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Crabtree/Warburg-like aerobic xylose fermentation by engineered *Saccharomyces cerevisiae*

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ABSTRACT

Bottlenecks in the efficient conversion of xylose into cost-effective biofuels have limited the widespread use of plant lignocellulose as a renewable feedstock. The yeast *Saccharomyces cerevisiae* ferments glucose into ethanol with such high metabolic flux that it ferments high concentrations of glucose aerobically, a trait called the Crabtree/Warburg Effect. In contrast to glucose, most engineered *S. cerevisiae* strains do not ferment xylose at economically viable rates and yields, and they require respiration to achieve sufficient xylose metabolic flux and energy return for growth aerobically. Here, we evolved respiration-deficient *S. cerevisiae* strains that can grow on and ferment xylose to ethanol aerobically, a trait analogous to the Crabtree/Warburg Effect for glucose. Through genome sequence comparisons and directed engineering, we determined that duplications of genes encoding engineered xylose metabolism enzymes, as well as *TKL1*, a gene encoding a transketolase in the pentose phosphate pathway, were the causative genetic changes for the evolved phenotype. Reengineered duplications of these enzymes, in combination with deletion mutations in *HOG1*, *ISU1*, *GRE3*, and *IRA2*, increased the rates of aerobic and anaerobic xylose fermentation. Importantly, we found that these genetic modifications function in another genetic background and increase the rate and yield of xylose-to-ethanol conversion in industrially relevant switchgrass hydrolysate, indicating that these specific genetic modifications may enable the sustainable production of industrial biofuels from yeast. We propose a model for how key regulatory mutations prime yeast for aerobic xylose fermentation by lowering the threshold for overflow metabolism, allowing mutations to increase xylose flux and to redirect it into fermentation products.

1. Introduction

Over the past few decades, lignocellulosic biomass derived from non-edible plants, such as switchgrass, have been developed as potentially sustainable feedstocks for the production of renewable biofuels, which can replace petroleum-based fuels (Narayanaswamy et al., 2011; Sun and Jin, 2021; Williams et al., 2019). Most lignocellulosic feedstocks require thermo-chemical pretreatment and enzymatic hydrolysis to break down cellulose and hemicellulose in plant cell walls into glucose and xylose, which are the dominant hexoses and pentoses, respectively (Pauly and Keegstra, 2008). However, native *Saccharomyces cerevisiae*, the main fermentative microbe used by the bioethanol industry, does not consume or ferment xylose, resulting in inefficient cellulosic biofuel production whose cost is not competitive with fossil fuels (Cunha et al., 2019; Kim et al., 2013).

To overcome the economic issues impeding the commercialization of lignocellulosic-based biofuels, genetic engineering and adaptive laboratory evolution (ALE) of *S. cerevisiae* have been widely used strategies for increasing the rates of xylose fermentation into ethanol, isobutanol, lactic acid, and other useful bioproducts (Brat and Boles, 2013; Reyes

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et al., 2014; Sandberg et al., 2019; Turner et al., 2015). Enabling xylose utilization by *S. cerevisiae* has been achieved by the introduction of xylose metabolism enzymes from other fungi or bacteria. Xylose reductase and xylitol dehydrogenase (XR-XDH) (Jin et al., 2000; Johansson et al., 2001) or xylose isomerase (XI) (Brat et al., 2009; Kuyper et al., 2005) enzymes convert xylose into xylulose. Both pathways require xylulokinase (XK) to phosphorylate xylulose into xylulose-5-phosphate, which can then be converted into ethanol via the pentose phosphate pathway and glycolysis (Hahn-Hagerdal et al., 2007). Individual insertions of these pathways into *S. cerevisiae* have not been sufficient for xylose-to-ethanol conversion at industrially relevant rates and yields in lignocellulosic hydrolysates, prompting many to explore additional genetic improvements. Since our work described here will focus on the XI pathway, we refer readers to more comprehensive reviews on genetic discoveries that enhance xylose consumption by the XR-XDH pathway (Kwak and Jin, 2017; Sun and Jin, 2021).

For the XI pathway, several groups have employed combinations of rational engineering and ALE to uncover genetic modifications that increase xylose consumption and conversion into ethanol. Mutations in *GRE3* (Lee et al., 2012, 2014; Sato et al., 2016; Traff et al., 2001), which encodes an aldose reductase capable of converting xylose into xylitol, an inhibitor of XI (Yamanaka, 1969); *PHO13* (Bamba et al., 2016; Lee et al., 2014); and *HXT7* (Reider Apel et al., 2016) were discovered by rational engineering and ALE to improve xylose consumption. Increased expression of XI by targeted or evolved integration of multiple XI copies into the genome has also been found to enhance xylose consumption (Dos Santos et al., 2016; Jeong et al., 2020). Finally, genome sequencing of strains from ALE identified synergistic interactions between loss-of-function mutations in *HOG1*, *ISU1*, and *IRA2* genes that enhance xylose consumption by *S. cerevisiae* (Dos Santos et al., 2016; Sato et al., 2016). These genetic changes impacted various metabolic pathways, including xylose catabolism, the pentose phosphate pathway, glycolysis, and respiration; these altered metabolic pathways together enhanced aerobic and anaerobic xylose consumption (Sato et al., 2016).

Despite the use of multiple genetic strategies, xylose fermentation by engineered *S. cerevisiae* strains has not attained nearly the same rate and yield as native glucose fermentation. Glucose uptake and catabolism occur with such high flux that *S. cerevisiae* ferments glucose at high concentrations, even in the presence of oxygen, despite the trade off in lower ATP yield compared to aerobic respiration. This phenomenon, known as the Crabtree/Warburg Effect (Crabtree, 1929; Warburg et al., 1927), may have emerged in *S. cerevisiae* through the evolution of intricate regulatory mechanisms, such as down regulation of genes functioning in respiration through glucose repression, and whole genome duplication (Conant and Wolfe, 2006; Lin and Li, 2011; Pfeiffer and Morley, 2014; Thompson et al., 2013). The funneling of glucose towards fermentative metabolism aerobically is thought to occur by the overflow of pyruvate from respiration into ethanol-producing fermentation, consequentially providing *S. cerevisiae* with a competitive advantage against ethanol-sensitive microbes in natural environments (Kotter and Ciriacy, 1993; Pronk et al., 1996).

In contrast to glucose, several studies have determined that strains of *S. cerevisiae* engineered for xylose metabolism primarily respire xylose in the presence of oxygen. For example, under aerobic conditions, we found that an engineered, xylose-fermenting strain with a mutation in *ISU1* upregulated proteins involved in mitochondrial respiration when grown on xylose (Sato et al., 2016). Furthermore, treatment of this strain with Antimycin A, an inhibitor of oxidative phosphorylation, blocks aerobic growth and consumption of xylose. Others have reported that xylose-metabolizing *S. cerevisiae* strains recognize xylose as a non-fermentable carbon source, resulting in the up-regulation of genes related to TCA cycle, glyoxylate pathway, respiratory metabolism, and gluconeogenesis when cultivated on xylose aerobically (Jin et al., 2004; Osiro et al., 2018; Runquist et al., 2009; Salusjarvi et al., 2008; Scalcinati et al., 2012). These results indicate that xylose flux in engineered strains may not be high enough to cause the overflow of pyruvate into ethanol aerobically, which happens naturally for glucose in yeasts demonstrating the Crabtree/Warburg Effect.

In this study, we sought to identify genetic changes that enable *S. cerevisiae* to ferment xylose into ethanol at high flux, such that it can ferment xylose aerobically without the need for respiration, a hallmark of the Crabtree/Warburg Effect for glucose. We first blocked respiration in a xylose-consuming *S. cerevisiae* strain by deleting *COX15*, which encodes a mitochondrial inner membrane protein involved in the synthesis of heme A, an essential prosthetic group for cytochrome *c* oxidase (Barros et al., 2001; Glerum et al., 1997). This respiration-deficient mutant strain was then subjected to aerobic ALE with xylose as the primary carbon source. Evolved clones selected for their abilities to ferment xylose into ethanol aerobically without respiration were then genome-sequenced to determine the causative genetic changes. By reengineering two genetic changes found in the evolved strains into the original parent strain, we generated a strain with increased xylose fermentation aerobically in the absence of respiration. Importantly, this reengineered strain fermented xylose into ethanol at faster rates anaerobically in both lab medium and lignocellulosic hydrolysate. Finally, these genetic changes increased xylose conversion in another strain background, suggesting that the combination of genetic modifications could be incorporated into existing industrial *S. cerevisiae* strains.

2. Materials and methods

2.1. Media

Standard undefined yeast lab media were prepared as previously described (Sherman, 2002). Briefly, media for culturing yeast contained 10 g/L yeast extract, 20 g/L peptone (YP), and various carbon source concentrations (X = 20–30 g/L xylose, D = 20 g/L dextrose/glucose, Gal = 20 g/L galactose, Gly = 20 g/L glycerol, EtOH = 15 g/L ethanol). Solid media also contained 2.5% agar and 200 μg/mL Geneticin (US Biological, Swampscott, MA), 200 μg/mL Hygromycin B (US Biological, Swampscott, MA), 100 μg/mL Nourseothricin (Jena Bioscience, Jena, Germany), or 200 μg/mL Zeocin (Thermo Fisher Scientific, Waltham, MA) as needed. AFEX-pretreated switchgrass hydrolysate (ASGH) was prepared as described elsewhere (Zhang et al., 2020).

2.2. Construction of engineered S. cerevisiae strains

Yeast strains used in this study are described in Tables 1 and S1. Deletions of *COX15*, *TAL1*, *xylA*, *XYL3*, *TKL1*, *GRE3*, *IRA2*, *HOG1*, and *ISU1* were performed by integration of polymerase chain reaction (PCR) products generated from *LoxP-KanMX-LoxP* (pUG6) or *LoxP-HphMX-LoxP* (pUG75) plasmid templates (Guldener et al., 1996), and primers containing 40–60 bp of homologous sequences flanking the targeted gene (Parreiras et al., 2014). PCR products were purified and transformed (Gietz and Schiestl, 2007) into the appropriate strains. Cre recombinase-mediated excision of *LoxP*-flanked antibiotic markers was carried out as published elsewhere (Guldener et al., 1996). Complementation of the *cox15Δ* mutation was performed with a modified pRS416 plasmid (Christianson et al., 1992) with the *URA3* auxotrophic marker replaced with the *HphMX* marker (pRSCENHygMX) and containing the *COX15* promoter, open reading frame (ORF), and terminator. Insertion of additional copies of *TAL1-xylA-XYL3* expression cassette (*XYL* cassette) and *TKL1* promoter, terminator, and ORF were performed using CRISPR/Cas9-mediated genome editing as previously described (Higgins et al., 2018) with some modifications. First, a *KanMX* antibiotic marker was used to replace the *GRE3* ORF (*gre3Δ::LoxP-KanMX)* or inserted between nucleotides 199,269 and 199,270 of *ChrI* ($ChrI^{199269}$) by homologous recombination in the desired strain. $ChrI^{199269}$ is the site of pseudogene, which has been previously used as a location for transgene insertion (Hittinger and Carroll, 2007). Selected strains were then transformed with the pXIPHOS plasmid (Higgins et al., 2018) to express Cas9 and a sgRNA sequence (ATGAAGGAGAAAACTCACCG), which was

Table 1
Saccharomyces cerevisiae strains used in this study.

Strain Name (GLBRCY#)	Name in Figure	Description	Reference
Y22-3		NRRL YB-210 MATa spore *HOΔ::ScTAL1-CpXylA-SsXYL3-LoxP-KanMX-LoxP*	Parreiras et al. (2014)
Y36	3WT	Y22-3 with *LoxP-KanMX-LoxP* maker excised by Cre	Parreiras et al. (2014)
Y286		Y36 *gre3Δ::LoxP ira2Δ::LoxP hog1Δ::LoxP-KanMX-LoxP isu1Δ::LoxP*	Sato et al. (2016)
Y560	Parent	Y286 with *LoxP-KanMX-LoxP* maker excised by Cre	This study
Y583	*cox15Δ*	Y286 *cox15Δ::LoxP-HphMX-LoxP*	This study
Y1031	Evo1	Y583 from aerobic xylose ALE flask B and without *KanMX* and *HphMX* cassettes	This study
Y1033	Evo2	Y583 from aerobic xylose ALE flask A and without *KanMX* and *HphMX* cassettes	This study
Y1031+Empty	Evo1+Empty	Y1031 transformed with pRSCENHygMX plasmid	This study
Y1033+Empty	Evo2+Empty	Y1033 transformed with pRSCENHygMX plasmid	This study
Y1031+*COX15*	Evo1+*COX15*	Y1031 transformed with pRSCENHygMX-*COX15* plasmid	This study
Y1033+*COX15*	Evo2+*COX15*	Y1033 transformed with pRSCENHygMX-*COX15* plasmid	This study
Y1176	Evo1 *xylA/xylaΔ*	Evo1 *ScTAL1-CpXylA-SsXYL3-LoxP/TAL1-xylaΔ::LoxP-KanMX-LoxP-SsXYL3*	This study
Y1183	Evo2 *xylA/xylaΔ*	Evo2 *ScTAL1-CpXylA-SsXYL3-LoxP/TAL1-xylaΔ::LoxP-KanMX-LoxP-SsXYL3*	This study
Y1185	Evo1 *TKL1/tkl1Δ*	Evo1 *TKL1/tkl1Δ::LoxP-KanMX-LoxP*	This study
Y1189	Evo2 *TKL1/tkl1Δ*	Evo2 *TKL1/tkl1Δ::LoxP-KanMX-LoxP*	This study
Y1327	+*XYL* +*TKL1*	Y560 *gre3Δ::ScTAL1-CpXylA-SsXYL3 ChrI::ScTKL1*	This study
Y1348	+*XYL* +*TKL1* 3WT	Y560 *gre3Δ::ScTAL1-CpXylA-SsXYL3 ChrI::ScTKL1* with wild-type *HOG1, ISU1* and *IRA2*	This study
Y1374	+*XYL* +*TKL1* *cox15Δ*	Y1327 *cox15Δ::LoxP-KanMX-LoxP*	This study
Y1376	+*XYL* +*TKL1* 3WT *cox15Δ*	Y1348 *cox15Δ::LoxP-KanMX-LoxP*	This study

designed by CRISpy-pop (Stoneman et al., 2020) to target *KanMX*. The *XYL* cassette and *TKL1* PCR products containing 50–60 bp of flanking sequences to *GRE3* and the *ChrI* locus, respectively, were co-transformed at 20-fold molar excess as repair templates. Transformed colonies were restreaked onto YPD twice to cure the pXIPHOS plasmid, followed by PCR and Sanger sequencing verification of *TAL1-xylA-XYL3* and *TKL1* insertions. All strains were confirmed for gene deletion and antibiotic marker excision by PCR with independent outside flanking primers. Sanger-sequencing of PCR products and DNA plasmids was performed by University of Wisconsin-Madison Biotechnology Center. All strains listed in Tables 1 and S1 are available under a Material Transfer Agreement with the Wisconsin Alumni Research Foundation.

2.3. Adaptive laboratory evolution (ALE)

ALE for selection of clones that ferment xylose aerobically without respiration was performed using the GLBRCY583 (Y583) strain, which lacks *COX15*. Three separate flasks containing 30 mL YP medium with 3% xylose and 0.05% glucose were inoculated with Y583 to optical density at 600 nm wavelength (OD_{600}) of 0.2 and shaken at 250 rpm and 30 °C. When the cell densities of each flask reached OD_{600} = 2–6, the cultures were subcultured with fresh medium to OD_{600} = 0.2. Medium for the second passage included 0.1% glucose, whereas all subsequent passages lacked glucose. Between 6 and 8 passages for each culture were performed until the maximum cell densities were reached within 48 h and all xylose was consumed from the medium as determined by high performance liquid chromatography (HPLC) and refractive index detection (RID) (Schwalbach et al., 2012). Two out of the three ALE cultures that displayed significant growth on xylose-only medium were plated onto YPX + Hygromycin B agar plates. Isolated colonies from the two evolved flasks were tested for growth on xylose aerobically in flask experiments described below. OD_{600} measurements were performed using 1-cm pathlength cuvettes and a Beckman Coulter DU720 spectrophotometer. Single clones (Evo1 and Evo2) from the two independently evolved flasks with the fastest cell growth and xylose consumption rates were selected for further study.

2.4. Genomic DNA library preparation and sequence analysis

Genomic DNA preparation and Illumina sequencing were performed as described previously (Sato et al., 2016) with some modifications. DNA was submitted to the University of Wisconsin-Madison Biotechnology Center. DNA concentration was verified using the Qubit® dsDNA HS Assay Kit (Life Technologies, Grand Island, NY). Samples were prepared according the TruSeq Nano DNA LT Library Prep Kit (Illumina Inc., San Diego, California, USA) with minor modifications. Samples were sheared using a Covaris M220 Ultrasonicator (Covaris Inc, Woburn, MA, USA) and were size selected for an average insert size of 550 bp using SPRI bead-based size exclusion. Quality and quantity of the finished libraries were assessed using an Agilent DNA1000 chip and Qubit® dsDNA HS Assay Kit, respectively. Libraries were standardized to 2 nM.

Cluster generation was performed using the Illumina PE Cluster Kits v4 and the Illumina cBot. Paired-end, 125-bp sequencing was performed, using v4 SBS chemistry on an Illumina HiSeq 2500 sequencer. Images were analyzed using the Illumina Pipeline, version 1.8.2. All DNA sequencing reads have been deposited in the NCBI SRA under BioProject PRJNA279877. Paired-end reads were mapped to GLBRCY22-3 (GCA_001634645.1) (McIlwain et al., 2016) using Bwa-mem 0.7.12-r1039 (Li, 2013). Genomic variants were identified using GATK (v3.4) (McKenna et al., 2010) with duplicate marking and indel realignment. Variants were called using Haplotype Caller without input of known sites/variants. GATK variant filtration was done using the GATK-recommended criteria: QD < 2, FS > 60, and MQ < 40. Coverage analysis was performed by summing unique counts in 500-bp non-overlapping windows using custom Python and R scripts and visualized using the sppIDer pipeline (Langdon et al., 2018).

2.5. Cell culturing and phenotypic growth analysis

Aerobic tube and anaerobic flask fermentation assays were performed as previously described (Parreiras et al., 2014) with some modifications. For aerobic and anaerobic growth assays, inoculum cultures were started from single colonies grown in YPD medium overnight and passaged to OD_{600} = 0.3. Log phase cultures were then diluted to OD_{600} = 0.1 in the appropriate medium at the start of the assay. Yeast cultures were grown in culture tubes containing 10 mL of medium shaken at 250 rpm at 30 °C or in 30 mL of medium stirred with a magnetic stir bar in flasks placed in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI), which was maintained with 2–5% H_2, 2–5% CO_2 and 90–95% N_2. For *COX15* complementation experiments, *cox15Δ* strains transformed with the pRSCENHygMX (Empty vector) or pRSCENHygMX-*COX15* plasmid were cultured in medium

containing 200 μg/mL Hygromycin B. For cell culture experiments using Antimycin A, yeast cells were grown to log phase in YPD medium aerobically, then shifted into tubes containing 10 mL fresh YPD or YPX medium treated with 10 μL DMSO or 10 μL of 0.5 mg/mL Antimycin A in DMSO (0.5 μg/mL final concentration, A8674, Sigma-Aldrich, St. Louis, MO), and incubated at 30 °C with shaking. For aerobic cell growth assay using glucosamine, yeast cells were grown to log phase in YPD medium aerobically, then shifted into tubes containing 10 mL fresh YPD or YPD medium treated with 250 μL of 200 mg/mL D-(+)-glucosamine hydrochloride (5 mg/mL final concentration, G4875, Sigma-Aldrich), and incubated at 30 °C with shaking.

For anaerobic bioreactor experiments, fermentations were conducted in 0.25 L Minibio bioreactors (Applikon Biotechnology Inc., Foster City, CA) containing 100 mL of 7% glucan-loading ASGH. Prior to fermentation, hydrolysates were adjusted to pH 5.0 and filtered through a 0.2 μm filter to remove precipitates and to ensure sterility. After transfer to the fermentation vessel, hydrolysates were sparged with 100% N_2 at the flow rate of ~20 mL/min overnight before the inoculation. *S. cerevisiae* strains were grown to early stationary phase in YPD aerobically, then diluted OD_{600} = 0.2 in fresh YPD for ~10 h. Cultures were then centrifuged at 3000×*g* for 5 min, the cell pellets were resuspended into ~10 mL of hydrolysate from the pre-sparged vessels, then inoculated back into each bioreactor to a starting OD_{600} of 0.5. Fermentations were conducted at 30 °C with continuous stirring (500 rpm) and sparged at ~20 mL/min with 100% N_2. During the fermentation, pH was maintained at 5.0 by automated addition of 5% NaOH. Cell density measurements were blanked with OD_{600} measurements from uninoculated hydrolysate diluted 1:10 or 1:25 with water. Extracellular glucose, xylose, ethanol, glycerol, and galactose concentrations for all experiments were determined by HPLC-RID. Calculations for xylose consumption and ethanol production rates and yields from anaerobic bioreactor fermentations were determined for the time period after all glucose was depleted and as described elsewhere (Sato et al., 2016).

3. Results

3.1. Evolved yeast mutants convert xylose into ethanol aerobically in the absence of respiration

Previously, we described a *S. cerevisiae* strain engineered to express single copies of xylose isomerase (*xylA* from *Clostridium phytofermentans*) and xylulokinase (*XYL3* from *Scheffersomyces stipitis*), as well as an additional copy of the native transaldolase *TAL1* by genomic insertion of a DNA cassette (henceforth called the "*XYL* cassette"). Informed by ALE (Sato et al., 2016), we further engineered deletion mutations in *HOG1*, *ISU1*, *GRE3*, and *IRA2* to enable rapid conversion of xylose into ethanol anaerobically. Interestingly, this xylose-fermenting strain (hereafter referred to as the "Parent" strain) converted xylose into ethanol in the presence of oxygen, albeit worse than the theoretically expected ethanol yield from xylose. However, this Parent strain could not grow on and ferment xylose aerobically in the presence

Of Antimycin A (Sato et al., 2016), or with the *cox15Δ* mutation, which is essential for the function of Electron Transport Chain Complex IV (Fig. S1). Thus, the Parent strain requires respiration to grow on xylose in the presence of oxygen.

To increase the flux of xylose to ethanol without aerobic respiration, we used ALE to select for respiration-deficient mutants that grow aerobically on xylose. Specifically, the Parent strain with the *cox15Δ* mutation was evolved aerobically on xylose in three separate flasks (Fig. S2A). Two out of the three flasks displayed significant growth during the third passage (Fig. S2B-C). From each of these two flasks, independent clones (Evo1 and Evo2) were isolated and confirmed to grow on and ferment xylose into ethanol aerobically (Fig. 1). The evolved strains grew on and consumed xylose more slowly than the Parent strain, but they produced higher levels of ethanol, likely due to their inabilities to respire. We confirmed that the Evo1 and Evo2 strains

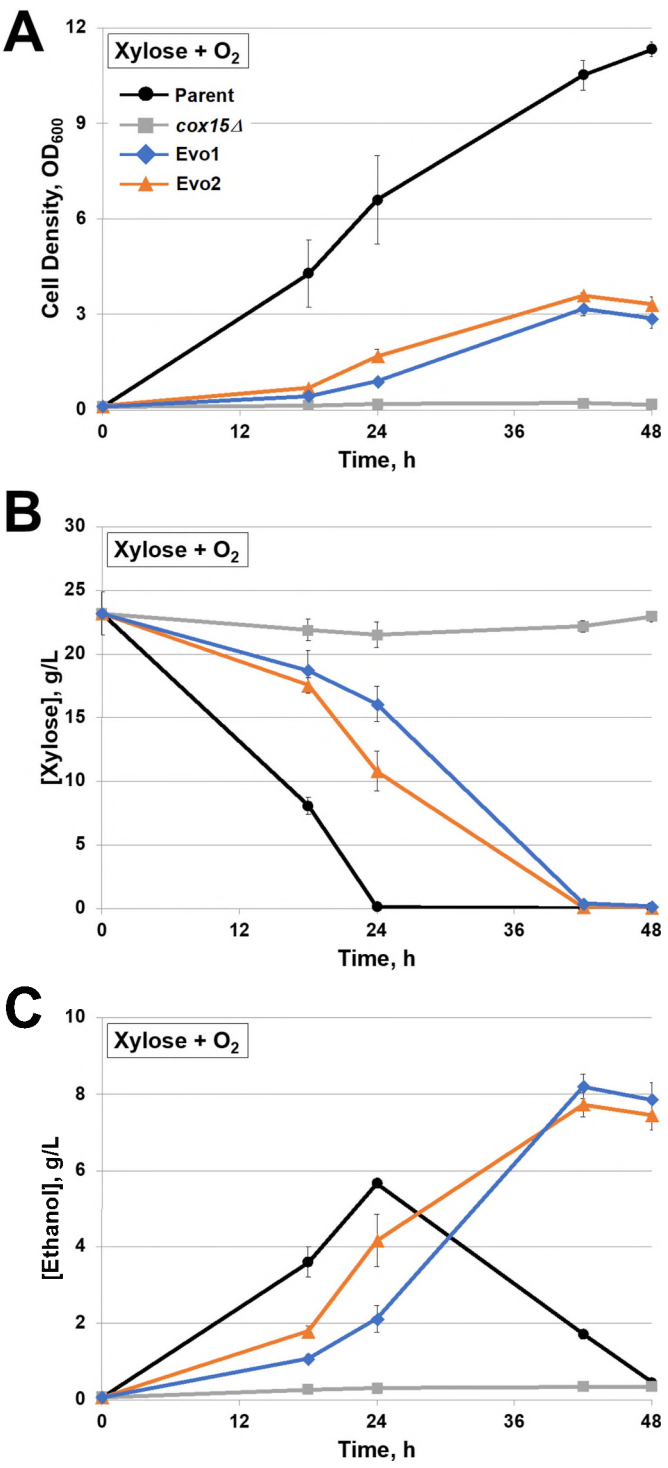

Fig. 1. Adaptive laboratory evolution enabled the engineered respiration-deficient yeast strains to ferment xylose into ethanol aerobically. The evolved (Evo1 and Evo2), respiration-deficient (*cox15Δ*), and parent containing wild-type *COX15* (Parent) strains were cultured aerobically in YPX medium. The evolved strains were evolved from the respiration-deficient strain. Average cell density (A), extracellular xylose (B), and ethanol (C) concentrations from independent triplicate experiments with SEM are reported.

did not evolve the ability to respire without *COX15* since both Evo1 and Evo2 strains did not grow on glycerol or galactose (Fig. S3). These results indicated that the evolved strains ferment xylose into ethanol aerobically without the requirement for respiration. A possible, albeit unlikely, mechanism for aerobic growth on xylose by the evolved strains

could be from bypassing the need for Cox15p. To test this, we first transformed a low-copy plasmid with or without *COX15* into the Evo1 and Evo2 strains. The evolved strains expressing *COX15* restored the ability to respire xylose and ethanol aerobically (Fig. 2). We then cultured these strains in xylose aerobically with the addition of Antimycin A, which inhibits oxidative phosphorylation by the Electron Transport Chain Complex III. The evolved strains, with or without *COX15*, could grow on and ferment xylose into ethanol aerobically in the presence of Antimycin A (Fig. 2), which is a phenotype analogous to how Crabtree/Warburg-positive yeasts metabolize glucose. Together, our results suggest that the evolved strains can metabolize xylose into ethanol aerobically without the requirements for Complex III and IV activities.

3.2. *Evolved mutations do not bypass glucose repression*

Another hallmark of Crabtree/Warburg-positive yeasts is glucose-mediated repression of metabolic pathways. In the presence of high levels of glucose, the Snf1p pathway is inactivated to repress the expression of genes involved in gluconeogenesis, respiration, and non-fermentable carbon metabolism, leading Crabtree/Warburg-positive yeasts to metabolize glucose first (Kayikci and Nielsen, 2015). Extracellular glucose is also bound by the sensors Rtg2p and Snf3p, leading to a signaling cascade that relieves repression by the transcription factor Rtg1p (Johnston and Kim, 2005). These regulatory pathways likely lead to inefficient fermentation of other sugars, such as xylose, present in lignocellulosic hydrolysates into ethanol; thus, overcoming glucose repression is important for the biofuel industry. We examined whether aerobic xylose fermentation by the evolved strains could be repressed by the non-metabolizable glucose analog, glucosamine. Aerobic growth and xylose consumption by both the Parent and the evolved strains were blocked in the presence of glucosamine (Fig. S4), which suggests that the fermentation of xylose into ethanol aerobically by the Evo1 and Evo2 strains requires genes and pathways that are repressed by glucose.

3.3. *Evolved mutations enhance the rate of anaerobic xylose fermentation*

The Crabtree/Warburg Effect in *S. cerevisiae* is a product of high glucose flux, which consequentially results in rapid glucose consumption aerobically and anaerobically. Thus, *S. cerevisiae* is used as an industrial workhorse for its ability to rapidly convert glucose into biofuels without the costly need for aeration. We next investigated if the evolved mutations positively impacted the rate of anaerobic xylose fermentation. Under anaerobic conditions, both the evolved strains fermented xylose into ethanol faster than the Parent strain, with or without *COX15* (Fig. S5). Unexpectedly, the evolved strains expressing *COX15* displayed slightly increased anaerobic xylose fermentation rates but had no differences in final ethanol titer (Fig. S6), suggesting that *COX15* may have

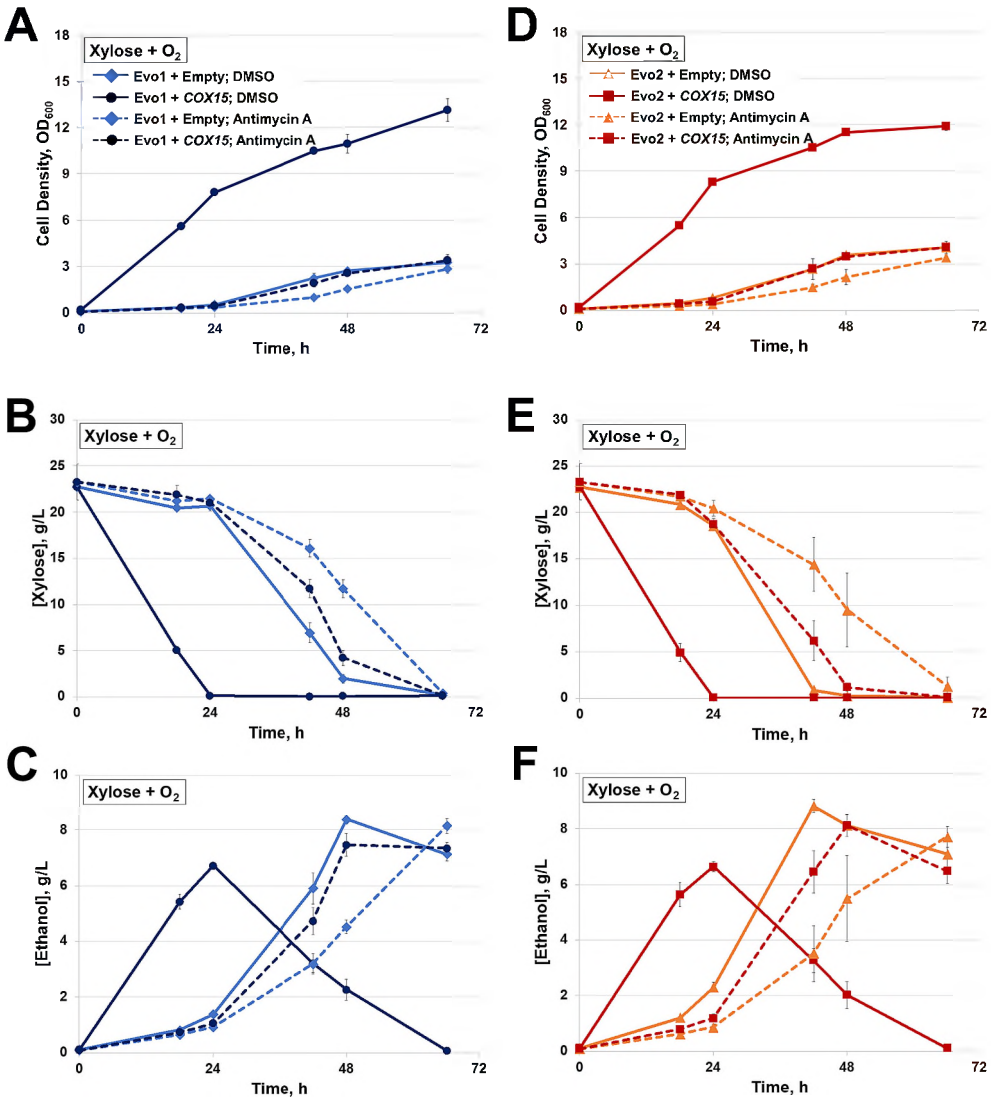

Fig. 2. Evolved strains can ferment xylose into ethanol aerobically in the presence of Antimycin A. The evolved strains containing the empty plasmid (Evo1+Empty or Evo2+Empty) or pRSCENHygMX-*COX15* plasmid (Evo1+*COX15* or Evo2+*COX15*) were cultured aerobically in YPX + Hygromycin B medium containing a DMSO control or 0.5 μg/mL Antimycin A, an inhibitor of Complex III. Average cell density (A and D), extracellular xylose (B and E), and ethanol (C and F) concentrations from independent triplicate experiments with SEM are reported.

direct or indirect anaerobic functions in xylose fermentation. Importantly, these studies further indicate that the evolved mutations increase the rate of xylose fermentation under anaerobic conditions.

3.4. Evolved strains contain overlapping duplications in ChrIV and XVI

To identify the evolved genetic changes that caused the Crabtree/Warburg-like phenotype, we sequenced the Evo1 and Evo2 strains using Illumina HiSeq technology and mapped their sequencing reads to the Y22-3 parental genome (McIlwain et al., 2016). Multiple single nucleotide changes in the evolved strains were identified that were not present in the unevolved *cox15Δ* strain (Supplementary File S1). However, the mutations in the two independently evolved strains did not overlap in the same genes or in genes within the same pathways or biological functions. Nevertheless, when comparing the read coverages across all chromosomes, we found that the read coverages across regions of ChrII, IV, and XVI were two-fold higher in the evolved strains than the unevolved *cox15Δ* strain, indicating that segmental duplications occurred during the directed evolution (Fig. 3A). Despite being evolved independently, both the evolved strains had duplications of a nearly identical 92-kb region of ChrIV and in a large and a 410-kb overlapping region of ChrXVI (Supplementary File S1), whereas the ChrII duplication was only present in the Evo1 strain. We found that the duplicated region of ChrIV contained the *XYL* cassette expressing *TAL1*, *xylA*, and *XYL3*, which had been inserted into the *HO* locus (Parreiras et al., 2014) (Fig. 3B). The overlapping region in ChrXVI notably included *TKL1*, which encodes transketolase, a pentose phosphate pathway enzyme involved in xylose catabolism (Fig. 3C).

3.5. Duplications of engineered xylose metabolism genes and TKL1 are required for robust aerobic xylose fermentation

We next sought to confirm whether duplications of the engineered xylose metabolism genes *TAL1*, *xylA*, and *XYL3* from ChrIV and *TKL1* from ChrXVI were important for the ability of the evolved strains to ferment xylose aerobically without respiration. Each of the four genes from the evolved strains were individually deleted and tested for their requirement in aerobic xylose fermentation. Deletion of the second copy of *xylA* blocked aerobic xylose fermentation by both evolved strains (Fig. 4A–C), whereas deletion of an extra copy of *TAL1* had no effect (Fig. S7). In contrast, deletion of an extra copy of *XYL3* had a modest effect on the Evo1 strain but little effect on the Evo2 strain, suggesting that the genetic bases for the Evo1 and Evo2 phenotypes are overlapping but not identical (Fig. S7). Consistent with this hypothesis, deletion of an extra copy of *TKL1* had a stronger effect on the Evo2 strain, significantly decreasing aerobic xylose fermentation rate, whereas the Evo1 strain with the extra copy of *TKL1* deleted displayed modestly decreased xylose fermentation (Fig. 4D–F). Together, these results suggest that duplications of *xylA*, *XYL3*, and *TKL1* are important for aerobic xylose fermentation without respiration in the evolved strains.

3.6. Duplicated genes synergize with hog1Δ, isu1Δ, and ira2Δ mutations to enhance xylose conversion aerobically and anaerobically

To directly test whether additional copies of the xylose metabolism genes and *TKL1* enable xylose fermentation aerobically without respiration, we engineered second copies of the *XYL* cassette and *TKL1* into the Parent strain containing the *cox15Δ* mutation. The *cox15Δ* strain engineered with additional copies of the xylose metabolism genes and *TKL1* displayed rapid consumption of and growth on xylose aerobically (Fig. 5A–C), similarly to the Evo1 and Evo2 strains. In contrast, insertion of an additional copy of *TKL1* into the *cox15Δ* strain did not enable growth on xylose aerobically, while insertion of a second copy of the *XYL* cassette caused modestly faster growth aerobically on xylose (Fig. S8). This result suggests that duplications of the *XYL* cassette and *TKL1* together are necessary for respiration-independent aerobic xylose

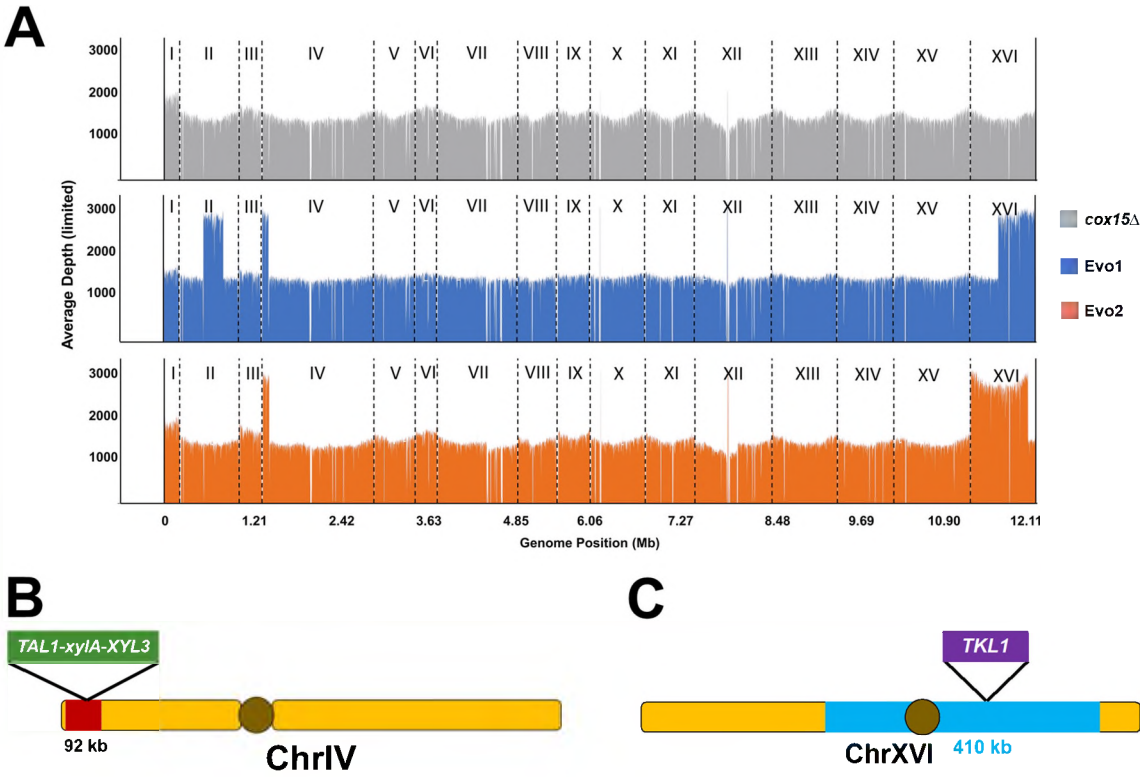

Fig. 3. Two independently evolved clones that ferment xylose aerobically contain overlapping duplications in ChrIV and ChrXVI. Average read depths between the pre-ALE strain (*cox15Δ*) and the evolved strains (Evo1 and Evo2) were analyzed using sppIDer (Langdon et al., 2018) (A). *TAL1-xylA-XYL3* expression cassette and *TKL1* gene are duplicated in ChrIV (B) and ChrXVI (C), respectively.

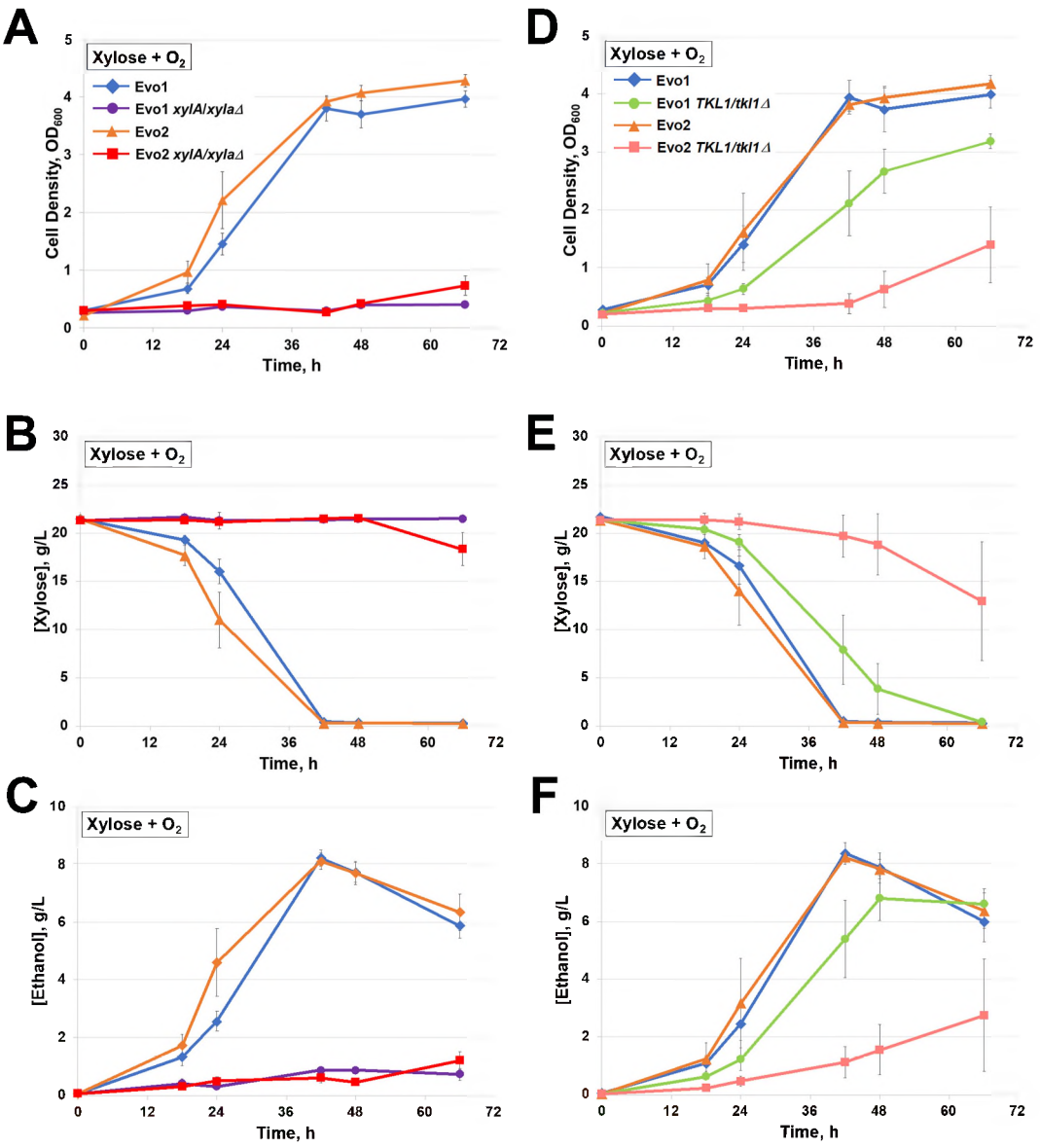

Fig. 4. Duplications of *xylA* (A–C) and *TKL1* (D–F) are responsible for aerobic xylose fermentation by the evolved strains. The evolved strains (Evo1 and Evo2) and the evolved strains with deletion of one copy of *xylA* or *TKL1* were cultured aerobically in YPX medium. Average cell density (A and D), extracellular xylose (B and E), and ethanol (C and F) concentrations from independent triplicate experiments with SEM are reported.

fermentation. When we engineered two copies of the *XYL* cassette and *TKL1* into the *cox15Δ* strain containing wild-type *HOG1*, *ISU1*, and *IRA2* (denoted as "3WT"), the strain did not metabolize xylose aerobically (Fig. 5A–C). This result further suggests that the *hog1Δ*, *isu1Δ*, and *ira2Δ* mutations, in combination with two copies of the *XYL* cassette and *TKL1*, are required to enable aerobic xylose fermentation. Previously, we showed that the increased rates of xylose conversion into ethanol by the evolved yeast strains were not dependent upon the deletion of *COX15* aerobically or anaerobically (Figs. S5 and S6). Similarly, we found that expressing second copies of the *XYL* cassette and *TKL1* in the *COX15*-containing Parent strain also increased the rates of xylose consumption and ethanol production aerobically (Fig. 6) and increased overall xylose fermentation anaerobically (Fig. 5D–F). Interestingly, when the Parent strain was engineered with a duplicated *XYL* cassette and *TKL1*, as well as wild-type *HOG1*, *ISU1*, and *IRA2*, it grew rapidly on xylose aerobically but produced very low amounts of ethanol, suggesting that this strain primarily respired xylose (Fig. 6). Indeed, aerobic growth and consumption of xylose, but not glucose, by this strain were completely blocked in the presence of Antimycin A. In contrast, the Parent strain engineered with the duplicated *XYL* cassette and *TKL1*, and containing deletion mutations in *HOG1*, *ISU1*, and *IRA2* maintained rapid xylose conversion into ethanol aerobically under Antimycin A treatment (Fig. S9). Together, these results suggest that additional copies of the *XYL* cassette and *TKL1* enhance aerobic respiration of xylose, while deletions of *HOG1*, *ISU1*, and *IRA2* redirect xylose metabolism from respiration to fermentation. The Parent strain engineered with both the additional *XYL* cassette and the additional copy of *TKL1*, along with wild-type *HOG1*, *ISU1*, and *IRA2* did not grow on xylose anaerobically (Fig. 5D–F). This result further supports the conclusion that two copies of the xylose metabolism genes and *TKL1* synergize with mutations in *HOG1*, *ISU1*, *GRE3*, and *IRA2* to promote both aerobic and anaerobic xylose fermentation. When the Parent strain was engineered with an additional copy of *TKL1* or the *XYL* cassette alone, we observed more modest increases in the rate of xylose conversion into ethanol anaerobically (Fig. S10).

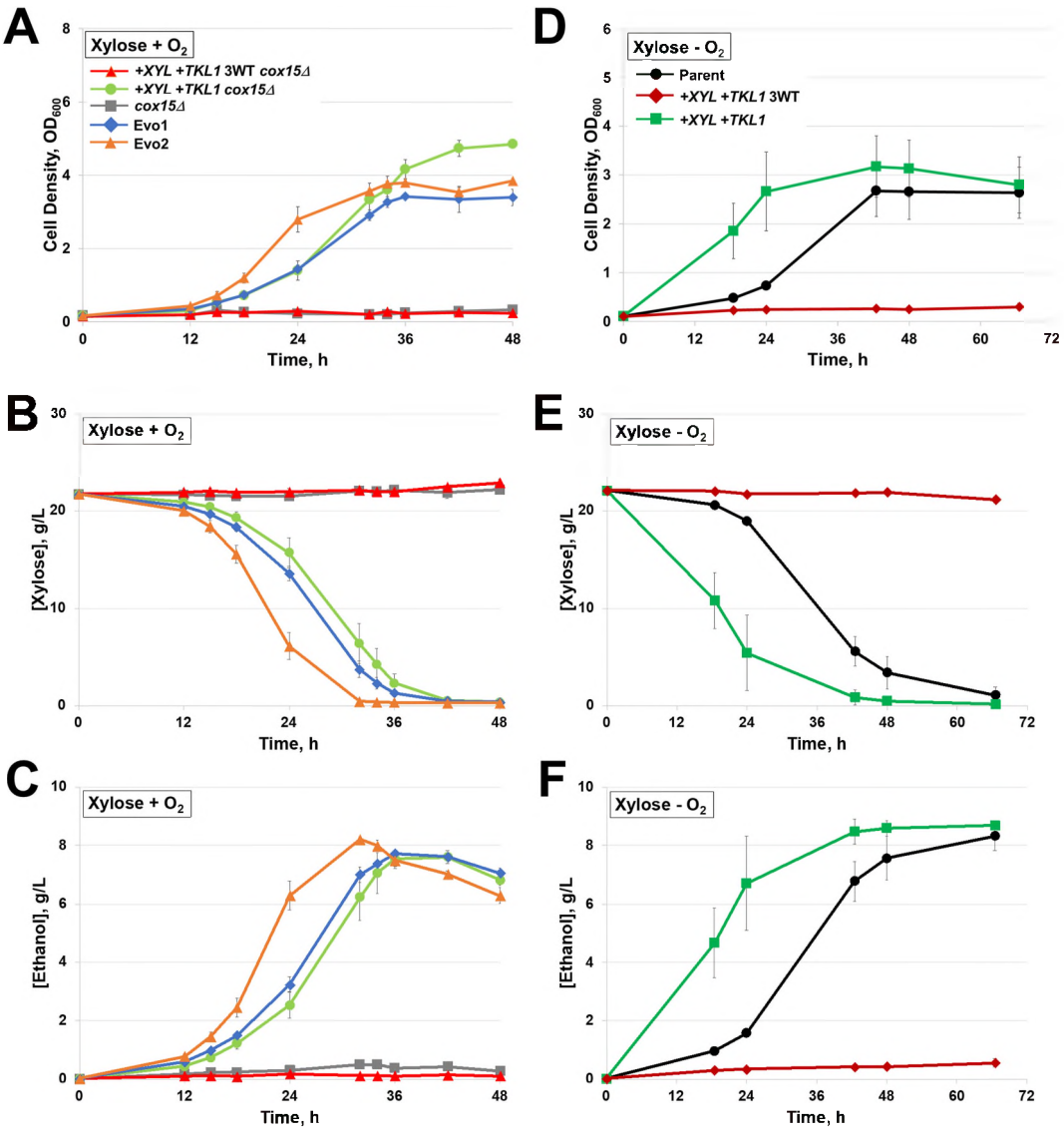

Fig. 5. Duplications of the *XYL* cassette and *TKL1* together with deletion mutations in *HOG1*, *ISU1*, and *IRA2* enhance fermentation of xylose aerobically and anaerobically. The evolved strains (Evo1 and Evo2) and the engineered strains with deletion of *COX15* were cultured aerobically in YPX medium (A–C). The engineered strains with wild-type *COX15* were cultured anaerobically in YPX medium (D–F). Average cell density (A and D), extracellular xylose (B and E), and ethanol (C and F) concentrations from independent triplicate experiments with SEM are reported. 3WT indicates a genotype of wild-type *HOG1*, *ISU1*, and *IRA2*.

3.7. Engineered gene duplications and deletion mutations function in another strain background

Mutations in *HOG1* and *IRA2*/PKA pathway genes, as well as *ISU1*, have been shown to enhance xylose metabolism in multiple strain backgrounds (Dos Santos et al., 2016; Osiro et al., 2019; Sato et al., 2016), suggesting that these genetic modifications have universal utility. Similarly, we tested the generality of our new genetic modifications in another strain background. First, we introduced additional copies of the *XYL* cassette and *TKL1* into the commonly used strain CEN.PK113-5D (Landi et al., 2011). As in the Y22-3 strain background, extra copies of the *XYL* cassette and *TKL1* significantly enhanced anaerobic xylose fermentation in the CEN.PK113-5D background also containing mutations in *HOG1*, *ISU1*, *GRE3*, and *IRA2* (Fig. S11). However, these genetic duplications were not effective in the CEN.PK113-5D background containing wild-type *HOG1*, *ISU1*, and *IRA2*. These results suggest that our genetic modifications can be applied to different strain backgrounds.

3.8. Engineering the duplications and deletion mutations improves the rate of xylose fermentation in industrially relevant switchgrass hydrolysate

As a final test of the utility of our genetic modifications, we investigated whether the combination of gene duplications and deletions enables accelerated xylose fermentation from pretreated and hydrolyzed lignocellulosic biomass to ethanol. Specifically, we compared the Parent strain containing single copies of the *XYL* cassette and *TKL1*, as well as deletion mutations in *HOG1*, *ISU1*, *GRE3*, and *IRA2*; the engineered Parent strain with extra copies of the *XYL* cassette and *TKL1*; and the modified Parent strain containing duplications of the *XYL* cassette and *TKL1*, but containing wild-type *HOG1*, *ISU1*, and *IRA2*. The three strains were cultured anaerobically in bioreactors containing AFEX-pretreated switchgrass hydrolysate (ASGH) (Tables 2 and S2), which contains inhibitory compounds that are known to inhibit cell growth and fermentation (Parreiras et al., 2014). As when cultured anaerobically in YPX media (Fig. 5D–F), the engineered Parent strain containing extra copies of the *XYL* cassette and *TKL1* consumed xylose and produced ethanol significantly faster than the Parent strain, whereas the ethanol

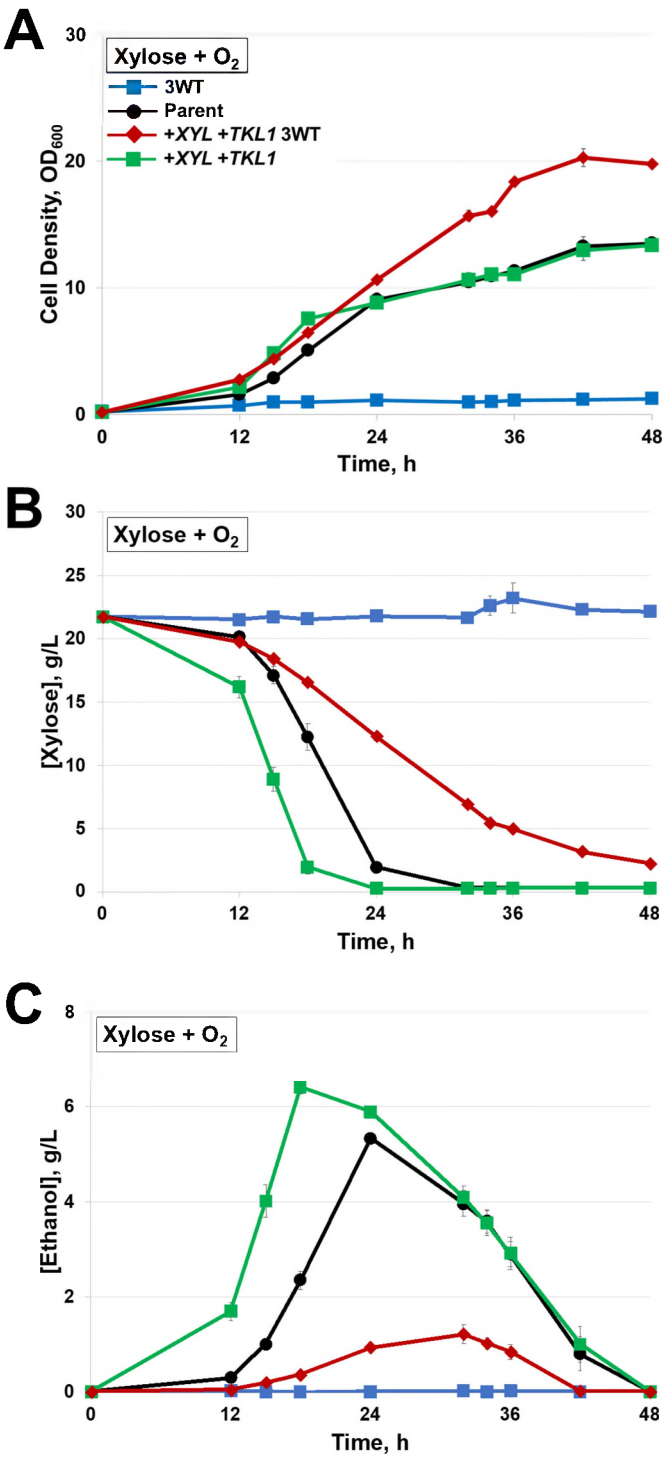

Fig. 6. Duplications of the *XYL* cassette and *TKL1* increase the xylose conversion rate, while deletion mutations in *HOG1*, *ISU1*, and *IRA2* reprogram aerobic xylose metabolism from respiration into fermentation. The engineered strains with wild-type *COX15* were cultured aerobically in YPX medium. Average cell density (A), extracellular xylose (B), and ethanol (C) concentrations from independent triplicate experiments with SEM are reported. All the strains contain at least one copy of the *XYL* cassette and *TKL1*. 3WT indicates a genotype of wild-type *HOG1*, *ISU1*, and *IRA2*.

yield from xylose was not significantly different between these two strains (Table 2). The modified Parent strain containing the duplications but wild-type *HOG1*, *ISU1*, and *IRA2* consumed little xylose and did not produce ethanol from xylose. These results suggest that extra copies of

Table 2
Xylose conversion kinetics of engineered *S. cerevisiae* strains from AFEX-pretreated switchgrass hydrolysate (ASGH) fermentations.

Strain	Y1348 (+*XYL* +*TKL1* 3WT)	Y560 (Parent)	Y1327 (+*XYL* +*TKL1*)
Specific xylose consumption rate[a]	**0.010 ± 0.008**[e]	**0.048 ± 0.008**[f]	**0.098 ± 0.004**
Specific ethanol production rate from xylose[b]	**ND**	**0.013 ± 0.002**[g]	**0.028 ± 0.004**
Ethanol productivity from xylose[c]	**ND**	**0.064 ± 0.011**[f]	**0.16 ± 0.01**
% of theoretical yield from xylose [d]	**ND**	**43.9 ± 8.2**	**55.9 ± 7.8**

ND = Not determined; the rate of ethanol production did not exceed the rate of stripping.
3WT = *HOG1 ISU1 IRA2*.
All the experiments were performed in biological triplicate.
[a] In g xylose consumed/OD_{600}/h ± SEM.
[b] In g ethanol produced/OD_{600}/h ± SEM.
[c] In g ethanol produced/L/h ± SEM.
[d] Percentage of maximum theoretical ethanol yield ±SEM.
[e] $p < 0.05$, Y1348 vs. Y1327.
[f] $p < 0.05$, Y560 vs. Y1327.
[g] $p < 0.07$, Y560 vs. Y1327.

the *XYL* cassette and *TKL1*, in combination with deletion mutations in *HOG1*, *ISU1*, and *IRA2*, enable accelerated xylose conversion from lignocellulosic hydrolysate into ethanol anaerobically. On the other hand, duplications of the *XYL* cassette and *TKL1* did not affect glucose conversion kinetics, whereas deletion mutations in *HOG1*, *ISU1*, and *IRA2* slightly improved glucose consumption and ethanol production rates (Table S2). Thus, our genetic modifications could improve industrial processes by significantly and specifically improving the conversion of xylose into ethanol from lignocellulosic biomass.

4. Discussion

Xylose has been targeted as a prospective carbon source for producing sustainable biofuels since it is the second most abundant sugar in plant-derived biomass (Lee et al., 2021). Thus, many studies have attempted to engineer *S. cerevisiae* to efficiently convert xylose into biofuels in a manner comparable to easily fermentable sugars like glucose (Demeke et al., 2015; Dos Santos et al., 2016; Feng et al., 2018; Lee et al., 2020; Parreiras et al., 2014; Sato et al., 2016; Zeng et al., 2017). The Crabtree/Warburg Effect was first described in tumor cells where lactate was aerobically fermented in high levels of glucose, along with decreased respiration (Crabtree, 1929; Warburg et al., 1927). This phenomenon also occurs in *S. cerevisiae*, which ferments high levels of glucose into ethanol under aerobic conditions (Pfeiffer and Morley, 2014). A similar effect has not been observed with xylose and other carbon sources in yeasts because it requires high metabolic flux, which is achieved for glucose partially through the repression of other metabolic pathways and the upregulation of hexose transporters and glycolytic enzymes (Kayikci and Nielsen, 2015; Pfeiffer and Morley, 2014). Through ALE and genetic manipulations, we determined a suite of genetic changes that are sufficient to enable strains to grow on xylose aerobically without respiration (Fig. 3). These genetic changes impart a trait that is analogous to the Crabtree/Warburg Effect for xylose, although repression by glucosamine (Fig. S4) indicates that the aerobic fermentation of xylose and glucose are still using different metabolic and regulatory pathways. Further studies will be needed to understand the biochemical and regulatory mechanisms that control the overflow metabolism of xylose.

Increased expression of metabolic enzymes by integrating multiple gene copies has been shown by others to increase the rate of xylose utilization and anaerobic fermentation. In particular, several studies engineered *S. cerevisiae* with genes overexpressing xylose isomerase

(*xylA*), xylulokinase (*XYL3*), and non-oxidative pentose phosphate pathway (*RKI1*, *RPE1*, *TAL1*, *TKL1*, *TKL2*, and *NQM1*) in combination with the deletion of the gene encoding aldose reductase, *GRE3* (Bracher et al., 2019; Demeke et al., 2013; Kuyper et al., 2005; Verhoeven et al., 2017). Deletions of several genes involved in iron-sulfur cluster biogenesis (*ISU1*) and various signaling pathways (*HOG1*, *IRA2*, *BCY1*, and *SSK1*) can also contribute to improved xylose metabolic flux in strains engineered with xylose isomerase, likely by reprogramming regulatory systems (Dos Santos et al., 2016; Myers et al., 2019; Sato et al., 2016; Wagner et al., 2019). Here, we discovered that increased copy number of the *XYL* cassette and *TKL1* played key roles for enabling aerobic xylose fermentation in a strain containing deletion mutations in *HOG1*, *ISU1*, and *IRA2* (Figs. 5A-C and 6), a trait not previously described in any yeast strain. Adding only a second copy of the *XYL* cassette resulted in increased xylose fermentation rate, whereas adding only a second copy of *TKL1* had no effect on xylose fermentation (Fig. S8). Thus, in addition to the regulatory mutations, at least two copies of *xylA* are required to reach the necessary level of enzymatic capacity to initiate xylose fermentation aerobically, while a second copy of *TKL1* further enhances this trait. Moreover, two copies of both the *XYL* cassette and *TKL1* led to increased xylose fermentation rate anaerobically in lab medium and switchgrass hydrolysate (Fig. 5D–F, S10, and Table 2), demonstrating that the genetic changes also increase xylose metabolic conversion in industrially relevant conditions.

The precise mechanism by which the mutations in *HOG1*, *ISU1*, and *IRA2* synergize to enable xylose fermentation has been unclear, but recent studies suggest that these mutations alter how *S. cerevisiae* senses xylose. Osiro and colleagues determined that the responses of three major sugar-signaling pathways, including cAMP/PKA, Snf3p/Rgt2p, and Snf1p/Mig1p, by wild-type strains cultured in high xylose concentrations were similar to the signals induced by low glucose concentrations (Osiro et al., 2018), which typically promote respiration under aerobic conditions. In contrast, the authors later demonstrated that *isu1Δ ira2Δ* double mutants cultured in high xylose concentrations displayed activation of some high glucose signaling pathways, suggesting that these mutations may promote the recognition of xylose as a fermentable sugar (Osiro et al., 2019). In support of this view, we showed that deletions of *HOG1*, *ISU1*, and *IRA2* caused the up-regulation of both the PKA and Snf1 pathways on xylose (Myers et al., 2019). PKA is normally activated in response to high glucose concentrations to promote growth and glucose fermentation, while Snf1 is normally activated when non-preferred carbon sources are consumed; this unusual regulatory combination seems to promote the fermentation of xylose with rapid uptake and high metabolic flux (Myers et al., 2019). Sugar uptake and growth rates also correlate with the hypothesized regulation of overflow metabolism by sensing glycolytic flux (Huberts et al., 2012; Litsios et al., 2018). When glycolytic flux surpasses a critical point of respiratory capacity, metabolism switches from respiration to fermentation due to the overflow of pyruvate flux to ethanol and other fermentation products (Dashko et al., 2014; Hagman and Piskur, 2015; Niebel et al., 2019). Thus, deletions of *HOG1*, *ISU1*, and *IRA2* together may cause *S. cerevisiae* to initiate similar, but not identical, metabolic activities with xylose.

Here, we showed that increasing xylose conversion in this primed regulatory background resulted in overflow metabolism of xylose into ethanol. Specifically, loss-of-function mutations in *HOG1*, *ISU1*, and *IRA2* significantly increased xylose consumption and ethanol production rates aerobically in strains containing either one or two copies of the *XYL* cassette and *TKL1* (Fig. 6), whereas the strain containing two copies of the *XYL* cassette and *TKL1* with wild-type *HOG1*, *ISU1*, and *IRA2* mostly respired xylose aerobically (Fig. 6) and did not grow on xylose anaerobically (Fig. 5D–F). These results indicate that overexpression of xylose isomerase and the pentose phosphate pathway enzymes may increase total metabolic flux, while the altered regulatory pathways prime yeast to ferment xylose. Collectively, these genetic changes increase the conversion of xylose into ethanol by enabling a form of xylose overflow metabolism that is analogous to how the Crabtree/Warburg Effect enables glucose fermentation under aerobic conditions (Fig. S9). Importantly, this increased metabolism also translates into faster rates of xylose fermentation in more industrially relevant anaerobic conditions.

To synthesize our observations with published work, we propose a model of aerobic, Crabtree/Warburg-like metabolic flux for xylose (Fig. 7). We assume that xylose-fermenting strains have a minimal capacity (*MC*) for enabling aerobic growth through respiration and a minimal switching capacity (*SC*) critical for changing from respiratory to fermentative flux. Once total metabolic flux exceeds the *MC* value, a strain can utilize xylose through respiration. (Fig. 7, second column). Regulatory pathways altered in *hog1Δ isu1Δ ira2Δ* triple mutants lower both *MC* and *SC*, causing the total metabolic flux (*MF*) of the strain to exceed both capacities. As xylose consumption rates and *MF* level increase beyond this lowered *SC* level, yeast begin to switch metabolic flux from respiration into fermentation. For yeast containing both the deletion mutations and the duplicated copies of the *XYL* cassette and *TKL1* (Fig. 7, right-most green column), the increased metabolic flux far exceeds the switching capacity, resulting in much greater conversion of xylose into ethanol aerobically.

5. Conclusion

To our knowledge, this is the first study to report defined genetic modifications that enable multiple xylose isomerase-expressing *S. cerevisiae* strains to successfully convert xylose from lab media and switchgrass hydrolysate into ethanol both aerobically and anaerobically. While the theoretical yield of ethanol from xylose was still lower than that from glucose, further investigation to better understand tolerance to lignocellulose-derived inhibitors, to improve xylose uptake, and to derepress genes involved in xylose metabolism will likely lead to further improvements. Furthermore, the evolved strains have slightly different aneuploidies (Fig. 3A), and the Evo2 strain is more efficient for aerobic and anaerobic xylose fermentation than the Evo1 strain (Figs. 1, 4, 5, S5, and S6), suggesting that there are additional unknown mutations that improve xylose conversion. Nonetheless, the yeast strains described here represent a significant advance; they have crossed the threshold not only of being able to ferment xylose anaerobically, but also of preferentially fermenting xylose in the presence of oxygen, a hallmark of the Crabtree/Warburg Effect previously only seen in yeast during the aerobic fermentation of glucose.

Declaration of competing interest

The Wisconsin Alumni Research Foundation is preparing a provisional patent application based on the results and strains described in this work. SBL, CTH and TKS are inventors. All other authors declare no competing interests.

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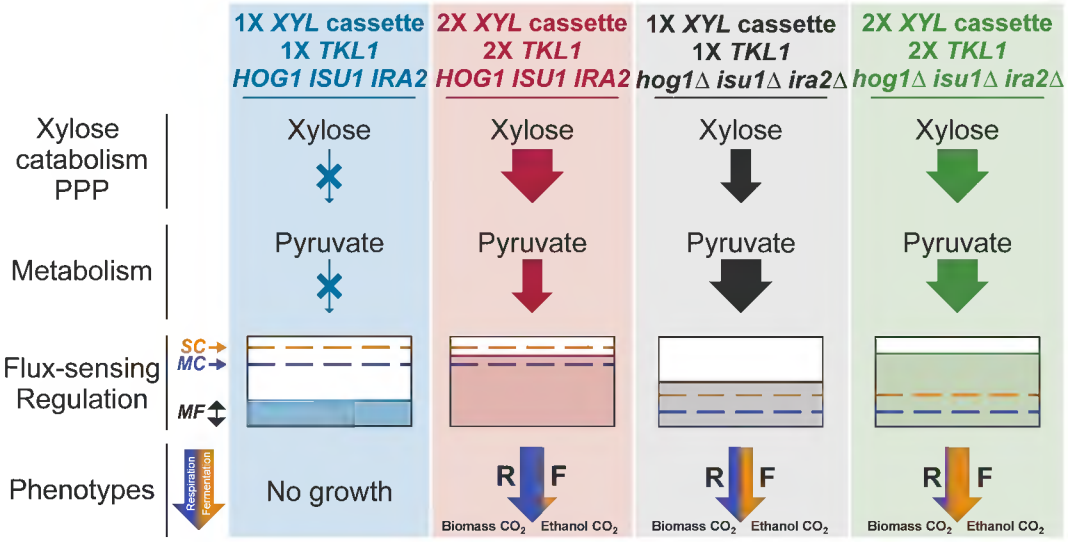

Fig. 7. Proposed model for aerobic xylose metabolic fluxes, including Crabtree/Warburg-like overflow into fermentation. The wild-type strain contains one copy of the *XYL* cassette and *TKL1* with wild-type copies of *HOG1*, *ISU1*, and *IRA2*. *SC*, the minimal switching capacity for changing metabolic flux from respiration to fermentation; *MC*, a minimal capacity for enabling respiratory metabolism; *MF*, total metabolic flux of the strains. R, respiratory flux; F, fermentative flux.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2021.09.008.

References

Bamba, T., Hasunuma, T., Kondo, A., 2016. Disruption of PHO13 improves ethanol production via the xylose isomerase pathway. Amb. Express 6, 4.
Barros, M.H., Carlson, C.G., Glerum, D.M., Tzagoloff, A., 2001. Involvement of mitochondrial ferredoxin and Cox15p in hydroxylation of heme O. FEBS Lett. 492, 133–138.
Bracher, J.M., Martinez-Rodriguez, O.A., Dekker, W.J.C., Verhoeven, M.D., van Maris, A. J.A., Pronk, J.T., 2019. Reassessment of requirements for anaerobic xylose fermentation by engineered, non-evolved *Saccharomyces cerevisiae* strains. FEMS Yeast Res. 19.
Brat, D., Boles, E., 2013. Isobutanol production from D-xylose by recombinant *Saccharomyces cerevisiae*. FEMS Yeast Res. 13, 241–244.
Brat, D., Boles, E., Wiedemann, B., 2009. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 75, 2304–2311.
Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., Hieter, P., 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene 110, 119–122.
Conant, G.C., Wolfe, K.H., 2006. Functional partitioning of yeast co-expression networks after genome duplication. PLoS Biol. 4, e109.
Crabtree, H.G., 1929. Observations on the carbohydrate metabolism of tumours. Biochem. J. 23, 536–545.
Cunha, J.T., Soares, P.O., Romani, A., Thevelein, J.M., Domingues, L., 2019. Xylose fermentation efficiency of industrial *Saccharomyces cerevisiae* yeast with separate or combined xylose reductase/xylitol dehydrogenase and xylose isomerase pathways. Biotechnol. Biofuels 12, 20.
Dashko, S., Zhou, N., Compagno, C., Piskur, J., 2014. Why, when, and how did yeast evolve alcoholic fermentation? FEMS Yeast Res. 14, 826–832.
Demeke, M.M., Dietz, H., Li, Y., Foulquie-Moreno, M.R., Mutturi, S., Deprez, S., Den Abt, T., Bonini, B.M., Liden, G., Dumortier, F., Verplaetse, A., Boles, E., Thevelein, J. M., 2013. Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol. Biofuels 6, 89.
Demeke, M.M., Foulquie-Moreno, M.R., Dumortier, F., Thevelein, J.M., 2015. Rapid evolution of recombinant *Saccharomyces cerevisiae* for xylose fermentation through formation of extra-chromosomal circular DNA. PLoS Genet. 11.
Dos Santos, L.V., Carazzolle, M.F., Nagamatsu, S.T., Sampaio, N.M., Almeida, L.D., Pirolla, R.A., Borelli, G., Correa, T.L., Argueso, J.L., Pereira, G.A., 2016. Unraveling the genetic basis of xylose consumption in engineered *Saccharomyces cerevisiae* strains. Sci. Rep. 6, 38676.
Feng, Q., Liu, Z.L., Weber, S.A., Li, S., 2018. Signature pathway expression of xylose utilization in the genetically engineered industrial yeast *Saccharomyces cerevisiae*. PloS One 13, e0195633.
Gietz, R.D., Schiestl, R.H., 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. 2, 31–34.
Glerum, D.M., Muroff, I., Jin, C., Tzagoloff, A., 1997. COX15 codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase. J. Biol. Chem. 272, 19088–19094.
Guldener, U., Heck, S., Fielder, T., Beinhauer, J., Hegemann, J.H., 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. 24, 2519–2524.
Hagman, A., Piskur, J., 2015. A study on the fundamental mechanism and the evolutionary driving forces behind aerobic fermentation in yeast. PloS One 10, e0116942.
Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M. F., 2007. Towards industrial pentose-fermenting yeast strains. Appl. Microbiol. Biotechnol. 74, 937–953.
Higgins, D.A., Young, M.K.M., Tremaine, M., Sardi, M., Fletcher, J.M., Agnew, M., Liu, L., Dickinson, Q., Peris, D., Wrobel, R.L., Hittinger, C.T., Gasch, A.P., Singer, S.W., Simmons, B.A., Landick, R., Thelen, M.P., Sato, T.K., 2018. Natural variation in the multidrug efflux pump *SGE1* underlies ionic liquid tolerance in yeast. Genetics 210, 219–234.
Hittinger, C.T., Carroll, S.B., 2007. Gene duplication and the adaptive evolution of a classic genetic switch. Nature 449, 677–681.
Huberts, D.H.E.W., Niebel, B., Heinemann, M., 2012. A flux-sensing mechanism could regulate the switch between respiration and fermentation. FEMS Yeast Res. 12, 118–128.
Jeong, D., Oh, E.J., Ko, J.K., Nam, J.O., Park, H.S., Jin, Y.S., Lee, E.J., Kim, S.R., 2020. Metabolic engineering considerations for the heterologous expression of xylose-catabolic pathways in *Saccharomyces cerevisiae*. PloS One 15, e0236294.

Jin, Y.S., Laplaza, J.M., Jeffries, T.W., 2004. *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. Appl. Environ. Microbiol. 70, 6816–6825.
Jin, Y.S., Lee, T.H., Choi, Y.D., Ryu, Y.W., Seo, J.H., 2000. Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae* containing genes for xylose reductase and xylitol dehydrogenase from *Pichia stipitis*. J. Microbiol. Biotechnol. 10, 564–567.
Johansson, B., Christensson, C., Hobley, T., Hahn-Hagerdal, B., 2001. Xylulokinase overexpression in two strains of *Saccharomyces cerevisiae* also expressing xylose reductase and xylitol dehydrogenase and its effect on fermentation of xylose and lignocellulosic hydrolysate. Appl. Environ. Microbiol. 67, 4249–4255.
Johnston, M., Kim, J.H., 2005. Glucose as a hormone: receptor-mediated glucose sensing in the yeast *Saccharomyces cerevisiae*. Biochem. Soc. Trans. 33, 247–252.
Kayikci, O., Nielsen, J., 2015. Glucose repression in *Saccharomyces cerevisiae*. FEMS Yeast Res. 15.
Kim, S.R., Park, Y.C., Jin, Y.S., Seo, J.H., 2013. Strain engineering of *Saccharomyces cerevisiae* for enhanced xylose metabolism. Biotechnol. Adv. 31, 851–861.
Kotter, P., Ciriacy, M., 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 38, 776–783.
Kuyper, M., Hartog, M.M., Toirkens, M.J., Almering, M.J., Winkler, A.A., van Dijken, J. P., Pronk, J.T., 2005. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res. 5, 399–409.
Kwak, S., Jin, Y.S., 2017. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective. Microb. Cell Factories 16, 82.
Landi, C., Paciello, L., de Alteriis, E., Brambilla, L., Parascandola, P., 2011. Effect of auxotrophies on yeast performance in aerated fed-batch reactor. Biochem. Biophys. Res. Commun. 414, 604–611.
Langdon, Q.K., Peris, D., Kyle, B., Hittinger, C.T., 2018. sppIDer: a species identification tool to investigate hybrid genomes with high-throughput sequencing. Mol. Biol. Evol. 35, 2835–2849.
Lee, J.W., Yook, S., Koh, H., Rao, C.V., Jin, Y.S., 2021. Engineering xylose metabolism in yeasts to produce biofuels and chemicals. Curr. Opin. Biotechnol. 67, 15–25.
Lee, M., Rozeboom, H.J., Keuning, E., de Waal, P., Janssen, D.B., 2020. Structure-based directed evolution improves *S. cerevisiae* growth on xylose by influencing in vivo enzyme performance. Biotechnol. Biofuels 13, 5.
Lee, S.M., Jellison, T., Alper, H.S., 2012. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 78, 5708–5716.
Lee, S.M., Jellison, T., Alper, H.S., 2014. Systematic and evolutionary engineering of a xylose isomerase-based pathway in *Saccharomyces cerevisiae* for efficient conversion yields. Biotechnol. Biofuels 7, 122.
Li, H., 2013. Aligning Sequence Reads, Clone Sequences and Assembly Contigs with BWA-MEM arXiv preprint arXiv:1303.3997.
Lin, Z.G., Li, W.H., 2011. Expansion of hexose transporter genes was associated with the evolution of aerobic fermentation in yeasts. Mol. Biol. Evol. 28, 131–142.
Litsios, A., Ortega, A.D., Wit, E.C., Heinemann, M., 2018. Metabolic-flux dependent regulation of microbial physiology. Curr. Opin. Microbiol. 42, 71–78.
McIlwain, S.J., Peris, D., Sardi, M., Moskvin, O.V., Zhan, F.J., Myers, K.S., Riley, N.M., Buzzell, A., Parreiras, L.S., Ong, I.M., Landick, R., Coon, J.J., Gasch, A.P., Sato, T.K., Hittinger, C.T., 2016. Genome sequence and analysis of a stress-tolerant, wild-derived strain of *Saccharomyces cerevisiae* used in biofuels research. G3-Genes Genom Genet. 6, 1757–1766.
McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M.A., 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303.
Myers, K.S., Riley, N.M., MacGilvray, M.E., Sato, T.K., McGee, M., Heilberger, J., Coon, J. J., Gasch, A.P., 2019. Rewired cellular signaling coordinates sugar and hypoxic responses for anaerobic xylose fermentation in yeast. PLoS Genet. 15, e1008037.
Narayanaswamy, N., Faik, A., Goetz, D.J., Gu, T., 2011. Supercritical carbon dioxide pretreatment of corn stover and switchgrass for lignocellulosic ethanol production. Bioresour. Technol. 102, 6995–7000.
Niebel, B., Leupold, S., Heinemann, M., 2019. An upper limit on Gibbs energy dissipation governs cellular metabolism. Nat Metab 1, 125–132.
Osiro, K.O., Borgstrom, C., Brink, D.P., Fjolnisdottir, B.L., Gorwa-Grauslund, M.F., 2019. Exploring the xylose paradox in *Saccharomyces cerevisiae* through in vivo sugar signalomics of targeted deletants. Microb. Cell Factories 18, 88.
Osiro, K.O., Brink, D.P., Borgstrom, C., Wasserstrom, L., Carlquist, M., Gorwa-Grauslund, M.F., 2018. Assessing the effect of d-xylose on the sugar signaling pathways of *Saccharomyces cerevisiae* in strains engineered for xylose transport and assimilation. FEMS Yeast Res. 18.
Parreiras, L.S., Breuer, R.J., Avanasi Narasimhan, R., Higbee, A.J., La Reau, A., Tremaine, M., Qin, L., Willis, L.B., Bice, B.D., Bonfert, B.L., Pinhancos, R.C., Balloon, A.J., Uppugundla, N., Liu, T., Li, C., Tanjore, D., Ong, I.M., Li, H., Pohlmann, E.L., Serate, J., Withers, S.T., Simmons, B.A., Hodge, D.B., Westphall, M. S., Coon, J.J., Dale, B.E., Balan, V., Keating, D.H., Zhang, Y., Landick, R., Gasch, A.P., Sato, T.K., 2014. Engineering and two-stage evolution of a lignocellulosic hydrolysate-tolerant *Saccharomyces cerevisiae* strain for anaerobic fermentation of xylose from AFEX pretreated corn stover. PloS One 9, e107499.
Pauly, M., Keegstra, K., 2008. Cell-wall carbohydrates and their modification as a resource for biofuels. Plant J. 54, 559–568.
Pfeiffer, T., Morley, A., 2014. An evolutionary perspective on the Crabtree effect. Front Mol Biosci 1, 17.
Pronk, J.T., Yde Steensma, H., Van Dijken, J.P., 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12, 1607–1633.
Reider Apel, A., Ouellet, M., Szmidt-Middleton, H., Keasling, J.D., Mukhopadhyay, A., 2016. Evolved hexose transporter enhances xylose uptake and glucose/xylose co-utilization in *Saccharomyces cerevisiae*. Sci. Rep. 6, 19512.
Reyes, L.H., Gomez, J.M., Kao, K.C., 2014. Improving carotenoids production in yeast via adaptive laboratory evolution. Metab. Eng. 21, 26–33.
Runquist, D., Hahn-Hagerdal, B., Bettiga, M., 2009. Increased expression of the oxidative pentose phosphate pathway and gluconeogenesis in anaerobically growing xylose-utilizing *Saccharomyces cerevisiae*. Microb. Cell Factories 8, 49.
Salusjarvi, L., Kankainen, M., Soliymani, R., Pitkanen, J.P., Penttila, M., Ruohonen, L., 2008. Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. Microb. Cell Factories 7, 18.
Sandberg, T.E., Salazar, M.J., Weng, L.L., Palsson, B.O., Feist, A.M., 2019. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. Metab. Eng. 56, 1–16.
Sato, T.K., Tremaine, M., Parreiras, L.S., Hebert, A.S., Myers, K.S., Higbee, A.J., Sardi, M., McIlwain, S.J., Ong, I.M., Breuer, R.J., Avanasi Narasimhan, R., McGee, M.A., Dickinson, Q., La Reau, A., Xie, D., Tian, M., Reed, J.L., Zhang, Y., Coon, J.J., Hittinger, C.T., Gasch, A.P., Landick, R., 2016. Directed evolution reveals unexpected epistatic interactions that alter metabolic regulation and enable anaerobic xylose use by *Saccharomyces cerevisiae*. PLoS Genet. 12, e1006372.
Scalcinati, G., Otero, J.M., Van Vleet, J.R., Jeffries, T.W., Olsson, L., Nielsen, J., 2012. Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption. FEMS Yeast Res. 12, 582–597.
Schwalbach, M.S., Keating, D.H., Tremaine, M., Marner, W.D., Zhang, Y., Bothfeld, W., Higbee, A., Grass, J.A., Cotten, C., Reed, J.L., da Costa Sousa, L., Jin, M., Balan, V., Ellinger, J., Dale, B., Kiley, P.J., Landick, R., 2012. Complex physiology and compound stress responses during fermentation of alkali-pretreated corn stover hydrolysate by an *Escherichia coli* ethanologen. Appl. Environ. Microbiol. 78, 3442–3457.
Sherman, F., 2002. Getting started with yeast. Methods Enzymol. 350, 3–41.
Stoneman, H.R., Wrobel, R.L., Place, M., Graham, M., Krause, D.J., De Chiara, M., Liti, G., Schacherer, J., Landick, R., Gasch, A.P., Sato, T.K., Hittinger, C.T., 2020. CRISpy-Pop: a web tool for designing CRISPR/Cas9-driven genetic modifications in diverse populations. G3-Genes Genom Genet. 10, 4287–4294.
Sun, L., Jin, Y.S., 2021. Xylose assimilation for the efficient production of biofuels and chemicals by engineered *Saccharomyces cerevisiae*. Biotechnol. J. 16, e2000142.
Thompson, D.A., Roy, S., Chan, M., Styczynsky, M.P., Pfiffner, J., French, C., Socha, A., Thielke, A., Napolitano, S., Muller, P., Kellis, M., Konieczka, J.H., Wapinski, I., Regev, A., 2013. Evolutionary principles of modular gene regulation in yeasts. Elife 2, e00603.
Traff, K.L., Otero Cordero, R.R., van Zyl, W.H., Hahn-Hagerdal, B., 2001. Deletion of the *GRE3* aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the *xylA* and *XKS1* genes. Appl. Environ. Microbiol. 67, 5668–5674.
Turner, T.L., Zhang, G.C., Kim, S., Subramaniam, V., Steffen, D., Skory, C.D., Jang, J.Y., Yu, B.J., Jin, Y.S., 2015. Lactic acid production from xylose by engineered *Saccharomyces cerevisiae* without *PDC* or *ADH* deletion. Appl. Microbiol. Biotechnol. 99, 8023–8033.
Verhoeven, M.D., Lee, M., Kamoen, L., van den Broek, M., Janssen, D.B., Daran, J.G., van Maris, A.J., Pronk, J.T., 2017. Mutations in *PMR1* stimulate xylose isomerase activity and anaerobic growth on xylose of engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis. Sci. Rep. 7, 46155.
Wagner, E.R., Myers, K.S., Riley, N.M., Coon, J.J., Gasch, A.P., 2019. PKA and HOG signaling contribute separable roles to anaerobic xylose fermentation in yeast engineered for biofuel production. PloS One 14, e0212389.
Warburg, O., Wind, F., Negelein, E., 1927. The metabolism of tumors in the body. J. Gen. Physiol. 8, 519–530.
Williams, D.L., Ong, R.G., Mullet, J.E., Hodge, D.B., 2019. Integration of pretreatment with simultaneous counter-current extraction of energy sorghum for high-titer mixed sugar production. Front Energy Res 6.
Yamanaka, K., 1969. Inhibition of D-xylose isomerase by pentitols and D-lyxose. Arch. Biochem. Biophys. 131, 502–506.
Zeng, W.Y., Tang, Y.Q., Gou, M., Sun, Z.Y., Xia, Z.Y., Kida, K., 2017. Comparative transcriptomes reveal novel evolutionary strategies adopted by *Saccharomyces cerevisiae* with improved xylose utilization capability. Appl. Microbiol. Biotechnol. 101, 1753–1767.
Zhang, Y., Serate, J., Xie, D., Gajbhiye, S., Kulzer, P., Sanford, G., Russell, J.D., McGee, M., Foster, C., Coon, J.J., Landick, R., Sato, T.K., 2020. Production of hydrolysates from unmilled AFEX-pretreated switchgrass and comparative fermentation with *Zymomonas mobilis*. Bioresour Technol Rep 11, 100517.