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Heat shock response in psychrophilic and psychrotrophic yeast from Antarctica

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Abstract The response to heat stress in six yeast species isolated from Antarctica was examined. The yeast were classified into two groups: one psychrophilic, with a maximum growth temperature of 20°C, and the other psychrotrophic, capable of growth at temperatures above 20°C. In addition to species-specific heat shock prote in (hsp) profiles, a heat shock (15°C–25°C for 3 h) induced the synthesis of a 110-kDa protein common to the psychrophiles, *Mrakia stokesii*, *M. frigida*, and *M. gelida*, but not evident in *Leucosporidium antarcticum*. Immunoblot analyses revealed heat shock inducible proteins (hsps) corresponding to hsps 70 and 90. Interestingly, no proteins corresponding to hsps 60 and 104 were observed in any of the psychrophilic species examined. In the psychrotrophic yeast, *Leucosporidium fellii* and *L. scottii*, in addition to the presence of hsps 70 and 90, a protein corresponding to hsp 104 was observed. In psychrotrophic yeast, as observed in psychrophilic yeast, the absence of a protein corresponding to hsp 60 was noted. Relatively high endogenous levels of trehalose which were elevated upon a heat shock were exhibited by all species. A 10 Celsius degree increase in temperature above the growth temperature (15°C) of psychrophiles and psychrotrophs was optimal for heat shock induced thermotolerance. On the other hand, in psychrotrophic yeast grown at 25°C, only a 5 Celsius degree increase in temperature was necessary for heat shock induced thermotolerance. Induced thermotolerance in all yeast species was coincident with hsp synthesis and trehalose accumulation. It was concluded that psychrophilic and psychrotrophic yeast, although exhibiting a stress response similar to mesophilic *Saccharomyces cerevisiae*, nevertheless had distinctive stress protein profiles.

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Introduction

Microorganisms inhabit diverse ecological niches including those that are extreme with respect to pH, temperature, pressure, nutrient content, and availability of water. Those extremophiles that dominate and play key roles in the ecology of both aquatic and terrestrial cold environments are termed psychrophiles and psychrotrophs. A psychrophile is defined as an organism that is capable of growth at or below 0°C but unable to grow above 20°C, whereas a psychrotroph, while capable of growth at around 0°C, can grow well above 20°C (Morita 1975; Watson 1987). Psychrophiles and psychrotrophs are widely distributed in nature, with 80% of the biosphere having temperatures below 5°C (Margesin and Schinner 1994). These types of microorganisms dominate the Antarctic continent with respect to biomass and function (Vishniac 1996). These microorganisms not only have crucial functions in their own environment such as cycling of essential elements and mineralizing of wastes, they also have the potential to be exploited for biotechnological processes. They can be utilized for low temperature fermentations, in bioremediation processes such as the cleaning up of oil spills, and they may also be a valuable and unique source of pharmaceuticals (for reviews see Gounot 1991; Russell 1992; Margesin and Schinner 1994). In current times of ozone depletion and global warming, it is important to understand microbial life in Antarctica and how the associated effects of increasing temperature and ultraviolet irradiation may challenge this diversity of life.

Temperature is undoubtedly one of the major factors affecting the growth and survival of any microorganism. Consequently, it is of considerable significance how psychrophilic and psychrotrophic microbes survive in their natural environment and how they adapt to temperatures exceeding their growth temperature range. In this respect, it

is noteworthy that all organisms studied to date acquire thermotolerance to a normally lethal heat stress when preexposed to a milder, nonlethal heat shock. This phenomenon is associated with the heat shock response (Lindquist and Craig 1988; Watson 1990; Piper 1993). There is a paucity of data concerning stress response systems and acquired stress tolerance in eukaryotic psychrophiles and psychrotrophs.

It is well established that induced stress tolerance is associated with concomitant synthesis of heat shock proteins (hsps) (Lindquist and Craig 1988) and the disaccharide trehalose (Attfield 1987; Hottiger et al. 1989), biochemical parameters that have been implicated as serving stress protective roles. Hsps are a set of highly conserved proteins that constitute a number of families based on protein molecular weight. In *Saccharomyces cerevisiae*, hsps fall into the following families: 100 kDa, 90 kDa, 70 kDa, 60 kDa, and a set of small molecular weight proteins (Watson 1990; Mager and Moradas-Ferreira 1993). They are located in different organelles and many function as molecular chaperones with respect to protein folding and assembly, as well as removal and repair of damaged proteins (Gething and Sambrook 1992; Craig et al. 1993). Heat shock protein (Hsp) 104 has been found to be essential for heat shock induced thermotolerance in *S. cerevisiae* (Sanchez et al. 1992). A proposed role for trehalose is in the stabilization of proteins and membranes during stress to enable the maintenance of cell structure (Hottiger et al. 1989; Weimken 1990). In the present studies, both hsp induction and trehalose accumulation in psychrophilic (*Mrakia frigida*, *Mrakia gelida*, *Mrakia stokesii*, and *Leucosporidium antarcticum*) and psychrotrophic (*Leucosporidium fellii* and *Leucosporidium scottii*) yeast from Antarctica were investigated to elucidate their possible contributions to intrinsic and induced thermotolerance.

Materials and methods

Yeast strains and culture conditions

All yeast strains used in the present studies were isolated from Antarctica. These were *Mrakia frigida* (CBS 5270, snow and soil), *M. gelida* (CBS 5272, soil), *M. stokesii* (CBS 5917, snow and soil), and *Leucosporidium antarcticum* (CBS 5942, ocean), all classified as psychrophiles on the basis of their maximum temperature (20°C) of growth (Fell et al. 1969; Watson 1987), and *Leucosporidium fellii* (CBS 7287, ocean and soil) and *L. scottii* (CBS 5930, soil), both classified as psychrotrophs on the basis that these strains were capable of growth above 20°C (Fell et al. 1969). Psychrophilic yeast were grown at 15°C and psychrotrophic yeast were grown at 15°C or 25°C on a rotary shaker (180 rpm) in YEP medium (0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, and 2% glucose). Experimental cultures were grown to an optical density of 0.2–0.3 (at 600 nm) corresponding to the logarithmic phase, with a cell density of approximately 2×10^6 to 5×10^6 cells ml^{-1}. *Saccharomyces cerevisiae* K7 (ATCC 26422), a mesophilic saké yeast, was grown at 25°C and heat shocked at 37°C (30 min) and used as a control. This strain has been extensively used over many years in this laboratory and exhibits a typical yeast heat shock response (Watson and Cavicchioli 1983; Lewis et al. 1993; Deegenaars and Watson 1995). All experiments were repeated a minimum of three times and produced consistent results.

Shock/stress conditions

For psychrophilic yeast, intrinsic thermotolerance was measured by rapidly heating cells grown at 15°C to either 35°C or 38°C in a 70°C waterbath and transferring them to either a 35°C or 38°C oscillating waterbath for the duration of a 60- or 120-min time course. Induced thermotolerance was measured by exposing cultures to a heat shock at 20°C or 25°C for 3 h prior to a heat stress at either 35°C or 38°C. Intrinsic and induced thermotolerance in psychrotrophic yeast grown at 15°C was measured in a similar manner to that of pychrophilic yeast with the exception of the heat stress temperature which was either 38°C or 42°C. In addition, a 37°C heat shock for 3 h was used to investigate induced thermotolerance. However, for psychrotrophic yeast grown at 25°C, intrinsic thermotolerance was measured to a heat stress at 42°C over a 60-min time course and induced thermotolerance was measured by exposing cultures to a 30°C or 37°C heat shock for 30 min prior to a heat stress at 42°C. Samples (0.5 ml) were taken at various time intervals, transferred to microfuge tubes, and cooled on ice to 15°C (psychrophilic and psychrotrophic yeast grown at 15°C) or 25°C (psychrotrophic yeast grown at 25°C). Samples were diluted in YEP medium, plated in duplicate on YEP agar and incubated at 15°C for 10–14 days. Thermotolerance was assessed as the percentage of colony forming units (cfu) after the appropriate treatment compared to an unstressed control (100% survivors).

Trehalose determination

Trehalose was extracted from 80 ml washed cells (5–7 mg dry weight) from three independent cultures. Control and heat shocked cells from psychrophilic and psychrotrophic yeast were extracted with 0.5 M trichloroacetic acid at 4°C and trehalose estimated by a modified anthrone method (Lewis et al. 1993).

[^{35}S]methionine labelling, protein extraction, and electrophoresis

Prior to [^{35}S]methionine labelling, 40 ml of culture was washed and resuspended in 2 ml YNB (0.67% yeast nitrogen base, 0.3% KH_2PO_4, and 2% glucose) medium without amino acids, essentially as described previously (Fuge et al. 1994). [^{35}S]methionine (100 μCi; specific activity 1150 Ci $mmol^{-1}$) was added to control and heat shock samples and

incubated at respective temperatures for 3 h (psychrophilic and psychrotrophic yeast grown at 15°C) or 30 min (psychrotrophic yeast grown at 25°C). Samples were then transferred to a microfuge tube containing 150 µl of 100 mg ml^{-1} unlabelled methionine. Cells were pelleted and extracted as previously described (M^{c}Alister et al. 1979). Protein concentration was determined using a Coomassie protein microassay procedure (Pierce, Rockford, IL, USA). Separation of proteins (10 µg) and low range molecular weight standards (Bio-Rad, Hercules, CA, USA) was achieved by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) utilizing a 10% separating and 4% stacking gel. Gels were silver stained, dried, and exposed to Hyperfilm-MP (Amersham, Buckinghamshire, UK) at −70°C for 5–7 days prior to developing.

Western immunoblotting analysis

Following extraction and electrophoresis as described, proteins were transferred to Hybond-C super nitrocellulose (Amersham). Western immunoblotting was carried out using the Amersham ECL western blotting detection kit according to the manufacturer's instructions. Final washes of membranes, prior to detection, were modified to 3 × 5 min in phosphate buffered saline (PBS)/0.3% Tween 20 followed by 3 × 5 min in PBS/0.1% Tween 20. Anti-hsp104 polyclonal antibody (Affinity BioReagents), anti-hsp90 monoclonal antibody (kind gift from P. Piper, University College London), anti-hsp70 monoclonal antibody (Affinity BioReagents), and anti-hsp60 monoclonal antibody (StressGen) were used at dilutions of 1:1000, 1:750, 1:5000, and 1:1000 respectively. The appropriate secondary antibody was used at a dilution of 1:1000. Membrane exposures to Hyperfilm-MP were from a few seconds to a few minutes prior to developing.

Results

Thermotolerance

Intrinsic and heat shock induced thermotolerance were measured to both a 35°C and a 38°C heat stress in the psychrophilic yeast species *Mrakia frigida*, *M. gelida*, *M. stokesii*, and *Leucosporidium antarcticum* (Fig. 1) grown at 15°C. Intrinsic tolerance levels were 10–100 fold greater after a heat stress at 35°C for 5 min as compared with a heat stress at 38°C for 5 min. Thermotolerance was induced to both a 35°C and a 38°C heat stress by exposing cells to a prior 3-h heat shock at either 20°C or 25°C. In all psychrophilic yeast, a 25°C heat shock for 3 h elicited maximal induction and duration of tolerance to a heat stress at 38°C. However, characteristic species-specific differences in the heat shock temperature eliciting maximal tolerance were observed at the slightly lower heat stress temperature of 35°C. In *M. stokesii* (Fig. 1c) and *L. antarcticum* (Fig. 1d),

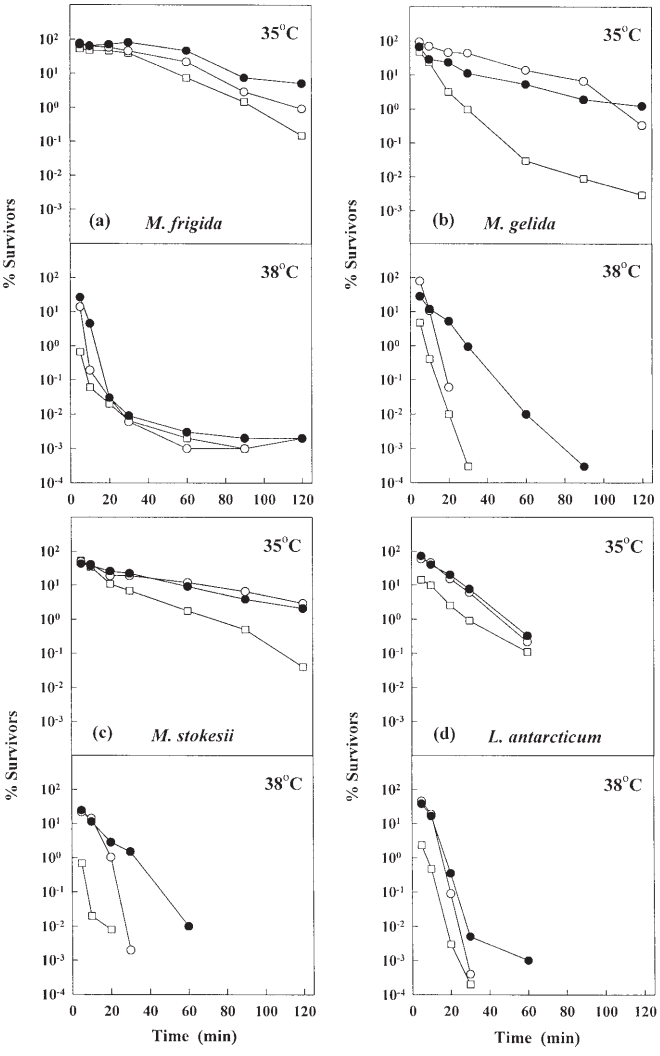

Fig. 1. Intrinsic and induced thermotolerance in mid-logarithmic phase cultures of **a** *Mrakia frigida*, **b** *M. gelida*, **c** *M. stokesii*, and **d** *Leucosporidium antarcticum*. Intrinsic tolerance (*open squares*) was measured by transferring cells, grown at 15°C, directly to 35°C (*top half* of each graph) or 38°C (*bottom half* of each graph). Induced thermotolerance was monitored at 35°C (*top half* of each graph) or 38°C (*bottom half* of each graph) following a heat shock for 3 h at 20°C (*open circles*) or 25°C (*closed circles*). Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample

both 20°C and 25°C heat shock temperatures induced the same level of tolerance to 35°C over the duration of the heat stress. On the other hand, in *M. frigida* (Fig. 1a) a heat shock at 25°C for 3 h evoked greater adaptation to a heat stress at 35°C than a heat shock at 20°C for 3 h. Conversely, in *M. gelida* (Fig. 1b) a heat shock at 20°C for 3 h rather than a heat shock at 25°C for 3h conferred maximal tolerance to a heat stress at 35°C. Duration and level of induced thermotolerance in all psychrophilic yeast was markedly greater for cells stressed to 35°C than for those exposed to a heat stress at 38°C.

The psychrotrophic yeast *L. fellii* and *L. scottii* were grown at 15°C and 25°C, corresponding to growth tempera-

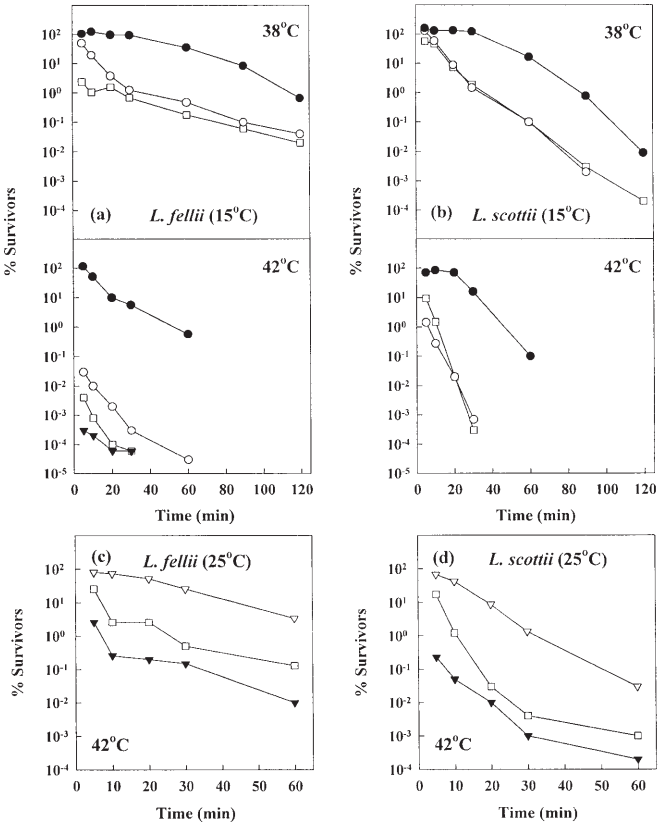

Fig. 2. Intrinsic and induced thermotolerance in mid-logarithmic phase cultures of *L. fellii* and *L. scottii*. For 15°C grown cultures of **a** *L. fellii* and **b** *L. scottii*, intrinsic tolerance (*open squares*) was measured by transferring cells directly to 38°C (*top half* of each graph) or 42°C (*bottom half* of each graph). Induced thermotolerance was monitored at 38°C (*top half* of each graph) or 42°C (*bottom half* of each graph) following a heat shock for 3 h at 20°C (*open circles*), 25°C (*closed circles*), or 37°C (*closed triangles*). For 25°C-grown cultures of **c** *L. fellii* and **d** *L. scottii*, intrinsic tolerance (*open squares*) was measured by transferring cells directly to 42°C. Induced thermotolerance was monitored at 42°C following a heat shock for 30 min at 30°C (*open triangles*) or 37°C (*closed triangles*). Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C or 25°C control sample

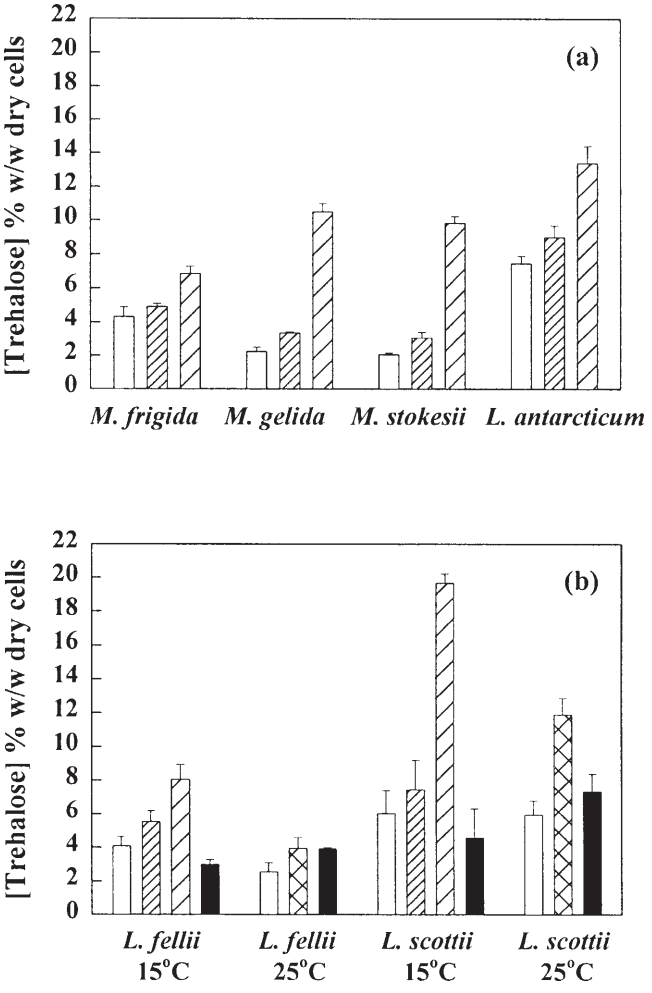

Fig. 3. Trehalose levels in control and heat-shocked cultures of **a** *M. frigida*, *M. gelida*, *M. stokesii*, and *L. antarcticum* (grown at 15°C) and **b** *L. fellii* and *L. scottii* (grown at 15°C or 25°C). Trehalose was extracted from a 15°C or 25°C control (□), a heat shock at 20°C for 3 h (▨), a heat shock at 25°C for 3 h (▨), a heat shock at 30°C for 30 min (▨) and a heat shock at 37°C (■) for 3 h (for 15°C grown cultures) or 30 min (for 25°C grown cultures). Results are presented as mean and standard deviation of measurements from three independent cultures

tures used in the current studies for psychrophilic and mesophilic yeast, respectively, and heat shocked (Fig. 2). In *L. fellii* (Fig. 2a) and *L. scottii* (Fig. 2b) grown at 15°C, a heat shock at 25°C for 3 h elicited maximal tolerance to heat stress temperatures of 38°C and 42°C. Generally, a heat shock at 20°C for 3 h did not confer any further tolerance to a heat stress above intrinsic levels. Intrinsic thermotolerance levels to a heat stress at 38°C for the duration of the 2-h time course were greater in psychrotrophic yeast than psychrophilic yeast. This prompted an examination of the stress response at a higher stress temperature of 42°C. Again, a heat shock at 25°C for 3 h induced maximal tolerance. However, neither a heat shock at 20°C or one at 37°C for 3 h induced any further thermotolerance to 42°C. Indeed, a heat shock at 37°C for 3 h was detrimental to cell viability, with complete loss of viability after the heat shock for *L. scottii* (hence the lack of data points on Fig. 2b for induced thermotolerance following a heat shock at 37°C). When *L. fellii* was grown at 25°C (Fig. 2c), intrinsic resistance to a 42°C heat stress was greater than when cultures were grown at 15°C. In *L. scottii* grown at 25°C (Fig. 2d) intrinsic tolerance levels were essentially the same as when grown at 15°C. A prior heat shock at 30°C for 30 min induced thermotolerance in both psychrotrophic yeast. However, a heat shock at 37°C for 30 min did not induce tolerance to a heat stress at 42°C over and above that of intrinsic thermotolerance levels.

Trehalose

Trehalose levels in control cultures of psychrophilic yeast (Fig. 3a) differ, with *L. antarcticum* having the greatest intrinsic levels (approximately 7% w/w). A 3-h, 25°C heat shock elicited maximal trehalose accumulation in all of the

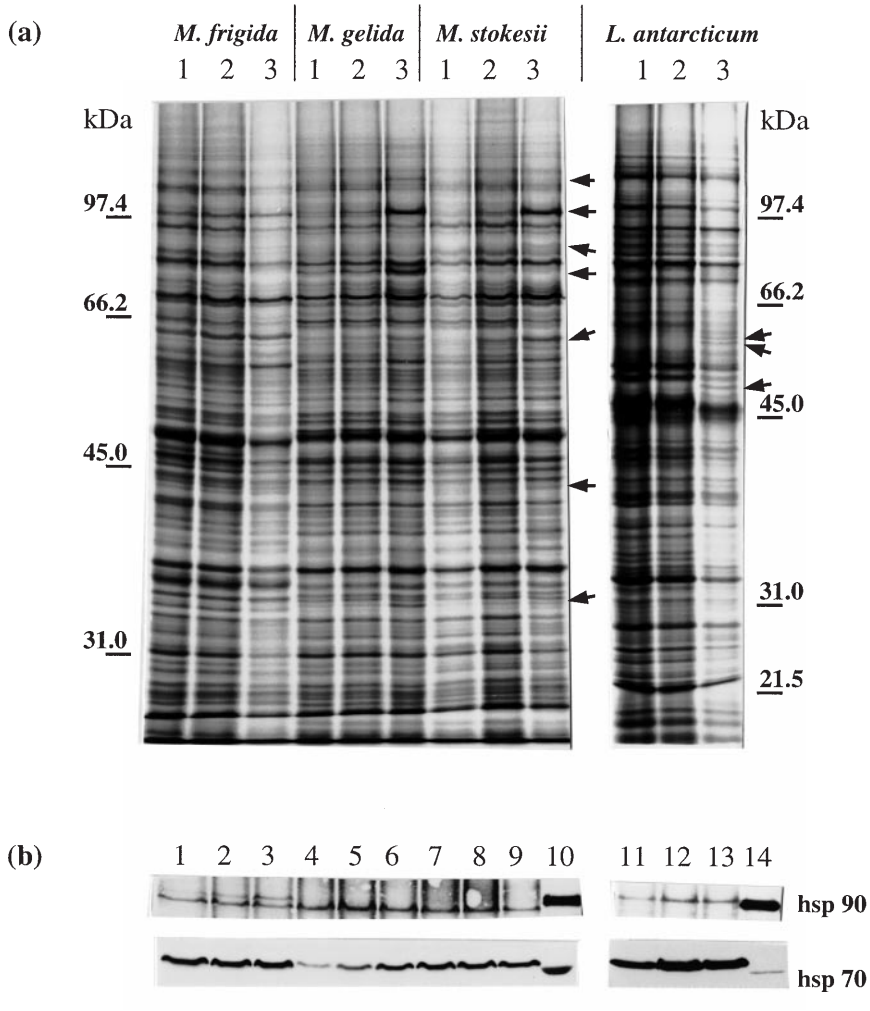

Fig. 4. **a** Sodium dodecyl sulfate (SDS)-polyacrylamide gel autoradiograms of [^{35}S]methionine-labelled protein extracts from *M. frigida*, *M. gelida*, *M. stokesii*, and *L. antarcticum*. Conditions were 15°C control (*lanes 1*), heat shock at 20°C for 3 h (*lanes 2*), and heat shock at 25°C for 3 h (*lanes 3*). *Arrows* indicate new or increased heat shock protein synthesis. Molecular mass standards (kDa) are as indicated. **b** Western blot analysis of *M. frigida*, *M. gelida*, *M. stokesii*, *L. antarcticum*, and *S. cerevisiae* K7. Proteins from 15°C control, heat shock at 20°C for 3 h, and heat shock at 25°C for 3 h from *M. frigida* (*lanes 1*, *2*, *and 3*, respectively), *M. gelida* (*lanes 4*, *5*, *and 6*, respectively), *M. stokesii* (*lanes 7*, *8*, *and 9*, respectively), and *L. antarcticum* (*lanes 11*, *12*, *and 13*, respectively) were probed with anti-hsp 90 (1:750) and anti-hsp 70 (1:5000). *Lanes 10 and 14* correspond to a heat shock (37°C for 30 min) protein sample from *S. cerevisiae* K7

psychrophilic yeast. However, a heat shock at 20°C for 3 h elicited a slight but obvious increase in trehalose above control levels in three of the four psychrophilic yeast.

In psychrotrophic yeast (Fig. 3b) grown at 15°C the pattern of trehalose accumulation resembled that of the psychrophilic yeast. Trehalose accumulation was maximal after a heat shock at 25°C for 3 h, and a heat shock at 20°C for 3 h increased levels slightly above control levels. The greatest increase was observed in *L. scottii* with a 3-fold increase in trehalose after a heat shock at 25°C for 3 h. A heat shock at 37°C for 3 h decreased trehalose levels below those of control cultures. For psychrotrophic yeast grown at 25°C, a heat shock at 30°C for 30 min induced the greatest increase in trehalose accumulation. In contrast to 15°C-grown cultures, a heat shock at 37°C for 30 min in 25°C-grown cultures increased trehalose levels slightly above control trehalose levels.

Heat shock proteins

Patterns of protein synthesis in control and heat shocked cells were examined using [^{35}S]methionine labelling. Changes in protein pattern were most pronounced in psychrophilic yeast subjected to a heat shock at 25°C for 3 h (Fig. 4a). In *M. gelida* and *M. stokesii*, a common 110-kDa protein was heat shock inducible. This protein was also visible in *M. frigida* but did not noticeably increase in synthesis upon a heat shock. Other species-specific, heat shock inducible proteins in addition to the 110-kDa protein were observed in the *Mrakia* genus. In *M. frigida*, a heat shock at 25°C for 3 h induced or increased the synthesis of a protein of approximately 90 kDa. Four additional heat shock inducible proteins of approximately 36, 44, 83, and 130 kDa were evident in *M. gelida* and the increased synthesis of heat shock proteins of approximately 64 and 90 kDa was seen in *M. stokesii*. The 110-kDa heat shock protein was not clearly evident with [^{35}S]methionine labelling in *L. antarcticum*. However, proteins of approximately 51, 58, and 59 kDa were increased upon a heat shock.

Different protein profiles were observed with [^{35}S]methionine labelling in psychrotrophic yeast grown at 15°C (Fig. 5a). In *L. fellii* and *L. scottii*, a heat shock (at 25°C for 3 h) inducible protein at about 100 kDa was identified as hsp 104 by western blot analysis (Fig. 5c). In addition, a heat shock at 25°C also induced proteins of 40, 42,

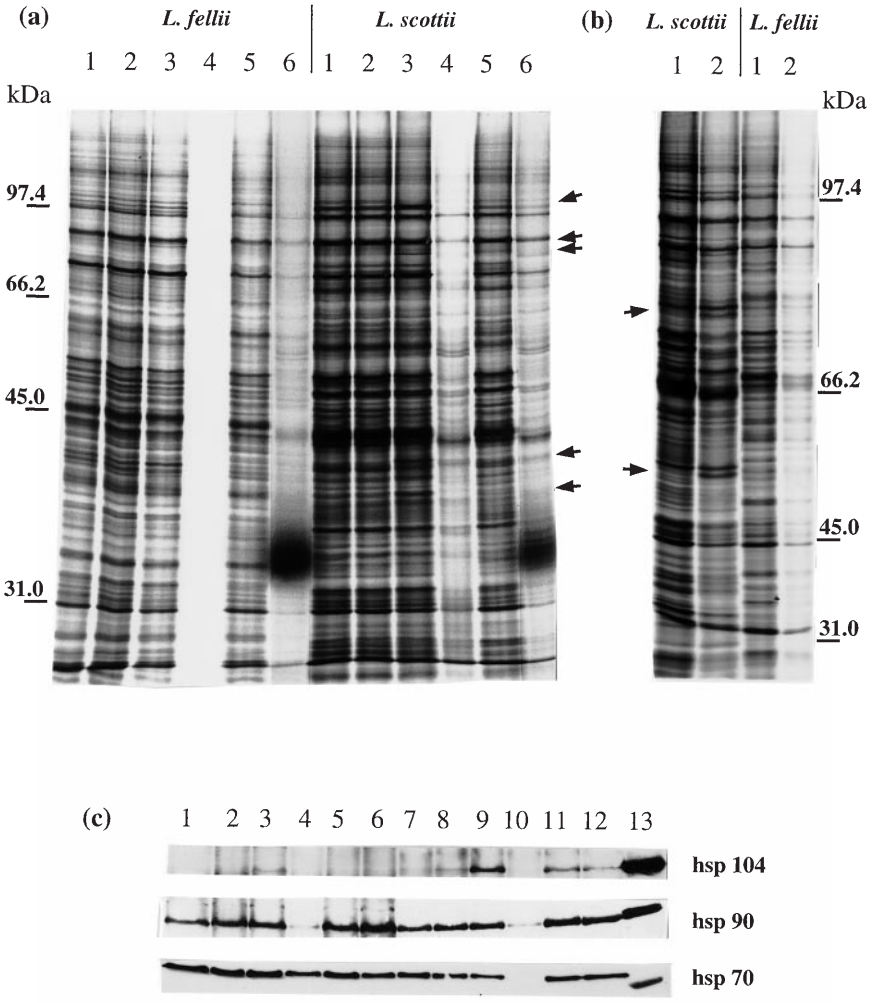

Fig. 5. SDS-polyacrylamide gel autoradiograms of [^{35}S]methionine-labelled protein extracts from *L. fellii* and *L. scottii* grown at 15°C and 25°C. Conditions for **a** were 15°C control (*lanes 1*), heat shock at 20°C for 3 h (*lanes 2*), heat shock at 25°C for 3 h (*lanes 3*), and heat shock at 37°C for 3 h (*lanes 4*) for cultures grown at 15°C. For cultures grown at 25°C, conditions were 25°C control (*lanes 5*) and heat shock at 37°C for 30 min (*lanes 6*). The *leftmost six lanes* are L. fellii; the *rightmost six lanes* are *L. scottii*. Conditions for **b** were 25°C control (*lanes 1)* and heat shock at 30°C for 30 min (*lanes 2*) for cultures grown at 25°C. The *left lanes* are *L. scottii*; the *right lanes* are *L. fellii*. For **a** and **b**, *arrows* indicate new or increased heat shock protein synthesis. Molecular mass standards (kDa) are as indicated. **c** Western blot analysis of *L. fellii*, *L. scottii*, and *S. cerevisiae* K7. Conditions were 15°C control, heat shock at 20°C for 3 h, heat shock at 25°C for 3 h, and heat shock at 37°C for 3 h for *L. fellii* (*lanes 1*, *2*, *3*, *and 4*, respectively) and *L. scottii* (*lanes 7*, *8*, *9*, *and 10*, respectively) grown at 15°C. Proteins were also extracted from 25°C control and heat shock (37°C for 30 min) samples of *L. fellii* (*lanes 5 and 6*, respectively) and *L. scottii* (*lanes 11 and 12*, respectively) grown at 25°C. All protein samples were probed with anti-hsp 104 (1:1000), anti-hsp 90 (1:750), and anti- hsp 70 (1:5000). *Lane 13* corresponds to a heat shock (37°C for 30 min) protein sample from *S. cerevisiae* K7

and 83 kDa in *L. scottii* and an 85-kDa protein in *L. fellii*. However, a heat shock at 20°C for 3 h did not increase the synthesis of any observable proteins in either psychrotrophic yeast. In addition, a heat shock at 37°C for 3 h completely inhibited de novo protein synthesis in *L. fellii* and decreased de novo protein synthesis in *L. scottii* (lanes 4).

Differences and similarities exist in the de novo protein profiles of psychrotrophic yeast grown at 25°C to those grown at 15°C (Fig. 5a,b). A 30-min, 37°C heat shock decreased de novo protein synthesis in both *L. fellii* and *L. scottii* (Fig. 5a, lanes 6). No clear heat shock inducible proteins were visible at either 104 or 110 kDa upon a heat shock at 30°C for 30 min (Fig. 5b). However, a heat shock at 30°C did increase the synthesis of proteins of approximately 37 and 58 kDa in *L. scottii*. In contrast in *L. fellii* grown at 25°C, a heat shock at 30°C inhibited de novo protein synthesis (Fig. 5b).

These observations were extended by western blot analysis using commercial antibodies to yeast hsp 60, 70, 90, and 104. As a control, protein extracts from 30-min, 37°C heat-shocked cells of the mesophilic yeast *S. cerevisiae*, strain K7, were used. As illustrated in Fig. 4b, hsp 70 and 90 were observed in protein extracts from control and heat-shocked cells of *M. frigida*, *M. gelida*, *M. stokesii*, and *L. antarcticum*. The hsp 70 observed in the psychrophilic yeasts was slightly greater in molecular weight than hsp 70 in *S. cerevisiae* K7. Levels of hsp 70 were equal in control and heat-shocked samples of *M. frigida* and *M. stokesii*. In *M. gelida* and *L. antarcticum*, hsp 70 levels increased upon a heat shock. In the case of hsp 90, a single band was observed in control and heat-shocked samples of all psychrophilic yeast species except *M. frigida*. In *M. frigida*, a second, slightly greater molecular weight hsp 90 was heat-shock inducible. In contrast to *S. cerevisiae*, there was no protein component detected by either anti-hsp 60 or anti-hsp 104 antibodies (results not shown) in any of the psychrophilic yeast examined. On the other hand, a protein recognised by the anti-hsp 104 antibody was found in psychrotrophic yeast (Fig. 5c). In *L. fellii* grown at 15°C, a hsp 104 homolog was induced by a heat shock at 25°C for 3 h. However, when *L. fellii* was grown at 25°C, no hsp 104 homolog was observed

in either control or heat-shocked samples. Anti-hsp 104 antibody also detected a protein counterpart in *L. scottii* grown at 15°C and heat-shocked at either 20°C or 25°C and also in cultures grown at 25°C and heat-shocked at either 30°C (results not shown) or 37°C (Fig. 5c). Hsp 60 counterparts were not detected by anti-hsp 60 antibody (results not shown) in psychrotrophic yeast grown at either temperature. With respect to hsp 70, single bands were observed in all protein samples of psychrotrophic yeast (Fig. 5c). A single band was also observed in all protein samples for hsp 90 with the exception of *L. scottii* grown at 15°C and heat-shocked at 37°C. In addition, a heat shock at 37°C decreased levels of hsp 70, 90, and 104 in psychrotrophic yeast grown at 15°C (Fig. 5c).

Discussion

This study and our previous investigations of the heat shock response in *C. psychrophila* (Deegenaars and Watson 1997) represent a significant contribution towards studies of stress response systems in yeast from Antarctica. In total, three out of five known psychrophilic yeast genera are represented; five out of seven known Antarctic, psychrophilic yeast, other than those in the genus *Cryptococcus*, have now been investigated.

It well known that microorganisms have unique hsps and temperatures that induce the heat shock response (Watson 1990). This also holds true for psychrophilic yeast. However, within a grouping such as yeast from Antarctica, similarities exist in the heat shock response. For instance, in all the psychrophilic yeast examined, a heat shock at 25°C for 3 h induced maximal tolerance to a heat stress at 38°C (Fig. 1). This heat shock temperature also stimulated maximal accumulation of trehalose (Fig. 3) and hsp synthesis (Fig. 4a,b). A heat shock at 25°C is 10 Celsius degrees above the optimum growth temperature of psychrophilic yeast. This agrees with previous observations by other researchers that a 10 Celsius degree increase in temperature above the growth temperature of an organism is required to elicit a heat shock response (Howarth and Ougham 1993; Cairns et al. 1995). It is of interest, however, that 25°C lies outside the normal growth temperature range of psychrophilic yeast. It has also been observed in *Trichosporon pullulans*, an Arctic psychrotrophic yeast, that a heat shock temperature outside its growth range is required to induce thermotolerance (Berg et al. 1987). In contrast, for *S. cerevisiae*, 37°C is used as an optimal temperature to elicit the heat shock response and this temperature lies within the normal growth temperature range of this mesophilic yeast (M^{c}Alister et al. 1979).

In psychrophilic yeast, it was observed that the temperature that induces thermotolerance varies depending on the heat stress temperature. In all psychrophilic yeast examined, a heat shock at 25°C for 3 h induced thermotolerance to a heat stress at 38°C (Fig. 1). However, different responses were observed amang the psychrophilic yeast when a heat stress at 35°C was used. In some instances, a heat shock at 20°C for 3 h elicited an equal or greater tolerance to a heat stress at 35°C than a heat shock at 25°C for 3 h (Fig. 1). These observations therefore suggest that induced tolerance depends not only on the shock conditions but also on the stress conditions.

With respect to hsp synthesis, both unique and overlapping proteins were observed amongst the Antarctic, psychrophilic yeast. Common to all psychrophilic yeast, with the exception of *L. antarcticum*, was the heat shock inducible 110-kDa protein. In addition, in all the psychrophiles, there was no protein counterpart to the *S. cerevisiae* hsp 104. Perhaps this highly conserved 110-kDa hsp in psychrophilic yeast, substitutes for the vital role that hsp 104 plays in thermotolerance in *S. cerevisiae* (Sanchez et al. 1992). In *M. frigida* and *M. stokesii*, a hsp of 90 kDa was also heat-shock inducible and common to both species (Fig. 4a). This heat shock protein probably falls into the yeast hsp 90 family as indicated by crossreactivity to the respective antibody (Fig. 4b). Despite the similarities amang psychrophilic yeast in these hsps, individual psychrophilic yeast also had their own characteristic heat shock protein profile. On this point, it was noteworthy that the individual heat shock protein profiles were most similar between species within the *Mrakia* genus (Fig. 4a) and *C. psychrophila* (Deegenaars and Watson 1997). The protein profile of *L. antarcticum* was very different from these other genera, with particular respect to the absence of the heat shock inducible 110-kDa protein and the molecular weights of its other hsps (Fig. 4a). The protein profile of hsps in *L. antarcticum* was most similar to that of the psychrotrophic yeast, *L. scottii* grown at 25°C and heat shocked at 30°C for 30 min (Fig. 5b).

Similarities exist in the heat shock temperature and time required for optimal hsp synthesis in other psychrophilic microorganisms. A heat shock at 25°C for 2 h elicited hsp synthesis in the psychrophilic pink snow mould fungus, *Monographella nivalis*, grown at 3°C (Cairns et al. 1995). Likewise, a heat shock at 20°C for 5 h induced hsp synthesis in the psychrophilic bacterium, *Aquaspirillum arcticum*, grown at 0°C (M^{c}Callum et al. 1986). Induced thermotolerance was also measured in *A. arcticum*, albeit at only one time point, after 15 min at a heat stress temperature of 36°C (M^{c}Callum and Inniss 1990).

In the psychrotrophic yeast, *L. fellii* and *L. scottii*, growth temperature influenced the heat shock response. When psychrotrophic yeast were grown at 15°C the response was very similar to that of psychrophilic yeast with maximal induction of thermotolerance and concomitant hsp synthesis and trehalose accumulation after a heat shock at 25°C for 3 h (Fig. 2a,b). However, a distinct difference was observed in hsp profiles. In psychrotrophic yeast grown at 15°C, there was no heat shock inducible 110-kDa protein but a 104-kDa hsp was induced as visualized by [^{35}S]methionine autoradiograms and western blots (Fig. 5c). Also in contrast to psychrophilic yeast, psychrotrophic yeast grown at 15°C were able to adapt to the slightly higher stress temperature of 42°C (Fig. 2a,b). Levels of intrinsic and induced thermotolerance as well as longevity of thermotolerance in psychrotrophic yeast grown at 15°C and stressed at 42°C (Fig. 2a,b) resembled those of psychro-

philic yeast stressed at 38°C (Fig. 1). In addition, psychrotrophic yeast grown at 15°C were more intrinsically resistant to a 38°C stress than psychrophilic yeast. A further difference between psychrophilic and psychrotrophic yeast grown at 15°C was the apparent lack of tolerance induction by a heat shock at 20°C for 3 h in psychrotrophic yeast (Fig. 2a,b).

In psychrotrophic yeast grown at 25°C, thermotolerance was induced by a 30-min, 30°C heat shock (Fig. 2c,d). This response was more rapid than in psychrophilic (Fig. 1) and psychrotrophic yeast grown at 15°C (Fig 2a,b) whereby a 3-h shock was required to elicit thermotolerance. Also, a temperature shift of only 5 Celsius degrees above the growth temperature (25°C) was sufficient to induce tolerance to a 42°C stress compared to an increase of 10 Celsius degrees in psychrophilic and psychrotrophic yeast grown at 15°C. Coincident with the 30°C heat shock was an increase in trehalose level in both psychrotrophic yeast (Fig. 3) and the induction of hsps in *L. scottii* (Fig. 5b). It was surprising that a heat shock at 37°C for 30 min decreased viability below intrinsic levels (Fig. 2c,d). This 12 Celsius degree increase in temperature above the growth temperature (25°C) also decreased de novo protein synthesis (Fig. 5a) and affected trehalose accumulation (Fig. 3). However, in contrast to 15°C-grown cells, cells given a heat shock at 37°C did not show decreased trehalose levels below those of control cultures. Comparison of 30°C and 37°C heat-shock induced responses in cultures grown at 25°C suggests that de novo protein synthesis and hsp synthesis per se have a more profound effect on induced thermotolerance than trehalose accumulation.

Western immunoblotting revealed hsp 90 and hsp 70 protein counterparts in all psychrophilic (Fig. 4b) and psychrotrophic yeast (Fig. 5c) examined. Psychrophilic yeast have an abundance of functional mitochondria (Watson 1987) and one would presume that hsp 60 would be abundant as a consequence of its highly conserved nature, chaperone function, and mitochondrial location (Cheng et al. 1989). It was somewhat surprising, therefore, that no hsp 60 homolog was observed in any of the psychrophilic and psychrotrophic yeast (results not shown) examined in this study, particularly since it had been detected in the Antarctic, psychrophilic yeast, *C. psychrophila* (Deegenaars and Watson 1997). Despite anti-hsp 104 antibody not detecting a 104 kDa protein in psychrophilic yeast, smaller protein bands of approximately 50 kDa did crossreact with the antibody in all psychrophilic yeast except *L. antarcticum* (results not shown). This crossreaction with smaller molecular weight proteins has also been observed in *Arabidopsis thaliana* (Schirmer et al. 1994) and the authors suggest that these smaller protein bands may represent lower molecular weight members of the Hsp 100 (Schirmer et al. 1996) family rather than proteolytic degradation products. Although heat shock proteins have been previously identified in psychrophilic (McCallum et al. 1986; McCallum and Inniss 1990; Cairns et al. 1995) and psychrotrophic microorganisms (McCallum et al. 1986; McCallum and Inniss 1990; Julseth and Inniss 1990), their relationship to known hsps remain obscure. The only other study, apart from the present report, to identify known hsp members was that in the Antarctic alga, *Plocamium cartilagineum* (Vayda and Yuan 1994) where hsp 70 and ubiquitin mRNA were induced.

Many of the Antarctic yeast examined were first designated as species within the genus *Candida* (di Menna 1966). *Candida gelida*, *C. frigida*, and *C. scottii* were reclassified by Fell et al. (1969) as *Leucosporidium* species. This genus also included the psychrophilic yeast, *L. antarcticum* and *L. stokesii*, and the psychrotrophic yeast, *L. fellii* (Giménez-Jurado and van Uden 1989). These species were reclassified again on the basis of their coenzyme Q systems, with *L. gelidum*, *L. frigidum*, and *L. stokesii* now classified as *Mrakia* species (Yamada and Komagata 1987). On the basis of classification, the lack of the 110-kDa hsp in psychrophilic *L. antarcticum* (Fig. 4a) and psychrotrophic *L. fellii* and *L. scottii* (Fig. 5a,b) may possibly be a genus-specific trait rather than a distinction between psychrophilic and psychrotrophic yeast. However, the presence of a protein counterpart to hsp 104 in psychrotrophic yeast (Fig. 5c) and an absence in all psychrophilic yeast may argue more favorably towards a distinction between psychrophily and psychrotrophy with respect to the heat shock response. This distinction could be resolved by examining the heat shock response and hsp synthesis in other Antarctic yeast including psychrophilic *Cryptococcus* species (Vishniac 1996), psychrophilic *M. nivalis*, and other psychrotrophic yeast.

We examined trehalose levels in psychrophilic and psychrotrophic yeast (Fig. 3), as trehalose accumulation has been correlated with the stress response in *S. cerevisiae* (Piper 1993). Heat shock induced accumulation of trehalose has also been observed in the yeast, *Schizosaccharomyces pombe* (De Virgilio et al. 1990) and *C. albicans* (Argüelles 1997). Concomitant trehalose accumulation (Fig. 3) and induced thermotolerance (Figs. 1,2) were observed with a heat shock at 25°C in psychrophilic yeast and psychrotrophic yeast grown at 15°C. However, this trend was less pronounced in psychrotrophic yeast grown at 25°C and heat shocked at 30°C. This suggests that trehalose alone is not responsible for heat shock induced thermotolerance.

The synthesis of the stress metabolites, hsps and trehalose, under heat shock conditions coinciding with induced thermotolerance suggests that Antarctic yeast have heat shock responses analogous to their mesophilic counterparts. In addition, their ability to adapt to stressful conditions could well favor them in their survival of an otherwise hostile environment.

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