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Metabolic engineering of a haploid strain derived from a triploid industrial yeast for producing cellulosic ethanol

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ABSTRACT

Many desired phenotypes for producing cellulosic biofuels are often observed in industrial *Saccharomyces cerevisiae* strains. However, many industrial yeast strains are polyploid and have low spore viability, making it difficult to use these strains for metabolic engineering applications. We selected the polyploid industrial strain *S. cerevisiae* ATCC 4124 exhibiting rapid glucose fermentation capability, high ethanol productivity, strong heat and inhibitor tolerance in order to construct an optimal yeast strain for producing cellulosic ethanol. Here, we focused on developing a general approach and high-throughput screening method to isolate stable haploid segregants derived from a polyploid parent, such as triploid ATCC 4124 with a poor spore viability. Specifically, we deleted the *HO* genes, performed random sporulation, and screened the resulting segregants based on growth rate, mating type, and ploidy. Only one stable haploid derivative (4124-S60) was isolated, while 14 other segregants with a stable mating type were aneuploid. The 4124-S60 strain inherited only a subset of desirable traits present in the parent strain, same as other aneuploids, suggesting that glucose fermentation and specific ethanol productivity are likely to be genetically complex traits and/or they might depend on ploidy. Nonetheless, the 4124-60 strain did inherit the ability to tolerate fermentation inhibitors. When additional genetic perturbations known to improve xylose fermentation were introduced into the 4124-60 strain, the resulting engineered strain (IIK1) was able to ferment a *Miscanthus* hydrolysate better than a previously engineered laboratory strain (SR8), built by making the same genetic changes. However, the IIK1 strain showed higher glycerol and xylitol yields than the SR8 strain. In order to decrease glycerol and xylitol production, an NADH-dependent acetate reduction pathway was introduced into the IIK1 strain. By consuming 2.4 g/L of acetate, the resulting strain (IIK1A) exhibited a 14% higher ethanol yield and 46% lower byproduct yield than the IIK1 strain from anaerobic fermentation of the *Miscanthus* hydrolysate. Our results demonstrate that industrial yeast strains can be engineered via haploid isolation. The isolated haploid strain (4124-S60) can be used for metabolic engineering to produce fuels and chemicals.

1. Introduction

Cellulosic ethanol production by yeast fermentation is a particular challenge because most fermenting microorganisms are unable to ferment each of the sugars that are present in plant cell wall hydrolysates, and lack of many other desirable traits, such as high ethanol productivity and tolerance to environmental stresses (Argueso et al., 2009; Garay-Arroyo et al., 2004). To efficiently produce ethanol

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from cellulosic hydrolysates, yeast must also utilize xylose, which often constitutes up to 40% of the total fermentable sugars in cellulosic hydrolysates, under the presence of a complex mixture of toxic compounds derived from plant biomass pretreatment (Kim et al., 2012b; Tomás-Pejó et al., 2014). Although native *Saccharomyces cerevisiae* cannot ferment xylose, the introduction of heterologous genes encoding a xylose pathway enables it to ferment xylose (Kim et al., 2013a,b; Kotter et al., 1990; Lee et al., 2014; Sarthy et al., 1987; Zhou et al., 2012). However, the xylose-fermenting capability of engineered *S. cerevisiae* varies significantly among different studies, and it was hypothesized that different strain backgrounds might be associated with the phenotypic variations (Kim et al., 2013a). A promising approach is to use industrial yeast strains, which have been employed for large-scale fermentations, as hosts for metabolic engineering (Bera et al., 2011; Demeke et al., 2013a,b; Diao et al., 2013; Fonseca et al., 2011; Hector et al., 2011; Kim et al., 2013a; Matsushika et al., 2009; Zhang et al., 2010). Industrial yeast strains possess many of the desired traits because they have already been subjected to selection in industrial environments. However, it is challenging to introduce designed genetic perturbations into industrial yeast strains because of their complex genetic structure, such as aneuploidy, polyploidy, or other chromosomal rearrangements that lead to poor spore viability (Akao et al., 2011; Argueso et al., 2009; Babrzadeh et al., 2012; Borneman et al., 2011, 2012; Dunn et al., 2012; Liti et al., 2009; Zheng et al., 2012). The versatile genetic systems that have been well-developed in laboratory strains to improve recombination, control mating behavior, and permit use of standard marker selections can be difficult or sometimes not possible to use when engineering an industrial yeast strain.

In the present study, we demonstrate an alternative way to modify an industrial yeast strain to make it easier to manipulate and characterize a phenotype of interest. We selected a polyploid industrial strain, *S. cerevisiae* ATCC 4124, which was originally isolated from a molasses distillery. The *S. cerevisiae* ATCC 4124 strain has been widely used for ethanol fermentation, and a number of studies reported that its engineered strains have industrially attractive phenotypes (Bera et al., 2011; Casey et al., 2010, 2013; Ho et al., 1998; Lau et al., 2010; Wang et al., 2014). Indeed, the present study confirms that the wild-type ATCC 4124 strain possesses a high tolerance to fermentation inhibitors, as well as superior glucose fermentation capability. In addition, one of the most efficient xylose-fermenting *S. cerevisiae* strains was developed from the ATCC 4124 strain (Casey et al., 2013), and the resulting 424 A (LNH-ST) strain has been widely used in many other studies (Bera et al., 2011; Casey et al., 2013; Kim et al., 2013a; Lau et al., 2010). However, the 424 A (LNH-ST) strain was made by multiple integrations of a xylose utilization pathway, and it was never compared to laboratory strains engineered in the same manner. Therefore, in this study, the industrial strain ATCC 4124 is directly compared with the laboratory strain D452-2 by engineering each with a heterologous xylose consumption pathway consisting of xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK). The resulting strain derived from the ATCC 4124 (4124-X123) converted xylose to ethanol more efficiently than the engineered laboratory strain derived from D452-2 (DX123). With all of these attractive phenotypes, the ATCC 4124 strain was selected for further metabolic engineering for xylose fermentation improvement. In order to engineer the industrial strain more efficiently in the laboratory for cellulosic ethanol applications, we isolated a stable haploid derivative of the ATCC 4124 strain. Next, by using genetic engineering and a series of high-throughput phenotypic evaluation, we successfully constructed a strain with better fermentation phenotypes than our best-existing strain (SR8), including increased ethanol yield, lower byproduct yield, and *in situ* detoxification of acetate, which is an inhibitor that is commonly present in cellulosic hydrolysates. Overall, this study demonstrates that deriving a haploid strain from a polyploid industrial parent is a promising approach for developing new metabolic engineering hosts for production of cellulosic biofuels.

2. Materials and methods

2.1. HO gene deletion, Illumina genome sequencing, and read depth analysis

Three different drug resistance markers were used sequentially to delete multiple copies of the *HO* gene in the ATCC 4124 strain. Primers JS1 and JS2 (Table S2) were used to amplify MX4 cassettes, with 80 bp flanking homology to the *HO* coding region, from plasmids pFA6, pAG32, and pAG25 (Goldstein and McCusker, 1999), and targeted deletion was performed by LiAc transformation (Gietz and Schiestl, 2007a, b) and selected on YP (10 g/L yeast extract, 20 g/L peptone) agar plates with 20 g/L glucose (YPD20) containing antibiotics, such as G418 (300 μg/mL), hygromycin B (300 μg/mL), and nourseothricin (120 μg/mL), respectively. Alternatively, an integrative plasmid (pAUR101; Clontech, Mountain View, CA, USA) containing the aureobasidin A resistance gene (AUR1-C) was used to disrupt the *HO* gene, and the resulting strain was selected on YPD20 containing 0.5 μg/mL aureobasidin A.

We constructed single, double, and triple deletions of the *HO* gene in the ATCC 4124 polyploid strain background (Table S1). Both the deletion of the *HO* gene and the presence of additional copies of the *HO* gene were confirmed by PCR analysis of each strain, using primers JS 5 – JS10 (Table S2). Illumina genome sequencing of the *HO* deletion strains was performed with small insert TruSeq libraries at the UC Davis Genome Center. Average read depth (sequencing coverage) of the genome, the *HO* gene, and each drug marker gene was analyzed by CLC Genomics Workbench 7, and the relative read depth of the *HO* and drug marker genes relative to the genome was calculated (Table S3). The *S. cerevisiae* genome R64 (GenBank accession GCF_000146045.2) was used for read mapping and calculation of read depth.

2.2. Strains and plasmids

All strains and plasmids used in this study are summarized in Table S1. The mutant allele (*ura3-52*) from the *S. cerevisiae* D452-2 strain was introduced into the 4124-S60 strain and selected on SC agar plates (synthetic complete media; 7 g/L yeast nitrogen base with ammonium sulfate, 0.77 g/L of complete supplement mixture with appropriate amino acid dropout) with 20 g/L glucose (SCD20) containing 1 g/L 5-fluoroorotic acid (5-FOA) yielding the auxotrophic mutant, 4124-S60u. Next, the xylose-fermenting strains were constructed by integrating the *Xcm*I-linearized pSR6-X123 plasmid containing *Scheffersomyces stipitis XYL1*, *XYL2*, and *XYL3* genes (Kim et al., 2012a) at the *URA3* locus of the 4124-S60u and ATCC4124 strains and selected on YP agar plates containing 40 g/L xylose (YPX40) yielding the S60-X123 and ATCC4124-X123 strains. The strain, S60u-X123, was selected on SCD20 agar plates minus uracil.

Since the S60-X123 and S60u-X123 strains have the identical phenotypes (data not shown), only one strain, S60-X123, was used for further genetic manipulation. The *PHO13* gene was disrupted by using primers Jin407 and Jin408 to amplify the *pho13*Δ::KanMX4 cassette from the genomic DNA of the BY4742 *pho13*Δ strain (clone ID: 13933) of the Yeast Knockout Collection (Open Biosystems). The cassette was introduced into S60-X123 with selection on YPD20 containing 300 μg/mL of G418, and yielding the S60-X123 *pho13*Δ strain. To knockout *ALD6* gene, a PCR fragment was generated using plasmid pAG25 (EUROSCARF) (Goldstein and McCusker, 1999) as the template DNA with primers Jin726 and Jin727, which amplified the nourseothricin (natMX4) resistance marker with additional 40 bp of DNA homologous to the *ALD6* gene. This PCR fragment was used to delete *ALD6* by homologous recombination and selected on YPD20 containing 120 μg/mL nourseothricin, to generate the IIK1 (S60-X123 *pho13*Δ *ald6*Δ) strain. The acetate and xylose co-consuming strain,

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