

Characterizing the in vivo role of trehalose in *Saccharomyces cerevisiae* using the *AGT1* transporter

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Trehalose is a highly stable, nonreducing disaccharide of glucose. A large body of research exists implicating trehalose in a variety of cellular phenomena, notably response to stresses of various kinds. However, in very few cases has the role of trehalose been examined directly in vivo. Here, we describe the development and characterization of a system in *Saccharomyces cerevisiae* that allows us to manipulate intracellular trehalose concentrations independently of the biosynthetic enzymes and independently of any applied stress. We found that many physiological roles heretofore ascribed to intracellular trehalose, including heat resistance, are not due to the presence of trehalose per se. We also found that many of the metabolic and growth defects associated with mutations in the trehalose biosynthesis pathway are not abolished by providing abundant intracellular trehalose. Instead, we made the observation that intracellular accumulation of trehalose or maltose (another disaccharide of glucose) is growth-inhibitory in a carbon source-specific manner. We conclude that the physiological role of the trehalose pathway is fundamentally metabolic: i.e., more complex than simply the consequence of increased concentrations of the sugar and its attendant physical properties (with the exception of the companion paper where Tapia et al. [Tapia H, et al. (2015) *Proc Natl Acad Sci USA*, 10.1073/pnas.1506415112] demonstrate a direct role for trehalose in protecting cells against desiccation).

yeast | trehalose | AGT1 | heat shock | carbon source

Trehalose is a nonreducing disaccharide consisting of two glucose monomers (α -D-glucopyranosyl-1,1- α -D-glucopyranoside). Because trehalose is relatively inert and very stable, it is thought to act as a protectant for subcellular structures against osmotic and other stress (1–4). These chemical properties render trehalose useful in vitro for a number of industrial purposes, such as cryo-storage of biological samples (5). In addition, trehalose seems to have multiple distinct physiological roles. Widely distributed organisms are able to produce trehalose, including bacteria, archaea, plants, fungi, and other invertebrates (6). The ability to degrade trehalose into two glucose monomers is even more widespread because vertebrate animals, including humans, are able to digest trehalose (6).

The described physiological roles of trehalose are as widely varied as its production. In bacteria, such as *Escherichia coli*, trehalose production is activated in response to cellular stress and is therefore thought to serve as a stress protectant (7, 8). In *Mycobacterium tuberculosis*, trehalose is the sugar component of the glycolipid that serves as cord factor—a major virulence determinant in this pathogen (9, 10). In archaea, the role of trehalose is less clear although there is evidence suggesting a role in osmotic and perhaps other stress protection (11). Trehalose synthesis is also essential for fungal virulence in *Candida albicans*, *Fusarium graminearum*, *Cryptococcus neoformans*, *Magnaporthe grisea*, and *Ustilago maydis* (12–15). In plants, trehalose is thought to be an osmoprotectant whereas its precursor, trehalose-6-phosphate, is a metabolic regulator of development (16, 17). In flying insects, trehalose serves as the main sugar component of hemolymph (18). A number of organisms, termed anhydrobiotes, are able to survive sustained periods of extreme desiccation, and it is thought that trehalose is a major mediator

of desiccation survival due to its proposed ability to stabilize membranes and/or proteins (19, 20).

A great deal of research has been performed over the last four decades to understand the physiological roles of trehalose in the model eukaryote *Saccharomyces cerevisiae*. In this system, trehalose has many ascribed roles: chemical chaperone, storage carbohydrate, membrane protectant, cell cycle regulator, metabolic regulator, etc. (4, 6, 21–24). Among these studies, a number of techniques are used to assess the role of trehalose: (i) in vitro analysis, (ii) observing transcriptional activation of trehalose biosynthetic genes and/or production of trehalose in response to a stimulus, or (iii) using mutations in biosynthetic genes and inferring the resulting phenotypes to be related to trehalose levels. Using the latter two approaches, it is difficult to reduce observed effects to trehalose itself versus another less obvious cause (metabolic flux, independent functions of biosynthetic enzymes, the trehalose precursor trehalose-6-phosphate, etc.). In the case of in vitro analysis, the effects can be attributed directly to trehalose, but the in vivo relevance remains unclear.

In this manuscript, we describe the development and characterization of a tool to directly assess the physiological role of trehalose using the Agt1p transporter. *AGT1* (alpha-glucoside transporter) is a gene that encodes a variant of the maltose transporter, with broader specificity for other sugars including trehalose (25–27). Previous work has demonstrated that *AGT1* expression allows yeast cells to import trehalose (28, 29). Using a constitutively expressed version of *AGT1* in combination with trehalose metabolism mutants, we were able to demonstrate that a number of phenotypes associated with trehalose biosynthesis mutants cannot be explained by the loss of trehalose alone (carbon source-based and temperature-based growth defects,

Significance

Trehalose is an important molecule for industrial and medical applications. These applications include use as a food additive to increase sweetness and promote freeze-dry preservation. Trehalose is also included in antibody preparations for stabilization during freezing or desiccation. Further, trehalose biosynthesis is required for virulence of fungal pathogens, and, because animal cells do not synthesize trehalose, trehalose biosynthesis is an attractive antifungal target. Despite all of its uses, the direct physiological roles of trehalose remain unclear. Here, we describe the development and characterization of a system in the model yeast *Saccharomyces cerevisiae* to directly assess the physiological roles of trehalose. We find that many of the roles traditionally ascribed to trehalose are not the result of trehalose accumulation per se.

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sporulation defects, and thermotolerance/acquired thermotolerance defects). Further, we demonstrate that disaccharide accumulation (both trehalose and maltose) inhibits growth in a carbon source-dependent fashion.

Results

Constitutively Expressed *AGT1* Allows for Trehalose Import. The biosynthesis of trehalose in yeast is highly regulated. Trehalose levels respond very strongly to diverse stresses, they vary in a cell cycle-dependent way, and the average intracellular level is strongly anticorrelated with growth rate (30). To study the physiological role(s) of trehalose, we needed a way to control the intracellular trehalose level independently from this regulation. Previous experiments indicated that the *Agt1p* transporter (also known as *Mal11p*) has a broader specificity range than other maltose transporters and included the ability to transport trehalose (25–28). For this reason, this transporter gene was renamed *AGT1* for alpha-glucoside transporter (26). Further, Plourde-Owobi et al. demonstrated that the product of the yeast *AGT1* gene is able to import trehalose into yeast cells in a manner dependent on the extracellular concentration (31). Most *S. cerevisiae* strains regulate *AGT1* expression along with the rest of the maltose regulon (glucose repressed, maltose activated). S288C-derived strains, such as those used in this work, have an inactive maltose regulator and therefore have no native *AGT1* expression. We reasoned that expressing the *AGT1* gene under the control of a strong, constitutive promoter would allow for intracellular trehalose levels to be precisely controlled by changing trehalose levels in the medium. This construct would function as a tool for directly examining the role of trehalose in the physiological processes for which it has a described role. We used this system in combination with mutations in the trehalose metabolism pathway to study the biological role(s) of trehalose and the biosynthetic pathway that leads to trehalose (Fig. 1A).

We inserted a copy of the *AGT1* gene driven by the promoter of the *TDH3* gene into the *CAN1* locus. This construct was then crossed into a variety of trehalose metabolism deletion mutants (*SI Appendix, Table S1*). To confirm that this construct was able to transport trehalose into the cell, we took advantage of the *ath1Δ* mutation. *ATH1* encodes the extracellular trehalase enzyme in yeast; when deleted, the cells are unable to grow on trehalose as a sole carbon source (Fig. 1B) (32). In contrast, when *ath1Δ* cells also express *AGT1*, they are now able to grow with the same doubling time as WT cells (Fig. 1C). Notably, both WT and *ath1Δ* cells expressing *AGT1* grow faster on trehalose than WT cells grow on trehalose without *AGT1*, suggesting that intracellular catabolism of trehalose is more efficient than extracellular catabolism. This growth experiment was also repeated in rich media, demonstrating that this system works in rich media as well (*SI Appendix, Fig. S1*).

To further validate that this transporter is functional, we measured the trehalose levels in both *AGT1*-expressing WT cells and cells missing both cytosolic trehalases (*nth1Δnth2Δ*). Over time, trehalose accumulated in both strains, although to a higher degree in the cells lacking cytosolic trehalases (Fig. 1D). This accumulation suggests that the active cytosolic trehalases in the WT cells are responsible for trehalose catabolism.

Intracellular Trehalose Accumulation Does Not Simply Reverse the Growth Defects of *tps1Δ* or *tps2Δ*. *TPS1* encodes the trehalose-6-phosphate synthetase enzyme, which catalyzes the joining of glucose-6-phosphate with the glycosyl unit from UDP-glucose (Fig. 1A) (6). Deletion of the *TPS1* gene is associated with a variety of growth phenotypes, including failure to grow on glucose or fructose, and failure to grow at 39 °C (Fig. 2A) (33–35). To test the idea that these phenotypes might simply be the result of the absence of intracellular trehalose, we combined the constitutively expressed *AGT1* transporter with *tps1Δ* to examine

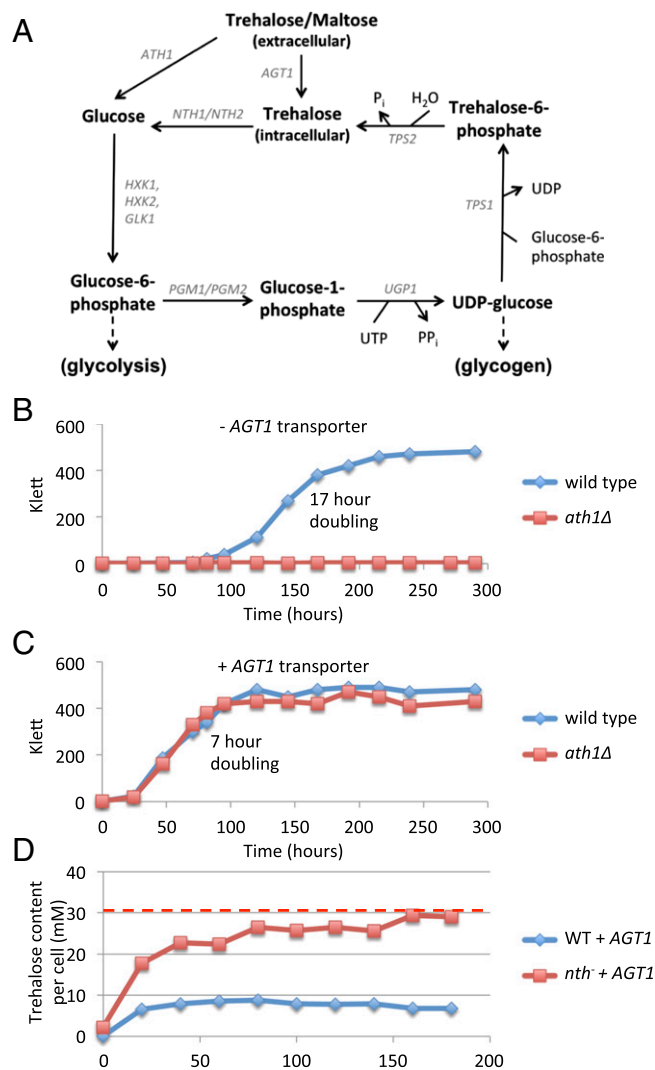


Fig. 1. Constitutively expressed *AGT1* functions as a trehalose transporter. (A) Schematic of trehalose metabolism. (B) Indicated strains (*AGT1* transporter not expressed) were grown in yeast nitrogen base (YNB) plus 1% trehalose at 30 °C. Calculated doubling time indicated to the right of each growth curve. (C) Indicated strains (*AGT1* transporter expressed in both strains) were grown in YNB plus 1% trehalose at 30 °C. Calculated doubling time indicated to the right of each growth curve. (D) Indicated strains were grown to midlog phase in YNB plus 2% glucose, and then 1% trehalose (29.2 mM) was added. Initial extracellular trehalose concentration is indicated by dashed red line. At each time point, cells were collected, and intracellular trehalose levels were measured.

whether accumulation of intracellular trehalose could by itself repair any of these growth defects. As shown in Fig. 2A, none of the growth defects associated with *tps1Δ* were fully repaired by addition of trehalose to the *AGT1*-expressing strain; most were not suppressed at all. There does however seem to be partial restoration of growth specifically on glucose at 30 °C and 37 °C.

Partial restoration of the *tps1Δ* glucose growth defect was recently described as a nongenetic “metabolically distinct sub-population” of cells (36). We also observed this phenomenon and note that it seems to be glucose-specific, as it does not occur for cells grown on fructose (*SI Appendix, Fig. S2*). We suspect that the growth restoration observed in Fig. 2A is related to this phenomenon. The 30 °C plate-based growth assays were also repeated in liquid culture with the same results, although notably some growth did occur in fructose at late time points (*SI Appendix, Fig. S3*). This growth may also be due to glucose-specific

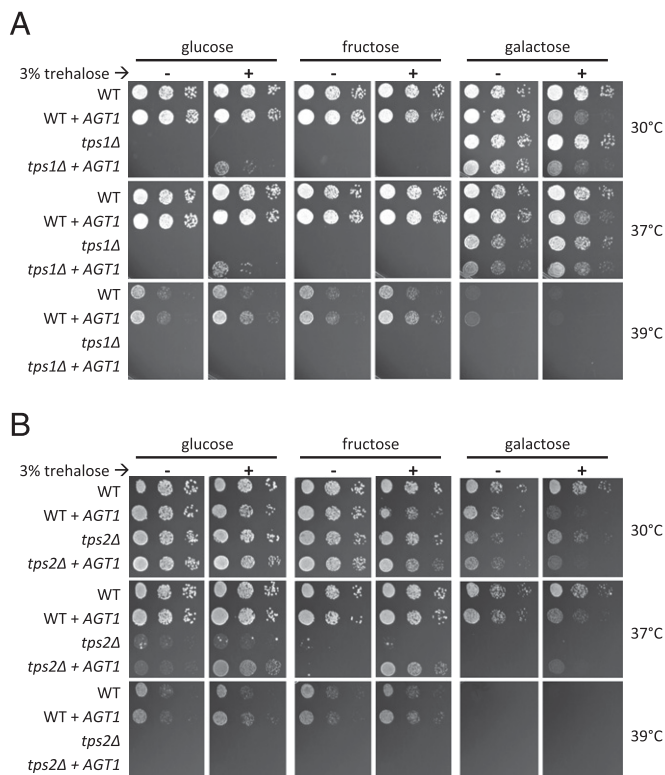


Fig. 2. Intracellular trehalose accumulation does not fully repair carbon-specific growth defects or high temperature growth defects of trehalose metabolism mutants. Serial dilutions (10-fold) of the indicated strains were spotted onto YNB plates with the indicated carbon sources with either no trehalose or 3% trehalose (as indicated). Initial well optical density for each strain was 0.1 at 600 nm. Plates were incubated at the indicated temperatures for 2–3 d. (A) Comparing *tps1Δ* and WT. (B) Comparing *tps2Δ* and WT.

suppression because this effect can be seen on fructose plates simply by adding glucose to the plates as well (*SI Appendix, Fig. S2*). In any case, it is clear that addition of trehalose does not fully repair these growth defects although there are some potentially confounding effects related to the catabolism of trehalose into intracellular glucose.

TPS2 encodes the trehalose-6-phosphate phosphatase enzyme, which catalyzes the production of trehalose from trehalose-6-phosphate (Fig. 1A) (6). Deletion of the *TPS2* gene is associated with a variety of growth phenotypes, including a mild growth defect on galactose at 30 °C and heat sensitivity at or above 37 °C (in contrast to *tps1Δ*, which has a minimal growth defect at 37 °C) (Fig. 2) (29, 37). Again, we combined the *AGT1* transporter with *tps2Δ* to examine whether or not trehalose import could repair any of these growth defects. As shown in Fig. 2B, the mild growth defect of *tps2Δ* at 30 °C on galactose seemed to be exacerbated by trehalose. In contrast, the 37 °C growth defects associated with *tps2Δ* were mostly repaired. Notably, increasing glucose in the medium repaired the 37 °C growth defect, again suggesting an uncharacterized role for glucose in this suppression, rather than of trehalose itself (*SI Appendix, Fig. S2*). Finally, trehalose did not restore growth to *tps2Δ* at 39 °C. The 30 °C and 37 °C plate-based growth assays were repeated in liquid culture with similar results (*SI Appendix, Fig. S3*).

Intracellular Trehalose Accumulation Does Not Repair Sporulation Defects of *tps1Δ* or *tps2Δ*. Homozygous deletions of either trehalose biosynthetic gene (*TPS1* or *TPS2*) are unable to sporulate in *S. cerevisiae* (38). We recapitulated this result and also determined that both *tps1Δ* and *tps2Δ* fail to complete the first step

of the meiotic program, DNA replication (*SI Appendix, Fig. S4*). The mechanistic basis of this defect is unclear. To determine whether or not intracellular trehalose alone could repair this sporulation defect, we examined WT and mutant cells expressing *AGT1* for their ability to sporulate after adding trehalose to the media and allowing it to accumulate for 1 h as described in *SI Appendix, Materials and Methods*. Addition of trehalose to the cells did not repair the sporulation defect associated with *TPS1* or *TPS2* deletion to any degree (Fig. 3).

Accumulation of Intracellular Trehalose Does Not Repair Thermotolerance or Acquired Thermotolerance Defects of *tps1Δ* but Does Repair *tps2Δ*. Yeast strains lacking *TPS1* or *TPS2* exhibit defects in both thermotolerance and acquired thermotolerance compared with WT (34, 39, 40). Thermotolerance refers to the ability of cells to survive when treated with a lethal dose of heat. Acquired thermotolerance refers to the ability of cells to survive a lethal dose of heat when pretreated first with a mild stress (which induces protective physiological effectors). In vitro, trehalose can protect denatured proteins from aggregation, suggesting a physical role for trehalose in protecting cells against heat stress, which is further bolstered by the in vivo observation that cells unable to produce trehalose are thermosensitive (34, 40, 41). These results are correlative; we used our system to assess directly the role of intracellular trehalose as a thermoprotectant and to distinguish this activity from potential roles of trehalose-6-phosphate, flux through the pathway, or uncharacterized properties of the trehalose metabolic enzymes.

The *tps1Δ* mutant is unable to make trehalose or the intermediate metabolite, trehalose-6-phosphate. If the absence of trehalose causes the thermotolerance defect of *tps1Δ*, addition of trehalose should repair that defect. Compared with WT, *tps1Δ* cells are much more sensitive to heat in the thermotolerance assay, yet addition of trehalose does not repair this defect to any degree (Fig. 4A). Similarly, *tps1Δ* is more sensitive to heat than WT cells in the acquired thermotolerance assay, and again intracellular trehalose does not repair this defect (Fig. 4B).

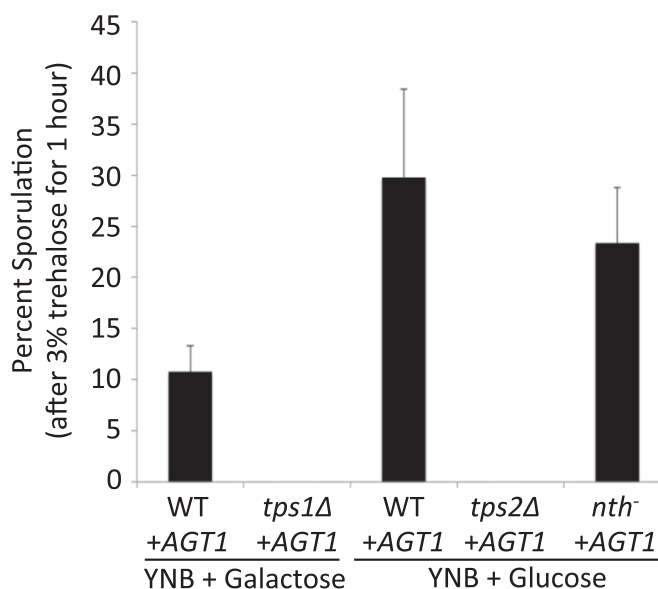


Fig. 3. Intracellular trehalose accumulation does not repair sporulation defects associated with trehalose metabolism mutants. Indicated strains were grown to log phase in minimal galactose or minimal glucose media, as indicated (*nth⁻* is a deletion of both *NTH1* and *NTH2*). Trehalose (3%) was then added to each culture for 1 h, and then cells were sporulated as described in *SI Appendix, Materials and Methods*.

increasing concentrations of maltose cause decreased growth rates, which is most severe in galactose-grown cells (Fig. 5B and *SI Appendix*, Fig. S6). Maltose-based growth retardation is equivalent in both WT and *nth1Δnth2Δ* cells expressing *AGTI*, likely because *nth1Δnth2Δ* cells have no basal maltose accumulation and therefore no impact on intracellular maltose levels (Fig. 5B and *SI Appendix*, Fig. S7). These results were also confirmed in rich medium (*SI Appendix*, Fig. S1).

Because both trehalose and maltose are disaccharides of glucose, we considered the possibility that accumulation of these molecules interferes with glucose metabolism and that adding more glucose could counteract the growth inhibition by these disaccharides. We tested this hypothesis by growing cells in several concentrations of glucose [1%, 2%, 4%, and 8% (wt/vol)] with no disaccharide, 2% trehalose, or 2% maltose. Although increasing glucose concentrations partially restores WT growth rate to cells in maltose, it has a lesser (if any) effect in trehalose (Fig. 5C and *SI Appendix*, Fig. S8).

To better understand the growth inhibitory phenomenon associated with accumulation of trehalose or maltose, we tested whether addition of galactose to cells that constitutively express the galactose transporter gene *GAL2* causes any growth defect. We observed no impact on growth rate for these cells, suggesting that growth inhibition is not a general phenomenon of sugar accumulation (*SI Appendix*, Fig. S9). We further demonstrated that this growth defect also occurs in *tps1Δ*, *tps2Δ*, and *ath1Δ*, which can all accumulate trehalose via *Agt1p*, suggesting that this effect is not specific to just WT and *nth1Δnth2Δ* cells (*SI Appendix*, Fig. S10).

Finally we performed gene expression analysis using microarrays and metabolomic profiling of WT or *nth1Δnth2Δ* cells expressing *AGTI* after addition of trehalose or maltose. There are many gene expression changes after addition of trehalose or maltose, but, for the most part, these changes seem to be related to the environmental stress response, and the magnitude of the changes is correlated to the level of growth inhibition (*SI Appendix*, Fig. S11). It is noteworthy that, in response to trehalose, but not maltose, there are two clusters of oscillating gene expression, which may represent the glucose-based oscillations in gene expression observed previously (*SI Appendix*, Fig. S11) (44, 45).

In contrast, there are very few time-correlated metabolism-wide changes in response to the addition of trehalose or maltose (*SI Appendix*, Fig. S11). When trehalose is added, the strongest metabolite change is an accumulation of trehalose. When maltose is added, a few metabolites change in level, including trehalose, indicative of the more severe inhibition of growth. At this point, the molecular mechanism underlying the growth-inhibitory role of trehalose (and maltose) remains unclear.

Discussion

We have described the development and characterization of a system to import trehalose into the cell with very precise experimental control—constitutive expression of the *AGTI* transporter gene. This is a useful approach to directly test the *in vivo* role of trehalose, both in yeast and in any organism capable of transgene expression. We have applied this system to demonstrate that trehalose may not play all of the cellular roles previously suggested. We specifically find that trehalose does not fully repair the carbon-based or high temperature-based growth defects associated with *tps1Δ* or *tps2Δ*, nor does trehalose repair the sporulation defect associated with the same gene deletions. We also demonstrate that trehalose is not the main determinant of thermotolerance or acquired thermotolerance in a *tps1Δ* mutant. Although the addition of trehalose does provide thermotolerance for WT and *tps2Δ* cells, we suggest that this protection is likely not due to trehalose itself; otherwise, *tps1Δ* would have also been protected (maltose also provides thermoprotection to WT cells). An alternative explanation for this behavior is that

trehalose addition activates the environmental stress response, which is able to provide thermoprotection to these strains.

In a companion paper, Tapia et al. (46) use *AGTI* to directly assess the role of trehalose on desiccation tolerance. Unlike the phenotypes described above, trehalose seems to have a strong, direct role in allowing cells to survive extreme desiccation. Comparing the results suggests that the ability of trehalose to function as a membrane or protein chaperone (as is the case in desiccation) requires very low water content, a situation not encountered by cells under the laboratory conditions we tested. These results further validate that this system allows researchers to distinguish the direct versus indirect roles of trehalose in the cell.

We also describe a carbon-specific growth rate reduction in response to intracellular trehalose accumulation. The molecular basis of this phenomenon remains unclear although there are some easily ruled-out possibilities. Because *Agt1p* is a proton symporter, cytosolic acidification could be responsible for growth inhibition. However, if cytosolic acidification were the cause, the magnitude of growth inhibition would not be dependent on carbon source, nor should addition of more glucose diminish the effect. Another possibility is that we are simply increasing the concentration of intracellular solutes to a point that normal cell biology is impeded. However, this possibility is unlikely, because adding more sugar in the form of glucose has a restorative effect on the growth rate in the maltose experiment. Another possibility is that the trehalose is being broken down into glucose, which has a strong effect in galactose due to induction of glucose repression. However, the effect is still observed in *nth1Δnth2Δ* cells, which are unable to produce glucose from trehalose and because maltose—which cannot be broken down by our strains—also causes growth inhibition. Interestingly, growth inhibition by trehalose has been recently observed in plants (47). Trehalose inhibits seedling growth in *Arabidopsis* via *SnRK1* (the *Arabidopsis* homolog of *Snf1*) through an unknown molecular mechanism (47). These results, taken together, suggest a potentially conserved role for the trehalose pathway in regulating cell growth through sugar sensing/signaling pathways, which has also been a proposed role for this pathway in yeast (23). Further work is required to better understand the role of the trehalose metabolism pathway in yeast and other organisms.

Increasing numbers of manuscripts are being published ascribing physiological roles to trehalose. For example, a number of recent manuscripts suggest that oral consumption of trehalose allows it to act as protective or curative in neurodegenerative disease models, or that topical application is curative for melanoma (48). However, in many of these cases, existing data are insufficient to demonstrate that trehalose itself is actually entering the cells of interest (rather than being first metabolized to glucose via trehalase enzymes expressed by intestinal villi cells, for example) or playing the described roles. Because of increased interest and other medical/industrial applications, it is important to accurately distinguish direct versus indirect effects of trehalose on biological systems. Using the *Agt1p* transporter will be useful in allowing researchers to directly assess the role of trehalose in modulating cellular physiology.

Materials and Methods

For additional information on yeast growth and manipulation, sporulation of yeast cultures, assessment of thermotolerance/acquired thermotolerance, measurement of trehalose, gene expression analysis, and metabolomics profiling, please see *SI Appendix*. Datasets associated with gene expression and metabolomics are available for download online ([Datasets S1](#) and [S2](#)).

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