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Trehalose synthesis is important for the acquisition of thermotolerance in *Schizosaccharomyces pombe*

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Summary

Yeast cells show an adaptive response to a mild heat shock, resulting in thermotolerance acquisition. This is accompanied by induction of heat-shock protein (hsp) synthesis and rapid accumulation of trehalose. Genetic approaches to determine the specific role of trehalose in heat-induced thermotolerance in *Saccharomyces cerevisiae* have been hampered by the finding that deletion of *TPS1*, the gene encoding trehalose-6-phosphate synthase, causes a variety of pleiotropic effects, including inability to grow on glucose-containing media. Here, we have studied a *tps1* mutant of the yeast *Schizosaccharomyces pombe* that reportedly has no such growth defects. We show that *tps1* mutants have a serious defect in heat shock-induced acquisition of thermotolerance if conditioned at highly elevated temperatures (40–42.5°C), which, in wild-type cells, prevent hsp but not trehalose synthesis. In contrast, hsp synthesis appears to become particularly important under conditions in which trehalose synthesis is either absent (in *tps1* mutant strains) or not fully induced (conditioning at moderately elevated temperatures, i.e. 35°C). In addition, *pka1* mutants deficient in cAMP-dependent protein kinase were examined. Unconditioned *pka1* cells had low levels of trehalose but a high basal level of thermotolerance. It was found that *pka1* mutant cells, contrary to wild-type cells, accumulated large amounts of trehalose, even during a 50°C treatment. *pka1 tps1* double mutants lacked this ability and showed reduced intrinsic thermotolerance, indicating a particularly important role for trehalose synthesis, which takes place during the challenging heat shock.

Introduction

Acquired thermotolerance of cells or organisms has been defined as a transient increase in resistance to heat induced by exposure to elevated temperatures or by various other stress-related treatments (Lindquist, 1986). During a conditioning heat shock, synthesis of a small set of highly conserved proteins, the heat-shock proteins (hsps), is dramatically increased. Some hsps have been shown to play a vital role in unstressed cells by associating with newly synthesized proteins (Beckman *et al*., 1990), assisting in their folding (Ang *et al*., 1991; Gething and Sambrook, 1992) and allowing these proteins to cross membranes (Cheng *et al*., 1989; Kang *et al*., 1990). However, analysis of hsp function in *Saccharomyces cerevisiae* has revealed that only a limited number of hsps have appreciable effects on the induction of thermotolerance during a conditioning heat shock (for a review, see Piper, 1993). In particular, Hsp104, Hsp70, Ctt1 and Ubi4 have been found to contribute to the acquisition of thermotolerance by promoting the resolubilization of aggregated proteins (Parsell *et al*., 1994), the sequestering and refolding of denatured proteins (for a review, see Mager and Ferreira, 1993), the prevention of oxidative damage (Wieser *et al*., 1991) and the proteolytic elimination of toxic proteins (Finley *et al*., 1987) respectively.

An additional element of the adaptive response of yeast to elevated temperature is the accumulation of the non-reducing disaccharide trehalose (Attfield, 1987; Hottiger *et al*., 1987; Ribeiro *et al*., 1994), which is thought to increase the cell's thermotolerance by enhancing the thermal stability of proteins and reducing the heat-induced formation of protein aggregates (Crowe *et al*., 1987; Hottiger *et al*., 1994; for a review, see Wiemken, 1990). Synthesis of trehalose is mediated by the trehalose-6-phosphate (Tre6P) synthase–phosphatase complex, which, in *S. cerevisiae*, consists of at least four subunits carrying either Tre6P synthase (Tps1), Tre6P phosphatase (Tps2) or regulatory activities (Tps3 and Tsl1; Bell *et al*., 1992; De Virgilio *et al*., 1993; Vuorio *et al*., 1993; Reinders *et al*., 1997). In accordance with the observation that heat-induced accumulation of trehalose is partially dependent on protein synthesis in *S. cerevisiae* (De Virgilio *et al*., 1991), both catalytic subunits (Tps1 and Tps2) have been shown to be hsps, and expression of all four genes encoding the subunits of the Tre6P synthase complex (*TPS1*, *TPS2*, *TPS3* and *TSL1*) has been found to be dramatically enhanced under heat-shock conditions (De Virgilio *et al*., 1993; Winderickx *et al*., 1996).

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Degradation of cytosolic trehalose is mainly mediated by the neutral trehalase (Londesborough and Varimo, 1984), which has attracted considerable attention because its potential activation by the cAMP-dependent protein kinase (cAPK) allows monitoring of the activation status of the Ras/cAMP pathway (for a review, see Thevelein, 1992). Interestingly, cAPK activity was found to exert a dual role in the regulation of trehalose levels in yeast, namely by post-translational activation of the neutral trehalase and by transcriptional repression of *TPS* genes via a novel *cis*-acting stress-responsive element (STRE), which is independent of the heat-shock factor (HSF; Jakobsen and Pelham, 1988; Sorger and Pelham, 1988). This element, which was shown to be negatively regulated by cAPK, mediates the heat-induced expression of several hsps, including *HSP12* (Varela *et al*., 1995), *CTT1* (Marchler *et al*., 1993; Schüller *et al*., 1994), *SSA3* (Boorstein and Craig, 1990), *TPS2* (Gounalaki and Thireos, 1994) and possibly also *TPS1*, *TPS3* and *TSL1* (Varela *et al*., 1995). Accordingly, studies using *S. cerevisiae* mutants of the Ras/cAMP pathway have shown that low cAPK activity correlates with high levels of *HSP* expression, trehalose synthesis and thermotolerance even in the absence of an inducing heat stress, whereas high cAPK activity reduces the levels of *HSP* expression, trehalose synthesis and thermotolerance even under heat-shock conditions (Hottiger *et al*., 1989; Iida, 1988; Shin *et al*., 1987).

Genetic approaches to determine the specific role of trehalose for the heat-induced thermotolerance in wild-type cells (De Virgilio *et al*., 1994), as well as for the intrinsic thermotolerance in mutants with attenuated cAPK activity, have been hampered by the finding that deletion of *TPS1*, the gene encoding Tre6P synthase, causes a variety of pleiotropic effects in *S. cerevisiae*. These effects include the inability to grow on rapidly fermentable sugars, such as glucose and fructose, and a deficiency in many glucose-induced regulatory mechanisms, indicating that trehalose biosynthesis, in addition to its role in the stress response, may also be involved in the general control of glycolysis (for a review, see Thevelein and Hohmann, 1995). Surprisingly, a *TPS1* homologue in the distantly related fission yeast, *Schizosaccharomyces pombe*, while also being essential for trehalose synthesis, was reported to be completely dispensable for growth on glucose (Blázquez *et al*., 1994), rendering this yeast species a much more attractive model system for studies of the role of trehalose in the acquisition of thermotolerance. Moreover, several elements of the cAPK pathway in *S. pombe*, including an adenylate cyclase (encoded by *cyr1*$^+$; Yamawaki-Kataoka *et al*., 1989; Young *et al*., 1989), a regulatory subunit of cAPK (encoded by *cgs1*$^+$; DeVoti *et al*., 1991) and a cAPK (encoded by *pka1*$^+$; Maeda *et al*., 1994), have recently been identified. The role of the cAMP signalling pathway in *S. pombe* is pleiotropic, as in *S. cerevisiae*, and involves the regulation of cell cycle events, sexual differentiation and gene expression (DeVoti *et al*., 1991; Hoffman and Winston, 1991; Mochizuki and Yamamoto, 1992). Moreover, it has been suggested that cAMP-dependent phosphorylation may also be involved in the post-translational regulation of neutral trehalase activity and in the modulation of the heat-shock response (Soto *et al*., 1995; Fernández *et al*., 1997).

Here, we report that deletion of *tps1*$^+$ in *S. pombe* seriously reduces the heat shock-induced acquisition of thermotolerance, especially if the conditioning heat shock is performed at very high temperatures (40–42.5°C), which prevent hsp synthesis but not trehalose accumulation in wild-type cells. We have also shown that general synthesis of hsps becomes particularly important under conditions in which trehalose synthesis is either absent (in *tps1* mutant strains) or not fully induced (in cells conditioned at 35°C). Moreover, even though an unconditioned *pka1* mutant cell was found to have low levels of trehalose, it had a high intrinsic level of thermotolerance, which was strongly dependent on the presence of a functional *tps1*$^+$ gene, indicating a particularly important role for the trehalose synthesis taking place during the challenging heat shock.

Results

Temperature limit for protein synthesis in S. pombe *defines the upper limit for a role of hsps in acquired thermotolerance*

To assess the relative importance of general protein synthesis, and the role of hsps in particular, in the acquisition of thermotolerance, we first determined the upper limit for (heat-shock) protein synthesis in *S. pombe*. The wild-type strain, PB003, showed sustained protein synthesis, as detected by the incorporation of L-[^{35}S]-methionine into TCA-precipitable material and visualization of the radiolabelled proteins following gel electrophoresis and autoradiography at 27°C, 35°C and 37.5°C, with the typical pattern of hsps at 35°C and 37.5°C (Fig. 1). At 40°C, protein synthesis was already dramatically reduced (13.1% when compared with the 35°C treatment) and at temperatures above 42.5°C virtually no protein synthesis could be detected. These results clearly indicate that hsp synthesis may only contribute significantly to the acquisition of thermotolerance in wild-type cells subjected to a conditioning heat shock at temperatures below 40°C.

Conditioning at moderate temperatures reveals an important role for hsp synthesis, but not of trehalose synthesis, for acquired thermotolerance

To study the role of trehalose and hsp synthesis for acquired thermotolerance, wild-type cells (PB003) were

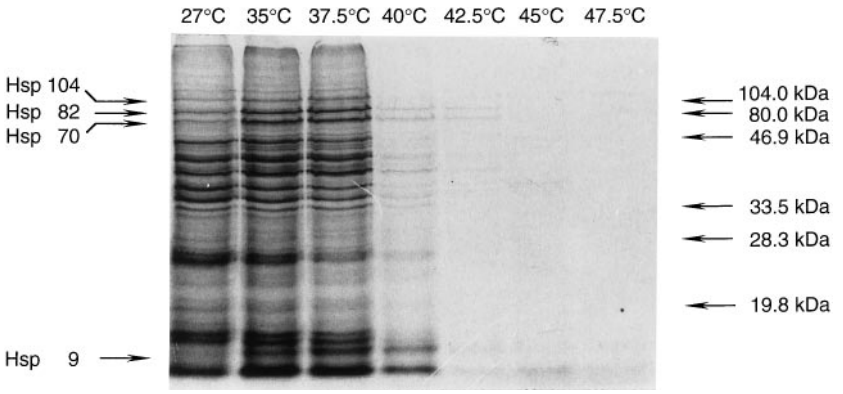

Fig. 1. Protein synthesis in *S. pombe* strain PB003 (wild type) during a conditioning heat shock at various temperatures. Cultures were labelled at the temperatures indicated by the addition of L-[^{35}S]-methionine for 1 h. Radiolabelled proteins were extracted and resolved by gel electrophoresis as described in *Experimental procedures*. The positions of molecular weight standards are indicated on the right (in kDa), and those of the hsps on the left.

exposed to 50°C for 8 min, either after growth at 27°C (unconditioned cells) or following a 1-h conditioning heat shock at various temperatures (35°C, 37.5°C, 40°C, 42.5°C, 45°C and 47.5°C; conditioned cells) in the presence or absence of cycloheximide (100 μg ml^{-1}). As shown in Fig. 2, unconditioned cells were extremely heat sensitive (less than 0.3% survivors after 8 min at 50°C; Fig. 2A) and did not contain appreciable amounts of trehalose (Fig. 2B), following a 1-h incubation at 27°C in the presence or absence of cycloheximide. However, conditioning of the cells for 1 h at 35°C, 37.5°C and 40°C did result in low (0.03 g g^{-1} protein), moderate (0.19 g g^{-1} protein) and high (0.42 g g^{-1} protein) accumulation of trehalose, respectively, while the levels of acquired thermotolerance achieved during conditioning at these three temperatures did not significantly differ from each other (about 55% survivors after 8 min at 50°C). These results suggest that trehalose accumulation may play a subordinate role in thermotolerance acquisition if the conditioning heat shock is carried out at 35°C, but may become increasingly important at higher temperatures (i.e. between 37.5°C and 42.5°C). In contrast, hsp synthesis may be particularly important during conditioning at a temperature of 35°C. In accordance with this suggestion, inhibition of protein synthesis (by the addition of cycloheximide before the conditioning heat shock) did significantly reduce the level of acquired thermotolerance (by about 58%) of cells conditioned at 35°C, but not of cells conditioned at 37.5°C or at higher temperatures (Fig. 2A). This is also in agreement with the above finding that wild-type cells are unable to synthesize significant amounts of proteins at temperatures higher than 40°C (Fig. 1), and that hsp synthesis may therefore not contribute to the acquisition of thermotolerance under these conditions. In accordance with previously published results (De Virgilio *et al*., 1990), cycloheximide did not affect the cell's ability to accumulate trehalose at any temperature tested, indicating that trehalose synthesis is mainly regulated at a post-translational level under heat-shock conditions. Cycloheximide addition also did not strongly affect the survival rate of cells during the conditioning heat shock (Fig. 2C). In control experiments, cycloheximide inhibited the incorporation of L-[^{35}S]-methionine into TCA-precipitable material by more than 90% in wild-type cells (strain PB003; data not shown). Except for a very small fraction of the cells (< 1.0%), 23% of which were able to survive the challenging heat shock at 50°C, the majority of the cells were unable to survive a 1-h conditioning heat shock at temperatures at or above 45°C, irrespective of the presence or absence of cycloheximide (Fig. 2C). Thus, a temperature of around 45°C constitutes the absolute physiological temperature limit within which *S. pombe* cells can acquire thermotolerance when pregrown at 27°C.

Conditioning at high temperatures reveals an important role for trehalose synthesis, but not for hsp synthesis, in acquired thermotolerance

Since the results in Fig. 2 suggested a potentially important role for trehalose synthesis in the acquisition of thermotolerance during a 1-h conditioning heat shock at temperatures between 40°C and 42.5°C, we decided to study the temperature-dependent induction of thermotolerance in a mutant defective in the gene coding for the Tre6P synthase (*tps1*$^{+}$). Unconditioned *tps1* mutant cells were as similarly heat sensitive as wild-type cells (less than 0.3% survivors after 8 min at 50°C), after a 1-h incubation at 27°C in the presence or absence of cycloheximide (Fig. 3A). While completely deficient for trehalose synthesis at any temperature tested (Fig. 3B), *tps1* mutant cells were able to acquire relatively high levels of thermotolerance during a heat shock at moderate temperatures (35°C and 37.5°C; about 70% survivors after 8 min at 50°C), but their ability to acquire thermotolerance was reduced if conditioned at 40°C or 42.5°C (Fig. 3A), when compared with the isogenic wild-type cells (Fig. 2A). Consequently, trehalose synthesis is an important factor for the acquisition of thermotolerance during conditioning at high temperatures between 40°C and 42.5°C. As in wild-type cells, the addition of cycloheximide before

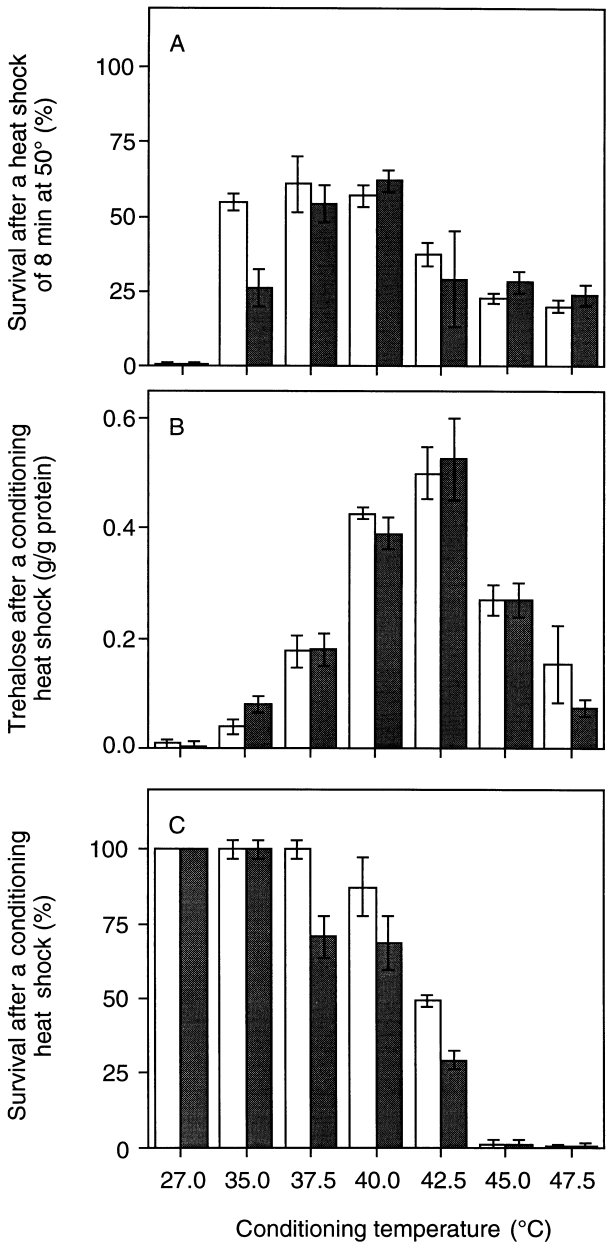

Fig. 2. Thermotolerance (A), trehalose levels (B) and survival (C) of *S. pombe* strain PB003 (wild type) after a conditioning heat shock at different temperatures in the absence or presence of cycloheximide. Cultures of wild-type cells were grown to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and subjected to a conditioning heat shock for 1 h at the temperatures indicated in the absence (open bars) or presence (shaded bars) of cycloheximide (100 $\mu g\,ml^{-1}$) added 5 min before initiation of the heat shock. Thermotolerance (A) was measured as the survival following a subsequent incubation for 8 min at 50°C. Trehalose levels (B) were determined after a 1-h conditioning heat shock. Survival after the conditioning heat shock (C) was determined as a percentage of the 27°C control culture. The error bar for each sample represents the SEM of at least three independent measurements.

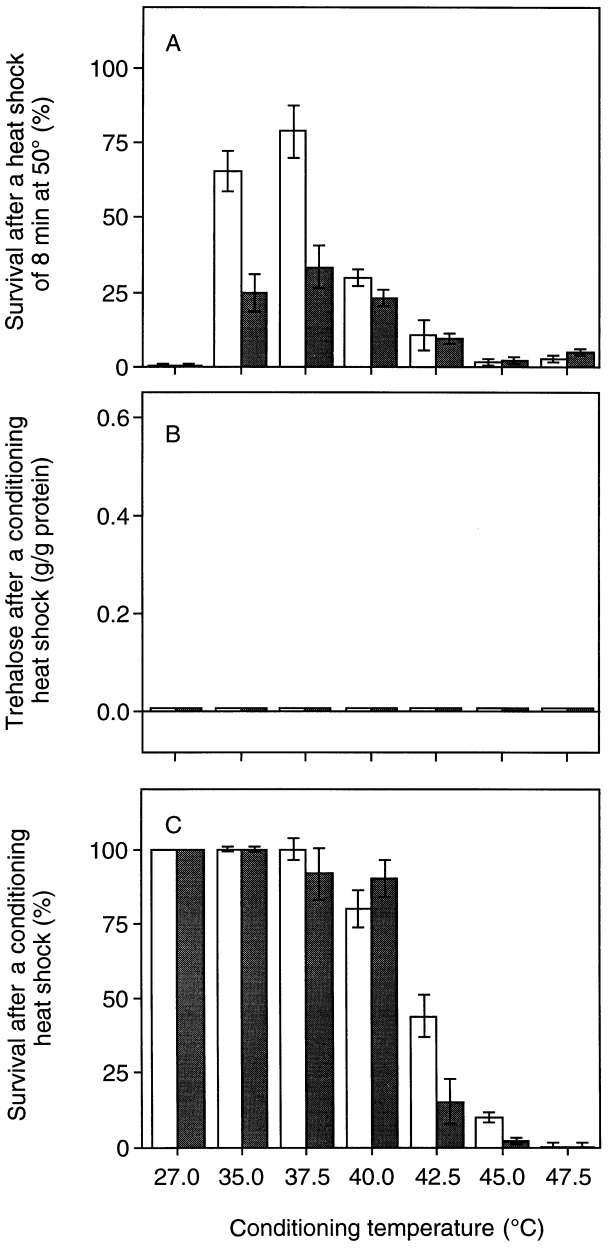

Fig. 3. Thermotolerance (A), trehalose levels (B) and survival (C) of *S. pombe* strain PBL-17 (*tps1*::*LEU2*) after a conditioning heat shock at different temperatures in the absence or presence of cycloheximide. Cultures of *tps1* mutant cells were grown to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and subjected to a conditioning heat shock for 1 h at the temperatures indicated in the absence (open bars) or presence (shaded bars) of cycloheximide (100 $\mu g\,ml^{-1}$) added 5 min before initiation of the heat shock. Thermotolerance (A) was measured as the survival following a subsequent incubation for 8 min at 50°C. Trehalose levels (B) were determined after a 1-h conditioning heat shock. All trehalose values were found to be below the detection limit indicated by the bars ($<0.001\,g\,g^{-1}$ protein). Survival after the conditioning heat shock (C) was determined as a percentage of the 27°C control culture. The error bar for each sample represents the SEM of at least three independent measurements.

Table 1. Trehalose content of *S. pombe* wild-type, *pka1*, *tps1* and *pka1 tps1* strains before and after a conditioning heat shock and after a challenging heat shock.

	Trehalose ($g\,g^{-1}$ protein)			
Relevant genotype	27°C	1 h at 40°C	27°C + 20 min at 50°C	1 h at 40°C + 20 min at 50°C
pka1$^+$ *tps1*$^+$	0.001	0.423	0.001	0.320
pka1::*ura4*$^+$ *tps1*$^+$	0.013	0.784	0.280	1.010
pka1$^+$ *tps1*::*LEU2*	< 0.001	< 0.001	< 0.001	< 0.001
pka1::*ura4*$^+$ *tps1*::*LEU2*	< 0.001	< 0.001	< 0.001	< 0.001

Cells were grown at 27°C to early exponential phase (< 4×10^6 cells ml^{-1}) on YES medium and assayed for their trehalose content either before or after a 1-h conditioning heat shock at 40°C. Unconditioned and conditioned cells were also tested for their ability to accumulate trehalose during the 20 min of the challenging heat shock at 50°C. Experiments were repeated at least three times, also using independent strains with the same relevant genotype (i.e. PB003, CHP421, MRP-7C and MRP-9C for wild-type strains; CHP453 and MRP-7A for *pka1*::*ura4*$^+$ *tps1*$^+$ strains; PBL-17 and MRP-7B for *pka1*$^+$ *tps1*::*LEU2* strains; and MRP-3A, MRP-7D and MRP-9D for *pka1*::*ura4*$^+$ *tps1*::*LEU2* strains). Accordingly, values represent the means of at least three independent cultures and at least two independent strains. The SEMs were less than 10% of the corresponding means in each case.

a conditioning heat shock at 40°C or at higher temperatures did not significantly affect the thermotolerance levels of *tps1* mutant cells when compared with the cycloheximide-untreated cells (Fig. 3A). This is in agreement with our finding that general protein synthesis rates at 40°C were also dramatically reduced in *tps1* mutant cells (> 86% reduction when compared with the 35°C treatment; data not shown) and that protein synthesis may therefore not be important for thermotolerance acquisition under these conditions. However, *tps1* mutant cells were impaired for their ability to acquire thermotolerance if cycloheximide was added during a conditioning heat shock at 35°C, and, unlike wild-type cells, also at 37.5°C. This indicates that protein synthesis (and especially hsp synthesis) is particularly important if trehalose synthesis is absent (i.e. conditioning at temperatures between 35°C and 37.5°C in a *tps1* mutant). In control experiments, cycloheximide inhibited the incorporation of L-[^{35}S]-methionine into TCA-precipitable material by more than 90% in *tps1* mutant cells (strain PBL-17; data not shown). As in wild-type cells, we detected a high sensitivity of *tps1* cells towards conditioning temperatures above 42.5°C, which was also barely altered by the presence of cycloheximide.

pka1 *mutant cells have a high level of basal thermotolerance that is partially dependent on the presence of* tps1$^+$

Studies using *S. cerevisiae* mutants of the Ras/cAMP pathway have shown that mutants with low cAPK activities have high levels of thermotolerance, even without a conditioning heat shock (Shin *et al*., 1987; Hottiger *et al*., 1989). While the presence of at least one of the three *TPK* genes encoding the catalytic subunits of cAPK is essential in *S. cerevisiae*, the single *pka1*$^+$ gene in *S. pombe* was reported to be dispensable for growth (Maeda *et al*., 1994). To study the role of the *pka1*$^+$ gene in basal and induced thermotolerance and in trehalose metabolism, *pka1* mutant and wild-type cells were exposed to a temperature of 50°C, either after growth at 27°C (unconditioned cells) or following a 1-h heat shock at 40°C (conditioned cells). Even though both unconditioned wild-type and *pka1* mutant cells did not contain appreciable amounts of trehalose (Table 1), the *pka1* mutant cells were extremely thermotolerant (about 10^4-fold more survivors after 20 min at 50°C) when compared with the wild-type cells (Fig. 4A). These results are consistent with data reported by Fernández *et al*. (1997) and may, at first glance, indicate that trehalose synthesis does not contribute to the high basal thermotolerance in *pka1* mutants. Surprisingly, however, we found that *pka1* mutant cells, in contrast to wild-type cells, were rapidly able to accumulate large amounts of trehalose during the 20 min of the challenging heat shock at 50°C (Table 1; 0.150 $g\,g^{-1}$ protein and 0.279 $g\,g^{-1}$ protein after 5 min and 10 min at 50°C respectively), suggesting that trehalose synthesis may in fact be important under these conditions. To test this assumption further, we constructed *pka1 tps1* double mutants and analysed their basal trehalose and thermotolerance levels. As expected, the *pka1 tps1* double mutants were completely defective for trehalose synthesis at 27°C or following an incubation for 20 min at 50°C (Table 1). In addition, thermotolerance levels of unconditioned *pka1 tps1* double mutants were found to be significantly lower than those of unconditioned *pka1* single mutants (about 40-fold fewer survivors after 20 min at 50°C; Fig. 4A), yet significantly higher than those of wild-type and *tps1* single mutant cells (about 200-fold more survivors after 20 min at 50°C), indicating that trehalose synthesis is an important, but not the only, element of the basal thermotolerance of *pka1* mutant cells.

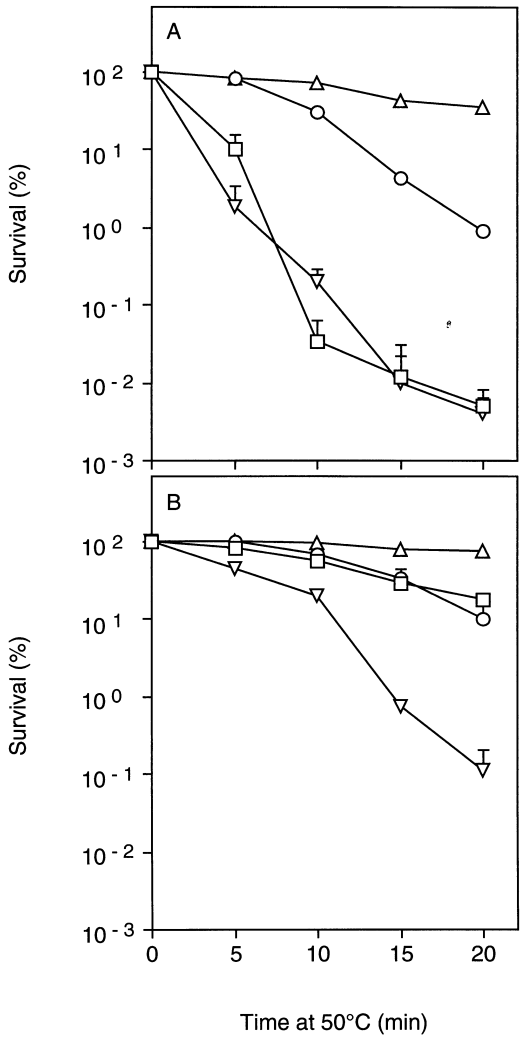

Fig. 4. Thermotolerance of *S. pombe* wild-type, *pka1*, *tps1* and *pka1 tps1* mutant strains before (A) and after (B) a conditioning heat shock. Cells were grown to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and subjected to a conditioning heat shock for 1 h at 40°C. Thermotolerance of wild-type (□) and *pka1* (Δ), *tps1* (∇) and *pka1 tps1* (○) mutant strains before or after the conditioning heat treatment was measured as the survival following incubation at 50°C for the times indicated. Experiments were repeated at least three times with two (*pka1*$^+$ *tps1*::*LEU2*, PBL-17 and MRP-7B; *pka1*::*ura4*$^+$ *tps1*$^+$, CHP453 and MRP-7A), three (*pka1*::*ura4*$^+$ *tps1*::*LEU2*, MRP-3A, MRP-7D and MRP-9D) or four (wild-type: PB003, CHP421, MRP-7C and MRP-9C) independent strains with the same relevant genotype. Accordingly, the error bars, some of which are smaller than the symbols, represent the SEM of at least three independent measurements.

Heat-induced acquisition of thermotolerance in wild-type and pka1 *mutant cells is partially dependent on the presence of* tps1$^+$

A conditioning heat shock for 1 h at 40°C not only induced significant thermotolerance levels in wild-type cells, but also led to a further increase of the basal thermotolerance in *pka1* mutant cells. Accordingly, conditioned *pka1* mutant cells had almost fourfold higher survival rates (74%) than wild-type cells (18%) after a challenging heat shock (20 min at 50°C). Remarkably, compared with wild-type cells, *pka1* mutant cells also had a considerably (twofold to threefold) higher capacity to accumulate trehalose during the conditioning heat shock (including the following challenging heat shock; Table 1), which coincided with twofold to threefold higher Tre6P synthase activity in these cells (Table 2). Deletion of *tps1* in either a wild-type or a *pka1* mutant background not only caused inability to synthesize trehalose (Table 1), but also significantly reduced the capability of the cells to acquire thermotolerance during a conditioning heat shock (about 165-fold reduction in the wild-type and sevenfold reduction in the *pka1* background after 20 min at 50°C), demonstrating also the particularly important role of trehalose synthesis in the full acquisition of heat-induced thermotolerance. However, as in unconditioned cells, the conditioned *pka1 tps1* double mutant cells acquired much higher thermotolerance levels than the single *tps1* mutant cells (about 100-fold more survivors after 20 min at 50°C), indicating the existence of additional *tps1*-independent elements of thermotolerance that are under the negative control of cAPK.

Different effects of pka1 *and* tps1 *mutations on enzymes of trehalose metabolism*

To study a possible involvement of cAPK in the activation/inactivation of the enzymes of trehalose metabolism in *S. pombe*, we determined the activities of neutral trehalase and Tre6P synthase in wild-type and *pka1* mutant cells before and after a 1-h heat shock at 40°C (Table 2). In accordance with our own previously reported results (De Virgilio *et al*., 1990), both neutral trehalase and Tre6P synthase activities in wild-type cells were found to increase during a heat shock at 40°C. Since these activity increases were previously shown to be unaffected by the addition of cycloheximide (De Virgilio *et al*., 1990), and since general protein synthesis rates at 40°C are dramatically reduced in *S. pombe* wild-type cells (Fig. 1), these activity increases are most probably caused by post-translational activation mechanisms and not by *de novo* synthesis of the corresponding enzymes. Interestingly, the heat-induced activity increases of both neutral trehalase and Tre6P synthase were also found in *pka1* mutant cells. Since general protein synthesis rates at 40°C were also dramatically reduced in *pka1* strains (>85% reduction when compared with the 35°C treatment; data not shown), both enzymes are likely to be activated by cAPK-independent post-translational mechanisms. This conclusion is in accordance with a previous report showing that the heat shock-induced activation of neutral trehalase and synthesis of trehalose are not affected by the prior addition of cycloheximide in an *S. pombe pka1* mutant (Fernández *et al*.,

Table 2. Trehalose-6-phosphate synthase and trehalase activities in *S. pombe* wild-type, *pka1*, *tps1* and *pka1 tps1* strains before and after a conditioning heat shock.

	Tre6P synthase (μkat g^{-1} protein)		Trehalase (μkat g^{-1} protein)	
Relevant genotype	27°C	40°C	27°C	40°C
pka1$^+$ *tps1*$^+$	0.71 ± 0.07	1.41 ± 0.31	0.39 ± 0.09	1.11 ± 0.23
pka1::*ura4*$^+$ *tps1*$^+$	2.42 ± 0.17	3.37 ± 0.08	0.28 ± 0.06	0.97 ± 0.17
pka1$^+$ *tps1*::*LEU2*	0.03 ± 0.02	0.03 ± 0.02	0.38 ± 0.05	0.13 ± 0.03
pka1::*ura4*$^+$ *tps1*::*LEU2*	0.06 ± 0.03	0.02 ± 0.01	0.14 ± 0.03	0.15 ± 0.01

Cells were grown to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and assayed for their trehalose-6-phosphate synthase and trehalase activities either before or after a conditioning heat shock of 1 h at 40°C. Experiments were repeated at least three times, also using independent strains with the same relevant genotype (i.e. CHP421, MRP-7C and MRP-9C for wild-type strains; CHP453 for a *pka1*::*ura4*$^+$ *tps1*$^+$ strain; PBL-17 and MRP-7B for *pka1*$^+$ *tps1*::*LEU2* strains; and MRP-3A, MRP-7D and MRP-9D for *pka1*::*ura4*$^+$ *tps1*::*LEU2* strains). Accordingly, values are means ± the SEM of at least three independent cultures and at least two independent strains.

1997). The same report, however, claims that the heat-induced activation of trehalase and the synthesis of trehalose are both significantly reduced in wild-type cells by the addition of cycloheximide before the conditioning heat shock, which is clearly at variance with our former results (De Virgilio *et al*., 1990) and the conclusions of the present study (see above). At present, we do not know the reasons for these discrepancies. However, our finding that trehalase activation and trehalose accumulation took place in wild-type cells even under heat-shock conditions that allow only residual protein synthesis (i.e. 1 h at 40°C) strongly indicates that these processes are not dependent on *de novo* protein synthesis and are mainly regulated at a post-translational level.

In this context, it is interesting that the putative gene product of a recently identified multicopy suppressor of a *pka1* mutation, namely *sck1*$^+$, was reported to display a high degree of homology to the catalytic domain of cAPK (Jin *et al*., 1995). Analysis of a *pka1 sck1* double mutant revealed that both gene products may share a redundant role during the exit from stationary phase and during spore germination, which may be associated with the post-translational activation of neutral trehalase. It will, therefore, be interesting to determine whether Sck1 may indeed be involved in trehalase activation under these conditions and whether it could also be involved in the alternative cAMP-independent pathway for heat shock-induced activation of neutral trehalase.

The observation that unconditioned *pka1* mutant cells had threefold to fourfold increased levels of Tre6P synthase activity indicates that cAPK may, as in *S. cerevisiae* (Winderickx *et al*., 1996), have a role in the negative regulation of *tps1*$^+$ transcription. As expected, *tps1*$^+$ was found to be essential for Tre6P synthase activity in wild-type and *pka1* mutant cells. Surprisingly, however, *tps1* mutants, as well as *pka1 tps1* double mutants, were also found to be defective for the heat-induced activation of neutral trehalase, indicating that the absence of trehalose may activate feed-back mechanisms preventing the unnecessary activation of neutral trehalase (Table 2).

Discussion

Upon exposure to a mild heat shock, yeast cells acquire thermotolerance, i.e. the capacity to survive a subsequent severe heat stress that would be lethal in the absence of the conditioning heat shock. There is substantial evidence from studies in *S. cerevisiae* that both hsp and trehalose synthesis may be important elements in acquired thermotolerance (for a review, see Piper, 1993). One central goal of the present study was to determine the relative contribution of trehalose synthesis to the acquisition of thermotolerance in the fission yeast *S. pombe*. Two observations make this yeast a particularly interesting object for studies of trehalose metabolism. First, the *S. pombe TPS1* homologue has been reported to be essential for Tre6P synthesis, but, unlike in *S. cerevisiae*, to be dispensable for growth on glucose (Blázquez *et al*., 1994). Analysis of a *tps1* mutation in *S. pombe* may therefore allow a more specific assessment of the role of trehalose for the acquisition of thermotolerance than in *S. cerevisiae* (De Virgilio *et al*., 1994). Second, using the protein translation inhibitor, cycloheximide, and temperature-sensitive mutants for protein synthesis, we have previously shown that the heat-induced increase in trehalose accumulation is partly dependent on protein synthesis in *S. cerevisiae*, whereas it is completely independent of protein synthesis in *S. pombe* (De Virgilio *et al*., 1990; 1991). Cycloheximide studies in *S. pombe* wild-type and *tps1* mutant cells may, therefore, allow a dissection of the specific roles of (heat-shock) protein and trehalose synthesis in the acquisition of thermotolerance.

One main conclusion of our studies is that the relative importance of hsp and trehalose synthesis in the acquisition of thermotolerance is strongly dependent on the temperature during the conditioning heat shock. The underlying

physiological reason for this is most probably the fact that the two adaptive responses have different temperature optima for maximal induction. Accordingly, in cells pregrown at 27°C, maximal induction of hsp synthesis is achieved by a heat shock at a temperature of around 35°C, and trehalose synthesis is maximally induced by a heat shock at a temperature of around 42.5°C. While conditioning heat shocks at temperatures between these maxima were found to result in partial activation of both responses, little overlap between the two responses was found at either 35°C or 42.5°C. It is therefore not surprising that cycloheximide treatment during a conditioning heat shock at 35°C revealed an important role for (heat-shock) protein synthesis, but not for trehalose synthesis, in the acquisition of thermotolerance. Similarly, it is not surprising that the studies of *tps1*$^+$ mutants revealed an important role for trehalose synthesis, but not for hsp synthesis, in the acquisition of thermotolerance if the conditioning heat shock was performed at 42.5°C (or 40°C). It is interesting to note, however, that, even in the absence of both trehalose synthesis and hsp synthesis (by the addition of cycloheximide), *tps1* mutant cells acquired significant levels of thermotolerance during a conditioning heat shock at 40°C, indicating the existence of additional post-translationally activated thermotolerance factors.

Previously, we have suggested that the function of trehalose and hsps may be complementary in that trehalose acts to prevent heat-induced inactivation and aggregation of proteins (protection), while hsps act in the resolubilization and refolding of aggregated and denatured proteins (repair) respectively (De Virgilio *et al*., 1991; Hottiger *et al*., 1994 and references therein). In accordance with such a model, it has recently been reported that trehalose and Hsp104 may have synergistic effects for thermotolerance in *S. cerevisiae* (Elliott *et al*., 1996). It would therefore be sensible to assume that hsp synthesis becomes particularly important if trehalose synthesis is absent. Interestingly, and in accordance with this assumption, we have found in this study that the acquisition of thermotolerance in *tps1* mutant cells conditioned at 37.5°C was particularly sensitive to cycloheximide addition before the conditioning heat shock.

A second important conclusion of this study is that rapid adaptive responses during the challenging heat shock may contribute significantly to the levels of thermotolerance. This conclusion is based on our surprising finding that unconditioned *pka1* mutants, in contrast to wild-type cells, were able to react to the challenging heat shock at 50°C with rapid synthesis of large amounts of trehalose. Moreover, the analysis of unconditioned *pka1 tps1* double mutants revealed that this rapid adaptive response is important for the high basal thermotolerance levels of *pka1* cells. Thus, future studies of acquired thermotolerance should undoubtedly also take into account the fact that certain cells may have the ability for rapid post-translational activation of protective mechanisms during the challenging heat shock.

Studies in *S. cerevisiae* have revealed that the transcription of a set of genes, including *TPS1* and other heat shock genes, is under the negative control of cAPK and that mutants with attenuated cAPK activity have high constitutive levels of the corresponding transcripts and/or proteins (Boorstein and Craig, 1990; Marchler *et al*., 1993; Schüller *et al*., 1994; Varela *et al*., 1995; Winderickx *et al*., 1996). If cAPK has a similar role in *S. pombe*, *pka1* mutants would be expected to have high constitutive levels of hsps, including also Tps1 (Blázquez *et al*., 1994; Degols *et al*., 1996). In accordance with this assumption, it has recently been reported that the level of *tps1*$^+$ mRNA in an unconditioned *pka1* mutant is about threefold higher than in the corresponding control strain (Fernández *et al*., 1997). This is also consistent with our finding that Tre6P synthase activity is enhanced about threefold in an unconditioned *pka1* mutant when compared with its wild-type parent. Interestingly, it has recently been suggested that *tps1*$^+$ transcription is positively regulated in response to heat shock through the Wis1–Spc1 mitogen-activated protein kinase (MAPK) pathway (Degols *et al*., 1996). This raises the possibility that two different pathways, the Wis1–Spc1 MAPK pathway and the cAPK signalling pathway, may act antagonistically to control the transcription of *tps1*$^+$. This presumably dual regulation of *tps1*$^+$ transcription by two different pathways may provide a basis for further analyses addressing the question of how these pathways interact to control elements of the heat-shock response.

Unconditioned *pka1 tps1* double mutants, despite their reduced basal thermotolerance levels when compared with *pka1* single mutants, were still found to be quite thermotolerant when compared with wild-type cells. As discussed above, it is possible that unconditioned *pka1* mutants may have elevated levels of various hsps, which may confer partial resistance to the challenging heat shock even if trehalose synthesis is absent (such as in *pka1 tps1* double mutants). While future studies should undoubtedly address this question in more detail (using also specific antibodies to determine the exact levels of various hsps), these results allow us to propose an interesting working model. The high basal level of thermotolerance in *pka1* mutants may be caused by at least two different processes, namely the constitutive synthesis of a set of hsps before the challenging heat shock and the rapid accumulation of trehalose during the challenging heat shock. A particularly interesting aspect of this model is the possibility that elevated levels of molecular chaperones may directly enhance the stability of the Tre6P synthase and therefore be a prerequisite for trehalose synthesis during the challenging heat shock at 50°C.

Table 3. *S. pombe* strains used in this study.

Strain	Genotype	Source (reference)
PB003	*h*$^+$ *ade6-M216 leu1-32 ura4-D18*	Blázquez *et al*. (1994)
PBL-17	*h*$^+$ *ade6-M216 leu1-32 ura4-D18 tps1::LEU2*	Blázquez *et al*. (1994)
CHP421	*h*$^-$ *his7-366 leu1-32 ura4-D18*	Jin *et al*. (1995)
CHP453	*h*$^-$ *his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$	Jin *et al*. (1995)
MRP-3A	*h*$^+$ *his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study
MRP-7A	*h*$^+$ *leu1-32 ura4-D18 pka1::ura4*$^+$	This study
MRP-7B	*h*$^+$ *ade6-M216 leu1-32 ura4-D18 tps1::LEU2*	This study
MRP-7C	*h*$^-$ *his7-366 leu1-32 ura4-D18*	This study
MRP-7D	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study
MRP-9C	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4-D18*	This study
MRP-9D	*h*$^+$ *leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study

Experimental procedures

Strains and growth conditions

The genotypes of all *S. pombe* strains used in this study are listed in Table 3. Strains MRP-3A, MRP-7A, MRP-7B, MRP-7C, MRP-7D, MRP-9C and MRP-9D are all haploid meiotic segregants of three independent diploid strains constructed by crossing strains PBL-17 and CHP453. Mating, sporulation and tetrad analysis of *S. pombe* were performed as described previously (Moreno and Nurse, 1991). Cells were grown in YES medium (0.5% yeast extract and 3% glucose), supplemented with the appropriate auxotrophic requirements and grown at 27°C on a rotary shaker (140 r.p.m.) to mid-exponential growth phase at a density of 4×10^6 cells ml^{-1}. For isotopic labelling studies, cells were transferred into minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose), supplemented with the appropriate auxotrophic requirements.

Heat-shock conditions and analysis of thermotolerance

Log-phase cells grown at 27°C in liquid medium were subjected to a conditioning treatment for 1 h at either 35°C, 37.5°C, 40°C, 42.5°C, 45°C or 47.5°C. Cycloheximide was used at a final concentration of 100 μg ml^{-1} and added 5 min before the conditioning heat shock where indicated (including the labelling experiments). For the determination of thermotolerance, aliquots of the cultures (1 ml) were transferred to prewarmed glass tubes, incubated at 50°C for the times indicated, rapidly cooled on ice, appropriately diluted with sterile water and plated on YES agar. Colonies were counted after 4 days at 27°C, and the percentage survival was assessed by comparison with controls not subjected to the 50°C treatment (100%).

Analytical procedures

Activities of both neutral trehalase and Tre6P synthase were measured in permeabilized cells. To this end, 20-ml aliquots of the log-phase cultures were filtered (Whatman GF/C), resuspended in 1 ml of 0.2 M Mes (K^+), pH 6.0, 0.05% Triton X-100, or in 1 ml of 0.2 M Tricine (Na^+), pH 7.0, 0.05% Triton X-100 and immediately frozen in liquid nitrogen. After thawing (3 min at 30°C), the cells were centrifuged, washed twice with ice-cold 0.2 M Mes (K^+), pH 6.0, or 0.2 M Tricine (Na^+), pH 7.0, and immediately used for the assays of trehalase and Tre6P synthase, respectively, as described previously (De Virgilio *et al*., 1990). For trehalose determination, 10–20 ml of exponentially growing cells were filtered (Whatman GF/C), washed four times with 5 ml of distilled water, resuspended in 1 ml of water and transferred to a boiling water bath for 10 min. After centrifugation (5 min at $15\,000\times g$), trehalose was determined in the supernatant by high-performance liquid chromatography (HPLC) analysis as described previously (De Virgilio *et al*., 1993). Protein was quantitated as described by Lowry *et al*. (1951) using BSA as standard.

For the analysis of protein synthesis at different temperatures (27–47.5°C), exponentially growing cultures were centrifuged for 5 min at $3000\times g$ and resuspended in minimal medium at a final concentration of 1×10^8 cells ml^{-1}. Cultures (1 ml) were labelled for 1 h at the temperatures indicated by the addition of 14 μCi of carrier-free L-[^{35}S]-methionine (1.1 μCi μl^{-1}). Radiolabelled proteins were extracted by adding NaOH (0.25 M final concentration) and subsequent precipitation with trichloroacetic acid (TCA; 12% final concentration); the pellets were washed three times with ice-cold acetone, resuspended in 0.1 M NaOH and mixed with an equal amount of twice-concentrated sample buffer (Laemmli, 1970). Radiolabelled proteins were resolved by one-dimensional SDS–PAGE; the gels were then stained, destained and dried before autoradiography at −70°C, using Kodak X-OMAT AR film.

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