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

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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 or-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).

Trehalose is a 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).

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.
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 grown 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 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 subpopulation” 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. 1](image-url) 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.
Intracellular trehalose accumulation does not repair sporulation defects associated with 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. 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.](https://www.pnas.org/content/112/36/10730/F1.large.jpg)

![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.](https://www.pnas.org/content/112/36/10730/F2.large.jpg)
The literature regarding tolerances of trehalase mutants is highly contradictory, with multiple papers demonstrating that these mutants are both more and less tolerant than WT cells (43). Therefore, we tested tolerances and acquired tolerances in nth1Δnth2Δ cells lacking any cytosolic trehalose-degrading enzymes but expressing the AGT1 transporter. These cells have a higher basal level of trehalose and accumulate more trehalose when exposed to higher extracellular levels (Fig. 1D). Our data indicate that nth1Δnth2Δ cells are slightly more thermosensitive than WT cells (SI Appendix, Fig. S5). Addition of trehalose results in tolerances at early time points in the killing curve although, at later time points, especially in the acquired tolerances assay, these cells seem to exhibit decreased survival compared with WT (SI Appendix, Fig. S5).

Intracellular Disaccharide Accumulation Negatively Impacts Growth. We were surprised by the observation that accumulation of intracellular trehalose in our strains correlated with a decreased growth rate, most notable on galactose, and sought to investigate this phenomenon further (Fig. 2). We first examined multiple concentrations of added trehalose [0%, 1%, and 3% (wt/vol)] for growth rate effects on WT cells expressing AGT1 grown on different carbon sources. Fig. S4 demonstrates that the addition of trehalose causes extremely slight growth retardation on glucose or fructose, which is more pronounced in galactose (Fig. S4 and SI Appendix, Fig. S6). Because nth1Δnth2Δ cells have a higher basal level of intracellular trehalose and accumulate higher levels of trehalose via Agt1p, we expected that these growth defects would be exacerbated by nth1Δnth2Δ. Indeed the growth defects were more pronounced (Fig. S4 and SI Appendix, Fig. S6).

Because Agt1p is also known to transport maltose, we tested growth inhibition using maltose as a carbon source. Again,
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 AGT1, 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 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 AGT1 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 AGT1 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 AGT1 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 macromolecule (as in the case of 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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