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.

Yeast is a model eukaryotic organism. Its simplicity makes this organism a suitable model for studying physiological processes, and the ease of genetic manipulation of its genome makes it a convenient organism for careful analysis of the properties of genes. Trehalose synthesis is essential for yeast cells, and its concentration is high during stationary phase and quiescence. We have shown that trehalose synthesis is necessary to maintain long-term cellular integrity under stress conditions. The stress-protecting function, which is the second major function ascribed to trehalose, was mainly deduced from correlations between trehalose levels and cell survival under adverse environmental conditions, such as high thermotolerance of stationary phase cells coincidently with high level of trehalose. Analysis of phenotypes associated with the deletion of the TPS1 gene encoding the trehalose-6-phosphate synthase that catalyzes the first enzymatic step of trehalose synthesis further supported this proposition. Indeed, tps1Δ mutants showed hypersensitivity to stress of various origins. The Tps1 protein is a key player in the stress response of the yeast cell. Using a yeast mutant unable to take up exogenous trehalose, we showed that the accumulated trehalose was readily consumed under carbon-starved conditions by a mechanism implicating the periplasmic acid trehalase (Atha1). Also, Shi et al. (11) reported a rapid trehalose mobilization upon exit of yeast cells from quiescent state. The stress-protecting function, which is the second major function ascribed to trehalose, was mainly deduced from correlations between trehalose levels and cell survival under adverse environmental conditions, such as high thermotolerance of stationary phase cells coincidently with high level of trehalose. Analysis of phenotypes associated with the deletion of the TPS1 gene encoding the trehalose-6-phosphate synthase that catalyzes the first enzymatic step of trehalose synthesis further supported this proposition. Indeed, tps1Δ mutants showed hypersensitivity to stress of various origins.

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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.
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TABLE 1

CEN.PK and BY background strains used in this study

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<th>Name in the text</th>
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<sup>a</sup> Strain genetic background. All the CEN.PK113-7D-derived strains bear the MAL2-8c and SUC2 alleles.

<sup>b</sup> The plasmids present in the strain are given in bold type.

<sup>c</sup> Referred to as strain TPS1 TPS2 in Fig. 2A.

<sup>d</sup> 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.

kinds, including heat, freezing shock, hydrostatic pressure, desiccation, and oxidative, ethanol and osmotic stresses (13–19). However, the loss of the TPS1 gene is also accompanied by several other phenotypes, apparently unrelated to the lack of trehalose, such as the inability to grow on rapidly fermentable sugars, hyperaccumulation of glycogen, sporulation and respiratory defects (20–22). In the rice blast fungus Magnaporthe oryzae, Talbot’s group (23, 24) succeeded in demonstrating that Tps1, in addition to trehalose-6P synthase enzymatic activity, possesses a regulatory function essential for the life cycle of the fungus.

These observations led us to suggest that this protein may have regulatory functions in addition to its enzymatic role in the synthesis of trehalose. Whether the trehalose and/or the Tps1 protein may help yeast to withstand stress exposure, we constructed and expressed catalytically inactive variants of Tps1 unable to synthesize trehalose, such as the inability to grow on rapidly fermentable sugars, hyperaccumulation of glycogen, sporulation and respiratory defects (20–22). In the rice blast fungus Magnaporthe oryzae, Talbot’s group (23, 24) succeeded in demonstrating that Tps1, in addition to trehalose-6P synthase enzymatic activity, possesses a regulatory function essential for the life cycle of the fungus.

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<sup>−</sup> 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 tps1-156 in pMP6). As a final step, the kanMX6 cassette was excised from the YCpLac33 backbone (27) using the In-Fusion system (Clontech) with primers 65 and 66. HSF1 gene was then deleted by homologous recombination in cells transformed by pMP20, after amplification of the KanMX6 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, YSc6413 (28)) and subcloning of the gene of interest in the same pGP564 backbone vector.

Culture Conditions—Unless otherwise stated, yeast cells were cultivated in YN synthetic medium (yeast nitrogen base without amino acids and ammonium at 1.7 g/liter (Difco)), supplemented with ammonium sulfate at 5 g/liter, complete drop-out when required (MP-Biomedicals CSM) and 2% (w/v) galactose (YN Gal) or 2% galactose plus 1% trehalose (YN GalTre medium). Cultures were conducted in shake flasks (0.5-liter Erlenmeyer flasks with a working volume of 100 ml) at 30 °C and 200 rpm on a rotary shaker. Cell plating for viability analysis (cfu assay) was performed on YP Gal medium (10 g/liter yeast extract, 10 g/liter bacto peptone, 20 g/liter bacto agar, and 20 g/liter galactose). For modulation of HSF1 transcript levels using the Tet-O<sub>2</sub> promoter, cells were cultivated according to Ref. 27 in the presence of 0.5 μg/ml tetracyclin (Sigma, stock solution of 5 mg/ml in 50% ethanol).

Stress Tolerance Assays—All stress experiments were carried out using exponentially growing cells (A<sub>600</sub> 0.5–0.7, corresponding to 1 × 10<sup>6</sup> cells/ml) cultivated on YN Gal or YN GalTre medium at 30 °C, after inoculation of the culture with a cell density corresponding to A<sub>600</sub> 0.025. For mild heat shock (37 °C to 42 °C), cells were incubated in an oven after initial immersion
in water bath set at the desired temperature for the heat transfer and temperature increase. Thermal shock (45 °C to 52 °C) was carried out for 1 h to acquire thermotolerance. Cells were incubated at 42 °C and then transferred and incubated at 30 °C to assess the presence of 5% ethanol (v/v), 5 mM H2O2, and 1 M NaCl, which stains only nucleated cells, and the 670-nm filter attached to the Attune Cytometer software. The percentage of cells determined from the forward scatter and side scatter plot. Cytometry-based viability assays presented in the text 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. Practicaly, 10 optical density units were collected in a centrifuge tube and washed twice with sterile water, and cell pellets were frozen at −20 °C. For protein extraction, 200 μl of extraction buffer (20 mM Heps, 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. Lendresque, 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-indolyl 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 supernatant was transferred to 1.5-ml microcentrifuge tubes for an additional centrifugation (1 min, 13,000 × g). Crude extract was kept on ice before their use, and total proteins were measured using the Bradford assay (Bio-Rad protein assay dye reagent concentrate; 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 blot analysis.

For Western blot analysis, proteins were loaded on a 4–15% precast polyacrylamide gel; proteins were loaded on a 4–15% precast polyacrylamide gel; gels were run in a gel apparatus from Bio-Rad (Bio-Rad; 456-8083), and were stained with Coomassie blue. The proteins were visualized using the ChemiDocTM XRS imaging system from Bio-Rad. Primary antibodies used were anti-mouse peroxidase-conjugated antibodies (diluted 1/2,000 in 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 supernatant was transferred to 1.5-ml microcentrifuge tubes for an additional centrifugation (1 min, 13,000 × g). Crude extract was kept on ice before their use, and total proteins were measured using the Bradford assay (Bio-Rad protein assay dye reagent concentrate; 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 blot analysis.

RNA Sampling and cDNA Synthesis for Quantitative PCR Assays—The cells were grown in YN Gal medium to A600~1.0 and submitted to heat shock as described above. Samples (~10⁸ cells) were collected (2,200 × g, 4 °C, 3 min) before (T0 control, calibrator sample) and 15 and 30 min after the shift to 40 °C. The pellets were immediately transferred in 2-ml microcentrifuge tubes, washed with 1 ml of cold water, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Frozen cells were mechanically disrupted for 3 min using the TissueLyserII apparatus from Qiagen, with one stainless steel bead per tube. Total RNA was extracted using the SV Total RNA purification kit from Promega. The quantification of the RNA samples was assessed by using the ND-1000 UV-visible light spectrophotometer (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 of ~2 and a 260/230-nm wavelength 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® (Fluidigm) 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 exonuclease treatment (NEB M0293S). To finalize samples preparation for IFC loading, a reagent mix consisting of 440 μl of Fluidigm Master Mix (Applied 430976), 44 μl of 20X PreAmp, and a 15 μl sample loading reagent (Fluidigm; 10X). A total of 7 μl of reagent was added to 6-μl aliquots were dispensed in units with 1 μl of water. Two microliters of primer sets were added to this reagent. For PCR preparation, each pair of primers was prepared in 100 nl of water (each primer) in the assay loading mix. 2 μl aliquots were dispensed into the 96 wells of the assay loading mix. The Dynamic Array™-controlled thermal cycling with 1 μl aliquots was dispensed into the inlets of the IFC. After loading the cDNA samples and primer sets into the IFC on the IFC Controller HX, the IFC was placed into the BioMark for thermal cycling with the following protocol: thermal mix steps at 50 °C for 2 min, 70 °C for 30 min, and 25 °C for 10 min and hot start at 50 °C for 2 min and 95 °C for 10 min, followed by 35 PCR cycles (95 °C for 15 s and 60 °C for 60 s). After completion of these cycles, melting analysis was performed to verify PCR specificity, contamination, and the absence of primer dimers.

qPCR 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

3The abbreviations used are: qPCR, quantitative PCR; IFC, integrated fluidic circuit.
control) was incorporated in each assay. Assay efficiencies of all primer sets analyzed on the IFC were re-evaluated by the dilution series method and applied to final calculations of transcript levels.

For normalization purposes, the transcript levels of six putative reference genes were evaluated in each sample (data not shown). For data analysis, individual Cq values were exported from the Fluidigm real time PCR analysis software version 4.1.2. They were used to assess normalized fold change values, using the wild type (TPS1) strain before heat shock as the calibrator sample, and robust normalization factors calculated from the geometric averaging of the four most stable reference genes in our experimental setup (i.e. TAF10, ALG9, IPP1, and UBC6). Final fold-change values are given as means ± S.D. from the two biological replicates (independent cultures) that were carried out for transcript level analysis in the CEN.PK strain background.

Other Analytical Procedures—Cell sampling and metabolites extraction were carried out as previously described (31), and ATP measurement was realized as described (32). The enzymatic measurement of ATP was carried out at 30 °C in Tris-HCl buffer with addition of 0.4 mM NAD+, 0.1 mM glucose, 4.5 units/ml glucose-6-phosphate dehydrogenase, and 1.5 units/ml hexokinase. NADH production was then assessed at 340 nm using a fluorescence spectrophotometer (Agilent G1103A). Trehalose measurement was done according to Ref. 33.

Statistical Analysis of the Data—Statistical analyses of “drop in viability” data were conducted by using the STATGRAPHICS Centurion 16 software. This included the analysis of variance, the Tukey’s test on the means, the nonparametric ICS Centurion 16 software. This included the analysis of variance in the CEN.PK strain background. The impact of different stresses was assayed on exponentially growing cells in a galactose synthetic medium, permissive for growth of the tps1 mutant that was not or with 1% exogenous trehalose. One set of strains was precisely measured by the flow cytometric method that allowed monitoring cells in a galactose synthetic medium, permissive for growth of the tps1 mutant that was not or with 1% exogenous trehalose. One set of strains was precisely measured by the flow cytometric method that allowed monitoring the cells in a galactose synthetic medium, permissive for growth of the tps1 mutant that was not or with 1% exogenous trehalose.

Results

Construction and Validation of Inactive Variants of the Tps1 Protein—To construct catalytically inactive variants of the S. cerevisiae Tps1 protein, we followed the strategy employed earlier by Wilson et al. (23) for Magnaporthe grisea Tps1. We mutated three key residues required for the interaction with glucose-6P substrate in the catalytic site of the protein, leading to Tps1Y102V, Tps1W111S, and Tps1D156G variants, respectively. After transformation of a tps1Δ mutant with CEN plasmids carrying the corresponding tps1 alleles, the loss of function of these strains relative to trehalose metabolism was validated in four ways. First, Western blotting against Tps1 validated that expression and stability of Tps1 variants were identical to wild type (not shown). Second, none of these strains exhibited measurable trehalose-6P synthase activity, whereas wild type (TPS1) cells led to a specific activity of 6.5 nmol/min/mg protein. Third, none of these strains could lead to trehalose-6P accumulation upon glucose addition to trehalose-grown cells according to experimental conditions described in (31). Contrary to the control strain that showed a burst of trehalose-6P 5 min after the glucose pulse (8 μmol/g DW), trehalose-6P was below the detection level in cells expressing the different tps1 alleles. As a final proof for trehalose-6P synthase deficiency in vivo, we assessed the inability to produce trehalose 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 egg glucose/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.

To directly assess the role of trehalose in response to various stresses (see below), we used the MAL- CEN.PK strain, able to import trehalose from the medium by the constitutively expressed Agt1 transporter (26, 36). The function of the catalytically inactive variants of Tps1 in yeast survival to stress was therefore examined in this CEN.PK strain background, as well as in the mal- BY4741 strain unable to import trehalose. The impact of different stresses was assayed on exponentially growing cells in a galactose synthetic medium, permissive for growth of the tps1 mutant that was not or with 1% exogenous trehalose. One set of strains was precisely measured by the flow cytometric method that allowed monitoring the cells in a galactose synthetic medium, permissive for growth of the tps1 mutant that was not or with 1% exogenous trehalose. One set of strains was precisely measured by the flow cytometric method that allowed monitoring the cells in a galactose synthetic medium, permissive for growth of the tps1 mutant that was not or with 1% exogenous trehalose.

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-6P 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-6P 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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FIGURE 1. Monitoring cell viability by flow cytometry. Representative BL-1–BL-3 scatter plots (log scale, BL-1 (525-nm filter), BL-3 (670-nm filter)), showing TPS1 (left column), tps1Δ (middle column), and tps1-156 (right column). Autofluorescence plots (A), or stained cells fluorescence plots (B), and viability determination plots of exponentially growing cells, after staining with the 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 autocorrelation analysis is shown in the middle column. A, viable cell autofluorescence plots (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 autocorrelation analysis is shown in the middle column. C, viability determination plots of exponentially growing cells, after staining with the 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 autocorrelation analysis is shown in the middle column. Legend is as in B.

TABLE 4

Influence of TPS1 alleles on the viability of exponentially growing cells

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<th>BY4741 background</th>
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<tr>
<td>Galactose 2%</td>
<td>91 ± 4</td>
<td>63 ± 10</td>
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<tr>
<td>Trehalose 2%</td>
<td>84 ± 5</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>Gal Tre</td>
<td>88 ± 6</td>
<td>66 ± 7</td>
</tr>
<tr>
<td>Gly Lac Eth</td>
<td>84 ± 5</td>
<td>59 ± 11</td>
</tr>
</tbody>
</table>

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 (p values < 10⁻⁵).

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 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 represented 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 eq glucose/10^7 cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>TPS1 (-)</th>
<th>TPS1 (+)</th>
<th>tps1Δ (-)</th>
<th>tps1Δ (+)</th>
<th>tps1-156 (-)</th>
<th>tps1-156 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h after upshift to the indicated temperature (unless otherwise stated in parentheses)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (30 °C; T₀)</td>
<td>0 ± 2</td>
<td>20 ± 7</td>
<td>0 ± 2</td>
<td>19 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 °C (1 h)</td>
<td>0 ± 1</td>
<td>45 ± 5</td>
<td>0 ± 2</td>
<td>49 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 °C (1 h)</td>
<td>35 ± 6</td>
<td>45 ± 6</td>
<td>47 ± 8</td>
<td>41 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 °C</td>
<td>22 ± 5</td>
<td>33 ± 7</td>
<td>51 ± 10</td>
<td>46 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 °C</td>
<td>13 ± 4</td>
<td>36 ± 7</td>
<td>25 ± 9</td>
<td>32 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 °C</td>
<td>3 ± 2</td>
<td>39 ± 9</td>
<td>33 ± 7</td>
<td>32 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 °C</td>
<td>0 ± 1</td>
<td>22 ± 8</td>
<td>8 ± 7</td>
<td>15 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52 °C</td>
<td>0 ± 1</td>
<td>25 ± 6</td>
<td>18 ± 6</td>
<td>17 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h after temperature upshift to 50 °C, following a preheat for 1 h at the given temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preheat at 37 °C</td>
<td>0 ± 2</td>
<td>35 ± 8</td>
<td>41 ± 12</td>
<td>35 ± 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preheat at 42 °C</td>
<td>0 ± 2</td>
<td>21 ± 8</td>
<td>26 ± 9</td>
<td>31 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under peroxide, salt or ethanol exposure for the indicated time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (30 °C; T₀)</td>
<td>0 ± 2</td>
<td>25 ± 7</td>
<td>23 ± 11</td>
<td>27 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM H₂O₂ for 2 h</td>
<td>0 ± 2</td>
<td>23 ± 6</td>
<td>9 ± 6</td>
<td>26 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M NaCl for 2 h</td>
<td>0 ± 2</td>
<td>26 ± 5</td>
<td>19 ± 4</td>
<td>23 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% EtOH 6 h</td>
<td>8 ± 3</td>
<td>9 ± 6</td>
<td>4 ± 3</td>
<td>7 ± 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 Thermostability Is Independent of Hsf1-dependent Transcription

To investigate the interaction between Hsf1 and Tps1, we used strains deleted for the essential HSF1 gene and rescued by expression of this gene under the tetracyclin-Tet-O2-repressible promoter. Western blot analysis confirmed that Hsf1 protein was no longer detected in cells treated with tetracyclin, whereas it was highly abundant in untreated cells because of the strength of Tet-O2 promoter (27) (Fig. 5A). Then we revisited a previous report, suggesting that trehalose is acquired thermotolerance. The Contribution of Tps1 to Thermotolerance Is Independent of Hsf1-dependent Transcription. Our finding that the Tps1 protein played a major role in resistance to high temperature upshifts raised the question of whether this role could be mediated through the heat shock transcriptional factor encoded by HSF1. Hsf1 is an essential protein in yeast, ascribed to be the primary regulator for heat-induced transcription of heat shock proteins (HSP). It is also involved in diverse cellular processes such as protein degradation, detoxification, energy generation, carbohydrate metabolism, and maintenance of cell wall integrity (40–42). To investigate the interaction between Hsf1 and Tps1, we used strains deleted for the essential HSF1 gene and rescued by expression of this gene under the tetracyclin-Tet-O2-repressible promoter. Western blot analysis confirmed that Hsf1 protein was no longer detected in cells treated with tetracyclin, whereas it was highly abundant in untreated cells because of the strength of Tet-O2 promoter (27) (Fig. 5A). Then we revisited a previous report, suggesting that trehalose is a positive regulator of the heat-induced phosphorylation and activity of Hsf1 (43, 44). Using yeast cells transformed with pTet-HSF1, we showed that a temperature upshift to 40 °C for 10 and 30 min led to the appearance of two bands on the SDS-PAGE detected by the anti-Hsf1 antibodies (Fig. 5B). As already shown, the upper band corresponded to the phosphorylated 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 of Hsf1 transcription factor. The 30-min tps1Δ sample (lane 1) was also treated by 1 unit of alkaline phosphatase (AP). C, RT-PCR analysis of HSF1 and HSP transcript levels after exposure of wild type TPS1 and tps1Δ mutants 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, IPI1, and UBC6). The data are represented as means ± S.D. of two biological replicates.
Role of Tps1 in Yeast Stress Tolerance

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, sensitized them to heat shock as illustrated by a 40% drop of viability after 2 h at 40 °C. Interestingly, viability profiles of the tps1-156 hsf1Δ pTet-HSF1 mutant and control strain (TPS1 hsf1Δ) show no strain sensitivity to heat shock. Expression of Hsf1, as illustrated by viability in the tps1Δ hsf1Δ strain, was not affected in the absence of tetraction inhibition of Tet-O promoter with the antibiotic, Hsf1, therefore, 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 reported (49), we determined the ATP content in yeast cells during the heat shock response. Remarkably, we found that the tps1Δ mutant overexpressing or not HSP104 became irreversibly depleted of its ATP content in less than 1 h at 42 °C (Fig. 8B). In contrast, in both wild type and tps1-156 cells, the ATP transiently dropped by ~50% during the first 20 min to regain initial levels after 40–60 min. The complete loss of ATP coincided with the time when the viability of the tps1Δ overexpressing HSP104 started to decline. However, this drop of viability was very slow as compared with that of tps1Δ alone, suggesting a protective effect of HSP104 overexpression, independent of ATP levels.

Discussion

Trehalose has long been proposed to play an important role during stress response, mainly as a chemical chaperone (2, 50–52). However, doubts about this stress-protecting function have been recently raised from contradictory results, notably in thermotolerance 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
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 sustain heat shock could be an interplay between Tps1 and key heat shock genes in the heat shock machinery, such as HSP104. We found that trehalose could contribute together, but in an independent manner, to the thermotolerance trait was impaired far more in a double tps1Δ hsp104Δ mutant than in either single mutant (38, 55). Taken together, these results favored a model in which Tps1 and Hsf1 contribute together, but in an independent manner, to the thermotolerance.

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
Role of Tps1 in Yeast Stress Tolerance

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 Pfk26, 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 researches aiming at optimizing the microbial cell factories on yeasts. In addition to their use in traditional processes, yeasts face growing interest in the field of white (synthetic) biotechnologies (64–66). All these processes are sources of many adverse situations for these microbial cell factories. Identifying the role of Tps1 in stress resistance is a prerequisite for robust strain engineering. The second perspective relies on the urgent need for new antifungal therapies. The trehalose metabolic pathway is widespread in the fungal kingdom where it can be part of the virulence system (23, 67–69), thus making Tps1 a potential antimicrobial target for weakening mammalian and plant pathogenic microorganisms.

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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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