Accumulation of Trehalose by Overexpression of *tps1*, Coding for Trehalose-6-Phosphate Synthase, Causes Increased Resistance to Multiple Stresses in the Fission Yeast *Schizosaccharomyces pombe*

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Recent studies have shown that heat shock proteins and trehalose synthesis are important factors in the thermotolerance of the fission yeast *Schizosaccharomyces pombe*. We examined the effects of trehalose-6-phosphate (trehalose-6P) synthase overexpression on resistance to several stresses in cells of *S. pombe* transformed with a plasmid bearing the *tps1* gene, which codes for trehalose-6P synthase, under the control of the strong thiamine-repressible promoter. Upon induction of trehalose-6P synthase, the elevated levels of intracellular trehalose correlated not only with increased tolerance to heat shock but also with resistance to freezing and thawing, dehydration, osmostress, and toxic levels of ethanol, indicating that trehalose may be the stress metabolite underlying the overlap in induced tolerance to these stresses. Among the isogenic strains transformed with this construct, one in which the gene coding for the trehalose-hydrolyzing enzyme, neutral trehalase, was disrupted accumulated trehalose to a greater extent and was more resistant to the above stresses. Increased trehalose concentration is thus a major determinant of the general stress protection response in *S. pombe*.

Considerable evidence has accumulated in recent years to indicate that the intracellular level of trehalose may determine the survival response of yeasts under extreme environmental conditions (43, 45). The protective function of trehalose against stress can be interpreted in terms of the water replacement hypothesis or the glass transition hypothesis (5, 8). As predicted for such a role, trehalose shows remarkable stress protection properties in vitro (9) and accumulates in vivo when cells approach stationary phase as well as during sublethal heat treatment and by the action of other stressors (2, 20, 35, 43, 45). The homeostasis of trehalose in yeasts is controlled by the synthesizing enzyme complex trehalose-6-phosphate (trehalose-6P) synthase and the hydrolyzing enzyme(s) trehalase(s).

Most studies showing strong correlations between trehalose content and stress resistance have been performed by using the budding yeast *Saccharomyces cerevisiae* as a model (45). This system, however, has several important drawbacks. First, the analysis that can be performed in this yeast with mutants in which *tps1* has been disrupted is limited. Cells unable to synthesize trehalose have a complex pleiotropic phenotype which includes lack of growth on readily fermentable sugars, loss of many regulatory responses, and deficient sporulation (42). Moreover, two distinct trehalases are present in *S. cerevisiae* (27), and their relative functions in trehalose metabolism are not clearly defined. The neutral (cytosolic) trehalase is generally considered to be involved in the intracellular degradation of trehalose (33), whereas the acid (vacuolar) trehalase appears to determine growth on exogenous trehalose (32). In addition, many studies on the role of trehalase in stress resistance include experimental strategies involving induced tolerance, by which cells become resistant to an otherwise lethal stress through adaptive treatment with a mild stress. Although this approach allows for the examination of particular changes accompanying tolerance acquisition, it also causes many different stress resistance determinants to be expressed and may mask the contribution from other stress-related factors. Indeed, several independent changes are known to occur during the adaptive period that induce tolerance to many types of stress (26, 34). These changes raise the question of whether an increase in trehalose content is merely a circumstantial event rather than a crucial factor. Others (1, 18) have found no obvious relationship between trehalose accumulation and tolerance and have questioned the relevance of trehalose as a determinant of resistance to some stresses.

In general, there is broad consensus that trehalose can serve as a stress protectant when yeast cells are challenged with high temperatures (2, 20). The correlation between trehalose accumulation and improved tolerance of stresses such as lyophilization (16), hyperosmotic shock (28), or freezing (19, 36) is much more limited. Fewer studies of trehalose as a resistance metabolite have been made with the fission yeast *Schizosaccharomyces pombe* than with *S. cerevisiae*. However, the *S. pombe* system has several advantages. Unlike *S. cerevisiae*, vegetative cells of this yeast contain only one trehalase (13), and mutants in which *tps1* has been deleted do not present the growth limitations shown by their baker’s yeast counterparts (3). Recently, evidence has been obtained from mutants defective in trehalose-6P synthase function to suggest that trehalose synthesis is required for the in vivo acquisition of thermotolerance in *S. pombe* subjected to severe heat preconditioning (39).

The simplest and most direct approach to evaluating the role of trehalose in protection against stress-induced injuries is to induce selective changes in trehalose content without simultaneously triggering significant changes in other cellular param-
eters. We addressed this problem by inducing overexpression, under normal physiological conditions, of the tps1 gene, which codes for trehalose-6P synthase, in *S. pombe* wild-type cells; cells in which tps1 had been deleted, which were unable to synthesize trehalose (3); and cells in which ntp1 had been deleted, which were devoid of neutral trehalase activity (6). The results indicate that modulation of tps1 function in *S. pombe* markedly affects tolerance not only of temperature upshifts but also of other stresses, including freezing and thawing, dehydration, and growth in the presence of normally toxic concentrations of ethanol and NaCl. Our procedure allows us to manipulate trehalose levels without prestressing the cells and demonstrates a central role for trehalose in fission yeast stress response.

### MATERIALS AND METHODS

#### Yeast strains and culture conditions

The *S. pombe* strains used in this study are shown in Table 1. Transformation of strains MM-1 (control), PBU13 (Δtps1), and MMT-3 (Δntp1) with plasmid pREP3X-tps1 was carried out by the lithium acetate method as described elsewhere (7). pREP3X-tps1 contains the gene encoding trehalose synthase in *S. pombe* under the control of the nmt1 thiamine-repressible promoter (7, 30). The overexpression phenotype in transformed strains was determined after 24 h of growth in the absence of thiamine. Cells were routinely grown with shaking (160 rpm) at 28°C in EMM2 with or without thiamine (5 mg/liter) (7). Culture media were supplemented with adenine and/or uracil (100 mg/liter) depending on the requirements of each particular strain.

#### Heat shock treatment

Cells were withdrawn from mid-exponential-phase cultures grown at 28°C for 24 h and transferred into fresh medium prewarmed at 38°C for the specified times. The heat-treated samples were then cooled in an ice bath. Afterwards, the cell suspensions were briefly vortexed, appropriately diluted, and spread in triplicate onto plates containing EMM2 solid medium with thiamine. The viability of the cells was measured by their ability to form colonies on EMM2 solid medium after incubation at 28°C for 8 days. The survival fraction was calculated, for each sample separately, as a percentage relative to the survival of control samples maintained without heat treatment.

#### Freezing and thawing conditions

Cells from mid-log-phase cultures were obtained by centrifugation, washed twice with sterile distilled water, and resuspended so that the final cell concentration was similar in all samples within the same experiment. Freeze-thaw resistance was measured according to reference 25. Essentially, 100-μl aliquots of the cell suspensions were transferred to 1.5-ml microcentrifuge tubes that were submerged in liquid nitrogen for 5 min. The tubes were thawed for 5 min in a 25°C water bath. The number of survivors was determined by plating dilutions onto EMM2 agar plates supplemented with thiamine. The viability of the cells was measured by their ability to form colonies on EMM2 solid medium after incubation at 28°C for 8 days. The survival fraction was calculated, for each sample separately, as a percentage relative to the survival of control samples that received no freeze-thaw treatment.

#### Dehydration resistance

Cells were removed from log-phase cultures after 24 h of growth, collected by centrifugation, washed twice with sterile distilled water, and finally resuspended in 1/100 of the original volume in 200 mM glycerol. Suspensions were frozen in liquid nitrogen for 1 min and kept for 16 h at −75°C before lyophilization. The level of the intracellular pool of trehalose was significantly higher when these strains were cultured in the same medium lacking thiamine (6). This result supports the involvement of the trehalase enzyme in trehalose breakdown in vivo. The level of the intracellular pool of trehalose was significantly higher when these strains were cultured in the same medium lacking thiamine (6). This result supports the involvement of the trehalase enzyme in trehalose breakdown in vivo. The level of the intracellular pool of trehalose was significantly higher when these strains were cultured in the same medium lacking thiamine (6).

#### Analytical determinations

Trehalose content in cells overexpressing thiamine-controlled trehalase-6P synthase. Trehalose levels were almost below the level of detection in cells from pREP3X-tps1-transformed strains of *S. pombe* when the cells were growing exponentially on glucose in EMM2 medium supplemented with thiamine (Table 2). Under conditions repressive for the expression of the plasmidic tps1 gene, trehalose accumulated to measurable levels only in cells from strain SS15, which carries a disruption in the gene encoding neutral trehalase (6). This result supports the involvement of the trehalase enzyme in trehalose breakdown in vivo. The level of the intracellular pool of trehalose was significantly higher when these strains were cultured in the same medium lacking thiamine. Northern blot analysis of tps1 mRNA confirmed that tps1 overexpression occurred under negative control by thiamine (data not shown).

### RESULTS

#### Trehalose content in cells overexpressing thiamine-controlled trehalase-6P synthase.

Table 2 shows the trehalose levels in *S. pombe* cells overexpressing the trehalase-6P synthase enzyme, which was induced by thiamine. The results indicate that trehalose levels were significantly higher in cells overexpressing the tps1 gene, compared to cells not expressing the gene. This suggests that the overexpression of tps1 is responsible for the increased trehalose levels observed in these cells.

#### Stress response to hyperthermic conditions in pREP3X-tps1-transformed cells.

As expected, disruption of tps1 blocked trehalase synthesis (Table 2) and also reduced the resistance of exponentially growing, glucose-repressed cells to the effects of a temperature upshift (Fig. 1). In contrast, overexpression of tps1 in different genetic backgrounds significantly increased trehalose concentration and cell survival during hyperthermia. For example, less than 0.01% of the cells in the wild-type strain SS10 survived a challenging heat shock (30 min at 48°C) when they were grown in a culture medium containing thiamine, which represses tps1 expression, but they were more resistant, by a factor of 2 to 3 log units, when grown in the presence of thiamine.

### TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>MM-1</td>
<td>h* ade6-M216 leu1-32 ura4-D18</td>
<td>6</td>
</tr>
<tr>
<td>PBU13</td>
<td>h* ade6-M216 leu1-32 ura4-D18 Δtps1:ura4</td>
<td>3</td>
</tr>
<tr>
<td>MMT-3</td>
<td>h* ade6-M216 leu1-32 ura4-D18 Δntp1:ura4</td>
<td>6</td>
</tr>
<tr>
<td>SS10</td>
<td>h* ade6-M216 leu1-32 ura4-D18 (pREP3X-tps1)</td>
<td>This work</td>
</tr>
<tr>
<td>SS1</td>
<td>h* ade6-M216 leu1-32 ura4-D18 Δtps1:ura4 (pREP3X-tps1)</td>
<td>This work</td>
</tr>
<tr>
<td>SS15</td>
<td>h* ade6-M216 leu1-32 ura4-D18 Δntp1:ura4 (pREP3X-tps1)</td>
<td>This work</td>
</tr>
</tbody>
</table>

### TABLE 2. Trehalose concentration in exponentially growing pREP3X-tps1-transformed cells of *S. pombe* under conditions of thiamine repression or derepression before and after a heat shock

<table>
<thead>
<tr>
<th>Strain</th>
<th>Thiamine addition</th>
<th>Trehalose concn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At zero time</td>
<td>After heat shock</td>
</tr>
<tr>
<td>SS10</td>
<td>+</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>16 ± 1.9</td>
</tr>
<tr>
<td>SS1</td>
<td>Δtps1</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18 ± 0.9</td>
</tr>
<tr>
<td>SS15</td>
<td>Δntp1</td>
<td>4.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>50 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>27 ± 1.6</td>
</tr>
</tbody>
</table>

* Cultures were grown in EMM2 at exponential phase for 24 h in the presence of thiamine (repression). These cells were used as inocula for media with or without thiamine, and the basal trehalose content was determined in nanomoles per 107 cells after a further 24 h of exponential growth (zero time) or following a heat stress. Values are the means from three independent determinations ± SD.

** wt, wild type; Defined as 48°C for 30 min.**
absence of thiamine, which allows for tps1 overexpression (Fig. 1).

Resistance to freeze-thaw stress as a function of tps1 expression. We compared the survival of cells overexpressing trehalose-6P synthase after a freeze-thaw cycle with the same cells under identical growth conditions but with plasmid-directed synthesis of trehalose repressed. While the viability of wild-type S. cerevisiae cells decreases by 99% when they are frozen directly (24), the S. pombe survival rate is even lower, less than 0.001% (Fig. 2A). Derepression of thiamine-controlled tps1 prior to freezing increased cell survival 20- to 200-fold, depending on the particular strain, demonstrating a role for trehalose in the adaptive process that confers protection against freezing (Fig. 2A). Incubation at 37°C for 1 h before freezing similarly increased cell survival in S. pombe wild-type and Δntp1 cells (data not shown). This type of thermal pretreatment induces an increase in trehalose concentration (12, 15, 39) (Table 2). Taken together, these results indicate that S. pombe strains overexpressing tps1 survive freezing better than control strains.

Effect of tps1 expression on resistance to dehydration stress. We examined the effect of tps1 expression on the survival of exponential-phase cells subjected to lyophilization. Freeze-drying is a very drastic procedure that results in high mortality in vegetative cells of S. pombe (Fig. 2B). Viability after freezing and drying was extremely low in control cells that had been grown in the presence of thiamine but increased noticeably upon derepression of tps1. The magnitude of the difference in viability due to tps1 overexpression was 2 to 3 log units, depending on the strain. Differences in the extent of survival were due to changes in intracellular trehalose levels and not to other physiological changes, since all strains were examined at similar stages of growth. These results demonstrate that trehalose increases tolerance of dehydration, possibly by acting as a compatible solute during dehydration.

Correlation between intrinsic resistance to exogenous ethanol and trehalose accumulation. Addition of 15% ethanol to exponentially growing S. pombe cultures drastically reduces viability (Fig. 2C). This reduction decreased if tps1 was induced, indicating that trehalose accumulation helps to alleviate ethanol toxicity. These results suggest that trehalose may have a common protective role in responses to diverse stress stimuli.

Trehalose content and osmotic stress resistance. Although the tolerance for other stresses in cells overexpressing tps1 and accumulating trehalose increased by several orders of magnitude compared to that in controls (see above), salt tolerance in such cells showed a less impressive increase that was, nevertheless, consistent in all experiments (Fig. 3). These results are consistent with the hypothesis that trehalose accumulation contributes to osmotic adjustment and increases the survival and growth rates of S. pombe cells under severe osmotic stress.

**DISCUSSION**

Following exposure to a mild stress, yeast cells become resistant to a subsequent, more-severe stress that would be lethal in the absence of the conditioning pretreatment. Studies of S. cerevisiae have revealed that this adaptive response relies primarily on the increased synthesis of specialized stress proteins and/or organic solutes such as trehalose and glycerol (37). High concentrations of these small metabolites do not perturb enzyme structure or function, and these compounds can stabilize proteins in vitro (9, 14, 20). In this study, we measured the tolerance of S. pombe to stress as a function of tps1 expression and function. Unlike previous investigators (2, 12, 20), we altered the levels of intracellular trehalose by turning thiamine-controlled synthesis of trehalose-6P on and off, instead of inducing changes in the trehalose pool with stresses that could complicate the interpretation of the results. In so doing, we could separate the phenotypic effects of tps1 modulation on various stress responses from other stress-dependent changes that might simultaneously contribute to resistance. Overexpression of tps1 did not result in the accumulation of trehalose in excess of the level found in heat-stressed control cells (Table
One representative result is shown for each experiment. Our results indicate that in during dehydration is well documented (11). Regardless of the nucleation is unclear, but its role as a substitute for water (31, 36). To our knowledge, the effect of trehalose on ice from freezing rather than thawing and is due primarily to (19). Much of the damage to cells in a freeze-thaw stress results against freeze-thawing (24, 36). Also, freeze-thaw-tolerant (36), but heat shock or osmotic stress do offer cross-protection (40). Our data correlate increased trehalose levels with ethanol endurance and support the observations made by others that trehalose mitigates the ethanol-induced leakage of electrolytes from intact yeast cells and liposomes (22, 29). In addition to protein denaturation and membrane disordering, other changes induced by ethanol are the same as those resulting from heat stress (10, 44). Thus, cells preadapted to one type of stress might acquire tolerance to the other. We found that cells overexpressing tps1, which were resistant to heat shock, also were comparatively more ethanol tolerant, while no change in temperature sensitivity or ethanol tolerance was shown when tps1 was repressed. Thus, trehalose appears to be a key determinant of thermostolerance, tolerance to freezing, and ethanol endurance in S. pombe. This conclusion may apply to other stresses as well because induction of trehalose synthesis also confers additional tolerance to dehydration and more resistance to NaCl. Although glycerol appears to be the main osmolyte accumulated in salt-stressed yeasts and filamentous fungi (4), a possible role for trehalose in osmotolerance has recently been recognized (17, 21).

Our results are consistent with others obtained with S. cerevisiae that indicate that heat-shocked cells have enhanced tolerance to salt and freezing (25) and that salt-adapted cells are more tolerant to ethanol than control cells (40). Collectively, these data are consistent with the hypothesis that induction of defenses against some stressors may increase a cell’s resistance to other stresses. These data also suggest that trehalose synthesis is a crucial part of the general stress response that helps cells withstand extreme conditions. In this context, some effects of tps1 overexpression resemble those observed following ntp1 disruption (6), as expected from the opposite functions of trehalose-6P synthase and trehalase in trehalose metabolism.

We previously reported that the expression of tps1 and ntp1 is enhanced during hyperthermia and by the action of other stressors (15). Consequently, trehalose-6P synthase and neutral trehalase may be considered stress proteins. Recent work (41) has shown that trehalose stabilizes proteins during heat shock but interferes with refolding. This finding suggests a rapid hydrolysis of trehalose during recovery and provides an explanation for the apparent paradox of the simultaneous increase in both enzymes. We do not know if the only protective function of trehalose-6P synthase is to produce trehalose. Our present results are insufficient to determine whether enhanced transcription of tps1 or trehalose accumulation is the critical determinant of stress resistance. However, cells in which ntp1 has been disrupted, which overexpress tps1, always have higher levels of trehalose and are more stress tolerant than their ntp-containing counterparts. We interpret these data to mean that trehalose accumulation, rather than trehalose-6P synthase synthesis, is the primary mechanism of resistance. An alternative explanation is cross talk between trehalose synthesis and hsp synthesis in stress response signaling. Although previous work does not favor this possibility (39), future studies are needed to address this question definitively.

While we support the conclusion that trehalose is an important stress tolerance factor, it is probably only one of several determinants of stress resistance (37). In any case, our observations will be useful for the baking and brewing industries and
for fuel production because yeast strains overexpressing tps1 can better survive temperature stress, freezing, dehydration, or the presence of high levels of salt or ethanol.

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