Eur. J. Biochem. 219, 179–186 (1994)

The role of trehalose synthesis for the acquisition of thermotolerance in yeast
I. Genetic evidence that trehalose is a thermoprotectant

Claudio DE VIRGILIO, Thomas HOTTIGER, Judith DOMINGUEZ, Thomas BOLLER and Andres WIEMKEN
Botanisches Institut der Universität Basel, Switzerland

(Received September 21, 1993) – EJB 93 1436/5

In the yeast *Saccharomyces cerevisiae*, accumulation of the non-reducing disaccharide trehalose is triggered by various stimuli that activate the heat-schock response. Several studies have shown a close correlation between trehalose levels and tolerance to heat stress, suggesting that trehalose may be a protectant which contributes to thermotolerance. In this study, we have examined mutants defective in genes coding for key enzymes involved in trehalose metabolism with respect to the heat-induced and stationary-phase-induced accumulation of trehalose and the acquisition of thermotolerance. Inactivation of either *TPS1* or *TPS2*, encoding subunits of the trehalose-6-phosphate synthase/phosphatase complex, caused an inability to accumulate trehalose upon a mild heat-shock or upon initiation of the stationary phase and significantly reduced the levels of heat-induced and stationary-phase-induced thermotolerance. Deletion of *NTH1*, the gene coding for the neutral trehalase, resulted in a defect in trehalose mobilization during recovery from a heat shock which was paralleled by an abnormally slow decrease of thermotolerance. Our results provide strong genetic evidence that heat-induced synthesis of trehalose is an important factor for thermotolerance induction. In an accompanying study [Hottiger, T., De Virgilio, C., Hall, M. N., Boller, T. & Wiemken, A. (1993) *Eur. J. Biochem. 219*, 187–193], we present evidence that the function of heat-induced trehalose accumulation may be to increase the thermal stability of proteins.

Upon exposure to a mild heat shock, cells of virtually all organisms exhibit an adaptive response, the heat-shock response. This response leads to the induction of thermotolerance, i.e. the ability to survive a subsequent severe heat stress that would be lethal in the absence of the preconditioning heat treatment. One of the key events of the heat-shock response is a dramatic increase in rates of synthesis of a small set of highly conserved proteins, the heat-shock proteins (hsp), while the synthesis of most other proteins is reduced (Lindquist and Craig, 1988; Schlesinger, 1990).

Whilst the mechanism of hsp induction has been elucidated in considerable detail (Craig and Gross, 1991; Sorger, 1991), the functions of hsp have been a matter for speculation for a long time. One of the most exciting concepts to emerge from the study of hsp was their role as molecular chaperones (Ellis, 1987); hsp have been found to play a vital role even in unstressed cells by associating with newly synthesized proteins (Beckman et al., 1990), assisting in their folding (Gething and Sambrook, 1992; Ang et al., 1991) and allowing these proteins to cross membranes (Deshaies et al., 1988; Cheng et al., 1989; Kang et al., 1990). Under conditions of heat stress, hsp are suggested to be involved in facilitating the disaggregation and refolding of heat-denatured proteins. In agreement with this concept, a close correlation between hsp synthesis and the induction of thermotolerance was established in many studies, providing indirect evidence for hsp involvement in the latter process (Lindquist and Craig, 1988). In yeast, it was shown that the induction of a particular hsp, Hsp104, which is not expressed during growth at normal temperatures, is required for the full development of induced thermotolerance (Sanchez and Lindquist, 1990). Nevertheless, whilst the function of hsp as molecular chaperones during growth at normal conditions is beyond doubt, the general conclusion of a causal relationship between heat-shock acquisition of thermotolerance and the synthesis of hsp is still a matter of debate. Moreover, in contradiction with the generally accepted concept of hsp-mediated thermotolerance, an increasing number of studies are providing evidence for hsp synthesis being neither sufficient (Finkelstein and Strausberg, 1983; Petko and Lindquist, 1986; Susek and Lindquist, 1989; Praekelt and Meacock, 1990) nor necessary (Hall, 1983; Watson et al., 1984; Wiedelitz et al., 1986; Van-

Correspondence to A. Wiemken, Botanisches Institut, Hebelstrasse 1, CH-4056 Basel, Switzerland

Abbreviations. Tre6*P*, trehalose-6-phosphate; hsp, heat-shock protein(s); TPS1, 56-kDa subunit of trehalose-6-phosphate synthase/phosphatase complex; TPS2, 102-kDa subunit of trehalose-6-phosphate synthase/phosphatase complex; TSL1, 123-kDa subunit of trehalose-6-phosphate synthase/phosphatase complex; TPS3, homolog of TSL1; NTH1, neutral trehalase.

Enzymes. Trehalose-6-*P* synthase [UDP-glucose: D-glucose-6-phosphate-1-glucosyltransferase] (EC 2.4.1.15); trehalose-6-*P* phosphatase [trehalose-6-phosphate phosphohydrolase] (EC 3.1.3.12); neutral trehalase (EC 3.2.1.28).

Bogelen et al., 1987; Lee and Dewey, 1988; Smith and Yaffe, 1991a,b) for the development of induced thermotolerance.

On the basis of these contradictory findings, it is plausible to assume that there may exist alternative ways to acquire thermotolerance that are independent of (heat-shock) protein synthesis. This is also supported by the general conceptual view that induction of thermotolerance may involve both protection from and repair of initial heat damage (De Virgilio et al., 1991a; Laszlo, 1992). One factor potentially important for the development of induced thermotolerance in yeast, is the heat-induced accumulation of the non-reducing disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside). In previous studies, we have shown that trehalose levels and thermotolerance are correlated under a variety of experimental conditions in both wild-type strains and regulatory mutants with altered pools of trehalose (Hottiger et al., 1987a,b, 1989; De Virgilio et al., 1990, 1991a; Hottiger et al., 1992). Also, we have shown that trehalose accumulation and the acquisition of thermotolerance during heat shock are partially independent of protein synthesis in *Saccharomyces cerevisiae* (De Virgilio et al., 1991a) and completely independent of protein synthesis in *Schizosaccharomyces pombe* (De Virgilio et al., 1990). Therefore, we suggested that trehalose, which was originally thought to function as a storage carbohydrate in yeast, may actually be a stress protectant involved in induced thermotolerance and other phenomena of stress protection (Wiemken, 1990).

The suggestion that trehalose has a role in thermotolerance has recently been substantiated by new independent lines of evidence: (a) TPS1 as well as TPS2, both subunits of the trehalose-6-phosphate (Tre6*P*) synthase/phosphatase complex with molecular masses of 56 kDa and 102 kDa, respectively, have been shown to be heat-shock proteins (Bell et al., 1992; De Virgilio et al., 1993); (b) consistent with these findings, *TPS1*, *TPS2*, as well as *TSL1*, encoding a third 123-kDa subunit of the Tre6*P* synthase/phosphatase complex, were found to contain canonical GAANNTTC heat-shock elements or the newly identified C_4T sequence (Vuorio et al., 1993); (c) deletion of the *otsA* and *otsB* genes of *Escherichia coli*, which encode Tre6*P* synthase and Tre6*P* phosphatase, respectively, resulted in a significant decrease of thermotolerance in stationary-phase *E. coli* cells (Hengge-Aronis et al., 1991).

In contrast to the situation in *E. coli*, genetic evidence for a contribution of trehalose to the thermotolerance of baker's yeast is still lacking. However, the recent cloning of three genes coding for subunits of the Tre6*P* synthase/phosphatase complex (Vuorio et al., 1992, 1993; Bell et al., 1992; De Virgilio et al., 1993) and the cloning of the gene encoding the neutral trehalase (Kopp et al., 1993) have provided tools to study the role of trehalose in the development of thermotolerance in *S. cerevisiae* also at the genetic level.

The present study was undertaken in order to clarify the relative contribution of heat-induced and stationary-phase-induced accumulation of trehalose to the acquisition of thermotolerance in yeast. Our studies show that specific deletions in either of two genes coding for subunits of the Tre6*P* synthase/phosphatase complex (*TPS1* and *TPS2*) cause an inability to accumulate trehalose either upon a mild heat shock or upon initiation of the stationary phase, and in both cases significantly reduce levels of thermotolerance. Our studies also show that the deletion of *NTH1*, the gene coding for the neutral trehalase, results in a defect in trehalose mobilization during recovery from a heat shock, which is paralleled by a slower decrease in thermotolerance.

MATERIALS AND METHODS

Yeast strains

The strain CJM169 (MATα, *ura3*, *his3-11,15*, *ade2-1*, *cif1::URA3*) and its isogenic wild-type parent CJM162 (MATα, *ura3*, *his3-11,15*, *ade2-1*) have been described previously (Gonzáles et al., 1992) and were kindly provided by C. Gancedo, Instituto de Investigaciones Biomédicas, Madrid. It is now well established that the mutations *byp1*, *cif1*, *fdp1*, *ggs1*, *glc6*, *tps1* and *tss1* are all alleles of the same gene (Bell et al., 1992; Cannon et al., 1992; Vuorio et al., 1992; Stucka and Blázquez, 1993; Van Aelst et al., 1993). For the sake of lucidity, we use the common name *TPS1* for the designation of this gene. This is justified by the finding that the gene product of *TPS1* was found to be a subunit of the Tre6*P* synthase/phosphatase complex (Bell et al., 1992; Vuorio et al., 1992). Therefore, in this study we refer to the *cif1* deletion mutant as the *Δtps1* mutant. The *tps2* disruption mutant CDV64 (MATα, *his4*, *ura3*, *leu2*, *bar1-1*, *tps2::URA3*) and its isogenic wild-type parent RH 144-3A (MATα, *his4*, *ura3*, *leu2*, *bar1-1*) have also been described previously (De Virgilio et al., 1993). The diploid strain GRD11-21, derived from the parental strains X2180-1A (MATα, *SUC2*, *mal*, *gal2*, *CUP1*) and MC333 (MATα, *leu2*, *trp1*, *met8*), was obtained from Gist Brocades, Delft, NL.

Yeast strains containing a deletion of *NTH1* were kindly supplied by P. Schoppink, P. Klaassen and K. Osinga (Gist-Brocades). They were prepared according to the following procedure. A yeast cDNA library in λgt11 (Clontech) was screened by the immunological technique described by Young and Davies (1983), using a monoclonal antibody against purified neutral trehalase from yeast. 300000 plaques were screened with the antibody, resulting in 22 positive plaques. Eight randomly picked plaques were purified and their inserts were amplified by polymerase-chain reaction in a Perkin-Elmer Cetus thermal cycler according to the manufacturers instructions, using primers complementary to the λgt11 sequence beside the *Eco*RI cloning site of the inserts. Isolation and manipulation of DNA was performed using standard procedures as previously described (Sambrook et al., 1989). One amplified insert, carrying the putative sequence of the neutral trehalase, was cloned at the *Eco*RI site of the multicloning site of the plasmid pTZ19R (Pharmacia), which additionally contained a cloned *Bgl*II−*Bgl*II fragment at the *Bam*HI site with the *ADH1* promotor sequence coupled to the G-418 resistance gene. The resulting plasmid pTRE16 G418-2 was treated with *Nsi*I to remove an internal part of the putative *NTH1* sequence and was subsequently transformed into strain GRD11-21 (Rothstein, 1983). Diploid transformants had approximately half of the neutral-trehalase activity when compared with wild-type cells (data not shown). Sporulation of the diploid transformant and tetrad analysis revealed that all four ascospores were viable and that G-418 resistance segregated in the ratio 2:2. As expected, all G-418-resistant segregants had no detectable neutral-trehalase activity, indicating that the G-418-resistance gene was integrated at the *NTH1* locus. The diploid strain PKC8 was constructed by crossing two G418 resistant, trehalase-negative segregants of the opposite mating type.

Culture conditions

Cells were grown in 1% yeast extract 2% bactopeptone media supplemented with either 2% maltose (YPM) for the strains CJM162 and CJM169 or 2% glucose (YPD) for all

other strains. Stock cultures were kept on YPD or YPM agar (strains CJM162 and CJM169) either with (PKC8) or without 300 μg/ml G-418 sulphate. Cell cultures were cultivated on a rotary shaker (140 rpm) at 27°C, transferred into fresh medium and allowed to grow under the same conditions for at least six generations, taking care that the cell densities were below 3×10^6 cells/ml at the beginning of the heat-shock experiments. Stationary phase cultures were obtained by growing cells for three days in YPM or YPD medium.

Heat-shock conditions and analysis of thermotolerance

The preconditioning heat-shock at 40°C was performed as previously reported (Hottiger et al., 1987a). For determination of thermotolerance, cultures were heated for different times either to 52°C (exponentially growing *Δtps1* cells), or to 50.4°C (exponentially growing *tps2* cells), or to 54°C (stationary-phase cells), rapidly cooled on ice, appropriately diluted with sterile water and plated on YPD agar or YPM agar as described (Hall, 1983).

Enzyme assays and determination of metabolite levels

The activity of neutral trehalase was measured in permeabilized cells (Miozzari et al., 1978). 40-ml aliquots of log-phase cultures were filtered (Whatman GF/C), suspended in 1 ml 0.2 M Tricine (Na^+), pH 7.0, 0.05% Triton X-100 and immediately frozen in liquid nitrogen. After thawing (2 min at 30°C), the cells were centrifuged (1 min at 3000 *g*), washed twice with ice cold 0.2 M Tricine (Na^+), pH 7.0 and immediately used for the assay of neutral trehalase according to De Virgilio et al. (1991b).

For determination of trehalose and Tre6*P*, 10 ml of exponentially growing cells were filtered (Whatman GF/C), washed four times with 5 ml distilled H_2O, resuspended in 1 ml H_2O and transferred to a boiling water bath for 5 min. After centrifugation (5 min at 15000 *g*), trehalose and Tre6*P* were determined in the supernatant by HPLC analysis as described earlier (De Virgilio et al., 1993). Protein was quantitated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

RESULTS

Heat-induced acquisition of tolerance to severe heat-shock and accumulation of trehalose in a *Δtps1* mutant

It has recently been reported that the product of the *TPS1* gene, encoding the 56-kDa subunit of the Tre6*P* synthase/phosphatase complex, is essential for Tre6*P* synthase activity (Bell et al., 1992). To investigate the role of the *TPS1* gene in basal and induced thermotolerance, a *Δtps1* mutant and its isogenic parent were exposed to a temperature of 52°C, either after growth at 27°C (unconditioned cells) or following a 60-min heat shock at 40°C (conditioned cells). As shown in Fig. 1A, unconditioned cells of both the wild type and the mutant did not contain appreciable amounts of trehalose. Both strains were extremely heat sensitive (less than 0.1% survivors after 20 min at the lethal temperature), with wild-type cells being significantly less susceptible to thermal killing (Fig. 1B).

After conditioning (1 h at 40°C), cells of the *Δtps1* strain were virtually free of trehalose, while the wild-type cells had accumulated considerable amounts of the disaccharide (Fig. 1A). The wild-type trehalose levels shown in Fig. 1A

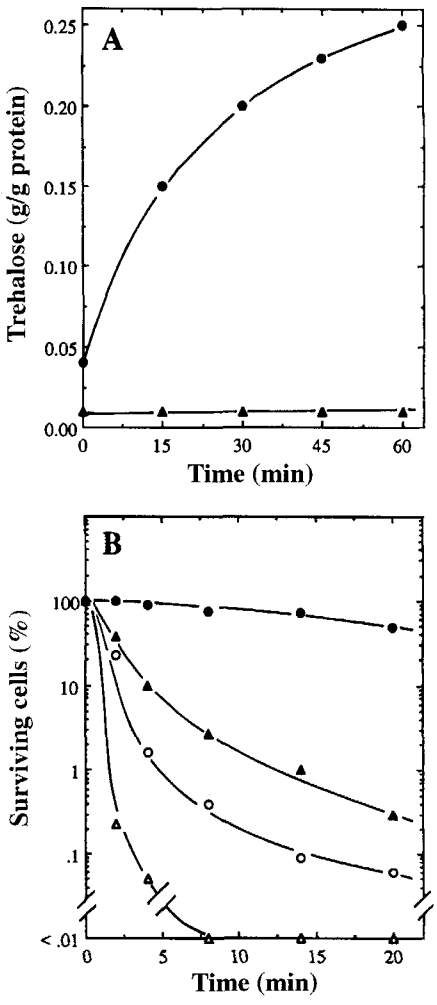

Fig. 1. Trehalose levels (A) and thermotolerance (B) of *S. cerevisiae* strains CJM162 (wild type) and CJM169 (*Δtps1*) during and after a preconditioning heat shock. (A) Cultures of wild-type (●) and *Δtps1* (▲) cells were grown to early-exponential phase ($<3\times10^6$ cells/ml) on YPM medium and, at time zero, were subjected to a preconditioning heat shock at 40°C. Samples were taken for trehalose determination at the desired time points. (B) The thermotolerance of wild-type (○,●) and *Δtps1* (△,▲) cells without (○,△) or with (●,▲) a preconditioning heat treatment for 60 min at 40°C was measured as the survival following incubation at 52°C for the times indicated.

are significantly lower than those reported in other studies from our laboratory, which is due to the use of maltose instead of glucose as the carbon source. This was necessary since *Δtps1* cells are unable to grow on glucose. The preconditioning heat shock induced thermotolerance in both the wild type and the *Δtps1* mutant. However, thermotolerance levels of the mutant were significantly lower than those of the wild type (about 100-fold less survivors after 20 min at 52°C; Fig. 1B). A similar phenotype has been described for a *Δhsp104* mutant (Sanchez and Lindquist, 1990).

Thermotolerance and accumulation of trehalose in a stationary *Δtps1* mutant

Stationary phase cells of the *Δtps1* strain (CJM169) were virtually free of trehalose (5 mg trehalose/g protein), while stationary wild-type cells (CJM162) had accumulated considerable amounts of the disaccharide (0.43 g trehalose/g

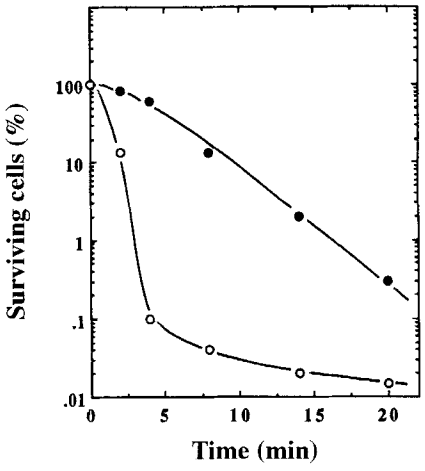

Fig. 2. Thermotolerance of stationary *S. cerevisiae* strains CJM162 (wild type) and CJM169 (*Δtps1*). Cultures of wild-type (●) and *Δtps1* (○) cells were grown to stationary phase (3 days) on YPM medium. Wild-type cells contained 0.43 g trehalose/g protein, while *Δtps1* cells were virtually free of trehalose (5 mg trehalose/g protein). Thermotolerance was measured as the survival following incubation at 54°C for the times indicated.

protein). To examine whether these differences in trehalose contents were also reflected in different degrees of thermotolerance, stationary-phase cells of both strains were subjected to a heat treatment at 54°C for different times. Whilst almost 80% of the wild-type cells were viable after 4 min at 54°C, only approximately 0.1% of the *Δtps1* cells survived the same heat treatment (Fig. 2). Accordingly, the absence of trehalose in *Δtps1* cells was accompanied by a more than 800-fold reduction of thermotolerance after 4 min at 54°C when compared with wild-type cells (Fig. 2).

Heat-induced acquisition of tolerance to a severe heat-shock and accumulation of trehalose or trehalose-6-phosphate in the presence and absence of *TPS2*

Disruption of *TPS2*, the gene coding for the 102-kDa subunit of the Tre6*P* synthase/phosphatase complex, causes loss of Tre6*P* phosphatase activity. Cells of a *Δtps2* disruption strain contained only approximately 8% of the trehalose formed in an isogenic wild-type strain after 1 h at 40°C, but accumulated large amounts of Tre6*P* at the same time (Fig. 3A; see also De Virgilio et al., 1993).

To test whether the accumulation of Tre6*P*, instead of trehalose, may affect the cell's ability to acquire induced thermotolerance, we subjected cells of a wild-type and a *tps2* disruption strain to a heat treatment at 50.4°C for different times, either with or without a preconditioning heat shock (1 h at 40°C). Untreated cells of both strains were unable to survive 4 min at 50.4°C (Fig. 3B). The preconditioning heat shock induced thermotolerance to a considerably different degree in the wild-type and in the *tps2* disruption strain. Whilst almost 100% of the wild-type cells were viable after 20 min at 50.4°C, only approximately 1% of the *tps2* disruption cells survived the same heat treatment (Fig. 3B). These data indicate an important role for the *TPS2* gene in thermotolerance induction and suggest that accumulation of Tre6*P* instead of trehalose reduces thermotolerance. A possible explanation for this is provided by the fact that trehalose, but not Tre6*P*, is able to protect proteins against thermal denaturation (Hottiger et al., 1993).

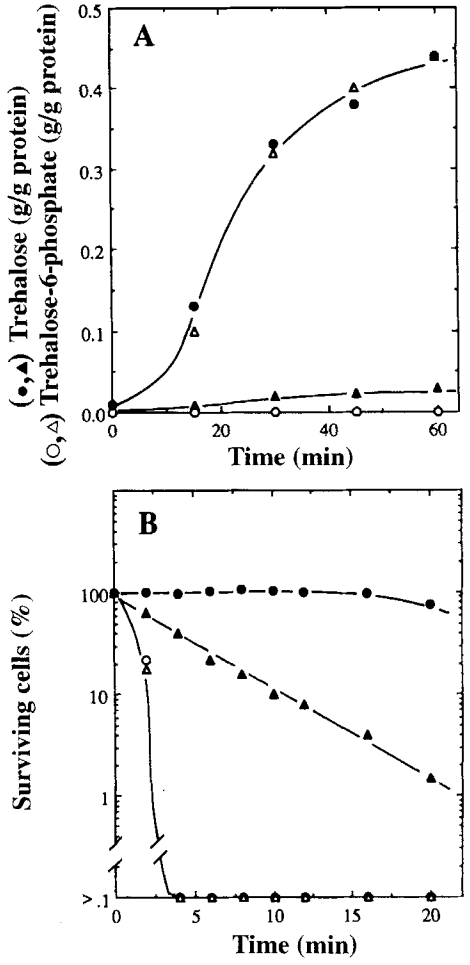

Fig. 3. Levels of trehalose and trehalose-6-phosphate (A) and thermotolerance (B) of *S. cerevisiae* strains RH 144–3A (wild type) and CDV64 (*tps2* disruption mutant) during and after a preconditioning heat shock. (A) Cultures of wild-type (●,○) and *tps2* disruption mutant (▲,△) cells were grown to early exponential phase (<3×10^6 cells/ml) on YPD medium and, at time zero, were subjected to a preconditioning heat shock at 40°C. Samples were taken for trehalose (●,▲) and trehalose-6-phosphate (○,△) determination at the desired time points. (B) The thermotolerance of wild-type (○,●) and *tps2* diruption mutant (△,▲) cells without (○,△) or with (●,▲) a preconditioning heat treatment for 60 min at 40°C was measured as the survival following incubation at 50.4°C for the times indicated.

Thermotolerance and accumulation of trehalose in a stationary *tps2* disruption mutant

While wild-type cells (RH144-3A) entering the stationary phase accumulated up to 1.0 g trehalose/g protein, cells of a *tps2* disruption mutant (CDV64) accumulated up to 1.4 g Tre6*P*/g protein but almost no trehalose upon initiation of the stationary phase (data not shown). To test whether the accumulation of Tre6*P*, instead of trehalose, may also affect the cell's ability to acquire stationary-phase-induced thermotolerance, we subjected cells of a wild-type and a *tps2* disruption strain to a heat treatment at 54°C for different times. Whilst almost 60% of the wild-type cells were viable after 8 min at 54°C, only approximately 0.06% of the *tps2* disruption cells survived the same heat treatment (Fig. 4). Thus, the accumulation of large amounts of Tre6*P* instead of trehalose in stationary *tps2* disruption cells was also paralleled by a significant decrease in thermotolerance.

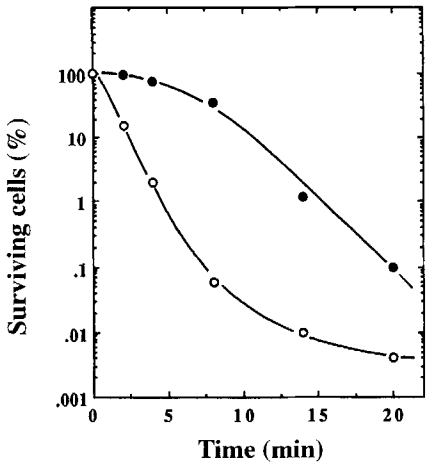

Fig. 4. Thermotolerance of stationary *S. cerevisiae* strains RH144-3A (wild type) and CDV64 (*Δtps2*). Cultures of wild-type (●) and *Δtps2* (○) cells were grown to stationary phase (3 days) on YPD medium. Wild-type cells contained 1.0 g trehalose/g protein, while *Δtps2* cells contained only traces of trehalose but high amounts of Tre6*P* (up to 1.4 g Tre6*P*/g protein). Thermotolerance was measured as the survival following incubation at 54°C for the times indicated.

Deletion of the gene coding for the neutral trehalase affects trehalose metabolism and the heat-induced acquisition of thermotolerance

The mobilization of trehalose in wild-type yeast strains recovering from a heat shock is brought about by a rapid activation of the neutral trehalase (De Virgilio et al., 1991b). As a consequence, a strain harbouring a deletion in the gene coding for the neutral trehalase (*NTH1*) is supposed to lack trehalose mobilization when allowed to recover from a heat shock. As this provides another means to manipulate trehalose levels, trehalose accumulation and mobilization as well as induction and decay of thermotolerance were studied in a *Δnth1* strain. As shown in Fig. 5A, a *Δnth1* mutant accumulated slightly more trehalose during a 1-h heat shock at 40°C when compared with its wild-type parent. Shifting the culture back to 27°C after 1 h at 40°C resulted in a rapid mobilization of trehalose in cells of the wild-type strain but not of the *Δnth1* mutant (Fig. 5A). The slow decrease in trehalose content observed in recovering *Δnth1* cells could be fully explained on the basis of dilution by growth (data not shown).

The observed differences in trehalose accumulation and mobilization patterns between the *Δnth1* strain and its wild type were paralleled by a different ability to acquire or to lose thermotolerance; without a preconditioning heat shock, cells of both strains were equally sensitive to thermal killing (Fig. 5B). A preconditioning heat shock for 1 h at 40°C induced thermotolerance to a slightly larger extent in the *Δnth1* strain than in the wild-type strain, reflecting the slightly higher amount of trehalose formed in the *Δnth1* strain after 1 h at 40°C. Having recovered for 30 min from the initial heat shock, cells of both strains began to lose their acquired thermotolerance (Fig. 5B). Remarkably, however, the decay of thermotolerance was less pronounced for *Δnth1* cells, survival rates being 10−20-fold higher than those of the wild-type cells after 20 min at 50.4°C.

To obtain a statistically reliable value for this observed difference and to circumvent any problems eventually arising from slight differences in the genetic backgrounds of the

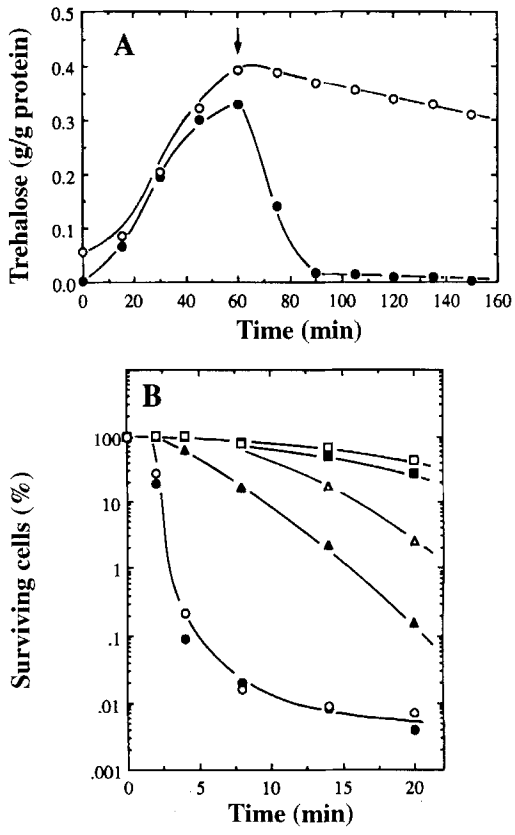

Fig. 5. Trehalose level (A) and thermotolerance (B) of *S. cerevisiae* strains GRD11-21 (wild type) and PKC8 (*Δnth1*) during a heat shock at 40°C followed by recovery at 27°C. (A) Cultures of wild-type (●) and *Δnth1* (○) cells were grown to early exponential phase (<3×10^6 cells/ml) on YPD medium and, at time zero, were subjected to a preconditioning heat shock. After 1 h at 40°C, the cultures were returned to 27°C (arrow). Samples were taken for trehalose determination at the desired time points. (B) Thermotolerance of wild-type (●,■,▲) and *Δnth1* (○,□,△) cells without a preconditioning heat shock (●,○), after a preconditioning heat shock of 60 min at 40°C (■,□), or after a preconditioning heat shock of 60 min followed by a recovery at 27°C for 30 min (△,▲). Thermotolerance was measured as the survival following incubation at 50.4°C for the times indicated.

Δnth1 and wild-type diploids (see also the Materials and Methods section), we examined ten spores of both diploid strains (PKC-8 and GRD11-21). Having recovered for 30 min from the initial heat shock, *Δnth1* spores had still 0.419 ± 0.03 g trehalose/g protein and 15.8 ± 4.8% of the cells survived the subsequent heat shock for 20 min at 50.4°C (Table 1). Under the same conditions, wild-type cells were already virtually free of trehalose (0.041 ± 0.02 g trehalose/g protein) and only 2.0 ± 1.0% of the cells were able to withstand the lethal heat shock for 20 min at 50.4°C (Table 1).

DISCUSSION

It is now well documented that heat shock leads to a dramatic increase in trehalose levels in yeast and a number of other microorganisms (Hottiger et al., 1993 and references therein). In the case of yeast, two lines of circumstantial evidence indicate that this response has a thermoprotective function. Firstly, a close correlation between trehalose levels and

Table 1. Trehalose content and thermotolerance of ten ascospores of the *Δnth1* (PKC-8) and wild-type (GRD11-21) diploids. Trehalose and thermotolerance (survival after 20 min at 50.4°C) were measured after a heat shock of 60 min at 40°C and a subsequent recovery phase at 27°C for 30 min.

Ascospore	Trehalose content and thermotolerance for			
	spores of the *Δnth1* mutant PKC-8		spores of the wild-type GRD11-21	
	trehalose	thermotolerance	trehalose	thermotolerance
	g trehalose/g protein	%	g trehalose/g protein	%
1A	0.427	11.6	0.061	0.8
1B	0.423	10.0	0.064	2.1
1C	0.423	13.0	0.057	3.1
1D	0.407	9.5	0.047	3.5
2A	0.460	19.0	0.048	1.1
2B	0.441	16.6	0.030	2.8
2C	0.375	22.1	0.028	1.1
2D	0.384	16.0	0.016	3.3
3A	0.407	24.6	0.017	1.0
3B	0.439	15.4	0.043	1.5
Mean value	0.419 ± 0.03	15.8 ± 4.8	0.041 ± 0.02	2.0 ± 1.0

thermotolerance has been found under a wide variety of experimental conditions (Wiemken, 1990). Secondly, at least two of the subunits of the Tre6*P* synthase/phosphatase complex have been shown to be hsp (Bell et al., 1992; De Virgilio et al., 1993) and three subunits of the complex contain the canonical GAANNTTC or the newly identified C_4T heat-shock elements (Vuorio et al., 1993). In this study, we provide direct genetic evidence supporting the view that trehalose acts as a thermoprotectant.

In a first set of experiments, we studied trehalose accumulation and thermotolerance induction in a *Δtps1* mutant. Disruption of the *TPS1* gene, that is essential for Tre6*P* synthase activity (Bell et al., 1992), abolished trehalose synthesis in response to heat shock and upon initiation of the stationary phase. In parallel, inactivation of *TPS1* resulted in a significant reduction of heat-induced thermotolerance (100-fold less survivors after 20 min at 52°C as compared to the wild type; Fig. 1) as well as of the intrinsic thermotolerance of stationary-phase cells (800-fold less survivors after 4 min at 54°C as compared to the wild type; Fig. 2). This is consistent with trehalose being required for full thermotolerance acquisition. There is, however, a caveat. The *TPS1* gene is allelic to the genes defined by the *fdp1*, *byp1*, *ggs1*, *cif1* and *glc6* mutations (Bell et al., 1992; Thevelein, 1992). The phenotype of the latter mutants is complex (Thevelein, 1992) and it was therefore suggested that the *TPS1* gene product, whilst being a component of the Tre6*P* synthase/phosphatase complex, has a primarily regulatory function (Bell et al., 1992). However, recent evidence indicates that *TPS1* may in fact be the structural gene for the Tre6*P* synthase. It has been reported that *E. coli* mutants lacking Tre6*P* synthase activity are able to accumulate trehalose when transformed with *TPS1* (McDougall et al., 1993). Also, a model has been presented that explains the pleiotropic phenotype of *Δtps1* mutants solely on the basis of their inability to synthesize trehalose (Hohmann et al., 1993). Regardless of whether *TPS1* is indeed the Tre6*P* synthase structural gene, its inactivation is expected to have far-reaching metabolic consequences. Therefore, our finding that *Δtps1* cells are defective in the induction of normal thermotolerance levels is not easily interpreted. This is further emphasized by the fact that unconditioned *Δtps1* cells were more heat sensitive than controls although both of them contained virtually no trehalose (Fig. 1).

In a second approach, we studied thermotolerance induction in a strain disrupted in the *TPS2* gene coding for Tre6*P* phosphatase. After a conditioning heat shock and also in the stationary phase, *tps2* disruption cells showed a significantly reduced ability to withstand exposure to either 50.4°C (60–100-fold less survivors after 20 min as compared to the wild type in heat conditioned cells) or 54°C (1000-fold less survivors after 8 min as compared to the wild type in stationary phase cells). Although the defect in full acquisition of thermotolerance in the *tps2* disruption mutant is most easily explained by the failure of the mutant to accumulate adequate amounts of trehalose, a point of caution again has to be made. The data shown in the accompanying paper (Hottiger et al., 1993) show that, in contrast to trehalose, Tre6*P* is apparently not a compatible solute, i.e. its accumulation in the cytosol is expected to be inhibitory to normal metabolism. This is also emphasised by the finding that *tps2* cells, in contrast to wild-type cells, are not able to resume growth under prolonged heat shock conditions at 40°C (possibly due to P_i depletion; De Virgilio et al., 1993). Therefore, the decreased thermotolerance of the *tps2* disruption mutant might simply reflect the toxicity of Tre6*P*.

The above considerations show that it is difficult to inhibit trehalose biosynthesis with sufficient specificity. We therefore chose to manipulate trehalose levels by inhibiting trehalose degradation. A mutant in the *NTH1* gene encoding neutral trehalase (Kopp et al., 1993) was used for this purpose. Disruption of *NTH1* resulted in a slight hyperaccumulation of trehalose during mild heat shock which is consistent with our previous finding of trehalose being subject to futile cycling under heat shock conditions (Hottiger et al., 1987a). Trehalose overproduction in *Δnth1* cells was paralleled by a small increase in thermotolerance as compared to the control (Fig. 3). During recovery from heat shock, the *nth1* mutant showed the expected defect in trehalose degradation (De Virgilio et al., 1991b) and a concomitant delay in the decay of thermotolerance, strongly suggesting that trehalose is a thermoprotective agent. Interestingly, a similar phenotype as

the one described here for the *nth1* strain was previously reported for a strain mutated in three genes of the *SSA1* subfamily of 70-kDa hsp (Hottiger et al., 1992). *ssa*$^-$ cells overproduced trehalose during mild heat shock and showed an abnormally slow decay of trehalose levels and of thermotolerance upon decreasing the temperature. This defect was ascribed mainly to the inability of *ssa*$^-$ cells to normally activate neutral trehalase during recovery from heat shock (Hottiger et al., 1992). The results presented in Fig. 4 are in agreement with this interpretation and suggest that the delayed decay of thermotolerance in *ssa*$^-$ cells may be a direct consequence of their altered trehalose metabolism.

To conclude, the data presented in this study strongly support the hypothesis that trehalose accumulation in yeast serves a thermoprotective function. However, our results also very clearly demonstrate the limitations of a genetic approach to establish the function of trehalose. In the case of the experiments involving the *tps1* and *tps2* mutants, we have shown that possible problems in the interpretation of results arise due to the complexity of the mutant phenotype. Similar restrictions may, in principle, apply to the experiments involving the *nth1* mutant strain. Finally, although trehalose synthesis is shown to be an important element of thermotolerance, our results suggest that trehalose accumulation is not the only mechanism by which yeast cells acquire thermotolerance and indeed a large number of alternatives have been suggested (Hottiger et al., 1993).

We thank Peter Schoppink, Paul Klaassen and Klaas Osinga (Gist-Brocades) for providing us the *Δnth1* mutant. This study was supported by the Swiss National Science Foundation

REFERENCES

Ang, D., Liberek, K., Skowyra, D., Zylic, M. & Georgopoulos, C. (1991) Biological role and regulation of the universally conserved heat shock proteins, *J. Biol. Chem. 266*, 24233–24236.

Beckman, R. F., Mizzen, L. A. & Welch, W. J. (1990) Interaction of Hsp70 with newly synthesized proteins: Implications for protein folding and assembly, *Science 248*, 850–854.

Bell, W., Klaassen, P., Ohnacker, M., Boller, T., Herweijer, M., Schoppink, P., van der Zee, P. & Wiemken, A. (1992) Characterization of the 56-kDa subunit of yeast trehalose-6-phosphate synthase and cloning of its gene reveal its identity with the product of *CIF1*, a regulator of carbon catabolite inactivation, *Eur. J. Biochem. 209*, 951–959.

Cannon, J. F., Morcos, P. A., Clemens, K. E. & Khalil, M. (1992) Functions of gene products that regulate glycogen metabolism in yeast, *Yeast 8*, S359.

Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. & Horwich, A. L. (1989) Mitochondrial heat shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria, *Nature 337*, 620–625.

Craig, E. A. & Gross, C. A. (1991) Is hsp70 the cellular thermometer? *Trends Biochem. Sci. 16*, 135–139.

De Virgilio, C., Piper, P., Boller, T. & Wiemken, A. (1991a) Acquisition of thermotolerance in *Saccharomyces cerevisiae* without heat shock protein hsp104 and in the absence of protein synthesis, *FEBS Lett. 288*, 86–90.

De Virgilio, C., Bürckert, N., Boller, T. & Wiemken, A. (1991b) A method to study the rapid phosphorylation-related modulation of neutral trehalase activity by temperature shifts in yeast, *FEBS Lett. 291*, 355–358.

De Virgilio, C., Simmen, U., Hottiger, T., Boller, T. & Wiemken, A. (1990) Heat shock induces enzymes of trehalose metabolism, trehalose accumulation and thermotolerance in *Schizosaccharomyces pombe*, even in the presence of cycloheximide, *FEBS Lett. 273*, 107–110.

De Virgilio, C., Bürckert, N., Bell, W., Boller, T. & Wiemken, A. (1993) Disruption of *TPS2*, the gene encoding the 100 kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae*, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity, *Eur. J. Biochem. 212*, 315–323.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. & Shekman, R. (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides, *Nature 332*, 800–805.

Ellis, J. (1987) Proteins as molecular chaperones, *Nature 328*, 378–379.

Finkelstein, D. B. & Strausberg, S. (1983) Identification and expression of a cloned yeast heat shock gene, *J. Biol. Chem. 258*, 1908–1913.

Gething, M. J. & Sambrook, J. (1992) Protein folding in the cell, *Nature 355*, 33–45.

Hall, B. G. (1983) Yeast thermotolerance does not require protein synthesis, *J. Bacteriol. 156*, 1363–1365.

Hengge-Aronis, R., Klein, W., Lange, R., Rimmele, M. & Boos, W. (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by *RpoS* and are involved in stationary phase thermotolerance in *Escherichia coli*, *J. Bacteriol. 173*, 7918–7924.

Hohmann, S., Neves, M. J., de Konig, W., Alijo, R., Ramos, J. & Thevelein, J. M. (1993) The growth and signalling defects of the *ggs1* (*fdp1/byp1*) deletion mutant on glucose are suppressed by a deletion of the gene encoding hexokinase PII, *Curr. Genet. 23*, 281–289.

Hottiger, T., Schmutz, P. & Wiemken, A. (1987a) Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*, *J. Bacteriol. 169*, 5518–5522.

Hottiger, T., Boller, T. & Wiemken, A. (1987b) Rapid changes of heat and desiccation tolerance correlated with changes of trehalose content in *Saccharomyces cerevisiae*, *FEBS Lett. 220*, 113–115.

Hottiger, T., Boller, T. & Wiemken, A. (1989) Correlation between trehalose content and heat resistance in yeast mutants altered in the Ras/adenylate cyclase pathway. Is trehalose a thermoprotectant? *FEBS Lett. 255*, 431–434.

Hottiger, T., De Virgilio, C., Bell, W., Boller, T. & Wiemken, A. (1992) The 70-kilodalton heat shock proteins of the SSA subfamily negatively modulate heat shock-induced accumulation of trehalose and promote recovery from heat stress in the yeast, *Saccharomyces cerevisiae*, *Eur. J. Biochem. 210*, 125–132.

Hottiger, T., De Virgilio, C., Hall, M. N., Boller, T. & Wiemken, A. (1993) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*, *Eur. J. Biochem. 219*, 187–193.

Kang, P.-J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. & Pfanner, N. (1990) Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins, *Nature 348*, 137–143.

Kopp, M., Müller, H. & Holzer, H. (1993) Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*, *J. Biol. Chem. 268*, 4766–4774.

Laszlo, A. (1992) The thermoresistant state: Protection from initial damage or better repair? *Exp. Cell Res. 202*, 519–531.

Lee, Y. Y. & Dewey N. C. (1988) Thermotolerance induced by heat, sodium arsenite or puromycin: Its inhibition and differences between 43°C and 45°C, *J. Cell. Physiol. 136*, 991.

Lindquist, S. L. & Craig, E. A. (1988) The heat shock proteins, *Annu. Rev. Genet. 22*, 631–677.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin reagent, *J. Biol. Chem. 193*, 265–275.

McDougall, J., Kaasen, I. & Strøm, A. R. (1993) A yeast gene for trehalose-6-phosphate synthase and its complementation of an *Escherichia coli otsA* mutant, *FEMS Microbiol. Lett. 107*, 25–30.

Miozzari, G. F., Niederberger, P. & Hütter, R. (1978) Permeabilization of microorganisms by Triton X-100, *Anal. Biochem. 90*, 220–233.

Petko, L. & Lindquist, S. L. (1986) Hsp26 is not required for growth at high temperatures, nor for thermotolerance, spore development, or germination, *Cell 45*, 885–894.

Praekelt, U. M. & Meacock, P. A. (1990) Hsp12, a new small heat shock gene of *Saccharomyces cerevisiae*: Analysis of structure, regulation and function. *Mol. & Gen. Genet. 223*, 97–106.

Rothstein, R. J. (1983) One step gene disruption in yeast, *Methods Enzymol. 101*, 202–211.

Sambrook, J., Fritsch, E. F. & Maniatis, F. (1989) *Molecular cloning: a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanchez, Y. & Lindquist, S. L. (1990) Hsp104 required for induced thermotolerance, *Science 248*, 1112–1115.

Schlesinger, M. J. (1990) Heat-shock proteins, *J. Biol. Chem. 265*, 12111–12114.

Smith, B. J. & Yaffe, M. P. (1991a) Uncoupling thermotolerance from the induction of heat shock proteins, *Proc. Natl Acad. Sci. USA 88*, 11091–11094.

Smith, B. J. & Yaffe, M. P. (1991b) A mutation in the yeast heat shock factor gene causes temperature-sensitive defects in both mitochondrial protein import and the cell cycle, *Mol. Cell. Biol. 11*, 2647–2655.

Sorger, P. K. (1991) Heat-shock factor and the heat-schock response, *Cell 65*, 363–366.

Stucka, R. & Blázquez, M. A. (1993) The *fdp1* and *cif1* mutations are caused by different single nucleotide changes in the yeast *CIF1* gene, *FEMS Microbiol. Lett. 107*, 251–254.

Susek, R. E. & Lindquist, S. L. (1989) Hsp26 of *Saccharomyces cerevisiae* is related to the superfamily of small heat shock proteins but is without a demonstrable function, *Mol. Cell. Biol. 9*, 5265–5271.

Thevelein, J. M. (1992) The RAS-adenylate cyclase pathway and cell cycle control in *Saccharomyces cerevisiae*, *Antonie Leeuwenhoek 62*, 109–130.

Van Aelst, L., Hohmann, S., Bulaya, B., de Koning, W., Sierkstra, L., Neves, M. J., Luyten, K., Alijo, R., Ramos, J., Coccetti, P., Martegani, E., de Magelhães-Rocha, N. M., Brandão, R. L., Van Dijck, P., Vanhalewyn, M., Durnez, P., Jans, A. W. H. & Thevelein, J. M. (1993) Molecular cloning of a gene involved in glucose sensing in the yeast *Saccharomyces cerevisiae*, *Mol. Microbiol. 8*, 927–943.

VanBogelen, R. A., Acton, M. A. & Neidhardt, F. C. (1987) Induction of the heat shock regulon does not produce thermotolerance in *Escherichia coli*, *Genes & Dev. 1*, 525–531.

Vuorio, O., Londesborough, J. & Kalkkinen, N. (1992) Trehalose synthase: Purification of the intact enzyme and cloning of the structural genes, *Yeast 8*, S626.

Vourio, O., Kalkkinen, N. & Londesborough, J. (1993) Cloning of two related genes encoding the 56 kDa and 123 kDa subunits of trehalose synthase from the yeast *Saccharomyces cerevisiae*, *Eur. J. Biochem. 216*, 849–861.

Watson, K., Dunlop, G. & Cavicchioli, R. (1984) Mitochondrial and cytoplasmic protein syntheses are not required for heat shock acquisition of ethanol and thermotolerance in yeast, *FEBS Lett. 172*, 299–302.

Wiedelitz, R. B., Magun, B. E. & Germer, E. W. (1986) Effects of cycloheximide on thermotolerance expression, heat shock protein synthesis and heat shock protein mRNA accumulation in rat fibroblasts, *Mol. Cell. Biol. 6*, 1088–1094.

Wiemken, A. (1990) Trehalose in yeast, stress protectant rather than reserve carbohydrate, *Antonie Leeuwenhoek 58*, 209–217.

Young, R. A. & Davis, R. W. (1983) Efficient isolation of genes by using antibody probes, *Proc. Natl Acad. Sci. USA 80*, 1194–1198.