Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH

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Incubation of *Saccharomyces cerevisiae* at sub-lethal temperatures results in an increase in thermotolerance. This process is dependent not only on the sub-lethal temperature but also on the duration of sub-lethal heating. This indicates that the mechanism inducing thermotolerance is a time/temperature dose response. Other factors that induce thermotolerance include exposure to ethanol, sorbic acid and low external pH values. These factors induce thermotolerance after incubation in the presence of protein synthesis inhibitors, and they are all known to affect the intracellular pH (pHi). The acquisition of increased thermotolerance is minimal with sub-lethal heating under neutral external pH conditions. However, when the external pH is reduced to 4.0 the level of induced thermotolerance increases to a maximum value. Using a specific ATPase inhibitor, diethylstilboestrol (DES), ATPase activity was shown to be essential for the cell to survive heat stress. In addition, measurement of acid efflux, or ATPase activity, revealed that proton pumping from the cell increased by approximately 50% at sub-lethal temperatures that induce thermotolerance. This work has clearly implicated pHi perturbation as the triggering mechanism conferring thermotolerance on *S. cerevisiae*.

**Introduction**

Mild heat stress, usually above the normal temperature range for growth, has been shown to increase the thermotolerance of a wide range of micro-organisms when subjected to a subsequent heat challenge (McAlister & Finkelstein, 1980; Plesofsky-Vig & Brambl, 1985; Mackey & Derrick, 1986). This stress response, more commonly known as the heat-shock response, may have implications for microbiological safety during food processing, particularly because the phenomenon is ubiquitous and almost certainly occurs in both food-spoilage and pathogenic organisms (Mackey & Derrick, 1987; Yamamori & Yura, 1982; Lindquist, 1986; Farber & Brown, 1990). In addition to the acquisition of thermotolerance, the stress response has been largely characterized by the expression and synthesis of a unique set of proteins: the heat-shock proteins (HSPs). However, the precise role of these proteins in the acquisition of thermotolerance is still unclear. Some have been implicated in cellular recovery following heat stress (Lindquist & Craig, 1988) and others appear to be directly involved in the thermotolerance mechanism (Borkovich et al., 1989; Finley et al., 1987). Perhaps the most convincing evidence for a specific protein involvement in thermotolerance comes from the HSP70 gene mutants in yeast (Sanchez & Lindquist, 1990). In cells with this deletion, full expression of thermotolerance is prevented but substantial protection is observed for the first 5 min of heat stress. Other evidence suggests that the synthesis of new proteins is not required for the acquisition of thermotolerance (Petko & Lindquist, 1986; Finkelstein & Strausberg, 1983; Hall, 1983; Watson et al., 1984; Barnes et al., 1990). These contradictions may be resolved if both protein-synthesis-dependent and independent mechanisms are involved in thermotolerance.

In addition to sub-lethal heating, many other stress factors are known to induce thermotolerance and the synthesis of HSPs for example, ethanol (Plesset et al., 1982), metal ions (Levinson et al., 1980), cellular trauma (White & Currie, 1982) and respiratory chain uncouplers (Ashburner & Bonner, 1979). Many of these factors are also known to cause perturbations in the intracellular pH (pHi) of the cell (Weitzen et al., 1987; Eras & Gancedo, 1987; Leao & van Uden, 1984). Changes in pHi may regulate important cellular functions (Busa & Nuccitelli, 1984). For example, pHi is critical to the activity of many enzymes with specific pH optima, including the mem-

**Abbreviations:** DES, diethylstilboestrol; HSP, heat-shock protein; MEB, malt extract broth; pHi, intracellular pH.
brane ATPase (Willsky, 1979) which is responsible for maintaining intracellular pH homeostasis in Saccharomyces cerevisiae (Serrano, 1980; Goffeau & Slayman, 1981). Recently therefore, it has been proposed that a decrease in pH correlates with the induction of HSPs (Weitzel et al., 1987). Hence, depression of pH is implicated as a trigger for stress-induced thermotolerance.

In this study, the effects of sub-lethal heat and other stress factors on the ability of S. cerevisiae to survive a subsequent 'lethal' temperature have been characterized and the necessity for protein synthesis examined.

Methods

Organism and growth conditions. Saccharomyces cerevisiae (strain CMC3236) was grown aerobically to late exponential phase at 25 °C in malt extract broth (MEB, Oxoid). Viability after various heat treatments was determined by resuscitation for 30 min followed by serial dilution of suspensions in MEB and plating on plate count agar (Oxoid).

Effect of pre-incubation time and temperature on thermotolerance. A cell suspension was prepared from an exponential culture grown in MEB which had been centrifuged, washed, resuspended in fresh broth at a concentration of approximately 10⁸ cells ml⁻¹ and allowed to resuscitate at room temperature for 30 min. Aliquots (0.5 ml) of suspension were heated in a modified thermocouple block calibrator (DB-40L) with a TP-16 time/temperature programmer (Techna) as previously described (Coote et al., 1991) at a range of sub-lethal temperatures between 34 and 45 °C for periods of 20–60 min. Sub-lethally heated cells were then immediately exposed to a lethal temperature of 52 °C for 10 min in the same apparatus. In control experiments, cells were heated as above at 52 °C for 10 min without pre-treatment. After heating, all samples were instantly cooled to 20 °C and viable counts were determined.

Induction of thermotolerance by exposure to other reagents. Thermotolerance after exposure to ethanol and sorbic acid was determined by resuspending the washed cell suspension (1 ml) in MEB containing either 6% (v/v) ethanol or 9 mM-sorbic acid. The pH of the broth was adjusted to either 6.0 or 4.5. Cells were resuspended in each reagent for 1, 2, 10, 20 or 60 min before being centrifuged and resuspended in fresh MEB (pH 6.0 or 4.5). The samples were then heated using a temperature programmer at 52 °C for 10 min and cell viability was determined.

Protein synthesis inhibition. Cultures grown in MEB were centrifuged and resuspended in fresh broth with or without cycloheximide (100 μg ml⁻¹, Sigma) and chloramphenicol (4 mg ml⁻¹, Sigma). Following incubation at 25 °C with shaking for 30 min, cells were centrifuged and resuspended in the original volume of fresh broth. Samples (1 ml) of each were centrifuged (MSE Microcentaur) at 13,000 r.p.m. for 30 s and subjected to the following pre-treatments prior to a lethal heat stress. Pre-treatment consisted of either resuspension in fresh broth plus sub-lethal heating (40 °C, 60 min), or resuspension in fresh broth plus sorbic acid (9 mM, 20 °C, 60 min) or resuspension in fresh broth plus ethanol (6%, 20 °C, 60 min). All samples were then centrifuged, resuspended in fresh broth and heated at 52 °C for 10 min as described above. The control culture was given no pre-treatment prior to heat stress at 52 °C.

Effect of pH on thermotolerance. Separate overnight cultures were centrifuged and resuspended in 90 ml of unbuffered MEB adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 or 8.0 with HCl/NaOH. After incubation at 25 °C with shaking for 1 h, samples (1 ml) were removed and separately heated in a time/temperature programmer for 25, 30, 35, 40, 50 or 60 min at 40 °C, then at 52 °C for 10 min in the same apparatus. Control cultures at each pH were heated at 40 or 52 °C only.

Determination of acid efflux rate. Overnight cultures were resuspended in sterile distilled water (1.5 to 3 mg dry wt ml⁻¹) and 15 ml samples placed in a magnetically stirred water-jacketed vessel at 25 °C. A Corning ion analyser 255 pH meter connected to a BBC Goerz Metrawatt SE 120 chart recorder (ABB Goerz) was used to monitor the suspension pH, which was adjusted to 4.5 with HCl. The system was calibrated using 50 μl aliquots of HCl and NaOH (0.01 M). Glucose (0.1%) was added to initiate proton pumping. Acid efflux was monitored for 5 min before the system was recalibrated and adjusted to pH 4.5. The rate of acid efflux was determined as described by Cole & Keenan (1987) and expressed in molar equivalents of HCl. To measure the rate of acid efflux after thermal stress, separate cell suspensions were incubated in a water bath at temperatures between 5 and 49 °C for 30 min. Prior to acid efflux measurements, the temperatures of stressed samples were allowed to equilibrate to 25 °C in the water-jacketed vessel.

The acid efflux rate was also determined in the presence of the membrane ATPase inhibitor diethylstilboestrol (DES, Sigma). Immediately after the addition of glucose, DES (2.7 mg ml⁻¹ in methanol) was added at a concentration of 5–100 μg ml⁻¹. The thermal inactivation of cells, when heated at 53 °C following exposure to DES and resuspension in fresh broth, was determined as described above.

Results

Induction of thermotolerance by exposure to sub-lethal heating

When S. cerevisiae was incubated at a series of sub-lethal temperatures (37, 40, 43, and 45 °C) for time periods varying from 0 to 60 min, an increased level of thermotolerance at 52 °C was observed (Fig. 1). A pre-incubation over 60 min at 34 °C resulted in no significant increase in thermotolerance. However, as the temperature of sub-lethal heating or the holding time at a sub-lethal temperature increased, there was a comparable increase in the levels of thermotolerance induced. This occurred at all sub-lethal temperatures above 34 °C with up to 40 min incubation, but after longer incubation times, thermotolerance started to decline. The maximum thermotolerance occurred after a 40 min incubation at 45 °C and represented a 300-fold increase in survivors compared to a non-sub-lethally heated control.

Induction of thermotolerance by exposure to other reagents

Prior exposure of S. cerevisiae to ethanol (6%) and sorbic acid (9%) resulted in the induction of increased thermotolerance of cells held at 52 °C for 10 min (Fig. 2).
This occurred when the external pH of the medium was either 6.0 or 4.5. However, with the exception of sorbic acid, thermotolerance was induced more rapidly and to a higher level at the lower pH of 4.5. Sorbic acid increased thermotolerance within 10 min of initial exposure at both pH 6.0 and 4.5 (Fig. 2a and b, respectively). The initial rate of induction by sorbic acid at both pH values was similar. However, the number of survivors after 60 min was higher at the lower pH value. In contrast to sorbic acid, the increase in thermotolerance as a result of exposure to ethanol occurred more slowly and was dependent on external pH. At pH 6.0 (Fig. 2a) thermotolerance was induced within 60 min, and at pH 4.5 (Fig. 2b) within 20 min. The effect of lowering pH alone was shown when cells were resuspended in fresh broth in the absence of sorbic acid or ethanol but at the lower pH of 4.5. Increased thermotolerance was induced within 20 min, but not to the same level as that induced by sorbic acid and ethanol. Clearly, reducing the external pH value enhances the induction of increased thermotolerance.

The role of protein synthesis

Pre-incubation for 30 min with the protein synthesis inhibitors cycloheximide (100 µg ml⁻¹) and chloramphenicol (4 mg ml⁻¹) (Watson et al., 1984) failed to prevent the induction of increased thermotolerance at 52 °C by sub-lethal heating, ethanol, or sorbic acid (Table 1).
Table 1. Effect of protein synthesis inhibition on the acquisition of thermotolerance in S. cerevisiae

Thermotolerance was measured at 52 °C for 10 min in S. cerevisiae after treatment with protein synthesis inhibitors, and compared to control cells. Thermotolerance was induced by sub-lethal heat (40 °C, 60 min), ethanol (6%, 60 min), and sorbic acid (9 mM, 60 min). Figures in parentheses indicate increase in log numbers due to exposure to sub-lethal heat, ethanol or sorbic acid.

<table>
<thead>
<tr>
<th></th>
<th>With inhibitors</th>
<th>No inhibitors</th>
</tr>
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<tbody>
<tr>
<td>Initial cell number</td>
<td>8.28</td>
<td>8.10</td>
</tr>
<tr>
<td>Cell number after lethal heating</td>
<td>5.77 (+0)</td>
<td>5.68 (+0)</td>
</tr>
<tr>
<td>6% ethanol</td>
<td>7.44 (+1.67)</td>
<td>6.57 (+0.89)</td>
</tr>
<tr>
<td>9 mM-sorbic acid</td>
<td>6.83 (+1.06)</td>
<td>6.99 (+1.31)</td>
</tr>
<tr>
<td>40 °C</td>
<td>7.57 (+1.80)</td>
<td>7.99 (+2.31)</td>
</tr>
</tbody>
</table>

* Cycloheximide (100 µg ml⁻¹) and chloramphenicol (4 mg ml⁻¹).

Effect of pH on thermotolerance

The effect of incubating S. cerevisiae for 1 h at different pH values on viability at a lethal temperature (52 °C) was determined both with and without pre-incubation at the sub-lethal temperature of 40 °C (Fig. 3). In both cases thermotolerance was expressed as the cell number above or below that surviving at pH 5.0, the starting pH of the culture medium (MEB). External pH changes alone had a marked effect on the degree of thermotolerance induced. A mild acidification to pH 4.0 increased the number of survivors approximately 100-fold. At all other pH values the number of survivors declined approximately tenfold. The effect of adding a sub-lethal incubation at 40 °C was to increase thermotolerance at all pH values. Maximum thermotolerance was again induced at the mild acid pH values of 4.0 and 5.0 (at least a 1000-fold increase in thermotolerance). Both decreasing the external pH to 3.0 and 2.0, and increasing it to 6.0 and 7.0, reduced the level of thermotolerance compared to the maximum induced at pH 4.0. However, unexpectedly, when the external pH was increased to 8.0, thermotolerance increased again. This clear effect of pH on the degree of thermotolerance induced prompted an examination of pH, and particularly the membrane ATPase which is involved in regulation of pH.

Thermotolerance at 52 °C after pre-incubation with the specific membrane ATPase inhibitor DES

Control cells exposed to 100 µg DES ml⁻¹ at 20 °C showed little loss in viability over a period of 6 h. Cells heated at 52 °C with no pre-treatment with DES started to lose viability after 8 min. However, cells pre-incubated in DES and then heated to 52 °C were dramatically less thermotolerant (Fig. 4). Exposure to 27 µg DES ml⁻¹ resulted in a sixfold log reduction in numbers after 4 min and exposure to 67 µg DES ml⁻¹ resulted in an eightfold log reduction within 1 min. These results suggest that the membrane ATPase has a key role in maintaining cell viability during heat stress.

Effect of sub-lethal heating and DES on acid efflux rate

Inhibition of glucose-stimulated acid efflux in the presence of DES was apparent with the lowest concentration tested (5 µg ml⁻¹) and increased to approximately 90% with the addition of 25 µg DES ml⁻¹, and to 100% with 50 µg DES ml⁻¹ and above (Fig. 5). When cells grown at 25 °C were subjected to a sub-lethal heat stress at 31 or 41 °C, their rate of acid efflux was increased by 40 and 46%, respectively, over the control rate measured at 25 °C (Fig. 6). However, after sub-lethal heat stress at 45 and 49 °C, acid efflux was reduced by 17 and 48%, respectively. Thermotolerance after the same tempera-
Mechanism of increased thermotolerance in yeast

Fig. 4. Effect of a 30 min pre-incubation with two concentrations of DES on the thermal inactivation of *S. cerevisiae*. Mid-exponential-phase cells were harvested and resuspended in fresh MEB with 27 μg DES ml⁻¹ (△), 67 μg DES ml⁻¹ (▽) or without DES (■). After 30 min, cells were resuspended in broth without DES and heated at 52 °C. The effect of incubation in 100 μg ml⁻¹ DES only is shown (■).

Fig. 5. Effect of the addition of DES on the percentage inhibition of the total acid efflux rate in *S. cerevisiae* following addition of glucose (10 g l⁻¹) (error bars indicate standard deviation of the mean of three experiments) measured at 25 °C.

Fig. 6. Effect of incubation temperature (30 min in sterile distilled water) on the acid efflux rate following addition of glucose (10 g l⁻¹) (error bars indicate standard deviation of the mean of 11 experiments), measured at 25 °C; and the acquisition of increased thermotolerance following addition of glucose (10 g l⁻¹) (error bars indicate standard deviation of the mean of three experiments) in *S. cerevisiae*. Log (increase in cell no.) indicates the increase in cell numbers due to sub-lethal heating when cells are subjected to a lethal temperature of 52 °C for 10 min.

Discussion

A number of studies have examined the range of pre-incubation temperatures which will afford the microbial cell an increased tolerance to a subsequent lethal temperature. The onset of the response generally occurs at temperatures close to the maximum for growth. The upper temperature inducing an increase in thermotolerance is limited as the incubation temperature approaches values that will have a lethal effect in their own right.

From this work the onset of the acquisition of increased thermotolerance in *S. cerevisiae* appears to be between 34 and 37 °C. At temperatures above 45 °C pre-incubation begins to have a lethal effect in its own right (data not shown). The degree of protection afforded to a population of cells is related not only to the sub-lethal
pre-incubation temperature, but also to its duration. Within the range of temperatures that give an increase in heat resistance, higher temperatures not only produce a greater response, with a greater number of cells surviving lethal temperatures, but also produce a quicker response than at lower pre-incubation temperatures. The fact that there is a kinetic relationship between incubation temperature and the degree of increased thermotolerance clearly indicates that the response to sub-lethal heating is not merely 'switched on' at a certain threshold temperature but that the molecular mechanism is a dose response relating to time as well as temperature of stress.

A number of stress factors have been shown to induce the acquisition of increased thermotolerance. These include those that are well known to induce thermotolerance such as mild heat (McAlister & Finkelman, 1980) and ethanol (Plisset et al., 1982), but also sorbic acid and low external pH, which has not been previously reported. Many of these factors, including mild heat, are known to reduce the pH value of yeast (Weitzel et al., 1987; Eraso & Gancedo, 1987; Cole & Keenan, 1987; Leao & van Uden, 1984). It follows therefore that a depression in pH is a strong candidate for the triggering mechanism of this response.

The kinetics with which pre-incubation with mild heat, ethanol, and sorbic acid induced increased thermotolerance in this study supports the notion that pH perturbation may play a key role as the stimulus for this response. Sorbic acid causes a rapid onset of increased thermotolerance and weak acids are also known to result in a rapid depression of pH (Cole & Keenan, 1987; Suomalainen & Oura, 1955; Ryan & Ryan, 1972; Krebs et al., 1983). The undissociated species of weak acids are uncharged and enter the yeast cell extremely rapidly (Macris, 1975). Upon entering the relatively high pH environment of the cell, the molecular species dissociates, releasing protons and hence depressing pH. Mild heat causes a less rapid reduction in pH (Weitzel et al., 1987) and we have shown that the acquisition of increased thermotolerance is also slower. Mild heat presumably reduces pH by making membranes 'leaky' and increasing proton permeability. As the external environment is more acidic than inside the cell, increased proton permeability will allow the passive influx of protons which will depress pH. Ethanol is thought to have a similar effect on membranes as mild heat, increasing membrane permeability, and thus affecting pH (Li et al., 1980; Leao & van Uden, 1984). The onset of increased thermotolerance with ethanol is slower than sorbic-acid-induced resistance but is more rapid when the external pH value is lower.

If the onset and magnitude of acquired thermotolerance is dependent upon a depression of pH, then we would expect to find the response to be minimal with mild heat under conditions where the net movement of protons into the cell will be least. The minimal effect of sub-lethal heating at a neutral external pH supports this. The pH value of S. cerevisiae, determined either by the dual radio-labelled isotope method (Conway & Downey, 1950) and the 31P-nuclear magnetic resonance (NMR) spectroscopy (Gillies et al., 1981), in MEB (pH 5.0) is between 6.5 and 7.0 (data not shown). When cells are sub-lethally heated at external pH values close to intracellular values (and hence where the net movement of protons across the membrane will be least) the acquisition of increased thermotolerance is low. As the external pH value is decreased, and the proton gradient increased, then thermotolerance is enhanced down to a pH of 4.0. At external pH values below 4.0 thermotolerance declines, presumably as low pH values begin to exert a lethal effect in their own right. Unexpectedly, the acquisition of thermotolerance at an external pH value of 8.0 was greater than that at pH 6.0-7.0, perhaps indicating that perturbation of pH in any direction may trigger the response.

The intracellular pH of S. cerevisiae is largely maintained by a membrane bound ATPase (Malpartida & Serrano, 1981; Serrano, 1980) and to a lesser extent by the secretion of organic acids which have been shown not to rapidly re-enter cells (Sigler et al., 1981). Yeast plasma membrane ATPase is regulated by a variety of factors including pH (Riemersma & Alsbach, 1974), extracellular pH, glucose, and ATP content of the cells (Serrano, 1980). Although the precise mechanism of acid-mediated activation is not known, synthesis of proteins can be excluded as cycloheximide did not prevent activation by acid pH (Eraso & Gancedo, 1987). It follows therefore that as intracellular acidification may be the trigger for an increase in thermotolerance, then the activity of this essential enzyme may be important in the response.

Using the specific ATPase inhibitor DES, (Eilam et al., 1984) ATPase activity has been demonstrated to be essential for the cell to survive heat stress. Perhaps more significantly, total acid efflux, a good measure of ATPase activity in yeast, increases when cells are incubated at some temperatures that confer thermotolerance upon the cell. A role of ATPase in the acquisition of thermotolerance is also supported by the recent work of Panareto & Piper (1990). pma1.1 mutants of S. cerevisiae and Schizosaccharomyces pombe, which show a decreased ATPase activity, also demonstrated a lower tolerance to heat following pre-incubation between 25 and 38 °C compared to the wild-type. It was suggested by these authors that the ATPase exerts an influence on the extent and duration of HSP synthesis following induction of the heat-shock response.

Whilst the precise mechanism of increased thermotolerance following exposure to sub-lethal heat, weak
acid and ethanol is not known, the necessity for the synthesis of the majority of HSPs must now be questioned. The present study, and previously reported work (Hall, 1983), has demonstrated that the increase in thermotolerance afforded by such stresses is unaffected by protein synthesis inhibitors. Two other pieces of information suggest that HSP synthesis is not required for increased thermotolerance. First, the rapidity of the response following exposure to sorbic acid is such that it is unlikely, in our belief, that sufficient protein synthesis could take place within this period to confer heat resistance. Second, pulse-labelling experiments (Piper et al., 1986) have shown that yeast cannot synthesize HSPs above 42–43°C but our work has demonstrated an increase in thermotolerance at temperatures as high as 45°C.

To conclude, the present work has clearly implicated pH, as the trigger for the acquisition of thermotolerance in S. cerevisiae. Furthermore, we postulate that one possible mechanism of increased thermotolerance may simply be due to an increased ability to maintain normal pH values. Recent measurements in our laboratory of pH₃ using ³¹P-NMR, indeed indicate that pre-incubation at sub-lethal temperatures allows the cell to maintain a higher pH value at a lethal temperature than unstressed cells. Increased thermotolerance following exposure to stress may not be directly related to the synthesis of HSPs, but also to the ability of the cell to maintain a reasonable pH value through the stimulation of the ATPase. Mild perturbation of pH may affect other metabolic functions such as cyclic AMP metabolism (Piper, 1990; Iida, 1988) which have been implicated in the heat-shock response. A unifying explanation of the mechanism of induced thermotolerance will therefore require an understanding of how these diverse metabolic factors interact.

References


