CHAPTER 3: Heat Shock Response in the Antarctic, Psychrophilic Yeast, *Candida psychrophila.*

3.1 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 (Franzmann, 1995;Vishniac, 1996). These microorganisms not only have crucial functions in their own environment such as cycling of essential elements and mineralising 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 at their optimum temperatures of growth and how they adapt to temperatures exceeding their growth 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 non-lethal heat shock. This phenomenon is associated with the heat shock response (Lindquist and Craig, 1988; Watson, 1990; Piper, 1993).

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.*, 1987), 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). 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).

There is a paucity of data concerning stress response systems and acquired stress tolerance in eukaryotic psychrophiles and psychrotrophs. Chapters 3 and 4 provide a comprehensive analysis of the heat shock response in psychrophilic and psychrotrophic yeast from Antarctica. Results presented in Chapter 3 established the optimal conditions required for induction of the heat shock response in the Antarctic, psychrophilic yeast *Candida psychrophila* as well as preliminary investigations of ethanol tolerance and the cold shock response. The analysis of the heat shock response in Antarctic yeast was extended in Chapter 4 to include a further four psychrophilic yeast, *Mrakia frigida*, *Mrakia gelida*, *Mrakia stokesii* and *Leucosporidium antarcticum* and two psychrotrophic yeast, *Leucosporidium fellii* and *Leucosporidium scottii*.

3.2 Results

3.2.1 Growth curves

Candida psychrophila was grown at its optimum temperature of 15°C (Goto *et al.*, 1969) in YEP media and subsamples were taken at various time intervals over a 120 h time course. Viable counts of subsamples enabled construction of a growth curve. Corresponding glucose and ethanol concentrations (% w/v) were also determined.

Figure 3.1 represents a typical growth curve for *C. psychrophila* grown at 15°C. *C. psychrophila* exhibits slow growth with a logarithmic phase of 20 – 30 h (spanning 1 x 10^6 to 1 x 10^7 cfu ml^{-1}) and a doubling time of approximately 7 h (as determined from the time taken for culture density to increase from 5 x 10^6 to 1 x 10^7 cfu ml^{-1}). Growth rate was further reduced when cell density reached 2 x 10^7 cfu ml^{-1}.

Glucose utilization paralleled the slow growth with less than half the available glucose content metabolised after 120 h. Available glucose was exhausted (result not shown) after approximately 170 h after inoculation. Only trace levels of ethanol were measured, over the 120 min time course, indicating that the end product of glycolytic fermentation of glucose in *C. psychrophila* was not ethanol.

Investigations of stress tolerance in *C. psychrophila* were carried out on mid-logarithmic phase cultures with an OD_{600} of 0.2 to 0.3 corresponding to 3 x 10^6 to 5 x 10^6 cfu ml^{-1} and stationary phase cultures defined as glucose exhausted media with a cell density of 1 to 5 x 10^8 cfu ml^{-1}.

3.2.2 Thermotolerance

Preliminary investigations of intrinsic resistance to a 5 min heat stress at 40°C, 35°C and 33°C were carried out on *C. psychrophila* cultures grown at 15°C to determine the heat stress temperature to be applied for examination of the heat shock response (Deegenaars, 1991). The results suggested that a lethal temperature of 40°C for 5 min was too high producing less than 0.05 % survivors. However, a 33°C and a 35°C heat stress for 5 min resulted in 84% and 40% survivors, respectively. It was concluded that although the

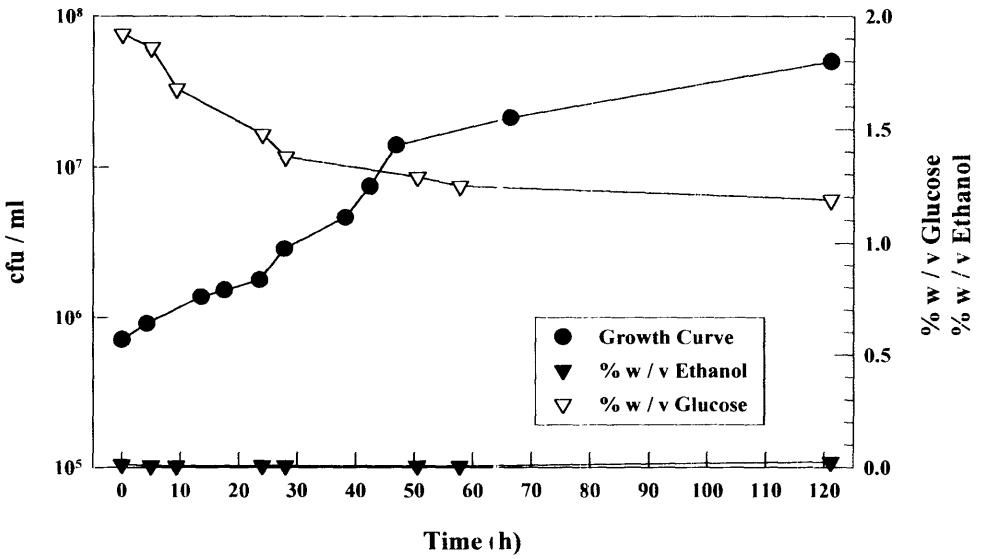

Figure 3.1. Growth curve of *C. psychrophila* at 15°C with corresponding glucose and ethanol measurements.

intrinsic tolerance to 35°C was initially high, when applied over a 60 min time course sufficient survivors were produced throughout and was a good starting point for the optimization of the heat shock response in *C. psychrophila*.

Figure 3.2A represents typical results for logarithmic phase cells of *C. psychrophila* grown at 15°C. Cells showed relative intrinsic thermosensitivity as compared to *S. cerevisiae* (with heat stress temperatures of 48°C to 52°C), producing less than 1% survivors after 30 min at 35°C. As we have previously reported (Deegenaars and Watson, 1992), exposure of cells to a mild heat shock at 20°C (the maximum temperature of growth for psychrophilic yeast) for 30 min prior to a heat stress (35°C) did not induce any significant thermotolerance (Fig. 3.2A). Similarly, a mild H_2O_2 shock (0.2 mM for 30 min) did not confer cross protection to a heat stress (Fig. 3.2A).

In contrast, stationary phase cultures, corresponding to depletion of glucose in the growth medium, exhibited higher intrinsic tolerance to heat stress over the 60 min time course (Fig. 3.2B). On the other hand, neither a 20°C heat shock nor a 0.2 mM H_2O_2 shock for 30 min conferred any further tolerance to a 35°C heat stress.

A possible explanation for the lack of tolerance induction by a 20°C heat shock with respect to logarithmic phase cells was that there was only a 5°C difference between the temperature of growth (15°C) and the heat shock temperature (20°C). Examination of the heat shock response in many microorganisms reveals a requirement for a difference in temperature of at least 10°C to elicit heat shock induced thermotolerance. Furthermore, 20°C was the upper temperature limit of growth for psychrophilic yeast and the heat shock temperature eliciting induced thermotolerance usually falls within the restrictive growth temperature range of a particular microorganism. Consequently, an alternative temperature regime for heat shock response analysis in *C. psychrophila* was tried. Cells were grown at 10°C, heat shocked for 30 min at 18°C (an 8°C difference) and stressed to 35°C (Fig. 3.3). The same scenario as for growth at 15°C resulted; that is, heat shock induced thermotolerance was not observed.

In the next series of experiments, a different tact was employed with respect to the heat shock temperature. It was thought that the duration of the heat shock might be the critical parameter. Figure 3.4 compares the induction of thermotolerance in a mid-

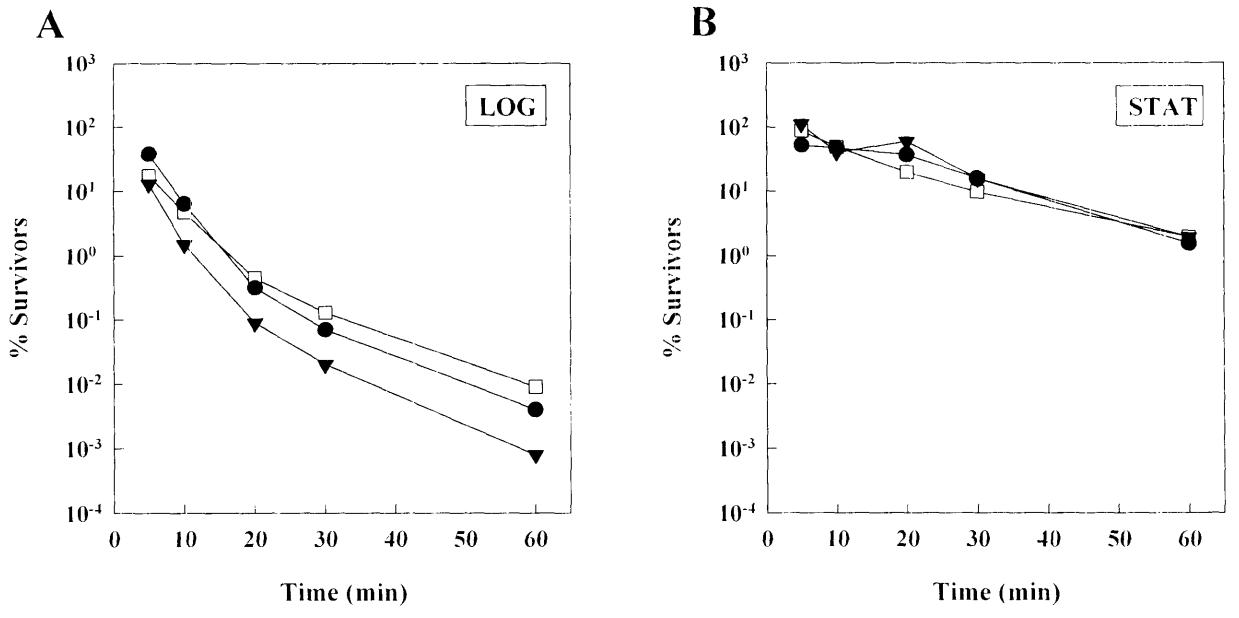

Figure 3.2. Intrinsic and induced thermotolerance to a 35°C heat stress in (A) mid-logarithmic and (B) stationary phase cultures of *C. psychrophila* grown at 15°C. Intrinsic thermotolerance (□) was measured by transferring cells directly to 35°C. Induced thermotolerance was monitored at 35°C following either a 30 min 20°C heat shock (●) or a 0.2 mM H_2O_2 shock (▼). Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

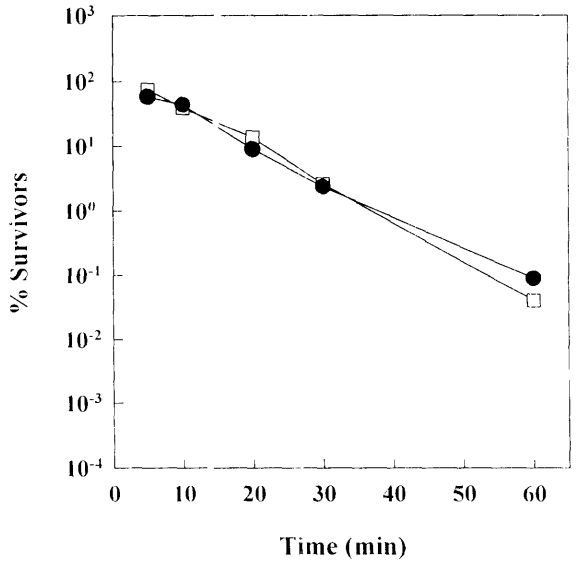

Figure 3.3. Intrinsic and induced thermotolerance to a 35°C heat stress in (A) mid-logarithmic and (B) stationary phase cultures of *C. psychrophila* grown at 10°C. Intrinsic thermotolerance (□) was measured by transferring cells directly to 35°C. Induced thermotolerance was monitored at 35°C following either a 30 min 18°C heat shock (●). Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 10°C control sample.

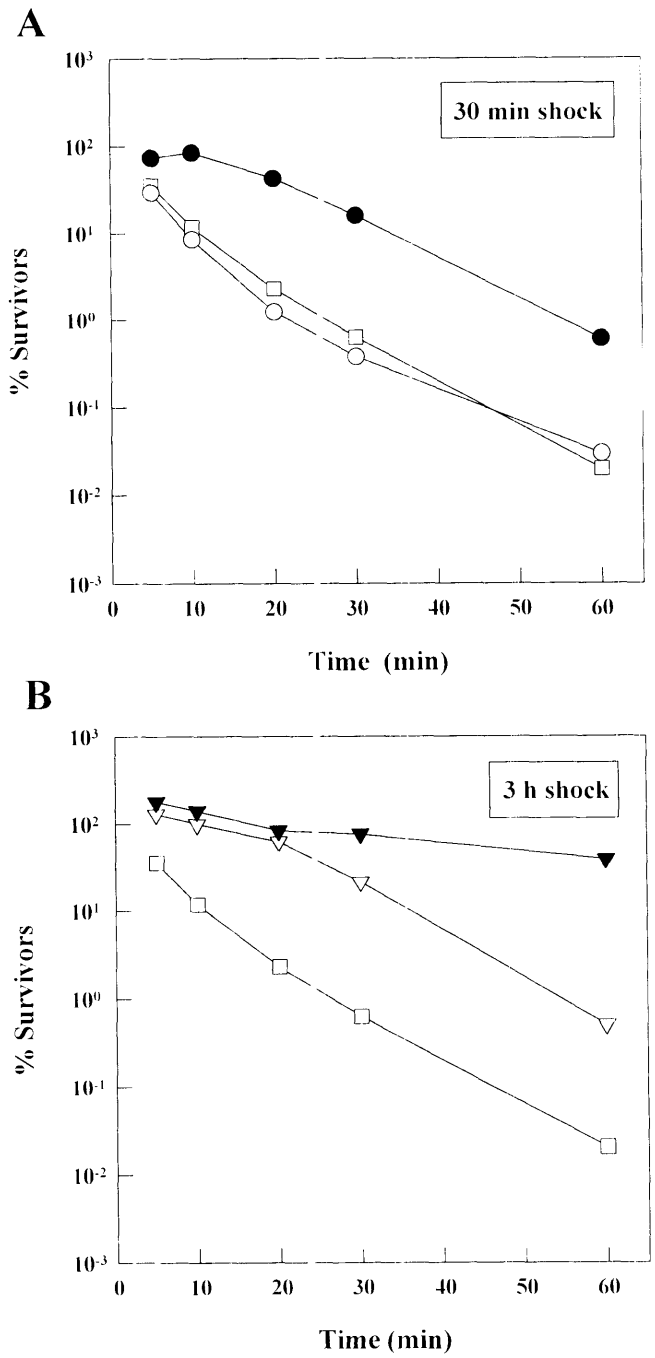

Figure 3.4. The effect of duration and temperature of heat shock on induced thermotolerance to a 35°C heat stress in mid-logarithmic phase cells of *C. psychrophila* grown at 15°C. Intrinsic thermotolerance (□) was measured by transferring cells directly to 35°C. Induced thermotolerance was monitored at 35°C following either a (A) 30 min (○, ●) or (B) 3 h (▽, ▼), 20°C (○ and ▽) or 25°C (● and ▼) heat shock. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

logarithmic phase culture of *C. psychrophila* by a 30 min (Fig. 3.4A) versus a 3 h heat shock (Fig. 3.4B). As previously shown (Fig. 3.2), a 30 min 20°C heat shock did not induce thermotolerance (Fig. 3.4A). However, a prior heat shock at 20°C for 3 h induced protection against a subsequent heat stress (Fig. 3.4B). Interestingly, a heat shock at the higher temperature of 25°C for 30 min (Fig. 3.4A) induced equivalent protection to a 3 h 20°C heat shock and a 3 h heat shock induced almost 100% protection against a 30 min heat stress at 35°C (Fig. 3.4B). Consequently, for subsequent experiments, 3 h shock treatments were employed for *C. psychrophila*.

Figure 3.5A illustrates the absence of a crossprotective effect by a 3 h, 0.2 mM H_2O_2 shock prior to a heat stress as compared to heat shock induced thermotolerance (3 h at 20°C) in logarithmic phase cells. Therefore, an increase in the duration of the peroxide shock did not alter its protective effect (Fig. 3.5A *cf.* Fig. 3.2A). Furthermore as expected, a 3 h, 20°C heat shock or 0.2 mM H_2O_2 did not confer any further tolerance above the high basal tolerance exhibited by stationary phase cells (Fig. 3.5B).

As mentioned previously, cells were relatively thermoresistant to a 35°C heat stress without prior exposure to a heat shock. Now that the temperature and duration of heat shock conferring thermotolerance had been optimized, were these parameters suitable to induce thermotolerance at a higher stress temperature? Firstly, intrinsic tolerance to higher stress temperatures of 38°C, 40°C and 42°C was examined over a 60 min time course (Fig. 3.6). Cells were extremely sensitive to 40°C and 42°C with less than 0.01% survivors and no survivors after 30 min, respectively. Cells were also more sensitive to incubation at 38°C, producing less than 0.1% survivors after 30 min than at 35°C. Consequently, a 38°C was chosen as a higher heat stress temperature. Figure 3.7 illustrates typical results for intrinsic and induced thermotolerance to a 38°c heat stress for mid-logarithmic phase cells of *C. psychrophila*. A marked difference was observed between the induction and duration of tolerance conferred by a 3 h 25°C heat shock as compared to a 3 h 20°C heat shock. After 60 min, a 25°C heat shock produced approximately 1% survivors whereas less than 0.001% survived with a 20°C heat shock

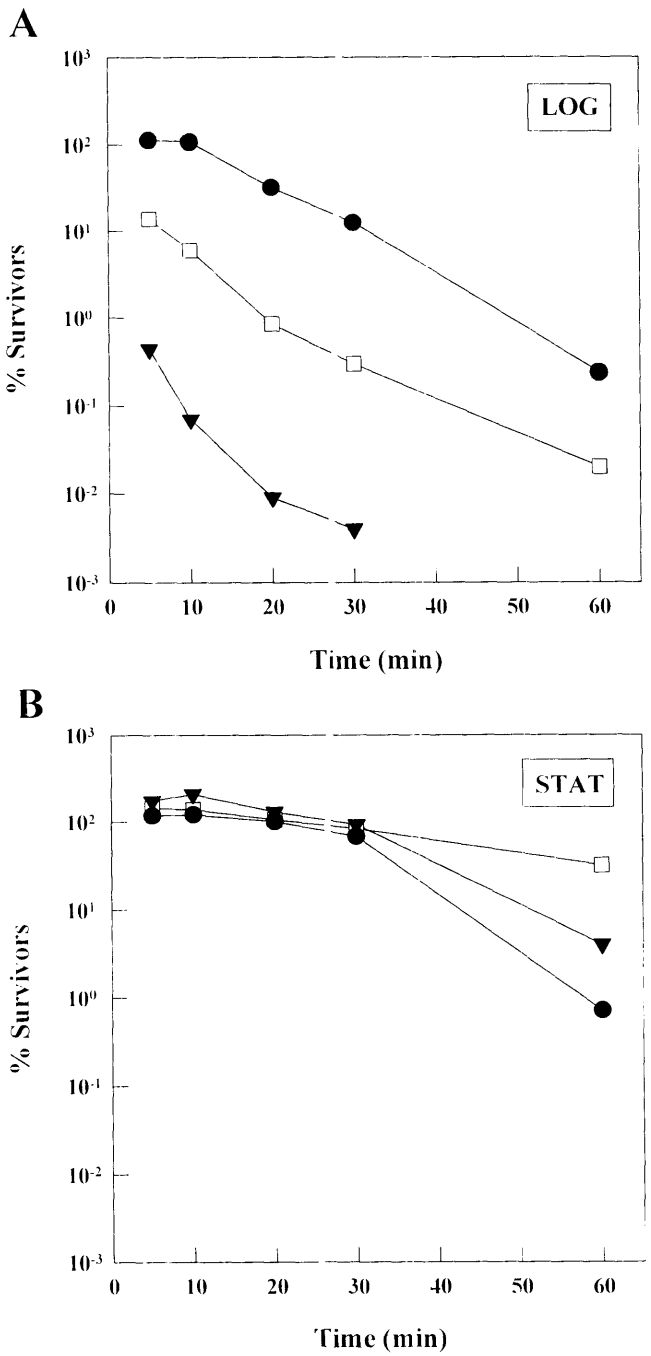

Figure 3.5. The effect of a 3 hour peroxide shock on induced thermotolerance to a 35°C heat stress in (A) mid-logarithmic and (B) stationary phase cultures of *C. psychrophila* grown at 15°C. Intrinsic thermotolerance (□) was measured by transferring cells directly to 35°C. Induced thermotolerance was monitored at 35°C following either a 3 h. 20°C heat shock (●) or a 0.2 mM H_2O_2 shock (▼). Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

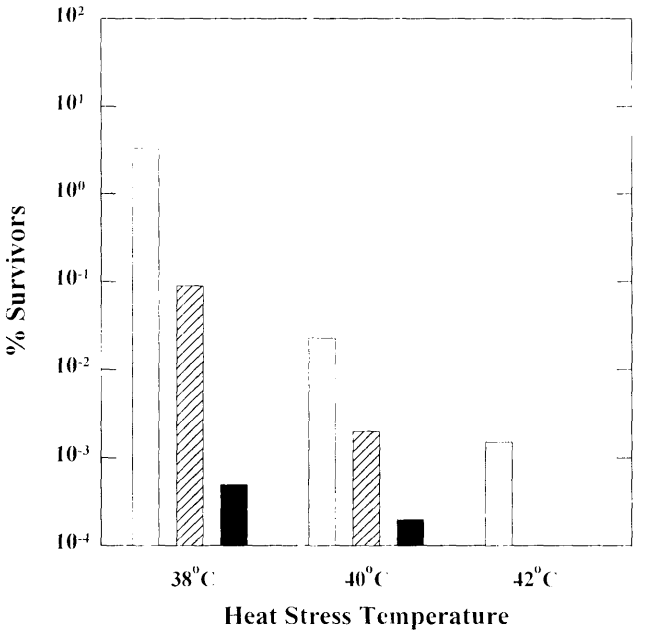

Figure 3.6. Intrinsic thermotolerance to a 38°C, 40°C or 42°C heat stress in mid-logarithmic phase cultures of *C. psychrophila* grown at 15°C. Intrinsic thermotolerance was measured by transferring cells directly to 38°C (▭), 40°C (▨) or 42°C (■). Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

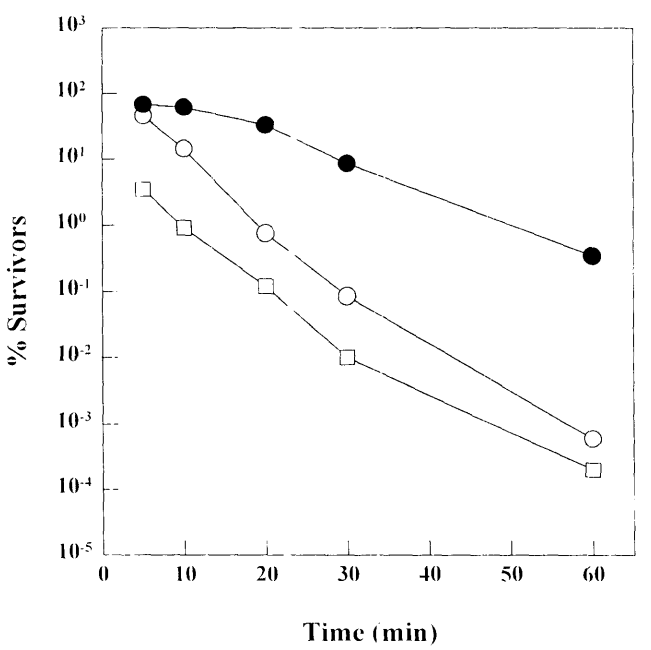

Figure 3.7. Intrinsic and induced thermotolerance to a 38°C heat stress in mid-logarithmic phase cultures of *C. psychrophila* grown at 15°C. Intrinsic thermotolerance (□) was measured by transferring cells directly to 38°C. Induced thermotolerance was monitored at 38°C following either a 3 h, 20°C (○) or 25°C (●) heat shock. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

3.2.3 Southern hybridization analysis

Plasmids harbouring *S. cerevisiae* heat shock genes (Table 2.2) (representing major heat shock gene families) were transformed into *E. coli* and isolated for use in probe construction for identification of homologous heat shock genes in *C. psychrophila* genomic DNA by Southern hybridization analysis. Results are listed in Table 3.1. Homology (under relatively low stringency hybridization and washing conditions) was observed to all *S. cerevisiae* heat shock genes with the exception of HSP 26.

3.2.4 Heat shock proteins

Patterns of protein synthesis in control and heat shocked cells were examined using ^{35}S-methionine labelling (Fig. 3.8). Changes in protein pattern were most pronounced in cells subjected to mild heat shock at 25°C for 3 h (lane 3). In these cells, prominent bands at about 110 and 80 kDa were observed as well as a less prominent heat shock inducible protein at 55 kDa. Protein bands corresponding to hsp 90, hsp 60, as well as a cluster of proteins corresponding to the yeast hsp 70 family were also observed (Fig. 3.8).

These observations were complimented by western blot analysis using antibodies to various *S. cerevisiae* hsps. As illustrated in Fig. 3.9, hsp 60 and 90 were detected in protein extracts from control and heat shocked cells of *C. psychrophila*. In the case of hsp 70, a single band was observed in protein extracts from control and heat shocked cells of *S. cerevisiae* K7 (Fig. 6.7), with a marked induction in the heat shocked cells. In contrast, two intrinsic and three distinct heat shock induced bands were observed in protein extracts from *C. psychrophila*. Of three hsp 70 proteins induced at 20°C, the two higher molecular weight bands showed more intense induction at 25°C. Again, in contrast to *S. cerevisiae* (Fig. 6.7), there was no marked increase in synthesis of hsp 90 or 60 in protein extracts from heat shocked *C. psychrophila* cells.

It was most significant that no protein component detected by anti-hsp104 antibody was evident in control or heat shocked protein extracts from *C. psychrophila*. On the other hand, very strong induction of hsp 104 was observed in protein extracts from the mesophile *S. cerevisiae* (Fig. 6.7).

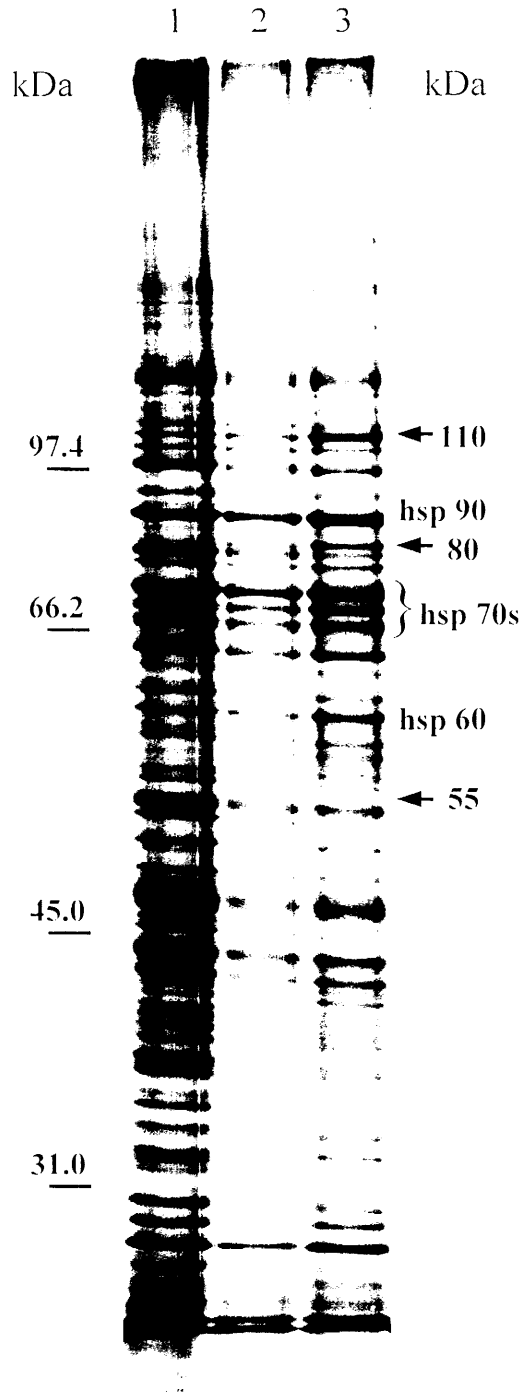

Figure 3.8. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control and heat shocked cells of *C. psychrophila*. Conditions were 15°C control (lane 1), 3 h at 20°C heat shock (lane 2) and 3 h at 25°C heat shock (lane 3). Arrows indicate new or increased heat shock proteins (kDa). Position of protein bands corresponding to hsp 90, 70 and 60 are marked. Molecular mass standards (kDa) are as indicated.

Table 3.1. Southern hybridization analysis with heat shock genes in *C. psychrophila*.

HSP gene probe	Homology[a]
HSP 104	+
SSA1	+
SSC1	+
HSP 60	+
HSP 30	+
HSP26	-
HSP 12	+

[a]Homology detected to HSP gene probes under relatively low stringency hybridization and washes (see section 2.2.7.10).

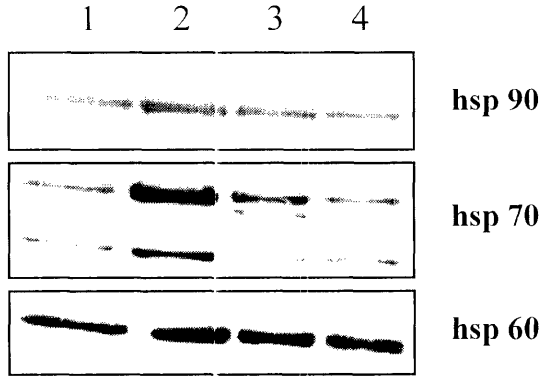

Figure 3.9. Western blot analysis of control and heat shock protein extracts from *C. psychrophila*. Proteins from 15°C control (lane 1), 3 h at 20°C heat shock (lane 2) and 3 h at 25°C heat shock (lane 3) were probed with anti-hsp 104 (1:1000), anti-hsp 90 (1:750), anti-hsp 70 (1:5000) and anti-hsp 60 (1:1000).

3.2.5 Trehalose

There was a two-fold increase in trehalose concentration in cells subjected to heat shock at 25°C for 3 hr (3% w/w) as compared to control cells at 15°C (1.5% w/w) or cells heat shocked at 20°C for 3 hr (1.5% w/w) (Fig. 3.10). The heat shock induced accumulation of trehalose in *C. psychrophila* appears less significant in comparison to *S. cerevisiae* K7 grown at 25°C and heat shocked for 30 min at 37°C where a 17-fold increase in trehalose was observed (Fig. 3.10).

3.2.6 Ethanol tolerance

Figure 3.1 demonstrated the absence of ethanol accumulation in *C. psychrophila* during growth on a 2% glucose source. It was therefore of interest to carry out preliminary examination presumed that this psychrophilic yeast might be ethanol stress sensitive. Tolerance to a 17%, 20% or a 23% ethanol stress was measured over a 4 h time course in *C. psychrophila*. *C. psychrophila* was extremely sensitive to 20% and 23% with no survivors being observed. Figure 3.11 shows results obtained for mid- to late logarithmic phase cells for a 17% ethanol stress. Mid-logarithmic phase cells were more ethanol stress sensitive (less than 0.05% survivors after 1 h) than late logarithmic phase cells (1% survivors after 1 h).

3.2.7 Cold shock

A decrease in temperature from 15°C to 5°C, constituting a cold shock, did not significantly affect growth or trehalose accumulation in *C. psychrophila* over a 6 h time course (Fig. 3.12A). Furthermore, ^{35}S-methionine labelling did not detect cold shock induced *de novo* protein synthesis or decreased synthesis of any particular protein band (Fig. 3.12B).

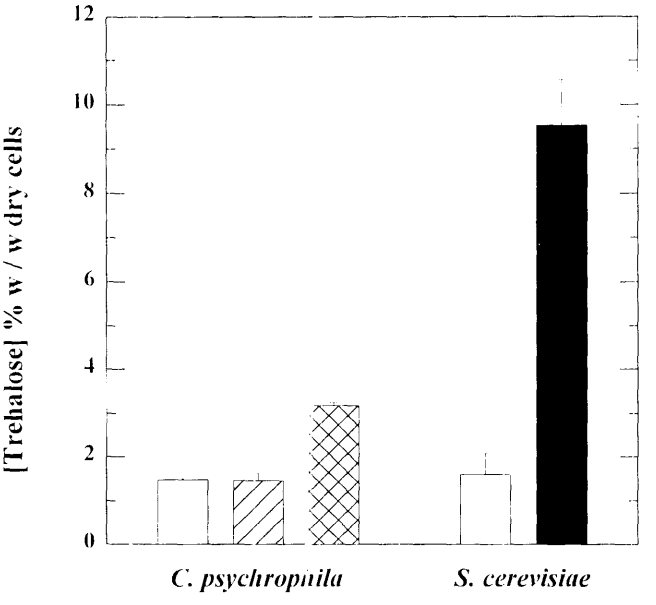

Figure 3.10. Trehalose levels in control and heat shock samples of *C. psychrophila* and *S. cerevisiae* K7. Trehalose was extracted from 15°C control ( ), 3 h, 20°C ( ) and 25°C ( ) heat shock samples from *C. psychrophila* and 25°C control ( ) and 30 min, 37°C ( ) heat shock samples of *S. cerevisiae* K7. Results are presented as mean and standard deviation of three measurements.

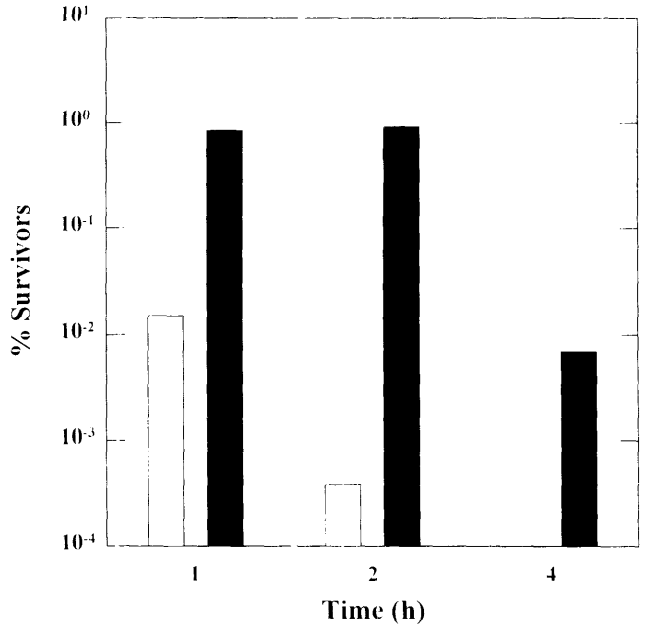

Figure 3.11. Intrinsic ethanol tolerance in mid-logarithmic ( ) and late-logarithmic cells ( ) of *C. psychrophila*. Intrinsic tolerance was measured after addition of 17% ethanol. Levels of tolerance are expressed as the percentage of survivors after treatment with respect to a 15°C control sample.

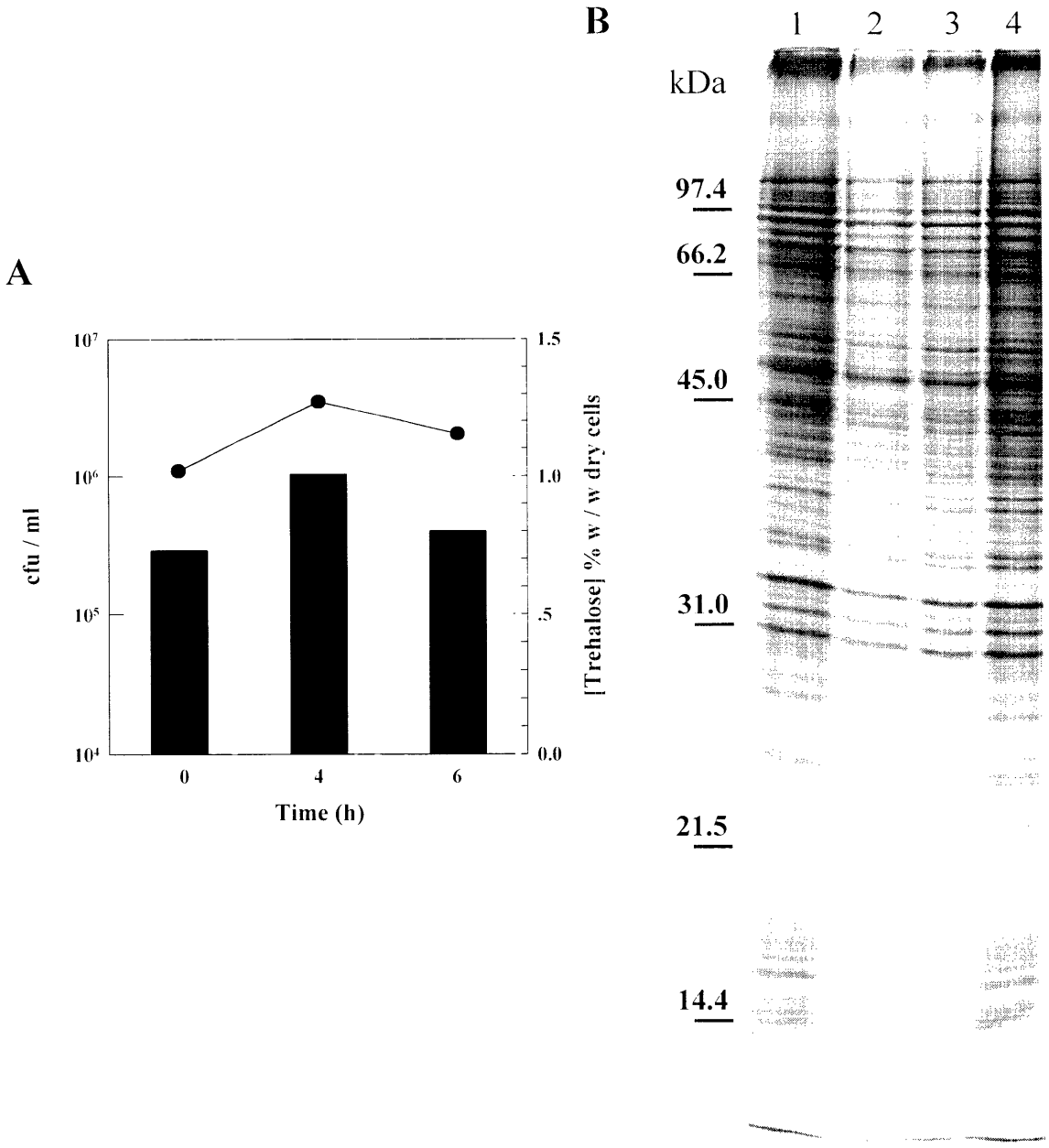

Figure 3.12. The effect of cold shock (5°C) on (A) growth (●), trehalose levels (■) and (B) *de novo* protein synthesis on mid-logarithmic phase cultures of *C. psychrophila* grown at 15°C. (A) Culture density was measured by viable plate counts at 0 h and after 4 and 6 h exposure to 5°C. Trehalose was extracted from control (0 h) and cold shocked samples after 4 and 6 h exposure to 5°C. (B) SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control and cold shock cells of *C. psychrophila*. Conditions were 15°C control (lane 1), 2 h at 5°C (lane 2), 4 h at 5°C (lane 3) and 6 h at 5°C (lane 4). Molecular mass standards (kDa) are as indicated.

3.3. Discussion

3.3.1 Heat shock response

It was of interest to study the heat shock response in psychrophilic yeast as it has been shown that in their natural environment they can survive temperatures above their normal growth temperature, for short periods of time (Inniss, 1975). The ability of psychrophiles to survive at such temperatures may be attributable to stress biomolecules. Investigations of stress tolerance were carried out at a growth temperature of 15°C, the optimum growth temperature of *C. psychrophila* (Goto *et al.*, 1969), rather than at 5°C or 10°C, temperatures normally experienced in the natural environment of an Antarctic psychrophilic yeast (Russell, 1992; Vishniac, 1993). This growth temperature was employed, as growth in liquid and on solid media, even at 15°C, proved to be relatively slow, as anticipated. Results from a single stress tolerance experiment required at least 10 to 14 days growth at 15°C on agar plates for enumeration. It should be pointed out that there are no precedents in the literature for studies on the stress response in psychrophilic yeast, defined herein as unable to grow above 20°C. It was therefore necessary to define conditions for heat shock induced acquisition of thermotolerance and heat shock protein synthesis.

It was noteworthy, although perhaps not surprising, that *C. psychrophila* was extremely sensitive to heat. Exposure of cells for 60 min at temperatures above 38°C rendered cells virtually 100% non-viable as measured by cfu. The relatively low heat stress temperatures of 35°C and 38°C adopted in the present studies, may be contrasted to heat stress temperatures from 48 to 52°C generally used in stress response studies in mesophilic yeasts. In addition, *C. psychrophila* cells were also relatively sensitive to ethanol (see section 3.3.2) and freeze-thaw stress (Hou, personal communication). On the other hand, this organism is relatively resistant to a 100 mM H_2O_2 stress (Fig. 7.1), 5-50 fold higher levels than used in oxidative stress studies in logarithmic phase cells of *S. cerevisiae* (Collinson and Dawes, 1992; Steels *et al.*, 1994).

With respect to heat shock induced thermotolerance, the present data was consistent with the observation that at least a 10°C increase in temperature above the growth temperature of an organism is required to induce thermotolerance (Lindquist, 1986;

Howarth and Ougham, 1993; Cairns *et al.*, 1995). In this regard, it was interesting that a 25°C heat shock provided maximal tolerance to a heat stress even though this temperature lies outside the normal growth temperature range of *C. psychrophila*. It has also been observed in *Trichosporon pullulans*, an Arctic psychrotrophic yeast, that a heat shock temperature outside of its growth range was required to induce thermotolerance (Julseth and Inniss, 1990). 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 (McAlister *et al.*, 1979).

Results presented in this chapter identified the importance of the duration and temperature of heat shock for acquired thermotolerance. A 3 h heat shock at 25°C (Fig. 3.4B) was found to elicit maximal tolerance and induce hsp synthesis in *C. psychrophila* whilst a 30 min 20°C heat shock did not confer tolerance (Fig. 3.4A) or induce *de novo* hsp synthesis (results not shown). Similarities exist in the heat shock temperature and time required for optimal hsp synthesis in other psychrophilic microorganisms. A 2 h, 25°C heat shock elicited hsp synthesis in the psychrophilic pink snow mould fungus, *Monographella nivalis*, grown at 3°C (Cairns *et al.*, 1995). Likewise, a 5 h, 20°C heat shock induced hsp synthesis in the psychrophilic bacterium, *Aquaspirillum arcticum*, grown at 0°C (McCallum *et al.*, 1986). Induced thermotolerance was also measured in *A. arcticum*, albeit at only one time point, after 15 min at a 36°C heat stress (McCallum and Inniss, 1990).

The magnitude of induced thermotolerance was dependent not only on the experimental conditions but also on the growth phase of the cells. In the case of logarithmic phase cells, a 25°C heat shock for 3 h elicited a 5-fold and 20-fold increase in tolerance to a 35°C (Fig. 3.4B) and 38°C (Fig. 3.7) 5 min heat stress, respectively. However, in stationary phase cells tolerance could not be further induced (Fig. 3.5B). These differences were a reflection of the higher intrinsic tolerance (90 – 100%) observed in stationary phase cells. These observations are consistent with previous studies in *S. cerevisiae* which have shown that stationary phase cells are intrinsically more resistant than logarithmic phase cells to a number of environmental stresses including heat (Schenberg-Frascino and Moustacchi, 1972; Parry *et al.*, 1976; Mitchel and Morrison, 1982; Watson, 1990). Also consistent with previous finding in *S. cerevisiae* (Collinson and Dawes, 1992;

Steels *et al.*, 1994) was the absence of peroxide shock induced thermotolerance (Fig. 3.5) in *C. psychrophila*.

With respect to heat shock proteins, ^{35}S- methionine labelling in *C. psychrophila* detected the *de novo* synthesis of three heat shock inducible proteins corresponding to 55, 80 and 110 kDa (Fig. 3.8). In one of the few reports on heat shock induced protein synthesis in eukaryotic microorganisms from polar regions, the induction of 12 proteins ranging from 32 to 84 kDa was reported in *T. pullulans* grown at 5°C and heat shocked at 29°C for 45 min (Julseth and Inniss, 1990a). These authors also reported heat shock induced thermotolerance, albeit tested at only one time point, 5 min at 45°C. 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; Berg *et al.*, 1987; Julseth and Inniss, 1990; McCallum 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.

Heat shock proteins, with the exception of small molecular weight proteins, are highly conserved among widely divergent organisms (Watson, 1990). It was not surprising therefore that Southern hybridization analysis of *C. psychrophila* genomic DNA (Table 3.1) indicated homology (under low stringency conditions) to numerous *S. cerevisiae* HSP genes, including HSP 104, 70 and 60. These results suggested that the use of antibodies directed against hsp 104, 90, 70 (ssa subfamily) and 60 from *S. cerevisiae* could be utilized for Western immunoblot analysis of control and heat shock proteins of *C. psychrophila*.

Western immunoblots (Fig. 3.9) dentified hsps 90, 70 and 60 in protein extracts from *C. psychrophila*, the position of these proteins were also identified with ^{35}S-methionine labelling. However the absence of a strong induction of these hsps (with the possible exception of hsp 70) upon a heat shock argues against their role in the heat shock induced thermotolerance observed in this Antarctic, psychrophilic yeast. Most intriguing was the absence of hsp 104, a key protein in thermotolerance and general stress tolerance in *S. cerevisiae* (Sanchez *et al.*, 1992). On the other hand, the psychrophilic yeast showed the presence of a strongly heat shock inducible protein of about 110 kDa. It may well be

that this protein plays a role in thermotolerance in psychrophilic yeasts, similar to that of hsp 104 in mesophilic species.

Trehalose levels were also examined in *C. psychrophila*, as trehalose has also been implicated as a stress tolerance factor in *S. cerevisiae*. Although trehalose levels did increase up to about 3% w/w upon a heat shock (but not at 20°C) these levels were relatively low as compared to *S. cerevisiae* (Fig. 3.10). Moreover, it has been suggested that a threshold level of around 5% w/w trehalose is required before a significant increase in stress tolerance is observed in mesophilic yeasts (Attfield *et al.*, 1992). Furthermore in *S. cerevisiae*, trehalose levels greater than 5% were not correlated to improved heat stress tolerance (Attfield *et al.*, 1992). Recent observations from our laboratory have strongly supported this concept (Lewis *et al.*, 1997). However, the slight increase in trehalose accumulation observed with a 25°C heat shock in *C. psychrophila* still correlated with maximal thermotolerance induction.

3.3.2 Ethanol tolerance

In contrast to *S. cerevisiae*, ethanol was not produced as a fermentation end product in *C. psychrophila* (Fig. 3.1) and hence this yeast may not have the capacity to adapt to an ethanol stress. The accumulation of ethanol to high concentrations in yeast results in a chemical stress which can be compared to extremes of temperature or pH (Ingram, 1986). The effect of heat shock and ethanol stress to *S. cerevisiae* are very similar with respect to protein synthesis (hsps), perturbation of the plasma membrane, trehalose accumulation and production of reactive oxygen species (reviewed in Mishra, 1993; Piper, 1995). Yeast are generally considered to be the most ethanol tolerant of all microorganisms however, there is a diverse range of tolerances amongst yeast species and even between *S. cerevisiae* strains (Rose, 1987). Results in our laboratory using 17% ethanol (for 1 h) in three *S. cerevisiae* strains show a 100- to 200-fold greater tolerance as compared to *C. psychrophila* (Swan, personal communication). Hence, *C. psychrophila*, a non-ethanologenic yeast, is relatively heat and ethanol stress sensitive. Ethanol sensitivity may in part be attributable to psychrophilic yeast membranes having a high content of polyunsaturated fatty acids (Arthur and Watson, 1976). Ethanol has been shown to increase membrane fluidity in *S. cerevisiae* and increase the concentration of unsaturated fatty acids in membranes (Beavan

et al., 1982; Mishra and Prasad; 1987; Mishra and Prasad; 1988). Consequently, it is possible that the already fluid membrane of *C. psychrophila* may not be able to withstand an ethanol-induced increase in fluidity.

The present preliminary work would benefit from further, more detailed investigations comprising heat or ethanol shock-induced ethanol stress tolerance, as documented in *S. cerevisiae* (Li and Hahn, 1978; Li *et al.*, 1980; Plesset *et al.*, 1982; Watson and Cavicchioli, 1983). In addition, further studies involving the role of hsps and trehalose, as well as the effect of ethanol on membrane composition and fluidity appear warranted.

3.3.3 Cold shock response

Many Antarctic yeast strains have been isolated from soil. Although the temperature remains fairly constant within such an environment, increases and decreases in temperature dependent on soil depth have been observed (Vishniac, 1993). In order to survive, psychrophilic yeast need to adapt not only to increases in temperature but also decreases in temperature. Results presented in Figure 3.12 on cold shock in *C. psychrophila* suggest that a decrease in temperature from 15°C to 5°C for 6 h has little or no effect on viability. Neither cold shock proteins (csps) nor trehalose appear to contribute to cold shock tolerance in *C. psychrophila*. Modification to experimental conditions such as decreasing the temperature to 0°C and increasing the duration of the shock together with two dimensional protein gels may identify a cold shock response in *C. psychrophila*. In *S. cerevisiae*, a decrease in temperature from 30°C to 10°C correlates with the synthesis of csps (Kondo and Inouye, 1991; Kondo and Inouye, 1992; Kondo *et al.*, 1992). The cold shock response has also been demonstrated in the Arctic, psychrotrophic yeast *Trichosporon pullulans* (Julseth and Inniss, 1990b). The synthesis of csps was dependent on the magnitude of the temperature shift with less csps observed when the shift was from 15°C to 5°C as compared to the synthesis of 26 csps when the temperature was decreased from 21°C to 5°C. The levels of csps was maximal 12 h after the downshift in temperature which was considerably more than the time required (45 min) to induce maximal synthesis of hsps (Julseth and Inniss, 1990ab) As for acquisition of thermotolerance, the adaptability of an organism to low temperature may depend not only on the synthesis of

csps but also on the regulation and interaction of a number of cellular components (Broeze *et al.*, 1978; Margesin and Schinner, 1994).

In contrast to cold shock studies in *C. psychrophila*, results from this laboratory have shown psychrophilic yeast to be particularly sensitive to freeze-thaw stress (-70°C) as compared to *S. cerevisiae* (Hou, personal communication). These findings were consistent with those of Meyer *et al.* (1975), who examined the survival and metabolic activity of psychrophilic and mesophilic yeast subjected to freeze-thaw stress and found that psychrophilic, *L. stokesii* (*M. stokesii*) was more freeze-thaw sensitive than the mesophilic yeast, *C. utilis*. However, resistance was growth phase dependent with stationary phase cells exhibiting more freeze-thaw stress resistance than logarithmic phase cells (Meyer *et al.*, 1975). The sensitivity of psychrophilic yeast appears somewhat surprising, given that these yeast are highly adapted to growth at low temperatures and in their natural habitat are subject to seasonal freeze-thaw stress with varying frequency (Wynn-Williams, 1980; Vishniac, 1993). However, it is possible that while psychrophilic yeast are ecologically more adapted for growth at constantly low temperatures than mesophilic yeast, their restricted growth temperature range increases their sensitivity to extremes of temperature and dramatic fluctuations in temperature, while mesophilic yeast may be able to adapt to large and rapid temperature changes more readily because of a less restricted growth temperature range.

CHAPTER 4: Heat Shock Response in Antarctic Psychrophilic and Psychrotrophic Yeast.

4.1 Introduction

Results from Chapter 3, recently published (Deegenaars and Watson, 1997), established the conditions under which an Antarctic psychrophilic yeast exhibits a heat shock response. The next question to be considered was whether the identified heat shock response was unique to *C. psychrophila* or whether the characteristics of and conditions inducing the response are common to other Antarctic psychrophilic yeast. This chapter extends the examination of the heat shock response to four additional psychrophilic yeast, *Mrakia frigida*, *M. gelida*, *M. stokesii* and *Leucosporidium antarcticum*. In addition, two psychrotrophic yeast, *Leucosporidium jellii* and *L. scottii* were also included in the analysis.

Psychrotrophic yeast are less restricted in their growth temperature ranges than psychrophilic yeast. Often the environment of psychrotrophic microorganisms is exposed to greater fluctuations in temperature whereas those of psychrophilic microorgansims are more likely to be permanently cold (Gounot, 1991). Studies of the heat shock response in psychrotrophic yeast were undertaken at two growth temperatures, 15°C and 25°C, corresponding to growth temperatures used for *C. psychrophila* (Chapter 3) and *S. cerevisiae* respectively. The relative contributions of the two key stress biomolecules, hsps and trehalose were also examined.

4.2 Experimental outline

Growth curves were constructed for all psychrophilic yeast grown at 15°C and psychrotrophic yeast grown at both 15°C and 25°C to observe any differences in growth rates (doubling time). Experimental cultures were grown to an OD_{600} of 0.2 to 0.3 corresponding to logarithmic phase cells of approximately 2×10^6 to 5×10^6 cfu ml^{-1}.

Psychrophilic yeast were grown at 15°C and heat stressed to 35°C and 38°C, to allow for inherent differences in intrinsic tolerance between species. Induced

thermotolerance to 35°C or 38°C was examined after a 20°C or 25°C heat shock for 3 h.

Likewise, psychrotrophic yeast grown at 15°C were heat stressed to 35°C and 38°C for investigations of intrinsic thermotolerance. The same conditions as for psychrophilic yeast were used in addition to a 37°C heat shock for 3 h for studies of induced thermotolerance.

In contrast, conditions for the analysis of the heat shock response in psychrotrophic yeast grown at 25°C differed from those grown at 15°C. Initial investigations were carried out to identify intrinsic resistance to higher heat stress temperatures. Consequently, both a 38°C and a 42°C heat stress were adopted for investigations of both intrinsic and induced stress tolerance. Induced tolerance was monitored following a 30°C or 37°C heat shock for 30 min.

Heat shock proteins and trehalose levels were examined for all control and heat shock samples. Isolation and preliminary characterization of hsp 110 from *M. stokesii* was also undertaken.

4.3 Results

4.3.1 Growth curves

Growth curves for the psychrophilic yeast, *M. frigida*, *M. gelida*, *M. stokesii* and *L. antarcticum* at 15°C are illustrated in Figure 4.1. All psychrophilic yeast exhibited relatively long logarithmic phases (approx. 30 h) similar to that observed for *C. psychrophila* (Fig. 3.1). Likewise, growth curves of the psychrotrophic yeast grown at 15°C showed slow growth (Fig. 4.2). When *L. fellii* was grown at 25°C a different growth profile was observed with even slower growth (Fig. 4.2A). However, this trend was not observed in the psychrotrophic yeast, *L. scottii* grown at 25°C (Fig. 4.2B), although growth was slightly slower at 25°C with lower maximal cell density than for cultures grown at 15°C. Doubling times of psychrophilic and psychrotrophic yeast grown at the different temperatures were calculated from growth curves and are presented in Table 4.1.

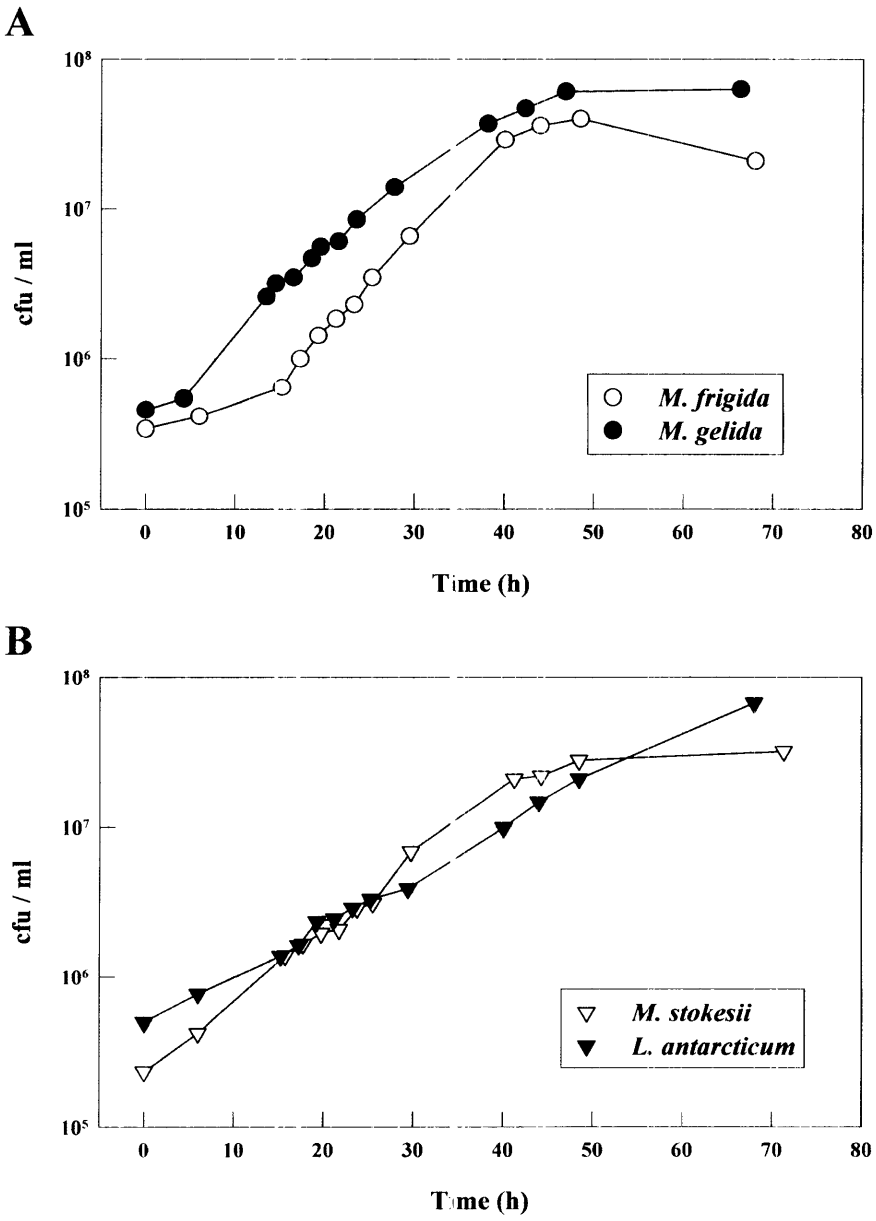

Figure 4.1. Growth curves of the Antarctic, psychrophilic yeast (A) *M. frigida*, (○), *M. gelida* (●), (B) *M. stokesii* (▽) and *L. antarcticum* (▼) grown at 15°C.

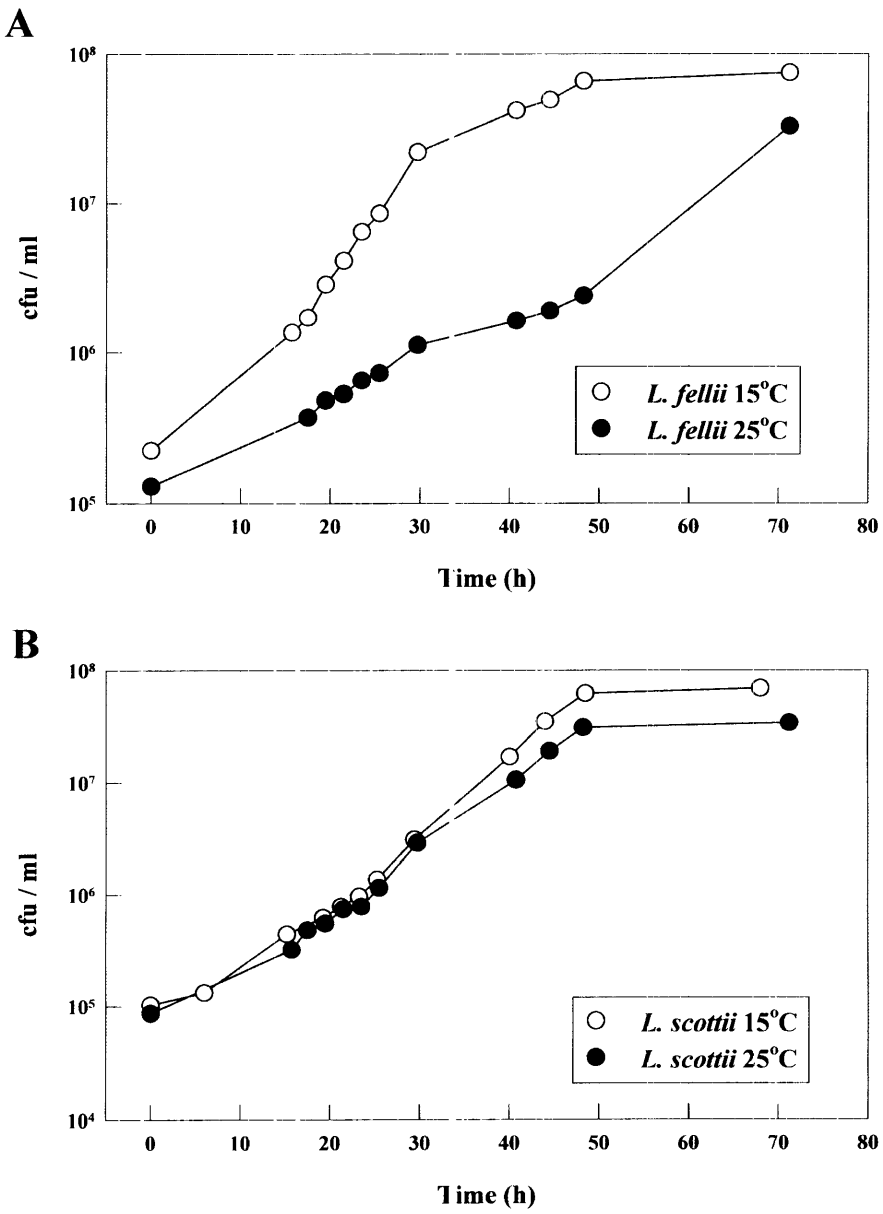

Figure 4.2. Growth curves of the Antarctic, psychrotrophic yeast (A) *L. fellii* and (B) *L. scottii* grown at 15°C (O) and 25°C (●).

Table 4.1. Culture doubling times for psychrophilic and psychrotrophic yeast.

Yeast	Growth temperature (°C)	Doubling Time (h)
C. psychrophila	15	7
M. frigida	15	6
M. gelida	15	5
M. stokesii	15	5
L. antarcticum	15	8
L. fellii	15	4
	25	7
L. scottii	15	4
	25	6

4.3.2 Thermotolerance

Intrinsic and heat shock induced thermotolerance were measured to both a 35°C and 38°C heat stress in the psychrophilic yeast *M. frigida*, *M. gelida*, *M. stokesii* and *L. antarcticum* (Fig. 4.3; Fig. 4.4) grown at 15°C. Intrinsic tolerance levels were 10 – 100 fold greater after a 5 min, 35°C heat stress as compared to a 5 min, 38°C heat stress. Thermotolerance was induced to both a 35°C and 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 3 h, 25°C heat shock elicited maximal induction and duration of tolerance to a 38°C heat stress (Fig 4.3B and D; Fig. 4.4B and D). However, characteristic species specific responses were observed at the slightly lower heat stress temperature of 35°C. In *M. stokesii* (Fig. 4.4A) and *L. antarcticum* (Fig. 4.4C), 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. 4.3A) a 3h, 25°C heat shock evoked greater adaptation to a 35°C heat stress than a 3h, 20°C heat shock. Conversely, in *M. gelida* (Fig. 4.3C) a 3 h, 20°C heat shock rather than a 3h, 25°C heat shock conferred maximal tolerance to a 35°C heat stress. Duration and level of induced thermotolerance in all psychrophilic yeast was markedly greater for cells stressed to 35°C than those exposed to a 38°C heat stress.

The psychrotrophic yeast *L. fellii* and *L. scottii* were grown at 15°C and 25°C corresponding to growth temperatures used for psychrophilic and mesophilic yeast, respectively. In *L. fellii* and *L. scottii* (Fig. 4.5) grown at 15°C, a 3h, 25°C heat shock

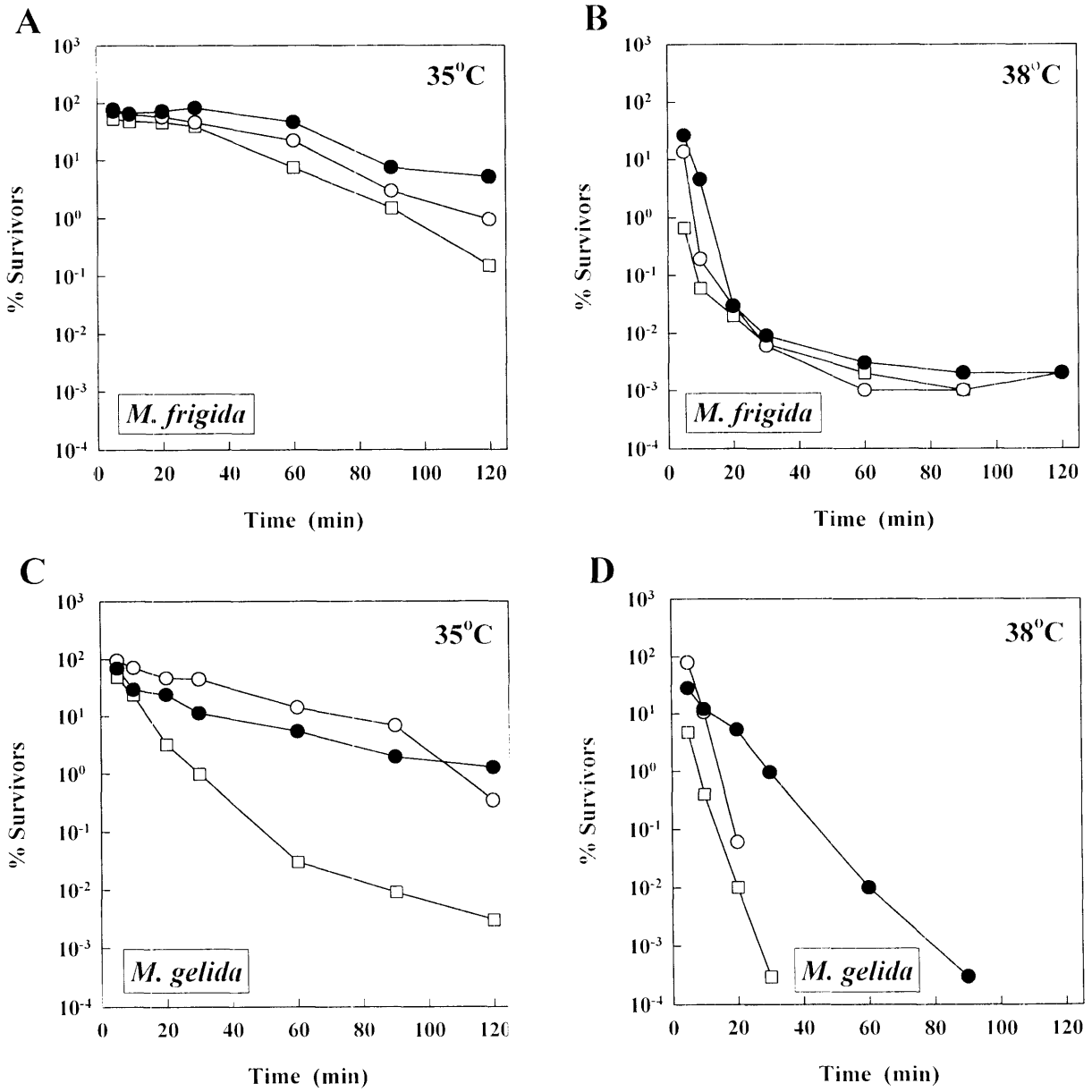

Figure 4.3. Intrinsic and induced thermotolerance in mid-logarithmic phase cultures of *M. frigida*. (A and B) and *M. gelida* (C and D) grown at 15°C. Intrinsic tolerance (□) was measured by transferring cells directly to 35°C (A and C) or 38°C (B and D). Induced thermotolerance was monitored at 35°C or 38°C following either a 20°C (○) or 25°C (●), 3 h heat shock. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

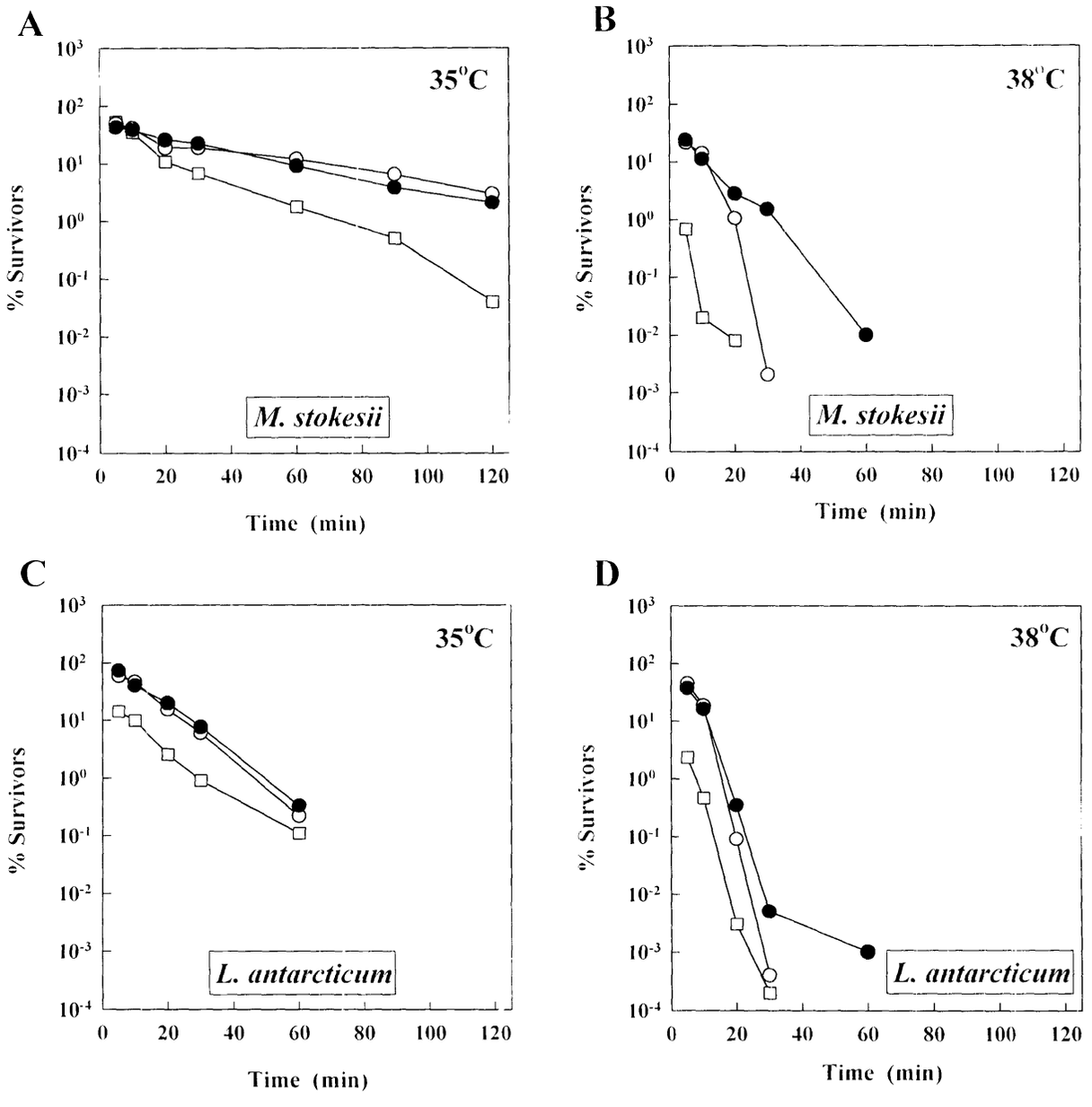

Figure 4.4. Intrinsic and induced thermotolerance in mid-logarithmic phase cultures of *M. stokesii*, (A and B) and *L. antarcticum* (C and D) grown at 15°C. Intrinsic tolerance (□) was measured by transferring cells directly to 35°C (A and C) or 38°C (B and D). Induced thermotolerance was monitored at 35°C or 38°C following either a 20°C (○) or 25°C (●), 3 h heat shock. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

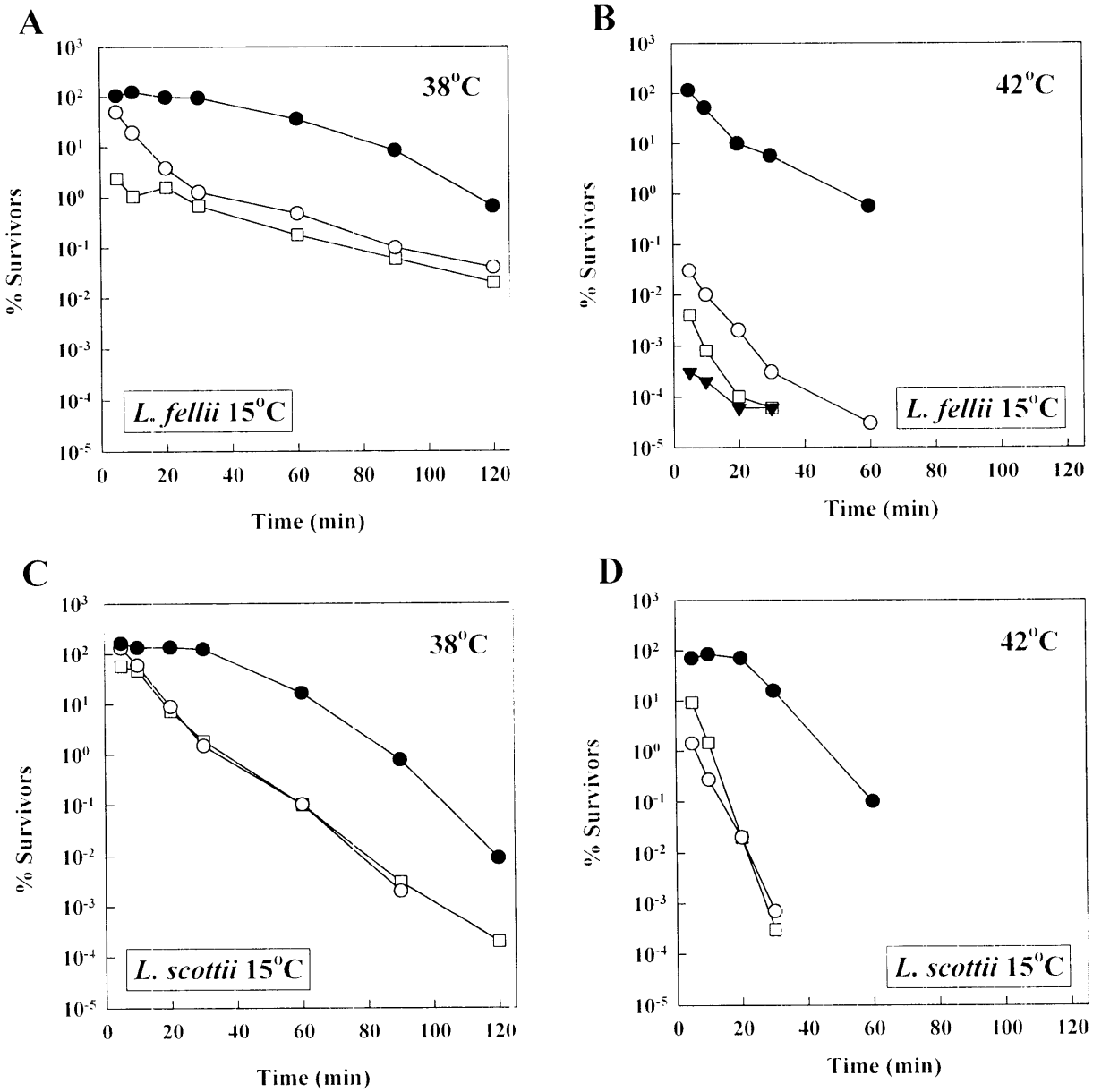

Figure 4.5. Intrinsic and induced thermotolerance in mid-logarithmic phase cultures of *L. fellii* (A and B) and *L. scottii* (C and D) grown at 15°C. Intrinsic tolerance (□) was measured by transferring cells directly to 38°C (A and C) or 42°C (B and D). Induced thermotolerance was monitored at 38°C or 42°C following either a 20°C (○), 25°C (●) or 37°C (▼), 3 h heat shock. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

elicited maximal tolerance to both a 38°C and 42°C heat stress. Generally, a 3h, 20°C heat shock did not confer any further tolerance to a heat stress above intrinsic levels. Intrinsic thermotolerance levels to a 38°C heat stress 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 corresponding to the heat stress temperature chosen for studies of intrinsic and induced thermotolerance in psychrotrophic yeast grown at 25°C. A heat stress temperature of 42°C was chosen for psychrotrophic yeast grown at 25°C following preliminary investigations of intrinsic tolerance to heat stress temperatures of 48°C, 45°C and 42°C. Intrinsic tolerance to a heat stress at 48°C for 5 min was lethal for *L. fellii* and resulted in only 0.001% survivors for *L. scottii*. Reducing the heat stress temperature to 45°C for 5 min resulted in 4.75% and 0.014% survivors for *L. scottii* and *L. fellii*, respectively whereas a 42°C heat stress produced survivors for the duration of the 60 min time course in both psychrotrophic yeast. Figures 4.5B and D exhibit intrinsic and induced thermotolerance to a 42°C heat stress in *L. fellii* and *L. scottii* grown at 15°C, respectively. A similar response to psychrophilic yeast was observed in that a 3h, 25°C heat shock induced maximal tolerance. However, neither a 3h, 20°C nor a 3h, 37°C heat shock induced any further thermotolerance to 42°C. Indeed, a 3 h, 37°C heat shock was detrimental to cell viability with complete loss of viability after the heat shock for *L. scottii* (hence lack of data points on Fig. 4.5D for induced thermotolerance following a 37°C heat shock). When *L fellii* was grown at 25°C (Fig. 4.6A), intrinsic resistance to a 42°C heat stress was greater than when cells were grown at 15°C. In *L. scottii* grown at 25°C (Fig. 4.6B) intrinsic tolerance levels were essentially the same as when grown at 15°C. A prior 30 min, 30°C heat shock induced thermotolerance in both psychrotrophic yeast. However, a 30 min, 37°C heat shock did not induce tolerance to a 42°C heat stress over and above that of intrinsic thermotolerance levels.

4.3.3 Trehalose

Trehalose levels in control cultures of psychrophilic yeast (Fig. 4.7A) differed, with *L. antarcticum* having the greatest intrinsic levels (approx. 7% w/w). A 3 h, 25°C heat shock elicited maximal trehalose accumulation in all of the psychrophilic yeast. However, a 3h, 20°C heat shock also increased trehalose levels slightly above control levels.

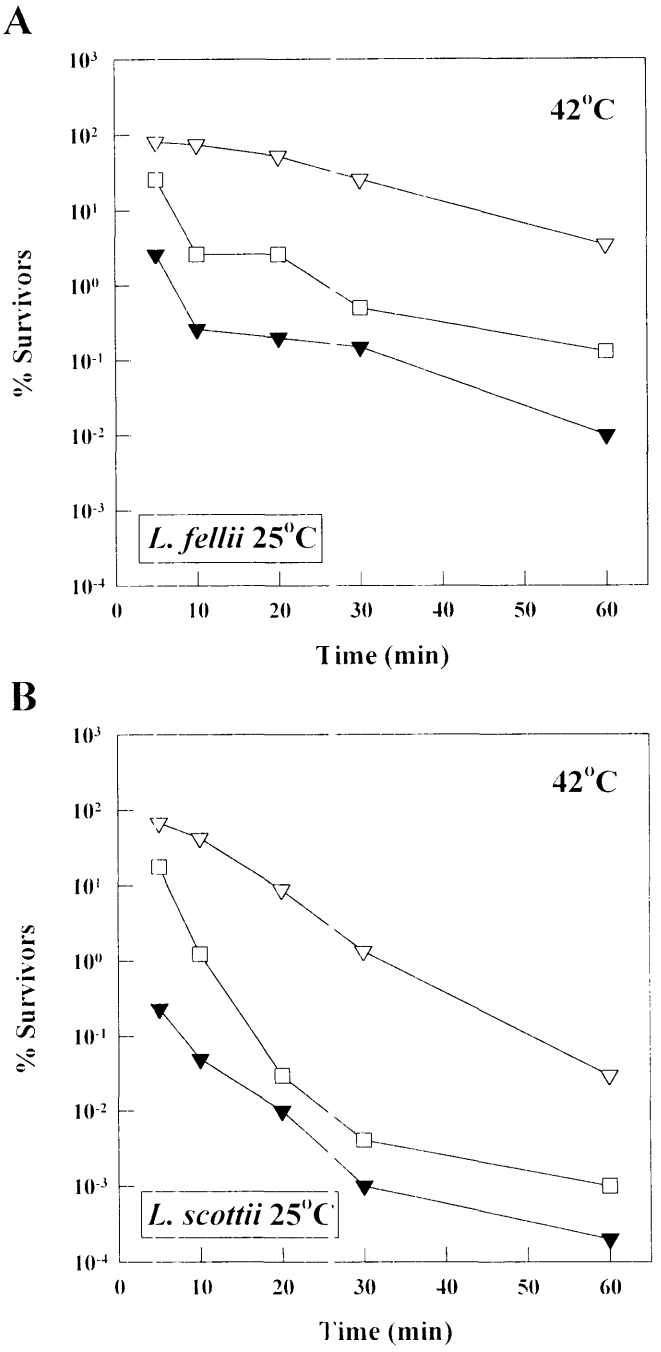

Figure 4.6. Intrinsic and induced thermotolerance in mid-logarithmic phase cultures of *L. fellii* (A and B) and *L. scottii* (C and D) grown at 25°C. Intrinsic tolerance (□) was measured by transferring cells directly to 42°C. Induced thermotolerance was monitored at 42°C following either a 30°C (▽) or 37°C (▼), 30 min heat shock. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 25°C control sample.

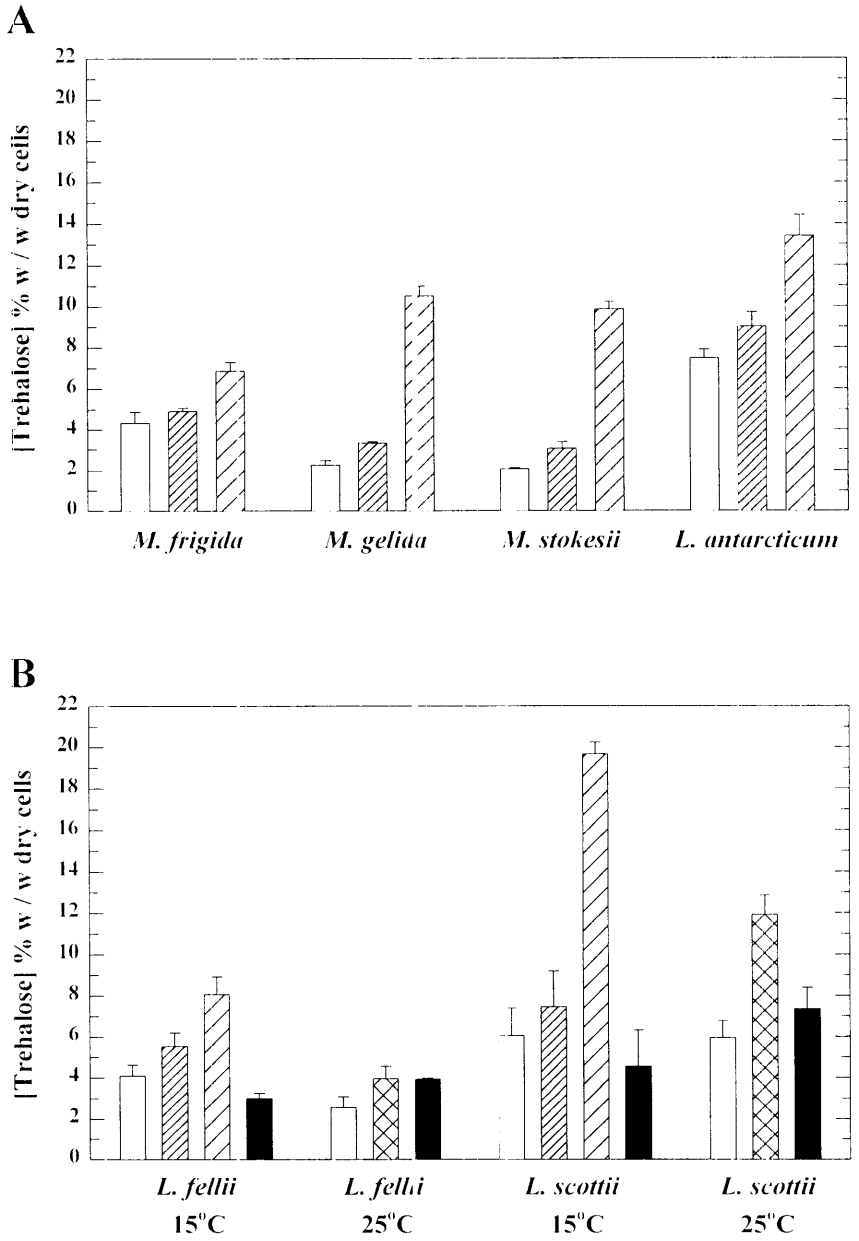

Figure 4.7. Trehalose levels in control and heat shock samples of (A) *M. frigida*, *M. gelida*, *M. stokesii* and *L. antarcticum* and (B) *L. fellii* and *L. scottii* (grown at either 15°C or 25°C). Trehalose was extracted from a 15°C or 25°C control ( ), a 3 h, 20°C heat shock ( ), a 3 h, 25°C heat shock ( ), a 30 min, 30°C heat shock ( ) and a 3 h (for 15°C grown cultures) or 30 min (for 25°C grown cultures), 37°C heat shock ( ). Results are presented as mean and standard deviation of three measurements.

In psychrotrophic yeast (Fig. 4.7B) grown at 15°C the pattern of trehalose accumulation resembled that of the psychrophilic yeast. Trehalose accumulation was maximal after a 3h, 25°C heat shock and a 3 h, 20°C heat shock increased levels slightly above control levels. The greatest increase was observed in *L. scottii* with a 3-fold increase in trehalose after a 3h, 25°C heat shock. Interestingly, a 3h, 37°C heat shock decreased trehalose levels below those of control cells. For psychrotrophic yeast grown at 25°C, a 30 min, 30°C heat shock induced the greatest increase in trehalose accumulation. In contrast to 15°C grown cells, a 30 min, 37°C heat shock in 25°C grown cells increased trehalose levels slightly above control trehalose levels.

4.3.4 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 25°C heat shock for 3 h (Fig. 4.8). 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 3 h, 25°C heat shock 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 4.9A). In *L. fellii* and *L. scottii*, a heat shock (25°C, 3 h) inducible protein at about 100 kDa was identified as hsp 104 by western blot analysis (Fig. 4.10B). In addition, a 25°C heat shock also induced proteins of 42 and 83 kDa in *L. scottii* and an 85 kDa protein in *L. fellii*. However, a 3 h, 20°C heat shock did not increase the synthesis of any observable proteins in either psychrotrophic yeast. In addition, a 3 h, 37°C heat shock inhibited *de novo* protein synthesis (lanes 4).

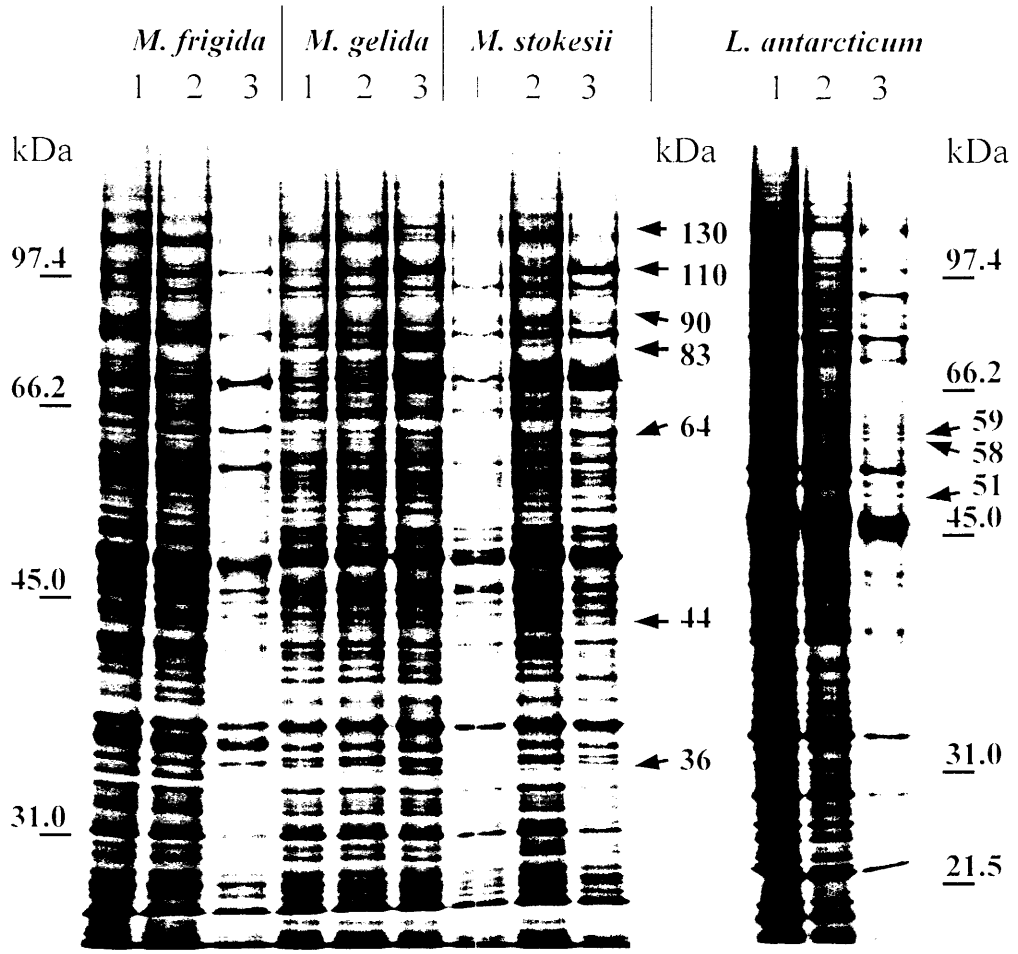

Figure 4.8. SDS-polyacrylamide gel autoradiograms of ^{35}S-methionine labelled protein extracts from control and heat shocked cells of *M. frigida*, *M. gelida*, *M. stokesii* and *L. antarcticum*. Conditions were 15°C control (lanes 1), 3 h at 20°C heat shock (lanes 2) and 3 h at 25°C heat shock (lanes 3). Arrows indicate new or increased heat shock protein synthesis (kDa). Molecular mass standards (kDa) are as indicated.

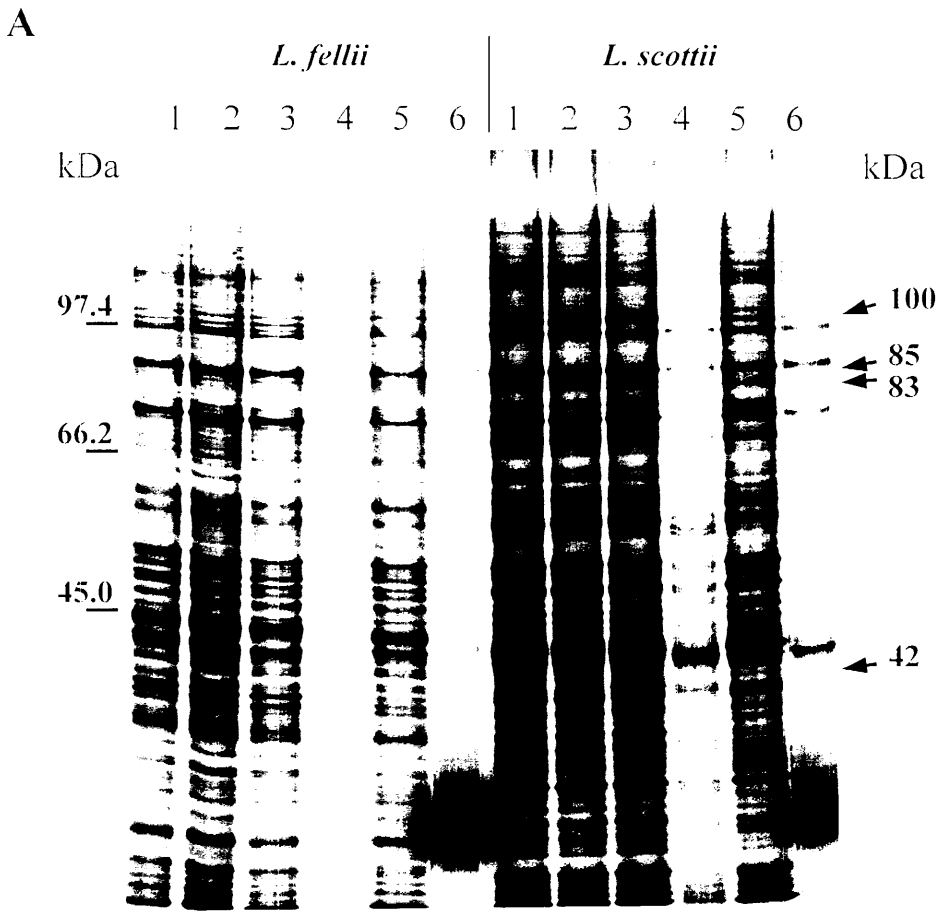

Figure 4.9. SDS-polyacrylamide gel autoradiograms of ^{35}S-methionine labelled protein extracts from control and heat shocked cells of *L. fellii* and *L. scottii* grown at 15°C and 25°C. Conditions for (A) were 15°C control (lanes 1), 3 h at 20°C heat shock (lanes 2), 3 h at 25°C heat shock (lanes 3) and 3 h a 37°C heat shock (lanes 4) for 15°C grown cultures. For 25°C grown cultures, conditions were 25°C control (lanes 5) and 30 min at 37°C heat shock (lanes 6). Conditions for (B) were 25°C control (lanes 1) and 30 min at 30°C heat shock (lanes 2) for 25°C grown cultures. For (A) and (B), arrows indicate new or increased heat shock protein synthesis (kDa). Molecular mass standards (kDa) are as indicated.

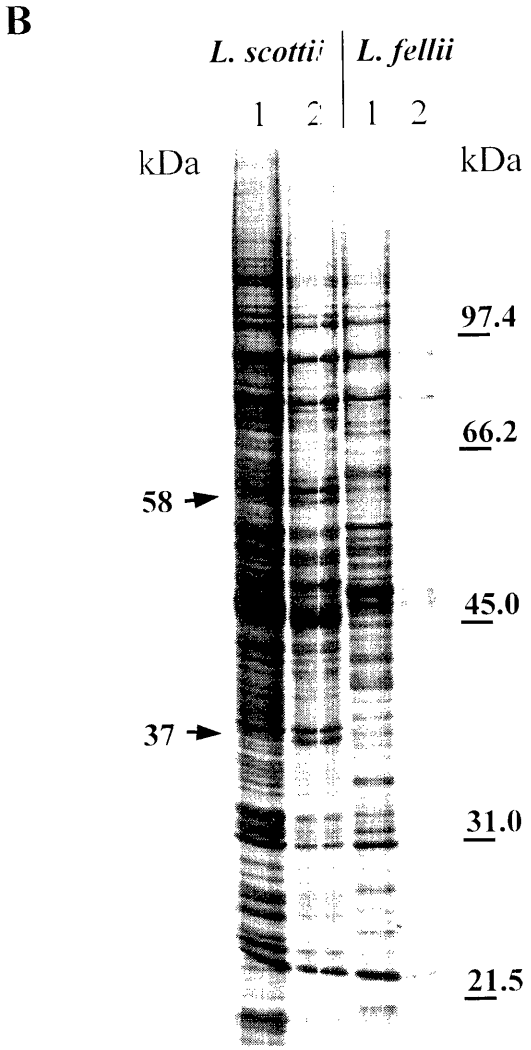

Figure 4.9. continued.

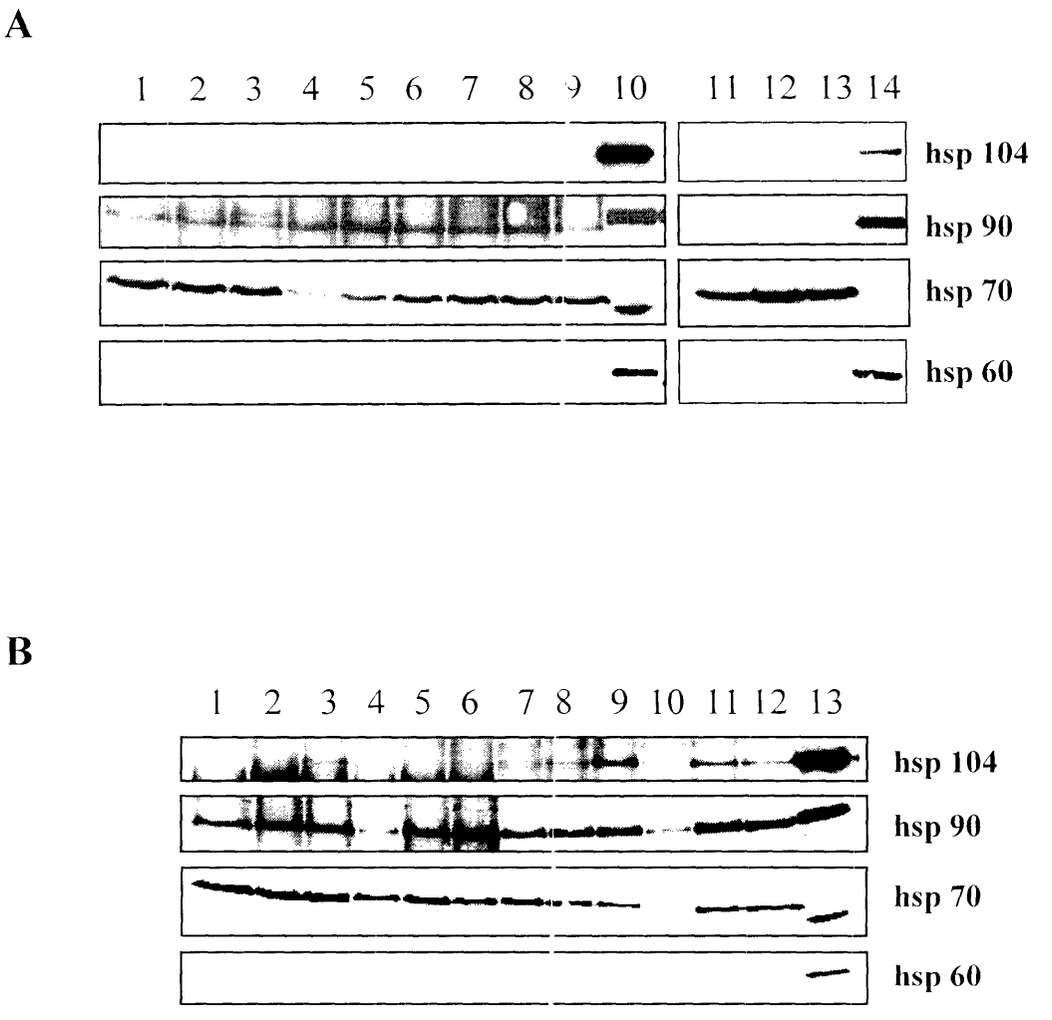

Figure 4.10. Western blot analysis of control and heat shocked protein extracts from (A) psychrophilic yeast and (B) psychrotrophic yeast. (A) Conditions were 15°C control, 3 h at 20°C heat shock and 3 h at 25°C heat shock for *M. frigida* (lanes 1,2 and 3, respectively), *M. gelida* (lanes 4, 5 and 6, respectively), *M. stokesii* (lanes 7, 8 and 9, respectively) and *L. antarcticum* (lanes 11, 12 and 13, respectively). Lanes 10 and 14 correspond to a 30 min 37°C heat shock protein sample from *S. cerevisiae* K7. (B) Conditions were 15°C control, 3 h at 20°C heat shock, 3 h at 25°C heat shock and 3 h at 37°C heat shock for 15°C grown *L. fellii* (lanes 1, 2, 3 and 4, respectively) and *L. scottii* (lanes 7, 8, 9 and 10, respectively). Proteins were also extracted from 25°C control and 30 min at 37°C heat shock samples of 25°C grown *L. fellii* (lanes 5 and 6, respectively) and *L. scottii* (lanes 11 and 12, respectively). Lane 13 corresponds to a 30 min 37°C heat shock protein sample from *S. cerevisiae* K7. All protein samples were probed with anti-hsp 104 (1:1000), anti-hsp 90 (1:750), anti- hsp 70 (1:5000) and anti-hsp 60 (1 in 1000).

Differences and similarities exist in the *de novo* protein profiles of psychrotrophic yeast grown at 25°C to those grown at 15°C (Fig. 4.9A and B). A 30 min, 37°C heat shock decreased *de novo* protein synthesis in both *L. fellii* and *L. scottii* (Fig. 4.9A, lanes 6). No clear heat shock inducible proteins were visible at either 104 or 110 kDa upon a 30 min, 30°C heat shock (Fig. 4.9B). However, a 30°C heat shock 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 30°C heat shock inhibited *de novo* protein synthesis (Fig. 4.9B).

These observations were extended by western blot analysis using 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. 4.10A, 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 shock 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 shock samples of all psychrophilic yeast 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 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. 4.10B). In *L. fellii* grown at 15°C, a hsp 104 homolog was induced by a 3 h, 25°C heat shock. However, when *L. fellii* was grown at 25°C, no hsp 104 homolog was observed in either control or heat shock samples. Anti-hsp 104 antibody also detected a protein counterpart in *L. scottii* grown at 15°C and heat shocked at both 20°C and 25°C and also in cultures grown at 25°C and heat shocked at both 30°C (results not shown) and 37°C (Fig. 4.10B). Hsp 60 counterparts were not detected by anti-hsp 60 antibody in psychrotrophic yeast grown at either temperature. With respect to hsp 70, single bands were observed in all protein samples of psychrotrophic yeast (Fig. 4.10B). 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 37°C heat shock decreased levels of hsp 70, 90 and 104 in 15°C grown psychrotrophic yeast (Fig. 4.10B).

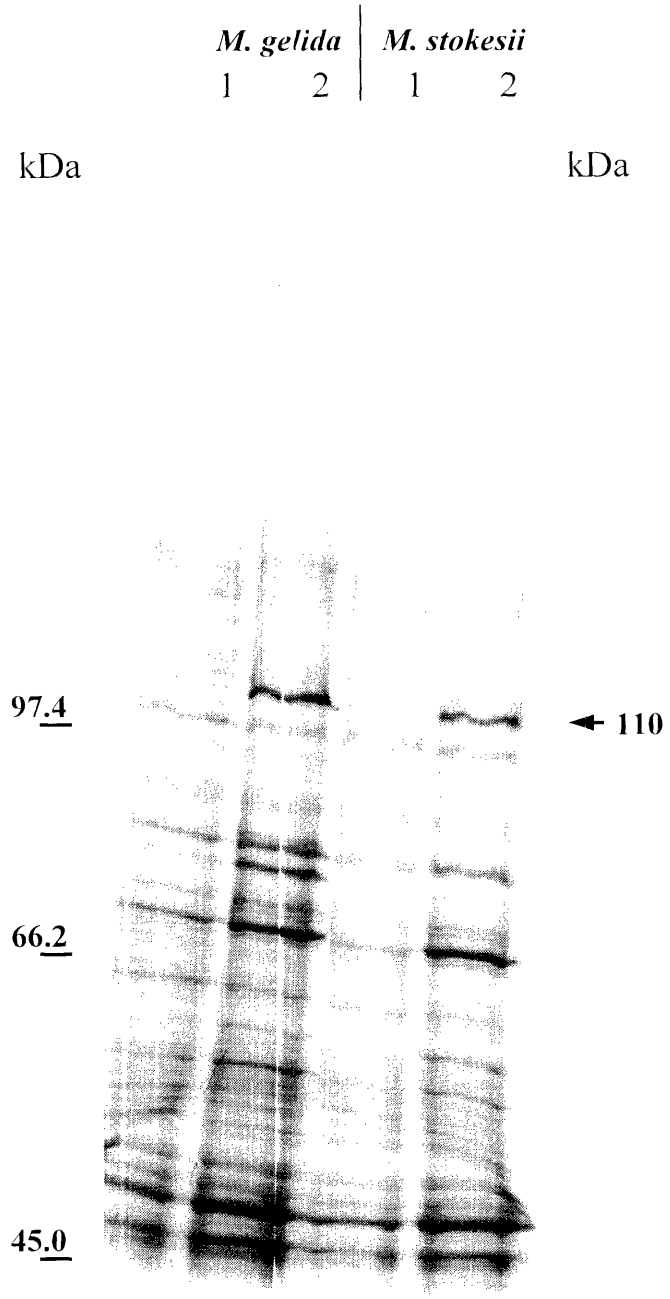

Figure 4.11. SDS-polyacrylamide gel (6%) autoradiograms of ^{35}S-methionine labelled protein extracts from control and heat shocked cells of *M. gelida* and *M. stokesii* used to help identify the position of the 110 kDa protein. Conditions were 15°C control (lanes 1), and 3 h at 25°C heat shock (lanes 2). Arrow indicates position of hsp 110. Molecular mass standards (kDa) are as indicated.

```
1. HSP 110:          1 NVLADNINGALXNKD 15
                       N+L D   +G L NKD
   ORF YKR021w:  442 NILLDEKSGDLVNKD 456
```

ORF YKR021w *Saccharomyces cerevisiae* chromosome XI

EMBL locus SCYKR021W, accession Z28246

Identities = 8/15 (53%), Positives = 10/15 (66%)

```
2. HSP 110:          2 VLADNINGALXNK 14
                       ++ DNI+G L NK
   F2,6BP:       267 IVKDNIHGYLVNK 279
```

Similar to *Saccharomyces cerevisiae* Fructose-2,6-bisphosphatase

GENBANK locus YSCL8300, accession U19028

Identities = 7/13 (53%), Positives = 10/13 (76%)

```
3. HSP 110:          1 NVLADNINGALXNKD 15
                       N+L D  NG L NK+
   ORF YJL084c   451 NLLQDPKNGDLVNKE 465
```

ORF YJL084c *Saccharomyces cerevisiae* chromosome X

EMBL locus SCYJL084C, accession Z49359

Identities = 8/15 (53%), Positives = 10/15 (66%)

Figure 4.12. BLASTP search of amino acid sequence homology of *M. stokesii* hsp 110 to *S. cerevisiae* protein sequences. Positions of homology to each sequence are shown by residue number and identical residues are indicated by the corresponding one letter amino acid code. Positive residues are indicated by a +.

4.3.5 Isolation and preliminary characterization of the 110 kDa hsp

To isolate the novel 110 kDa heat shock inducible protein, 6% polyacrylamide gels were used to allow separation of higher molecular weight proteins. As shown in Figure 4.11, ^{35}S-methionine labelled control and heat shock samples of *M. gelida* and *M. stokesii* were used to help identify the position of the 110 kDa protein on a 6% gel. Numerous unlabelled heat shock protein samples (50 µg) from *M. stokesii* were electrophoresed on 6% polyacrylamide gels, electroblotted onto PVDF membrane, stained with Coomassie brilliant blue, isolated and pooled. N-terminal sequence analysis was carried out by the Australian Proteome Analysis Facility (Macquarie University Centre for Analytical Biochemistry, NSW, Australia). The predicted 15 amino acid sequence was N V L A D N I N G A L X N K D. Figure 4.12 shows the results obtained for a BLASTP (Altschul *et al.*, 1990) search of amino acid sequence homology to *S. cerevisiae* protein sequences. Homology was observed to two open reading frames coding for proteins of unknown function as well as a protein having similarities to fructose-2, 6-bisphosphatase.

4.4 Discussion

4.4.1 Thermotolerance

This Chapter and Chapter 3 represent a significant analysis of the heat shock response in yeast from Antarctica. In total, five out of the seven known Antarctic psychrophilic yeast species, other than those in the genus *Cryptococcus*, constituting three out of the five known psychrophilic yeast genera have now been investigated with respect to the heat shock response.

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 3 h, 25°C heat shock (a heat shock temperature 10°C above the optimum growth temperature) induced maximal tolerance to a 38°C heat stress (Fig. 4.3B and D; Fig. 4.4B and D.). This heat shock temperature also stimulated maximal accumulation of trehalose (Fig. 4.7A) and hsp synthesis (Fig. 4.8; Fig. 4.10A). These results are consistent with the observed findings in *C. psychrophila* (Chapter 3).

In psychrophilic yeast, it was observed that the temperature that induces thermotolerance varies depending on the heat stress temperature. For a 38°C heat stress, a 3h, 25°C heat shock elicited maximal tolerance in all psychrophilic yeast (Fig. 4.3B and D; Fig. 4.4B and D). However, different responses were observed between the psychrophilic yeast when a 35°C heat stress was used. In some instances, a 3 h, 20°C heat shock elicited an equal or greater tolerance to a 35°C heat stress than a 3h, 25°C heat shock (Fig. 4.3A and C; Fig. 4.4A and C). These observations therefore suggest that induced tolerance not only depends on the shock conditions but also 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 (Fig. 4.8). In addition, in all the psychrophiles, there was no protein counterpart to the *S. cerevisiae* hsp 104 (Fig. 4.10A). In *M. frigida* and *M. stokesii*, a hsp of 90 kDa was also heat shock inducible and common to both species (Fig. 4.8). This heat shock protein likely falls into the yeast hsp 90 family as indicated by crossreactivity to the respective antibody (Fig. 4.10A). Despite the similarities between 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. 4.8) and *C. psychrophila* (Fig. 3.8). 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. 4.8). The protein profile of hsps in *L. antarcticum* were most similar to the psychrotrophic yeast, *L. scottii* grown at 25°C and heat shocked at 30°C for 30 min (Fig. 4.9B).

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 (Fig. 4.5A and C) and concomitant hsp synthesis (Fig. 4.9A) and trehalose accumulation (Fig. 4.10B) after a 3 h, 25°C heat shock. 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 visualised by ^{35}S-methionine autoradiograms (Fig. 4.9A) and western blots (Fig. 4.10B). 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. 4.5B and D). 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. 4.5B and D) resembled those of psychrophilic yeast stressed at 38°C (Fig. 4.3B and D; Fig. 4.4B and D). 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 3 h, 20°C heat shock in *L. scottii* and only minimal induction in *L. fellii* (Fig. 4.5). This trend was also observed in studies of induced thermotolerance in the mesophilic yeast, *S. cerevisiae* K7 grown at 15°C (Fig. 6.3A).

In psychrotrophic yeast grown at 25°C, thermotolerance was induced by a 30 min, 30°C heat shock (Fig. 4.6A and B). This response was more rapid than in psychrophilic (Fig. 4.3 and 4.4) and psychrotrophic yeast grown at 15°C (Fig. 4.5) whereby a 3 h shock was required to elicit thermotolerance. This observation is consistent with that noticed by M^{c}Callum and Inniss (1990) whereby a 1 h heat shock induced thermotolerance in the psychrotrophic bacterium, *Aquaspirillum arcticum* compared to a 5 h heat shock in the psychrophilic bacterium, *Bacillus psychrophilus*. The present results also indicated that a temperature shift of only 5°C above the growth temperature (25°C) was sufficient to induce tolerance to a 42°C stress compared to an increase of 10°C in psychrophilic and psychrotrophic yeast grown at 15°C. Coincident with the 30°C heat shock was increases in trehalose levels in both psychrotrophic yeast (Fig. 4.7B) and induction of hsps in *L. scottii* (Fig. 4.9B). It was surprising that a 30 min, 37°C heat shock decreased viability below intrinsic levels (Fig. 4.6A and B) particularly since in *S. cerevisiae* K7 grown at 15°C, these heat shock conditions elicited maximal induction of thermotolerance (Fig. 6.3A). This 12°C increase in temperature above the growth temperature (25°C) also decreased *de novo* protein synthesis (Fig. 4.9A) and affected trehalose accumulation (Fig. 4.7B). However, in contrast to 15°C grown cells, a 37°C heat shock did not decrease trehalose levels below those of control cells. Comparison of 30°C and 37°C heat shock induced responses in 25°C grown cells suggests that *de novo* protein synthesis and hsp synthesis *per se* have a more profound effect on induced thermotolerance than trehalose accumulation. A 3 h, 37°C heat shock in psychrotrophic yeast grown at 15°C elicited similar yet more marked responses in decreased viability (Fig. 4.5B and D), *de novo*

protein synthesis (Fig. 4.9A), trehalose concentration (Fig. 4.7B) and levels of hsp 70, hsp 90 and hsp 104 (Fig. 4.10B).

Western immunoblotting revealed hsp 90 and hsp 70 protein counterparts in all psychrophilic (Fig. 4.10A) and psychrotrophic yeast (Fig. 4.10B) 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 examined in this chapter especially since a hsp 60 protein counterpart was detected in the Antarctic, psychrophilic yeast, *C. psychrophila* (Fig. 3.9).

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 *Mrakia* species but not in *L. antarcticum* or *C. psychrophila* (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.

Many of the Antarctic yeast examined were first designated as species within the genus *Candida* (di Menna, 1966a). *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 apparent lack of the 110 kDa hsp in psychrophilic *L. antarcticum* (Fig. 4.8A) and psychrotrophic *L. fellii* and *L. scottii* (Fig. 4.9A) 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. 4.10B) and an absence in all psychrophilic yeast (Fig. 4.10A) may argue more favourably 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 and Kurtzman, 1992; Vishniac, 1996), psychrophilic *Mrakia nivalis* and other psychrotrophic yeast.

Indeed, in our laboratory, examination of the heat shock response has recently been extended to include *Cryptococcus vishniacii* (psychrophilic) and *Cryptococcus antarcticus* (some strains psychrotrophic). Both yeast lack hsp 104 and 60 counterparts but do have hsp 70 and 90 counterparts as ascertained by western immunoblotting, and it also appears from ^{35}S-methionine labelling that a 1 0 kDa protein was not heat shock inducible (Thomas-Hall, personal communication). One can surmise that psychrophilic yeast are characterized by the absence of proteins with homology to *S. cerevisiae* hsp 104 and 60 antibodies.

The affect of heat shock on trehalose accumulation was measured in psychrophilic and psychrotrophic yeast (Fig. 4.7). Heat shock induced accumulation of trehalose has been observed and linked to induced thermotolerance in the yeast, *S. cerevisiae* (Piper, 1993), *Schizosaccharomyces pombe* (De Virgilio *et al.*, 1990) and *C. albicans* (Argüelles, 1997). Concomitant trehalose accumulation (Fig. 4.7) and induced thermotolerance were observed with a 25°C heat shock in psychrophilic yeast (Fig. 4.3; Fig. 4.4) and psychrotrophic yeast grown at 15°C (Fig. 4.5). However, this trend was less pronounced in psychrotrophic yeast grown at 25°C and heat shocked at 30°C, despite this heat shock temperature eliciting maximal thermotole ance. This suggests that trehalose alone is not responsible for heat shock induced thermo olerance.

Constitutive and heat shocked levels of trehalose were greater in all psychrophilic and psychrotrophic yeast than those observed in *C. psychrophila* (Fig. 3.10). The psychrophilic yeast *L. antarcticum* has the highest intrinsic level of trehalose (Fig. 4.7) and the lowest intrinsic tolerance to a 35°C heat stress (Fig. 4.4C). Furthermore, the magnitude and duration of heat shock (25°C) induced thermotolerance was less marked for *L. antarcticum* than other psychrophilic yeast. This further supports the notion that the role of trehalose in thermotolerance is less significant than other stress factors such as hsps. An alternative explanation as discussed in Chapter 3 could be that a threshold level exists for trehalose, above which any further trehalose accumulation does not confer tolerance.

Relative heat sensitivity and lower heat shock protein inducible temperatures in psychrophilic and psychrotrophic eukaryotes compared to mesophiles may correlate with their lower optimum temperature for grow h and thermal sensitivity of cellular constituents

including proteins. In the psychrophilic, filamentous fungus, *Monographella nivalis* grown at 3°C, studies showed that general protein synthesis was suppressed at 35°C with complete cessation of protein synthesis at 40°C (Cairns *et al.*, 1995). Nash *et al.* (1968) found that the thermolability of protein synthesis after 30 min at 35°C in *C. gelida* (*M. gelida*), was in part due to the presence of temperature-sensitive aminoacyl-tRNA synthetases and thermolabile soluble enzymes used in the formation of polypeptide chains on the ribosome. In addition, the rates of protein degradation in microorganisms from extreme environments, such as psychrophiles and psychrotrophs, are much higher than mesophiles (Potier *et al.*, 1987; Araki, 1992).

All psychrophilic and psychrotrophic yeast exhibited slow growth in comparison to both the mesophilic yeast, *S. cerevisiae* grown at 25°C (Fig. 6.1B) and thermophilic yeast grown at 35°C (Fig. 5.1). In *S. cerevisiae*, slow growth has been correlated with a higher intrinsic thermotolerance (Ellliot and Futcher, 1993). However, the present studies in both psychrophilic and psychrotrophic yeast generally exhibits the inverse correlation. Psychrotrophic yeast grown at 15°C had a faster doubling time than psychrophilic yeast (Table 4.1), yet exhibited the greatest intrinsic tolerance to a 38°C heat stress (Fig. 4.4 *cf.* Fig. 4.2 and Fig. 4.3). Furthermore, the psychrophilic yeast *C. psychrophila* (Fig. 3.5 and Fig. 3.7) and *L. antarcticum* (Fig. 4.4C and D) exhibiting the slowest doubling times also were the least intrinsically thermotolerant. However, the doubling times of psychrotrophic yeast grown at 25°C, exhibiting a higher intrinsic tolerance to a heat stress at 42°C, was longer than when these yeast were grown at 15°C. These results together with those discussed in Chapter 6 suggest a correlation between growth rate and thermotolerance within a species however the correlation diminishes when comparing between species within a genus or between microorganisms differing in their natural environmental temperatures.

Psychrophilic and psychrotrophic yeast appear to be very well adapted to their environment, as evidenced in these studies. They were able to adapt to temperature fluctuations outside of their natural temperature growth range, a normal phenomenon in their own ecological niche (Margesin and Schinner, 1994). 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 favour them in their survival of an otherwise hostile environment. One may speculate that these extremophilic microorganisms could well adapt to harsh conditions resulting from ozone depletion and subsequent global warming.

4.4.2 Preliminary characterization of hsp 110

Preliminary characterization of the N-terminal amino acid sequence has identified hsp 110 as a protein exhibiting similarity to fructose-2,6-bisphosphatase (f2,6bpase). This enzyme regulates levels of fructose-2,6-bisphosphate (f2,6bp) which itself is a key regulator controlling glycolysis and gluconeogenesis (see Fig. 4.13). F2,6bpase is activated by cAMP dependent phosphorylation to cleave f2,6bp to fructose-6-phosphate (f6p) which in turn inhibits f2,6bpase. F2,6bp is an allosteric activator of phosphofructokinase-1 (pfk1) and inhibits fructose-1,6-bisphosphatase (f1,6bpase) (Stryer, 1988; Mathews and van Holde, 1990).

In unstressed yeast grown on glucose, phosphofructokinase-2 (pfk2) is activated by cAMP dependent phosphorylation, causing an increase in f2,6bp (François *et al.*, 1984; Piper, 1993). The resultant increase in f2,6bp activates pfk1 which in turn phosphorylates f6p to fructose-1,6-bisphosphate (f1,6bp) thus increasing glycolysis. However, when yeast is heat shocked, a resultant increase in intracellular glucose, a partial inhibition of glycolysis and a decrease in hexose phosphates and f2,6bp ensues (Neves and François, 1992; Piper, 1993). A decrease in f2,6bp would result in activation of f1,6bpase and inhibition of pfk1 thus decreasing glycolytic flux and increasing gluconeogenesis. Consistent with a heat shock induced increase in glucose is an accumulation of trehalose, synthesized from UDP-glucose and glucose-6-phosphate (g6p) (Vandercammen *et al.*, 1989; Neves and François, 1992; Piper, 1993).

It is therefore of interest that hsp 110 has similarities to f2,6-bpase in psychrophilic yeast, as a heat shock induced increase in f2,6bpase would decrease the levels of f2,6bp resulting in the aforementioned key characteristics of the heat shock response. The next step in characterization of this novel hsp would constitute its isolation using two-dimensional gel electrophoresis, with subsequent confirmation of the amino acid sequence. This would enable construction of an oligonucleotide probe for use in isolation and characterization of the gene encoding hsp 110 in *M. stokesii*.