labelled $(NH_4)_2SO_4$ as the sole nitrogen source. Cells were harvested at difference phases of growth, namely during exponential growth, after the diauxic shift and in the late stationary phase. Aliquots from all conditions were mixed to maximize the coverage of all potential present proteins. The LC-MS traces of the targeted peptides were manually curated using Skyline software. The sum of all transition peak areas for the endogenous and standard (^{15}N-labelled) peptide were used to calculate the ratio between the endogenous and standard peptides. The list of targeted peptides are provided in Table S8 (Supporting Information).

In vivo phosphorylation of Hexokinase 2

For detection of phosphorylation of Hxk2 on serine-15 (S15) and serine-158 (S158), proteomic LC-MS measurements were done similar to the above described workflow except that isotopically labeled synthetic peptides (AQUA Ultimate grade with ^{13}C^{15}N-labelling on the C-terminal lysine, Thermo Scientific, CA, USA) were used for detection and quantification of the phosphorylated and non-phosphorylated peptides. From the quantification of both the non-phosphorylated and phosphorylated peptides, the percentage of phosphorylation was calculated.

For S15-quantification an amount of the digested peptides equivalent to 1 μl yeast lysate starting material (as described for the glycolytic protein levels) was injected together with 55 fmol standard peptides. For the peptide covering the sequences GSMADVPK and KGSMADVPK, screening and quantification included potential N-terminal mis-cleavage and methionine oxidation. For quantification of S158, purified protein samples were used, to improve detection of the long and hydrophobic peptide covering the sequence of S158. Detection and quantification was done after in-solution digestion of the purified protein with trypsin followed by clean-up using mixed anion exchange columns (OASIS MAX 1 cc (30 mg), Waters). For the peptide covering this sequence AFIDEQFPQGISEPIPLGFTFSFPASQNK, both the phosphorylated and non-phosphorylated peptide was screened to calculate the percentage phosphorylation.

RESULTS

Hxk2 complementation of evolved D-xylose consuming strains

The quadruple hexokinase deletion mutant *S. cerevisiae* DS71054-evoB strain was previously evolved by evolutionary engineering selecting for improved growth on D-xylose in the presence of increasing D-glucose concentrations (Nijland *et al.* 2014). This resulted in D-glucose-tolerant growth on D-xylose, a phenotype that could be assigned to a mutation at position N367 in the endogenous chimeric Hxt36 transporter causing a defect in D-glucose transport while still allowing uptake of

D-xylose (Nijland *et al.* 2014). This strain was subsequently used as starting point for a second evolutionary engineering aimed at developing a strain that grows on D-xylose in the presence of high D-glucose concentrations at maximum growth rates. Herein, cells were grown aerobically in a turbidostat on 1% D-xylose in the presence of 10% D-glucose. Throughout the evolutionary engineering single colony isolates were obtained after 31, 52 and 85 days and named DS71054-evo3, DS71054-evo4 and DS71054-evo6, respectively (Nijland *et al.* 2019a). This second evolutionary engineering resulted in D-glucose-insensitive growth and consumption of D-xylose, which could be attributed to glucose insensitive D-xylose uptake via a novel chimeric Hxt37 N367I transporter that emerged from a fusion of the HXT36 and HXT7 genes, and the down regulation of the main glucose transporters Hxt1 and Hxt2.

To investigate if the improved D-xylose uptake and consumption would remain also under D-glucose consuming conditions, hexokinase (Hxk2) of *S. cerevisiae* was re-introduced via overexpression on plasmid pRS313-P7T7 in the parental DS71054 strain and the evolved strain lineage. Hxk2 complemented strains were grown anaerobically on minimal medium containing 7% D-glucose and 3% D-xylose. Under these conditions, D-xylose consumption by the DS71054-evo6-Hxk2 strain remained improved as compared to the parental DS71054-Hxk2 strain. Furthermore, in the presence of D-glucose consumption, the same order of improved D-xylose consumption (in g/l) was observed as in the evolutionary engineering experiment, *i.e.* DS71054-Hxk2 < DS71054-evoB-Hxk2 < DS71054-evo3-Hxk2 < DS71054-evo4-Hxk2 < DS71054-evo6-Hxk2 (Fig. S1B, Supporting Information). Remarkably, under co-fermentation conditions, D-glucose consumption (in g/l) reciprocally decreased with the increasing D-xylose consumption (Fig. S1A, Supporting Information) causing an overall decreased growth rate. However, when the sugar consumption rates were corrected for the biomass (in mmol/gDW.hr), a stable total sugar consumption rate was obtained which amounted to 2.8 ± 0.4 mmol/gDW.hr (Fig. 1A). Since the absolute D-xylose consumption rate (in mmol/l.h) was improved in the lineage whereas the D-glucose consumption rate reduced, the absolute total sugar consumption rates (in mmol/l.h) (Fig. 1B) decreased in the lineage. As a control, the same anaerobic fermentation was performed in minimal medium with 7% D-glucose, showing similar D-glucose consumption rates (in mmol/l.h) for all strains (Fig. 1B, open triangles). The total sugar consumption rate of 2.7 ± 0.4 mmol/gDW.hr (Fig. 1A, open triangles) is comparable to the rates obtained on 7% D-glucose and 3% D-xylose. In addition, the rates of ethanol production and sugar consumption were determined for the DS71054-Hxk2 and DS71054-evo6-Hxk2 strains grown anaerobically with 3% D-xylose and 7% D-glucose, 7% D-glucose or 3% D-xylose. The sugar consumption profiles of both strain on singles carbon sources are identical showing that D-glucose or D-xylose consumption *per se* is not impaired or altered (Fig. S2, Supporting Information). The ethanol yields (in g produced ethanol/g consumed sugar) of DS71054-Hxk2 and DS71054-evo6-Hxk2 is significantly higher on D-xylose as sole carbon source (0.401 ± 0.005 g/g and 0.413 ± 0.005 g/g, respectively) as compared to D-glucose as sole carbon source (0.387 ± 0.001 g/g and 0.382 ± 0.003 g/g, respectively). Since DS71054-evo6-Hxk2 consumes relatively more D-xylose and some D-glucose remained after 30 hours of fermentation the ethanol yield of DS71054-evo6-Hxk2 in the medium containing 7% D-glucose and 3% D-xylose is slightly higher (0.408 ± 0.004 g/g) as compared to DS71054-Hxk2 (0.398 ± 0.007 g/g) (Table S4, Supporting Information).
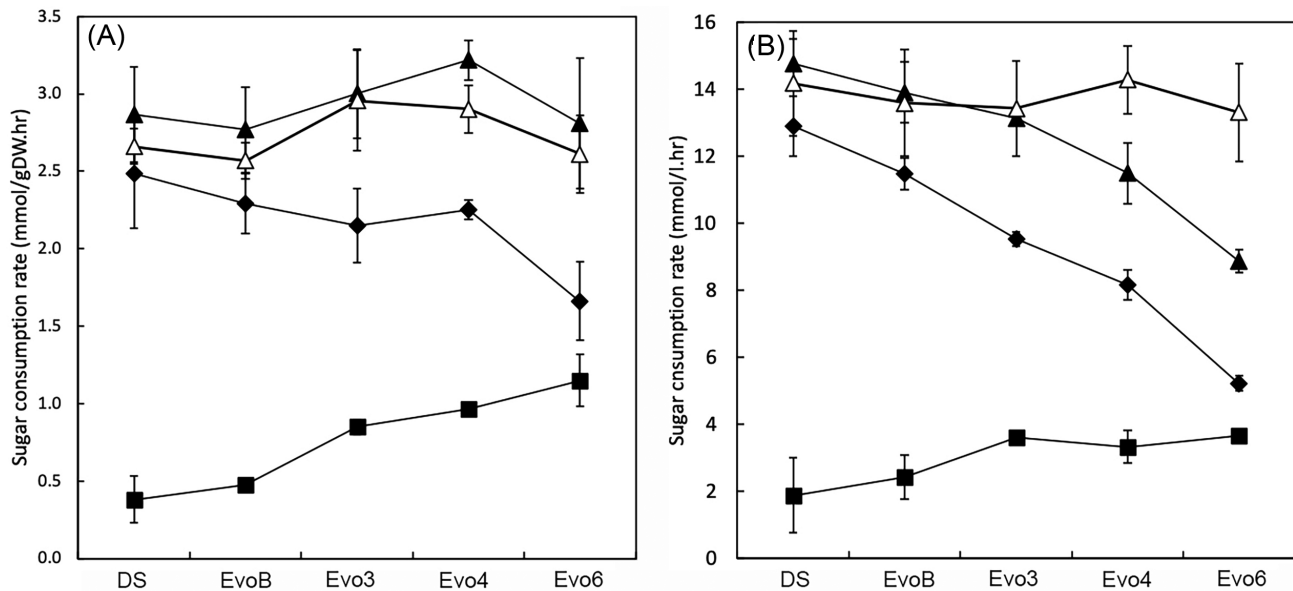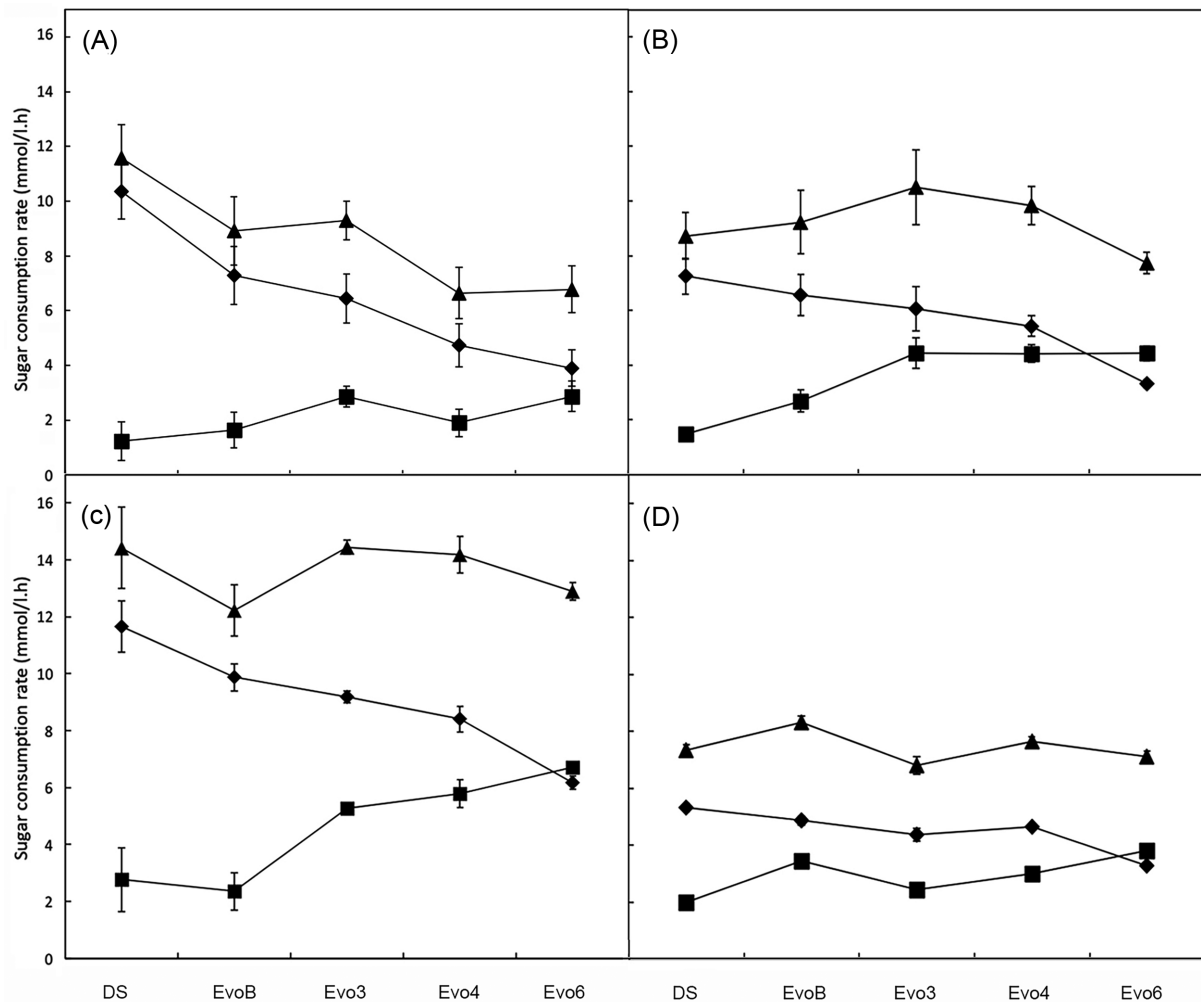
Figure 1. D-glucose (♦), D-xylose (■) and total sugar (▲) consumption rates in mmol/gDW.hr **(A)**, and in mmol/l.hr **(B)**, of the DS71054 hexokinase deletion strain (DS) and the evolved derivatives DS71054-evoB, DS71054-evo3, DS71054-evo4 and DS71054-evo6, all complemented with the *HXK2* gene. Cells were grown anaerobically on minimal medium with 7% D-glucose and 3% D-xylose, or with only 7% D-glucose (△). Error bars were obtained from biological triplicates.

Previously, we observed that the initial D-glucose uptake rate (performed at 380 mM [7%] D-glucose) remained constant in the evolved lineage (Nijland *et al.* 2019a). D-glucose uptake experiments were performed in the presence of D-xylose were performed using 380 mM ^{14}C D-glucose (7%) and 200 mM D-xylose (3%) to mimic the conditions in the growth experiments. Although the variation in measured ^{14}C D-glucose uptake rates in the various strains is large due to the low specific radioactivity of the ^{14}C D-glucose, the overall trend is that D-glucose uptake in the presence of D-xylose increased within the lineage (Fig. S3, Supporting Information). Importantly, this demonstrates that D-glucose transport and consumption in the DS71054-evo6-Hxk2 strain *per se* is not decreased and that the reduced expression of Hxt1 and Hxt2 (and the deletion of Hxt7 due to the new fusion with Hxt36) does not result in lower D-glucose consumption rate when cells are grown on D-glucose only. These data suggests that the reduced D-glucose consumption within the lineage observed during sugar co-fermentation is linked to the improved D-xylose consumption.

Complementation with alternative hexokinases

The reduced D-glucose consumption in the evolved strains in the presence of D-xylose suggests an inhibition of glycolysis. *In vitro* studies have shown that Hxk2 activity is inhibited by D-xylose through an irreversibly inactivation involving an autophosphorylation mechanism (DelaFuente 1970; Fernández *et al.* 1984; Fernandez *et al.* 1986). In the presence of Mg-ATP and D-xylose, a decreased Hxk2 activity was observed (Fernandez *et al.* 1986; Katja Heidrich *et al.* 1997). Furthermore, Hxk2 is inhibited by trehalose-6-phosphate (Blázquez *et al.* 1993). To examine if these phenomena explain the reduced D-glucose consumption rates observed, as a first approach various alternative hexokinases and mutants thereof were expressed in the DS71054 strain and the evolved lineage and growth and sugar consumption were monitored on 7% D-glucose and 3% D-xylose. Complementation of DS71054 with Hxk1 of *S. cerevisiae* yielded

slightly decreased D-glucose consumption rates (in mmol/l.h) as compared to Hxk2 (compare Fig. 2A with Fig. 1), and only a minor improvement in D-glucose consumption was observed in DS71054-evo6-Hxk1 as compared to DS71054-Hxk1 (Fig. 2A). Next, the expression of the glucokinase, Glk1, was tested which is insensitive to trehalose 6-phosphate inhibition (Blázquez *et al.* 1993). This resulted in significantly lower D-glucose consumption rates in DS71054-Glk1 (7.25 ± 0.66 mmol/l/h, Fig. 2B) as compared to the DS71054-Hxk2 strain (12.90 ± 0.90 mmol/l/h, Fig. 1B), a phenomenon that has been reported before (Walsh *et al.* 1991). A similar phenomenon was observed in all strains of the lineage, but with the DS71054-evo6 strain, the reduction of the D-glucose consumption rates was significantly with Glk1 as compared to the Hxk2 complementation (47.1% reduction with Glk1 as compared to 59.5% with Hxk2). *In-vitro* phosphorylation analysis suggested that the Hxk2 mutant (Hxk2-Y), in which phenylalanine at position 159 was mutated to tyrosine, is less sensitive (40%) to D-xylose inhibition (Bergdahl *et al.* 2013). This Hxk2-Y mutant showed a slightly lower glucose consumption rate when expressed in the various strains as compared to Hxk2, but the decline is less with Hxk2-Y (42.9% reduction, Fig. 2C) as compared to Hxk2 (59.5% reduction, Fig. 1B) when cells were grown on D-glucose and D-xylose. The limited effect of the Hxk2-Y mutants could be due to the high D-xylose concentration in the DS71054-evo6 strain as compared to the CEN.PK2–1C strain used by Bergdahl and coworkers which does not have any specific D-xylose transporters and therefore also does not support co-consumption of D-glucose and D-xylose (Bergdahl *et al.* 2013). Next, the proposed trehalose-6-phosphate insensitive hexokinase from *Schizosaccharomyces pombe* (Blázquez *et al.* 1994; Bonini, Van Dijck and Thevelein 2003) was codon optimized and expressed in the DS71054 lineage. Unfortunately, the DS71054 strain expressing *sp*Hxk2 showed a significantly reduced D-glucose consumption rate (∼40%) as compared to Hxk2 (Fig. 2D), which may relate to reduced RNA or protein stability in *S. cerevisiae*. Also with *sp*Hxk2 a decrease in D-glucose consumption rate was noted within the lineage but this could

Figure 2. D-glucose (♦), D-xylose (■) and total sugar consumption rates (in mmol/l.h) (▲) by the parental DS71054 hexokinase deletion strain (DS) and the evolved derivatives DS71054-evoB, DS71054-evo3, DS71054-evo4 and DS71054-evo6 grown anaerobically in minimal medium supplemented with 7% D-glucose and 3% D-xylose. The strains were complemented with Hxk1 **(A)**, Glk1 **(B)**, Hxk2-Y **(C)** (Bergdahl *et al.* 2013) and *sp*Hxk2 **(D)** (Bonini, Van Dijck and Thevelein 2003). Error bars were obtained from biological duplicates.

not be reliably quantified because of the overall reduced performance. Moreover, although *sp*Hxk2 was claimed not to be inhibited by trehalose-6-phosphate, *in vitro* experiments showed that at the highest concentration of trehalose-6-phosphate (3 mM) tested, still some inhibition occurred (Blázquez *et al.* 1993). Overall, the alternative hexokinases and mutants all showed reduced D-glucose consumption rates as compared to Hxk2, both in the parental strain and evolved strains. With Glk1 and Hxk2-Y, the decline in glucose consumption within the lineage was less as compared to Hxk2.

Transcriptomic analysis

To investigate if the decreased D-glucose consumption rate in the evolved lineage under co-consumption conditions is due to alterations in the transcriptome, RNA sequencing was performed of the evolved strains complemented with Hxk2. In order to keep the differences in extracellular D-glucose concentrations to a minimum, the strains were grown anaerobically with 7% D-glucose and 3% D-xylose for only 7 hours. The remaining D-glucose concentration after this time varied between 5.4% and 4.2% in DS71054-evo6-Hxk2 and DS71054-Hxk2, respectively

(data not shown). The 3' mRNA was sequenced in duplicate and the fold changes (FC) were determined using the DS71054-Hxk2 strain as reference. In the DS71054-evo6-Hxk2 strain, 169 genes were at least 3-fold up-regulated (Table S5, Supporting Information) and 31 genes were at least 3-fold down-regulated (Table S6, Supporting Information) in DS71054-evo6-Hxk2 as compared to the DS71054-Hxk2 strain. Three glycolytic proteins show upregulation in all evolved DS71054-Hxk2 strains: the 3-phosphoglycerate kinase Pgk1, triose phosphate isomerase Tpi1, and the alcohol dehydrogenase Adh1. In DS71054-evo6-Hxk2, the fold-change in expression levels were 13.2, 13.1 and 9.9 for Pgk1, Tpi1 and Adh1, respectively. Furthermore, fitting with the diploid phenotype of DS71054-evo6-Hxk2 (Nijland *et al.* 2019a), genes involved in mating (e.g. *MFA1*, *MFα1*) are upregulated in DS71054-evo6-Hxk2. Importantly, in the Hxk2 complemented strain, the Hxt family of genes shows a remarkable down-regulation: Hxt1 (88-fold), Hxt7 (17-fold) and Hxt2 (6.9-fold) in line with previous observations in the DS71054-evo6 strain (Nijland *et al.* 2019a). The apparent down-regulation of Hxt7 in DS71054-evo6-Hxk2 is due to the formation of the chimeric Hxt37 (Nijland *et al.* 2019a). The transcriptional analysis of the evolved lineage was mainly performed to investigate if

the expression of genes involved in D-glucose metabolism were altered which could explain the decreased D-glucose consumption rate. However, none of the genes of glycolysis or the TCA-cycle were down-regulated.

Interestingly, various genes of the trehalose pathway were upregulated in the evolved lineage. In DS71054-evo6-Hxk2, as compared to DS71054-Hxk2, *TPS1*, *TSL1* and *NTH1* were upregulated 3.5, 5.0 and 2.5 times, respectively. Remarkably, like many genes of the glycogen pathway (*e.g. GDB1*, *GPH1*, *SGA1* and *UGP1*) and the oxidative pentose phosphate pathway (*e.g. GND1* and *GND2*), the trehalose pathway genes are solely upregulated in the Hxk2 complemented strain and not in the parental DS71054-evo6 strain (Fig. S4, Supporting Information) although the medium composition (7% D-glucose and 3% D-xylose) and experimental set-up was similar. To investigate if the increased expression of the trehalose pathway genes (and other genes related to glucose-6-phosphate metabolism) is specific for D-glucose and D-xylose co-consumption conditions, strains DS71054-Hxk2 and DS71054-evo6-Hxk2 were grown anaerobically on solely 7% D-glucose or 3% D-xylose for 7 hours and the expression of Tps1, Tps2, Tps3, Tsl1, Pgi1, Zwf1, Pgm1, Pgm2, Sga1 and Ugp1 was analyzed by qPCR. For comparison, the expression of the respective genes in DS71054-Hxk2 grown on 7% D-glucose or 3% D-xylose was set at 1.0. Similar to the data obtained in the transcriptomic analysis, Tps1 and Tsl1 were upregulated 2.56 ± 0.4 times and 2.44 ± 0.65 times in DS71054-evo6-Hxk2 as compared to DS71054-Hxk2 when grown with 7% D-glucose and 3% D-xylose. However in medium containing solely 7% D-glucose or 3% D-xylose, no upregulation of these genes was observed in the DS71054-evo6-Hxk2 strain (Fig. S5, Supporting Information). Sga1, involved in the conversion of $(glycogen)_n$ to D-glucose, was upregulated 3.52 ± 0.64 times in the DS71054-evo6-Hxk2 strain grown on 7% D-glucose and 3% D-xylose. A similar level of upregulation was observed when cells were grown on 7% D-glucose, i.e. 2.59 ± 0.13, and thus this phenomenon is not specific for the co-consumption conditions. Of all other genes (*TPS2*, *TPS3*, *PGI1*, *ZWF1*, *PGM1*, *PGM2* and *UGP1*) no increased in expression was observed in DS71054-evo6-Hxk2 grown on any of the carbon sources (Fig. S5, Supporting Information). The data shows that the upregulation of genes belonging to the trehalose pathway is restricted to the DS71054-evo6 strain complemented with Hxk2 and observed only under co-consumption conditions. This implies that the upregulation of the trehalose pathway is not the result of the initial evolutionary engineering experiment, and suggests that it is due to a metabolic response unique for the improved co-metabolism conditions.

Proteomics and phosphorylation of Hxk2

The reduced rate of D-glucose consumption in the co-consumption experiments employing the evolved strain restored with Hxk2 may indicate limitations of the glycolytic pathway at the level of hexokinase. There are two known phosphorylation sites on Hxk2: serine 15 and serine 158. Phosphorylation at serine 15 causes a conformational change which affects dimerization (Kettner *et al.* 2012), influences its regulatory function (Vojtek and Fraenkel 1990) and regulates its nucleocytoplasmic shuttling (Fernández-García *et al.* 2012). Auto-phosphorylation of the serine at residue 158 has been observed only *in vitro* in the presence of D-xylose (Fuente, Lagunas and Sols 1970; Menezes and Pudles 1976; Vojtek and Fraenkel 1990) and causes a reversible inactivation of the enzyme which is also linked to a change in the ATP/ADP ratio (DelaFuente 1970;

Menezes and Pudles 1977; Salusjärvi *et al.* 2008; Souto-Maior, Runquist and Hahn-Hägerdal 2009). Inactivation of Hxk2 has also been observed in yeast incubated with xylose and in genetically engineered strains that can metabolize xylose, and this results in a reduced Crabtree effect (Menezes and Pudles 1977; Fernández *et al.* 1984; Schuddemat, van den Broek and van Steveninck 1986; Salusjärvi *et al.* 2008; Souto-Maior, Runquist and Hahn-Hägerdal 2009). Both phosphorylation sites on Hxk2 were previously detected by MALDI-TOF analysis and verified by peptide sequencing (Kriegel *et al.* 1994; Katja Heidrich *et al.* 1997). To examine the phosphorylation levels, we used LC-MS to separate and quantify peptide fragments which harbor the S15 and S158 residues. Cell free extracts were obtained from DS71054-Hxk2 and the evolved lineage strains after 16 h of growth on 7% D-glucose and 3% D-xylose. Although DS71054-evoB-Hxk2 showed a 3-fold increase in phosphorylation of S15, no significant altered phosphorylation levels were observed for S15 and S158 in DS71054-evo6-Hxk2 (~10%–13%; Fig. S6, Supporting Information) as compared to DS71054-Hxk2. These data suggest that inactivation of Hxk2 by phosphorylation is not a major cause of the reduced rates of D-glucose metabolism under co-consumption conditions.

Proteomic analysis of the major glycolytic proteins was performed using the same cell free extracts as described above. Among the highest upregulated proteins in DS71054-evo6-Hxk2, compared to DS71054-Hxk2, are the enzymes involved in the glycogen pathway, in which *e.g.* Gph1, Glc3 and Gdb1 were upregulated 3.9 ± 0.5, 3.1 ± 0.2 and 2.8 ± 0.9 times, respectively. A similar fold-change of these proteins was observed in all strains of the evolved lineage (Table S7, Supporting Information), and these data align well with the transcriptomic data (e.g. *GPH1*, *GLC3* and *GDB1* were upregulated 5.4 ± 0.2, 2.0 ± 0.5 and 6.5 ± 1.2 times, respectively). However, many other proteins whose genes are significant upregulation at the transcriptional level do not show a similar behavior at the protein level in line with previous observations (Ideker *et al.* 2001; Griffin *et al.* 2002). Interestingly, as in the transcriptomic data, the proteomics data indicate that in the evolved strains, the levels of the trehalose pathway enzymes Tps1, Tps2 and Tps3 (Tsl1 was not analyzed) increased about 2-fold (Table S7, Supporting Information). Furthermore, a small decrease in the level of Hxk2 is noted in the DS71054-evo6-Hxk2 strain, but there is no clear trend in the lineage.

Intracellular metabolites and trehalose-6-phosphate accumulation

Since several of the trehalose pathway genes are upregulated in the evolved strains when grown on both D-glucose and D-xylose, the intra- and extracellular concentrations of glucose-6-phosphate, trehalose and trehalose-6-phosphate were determined. In addition, ATP and NAD(H) levels were determined. All intracellular metabolites were isolated from cells grown for up to 12 hours in minimal medium containing 7% D-glucose and 3% D-xylose. A minor elevation of ATP levels was observed in DS71054-evo6-Hxk2 as compared to DS71054-Hxk2 increasing 1.9 ± 0.2 and 1.6 ± 0.3 fold after 2 and 4 hours, respectively (data not shown). The NAD level was 1.7 ± 0.3 fold increased in DS71054-evo6-Hxk2 compared to DS71054-Hxk2 whereas the NADH/NAD ratio increased almost 2 times in DS71054-evo6-Hxk2. However, the NADH/NAD ratios were not consistently altered in the other evolved DS71054-Hxk2 strains (data not shown). Also, no difference in intracellular glucose-6-phosphate concentration was observed in DS71054-evo6-Hxk2 as compared to DS71054-Hxk2

(data not shown). However, both the extracellular and intracellular trehalose concentration was increased in DS71054-evo6-Hxk2 compared to the DS71054-Hxk2 strain and also, albeit to a lesser extent, in all evolved DS71054-Hxk2 strains (Figs S7A and B, Supporting Information). The increase in trehalose could be the result of an increased trehalose-6-phosphate concentration which inhibits the hexokinase (Blázquez *et al.* 1993). Therefore, LC-MS was used to analyze the trehalose-6-phosphate concentration in the strains. The amount of trehalose-6-phosphate was normalized using the total ion count (TIC) of the isolated intracellular content. DS71054-evo6-Hxk2 showed significantly increased levels of trehalose-6-phosphate (977 ± 80 ppm) compared to DS71054-Hxk2 (15.6 ± 0.6 ppm), and the other evolved strains showed a similar trend (Fig. S8, Supporting Information). These data agree with the transcriptome and proteomic analysis which show an up-regulation of the *TPS1*, *TSL1* and *NTH1* genes of the trehalose pathway. Thus, the expression of Hxk2 under conditions of co-consumption of D-glucose and D-xylose causes an upregulation of the trehalose biosynthetic genes yielding elevated levels of trehalose-6-phosphate. Since the latter is an inhibitor of hexokinase, accumulation of trehalose-6-phosphate may explain the reduced D-glucose consumption under co-metabolism conditions. Importantly, the DS71054-evo6-Hxk2 strain grown anaerobic on solely 7% D-glucose showed similarly low levels of trehalose-6-phosphate as compared to DS71054-Hxk2. Trehalose-6-phosphate accumulated slightly in the DS71054-evo6-Hxk2 strain when grown on solely 3% D-xylose as compared to 7% D-glucose only (Fig. 4, white bars), but these levels were significantly lower as observed with 7% D-glucose and 3% D-xylose in the growth medium. Thus, the increased levels of trehalose-6-phosphate in the lineage of evolved strains is limited to the sugar co-consumption conditions and only observed when glucose consumption is restored by the re-introduction of Hxk2.

Genetic inactivation of the trehalose pathway

Since the intracellular trehalose-6-phosphate levels were elevated in the Hxk2-complemented evolved lineage (Fig. 4; Fig. S8, Supporting Information), the trehalose pathway was targeted for deletions in DS71054-evo6-Hxk2. Here, the DS71054-evo6 strain was used to decrease the variation in growth due to the flocculation. This strain shows the same D-glucose and D-xylose consumption rates compared to DS71054-evo6 (Nijland *et al.* 2019a). Comparable to other studies (Bell *et al.* 1992; Bonini, Van Dijck and Thevelein 2003; Jules *et al.* 2008), deletion of Tps1, the trehalose-6-phosphate synthase, yielded strains unable to grow on D-glucose, most likely due to substrate accelerated death in which D-glucose is rapidly converted to glucose-6-phosphate causing an instant lowering of the ATP levels below a certain threshold. Deletion of Tps2, the trehalose phosphatase, has been reported to result in a temperature sensitivity phenotype and a loss of trehalose-6-phosphate phosphatase activity (De Virgilio *et al.* 1993). The deletion of the *TPS2* gene in DS71054-evo6-Hxk2, however, yielded unaltered D-xylose consumption rates, and D-glucose consumption was still decreased as compared to the parental DS71054-Hxk2 strain (data not shown). In this strain, however, trehalose-6-phosphate can still be formed. Next to Tps1 and Tps2, the trehalose enzymatic complex has two partially redundant subunits, Tps3 and Tsl1, that fulfil a structural and/or regulatory role (Bell *et al.* 1992; Reinders *et al.* 1997; Trevisol *et al.* 2014). Although there is no direct interaction between Tps3 and Tsl1, both enzymes interact with Tps1 and Tps2 (Trevisol *et al.* 2014). Whereas in the strain in which *TPS1* and *TPS2*

were deleted, D-glucose consumption was still reduced in the presence of D-xylose, deletion of the *TSL1* gene resulted in significantly improved D-glucose consumption rates and improved growth on minimal medium containing 7% D-glucose and 3% D-xylose. Furthermore, the DS71054-evo6-ΔTsl1-Hxk2 strain also showed an increased D-xylose consumption rate (in mmol/l.hr; Fig. 3). Deletion of *TPS3* also caused improved D-glucose consumption but not as pronounced as the deletion of *TSL1* (Fig. 3). Whereas the single deletions of *TSL1* and *TPS3* increased the D-glucose consumption rate, the double deletion of *TPS3* and *TSL1* caused a marked decrease in the consumption rate of both D-glucose and D-xylose (data not shown). In contrast to DS71054-evo6-Hxk2, the individual *TSL1* and *TPS3* deletions in the parental DS71054-Hxk2 strain showed unaltered consumption rates for D-glucose and D-xylose (data not shown). The improved D-glucose consumption of the *TSL1* and *TPS3* deletions in DS71054-evo6-Hxk2 did not alter the ethanol yield (in g ethanol produced/g sugar consumed) on minimal medium containing 7% D-glucose and 3% D-xylose, solely 7% D-glucose or solely 3% D-xylose (Table S4, Supporting Information). Also the growth rates and sugar consumption rates on single sugar fermentations of the *TSL1* and *TPS3* deletions were unaltered as compared to the DS71054-evo6-Hxk2 and DS71054-Hxk2 strains.

To investigate if the increased D-glucose consumption rates in DS71054-evo6-ΔTps3-Hxk2 and DS71054-evo6-ΔTsl1-Hxk2 is due to reduced trehalose-6-phosphate levels, the intracellular metabolites of all strains were isolated after 12 hours of anaerobic growth in minimal medium containing 7% D-glucose and 3% D-xylose. Indeed, a marked reduction in the accumulation of trehalose-6-phosphate was observed in the strains with a deletion of *TPS3* and *TSL1*. In contrast, the trehalose-6-phosphate level increased 57- and 53-fold in DS71054-evo6-Hxk2 and DS71054-evo6-Hxk2, respectively (Fig. 4).

These data strongly suggest that the accumulation of trehalose-6-phosphate in cells grown on both D-glucose and D-xylose is at least one of the factors causing the reduction of D-glucose consumption in evolved strains that have been complemented with Hxk2.

Acetate formation

We noted that the acetic acid production in the DS71054-evo6-ΔTsl1-Hxk2 strain during mixed sugar metabolism was significantly decreased as compared to the parental DS71054-Hxk2 strain. Interestingly, already in DS71054-evo6-Hxk2, reduced acetic acid production occurred (Fig. S9, Supporting Information). The decreased acetic acid production could result from glucose-6-phosphate accumulation in the evolved strains, even though increased levels of this metabolite were not detected. The glucose-6-phosphate can be redirected into the oxidative pentose phosphate pathway in which it is converted into ribulose-5-phosphate that subsequently enters the pentose phosphate pathway. In these conversions, NADPH is produced and we speculate that the production of NADPH via the acetaldehyde dehydrogenase Ald6, which converts acetaldehyde into acetic acid, is no longer needed for a proper redox balance. The expression levels of some of the genes involved in the oxidative pentose phosphate pathway (*e.g. GND1* and *GND2*) show a minor increase in DS71054-evo6-Hxk2 grown in minimal medium with 7% D-glucose and 3% D-xylose relative to the DS71054-Hxk2 strain (Fig. S4, Supporting Information). Increased expression was confirmed in the proteomics analysis showing a fold change in DS71054-evo6-Hxk2 of 1.7 ± 0.1 and 2.4 ± 0.9 for Gnd1 and Gnd2, respectively (Table S6, Supporting Information) and was
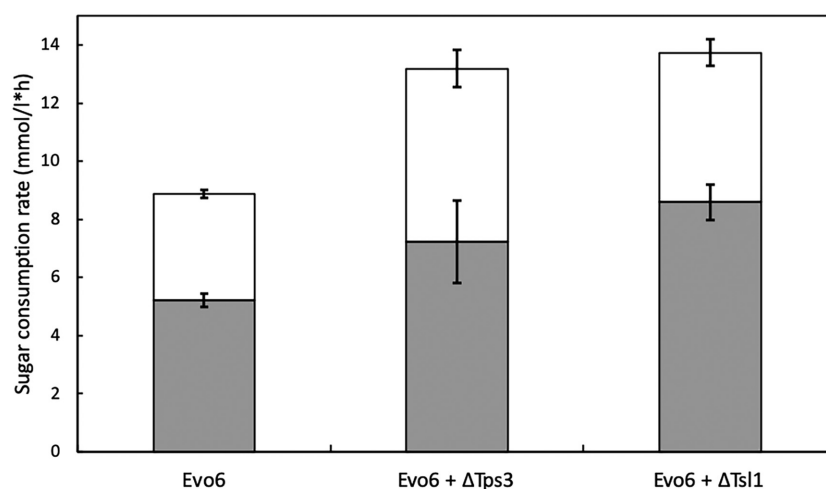
Figure 3. D-glucose (grey bars) and D-xylose (white bars) consumption rates (in mmol/l.hr) by DS71054-evo6, DS71054-evo6-∆Tps3 and DS71054-evo6-∆Tsl1 complemented with the *HXK2* gene. Cells were grown anaerobically on 7% D-glucose and 3% D-xylose. Error bars were obtained from biological duplicates.
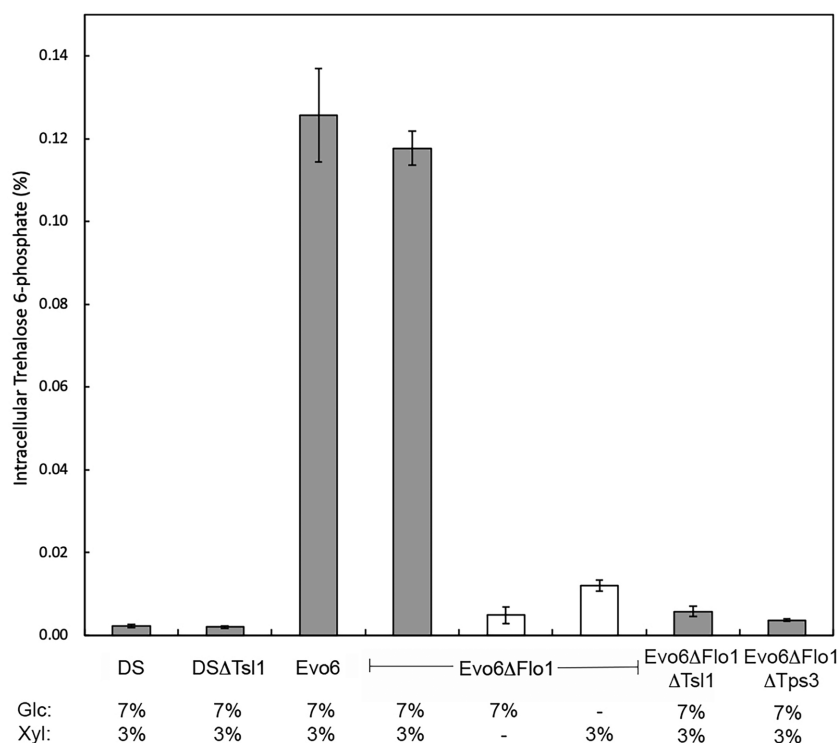
Figure 4. Intracellular trehalose-6-phosphate in the DS71054 hexokinase deletion strain (DS), the evolved derivative DS71054-evo6, and indicated deletion mutants of the enzymes of the trehalose pathway, all complemented with the *HXK2* gene. The strains were grown anaerobically with 7% D-glucose and 3% D-xylose (grey bars), 7% D-glucose (white bar) or 3% D-xylose (white bar). The intracellular concentration was measured after 12 h and signal normalized for the total ion count. Error bars were obtained from biological duplicates.

not observed in the DS71054-evo6 strain that is unable to consume D-glucose. The expression of *ALD6* remains unaltered in DS71054-evo6-Hxk2 as compared to DS71054-Hxk2 (data not shown).

DISCUSSION

In the development of yeast for second generation ethanol production there is a continued need for high performance sugar metabolizing strains. Major issues in pentose transport, in particular D-xylose uptake have been solved through genetic engineering of the endogenous Hxt transport landscape (Farwick *et al.* 2014; Nijland *et al.* 2014; Shin *et al.* 2015; Verhoeven *et al.* 2018a). However, the introduced mutations in Hxt transporters not only abolishes D-glucose transport, but also partially reduce the D-xylose transport rate, whereas high rates are required for efficient D-xylose utilization. In a previous study to elevate the D-xylose transport rate, we have conduced evolutionary engineering of the hexokinase deletion strain DS71054-evoB that contains the N367I mutation in the chimeric Hxt36 transporter (Nijland *et al.* 2014). More stringent conditions were imposed to allow these cells to grow with high rates on D-xylose

in the presence of excess D-glucose. This yielded the DS71054-evo6 strain which shows high growth rates on D-xylose in the presence of a 10-fold concentration of D-glucose. These growth rates are nearly identical to the growth rate on D-xylose only. Transcriptome and genome analysis demonstrated that in this strain the transporter landscape has been altered dramatically. First, Hxt36 was converted into Hxt37 N367I, resulting in a loss of the Hxt7 transporter. Second, due to mutations in Pbs2 in DS71054-evo4 and DS71054-evo6, the expression of Hxt1 and Hxt2 is dramatically reduced (Nijland *et al.* 2019a). In this strain, Hxt37 N367I supports D-xylose uptake, whereas the remaining hexose transporters Hxt4 and Hxt5 still allow for unaltered initial D-glucose uptake rates.

Here, we restored D-glucose metabolism in the lineage of evolved D-xylose metabolizing DS71054 strains by re-introduction of the hexokinase Hxk2. Importantly, this allowed all strains of the evolved lineage to grow and consume D-glucose with identical rates (Fig. 1, Fig. 2, Supporting Information) even though with the lineage the Hxt landscape was progressively reduced. In this respect, it should be noted that the expression of a single Hxt transporter is sufficient to sustain maximal growth rates on D-glucose (Reifenberger, Freidel and Ciriacy 1995; Reifenberger, Boles and Ciriacy 1997; Diderich *et al.* 1999). Nevertheless, intracellular D-glucose concentrations might be significantly decreased due to the reduced Hxt transporter landscape. Remarkably, the D-glucose consumption rates decreased progressively within the lineage when cells were grown on both D-xylose and D-glucose. This decrease in D-glucose consumption is only evident under conditions of co-consumption of D-glucose with D-xylose, and not observed when cells are grown on D-glucose only (Fig. S2, Supporting Information) indicative of a functional link with the improved D-xylose metabolism. Importantly, the inhibition is not due to a transport deficiency as the initial D-glucose transport rates in the evolved strains was not affected by the presence of D-xylose (Nijland *et al.* 2019b). Also, transcriptome and proteomic data of cells grown on D-glucose and D-xylose, showed no major down-regulation of genes/proteins involved in glycolysis or the TCA-cycle. Moreover, in the DS71054-evo6-Hxk2 strain, the total sugar consumption rate corrected for the biomass (in mmol/gDW.h) remained unaltered (Fig. 1A) as compared to the parental DS71054-Hxk2 strain, but the overall sugar flux improved dramatically in favor of D-xylose consumption. In the co-fermentation, the collective sugar consumption rate (in mmol/l.h) decreased and this is paralleled with a decreased growth rate as expected when D-xylose consumption becomes a more dominant factor.

A major question is how improved D-xylose consumption can lead to a concomitant decrease in the rate of D-glucose consumption. In the D-xylose consuming strains, D-xylose enters glycolysis via the overexpressed pentose phosphate pathway as fructose-6-phosphate and glyceraldehyde-3-phosphate in a ratio of 2:1. Thus, the metabolites arising from the improved D-xylose consumption in the DS71054-evo6-Hxk2 strain at the level of the aforementioned metabolites will collide with the regular D-glucose flux in the glycolytic pathway. Fructose-6-phosphate is further metabolized by the hetero-octameric phosphofructokinase (Pfk). Studies on the regulation of glycolytic genes indicate that glucose induces *PFK1* and *PFK2* mRNA synthesis (Moore *et al.* 1991). Our proteomic studies suggests that the levels of both enzymes, and any of the other glycolytic enzymes, are similar in the parental and evolved strain when grown on the mixture of D-glucose and D-xylose. Thus the increased rate of D-xylose consumption seems not to be accompanied by an overall increased performance of the glycolytic
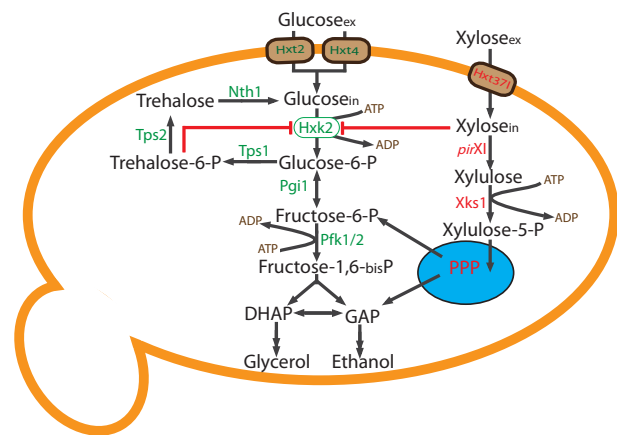
Figure 5. Schematic view of the DS71054-evo6 strain co-consuming D-glucose and D-xylose. D-glucose is transported into the cell via Hxt2 and Hxt4. Hxt37 N367I transports solely D-xylose. The metabolism of D-glucose is mediated via the enzymes hexokinase (Hxk2) and glucose-6-phosphate isomerase (Pgi1) to yield fructose-6-phosphate, which is further converted in the glycolytic pathway into ethanol. Accumulation of glucose-6-phosphate leads to accumulation of trehalose-6-phosphate which inhibits Hxk2 therefore decreasing the glycolysis rate. Furthermore, in *in vitro* studies, Hxk2 can also be inhibited by accumulation of D-xylose in the presence of MgATP, but in this study an expected increase in the phosphorylation of Hxk2 was not observed. The conversion of D-xylose into xylulose is in *S. cerevisiae* possible via the introduction of xylose isomerase (*pir*XI), followed by the phosphorylation of xylulose into xylulose-5-phosphate by xylulose kinase (Xks1). Xylulose-5-phosphate enters the pentose phosphate pathway to eventually yield fructose-6-phosphate and glyceraldehyde-3-phosphate (GAP) that can be converted into ethanol.

pathway, creating a bottleneck for an increase in the overall sugar consumption rate. Fructose-6-phosphate can also be converted into glucose-6-phosphate via the bidirectional phosphoglucose isomerase (Pgi1) (Fig. 5), which is expressed at increased levels in the evolved strain when cells are grown on glucose and xylose (Fig. S4, Supporting Information). The glucose 6-phosphate can subsequently be converted into trehalose-6-phosphate and trehalose. Importantly, this is only observed in the cells that contain Hxk2 and that are grown on D-glucose and D-xylose demonstrating that besides D-xylose metabolism, D-glucose metabolism plays a pivotal role in the formation of trehalose-6-phosphate. Trehalose is either secreted or re-converted into D-glucose. The latter reaction is catalyzed by Nth1, and also this enzyme is upregulated in the evolved strain but only when the cells express Hxk2 (Fig. S4, Supporting Information). Overall, this leads to a futile cycle in which ATP is consumed, and the glucose is recycled instead of consumed. Interestingly, accumulation of trehalose has been observed before under conditions of reduced D-glucose consumption causing an accumulation of hexose phosphates upstream of the phosphofructokinase (Pfk) reaction (Bosch *et al.* 2008). Mathematical modelling showed that in these strains, the effects may be explained by changes in the kinetics of phosphofructokinase and phosphoglucose isomerase. The same principle may be the underlying cause of the observed re-distribution of D-xylose and D-glucose metabolic flux observed in this study as Pgi1 is upregulated. The evolved DS71054-evo6 strain used shows the highest D-xylose consumption rates in the presence of D-glucose reported to date. Therefore, the proposed collision of pathways in the DS71054-evo6 strain may not have been observed before.

The accumulation of trehalose-6-phosphate is likely a consequence of the aforementioned limitation in the overall performance of glycolysis. The exclusive accumulation of

intracellular trehalose-6-phosphate in the DS71054-Hxk2 lineage when grown on mixed sugar fits well with the transcriptomics data showing the upregulation of Tps1 and the regulatory subunit Tsl1 of the trehalose biosynthetic pathway. Trehalose-6-phosphate is an inhibitor of Hxk2 (Blázquez *et al.* 1993; Thevelein and Hohmann 1995), and thus accumulation of this metabolite may result in inhibition of glucose metabolism in the evolved strains further limiting D-glucose metabolism. Indeed, accumulation of trehalose-6-phosphate is observed in the entire lineage of evolved DS71054-Hxk2 strains but is 53.5 times increased in DS71054-evo6-Hxk2 as compared to the parental DS71054-Hxk2 strain. Importantly, when DS71054-evo6-Hxk2 was grown on solely D-glucose or D-xylose no major accumulation of trehalose-6-phosphate was observed, nor did trehalose-6-phosphate accumulate in the parental DS71054-Hxk2 strain under any given condition. It is important to note that the accumulation of trehalose-6-phosphate is not a direct result of the evolutionary engineering that screened for improved D-xylose metabolism in presence of high concentrations of D-glucose in a strain defective in D-glucose metabolism. Rather, it is linked to the restoration of D-glucose metabolism by the re-introduction of Hxk2. Elevated levels of trehalose 6-phosphate and trehalose, as well as the upregulation of the involved genes in the trehalose pathway (specifically *TPS1* and *TSL1*) are observed only upon sugar co-consumption in the Hxk2 complemented strains. For instance, in the absence of Hxk2 (Fig. S4, Supporting Information) or during fermentations with D-glucose or D-xylose alone (Fig. S5, Supporting Information), no upregulation was observed. To prevent accumulation of trehalose-6-phosphate in the evolved strains, *TPS1*, *TPS2*, *TPS3* and *TSL1* were individually deleted in DS71054-evo6-Hxk2. Deletion of *TPS1* led to a growth defect on D-glucose in agreement with earlier reports (Bell *et al.* 1992), likely because of substrate accelerated death where all ATP is consumed to produce glucose-6-phosphate from glucose. Deletion of *TPS2* led to a severe growth defect as shown before (De Virgilio *et al.* 1993; Bell *et al.* 1998). However, deletion of *TSL1*, and to a lesser extent *TPS3*, in DS71054-evo6-Hxk2 resulted in improved D-glucose consumption rates when cells are grown D-glucose and D-xylose (Fig. 3). Due to improved biomass formation under those conditions, also the D-xylose consumption rate improved. In these two deletion strains, levels of trehalose-6-phosphate were as expected reduced (Fig. 4). We hypothesize that, in the *TSL1* and *TPS3* deletion strains, glucose-6-phosphate can no longer be converted into trehalose-6-phosphate and instead enters the oxidative part of the pentose phosphate pathway in which NADPH is produced. This may also explain the reduced levels of acetic acid production in DS71054-evo6-ΔTsl1-Hxk2 and DS71054-evo6-Hxk2 (Fig. S9, Supporting Information) as NADPH formation via acetic acid from acetaldehyde is no longer required to maintain the redox balance.

Another hypothesis for the decreased D-glucose consumption in the evolved DS71054 lineage could be direct inhibition of Hxk2 by D-xylose. *In vitro* studies have shown an irreversible inactivation of Hxk2 activity via protein phosphorylation in the presence of D-xylose and MgATP (DelaFuente 1970; Fernandez *et al.* 1986). Increased D-xylose transport in the evolved strains, and thus potentially higher levels of intracellular D-xylose may directly affect D-glucose consumption by the aforementioned mechanism. According to this hypothesis, inhibition of Hxk2 by D-xylose should result in auto-phosphorylation of serine 15. Although some phosphorylation of Ser15 could be detected in one of the strains, there was no clear trend within the lineage (Fig. S6, Supporting Information). The introduction of a mutant of Hxk2, i.e. Hxk2-Y, which is less sensitive to D-xylose slightly improved D-glucose consumption in the evolved lineage relative to the parental strain, but the overall trend of improved D-xylose consumption at the expense of D-glucose consumption remained. This suggests that in the evolved strains, inhibition of Hxk2 by D-xylose plays only a minor role. The replacement of Hxk2 for Glk1, which is insensitive to trehalose-6-phosphate inhibition slightly impacted the aforementioned trend. Since with Glk1 lower glucose consumption rates were observed as compared to Hxk2, the use of glucokinase did not improve overall sugar consumption.

A further hypothesis relevant to co-metabolizing conditions it that D-glucose inhibits the xylose isomerase (XI) or other pentose phosphate pathway enzymes. To test this hypothesis we over-expressed Hxt1 on the pRS313-P7T7 plasmid in the DS71054-evo6-Hxk2 strain and compared the sugar consumption with the DS71054-evo6-Hxk2 strain grown on a medium containing 7% D-glucose and 3% D-xylose. The Hxt1 overexpression increased the D-glucose consumption while D-xylose consumption decreased, resulting in increased growth rates (Fig. S10, Supporting Information). This suggests that D-glucose inhibits D-xylose consumption, as previously proposed in a study where D-xylose metabolism was inhibited by maltose in a co-fermentation experiment (Subtil and Boles 2012). Since maltose is transported via the Mal transporters (Lagunas 1993), inhibition of D-glucose at the transport level can be excluded. Rather the inhibitory effect was explained by the accumulation of intracellular D-glucose, although the mechanism was not resolved. In general, xylose isomerases are also known to act as *bona fide* glucose isomerases converting D-glucose into D-fructose (Bhosale, Rao and Deshpande 1996). Thus intracellular D-glucose might compete with D-xylose at the level of xylose isomerase. However, *in vitro*, the D-xylose isomerase activity of XI was decreased only by $8.2 \pm 0.2\%$ by an 6-fold excess of D-glucose (Lee M *et al.*, personal communications), and thus inhibition of xylose isomerase activity by D-glucose seem not a likely possibility.

Summarizing, through the re-introduction of the hexokinase Hxk2, we could restore D-glucose consumption in evolved strains of *S. cerevisiae* that exhibit high performance D-glucose insensitive growth on D-xylose. Within the lineage of evolved strains, metabolism of D-glucose was gradually reduced concomitantly with increased D-xylose consumption, while the overall sugar conversion rate normalized on biomass remains relatively constant. The observed redistribution of the sugar flux into the glycolytic pathway involves the D-xylose dependent inhibition of D-glucose metabolism likely through accumulation of trehalose-6-phosphate which inhibits the Hxk2 activity and thereby limits D-glucose metabolism. Interfering with trehalose-6-phosphate accumulation by disrupting the function of the trehalose synthase complex partly alleviated this bottleneck. However, for higher performance in D-glucose and D-xylose co-metabolism, intrinsic limitations in the glycolytic pathway need to be resolved.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

AUTHOR CONTRIBUTIONS

JN, HS, PW and AD conceived and designed the research; JN, ED and RD performed the experiments; PW constructed the strains; PW and AD supervised the project; the manuscript was written by the contributions of JN and AD.

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