Characterizing phenotypic diversity of trehalose biosynthesis mutants in multiple wild strains of *Saccharomyces cerevisiae*

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**Abstract**

In the yeast *Saccharomyces cerevisiae*, trehalose-6-phosphatase synthase (Tps1) and trehalose-6-phosphate phosphatase (Tps2) are the main proteins catalyzing intracellular trehalose production. In addition to Tps1 and Tps2, 2 putative regulatory proteins with less clearly defined roles also appear to be involved with trehalose production, Tps3 and Tsl1. While this pathway has been extensively studied in laboratory strains of *S. cerevisiae*, we sought to examine the phenotypic consequences of disrupting these genes in wild strains. Here we deleted the TPS1, TPS2, TPS3, and TSL1 genes in 4 wild strains and 1 laboratory strain for comparison. Although some tested phenotypes were not shared between all strains, deletion of TPS1 abolished intracellular trehalose, caused inability to grow on fermentable carbon sources and resulted in severe sporulation deficiency for all 5 strains. After examining tps1 mutant strains expressing catalytically inactive variants of Tps1, our results indicate that Tps1, independent of trehalose production, is a key component for yeast survival in response to heat stress, for regulating sporulation, and growth on fermentable sugars. All tps2Δ mutants exhibited growth impairment on nonfermentable carbon sources, whereas variations were observed in trehalose synthesis, thermosensitivity and sporulation efficiency. tps3Δ and tsl1Δ mutants exhibited mild or no phenotypic disparity from their isogenic wild type although double mutants tps3Δ tsl1Δ decreased the amount of intracellular trehalose production in all 5 strains by 17–45%. Altogether, we evaluated, confirmed, and expanded the phenotypic characteristics associated trehalose biosynthesis mutants. We also identified natural phenotypic variants in multiple strains that could be used to genetically dissect the basis of these traits and then develop mechanistic models connecting trehalose metabolism to diverse cellular processes.

**Keywords:** trehalose biosynthesis; *Saccharomyces cerevisiae*; tps1 catalytically inactive alleles; genetic heterogeneity; TPS1; TPS2; TPS3; TSL1

**Introduction**

*Saccharomyces cerevisiae* is one of the most extensively characterized eukaryotes (Botstein and Fink 2011; Duina et al. 2014). However, research on *S. cerevisiae* has historically focused on a limited number of strains, or genetic backgrounds. In particular, derivatives of the strain S288C are some of the most commonly used laboratory strains, including the derivative used to produce the first completely sequenced, assembled, and annotated eukaryotic genome (Mortimer and Johnston 1986; Goffeau et al. 1996; Brachmann et al. 1998; Engel et al. 2014). These laboratory strains have also been used as powerful tools for functional genome analyses (Botstein and Fink 2011; Giaever and Nislow 2014). However, there are many different *S. cerevisiae* strains, and much of the phenotypic variation between strains remains unexplored. Extensive analysis has been performed to examine the nucleotide sequence diversity between *S. cerevisiae* strains from diverse backgrounds and found that the genomes of wild and laboratory strains have significant genetic variability (Dunn et al. 2005; Liti et al. 2009; Borneman et al. 2016; Gallone et al. 2016; Peter et al. 2018). While laboratory strains of *S. cerevisiae* have been, and continue to be, useful tools for understanding fundamental aspects of eukaryotic biology, comparative genetic analysis between wild and lab strains, or simply examining wild strains, can provide further biological insight (Ruderfer et al. 2006; Botstein and Fink 2011; Hittinger 2013; Peter et al. 2018). For example, the list of "essential" genes in *S. cerevisiae* varies somewhat from strain to strain (Chin et al. 2012). Strain-specific phenotypic variations can provide insight into the prevalence of selected phenotypes across strains and provide tools for characterizing the genetic basis of observed phenotypic variance. Trehalose metabolism is an example of a biological process in yeast that has been thoroughly characterized in lab strains; however, an in-depth analysis of phenotypic variations in wild yeasts has yet to be performed (Van Aelst et al. 1993; Gibney et al. 2015, 2020).

Trehalose is a nonreducing disaccharide of α-(1,1)-linked glucose present in many organisms, including bacteria, fungi,
insects, and plants (Elbein et al. 2003). In the yeast S. cerevisiae, trehalose synthesis involves 2 main enzymes: trehalose-6-phosphate synthase (Tps1), which catalyzes the synthesis of trehalose-6-phosphate (T6P), and trehalose-6-phosphate phosphatase (Tps2), which dephosphorylates T6P to trehalose (Fig. 1). The trehalose synthesis complex (TPS) in yeast includes 2 other proteins, Tps3 and Tsl1, which are 2 putative regulatory subunits (Reinders et al. 1997; Bell et al. 1998). The TPS complex was thought to include Tps1 (56 kDa), Tps2 (102 kDa), and Tsl1 (123 kDa), whereas Tps3 (118 kDa) is a paralog of Tsl1 remaining from an ancestral whole genome duplication in the S. cerevisiae lineage and was discovered later (Panek et al. 1987; Vander CAMMEN et al. 1989; LONDONSHOROUGH and VUORIO 1993; DE MESQUITA et al. 2003; KELLIS et al. 2003; VOIT 2003; BYRNE and WOLFE 2005; MAHMUD et al. 2010; TREVISOL et al. 2014). The yeast 2-hybrid approach revealed that there is no interaction between Tps3 and Tsl1, but both interact with Tps1 and Tps2 which, in turn, interact with each other (REINDERS et al. 1997). These observations, along with cofractionation experiments for detecting physical association of proteins, suggest a model where the TPS complex may be stabilized by protein–protein interactions with either Tsl1 or Tps3, though comprehensive testing of this model remains to be performed. Interestingly, all 4 protein components of the TPS complex have blocks of amino acid homology, potentially suggesting a common ancestor (DE VIRGILIO et al. 1993; VUORIO et al. 1993).

Mutants of trehalose biosynthesis genes in S. cerevisiae have been characterized in many previous studies in multiple laboratory strains, including S288C derivatives, and diverse phenotypes have been observed (Gancedo and Flores 2004). For example, tps1 mutants not only lack metabolic ability to synthesize T6P or trehalose but also exhibit several pleiotropic defects including inability to utilize fermentable carbon sources, thermosensitivity, alterations in glycogen levels, and sporulation deficiency (HOTTIGER et al. 1989; GONZÁLEZ et al. 1992; HOHMANN et al. 1993, 1996; VAN AELST et al. 1993; ARGÜELLES 1994; NEVES et al. 1995; SINGER and LINDQUIST 1998a; ENYENIHI and SAUNDERS 2003; GANCEDO and FLORES 2004; SHI et al. 2010; VAN HEERDEN et al. 2014; GIBNEY et al. 2015; LIU et al. 2020). While several hypotheses and metabolic models have been proposed, the molecular mechanism underlying these unrelated phenotypes associated with this mutant remains elusive. A number of observations suggest that components of the trehalose metabolism pathway may have other functions beyond production of trehalose. For example, in the rice blast fungus Magnaporthe grisiæa, Tps1 possesses an independent regulatory function essential for plant infection by the fungus, in addition to T6P synthase enzymatic activity (WILSON et al. 2007). Additionally, in S. cerevisiae, accumulated intracellular trehalose failed to repair many of the phenotypes associated with tps1, suggesting that absence of this disaccharide does not cause these phenotypes (RATNAKUMAR and TUNNACLIFFE 2006; CALAHAN et al. 2011; GIBNEY et al. 2015). Other components in the TPS complex also exhibit diverse phenotypes: similar to tps1 mutants, tps2 mutants are also heat sensitive and sporulation defective, but often exhibit growth defects on non-fermentable sugars (PIPER and LOCKHEART 1988; DE VIRGILIO et al. 1993; THEVELEIN and HOHMANN 1995; ELLIOTT et al. 1996; ENYENIHI and SAUNDERS 2003; GIBNEY et al. 2015). The other 2 proteins involved in trehalose biosynthesis, Tps3 and Tsl1, have been the least studied. In the W303 genetic background, both single mutants of TPS3 or TSL1 decreased activity of Tps1 and Tps2, while trehalose levels were reduced to approximately 50% in the tps3 tsl1 double mutant (BELL et al. 1998).

![Fig. 1. Schematic of trehalose metabolism in S. cerevisiae. Main pathway metabolites are shown in bolded text. Enzymes are shown in gray text. Dashed arrows associated with Tps3/Tsl1 indicate unknown mechanisms associated with supporting Tps1 and Tps2 function. To provide metabolic context, the glycolysis and glycogen synthesis pathways are also indicated.](image-url)
To evaluate phenotypic diversity of trehalose biosynthesis mutants in wild strains compared to lab strains, we deleted each of the 4 biosynthetic genes in 5 different strains. We included 2 commercial wine strains (Simi White and CSM), 1 vineyard isolate (Bb32(3)), 1 oak-tree isolate (YPS1000), and an S288C derivative for comparison. After examining multiple phenotypes for these mutants, we were able to identify common phenotypes shared amongst most strains, in addition to strain-specific variations that could be used for future genetic analysis. To further investigate the pleiotropic phenotypes associated with tps1Δ, we also examined a number of mutant TPS1 alleles that produce protein variants unable to synthesize T6P. Using these alleles, we provide further evidence that a number of the phenotypes associated with trehalose metabolism are not directly related to intracellular trehalose levels. This strengthens the notion that some other aspect of trehalose metabolism has an independent function. Together these results provide a comparison of trehalose biosynthesis mutant phenotypes from diverse strains, which was used to both confirm and expand our understanding of how trehalose metabolism is integrated into the cellular growth, stress response, and signaling network.

Materials and methods

Yeast growth media
Yeast cell growth and standard laboratory manipulations were performed using standard approaches (Botstein and Fink 2011). All media used were either minimal (YNB: 0.67% w/v yeast nitrogen base without amino acids plus 2% w/v indicated carbon sources), or rich (YP: 2% w/v Bacto peptone, 1% w/v yeast extract, 2% w/v indicated carbon sources). Solid media formulations included 2% w/v agar and were poured into standard 10 cm plastic Petri dishes or rectangular plates (Petri 1887). Exceptions are YPGE and SGE media, rich and minimal formulations, respectively, containing 3% w/v glycerol and 2% w/v ethanol as respiratory carbon sources.

Yeast growth
Measurements of cell density were performed by measuring absorbance at 600 nm using a Genesy 6 UV-Vis spectrophotometer (Thermo Fisher). Measurements of cell density and cell size were performed using a Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter) with a 100-μm aperture. For comparative growth assays, cells were spotted onto relevant solid growth media. Cell spotting was performed by dilution of a stationary phase cell culture were removed into microcentrifuge tubes. For the replicates, cell dilutions were spotted onto solid media using a Replica Plater for 96-well Plate, either the 8 x 6 or 12 x 8 array as needed (Sigma-Aldrich). Plates were incubated at indicated temperatures for indicated times as noted in the figures and legends. At least 3 independent biological replicates were performed on different days for spotting assays shown in figures, and a representative image is shown.

Yeast strain construction
The strains used in this study are listed in Supplementary Table 1. Gene deletions were constructed by transforming PCR products amplified from plasmids containing different deletion cassettes: pFA6a-kanMX for kanMX, pAG32 for hphMX, and pAC372 for natAC (Supplementary Table 2). Primers were designed with 40 flanking base pairs identical to the upstream and downstream region of genes to be deleted by homologous recombination. All gene deletions in the S288C background were made by transformation into a diploid to get a heterozygote, which was confirmed by PCR then dissected to get MATα and MATα segreagants before mating together to obtain a homozygous diploid, which was also confirmed by PCR. Similarly, mutant strains constructed from non-S288C strains (Simi White, YPS1000, CSM, and Bb32) were made in the same way although homozygous diplods were obtained directly after dissection (the wild strains are homothallic; spores germinate, then cells switch mating types, and mate with each other to produce colonies that are essentially all diploid cells). All combinatorial gene deletion/insertion strains were made by mating, sporulating, and tetrads dissection. Sporulation was performed by growing cells to log phase in rich media, collecting cells by centrifugation, washing once in 1% w/v potassium acetate, and then resuspending in 1% w/v potassium acetate. Cells were then incubated at room temperature on a roller wheel for at least 6 days before tetrads dissection.

Plasmid construction
Plasmids were built using pRS-series shuttle vector backbones containing the THD3 promoter and CYC1 3’ UTR (Sikorski and Hieter 1989; Mumberg et al. 1995). All inserts were amplified using primers from Integrated DNA Technologies (IDT), and final PCR products contained a 5’-end SpeI and 3’-end XhoI sites to use for restriction enzyme-based confirmation of insertion during cloning (Supplementary Table 2). To construct plasmids containing different tps1 variants (TPS1124V, TPS1Y102V, TPS1W111S, TPS1DISG, and TPS1Y102V, W111S) and tsaA, linearized p416GPD (using SpeI and XhoI) was transformed into ura3Δ yeast cells along with different PCR inserts for each gene/allele containing 40bp of flanking sequence identical to the 3’ end of the THD3 promoter or to the 5’ end of the CYC1 CYC1 3’ UTR present in p416GPD. Plasmids assembled by homologous recombination in yeast after transforming both fragments were then extracted using Zymoprep Yeast Plasmid MiniPrep II kit (Zymo Research). Extracted plasmids were transformed into TOP10 E. coli for storage and insert confirmation. During construction of these mutant-allele-containing plasmids, we inadvertently also constructed an allele containing 2 amino acid substitutions (Y102V and W111S). As this allele would also be predicted to be nonfunctional for T6P synthesis, we decided to include it for further analysis. MKT1 expression plasmids were cloned using Gibson Assembly (Supplementary Table 2) (Gibson et al. 2009). Linearized p426GPD (using SpeI and XhoI) was incubated with MKT1 alleles amplified from their respective strain backgrounds along with the Gibson Assembly mixture at 50°C for 1 h before transforming into TOP10 E. coli. After transformation, individual colonies were screened for correct insertion using restriction digest. All gene insertion and allele-specific mutations were confirmed by Sanger Sequencing at the Cornell Institute of Biotechnology sequencing core facility.

Assessment of thermotolerance
To assess thermotolerance, minimal media cultures were inoculated with a single colony and grown overnight. Cells were then diluted into the same minimal medium to an OD600 = 0.05 and grown another 24 h to stationary phase. Two aliquots of 0.8 ml culture were removed into microcentrifuge tubes. For the heat shock, one of the aliquots was incubated in a 42°C thermomixer for 2 h. Both pre- and post-heat shocked cell dilutions were plated on rich media (YPD or YPgal, depending on strain growth requirements) and incubated at 30°C for 2–3 days to measure viability by counting colony forming units. At least 3 independent biological replicates were performed for each thermotolerance assay.
Measurement of sporulation efficiency

Sporulation was performed by growing cells to log phase in rich media (except cells containing plasmid-based tps1 catalytically inactive alleles which were grown in minimal media), collecting cells by centrifugation, washing twice in 1% w/v potassium acetate, then resuspending in 1% w/v potassium acetate. Cells were then incubated at room temperature on a roller wheel for at least 6 days before calculating % sporulation by counting at least 300 cells. Sporulation efficiency was calculated as the proportion of observed tetrads compared to the total number of observed cells. At least 3 independent biological replicates were performed for each sporulation efficiency assay.

Measurement of trehalose and glycogen

Trehalose and glycogen levels were measured essentially as described (Parrou andFrançois 1997). Briefly, 10 OD600 units of stationary phase cells were isolated, washed in cold water, and resuspended in 250 μl of 0.25 M sodium carbonate. Cell mixtures were then stored at −80°C until ready to perform the assay. To begin the assay, cells were boiled at 95°C for 4 h with occasional agitation—this step extracts the trehalose and glycogen, as both are highly stable and not degraded. Next, 150 μl of acetic acid was added to the sample, followed by 600 μl of 0.2 M sodium acetate. After mixing, 350 μl was removed to a fresh tube, and 5 μl of 70 U/ml trehalase (Megazyme) or 70 U/ml amyloglucosidase (Sigma Aldrich) was added. This was incubated overnight at 37°C or 57°C, respectively, in a thermomixer set at 550 rpm (Eppendorf). Next, the sample was centrifuged at maximum speed for 3 min, and 200 μl of each sample was used to measure the amount of glucose liberated from trehalose or glycogen using the Glucose (GO) Assay Kit (Sigma Aldrich).

Statistical analysis

All experiments were conducted using at least 3 independent biological replicates. Mutants were evaluated for statistical significance compared to their isogenic wild type strains using a paired t test and are presented as the mean and standard deviation. The asterisks (*) indicate the mutant phenotype showed a difference (P < 0.05) compared to its isogenic wild type (P-values were not corrected for multiple hypothesis testing). Separately, to evaluate statistically significant phenotypic differences between the wild type strains examined, or also between strains with identical gene deletions, 1-way ANOVA with post-hoc Tukey HSD tests were performed (Supplementary Tables 3-8). Indicated P-values corresponding to the F-statistic of 1-way ANOVA lower than 0.05, suggest that the one or more evaluated treatments are significantly different.

Results and discussion

Trehalose mutant phenotypes under consideration

Multiple phenotypes have been ascribed to different trehalose mutants. To systematically examine the phenotypic diversity of each trehalose biosynthesis gene deletion mutant (tps1, tps2, TPS3, TSL1) and a double deletion mutant (TPS3 and TSL1), we deleted each gene in 5 different strains of S. cerevisiae. These strains included 2 commercial wine strains (Simi White and CSM), 1 vineyard isolate (Bb32(3)), 1 oak-tree isolate (YPS1000), and an S288C derivative for comparison. Regarding phenotypes, we opted to focus on a core set of relevant phenotypes that could be assayed for each mutant. Because trehalose mutants have variations in both trehalose and glycogen levels, we quantified both. Further, stress sensitivity and sporulation defects are common phenotypes for tps1A and tps2A deletion mutants, so we assessed thermotolerance (42°C for 2 h), ability to grow at an elevated temperature (growth at 37°C), and ability to perform meiosis/sporulation. We also assessed cell size and growth on multiple carbon sources in both rich and minimal medium.

Construction and phenotypic characterization of tps1A

TPS1 encodes the trehalose-6-phosphate synthetase enzyme, which catalyzes the joining of glucose-6-phosphate with the glycosyl unit from UDP-glucose (Fig. 1). Deletion of the TPS1 gene is associated with a variety of growth phenotypes, including failure to grow on fermentable carbon sources (e.g. glucose, fructose), failure to grow at elevated temperatures, failure to sporulate, and higher levels of glycogen accumulation (Gancedo and Flores 2004). Variations in some of these phenotypes, such as carbon source utilization, have been reported previously. For example, while a small subpopulation of S288C tps1A cells have the ability to grow on glucose at roughly 1 in 1,000, deleting TPS1 in W303 genetic background almost completely abolishes their growth on the same sugar (Van Heerden et al. 2014; Gibney et al. 2020). Elucidating the genetic underpinnings regulating such differences will have a significant impact in our understanding of the tested cellular processes.

Trehalose and glycogen levels were both determined using stationary phase cells because the cellular content of both carbohydrates is barely detectable in exponential phase even in wild type cells (Yi et al. 2016). As expected, no trehalose was produced in all 5 tps1A strains whereas the wild type strains showed a variety of intracellular trehalose levels (Fig. 2a, Supplementary Table 4). On the other hand, previous work demonstrated that glycogen levels are increased compared to wild type in S288C, CEN.PK, and other lab tps1A strains (Cannon et al. 1994; Guillou et al. 2004; Shi et al. 2010). One potential explanation is that absence of Tps1 results in excess UDP-glucose being shunted to glycogen production (Fig. 1) (Shi et al. 2010). While most tps1A strains did have significantly higher glycogen levels than wild type, Bb32 tps1A was notable in that glycogen levels for this strain remained identical to wild type cells (Fig. 2b), demonstrating that trehalose and glycogen levels are not necessarily negatively correlated. While characterizing these strains, we also noted a slight increase in cell size in Simi White tps1A and CSM tps1A, 4% and 6% larger, respectively, though no significant differences in cell size were detected in the other strain backgrounds (Supplementary Fig. 2a).

Heat sensitivity of lab tps1A strains has been reported in a number of previous studies (Hottiger et al. 1989; Eleutherio et al. 1993; Arguelles 1994; Singer and Lindquist 1998b; Gibney et al. 2015). Thermotolerance refers to the ability of cells to survive when treated with a lethal heat dosage. Here, thermotolerance of tps1A mutants was assessed by incubating stationary phase cells at 42°C for 2 h. In response to this heat shock, the viability of most tps1A mutants dropped significantly. Interestingly, Bb32 tps1A did not exhibit heat sensitivity and maintained wild type levels of viability (Fig. 2c). Trehalose has been proposed to protect denatured proteins from aggregation and therefore protect cells against thermal stress (Singer and Lindquist 1998a; Jain and Roy 2009). This model for trehalose function is based on a correlation of trehalose production during cellular stress along with direct observation of trehalose-mediated prevention of denatured protein aggregation in vitro (Singer and Lindquist 1998a). However, a direct test of intracellular trehalose as a thermoprotectant
indicated that accumulation of this disaccharide inside cells did not repair tps1Δ thermotolerance defect (Gibney et al. 2015). Similarly, Bb32 tps1Δ cells do not produce trehalose but have wild type levels of thermotolerance, providing further evidence that high intracellular trehalose levels are not required for heat resistance. To further understand this particular phenotype, Bb32 tps1Δ could be used as a tool for future work to dissect the genetic underpinnings of tps1Δ thermosensitivity. In addition to examining acute thermotolerance at a temperature too high for long-term cellular survival, we also examined the ability of these mutants to grow at 37°C—a milder heat stress that each wild type strain can adapt to and grow (Fig. 2f and Supplementary Fig. 3). The viability and/or growth rate of S288C tps1Δ and Simi White tps1Δ were noticeably compromised compared to their wild types, though the tps1Δ mutants in the remaining 3 strains did not exhibit an obvious temperature-sensitive growth phenotype.

Homozygous TPS1 deletion mutants are unable to sporulate in S288C-derived and W303 laboratory strains (Van Aelst et al. 1993; de Silva-Udawatta and Cannon 2001; Enyenihi and Saunders 2001; A. Chen et al. 2017).

**Fig. 2.** tps1Δ mutant phenotypes. a) Intracellular trehalose, b) intracellular glycogen, c) thermotolerance, d) sporulation efficiency, e) growth at 30°C for 2 days, and f) growth at 37°C for 3 days. For panels (e) and (f), indicated strains were grown overnight in YNB + 2% galactose liquid before performing assays as described in Materials and Methods. For plate images, 10-fold serial dilutions were prepared and spotted onto the indicated media. The initial dilution had an OD600 of 1.0. Three biological replicates were performed for all tested phenotypes. Asterisks represent statistical difference (P < 0.05) between the mutants and their isogenic wild types. Strain legend below (c) and (d) applied to (a)–(d).
2003; Gibney et al. 2015; Liu et al. 2020). However, this is not a shared phenotype to all laboratory strains as in the SK1 genetic background, we observed that 60% of tps1Δ mutant cells are still able to sporulate, suggesting that the sporulation defect associated with this mutant may be specific to certain lab strains (Supplementary Fig. 4). In contrast, all 5 tested tps1Δ strains exhibited sporulation defects compared to their isogenic wild type strains which had sporulation efficiencies between 60% and 100% (Fig. 2d). Four out of 5 did not sporulate at all (Fig. 2d).

Roughly 20% of YPS1000 tps1Δ cells were able to sporulate, though this was still significantly lower than the YPS1000 wild type strain (Fig. 2d). One hypothesis to explain this defect is the absence of trehalose. However, Gibney et al. demonstrated that the sporulation defect cannot be fixed by adding intracellular trehalose to an S288C tps1Δ before sporulation (Gibney et al. 2015). The mechanistic basis connecting trehalose metabolism to sporulation remains unexplained, though overexpression of either IME1 or IME2, transcription factors that regulate meiosis, is able to suppress the tps1Δ sporulation defect (de Silva-Udawatta and Cannon 2001). As with thermosensitivity, strain-to-strain variation in this phenotype could be a useful tool for future study to dissect the mechanistic basis of this phenotype.

The most widely studied phenotype of tps1Δ is its inability to grow on rapidly fermentable carbon sources such as glucose and fructose (Navon et al. 1979; González et al. 1992; Stucka and Blázquez 1993; Van Aelst et al. 1993; Hohmann et al. 1994, 1996; Luyten et al. 1995; Neves et al. 1995). Two main models have been historically proposed to explain this deficiency (Thevelein and Hohmann 1995; Walther et al. 2013). First, trehalose-6-phosphate (T6P) slows down glycolysis by inhibiting Hxk2, the major fermentative hexokinase enzyme, and that without Tps1 or T6P, metabolic flux through upper glycolysis consumes more ATP than lower glycolysis can produce, causing cessation of growth (Blázquez et al. 1993; Hohmann et al. 1996). Second, the trehalose pathway is important for releasing inorganic phosphate during fermentation that can be used as a substrate for glyceraldehyde-3-phosphate dehydrogenase in lower glycolysis (Van Aelst et al. 1993; Van Heerden et al. 2014). However, contrary experimental evidence demonstrates that neither of these models can fully explain the observed phenotypes. For example, T6P inhibition of Hxk2 has been demonstrated in vitro, but the in vivo relevance has not been directly examined (Hohmann et al. 1993). Further, overexpression of a T6P-insensitive hexokinase enzyme from Schizosaccharomyces pombe in S. cerevisiae did not affect glucose growth and only had minor effects on the short-term response to glucose exposure (Bonini et al. 2003). Regarding phosphate release for lower glycolysis, tps2Δ mutants also accumulate phosphate in T6P, yet are able to grow well on glucose and fructose (Van Heerden et al. 2014; Gibney et al. 2015). More recent work has implicated persistent decreased intracellular pH associated with glucose-exposed tps1Δ cells as at least partially responsible for the metabolic block at glyceraldehyde-3-phosphate dehydrogenase (van Leemputte et al. 2020). Another recent proposal to explain this fermentable carbon source growth defect is based on the observation that glucose-exposed tps1Δ mutants hyperaccumulate fructose-1,6-bisphosphate, which subsequently activates Ras and triggers apoptosis (Peeters et al. 2017). Taken together, these models and some of the differences between each model indicate a need to develop a more comprehensive model for the phenotypic consequences of trehalose metabolism that can explain observed phenotypes.

When testing carbon source utilization, all 5 tps1Δ strains grew well on media containing respiratory carbon sources galactose (preferred respiration) or glycerol/ethanol (obligate respiration) (Fig. 2e). None of the tps1Δ strains grew on fructose, as observed with most laboratory strains, though notably Bb32 tps1Δ exhibited a small number of cells able to grow in rich, fructose-containing medium. Interestingly, all 5 tps1Δ strains showed varying levels of growth on rich glucose medium, and a few strains showed slight growth on minimal glucose medium (Fig. 2e). We were somewhat surprised by the varying levels of tps1Δ growth observed on glucose. Multiple laboratory strains are completely unable to grow on glucose in rich or minimal media (Van Aelst et al. 1993; Vuorio et al. 1993). In contrast, we and others recently demonstrated a subpopulation of S288C tps1Δ mutant cells able to grow on glucose, that this phenotype is not stably genetically propagated, is enhanced by peptone, and does not confer the ability to grow on fructose (Van Heerden et al. 2014; Gibney et al. 2020). We termed these persister-like cells and demonstrated that robust S288C tps1Δ persister-like cell formation requires a loss-of-function MKT1 allele common to the S288C background (Gibney et al. 2020). To characterize glucose growth in these wild tps1Δ strains, we tested whether glucose growth was stably, genetically propagated to all daughter cells and found persister-like cell behavior instead (Supplementary Fig. 5). Further, similar to persister-like cells, this phenotype is enhanced in rich media and absent in fructose-containing media (Fig. 2e and Supplementary Fig. 5). Finally, we used S288C tps1Δ to evaluate the function of the MKT1 alleles from each yeast strain—functional alleles of MKT1 are able to reduce the frequency of persister-like cell formation in S288C tps1Δ. Each MKT1 allele was fully sequenced, and the corresponding amino acid sequence was compared to the reference strain S288C (Supplementary Fig. 6).

As observed previously, when the W303 allele of MKT1 was introduced to the S288C tps1Δ strain, formation of persister-like cells was 10-fold less than on YPD (Fig. 3a) and was completely abolished on SD (Fig. 3b) (Gibney et al. 2020). Introducing any of the other 4 wild type alleles to S288C also decreased persister-like cell formation in S288C tps1Δ. These results suggest that persister-like cell formation in these strains is regulated by other factors beyond MKT1.

Taken together, it is clear that there are a number of common tps1Δ phenotypes shared among the strains tested. For example, none of the strains accumulated trehalose during stationary phase as expected. Most strains accumulated higher levels of glycogen, were sensitive to acute heat shock, exhibited failed meiosis/sporulation, and were unable to grow on fructose. However, for each phenotype there is at least 1 tps1Δ strain that maintains a wild type phenotype; these exceptions to the phenotypic rule could be useful tools to examine the genetic underpinnings of each phenotype. Finally, we also demonstrated that the surprising level of glucose growth by these tps1Δ mutants is akin to the described persister-like cells. As persister-like cell regulation in these wild strains appears uncoupled to MKT1, our results suggest another regulatory mechanism allows some fraction of tps1Δ cells to survive and thrive in glucose. It is clear that there are still uncharacterized mechanisms connecting the function of Tps1 to associated phenotypes, and perhaps the genetics of nonlaboratory strains will be useful in filling this knowledge gap.

**Variable restoration of tps1Δ phenotypes by metabolically inactive alleles of TPS1**

While the molecular mechanisms underlying the diverse phenotypes of tps1Δ mutants have remained unclear, in addition to the work described above, multiple publications have demonstrated that intracellular trehalose levels are unrelated to a number of these phenotypes. For example, yeast cells provided with
Methods

allele combining 2 of the mutations as described in homologous amino acid positions in [Wilson et al. 2000; Deroover et al. 2016; Vicente et al. 2018]. Each of these alleles was cloned into a low-copy plasmid and expressed in S288C tps1Δ from the strong, constitutive TDH3 promoter.

By measuring trehalose levels, we confirmed that stationary phase tps1Δ cells containing the metabolically inactive alleles exhibited severely impaired trehalose production (Fig. 4a). Only Tps1Y102V produced detectable levels of trehalose, though still significantly lower than wild type or complemented tps1Δ, producing roughly 20% of wild type trehalose levels. Similar to complementation with plasmid-based wild type TPS1, E. coli otsA was able to fully restore trehalose to wild type levels as well (Fig. 4a). On the other hand, glycerol levels did not follow the expected trends. As seen in 4 of 5 wild strains, and previously in lab strains, deletion of TPS1 leads to increased glycerol levels (Fig. 2b) (Parrou et al. 1997). Expression of otsA, tps1ΔR24G, and tps1ΔY102V resulted in wild type glycerol levels, despite the 2 metabolically inactive alleles failing to produce trehalose (Fig. 4b). Expression of tps1ΔD156G, tps1ΔY102V, W111S, and tps1ΔW111S accumulated similar amounts of glycerol to tps1Δ, while tps1ΔW111S produced an intermediate level of glycerol that was significantly higher than wild type (Fig. 4b). These results again suggest that metabolic overflow of the shared substrate UDP-glucose does not fully explain the glycerol accumulation phenotypes observed here. Survival after a 2 h heat shock at 42°C was largely uncorrelated with trehalose or glycerol levels, with expression otsA, tps1ΔR24G, and tps1ΔY102V each partially restoring heat resistance, though still significantly less than wild type levels (Fig. 4e). In contrast to glycerol levels and heat survival, none of the TPS1 alleles were able to fully restore wild type sporulation levels, though otsA expression did increase sporulation efficiency to roughly 30% of wild type levels (Fig. 4d). Notably even wild type has lower sporulation efficiency likely due to pre-sporulation culture conditions: to maintain the plasmids, cells were sporulated from minimal medium cultures. Utilization of fermentable carbon sources demonstrated a similar pattern to glycerol accumulation and heat survival, as expression of otsA, tps1ΔR24G, and tps1ΔY102V exhibited wild type growth on glucose and fructose, unlike the other alleles except for tps1ΔW111S (Fig. 4e). Expression of tps1ΔW111S resulted in an intermediate growth phenotype on glucose and fructose, similar to its intermediate phenotype in glycerol accumulation, despite maintaining a null phenotype for trehalose accumulation and heat survival. Finally, we also examined each allele for the ability to restore wild type growth rates at slightly elevated temperatures (37°C and 39°C). Only expression of otsA was able to restore high temperature growth to levels similar to wild type and complemented tps1Δ (Fig. 4f). Additionally, our results suggest that the cif1 allele of TPS1, tps1Δ-W183*, is nonfunctional and that previous reports of variable growth on glucose may be more related to the tps1Δ persister-like phenotype than to any residual metabolic activity of the cif1 allele (Fig. 4) (Bell et al. 1992; Stucka and Blázquez 1993). Taken together, as seen in M. grisea, multiple metabolically inactive alleles of TPS1 are able to partially or fully complement a number of tps1Δ phenotypes despite inability to produce trehalose (Table 1). Further, expression of otsA results in production of wild type levels of trehalose, restored growth on fructose, but is

intracellular trehalose fail to restore wild type carbon source utilization, sporulation, or heat resistance (Gibney et al. 2015). Though notably intracellular trehalose promotes high levels of desiccation tolerance, suggesting that intracellular trehalose does play roles in stabilization of biomolecules in conditions of low water activity (Tapia et al. 2015). In the yeast Magnaporthe grisea, catalytically inactive alleles of Tps1 unable to produce T6P complemented phenotypes associated with tps1Δ, further suggesting that at least some tps1Δ phenotypes are unrelated to trehalose content (Wilson et al. 2007). We demonstrate that variations in trehalose levels (Fig. 2a) among different yeast strains also fail to correlate with cell type (Fig. 2c), sporulation efficiency (Fig. 2d), or carbon source utilization (Fig. 2e).

To further investigate the pleiotropic nature of tps1Δ phenotypes in S. cerevisiae, we took advantage of multiple, published allelic variations and tested each of these alleles for their ability to complement loss-of-function phenotypes associated with tps1Δ in our laboratory strain of yeast. These catalytically inactive alleles of Tps1 were originally generated in M. grisea to disrupt 4 key residues (R22G, Y99V, W108S, and D153G) required for interaction with glucose-6-phosphate in the catalytic site of the enzyme (Wilson et al. 2007). We constructed each of those 4 alleles at the homologous amino acid positions in S. cerevisiae, along with a fifth allele combining 2 of the mutations as described in Materials and Methods (tps1ΔR24G, tps1ΔY102V, tps1ΔW111S tps1ΔD156G, and tps1ΔY102V, W111S). For example, based on amino acid alignment, R22 in M. grisea corresponds to R24 in S. cerevisiae. We also included a truncated allele of TPS1 by inserting a stop codon at amino acid 183, resulting in a C-terminal truncation that removes 63% of the Tps1 protein (tps1ΔW183*). This truncation was originally described as the cif1 allele, and reportedly exhibited phenotypic variability in different strains based on glucose and fructose growth compared to a complete TPS1 gene deletion, in addition to wild type sporulation (González et al. 1992; Stucka and Blázquez 1993). Finally, we included the E. coli homolog of TPS1, otsA, which has 53% amino acid similarity to Tps1. This allele has been previously used to restore trehalose-6-phosphate synthase activity to yeast cells (Ruhl et al. 2013). Further, expression of E. coli otsA in W303 (tps1Δ and CEN.PK tps1Δ partially restored glucose growth (Bonini et al. 2000; Deroover et al. 2016; Vicente et al. 2018).
only partially able to restore high temperature survival and sporulation (Table 1). The observation that \textit{otsA} is able to suppress or partially suppress a number of these phenotypes suggests potential regulatory models: T6P levels, metabolic flux, or UDP-glucose levels may be involved in regulating these phenotypes, though there are likely other possibilities. It is noteworthy that a \textit{tps1Δ} mutant expressing \textit{otsA} still hyperaccumulates sugar-phosphates despite having restored glucose growth, suggesting...
glycolytic regulation is not completely normal in the otsA expression strain (Bonini et al. 2000). Considering multiple observations that disconnect trehalose production from a number of trehalose metabolism mutant phenotypes, one possibility is that Tps1, or another protein in trehalose metabolism, has an independent role or roles in the cell. This notion is supported by restoration of carbon source utilization by catalytically inactive variants (R24V and Y102V), and also by the observation that some fraction of Tps1 appears to be free from the rest of the TPS complex (Bell et al. 1998). Future studies geared toward using evidence based on natural genetic variants combined with these allelic variations could shed light on the molecular mechanisms underlying these diverse phenotypes.

Construction and phenotypic characterization of tps2Δ

TPS2 encodes the trehalose-6-phosphate phosphatase enzyme, which catalyzes the production of trehalose by dephosphorylation of trehalose-6-phosphate (Fig. 1). Disruption of TPS2 causes inability to effectively synthesize trehalose and excessive accumulation of the intermediate metabolite trehalose-6-phosphate (de Virgilio et al. 1993; Bell et al. 1998). Apart from losing metabolic activity, a variety of pleiotropic phenotypes are associated with tps2Δ from different genetic backgrounds, including acute thermosensitivity, inability to proliferate at elevated temperature, defective growth on galactose and glycerol/ethanol, and defective sporulation (de Virgilio et al. 1993; Elliott et al. 1996; Hohmann et al. 1996). As with tps1Δ, the mechanism explaining these unrelated phenotypes is not yet elucidated.

We constructed tps2Δ mutants in the same 5 strains and subjected them to the same phenotypic tests as performed for tps1Δ mutants. All 5 tps2Δ strains synthesized lower amount of trehalose than their isogenic wild type, though none exhibited total failure to produce trehalose (Fig. 5a). In fact, some of them accumulated comparatively high levels of trehalose such as the Simi White tps2Δ and Bb32 tps2Δ, with 79% and 48% trehalose levels compared to their isogenic wild type strains, respectively. Trehalose accumulation has also been observed in other strain background tps2Δ mutants, including W303, and might be explained by the presence of other nonspecific phosphatases independent of the trehalose synthase complex, hydrolyzing T6P into trehalose with lower efficiency than T6P phosphatase (Bell et al. 1998; Ratnakumar and Tunnacliffe 2006). The possibility that other proteins in the TPS complex (Tps1, Tps3, and Tsl1) might contribute to this accumulation was refuted when a residual level of T6P phosphatase activity was found in a quadruple tps1Δtps2Δtps3Δtsl1Δ strain (Reinders et al. 1997). This further indicates that there should be significant T6P phosphatase capacity in vivo to sustain observed trehalose accumulation in tps2Δ. Four out of 5 strains accumulated higher levels of glycogen in the absence of TPS2, ranging from 336% to 158% of the isogenic wild types, while YPS1000 tps2Δ did not (Fig. 5b). Similar to tps1Δ observations, intracellular trehalose and glycogen levels are not necessarily anticorrelated. Only Simi White tps2Δ was larger in cell size compared to its isogenic wild type, which was also the case with Simi White tps1Δ (Supplementary Fig. 2b).

Acute thermosensitivity (42°C for 2 h) in the 5 tested stationary tps2Δ strains varied: 4 of 5 strains exhibited significantly lower survival rates than their isogenic wild type, though the degree of survival ranged from 5% to 60% (Fig. 5c). YPS1000 tps2Δ heat sensitivity did not significantly differ from its isogenic wild type strain, suggesting genetic variation in this strain background can suppress the typical tps2Δ thermosensitivity phenotype (Fig. 5c). Temperature-sensitive growth is another stress phenotype associated with tps2Δ in multiple previous studies (Piper and Lockheart 1988). All 5 tps2Δ strains observable detectable growth defects at elevated temperature (Fig. 5f and Supplementary Fig. 7). Complete cessation of growth was found in tps2Δ strains in the S288C, Simi White, CSM, and Bb32 genetic backgrounds. As with acute thermosensitivity, YPS1000 tps2Δ cells were less affected by the elevated temperature and had only slightly smaller colonies compared to wild type at similar dilutions (Fig. 5f and Supplementary Fig. 7). Trehalase, as the end-product of the trehalose biosynthesis pathway, has been long regarded as a chemical chaperone to protect cells against various types of stresses, including heat stress (Hottiger et al. 1987; Herdeiro et al. 2006; Crowe 2007). However, as observed in tps1Δ cells, increased intracellular trehalase accumulation failed to repair tps2Δ heat sensitivity, raising the doubt that it can stabilize intact, hydrated cells during thermal stress (Gibney et al. 2015). Another hypothesis is that excessive accumulation of T6P at elevated temperature is likely to disturb energy metabolism and might be the cause of thermosensitive growth in tps2Δ (Thevelein and Hohmann 1995). However, thermosensitivity was not a general feature of sugar-phosphate accumulation, though it might be a T6P-specific phenotype (Gibney et al. 2018). Similar to tps1Δ mutants, the

### Table 1. Allele-specific complementation of tps1Δ phenotypes.

| Phenotype             | Wild type Empty vector | tps1Δ + p416GPD- |
|-----------------------|------------------------|------------------|
| Glucose growth        | +                      | +                |
| Fructose growth       | +                      | +                |
| High temp. growth (39°C) | +               | +                |
| High temp. survival (42°C—2h) | +               | +                |
| Sporulation efficiency| +                      | +                |
| Trehalose level       | +                      | +                |
| Glycogen level        | +                      | +                |
| M. grisea trehalose   | +                      | +                |
| M. grisea sporulation | +                      | +                |
| M. grisea virulence   | +                      | +                |

*All scoring is based on Figure 4 data, and each score is relative to wild type levels (+: absent (−), intermediate (−/+), increased (+/+)). M. grisea data are from Wilson et al., though the different alleles were genomically integrated rather than being expressed from a plasmid, and the homologous amino acid positions are slightly different in M. grisea (R22G, Y99V, W108S, and D155C).*
mechanism underlying thermosensitivity associated with tps2Δ remains unclear.

Another shared phenotype between tps1Δ and tps2Δ mutants is an inability to sporulate in the S288C background (Sur et al. 1994; Gibney et al. 2015; Liu et al. 2020). Here we similarly show that S288C tps2Δ, Simi White tps2Δ, and Bb32 tps2Δ are unable to sporulate (Fig. 5d). However, compared to their own wild type strains, CSM tps2Δ and YPS1000 tps2Δ reached 26% and 74% sporulation, respectively (Fig. 5d). As shown in Fig. 2d, YPS1000 tps1Δ also exhibited a higher sporulation efficiency compared to those

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**Fig. 5.** tps2Δ mutant phenotypes. a) Intracellular trehalose; b) intracellular glycogen; c) thermotolerance; d) sporulation efficiency; e) growth at 30°C for 2 days; f) growth at 37°C for 3 days. Indicated strains were grown overnight in YNB + 2% glucose liquid before performing assays as described in Materials and Methods. For plate images, 10-fold serial dilutions were prepared and spotted onto the indicated media. The initial dilution had an OD₆₀₀ of 1.0. Three biological replicates were used for all tested phenotypes. Asterisks represent statistical difference (P < 0.05) between the mutants and their isogenic wild type strains. The strain legend below (c) and (d) applies to (a)–(d).
from other genetic backgrounds, suggesting an allelic difference in the gene(s) regulating sporulation of trehalose biosynthesis mutants in YPS1000. Interestingly and in contrast to these results, a tps2Δ mutant in the W303 genetic background reportedly exhibited identical sporulation efficiency compared to its wild type (Neves et al. 1995; Hohmann et al. 1996).

Abnormal growth on respiratory carbon sources has been observed for multiple tps2Δ lab strains (S288C, CEN PK) (Walther et al. 2013; Gibney et al. 2015). While tps1Δ mutants did not exhibit growth defects on respiratory carbon sources (Fig. 2e), all 5 tps2Δ mutant strains grew slower than wild type cells on respiratory carbon sources including galactose (preferred respiration substrate) and combined glycerol/ethanol (obligate respiration substrate) (Fig. 5e). On preferentially fermented carbon sources glucose and fructose, all 5 mutants grew similarly to their isogenic wild type strains (Fig. 5e). One model for this respiratory growth impairment is that increased accumulation of T6P in the tps2Δ mutant causes an increase in cytosolic pH and therefore strongly reduced growth rates on nonfermentable carbon sources (Walther et al. 2013). Evidence supporting this hypothesis is that normal growth was observed in tps1Δtps2Δ, which does not produce T6P (Bonini et al. 2000). However, despite the observation that T6P inhibits hexokinase in vitro, suggesting prevention of uncontrollable sugar phosphorylation and fatal depletion of ATP, yeast cells can tolerate 10-fold decreases and 4-fold increases in T6P without any noticeable impact on glycolytic flux or on the ability to consume fermentable carbon sources (Blázquez et al. 1993; Thevelein and Hohmann 1995; Hohmann et al. 1996; Walther et al. 2013). Further, in a strain lacking TPS1, expression of the bacterial T6P synthase otsA or the leaky TPS1 allele (hyp1-3) resulted in no difference on fermentable carbon source growth or fermentation rate, even though significant amounts of T6P were produced (Hohmann et al. 1992, 1996; Bonini et al. 2000). Overall, the pleiotropic phenotypes observed in tps1Δ and tps2Δ further emphasize the crucial, but unclear role of the trehalose biosynthesis pathway in regulation of respiratory and fermentative metabolism.

Construction and phenotypic characterization of tps3Δ, tsl1Δ, and tps3Δtsl1Δ

In S. cerevisiae, 2 additional proteins in the trehalose synthase complex, Tps3 and Tsl1, were identified based on a high degree of amino acid sequence similarity with Tps1 and Tps2, despite not having defined trehalose synthesis catalytic activities. The TPS3 and TSL1 genes are paralogs resulting from a whole genome duplication in the S. cerevisiae lineage (Ferreira et al. 1996; Bell et al. 1998). Using the yeast 2-hybrid approach, it was demonstrated that Tsl1 and Tps3 can interact with Tps1 and Tps2 in vivo, while the latter 2 proteins also interact with each other (Reinders et al. 1997; de Silva-Udawatta and Cannon 2000). These 4 proteins share roughly 33% identity over a stretch of 500 amino acids, but it is not known whether physical interactions between these proteins occur through this common sequence (François and Parrou 2001). TPS3 and TSL1 mRNA expression levels indicate that these proteins may be regulated differently under specific growth conditions: TPS3 is expressed at a constant rate in exponentially growing and stationary-phase cells, while TSL1 expression is greatly enhanced upon entrance into stationary phase (Winderickx et al. 1996). Although tps3Δ, tsl1Δ, or tps3Δtsl1Δ mutants have not been as extensively studied as tps1Δ and tps2Δ, Tps3 and Tsl1 are seemingly important contributors to trehalose metabolism as both paralogs have been maintained in the yeast genome and both encode proteins that interact with the other trehalose synthesis proteins. Here, we constructed both single deletion mutants and the double deletion mutant in all 5 genetic backgrounds and performed the same phenotype tests used for tps1Δ and tps2Δ. Most single tps3Δ or tsl1Δ deletions had a minor effect on trehalose levels, if any (Fig. 6a). However, deleting both TPS3 and TSL1 reduced trehalose production to levels ranging from 55.3% to 88.4% of wild type production levels (Fig. 6a). This was also reported in the W303 lab strain where the single deletion of TPS3 or TSL1 slightly lowered the trehalose content, and the double deletion had <50% trehalose compared to the wild type strain (Reinders et al. 1997; Bell et al. 1998). Glycogen levels were slightly higher in S288C, Simi White, and YPS1000 tsl1Δ and tps3Δtsl1Δ strains compared to their isogenic wild types (Fig. 6b). We did not observe any significant cell size variations with any of these mutants (Supplementary Fig. 2). It is not clear why deleting both of these genes only results in mild to moderate changes to intracellular trehalose content. It is possible that these proteins are not very important for trehalose production, or that we have not identified an appropriate condition that strongly requires one or both of these genes.

Almost every single and double mutant in all 5 strains had wild type levels of thermotolerance when exposed to 42°C for 2 h (Fig. 6c). Only 1 strain, Simi White tps3Δtsl1Δ, exhibited mild but statistically significant thermosensitivity (Fig. 6c). It is possible that the slight thermosensitivity of Simi White tps3Δtsl1Δ results from destabilized TPS complex, and subsequently developing tps1Δ-like thermosensitivity. Notably, in the W303 background after a heat shock, tps3Δtsl1Δ cells exhibited moderately reduced Tps1 activity compared to wild type (Reinders et al. 1997). This was further supported by another study demonstrating that deletion of TPS3 and TSL1 appeared to destabilize the trehalose synthase complex (Bell et al. 1998). However, it is also notable that the double deletion and presumably destabilized TPS complex has no effect on thermotolerance in 4 of 5 strains. Similar to observations of thermotolerance, none of the deletion mutants exhibited any growth defect when incubated at high temperature, 37°C (Fig. 6f). Surprisingly, we noticed a mild growth enhancement at 37°C on both rich and minimal media for Simi White tsl1Δ and Simi White tps3Δtsl1Δ compared to their isogenic wild type and the single tps3Δ mutant (Fig. 6f and Supplementary Fig. 7). To further confirm this phenotype, we constructed multiple, independent Simi White tsl1Δ deletion mutants by dissecting independent heterozygous transformants, collecting resulting segregants from each dissection, and retesting their ability to grow 37°C. All tested tsl1Δ strains exhibited enhanced growth at 37°C compared to wild type (Supplementary Fig. 8). It is possible that a genetic variant between Simi White and these other strains could reveal the genetic basis of this mildly enhanced thermotolerance and illuminate the connections between trehalose metabolism and cellular stress response.

Deleting TPS3, TSL1, or both did not significantly compromise sporulation efficiency of any tested strain (Fig. 6d). Similarly, none of these mutants in any of these strains exhibited growth defects on tested carbon sources (Fig. 6e). Although there are few published studies examining tps3Δ or tsl1Δ mutants, a published dissertation demonstrated that in the lab strain CEN.PK, tps3Δ, tsl1Δ, and tps3Δtsl1Δ all exhibited a severe deficiency in ascus formation (Karabulut 2012). This was not the case with the 5 strains tested here, which all had wild type levels of sporulation, suggesting that the CEN.PK phenotypes may be strain-specific and also useful for future studies to investigate the genetic connections between trehalose metabolism and sporulation. Taken together, phenotypes associated with tps3Δ, tsl1Δ, or tps3Δtsl1Δ mutants
are mild if present at all. Further work will be required to determine the precise role(s) and significance that these proteins play in trehalose metabolism or other cellular processes.

**Conclusions**

In this study, we constructed mutants of the trehalose biosynthesis pathway (TPS1, TPS2, TPS3, and TSL1) in 5 diverse *S. cerevisiae* strains to rigorously examine whether published lab strain phenotypes are also exhibited by wild strains. For each mutant, we assessed trehalose production, glycogen production, cell size, acute thermotolerance, high temperature growth, sporulation efficiency, and growth on a variety of carbon sources in rich and minimal medium.

Regarding deletion of TPS1 and TPS2, many reported laboratory phenotypes were observed with some variations in magnitude. However, for most phenotypes at least 1 mutant strain exhibited little to no defect—these variants have potential value to dissect the underlying genetic basis for these phenotypic variations, and further our understanding of how trehalose metabolism is integrated into these diverse cellular processes. We further demonstrated that *otsA* and a subset of published, catalytically inactive alleles of *tps1* are able to selectively complement *tps1D* phenotypes (Wilson et al. 2007). These observations align with similar findings in *M. grisea*, and further support the notion that trehalose itself does not explain many of the pleiotropic phenotypes associated with *tps1D*. We observed very mild, if any, phenotypes associated with deletion of either TSL1 or

**Fig. 6.** *tps3Δ, tsl1Δ, and tps3Δtsl1Δ* mutant phenotypes. (a) Intracellular trehalose; (b) intracellular glycogen; (c) thermotolerance; (d) sporulation efficiency; (e) growth at 30°C for 2–3 days; (f) growth at 37°C for 3 days. Indicated strains were grown overnight in YNB + 2% glucose liquid before performing assays as described in Materials and Methods. For plate images, 10-fold serial dilutions were prepared and spotted onto the indicated media. The initial dilution had an OD_{600} of 1.0. Three biological replicates were used for all tested phenotypes. Asterisks represent statistical difference (*P* < 0.05) between the mutants and their isogenic wild type strains.
Though the double deletion consistently has moderately impaired trehalose production in each strain, no other strong or even mild phenotype was commonly observed for these mutants, suggesting either a minor role for these proteins, an undefined conditional role, or an undefined role outside of trehalose production. One exception to these observations included a mild resistance to thermal stress in Simi White associated with tsl1Δ, though the mechanism behind this phenotype is unknown. Future studies taking advantage of these natural and engineered phenotypic variants could provide deeper mechanistic insight into the multiple cellular roles of the trehalose metabolic pathway.

**Fig. 6.** Continued.
Data availability
Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material is available at G3 online.

Acknowledgments
We thank members of the Gibney lab for helpful discussions and critical reading of this manuscript.

Funding
This research was supported by startup funds provided to P.A.G. by the Department of Food Science in the College of Agriculture and Life Sciences at Cornell University.

Conflicts of interest
None declared.

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*Communicating editor C. Hoffman*