

The Thermophilic Yeast *Hansenula polymorpha* Does Not Require Trehalose Synthesis for Growth at High Temperatures but Does for Normal Acquisition of Thermotolerance

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The *TPS1* gene from *Hansenula polymorpha*, which encodes trehalose-6-phosphate (Tre6P) synthase, has been isolated and characterized. The deletion of *TPS1* rendered *H. polymorpha* cells incapable of trehalose synthesis under conditions where wild-type cells normally accumulate high levels of trehalose. Interestingly, the loss of Tre6P synthase did not cause any obvious growth defects on a glucose-containing medium, even at high temperatures, but seriously compromised the cells' ability to acquire thermotolerance.

The accumulation of the nonreducing disaccharide trehalose is an element of the adaptive response of various microorganisms to stress conditions such as nutrient starvation and heat shock. Consistent with the suggestion that trehalose may, therefore, act as a stabilizer of cellular structures, *in vitro* studies have revealed the exceptional capability of trehalose in protecting biological membranes and enzymes from stress-induced damage (for reviews see references 5, 18, and 19). Moreover, genetic studies in yeast have shown that the loss of trehalose-6-phosphate (Tre6P) synthase activity severely compromises the cells' ability to acquire tolerance towards heat stress (references 6 and 16 and references therein). In agreement with these findings, it was recently demonstrated that trehalose is important for both maintaining proteins in their native forms and suppressing the aggregation of denatured proteins during heat shock in living cells (17). In this context, we chose to study the trehalose metabolism in the thermophilic, methylotrophic budding yeast *Hansenula polymorpha*, because this yeast is particularly well adapted to growth at very high temperatures (up to 48°C) and may therefore provide further insight into the role of trehalose as a stress protectant.

Trehalose metabolism in *H. polymorpha*. Trehalose accumulation in fungi has been reported to occur particularly during starvation conditions (e.g., entry into stationary phase) or during a mild heat shock (for reviews see references 13, 22, and 24). Consistent with these observations, cells of a thermophilic, homothallic *H. polymorpha* wild-type strain were found to have low trehalose levels during exponential growth and to accumulate trehalose when grown to stationary phase at 27 and 37°C (Fig. 1A). In parallel, the levels of Tre6P synthase activity (Fig. 1A), Tps1 protein (Fig. 1B), and *TPS1* mRNA (Fig. 1C) also increased upon entry of cells into stationary phase when grown at 27 and 37°C. Interestingly, cells grown at 47°C were found to reach high levels of trehalose during exponential growth and to increase levels of Tre6P synthase activity (Fig. 1A), Tps1 protein (Fig. 1B), and *TPS1* mRNA (Fig. 1C) during late exponential growth phase, just before entry into stationary phase. Taken together, these results show that trehalose accumulation in *H. polymorpha*, as in other fungi, is part of the carbon source starvation response and that trehalose synthesis is at least

partially controlled at a transcriptional level under these conditions.

Similar to other fungi, *H. polymorpha* cells were also found to accumulate trehalose under heat shock conditions. However, as expected on the basis of the higher temperature optimum for growth (than that of other yeast species such as *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*), *H. polymorpha* cells accumulated large amounts of trehalose only when subjected to a temperature shift from 27 to 47°C (instead of 40°C). Accordingly, cells were found to accumulate large amounts of trehalose during a 2-h heat shock at 47°C (Fig. 2A). In parallel, the levels of Tre6P synthase activity (Fig. 2A), Tps1 protein (Fig. 2B), and *TPS1* mRNA (Fig. 2C) also increased significantly under these conditions. Notably, while the levels of both Tre6P synthase activity (Fig. 2A) and Tps1 protein (Fig. 2B) increased with relatively slow kinetics, the level of *TPS1* mRNA increased dramatically shortly after the temperature shift (5 min), reaching a maximum after 20 to 40 min and decreasing slightly after 2 h at 47°C (Fig. 2C). Thus, similar to the situation in other fungi (4, 25, 26), trehalose synthesis is an element of the heat shock response in *H. polymorpha*, which is at least partially controlled by transcriptional mechanisms. In accordance with this notion, the *TPS1* mRNA levels (Fig. 2C) decreased to almost pre-heat shock levels (time zero) when cells were allowed to recover from the heat shock for 30 min at 27°C. Despite the fact that the level of Tps1 protein remained high and that even the *in vitro*-measured Tre6P synthase activity increased (Fig. 2A and B), trehalose was found to be completely and rapidly mobilized, possibly due to the activation of trehalases, when heat-shocked cells were allowed to recover for 30 min at 27°C (Fig. 1A).

Isolation of the *H. polymorpha* *TPS1* gene. Based on a comparison of known *TPS1* genes from *S. cerevisiae*, *S. pombe*, *Kluyveromyces lactis*, and *Aspergillus niger*, two degenerate oligonucleotides were designed which correspond to highly conserved regions in these genes. These two oligonucleotides were used as primers in a PCR with genomic DNA from *H. polymorpha* as a template. The resulting 650-bp fragment was subsequently used as a probe to screen an *H. polymorpha* genomic library (8) by colony filter hybridization. Two positive clones (pHRP20-1 and pHRP21-3) were found to contain the entire *H. polymorpha* *TPS1* gene. A segment of 2,695 bp containing the entire *H. polymorpha* *TPS1* gene, including flanking sequences, was sequenced on both strands by the cycle sequencing method with AmpliTaq DNA polymerase (FS), BigDye

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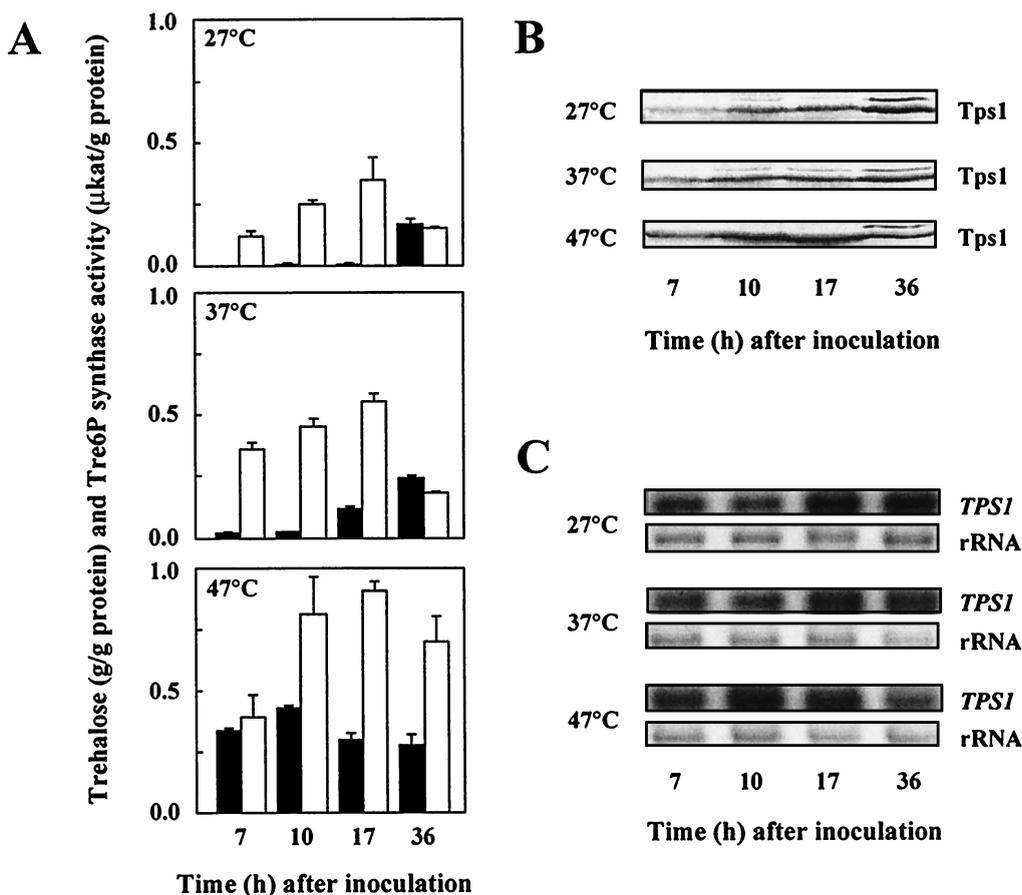


FIG. 1. Levels of trehalose and Tre6P synthase activity (A), Tps1 protein (B), and *TPS1* mRNA (C) of wild-type *H. polymorpha* cells grown to stationary phase at 27, 37, and 47°C. Cultures were pregrown overnight at the temperatures indicated and diluted (at time zero) with fresh YPD medium to an initial optical density at 600 nm of 0.2. Samples were taken in exponential phase (7 h), late exponential phase (10 h), early stationary phase (17 h), and late stationary phase (36 h). Glucose was consumed after 12 h (at 27°C), 11 h (at 37°C), or 12 h (at 47°C). (A) Shaded bars denote trehalose levels (determined as described in reference 7), and open bars denote Tre6P synthase activities (assayed as described in references 6 and 7). Error bars indicate standard deviations. (B) Immunoblot analysis of the Tps1 protein levels by using polyclonal rabbit antibodies raised against a peptide (Gly-Val-Asp-Arg-Leu-Asp-Tyr-Ile-Lys-Gly-Val-Pro-Gln-Lys) corresponding to a highly conserved amino acid sequence in Tps1 proteins of various fungi. Several proteins cross-reacted weakly with these antibodies, and the identification of the correct band was, therefore, verified in each case by the use of an extract from an *H. polymorpha* $\Delta tps1$ strain (data not shown). (C) Total RNAs were extracted (14), and equal amounts (10 μ g) were probed with an internal 650-bp fragment of the *H. polymorpha* *TPS1* gene after electrophoresis and blotting. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

terminators, and the ABI Prism 310 capillary sequencer (Perkin-Elmer Applied Biosystems). Analysis of the *H. polymorpha* *TPS1* sequence revealed an open reading frame of 1,428 bp coding for a putative protein of 476 amino acids (54.4 kDa). As expected, the predicted *H. polymorpha* Tps1 sequence exhibits similarity to Tps1 sequences from other organisms. Sequence identities range from 35% (with *Rhizobium* sp. Tps1) to 74% (with *Candida albicans* Tps1) over the full length of the shorter protein. Analysis of the DNA sequence (including 972 bp) immediately upstream of the putative *H. polymorpha* *TPS1* start codon revealed the existence of two elements (TGAAG CCTCTTGAAA and TGAATATAAAGGAAA at positions -94 and -166, respectively) with high similarity to *S. cerevisiae* and *Drosophila* heat shock elements which are typically composed of multiple, contiguous, and possibly inverted 5-bp NGAAN repeats (9).

Complementation of *S. cerevisiae* and *S. pombe* *tps1* mutants by *H. polymorpha* *TPS1*. In order to test whether we had isolated a functional *TPS1* gene, *H. polymorpha* *TPS1* was analyzed for its ability to complement the phenotypes of *S. cerevisiae* and *S. pombe* *tps1* mutants. To this end, a 2.6-kb

HindIII/HindIII fragment containing the full-length *H. polymorpha* *TPS1* gene, including 0.6 and 0.2 kb of the immediate up- and downstream flanking regions, respectively, was excised from plasmid pHRP21-3, isolated, and cloned at the *HindIII* sites of YEplac195 (10) and pUR19 (1), thus creating YE-*HpTPS1* and pUR-*HpTPS1*, respectively. Using these plasmids, we found that *H. polymorpha* *TPS1* restored, albeit to a different extent, the ability to synthesize trehalose in *S. cerevisiae* and *S. pombe* *tps1* mutants (Table 1). In a further experiment, the *S. cerevisiae* $\Delta tps1$ strain YSH 6.106.-1A (15) was transformed with either YE-*HpTPS1* or the corresponding control plasmid (YEplac195). As expected, *S. cerevisiae* $\Delta tps1$ cells containing the control plasmid were able to grow on a galactose-containing medium but not on a glucose-containing medium (data not shown). In contrast, *S. cerevisiae* $\Delta tps1$ cells containing YE-*HpTPS1* were able to grow on both galactose- and glucose-containing media. Accordingly, *H. polymorpha* *TPS1* also complements the growth defect of an *S. cerevisiae* $\Delta tps1$ mutant on glucose. Together, these results show that *H. polymorpha* *TPS1* codes for a functional Tre6P synthase.

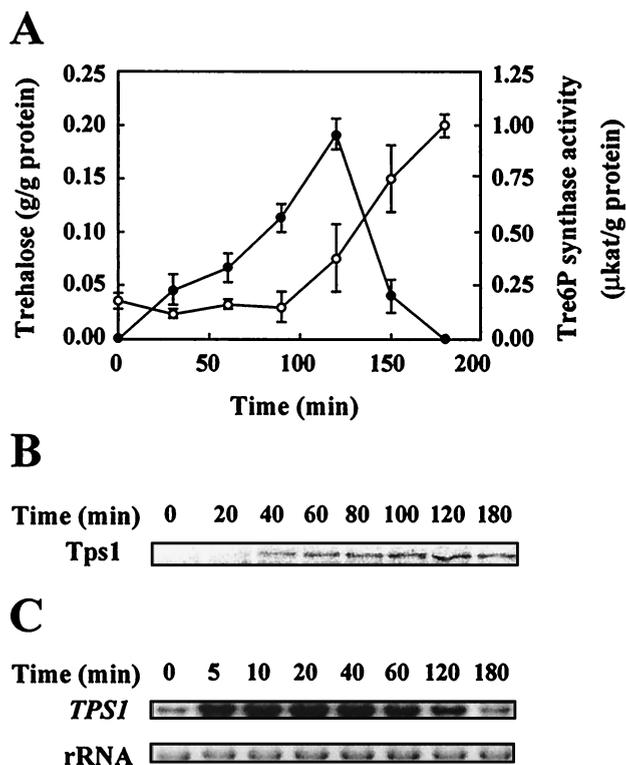


FIG. 2. Levels of trehalose and Tre6P synthase activity (A), Tps1 protein (B), and *TPS1* mRNA (C) of wild-type *H. polymorpha* cells during heat shock and subsequent recovery. Cultures were grown at 27°C on YPD medium to early exponential phase, shifted to 47°C at time zero, and transferred back to 27°C after 120 min. (A) Black circles, trehalose levels; white circles, Tre6P synthase activities. Error bars indicate standard deviations. (B) Protein samples were extracted at the times indicated and subjected to immunoblot analysis of Tps1 protein levels (for further details see the legend for Fig. 1). (C) Total RNAs were extracted (14) at the times indicated, and equal amounts (10 μg) were probed as described in the legend for Fig. 1.

Effects of the *H. polymorpha* *TPS1* deletion. To determine the consequences of the loss of Tps1, we replaced the complete *H. polymorpha* *TPS1* coding region with the *kanMX2* module coding for Geneticin resistance (23). To this end, two DNA fragments of 0.6 and 1.0 kb, containing sequences immediately up- and downstream of the *TPS1* start and stop codons, respectively, were amplified by PCR. *NotI-SpeI* and *Sall-EcoRI* restriction sites were introduced with the PCR primers at the ends of the 5' and 3' fragments, respectively. The resulting fragments were purified, digested with *NotI/SpeI* or *Sall/EcoRI*, and coligated with an *SpeI-Sall* fragment containing the *kanMX2* module to *NotI/EcoRI*-digested pBluescript KS(+) (Stratagene). The deletion cassette with flanking sequences of *TPS1* separated by the *kanMX2* module was then excised from the resulting plasmid with *NotI* and *EcoRI* and transformed into the *H. polymorpha* wild-type strain. Two independent Geneticin-resistant colonies were isolated, and the correct integration of the marker at the *TPS1* locus was verified by PCR and Southern blot analysis (data not shown). On plates, the *H. polymorpha* Δ *tps1* strains had no obvious growth defect at 27°C on all carbon sources we tested, including glucose, fructose, sucrose, galactose, glycerol, and ethanol. In addition, we did not observe any growth defect on glucose-containing plates or liquid media for the *H. polymorpha* Δ *tps1* mutant at any temperature tested (i.e., 27, 37, and 47°C). Thus, the *TPS1* gene is not essential for growth in *H. polymorpha*.

As expected, and in contrast to wild-type cells, *H. polymor-*

pha Δ *tps1* mutant cells grown on 1% yeast extract–2% peptone–2% glucose (YPD) medium had no in vitro-detectable Tre6P synthase activity during exponential growth, in stationary phase, or under heat shock conditions (detection limit, 0.001 μkat/g of protein). Consistent with these findings, *H. polymorpha* Δ *tps1* mutants were unable to synthesize detectable amounts of trehalose upon entry into stationary phase and during heat shock, while wild-type cells accumulated large amounts of trehalose under the same conditions (detection limit, 0.001 g/g of protein). Together, these findings indicate that the isolated *H. polymorpha* *TPS1* gene encodes the only functional Tre6P synthase in *H. polymorpha*.

Previous genetic approaches to determine the specific role of trehalose for the heat-induced thermotolerance in *S. cerevisiae* have been hampered by the finding that the deletion of *TPS1* causes a variety of pleiotropic effects, including the inability to grow on glucose-containing media (6). However, studies of an *S. pombe* *tps1* mutant, which reportedly has no such growth defects, showed that trehalose synthesis is indeed important for the acquisition of thermotolerance (16). Since the loss of Tps1 causes no obvious growth defect in *H. polymorpha* on glucose-containing media, we could also assess the role of trehalose synthesis in this particularly thermophilic yeast species. We found that unconditioned *H. polymorpha* Δ *tps1* cells were as heat sensitive to a 40-min challenging heat shock at 56.5°C as wild-type cells. However, while a 1-h conditioning heat shock at 47°C increased the survival rate of wild-type cells towards the challenging heat shock (40 min at 56.5°C) more than 1,000-fold, *H. polymorpha* Δ *tps1* mutant cells remained similarly sensitive to the challenging heat shock with or without a prior conditioning heat shock (data not shown). These results clearly indicate that trehalose synthesis is an important factor for the acquisition of thermotolerance in *H. polymorpha*. Notably, in this context, while trehalose synthesis was found to be important for the acquisition of thermotolerance in *H. polymorpha*, it was completely dispensable for growth at high temperatures. Thus, the high levels of trehalose found in wild-type cells growing exponentially at 47°C are not essential for growth at these temperatures but rather

TABLE 1. Complementation of the trehalose synthesis defect of *S. cerevisiae* and *S. pombe* Δ *tps1* mutants by *H. polymorpha* *TPS1*^a

Strain	Relevant genotype	Mean amt of trehalose ± SE (g/g of protein)	
		After heat shock	In stationary phase
<i>S. cerevisiae</i>			
YSH 6.106.-3A	<i>TPS1</i>	0.150 ± 0.012	0.008 ± 0.001
YSH 6.106.-1A	Δ <i>tps1</i> :: <i>TRP1</i>	<0.001	<0.001
YSH 6.106.-1A/ YE _p - <i>HpTPS1</i>	Δ <i>tps1</i> :: <i>TRP1/TPS1</i>	0.015 ± 0.006	0.009 ± 0.004
<i>S. pombe</i>			
PB003	<i>tps1</i> ⁺	0.255 ± 0.015	0.225 ± 0.018
PBL-17	<i>tps1</i> :: <i>LEU2</i>	<0.001	<0.001
PBL-17/pUR- <i>HpTPS1</i>	<i>tps1</i> :: <i>LEU2/TPS1</i>	0.187 ± 0.018	0.050 ± 0.010

^a Cells were grown at 27°C to exponential phase (for heat shock experiments) or stationary phase (4 days) on synthetic defined media containing either 2% galactose (YSH 6.106.-1A [*MAT*α *leu2* *ura3* *trp1* *his3* *ade2* Δ *tps1*::*TRP1*]) and its isogenic wild type YSH 6.106.-3A [*MAT*α *leu2* *ura3* *trp1* *his3* *ade2*]) (15) or 2% glucose (PBL-17 [*h*⁺ *ade6-M216* *leu1-32* *ura4-D18* *tps1*::*LEU2*]) and its isogenic wild type PB003 [*h*⁺ *ade6-M216* *leu1-32* *ura4-D18*]) (16) as the carbon source. For heat shock experiments, cells growing exponentially at 27°C were shifted to 40°C for 2 h. Results are the means ± standard errors from three independent experiments. The detection limit for trehalose levels was 0.001 g/g of protein.

may function as a guard against potentially harsher heat conditions.

Effect of Tre6P on the *H. polymorpha* glucose-phosphorylating activity. The deletion of *TPS1* in *H. polymorpha*, unlike deletions of the corresponding genes in *S. cerevisiae* and *K. lactis* but similar to the *tps1*⁺ deletion in *S. pombe*, did not result in any obvious growth defect on a glucose-containing medium. A somewhat intermediate situation has been reported for *A. niger* and *C. albicans*, in which the loss of Tre6P synthase(s) caused an apparent growth defect only at very high glucose concentrations and/or at elevated temperatures, respectively (26, 27). A model which may explain these different phenotypic consequences of the loss of Tre6P synthase suggests that they arise from the different susceptibilities of the corresponding hexokinases to inhibition by Tre6P, which in some Tre6P synthase mutants would lead to an unrestrained flux through hexokinases and consequently the inhibition of growth on glucose (4; for a review including further models see reference 20). Accordingly, while the major hexokinase activities of *S. pombe*, *A. niger*, and *S. cerevisiae* (and *K. lactis*) were found to be insensitive, weakly sensitive, and strongly sensitive, respectively, to Tre6P, the deletion of the Tre6P synthase-encoding genes in the corresponding fungi caused no defect, a weak defect (see above), and a strong defect, respectively, for growth on glucose-containing media (2, 3, 4, 11, 12, 21, 26). Surprisingly, and at variance with this model, the loss of Tre6P synthase in *H. polymorpha* did not impair growth on glucose even at elevated temperatures, despite the fact that we found glucose-phosphorylating activity from *H. polymorpha* to be rather strongly inhibited by 1 mM Tre6P (53.7 and 9.6% inhibition in the presence of 1 and 10 mM glucose, respectively; for a description of the assay see reference 3). These results may be reconciled with the above-presented model, however, if *H. polymorpha* did not rely exclusively on Tre6P synthase-dependent control mechanisms but also on Tre6P synthase-independent control mechanisms to restrict the initial steps of glycolysis. Possibly, since the demand for a high glycolytic flux is particularly strong during fermentative growth, the loss of the Tre6P synthase-mediated control mechanisms may pose a severe problem only for fungi with a high fermentative capacity (*S. cerevisiae* and *K. lactis*) and not for fungi with prevalently respiratory metabolisms, such as *A. niger*, *C. albicans*, and *H. polymorpha*. Thus, the importance of Tre6P synthase for the control of glycolysis in fungi may be determined not only by the susceptibility of hexokinases to Tre6P-mediated inhibition but also by the general (fermentative or respirative) nature of the carbohydrate metabolism.

Nucleotide sequence accession number. A segment of 2,695 bp was submitted as *H. polymorpha TPS1* to the EMBL database under accession no. AJ010725.

Anke Reinders and Ivano Romano contributed equally to the manuscript.

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