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Adjustment of Trehalose Metabolism in Wine *Saccharomyces cerevisiae* Strains To Modify Ethanol Yields

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The ability of *Saccharomyces cerevisiae* to efficiently produce high levels of ethanol through glycolysis has been the focus of much scientific and industrial activity. Despite the accumulated knowledge regarding glycolysis, the modification of flux through this pathway to modify ethanol yields has proved difficult. Here, we report on the systematic screening of 66 strains with deletion mutations of genes encoding enzymes involved in central carbohydrate metabolism for altered ethanol yields. Five of these strains showing the most prominent changes in carbon flux were selected for further investigation. The genes were representative of trehalose biosynthesis (*TPS1*, encoding trehalose-6-phosphate synthase), central glycolysis (*TDH3*, encoding glyceraldehyde-3-phosphate dehydrogenase), the oxidative pentose phosphate pathway (*ZWF1*, encoding glucose-6-phosphate dehydrogenase), and the tricarboxylic acid (TCA) cycle (*ACO1* and *ACO2*, encoding aconitase isoforms 1 and 2). Two strains exhibited lower ethanol yields than the wild type (*tps1Δ* and *tdh3Δ*), while the remaining three showed higher ethanol yields. To validate these findings in an industrial yeast strain, the *TPS1* gene was selected as a good candidate for genetic modification to alter flux to ethanol during alcoholic fermentation in wine. Using low-strength promoters active at different stages of fermentation, the expression of the *TPS1* gene was slightly upregulated, resulting in a decrease in ethanol production and an increase in trehalose biosynthesis during fermentation. Thus, the mutant screening approach was successful in terms of identifying target genes for genetic modification in commercial yeast strains with the aim of producing lower-ethanol wines.

Reducing the ethanol yields produced by yeast during the fermentation process has become an important biotechnological focus in the past decade, driven by consumer and industry demand for lower-alcohol wines (1, 2, 3). This demand stems from health issues linked to excessive alcohol consumption, as well as concerns related to wine quality, as high alcohol levels have a masking effect on the flavor and aroma bouquet of wine (4). Other practical problems arise as restrictions are placed on the ethanol content in wines in certain countries and additional taxes are levied according to ethanol concentration (2, 5).

The existing procedures for the removal or reduction of ethanol postfermentation, including spinning cone columns and reverse osmosis, are costly and have a negative impact on wine quality. An alternative solution is the development of yeast strains that produce lower levels of ethanol during fermentation. Several such studies have been attempted in the past, with measured success. Most of these studies have employed metabolic engineering strategies, and Varela et al. (3) have recently evaluated and compared several of these strategies.

Several applications have focused on increasing glycolytic flux to glycerol as opposed to ethanol (6). The target genes include *GPD1* and -2 (7, 8, 9), the *ADH* gene family (10, 11), and the *PDC* gene family (12). However, fermentations conducted with these genetically modified yeast strains are sometimes sluggish and are typically characterized by the formation of unwanted by-products, mostly due to the disruption of redox balance imposed by the genetic modifications.

All approaches have been hampered by our limited knowledge of the global and specific regulation of fermentative metabolism in yeast. The available data are derived from investigations of selected gene expression (13), global gene expression (14, 15, 16), proteomic responses to wine fermentation (17), and metabolite profiling (18), as well as the integration of transcript and proteome data (19, 20) and transcriptome and aroma metabolite data

(21, 22, 23). Very little information pertaining to the link between genetic regulation and metabolite yields, particularly the flux toward ethanol during fermentation, can be derived from these limited data sets. The most insightful gene expression studies in line with this area of interest, all conducted under simulated wine fermentation conditions, have shown that the bulk of glycolytic genes are slowly downregulated as fermentation progresses, with only a few exceptions where isoforms of the same protein are differentially expressed (15, 16). A comparison of gene expression levels of glycolytic genes at the same stage in fermentation showed large variations between genes in this pathway, confirming the complex regulation governing this central metabolic pathway (15). As would be expected under glucose-repressed fermentative conditions, the bulk of tricarboxylic acid (TCA) genes appear to be expressed at low levels during fermentation. Further investigation into metabolic fluxes under simulated wine fermentation conditions (24) drew attention to discrepancies between these fluxes and the corresponding gene expression patterns (15).

In our study, we opted for a novel, untargeted approach to screen for genes which may be candidates for genetic modification in the quest for low-ethanol fermentations. In this endeavor, we made use of the EUROSCARF deletion library, allowing us to select strains with a single deletion in genes involved in the various categories of central carbon metabolism. The 66 selected strains

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were used to ferment synthetic wine must, and the must was analyzed for key metabolites during and at the end of fermentation. For the purpose of this study, central carbohydrate metabolism was defined as the pathways of glycolysis, ethanol production, TCA cycle, oxidative pentose phosphate pathway (OPPP), trehalose and glycogen metabolism, glycerol synthesis and anapleurotic reactions of glycolysis, and the TCA cycle. By using this unbiased initial screen of the 66 deletion mutants, we were able to identify several candidate genes associated with the predetermined parameters of altered ethanol yield. Of these, 5 genes of interest were selected for validation and further in-depth analysis. Based on the results, one gene (*TPS1*) was selected for proof-of-concept validation in an industrial yeast strain. A novel strategy for modifying *TPS1* expression levels in a growth phase-specific manner using promoters of medium strength was implemented. The impact of these constructs was assessed in an industrial wine yeast strain, VIN13. The data show that this strategy of moderately increasing *TPS* gene expression at selected stages during fermentation avoided the complications and unrelated phenotypic effects that are caused by the metabolic burden of multicopy plasmids and highly expressed transgenes. Most importantly, the modified strains also showed higher trehalose accumulation and significantly lower ethanol yields than the corresponding control strains. The strategy therefore adds a useful additional target in achieving lower ethanol yields in wine fermentations. The strategy can be expanded to include additional targets identified in the first- and second-round screens.

MATERIALS AND METHODS

Selection of deletion strains. *Saccharomyces cerevisiae* strains from the EUROSCARF library were selected from the haploid BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) background (Table 1). Homozygous deletion strains for essential genes were selected from the diploid BY4743 (*MATa/MATa his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*) background. For the industrial yeast, the commercial strain VIN13 (Anchor yeast) was used.

Deletion strain verification. All strains from the EUROSCARF library were confirmed either by PCR with gene-specific primers and size graduation on an agarose gel or by sequencing the unique UPTAGs and DOWNTAGs coded in each deletion strain as detailed by the *Saccharomyces* Genome Deletion Project.

Construction of deletion and overexpression laboratory strains. Five genes were selected for further investigation and knocked out in *Saccharomyces cerevisiae* BY4742. Deletion cassettes, including the *KANMX4* cassette, were amplified from the corresponding EUROSCARF strain using the following gene-specific primers (5'-to-3' direction): *TPS1*_f, GTCCAAGCACGTCAGCGCTG, and *TPS1*_r, GTCGCTGTTCACACCGCAT; *TDH3*_f, GAGAACAGGGGCACAAACAGG, and *TDH3*_r, GCGTTCCTATCGGTACAGCC; *ZWF1*_f, GATGCATACTCCGGCGTCTT, and *ZWF1*_r, GGCATCTTCCCCCACCAA; *ACO1*_f, AGAGCCGCAAAAGGGAGGTC, and *ACO1*_r, GACGTTCCGGCTGGA GAAGTC; and *ACO2*_f, CATACAGCTCTCACATCGTAG, and *ACO2*_r, CAACTACGGCTTAACCTCAAGG.

PCR products were integrated into the BY4742 background strain using a standard lithium acetate transformation protocol. Transformation mixes were plated onto agar plates containing yeast extract-peptone-dextrose (YPD) plus the aminoglycoside antibiotic G418 (300 µg · ml⁻¹). Putative colonies were restreaked onto fresh selection plates before strain verification. Verification was done by genomic DNA (gDNA) PCR using a *KANMX4*-specific primer (ACAGTCTTGACGTGCGCAG) and the following gene-specific primers binding downstream from the integration site: *TPS*_i, GTCAGGGGTGATAGCCAT; *TDH3*_i, GGAGCCCGCTTT

TTAAGCT; *ZWF1*_i, GTAGCGCTACTGGAAGCA; *ACO1*_i, GGTGCC TGATTCTCGATTGTG; and *ACO2*_i, CACGCTCTTGAGTCATCGC.

Overexpression of the five genes of interest was achieved by cloning into the multicopy 2µ episomal vector pPVD1. All genes were under the regulation of the *PGK1* promoter. Transformations were carried out using a standard lithium acetate protocol with selection on minimal medium lacking histidine, including 2% (wt/vol) glucose as the carbon source.

Construction of *TPS* overexpression plasmids and transformation of industrial yeast. A centromeric plasmid was constructed by inserting the centromere and ARS sequence from the Ycplac22 plasmid into the pTEF/Zeo expression vector (Invitrogen). Next, the two promoter regions (upstream from the *DUT1* and *GIP2* genes) and the gene of interest (*TPS1*) were cloned from the genomic DNA of the industrial *S. cerevisiae* strain VIN13 and inserted into the centromeric expression vector. The trehalose-6-phosphate synthase (*TPS1*) coding sequence and terminator regions were amplified from VIN13 genomic DNA. Construct integrity was confirmed by PCR and sequencing. The primers used to amplify target sequences were as follows: *TPS1*f, GATCCAGCTGATGACTACGG ATAACGCTAAGG; *TPS1*r, GATCGGCGCCTAACAGCGCTACAGACA GGC; *DUT1*f, GATCGCATGCACTATGTACATACACACGCACC; *DUT1*r, GATCCAGCTGTTGGTTATTTTTGGCTCGCTGTA; *GIP2*f, GATCGCATGCGCTGTCTAGATGCATTTTTCCTCA; and *GIP2*r, GATC CAGCTGTGTGTGCGTTGATGAATCCTAA.

The final constructs were transformed into the VIN13 yeast strain by electroporation (25, 26). Positive transformants were selected by plating on selective medium containing 1 g · liter⁻¹ zeocin. To verify transformants, plasmid isolation from yeast was performed using the Zymoprep yeast plasmid miniprep II kit (Inqaba Biotech, Johannesburg, South Africa). The plasmids isolated from the various transformed strains were used in a back transformation in *Escherichia coli* strain DH5α and plated on zeocin selective medium. Restriction digests of isolated plasmids were also performed to confirm the identities of constructs isolated from the yeast.

Four strains were generated in this part of the study. Two were control strains transformed with the plasmids containing only the *DUT1* or *GIP2* promoter regions (named DUT-control and GIP-control), and two were *TPS1* overexpression strains transformed with plasmids containing the promoter regions as well as the *TPS1* gene (named DUT-TPS and GIP-TPS).

Fermentation conditions. All strains were maintained on YPD plates, and overnight cultures grown in YPD broth. For the initial screen, small-scale fermentations were conducted in 100-ml tubular bottles sealed with a rubber bung and an S-bend airlock. These fermentations were conducted in 80 ml synthetic wine must MS300 (27) with 100, 200, or 250 g · liter⁻¹ initial hexoses as indicated in the text. Glucose and fructose were added in equimolar quantities. All fermentations were inoculated to an initial cell density of an optical density at 600 nm (OD₆₀₀) of 0.1. All fermentations were conducted with at least three independent replicates. The progression of fermentations was monitored by weight loss (indicative of CO₂ generation) and allowed to run to completion at room temperature.

Fermentations conducted with control and transformed industrial yeast strains were carried out in 250-ml Erlenmeyer flasks containing 200 ml MS300 (27) and sealed with rubber bungs and S-bend airlocks. Glucose and fructose were added in equal amounts (125 g · liter⁻¹). Preinoculated cultures were grown overnight at 30°C in YPD broth containing 1 g · liter⁻¹ zeocin and used to inoculate fermentations to a cell density (OD₆₀₀) of 0.1. All fermentations were carried out in triplicate. Zeocin was not included in the must under fermentation to avoid metabolic impacts related to stress imposed by high antibiotic concentrations. Fermentations were monitored by weight loss, and cell proliferation was determined by OD₆₀₀ readings (Shimadzu UV-1601PC UV-visible scanning spectrophotometer; Shimadzu, Japan).

Real-time PCR. To verify gene expression in the transformed and control strains, RNA isolations were performed on samples taken at time

TABLE 1 Deletion mutations of strains from the EUROSCARF collection used to screen for lower ethanol yield under simulated wine fermentation conditions

Category, protein	Standard name of gene	Systematic name of gene	EUROSCARF strain ^a
Glycolysis			
Hexokinase I	<i>HXK1</i>	YFR053c	Y15867
Hexokinase II	<i>HXK2</i>	YGL253w	Y14620
Phosphofructokinase I	<i>PFK1</i>	YGR240c	Y15893
Phosphofructokinase II	<i>PFK2</i>	YMR205c	Y10791
Glyceraldehyde-3-phosphate dehydrogenase I	<i>TDH1</i>	YJL052w	Y11371
Glyceraldehyde-3-phosphate dehydrogenase II	<i>TDH2</i>	YJR009c	Y16806
Glyceraldehyde-3-phosphate dehydrogenase III	<i>TDH3</i>	YGR192c	Y14822
Pyruvate kinase II	<i>PYK2</i>	YOR347c	Y11644
Anapleurotic reactions			
Pyruvate decarboxylase I	<i>PDC1</i>	YLR044c	Y12655
Pyruvate decarboxylase V	<i>PDC5</i>	YLR134w	Y14091
Pyruvate decarboxylase VI	<i>PDC6</i>	YGR087c	Y14717
Pyruvate carboxylase 1	<i>PYC1</i>	YGL062w	Y14429
Pyruvate carboxylase 2	<i>PYC2</i>	YBR218c	Y13358
Phosphoenolpyruvate carboxykinase	<i>PCK1</i>	YKR097w	Y16013
Fermentation			
Alcohol dehydrogenase I	<i>ADH1</i>	YOL086c	Y16236
Alcohol dehydrogenase II	<i>ADH2</i>	YMR303c	Y10891
Alcohol dehydrogenase III	<i>ADH3</i>	YMR083w	Y16217
Alcohol dehydrogenase IV	<i>ADH4</i>	YGL256w	Y14623
Alcohol dehydrogenase V	<i>ADH5</i>	YBR145w	Y13284
Aldehyde dehydrogenase VI (major cytoplasmic)	<i>ALD6</i>	YPL061w	Y12767
Aldehyde dehydrogenase IV (major mitochondrial)	<i>ALD4/ALD3</i>	YMR169c	Y10752
Aldehyde dehydrogenase V (minor mitochondrial)	<i>ALD5/ALD2/ALD3</i>	YMR170c	Y10753
Aldehyde dehydrogenase VII	<i>ALD7</i>	YOR374w	Y11671
Trehalose metabolism			
Trehalose-6-phosphate synthase in TPS complex	<i>TPS1</i>	YBR126c	Y13265
Trehalose-6-phosphate phosphatase in TPS complex	<i>TPS2</i>	YDR074w	Y16692
Trehalose synthase long chain in TPS complex	<i>TSL1</i>	YML100w	Y16498
TPS regulatory unit	<i>TPS3</i>	YMR261c	Y10847
Neutral trehalase	<i>NTH1</i>	YDR001c	Y13941
Acid trehalase	<i>ATH1</i>	YPR026w	Y17145
Glycogen metabolism			
Glycogen synthase initiator	<i>GLG2</i>	YJL137c	Y17003
Glycogen synthase 1	<i>GSY1</i>	YFR015c	Y15694
Glycogen synthase 2	<i>GSY2</i>	YLR258w	Y15167
Glycogen branching enzyme	<i>GLC3</i>	YEL011w	Y16388
Sporulation-specific glycoamylase	<i>SGA1</i>	YIL099w	Y12258
Glycogen phosphorylase 1	<i>GPH1</i>	YPR160w	Y15575
Glycogen debranching enzyme 1	<i>GDB1</i>	YPR184w	Y15599
Hexose phosphate and OPPP			
Phosphoglucosyltransferase 1	<i>PGM1</i>	YKL127w	Y14977
Phosphoglucosyltransferase 2	<i>PGM2</i>	YMR105c	Y16545
Homologous to UDP-glucose pyrophosphorylase		YHL012W	Y10951
Glucose-6-phosphate dehydrogenase	<i>ZWF1</i>	YNL241c	Y11971
Transketolase	<i>TKL1</i>	YPR074c	Y15493
Glycerol metabolism			
Glycerol kinase 1	<i>GUT1</i>	YHL032c	Y10931
Glycerol kinase 2	<i>GUT2</i>	YIL155c	Y12314
Glycerol-3-phosphate dehydrogenase 1	<i>GPD1</i>	YDL022w	Y13718
Glycerol-3-phosphate dehydrogenase 2	<i>GPD2</i>	YOL059w	Y11751

(Continued on following page)

TABLE 1 (Continued)

Category, protein	Standard name of gene	Systematic name of gene	EUROSCARF strain ^a
TCA metabolism			
Citrate synthase 1	<i>CIT1</i>	YNR001c	Y15376
Citrate synthase 2	<i>CIT2</i>	YCR005c	Y13485
Citrate synthase 3	<i>CIT3</i>	YPR001w	Y12828
Aconitase 1	<i>ACO1</i>	YLR304c	Y15212
Aconitase 2	<i>ACO2</i>	YJL200C	Y17022
Isocitrate dehydrogenase NAD ⁺ subunit 1	<i>IDH1</i>	YNL037c	Y15362
Isocitrate dehydrogenase NAD ⁺ subunit 2	<i>IDH2</i>	YOR136w	Y12392
α-Ketoglutarate dehydrogenase (KGDH) complex component	<i>KGD1</i>	YIL125w	Y12284
Dihydrolipoyl transsuccinylase α-KGDH complex component	<i>KGD2</i>	YDR148c	Y13506
α-Subunit of succinyl-coenzyme A (CoA) ligase	<i>LSC1</i>	YOR142w	Y12398
β-Subunit of succinyl-CoA ligase	<i>LSC2</i>	YGR244c	Y15897
Diploids (essential genes)			
Glucose-6-phosphate isomerase	<i>PGI1</i>	YBR196c	Y23336
Aldolase	<i>FBA1</i>	YKL060c	Y24909
Triosephosphate isomerase	<i>TPI1</i>	YDR050c	Y23986
3-Phosphoglycerate kinase	<i>PGK1</i>	YCR012w	Y23492
Phosphoglycerate mutase	<i>GPM1</i>	YKL152c	Y25002
Enolase I	<i>ENO1</i>	YGR254w	Y27286
Pyruvate kinase I	<i>PYK1/CDC19</i>	YAL038w	Y20368
Pyruvate decarboxylase	<i>PDC2</i>	YDR081c	Y24016
Transcription factor	<i>TOA2</i>	YKL058w	Y24907
UDP-glucose pyrophosphorylase	<i>UGP1</i>	YKL035w	Y24884

^a Strains in all but the last category are all mutants in the haploid BY4742 background. Strains in the last category are mutants in the diploid BY4743 strain.

points (corresponding to days) T_2 , T_5 , T_{11} , and T_{18} to cover the range of different growth phases of the yeast during fermentation. RNA was extracted using the hot phenol extraction protocol (28). RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and integrity assessed on denaturing and nondenaturing gels. The ImProm-II reverse transcription system was used to synthesize cDNA using a random primer set.

Primers were designed using Primer Express software, version 3 (Applied Biosystems), and a KAPA SYBR fast quantitative PCR (qPCR) kit was used to perform quantitative real-time (qRT)-PCR analysis. Spectral data were captured with the Applied Biosystems 7500 cycler (Life Technologies, Foster City, CA, USA). Data analyses were conducted using Signal Detection Software (SDS), version 1.3.1 (Applied Biosystems), to determine the corresponding cycle threshold (C_T) values and PCR efficiencies for the samples analyzed (29). The primer sequences used for qRT-PCR were as follows: TPS1f, TTGCACGCCATGGAAGTG; TPS1r, AACAACTTGCCCTCCATT; ACTf, GCCGAAAGAATGCAAAA GGA; and ACTr, TCTGGAGGAGCAATGATCTTGAC.

Metabolite analysis. Glucose, fructose, glycerol, and ethanol were monitored by high-performance liquid chromatography (HPLC). Components were separated on a Bio-Rad Aminex HPLC-87H (300- by 7.8-mm) column at 55°C with 0.5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 ml · min⁻¹. Data are represented as either concentration (g · liter⁻¹) or yield (g · g_{sugar consumed}⁻¹ × 100). All data are reported as the means of at least three replicates ± standard deviations.

Trehalose extraction (30) and quantification were performed at selected time points during fermentation by harvesting 5 ml of fermentation culture. The cells were dried and weighed. For the extraction, 500 μl of 0.25 M Na₂CO₃ was added for every 25 mg of cells. The buffer/cell mixture was vortexed and incubated at 95°C for 20 min, followed by centrifugation to pellet cell residues. Supernatants were analyzed for trehalose using the Megazyme trehalose assay kit (Megazyme International, Ireland) according to the supplier's specifications.

RESULTS

Initial screen for altered ethanol yield. Sixty-six single-deletion mutants (in the haploid background BY4742 with the exception of essential genes, in which case, the diploid BY4743 background deletion was used; all from EUROSCARF) were selected with the aim of identifying steps in central carbohydrate metabolism that could potentially cause changes in carbon flux and metabolite yields under simulated wine fermentation conditions (synthetic grape must containing 100 g · liter⁻¹ glucose and 100 g · liter⁻¹ fructose) (Table 1). Of particular interest were strains with dual abilities to consume all sugars and increase flux either toward or away from ethanol. Deletion mutants in the BY4742 genetic background showed huge variations in improved or inferior abilities to yield ethanol (Fig. 1) and utilize sugars (Fig. 2). The diploid strains (BY4743) performed well with regard to consuming glucose and fructose.

A number of knockouts led to higher ethanol yields. Of these, three genes showing the most extreme yield increases were selected for further analysis (*zwf1Δ*, *aco1Δ*, and *aco2Δ*). Only 7 gene knockouts led to fermentations with lower ethanol yields. Of these, the most interesting two (*tdh3Δ* and *tps1Δ*) were selected for preliminary follow-up. All five strains fermented more sugars than the wild type, with the *tdh3Δ* strain fermenting completely to dryness (Fig. 1 and 2). For these genes, knockouts were generated in the wild-type BY4742 background to confirm phenotypes. Fermentations with these strains were repeated, and the results shown in Fig. 1 and 2 were reproduced using the newly generated knockout strains (see Table S1 in the supplemental material).

Overexpression of selected genes of interest. Overexpression of the five selected genes of interest (*TDH3*, *ZWF1*, *ACO1*, *ACO2*,

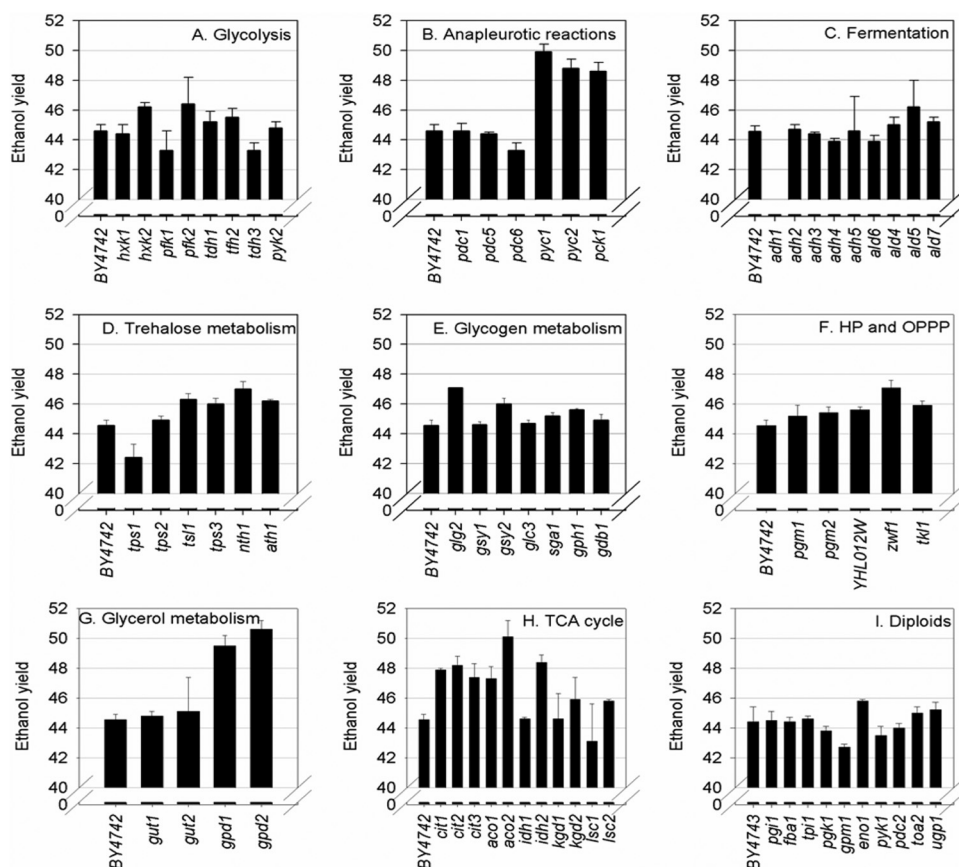


FIG 1 Ethanol yield ($\text{g ethanol} \cdot \text{g}^{-1} \text{ sugar consumed} \times 100$) of EUROSCARF deletion strains fermented in synthetic grape must ($100 \text{ g} \cdot \text{liter}^{-1}$ glucose and $100 \text{ g} \cdot \text{liter}^{-1}$ fructose). (A to H) Results for indicated deletion mutant strains in the haploid BY4742 background. The carbon metabolism category is indicated for each panel. (I) Results for indicated deletion mutant strains in the diploid BY4743 background.

and *TPS1*) was carried out in wild-type BY4742 and BY4742 $\Delta tps1::KANMX4$ genetic backgrounds. As the *aco1*, *aco2*, and *zwf1* deletions led to increased ethanol yields, it was necessary to investigate whether their overexpression could present the opposite phenotype of decreased ethanol yields. For this reason, these genes were overexpressed in the wild-type BY4742 genetic background, as well as in the *tps1* and *tdh2* deletion mutants (which showed the lowest ethanol yields). It was hypothesized that the combined effect of the overexpression and deletion mutations, both of which potentially have a reductive effect on ethanol yields, could reduce the ethanol yield even further. The overexpression of the *TPS1* and *TDH3* genes in their respective null backgrounds served as a control. Only *TDH3* and *TPS1* were overexpressed in the BY4742 $\Delta tdh3::KANMX4$ strain. Genes were overexpressed under the control of the *PGK1* promoter in a multicopy 2μ plasmid (pPVD1). This system was chosen to represent the extreme opposite of a complete deletion of the gene. Fermentations were performed with an initial sugar concentration of $100 \text{ g} \cdot \text{liter}^{-1}$ and allowed to run to completion before analysis of the final parameters (Table 2). All data were calculated relative to the results for the empty-plasmid control.

It was evident that the plasmid itself had an effect on fermentation characteristics, as seen by the difference between the results for the background strain and the empty-plasmid control. For this reason, comparisons were made between overexpressing strains

and the empty-plasmid control as an indication of the effect of the overexpressed gene on metabolism. The two most-significant phenotypes from the overexpression set were those of the *TDH3* and *TPS1* mutants. Both strains demonstrated a reduced fermentation capacity (see Fig. S1 in the supplemental material) as defined by lower rates of CO_2 production. The BY4742:pPVD1-*TPS1* strain was the only one to render a significantly lower ethanol yield (Table 2). All overexpression strains in the wild-type and *tps1* Δ background, with the exception of BY4742:pPVD1-*ACO2*, had higher residual sugar contents at the end of fermentation. None of the overexpression strains in the *tdh3* Δ background showed significant changes in carbohydrate levels at the completion of fermentation.

Overexpression of the *TPS1* gene in commercial yeast. Based on the results of the mutant screen in the haploid laboratory yeast, several genes would be potential targets for future modification in wine yeast. *TPS1* was selected for overexpression in an industrial yeast genetic background (strain VIN13) as proof of concept to extend the results from experiments conducted with the laboratory strains to diploid wine yeast strains used in industry. For this purpose, two different low-strength promoters were selected to drive the expression of the transgene. The promoters (*DUT1* and *GIP1*) drive gene expression during early exponential growth of the yeast and early stationary phase, respectively. The relative levels of gene expression were determined at four time points during

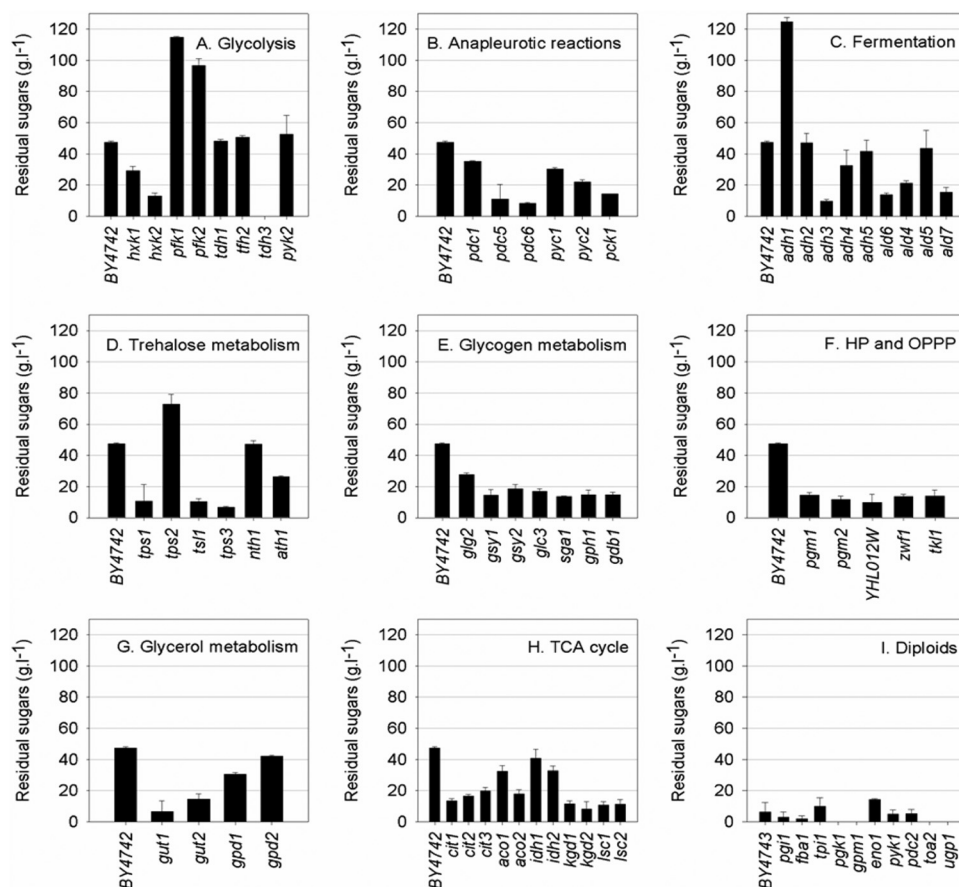


FIG 2 Residual sugar (g · liter⁻¹) in EUROSCARF deletion strains fermented in synthetic grape must (100 g · liter⁻¹ glucose and 100 g · liter⁻¹ fructose). (A to H) Results for indicated deletion mutant strains in the haploid BY4742 background. The carbon metabolism category is indicated for each panel. (I) Results for indicated deletion mutant strains in the diploid BY4743 background.

fermentation: T_2 , T_5 , T_{11} , and T_{14} (Fig. 3). The first time point, T_2 , is representative of the exponential growth phase. A significant (>2-fold) overexpression of the *TPS1* gene under the control of the *DUT1* promoter was observed during the exponential growth phase compared to its expression in the wild type (VIN13) and the DUT control (plasmid containing promoter only). The second time point, T_5 , is representative of early stationary phase, and a significant overexpression of the *TPS1* gene under the control of the *GIP2* promoter is observed. As the fermentations were conducted in the absence of selection pressure (i.e., without zeocin), the plasmid retention was assayed at the end of fermentation and found to be upward of 95%.

Primary fermentation kinetics of *TPS1*-overexpressing VIN13 strains. Samples of the fermentation must were taken at various time points during fermentation to determine the fermentation kinetics of transformed strains. The glucose consumption curves for all transformed and control strains appeared similar, while the transformed strains displayed slightly lower rates of fructose utilization (see Table S2 in the supplemental material). All *TPS1*-overexpressing strains, as well as the plasmid-only transformed strains, showed significant increases in glycerol levels (Fig. 4). The DUT-TPS and GIP-TPS test strains showed significantly reduced ethanol levels, as well as reduced ethanol yields, at the end of fermentation (Fig. 4). While the ethanol yields were close to or slightly over the theoretical maximum ethanol yield of 0.5 for

some data points, the trends were consistent between independent biological repeats within experiments and between sequential repeats of entire fermentation sets.

Trehalose production by *TPS1*-overexpressing strains. The trehalose levels showed typical trends throughout fermentation, increasing as fermentation progressed through to the stationary growth phase of the yeast and subsequently declining toward the end of fermentation. Sampling for trehalose was also carried out at time points T_2 , T_5 , T_{11} , and T_{18} . At T_2 , the DUT-TPS strain showed significantly higher levels of trehalose production than all other strains and the wild-type control (Fig. 5). This increase in trehalose in the DUT-TPS strain persisted throughout fermentation, though the difference was less obvious and not statistically significant for the T_{18} time point. The trehalose levels in the GIP-TPS strain increased beyond the wild-type levels by T_5 (Fig. 5), aligning with the increase in TPS gene expression observed for this time point (Fig. 3). This increase appeared to be maintained throughout fermentation, as the increases in trehalose levels for this strain (relative to the levels in the wild-type and empty plasmid controls) were statistically significant at all subsequent sampling points.

DISCUSSION

Screening for reduced ethanol yield. Identification of key steps in the pathways of central carbohydrate metabolism contributing to

TABLE 2 Final fermentation characteristics of overexpressing strains

Strain	Mean concn (g · liter ⁻¹) or yield (g · g _{sugar consumed} ⁻¹ × 100) ± SD ^a				
	Residual sugars	Glycerol	Glycerol yield	Ethanol	Ethanol yield
BY4742	0.90 ± 0.03	1.03 ± 0.08	1.02 ± 0.08	1.21* ± 0.00	1.20* ± 0.00
BY4742:pPVD1	1.00 ± 0.22	1.00 ± 0.12	1.00 ± 0.14	1.00 ± 0.06	1.00 ± 0.09
BY4742:pPVD1->ZWF1	1.18 ± 0.07	1.05 ± 0.06	1.07 ± 0.07	1.03 ± 0.06	1.05 ± 0.07
BY4742:pPVD1-TDH3	1.36* ± 0.07	0.76* ± 0.11	0.79 ± 0.11	0.93 ± 0.06	0.96 ± 0.07
BY4742:pPVD1-ACO1	1.21 ± 0.14	0.95 ± 0.04	0.97 ± 0.05	1.04 ± 0.04	1.06 ± 0.06
BY4742:pPVD1-ACO2	0.97 ± 0.08	1.03 ± 0.07	1.02 ± 0.08	1.08 ± 0.04	1.07 ± 0.04
BY4742:pPVD1-TPS1	1.29* ± 0.09	0.73* ± 0.04	0.75* ± 0.04	0.88* ± 0.01	0.90* ± 0.01
BY4742 <i>tps1</i> Δ	0.96 ± 0.33	0.90 ± 0.11	0.90 ± 0.10	1.06 ± 0.06	1.06 ± 0.06
BY4742 <i>tps1</i> Δ:pPVD1	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.07
BY4742 <i>tps1</i> Δ:pPVD1-ZWF1	1.60 ± 0.53	0.91 ± 0.13	0.94 ± 0.12	0.95 ± 0.07	0.97 ± 0.05
BY4742 <i>tps1</i> Δ:pPVD1-TDH3	2.11* ± 0.64	0.79* ± 0.09	0.83* ± 0.07	0.94 ± 0.05	1.00 ± 0.06
BY4742 <i>tps1</i> Δ:pPVD1-ACO1	2.06* ± 0.60	0.97 ± 0.13	1.03 ± 0.11	1.02 ± 0.05	1.08 ± 0.07
BY4742 <i>tps1</i> Δ:pPVD1-ACO2	1.72 ± 1.08	0.92 ± 0.09	0.95 ± 0.05	1.04 ± 0.06	1.09 ± 0.10
BY4742 <i>tps1</i> Δ:pPVD1-TPS1	1.26 ± 0.50	0.86 ± 0.16	0.86 ± 0.14	0.98 ± 0.08	0.99 ± 0.06
BY4742 <i>tdh3</i> Δ	0.49 ± 0.10	1.33 ± 0.06	1.25 ± 0.05	1.33 ± 0.03	1.25 ± 0.03
BY4742 <i>tdh3</i> Δ:pPVD1	1.00 ± 0.11	1.00 ± 0.13	1.00 ± 0.14	1.00 ± 0.09	1.00 ± 0.10
BY4742 <i>tdh3</i> Δ:pPVD1-TDH3	1.01 ± 0.14	1.09 ± 0.13	1.09 ± 0.13	1.13 ± 0.06	1.13 ± 0.07
BY4742 <i>tdh3</i> Δ:pPVD1-TPS1	0.85 ± 0.14	1.16 ± 0.14	1.14 ± 0.15	1.01 ± 0.07	0.99 ± 0.09

^a Fermentations were started at 100 g · liter⁻¹ sugar in a synthetic grape must. All results were calculated relative to the results for the empty plasmid control (pPVD1). Values were normalized to those of the empty-plasmid control. *, value is significantly different from the value for the empty plasmid control.

ethanol yield could be achieved by various approaches. As a first attempt, one could choose genes whose transcripts or enzymes have been highlighted by one of the -omics approaches mentioned in the introduction. However, these are not without their challenges, particularly considering that comparing data sets from studies of different yeast strains may result in incorrect selections. Second, in this *in silico* age, it is appealing to pursue a modeling and prediction technique. A kinetic model of yeast glycolysis has been successfully constructed and applied to aerobic, glucose-limited cultures (31). Although this model is of great use, its experi-

mental growth conditions are far removed from those in wine fermentations. Multiple parameters would need to be determined experimentally and substituted into the model toward the purpose of identifying key steps in the regulation of flux toward ethanol in wine fermentations. Therefore, the current study followed a different strategy: the availability of the EUROSCARF deletion library has allowed for a significant amount of research to be carried out in the global yeast community. Strains with gene deletions in central carbohydrate metabolism (Table 1) were systematically selected with a view to identifying key steps regulating flux toward

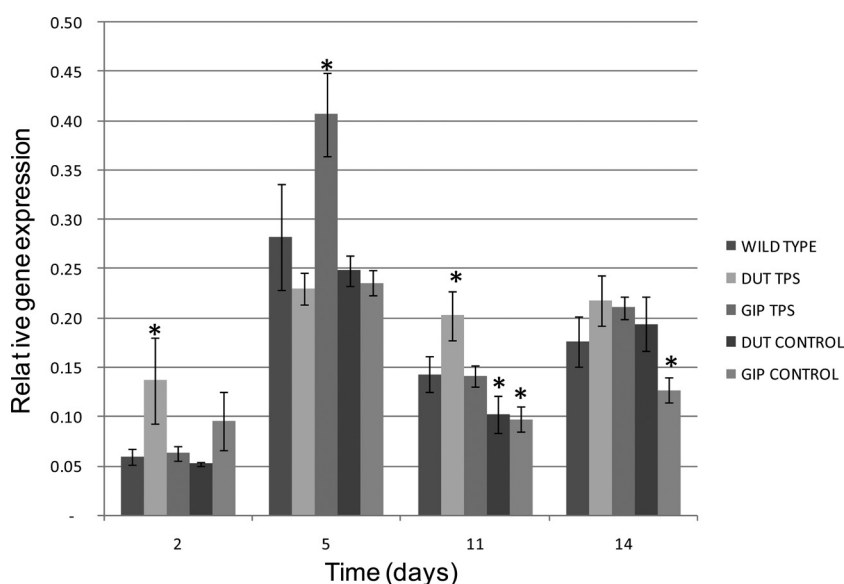


FIG 3 Relative gene expression levels at different stages of wine fermentation. Values are the average of three biological repeats ± standard deviation. *, $P < 0.05$ (95% confidence).

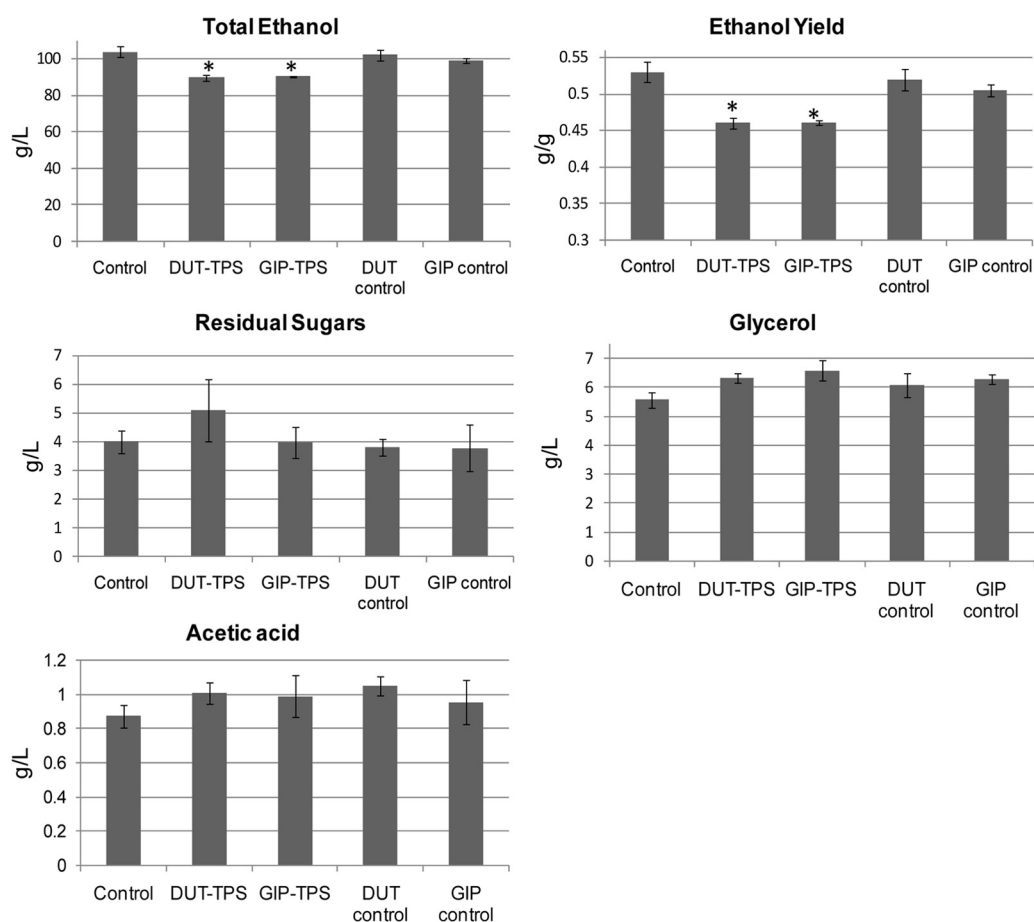


FIG 4 Ethanol, acetic acid, and glycerol concentrations at the end of fermentation (T_{18}). Sugar utilization and ethanol yield as determined at the end of fermentation are also depicted. Values are the average of three biological repeats $\pm$ standard deviation. The Student t test was used to establish significant differences between fermentations conducted with the *TPS1*-overexpressing strains and their respective empty plasmid controls. *, $P < 0.05$ (95% confidence).

ethanol under simulated wine fermentation conditions. Of particular interest were strains that fermented more sugars than the wild type and had altered ethanol yields.

Under these conditions of high initial sugar concentration, the

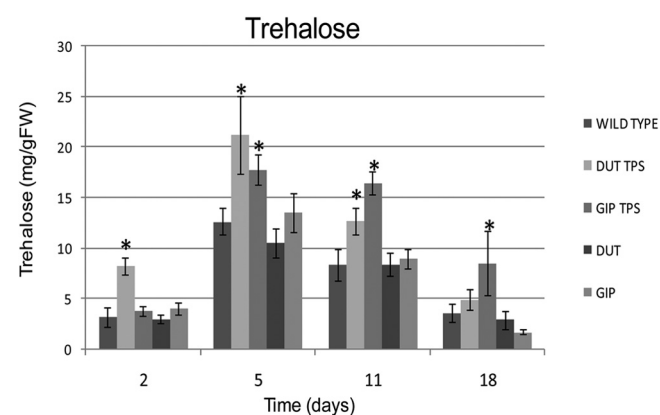


FIG 5 Trehalose levels (normalized relative to cell biomass) at different stages of wine fermentation. Values, calculated as milligrams of trehalose per gram of fresh weight, are the average of three biological repeats $\pm$ standard deviation. *, $P < 0.05$ (95% confidence).

wild-type haploid BY4742 laboratory strain was unable to ferment to dryness (Fig. 2). These poorer fermentation characteristics of BY4742 provided an appropriate background for screening selected single-deletion mutants for improved fermentation characteristics and performance.

The results from small-scale fermentations of all 66 strains showed their differing abilities to yield ethanol (Fig. 1) and ferment hexoses (Fig. 2). Of the haploid strains, there were 5 with significantly lower and 25 with significantly higher ethanol yields. Forty-three fermented significantly more sugar and 5 significantly less sugar than BY4742. The deletion strain yielding the lowest ethanol was the *adh1Δ* knockout. However, this strain could not complete fermentation and was stuck at a residual sugar level of $124.6 \pm 2.8 \text{ g} \cdot \text{liter}^{-1}$ residual sugars. This is easily explained by the facts that *ADH1* is the major cytosolic alcohol dehydrogenase responsible for the production of ethanol during fermentation (11) and that, when it is deleted, the strain grows slowly under anaerobic growth on glucose. Knockout strains with mutations of *TPS1* and *TDH3* had the next-lowest ethanol yields after the *adh1Δ* knockout (Fig. 1). The combined phenotype of lower ethanol yield and improved ability to utilize sugars made these strains attractive for further study. The glycerol levels also differed between the wild-type and knockout strains. The *tps1Δ* strain pro-

duced nearly double the amount of glycerol as BY4742 at the end of fermentation (see Table S1 in the supplemental material). This property could also be an advantage in the wine-making context, as increased glycerol levels could contribute to an overall improvement in sensory quality (32).

Apart from the strains yielding lower ethanol, those yielding higher ethanol were also of interest. It was tentatively hypothesized that overexpressing these genes could reverse the ethanol yield phenotype. Strains selected toward this purpose were representative of the oxidative pentose phosphate pathway (*ZWF1*, encoding glucose-6-phosphate dehydrogenase) and the TCA cycle (*ACO1*, encoding aconitase 1, and its putative isozyme, *ACO2*). Interestingly, both *ZWF1* and *ACO1* had been identified in transcript expression studies (15, 17) as being upregulated under simulated wine fermentation conditions. The phenotypes were confirmed in independent knockout experiments (see Table S1 in the supplemental material), which was particularly pertinent for the *tps1Δ* deletion as this phenotype reportedly varies in different genetic backgrounds (33).

Overexpression of five genes of interest. The five genes selected from the initial screen (*ZWF1*, *TDH3*, *ACO1*, *ACO2*, and *TPS1*) were overexpressed in BY4742 under the control of the *PGK1* promoter in a multicopy plasmid (pPVD1). The empty-plasmid control showed altered fermentation characteristics compared to those of the wild type. This may be a result of the metabolic load of such a multicopy expression system, and in consequence, all comparisons will be discussed relative to the results for this control. None of the overexpression strains presented an opposite ethanol yield phenotype in comparison to the corresponding deletion phenotype (Table 2). In fact, the overexpression strains for *ZWF1*, *ACO1*, and *ACO2* showed no significant changes in any measured fermentation characteristic in the wild-type BY4742 background. This could be attributed to strict post-transcriptional and posttranslational regulation of these metabolic steps. However, both the *TPS1* and *TDH3* overexpression strains fermented at reduced rates and exhibited higher residual sugar levels than the control (Table 2). This was in contrast to their deletion counterparts' improved ability to utilize sugars.

Consequences of altering *TDH3* expression. *Tdh3p* catalyzes the reduction of glyceraldehyde-3-phosphate and is the first reductive step in glycolysis, using NAD^+ as the reducing equivalent. It is one of three isozymes and accounts for between 50 and 60% of glyceraldehyde-3-phosphate dehydrogenase activity (34). It is conceivable that V_{max} was increased due to the overexpression of *TDH3* perturbing the redox balance. The glycerol levels in the *TDH3* overexpression strain were lower than in the control (perhaps as a result of substrate competition at the triose-phosphate level), and the ethanol levels were unchanged (Table 2), indicating that these two pathways were not employed to restore the NAD^+/NADH ratio. This potential redox imbalance may explain the higher residual sugars at the end of fermentation, caused by a slowdown in glycolytic flux. These results highlight the fine control of glycolytic flux by the expression and regulation of the pathway's enzymes in their endogenous state and that perturbations to any one step may affect other steps in an unpredictable manner.

Altered *TPS1* expression causes modifications in flux and carbohydrate distribution. The overexpression of *TPS1* yielded a more-predictable fermentation phenotype. Trehalose-phosphate-synthase 1 (*TPS1*) catalyzes the first step in trehalose biosynthesis, converting UDP-glucose and glucose-6-phosphate to

trehalose-6-phosphate (35). Trehalose-6-phosphate is responsible for the inhibition of hexokinase-mediated phosphorylation of glucose at the entry point of glycolysis. It has been proposed that this regulation is crucial in the control of flux through glycolysis (34). The reduced fermentative capacity of the *TPS1* overexpression strain would suggest that glycolytic flux has been partially inhibited. Although we did not measure trehalose-6-phosphate levels, we hypothesize that they were increased, causing inhibition of Hxkp2 (the major isoform of hexokinase) and, thus, an attenuation in the amount of glucose entering glycolysis. The effect was not purely in reducing overall flux through glycolysis but also in the distribution of carbohydrates. This is evident by reductions in the amounts of both absolute and yielded ethanol and glycerol, both metabolites representing the endpoint of a pathway under fermentative conditions remote from the initial step of hexose assimilation (Table 2).

Regulated *TPS1* overexpression in a commercial yeast. The part of the study discussed here focused on the slight overexpression of the *TPS1* gene under the control of a low-strength promoter (of the *DUT1* gene) that has been linked to gene expression during the exponential growth phase of yeast, as well as the promoter of the *GIP2* gene (linked to gene expression during the stationary growth phase of yeast). The aim was to slightly increase *TPS1* gene expression and enzyme activity without burdening the yeast cell and challenging the redox balance. The expression of *TPS1* under the control of the *DUT1* promoter was up to 2-fold higher than in the wild-type strain (VIN13) during early exponential growth, whereas the expression of *TPS1* under the control of the *GIP2* promoter was up to 60% higher than in the wild type during early stationary phase (Fig. 3). It appears that the expression of *TPS1* both under the control of the *DUT1* promoter during the early exponential growth phase and of the *GIP2* promoter in early stationary phase has a significant metabolic impact in terms of decreasing total ethanol and ethanol yield (Fig. 4). Expression under the control of the *GIP2* promoter seems to reduce the impact on fermentation capacity and the slower sugar utilization to a lesser extent than *DUT1* promoter-driven expression. This might suggest that the expression of the *TPS1* gene during early stationary phase (*GIP2* promoter induced) is more effective and causes less stress to the cell than expression during early exponential growth.

There is also a slight, though not statistically significant increase in glycerol production observed for all transformed strains (Fig. 4). In *S. cerevisiae*, glycerol production plays an important role in stress tolerance and maintaining the intracellular redox balance (36, 37). No increase in acetic acid levels was observed for either of the test strains compared to the levels in their empty plasmid controls (Fig. 4). This is a positive outcome, suggesting that the slight overexpression of the *TPS1* gene by these specific promoters can successfully shift carbon flux with minimal impact on redox balance and without any negative impact in terms of unwanted fermentation by-products.

The trehalose data (Fig. 5) align well with the patterns of *TPS1* overexpression by the transformed strains (Fig. 3). The increases in trehalose levels in these strains show that carbon was likely redirected to this alternative carbon sink in the transformed strains, accounting for the decrease in ethanol yield (Fig. 4). Furthermore, the increased trehalose levels may have inhibited hexokinase activity, thus restricting associated flux through glycolysis. This would explain the reduced fermentation rate and

slightly higher residual sugar levels in fermentations conducted with these strains.

In terms of carbon balance, the difference in ethanol levels is not accounted for entirely by the increase in trehalose. In the first place, it is unlikely that the particular discrete time points that we selected for sampling represent the points of maximum, or optimum, trehalose accumulation. Detailed flux balance analysis is also not feasible when extracellular metabolite measurements (ethanol) need to be compared with intracellular metabolite (trehalose) levels, particularly when the trehalose levels are in constant flux, in contrast to the ethanol levels, which represent a “pooled” end product. In the present study, we did not attempt to determine how much trehalose was reutilized, converted to glycogen and other sinks, or leaked into the medium toward the end of fermentation.

Conclusions. This study has shown that selective screening of a deletion library for altered ethanol yields under simulated wine fermentation conditions is an ideal starting point for the identification and development of *S. cerevisiae* strains with altered flux in central carbohydrate metabolism. The results highlighted that key steps in multiple parts of this network of reactions are responsible for the regulation of flux and the final accumulated metabolite pools. Of the five genes selected for further investigation, alterations in *TDH3* and *TPS1* expression proved to be the most stable, reproducible, and insightful. *TPS1* overexpression was extended to an industrial yeast genetic background, using graded expression in a growth stage-specific vector system. The results from fermentations conducted with these strains show successful (moderate and stage-specific) overexpression of the *TPS1* gene leading to increased trehalose accumulation by these strains. The downstream effect of this modification was the desired metabolic target of reduced final ethanol and ethanol yield. This corroborates the findings of the deletion screen and provides the first example of a successful commercial wine yeast engineering strategy targeting trehalose as a means for lowering ethanol levels. The significant decreases in ethanol in these fermentations were not associated with any of the usual pitfalls related to redox disruption and stuck fermentations, confirming the suitability of the controlled promoter strategy for genetic engineering strategies in wine yeast. The current study was thus successful in terms of validating the screening method for the identification of genes that affect carbohydrate flux, the identification of deletion strains that alter final fermented ethanol yield, and the provision of a platform for application to industrial yeast tailored for lower-ethanol fermentations. The data also suggest that specifically targeting trehalose metabolism as part of a breeding and selection strategy is an option for generating non-genetically modified wine yeast strains with lower ethanol yields. The present study can be extended to include other potential targets identified in the initial mutant screens.

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