CHAPTER 7: Oxidative Stress Tolerance.

7.1 Introduction

A key factor to the survival of microbes is their adaptation to oxygen toxicity. Oxygen is capable of undergoing a series of one-electron reductions, generating reactive oxygen species (ROS) including superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) and when the levels of ROS exceeds the antioxidant capacity of the cell, an oxidative stress ensues (Burdon *et al.*, 1990; Pahl and Baeuerle, 1994; Moradas-Ferreira *et al.*, 1996). ROS are generated as a consequence of normal respiratory metabolism, exposure to ionizing radiation, stimulated macrophages, increased oxygen pressure and the presence of hydrogen peroxide, menadione or paraquat in the growth medium (Kullik and Storz, 1994; Moradas-Ferreira *et al.*, 1996). Most intensive studies of oxidative stress systems in microorganisms have been carried out in *Escherichia coli* (Farr and Kogama, 1991), *Salmonella typhimurium* (Farr and Kogoma, 1991), *Bacillus subtilis* (Dowds, 1994) and *S. cerevisiae* (Moradas-Ferreira *et al.*, 1996). At low temperatures, oxygen solubility and availability increases (Sinclair and Stokes, 1963). In low temperature environments in which psychrophiles and psychrotrophs are found, oxidative stress resistance may, therefore, be necessary for life itself. In contrast, thermophilic enteric yeast are facultative anaerobes (Watson *et al.*, 1978) and they inhabit environments which have low oxygen levels. Furthermore, thermophilic enteric yeast most probably are confronted by an onslaught of reactive oxygen species with respect to the host immune system responding to potential pathogenicity. Consequently, it was of particular interest to examine the oxidative stress response in yeast from these environmental extremes. In this chapter, intrinsic and induced tolerance to a hydrogen peroxide stress in psychrophilic, psychrotrophic and thermophilic yeast were examined. In addition, the crosstolerance effect of a prior heat shock on induced peroxide stress tolerance was also examined.

7.2 Experimental outline

Experimental cultures were grown to an OD_{600} of 0.2 to 0.3 corresponding to logarithmic phase cells of approximately 2 x 10^6 to 4 x 10^6 cfu ml^{-1} for psychrophilic yeast, 5 x 10^6 cfu ml^{-1} for psychrotrophic yeast and 2 x 10^6 to 5 x 10^6 for thermophilic yeast. Stationary phase cultures were defined as glucose exhausted media with a cell density of 1 x 10^8 cfu ml^{-1} for *C. psychrophila* and 2.5 x 10^7 cfu ml^{-1} for *A. telluris* strain 2760.

Preliminary experiments of intrinsic peroxide tolerance for psychrophilic yeast were carried out in *C. psychrophila* grown at 15°C. Cultures were exposed to 10, 50 or 100 mM H_2O_2 over a 2 h time course. Subsequently both 50 and 100 mM H_2O_2 were used to examine induced peroxide tolerance in *C. psychrophila*. Intrinsic peroxide tolerance to 100 mM H_2O_2 was measured for the psychrophilic yeast, *M. frigida*, *M. gelida*, *M. stokesii* and *L. antarcticum* grown at 15°C and the psychrotrophic yeast, *L. fellii* and *L. scottii* grown at 15°C and 25°C. Induced peroxide tolerance to 100 mM H_2O_2 was measured following a 3 h, 0.2 mM H_2O_2 shock, 20°C heat shock or 25°C heat shock for psychrophilic and psychrotrophic yeast grown at 15°C or a 30 min, 0.2 mM H_2O_2 shock or 37°C heat shock for psychrotrophic yeast grown at 25°C.

Preliminary analysis of intrinsic peroxide tolerance was carried out in the thermophilic yeast strain, *A. telluris* 2760 (respiratory-competent) by exposing cultures grown at 35°C to 1, 2, 5, or 10 mM H_2O_2. Subsequently, intrinsic peroxide tolerance was examined after exposure to 2 mM H_2O_2 for *A. telluris* 1787 (respiratory-deficient) and *A. telluris* 2760 (respiratory-competent) for direct comparison between the two strains. *A. telluris* 2760 was also exposed to 5 and 10 mM H_2O_2. Induced peroxide tolerance to 2, 5 or 10 mM H_2O_2 was examined following a 30 min, 0.2 mM H_2O_2 shock or a 40°C heat shock.

Heat shock proteins and trehalose levels were examined for control and peroxide shock samples of *C. psychrophila*, *A. telluris* 2760 (respiratory-competent) and *A. telluris* 1787 (respiratory-deficient). Peroxide shock inducible *de novo* protein synthesis was monitored for all psychrophilic yeast grown at 15°C, psychrotrophic yeast grown at 15°C and 25°C and thermophilic yeast grown at 35°C.

7.3 Results

7.3.1 Peroxide stress tolerance

7.3.1.1 Antarctic yeast

Figure 7.1 illustrates preliminary results on intrinsic peroxide stress tolerance for the psychrophilic yeast, *C. psychrophila*. As the concentration of hydrogen peroxide increased from 10 mM to 100 mM H_2O_2 culture viability decreased. However, culture viability was able to be measured throughout the time course after exposure to both 50 and 100 mM H_2O_2. These two concentrations were used for initial investigations of intrinsic and induced peroxide stress tolerance in *C. psychrophila* (Fig. 7.2). Cross-tolerance against H_2O_2 stress was noted, in that a heat shock at 20°C for 3 h protected against a lethal peroxide stress (Fig. 7.2). Moreover, a mild H_2O_2 shock (0.2 mM for 30 min) also protected against a lethal peroxide stress (Fig. 7.2) but not against lethal heat stress (Chapter 3, Fig. 3.5). However, a 25°C heat shock did not induce peroxide stress tolerance (Fig. 7.3A). Stationary phase cultures were intrinsically more resistant to a peroxide stress and retained this resistance for a longer duration than logarithmic phase cultures (Fig. 7.3B).

Intrinsic and induced peroxide stress tolerance to a 100 mM H_2O_2 stress in the other Antarctic psychrophilic yeast are depicted in Fig. 7.4. Intrinsic peroxide tolerance levels differed in the four psychrophilic species examined. *L. antarcticum* (Fig. 7.4D) showed the least tolerance followed by *M. frigida* (Fig. 7.4A) with *M. gelida* (Fig. 7.4D) and *M. stokesii* (Fig. 7.4C) showing the most intrinsic tolerance to a 100 mM H_2O_2 stress. Peroxide stress tolerance was induced by both a 3 h, 20°C heat shock and a 3 h, 25°C heat shock in all psychrophilic yeast. A 3 h, 20°C heat shock induced maximal peroxide stress tolerance in all *Mrakia* species examined. Conversely, a 3 h, 25°C heat shock rather than a 3 h, 20°C heat shock induced maximal tolerance in *L. antarcticum*. In all psychrophilic yeast, a 3 h, 0.2 mM H_2O_2 shock induced peroxide tolerance to a 100 mM H_2O_2 stress. In *M. frigida*, *M. gelida* and *M. stokesii* the level of peroxide tolerance induced by a peroxide shock was essentially equal to levels induced by a 3 h, 20°C heat shock. In *L. antarcticum* however, a peroxide shock marginally induced peroxide tolerance levels over those of intrinsic levels.

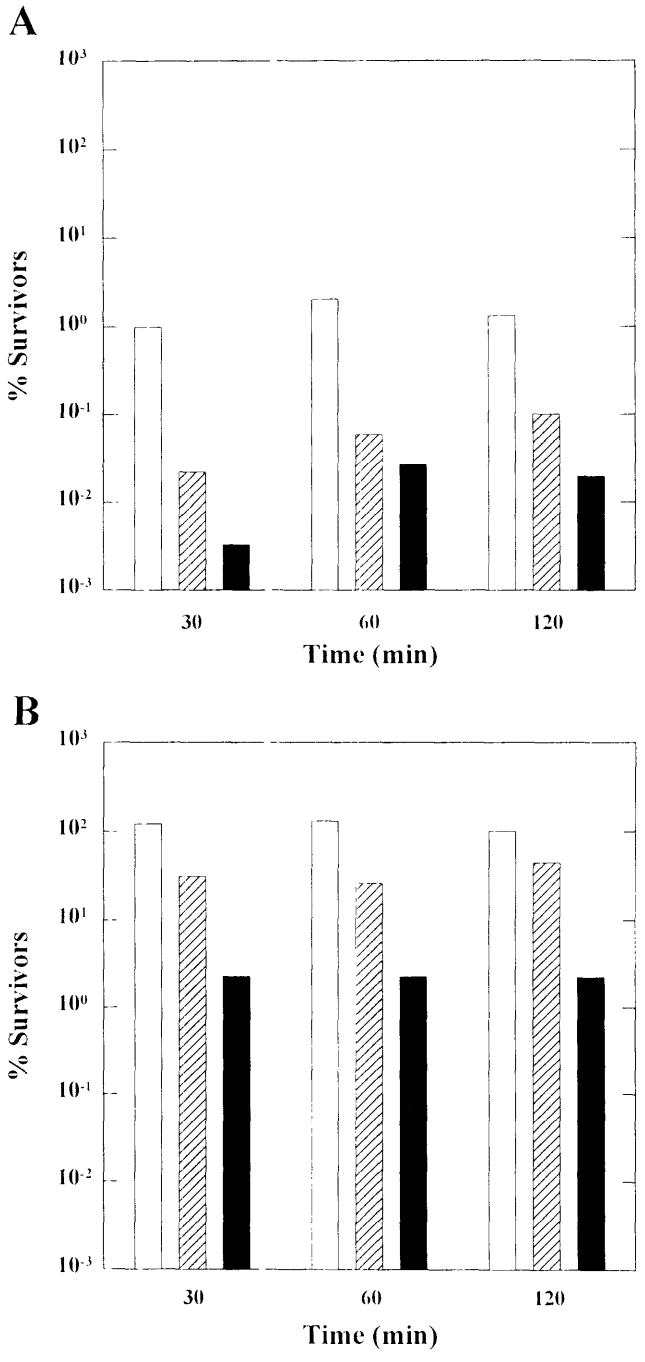

Figure 7.1. Intrinsic peroxide tolerance in (A) mid-logarithmic and (B) late logarithmic phase cultures of *C. psychrophila* grown at 15°C. Intrinsic tolerance was measured at the times indicated following addition of 10 (▭), 50 (▨) or 100 (■) mM H_2O_2. Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

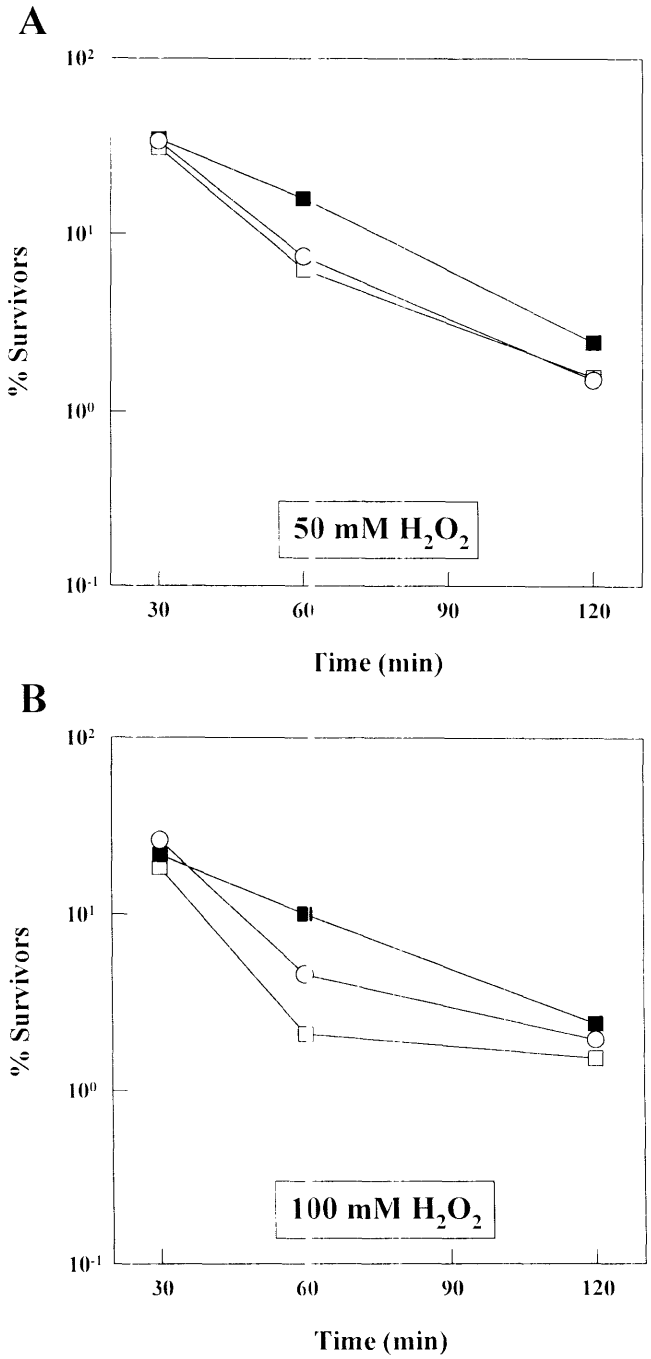

Figure 7.2. Intrinsic and induced peroxide tolerance in mid-logarithmic phase cultures of *C. psychrophila* grown at 15°C. Intrinsic tolerance (□) was measured at the times indicated after the addition of (A) 50 or (B) 100 mM H_2O_2. Induced peroxide tolerance to (A) 50 or (B) 100 mM H_2O_2 was monitored following a 3 h, 0.2 mM H_2O_2 shock (■) or 20°C heat shock (○). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

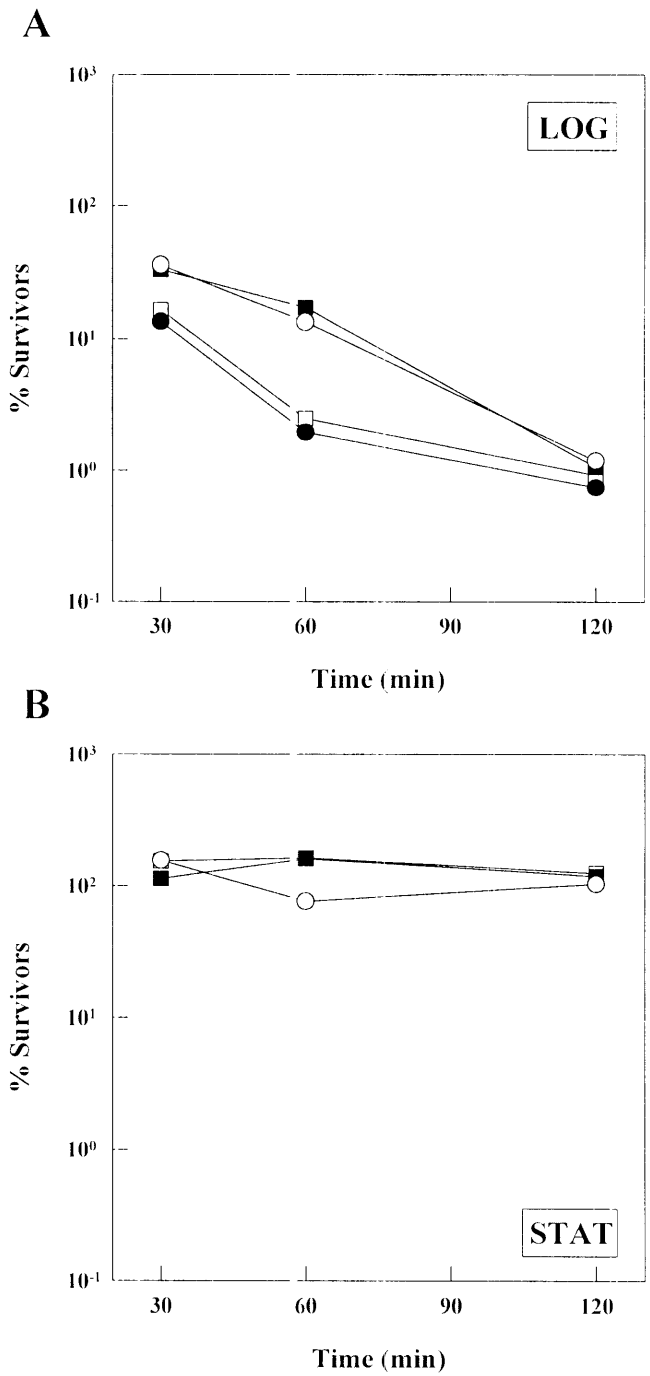

Figure 7.3. Intrinsic and heat shock induced peroxide tolerance in (A) mid-logarithmic and (B) stationary phase cultures of *C. psychrophila* grown at 15°C. Intrinsic tolerance (□) was measured at the times indicated after the addition of 100 mM H_2O_2. Induced peroxide tolerance to 100 mM H_2O_2 was monitored following a 3 h, 0.2 mM H_2O_2 shock (■), 20°C heat shock (○) or 25°C heat shock (●). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

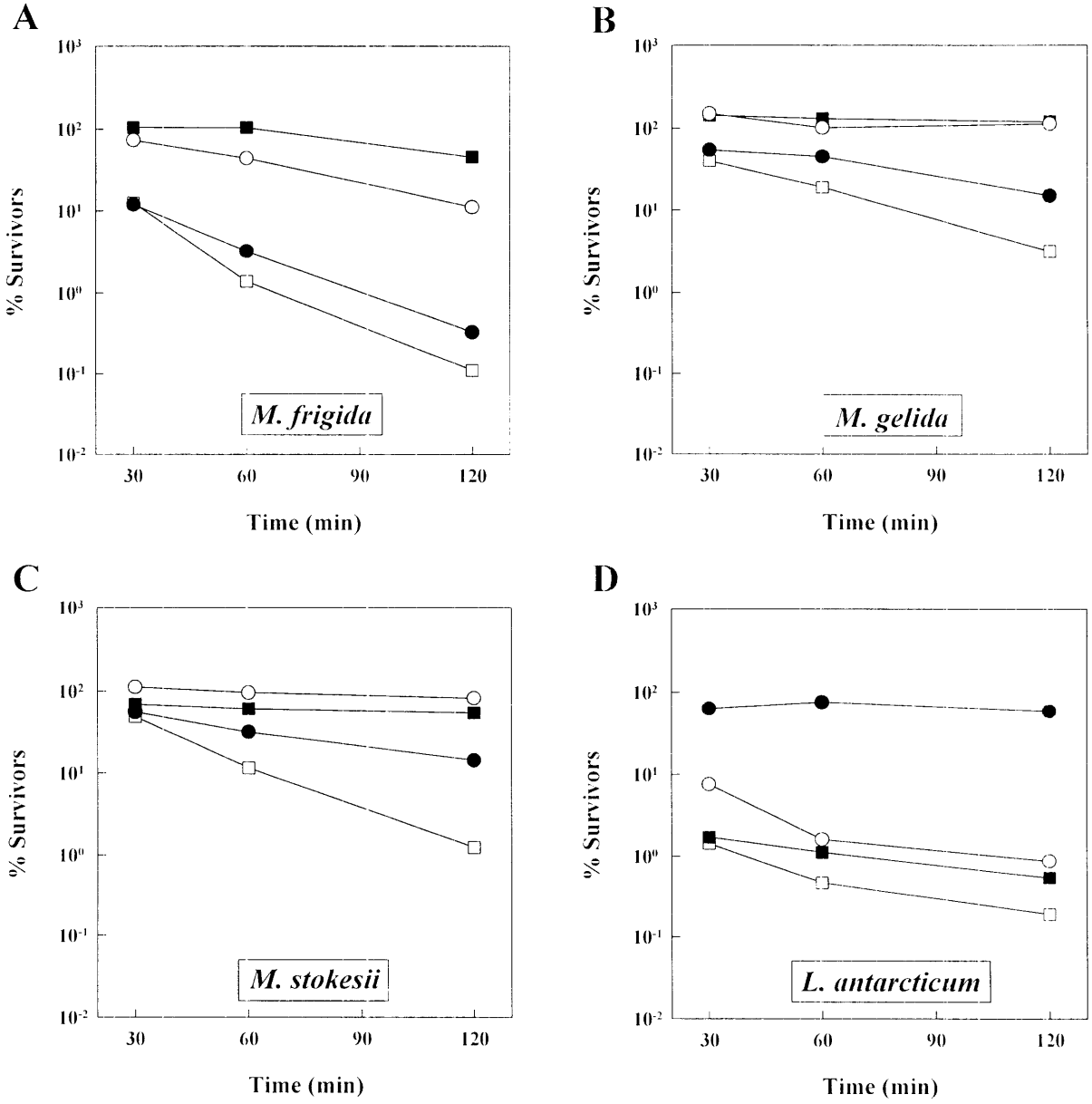

Figure 7.4. Intrinsic and induced peroxide tolerance in mid-logarithmic phase cultures of (A) *M. frigida*, (B) *M. gelida*, (C) *M. stokesii* and (D) *L. antarcticum* grown at 15°C. Intrinsic tolerance (□) was measured at the times indicated after the addition of 100 mM H_2O_2. Induced peroxide tolerance to 100 mM H_2O_2 was monitored following a 3 h, 0.2 mM H_2O_2 shock (■), 20°C heat shock (○) or 25°C heat shock (●). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

The psychrotrophic yeast *L. scottii* grown at 15°C (Fig. 7.5B) had high levels of intrinsic peroxide tolerance to a 100 mM H_2O_2 over a 2 h time course as compared to *L. fellii* (Fig. 7.5A) and the other psychrophilic yeast. Peroxide stress tolerance was increased in both *L. fellii* and *L. scottii* grown at 15°C by exposure to a prior 3 h, 20°C heat shock, a 3 h, 25°C heat shock or a 3 h 0.2 mM H_2O_2 shock. In *L. fellii* grown at 15°C (Fig. 7.5A) a 3 h, 25°C heat shock or a 3 h, 0.2 mM H_2O_2 peroxide shock elicited maximal peroxide stress tolerance. Interestingly, all three shock treatments elicited equivalent peroxide stress tolerance in *L. scottii* grown at 15°C. However, when the psychrotrophic yeast were grown at 25°C (Fig. 7.5C and D) their stress response patterns were very similar. In both *L. fellii* and *L. scottii*, a 30 min 0.2 mM H_2O_2 shock induced peroxide stress tolerance but a 30 min, 37°C heat shock decreased tolerance below intrinsic levels. Intrinsic peroxide stress tolerance in *L. scottii* was greater when grown at 15°C. In *L. fellii*, intrinsic peroxide tolerance levels were slightly greater when grown at 25°C.

7.3.1.2 Thermophilic yeast

Preliminary results on intrinsic peroxide stress tolerance for *A. telluris* strain 2760 (respiratory-competent) are illustrated in Fig 7.6. Culture sensitivity increased with increasing peroxide concentration and duration of stress. A 2 mM H_2O_2 concentration was used for initial examination of oxidative stress tolerance in both thermophilic yeast strains as it was presumed intuitively that the respiratory-deficient strain 1787 would exhibit a more marked sensitivity to a peroxide stress as compared to the respiratory-competent strain 2760. In fact there was a significant difference between logarithmic phase cultures of strains 2760 and 1787 exposed to a 2 mM H_2O_2 stress with strain 2760 showing greater basal tolerance over a 120 min time course than strain 1787 (Fig 7.7). Hence, for the purposes of this study, higher concentrations of hydrogen peroxide (5 and 10 mM) were also used to examine intrinsic and induced peroxide stress tolerance in the respiratory-competent strain 2760 (Fig. 7.8).

In both strains, a 40°C heat shock (30 min) and a 0.2 mM H_2O_2 (30 min) shock pretreatment resulted in increased resistance to the respective peroxide stress. However, the inducer of maximal peroxide stress resistance differed with a peroxide shock conferring maximal tolerance in the respiratory-competent strain 2760 (Fig. 7.7A: Fig. 7.8A and B)

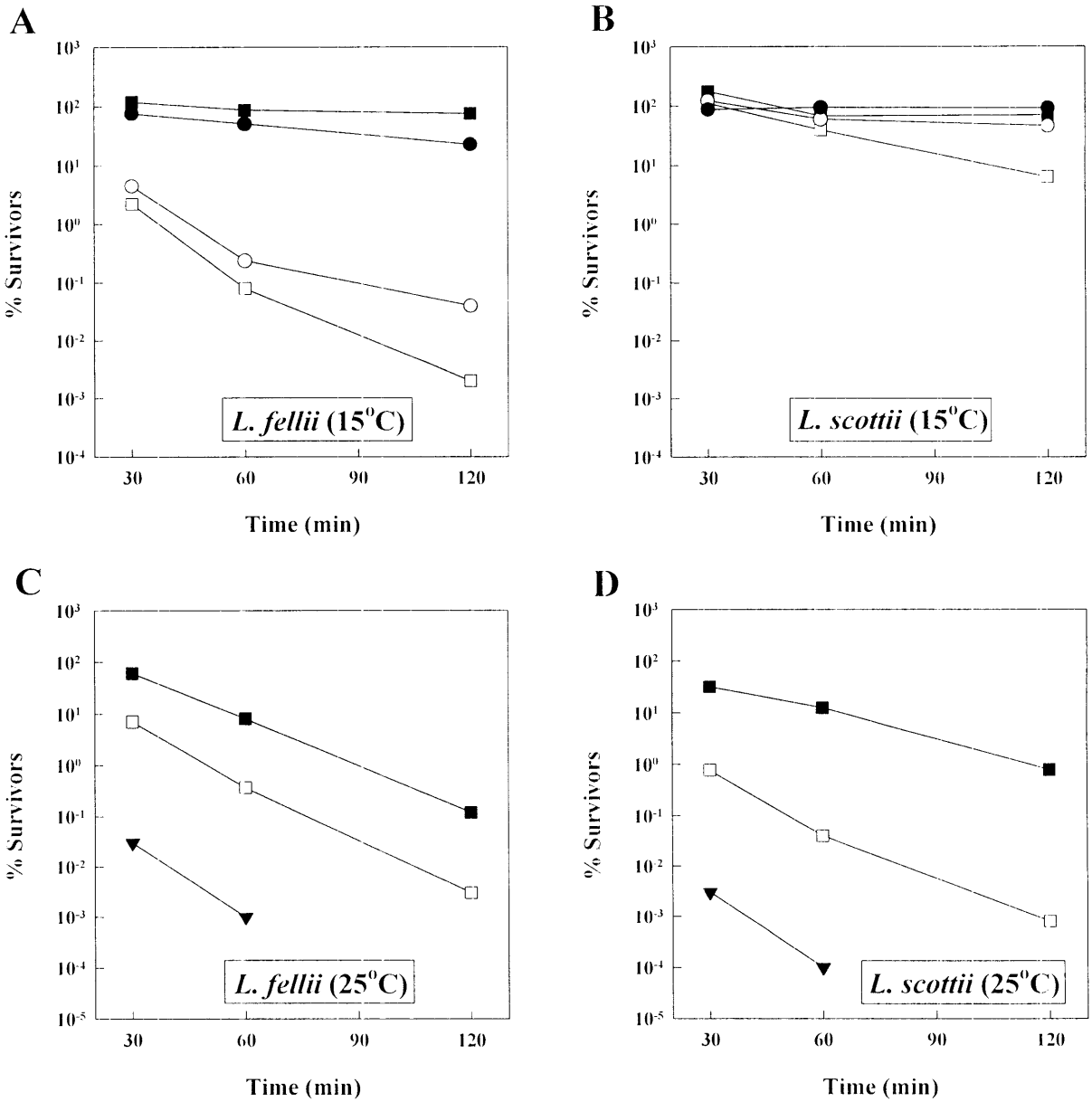

Figure 7.5. Intrinsic and induced peroxide tolerance in mid-logarithmic phase cultures of *L. fellii* and *L. scottii* grown at 15°C and 25°C. Intrinsic tolerance (□) was measured at the times indicated after the addition of 100 mM H_2O_2. Induced peroxide tolerance to 100 mM H_2O_2 was monitored following a 3 h, 0.2 mM H_2O_2 shock (■), 20°C heat shock (○) or 25°C heat shock (●) for (A) *L. fellii* and (B) *L. scottii* cultures grown at 15°C. For 25°C grown cultures of (C) *L. fellii* and (D) *L. scottii*, induced peroxide tolerance to 100 mM H_2O_2 was monitored following either a 30 min, 0.2 mM H_2O_2 shock (■) or 37°C heat shock (▼). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C or 25°C control sample.

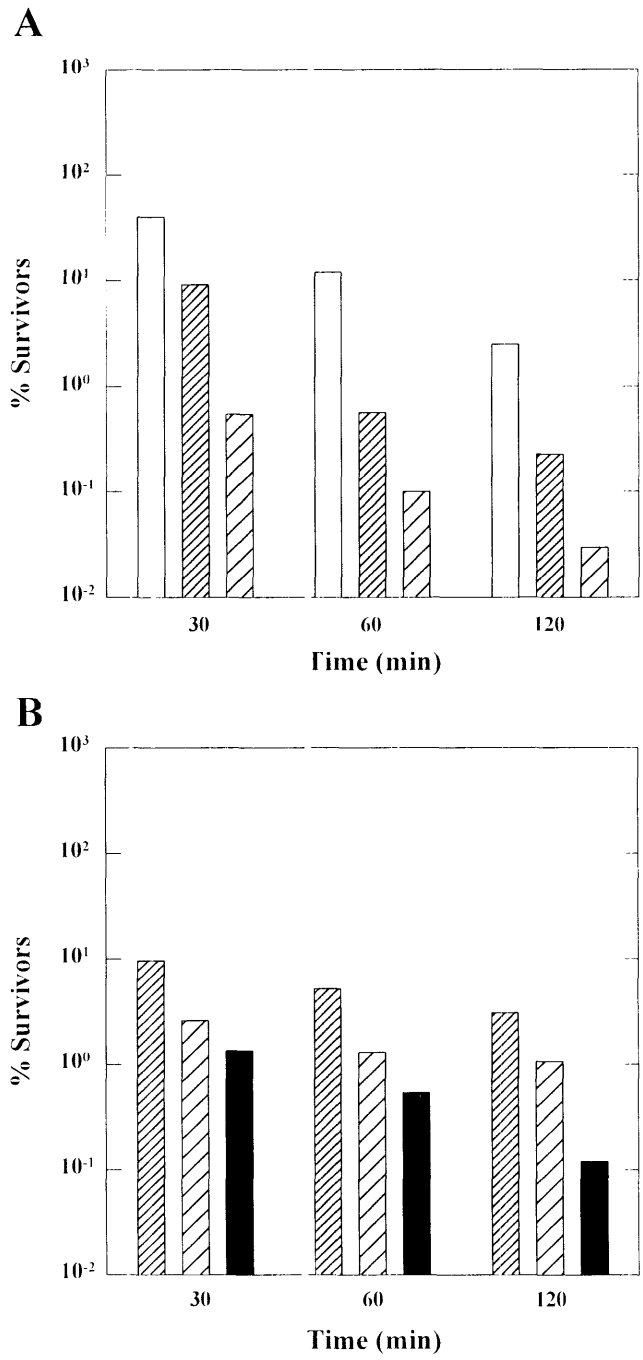

Figure 7.6. Intrinsic peroxide tolerance in (A) mid-logarithmic and (B) late logarithmic phase cultures of *A. telluris* 2760 (respiratory-competent) grown at 35°C. Intrinsic tolerance was measured at the times indicated following addition of 1 (▭), 2 (▨), 5 (▨) or 10 (■) mM H_2O_2. Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

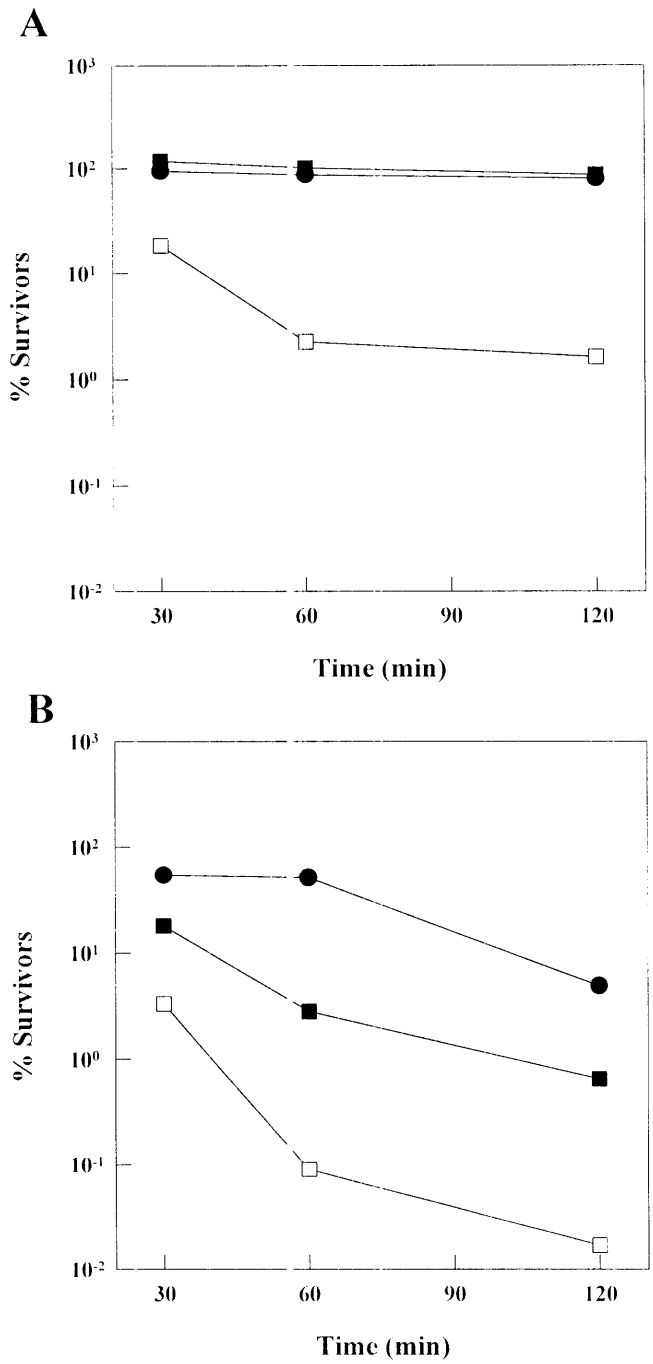

Figure 7.7. Intrinsic and induced peroxide tolerance to a 2 mM H_2O_2 stress in mid-logarithmic phase cultures of (A) *A. telluris* 2760 (respiratory-competent) and (B) *A. telluris* 1787 (respiratory-deficient) grown at 35°C. Intrinsic tolerance (□) was measured at the times indicated after the addition of 2 mM H_2O_2. Induced peroxide tolerance to 2 mM H_2O_2 was monitored following a 30 min, 0.2 mM H_2O_2 shock (■) or 40°C heat shock (●). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

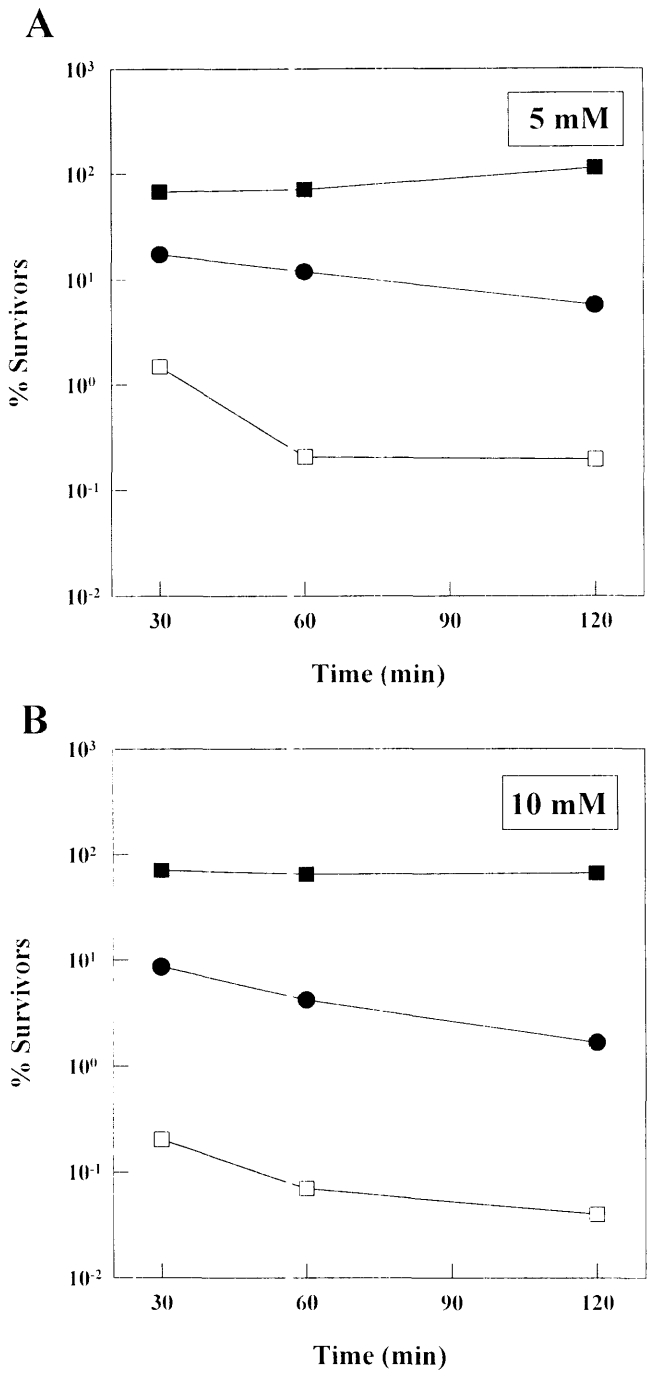

Figure 7.8. Intrinsic and induced peroxide tolerance to a 5 or 10 mM H_2O_2 stress in mid-logarithmic phase cultures of *A. tellurix* 2760 (respiratory-competent) grown at 35°C. Intrinsic tolerance (□) was measured at the times indicated after the addition of (A) 5 or (B) 10 mM H_2O_2. Induced peroxide tolerance to 5 or 10 mM H_2O_2 was monitored following a 30 min, 0.2 mM H_2O_2 shock (■) or 40°C heat shock (●). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

and a heat shock eliciting maximal tolerance in the respiratory-deficient strain 1787 (Fig. 7.7B). Stationary phase cultures of the respiratory-competent strain 2760 (Fig. 7.9) were intrinsically more resistant than logarithmic phase cultures with a 50-fold increase after 30 min and a 190-fold increase after 60 min. Slight increases in peroxide stress tolerance were induced by a 0.2 mM H_2O_2 shock and a 40°C heat shock.

7.3.2 Trehalose

In contrast to heat shock induced trehalose accumulation, a peroxide shock did not correlate with increased trehalose levels in *C. psychrophila* (Fig. 7.10A) or either thermophilic yeast strain (Fig. 7.10B and C), in fact, a slight decrease below constitutive levels was observed in both thermophilic yeast strains.

7.3.3 Stress proteins

7.3.3.1 Antarctic yeast

A peroxide shock did not induce the synthesis of hsps 104, 90, 70 or 60 as observed by western immunoblot analyses of protein extracts from *C. psychrophila* (Fig. 7.11). These results were confirmed using ^{35}S-methionine labelling (Fig. 7.12). However, proteins corresponding to 51, 43, 41, 35 and 30 kDa were induced by a mild peroxide shock (Fig. 7.12).

^{35}S-methionine labelling of control and peroxide shock samples in other psychrophilic yeast indicated no peroxide shock inducible proteins (psps) in either *M. frigida* (Fig. 7.13A) or *L. antarcticum* (Fig. 7.13D). However, a 3 h, 0.2 mM H_2O_2 shock resulted in the slight induction of a common 90 kDa protein in both *M. gelida* (Fig. 7.13B) and *M. stokesii* (Fig. 7.13C). In addition, a further two psps at approximately 70 and 100 kDa were observed in *M. gelida* (Fig. 7.13B). No psps were identified in either 15°C or 25°C grown *L. scottii* cultures (Fig. 7.14A). On the other hand, a peroxide shock resulted in the slight induction of an 80 kDa protein in *L. fellii* grown at 15°C (Fig. 7.14B, lane 2). When *L. fellii* was grown at 25°C and exposed to a peroxide shock, six (31, 33, 41, 45, 47 and 53 kDa) increased in synthesis (Fig. 7.14B, lane 4).

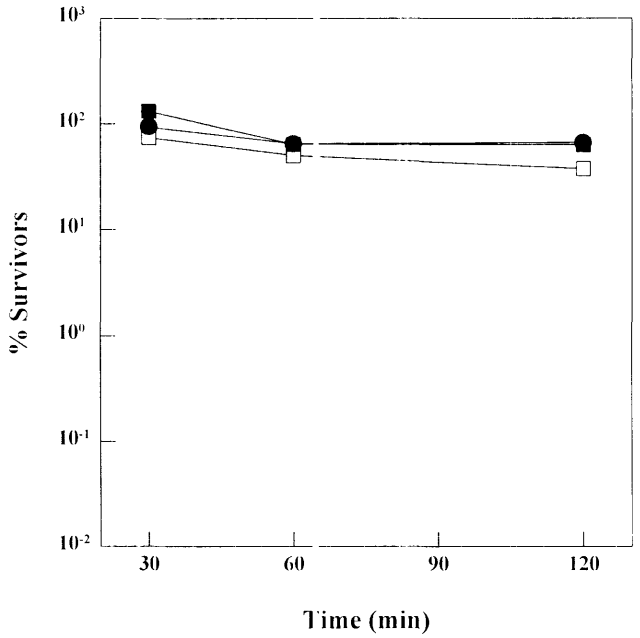

Figure 7.9. Intrinsic and induced peroxide tolerance to a 10 mM H_2O_2 stress in stationary phase cultures of *A. telluris* 2760 (respiratory-competent) grown at 35°C. Intrinsic tolerance (□) was measured at the times indicated after the addition of 10 mM H_2O_2. Induced peroxide tolerance to 10 mM H_2O_2 was monitored following a 30 min, 0.2 mM H_2O_2 shock (■) or 40°C heat shock (●). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

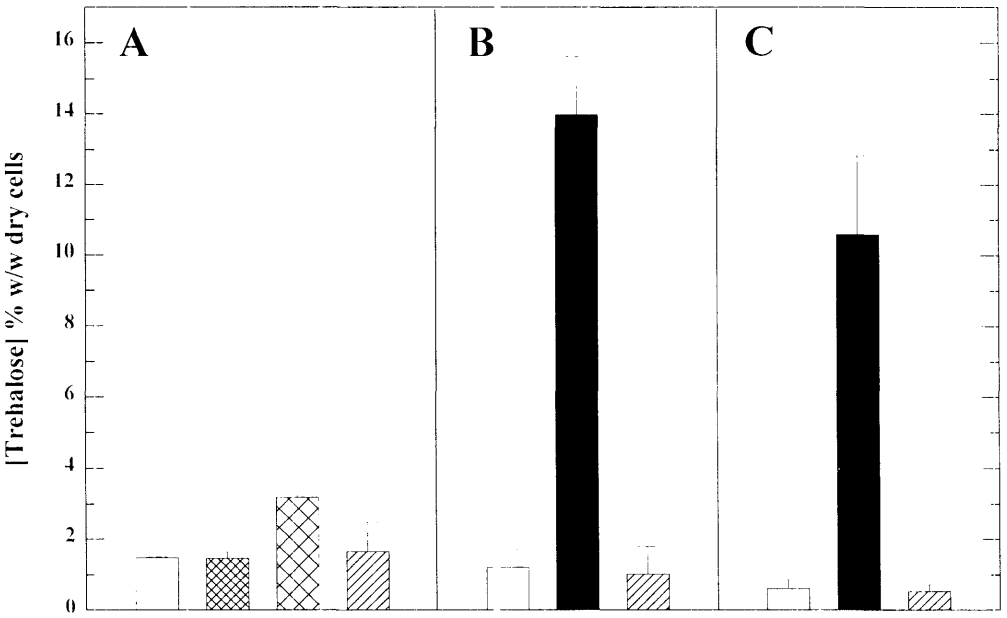

Figure 7.10. Trehalose levels in control, heat shock and peroxide shock samples of (A) *C. psychrophila*, (B) *A. telluris* 2760 (respiratory-competent) and (C) *A. telluris* 1787 (respiratory-deficient). Trehalose was extracted from control (□), 20°C heat shock (▩), 25°C heat shock (⊠), 40°C heat shock (■) and 0.2 mM H_2O_2 shock (▨) samples. Results are presented as mean and standard deviation of three measurements.

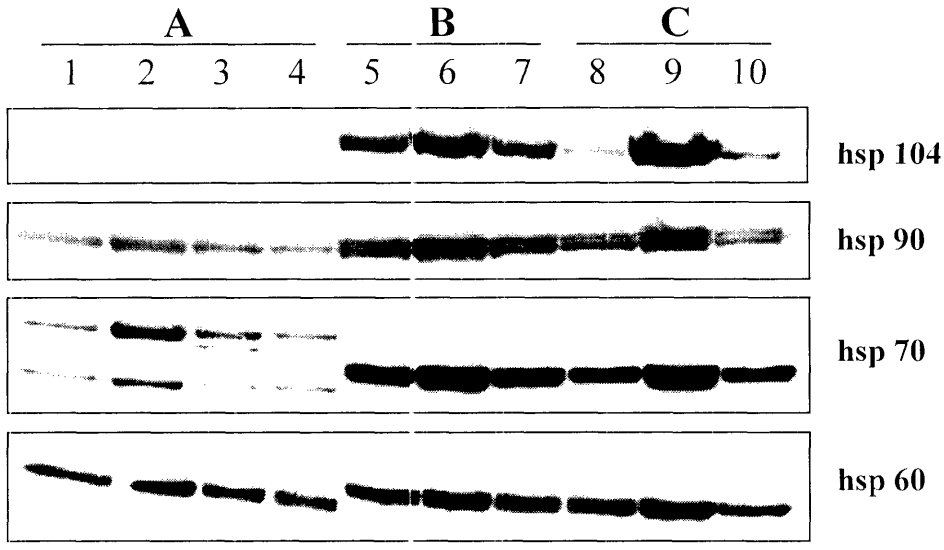

Figure 7.11. Western blot analysis of control, heat shock and peroxide shock extracts from (A) *C. psychrophila*, (B) *A. telluris* 2760 (respiratory-competent) and (C) *A. telluris* 1787 (respiratory-deficient). Proteins from 15°C control (lane 1), 20°C heat shock (lane 2), 25°C heat shock (lane 3), 35°C control (lanes 5 and 8), 40°C heat shock (lanes 6 and 9) and 0.2 mM H_2O_2 shock (lanes 4, 7 and 10) were probed with anti-hsp 104 (1:1000), anti-hsp 90 (1:750), anti-hsp 70 (1:5000) and anti-hsp 60 (1:1000).

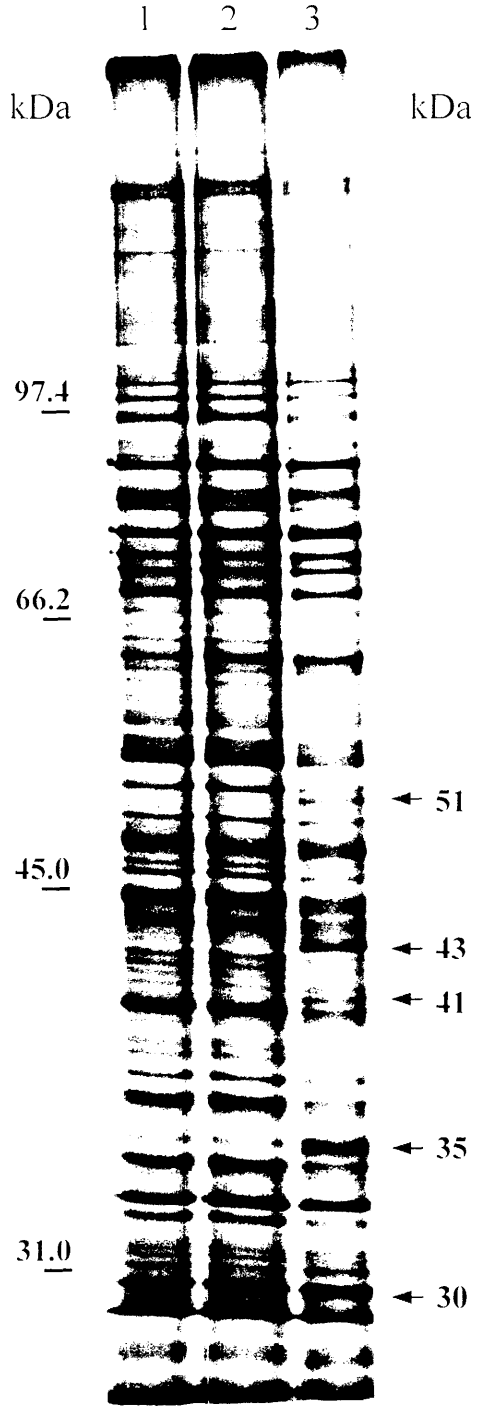

Figure 7.12. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control, heat shocked and peroxide shocked cells of *C. psychrophila*. Conditions were 15°C control (lane 1), 3 h at 20°C heat shock (lane 2) and 3 h at 0.2 mM H_2O_2 shock (lane 3). Arrows indicate new or increased peroxide shock inducible proteins (kDa). Molecular mass standards (kDa) are as indicated.

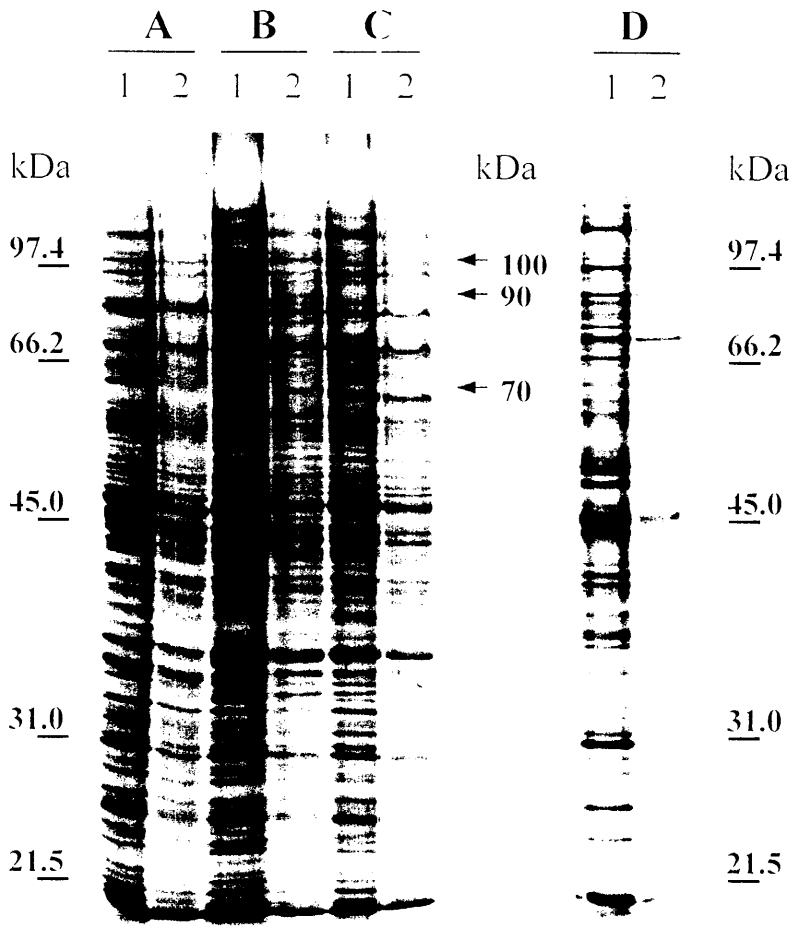

Figure 7.13. SDS-polyacrylamide gel autoradiograms of ^{35}S-methionine labelled protein extracts from control and peroxide shocked cells of (A) *M. frigida*, (B) *M. gelida*, (C) *M. stokesii* and (D) *L. antarcticum*. Conditions were 15°C control (lanes 1) and 3 h at 0.2 mM H_2O_2 shock (lanes 2). Arrows indicate new or increased peroxide shock inducible proteins (kDa). Molecular mass standards (kDa) are as indicated.

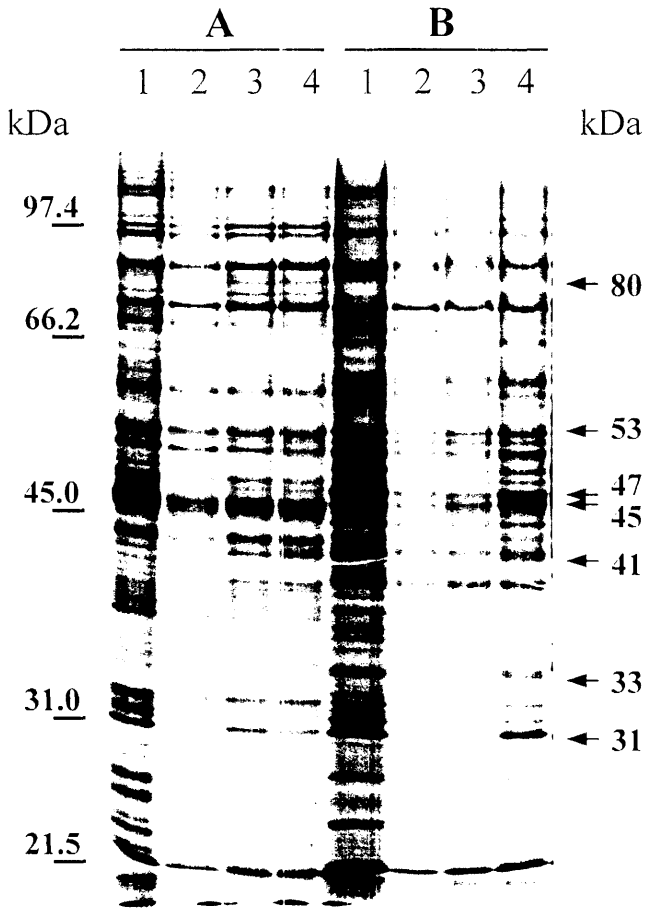

Figure 7.14. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control and peroxide shocked cells of (A) *L. scottii* and (B) *L. fellii* grown at 15°C and 25°C. Conditions were 15°C control (lanes 1), 3 h at 0.2 mM H_2O_2 shock (lanes 2), 25°C control (lanes 3) and 30 min at 0.2 mM H_2O_2 (lanes 4). Arrows indicate new or increased peroxide shock inducible proteins (kDa). Molecular mass standards (kDa) are as indicated.

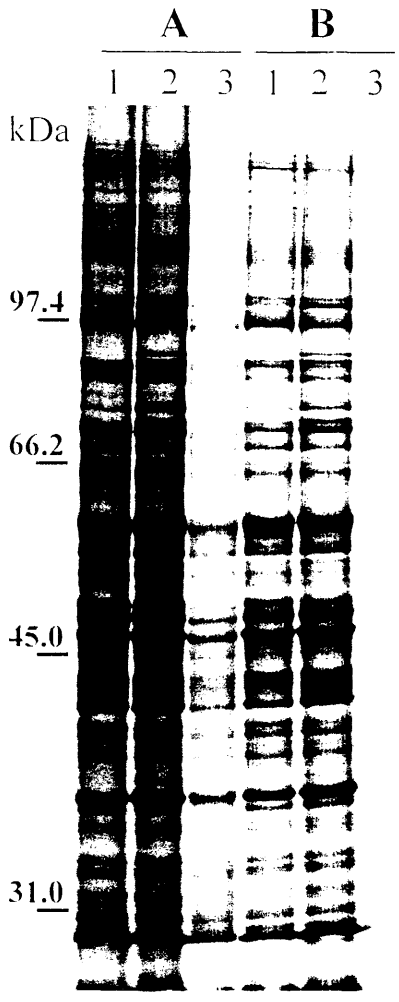

Figure 7.15. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control, heat shocked and peroxide shocked cells of (A) *A. tellurix* 2760 (respiratory-competent) and (B) *A. tellurix* 1787 (respiratory-deficient). Conditions were 35°C control (lanes 1), 40°C heat shock (lanes 2) and 0.2 mM H_2O_2 (lanes 3). Molecular mass standards (kDa) are as indicated.

7.3.3.2 Thermophilic yeast

It was of interest to discover a concomitant decrease in overall *de novo* protein synthesis with a peroxide shock in both thermophilic yeast strains (Fig. 7.15). Furthermore, no individual psps were observed in either strain. The effect of inhibition of protein synthesis by a peroxide shock was most pronounced in the respiratory-deficient strain, 1787 (Fig. 7.15B). Moreover, a peroxide shock did not induce the synthesis of hsp 104, 90, 70 or 60 in either strain, as evidenced by western immunoblot analyses (Fig. 7.11B and C).

7.4 Discussion

Psychrophilic and psychrotrophic yeast grown at 15°C were relatively resistant to a 100 mM H_2O_2 stress, at levels 5-50 fold greater than used in oxidative stress studies in logarithmic phase cells of *S. cerevisiae* grown at 25°C (Collinson & Dawes, 1992; Steels *et al.*, 1994). Furthermore, the resistance to such a high concentration of H_2O_2 is unusual compared to other microorganisms such as 1-3 mM H_2O_2 in *E. coli* (Imlay & Lim, 1988), 20 mM H_2O_2 in *Streptomyces coelicolor* (Lee *et al.*, 1993) and 40 mM H_2O_2 in *Schizosaccharomyces pombe* (Lee *et al.*, 1995). However, peroxide stress tolerance in yeast has also been observed to higher hydrogen peroxide concentrations under certain conditions of growth. For example, stationary phase cultures (defined as ethanol exhausted media) of industrial baking strains of *S. cerevisiae* grown at 25°C exhibited peroxide stress resistance ranging from 14% to 90% survivors after 1 h exposure to 300 mM H_2O_2 (Lewis *et al.*, 1997). Also logarithmic phase cultures of the pathogenic yeast *C. albicans* grown at 30°C exhibited intrinsic resistance to hydrogen peroxide concentrations ranging from 25 mM to 100 mM (Jamieson *et al.*, 1996). Nevertheless, the level of resistance to a 100 mM H_2O_2 stress (after 1 h) in logarithmic phase cultures of all psychrophilic and psychrotrophic yeast examined in this chapter was greater than that reported for the same time duration in *C. albicans* (Jamieson *et al.*, 1996). The psychrotrophic yeast, *L. scottii* grown at 15°C exhibited the highest intrinsic peroxide tolerance (Fig. 7.5B). The general trend suggested by the present studies indicate that growth temperature influences peroxide stress tolerance, with 15°C grown yeast cultures (Fig. 7.4; Fig. 7.5A and B) having more intrinsic resistance to a peroxide stress than 25°C or 35°C grown cultures (Fig. 7.5D; Fig. 7.8). This trend has also been observed in peroxide tolerance experiments in *S. cerevisiae*

K7 grown at different temperatures (Chapter 8, Fig. 8.1).

In contrast to psychrophilic yeast, thermophilic yeast grown at 35°C were quite intrinsically sensitive to a peroxide stress. The respiratory-deficient strain 1787 was less resistant to a 2 mM H_2O_2 stress than the respiratory-competent strain 2760 (Fig. 7.7). Moreover, the peroxide stress viability time courses were similar for a 10 mM H_2O_2 stress in strain 2760 (Fig. 7.8B) and a 2 mM H_2O_2 stress in strain 1787 (Fig. 7.7) particularly for the 60 and 120 min time points. To further attest to the peroxide sensitivity of the respiratory-deficient strain 1787 was the observation that the peroxide shock induced adaptive response was less than that acquired by a heat shock (Fig. 7.7B) and less than the peroxide shock inducible response in the respiratory-competent strain 2760 (Fig. 7.7). Nonetheless, a peroxide shock did induce peroxide stress tolerance (Fig. 7.7B). These results comply with finding in *S. cerevisiae* whereby an ethidium bromide induced petite and a *hap1* disruption mutant (affecting mitochondrial cytochromes and catalase T) were indeed more intrinsically sensitive to either a peroxide or menadione stress compared to the wild type strain. Furthermore, both of these strains were able to acquire oxidative stress tolerance when preexposed to a mild peroxide or menadione shock (Collinson and Dawes, 1992; Flattery O'Brien *et al.*, 1993). Results presented in this chapter agree with these previous findings and suggest that impaired mitochondrial function as observed in respiratory-deficient yeast increases peroxide stress sensitivity but still allows peroxide shock acquisition of peroxide stress tolerance albeit to a lesser extent as compared to respiratory-competent yeast.

In all the psychrophilic and psychrotrophic yeast examined, both a heat shock and a peroxide shock conferred resistance to a peroxide stress (Fig. 7.4 and Fig. 7.5). Therefore, sub-lethal exposure to a chemical or heat shock can enable adaptation to a lethal dose of the same agent or a cross-protective response to an unrelated agent. This cross-tolerance response with a heat shock eliciting peroxide stress tolerance has also been observed *S. cerevisiae* (Collinson and Dawes, 1992; Steels *et al.*, 1994) and *Neurospora crassa* (Kapoor and Lewis, 1987). Interestingly, the temperature of heat shock that induced maximal peroxide stress tolerance was not always the same heat shock temperature that elicited maximal thermotolerance (Chapter 4). All psychrophilic yeast in the *Mrakia* genus showed that a 3 h, 20°C heat shock elicited equivalent peroxide stress tolerance as a 3 h, 0.2 mM H_2O_2 shock to a 100 mM H_2O_2 stress (Fig. 7.4A, B and C). In contrast, in *L.*

antarcticum (Fig. 7.4D), *L. fellii* (grown at 15°C) and *L. scottii* (grown at 15°C) a 3 h, 25°C heat shock rather than a 3 h, 20°C heat shock induced greater tolerance to a peroxide stress (Fig. 7.5A and B). In *C. psychrophila*, a 3 h, 20°C heat shock induced peroxide stress tolerance to 100 mM H_2O_2 less than or equal to the same level as that induced by a peroxide shock however, a 3 h, 25°C heat shock although eliciting maximal thermotolerance induction (Chapter 3, Fig. 3.7) did not increase peroxide stress resistance. These trends indicate that the variations in peroxide stress tolerance, as for thermotolerance, may be genus specific.

Similarly, peroxide stress tolerance in the thermophilic yeast strains (Fig. 7.7; Fig. 7.8) was induced not only by a peroxide shock but also by a heat shock. This may well be an important defence mechanism for the thermophilic enteric yeast whereby initial entry into a warm-blooded host induces a heat shock response thus enabling the organism, at least for a short time, to survive an encounter with reactive oxygen species directed against it by the host. It is noteworthy in this respect that the opportunistic human pathogen *C. albicans* has also been reported to resist oxidative stress upon a heat shock (Jamieson *et al.*, 1996).

Consistent with recent findings in *S. cerevisiae* (Jamieson, 1992; Steels *et al.*, 1994; Lewis *et al.*, 1997) and *S. pombe* (Lee *et al.*, 1995) stationary phase cultures of the psychrophilic yeast, *C. psychrophila* and the thermophilic yeast, *A. telluris* (respiratory-competent strain) exhibited higher constitutive tolerance and duration of tolerance to the respective peroxide stress (Fig. 7.3B; Fig. 7.9). The increased oxidative stress tolerance of stationary phase cells in *S. cerevisiae* correlates with the increased synthesis of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and cytochrome c peroxidase, coincident with respiratory metabolism following glucose exhaustion (Moradas-Ferreira *et al.*, 1996). Similarly, stationary phase cultures of *S. pombe* displayed an increased synthesis of catalase, glucose-6-phosphate dehydrogenase and glutathione reductase (Lee *et al.*, 1995).

In *S. cerevisiae*, a 0.4 mM H_2O_2 shock for 1 h induces the synthesis of twelve psps including hsp 12, hsp 60 and three 70 kDa proteins (Jamieson *et al.*, 1994). Furthermore, a SSA1 promoter is induced by a peroxide shock (Jamieson *et al.*, 1994) suggesting that in *S. cerevisiae* an overlap may exist between heat shock and oxidative stress response systems. Although psps (Fig. 7.12) were detected in *C. psychrophila*, western analysis (Fig. 7.11A)

indicated that hsp 104, 90, 70 and 60 were not peroxide shock inducible. Consistent with these findings was the absence of peroxide-shock inducible hsp synthesis (Fig. 7.11B and C) in the respiratory-competent and respiratory-deficient strains of the thermophilic yeast, *A. telluris*, despite exhibiting strong heat shock induced synthesis of hsps (Chapter 5, Fig. 5.4).

With respect to psps in psychrophilic yeast, it was interesting that a 90 kDa protein exhibited slight peroxide shock inducibility in both *M. gelida* (Fig. 7.13B) and *M. stokesii* (Fig. 7.13C) and that a protein of approximately the same molecular weight was heat shock inducible in *M. frigida* and *M. stokesii* (Chapter 4, Fig. 4.8). This supports an overlapping mechanism for peroxide stress tolerance and thermotolerance. However, it was somewhat surprising that a peroxide shock did not increase *de novo* protein synthesis of any particular protein in either the psychrophilic yeast, *M. frigida* (Fig. 7.13A) and *L. antarcticum* (Fig. 7.13D), the psychrotrophic yeast, *L. scottii* grown at either 15°C or 25°C (Fig. 7.14) or either thermophilic yeast strain (Fig. 7.15). Indeed a peroxide shock inhibited *de novo* protein synthesis in *A. telluris* especially in the case of the respiratory-deficient strain 1787 (Fig. 7.15) and to differing degrees in most of the Antarctic yeast (Fig. 7.13; Fig. 7.14). The absence of peroxide shock inducible protein synthesis and inhibition of *de novo* protein synthesis suggests that psps are not solely responsible for induced peroxide stress tolerance. An alternative explanation for the absence of psps may be that a higher concentration of hydrogen peroxide or an increase in the duration of the shock treatment may be required to induce psps although a 0.2 mM H_2O_2 did induce peroxide stress tolerance in all of the yeast examined.

The disaccharide trehalose also does not appear to play a significant role in peroxide stress tolerance in psychrophilic yeast, as observed in *C. psychrophila* (Fig. 7.10A) or in either thermophilic yeast strain (Fig. 7.10B and C). In contrast, a 5 mM H_2O_2 treatment was reported to increase trehalose levels in *S. cerevisiae* (Attfield, 1987). This hydrogen peroxide concentration with respect to oxidative stress studies in *A. telluris* would constitute a peroxide stress rather than a peroxide shock and be deleterious to cell viability.

Other factors including superoxide dismutase, catalase, cytochrome c peroxidase and glutathione, that have been implicated in oxidative stress tolerance in *S. cerevisiae* (reviewed in Chapter 1), may be of equal or greater importance than peroxide shock

proteins as antioxidant defence mechanisms for psychrophilic, psychrotrophic and thermophilic yeast. However, there are no known reports of antioxidant levels in either Antarctic or thermophilic yeast and this avenue of research would warrant further investigation.

Psychrophilic and psychrotrophic yeast appear to be very well adapted to their environment, as evidenced in these studies. Not only were they able to adapt to temperature fluctuations outside of their natural temperature growth range (Chapter 3 and 4), a normal phenomenon in their own ecological niche (Margesin & Schinner, 1994), but they were also intrinsically resistant to relatively high peroxide levels. The high peroxide tolerance observed could equate with the high oxygen levels (as a consequence of increased oxygen solubility) in low temperature environments (Sinclair & Stokes, 1963).

Considering the anaerobic nature of the environment in which *A. telluris* is found, it is interesting that this thermophilic yeast exhibited an oxidative stress response. This adaptive response would enable thermophilic yeast to survive an encounter with reactive oxygen species directed against it by the host's macrophage defence system and an oxygen rich environment outside of the host as in soil, an environment in which *A. telluris* has been isolated (Travassos and Cury, 1971). On this note, the anaerobic, opportunistic pathogen *Bacteroides fragilis* also exhibits a peroxide shock induced oxidative stress response with concomitant increases in catalase, superoxide dismutase and *de novo* protein synthesis (Slade *et al.*, 1984; Rocha *et al.*, 1996). Furthermore, the pathogenic yeast *C. albicans* has also been shown to exhibit an oxidative stress response to both menadione (a superoxide radical generator) and hydrogen peroxide (Jamieson *et al.*, 1996) coincident with *de novo* protein synthesis.

CHAPTER 8: Oxidative Stress Response as Influenced by Temperature.

8.1 Introduction

Chapter 7 examined oxidative stress response in Antarctic psychrophilic and psychrotrophic yeast and thermophilic enteric yeast. The results suggested that growth temperature influences oxidative stress resistance with increasing basal tolerance as growth temperature decreases. Furthermore intrinsic thermotolerance in *S. cerevisiae* was found to be growth temperature dependent (Chapter 6). Consequently the experiments outlined in this chapter were designed to examine the effect of growth temperature on intrinsic and induced peroxide stress tolerance in *S. cerevisiae*. During the course of these experiments it was realised that the temperature at which the peroxide stress was applied may influence peroxide stress resistance. Subsequently, this hypothesis was tested in *S. cerevisiae* grown at different temperatures as well as the previously studied psychrophilic, psychrotrophic and thermophilic yeast strains grown at their respective temperatures.

8.2 Experimental outline

Experimental cultures were grown to an optical density of 0.2 to 0.3 (at 600 nm) corresponding to logarithmic phase cells of approximately 1 x 10^6 to 2 x 10^6 cfu ml^{-1} for 15°C and 35°C grown cultures and 3 x 10^6 to 4 x 10^6 cfu ml^{-1} for 25°C grown cultures of *S. cerevisiae* K7. Cell densities of psychrophilic, psychrotrophic and thermophilic yeast cultures were as described in Chapter 7.

Preliminary experiments of intrinsic peroxide stress tolerance were carried out for *S. cerevisiae* K7 grown at 15°C, 25°C and 35°C. Cultures were directly exposed to 10, 50 or 100 mM H_2O_2 for 2 h. Subsequently, intrinsic peroxide stress tolerance to 100 mM H_2O_2 was measured for 15°C grown cultures and to 10 mM H_2O_2 for both 25°C and 35°C grown cultures.

Induced peroxide stress tolerance to 100 mM H_2O_2 was measured following a 30 min, 0.2 mM H_2O_2 shock, 20°C heat shock, 25°C heat shock or 37°C heat shock and return

Table 8.1. Experimental conditions used to examine the influence of growth temperature and incubation temperature (during peroxide stress) on intrinsic peroxide stress tolerance.

Yeast	Growth temperature (°C)	$[H_2O_2]$ (mM)	Incubation temperature (°C)
***S. cerevisiae* K7**	25	10	15
	25	10	25
	35	10	15
	35	10	35
	15	50	15
	15	50	25
	15	50	35
	15	100	15
	15	100	25
	15	100	35
	25	50	15
	25	50	25
	25	100	15
	25	100	25
	35	50	15
	35	50	35
	35	100	15
	35	100	35
Psychrophiles (*C. psychrophila*, *M. frigida*, *M. gelida*, *M. stokesii*, *L. antarcticum*)	15	100	15
	15	100	25
Psychrotrophs (*L. fellii*, *L. scottii*)	15	100	15
	15	100	25
	25	100	15
	25	100	25
Thermophiles (*A. telluris* 2760, *A. telluris* 1787)	35	10	15
	35	10	25
	35	10	35
	35	50	15
	35	50	25
	35	50	35
	35	100	15
	35	100	25
	35	100	35

of cultures to 15°C for 15°C grown *S. cerevisiae* K7 cultures. For 25°C grown cultures, induced peroxide stress tolerance was measured by either subjecting cells to a 0.2 mM H_2O_2 shock or 37°C heat shock for 30 min followed by addition of 10 mM H_2O_2 and return of cultures to 25°C. Similarly, for 35°C grown cultures, induced peroxide stress tolerance was measured by either subjecting cells to 0.2 mM H_2O_2 shock or 40°C heat shock for 30 min followed by addition of 10 mM H_2O_2 and return of cultures to 35°C.

In addition, to determine if the temperature at which the peroxide stress was applied had an effect on peroxide stress tolerance, experiments were carried out as indicated in Table 8.1.

Peroxide shock inducible *de novo* protein synthesis was monitored for *S. cerevisiae* K7 grown at 15°C, 25°C and 35°C.

8.3 Results

8.3.1 Peroxide stress tolerance in S. cerevisiae

Initial peroxide stress experiments were carried out to ascertain differences in intrinsic peroxide tolerance in *S. cerevisiae* K7 grown at different temperatures (Fig. 8.1). These experiments were undertaken by exposing cells directly to 10, 50 or 100 mM H_2O_2 at 15°C, 25°C or 35°C corresponding to the temperature at which the culture was grown. Results presented in Fig. 8.1 indicate an increased peroxide sensitivity as the growth temperature of the culture increased and duration of the stress progressed. 15°C grown cultures were relatively resistant to 10, 50 and 100 mM H_2O_2 for the duration of the time course. However, cultures grown at 25°C showed intermediate resistance with increased sensitivity to the higher concentrations of H_2O_2 whilst cultures grown at 35°C exhibited extreme peroxide stress sensitivity. Consequently, examination of intrinsic and induced peroxide stress tolerance was pursued at 10 mM H_2O_2 for 25°C and 35°C grown cultures and at 100 mM H_2O_2 for 15°C grown cultures (Fig. 8.2). A mild peroxide shock (0.2 mM H_2O_2 for 30 min) induced tolerance to the respective peroxide stress (carried out at the growth temperature of the culture) for *S. cerevisiae* K7 grown at 15°C, 25°C and 35°C (Fig. 8.2). ^{35}S-methionine labelling however, did not reveal any peroxide shock inducible proteins for cultures grown at 15°C, 25°C or 35°C (Fig. 8.3). In fact, a peroxide shock (0.2

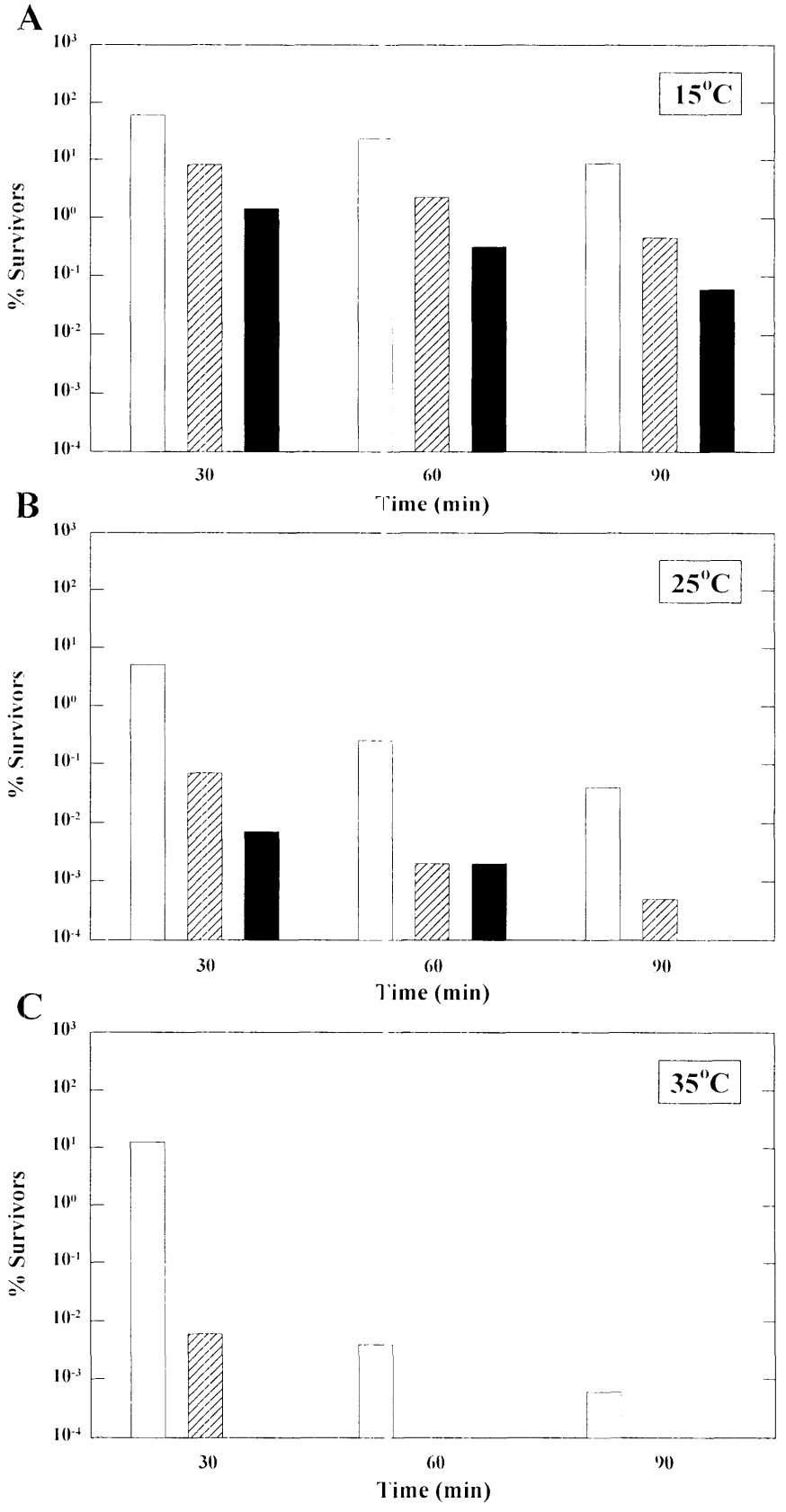

Figure 8.1. Intrinsic peroxide tolerance in mid-logarithmic phase cultures of *S. cerevisiae* grown at (A) 15°C, (B) 25°C and (C) 35°C. Intrinsic tolerance was measured at the times indicated following addition of either 10 (▭), 50 (▨) or 100 (■) mM H_2O_2. Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C, 25°C or 35°C control sample.

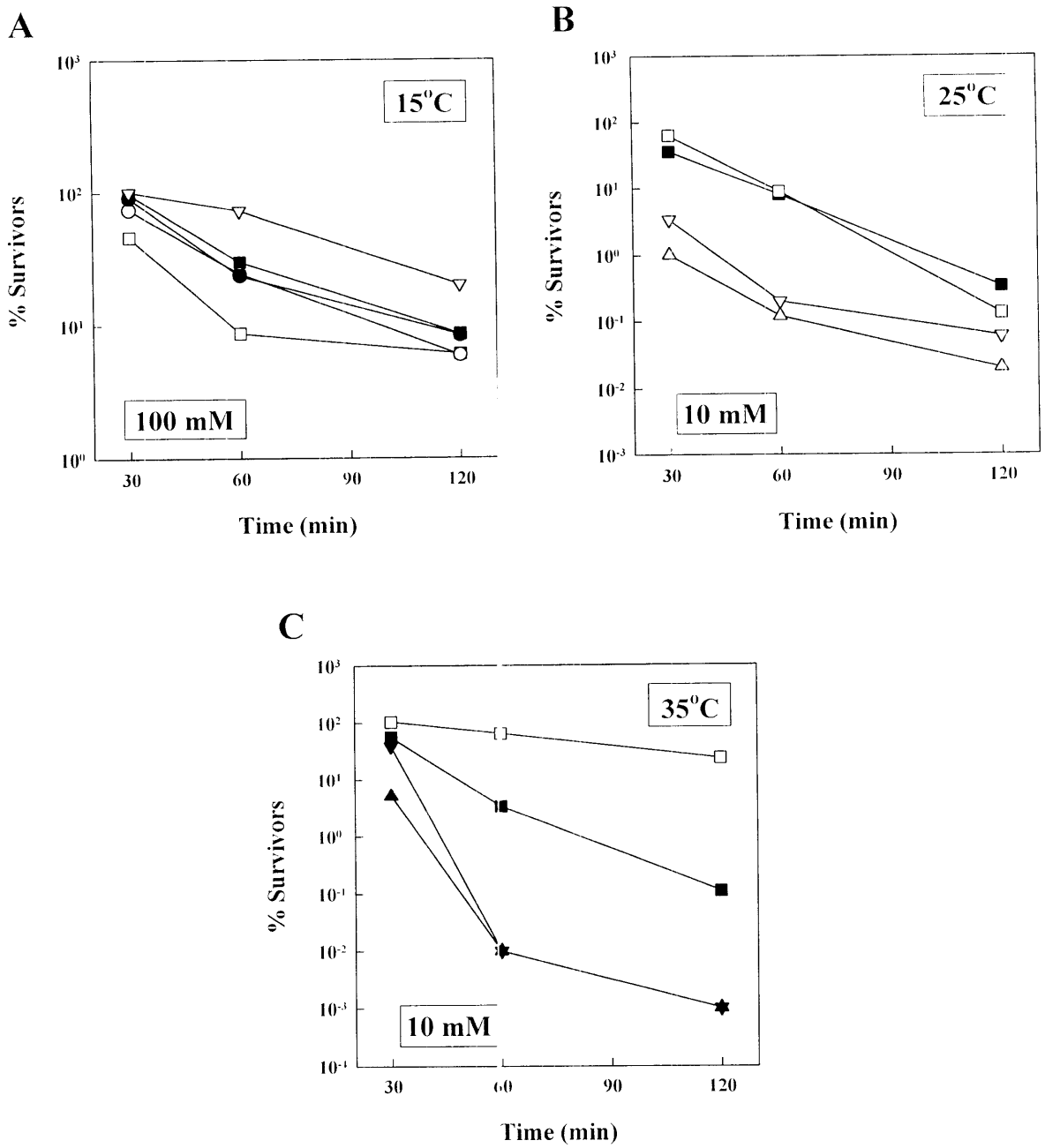

Figure 8.2. Intrinsic and induced peroxide tolerance in mid-logarithmic phase cultures of *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C and (C) 35°C. Intrinsic tolerance was measured at the times indicated following addition of 100 mM H_2O_2 for (A) and 10 mM H_2O_2 for (B) and (C) and incubating cultures for duration of peroxide stress at 15°C (□). Intrinsic tolerance was also measured at the times indicated following addition of 10 mM H_2O_2 for (B) and (C) and incubating cultures for duration of peroxide stress at 25°C (△) for (B) and at 35°C (▲) for (C). Induced peroxide tolerance to the respective peroxide stress was monitored following a 30 min, 0.2 mM H_2O_2 shock (■), 20°C heat shock (○), 25°C heat shock (●), 37°C heat shock (▽) or a 40°C heat shock (▼). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C, 25°C or 35°C control sample.

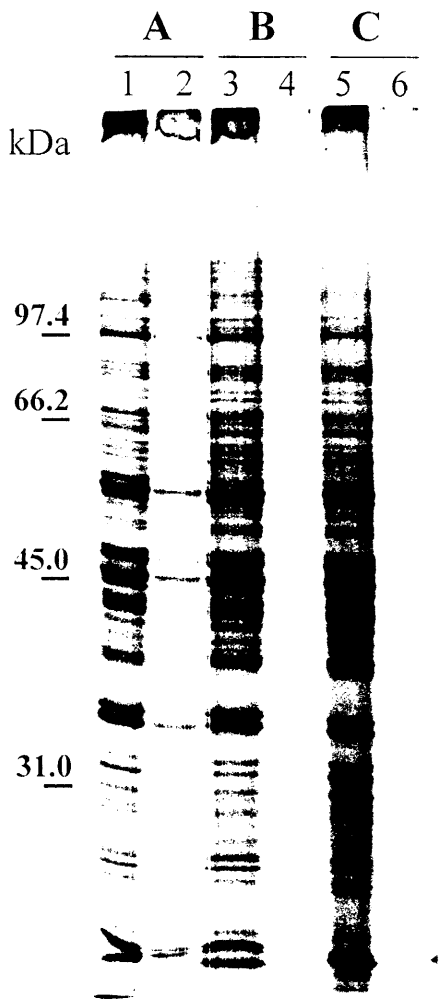

Figure 8.3. SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine labelled protein extracts from control and peroxide shocked cells of *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C or (C) 35°C. Conditions were 15°C control (lane 1), 25°C control (lane 3), 35°C control (lane 5) and 0.2 mM H_2O_2 shock (lanes 2, 4, and 6 corresponding to 15°C, 25°C and 35°C grown cultures, respectively). Molecular mass standards (kDa) are as indicated.

mM H_2O_2) decreased *de novo* protein synthesis and this effect was most pronounced in cultures grown at 35°C.

Crosstolerance was also observed, with a 20°C, 25°C and 37°C heat shock for 30 min conferring peroxide stress tolerance in cultures grown at 15°C (Fig. 8.2). Surprisingly, a 37°C heat shock rather than a peroxide shock induced maximal tolerance induction for 15°C grown cultures. In contrast, although a 37°C and 40°C heat shock for 30 min induced crosstolerance to a peroxide stress for cultures grown at 25°C and 35°C respectively, a peroxide shock conferred maximal tolerance at both growth temperatures. Furthermore intrinsic peroxide tolerance was markedly increased and surpassed heat or peroxide shock induced tolerance in 25°C and 35°C grown cultures when the peroxide stress was applied at the lower temperature of 15°C (Fig. 8.2).

The increased constitutive stress tolerance observed in cultures grown at 25°C and 35°C and exposed to a 10 mM H_2O_2 peroxide stress at 15°C prompted further examination of the influence of the incubation temperature on peroxide stress tolerance in *S. cerevisiae*. Cultures were grown at 15°C, 25°C and 35°C and exposed to 50 and 100 mM H_2O_2 at 15°C, 25°C and 35°C (Fig. 8.4). As mentioned previously, cultures grown at 25°C and 35°C were intrinsically quite sensitive to these higher concentrations of hydrogen peroxide when the stress was applied at the same temperature as the temperature at which the culture was grown (Fig. 8.1). However, intrinsic peroxide stress tolerance increased markedly as the temperature, at which the peroxide stress was applied, decreased (Fig. 8.4B and C). Cultures grown at 15°C and stressed at 15°C remained the most intrinsically resistant to a 50 and 100 mM H_2O_2 stress (Fig. 8.4A). However, intrinsic peroxide stress tolerance, in cultures grown at 15°C, declined when the temperature at which the stress was applied increased, with the most pronounced effect observed at an incubation temperature of 35°C (Fig. 8.4A).

8.3.2 Peroxide stress tolerance in psychrophilic and psychrotrophic yeast

The above observations prompted further investigations of intrinsic peroxide stress tolerance in psychrophilic (Fig. 8.5) and psychrotrophic (Fig. 8.6) yeast. Psychrophilic yeast cultures of *C. pychrophila*, *M. frigida*, *M. gelida*, *M. stokesii* and *L. antarcticum* were

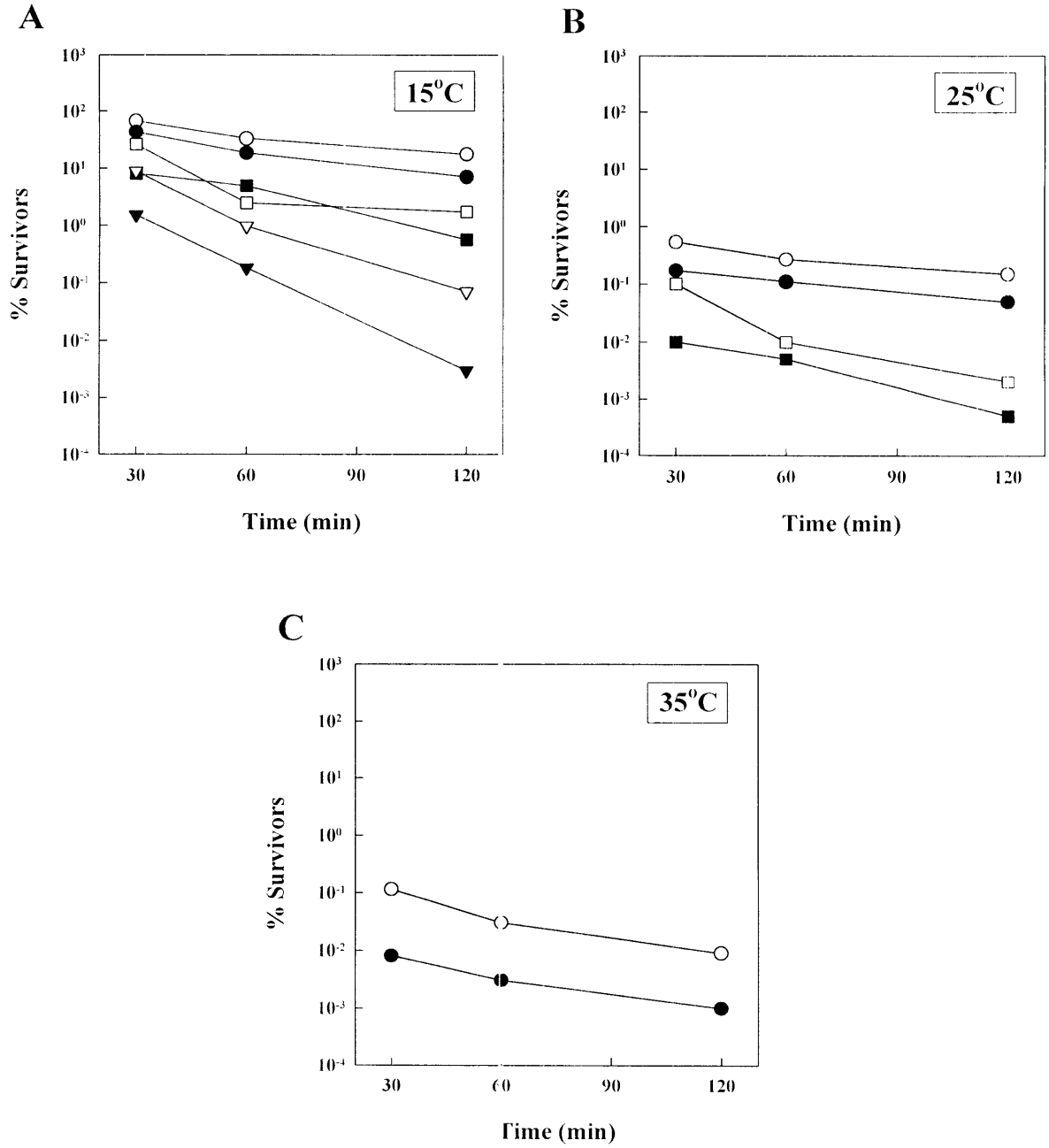

Figure 8.4. Intrinsic peroxide tolerance in mid-logarithmic phase cultures of *S. cerevisiae* K7 grown at (A) 15°C, (B) 25°C and (C) 35°C and peroxide stressed at different temperatures. Intrinsic tolerance was measured at the times indicated following addition of either 50 mM H_2O_2 (open symbols) or 100 mM H_2O_2 (closed symbols) and incubating culture for duration of peroxide stress at either 15°C (○, ●), 25°C (□, ■) or 35°C (▽, ▼). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C, 25°C or 35°C control sample.

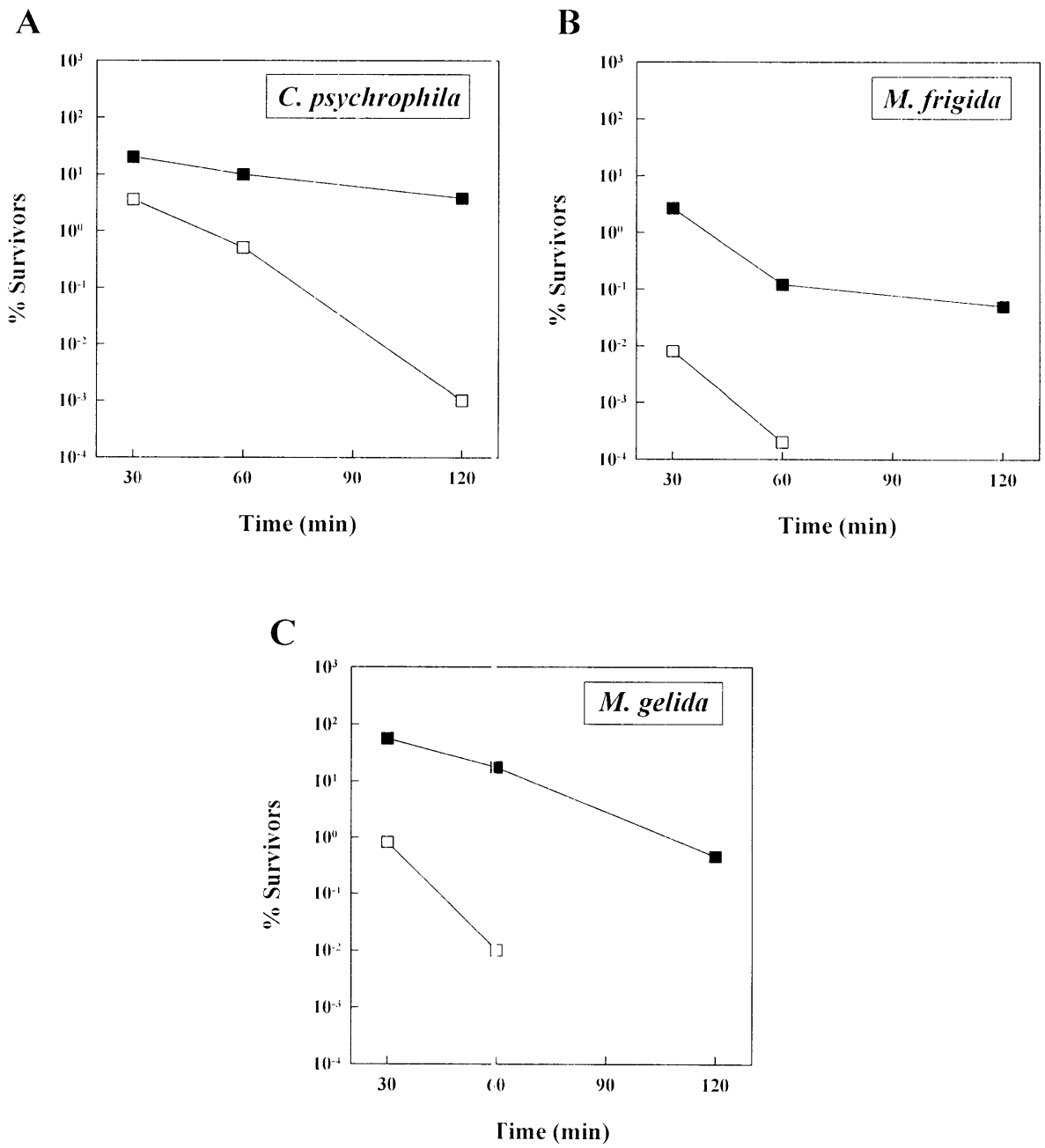

Figure 8.5. Intrinsic peroxide tolerance in mid-logarithmic cultures of (A) *C. psychrophila*, (B) *M. frigida*, (C) *M. gelida*, (D) *M. stokesii* and (E) *L. antarcticum* grown at 15°C and peroxide stressed at different temperatures. Intrinsic tolerance was measured at the times indicated following addition of 100 mM H_2O_2 and incubating culture for duration of peroxide stress at either 15°C (■) or 25°C (□). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C control sample.

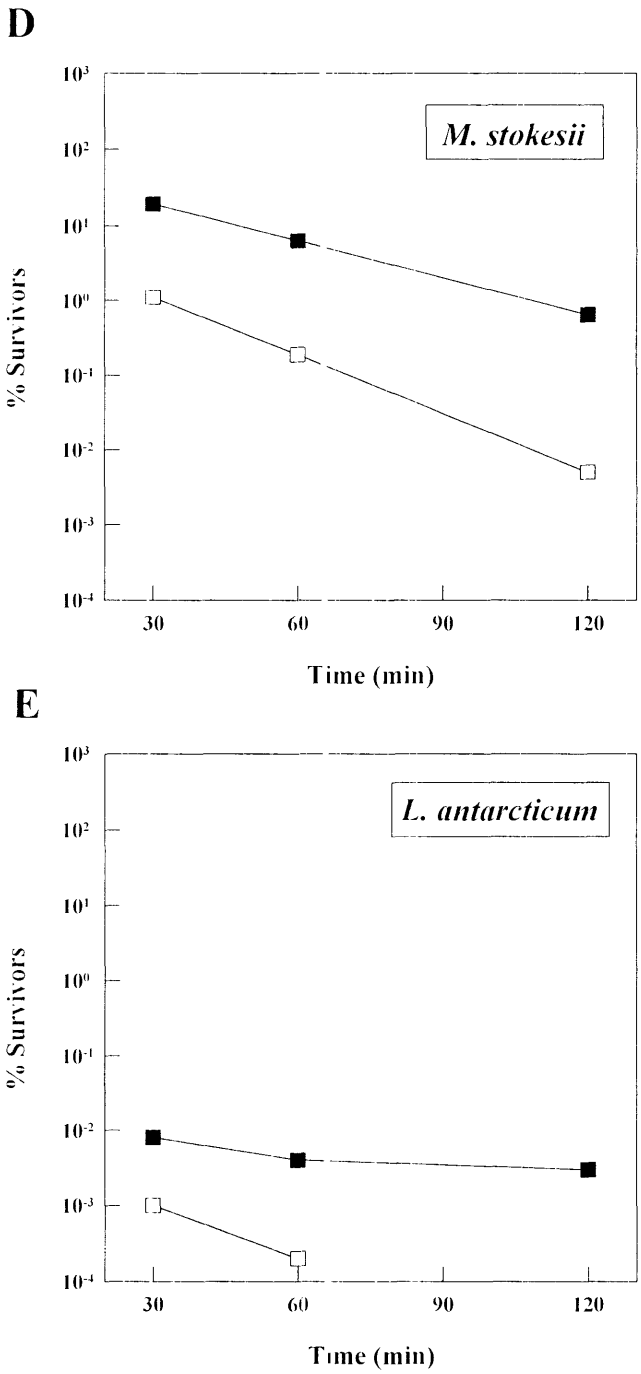

Figure 8.5. continued

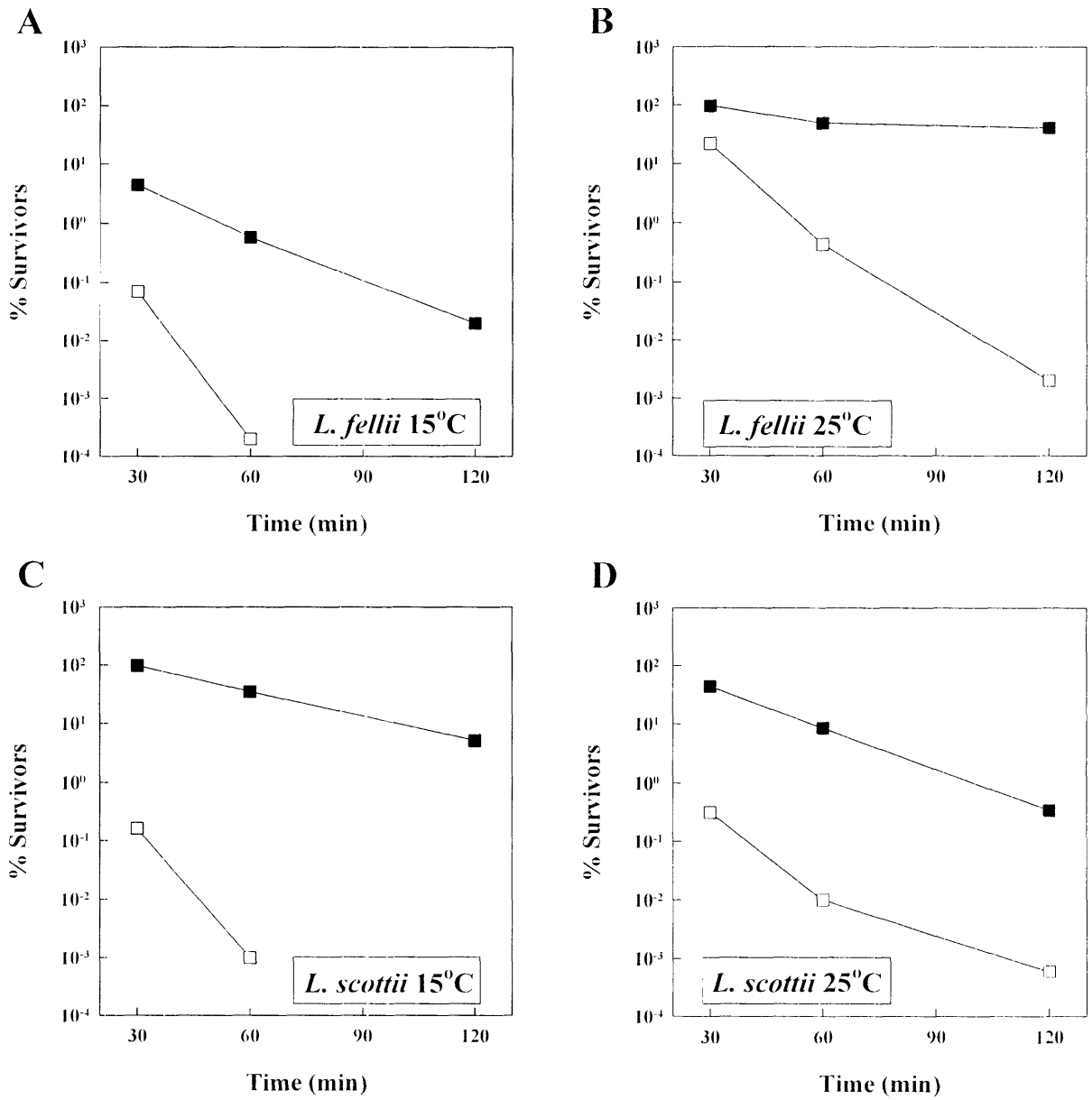

Figure 8.6. Intrinsic peroxide tolerance in mid-logarithmic cultures of *L. fellii* (A and B) and *L. scottii* (B and D) grown at 15°C (A and C) or 25°C (B and D) and peroxide stressed at different temperatures. Intrinsic tolerance was measured at the times indicated following addition of 100 mM H_2O_2 and incubating culture for duration of peroxide stress at either 15°C (■) or 25°C (□). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 15°C or 25°C control sample.

grown at 15°C and exposed to a 100 mM H_2O_2 stress for 2 h at 15°C and 25°C (Fig. 8.5). For all psychrophilic yeast examined an increase in the temperature at which the stress was applied from 15°C to 25°C resulted in a marked decrease in constitutive peroxide stress tolerance (ranging from 5-fold to 300-fold after 30 min exposure). The psychrophilic yeast, *M. frigida* (Fig. 8.5B), *M. gelida* (Fig. 8.5C) and *L. antarcticum* (Fig. 8.5E) were the most affected with no tolerance after a 2 h exposure to 100 mM H_2O_2 at 25°C.

Psychrotrophic yeast, *L. fellii* and *L. scottii*, were grown at 15°C and 25°C and exposed directly to 100 mM H_2O_2 at 15°C and 25°C (Fig. 8.6). Intrinsic peroxide stress resistance of 25°C grown cultures increased when stressed at 15°C whilst cultures grown at 15°C displayed a marked decrease in stress tolerance with no survivors after a 2 h peroxide stress at 25°C.

8.3.3 Peroxide stress tolerance in thermophilic yeast

Peroxide stress tolerance experiments in the respiratory-competent (strain 2760) and respiratory-deficient (strain 1787) strains of the thermophilic yeast, *A. telluris* described in chapter 7 indicated relative peroxide stress sensitivity as compared to psychrophilic and psychrotrophic yeast. The increases in peroxide stress resistance by decreasing the incubation temperature during the peroxide stress exhibited by *S. cerevisiae* K7 cultures grown at 25°C and 35°C (Fig. 8.4B and C) prompted examination of this effect in both thermophilic yeast strains (Fig. 8.7; Fig. 8.8). Thermophilic yeast were grown at 35°C and exposed to 2 mM (strain 1787, Fig. 8.8A), 10 mM (strain 2760, Fig. 8.7A), 50 mM (Fig. 8.7B; Fig. 8.8B) or 100 mM (Fig. 8.7C; Fig. 8.8C) H_2O_2 and incubated for the duration of the peroxide stress at temperatures of 15°C, 25°C and 35°C. Peroxide stress tolerance increased as the incubation temperature, at which the stress was applied, decreased for all peroxide stress concentrations. The most pronounced effect of decreased incubation temperature on intrinsic peroxide stress tolerance was observed after exposure to 100 mM H_2O_2 with a 500-fold and 1000-fold increase (after 30 min) when incubated at 15°C as compared to incubation at 35°C for *A. telluris* strains 2760 (Fig. 8.7C) and 1787(Fig. 8.8C), respectively.

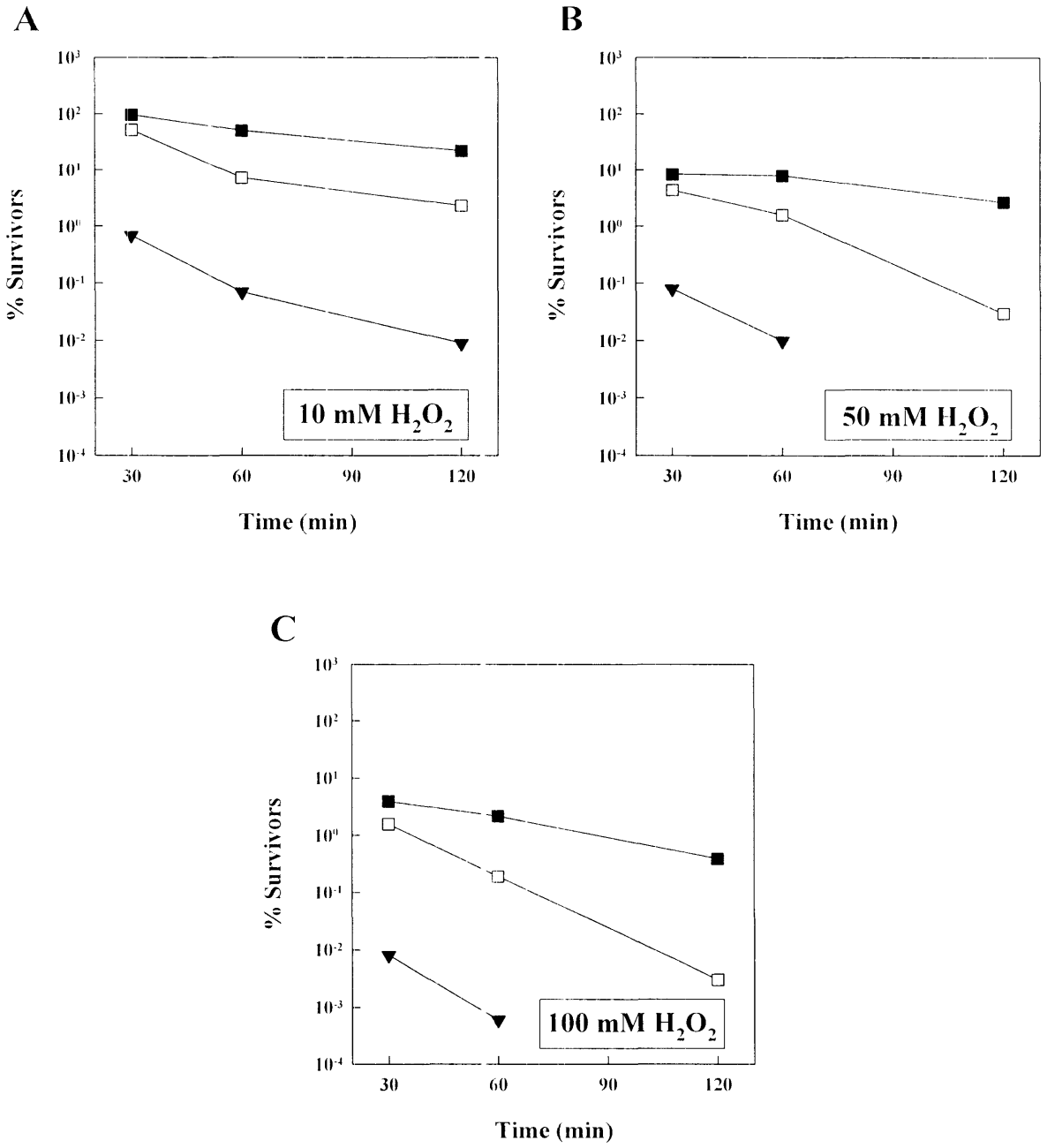

Figure 8.7. Intrinsic peroxide tolerance in mid-logarithmic cultures of *A. telluris* 2760 (respiratory-competent) grown at 35°C and peroxide stressed at different temperatures. Intrinsic tolerance was measured at the times indicated following addition of (A) 10 mM, (B) 50 mM or (C) 100 mM H_2O_2 and incubating culture for duration of peroxide stress at either 15°C (■), 25°C (□) or 35°C (▼). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

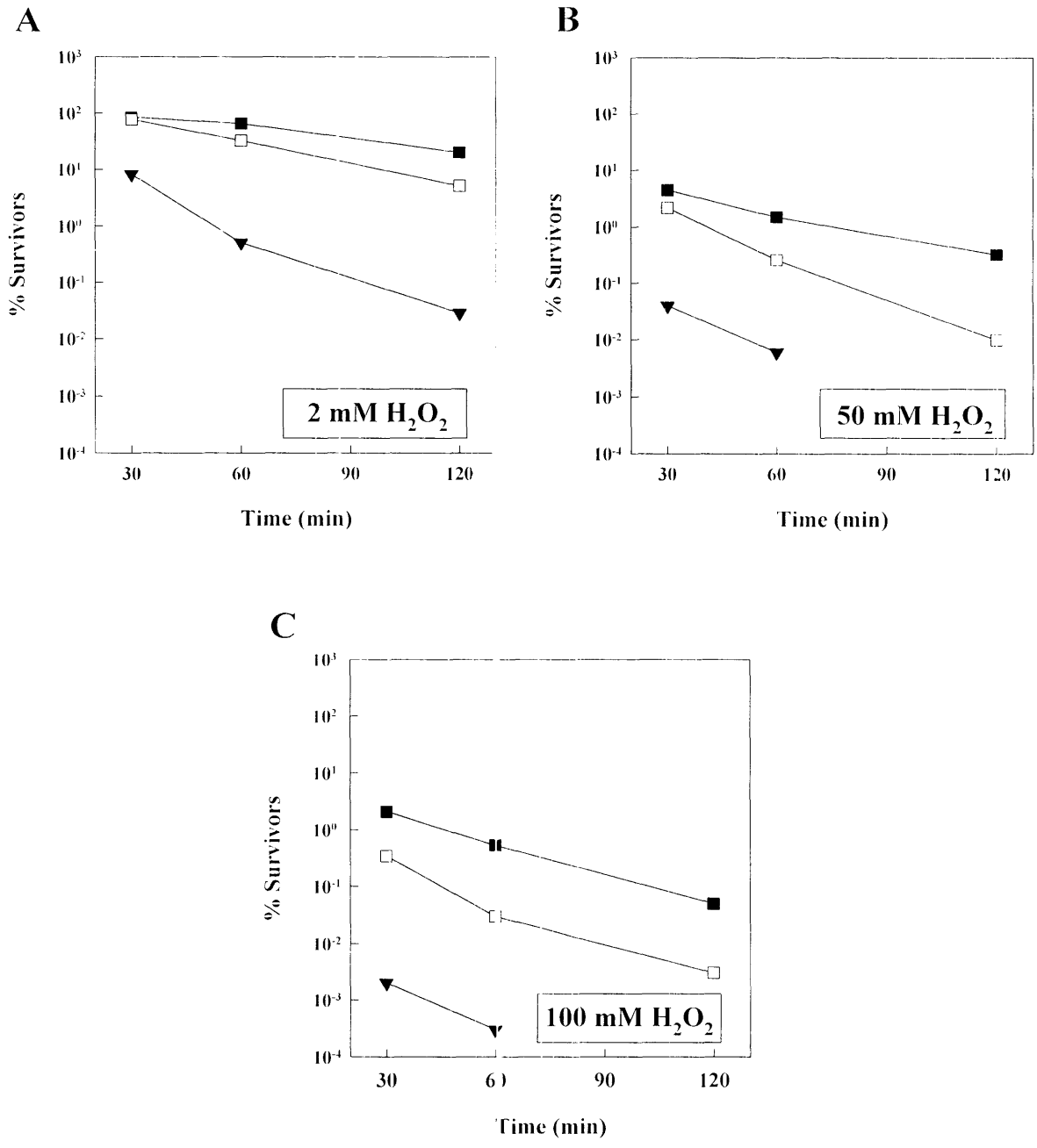

Figure 8.8. Intrinsic peroxide tolerance in mid-logarithmic cultures of *A. tellurix* 1787 (respiratory-deficient) grown at 35°C and peroxide stressed at different temperatures. Intrinsic tolerance was measured at the times indicated following addition of (A) 2 mM, (B) 50 mM or (C) 100 mM H_2O_2 and incubating culture for duration of peroxide stress at either 15°C (■), 25°C (□) or 35°C (▼). Levels of peroxide tolerance are expressed as percentage of survivors after the appropriate treatment with respect to a 35°C control sample.

8.4 Discussion

Although hydrogen peroxide can elicit an oxidative stress, it is not a free radical itself as it has no unpaired electrons and has poor reactivity. Its effect as a reactive oxygen species is primarily through the production of the highly reactive hydroxyl radical ($OH^{\bullet}$) via the metal-dependent Fenton/Haber-Weiss reaction (M^{c}Cord and Day, 1978). Furthermore, H_2O_2 can readily traverse biological membranes (Halliwell and Gutterodge, 1986) thus enabling the potent $OH^{\bullet}$ product to affect all of the major cellular macromolecules. DNA damage includes chemical alteration of deoxyribose as well as purines and pyrimidines leading to increased mutations and DNA strand breakage (Breimer, 1988; Aruoma *et* al., 1989). Lethal concentrations of hydrogen peroxide have been observed to induce DNA base damage in yeast (Frankenberg *et al.*, 1993) with an oxidative damage marker, 8-hydroxyguanine being detectable (Woodford *et al.*, 1995). Oxidative damage to proteins includes oxidation of amino acids, particularly those with sulfhydryl groups (Brot *et al.*, 1981; Sies, 1986), and protein cross-linking causing increased proteolysis and decreased biological activity (Dean and Pollak, 1985; Davies, 1986; Wolff and Dean, 1986; Levine *et al.*, 1994). Lipids, particularly those associated with biomembranes are also susceptible to attack by ROS. Hydrogen abstraction of unsaturated fatty acids results in a self-perpetuating cycle of lipid peroxidation (Slater, 1979; Kappus, 1985). Lipid peroxidation thus results in decreased fatty acyl chain length causing an increase in membrane fluidity as well as an accumulation of destructive end products including epoxides, aldehydes and alkanes which also damage DNA and inactivate proteins (Halliwell and Gutteridge, 1989; Moradas-Ferreira *et al.*, 1996). Peroxide stress induced membrane lipid peroxidation has been observed in yeast by measuring the levels of thiobarbituric acid reactive substance (TBARS) which are indicative of malondialdehyde formation (Steels *et al.*, 1994).

8.4.1 Intrinsic peroxide stress tolerance

Results presented in Chapter 7 showed that psychrophilic and psychrotrophic yeast grown at 15°C were intrinsically resistant to a relatively high oxidative stress while thermophilic yeast grown at 35°C were relatively oxidative stress sensitive. These results are quite surprising considering that psychrophilic and psychrotrophic yeast have very fluid membranes with a high polyunsaturated fatty acid content in contrast to thermophilic

yeast membranes which consist predominantly of monounsaturated and saturated fatty acids (Watson *et al.*, 1978). This would suggest that psychrophilic and psychrotrophic yeast biomembranes would be more susceptible to free radical attack and lipid peroxidation than thermophilic yeast yet the former were more oxidative stress resistant. Furthermore, the membranes of psychrotrophic yeast grown at 25°C also differed from those grown at 15°C with a concomitant 50% decrease in the proportion of 18:3 polyunsaturated fatty acids as temperature increased from 15°C to 25°C (Swan, personal communication), yet *L. scottii* was more sensitive at 25°C. Furthermore, the membranes of psychrotrophic yeast grown at 25°C also differ from those grown at 15°C with a concomitant 50% decrease in the proportion of 18:3 polyunsaturated fatty acids as temperature increased from 15°C to 25°C (Swan, personal communication).. These findings suggest that psychrophilic and psychrotrophic yeast must have powerful antioxidant mechanisms to minimize membrane damage. Nevertheless, oxidative stress tolerance was well correlated with psychrophilic and psychrotrophic yeast existing as obligate aerobes while thermophilic yeast are facultative anaerobes. Similarly, in *S. cerevisiae*, anaerobic yeast cultures were also found to be more oxidative stress sensitive than aerobically grown cultures (Steels *et al.*, 1994).

In the current Chapter, a similar picture emerges for *S. cerevisiae* grown at different temperatures, that is, as growth temperature decreased intrinsic peroxide stress tolerance increased (Fig. 8.1). However, a decrease in growth temperature in *S. cerevisiae* K7 has also been correlated with an increase in monounsaturated fatty acids (Swan, personal communication). It is well established that yeast alter their membrane fatty acid composition with growth temperature (Kates and Baxter, 1962; Arthur and Watson, 1976; Watson *et al.*, 1976) with an increase in fatty acid unsaturation as temperature decreases (Kates, 1964, Watson, 1980; Watson, 1984; Watson, 1987). It has also been observed that the higher the polyunsaturated fatty acid content of membrane phospholipids in anaerobically grown cultures of *S. cerevisiae*, the greater the degree of membrane lipid damage caused by an oxidative stress (Steels *et al.*, 1994). Culture viability after a peroxide stress was also negatively correlated with increased polyunsaturated fatty acid content (Steels *et al.*, 1994). However, in the same study, aerobically grown yeast cells which have a high content of monounsaturated fatty acids were the most oxidative and heat stress tolerant. This correlates with 15°C grown *S. cerevisiae* K7 cultures (increased

monounsaturated fatty acid content) exhibiting the highest intrinsic peroxide (Fig. 8.1) and heat stress tolerance (Chapter 6, Fig. 6.3).

Results presented in this chapter revealed that *S. cerevisiae* cultures grown at 35°C and peroxide stressed at 35°C were the most intrinsically sensitive to an oxidative stress (Fig. 8.1; Fig. 8.2). A growth temperature of 35°C may also increase the incidence of respiratory-deficient petite mutations as previously reported for yeast grown at 38°C (Sherman, 1956; Ycas, 1956; Sherman, 1959). Previous investigations for *S. cerevisiae* (Collinson and Dawes, 1992; Flattery O'Brien *et al.*, 1993) and thermophilic yeast (discussed in Chapter 7) revealed increased oxidative stress sensitivity for respiratory-deficient yeast as compared to respiratory-competent yeast. Furthermore, an increase in growth temperature would result in increased membrane permeability (Piper, 1995) with a resultant increased flux of H_2O_2 into the cell. The increase in membrane permeability is somewhat counteracted by a concomitant decrease in the unsaturation of lipids (Watson, 1984) and sterol content (Travassos and Cury, 1971). It has also been suggested that a rise in temperature would correlate with an increase in molecular oxygen from the aqueous to lipid phase of cells thus resulting in enhanced membrane lipid peroxidation (Steels *et al.*, 1994) and consequently increased oxidative stress sensitivity. Heat and oxidative stress may act in synergism as observed for heat and ethanol stress (reviewed in Piper, 1995), that is, at higher temperatures the effect of the respective stress is more pronounced (Freeman *et al.*, 1990). In this respect, it of interest that heat, oxygen and ethanol produce reactive oxygen species (Piper, 1995) and antioxidant defence mechanisms (Jamieson, 1992; Costa *et al.*, 1993; Flattery O'Brien *et al.*, 1993, Mager and Moradas-Ferreira, 1993; Steels *et al.*, 1994, Moradas-Ferreira *et al.*, 1996). Heat and ethanol stress have been shown to increase the levels of the superoxide anion as a result of incomplete reduction of molecular oxygen by the mitochondrial respiratory chain (Moradas-Ferreira *et al.*, 1996). Furthermore, an increase in catalase T and Mn-SOD (enzymatic antioxidant defences) has been observed in response to all three stress inducers, indicating similar mechanisms for stress tolerance induction (Bilinski *et al.*, 1985; Wieser *et* al., 1991; Ruis and Hamiliton, 1992; Costa *et al.*, 1993; Moradas-Ferreira *et al.*, 1996). Therefore one may presume that a heat stress or an increase in growth temperature would already cause an oxidative stress, with any further increase in oxidant resulting in a cumulative effect as observed by the increased oxidative sensitivity of yeast grown at 35°C and peroxide stressed at 35°C.

Overall, intrinsic tolerance to a peroxide stress correlated inversely to growth temperature. These findings were in contrast to observations of the influence of growth temperature on thermotolerance reported in Chapter 6, where 15°C grown cells exhibited the highest basal tolerance followed in order by 35°C and 25°C grown cultures. This difference may again be attributable to the compounded effect of both heat and peroxide as discussed above.

A striking similarity between *S. cerevisiae* grown at 15°C (Fig. 8.2A) and psychrophilic and psychrotrophic yeast grown at 15°C (Chapter 7) was observed when comparing intrinsic peroxide stress tolerance to a 100 mM H_2O_2 stress. However, species specific differences were observed in the extent and duration of peroxide stress tolerance with the psychrotrophic yeast, *L. scottii* (Fig. 7.5B) exhibiting the highest basal tolerance. The high basal tolerance suggests that growth at a relatively low growth temperature (15°C) correlates with increased peroxide stress tolerance regardless of the natural environmental domains of the yeast studied. With respect to a growth temperature of 25°C, psychrotrophic yeast exhibited 2 - 3 orders of magnitude greater tolerance to 100 mM H_2O_2 than *S. cerevisiae*. This may be attributable to the ability of psychrotrophic yeast to also grow at lower temperatures and thus be more intrinsically able to tolerate increased oxygen levels in comparison to mesophilic yeast. At a growth temperature of 35°C, thermophilic yeast were found to be more intrinsically tolerant to a 100 mM H_2O_2 stress than *S. cerevisiae* however the converse was exhibited when exposed to a lower concentration of H_2O_2 (10 mM). This can be accounted for by the combined effect of temperature and an oxidative stress having a more pronounced effect on the mesophilic yeast. In contrast, thermophilic yeast are suited to growth at higher temperatures and consequently the increased temperature effect may be nullified resulting in an oxidative stress being the only stressor, with the effect being more discernible at higher H_2O_2 concentrations.

A dramatic effect was observed when examining the influence of the temperature at which the peroxide stress was applied (incubation temperature) in *S. cerevisiae*. Not only did a decrease in incubation temperature correlate with an increase in basal tolerance to a 10 mM H_2O_2 stress in *S. cerevisiae* grown at 25°C and 35°C (Fig. 8.2B and C) it also stimulated a dramatic increase in survival at a higher concentration of H_2O_2 (100 mM) (Fig. 8.4B and C). Conversely, an increase in incubation temperature decreased viability

for 15°C grown cultures (Fig. 8.5A). These results further support the hypothesis that increased susceptibility to a peroxide stress is directly correlated to increased growth or incubation temperature. Therefore an increase in growth or incubation temp constitutes a further stress, perhaps producing more detrimental oxygen derived free radicals (Steels *et al.*, 1994). Similar findings for psychrophilic (Fig. 8.5), psychrotrophic (Fig. 8.6) and thermophilic yeast (Fig. 8.7; Fig. 8.8) further substantiate this theory. Lowering the incubation temperature substantially increased basal tolerance in psychrotrophic yeast grown at 25°C and thermophilic yeast grown at 35°C. A dramatic increase in peroxide stress tolerance was particularly observed in thermophilic yeast when exposed to a 100 mM H_2O_2 at 15°C as compared to incubation at 35°C (Fig. 8.7C; Fig. 8.8C). Similarly, in the facultative pathogen, *Listeria monocytogenes*, increased acid stress tolerance was correlated with a decrease in the temperature from 30°C to 10°C at which the acid stress was applied (Patchett *et al.*, 1996). Conversely in psychrophilic (Fig. 8.5) and psychrotrophic yeast grown at 15°C (Fig. 8.6A and C), incubation at 25°C throughout the peroxide stress substantially decreased tolerance levels.

To further corroborate the theory of increased temperature increasing peroxide stress sensitivity, further investigations need to be undertaken to determine the extent of cellular damage to proteins, lipids and DNA at the different growth and incubation temperatures in psychrophilic, psychrotrophic, mesophilic and thermophilic yeast as outlined in these studies. For example, this would necessitate measuring levels of 1) antioxidant enzymes including SOD (McCord and Fridovich, 1969), glutathione peroxidase (Lawrence and Burk, 1976; Carmognol *et al.*, 1983) and catalase (Aebi, 1984; Verduyn *et al.*, 1984); 2) lipid peroxidation via malondialdehyde formation (Jain, 1988; Halliwell and Gutteridge, 1989); 3) carbonyl groups on proteins as an indicator of protein oxidation (Levine *et* al., 1994) and 4) DNA damage using 8-hydroxyguanine as a marker (Halliwell and Aruoma, 1993).

8.4.2 Induced peroxide stress tolerance

With respect to induced peroxide stress tolerance, a mild peroxide shock pretreatment increased peroxide stress tolerance at all growth temperatures (Fig. 8.2). Furthermore, heat shock conferred oxidative stress resistance however the converse did not apply (Collinson and Dawes, 1992; Steels *et al.*, 1994). As previously mentioned there

appears to exist a correlation between heat, oxidative and ethanol stress. In keeping with this, a heat shock has also been reported to confer ethanol stress tolerance however thermotolerance is not induced by ethanol (van Uden, 1984b; Costa *et al.*, 1993). Some hsps are also induced by a peroxide or ethanol pretreatment (Parsell and Lindquist, 1993; Jamieson *et al.*, 1994; Piper *et al.*, 1994) This appears to contradict the aforementioned findings where increased temperature resulted in increased peroxide sensitivity. However, hsps induced by a mild increase in temperature (heat shock) would through their chaperone function most likely facilitate repair and recovery from a subsequent oxidative stress.

Davidson *et al.* (1996) suggested that heat-induced protein denaturation would result in decreased levels of antioxidant enzymes and hence render cells defenceless against an oxidative stress. Therefore, at 35°C one would expect to observe more protein damage. This theory complies with results obtained using ^{35}S-methionine labelling, that is, a more pronounced peroxide shock-induced inhibition of *de novo* protein synthesis in parallel with an increase in growth temperature (Fig. 8.3). Furthermore, the same effect was observed when thermophilic yeast were exposed to a peroxide shock (Chapter 7, Fig. 7.15). Moreover, Davies and Lin (1988) also reported a decrease in the rate of protein synthesis and an increase in protein degradation when *E. coli* was exposed to hydrogen peroxide or oxidative free radicals. In contrast to previous findings (Jamieson *et al.*, 1994), no peroxide shock inducible proteins (psps) were observed at any growth temperature (Fig. 8.3). This observation was consistent with previous findings in our laboratory with *S. cerevisiae* strain K7, however the concentration and duration of the peroxide shock differ from those used by Jamieson *et al.* (1994). Furthermore, one-dimensional SDS-PAGE is somewhat limited and perhaps utilization of two-dimensional gel electrophoresis may reveal psps. Nevertheless, at all growth temperatures, a 0.2 mM H_2O_2 shock did elicit increased resistance to a peroxide stress (Fig. 8.2).

The present studies show that at all growth temperatures the heat shock temperature inducing peroxide stress tolerance (Fig. 8.2) was the same as that eliciting maximal heat shock induction of thermotolerance (Chapter 6, Fig. 6.3). This situation is in direct contrast to that observed for psychrophilic and psychrotrophic yeast, where the heat shock temperature inducing the greatest peroxide tolerance was species specific (Chapter 7). Although, peroxide stress tolerance was induced by a 40°C heat shock for *S. cerevisiae* cultures grown at 35°C, the duration was comparatively short-lived (tolerance waned after

30 min time point). This observation lends further support to an increase in temperature causing increased peroxide stress susceptibility.

In summary, the present studies indicate a definite correlation with growth temperature with lower growth temperatures correlating with increased peroxide stress tolerance. Furthermore, the incubation temperature during the stress has a profound effect on basal tolerance, with a decrease in incubation temperature corresponding with increased tolerance regardless of growth temperature. However, growth temperature does not significantly influence the inducibility of peroxide stress tolerance by a heat or peroxide shock.