CHAPTER 5: Heat Shock Response in the Thermophilic Enteric Yeast, *Arxiozyma telluris*.

5.1 Introduction

In terms of growth temperature, microorganisms can be divided into psychrophiles, mesophiles and thermophiles. The vast majority of microorganisms are mesophiles and occupy temperature niches that are not regarded as extreme. On the other hand there are microorganisms that are associated with low or high temperature environments, and these are known as psychrophiles and thermophiles respectively. Psychrophilic microbes have been defined as organisms that are capable of growth below 0°C and have a maximum growth temperature of 20°C (Morita, 1975). Prokaryotic thermophiles have optimum growth temperatures of 65 to 80°C (Kristjansson, 1989) while those with optimum growth temperatures around 100°C have been termed hyperthermophiles (Gottschal and Prins, 1991; Herbert, 1992; Kelly and Brown, 1993). The definition of a thermophile with respect to eukaryotic microorganisms is more ambiguous. The maximum temperature for growth of thermophilic fungi is 61-62°C (Tansey and Brock, 1972; Tansey and Brock, 1978) and the minimum temperature for growth is equal to or greater than 20°C (Cooney and Emerson, 1964). The upper temperature limit of eukaryotic algae falls between 55 to 60°C (Cairns *et al.*, 1995). However the maximum temperature for growth of yeast is generally between 46°C and 48°C (Stokes, 1971). The restrictive growth temperature range of thermophilic yeast lies between 20°C and 46°C (Travassos and Cury, 1971; van Uden, 1984a; Watson, 1987; Maresca and Kobayashi, 1996) and therefore the definition of a thermophilic yeast differs considerably from that of prokaryotes, algae or fungi. Watson (1987) proposed that a thermophilic yeast be considered as having a minimum temperature for growth of 20°C, with no restriction to maximum temperature of growth. This concept is useful to the extent that few yeast species fall into this definition (Watson, 1987) and those that do are enteric yeast, isolated from digestive tracts of domestic and wild animals (Travassos and Cury, 1971; Watson *et al.*, 1980; Watson, 1987). As such, thermophilic yeast can be considered potential opportunistic pathogens of animals and humans if the balance between host and commensal is compromised (Hurley *et al.*, 1987). The thermophilic respiratory-deficient yeast *Candida slooffii* and *Torulopsis pintolopesii* as

well as the thermophilic respiratory-competent yeast *Saccharomyces telluris* and *Torulopsis bovina* (Travassos and Cury, 1971; Watson, 1987) are now classified as strains of *Arxiozyma telluris* (van der Walt and Yarrow, 1984; Barnett *et al.*, 1990). Thermophilic yeast have been further characterized as being facultative anaerobes (Watson *et al.*, 1978) and are either naturally respiratory-deficient or are capable of giving rise to stable respiratory-deficient mutants either spontaneously or by ethidium bromide induction (Arthur *et al.*, 1978; Watson *et al.*, 1980).

Although thermophilic yeast usually inhabit the digestive tract of animals they have also been isolated outside of the animal host, most commonly in soil samples (Travassos and Cury, 1971). This suggests that thermophilic yeast can survive outside of their normal environment, at least for short periods of time. In this respect, two major factors apart from nutrient availability that affront them in their new environment would be a change in temperature (discussed in this chapter) and a change in the availability of oxygen (discussed in Chapter 7). As a consequence, it was of interest to examine how thermophilic yeast adapt to changes in these environmental parameters to enable survival.

In comparison to mesophilic microorganisms, there are relatively few reports relating to stress response systems in thermophilic microorganisms. A recent review summarized the characterization of the heat shock response in archaebacteria (Trent, 1996) and there are a few reports related to the stress response in eukaryotic thermophiles (Trent *et al.*, 1994). This chapter examines the heat shock response in two strains of the thermophilic enteric yeast, *A. telluris*, one respiratory-competent CBS 2760 (formerly *T. bovina*) and the other a naturally occurring respiratory-deficient strain CBS 1787 (formerly *T. pintolopesii*).

5.2 Results

5.2.1 Growth curves

Representative growth curves for both thermophilic yeast strains grown at 35°C are illustrated in Figure 5.1. Cells reached logarithmic phase only 4 h after inoculation and remained logarithmic for approximately 10 h. Doubling time of both strains was

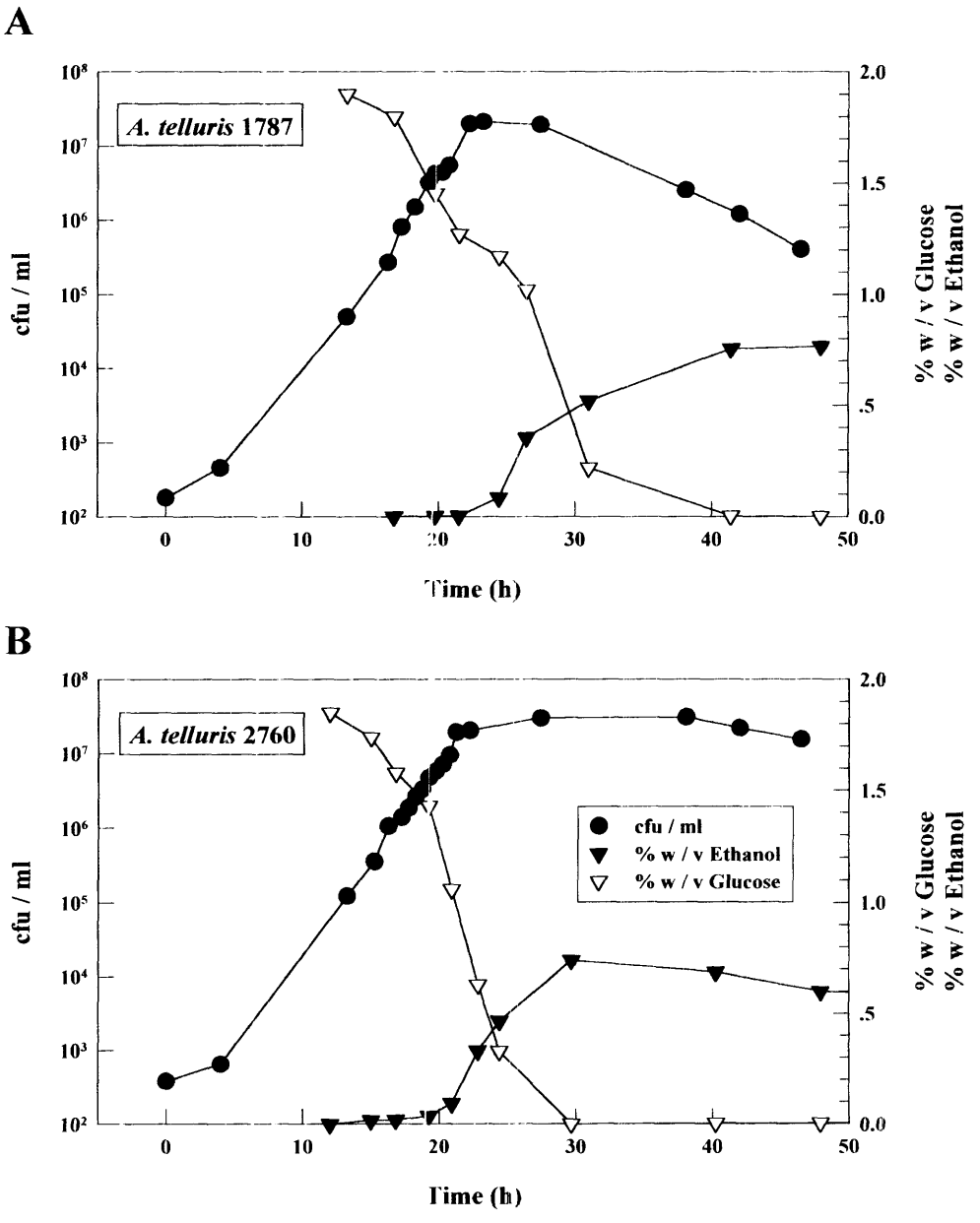

Figure 5.1. Growth curves of (A) *A. telluris* 1787 (respiratory deficient) and (B) *A. telluris* 2760 (respiratory competent) grown at 35°C with corresponding glucose and ethanol measurements.

approximately 1 h. Glucose (2%) was depleted after approximately 30 h for the both strains. Cell death in the respiratory-deficient strain 1787 paralleled glucose depletion and ethanol accumulation, which is not surprising considering the strain's inability to grow on ethanol, whereas the respiratory-competent strain remained in stationary phase (approximately 2 x 10^7 cfu ml^{-1}). Respiratory competency in strain 2760 and lack thereof in strain 1787 was checked using an oxygen electrode.

Investigations of stress tolerance were carried out using logarithmic phase cultures at an OD_{600} of 0.2, corresponding to 2 x 10^6 cfu ml^{-1} and stationary phase cultures (defined as glucose exhausted media) of strain 2760 with a cell density of approximately 2 x 10^7 cfu ml^{-1}.

5.2.2 Thermotolerance

Preliminary investigations of intrinsic thermotolerance in *A. telluris* 1787 grown at 37°C to heat stress temperatures ranging from 47°C to 70°C for a 5 min duration resulted in 1.10% and 0.01% survivors respectively (Deegenaars, 1991). Subsequently a 47°C heat stress temperature was chosen for investigation of intrinsic and induced thermotolerance in both thermophilic yeast. Previous experiments (Deegenaars, 1991; Deegenaars and Watson, 1992) determined that a 42°C heat shock resulted in acquired tolerance to a 47°C heat stress. Figure 5.2 represents heat shock acquisition of thermotolerance under these conditions in *A. telluris* 1787. Intrinsic tolerance to a 47°C heat stress for 5 min was 1.9% and 2.7% for mid and late logarithmic cultures respectively. To determine whether the heat shock response could be further optimized, cultures were grown at 30°C to decrease the growth rate and 37°C was used as the heat shock temperature. As illustrated in Figure 5.3, a 37°C heat shock for 30 min (a heat shock temperature that induces thermotolerance in *S. cerevisiae*) did not induce thermotolerance to a 45°C heat stress in *A. telluris* 1787. Subsequently, a refinement of the original heat shock and growth temperature conditions resulted in a more marked difference being observed between intrinsic and induced thermotolerance to a 47°C heat stress (Fig. 5.4). A 40°C heat shock for 30 min prior to a 47°C heat stress conferred tolerance in both the respiratory-competent (2760) and respiratory-deficient (1787) strains grown at 35°C, with close to 100% viability for the duration of the 60 min time course (Fig. 5.4). The magnitude of induction between intrinsic and induced thermotolerance was similar in both strains.

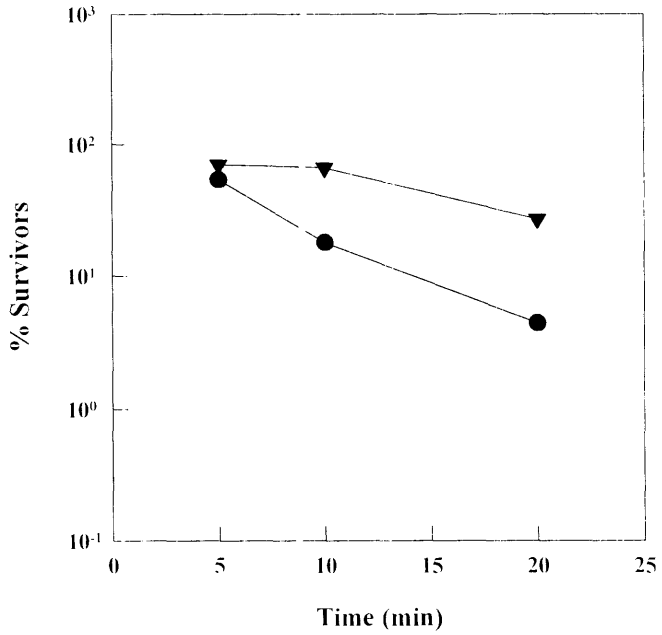

Figure 5.2. Induced thermotolerance to a 47°C heat stress in mid-logarithmic phase cultures (●) and late-logarithmic cultures (▼) of *A. telluris* 1787 (respiratory-deficient) grown at 37°C. Induced thermotolerance was monitored at 47°C following a 42°C heat shock for 30 min. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 37°C control sample.

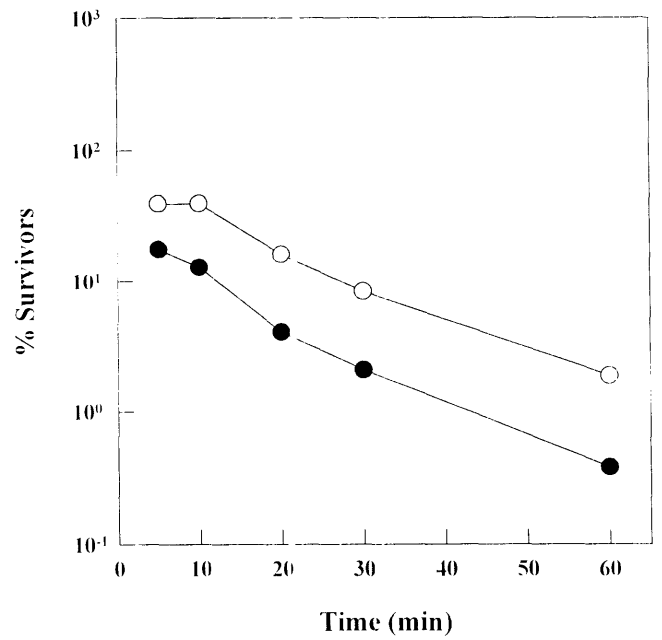

Figure 5.3. Intrinsic and induced thermotolerance to a 45°C heat stress in mid-logarithmic phase cultures of *A. telluris* 1787 (respiratory-deficient) grown at 30°C. Intrinsic thermotolerance (○) was measured by transferring cells directly to 45°C. Induced thermotolerance (●) was monitored at 45°C following a 37°C heat shock for 30 min. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 30°C control sample.

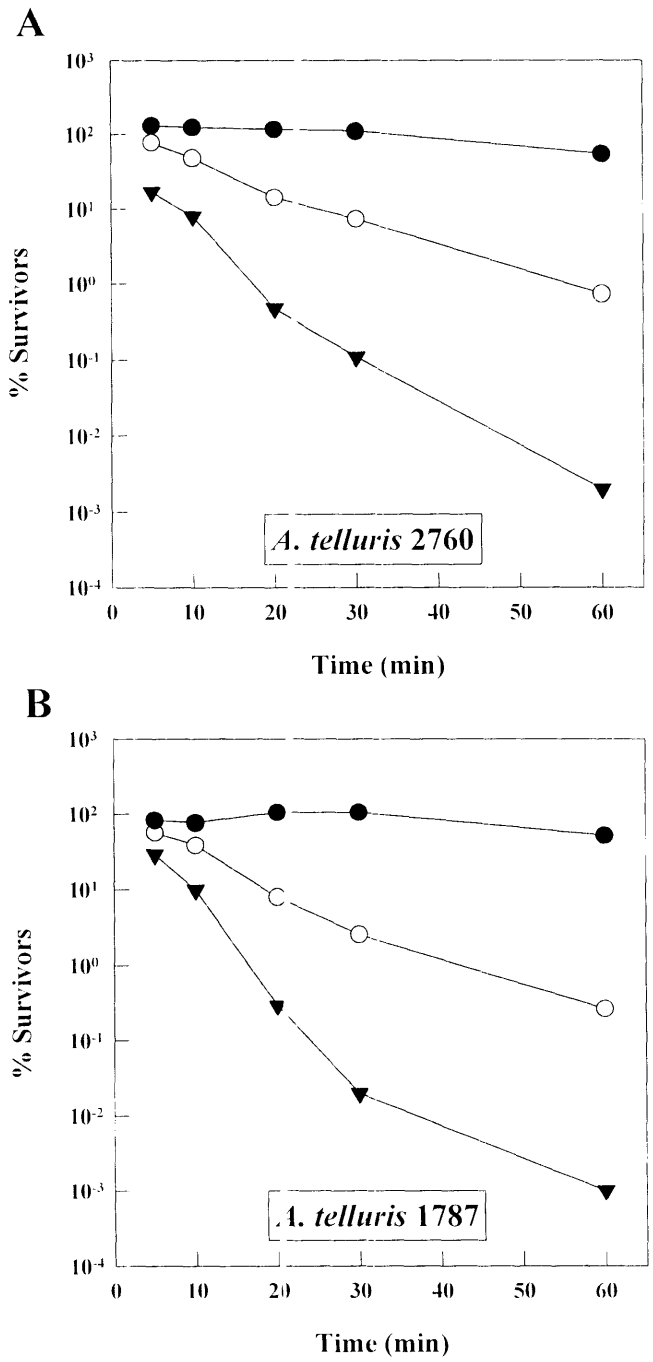

Figure 5.4. Intrinsic and heat or peroxide shock induced thermotolerance to a 47°C heat stress in mid-logarithmic phase cultures of (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient) grown at 35°C. Intrinsic thermotolerance (○) was measured by transferring cells directly to 47°C. Induced thermotolerance was monitored at 47°C following either a 40°C heat shock (●) or a 0.2 mM H_2O_2 shock (▼) for 30 min. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

A higher heat stress temperature of 50°C was also used to examine intrinsic and induced tolerance in the respiratory-competent strain 2760 (Fig. 5.5). Both mid and late logarithmic cultures were extremely sensitive to a 50°C heat stress with levels 40-fold less after 5 min as compared to cultures exposed to a 47°C heat stress (Fig. 5.4). No survivors were obtained after 10 min at 50°C. Nonetheless, a 40°C heat shock for 30 min did induce thermotolerance, albeit to levels considerably lower than that when a 47°C heat stress was used (Fig. 5.5). Intrinsic and induced tolerance to a 47°C heat stress was also examined in stationary phase (glucose-depleted) cultures of strain 2760 (Fig. 5.6). Cultures were constitutively more thermoresistant than logarithmic phase cultures (Fig. 5.4A) over the 60 min time course. As to be expected because of the high constitutive resistance displayed in stationary phase cultures, neither a peroxide shock nor a heat shock induced further thermotolerance. Similarly, as *A. telluris* strain 1787 cultures approached stationary phase a concomitant increase in intrinsic resistance to a heat stress was observed (results not shown).

Crosstolerance to a 47°C heat stress was not observed when cultures were preexposed to a 0.2 mM H_2O_2 shock for 30 min (Fig. 5.4), in fact, there was a 350-fold and 260-fold decrease after 60 min for strains 2760 and 1787, respectively.

5.2.3 Southern hybridization analysis

Table 5.1 identifies genomic DNA homology, under relatively low stringency hybridization and washing conditions, between both thermophilic yeast strains and representatives of heat shock gene families of *S. cerevisiae*.

5.2.4 Heat shock proteins

As evidenced from ^{35}S-methionine labelled *de novo* protein synthesis, a 40°C heat shock for 30 min induced the synthesis of proteins corresponding in molecular mass to hsp 104, 90 and members of the hsp 70 family in both 2760 and 1787 (Fig. 5.7). Furthermore, a heat shock inducible protein was identified at approximately 35 kDa in both strains (Fig. 5.7).

Western immunoblot analysis (Fig. 5.8) using *S. cerevisiae* anti-hsp 104, anti-hsp 90, anti-hsp 70 (crossreacts with ssa hsp 70 subfamily) and anti-hsp 60 antibodies

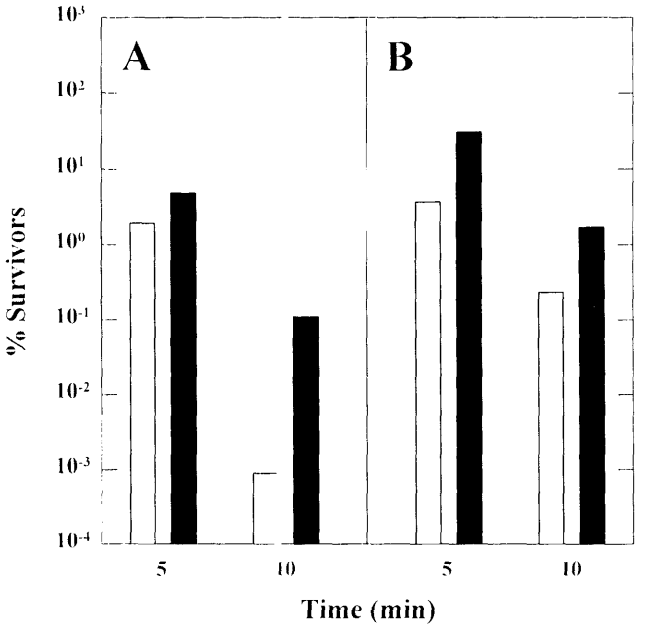

Figure 5.5. Intrinsic and induced thermotolerance to a 50°C heat stress in (A) mid-logarithmic and (B) late logarithmic phase cultures of *A. telluris* 2760 (respiratory-competent) grown at 35°C. Intrinsic thermotolerance (▭) was measured by transferring cells directly to 50°C. Induced thermotolerance (▬) was monitored at 50°C following a 40°C heat shock for 30 min. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

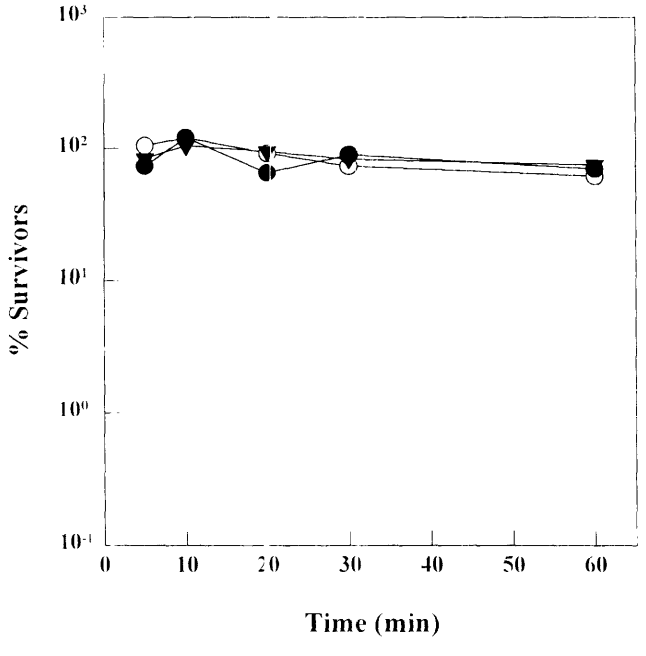

Figure 5.6. Intrinsic and heat or peroxide shock induced thermotolerance to a 47°C heat stress in stationary phase cultures of *A. telluris* 2760 (respiratory-competent) grown at 35°C. Intrinsic thermotolerance (○) was measured by transferring cells directly to 47°C. Induced thermotolerance was monitored at 47°C following either a 40°C heat shock (●) or a 0.2 mM H_2O_2 shock (▼) for 30 min. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

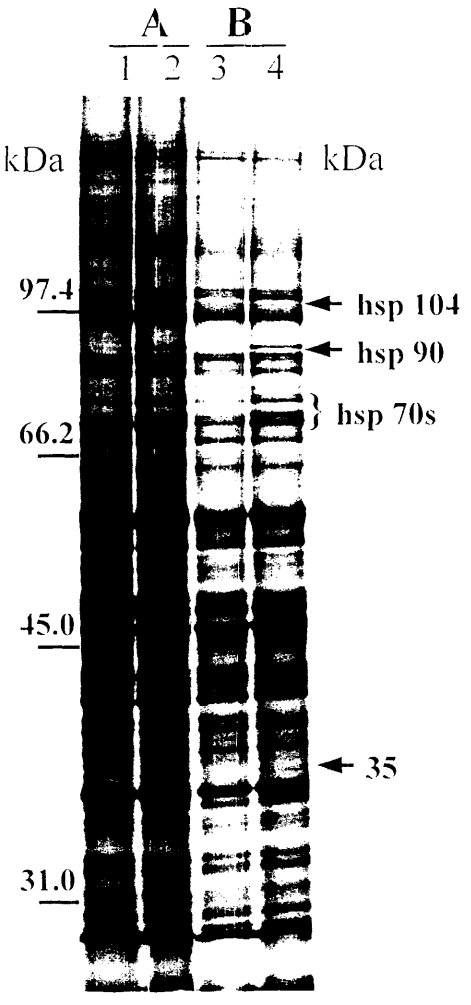

Figure 5.7. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control and heat shocked cells from (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient). Conditions were 35°C control (lanes 1 and 3) and 40°C heat shock (lanes 2 and 4). Arrows indicate new or increased heat shock protein synthesis (kDa). Molecular mass standards (kDa) are as indicated.

Table 5.1. Southern hybridization analysis with heat shock genes in *A. telluris*.

HSP gene probe	Homology[a]	
	A. telluris 1787	*A. telluris 2760*
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).

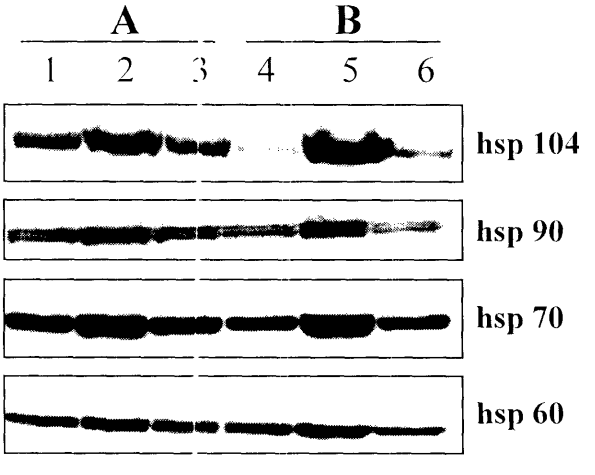

Figure 5.8. Western blot analysis of control and heat shock protein extracts from (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient). Proteins from 35°C control (lanes 1 and 4) and 40°C heat shock (lanes 2 and 5) were probed with anti-hsp 104 (1:1000), anti-hsp 90 (1:750), anti-hsp 70 (1:5000) and anti-hsp 60 (1:1000).

confirmed the identity of the hsps observed by ^{35}S-methionine labelling (Fig. 5.7). Overall, constitutive hsp levels were greater in strain 2760 as compared to strain 1787, with a marked difference in hsp 104 levels between the strains. In addition, two protein bands were recognised by the anti-hsp 90 antibody, corresponding to the *S. cerevisiae* hsp 90 proteins, hsc 82 and hsp 82 (upper band).

5.2.5 Trehalose

A 40°C heat shock for 30 mir resulted in a marked increase in trehalose accumulation with an approximate 12-fold and 17-fold increase for strains 2760 and 1787, respectively (Fig. 5.9). The absolute levels of trehalose were less for both control and heat shock samples in strain 1787 (Fig. 5.9B) as compared to strain 2760 (Fig. 5.9A).

5.2.6 Cycloheximide treatment

Cycloheximide treatment (50 μg ml^{-1}) prior to a 40°C heat shock resulted in inhibition of protein synthesis (Fig. 5.11) concomitant with a decrease in induced thermotolerance (Fig. 5.10) and a reduction (3-fold and 4-fold for strains 2760 and 1787, respectively) in heat shock accumulation of trehalose (Fig. 5.12).

5.2.7 Cold shock

A decrease in temperature from 35°C to 5°C, constituting a cold shock, resulted in reduced viability in both strains after 2 h and a slight increase in trehalose accumulation in the respiratory-competent strain 2760 after 4 h (Fig. 5.13A). ^{35}S-methionine labelling did not resolve any cold shock inducible proteins in either strain over the time course (Fig. 5.13B).

5.3 Discussion

The microorganisms examined in this chapter belong to a highly specialized group of yeast that occupy distinct ecological niches. These yeast are found in the digestive tract of warm-blooded domestic and wild animals and are obligate or facultative saprophytes (Travassos and Cury, 1971). The low temperature sensitivities of these yeasts that are unable to grow below 20 to 28°C are in marked contrast to mesophilic and psychrophilic

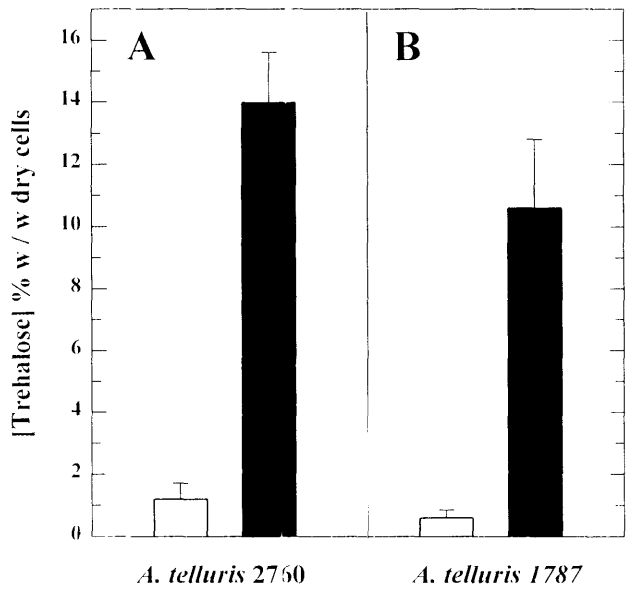

Figure 5.9. Trehalose levels in control a ıd heat shock samples of (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient). Trehalose was extracted from control (▭) and 40°C heat shock (▬) samples. Results are presented as mean and standard deviation of three measurements.

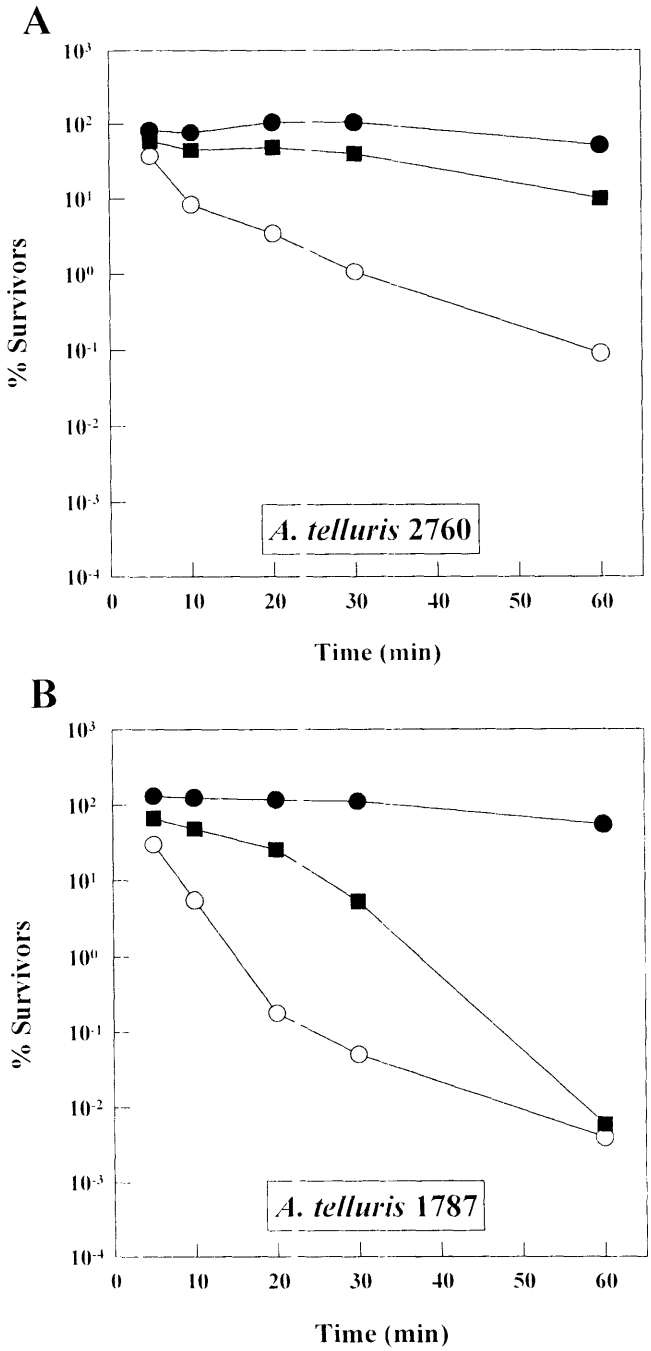

Figure 5.10. The effect of cycloheximide treatment on induced thermotolerance to a 47°C heat stress in mid-logarithmic phase cultures of (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient) grown at 35°C. Intrinsic thermotolerance (○) was measured by transferring cells directly to 47°C. Cycloheximide (50 μg ml^{-1}) was added 20 min prior to a heat shock. Induced thermotolerance was monitored at 47°C following a 30 min, 40°C heat shock with (■) or without (●) cycloheximide treatment. Levels of thermotolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

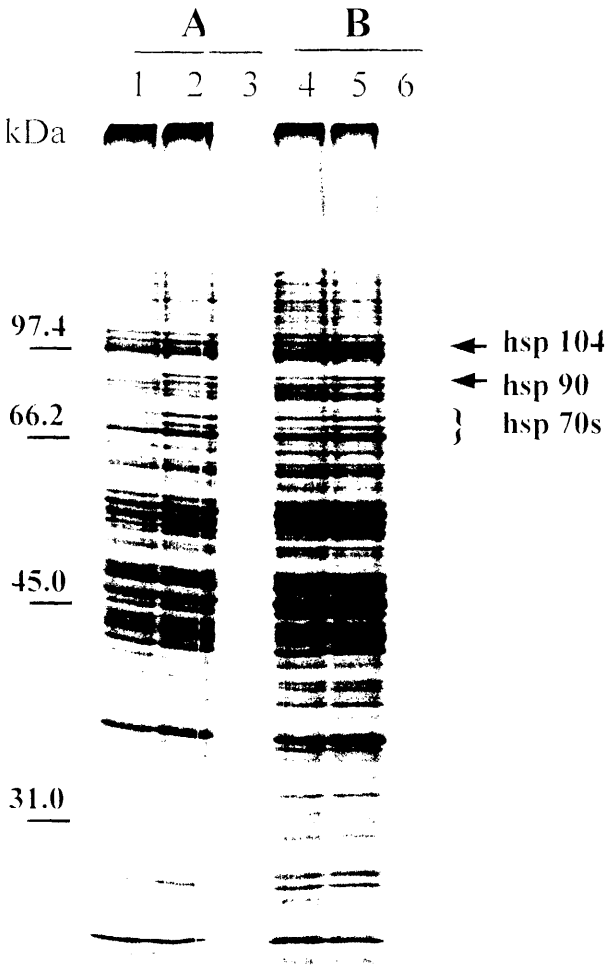

Figure 5.11. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control, heat shocked and cycloheximide treated cells of (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient). Cycloheximide (50 μg ml^{-1}) was added 20 min prior to a heat shock. Conditions were 35°C control (lanes 1 and 4), 40°C heat shock (lanes 2 and 5) and cycloheximide treated (lanes 3 and 6). Arrows indicate new or increased heat shock protein synthesis. Lanes 3 and 6 show inhibition of protein synthesis by cycloheximide. Molecular mass standards (kDa) are as indicated.

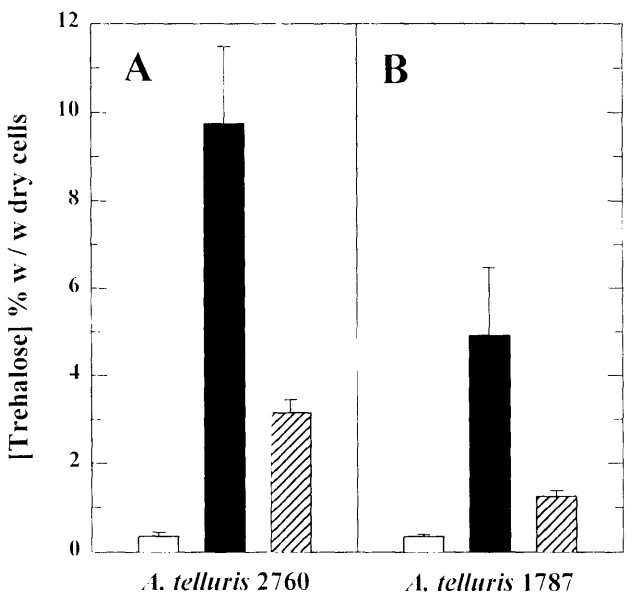

Figure 5.12. The effect of cycloheximide treatment on heat shock induced trehalose accumulation in (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient) grown at 35°C. Cycloheximide (50 μg ml^{-1}) was added 20 min prior to a heat shock. Trehalose was extracted from control ( ) and 40°C heat shock samples with ( ) or without ( ) cycloheximide treatment. Results are presented as mean and standard deviation of three measurements.

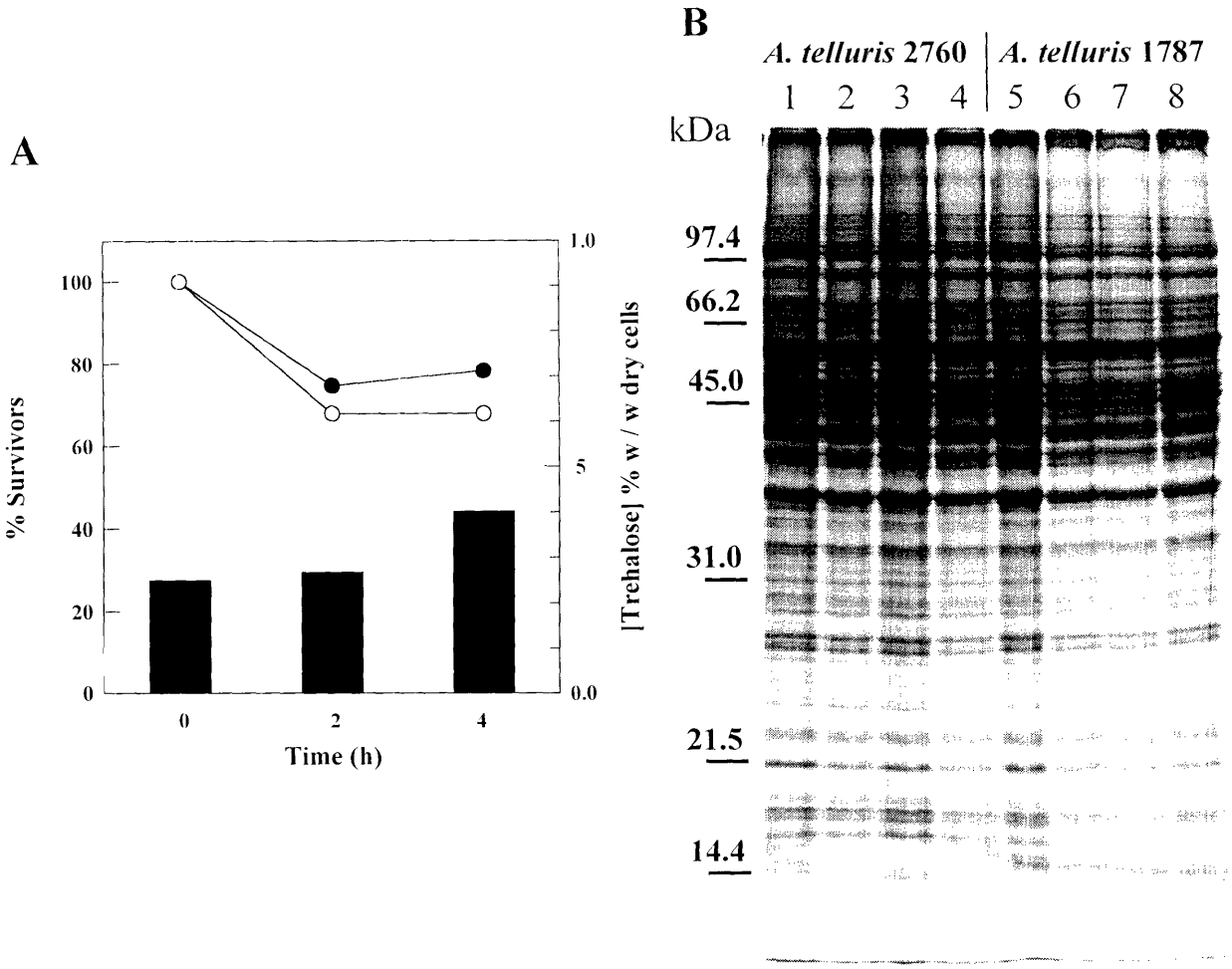

Figure 5.13. The effect of cold shock (5°C) on (A) growth (○ , ●), trehalose levels (■) and (B) *de novo* protein synthesis on mid-logarithmic phase cultures of *A. telluris* grown at 35°C. (A) Cold shock tolerance was measured by transferring cells directly to 5°C. Levels of tolerance for *A. telluris* 2760 (●) and 1787 (○) are expressed as the percentage of survivors after treatment with respect to a 35°C control sample (0 h). Trehalose was extracted from control (0 h) and cold shocked samples of *A. telluris* 2760 (respiratory-competent) after 2 and 4 h exposure to 5°C. (B) SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control and cold shock cells of *A. telluris* 2760 and 1787. Conditions were 35°C control (lanes 1 and 5), 2 h at 5°C cold shock (lanes 2 and 6) and 4 h at 5°C cold shock (lanes 3 and 7). Molecular mass standards (kDa) are as indicated.

species (Watson, 1987). It is primarily on the basis of low temperature sensitivity that these yeast have been classified as thermophilic enteric yeast (Travassos and Cury, 1971; Watson *et al.*, 1980; van Uden, 1984a).

Results presented in this chapter de nonstrate that, despite their relatively narrow temperature range for growth, both respiratory-competent and respiratory-deficient strains of *A. telluris* were capable of a heat shock response that in many respects parallels that of the mesophilic yeast, *S. cerevisiae*. Key heat shock proteins of 104, 90, 70 and 60 kDa were synthesized (Fig. 5.7 and Fig. 5.8) and trehalose levels markedly increased (Fig. 5.9) upon exposure to a heat shock, coincident with an increase in thermotolerance (Fig. 5.4). Moreover, low stringency Southern hybridization analyses detected homology to *S. cerevisiae* HSP 104, SSA1 and SSC1 (HSP 70 family members), HSP 60, and the genes for the small hsps 30, 26 and 12 (Table 5.1). The 35 kDa heat shock inducible protein observed in both thermophilic yeast strains (Fig. 5.7) could be analogous to the heat shock inducible glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. (M^{c}Alister and Holland 1985a,b; Lindquist and Craig, 1988; Craig, 1992; Boucherie *et al.*, 1995).

Acquired thermotolerance has also been characterized in the hyperthermophilic archaea *Sulfolobus shibatae* and ES4 (Trent *et al.*, 1990; Holden and Baross, 1993). *S. shibatae* grown at 70°C, heat shocked at 88°C for 1 h acquired tolerance to an otherwise lethal temperature of 92°C (Trent *et al.*, 1990). A major heat shock inducible protein of 55 kDa was identified and further investigations suggest that it exhibits a double-ring structure similar to bacterial hsp 60 (Trent *et al.*, 1990; Trent, 1996). In ES4, a heterotrophic sulphur-reducing hyperthermophilic archaea, thermotolerance to a lethal temperature of 105°C was acquired by preexposing cells grown at 95°C to a 102°C heat shock for 90 min. Coincident with heat shock was the increased synthesis of proteins of approximately 90 and 150 kDa (Holden and Baross, 1993). Heat shock protein synthesis has also been identified in other archaea including halophiles and methanogens, many corresponding to bacterial hsp 60 and hsp 70 (Trent, 1996). Thus, hyperthermophilic archaea resemble *A. telluris* in having restricted temperature growth ranges and also in their homology to known hsps.

Although *A. telluris* can be defined as a thermophile, thermophilic yeast differ significantly in growth temperature from hyperthermophilic microorganisms, with *A. telluris* strain 2760 exhibiting sensitivity to a 50°C heat stress (Fig. 5.5). In this respect, it

may be more appropriate to compare the heat shock response of thermophilic yeast to other thermophilic microorganisms such as fungi, as opposed to hyperthermophiles.

In both thermophilic yeast strains, a 5°C increase in temperature above the growth temperature (35°C) was sufficient to induce tolerance to a 47°C heat stress (Fig. 5.4). In organisms that grow over a broad range of temperatures, an increase of at least 10°C is required to elicit the heat shock response (Lindquist, 1986; Howarth and Ougham, 1993; Cairns *et al.*, 1995). However, in organisms exhibiting a more restricted growth temperature range, a maximal heat shock response can occur with a 5°C increase above the optimal growth temperature of the organism (Lindquist, 1986) as reported herein for the thermophilic yeast, *A. telluris*. These observations are in agreement with reports concerning the thermophilic eukaryotic fungus *Thermomyces langulinosus* (Trent *et al.*, 1994) and the thermophilic acidophilic bacterium *Streptococcus thermophilus* (Auffray *et al.*, 1995). The former was grown at 50°C, heat shocked at 55°C for 60 min with concomitant hsp synthesis resulting in acquired tolerance to a 58°C heat stress. The latter acquired thermotolerance with an increase of temperature 5°C to 10°C above its optimal growth temperature of 42°C. SDS-PAGE and western immunoblot analyses of heat shocked *S. thermophilus* identified twenty-two hsps with five immunologically related to *E. coli* and one exhibiting similarity to *B. subtilis* σ^{43} factor (Auffray *et al.*, 1995). Furthermore, the heat shock response could be induced by a 5°C increase in temperature for *S. cerevisiae* K7 grown at 35°C and heat stressed at 48°C (Fig. 6.3C). All attributes of the response in *S. cerevisiae* K7 grown at 35°C closely resemble those of 2760, the respiratory-competent strain of *A. telluris* (Fig. 5.4) suggesting that growth temperature influences thermotolerance (see Chapter 6).

Trehalose induction has been closely correlated to acquired thermotolerance in the yeast, *S. cerevisiae* (Attfield, 1987; Hottiger *et al.*, 1989; Wiemken, 1990), *Schizosaccharomyces pombe* (De Virgilio *et al.*, 1990) and *C. albicans* (Argüelles, 1997) and in the filamentous fungi, *Neurospora crassa* (Neves *et al.*, 1991). There are however no investigations other than the present studies of heat shock induced accumulation of this stress biomolecule in thermophilic microorganisms. Nevertheless, trehalose-synthesizing enzymes have been isolated from the hyperthermophilic archae *Sulfolobus solfataricus* KM1 (Kato *et al.*, 1996). Our studies suggest that heat shock induced trehalose

accumulation (Fig. 5.9) may contribute to acquired thermotolerance in thermophilic yeast with the role of trehalose being similar to that proposed for *S. cerevisiae*.

Increases in temperature have been correlated with the induction of petite (respiratory-deficient) cells in *S. cerevisiae* (Sherman, 1959). The respiratory-deficient strain 1787 (formerly *T. pintolopesii*) has been characterised as a naturally occurring respiratory-deficient thermophilic yeast. It has aberrant mitochondrial profiles as observed by electron microscopy (Arthur, 1979), lacks cytochrome aa_3, is respiratory-deficient and is capable of growth under strict anaerobic conditions (Watson *et al.*, 1980). In this respect 1787 resembles the cytoplasmic petites of *S. cerevisiae*. Possibly, the natural habitats of thermophilic yeast within the digestive tract of domestic and wild animals selects for respiratory deficiency (Arthur *et al.*, 1978). In the present studies there was no marked difference in hsp profiles (Fig. 5.7 and Fig. 5.8) nor in the levels of induced thermotolerance (Fig. 5.4) between the respiratory-competent strain and the respiratory-deficient strain. These results were consistent with previous findings in *S. cerevisiae*. Ethidium bromide induced petites and chloramphenicol treated cells of *S. cerevisiae* have been shown to exhibit similar heat shock responses to respiratory-competent yeast with respect to their ability to acquire thermotolerance and hsp profiles (Lindquist *et al.*, 1982; Watson *et al.*, 1984; Weitzel *et al.*, 1987). This suggests that functional mitochondria or mitochondrial protein synthesis were not required for induced thermotolerance. Also consistent with previous findings in *S. cerevisiae* petite cells, was the presence of the mitochondrial chaperone hsp 60 in both respiratory-competent and respiratory-deficient strains of *A. telluris* (Fig. 5.8) (M^{c}Mullen and Hallberg, 1988).

There is evidence in the literature for hsp 104 involvement in intrinsic thermotolerance in *S. cerevisiae* (Lindquist and Kim, 1996). In contrast, higher constitutive levels of hsp 104 in the respiratory-competent strain as compared with the respiratory-deficient strain (Fig. 5.8) did not confer higher levels of intrinsic tolerance on the former (Fig. 5.4). However, induced thermotolerance in both strains was correlated with an increase in the level of hsp 104 above constitutive levels (Fig. 5.8), suggesting that in thermophilic yeast as in *S. cerevisiae*, hsp 104 plays a more significant role in induced thermotolerance. Presumably, heat shock induction of hsp 104 was more marked in the respiratory-deficient strain to compensate for lower constitutive levels.

Despite observing a crosstolerance effect between heat shock and peroxide stress tolerance in thermophilic yeast (Chapter 7, Fig. 7.8) a peroxide shock did not confer thermotolerance (Fig. 5.4) in either thermophilic yeast strain. This finding agrees with those in the yeast, *S. cerevisiae* (Collinson and Dawes, 1992; Steels *et al.*, 1994) and *C. psychrophila* (Deegenaars and Watson, 1997) as well as in *Xanthomona oryzae* pv. *oryzae* (Mongkolsuk *et al.*, 1997). In contrast, a peroxide shock has been observed to induce thermotolerance in *Schizosaccharomyces pombe* (Mutoh *et al.*, 1995), *Neurospora crassa* (Kapoor and Lewis, 1986), *E. coli* (van Bogeln *et al.*, 1987), *Salmonella typhimurium* (Christman *et al.*, 1985) and *Bacillus subtilis* (Dowds, 1994). In fact, a peroxide shock increased thermosensitivity in both thermophilic yeast strains (Fig. 5.4). This may be a consequence of hydrogen peroxide induced free radical damage which is enhanced at the heat stress temperature (see Chapter 8).

Consistent with findings in *S. cerevisiae* (Schenberg-Frascino and Moustacchi, 1972; Parry *et al.*, 1976; Mitchel and Morrison, 1982) was the observation that stationary phase cultures of *A. telluris* strain 2760 (Fig. 5.6) were more intrinsically resistant to a heat stress than logarithmic phase cultures (Fig. 5.4A). This trend was also observed with the respiratory-deficient strain 1787 as cell density of cultures increased (Fig. 5.2). Also in accordance with previous findings in *S. cerevisiae*, cycloheximide treatment resulted in a decrease in heat shock induced trehalose accumulation and thermotolerance (Hall, 1983; Watson *et al.*, 1984; De Virgilio *et al.*, 1991; Cootes *et al.*, 1992; Neves and François, 1992; Gross and Watson, 1996). These results suggest the requirement of *de novo* hsp synthesis, full induction of trehalose accumulation or a heat shock inducible signal resulting from abnormal protein accumulation for optimal induced thermotolerance in thermophilic yeast.

In some respects the thermophilic enteric yeast resemble a number of important human fungal pathogens, such as *Candida albicans* and *Histoplasma capsulatum*. These similarities include warm-blooded host habitats and the ability to undergo morphological changes (Travassos and Cury, 1971). The latter ability depends on environmental conditions. The opportunistic fungal pathogen, *C. albicans* resembles yeast cells when grown at 25°C, however nutritional stimuli and a temperature increase to 37°C causes germ tube formation which can initiate host-tissue invasion (Zeuthen and Howard, 1989; Matthews, 1995). In contrast, the dimorphic fungal pathogen, *H. capsulatum* exists as multicellular filamentous fungi in soil and as unicellular budding yeast in human tissue

(Maresca and Carratù, 1990). In recent years there has been much research and speculation as to the potential role of hsps in the pathogenesis and morphogenesis of these important opportunistic human pathogens. There is compelling evidence, for example, that hsps associated with these pathogens induce cellular immune responses in animal and human hosts (Mathews and Burnie, 1995). Moreover, it is now well established that in certain microbial diseases hsps, such as GroEL (hsp 60) and DnaK (hsp 70) are key immunodominant antigens (van Eden and Young, 1996).

The heat shock response has been characterized in both fungal pathogens. In *C. albicans* grown at 37°C heat shock induced thermotolerance with coincident hsp synthesis has been observed when cells were heat shocked for 30 min at 45°C and exposed to an otherwise lethal temperature of 55°C (Zeuthen and Howard, 1989). In *H. capsulatum*, morphogenesis and the heat shock response are induced by a temperature shift from 25°C to 37°C. However, an increase in the heat shock temperature to 40°C is required to elicit a heat shock response in *H. capsulatum* existing as yeast at 37°C within the host (Maresca and Carratù, 1990; Maresca and Kobayashi, 1996.). This latter response closely parallels results reported herein for the thermophilic enteric yeast, *A. telluris*. Although the thermophilic enteric yeast have not been reported as human pathogens, it is nevertheless tempting to speculate, given their natural and specialized ecological environments in warm-blooded animals, that a similar role for hsps exists in host parasite interactions with these yeast.

Thermophilic yeast in their natural environment (warm-blooded host) would be highly unlikely to experience sudden decreases in temperature and hence it is not surprising that thermophilic yeast do not exhibit a characteristic cold shock response (Fig. 5.13). However, thermophilic yeast are more likely to experience an increase in temperature either as an opportunistic pathogen invading their host or within the host if the host organism is compromised by infection or fever. The characteristics of the heat shock response exhibited by thermophilic yeast would certainly contribute towards their survival in their natural habitats.

CHAPTER 6: Influence of Growth Temperature on the Heat Shock Response in *Saccharomyces cerevisiae*.

6.1 Introduction

S. cerevisiae has been extensively investigated with respect to metabolism at experimental growth temperatures of 25°C to 30°C (Watson, 1987). Relatively little research on *S. cerevisiae* has been carried out at experimental growth temperatures outside of the range 25°C to 30°C. The majority of studies on stress tolerance and, in particular, thermotolerance in *S. cerevisiae* have also been confined to growth at temperatures around 25°C. In the case of heat shock acquisition of thermotolerance, cells are typically grown at 25°C to mid-logarithmic phase, heat shocked at 37°C for 30 to 60 min and then exposed to heat stress temperatures of 48°C to 52°C. Under these conditions it is well established that a heat shock results in acquisition of thermotolerance coincident with the synthesis of key stress biomolecules, namely, the disaccharide trehalose (Attfield, 1987; Hottiger *et al.*, 1989) and a set of heat shock proteins (hsps) (Lindquist and Craig, 1988; Watson, 1990; Piper, 1993). The level of induced thermotolerance is quite pronounced in logarithmic phase cells, characteristically showing a 100 to 1000-fold increase as compared to intrinsic thermotolerance observed in non-heat shocked control cells (Sanchez *et al.*, 1992). Hsp 104 has been ascribed a key role in various stress response systems and primarily in the acquisition of thermotolerance in *S. cerevisiae* (Sanchez *et al.*, 1992). The proposed function of trehalose in heat-stressed yeast is to maintain the structure and integrity of cell membranes and proteins (Hottiger *et al.*, 1989; Wiemken, 1990; Piper, 1993). Furthermore, recent investigations by Elliot *et al.* (1996) reveal that trehalose and hsp 104 act in concert to account for the majority of the observed intrinsic thermotolerance of stationary phase cells.

However, stress response studies at a growth temperature of 25°C, may not be particularly relevant to the natural and diverse environments in which mesophilic yeast may be found (Watson, 1987). Walton and Pringle (1980) have previously reported that intrinsic thermotolerance to a 52°C heat stress varies with growth temperature. A direct correlation was observed between an increase in constitutive thermotolerance and an increase in growth temperature. They postulated that the cell membrane of cultures grown

at the lower temperature of 23°C were more prone to thermal damage as compared to cultures grown at 36°C. It has also been previously shown that yeast adjust their membrane fatty-acyl composition with temperature (Watson, 1987). The lower the temperature the greater the membrane lipid unsaturation. It is however, unresolved as to whether induced thermotolerance, hsp synthesis and trehalose accumulation in *S. cerevisiae* are affected by changes in growth temperature, not only at higher growth temperatures but also at lower growth temperatures.

The present chapter examines the influence of yeast growth temperature on the heat shock response in the mesophilic yeast, *S. cerevisiae* and compares the response to the heat shock response in psychrophilic, psychrotrophic (Chapter 3) and thermophilic yeast (Chapter 4).

6.2 Experimental outline

Growth curves were constructed for cultures of *S. cerevisiae* K7 grown at 15°C, 25°C and 35°C and culture doubling times calculated. Experimental cultures were grown to an OD_{600} of 0.2 to 0.3 corresponding to logarithmic phase cells of approximately 1 x 10^6 (for 15°C and 35°C grown cultures) to 3 x 10^6 cfu ml^{-1} (for 25°C grown cultures).

Initially, a heat stress temperature of 35°C was chosen for *S. cerevisiae* cultures grown at 15°C to enable direct comparison of intrinsic and induced (20°C heat shock) thermotolerance to the heat shock response in *C. psychrophila* (Chapter 3) and other psychrophilic/psychrotrophic (Chapter 4) yeast.

Subsequently, intrinsic and induced thermotolerance were measured to a heat stress temperature of 48°C for all *S. cerevisiae* cultures irrespective of growth temperature:

1. For 15°C grown cultures, induced tolerance to 48°C was measured following a 30 min heat shock at 20°C, 25°C or 37°C. The heat shock temperatures of 20°C and 25°C were chosen as these temperatures elicit thermotolerance in psychrophilic yeast (Chapter 3 and 4). A 37°C heat shock was also included as this temperature induces a heat shock response in *S. cerevisiae* cultures grown at 25°C.
2. Cultures grown at 25°C were heat shocked at 37°C for 30 min prior to a 48°C heat stress. This regime represents a typical heat shock response protocol in *S. cerevisiae*.

3. Induced thermotolerance for cultures grown at 35°C was measured by exposing cultures to a 40°C heat shock for 30 min prior to a 48°C heat stress. This regime is analogous to that used for thermophilic yeast in Chapter 5.

4. To examine the effect of inhibition of protein synthesis on induced thermotolerance, cycloheximide was added 20 min prior to a 37°C heat shock for 15°C and 25°C grown cultures and prior to a 40°C heat shock for 35°C grown cultures.

Heat shock proteins and trehalose levels were examined for all control and heat shock samples.

6.3 Results

6.3.1 Growth curves

The doubling time was determined from growth curves (Fig. 6.1) of cultures grown at 15°C, 25°C and 35°C and found to be 5 h, 2 h and 3.5 h, respectively. It was also observed that the maximum culture density reached during growth was less for 35°C grown cells (Fig. 6.1C; 6 x 10^6 cfu ml^{-1}) as compared to 15°C (Fig. 6.1A; 5 x 10^7 cfu ml^{-1}) and 25°C grown cells (Fig. 6.1B; 5 x 10^7 cfu ml^{-1}).

6.3.2 Thermotolerance

Figure 6.2 compares the intrinsic and induced thermotolerance to a 35°C heat stress in *S. cerevisiae* grown at 15°C (Fig. 6.2A) to the psychrophilic yeast, *C. psychrophila* (Fig. 6.2B). *S. cerevisiae* exhibits no loss in viability to a 35°C heat stress over the 60 min time course even in non-heat shocked cultures whereas *C. psychrophila* was relatively intrinsically thermosensitive. Heat shocked cultures (20°C heat shock for 3 h) of *C. psychrophila* initially attain levels of thermotolerance exhibited intrinsically in *S. cerevisiae* grown at 15°C, however the duration of tolerance is short-lived.

Consequently, a higher heat stress temperature of 48°C was used to examine intrinsic and induced thermotolerance for *S. cerevisiae* K7 grown at 15°C, 25°C and 35°C (Fig. 6.3). Intrinsic thermotolerance to a 48°C heat stress was greater for a 15°C grown

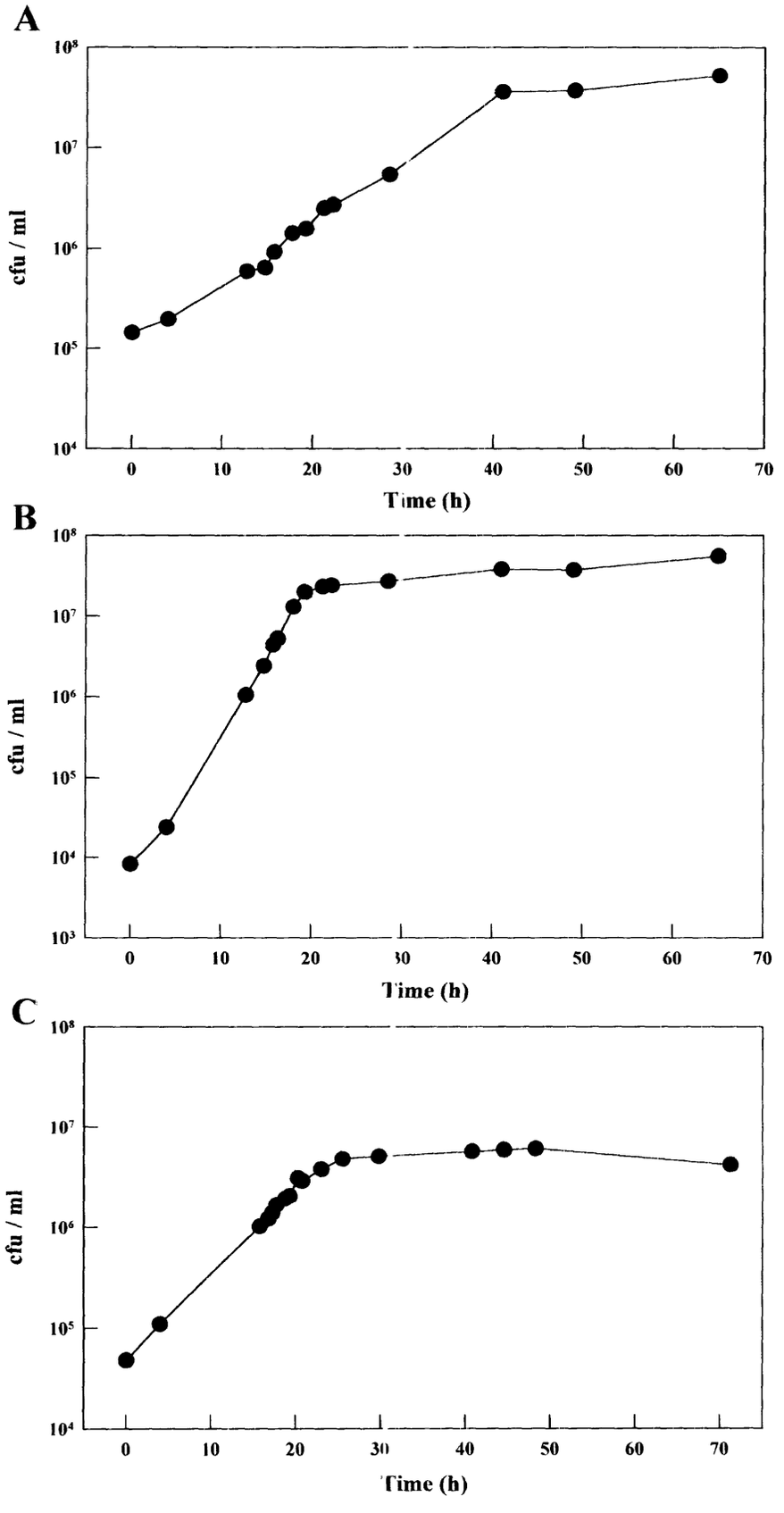

Figure 6.1. Growth curves of *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C and (C) 35°C.

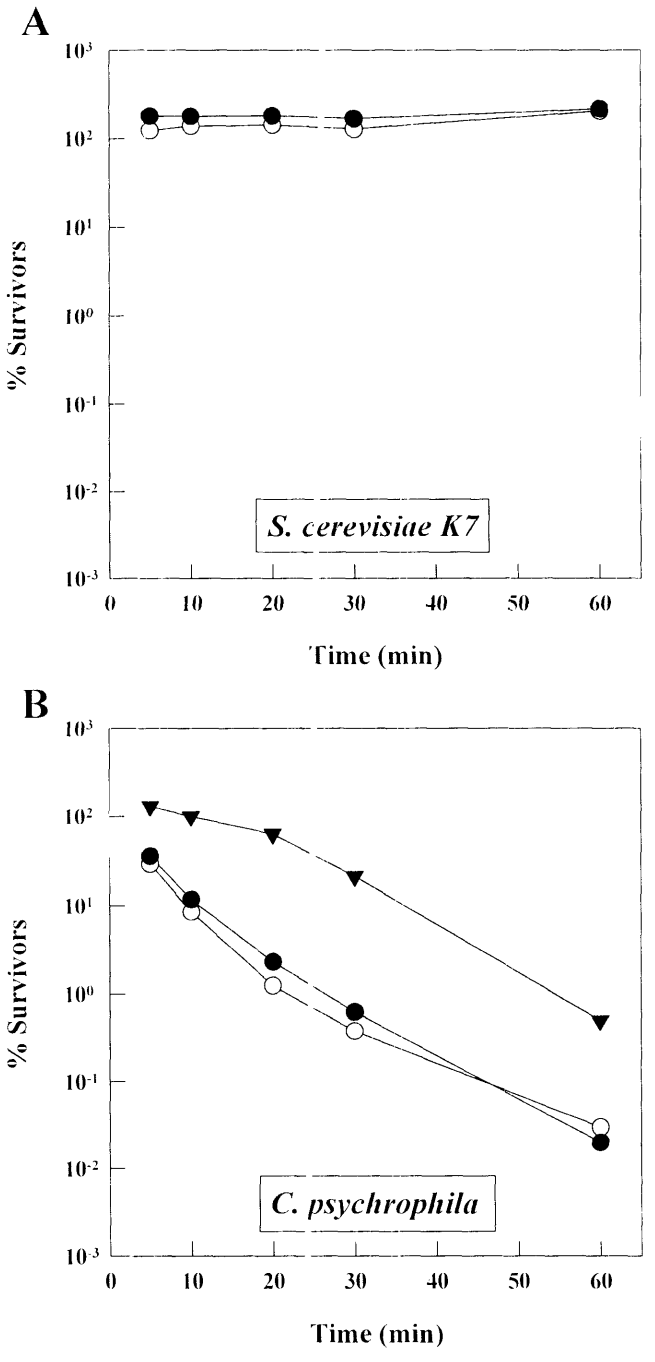

Figure 6.2. Intrinsic and induced thermotolerance to a 35°C heat stress in mid-logarithmic phase cultures of (A) *S. cerevisiae* K7 and (B) *C. psychrophila* grown at 15°C. Intrinsic thermotolerance (○) was measured by transferring cells directly to 35°C. Induced thermotolerance was monitored at 35°C following a heat shock at 20°C for 30 min (●) or 3 h (▼). Levels of thermotolerance were expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

culture (Fig. 6.3A) than either 25°C (Fig. 6.3B) or 35°C (Fig. 6.3C) grown cultures. Intrinsic thermotolerance levels in cultures grown at 15°C were double those of 35°C grown cultures and approximately 60-fold greater than those of 25°C grown cultures, after a 5 min, 48°C heat stress. Also, the maintenance of thermotolerance for the duration of the 60 min time course was greater in 15°C grown cultures, with a 400-fold increase in survivors over 25°C and 35°C grown cultures at the 60 min time point.

For cells grown at 15°C (Fig. 6.3A), both 25°C and 37°C heat shocks for 30 min induced thermotolerance over and above intrinsic thermotolerance levels for the duration of the 60 min stress time course. However, induced thermotolerance was not elicited by a 20°C heat shock for 30 min prior to a heat stress. A 37°C, 30 min heat shock was optimal for induction of thermotolerance, with a 1.5-fold increase in survivors after a 5 min exposure and a 3.5-fold increase after a 60 min exposure to a 48°C heat stress, as compared to intrinsic tolerance observed in control cells.

A typical heat shock response is depicted in Fig. 6.3B. A 25°C grown culture was heat shocked at 37°C for 30 min prior to a 48°C heat stress resulting in marked increases in thermotolerance (125-fold increase for 5 min and 700-fold increase for 60 min) over intrinsic levels of thermotolerance.

Similarly, for cells grown at 35°C, a 40°C heat shock for 30 min increased the percentage of survivors by approximately 4.5-fold and 200-fold after a 5 min and 60 min, 48°C heat stress, respectively (Fig. 6.3C).

Cycloheximide treatment (50 μg ml^{-1}) prior to a 37°C heat shock for 15°C or 25°C grown cultures and a 40°C heat shock for 35°C grown cultures resulted in decreased viability to a 48°C heat stress (Fig. 6.3). However, levels of induced thermotolerance in all cultures were still greater than intrinsic thermotolerance. In addition, in 15°C grown cultures (Fig. 6.3A), the levels of tolerance exhibited after cycloheximide treatment were less than those induced by a 25°C heat shock.

6.3.3 Trehalose

At all growth temperatures, trehalose levels increased markedly upon a heat shock (Fig. 6.4). Maximal accumulation was observed by a 37°C heat shock in 15°C (13-fold

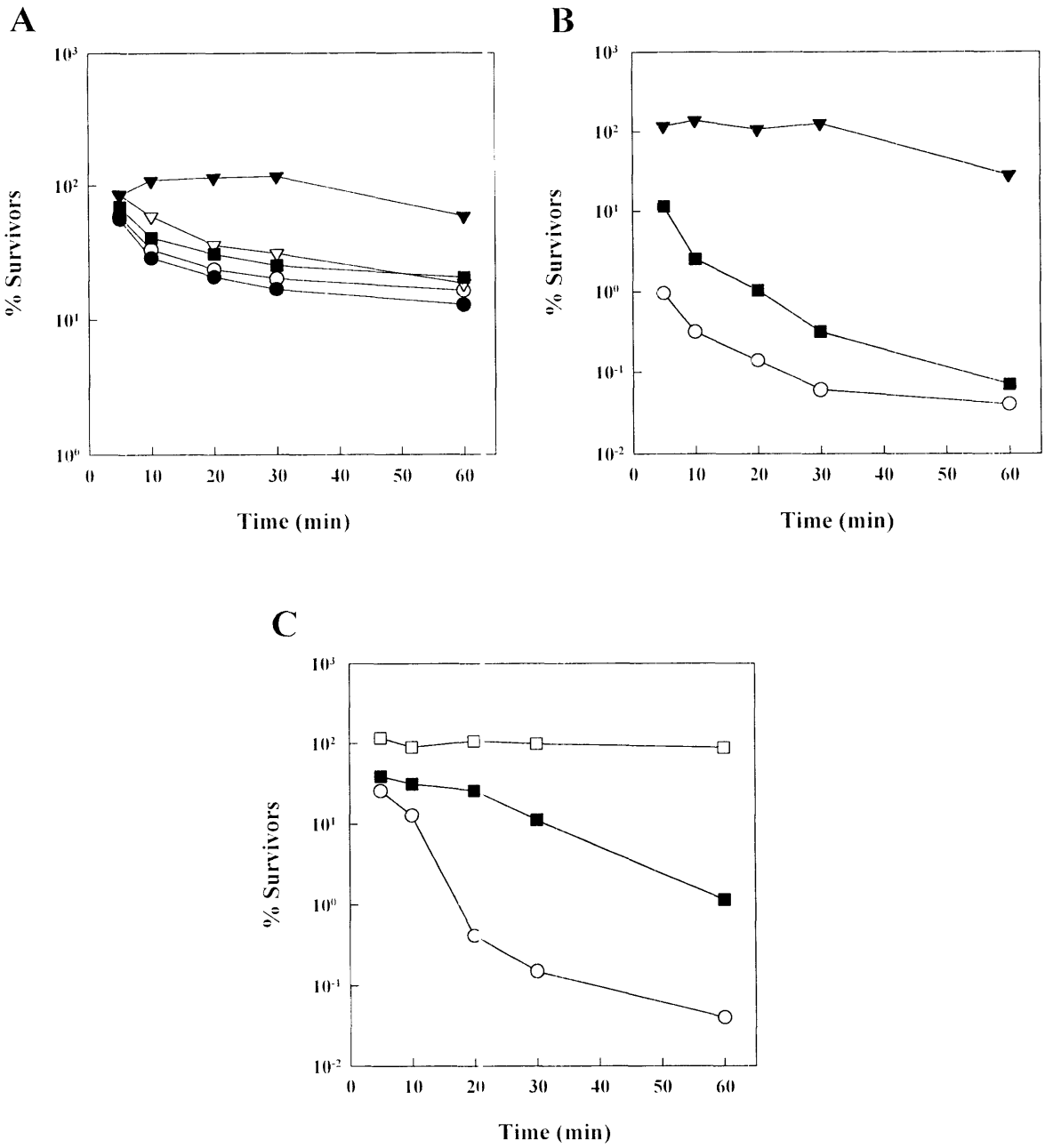

Figure 6.3. Intrinsic and induced thermotolerance in mid-logarithmic phase cultures of *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C or (C) 35°C. Intrinsic tolerance (○) was measured by transferring cells directly to 48°C. Induced thermotolerance was monitored at 48°C following a 30 min heat shock at 20°C (●), 25°C (▽), 37°C (▼) or 40°C (□) in the absence or presence (■) of 50 μg ml^{-1} cycloheximide. Levels of thermotolerance were expressed as percentage of survivors after the appropriate treatment with respect to a 15°C, 25°C or 35°C control sample.

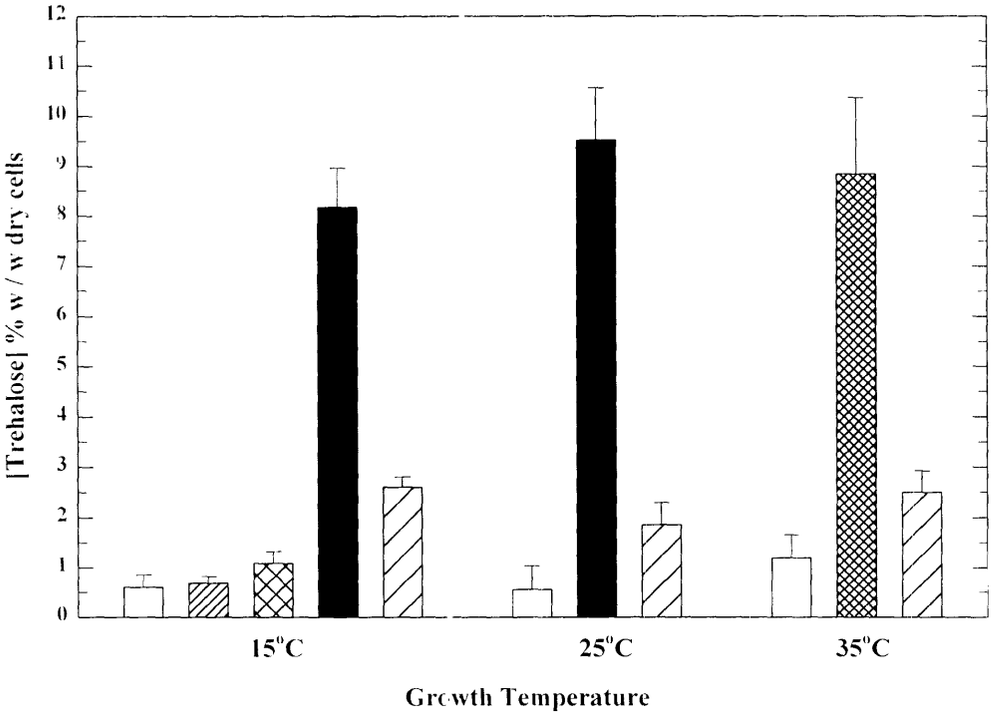

Figure 6.4. Trehalose levels in control and heat shock samples of *S. cerevisiae* K7 grown at 15°C, 25°C or 35°C. Trehalose was extracted from a 15°C, 25°C or 35°C control (▭), 20°C heat shock (▨), 25°C heat shock (▩), 37°C heat shock (■), 40°C heat shock (▩) and 50 μg ml^{-1} cycloheximide treated heat shock samples (▨). Results are presented as mean and standard deviation of three measurements.

increase) and 25°C (17-fold increase) grown cultures and a 40°C heat shock in 35°C (7-fold increase) grown cultures. Cycloheximide treatment partially inhibited heat shock accumulation of trehalose in all cultures. Nevertheless, trehalose levels were still greater than those observed in control cells with a 4-fold, 3-fold, and 2-fold increase for 15°C, 25°C and 35°C grown cultures, respectively.

6.3.4 Heat shock proteins

De novo protein synthesis in control and heat shocked samples of cells grown at 15°C, 25°C or 35°C were examined by ^{35}S-methionine labelling (Fig. 6.5). Hsp 104, 90 and 70 were observed in 37°C heat shocked samples of 15°C and 25°C grown cultures and 40°C heat shocked samples of 35°C grown cultures. Protein profiles of control cells were remarkably similar despite the differences in growth temperature. In 35°C grown cultures, a protein of approximately 80 kDa was more pronounced as compared to 15°C or 25°C grown cultures. Furthermore, a protein at approximately 40 kDa exhibited increased synthesis in a 15°C grown culture heat shocked at 20°C and 25°C. This protein was also visible in control samples of 25°C and 35°C grown cultures at greater levels than those of control samples of 15°C grown cultures. However, a heat shock temperature of 37°C or 40°C decreased levels of the 40 kDa protein below the growth temperature control samples at 25°C and 35°C, respectively.

The efficacy of cycloheximide treatment to inhibit protein synthesis was confirmed by the absence of protein profiles in ^{35}S-methionine labelled cycloheximide treated samples at all growth temperatures (Fig. 6.6). It should be noted that cycloheximide treatment was extended only to a 37°C heat shock for 15°C grown cells as *de novo* hsp synthesis was limited to this heat shock temperature.

Western blot analyses of control and heat shock samples for cells grown at 15°C, 25°C or 35°C are depicted in Fig. 6.7. The results obtained using antibodies directed against hsps 104, 90, 70 and 60 confirm the above observations in heat shocked samples using ^{35}S-methionine labelling. In addition to a heat shock increasing the levels of all hsps at all growth temperatures, an increase in growth temperature correlated with increased levels of hsps 90, 70 and 60 in control samples. It should be noted that western blots

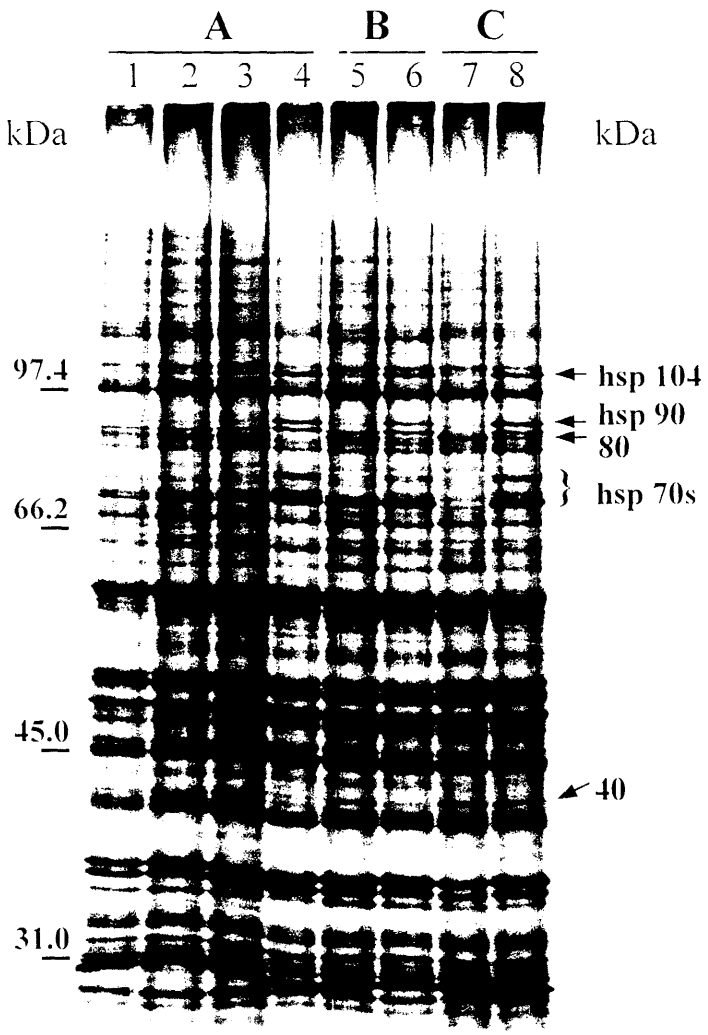

Figure 6.5. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control and heat shocked cells of *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C or (C) 35°C. Conditions were 15°C control (lane 1), 20°C heat shock (lane 2), 25°C heat shock (lane 3), 37°C heat shock (lanes 4 and 6), 25°C control (lane 5), 35°C control (lane 7) and 40°C heat shock (lane 8). Arrows indicate new or increased heat shock protein synthesis. Molecular mass standards (kDa) are as indicated.

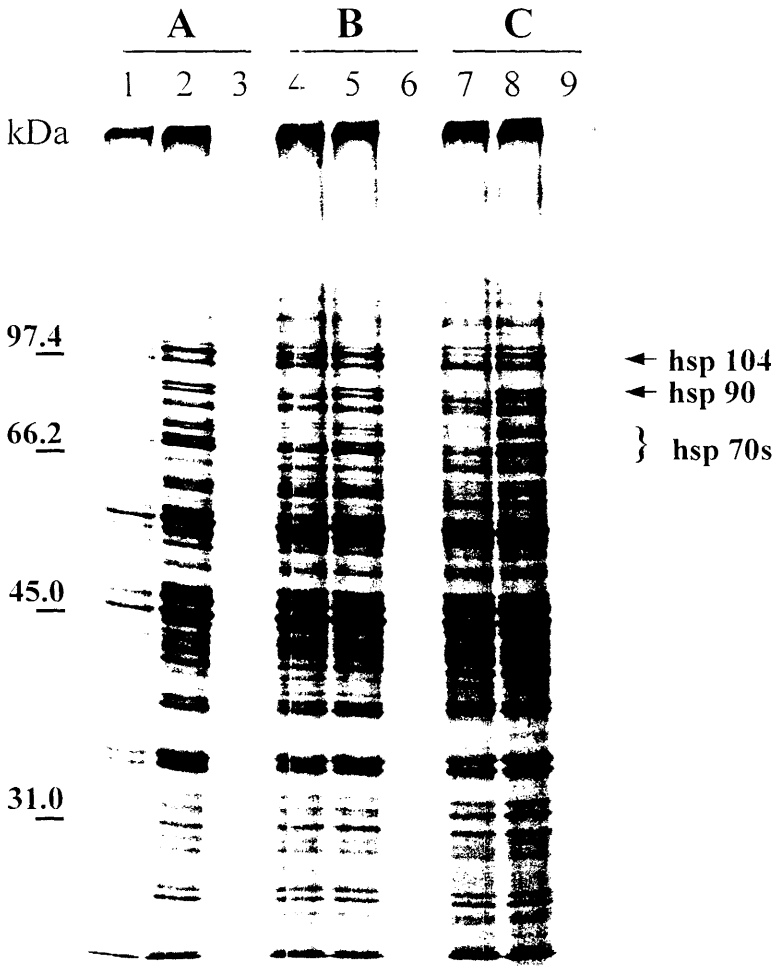

Figure 6.6. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control, heat shocked and cycloheximide treated cells of *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C or (C) 35°C. Conditions were 15°C control (lane 1), 37°C heat shock (lanes 2 and 5), cycloheximide treated (lanes 3, 6 and 9), 25°C control (lane 4), 35°C control (lane 7) and 40°C heat shock (lane 8). Arrows indicate new or increased heat shock protein synthesis. Lanes 3, 6 and 9 show cycloheximide induced inhibition of protein synthesis. Molecular mass standards (kDa) are as indicated.

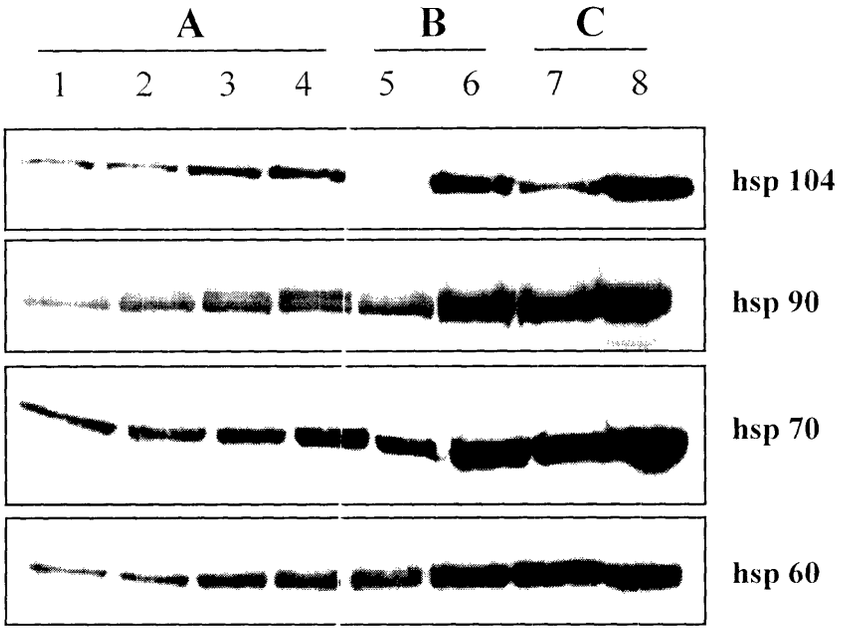

Figure 6.7. Western blot analysis of control and heat shock protein extracts from *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C or (C) 35°C. Proteins from 15°C control (lane 1), 20°C heat shock (lane 2), 25°C heat shock (lane 3), 37°C heat shock (lanes 4 and 6), 25°C control (lane 5), 35°C control (lane 7) and 40°C heat shock (lane 8) were probed with anti-hsp 104 (1:1000), anti-hsp 90 (1:750), anti-hsp 70 (1:5000) or anti-hsp 60 (1:1000).

probed with anti-hsp 90 antibody (Fig. 6.7) detected two protein bands, the lower molecular mass band corresponding to hsc 82 and the top band corresponding to hsp 82 (Borkovich *et al.*, 1989). Both hsp 82 and hsc 82 follow the same pattern of expression as hsp 70 and 60 (Fig. 6.7). In contrast to this pattern, 25°C grown cultures had the lowest intrinsic level of hsp 104 as compared to either 15°C or 35°C grown cultures. Furthermore, intrinsic levels of hsps 90, 70 and 60 in 35°C grown cultures were equal to those of 37°C heat shocked samples of 25°C grown cultures.

6.4 Discussion

This chapter examines intrinsic and induced thermotolerance in *S. cerevisiae* grown at different temperatures in an attempt to resolve whether growth temperature influences the heat shock response. Furthermore, the relative contributions of the stress biomolecules, hsps and trehalose, to both constitutive and acquired thermotolerance for cultures grown at different temperatures were measured.

In one respect our results confirmed earlier findings that growth at 35°C (Fig. 6.3C) related to higher intrinsic thermotolerance as compared to growth at 25°C (Fig. 6.3B) (Walton and Pringle, 1980). These authors suggested that membrane damage was primarily responsible for differences in intrinsic resistance to a 52°C heat stress in *S. cerevisiae* cultures grown at 23°C, 30°C or 36°C. However, recent findings in our laboratory have indicated no clear relationship between membrane fatty acid composition and membrane fluidity with stress tolerance in *S. cerevisiae* (Swan and Watson, 1997). On the other hand, results presented herein suggest that constitutive levels of hsps influence intrinsic thermotolerance at a growth temperature of 35°C. Western blots (Fig. 6.7) showed high constitutive levels of hsp 90, 70 and 60 in 35°C grown cells equal to 25°C grown cells heat shocked at 37°C for 30 min. In addition, constitutive levels of hsp 104 were higher in 35°C grown cells than cells grown at 15°C or 25°C. Previous studies using hsp 70 (Craig and Jacobsen, 1984) and hsp 90 (Borkovich *et al.*, 1989) mutants revealed the necessity of ssa1/ssa2 (hsp 70, stress seventy family A) and hsp 90 for growth at normal temperatures (23°C) and, in particular, for growth at high temperatures (37°C). Hsp 82 and hsc 82 constitute an essential, highly conserved gene family in *S. cerevisiae* and both proteins are required at higher concentrations for growth at higher temperatures (Borkovich *et al.*, 1989). The present studies were consistent with these observations in

that constitutive levels of hsp 82 and hsc 82 correlated with growth temperature, increasing from 15°C to 25°C to 35°C (Fig. 6.7). Cheng *et al.* (1992) carried out studies on the effect of overexpression of HSP 90 on high temperature growth and thermotolerance. They reported a resultant reduction in growth rate at 37.5°C when HSP 90 is overexpressed and suggest that there is an optimal level of hsp 90 for growth at high temperatures. Studies using *ssa1 ssa2* double mutants revealed an obligatory requirement for both ssa1 and ssa2 hsp 70 proteins for growth at high temperatures (Craig and Jacobsen, 1984). However, *ssa1 ssa2* double mutants, grown at 23°C, showed greater intrinsic thermotolerance than wild-type cells and induced thermotolerance levels compared favourably to those observed in wild-type cells. The anti-hsp 70 antibody used in our investigations showed the highest amino acid sequence complimentarity to the ssa hsp 70 family. The western immunoblot results at a growth temperature of 35°C (Fig. 6.7) comply with the reported findings for hsp 82, hsc 82 and hsp 70. Furthermore, the western immunoblots (Fig. 6.7) also display strong expression of hsp 60 at high temperatures.

The higher intrinsic thermotolerance of cells grown at 35°C (Fig. 6.3C) as compared with cells grown at 25°C (Fig. 6.3B) can perhaps be explained by the suggestion that cultures grown at the higher temperature already exist in a heat shock state in that the growth temperature of 35°C is close to the 37°C heat shock temperature that elicits a heat shock response in 25°C grown cells. However, in the case of trehalose, constitutive levels in 35°C grown cells, although comparable to 15°C or 25°C grown cells, were substantially lower than levels attained upon a 37°C heat shock (Fig. 6.4). Moreover, thermotolerance could be further induced in cells grown at 35°C (Fig. 6.3C) with concomitant increases in hsp 104, 90, 70 and 60 (Fig. 6.5 and 6.7) and trehalose (Fig. 6.4) following a 40°C heat shock for 30 min.

It was somewhat surprising that the present studies revealed that a growth temperature of 15°C (Fig. 6.3A) correlated with the highest intrinsic tolerance. This may be related to a high intrinsic level of hsp 104 (Fig. 6.7) relative to cells grown at 25°C. There is evidence in the literature for hsp 104 involvement in intrinsic thermotolerance. Sanchez *et al.* (1992) observed an increase in expression of hsp 104 in respiring as compared to fermenting yeast concurrent with a lower intrinsic thermotolerance in the latter cells. More recently, Gross and Watson (1996) correlated higher constitutive HSP 104 transcription in Yres, an intrinsically thermoresistant *S. cerevisiae* strain as compared

to Ysen, an inherently thermosensitive strain. Hsp 104 may also play a role at lower growth temperatures as indicated by 100% viability of hsp 104 wild type spores stored at 4°C for 6 months in contrast to 25 to 50% viability in spores of a mutant defective in hsp 104 (Sanchez *et al.*, 1992). Although these results suggest a role for hsp 104 for low temperature growth and intrinsic thermotolerance, a previous report showed that intrinsic thermotolerance (for cells grown at 25°C and stressed to 50°C) nor ability to grow at 25°C or 37°C was greatly effected by a hsp 104 mutation (Sanchez and Lindquist, 1990). However, recent studies showed that a higher basal level of hsp 104 in *hsf1-m3* cells (mutation lacking heat shock transcription factor) and increased levels of hsp 104 in W303Δ104 cells (carrying an estrogen-regulated Hsp 104 expression system) were sufficient for thermotolerance in the absence of a heat shock (Lindquist and Kim, 1996). Nonetheless, an apparent contradiction exists with our findings showing intrinsic thermotolerance being greater in 15°C grown cells (Fig. 6.3A) despite constitutive levels of hsp 104 being significantly higher in 35°C grown cells as compared to 15°C grown cells (Fig. 6.7). The only other hsps that have been reported to be necessary for growth at a colder temperature (19°C) were ssb1 and ssb2, members of the hsp 70 family. Mutations in both ssb1and ssb2 genes resulted in a cold sensitive phenotype (Craig and Jacobsen, 1984; Craig and Jacobsen, 1985; Iwahashi *et al.*, 1995).

Growth rates were well correlated with observed differences in intrinsic thermotolerance. Cultures grown at 15°C were the slowest growing (5 h doubling time) followed by those grown at 35°C (3.5 h doubling time) and optimum growth (2 h doubling time) was observed (for the growth temperatures examined) for cultures grown at 25°C (Fig. 6.1). Slow growth in this instance parallels with intrinsic thermotolerance, that is, 15°C grown cells showed the highest basal tolerance, followed by 35°C grown cells and then 25°C grown cells. The increase in doubling time with an increase in growth temperature from 25°C to 35°C, in addition to a decrease in final culture density (6×10^6 cfu ml^{-1} *cf.* 5×10^7 cfu ml^{-1}) suggests that a growth temperature of 35°C (Fig. 6.1) is close to the maximum growth temperature of this *S. cerevisiae* strain. It has long been observed by researchers of the heat shock response in *S. cerevisiae* that stationary phase cells (slower growing) are more intrinsically resistant to a heat stress compared to exponentially growing cells (Watson, 1990). Furthermore, Elliot and Futcher (1993) reported a correlation between slow growth and carbon catabolite derepression with an increased

intrinsic resistance to a heat stress. The authors also suggest that exponentially growing cells (without a preconditioning treatment) are always stress sensitive. Our studies agree with the findings that slow growth correlates with increased thermotolerance. Furthermore, our results indicated that the temperature at which exponential cells were grown (affecting growth rate) had a dramatic influence on intrinsic thermotolerance (Fig. 6.2).

Similar experiments carried out in *Listeria monocytogenes* differ from our findings (Patchett *et al.*, 1996). To differentiate between the effect of growth temperature and growth rate both continuous and batch cultures were employed. Results suggested that neither growth temperature or growth rate affected susceptibility to a heat stress. However, in *L. monocytogenes*, acid stress tolerance was dependent on both growth temperature and growth rate. An increased growth rate or an increase in growth temperature correlated with an increased resistance to an acid shock at pH 2.5.

With respect to heat shock induced thermotolerance, cultures grown at all temperatures (Fig. 6.3) attained essentially 100% viability over 30 min of the time course to a 48°C heat stress coincident with *de novo* hsp synthesis and trehalose accumulation. However, levels of hsps 104, 90, 70 and 60 (Fig. 6.7) in 37°C heat shocked cells of 15°C grown cultures were significantly less than hsp levels in heat shocked 25°C and 35°C grown cells. These results suggest that only a critical or threshold amount of hsp may be required for optimum levels of heat shock induced thermotolerance. This was particularly evident in the case of cells grown at 15°C and heat shocked at 37°C where hsp 104 levels were less than those of heat shocked 25°C and 35°C grown cells (Fig. 6.7) but obviously sufficient to elicit heat shock induced thermotolerance. Our results suggest that *de novo* hsp synthesis resulting in an increase of hsp over respective constitutive levels is sufficient to induce thermotolerance.

The disaccharide trehalose does not appear to function in intrinsic thermotolerance of *S. cerevisiae* grown at 15°C, 25°C or 35°C (Fig. 6.4). Although the levels of trehalose in control cells at all three growth temperatures were similar, there were marked differences in intrinsic thermotolerance (Fig.6.3). On the other hand, trehalose accumulation was concomitant with heat shock induced thermotolerance at all growth temperatures examined (Fig. 6.4). Interestingly, the magnitude of trehalose induction upon a heat shock was greatest in 25°C grown cultures that were intrinsically the most

thermosensitive (Fig. 6.3B). A 37°C heat shock also elicited the greatest hsp 104 induction (ie the most difference between control and heat shock samples) in 25°C grown cells (Fig. 6.7). This may support the concept of a synergistic relationship between trehalose and hsp 104 in heat shock induced thermotolerance in 25°C grown yeast cells to compensate for the low intrinsic thermotolerance observed (Fig. 6.3B). A synergistic relationship between trehalose and hsp 104 has been previously correlated with intrinsic thermotolerance in stationary phase cells (Elliot *et al.*, 1996). The authors reported that a null mutation at the TIPS2 locus (normally encoding trehalose-6-phosphate phosphatase hence resulting in cessation of trehalose synthesis) or a *HSP 104* mutant caused only moderate heat stress sensitivity in stationary phase cells. However, the *tps1 hsp104* double mutation resulted in essentially no stationary phase induced thermotolerance and hence suggested a synergistic interplay between trehalose, hsp 104 and stationary phase thermotolerance.

Previous studies on carbohydrate composition of fungi from different growth temperature groups indicated higher trehalose levels in spores of thermophilic fungi as compared to psychrophilic fungi (Feofilova *et al.*, 1994). Furthermore, trehalose levels increased upon a heat shock and decreased upon a downshift in temperature. The authors suggest that trehalose is a "high-temperature" sugar whereas at low temperatures mannitol and glucose are more prevalent. Results presented in Chapters 3, 4, 5 and the current chapter examining trehalose levels in psychrophilic, mesophilic and thermophilic yeast confirm heat shock induced trehalose accumulation. However, psychrophilic and psychrotrophic yeast generally had higher constitutive levels of trehalose (Fig. 4.7) than either *S. cerevisiae* (Fig. 6.4) or thermophilic yeast (Fig. 5.11) and growth temperature did not markedly effect constitutive trehalose levels in *S. cerevisiae* (Fig. 6.4).

Results presented in this chapter also suggest that the heat shock temperature eliciting *de novo* hsp synthesis, trehalose accumulation and optimal induction of thermotolerance may be "predetermined". For example, irrespective of growth temperature, a 37°C heat shock evokes hsp synthesis (Fig. 6.5 and 6.7), trehalose accumulation (Fig. 6.4) and induction of thermotolerance in both 15°C (Fig. 6.3A) and 25°C (Fig. 6.3B) grown cultures. It appears therefore that heat shock temperature correlates with a temperature conferring optimal growth (25°C-30°C) of *S. cerevisiae*. However, similar studies in the eurythermal goby fish, *Gillichthys mirabilis* indicated that the heat shock temperature inducing hsp 90 synthesis differed for various temperature

acclimated individuals (Dietz, 1994; Somero, 1995). Furthermore, Dietz and Somero (1992) found that hsp 90 concentration increased with acclimation temperature. Our results for *S. cerevisiae* agree with the latter finding not only for hsp 90 but also for hsp 70 and hsp 60 (Fig. 6.7).

The observed reduction in heat shock induced trehalose accumulation (Fig. 6.4) and induced thermotolerance (Fig. 6.3B) with cycloheximide treatment for 25°C grown cells was consistent with previous findings (Hall, 1983; Watson *et al.*, 1984; De Virgilio *et al.*, 1991, Coote *et al.*, 1992; Neves and François, 1992; Gross and Watson, 1996). This same trend was also observed for 15°C and 35°C grown cells (Fig. 6.3A and C, Fig. 6.4). The effect of cycloheximide was most pronounced for 15°C grown cells heat shocked at 37°C with minimal induction of tolerance to a heat stress (with absolute values less than those induced by a 25°C heat shock) as compared to untreated cells (Fig. 6.3A). However, despite this reduction, the levels of induced thermotolerance with cycloheximide treatment in 15°C grown cells was still greater than those measured for cycloheximide treated 25°C and 35°C grown cells over the 60 min time course (Fig. 6.3). This may be attributable to the greater basal tolerance exhibited by 15°C grown cells (Fig. 6.3) as compared to 25°C or 35°C grown cells. The marked inhibition of induced thermotolerance in cycloheximide treated 15°C grown cells suggests a necessity for either *de novo* protein synthesis, a heat shock inducible signal produced by accumulation of aberrant proteins during the heat shock, increased levels of hsps over constitutive levels or an increase in trehalose accumulation. *De novo* hsp synthesis may be of particular relevance because of lower basal levels of hsp 60, hsp 70 and hsp 90 (Fig. 6.7).

In addition to examining the influence of growth temperature on known hsps, two novel growth temperature dependent proteins, at approximately 40 and 80 kDa, were identified (Fig. 6.5). The *de novo* synthesis of both proteins was coincident with increased growth temperature. Furthermore, a heat shock temperature eliciting hsp synthesis negatively regulated the synthesis of these two growth temperature dependent proteins. The latter observation suggests down-regulation of these proteins by hsps. This is in line with previous studies which have shown hsps regulating their own synthesis (Stone and Craig, 1990; Craig and Gross, 1991), the synthesis of other hsps (Lindquist and Craig, 1988) and trehalose accumulation (Hottiger *et al.*, 1992).

It is useful to compare psychrophiles and psychrotrophs with mesophiles and thermophiles to help identify key features of temperature adaptation and to ascertain whether various aspects of the heat shock response are affected by growth temperature. In comparison to psychrophilic and psychrotrophic yeast (Chapters 3 and 4), mesophilic *S. cerevisiae* grown at 15°C was able to withstand a higher heat stress temperature of 48°C (Fig. 6.3A). At a heat stress temperature of 35°C, a stress temperature used in analyses of psychrophilic yeast, *S. cerevisiae* showed no loss of viability, with or without a preconditioning heat shock, over a 60 min time course (Fig. 6.2A). Furthermore, the heat shock temperature and duration of heat shock eliciting *de novo* hsp synthesis and trehalose accumulation differed. A 3 h heat shock at 25°C induced hsps and trehalose for psychrophilic and psychrotrophic yeast grown at 15°C (Chapter 3) while a 30 min heat shock at 37°C was required for *S. cerevisiae* grown at 15°C. Another apparent difference between the heat shock response in psychrophilic yeast and *S. cerevisiae* was the specific hsps synthesized during the respective heat shock. Regardless of growth temperature, heat shocked cells of *S. cerevisiae* synthesized hsps 104, 90, 70 and 60 (Fig. 6.5 and 6.7). On the other hand, psychrophilic yeast exhibited species specific hsp profiles with a common 110 kDa heat shock inducible protein and characteristic absence of a hsp 104 homologue to a *S. cerevisiae* anti-hsp 104 antibody (Chapters 3 and 4). In contrast, the heat shock response in 35°C grown *S. cerevisiae* cultures was remarkably similar to thermophilic yeast (grown at 35°C, heat shocked at 40°C for 30 min and heat stressed to 48°C) with respect to viable counts for intrinsic and induced thermotolerance, trehalose accumulation and the heat shock induced synthesis of hsps 104, 90, 70 and 60 (Chapter 5). The heat shock temperature evoking hsp synthesis and induced thermotolerance correlated with the growth temperature of environmental temperature-adapted yeast.

A study of heat shock tolerance in *Drosophila melanogaster* showing long term (15 yr) adaptation to different temperatures (18°C, 25°C and 28°C) (Cavicchi *et al.*, 1995) provided similar findings to environmental temperature-adapted psychrophilic (Chapters 3 and 4) and thermophilic yeast (Chapter 5). Flies at 28°C had a greater intrinsic tolerance to a heat stress and flies at 18°C exhibited a thermosensitive phenotype. Distinct differences in the heat shock response were observed between long term temperature-adapted yeast (psychrophilic and thermophilic yeast) and *S. cerevisiae* grown at different temperatures. This was particularly evident when comparing the heat shock response in Antarctic yeast

to *S. cerevisiae* grown at 15°C. However, it was of interest to observe a very similar heat shock response in thermophilic yeast and *S. cerevisiae* grown at 35°C. This suggests that growth temperature alone can not account for the observed differences in intrinsic or induced thermotolerance between psychrophilic, mesophilic and thermophilic yeast.

In summary, the present studies reveal a relationship between growth temperature and heat shock response in *S. cerevisiae*. The results indicate that the temperature at which *S. cerevisiae* is grown influences intrinsic thermotolerance, growth rate and constitutive hsp levels but does not greatly influence heat shock induced thermotolerance or trehalose accumulation. The findings suggest that hsp 104 and slow growth contribute to the higher basal tolerance observed in cells grown at 15°C and 35°C. Furthermore, two novel temperature dependent proteins (40 kDa and 80 kDa) were identified which appear to be down regulated by a heat shock.