Yeast Tolerance to Various Stresses Relies on the Trehalose-6P Synthase (Tps1) Protein, Not on Trehalose*

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Background: Decades of observations strengthened the idea that trehalose is a chemical chaperone.

Results: A catalytically inactive variant of the trehalose-6P synthase (Tps1) maintains cell survival and energy homeostasis under stress exposure.

Conclusion: The Tps1 protein itself, not trehalose, is crucial for cell integrity.

Significance: This work provides unbiased evidence for an alternative function of Tps1, a new "moonlighting" protein.

Trehalose is a stable disaccharide commonly found in nature, from bacteria to fungi and plants. For the model yeast Saccharomyces cerevisiae, both trehalose and trehalose-6P synthase (Tps1) genes were based on the yeast genome project and encoded the yeast Tps1p. Our goal was to generate mutants from which trehalose and/or ATP levels during heat shock. Altogether, these findings suggested that the Tps1 protein is essential to maintain ATP levels during heat shock.

Materials and Methods section of the article and major flaws in cytometry data analysis to data fabrication on the part of one of the authors. Given these errors, the withdrawing authors state that the only responsible course of action would be to withdraw the article to respect scientific integrity and maintain the standards and rigor of literature from the withdrawing authors' group as well as the Journal. The withdrawing authors sincerely apologize to the readers and editors.

This article has been withdrawn by Marie-Ange Teste, Jean M. François, and Jean-Luc Parrou. Marjorie Petitjean could not be reached. The corresponding author identified major issues and brought them to the attention of the Journal. These issues span significant errors in the Materials and Methods section of the article and major flaws in cytometry data analysis to data fabrication on the part of one of the authors. Given these errors, the withdrawing authors state that the only responsible course of action would be to withdraw the article to respect scientific integrity and maintain the standards and rigor of literature from the withdrawing authors' group as well as the Journal. The withdrawing authors sincerely apologize to the readers and editors.

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a highly stable, nonreducing disaccharide found in many organisms including bacteria, archa, plants, invertebrates, and fungi.
were added by recombinant PCR to the extremities of kanMX4 selection marker, yielding to pMP2. This vector was then used as template for site-directed mutagenesis by inverse PCR, yielding to different alleles (tps1-102 in pMP8, tps1-111 in pMP7, and tps1-147 in pMP6). As a final step, the kanMX-tagged alleles were excised from the YCpLac33 backbone by homologous recombination in cells transformed by pMP20, after amplification of the KanMX cassette on the pFA6a plasmid with primers 82 and 83. Strain genotypes were confirmed by PCR. Overexpression of HSPs was carried out using the 2µ plasmid from the yeast tilling collection (Open Biosystems, YSC4613 (28)) and subcloning of the gene of interest in the same pGP564 backbone vector.

**Experimental Procedures**

**Construction of Yeast Strains and Plasmids**—The *S. cerevisiae* strains used in this study (Table 1) were derived from the CEN.PK113–7D, a prototrophic MAL constitutive strain (25), or from the BY4741, a *mal* strain (EUROSCARF collection). Primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The *TPS1* alleles were obtained by site-directed mutagenesis. As an overview of the construction process, 139-bp sequences corresponding to 5’ and 3’ ends of *hisG* were added by recombinant PCR to the extremities of *TPS1*. This mosaic PCR product was cloned into a yeast shuttle plasmid (pMP1, YCpLac33 backbone) and marked with the kanMX4 selection marker, yielding to pMP2. This vector was then used as template for site-directed mutagenesis by inverse PCR, yielding to different alleles (*tps1*-102 in pMP8, *tps1*-111 in pMP7, and *tps1*-147 in pMP6). As a final step, the kanMX-tagged alleles were excised from the YCpLac33 backbone by homologous recombination in cells transformed by pMP20, after amplification of the KanMX cassette on the pFA6a plasmid with primers 82 and 83. Strain genotypes were confirmed by PCR. Overexpression of HSPs was carried out using the 2µ plasmid from the yeast tilling collection (Open Biosystems, YSC4613 (28)) and subcloning of the gene of interest in the same pGP564 backbone vector.

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| Name in the text | Origin | Genotype | Reference | Source |
|------------------|--------|----------|-----------|--------|
| **TPS1**        | CEN.PK | MATa ara3-52 | JF1094    | Ref. 25 |
| tps1Δ           | CEN.PK | MATa ara3-52 tps1::hisG | JF1476 | Lab collection |
| tps1-156        | CEN.PK | MATa ara3-52 tps1::tp1-156 KanMX4 | JF118 | This study |
| tps2Δ           | CEN.PK | MATa tsp2::KanMX4 | JF248 | This study |
| tps1Δ tps2Δ     | CEN.PK | MATa ara3-52 tps1::hisG tps2::KanMX4 | JF87 | This study |
| tps1-156 tps2Δ  | CEN.PK | MATa ara3-52 tps1::hisG tps2::KanMX4, YCP.Lac33 tps1-156 | JF451 | This study |
| tps1-156 hsf1    | CEN.PK | MATa ara3-52 tps1::hisG hsfin1::KanMX6, pTet-O, HSF1 | JF91 | This study |
| tps1-156 hsfl    | CEN.PK | MATa ara3-52 tps1::hisG hsfl::KanMX6, pTet-O, HSF1 | JF90 | This study |
| tps1-156 hsf1 hsf1| CEN.PK | MATa ara3-52 tps1::hisG hsfl::KanMX6, pTet-O, HSF1, YCP.Lac33 tps1-156 | JF101 | This study |
| tps1Δ pHSP104   | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 HSP104 | JF154 | This study |
| tps1Δ pHSP92    | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 HSP92 | JF155 | This study |
| tps1Δ pHSC82    | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 HSC82 | JF156 | This study |
| tps1Δ SSA3      | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 SSA3 | JF160 | This study |
| **TPS1**        | BY4741 | MATa | JF412 | Euroscarf |
| tps1Δ           | BY4741 | MATa tps1::hisG | JF1927 | Lab collection |
| tps1-156        | BY4741 | MATa tps1::tp1-156 KanMX4 | JF173 | This study |

| Strain genetic background. All the CEN.PK113–7D-derived strains bear the MAL2-8c and SUC2 alleles. |
| The plasmids present in the strain are given in bold type. |
| Referred to as strain TPS1 TPS2 in Fig. 2A. |
| This strain is the BY4741 reference strain. All BY strains bear the his3-Δ1 leu2-Δ0 ara3-Δ0 met15-Δ0 ade2 can1 lys2 trp1 alleles. |

**CEN.PK and BY background strains used in this study**

**Table 1**

| Name in the text | Origin | Genotype | Reference | Source |
|------------------|--------|----------|-----------|--------|
| **TPS1**        | CEN.PK | MATa ara3-52 | JF1094    | Ref. 25 |
| tps1Δ           | CEN.PK | MATa ara3-52 tps1::hisG | JF1476 | Lab collection |
| tps1-156        | CEN.PK | MATa ara3-52 tps1::tp1-156 KanMX4 | JF118 | This study |
| tps2Δ           | CEN.PK | MATa tsp2::KanMX4 | JF248 | This study |
| tps1Δ tps2Δ     | CEN.PK | MATa ara3-52 tps1::hisG tps2::KanMX4 | JF87 | This study |
| tps1-156 tps2Δ  | CEN.PK | MATa ara3-52 tps1::hisG tps2::KanMX4, YCP.Lac33 tps1-156 | JF451 | This study |
| tps1-156 hsf1    | CEN.PK | MATa ara3-52 tps1::hisG hsfin1::KanMX6, pTet-O, HSF1 | JF91 | This study |
| tps1-156 hsfl    | CEN.PK | MATa ara3-52 tps1::hisG hsfl::KanMX6, pTet-O, HSF1 | JF90 | This study |
| tps1-156 hsf1 hsf1| CEN.PK | MATa ara3-52 tps1::hisG hsfl::KanMX6, pTet-O, HSF1, YCP.Lac33 tps1-156 | JF101 | This study |
| tps1Δ pHSP104   | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 HSP104 | JF154 | This study |
| tps1Δ pHSP92    | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 HSP92 | JF155 | This study |
| tps1Δ pHSC82    | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 HSC82 | JF156 | This study |
| tps1Δ SSA3      | CEN.PK | MATa leu2,3-112 tps1::hisG, pGP564 SSA3 | JF160 | This study |
| **TPS1**        | BY4741 | MATa | JF412 | Euroscarf |
| tps1Δ           | BY4741 | MATa tps1::hisG | JF1927 | Lab collection |
| tps1-156        | BY4741 | MATa tps1::tp1-156 KanMX4 | JF173 | This study |
TABLE 2

Plasmids used in this study

| Column 1 | Column 2 | Column 3 | Column 4 |
|----------|----------|----------|----------|
| pMP 6    | YcpLac33 tsps1-J56 | YcpLac33; CEN origin; TSPI promoter | This study |
| pMP 20   | pTet-HSF1 | pCM173 backbone (27); CEN origin; Tet-O2 promoter | This study |
| pMP 38   | pHSPI104 | pGPl64 backbone (28); 2µ origin; native promoters | This study |
| pMP 39   | pHS28    | Same as pMP 38 | Same as pMP 38 |
| pMP 40   | pHS28    | Same as pMP 38 | Same as pMP 38 |
| pMP 41   | pSSA3    | Same as pMP 38 | Same as pMP 38 |

* Restriction sites are underlined.

WITHDRAWN

March 26, 2019

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in water bath set at the desired temperature. Transfer and temperature increase. Heat shock (45 °C to 52 °C) was carried out to acquire thermotolerance. After 42 °C and then transferred to the presence of 5% ethanol, oxidative stress, and osmotic stress was analyzed in parent cell populations (log scale) according to the cytometer software. The percentage of the total number of cells determined from the forward scatter plot. Cytometry-based viability assays presented in this work were repeated at least three times, from independent cultures. The data were then exported to Excel for graphics preparation and to XL-Stat or Statgraphic software for statistical analysis. Viability was also determined by measuring cfu after 2 days on YP Gal plates at 30 °C to assess the correlation between the cytometry-based viability assay and cfu results.

Assay of Trehalose-6P Synthase—Crude extracts were prepared according to Ref. 29. Practically, 10 optical density units were collected in a centrifuge tube and washed once with sterile water, and cell pellets were frozen at −20 °C. For protein extraction, 200 µl of extraction buffer (20 mM Hepes, pH 7.1, 1 mM EDTA, 100 mM KCl, 1 mM DTT, and 1 mM PMSF) and 0.3-g glass beads (0.5-mm diameter; BioSpec 11079105) were added to the cell pellets. Crude extracts were kept on ice before use, and total proteins were quantified using the Bradford assay (Bio-Rad protein assay dye reagent concentrate, 500-0006). The activity of trehalose-6P synthase was carried out according to Ref. 29.

Western Blot Analysis of Tps1—Crude extract preparations were carried out as for trehalose-6P synthase assay. For protein electrophoresis, 300 ng of proteins were loaded on Stain-Free precast SDS-PAGE gels 10% (Bio-Rad; 456-8033), and the migration was done at 180 V for 25 min. Transfer to nylon membrane (Bio-Rad; 170-4158) was done using the Trans-Blot® Turbo™ blotting system. Membrane saturation was done in 50 ml of 10× TBS and 0.1% Tween 20, plus 2.5 ml of
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blocking reagent for TBS (Thermo; 37535), for 1 h with gentle agitation and at room temperature. Primary antibodies were raised against the 56-kDa subunit of Tps1 (KH-1142, rabbit serum, kind gift from J. Londsborough, Technical Research Center of Finland, Espoo, Finland (6)) and diluted 1/10,000 times in TBS Tween solution. Hybridization with secondary antibodies was performed with anti-rabbit alkaline phosphatase-conjugated antibodies (diluted 1/2,000 in 1× TBS) (Sigma; A3687). Revelation was monitored by 5-bromo-4-chloro-3-indoly phosphate/nitro blue tetrazolium substrate (Sigma; B3679).

Western Blot Analysis of Hsf1—For crude extract preparation, 50 optical density units were collected from cells grown to exponential phase, before (T0) and during heat shock to 40 °C at the indicated times. Cells were washed once with cold water and frozen in liquid nitrogen. The extraction was carried out immediately after at 4 °C in a buffer containing 50 mm Tris-HCl, pH 6, 0.5 mm NaCl, 1 mm EDTA, pH 7, 0.1% SDS, 1% Triton, 1% mixture protease inhibitor, 20 mm NaF, 1 mm Na2VO3, 50 mm β-glycerophosphate, and 1 mm PMSE. For the control lane with phosphatase, the buffer contained 50 mm Tris-HCl, pH 6, 0.5 mm NaCl, 1 mm EDTA, pH 7, 0.1% SDS, 1% Triton, and 10 units of alkaline phosphatase. The cells were broken in the presence of 0.5-g glass beads for 30 runs of 30 s at 4 °C. The samples were centrifuged for 5 min at 500 × g, and the supernatants were transferred to 1.5-ml microcentrifuge tubes for an additional centrifugation (1 min, 13,000 × g). Crude extracts were stored at −80 °C before their use, and total proteins were quantified using the Pierce; 34075), using the ChemiDocTM XRS imaging system (Bio-Rad); 456-8083), and the migration was carried out at 4 °C at 100 V. Procedure was done according to Tps1 Western blotting. Revelation for Hsf1 was monitored by chemiluminescence using a Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent) was used to certify RNA integrity. Only RNA samples with a 260/280-nm wavelength ratio >2 and a 260/230-nm wave-length ratio >2 were retained for analysis. One microgram of total RNA was reverse-transcribed into cDNA in a 20-μl reaction mixture, using the iScript cDNA synthesis kit (Bio-Rad).

Quantitative PCR Assay on the Biomark—High throughput quantitative PCR was performed using the Fluidigm® Biomark microfluidic system. Every sample-gene combination is quantified using 96.96 Dynamic Array™ integrated fluidic circuits (IFC), which ensure performing up to 9,216 parallel qPCRs in nanoliter scale volumes. Preamplification of cDNA samples, chip loading, and quantitative PCR were performed according to the manufacturer’s protocols. cDNA sample preparation was as follows: 5 ng of each cDNA were initially preamplified (activation at 95 °C for 10 min and 14 PCR cycles (95 °C for 15 s and 60 °C for 4 min)) with PreAmp Master Mix (Fluidigm) and a primer mix at 200 nM containing all the primers targeting all the genes analyzed on the array. Preamplified samples were then diluted after an exogenous treatment (NEB M0293S). To finalize samples preparation for IFC loading, a reagent mix consisting of 440 μl of PreAmp Master Mix (Applied 430976), 44 μl of 20× Enhancer (Fluidigm; 102000), 44 μl of 1:10,000 diluted cDNA sample loading reagent (Fluidigm; 101000) and 132 μl of NanoDrop Technologies, whereas the Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent) was used to certify RNA integrity. Only RNA samples with a 260/280-nm wavelength ratio >2 and a 260/230-nm wave-length ratio >2 were retained for analysis. One microgram of total RNA was reverse-transcribed into cDNA in a 20-μl reaction mixture, using the iScript cDNA synthesis kit (Bio-Rad).

PCR Assays Quality Control and Data Analysis—Most of the primer sets for qPCR, especially the reference genes, were previously described and validated (30). New pairs of primers were designed using Vector NTI advance v11 (Life Technologies, Inc.). A BLAST analysis against the S. cerevisiae genome sequence was included for specificity confidence. Reaction efficiency for each primer pair was evaluated by the dilution series method using a mix of cDNA samples as the template. The absence of genomic DNA in RNA samples was checked by qPCR before cDNA synthesis. All these quality control assays were performed on a classical qPCR machine (MyIQ real time PCR system from Bio-Rad) as previously described (30). The qPCR assays on the Biomark were performed in technical duplicates. A negative control (No Template Con-
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ABSTRACT

The non-fermentative yeast S. cerevisiae was used to validate the role of Tps1, a catalytic subunit of trehalose-6-P phosphatase, in stress tolerance. Three Tps1 catalytically inactive variants were constructed, each with a different residue changed to the corresponding position in the Tps2 protein. They were functionally validated in wild type and mutant strains. Their function was analyzed by drop viability, Western blotting, and transcript levels. Each variant, when expressed in a tps1 mutant, showed a specific activity of 6.5 nmol/min/mg protein. Third, none of these strains could lead to trehalose production under conditions known to readily trigger the accumulation of this sugar, such as heat shock (6, 34, 35). When the cells were incubated for 2 h at 42 °C, which is the optimum temperature for Tps1 catalytic activity and trehalose accumulation, wild type cells accumulated a huge amount of trehalose (22 ± 5 μg eggglucose/107 cells), whereas no trace of this disaccharide could be detected in cells expressing the catalytic variants. Because the three variants gave identical results, including for stress experiments carried out below (data not shown), we arbitrarily used and thoroughly present results obtained with the Tps1D156G variant.

The drop of viability was not prevented by preload of trehalose (see Table 5 for intracellular trehalose content) or by glucose/107 cells, whereas no trace of this disaccharide was detected or not with 1% exogenous trehalose. The strain was precisely measured by a quantitative flow method that allowed cell counting up to 100,000 cells/sample (Fig. 1). For each strain, a typical pattern of the viability was observed in tps1-156 mutant cells expressing the catalytically inactive Tps1D156G variant.

Tps1, Not Trehalose, Is Important for Yeast Viability in Response to Various Kinds of Stresses—In response to temperature upshift to 42 °C, the viability of the tps1Δ mutant sharply dropped by several decades, whereas the viability of the wild type TPS1 strain remained almost unchanged as could be seen from this logarithmic graph (Fig. 2A). Interestingly, the viability pattern of the tps1-156 strain was exactly the same as wild type. In none of these strains, preload of trehalose (see Table 5 for intracellular trehalose content) had a protective effect and changed strain sensitivity to heat. We then investigated the impact of the catalytically inactive Tps1D156G variant on heat resistance of a tps2Δ mutant (Fig. 2A), defective in trehalose-6-P phosphatase and also known to exhibit temperature-sensitive growth (37, 38). When exposed to 42 °C, we found that this mutant was much less sensitive than the tps1Δ mutant, whereas heat sensitivity of the tps1Δ tps2Δ double mutant was almost the same as tps1Δ. In contrast, upon expression of the tps1-156 allele, the tps2Δ mutant recovered the survival rate of wild type TPS1 cells. These data indicated that the heat sensitivity of a tps2Δ mutant strain was likely due to the overaccumulation of trehalose-6-P during heat shock and not to the absence of the Tps2 protein nor to the lack of trehalose.

We then analyzed cell viability in response to desiccation, another relevant adverse condition for yeast. As is shown in Fig. 2B, the loss of TPS1 gene markedly increased the sensitivity of
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yeast cells to dehydration, with less than 2% residual viability after 40 h. As for heat shock to 42 °C, this marked sensitivity was not observed in tps1-156 cells. However, when the strains were loaded with trehalose prior to desiccation, a protective effect of the disaccharide was observed only in the tps1Δ mutant.

These unpredicted results led us to extend investigation into the impact of the Tps1D156G variant and/or of trehalose on the drop of viability in response to various stress conditions. As is shown in Fig. 3, no statistically significant difference could be noticed between TPS1 (wild type) and tps1-156 cells. In none of these stresses, preload of trehalose had a protective effect, contrary to what was observed during desiccation for tps1Δ fragile cells. However, different patterns could be observed according to the stress applied. As previously seen in Fig. 2A, the drop of viability of a tps1Δ mutant was 5 times higher than that of wild type TPS1 and tps1-156 in response to temperature upshift to 42 °C (50% versus 10% drop of viability after 2 h). Likewise, the tps1Δ mutant was two times more sensitive than wild type TPS1 and tps1-156 cells, following a 5 mM H₂O₂ stress. Unexpectedly, we found that exposure to a mild temperature shock at 37 °C for 2 h was more detrimental than at 42 °C, even for the wild type TPS1 strain. Also, this drop of viability at 37 °C was comparable between TPS1, tps1-156 and tps1Δ cells. Similar results were obtained upon treatment of these three strains with 5% ethanol for 6 h.

Very similar profiles were obtained with strains from the BY4741 background (data not shown). A notable exception was the extreme sensitivity to desiccation stress of the BY derived

FIGURE 1. Monitoring cell viability by flow cytometry. A, auto-fluorescence. Representative BL-1–BL-3 scatter plots (log scale, BL-1 [525-nm filter], BL-3 [670-nm filter], showing right column, tps1Δ (middle column), and tps1-156 (left column). Auto-fluorescence plots (A), or stained cells fluorescence plots for viability determination, before (A) or after exposure of the cells to 42 °C for 2 h (B). A, cell auto-fluorescence plots. Voltage calibration was set for optimal positioning of the whole cell population below 103 thresholds (red gate), for both the x axis (BL-1, 525-nm filter) and the y axis (BL-3, 670-nm filter). B, viability determination plots of exponentially growing cells, after staining with the Guava ViaCount reagent. x axis, fluorescence intensity of the nuclear dye, which stains only nucleated cells; y axis, fluorescence intensity of the viability dye, which brightly stains dying cells. Positioning of gates for "living" cells (blue gate) and "nonviable" cells (red gate), according to manufacturer's recommendations. Numbers (in %) indicate the fraction of the population in the gate. C, viability determination plots of cells exposed for 2 h to 42 °C. Legend is as in B.

TABLE 4

Influence of TPS1 alleles on the viability of exponentially growing cells

| Carbon source | CEN.PK113–7D background | BY4741 background |
|---------------|--------------------------|-------------------|
|               | TPS1 | tps1Δ | tps1-156 | TPS1 | tps1Δ | tps1-156 |
| Galactose 2%  | 91 ± 4 | 63 ± 10 | 89 ± 4 | 86 ± 5 | 61 ± 12 | 83 ± 6 |
| Trehalose 2%  | 84 ± 5 | 61 ± 8 | 86 ± 5 | NG | NG | NG |
| Gal Tre       | 88 ± 6 | 66 ± 7 | 86 ± 4 | 81 ± 6 | 55 ± 8 | 84 ± 3 |
| Gly Lac Eth   | 84 ± 5 | 59 ± 11 | 85 ± 7 | ND | ND | ND |

Shown are results from flow cytometric analysis of viability of exponentially growing cells (A₅₀₅ = 1.0 unit) cultivated in YN synthetic medium supplemented with different carbon sources at 30 °C. The values are the means ± S.D. of five independent experiments. Gal Tre, 2% galactose + 1% trehalose; Gly Lac Eth, 3% glycerol + 2% lactate + 2% ethanol; ND, not determined; NG, no growth.
FIGURE 2. Time-dependent evolution of viability during exposure to heat shock or desiccation. The figure shows the viability of CEN.PK background strains (A and B) in the absence (YN Gal medium, (−)) or in the presence of trehalose (YN GalTre medium, (+)), BY background strains (C) were only grown on YN Gal medium because their mal/H11002 genotype precluded the import of trehalose from the medium. The data are presented as means ± S.D. of at least three independent biological replicates. S.D. not plotted on the log scale graphics.

A, viability (log scale) as a function of time, in response to heat shock to 42 °C. The dotted line-delimited area in the left panel highlights the scaling for data presented in the right panel. B, viability (log scale) as a function of desiccation time. After desiccation at 30 °C for the indicated time, cell viability was measured after 1 h of rehydration in PBS solution (see Ref. 18 for further details). Control at time 0 corresponds to exponentially growing cells at 30 °C. The dotted line-delimited area highlights the scaling for data presented in C. C, same as in B with BY background strains.

TABLE 5
Intracellular trehalose quantification in the CEN.PK113–7D background strains

Tre and Tre indicate whether growth was performed in YN Gal or in YN GalTre medium, respectively. The values are the means ± S.D. of three biological replicates and are expressed in μg eqg glucose/10^7 cells.

| Condition | TPS1 − Tre | TPS1 + Tre | tps1∆ − Tre | tps1∆ + Tre | tps1-156 − Tre | tps1-156 + Tre |
|-----------|------------|------------|-------------|-------------|----------------|----------------|
| 2 h after upshift to the indicated temperature (unless otherwise stated in parentheses) | | | | | | |
| Control (30 °C; T_0) | 0 | 28 ± 8 | 0 | 20 ± 7 | 0 | 19 ± 6 |
| 37 °C (1 h) | 0 | 46 ± 6 | 0 | 50 ± 7 | 0 | 49 ± 7 |
| 42 °C (1 h) | 35 ± 6 | 45 ± 6 | 0 | 47 ± 8 | 0 | 41 ± 9 |
| 42 °C | 22 ± 5 | 33 ± 7 | 0 | 51 ± 10 | 0 | 46 ± 9 |
| 45 °C | 13 ± 4 | 36 ± 7 | 0 | 25 ± 9 | 0 | 32 ± 7 |
| 48 °C | 3 ± 2 | 39 ± 9 | 0 | 33 ± 7 | 0 | 32 ± 6 |
| 50 °C | 0 | 22 ± 8 | 0 | 8 ± 7 | 0 | 15 ± 6 |
| 52 °C | 0 | 25 ± 6 | 0 | 18 ± 6 | 0 | 17 ± 3 |
| 2 h after temperature upshift to 50 °C, following a preheat for 1 h at the given temperature | | | | | | |
| Preheat at 37 °C | 3 ± 2 | 35 ± 8 | 0 | 41 ± 12 | 0 | 35 ± 11 |
| Preheat at 42 °C | 0 | 21 ± 8 | 0 | 26 ± 9 | 0 | 31 ± 5 |
| Under peroxide, salt or ethanol exposure for the indicated time | | | | | | |
| Control (30 °C; T_0) | 0 | 25 ± 7 | 0 | 23 ± 11 | 0 | 27 ± 8 |
| 50 mM H_2O_2 for 2 h | 0 | 23 ± 6 | 0 | 9 ± 6 | 0 | 26 ± 9 |
| 1 M NaCl for 2 h | 0 | 26 ± 5 | 0 | 19 ± 4 | 0 | 23 ± 5 |
| 5% EtOH for 6 h | 8 ± 3 | 9 ± 6 | 0 | 4 ± 3 | 0 | 7 ± 5 |
strains (Fig. 2, compare C and B). Altogether, these results strengthened the contribution of the Tps1 protein, but not of trehalose, in the tolerance of yeast to oxidative stress and high temperature.

**Tps1 Is Required for Thermotolerance and Acquired Thermotolerance**—Literature data are relatively inconsistent with respect to the conditions carried out to investigate physiological responses to heat shock, because temperature upshifts ranging from moderate (i.e. 37 °C) to extreme temperatures (i.e. up to 52 °C) can be found. Thus, based on the above results, we sought to reassess the potential role of either trehalose or Tps1 protein in yeast subjected to different temperature upshifts. Results presented in Fig. 4A showed that the deletion of TPS1 rendered the cells extremely sensitive to temperature stress above 40 °C. They also established the importance of the Tps1 protein in protecting the cells, because the sole presence of the Tps1D156G variant allowed yeast cells to resist like wild type to these heat shocks. These results finally confirmed the ineffectiveness of trehalose to protect cells against high temperature stress. Preloading TPS1, tps1Δ, and tps1-156 cells with the disaccharide did not provide any positive impact on the heat resistance of these cells (Fig. 4A), despite similar accumulation of trehalose in the different strains for all tested temperatures (Table 5). Comparable behavior was obtained with strains from the BY4741 background (data not shown), but, as reported above with other stresses, these BY strains showed a slightly higher thermosensitivity than the CEN.PK strains.

We then investigated whether the adaptive or acquired thermotolerance required Tps1 rather than trehalose. Acquired thermotolerance is defined as the ability of proliferating yeast cells to withstand a potentially lethal heat shock (e.g. 50 °C), provided they are previously submitted to gentle stress such as a moderate temperature rise (39). As compared with a direct shift of yeast cells from 30 °C to 50 °C, a preheat at 37 °C for 1 h did not help the cells to better survive at this high temperature (Fig. 4B). These data reinforced the observation made above that the TPS system hardly contributes to cell resistance in response to a mild temperature shift to 37 °C. In contrast, a preincubation of yeast cells at 42 °C for 1 h clearly increased the potency of both wild type TPS1 and tps1-156 cells to endure exposure to 50 °C, whereas the viability of tps1Δ cells remained extremely low under this condition (Fig. 4B). Again, preloading the cells with trehalose did not bring any positive effect on the adaptive thermotolerance of these cells. Similar results were obtained using the BY4741 strain background (data not shown). We could therefore conclude that the Tps1 protein itself, and not trehalose, is part of the molecular machinery necessary to survive to high temperature stresses.
The Contribution of Tps1 to Thermotolerance Is Independent of Hsf1-dependent Transcriptional Upregulation. To determine whether the Tps1 protein played a major role in the acquisition of thermotolerance, we used strains deleted for the essential gene and rescued by expression of this gene under the tetracycline-regulable promoter. Western blot analysis confirmed that Hsf1 protein was no longer detected in cells treated with 0.5 μg/liter tetracycline (+, expression of HSF1 is down) or in the absence of the antibiotic (−, expression of HSF1 is up). B, mobility shift of Hsf1 upon exposure of yeast cells to 40 °C for 10–30 min. Strains were transformed with pTet-HSF1, leading to high expression of the protein. The 30-min ptps1Δ sample (lane 1) was also treated by 1 unit of alkaline phosphatase (+). C, RT-qPCR analysis of HSF1 and HSP transcript levels after exposure of wild type TPS1 and ptps1Δ cells to 40 °C for 15–30 min. The values give normalized fold changes (log scale) relative to the TPS1 strain before heat shock (time 0), used as the calibrator sample. Normalization was carried out using multiple, validated reference genes (TAF10, ALG9, IP11, and UBC6). The data are represented as means ± S.D. of two biological replicates.

The Contribution of Tps1 protein to Thermotolerance. The Contribution of Tps1 to thermotolerance is not mediated by Hsf1 protein. Western blot analysis of Hsf1 in yeast crude extracts (lanes 1 and 2) showed the native form of this protein, because it disappeared after incubation of the cell extract with alkaline phosphatase prior to gel electrophoresis. The intensity of this upper, phosphorylated band was proportional to the phosphorylation state of Hsf1 upon exposure of yeast cells to 40 °C for 10–30 min, leading to high expression of the protein. The 30-min ptps1Δ sample (lane 1) was also treated by 1 unit of alkaline phosphatase (+). C, RT-qPCR analysis of HSF1 and HSP transcript levels after exposure of wild type TPS1 and ptps1Δ cells to 40 °C for 15–30 min. The values give normalized fold changes (log scale) relative to the TPS1 strain before heat shock (time 0), used as the calibrator sample. Normalization was carried out using multiple, validated reference genes (TAF10, ALG9, IP11, and UBC6). The data are represented as means ± S.D. of two biological replicates.

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(30), whereas previous works were done by Northern blot analysis, a much less accurate and sensitive method.

Finally, we explored a genetic interaction between TPS1 and HSF1 on the cell viability in response to 40 °C heat shock (Fig. 6). The strong expression of Hsf1 (TPS1 hsf1Δ pTet-HSF1 strain) led to similar results as for the TPS1 strain (compare data in Figs. 3 and 6). Lowering the expression of Tet-O promoter with the antibiotic barely affected the viability of these cells at 30 °C. Interestingly, overexpression of Hsf1 sensitized them to heat shock as illustrated by a moderate 10% drop of viability after 2 h at 40 °C. Interestingly, viability profiles of the tps1-156 hsf1Δ pTet-HSF1 strain were strictly identical to the control strain (TPS1 hsf1Δ). To determine whether the high strain sensitivity to heat shock observed upon overexpression of Hsf1, as illustrated by the drop of viability in the tps1Δ hsf1Δ mutant (Fig. 6A), could be recapitulated in the absence of tetracyclin, we determined the viability of this strain transformed with the plasmid bearing Tet-O promoter with the antibiotic barely affecting the viability of these cells at 30 °C but considerably dropped significantly to reach less than 10% viable cells after 8 h of Tet-O extinction. Taken together, these results further indicated that trehalose had no role in thermotolerance and adaptive thermotolerance, which actually implicated Tps1, independently of the Hsf1 transcriptional activity.

Tps1 Protects Cells from ATP Depletion upon Exposure to Heat Shock—Based on the above results, we investigated whether overexpression of different heat shock proteins can suppress the tps1Δ sensitivity to heat shock. Accordingly, cell survival of this mutant after exposure to 42 °C for 2 h increased from 10 to ~70% upon overexpression of HSP104, SSA3, HSP82, and HSC82 (Fig. 7A). Among the different HSPs found to suppress this tps1Δ phenotype, HSP104 turned out to be the most efficient suppressor able to restore thermotolerance and adaptive thermotolerance similar to wild type cells (Fig. 7, A and B). However, upon longer exposure, the cell viability of the tps1Δ transformed with a high copy vector containing HSP104 dropped significantly to reach less than 10% viable cells after 8 h (Fig. 8A). Because of the nature of the Hsp104 protein that belongs to the Clp/Hsp100 family of AAA+ proteins (ATPases associated with various cellular activities) (48) and because energy deficiencies in the tps1Δ mutant have been recently raised from contradictory results, notably in thermostolerance and desiccation (51–53). A main reason for this ambiguity was the inability to directly assess the physiological function of trehalose in yeast cells without interfering with its metabolism, as well as with stress applied. In this report, we achieved this goal by exploiting our previous finding that yeast
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The most remarkable finding in this report was to show that the Tps1 protein is indispensable in yeast to withstand high temperature and oxidative stresses. Indeed, contrary to a tps1Δ mutant that is stress hypersensitive, a mutant strain expressing a catalytically inactive Tps1 variant exhibited the same sensitivity to stresses as wild type cells. We nevertheless made the observation that Tps1 had no role in protecting cells against a mild heat stress carried out at 37 °C or a severe stress to 50 °C after a preconditioning at 37 °C, whereas this protein became determinant in yeast survival to temperature upshift to 40 °C or higher. This result indicated that the function of Tps1 to protect cells against severe heat stress relies on low amounts of the protein present in exponentially growing cells before stress exposure (8) and does not require its transcriptional activation, which only takes place at temperature below 40 °C (6). As a conclusion, our finding strongly supports the notion that the yeast S. cerevisiae Tps1 protein, similar to what was found for the M. grisea protein (23), has regulatory functions, independent from its enzymatic role in the trehalose biosynthetic pathway.

A system for yeast to maintain heat shock could be an interplay between stress genes in the heat shock machinery. We showed that trehalose could contribute together, but in an independent manner, to the thermotolerance. This result indicated that the function of Tps1 to protect cells against severe heat stress relies on low amounts of the protein present in exponentially growing cells before stress exposure (8) and does not require its transcriptional activation, which only takes place at temperature below 40 °C (6). As a conclusion, our finding strongly supports the notion that the yeast S. cerevisiae Tps1 protein, similar to what was found for the M. grisea protein (23), has regulatory functions, independent from its enzymatic role in the trehalose biosynthetic pathway.

The question was therefore to know how Tps1 contributes to thermotolerance of the cells in interaction with Hsf1-dependent heat shock proteins. Part of the explanation came from the finding that the ATP content in a mutant lacking Tps1 protein was completely depleted within 1 h after heat shock to 42 °C, whereas this loss of ATP was fully prevented in cells expressing the catalytically inactive Tps1 variant. The Tps1 dependence of ATP maintenance could therefore explain that the suppression of the heat sensitivity of tps1Δ by overexpression of HSP104 did not persist upon long term exposure to 42 °C, because ATP is indispensable for the disaggregation and refolding activity of Hsp104 protein (56–58). In addition, this function of Tps1 in the maintenance of energy may reconcile previous reports showing that the conformational repair of a glycoprotein in ER lumen or the solubilization of a mutant huntingtin protein in heat shocked cells is compromised in a tps1Δ mutant (59, 60).

Finally, we found that the absence of the Tps1 protein resulted in a dramatic 30–40% drop of viability in exponentially

FIGURE 8. The tps1Δ mutant rapidly loses viability in response to heat shock. A, loss of viability (log scale) of the tps1Δ mutant in response to heat shock to 42 °C. The dotted line refers to the wild type tps1Δ mutant. The ATP levels data presented in FIGURE 8 are represented as means ± S.D. of three independent biological replicates. B, ATP levels in response to heat shock. The strains are the wild type tps1Δ, tps1-156, tps1Δ + pHSP104, tps1-156 + pHSP104, and tps1Δ + pHSP104. The results are the mean ± S.D. of three independent biological replicates.
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growing cells without exposure to any harmful conditions. Because these tps1Δ cells exhibited an ATP content similar to wild type cells in all permissive growth conditions for tps1Δ mutant (Ref. 49 and unpublished data), it is likely that Tps1 must have additional regulatory function(s) indispensable for cell viability in dividing cells. Moreover, Tps1 is a relatively low abundance protein in exponentially growing yeast cells on glucose (6, 8, 61), with similar levels to Pkh26, which synthesizes the glycolytic effector Fru-2,6-P₂ (62). We then favor the idea that Tps1, in addition to its catalytic function in trehalose synthesis, is a sensing/signaling intermediate with regulatory function(s), at least in energy homeostasis. Such function in preventing energy depletion should be essential to withstand adverse conditions.

To conclude, beyond the simple metabolic function of Tps1 via trehalose-6P and trehalose synthesis, our results may allow considering Tps1 as a new, attractive example of “moonlighting” proteins, which are characterized by their ability to perform completely unrelated tasks utilizing regions outside the active site for other functions, mostly regulatory and structural (63). Also, in light of this crucial role of Tps1 in stress resistance, probably through mechanisms that maintain energy homeostasis, our view of survival mechanisms to stress has to be revised and will likely be extendable to other organisms that express Tps1 homologs. These findings finally appear strategic for two relevant yet divergent perspectives. The first one fits with the current interest of many biotechnologically oriented efforts aiming at optimizing the microbial cell factories yeasts. In addition to their use in traditional processes, yeasts face growing interest in being an appealing target for strain selection and/or engineering. Considering Tps1 as a new, attractive example of “moonlighting” proteins, which are characterized by their ability to perform completely unrelated tasks utilizing regions outside the active site for other functions, mostly regulatory and structural (63).

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Note Added in Proof—The autofluorescence scatter plots shown in Fig. 1A were not correct in the version of this article that was published on May 1, 2015 as a Paper in Press. Specifically, data from the TPS1 strain was mistakenly duplicated and used to represent results from the tps1-156 strain. Furthermore, the scatter plots shown in Fig. 1 (B and C) represented results of independent experiments with possible experimental variation. The corrected version of Fig. 1 presents data obtained for TPS1, tps1Δ, and tps1-156 strains from experiments performed on the same day to avoid variations caused by the environment (medium and culture conditions). This correction does not affect the interpretation of the results or the conclusions.
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