Chapter 3: Thermotolerance, heat shock protein synthesis and trehalose accumulation in repressed and derepressed cells

3.1 Introduction

In *Saccharomyces cerevisiae* a key feature of the heat shock response is induced thermotolerance, in which cells that have been subjected to a mild heat shock acquire tolerance to additional, more severe stress conditions that would otherwise prove fatal. It has long been accepted that thermotolerance of yeast varies with growth phase. Budding yeast prefers to grow by fermentation of glucose and when this hexose is abundant, cells divide exponentially. During the exponential or logarithmic growth phase, glycolysis results in the formation of pyruvate, which is decarboxylated and excreted from the cell in the form of ethanol (Lagunas, 1986; Kappeli, 1986; Alexander and Jeffries, 1990). Reduction of pyruvate to ethanol facilitates regeneration of the NAD consumed in glycolysis (Ronne, 1995). During this exclusively fermentative growth, genes required for oxidative metabolism and for the use of non-fermentable carbon sources are repressed (reviewed in Wills, 1990). The phenomenon is commonly referred to as glucose repression, having been originally coined 'carbon catabolite repression' for a related but mechanistically distinct response in bacteria (Magasanik, 1961). Once the supply of fermentable carbohydrate in the medium becomes limiting, cells enter the diauxic shift where the enzymes of oxidative metabolism are derepressed or induced to reflect changing nutrient availability and utilization (Entian and Barnett, 1992; Gancedo, 1992). The non-fermentable carbon sources are assimilated through gluconeogenesis, with ethanol and its intermediate, acetate, being converted to acetyl-CoA, which is either catabolized by the tricarboxylic acid cycle or assimilated by the glyoxylate cycle (McCammon, 1996). At this stage, commonly referred to as stationary phase, the growth rate of cells slows to accommodate this alternative metabolism.

It has been well established that post-fermentative or stationary phase cells exhibit higher levels of resistance to a temperature challenge (Schenberg-Frascino and Moustacchi, 1972; Parry *et al.*, 1976; Mitchel and Morrison, 1982). However, growth phase *per se* may

not be the limiting factor conferring tolerance, and other morphological, physiological and metabolic parameters within its bounds have been previously investigated in this regard. A prevailing example constitutes the position of cells in the cell cycle. Throughout exponential growth, a mother cell begins the cycle in G1 phase, during which growth is coordinated with division. Under favourable conditions, cells pass a transition known as start and produce a new bud, concomitant with S phase. The bud grows throughout S and G2, with nuclear division occurring at M (Pringle and Hartwell, 1981). Unbudded cells, those residing in G1 (Schenberg-Frascino and Moustacchi, 1972) or even an out of cycle phase termed G0 (Plesset *et al.*, 1987), often associated with nutrient depleted stationary phase cells (Werner-Wasburne *et al.*, 1993), have been shown to be maximally thermotolerant. In addition, other factors such as nutrient limitation (Lillie and Pringle, 1980), intracellular levels of cAMP (Shin *et al.*, 1987; Werner-Wasburne *et al.*, 1989) and growth rate (Elliot and Futcher, 1993), which is inextricably linked to other growth phase parameters, have been investigated with respect to their influence on stress tolerance. However, at the time of instigation of the present work, which has since been published (Gross and Watson, 1996a), the affect of catabolite derepression on thermotolerance had received little attention. Notable exceptions were the work of Sanchez *et al.* (1992), Weitzel and Li, (1993) and a third, comprehensive analysis, coinciding with the present studies, by Elliot and Futcher, (1993). Whilst all these parameters could have a collective influence on thermotolerance, they are physiological manifestations of growth phase. At a mechanistic level they have been invariably related to hsps and trehalose (reviewed in Thevelein, 1984; Piper, 1993).

The hsps and the disaccharide trehalose, are the two major, most widely studied biochemical aspects of the adaptive stress response (see Chapter 1). There is evidence which suggests a strong, positive correlation between the extent of induced thermotolerance and the induction of hsps (McAlister *et al.*, 1979; Lindquist and Craig, 1988) and the rapid accumulation of trehalose (Attfield, 1987; Hottiger *et al.*, 1987) during heat shock treatment.

Close association between specific hsp and thermotolerance has been previously reported (Lindquist and Craig, 1988). Recent results have demonstrated that hsp 104 is required for both induced tolerance (Sanchez and Lindquist, 1990; Winkler *et al.*, 1991;

Sanchez *et al.*, 1992) and the higher levels of intrinsic thermotolerance exhibited by respiring cells growing on acetate (Sanchez *et al.*, 1992). In addition, various results suggest a relationship between the synthesis of hsp and nutrient limitation (Bataille *et al.*, 1991) and between heat shock resistance and carbon catabolite derepression (Hou *et al.*, 1990; Elliot and Futcher, 1993; Weitzel and Li, 1993). It has been further suggested that slow growth associated with, for example, growth of cells on a catabolite derepressive carbon source, invokes general stress tolerance (Weitzel and Li, 1993).

Conflicting data concerning the relevance of hsp in stress tolerance is also evident. Some reports indicate a lack of tolerance despite the presence of hsp (Cavicchioli and Watson, 1986; Barnes *et al.*, 1990) while others demonstrate acquisition of tolerance in the absence of newly synthesized hsp (Hall, 1983; Watson *et al.*, 1984; Smith and Yaffe, 1991). In this context, some researchers assign greater importance to trehalose as a thermoprotectant (Hottiger *et al.*, 1989; De Virgilio *et al.*, 1990; De Virgilio *et al.*, 1991; Hottiger *et al.*, 1992). Interestingly, in *S.cerevisiae*, trehalose also accumulates during periods of reduced growth attributable to nutrient limitation (Lillie and Pringle, 1980) and during stationary phase (Panek and Matoon, 1977; Francois *et al.*, 1991), presumably due to the observation that enzymes of trehalose metabolism are regulated by cAMP (Thevelein, 1988; Francois *et al.*, 1991). Although it appears now well established that both hsp and trehalose are of considerable importance to normal cellular metabolism, their relative contributions to intrinsic and induced thermotolerance and factors regulating their modes of action remain unresolved (Piper, 1993). It is noteworthy that an increasing number of reports provide evidence that both hsp and trehalose may facilitate cell survival following hyperthermia (Panek and Panek, 1990; Panek *et al.*, 1990; De Virgilio *et al.*, 1991; Hottiger *et al.*, 1992; Piper, 1993; Gross and Watson, 1994). This concept is investigated in detail in Chapter 5.

Despite abundant data relating hsp and trehalose to the stress response, few reports, if any, have compared the requirement of both parameters for intrinsic and induced thermotolerance, in both repressed and derepressed cells. In this chapter, the interrelationship between thermotolerance, trehalose accumulation and hsp induction was investigated in two naturally occurring strains of *S. cerevisiae* exhibiting contrasting thermotolerance phenotypes (Gross and Watson, 1992), under conditions of carbon

catabolite repression and derepression. Results presented imply that a major influence on the thermotolerance of *S. cerevisiae* may be the derepression of enzymes associated with respiration, which occurs when glucose is exhausted or when cells are grown on substrates that do not repress respiration. Also the data indicated that increased hsp synthesis and trehalose accumulation is not required for induced thermotolerance in derepressed cultures, while the contribution of the latter in repressed cultures was tenuous.

3.2 Results and Discussion

3.2.1 Affect of carbon source on thermotolerance

The present studies have shown that both intrinsic and heat shock induced thermotolerance of rapidly growing *S. cerevisiae* was affected by carbon source and nutrient availability in the growth medium. Generally, cells grown on a non-fermentable carbon source (acetate) were several orders of magnitude more heat tolerant than cells grown on a fermentable carbon source (glucose). This was particularly evident for Ysen, a relatively thermosensitive strain. Cells of Ysen grown on glucose were intrinsically sensitive to heat stress (Fig. 3.1 A, B) while cells grown on acetate were more resistant, by a factor of 2-3 logs, to a heat stress (Fig 3.1 C, D). It was noteworthy that the affect of carbon source was less marked, to the order of one log or less, with respect to heat shock induced thermotolerance.

In glucose medium, particularly YEPG, induced thermotolerance for Yres was significantly higher than intrinsic thermotolerance (Fig. 3.1 A, B). However, in acetate medium, in which intrinsic tolerance was as high as 90-100 %, there was little difference between intrinsic and heat shock induced thermotolerance, under the present conditions (Fig. 3.1 C, D). Consequently, in subsequent chapters, higher stress temperatures were employed for acetate grown cultures. A further, notable difference for both strains was the period of maintenance of acquired thermotolerance over the 60-minute time course. Levels of induction declined considerably in glucose supplemented YEP, an effect that was less obvious in YNBA, particularly for Yres. Several recent studies (Sanchez *et al.*, 1992; Elliot and Futcher, 1993; Weitzel and Li, 1993) have reported an association between carbon catabolite derepression and stress tolerance. Slower growth, on relatively poor

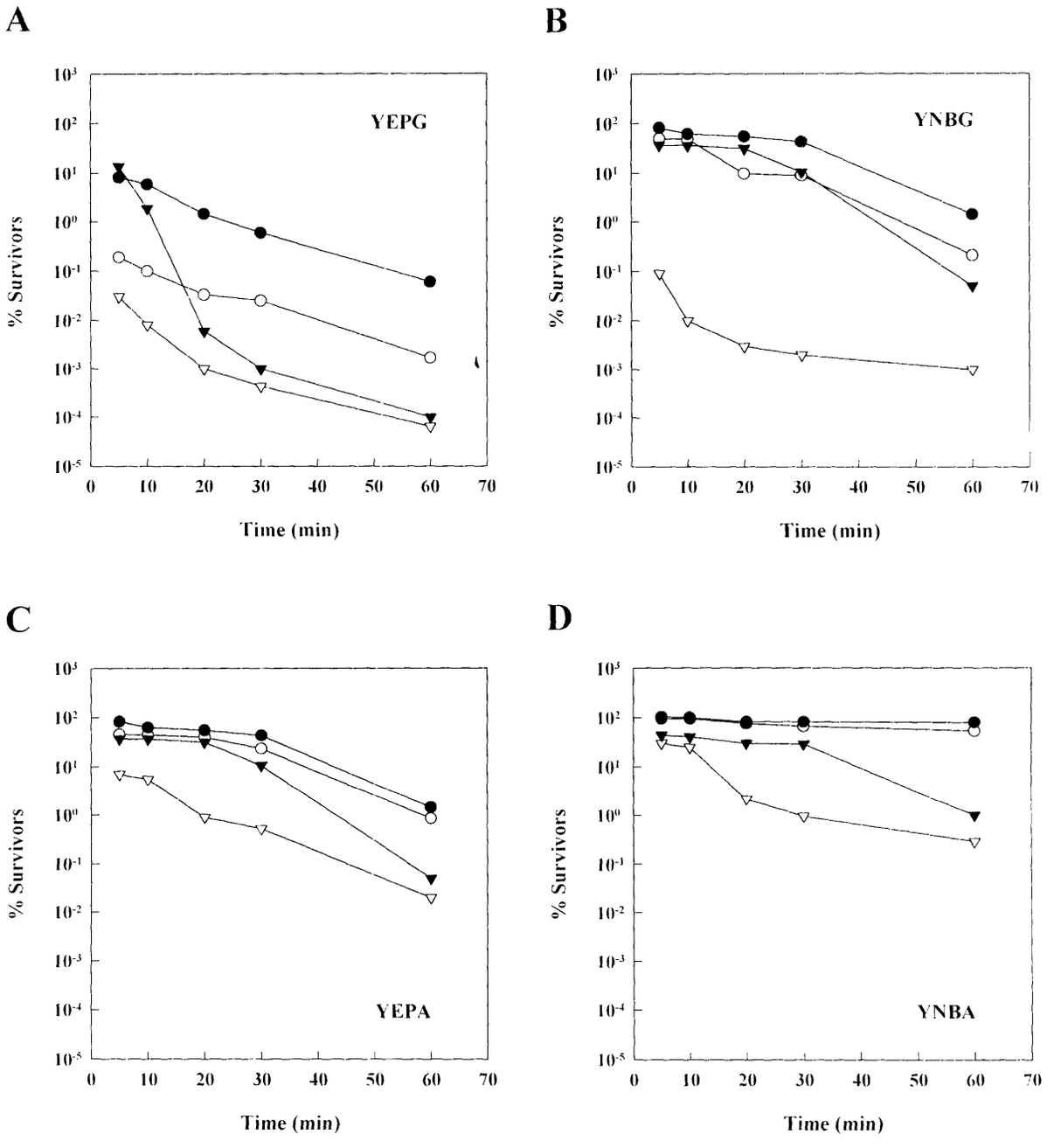

Fig. 3.1. Intrinsic (open symbols) and induced (closed symbols) thermotolerance in mid-log phase cultures of *S. cerevisiae* Yres (circles) and Ysen (triangles) grown on (A) YEPG, (B) YNBG, (C) YEPA and (D) YNBA media. Intrinsic tolerance was assessed following incubation at 50°C for the times indicated Induced tolerance was monitored at 50°C following a 30 min incubation at 37°C. Levels of thermotolerance are expressed as the percentage of viability with respect to a 25°C control sample.

medium and/or non-fermentable carbon source, has been proposed as a possible determinant of stress resistance (Elliot and Futcher, 1993).

To assess the relative growth rates of glucose repressed and derepressed cultures of Yres and Ysen, growth curves were constructed. Both *S. cerevisiae* strains exhibited initial rapid growth on glucose, in YEPG medium, followed by aerobic catabolism of accumulated ethanol upon glucose exhaustion (Fig. 3.2 A, B). Glucose assimilation occurred at a slightly decreased rate in synthetic YNBG medium (Fig. 3.3), which was consistent with an increase in doubling time (Table 3.1). Growth curves for both strains in YEPA (Fig. 3.2 C) and YNBA media (Fig. 3.4) indicated lower growth rates as compared with glucose repressed cultures. Doubling times in YNBA medium were approximately 2.6- and 4-fold greater than in YEPG, for Yres and Ysen respectively (Table 3.1). This marked decrease in growth rate was reflected in the slow rate of assimilation of acetate (Fig. 3.4). Although both strains attained plateau densities of approximately 3 x 10^7 cells ml^{-1} within 70 h, only one third of the available carbon source had been utilized following 190 h of growth. In addition, derepressed cultures exhibited an absence of the characteristic decline in cell numbers coincident with the onset of respiratory metabolism in glucose supplemented medium (Fig. 3.2, Fig. 3.3). The latter phenomenon most likely represents the period of metabolic adjustment to respiratory growth on ethanol, which once accumulated in the growth medium exerts a toxic effect on cells (Ingram, 1986; reviewed in Mishra, 1993). A number of ethanologenic microorganisms, including bacteria and fungi, are faced with this environmental challenge and need to employ adaptive mechanisms to overcome the chemical stress which has been shown to affect growth, survival and morphological transitions. In *S. cerevisiae*, studies have indicated that ethanol noncompetitvely inhibits uptake of glucose (Thomas and Rose, 1979; Leão and Van Uden, 1982), maltose (Loureiro-Dias and Peinado, 1982), ammonium ions (Leão and Van Uden, 1983) and amino acids (Thomas and Rose, 1979; Leão and Van Uden, 1984). Adaptive mechanisms known to be employed by *S. cerevisiae* include alteration of plasma membrane lipid composition (Beavan *et al.*, 1982; Walker-Caprioglio *et al.*, 1990), induction of stress proteins (Pelham, 1987) and an increase in the rate of ethanol metabolism (Blatiak *et al.*, 1987). Subsequent to this metabolic adjustment, accumulated ethanol supports a further period of growth, albeit exclusively respiratory. However, as evident from the relatively slight and gradual increase in cell numbers (Fig. 3.2, Fig. 3.3)

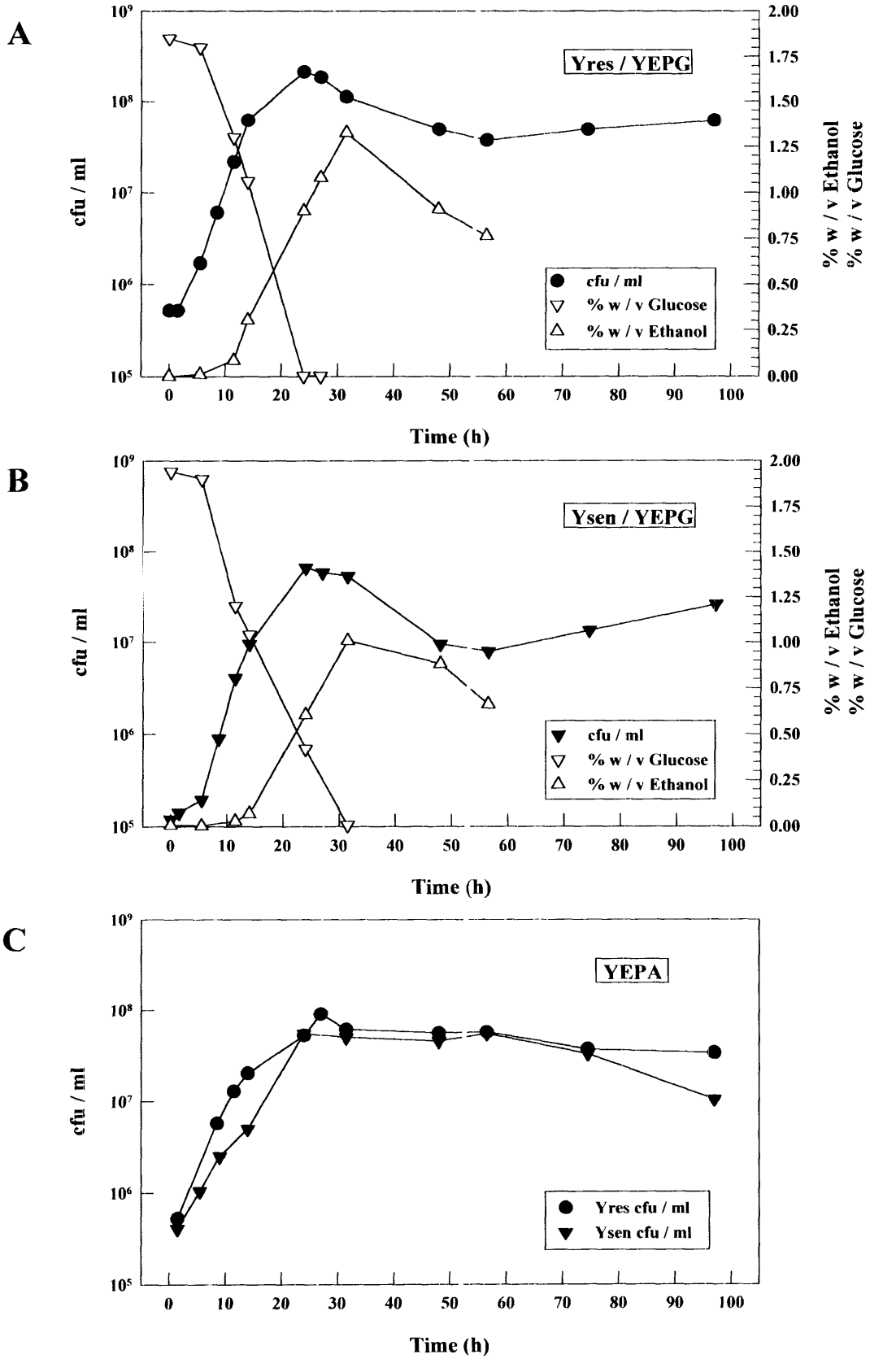

Fig. 3.2. Growth of *S. cerevisiae* Yres (A) and Ysen (B) in YEPG and YEPA (C) media. Corresponding glucose and ethanol concentrations are shown for glucose supplemented cultures.

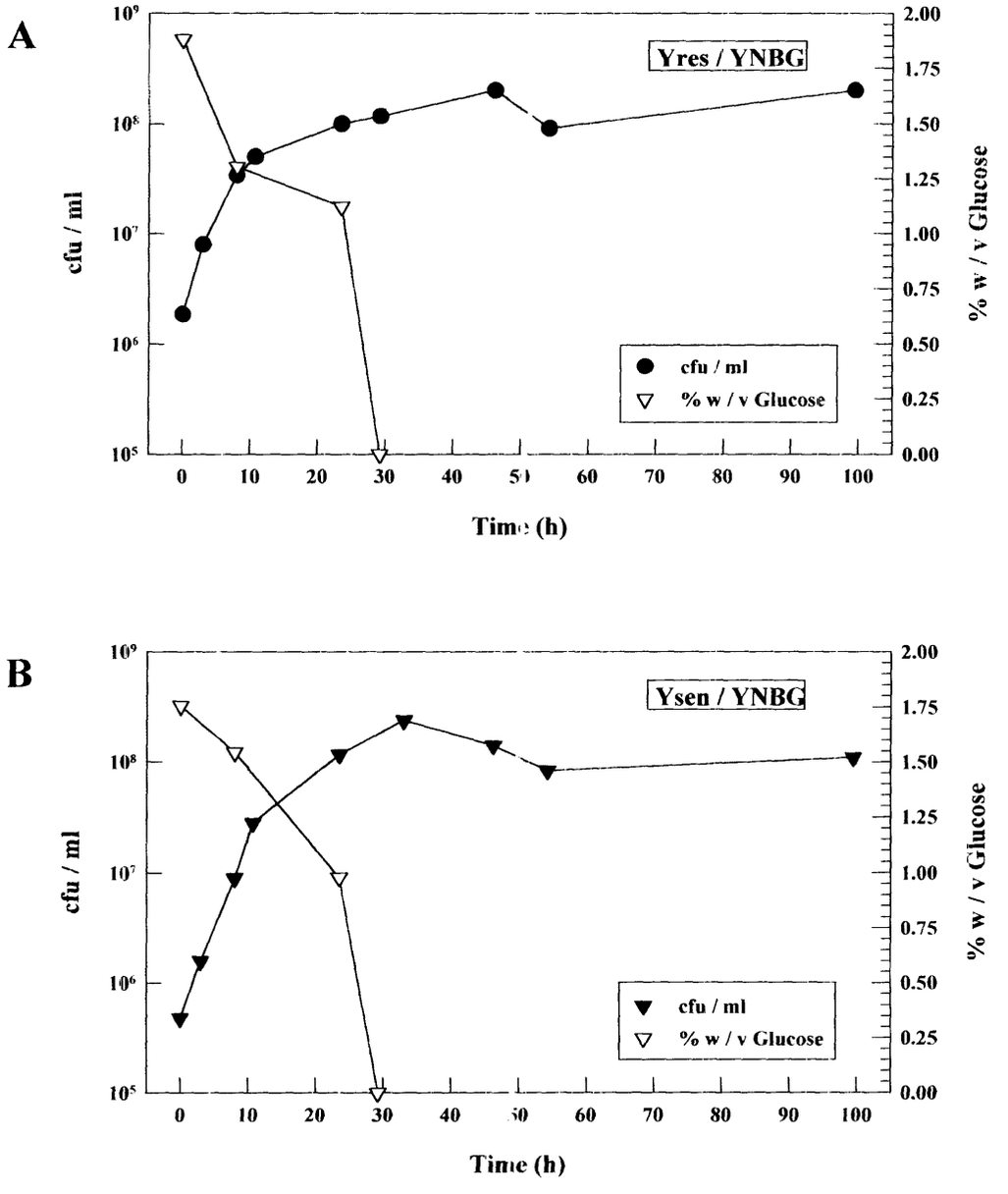

Fig. 3.3. Growth of *S. cerevisiae* Yres (A) and Ysen (B) in YNBG medium with corresponding glucose concentration.

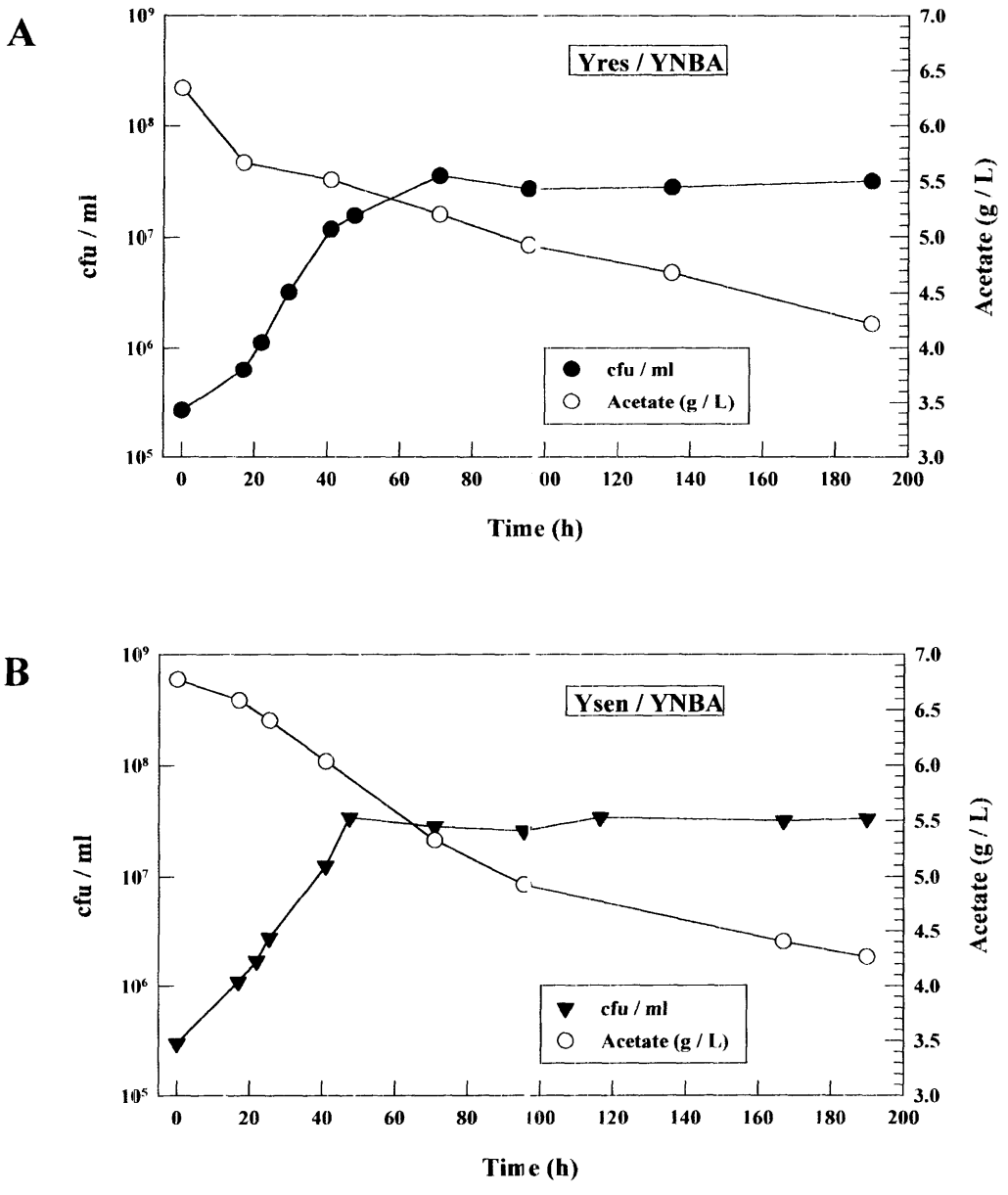

Fig. 3.4. Growth of *S. cerevisiae* Yres (A) and Ysen (B) in YNBA medium with corresponding acetate concentration.

Table 3.1. Culture doubling times for Yres and Ysen grown in various media.

Medium	Doubling Time (min) at 25°C	
	Yres	Ysen
YEPG	90	90
YNBG	120	120
YEPA	150	180
YNBA	360	360

Doubling time was defined as the time taken for the cell density of logarithmic phase cultures to double and estimated from respective growth curves (Figs. 3.2-3.4).

Table 3.2. Culture viability following a 50°C heat stress for logarithmic and post-logarithmic phase cultures of Yres and Ysen in various media.

Medium	% Survivors (50°C / 5min)			
	Yres		Ysen	
	Logarithmic	Post-log	Logarithmic	Post-log
YEPG	0.16	4.40	0.03	0.05
YNBG	32.0	48.4	0.05	1.50
YEPA	45.2	106.0	4.90	48.50
YNBA	92.3	135.0	24.20	127.0

Post-logarithmic phase cells were assessed by depletion of glucose in the culture medium (see section 2.2.2).

the growth rate is slow, limited by the low capacity of *S. cerevisiae* to respire (Lagunas, 1986; Alexander and Jeffries, 1990). The specific growth rate during respiration has been shown to be approximately 10% of that during fermentation, despite the energetic efficiency of this form of catabolism (Blomberg *et al.*, 1988).

Overall, there appeared to be a stepwise decrease in the rate of growth of both strains under the constraints of available carbon source and nutrient limitation, from the highest rate in YEPG decreasing in the order YNBG, YEPA and YNBA. This is indicative of the fact that carbon source is the critical nutrient which stimulates growth in *S. cerevisiae* (Granot and Snyder, 1993) with fermentable carbohydrates being preferred. *S. cerevisiae* will utilize glucose, other hexoses, disaccharides and finally non-fermentable substrates, in that order (reviewed in Wills, 1996). In an attempt to distinguish between the affects of carbon source and altered growth rates on thermotolerance, cells further advanced in growth phase were also examined for intrinsic and induced thermotolerance characteristics. Glucose grown cultures were examined during post-fermentative growth in the diauxic lag phase (after 30 h growth, Fig. 3.2, 3.3), subsequent to exhaustion of glucose. Post-exponential acetate cultures were examined after 50-60 h growth, corresponding to maximal cell densities (Fig. 3.2, 3.4). Generally, it was noted that levels of intrinsic and heat shock induced tolerance for both strains increased with the age of the culture for each medium (Table 3.2, Fig. 3.5). These results confirmed trends apparent in previously published work (Sherman, 1956; Schenberg-Frascino and Moustacchi, 1972; Parry *et al.*, 1976; Paris and Pringle, 1983). In addition, as was the case for exponentially dividing cells, increased intrinsic resistance in older cultures grown in acetate supplemented media was exhibited by both strains. Most notable was the 2500-fold increase in basal resistance exhibited by Ysen in YNBA medium, as compared with YEPG. This stands in marked contrast to the less dramatic, 30-fold increase observed for Yres, attributable to the higher levels of intrinsic resistance exhibited by this strain in YEPG.

The contrasting phenotypes of the two strains, in terms of thermoresistance, were most obvious in both exponential YNBG cultures, where Yres attained 600-fold greater resistance, and post-fermentative YEPG and YNBG cultures, where Yres exhibited 90- and 30-fold greater capacity for survival than Ysen, respectively (Table 3.2). Clearly, this trend

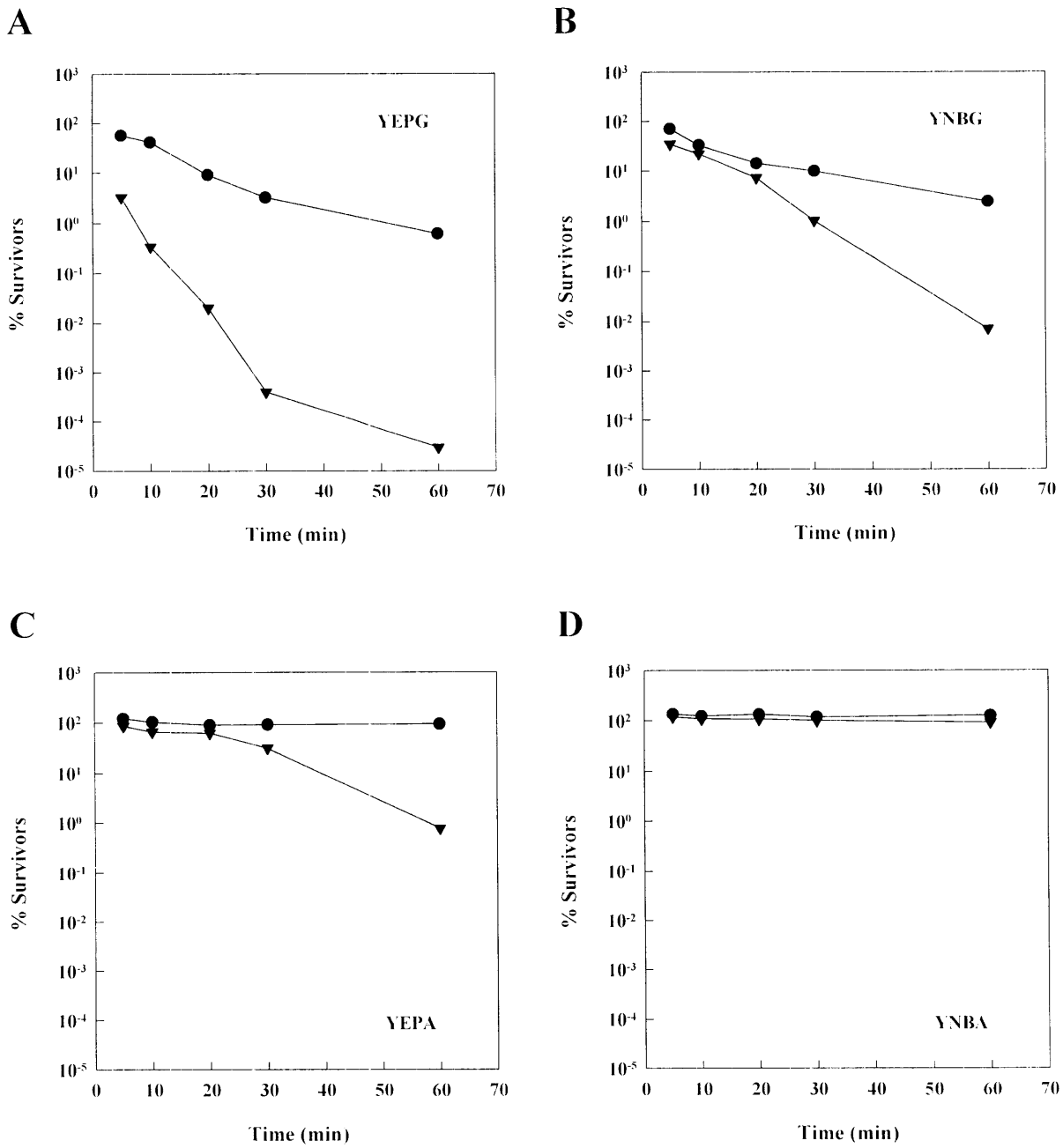

Fig. 3.5. Induced thermotolerance in post-fermentative cultures of *S. cerevisiae* Yres (circles) and Ysen (triangles) grown on (A) YEPG, (B) YNBG, (C) YEPA and (D) YNBA media. Induced tolerance was monitored at 50°C following a 30 min incubation at 37°C. Levels of thermotolerance are expressed as the percentage of viability with respect to a 25°C control sample.

was a result of the fact that whilst exponential growth in YNBG and residence in diauxic lag in both YEPG and YNBG elicited higher innate resistance in Yres, Ysen remained relatively unaffected. This was an important observation in terms of the influence of growth rate on tolerance and its implications are further considered below. Furthermore, the difference in intrinsic resistance between the two strains diminished during growth on acetate, particularly in more advanced cultures (Table 3.2) which presumably, as acetate assimilation progressed (Fig. 3.4), were in a greater state of catabolite derepression. It has been demonstrated that derepression of catabolite repressed proteins is not an all or none phenomenon but that some proteins are expressed earlier than others as the culture advances (Perlman and Mahler, 1974; Boucherie, 1985; Bataille *et al.*, 1991). This may also serve to explain the differences in growth rate between YEPA and YNBA cultures, the former comprising a less defined medium with a richer source of nutrients. Further work examining the temporal combinations of derepressed protein expression and its affect on thermotolerance appears warranted. In Chapter 7 of this thesis, novel work is presented involving differential display of reverse transcribed mRNA from heat shocked repressed and derepressed cells. Data suggests that this technique would lend itself favourably not only to investigations regarding stress tolerance but derepressive metabolism in general.

While many studies have indicated that rapidly growing, fermentative cells are stress sensitive compared to respiratory or stationary phase cells, few attempts have been made on comparing or differentiating between the effects of growth phase and catabolite derepression. However, such an endeavour presents inherent difficulties because the two parameters are interrelated; in essence, slow growth is effectuated by catabolite derepression. Indeed, the present data were indicative of a correlation between the level of intrinsic thermoresistance of a culture and both its rate of growth and state of catabolite repression. In the case of Yres, logarithmic phase cells were least resistant in YEPG during rapid growth (Fig. 3.1, Table 3.2) and most resistant in later stages of growth in YNBA, where lower growth rates were exhibited (Fig. 3.5, Table 3.1). Resistance levels in exponential YNBG and diauxic shift YEPG cultures were intermediate (Fig. 3.3, 3.5) corresponding to intermediate growth rates (Table 3.1). However, significantly, results obtained for Ysen did not comply with this trend. The same degree of thermosensitivity was maintained in exponential YNBG and post-fermentative YEPG cultures despite slower growth rates maintained by these cells (Fig. 3.1, Table 3.1, Table 3.2). Ysen only exhibited

a considerable increase in thermoresistance at diauxic lag in YNBG and in acetate supplemented media (Table 3.2). Clearly, this result casts uncertainty on the significance of growth rate effects on hyperthermia.

During the diauxic shift of yeast cells growing on glucose, unbudded cells start to accumulate and many morphological changes occur not after but already well before the glucose in the medium is exhausted (Francois *et al.*, 1987; Werner-Washburne *et al.*, 1989; Bataille *et al.*, 1991). As cells respond to glucose exhaustion, they complete the cell cycle and finally come to rest in G1 phase (Herskowitz, 1988). Consequently, at this stage in the growth curve, cells become partially synchronised prior to recommencement of the cell cycle following diauxic lag. An association between cell cycle position and thermotolerance has been previously reported. Schenberg-Frascino and Moustacchi (1972) demonstrated that tolerance was maximal during the latent period before budding (corresponding to G1 or G0) and minimal during budding, concomitant with synthesis of nuclear DNA (corresponding to early S phase (Herskowitz, 1988)). Plesset *et al.* (1987) arrived at similar conclusions employing centrifugal elutriation to separate different stages of cell cycle in exponential cells. Importantly, they demonstrated isolation of resistant cells in G1 or even G0 from rapidly growing, repressed cells. Therefore, in the context of the present work, cell cycle rather than growth phase *per se* may have affected thermotolerance. At division the daughter cell is normally smaller than the mother, especially under conditions of slow growth (Hartwell, 1994). The daughter cell delays more than the mother in the G1 phase of the next cell cycle before producing a bud, giving itself time to grow to the size characteristic of a mother cell required to progress through start (Lord and Wheals, 1980). Incidentally, the critical size that characterizes the G1/S transition varies according to the carbon source (Lorincz and Carter, 1979). It follows that in slowly growing, thermoresistant acetate grown cells, either a greater majority of the population may have been in G1 phase or that residence in G1 was sustained to a greater extent. Further support for such a notion comes from observations that, under sugar limitation, aerobic cultures tend to spontaneously synchronize their cell cycle (von Meyenburg, 1969; Parulekar *et al.*, 1986).

Although the greater basal thermotolerance of both strains in acetate supplemented media was coincident with a decline in the growth rate (Fig. 3.2, Table 3.1), several aspects

of the present data enable inference of a distinction between the affects of slow growth or catabolite derepression on intrinsic resistance. While cultures of both strains in YEPA and YNBA are derepressed, the substantial 2-fold decrease in growth rate evident in YNBA (Table 3.1) was not accompanied by a marked increase in resistance (Table 3.2). Particularly pertinent was the observation that for Ysen, the 5-fold increase in resistance between YEPA and YNBA did not appear to correlate with the 2-fold increase in doubling time, when compared with the 100-fold increase observed from YNBG to YEPA for only a 60 min increase in doubling time. Moreover, an increase in basal resistance in post-fermentative YEPG cultures, maintaining lower growth rates than exponential YNBG cultures, was not evident for either strain. Similarly, despite lower growth rates in post-fermentative YNBG cultures (Fig. 3.3) greater levels of basal resistance than those exhibited by exponential YNBA cultures (Fig. 3.4) were not demonstrated. In addition, although further advanced YEPA and YNBA cells were more resistant than their exponential counterparts, they were also more resistant than post-fermentative YEPG and YNBG cells (Table 3.2). The latter was particularly evident in the case of Ysen where a 1000-fold increase in tolerance was noted between post-exponential YEPG and YEPA cultures, despite negligible difference in the growth rate of these cells (Fig. 3.2). However, in either case, post-exponential YEPA and YNBA cultures would have been in a greater state of catabolite derepression, as discussed above. Growth rate was therefore unlikely to be a factor with respect to heat tolerance in the present studies, whilst catabolite derepression was of greater significance.

A variety of mutations that inactivate either production of cAMP or cAMP-dependent protein kinases result in cessation of growth and arrest of the cell cycle in G1 (Matsumoto *et al.*, 1982). Mutations that inhibit the pathway such as temperature sensitive alleles of *cdc25* or *cyr1* (adenylate cyclase) are stress resistant even in rich medium (reviewed in Broach and Deschenes, 1990). Conversely, mutations that hyperactivate the pathway, such as $RAS2^{val19}$ and *bcy1*, lead to thermosensitivity. It has recently been shown that steady state cAMP levels in exponential cells growing under different conditions are inversely related to cell density (Wheals *et al.*, 1995). In addition, as evident from a variety of other genetic (Baroni *et al.*, 1992; Mitsuzawa, 1994) and biochemical experiments (Russell *et al.*, 1993), low cAMP levels equate with the derepressed state. In this context, past reports have shown that cells of the constitutively derepressed *hxk2-202* mutant exhibit

constitutive heat shock resistance (Elliot and Futcher, 1993) while cells of the *snf1-172* mutation, which are constitutively repressed, are intrinsically heat shock sensitive (Thompson-Jaeger *et al.*, 1991). The opposite phenotypes of the latter two mutants present a strong argument in favour of the influence of catabolite derepression on thermotolerance. Data from the present studies concerning comparably high levels of tolerance in exponential acetate grown cultures and derepressed, post-fermentative YNBG cultures confirm these previous observations. However they extend such observations by enabling a clearer distinction between affects of slow growth or derepression. Further experiments conducted by Elliot and Futcher (1993) using various nitrogen sources rather than carbon source to control growth rate, suggested a poor correlation between slow growth and stress tolerance. Moreover, there was little correlation in thermotolerance at similar growth rates for different carbon sources. Again it would appear that perhaps the extent of derepression in cells is a major determinant of thermotolerance.

Subsequent to exhaustion of glucose, cells recommence growth on ethanol, as evident from increased growth rate following the ethanol peak (Fig. 3.2). Despite an active engagement in metabolic cycling, such cells exhibited high levels of thermotolerance (Table 3.2). This appears to indicate that the position in the cell cycle was not a major influence on thermotolerance in the present studies. Using cell cycle inhibitors, Barnes *et al.* (1990) demonstrated that G0 was the only position in the cell cycle that was particularly stress tolerant and that other cycle positions, including unbudded G1 cells, were equally stress sensitive. Moreover, it has recently been shown that G1 arrest results from failure of cyclin protein (products of *CLN1* and *CLN2*) to accumulate, under the negative control of cAMP (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994). It follows that in derepressed cells, which exhibit low cAMP levels, cyclins will be present, inhibiting G1 arrest. The aforementioned speculation concerning sustained residence in G1 by acetate cells can therefore be discounted, with the effect of cell cycle on thermotolerance also appearing to be of less significance than the state of catabolite repression.

In summary, in the present studies, Yres and Ysen had similar generation times at 25° C in glucose (approx. 2 h) and on acetate (approx. 6 h) (Table 3.2) but markedly different heat tolerance. Coupled with other published findings, the evidence presented suggests that growth rate was unlikely to be determinant of heat tolerance whilst catabolite

derepression was of greater significance. Furthermore, results indicated that while YNB medium induced higher levels of tolerance, presumably an attribute of nutrient limitation, trends in thermotolerance data generated for cells grown on YEP or YNB were similar. Historically, labelling of yeast proteins in synthetic medium lacking methionine has been standard procedure. Consequently, in ensuing chapters of this thesis, all investigations regarding Yres and Ysen were carried out with cells grown in YNB medium, to maintain consistency in culture conditions between protein extraction and other experimental procedures. As an unforseen advantage, growth in YNB also elicits expression of a greater number of genes of unknown function in *S. cerevisiae* (Dujon *et al.*, 1994). This was of particular relevance for work conducted in Chapter 7, where a search of the genomic profile was undertaken for novel genes involved in conferral of the thermotolerance phenotype.

3.2.2 Influence of hsps and trehalose on thermotolerance

Results discussed thus far have concerned, to a large extent, the affect of cell metabolism on intrinsic thermotolerance. However, as was implicit from the data, rapidly growing, vegetative cells, with the capacity to acquire high levels of induced thermotolerance (Fig. 3.1), mounted the classical heat shock response. Both Yres and Ysen exhibited increased synthesis of typical heat shock inducible proteins (Nicolet and Craig, 1991) at approximately 100, 90 and 70 kDa, in both media types (Fig. 3.6, indicated by arrows). Of particular note was the heat shock induction of a high molecular weight protein, approx 130-150 kDa, in both strains grown in either medium (indicated by top arrow in Fig. 3.6). A heat shock gene from *S. cerevisiae* encoding a 150 kDa secretory glycoprotein (Russo *et al.*, 1992) has been shown to be under dual regulation by heat and nutrient stress (Russo *et al.*, 1993). In addition, variations were noted in protein profiles of YNBG and YNBA cultures, presumably due to the different metabolic modes induced by the repressive (glucose) and derepressive (acetate) carbon substrates. In particular, protein bands corresponding to approximately 66 and 64.5 kDa showed pronounced expression in YNBA whereas a 60 kDa band was more intense in YNBG.

A role for hsp 104 in yeast tolerance to a number of stresses has been proposed (Sanchez *et al.*, 1992) and it was reported that levels of hsp 104 were significantly higher

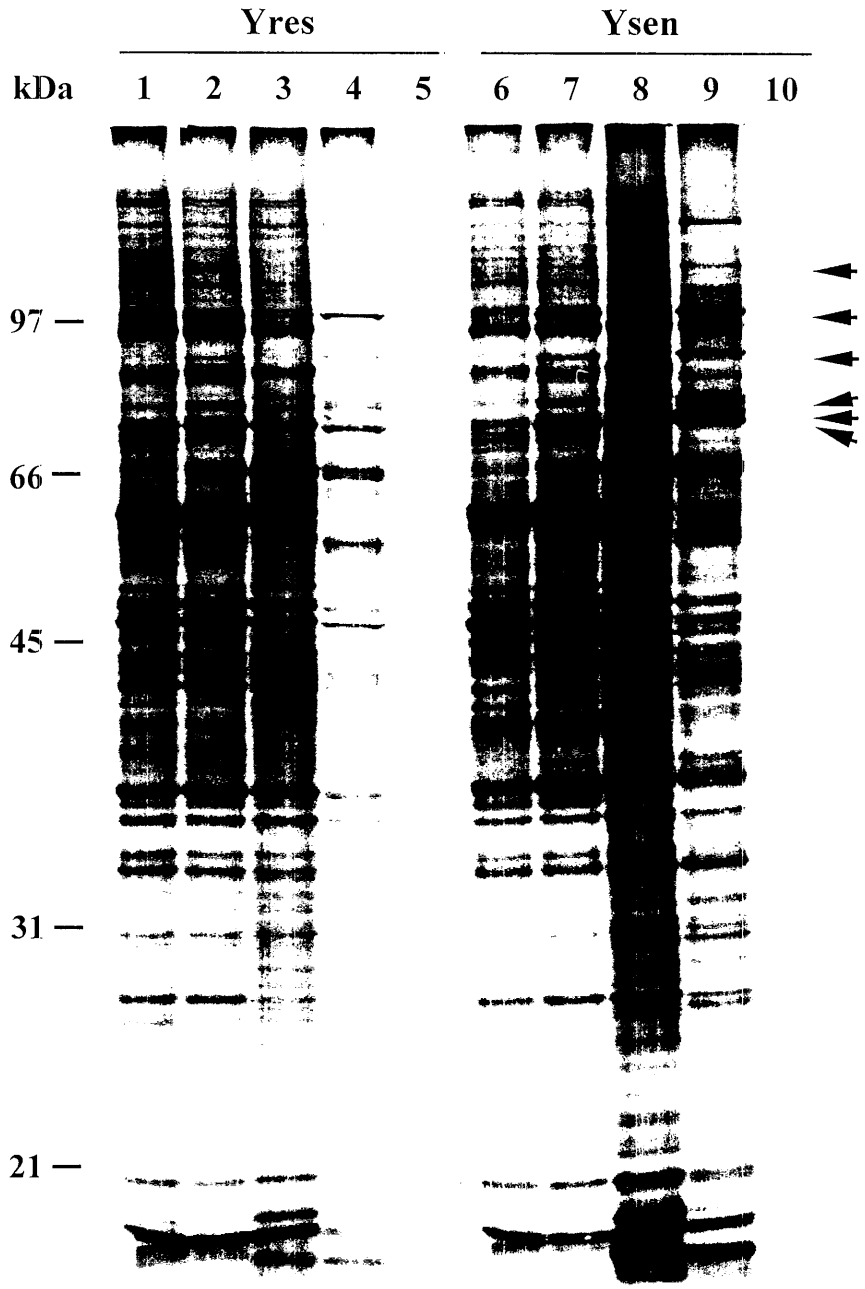

Fig. 3.6. SDS-polyacrylamide gel autoradiogram of [^{35}S]-methionine labelled protein extracts from Yres and Ysen. Lanes 1-4: control and heat shock samples of Yres grown on glucose and acetate, respectively. Lanes 6-9: control and heat shock samples of Ysen grown on glucose and acetate, respectively. Lanes 5 and 10: heat shock in the presence of 20μg ml^{-1} cycloheximide. Arrows indicate new or increased protein bands in heat shocked samples compared with controls. Molecular mass standards (kDa) are as indicated.

in carbon catabolite derepressed cells than in cells grown on glucose. Whilst major differences in intrinsic levels of hsp 104 in the two strains of yeast grown on glucose or acetate were not obvious at the translational level in the present experiments (Fig. 3.6), Yres consistently exhibited greater constitutive transcription of *HSP 104* than Ysen, in both media (Fig. 5.3, Chapter 5; Fig. 6.5, Chapter 6). Pre-incubation of cells with cycloheximide (20 $\mu g\ ml^{-1}$) had no significant effect on intrinsic resistance in glucose or acetate supplemented media (see Chapter 5). Moreover, heat shock induced thermotolerance was essentially not inhibited by cycloheximide in acetate medium (Fig. 3.7). Thus, under conditions in which cells exhibit high intrinsic tolerance, such as growth on a non-fermentable carbon source, hsp synthesis may not be essential for thermotolerance acquisition. Conversely, for Ysen grown on glucose medium, thermotolerance acquisition was impaired by cycloheximide treatment (Fig. 3.7). This was not the case for Yres, which exhibited a relatively high intrinsic thermotolerance on glucose. The mechanism responsible for induced thermotolerance, which is a transient, non-heritable trait, may well be different to that for intrinsic thermotolerance. In support of such a concept are findings by the group of Coote who demonstrated that membrane ATPase activity was essential for basal heat resistance but induced tolerance was independent of this factor (Coote *et al.*, 1994). In addition, heat-induced protein damage has been proposed to be the trigger for hsp induction (Craig and Gross, 1991). It follows that in cultures treated with cycloheximide during heat shock, this signal may be partly abolished. In this context, cells that exhibited high intrinsic thermotolerance and were not impaired in tolerance induction, such as Yres in glucose medium, may rely on additional, pre-existing factors that are modified during heat treatment. Results presented in Chapter 7 using differential display of PCR amplified cDNA from Ysen and Yres show promise toward identification of such factors (Gross and Watson, 1995; 1996b; 1998a).

Previous studies have presented conflicting conclusions with respect to the role of hsps and induced thermotolerance (Watson, 1990). These studies include reports on high levels of induced thermotolerance in temperature-sensitive *Δhsp104* (DeVirgilio *et al.*, 1991), a mutant strain defective in the synthesis of the major heat shock proteins (Smith and Yaffe, 1991), and in cycloheximide treated cells (McAlister and Finkelstein, 1980; Hall, 1983; Watson *et al.*, 1984; DeVirgilio *et al.*, 1990). Moreover, it has been suggested

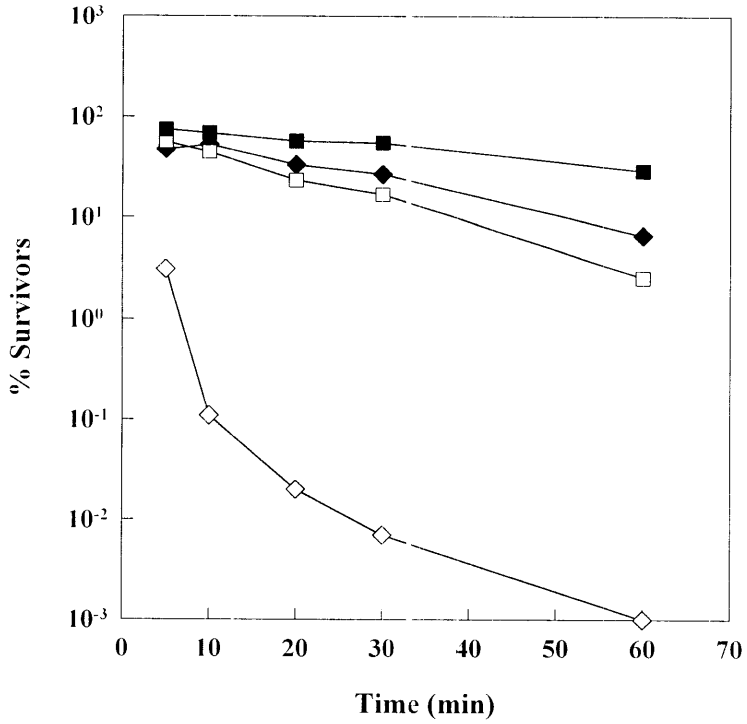

Fig. 3.7. Effect of cycloheximide on heat shock induced thermotolerance in mid-log phase cultures of Yres and Ysen. Cells were incubated with cycloheximide (20μg ml^{-1}) for 20 min prior to and during a 37°C (30 min) heat shock. Induced thermotolerance was monitored at 50°C for the times indicated. Cells grown or glucose, □ Yres, ◇ Ysen; on acetate ■ Yres, ◆ Ysen.

that heat shock induction of thermotolerance in the absence of protein synthesis is the result of the accumulation of trehalose (Hottiger *et al.*, 1989; DeVirgilio *et al.*, 1990; DeVirgilio *et al.*, 1991).

In the present work, blocking protein synthesis by cycloheximide had little effect on heat shock induced thermotolerance in cells grown on a non-fermentable carbon source (Fig. 3.7). Trehalose accumulation in cycloheximide treated, heat shocked cells was however, inhibited by at least 50%, to approximately 5% w/w dry cells and 1% w/w dry cells for Yres and Ysen respectively (Fig. 3.8). Cycloheximide inhibited trehalose accumulation has been previously reported in *S. cerevisiae* (Neves and François, 1992; Coote *et al.*, 1992; Lewis *et al.*, 1995). It can therefore be concluded that induced thermotolerance and trehalose accumulation are not closely correlated in these strains. Further support for this conclusion comes from data for trehalose (Fig. 3.8) and thermotolerance levels in glucose and acetate grown cells (Fig. 3.1b,d). There is no obvious relationship between trehalose levels and either intrinsic or induced thermotolerance. For example, in the case of heat shock induced thermotolerance, glucose grown cells consistently had substantially higher trehalose levels than acetate grown cells, yet the latter cell type was the more thermotolerant. In addition, the greater magnitude of tolerance induction in glucose grown Ysen compared to Yres, was accompanied by markedly lower levels of trehalose accumulation, 6-fold compared to 20-fold respectively. It is interesting to note that recent work has indicated an increased activity of the glucose transport system during heat shock in *S. cerevisiae* (Ribeiro *et al.*, 1994). The increase was found to be due to the kinetic affect of temperature upon its activity and increased *de novo* synthesis of transporter molecules, an effect that was inhibited by cycloheximide. In support of this, temperature dependent changes in levels of glucose-6-phosphate and UDP glucose, substrates of trehalose-6-phosphate synthase, have also been reported (Winkler *et al.*, 1991). Conceivably, these results may serve to explain the present data concerning limited trehalose accumulation in derepressed cultures and decreased accumulation in cycloheximide treated cultures.

Although there is increasing evidence that trehalose may play a role in yeast stress tolerance, not only as a cryoprotectant (Coutinho *et al.*, 1988) but also in intrinsic and

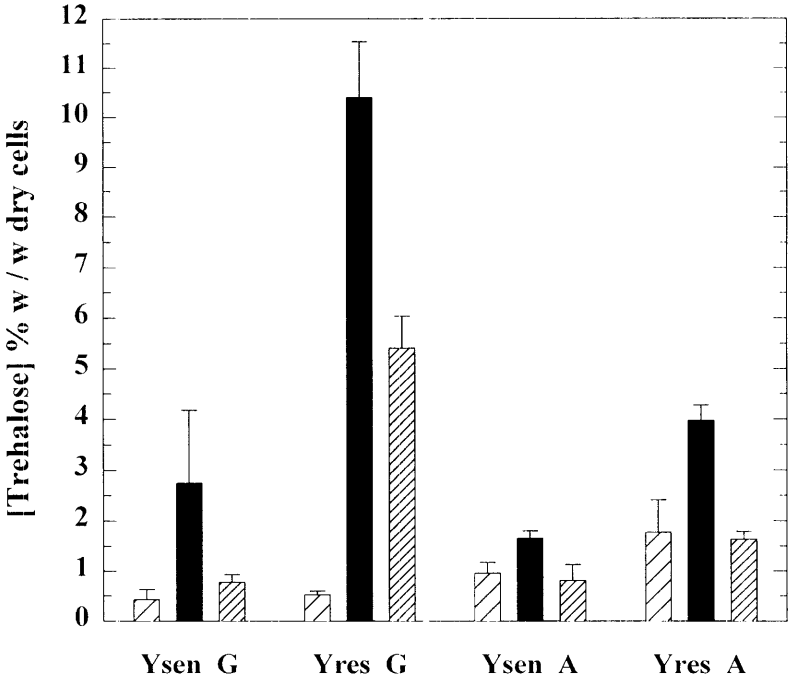

Fig. 3.8. Trehalose levels in control (▨) and heat shocked (■) cultures of Yres and Ysen. Cultures were grown to mid-log phase on either glucose (G) or acetate (A) and incubated at 37°C (30 min) in the absence or presence of 20μg ml^{-1} cycloheximide (▨). Error bars represent the standard deviation of measurements from three experiments.

induced thermotolerance (Piper, 1993) there are however, contradictory reports. In a *ubc4ubc5* mutant, in which the trehalose concentration in logarithmic phase cells is as low as in the corresponding wild-type, intrinsic thermotolerance is very high when compared to the wild type (Nwaka *et al.*, 1994). This higher thermotolerance could be attributed to the higher constitutive expression of heat shock proteins exhibited by the mutant (Seufert and Jaentsch, 1990). On the other hand, it should be noted that *ubc4ubc5* cells grow substantially slower than the wild type (Nwaka *et al.*, 1994) and as noted previously, slowly growing cells are relatively, more intrinsically thermotolerant (Elliot and Futcher, 1993). With respect to induced thermotolerance, in a trehalase-negative mutant Δ*nth1*, trehalose levels remain high following a heat shock at 40°C and recovery at 30°C for 40 min (Nwaka *et al.*, 1994). However, both the wild type, in which trehalose rapidly declines to low levels following a heat shock and recovery, and Δ*nth1* cells lose induced thermotolerance upon recovery. In addition, cells of the *tps1* mutant are not deficient in thermotolerance acquisition, despite the fact that this strain is unable to synthesize trehalose (Argüelles, 1994). The relationship among hsp 104, trehalose and induced thermotolerance remains unclear. One report indicates that although heat shock induces high levels of trehalose in a mutant deficient in hsp 104, the cells are thermosensitive (Winkler *et al.*, 1991) and another report records that the same mutant is thermotolerant following a heat shock (DeVirgilio *et al.*, 1991). There are subtle but perhaps important differences in the heat shock protocols adopted by the two groups. The former used a heat shock at 39°C for 40 min, while the latter used 40°C for 60 min. These variations may well account for the differences in survival reported by the two groups. Work undertaken in Chapter 4 attempts to clarify the relative contribution of trehalose to induced thermotolerance using a mutant deficient in heat shock induced trehalose accumulation. Results presented support the conclusion that trehalose is of less significance as compared with *de novo* protein synthesis and, by inference, hsps.

Overall, the present results indicated that the marked differences in thermotolerance exhibited by fermenting and non-fermenting yeast cells are not closely correlated with either levels of hsp or trehalose. Furthermore, the mechanisms of intrinsic and induced thermotolerance appear to be different. However, both hsp and trehalose are likely to be major factors in some aspect of stress tolerance. The possible contribution of each to recovery from hyperthermia is investigated in Chapter 5.

Chapter 4: Thermotolerance, trehalose accumulation and heat shock protein synthesis in a *S. cerevisiae* trehalose synthase mutant.

4.1 Introduction

There is a large body of published evidence that suggests trehalose may act as a stress protectant under a variety of conditions. This evidence was discussed in Chapter 1 (section 1.6) and drawn on in discussion of results presented in Chapter 3, as well as Chapters 5 and 6. However, results presented in Chapter 3 appeared to suggest that the contribution of trehalose to protection from heat stress, particularly in derepressed cells, is limited (Gross and Watson, 1996a). Other published accounts have also indicated that the correlation between trehalose content and thermotolerance in yeast is equivocal (Panek *et al.*, 1990; Neves *et al.*, 1991; Winkler *et al.*, 1991; Lewis *et al.*, 1997).

In addition to the equivocal data concerning trehalose, there exists an apparent contention in the literature concerning the relative significance between hsps and trehalose as thermoprotectants. Despite this, few reports, with the exception of De Virgilio *et al.*, (1991), Hottiger *et al.*, (1992) and results presented in Chapter 3 (Gross and Watson, 1996a) have considered their action as complementary (discussed further in Chapter 6). Most evidence implicating the involvement of either hsps or trehalose in thermotolerance is fundamentally by association, however, many reports concentrate on one, ignoring the possible validity of the other. For example, in a recent study, Panek's group attributed the high stress resistance of an *hsr1* mutant largely to the affects of trehalose (Eleutherio *et al.*, 1995), while dismissing the affects of high levels of constitutive hsp expression, which were not investigated, exhibited by this strain (Iida and Yahara, 1984). Furthermore, in several papers describing the role of hsp 104 in stress tolerance, trehalose is not measured in the strains (Sanchez and Lindquist, 1990 Sanchez *et al.*, 1992, 1993; Lindquist and Kim, 1996). Also, evidence has been presented to support the greater importance of hsps over trehalose (Sanchez and Lindquist, 1990. Winkler *et al.*; 1991; Sanchez *et al.*, 1992) and *vice versa* (Hottiger *et al.*, 1989, 1992; De Virgilio *et al.*, 1990, 1991). Therefore, reconciliation of these two factors appears to necessitate comparison of both metabolites under the same conditions, in the same strain. However, it was evident from results

presented in Chapter 3, that the influences of hsps and trehalose were difficult to separate under stress conditions due to a decline in trehalose accumulation in cells heat shocked in the presence of the protein synthesis inhibitor cycloheximide.

In 1979 Navon *et al.* described strain Klg 102 as harbouring a recessive mutation on chromosome II that was linked to *LYS2*. This mutation was considered to be pleiotropic, and designated *cif1* due to the fact that it is deficient in catabolite inactivation of fructose-1-6 bisphosphate. The original mutant does not grow on fructose and exhibits a partial growth defect on glucose. Charlab *et al.* (1985) demonstrated that Klg 102 had no UPDG-dependent trehalose synthase activity. Previously, it had been shown that the product of the $MAL4^c$ (constitutive) gene is a pre-requisite of the phenomenon of trehalose accumulation during growth on glucose (Panek *et al.*, 1979; Operti *et al.*, 1982). Little or no disaccharide is accumulated during growth on glucose in *mal* strains, which cannot ferment maltose, or in strains which bear other inducible maltose fermentation genes: *MAL2* (Zimmermann and Eaton, 1974) and *MAL6* (Zimmermann and Eaton, 1974; Panek *et al.*, 1979). Strain Klg 102 is therefore *mal cif1* and consequently, deficient in trehalose accumulation. Other mutant strains had been observed that also exhibited no detectable UDPG-dependent trehalose synthase activity and yet accumulated trehalose when grown on maltose (Operti *et al.*, 1982). To investigate this apparent paradox, Paschoalin *et al.* (1989) performed a cross between strains 1403-7A ($MAL4^c$ *CIF*) and Klg 102, in order to introduce a constitutive *MAL* gene into the *cif1* strain. The resulting segregant was $MAL4^c$ *cif1* and designated VFP1-8C. Although VFP1-8C was unable to grow on fructose, trehalose accumulation was exhibited during growth on glucose, maltose, galactose and glycerol. Characterization of this strain permitted identification of a second enzymatic activity that uses ADPG, rather than UPDG, and G-6-P as substrates for trehalose synthesis (Paschoalin *et al.* 1989).

In an attempt to determine whether the significance of the two putative thermoprotectants could be more definitively separated, VFP1-8C and its trehalose deficient progenitor Klg 102, were investigated with regard to heat shock induced thermotolerance, trehalose accumulation and hsp profiles.

4.2 Results

4.2.1 Thermotolerance

Strains VFP1-8C and Klg 102 exhibited similar levels of intrinsic thermotolerance to a 50°C heat challenge (Fig. 4.1). However, subsequent to 5 min heat stress exposure, resistance declined substantially in Klg 102, resulting in at least a 100-fold difference between the two strains for the remainder of the time course. Likewise, in the case of induced thermotolerance, for which an approximate 10-fold increase was observed in both strains, Klg 102 exhibited a more dramatic decline as compared with VFP1-8C. Maintenance of acquired thermotolerance was therefore substantially greater in VFP1-8C (Fig. 4.1). The absence of protein synthesis during heat shock was found to inhibit development of thermotolerance in both strains. In addition, cell viability declined even more rapidly, throughout the duration of the 60 min time course, as compared with viability monitored for intrinsic tolerance. The efficacy of cycloheximide treatment was verified by the absence of *de novo* protein profiles in [^{35}S]-methionine labelled extracts (Fig. 4.3).

4.2.2 Trehalose content

Trehalose content was determined in VFP1-8C and Klg 102 to ensure agreement with previously reported data (Eleutherio *et al.*, 1993). Klg 102 did not exhibit substantial trehalose accumulation following either a 37°C or a 40°C heat shock (Fig. 4.2). However, the control strain exhibited 2.5- and 3.5-fold increases in trehalose content subsequent to 37°C and 40°C heat shock, respectively. In the case of VFP1-8C, incubation of cells prior to and during heat shock with 20 μg ml^{-1} cycloheximide was observed to affect a significant reduction in the extent of heat shock induced trehalose accumulation (Fig. 4.2).

4.2.3 Protein synthesis

Figs. 4.3 and 4.4 show autoradiograms of [^{35}S]-methionine labelled protein extracts from VFP1-8C and Klg 102. Increased synthesis of typical heat shock inducible proteins at approximately 100, 90 and 70 kDa was noted in both strains, with particularly marked induction in Klg 102 (Fig. 4.3 indicated by arrows). Patterns of *de novo* hsp synthesis induced during either 37°C or 40°C heat shock (Fig. 4.4) were consistent with immuno

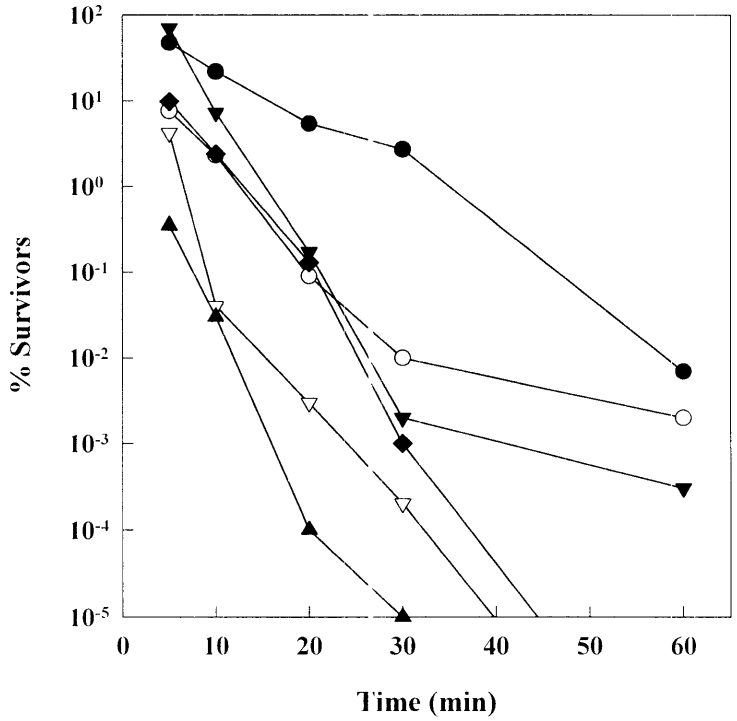

Fig. 4.1. Intrinsic (open symbols) and induced (closed symbols) thermotolerance in mid-log phase cultures of *S. cerevisiae* strains VFP1-8C (○,●) and Klg 102 (▽,▼). Intrinsic tolerance was assessed following incubation at 50°C for the times indicated. Induced tolerance was determined at 50°C following a 30 min heat shock at 37°C. Induced tolerance was also monitored in cells incubated with cycloheximide (20 μg ml^{-1}) for 20 min prior to and during heat shock (◆VFP1-8C, ▲Klg 102).

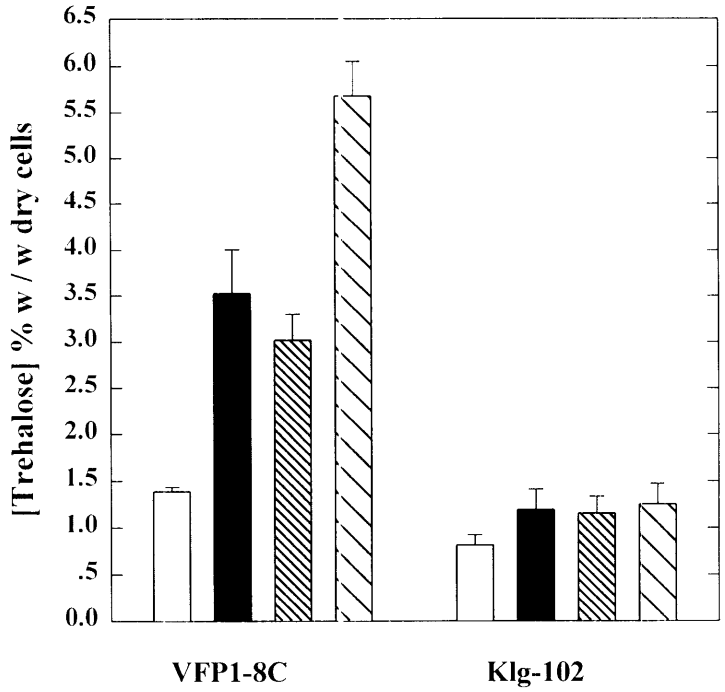

Fig. 4.2. Trehalose levels in control (▭) and heat shocked cultures of *S. cerevisiae* strains VFP1-8C and Klg 102. Cells were grown to mid-log phase in glucose medium (25°C) and heat shocked at 37°C (▬) or 40°C (▨) (30 min). Heat shock at 37°C was also carried out in the presence of 20 µg ml^{-1} cycloheximide (▩). Error bars represent the standard deviation of measurements from three experiments.

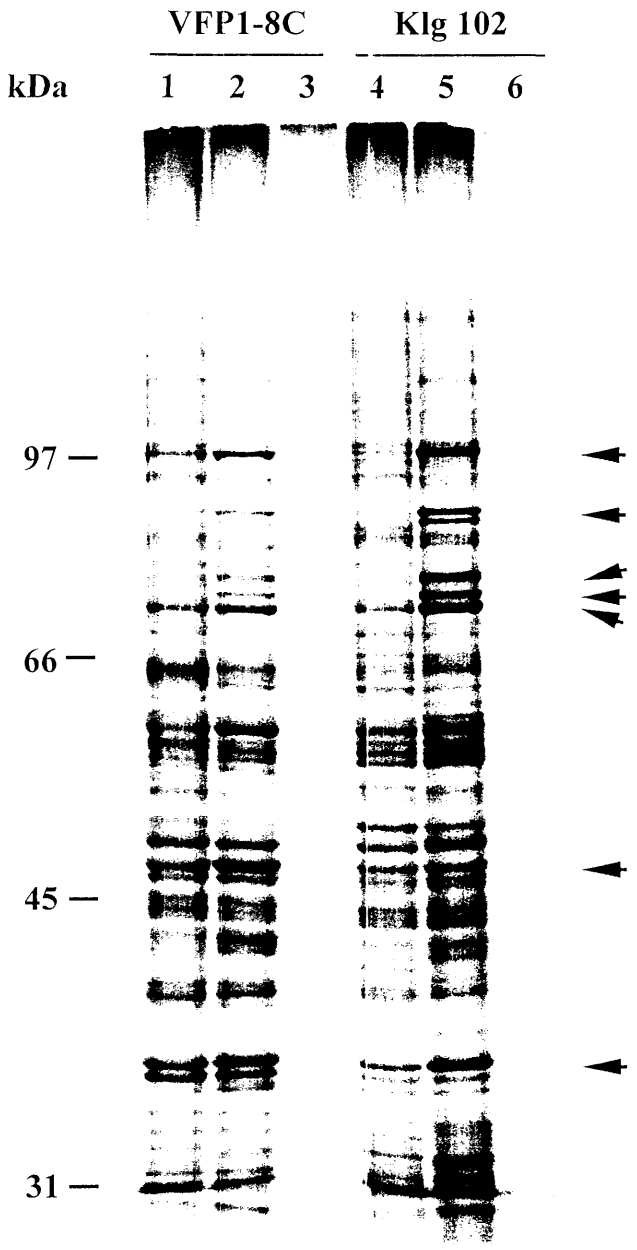

Fig. 4.3. SDS-polyacrylamide gel autoradiogram of [^{35}S]-methionine labelled protein extracts from heat shocked (37°C / 30 min) cells of *S. cerevisiae* strains VFP1-8C and Klg 102 in the presence or absence of protein synthesis. Lanes 1 and 2: control (25°C) and heat shocked samples of VFP1-8C. Lanes 4 and 5 control (25°C) and heat shocked samples of Klg 102. Lanes 3 and 6: heat shock in the presence of 20μg ml^{-1} cycloheximide. Arrows indicate new or increased protein bands in heat shocked samples compared with controls. Molecular mass standards (kDa) are as indicated.

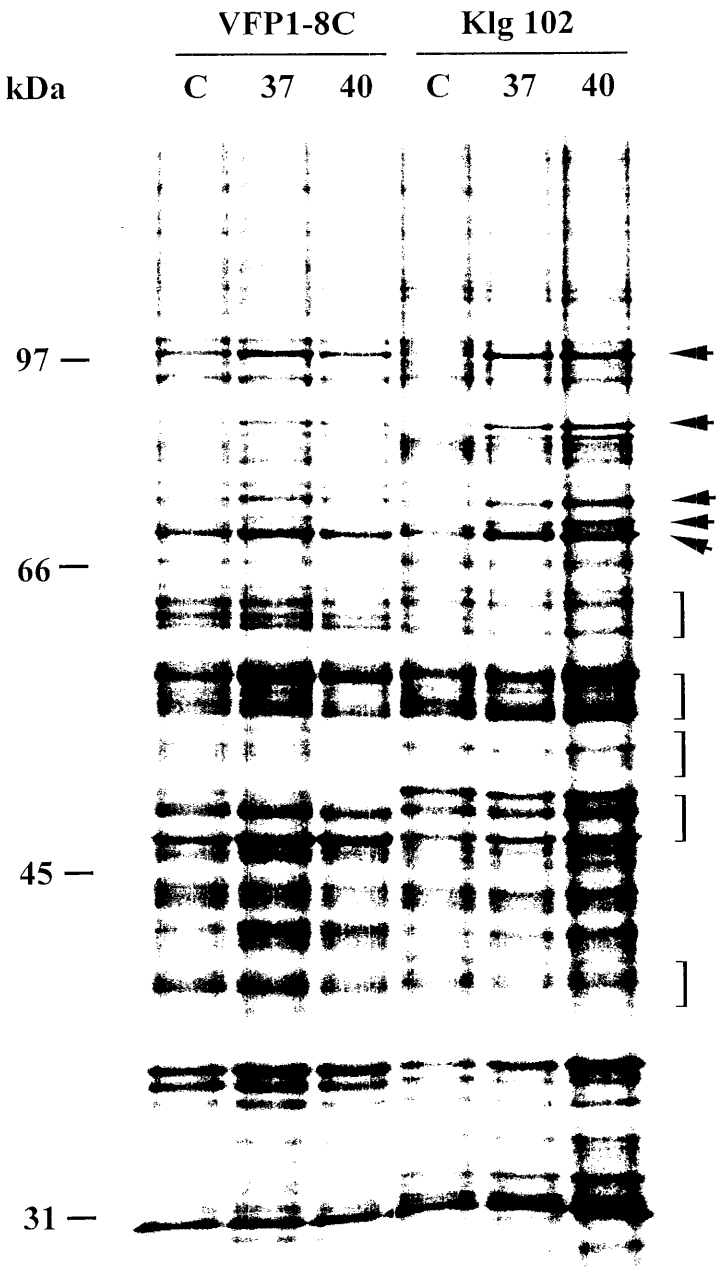

Fig. 4.4. SDS-polyacrylamide gel autoradiogram of [^{35}S]-methionine labelled protein extracts from *S. cerevisiae* strains VFP1-8C and Klg 102 grown at 25°C (C) and heat shocked at 37°C or 40°C (30 min). Arrows indicate new or increased protein bands in heat shocked samples compared with controls. Differences between control protein profiles of VFP1-8C and Klg 102 are denoted by]. Molecular mass standards (kDa) are as indicated.

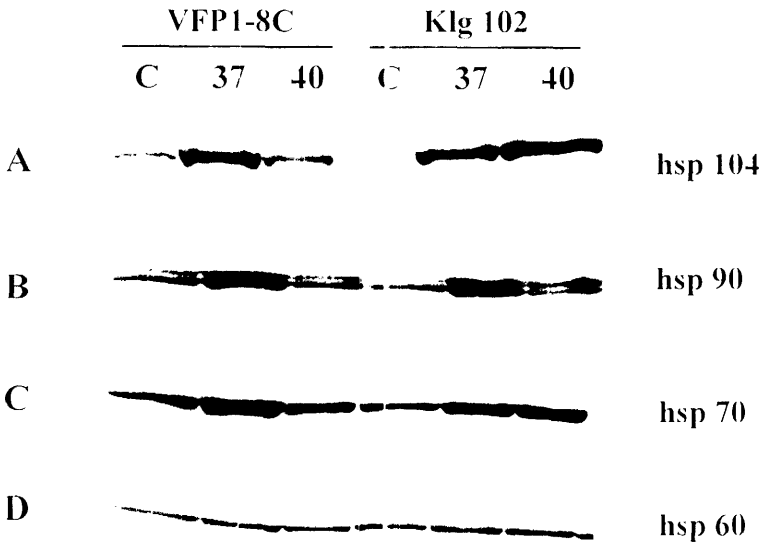

Fig. 4.5. Western immunoblot analysis of protein extracts from 25°C control (C) and heat shocked cells of *S. cerevisiae* strains VFP1-8C and Klg 102. Heat shock was carried out at either 37°C or 40°C (30 min). Immune complexes were generated for anti-hsp 104, 90, 70 and 60 antibodies.

complexes generated under the same conditions for hsps 104, 90, 70 and 60 (Fig. 4.5). Notably, levels of induction were similar, if not more intense, in Klg 102 following a 40°C heat shock as compared with heat shock at 37°C. By contrast, a 40°C heat shock was observed to elicit less intense induction in the control strain VFP1-8C, especially in the case of hsp 104. Overall, VFP1-8C exhibited greater constitutive expression of hsps 104, 70 and the inducible form of hsp 90 (Fig. 4.5). On the other hand, Klg 102 exhibited pronounced heat shock induced hsp synthesis, at both heat shock temperatures (Figs. 4.3, 4.4). Both strains exhibited the presence of heat shock inducible proteins of approximately 48 and 35 kDa (Fig. 4.3). It should be noted however, that distinct differences were detected between control protein profiles (Fig. 4.4).

4.3 Discussion

In agreement with a previous report (Eleutherio *et al.*, 1993), the intrinsic thermotolerance of mutant Klg 102 and its derivative control strain VFP1-8C was observed to be similar in magnitude (Fig. 4.1). In contrast to the previous findings however, absolute values of intrinsic tolerance established in the present studies were 4 to 5-fold lower. In addition, Klg 102 exhibited a significant level of heat shock induced thermotolerance, compatible in fact with that acquired by the control strain. Several factors could account for these apparent discrepancies. During the course of experimentation in the present work, it was observed that older, late logarithmic phase cultures of both VFP1-8C and Klg 102 resembled more closely the higher values of intrinsic tolerance reported (Eleutherio *et al.*, 1993). This was consistent with the well-established fact, and a result presented in Chapter 3, that intrinsic thermotolerance increases in a temporal manner, with respect to growth phase (Table 3.2). As a result, the magnitude of induced thermotolerance reported by Eleutherio *et al.* (1993) appears less dramatic, 2-fold for VFP1-8C as compared with the greater than 10-fold induction observed in the present work (Fig. 4.1). Furthermore, the authors in question assessed both intrinsic and induced tolerance at only a single time point, 8 min, following exposure to 50.5°C. Considering acquired tolerance declined rapidly in Klg 102, these workers may have sampled the culture too late to observe its acquisition, which, in addition, remained significantly higher than intrinsic values for the entire duration of the time course (Fig. 4.1). It should also be noted that Eleutherio *et al.* (1993) employed a 40°C heat shock for 60 min. Consequently, further experiments were conducted where cultures of both strains

were divided and exposed to 37°C and 40°C pre-treatments in parallel. The difference in heat shock temperature was found to have negligible affect on the extent or kinetics of induced tolerance to a subsequent 50°C challenge (results not shown). A notable difference in the kinetics of tolerance between the two strains however, was that of prolonged durability in VFP1-8C (Fig. 4.1). A tenuous correlation may thus be inferred between trehalose and the maintenance of thermotolerance, as distinct from conferral, throughout the time course. However, in the case of Klg 102 the pronounced decline in acquired tolerance and loss of viability at 50°C may be attributed not only to a trehalose deficiency but also, low constitutive expression of hsp 104 (Fig. 4.5). Pre-existing hsp 104 in VFP1-8C may be sufficient to confer the extra margin of intrinsic tolerance upon this strain, although not as effective as its possible activation and increased synthesis at 37°C (Figs. 4.3, 4.5 this work; Lindquist and Kim, 1996). It was not surprising therefore, that the absence of protein synthesis during the 37°C pre-treatment (Fig. 4.3) was found to inhibit the development of thermotolerance in both strains (Fig. 4.1). However, heat shock protein profiles and the effect of cycloheximide on thermotolerance acquisition have not been previously described in these strains and consequently, this was a key result. Its implications are elaborated on below.

Trehalose content of cells under control and heat shock conditions (Fig. 4.2) was found to be in general agreement with those reported previously (Eleutherio *et al.*, 1993). However, significant accumulation was still observed in heat shocked cells of VFP1-8C in the absence of protein synthesis, despite the obvious compromise in tolerance acquisition (Fig. 4.1). It has been suggested that a 'threshold' level of trehalose be required to induce significant levels of stress tolerance (Gadd *et al.*, 1987). This concept suggests that the protective effect of trehalose is influenced by its intracellular concentration, but that above a critical value, in the order of 4-5 % w/w in *S. cerevisiae* (Attfield *et al.*, 1992), further accumulation will not further increase stress tolerance. This may explain why induction of tolerance in VFP1-8C in the absence of protein synthesis is abolished while the concentration of trehalose is only slightly reduced, to approximately 3 % w/w, presumably below the critical value. In support of the 'threshold' concept, trehalose accumulation following a 40°C heat shock was observed to be substantially greater than the level induced at 37°C (Fig. 4.2) but was not accompanied by an increase in the degree of induced tolerance (results not shown). In fact, in this context, it was interesting to note a certain parallelism between VFP1-8C and Ysen. Both strains were compromised in

tolerance acquisition in the absence of protein synthesis (for Ysen see Fig. 3.7), accompanied by a decrease in trehalose accumulation to below the putative threshold concentration (for Ysen see Fig. 3.8). Whereas, Yres was not deficient in tolerance induction and the accompanying decrease in trehalose content, to approximately 6 % w/w, remained above the critical value (Fig. 3.8). Heat shocked induced trehalose accumulation in excess of the threshold value may therefore be a consequence of the kinetic affect of temperature (Ribeiro *et al.*, 1994; discussed in Chapter 3), which would otherwise appear to comprise a futile investment in energy expenditure for the cell. However, the 'threshold' theory fails to provide an explanation for high levels of thermoresistance exhibited by acetate grown cells, which occur despite trehalose concentrations well below the suggested critical value. Perhaps other factors or mechanisms (discussed in Chapter 3) come into play under these conditions. The salient feature of the present results however, was the acquisition of thermotolerance by trehalose deficient Klg 102 and its total inhibition in the absence of protein synthesis (Fig. 4.1). Clearly, *de novo* protein synthesis, and by inference induction of hsps, is a crucial component of the tolerance mechanism in this strain. Moreover, threshold theory aside, VFP1-8C was also compromised in acquisition of tolerance, even in the presence of significant trehalose accumulation. It seems unlikely, intuitively, that the small difference in trehalose content in cells heat shocked in the absence or presence of protein synthesis would be solely responsible for conferral of thermotolerance. Thus, hsps appear to be of greater significance. However, the concept that the combined action of hsps and trehalose confer thermotolerance (as described in the introduction to this chapter) remains valid in the case of VFP1-8C, which exhibited a greater extent of intrinsic and heat shock induced thermotolerance as compared with Klg 102. Nevertheless, while results presented in Chapter 3 could not conclusively dismiss a role for trehalose in conferral of thermotolerance, the present data provided strong evidence that its role is less significant than that of hsps.

Having arrived at what appeared to be a feasible conclusion, the differences in protein profiles of the two mutants should not be ignored (Fig. 4.4). In addition, electron microscopic investigations have revealed the strains to be morphologically distinct, with VFP1-8C exhibiting the classical oval shape typical of yeast cells and Klg 102 appearing comparatively small and torpedo shaped (Webster, 1995). Phenotypic differences between the strains therefore extend beyond a mere proficiency or deficiency in trehalose accumulation. In this context, it was noteworthy that despite the fact that VFP1-8C is a

genetic derivative of Klg 102, fewer differences were apparent between the non-related strains Yres and Ysen (Fig. 3.6, Chapter 3). Conclusions attained comparing any two given strains, especially mutants should probably be treated with circumspect, not necessarily providing a definitive model for the species as a whole. Many inconsistencies in the literature concerning a role for trehalose or hsps in thermotolerance (as described in the introduction of the present and preceding chapters) could be attributable to the fact that most work is undertaken with only one or two strains, leading to erroneous conclusions. On the other hand, in a report concerning heat and rapid freezing tolerance of 12 closely related strains of *S. cerevisiae*, lack of a clear relationship between trehalose and stress tolerance was still evident (Attfield *et al.*, 1992). Results demonstrated the inconsistency of trehalose content with heat and rapid freezing tolerance under some circumstances and supported the concept that trehalose content and stress tolerance are not strictly related. A further, significant factor likely to contribute to inconsistencies in the literature, specifically concerning studies of pleiotropic yeast mutants, is that different alleles are often obtained in different genetic backgrounds. This is also true of the *cif1* type mutants under investigation in the present chapter. In *S. cerevisiae*, *CIF1* is one of several alleles, *FDP1*, *BYP1*, *SST1*, *GLC1* and *TSS1*, of the *GGS1/TPS1* gene, that have been isolated using different approaches (van de Poll *et al.*, 1974; Navon *et al.*, 1979; Charlab *et al.*, 1985; Hohmann *et al.*, 1992; Stucka and Blàzquez, 1993). The *GGS1* gene was cloned independently as complementing the *fdp1* and *byp1* mutants (Hohmann *et al.*, 1992; Van Aelst *et al.*, 1993), the *cif1* mutant (Gonzàlez *et al.*, 1992) and the *glc6* mutant (Cannon *et al.*, 1994). The same gene was also cloned by two other groups as encoding the 56kDa subunit of the trehalose-6-phosphate synthase (t-6-P) complex and designated *TPS1* (Bell *et al.*, 1992) and TSS1 (Vuorio *et al.*, 1993) accordingly. As noted in the introduction to this chapter, generally, non-reverted or unsuppressed *cif1* mutants exhibit an inability to adapt to rapidly fermentable carbon sources, have impaired catabolite inactivation of fructose-1,6-bisphosphate and are unable to accumulate trehalose (Van De Poll and Schamhart, 1977; Navon *et al.*, 1979; Panek *et al.*, 1988). Thus, *CIF1* and *GGS1/TPS1* are alleles that encode a protein constituting a crucial component of t-6-P synthase, as well as performing functions essential to the sensing and regulation of glucose metabolism (Hohmann *et al.*, 1993). Specifically, the pleiotropic effects of the *cif1* mutation comprise a deficiency in many glucose-induced regulatory phenomena including induction of cAMP synthesis, induction of glycolytic enzymes, inactivation of gluconeogenic enzymes,

phosphatidyl inositol turnover, activation of cation transport and stimulation of H^+-ATPase (Van Aelst *et al.*, 1993; Thevelein and Hohmann, 1995). In this context it was interesting to note that both VFP1-8C and Klg 102 exhibited pronounced heat shock inducible synthesis of proteins at approximately 35 and 48 kDa (Fig. 4.3). The molecular weights of these bands corresponded to those of glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and enolase, respectively, both of which have been previously observed to be induced following mild heat treatment (McAlister and Holland, 1985a, 1985b; Iida and Yahara, 1985; Boucherie *et al.*, 1995a).

With the exception of a recent report (Neves *et al.*, 1995), few systematic studies have been performed on the influence of the genetic background on pleiotropic phenotypes of *cif1* mutants. The latter report presented evidence that the extent of specific phenotypic traits evoked by *fdp1*, *byp1*, *glc6* and *cif1* varied significantly in three different wild-type strains of *S. cerevisiae*. In the context of the present discussion, a further notable example of inconsistency among publications constitutes a report by Petit and François (1994) contesting observations of Panek's group concerning synthesis of trehalose in *cif1* or *fdp1* mutants (Operti, *et al.*, 1982; Paschoalin *et al.*, 1989). However, mutants constructed by Petit and François (1994) were in different strains to those employed in the earlier studies. Influences of genetic background also serve to explain an apparent inconsistency between the present results concerning heat shock induced hsp synthesis in VFP1-8C and Klg 102 and contrary data reported for a WDC-3A *cif1* mutant (Hazell *et al.*, 1995). These authors demonstrated a lack or only low levels of heat shock induced HSP transcription in mutant cells as opposed to control cells harbouring a functional *CIF1* gene. It was concluded that efficient heat shock induced expression of stress genes is generally dependent on *CIF1* function. Clearly, this was not the case in the present studies (Fig. 4.3, Fig. 4.4). It is noteworthy however, that results presented in Chapters 5 and 6 of this thesis demonstrated that heat shock inducible transcription, in the case of Ysen, did not necessarily equate with levels of translation (Figs. 5.3, 5.5 and Figs. 6.2, 6.5). Consequently, transcriptional data presented by Hazell *et al.* (1995), in the absence of translational data, may not have provided the full picture.

Although a clear lack of correlation between trehalose and induced thermotolerance was evident from the current results, it may still be argued that its affects are merely outweighed by combined affects of other factors contributing to tolerance. Such factors as may arise as a consequence of pleiotropic effects of the *cif1* mutation or undefined effects

arising from the *mal* lesion in Klg 102. Indeed, as discussed above, distinct differences were observed in control protein profiles of the two strains (Fig. 4.4). On the other hand, a lack of correlation with trehalose and acquired thermotolerance extends to other stresses as well. For example, exposure to some toxic chemicals has been observed to induce hsp synthesis but not trehalose accumulation (Nwaka *et al.*, in press). Furthermore, hsp induction, in contrast to trehalose accumulation, has been reported in yeast cells exposed to the potential mutagen tetrachloroisophthalonitrile (TPN) (Fujita *et al.*, 1995). It was demonstrated that the minimal concentration of TPN required for induction of hsp synthesis leads to acquisition of tolerance to a 51°C challenge without an increase in trehalose.

Taken together, published accounts and the present results indicate that while relative contributions of the two factors are difficult to separate, trehalose may only be required for prolonged stress protection while hsps clearly play a predominant role in conveying thermotolerance. However, since this evidence was not definitive, a role for trehalose in tolerance conferral may not be discounted and work conducted in subsequent chapters includes further investigations concerning the disaccharide.