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Acquisition of thermotolerance in *Saccharomyces cerevisiae* without heat shock protein hsp104 and in the absence of protein synthesis

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Acquisiton of thermotolerance in response to a preconditioning heat treatment at 40°C was studied in mutants of the yeast *Saccharomyces cerevisiae* lacking a specific heat shock protein or the ability to synthesize proteins at 40°C. A mutant carrying a deletion of heat shock protein hsp104 and the corresponding wildtype strain were both highly sensitive to heat stress at 50.4°C without preconditioning but both acquired almost the same level of thermotolerance after 60 min of preconditioning. Both strains showed equal induction of trehalose-6-phosphate synthase and accumulated equal levels of trehalose during the treatment. The conditional mutant ts – 187 synthesized no proteins during the preconditioning heat treatment but nevertheless acquired thermotolerance, albeit to a lesser degree than the corresponding wildtype strain. Induction of trehalose-6-phosphate synthase and accumulation of trehalose were reduced to a similar extent. These results show that acquisition of thermotolerance and accumulation of trehalose are closely correlated during heat preconditioning and are modulated by protein synthesis but do not require it.

Trehalose; Heat shock protein; Thermotolerance; *Saccharomyces cerevisiae*

1. INTRODUCTION

Upon exposure to a mild heat treatment, a variety of organisms acquire thermotolerance, i.e. the ability to survive a subsequent severe heat stress that would be lethal in the absence of the preconditioning heat treatment [1]. During the preconditioning heat shock, a small set of polypeptides known as heat shock proteins (hsp) is highly induced [1–3]. Many hsps are also formed constitutively and are essential at normal growth temperatures [3,4]. Some of these are known to act as molecular chaperones in the assembly and proper folding of proteins [5–7]. However, the function of most other hsps is unknown.

In yeast in particular, despite the common assumption that hsps play a role for acquired thermotolerance, a number of reports suggest that this might not be the case [8–13]. However, it has recently been shown that in *Saccharomyces cerevisiae*, the induction of heat shock protein hsp104, which is not expressed during growth at normal temperature, is required for acquired thermotolerance to prolonged (>5 min) heat treatments [14]. Subsequently, hsp104 has been found to facilitate recovery of RNA splicing once this process has been disrupted following a heat shock [15]. However, at least with respect to this function, hsp104 appears not to be an element of acquired thermotolerance: cells became thermotolerant with respect to RNA splicing even when protein synthesis was blocked during the preconditioning heat treatment [15]. Thus, hsps like hsp104 [15] or the molecular chaperones [3–7] may be involved in the **repair** of damaged structures following heat stress.

Another factor potentially important for acquired thermotolerance in yeast is the non-reducing disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside). Several studies have shown a close correlation between accumulation of trehalose and tolerance to short-term heat stress [13,16–20]. Trehalose is also present in particularly large quantities in the most stress-tolerant cells of fungi and other microorganisms such as spores and stationary phase cells [19]. There is a vast literature showing that trehalose is an outstanding protecting agent for membranes and proteins (for review see [20]). This suggests that trehalose may be involved in **protection** from heat-induced damage to membranes and proteins.

The present work has been undertaken in order to further clarify the role of heat-induced synthesis of hsps and accumulation of trehalose for the acquisition of thermotolerance. Our studies demonstrate that short-term thermotolerance is acquired in the absence of hsp104 and even in the absence of general protein synthesis during the preconditioning heat treatment. They also show a close correlation between trehalose accumulation and induction of thermotolerance under all conditions, supporting further the hypothesis that trehalose acts as a thermoprotectant.

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2. MATERIALS AND METHODS

2.1. *Yeast strains and culture conditions*

The following strains of *S. cerevisiae* were used: A364A (a, *lys2, tyr1, his7, gal1, ade1, trp1, ura1*), and ts⁻187 derived from A364A as reported in [21,22], both provided by the Yeast Genetic Stock Center, Berkeley, CA; W303 (a *ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*), and Δhsp104 derived from W303 as reported in [14], both kindly provided by Dr S. Lindquist, University of Chicago.

Stock cultures were kept on YPDA (1% yeast extract, 2% bactopeptone, 2% glucose, 2% agar). Cells were grown to stationary phase in liquid YM-1 medium [21] on a rotary shaker (140 rpm) at 27°C, transferred into fresh medium and allowed to grow under the same conditions for at least 6 generations, taking care that the cell densities were below 3×10^6 cells/ml at the beginning of the heat shock experiments. For isotopic labeling studies, cells were transferred into YM-5 medium [21,23].

2.2. *Heat shock conditions and analysis of thermotolerance*

The preconditioning heat shock (40°C) was performed as previously reported [13]. For determination of thermotolerance, cultures (1 ml) were heated to 50.4°C for different times, rapidly cooled on ice, appropriately diluted with sterile water and plated on YPDA as described [8].

2.3. *Analytical procedures*

Trehalose was extracted by trichloroacetic acid and determined by the anthrone procedure as described [16]. Its identity was verified in all types of experiments by thin layer chromatography [16]. In no case were carbohydrates other than trehalose present in significant amounts in the extracts. Enzyme extraction was performed as in [13]. Trehalose-6-phosphate synthase (T6PS) was measured at 50°C (its temperature optimum) using a coupled enzyme assay [16]. Protein synthesis was measured by the incorporation of [^{3}H]isoleucine (NEN-Du Pont, Boston, MA) as follows. Exponentially growing cells were centrifuged for 5 min at $3000 \times g$ and resuspended in YM-5 medium at a final concentration of 1×10^8 cells/ml. Cultures (1ml) were labeled by the addition of 1-10 μCi of [^{3}H]isoleucine. Radiolabeled proteins were extracted with 0.25 M NaOH and subsequent precipitation with 12% (w/v) trichloroacetic acid; the pellets were washed 3 times with ice cold acetone, resuspended in 0.1 mM NaOH, and mixed with an equal amount of twice concentrated sample buffer [24]. Radiolabeled proteins were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis [24]; the gels were then stained, destained, incubated with enhancer (ENLIGHTNING, NEN-Du Pont) according to the manufacturer's description and dried prior to autoradiography at -70°C, using Kodak X-OMAT AR film.

3. RESULTS

3.1. *Heat-induced acquisition of tolerance to a severe heat shock and accumulation of trehalose in the presence and absence of hsp104*

The presence of hsp104 has been reported to be crucial for the induction of thermotolerance [14]. We used the deletion mutant Δhsp104 and the corresponding wildtype strain to compare acquisition of thermotolerance and accumulation of trehalose in response to a preconditioning heat treatment at 40°C for 30 or 60 min (Fig. 1). Without preconditioning, cells of both strains were unable to survive 4 min at 50.4°C (Fig. 1A). In accordance with previously published results [14], a preconditioning heat treatment at 40°C for 30 min induced thermotolerance to a different degree in the wildtype and in the mutant Δhsp104 (Fig. 1B). However, the difference became apparent only after a long exposure (>8 min) to 50.4°C; both strains acquired complete tolerance to 4–8 min at 50.4°C (Fig. 1B). When the preconditioning heat treatment at 40°C was performed for 60 min, both strains became even more tolerant to long exposure (15–20 min) to 50.4°C (Fig. 1C). When similarly preconditioned cells of the 2 strains were exposed to 50.8 and 52.2°C for 8 min, both displayed the same thermotolerance, >95% surviving 50.8°C and >90% being killed at 52.2°C (data not shown).

Both wildtype and Δhsp104 mutant cells accumulated trehalose with the same kinetics during the preconditioning heat treatment at 40°C (Fig. 1D). As observed previously in wildtype cells [17], the activity of T6PS increased about five-fold in both strains during 60 min of the heat treatment at 40°C (Fig. 1E).

3.2. *Acquisition of thermotolerance and accumulation of trehalose by a preconditioning heat treatment in the presence and absence of protein synthesis*

To investigate the role of protein synthesis in the acquisition of thermotolerance during a preconditioning heat treatment, we made use of a temperature-sensitive mutant, ts⁻187, deficient in protein synthesis at elevated temperatures [22,23]. Strain ts⁻187 showed complete arrest of protein synthesis within 1 min of the heat treatment at 40°C, as measured by the incorporation of [^{3}H]isoleucine, whilst its wildtype parent incorporated [^{3}H]isoleucine with linear kinetics during the heat shock (Fig. 2A). Both ts⁻187 and its wildtype parent showed the same pattern of labeled proteins when grown at 27°C (Fig. 2B, lanes 1 and 2). While the wildtype strain showed the typical pattern of hsps after 30 min or 60 min at 40°C (Fig. 2B, lanes 3 and 5), no radiolabeled proteins could be detected in ts⁻187 under the same conditions (Fig. 2B, lanes 4 and 6). These results show that ts⁻187 is unable to synthesize heat shock proteins or any new proteins during the preconditioning heat treatment and consequently also during the subsequent challenging heat treatment.

Cells of both ts⁻187 and isogenic wildtype were unable to survive 4 min at 50.4°C (Fig. 3A). A preconditioning heat treatment at 40°C for 20 min or 60 min induced thermotolerance to a larger extent in wildtype than in mutant ts⁻187, indicating that functional protein synthesis contributes to the development of thermotolerance (Fig. 3B,C). However, the degree of thermoprotection increased further between 20 and 60 min of the preconditioning heat treatment not only in the wildtype but also in the mutant, and both finally survived 4 min at 50.4°C almost completely (Fig. 3C).

Remarkably, compared with its wildtype parent, mutant ts⁻187 had a considerably lower capacity to accumulate trehalose during the preconditioning heat treatment at 40°C (Fig. 3D). The reduction in trehalose accumulation coincided with a reduction in the increase of T6PS activity by about 60% during the precondition-

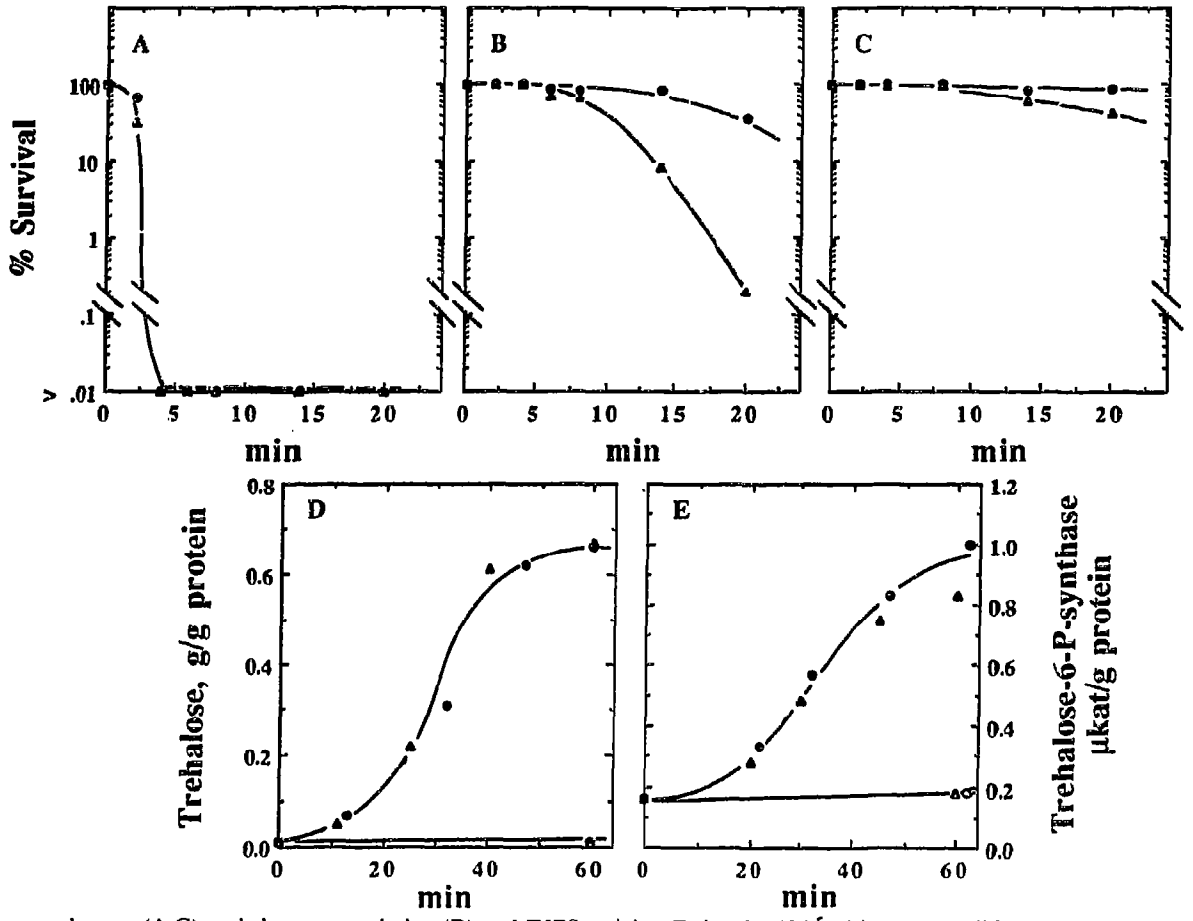

Fig. 1. Thermotolerance (A-C), trehalose accumulation (D) and T6PS activity (E) in Δhsp104 and its parent wildtype, W303. Log-phase cells ($<3\times10^6$ cells/ml) of W303 (●,○) and Δhsp104 (▲,△) were subjected to a preconditioning heat treatment at 40°C for various times. Thermotolerance of cells without preconditioning (A) or after 30 min (B) or 60 min (C) preconditioning was measured as the survival following incubation at 50.4°C for the times indicated. Trehalose accumulation (D) and T6PS activity (E) during the preconditioning heat treatment at 40°C. Open symbols in (D) and (E) designate controls incubated at 27°C.

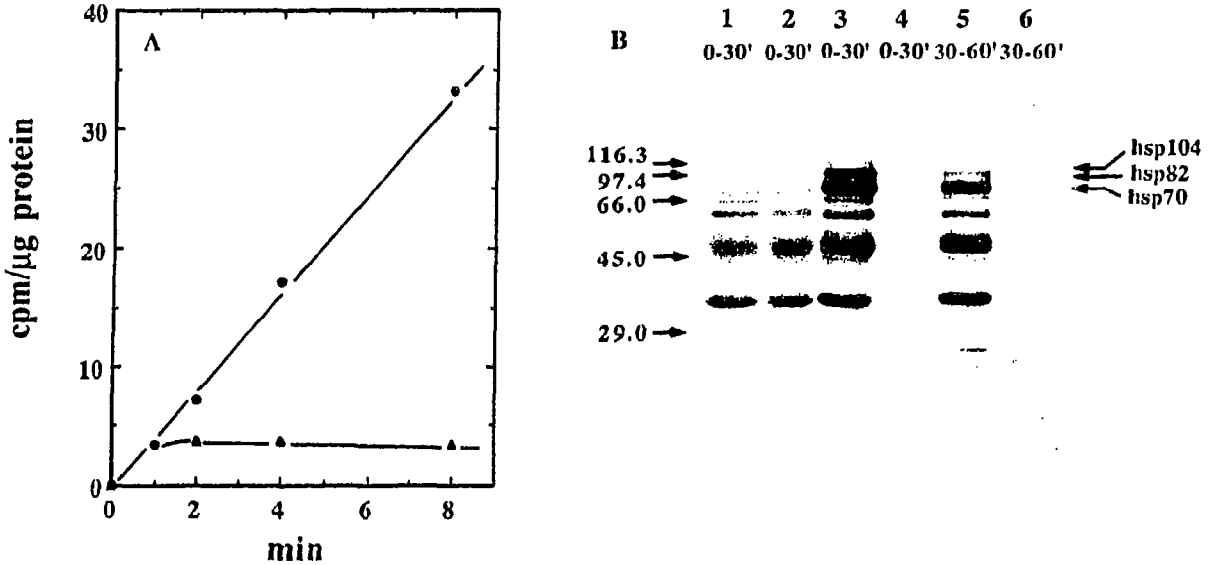

Fig. 2. Protein synthesis in mutant ts$^-$187 and its wildtype parent, A364A, during the preconditioning heat treatment at 40°C. A: [^{3}H]isoleucine incorporation into TCA-precipitable material in A364A (●) and ts$^-$187 (▲). Cells were brought from 27°C to 40°C at time zero and simultaneously received [^{3}H]isoleucine. B: analysis of the radiolabeled proteins of A364A (lanes 1,3,5) and ts$^-$187 (lanes 2,4,6) after incubation at 27°C (lanes 1 and 2) or 40°C (lanes 3-6) for the times indicated. The position of molecular weight standards are indicated on the left (in kDa), and those of the hsps on the right.

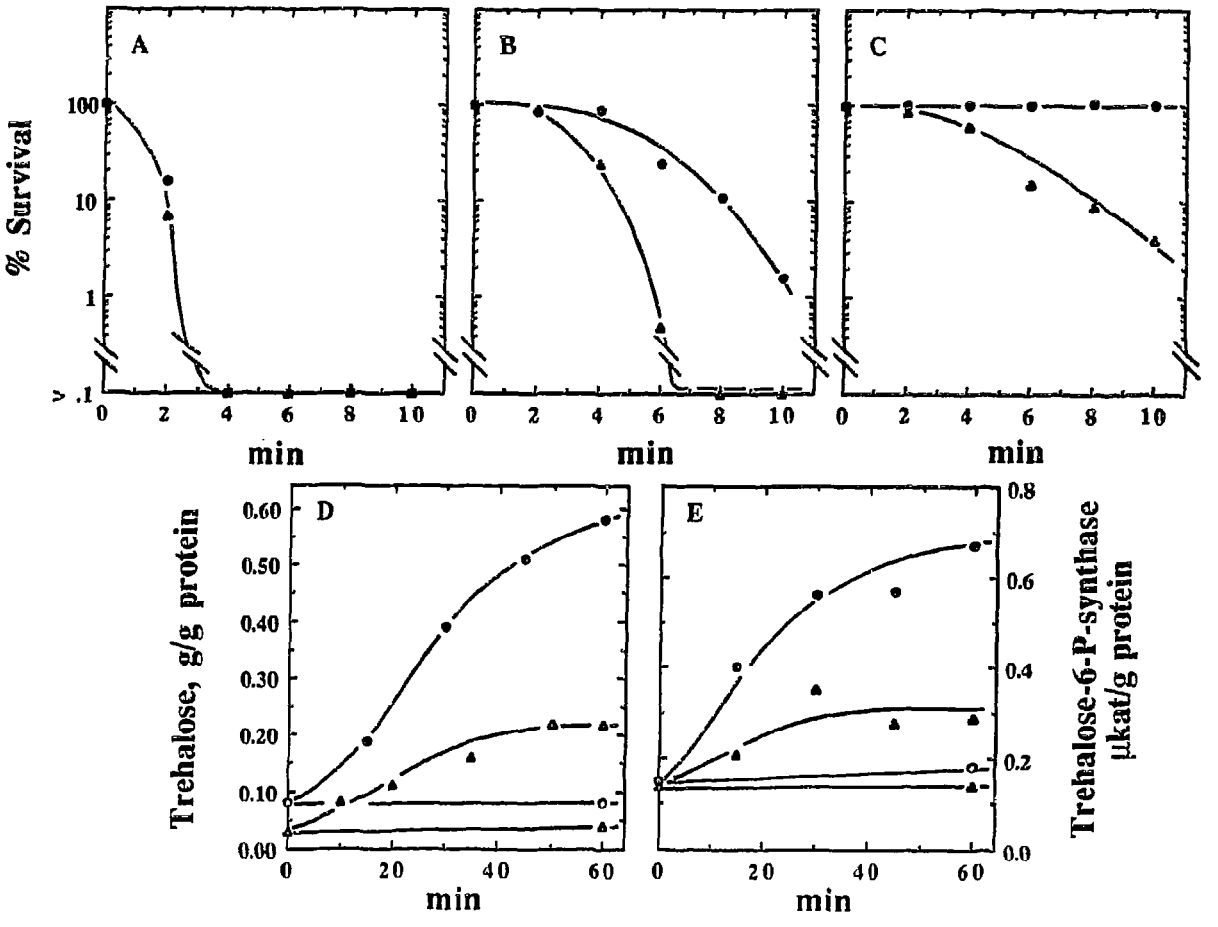

Fig. 3. Thermotolerance (A–C), trehalose accumulation (D) and T6PS activity (E) in ts⁻187 and its parent wildtype, A364A. Log-phase cells (< 3×106 cells/ml) of A364A (●,○) and ts⁻187 (▲,△) were subjected to a preconditioning heat treatment at 40°C for various times. Thermotolerance of cells without preconditioning (A) or after 20 min (B) or 60 min (C). Preconditioning heat treatment at 40°C (C) was measured as the survival following incubation at 50.4°C for the times indicated. Trehalose accumulation (D) and T6PS activity (E) during the preconditioning heat treatment at 40°C. Open symbols in (D) and (E) designate controls incubated at 27°C.

ing heat treatment (Fig. 3E). The fact that the activity increased about two-fold in the absence of protein synthesis suggests a contribution of posttranslational mechanisms in T6PS regulation.

4. DISCUSSION

In accordance with the published results [14], we found a clear impairment in thermotolerance for Δhsp104 in comparison to its wildtype parent. Interestingly, cells of Δhsp104 had also a considerably reduced tolerance to severe heat stress in the absence of a preconditioning heat treatment (data not shown). However, the reduction of acquired thermotolerance to 50.4°C caused by this deletion was evident only when the cells were challenged by a long-term heat shock (> 5 min). Moreover, the reduction observed at this challenging temperature almost disappeared when the time of the preconditioning heat treatment was prolonged from 30 min to 60 min. These results imply that the importance of hsp104 for thermotolerance diminishes upon prolongation of the preconditioning heat treatment and that another factor becomes important. The fact that Δhsp104 accumulates trehalose and induces T6PS like the wildtype during the preconditioning heat treatment is compatible with the idea that one such factor might be trehalose.

A number of previous studies have used inhibitors like cycloheximide to support the notion that protein synthesis is not necessary for acquisition of thermotolerance [8,9,12,13,25]. Considering the drawbacks inherent in inhibitor studies, we chose to investigate a well-characterized temperature-sensitive mutant failing to initiate new polypeptide chains at the restrictive temperature [22]. We verified that ts⁻187 immediately stopped protein synthesis upon the shift to 40°C. Measurement of thermotolerance revealed that the preconditioning heat treatment considerably induced acquired thermotolerance in ts⁻187, particularly to short-term heat stress, even in the complete absence of protein synthesis. However, the degree of tolerance was

lower in the mutant than in the wildtype parent, suggesting that protein synthesis is necessary for full induction of thermotolerance. It should be noted, however, that this reduction is not necessarily due to the lack of hsps. Instead, our data show that the reduced thermotolerance in ts⁻187 is again closely correlated with a reduction in the accumulation of trehalose. Remarkably, on the basis of trehalose accumulation, the mutant reached almost exactly the same degree of thermotolerance as the wildtype parent strain: the mutant accumulated the same amount of trehalose and acquired the same degree of thermotolerance after 60 min of the preconditioning heat treatment (Fig. 3C) as did the wildtype after 20 min (Fig. 3B). These results lend further correlative support to the suggestion that trehalose acts as a thermoprotectant.

On the basis of our results, we suggest that acquired thermotolerance is due to 2 different, independent mechanisms, namely to induced **protection** from and to induced **repair** of heat stress damage. The hsps appear to be required for repair of heat-induced damage [4,5]. These repair functions are important primarily for recovery **after** severe heat stress during which damages to macromolecules have accumulated, with lethal consequences in the absence of repair. On the other hand, hsps-independent mechanisms like the accumulation of trehalose may be important for protection **during** the severe heat stress, preventing the occurrence of damages [13,16-20].

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