# **CHAPTER 1:** Introduction

### 1.1 The heat shock response

The heat shock response refers to the synthesis of a set of proteins, the so-called heat shock proteins (hsps), by cells subjected to a sublethal heat shock. This response appears to be universal and has been characterized in all organisms so far examined including bacteria, archaea, fungi, plants, animals and humans.

The heat shock proteins (hsps) or closely related proteins are also induced when organisms are exposed to diverse environmental stresses other than heat. Although the particular inducers of the response vary somewhat from organism to organism, the induction of the stress response can also be achieved by diverse agents including sodium arsenite, sulfhydryl reagents, hydrogen peroxide, transition metal ions, amino acid analogues, viral infection and abnormal protein synthesis (Lindquist and Craig, 1988). It is generally thought that stress proteins provide protection, at the intracellular level, from various stresses allowing cells to recover and survive. The disaccharide trehalose is also synthesized in response to various stresses in yeast and may play a role in thermotolerance as a stress protectant in that organism (Attfield, 1987; De Virgilio *et al.*, 1991; Hottiger *et al.*, 1992).

### **1.2** Cellular events during heat shock

In addition to the synthesis of hsps, stress results in changes at the cellular level. Perturbations at the cellular level include the cessation of mitosis and DNA synthesis (Kramhoft and Zeuthen, 1971), actin filaments in nuclei are affected, transient damage in nucleoli, and blockage of the assembly and export of ribosomes from the nucleus (Pelham, 1984; Welch and Suhan, 1985). Changes at the molecular level include inhibition of most genes which are expressed under nonstressful conditions and alterations of gene expression by changes in RNA processing, mRNA stability and translation, and transcriptional termination (Lindquist, 1980; Morimoto *et al.*, 1990).

### **1.3 History of the stress response**

Interest in the heat shock response was initiated by Ritossa in 1962 when he reported the transient induction of puffing in the salivary gland polytene chromosome of *Drosophila* larvae (*D. buskii* and *D. melanogaster*) after a brief heat shock. It was also reported that, in the case of prolonged heat shock treatment, the puffs that were active prior to the heat shock treatment regressed or disappeared altogether. Puffing in salivary gland polytene chromosomes is indicative of areas of intense RNA transcription of active genes.

It was twelve years later that Tissières *et al.* (1974) recognized the significance of Ritossa's observations when they analyzed radiolabeled proteins from heat shocked *Drosophila* salivary glands using SDS-slab gel electrophoresis. The results indicated the induction of a set of proteins coinciding with the heat shock induced puffing of polytene chromosomes in *Drosophila*.

Following these initial observations, research on the heat shock response was concentrated on *Drosophila* (Ashburner and Bonner, 1979) and it wasn't until the early 1980s that other organisms were examined with respect to heat shock. The number of papers currently relating to hsps exceeds 800 a year (Somero, 1995). To date, every organism so far examined has been shown to respond to a mild temperature shock, with respect to normal growth temperature, by increased synthesis of specific proteins.

### **1.4 Heat shock protein families**

Despite the universal nature of the stress response, differences are evident in the number and molecular weight of hsps synthesized in various organisms. The hsps can be divided into six main groups according to their molecular mass. These groups are large hsps of 100-110 kDa, 82-90 kDa, 70 kDa family. 60 kDa family, 40-50 kDa and a diverse group of small hsps approximately 15 - 30 kDa, and ubiquitin, a 8.5 kDa protein (Morimoto *et al.*, 1990; Watson, 1990). The number after a hsp refers to the molecular mass as estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

Within these groups, with the exception of the small molecular weight hsps, the hsps are highly conserved among widely divergent organisms. For example, hsp 70 has about 50% of its sequence conserved between *Escherichia coli* and human, with some

domains showing 96% sequence conservation (Schlesinger, 1990). In eukaryotes, the most highly conserved stress-inducible protein is ubiquitin. The monomeric unit is a 76 amino acid residue polypeptide of 8.5 kDa found in all eukaryotic organisms and with almost total amino acid sequence homology in diverse organisms (Watson, 1990). This high evolutionary conservation of sequence suggests that the function of these hsps in different organisms is likely to be identical or very similar and essential for survival (Lindquist and Craig, 1988; Schlesinger, 1990: Watson, 1990; Craig and Gross, 1991; Panaretou and Piper, 1992). The fact that heat shock proteins are also developmentally regulated (Bienz, 1984; Bond and Schlesinger, 1987) and are synthesized in unstressed cells (Lindquist and Craig, 1988) further attest to their fundamental importance in basic cell physiology.

# 1.5 Heat shock genes in Saccharomyces cerevisiae

#### 1.5.1 HSP 104

Hsps of approximately 100 kDa are synthesized in most organisms exposed to a heat shock (Plesofsky-Vig and Brambl, 1993). In yeast, a HSP 104 gene has been isolated (Sanchez and Lindquist, 1990) and sequenced (Parsell et al., 1991). Increased HSP 104 gene expression is increased markedly during heat shock, entry into stationary phase, sporulation and growth on acetate (Sanchez et al., 1992). The HSP 104 gene encodes a 908-amino acid protein with a predicted relative mass (Mr) of 102 100 containing two putative nucleotide binding sites and exhibiting high sequence homology to the highly conserved ClpA/ClpB protein family whose members are involved in ATP-dependent Hsp 104 expression is not only required for proteolysis (Parsell et al., 1991). thermotolerance (Sanchez and Lindquist 1990) but is also important for tolerance to ethanol, arsenite, long term cold storage (Sanchez et al., 1992) and temperature-dependent barotolerance (Iwahashi et al., 1997). Hsp 104 mutant cells grow equally as well as wildtype cells at low temperatures, however, a dramatic decrease in viability is observed at a growth temperature of 50°C (Vogel et al., 1995). Examination of the phenotypes of combined hsp 104 and hsp 70 mutants suggested a functional relationship between hsp 104 and hsp 70. Hsp 70 expression partially compensates for hsp 104 in thermotolerance and hsp 104 expression compensates for hsp 70 during growth (Sanchez et al., 1993). Nevertheless, constitutive expression of hsp 104 does not regulate the expression of other heat shock genes (Vogel et al., 1995) nor affect trehalose accumulation (see Section 1.8 below) in exponentially growing cells (Winkler *et al.*, 1991). However, in stationary phase cells a synergy has been observed between hsp 104 and trehalose. Double mutants lacking hsp 104 and trehalose exhibit extreme heat shock sensitivity whereas a single mutation in either gene has little effect (Elliot *et al.*, 1996).

Intron splicing of yeast mRNA precursors is particularly heat sensitive. However, most HSP genes do not have intervening sequences (Yost *et al.*, 1990) and are therefore not affected by heat shock. One function of hsp 104, with the cooperation of hsp 70, is in the ATP-dependent repair of mRNA splicing subsequent to a heat shock (Vogel *et al.*, 1995). Another proposed function of hsp 104 is to disaggregate and resolubilize heat damaged proteins as ascertained from *in vitro* experimentation using heat-inactivated luciferase aggregates (Parsell *et al.*, 1994). More recently, hsp 104 has also been observed to influence the fidelity of protein synthesis by regulating the inheritance of the extrachromosomal genetic element, *PSI*+, a yeast prion (Lindquist *et al.*, 1995; Schirmer *et al.*, 1996).

Studies of a heat shock transcription factor mutant, *hsf1-m3*, which reputedly blocked the induction of hsps but did not inhibit induced thermotolerance disputed the role of heat shock proteins in thermotolerance Smith and Yaffe, 1991). This finding seemed at odds with previous findings that hsp 104 played an essential role in thermotolerance (Sanchez and Lindquist, 1990). This contradiction prompted further examination of hsp 104 expression in *hsf1-m3* (Lindquist and Kim, 1996). Results showed a higher constitutive expression of hsp 104 in this *S. cerevisiae* strain as compared to other strains, and despite blocking heat shock induction of hsp 26, hsp 104 was still induced by a heat shock. albeit at a slightly reduced level. Furthermore, regulation of hsp 104 expression using heterologous promoters revealed that high levels of hsp 104 without any conditioning pretreatment were sufficient for thermotolerance (Lindquist and Kim, 1996).

# 1.5.2 HSP 90 gene family

The HSP 90 gene family in *S. cerevisiae* has two members, which exhibit 97% similarity (Craig, 1992). One, HSC 82, is constitutively expressed at high levels and is moderately heat shock inducible with an increase of 1.5 to 2-fold over basal levels. The other HSP 82, is constitutively synthesized at a lower level and is more strongly heat shock inducible exhibiting a 20-fold increase (Lindquist and Craig, 1988; Borkovich *et al.*, 1989;

Craig, 1992). HSP 82 is also expressed on entry into stationary phase or transfer into sporulation medium (Kurtz and Lindquist, 1984; Borkovich *et al.*, 1989).

The HSP 90 gene products, hsp 82 ( $M_r$  81 419) and hsc 82 ( $M_r$  80 885) are highly conserved with 50% amino acid identity between eukaryotes and greater than 40% identitity to *E. coli* HTPG (Farrelly and Finkelstein, 1984; Lindquist and Craig, 1988; Borkovich *et al.*, 1989). They are synthesized abundantly in the cytosol of eukaryotes (Borkovich *et al.*, 1989). The function of the two proteins appears to be equivalent, as a mutation of either gene does not affect growth at 25°C. However, site-directed mutagenesis of both genes resulted in cessation of growth at any temperature, indicating that HSP90 constitutes an essential gene family (Borkovich *et al.*, 1989). On the other hand, HSP90 overexpression correlated with decreased thermotolerance and decreased growth at 37.5°C (Cheng *et al.*, 1992).

In vertebrates, hsp 90 has been found to bind to a variety of proteins including steroid hormone receptors (Picard *et al.*, 1990; Pratt *et al.*, 1992) and viral tyrosine kinases (Brugge, 1986). More recently, the function assigned to yeast hsp 90 is as a molecular chaperone, binding to and assisting target proteins to fold into their active configurations and preventing the aggregation of denatured proteins. Hsp 90 does not act alone but forms a supercomplex with other proteins inclucing hsp 70 (Ssa subfamily and Sti1p) and DnaJ proteins to direct protein folding and renature damaged proteins in the cytosol of eukaryotes (Wiech *et al.*, 1993; Chang and Lindquist, 1994; Bose *et al.*, 1996; Schumacher *et al.*, 1996).

### 1.5.3 HSP 70 gene family

The HSP 70 genes are members of multigene families in eukaryotes. The HSP 70 gene family of *S. cerevisiae* is the most thoroughly studied HSP 70 gene family and consists of at least ten members differing in sequence, cellular localization and in the conditions required for their synthesis.

The ten members of the gene family have been placed into functional groups based on structural and genetic analysis. The ten genes have been call stress seventy (SS) genes and have been divided into subgroups SSA, SSB, SSC, SSD and SSE (Craig, 1989; Mukai *et al.*, 1993). The nucleotide identities of the HSP 70 genes range from 50% to 97% (Craig *et al.*, 1990).

Hsp 70 proteins are highly conserved exhibiting approximately 50% amino acid identity among all species characterized (Parsell and Lindquist, 1994). All identified hsp 70 proteins contain two conserved domains, an ATP-binding domain (Flaherty et al., 1990: M<sup>c</sup>Kay, 1991; M<sup>c</sup>Kay et al., 1994) and a carboxy-terminal substrate domain (Hightower et al., 1994). They have been found to bind to diverse substrates, and actively assist protein folding, unfolding, assembly and disassembly (Gething and Sambrook, 1992). Furthermore, hsp 70 proteins in yeast have been found to down-regulate the expression of other hsps (Nelson et al., 1992a; Craig et al., 1994). Previous investigations have elucidated that hsp 70s do not function in isolation, other hsps are involved. In E. coli, the hsp 70 homolog DnaK carries out its function with two other hsps, DnaJ and GrpE (Georgopoulos et al., 1994). Eukaryotes also have numerous DnaJ homologues including the gene products of SIS1 (Luke et al., 1991) and YDJ1 (MAS5) genes which are both cytosolic (Caplan and Douglas, 1991; Atencio and Yaffe, 1992). YDJ1 expression is essential for growth at high temperature, moderately heat shock inducible and the protein product functions in protein import into mitochondria (Atencio and Yaffe. 1992). The Ydj1p is associated with ssa proteins and Sis1p interacts with ssb1p (Atencio and Yaffe. 1992; Caplan et al., 1992). A mitochondrial yeast DnaJ homologue, SCJ1, has also been identified with overexpression studies suggesting a protein translocation function together with hsp 70 into mitochondria, as well as an involvement in protein secretion via the endoplasmic reticulum (ER) (Blumberg and Silver, 1991). The gene product of SEC63. another DnaJ-homologue, is required for translocation of proteins into the ER (Sadler et SEC63 gene disruption mutants exhibited growth inhibition at high al., 1989). temperatures and accumulated secretory precursors into the cytoplasm (Rothblatt et al., 1989).

### 1.5.3.1 Subgroup SSA

Subgroup SSA is the most complex and contains four genes, SSA1 to SSA4 whose cytoplasmic products are needed at relat vely high levels for survival (Deshaies *et al.*, 1988; Craig, 1989: Craig *et al.*, 1990). The four SSA genes share 84 to 99% amino acid identity but differ in their regulation (Boorstein *et al.*, 1994; Craig *et al.*, 1994). Both SSA1 and SSA2 are expressed constitutively in unstressed cells with increased expression of only SSA1 upon heat shock (Craig, 1989; Craig *et al.*, 1990). The constitutive expression of SSA2 accounts for 75% of total SSA expression (Sanchez *et al.*, 1993). On

the other hand, SSA3 and SSA4 expression is very low under normal conditions but expression is markedly increased upon heat shock (Craig, 1989: Craig *et al.*, 1990). Mutations of the SSA1 and SSA2 genes confer temperature sensitive growth, with a decreased growth rate at temperatures form 23 to 35°C and an inability to form colonies at 37°C (Craig and Jacobsen, 1984).

*In vitro* and *in vivo* studies of the gene products of the SSA subgroup suggest that they function in translocation of proteins across membranes (Craig *et al.*, 1990). Their role appears to involve refolding of proteins in cooperation with other heat shock proteins such as ssd1p (kar2p) in posttranslational translocation of proteins from the cytosol across the ER into the lumen of the ER (Deshaies *et al.*, 1988; Sanders *et al.*, 1992; Bush and Meyer, 1996). However, despite their refolding function, ssa1p and ssa2p do not appear to function in the folding of nascent polypeptides (Bush and Meyer, 1996). Ssa proteins have also been shown to self-regulate the heat shock response by negatively regulating heat shock gene expression (Stone and Craig, 1990; Craig *et al.*, 1994). This class of hsp 70 has also been associated with ubiquitin-dependent protein degradation. It appears that ssa proteins by virtue of their protein-binding ability temporarily protect aberrant proteins from proteins for proteins for more proteins.

### 1.5.3.2 Subgroup SSB

Subgroup SSB consists of two genes SSB1 and SSB2. Both members of this subgroup are expressed at moderately high levels during exponential growth and the gene products, ssb1 and ssb2, are 99.3% identical (Craig, 1989; Craig *et al.*, 1994). In contrast to the SSA genes, the expression of SSB1 and SSB2 is turned off when cells are heat shocked at 37°C (Craig, 1989). Double mutation studies suggest that ssb1 and ssb2 are required for optimal growth at low temperatures (Craig, 1989; Craig *et al.*, 1994). The SSB protein of *S. cerevisiae* has been demonstrated to be a nucleolar-specific protein that is associated with the snR10 and snR11 small nuclear RNAs (Clark *et al.*, 1990). Nelson *et al.* (1992b) proposed that SSB gene products, because of their close association with translating ribosomes, were likely to be involved in protein synthesis, facilitating passage of nascent chains through ribosomes.

### 1.5.3.3 Subgroup SSC

Subgroup SSC contains only one gene, SSC1, an essential gene whose product is located in the matrix of the mitochondria (Craig *et al.*, 1987; Craig, 1989). The gene is moderately expressed under normal physiological conditions and is markedly increased upon heat shock (Craig *et al.*, 1990). The ssc1 protein is distantly related to other hsp 70s with amino acid identities ranging from 54.5% to 49.6% (Boorstein *et al.*, 1994). Ssc1p appears to aid in the transport of precursor proteins into mitochondria and facilitate protein folding and unfolding as inactivation blocks mitochondrial import (Craig *et al.*, 1990; Kang *et al.*, 1990). More recently. Nwaka *et al.* (1996) examined a *ssc1-3* temperature sensitive mutant containing a mutation in the ATPase binding domain which identified a recovery function for ssc1p in binding heat shock denatured proteins.

#### 1.5.3.4 Subgroup SSD

Subgroup SSD consists of another essential gene, SSD1 (also known as KAR2). KAR2 was identified in a yeast mutant blocked in nuclear fusion after mating of haploid cells to form diploids (Polain and Conde. 1982; Normington *et al.*, 1989; Rose *et al.*, 1989). It is expressed at moderate levels during exponential growth and an increase in expression is observed after a heat shock (Craig, 1989). The gene product ssd1p is situated in the lumen of the ER and functions in the translocation of proteins into this organelle (Craig *et al.*, 1990; Vogel *et al.*, 1990). The mammalian homologue of ssd1p is BiP (immunoglobulin heavy chain binding protein) which is very similar if not identical to grp78, a glucose regulated protein (Vogel *et al.*, 1990). Although the function of BiP is still ambiguous, it appears to inhibit the transport of either malfolded or damaged glycosylated secretory proteins from the ER to the golgi body (Deshaies *et al.*, 1988; Pelham, 1989).

#### 1.5.3.5 Subgroup SSE

Previous investigations identified a highly conserved calmodulin-binding domain in many hsp 70 proteins suggesting that calmodulin-binding may be important for hsp 70 function (Stevenson and Calderwood, 1990). This finding led to the discovery of an additional, essential HSP 70 gene family in *S. cerevisiae*, the SSE subfamily. The two members, SSE1 and SSE2, were isolated on the basis of their protein product  $Ca^{2^{-}}$ -

dependent calmodulin-binding properties (Mukai *et al.*, 1993). SSE1 is moderately expressed at 23°C and increases slightly upon a 37°C heat shock. In contrast, SSE2 is constitutively expressed at low levels and exhibits a marked increased in expression with a heat shock. Sse1p ( $M_r$  77 408) and sse2p ( $M_r$  77 619) have 76% amino acid identity to each other, with only a 13 to 28 % amino acid identity to other hsp 70 family members. Mutations of SSE1 resulted in slow growth at any temperature while disruption of SSE2 had no observable phenotypic effect. Phenotypically, a *sse1sse2* double mutant resembled that of the *sse1* disruption mutant (Mukai *et al.*, 1993). The function of either SSE gene product has not been characterized.

### 1.5.4 HSP 60

An antibody directed against hsp 58 of Tetrahymena thermophila enabled identification of a S. cerevisiae hsp 60 (M<sup>c</sup>Mullen and Hallberg, 1988). Reading et al. (1989) isolated a HSP 60 gene (MIF gene) encoding a 572 amino acid polypeptide (Mr 60 830). The expression of HSP 60 mRNA is induced 2-3 fold above basal levels when cells are heat shocked (Reading et al., 1989). Nucleotide sequence analysis revealed that the yeast HSP 60 gene product, hsp 60. is structurally related to the groEL protein of E. coli and the RUBISCO-binding protein (RBP) of chloroplasts (Ostermann et al., 1989). These two proteins and hsp 60 are members of the "chaperonin" class of protein factors (Cheng et al., 1989). Hsp 60 is a mitochondrial heat shock protein, encoded in the nucleus, which is essential for assembly of oligomeric protein complexes imported into the mitochondrial matrix (Cheng *et al.*, 1989). It is has been proposed that ssc1p (mitochondrial hsp 70) and hsp 60 function sequentially during protein translocation and folding (Neupert et al., 1990). The close association of both hsp 60 and hsp 70 with various proteins at high temperatures most probably prevents protein aggregation during the heat stress and enables recovery via ATP-dependent protein refolding (Martin et al., 1992: Parsell and Lindquist. 1994; Frydman and Hartl, 1994). Hsp 60 (chaperonin 60 or cpn 60. Hemmingsen et al., 1988) forms large oligomeric complexes arranged as two stacked heptameric rings (Hendrix, 1979; Hohn et al., 1979; Frydman and Hartl, 1994). In close association with hsp 60s are the highly conserved hsp 10s (chaperonin 10 or cpn 10) which also form a single heptameric ring that binds hsp 60 (Lubben et al., 1990; Hartman et al., 1992; Frydman and Hartl, 1994). This complex structure in conjunction with ATP enables the folding and release of nascent polypeptides and denatured proteins (Parsell and Lindquist, 1994).

A cytosolic hsp 60 homologous to mouse Tcp-1 (tailless complex polypeptide) has also been identified in yeast (Ursic and Culbertson, 1991). A slight sequence similarity exists between cpn 60 and TCP-1 (Ellis, 1990; Gupta, 1990). To differentiate between the hsp 60s it has been suggested that these chaperonins be divided into two groups, with the groE subfamily found in eubacteria, plastids and mitochondria and the TCP-1 family found in archaea and the eukaryotic cytosol (Ellis, 1992; Ellis, 1996). In eukaryotes, TCP-1 is not heat shock inducible. In contrast, heat shock strongly induces the expression of GroE in eubacteria and mitochondria as well as TCP-1 in archaea. (Ellis, 1996).

### 1.5.5 HSP 30

Hsp 30 was originally isolated from cell membrane fractions and is the only membrane-localized hsp identified to date (Panaretou and Piper, 1992). HSP 30 was independently identified and sequenced by Régnacq and Boucherie (1993). HSP 30 expression is induced by entry into stationary phase, nutrient limitation and 6% ethanol (Régnacq and Boucherie, 1993; Piper *et al*, 1994). It encodes a 332-amino acid protein with a  $M_r$  of 37 044. Total cellular concentration of hsp 30 is significantly less than soluble hsps such as hsp 104, 90, 70 or hsp 26 (Piper *et al.*, 1994). Hsp 30 has a putative glycosylation site, three protein kinase C phosphorylation sites and a cAMP-dependent protein kinase site and is highly hydrophobic (Régnacq and Boucherie, 1993). The proposed function for hsp 30 is in reducing plasma membrane damage during and subsequent to a heat shock by either contro ling membrane structure or interacting directly with essential membrane proteins such as ATPase (Panaretou and Piper, 1992).

# 1.5.6 Small HSPs

Small heat shock proteins are synthesized in most organisms studied in response to heat shock and normal developmental cues. They are a relatively diverse group conserved more in structural properties than amino acid sequence and they show homology to mammalian  $\alpha$ -crystallin (Ingolia and Craig, 1982; Plesofsky-Vig *et al.*, 1992). The proteins have similar hydropathy profiles and small regions of amino acid identity (Rossi and Lindquist, 1989). Small hsps also assemble into large aggregates, with a molecular mass between 200 and 800 kDa, following heat shock and upon entry into stationary phase (Tuite *et al.*, 1990: Bentley *et al.*, 1992).

### 1.5.6.1 HSP 26

The HSP 26 gene of *S. cerevisiae* is transcribed upon entry into stationary phase, during sporulation and following heat shock (Kurtz et al., 1986). In contrast to other heat shock genes, transcription of HSP 26 appears to be regulated by a mechanism of basal repression during growth at normal temperatures and derepression during heat shock (Susek and Lindquist, 1990). Almost all other heat shock genes studied to date are regulated by transcriptional activation. HSP 26 encodes a 213-amino acid protein (Mr 27 000) with the N-terminal methionine cleaved posttranslationally (Bossier et al., 1989). Studies by Rossi and Lindquist (1989) suggest that the intracellular localization of hsp 26 in S. cerevisiae is dependent upon the physiological state of the cell and not just simply upon the presence or absence of heat stress. In heat shocked exponentially growing cells hsp 26 is localized in the nucleus of the cell whereas, in stationary phase hsp 26 is no longer located in the nucleus (Rossi and Linquist, 1989). Furthermore, it was demonstrated that hsp 26 has no obvious regulatory role, no effect on thermotolerance, ethanol tolerance, spore formation, germination, or aerobic and anerobic growth at any temperature on various carbon sources (Kurtz et al., 1986; Petko and Lindquist, 1986; Susek and Lindquist. 1989; Tuite et al., 1990). More recently, it has been shown that hsp 26 is also induced by an osmostress (Vare a et al., 1992).

## 1.5.6.2 HSP 12

Praekelt and Meacock (1990) isolated and characterized a second small heat shock gene. HSP 12 from *S. cerevisiae*. HSP 12 was expressed in stationary phase cells and following a heat shock but was not induced in logarithmic phase cells. Its expression appeared to be regulated by cAMP-mediated protein phosphorylation and heat shock (Praekelt and Meacock, 1990). HSP 12 encodes a protein with a relative mass of 14 kDa, which has structural similarity but limited sequence similarity to other small heat shock genes. Disruption of HSP 12 did not effect growth at various temperatures nor the ability to acquire thermotolerance (Praekelt and Meacock, 1990). Hsp 12 is also induced by oxidative stress (Jamieson *et al.*, 1994), ethanol (Piper *et al.*, 1994) and osmostress via the

high-osmolarity glycerol pathway (HOG; Varela *et al.*, 1992; Varela *et al.*, 1995). The functions of hsp 12 and hsp 26 have as yet not been elucidated.

#### 1.5.7 Other S. cerevisiae heat shock proteins

Approximately 80 out of 500 S. cerevisiae proteins have been shown to be heat shock inducible (Miller et al., 1982) of which many have not been characterized. However, a few other heat shock proteins have been identified and characterized to some extent including hsp 150, a secretory glycoprotein that is induced by both heat and nitrogen limitation (Russo et al., 1992a,b; Russo et al., 1993). Hsp 118, an acidic glycoprotein is induced upon sulfur starvation, appears to be cAMP regulated and exhibits an 11-fold increase in expression after a heat shock (Verma et al., 1988a,b). STI1 encodes a 589amino acid protein with a Mr 66 246, that when overexpressed activates the expression of SSA4 (Nicolet and Craig, 1989). Stilp is also induced in response to canavanine and entry into stationary phase but not ethanol shock. A stilp disruption mutant grows normally at 30°C but exhibits impaired growth at higher and lower temperatures (Nicolet and Craig, 1989). Stilp is related to MAP kinase kinase kinases and is part of the HSP 90 (see section 1.5.2) supercomplex (Chang and Lindquist, 1994; Chang et al., 1997). Tip1 is a temperature shock inducible protein that is induced by both a heat and cold shock (Kondo and Inouye. 1991) and it has recently been identified as a cell wall mannoprotein possibly functioning in stress protection of the cell membrane (Kowalski et al., 1995; van der Vaart et al., 1995). Three glycolytic enzymes, enolase (hsp 48). phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (hsp 35) have also been observed to increase in synthesis when exposed to a mild heat shock (Iida and Yahara, 1985; M<sup>c</sup>Alister and Holland, 1985a,b; Piper et al., 1986; Lindquist and Craig, 1988; Boucherie et al., 1995). Enolase is also induced upon entry into stationary phase and when starved for sulfur (lida and Yahara, 1985). Two DNA damage proteins, DDR2 and DDR48, are induced by heat, DNA damaging agents and UV irradiation (M<sup>c</sup>Clanahan and M<sup>c</sup>Entee, 1986). The *de novo* synthesis and activity of TPS1 and TPS2, encoding trehalose-6-phosphate synthase (56 kDa) and trehalose-6-phosphate phosphatase (103 kDa) respectively, increases with a heat shock (Hottiger et al., 1987; Neves and François, 1992). Two antioxidant enzymes. cytosolic catalase T and MnSOD also show heat shock inducibility (Belazzi et al., 1991; Costa et al., 1993). The CTT1 gene encoding cytosolic catalase T is also induced in response to oxidative and osmotic stress and is negatively regulated by cAMP (Belazzi et *al.*, 1991; Wieser *et al.*, 1991; Miralles *et al.*, 1995). MnSOD is the yeast mitochondrial manganese form of superoxide dismutase (SOD) whose synthesis is also increased upon exposure to ethanol and presumably functions in trapping superoxide radicals within the mitochondria, limiting release into the cytosol (Costa *et al.*, 1993; Piper, 1993).

Ubiquitin, a highly conserved 70-amino acid protein is also induced by heat (Watson, 1990). Covalent binding of ubiquitin to various acceptor proteins helps promote regulation of many cellular processes that are related to a stress response. These include selective protein degradation, DNA repair and response of cells to heat, starvation and amino acid analogues (Finley *et al.*, 1987). Ubiquitin is also required for sporulation and maintenance of spores (Finley *et al.*, 1987, Tanaka *et al.*, 1988). Overexpression of ubiquitin elicited increased tolerance to os nostress, ethanol and canavanine (an amino acid analogue), did not effect thermotolerance and decreased tolerance to cadmium, arsenite and paromycin (accumulates aberrant proteins) (Chen and Piper, 1995). The four yeast ubiquitin genes. UBI1, UBI2, UBI3 and UBI4, have been extensively studied and are expressed in exponential cells (Ozkaynak *et al.*, 1987). An important function for ubiquitin (see also section 1.10), in the stress response of eukaryotic organisms, is in the removal of abnormal or denatured proteins (Parag *et al.*, 1987; Grant *et al.*, 1989).

# **1.6 Functions of heat shock proteins**

The functions of hsps have been mainly elucidated from studies on constitutively expressed proteins rather than proteins that are induced following heat shock. Three major function for hsps have been proposed molecular chaperones, thermotolerance and immunity.

### 1.6.1 Molecular chaperones

The term molecular chaperone was first used to describe the role of the nuclear protein nucleoplasmin in assisting the *in vitro* assembly of nucleosomes from isolated histones and DNA (Laskey *et al.*, 1978). Ribulose bisphosphate carboxylase-oxygenase (rubisco) binding protein was also described as a molecular chaperone because of its requirement for assembly of rubisco in chloroplasts from higher plants (Barraclough and Ellis, 1980: Musgrove *et al.*, 1987). In 1986, Pelham suggested that hsp 70 and 90 families

were an essential requirement for protein assembly and disassembly in the cytosol, nucleus and ER under nonstressful conditions. He also proposed that during stress, an increase in hsp synthesis was required for dissolution of protein aggregates and in protein refolding as well as preventing any further aggregation by binding to exposed hydrophobic surfaces. Subsequently, the term "chaperonin" was coined for GroEL of *E. coli* and the related mitochondrial hsp 60 of eukaryotes due to their high sequence conservation to rubisco binding protein (Hemmingsen *et al.*, 1988). A molecular chaperone is currently defined as "a protein that binds to and stabilizes an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein facilitates its correct fate *in vivo*, be it folding, oligomeric assembly, transport to another subcellular compartment, or controlled switching between active/inactive conformations" (Hendrick and Hartl, 1993).

As mentioned in section 1.5, many of the major heat shock protein families, in particular hsp 60, 70, 90 and 104, function as molecular chaperones in different cellular locations. For further discussion, the interested reader is referred to recent reviews by Craig *et al.* (1993), Becker and Craig (1994), Hartl (1995), Ellis (1996), Hartl (1996), Hayes and Dice (1996) and Langer *et al.* (1996).

### 1.6.2 Thermotolerance

A possible function of hsps is ir the acquisition of thermotolerance, that is, a transient resistance to a normally lethal temperature induced upon exposure to a prior heat shock. However, it should be pointed out that unequivocal evidence has not resolved the relationship between hsps and induced thermotolerance. Although positive correlations exist between the amount of heat shock proteins present and the degree of tolerance, there are notable exceptions. For instance, in *S. cerevisiae*, cycloheximide treated cells still acquired thermotolerance despite inhibition of protein synthesis (Hall, 1983). Furthermore, cytoplasmic and mitochoncrial protein synthesis inhibition did not affect either induced thermotolerance or ethanol tolerance (Watson *et al.*, 1984). Moreover, disruption mutants of all hsps, with the notable exception of hsp 104, still acquired thermotolerance (Sanchez and Lindquist, 1990). The problem that exists when examining the effect of inhibition of *de novo* protein synthesis on thermotolerance acquisition. is the resultant prevention of abnormal protein accumulation. The presence of aberrant proteins has been previously proposed to be a trigger of the heat shock response (Craig and Gross, 1991; Piper, 1993). It appears, therefore, that some hsps are necessary but may not be

sufficient for thermotolerance and that other factors, such as trehalose (see section 1.8), may contribute to the thermotolerant state. Heat shock proteins may aid the acquisition of thermotolerance by way of their normal cellular roles, they may help dissociate and remove denatured proteins and protein ccmplexes and they may facilitate folding and assembly of some proteins under stressful conditions. De Virgilio *et al.* (1991) proposed that the role of hsp 104 and molecular chaperones was in the repair of damaged structures following heat stress whilst trehalose was involved in protection of membranes and proteins during stress.

#### 1.6.3 Immune system

Stress proteins have been identified as immune targets in most major human parasite infections and antibodies to hsp 60, hsp 70 or hsp 90 have been detected in the sera of patients suffering from malaria, trypanosomiasis, leishmaniasis, tuberculosis, schistosomiasis and filariasis (Cohen and Young, 1991). Furthermore, antigens from a large number of infectious bacterial agents (including Mycobacterium, Shigella and Salmonella) and pathogenic fungi (including Aspergillus fumigatus, Fonsecaea pedrosoi and Histoplasma capsulatum) have been identified as members of stress protein families (Maresca and Kobayashi, 1996). The major immunodominant stress antigen target of both antibody and T cell responses is hsp 60 (Shinnick et al., 1988; Young et al., 1988; Kaufmann, 1992). Immunological reactions to hsp 60 and hsp 70 have been implicated in neurogenerative diseases, cancer and inflammatory processes associated with pathogens (Garbe, 1992; Maresca and Kobayashi, 1996). The highly conserved nature of stress proteins has further raised the question of an anti-self stress protein immune response leading to the risk of autoimmune disease such as rheumatoid arthritis and multiple sclerosis (Georgopoulos and M<sup>c</sup>Farland, 1993). Despite adjuvant arthritis in rats being associated with a mycobacterial hsp (van Eden et al., 1988) no direct involvement of hsps in human rheumatoid arthritis have been shown (Mollenhauer and Schulmeister, 1992; Yang and Feige, 1992; Gaston and Pearce, 1996). It has been suggested that the protein folding and translocation functions associated with stress proteins could assist in several steps of antigen processing. These functions could aid antigen processing by recognising abnormal or partially unfolded proteins, by targeting antigens to intracellular organelles, in the stimulation of membrane translocation and by enhancement of protein unfolding and degradation causing the release of peptides suitable for binding to major histocompatibility

complex molecules and subsequent T cell recognition. Hsp deletions could be useful for attenuating pathogens to be used as vaccines. However, the sequence similarity between host and pathogen hsps proves problematic for vaccine development (Schoel and Kaufmann, 1996). The role of stress proteins in the immune response has been reviewed by Young (1990). Kaufmann (1991), Feige and Mollenhauer (1992), and van Eden and Young (1996).

# 1.7 Heat shock gene regulation

### 1.7.1 Heat shock element (HSE)

All heat shock genes have a highly conserved regulatory sequence termed the heat shock regulatory element or HSE. HSEs are located upstream of the TATA box in eukaryotic heat shock genes and have a consensus sequence C-GAA-TTC-G (Bienz and Pelham, 1987). The HSE is often found in multiple copies in the promoter regions of heat shock genes (Bienz and Pelham, 1987) and they function as both promoters and enhancers. HSEs are binding sites of a specific transcription factor, heat shock transcription factor or HSF. The regulation of heat shock genes is different in animal and yeast cells (Sorger and Nelson, 1989). In mammalian and Drosophila cells, the HSF is phosphorylated upon heat shock genes (Sorger and Pelham, 1988). However, in the yeast *S. cerevisiae* the HSF (833-amino acid protein) is bound as a trimer to DNA before and after heat shock and transcriptional activation appears to involve the modification of DNA-bound factors by phosphorylation (Sorger *et al.*, 1987; Sorger and Pelham, 1988; Perisic *et al.*, 1989).

#### 1.7.2 Stress response element (STRE)

In *S. cerevisiae*, an alternative HSF-independent heat stress control element, the stress response element (STRE), was identified in the promoter region of cytoplasmic catalase T (encoded by CTT1) and DNA responsive genes DDRA2 and DDR2 (Kobayashi and M<sup>c</sup>Entee, 1990; Wieser *et al.*, 1991; Kobayashi and M<sup>c</sup>Entee, 1993; Marchler *et al.*, 1993). STREs contain an AGGGG or CCCCT consensus sequence (Koybayashi and M<sup>c</sup>Entee, 1990; Wieser *et al.*, 1991) and bind a 140 kDa polypeptide (Koyabashi and M<sup>c</sup>Entee, 1993). STREs are activated by a variety of stresses including N<sub>2</sub> starvation,

osmotic stress, oxidative stress (Marchler *et al.*, 1993), low external pH, weak organic acids and ethanol (Schüller *et al.*, 1994). Subsequent to identification of STREs, various STRE-like elements have been identified in promoter regions of heat shock genes including TPS2 (Gounalaki and Thireos, 1994), SSA3 (Boorstein and Craig, 1990). HSP 12, HSP 104 (Ruis and Schüller, 1995) and UBI4 (Kobayashi and M<sup>c</sup>Entee, 1993; Schüller *et al.*, 1994). The HOG pathway (Schüller *et al.*, 1994) and RAS-protein kinase A pathway (Thevelein, 1991; Durnez *et al.*, 1994) have both been implicated in the regulation of STRE activity.

For more detailed discussion on stress-induced transcriptional activation in *S. cerevisiae* refer to reviews by Mager and de Kruijff (1995) and Ruis and Schüller (1995).

### 1.8 Trehalose

The non-reducing disaccharide of glucose, trehalose ( $\alpha$ -D-glucopyranosyl (1-1)- $\alpha$ -D-glucopyranoside), has also been proposed to be a stress protectant. In yeast, trehalose is synthesized in a two step reaction (Cabib and Leloir, 1958) involving an enzyme complex consisting of three subunits encoded by TPS1, TPS2 and TSL1. In the first step, trehalose-6-phosphate synthase catalyses the synthesis of trehalose-6-phosphate (t6p) by transferring the glucosyl residue from UDP-glucose to glucose-6-phosphate. In the second step, t6p phosphatase cleaves phosphate from t6p producing trehalose. The TPS1 (or CIF1) gene encodes the 56 kDa subunit corresponding to t6p synthase (Bell *et al.*, 1992) while TPS2 encodes the 100 kDa subunit that is t6p phosphatase (De Virgilio *et al.*, 1993). The 123-kDa subunit is encoded by TSL1 and has been proposed to have a regulatory function as well as some t6p phosphatase activity (Vuorio *et al.*, 1993). As mentioned previously (section 1.5.7), TPS1 and TPS2 mRNAs are heat shock inducible (Bell *et al.*, 1993).

Originally, trehalose was thought to function as a storage carbohydrate (Thevelein, 1984). However, trehalose accumulation has now been linked to exposure to heat shock and noxious chemicals, growth on respiratory carbon sources and nutrient starvation (Lillie and Pringle, 1980: Attfield, 1987; Hottiger *et al.*, 1987; Hottiger *et al.*, 1989; Attfield *et al.*, 1992). The fact that trehalose concentration parallels induction of thermotolerance led to the proposal of a stress protection function (Attfield, 1987; Van Laere, 1989: Wiemken,

1990; Hottiger et al., 1992; De Virgilio et al., 1994). Trehalose increase can be accounted for by an increase in t6p synthase (Neves and François, 1992) and substrates, UDP-glucose and glucose-6-phosphate (Winkler et al., 1991). During respiratory growth, trehalose has been demonstrated as a significant intrinsic stress protectant (Attfield *et al.*, 1994; De Virgilio et al., 1994; van Dijck et al., 1995). However, the role of trehalose in thermotolerance of exponentially growing veast is equivocal. Many researchers favour trehalose playing a role in thermotolerance while other researchers have shown that trehalose accumulation alone is insufficient to explain acquired thermotolerance (Winkler et al., 1991; Argüelles et al., 1994; Nwaka et al., 1994; van Dijck et al., 1995). For example, Winkler et al. (1991) showed that a hsp 104 mutant despite having high heat shock induced accumulation of trehalose was unable to acquire thermotolerance. However, in vitro, trehalose has been shown to protect proteins and lipids against damage by freeze-thaw, high temperature shift and desiccation (Hottiger *et al.*, 1994). Trehalose has also been shown to stabilize isolated proteins, like phosphofructokinase, lactate dehydrogenase, and the restriction enzyme Pst I (Carpenter et al., 1987; Carpenter, 1993; Sen et al., 1993). Moreover, in stationary phase cells a synergistic role between trehalose and hsp 104 has been proposed (Elliot et al., 1996). Mutants lacking both hsp 104 and trehalose exhibited extreme heat shock sensitivity even at moderate temperatures where neither hsp 104 mutants nor trehalose mutants were affected. Recently, a role for trehalose in barotolerance has also been suggested (Fernandes et al., 1997) with trehalose accumulation correlating with increased tolerance. Furthermore, t6p synthase mutants unable to produce trehalose exhibited greater sensitivity to hydrostatic pressure. Trehalose may also play a role in the regulation of glycolysis and sugar-phosphate/free phosphate balance (Panek and Matoon. 1977; Thevelein and Hohmann, 1995). Recently, Van Aelst et al. (1993) suggested a role for trehalose as a glucose sensor, as growth on glucose was inhibited in a TPS1 mutant unable to synthesize trehalose.

The disaccharide trehalose is hydrolysed by the enzyme, trehalase. In yeast three trehalases have been identified. One, cytosolic neutral trehalase encoded by the NTH1 gene, responsible for intracellular hydrolysis of trehalose, is regulated by nutrient supply, cAMP-dependent phosphorylation and temperature (Londesborough and Varimo, 1984; App and Holzer, 1989; Kopp *et al.*, 1993). The second, vacuolar acid trehalase encoded by the ATH1 gene, responsible for hydrolys s of extracellular trehalose, is also regulated by nutrient supply (Mittenbühler and Holzer, 1988: Destruelle *et al.*, 1994; Destruelle *et al.*,

1995). A putative trehalase nth1p encoded by the NTH2 gene (homologue of NTH1) is regulated by both temperature and nutrients (Wolfe and Lohan, 1994). The function of nth2p in trehalose breakdown is at present unknown. NTH1 and NTH2 genes are also heat shock inducible and are essential for recovery of cells following a 50°C heat stress. Hydrolysis of trehalose is also important for spore germination and resumption of growth on ethanol and other nonfermentable carbon sources (Souza and Panek, 1968; Thevelein, 1984). For further discussion on trehalose and its role in thermotolerance refer to recent reviews by De Virgilio *et al.* (1994), Hottiger *et al.* (1994), Van Dijck *et al.* (1995), and Nwaka and Holzer, (1998).

# 1.9 Other factors involved in the heat shock response

Numerous other factors besides hsps and trehalose have been implicated in the heat shock response of yeast (reviewed by Piper, 1993). A heat shock results in an increase in intracellular glucose and inhibition of glycolysis (Neves and François, 1992): increased membrane permeability with a resultant decrease in intracellular pH (pH<sub>i</sub>) and increased proton influx (Eraso and Gancedo, 1987; Weitzel *et al.*, 1987); and transient arrest in G1 phase of the cell cycle (Plesset *et al.*, 1987; Barnes *et al.*, 1990). A decrease in pH<sub>i</sub> and increase in intracellular ions stimulates cAMP-dependent protein kinase A (Thevelein, 1992) and plasma membrane ATPase activity. The increased plasma membrane ATPase activity counteracts proton influx (Eraso and Gancedo, 1987; Weitzel *et al.*, 1987); weitzel *et al.*, 1987), influences thermotolerance and is essential for heat shock induced *de novo* synthesis of hsps (Panaretou and Piper, 1990; Coote *et al.*, 1994). Low external pH resulting in a decrease in pHi (Cole and Keenan, 1986) leads to inhibition of glycolysis. decreased levels of fructose-2,6-bisphosphate and increases in cAMP and trehalase (François *et al.*, 1986). The intracellular levels of cAMP have also been implicated in thermotolerance (Cameron *et al.*, 1988; Piper, 1990).

### **1.10** Stress induced proteolysis

Stress induced proteolysis is an integral part of the stress response system. During stress, molecular chaperones restrict incorrect interactions between abnormal proteins and aid in the refolding of proteins (Rothman, 1989; Ellis and van der Vies, 1991). Damaged

proteins that can not be rescued by molecular chaperones as well as proteins that are deleterious during stress or no longer required need to be eliminated.

Yeast has two proteolytic systems. a vacuolar (lysosomal) system containing nonspecific peptidases and a non-lysosomal system utilizing highly specific peptidases located throughout the cell (Hilt and Wolf, 1992), such as proteinase yscE (Achstetter *et al.*, 1984, Heinemeyer *et al.*, 1991). Proteolysis is a selective process achieved by selective uptake into vacuoles for lysosomal proteolysis and degradation of selected proteins either by direct recognition of signals within the protein or by an intermediatory system such as the ubiquitin system that tags specific proteins for proteolysis (Hilt and Wolf, 1992). In yeast, 86% of protein degradation is carried out by the vacuolar proteinases, yscA and yscB (Teichert *et al.*, 1989). The yeast proteinase yscE has three distinct proteolytic activities, chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolysing (Heinemeyer *et al.*, 1991; Hilt and Wolf, 1992).

Two postulated degradation signals within proteins for non-lysosomal degradation have been postulated. Either, PEST (Pro. Glu, Ser, Thr) sequences are contained within the selected protein (Rechsteiner *et al.*, 1987) or the N-end rule, where the N-terminal amino acid regulates protein half-life, signals a protein for proteolysis (Bachmair *et al.*, 1986). Multiubiquitination also marks proteins for non-lysosomal proteolysis via proteinase yscE (Chau *et al.*, 1989; Heinemeyer *et al.*, 1991; Hilt *et al.*, 1992; Richter-Ruoff *et al.*, 1992). Ubiquitin is covalently attached to a lysine residue of the selected protein by an ATP-dependent process involving ubiquitin-activating enzyme, E1; ubiquitin conjugating enzyme family, E2; and in some cases an additional ubiquitin-protein ligase. E3 to aid conjugation (Reviewed by Hershko, 1991; Jentsch, 1992; Hilt and Wolf, 1992).

# **1.11** Oxidative stress response

Yeast are facultative anaerobes and prefer to grow in the presence of oxygen, as ATP generation is higher during aerobic oxidation of glucose. There are no known obligate anaerobic yeast, however yeast can grow in the complete absence of oxygen if supplemented with unsaturated fatty acids, sterol and nicotinic acid (Rose, 1987). As a consequence yeast must adapt to harmful natural by-products of oxygen metabolism, the so-called reactive oxygen species (ROS). A certain concentration of ROS is a physiological consequence of respiration and is primarily counteracted by enzymatic and

non-enzymatic antioxidant defence mechanisms. A secondary defence mechanism involves the repair or removal of damaged macromolecules. (Kullik and Storz, 1994; Pahl and Bauerle, 1994; Moradas-Ferreira *et al.*, 1996). However, excess production of ROS, constituting an oxidative stress, is harmful to the cell as ROS damage DNA, proteins and lipids (Moody and Hassan, 1982). Yeast display an adaptive oxidative stress response, similar to the heat shock response, if treated with a low concentration of the respective oxidant prior to a subsequent challenge with a higher concentration of the oxidant (Collinson and Dawes, 1992; Jamieson, 1992; Steels *et al.*, 1994). In *S. cerevisiae*, peroxide stress tolerance has also been correlated with peroxide shock inducible proteins (psps) including hsp 12, hsp 60 and hsp 70 (Jamieson, 1994).

#### 1.11.1 Non-enzymatic antioxidant defences

The antioxidant defence molecule glutathione (tripeptide  $\gamma$ -L-glutamyl-Lcystinylglycine) is one of the most important non-enzymatic defence mechanism against oxidative stress in yeast (Meister and Anderson, 1983; Grant et al., 1996; Stephen and Jamieson, 1996). Glutathione (GSH) is synthesized in two ATP-dependent steps: firstly,  $\gamma$ glutamylcysteine synthetase (GSH1) catalyses the formation of  $\gamma$ -glutamylcysteine from glutamic acid and cysteine and secondly, glutathione synthase catalyses the ligation of glycine to γ-glutamylcysteine to form glutathione (Meister, 1988; Grant et al., 1996). A concomitant increase in GSH1 gene expression has been observed when yeast cells are exposed to oxidants, particularly menadione (Stephen et al., 1995). The constitutive expression of GSH1 mRNA is partially dependent on the transcription factor Yap1 (see below) (Wu and Moye-Rowlely, 1994; Stephen et al., 1995). GSH1 mutants unable to synthesize glutathione exhibit extreme sensitivity to H<sub>2</sub>O<sub>2</sub>, superoxide radical generators (menadione and plumbagin) and tert-butyl hydroperoxide (t-BOOH) in both exponential and stationary phase (Grant et al., 1996; Stephen and Jamieson, 1996). These mutants however, were still able to induce tolerance to oxidants (Stephen and Jamieson, 1996) indicating the requirement of other factors for acquired stress tolerance. GSH functions as a free radical scavenger, by reacting with oxidants via the redox-active sulfhydryl group to produced oxidised glutathione (GSSG), and also reduces protein disulphides (Moradas-Ferreira. 1996; Stephen and Jamieson. 1996).

Other non-enzymatic oxidative defence molecules include thioredoxin involved in reduction of protein disulphides, metallothionein which binds Cu thus preventing the Fenton reaction (see below) and polyamines which protect lipids from oxidation (for review see Moradas-Ferreira *et al.*, 1996).

### 1.11.2 Enzymatic antioxidant defences

During respiration, single electron transfer reactions reduce oxygen to ROS (reviewed by Burdon *et al.*, 1990; Pahl and Baeuerie, 1994; Moradas-Ferreira *et al.*, 1996). Initially, oxygen is reduced to form superoxide radicals ( $O_2^{\bullet}$ ) which are sequentially converted by superoxide dismutase (SOD) to hydrogen peroxide ( $H_2O_2$ ). Catalase enzymes and glutathione peroxidase then decompose  $H_2O_2$  to water and oxygen. In the presence of iron, copper and nickel,  $H_2O_2$  may give rise to the highly reactive hydroxyl radical (OH<sup>•</sup>) via the Fenton or Haber/Weiss reaction (M<sup>c</sup>Cord and Day, 1978).

Yeast contains two SOD genes, SOD1 encoding the cytosolic, copper-containing CuZnSOD and SOD2 encoding the manganese-containing MnSOD, and two catalase genes, CTT1 encoding cytosolic catalase T and CTA1 encoding peroxisomal catalase A (Kullik and Storz, 1994).

Other enzymes involved in antioxidant defence include cytochrome c peroxidase and thioredoxin peroxidase involved in the reduction of hydrogen peroxide, glutathione reductase which catalyzes the reduction of oxidized glutathione and glucose-6-phosphate dehydrogenase which through the pentose phosphate pathway synthesizes NADPH. NADPH is required for peroxide decomposition and reduction of oxidized glutathione (Moradas-Ferreira *et al.*, 1996).

### 1.11.3 Transcriptional regulation of the oxidative stress response

Several *S. cerevisiae* transcription factors have been characterized that respond to exposure to antioxidants and ROS to help regulate the intracellular ROS concentration (reviewed by Kullik and Storz, 1994; Pahl and Baeuerie, 1994; Moradas-Ferreira, 1996).

It has been proposed that in *S. cereviside*, heme functions as an oxygen sensor (Labbe-Rosine and Labbe, 1990) and activates the transcriptional factors, HAP1 and HAP2/3/4 (Zitomer and Lowry, 1992). EAP1 and HAP2/3/4 have been implicated in the regulation of CTT1 and SOD2 genes that are induced by oxygen (Gralla and Kosmann,

1992; Zitomer and Lowry, 1992). Whereas the ADR1 transcription factor regulates fatty acid induction of CTA1 gene expression (Simon *et al.*, 1991).

The YAP1 transcription factor was originally identified by its ability to bind the AP-1 eukaryotic transcription factor binding site (Moye-Rowley *et al.*, 1989) YAP1 and YAP2 genes were also isolated as genes whose overexpression was required to initiate growth after drug-induced growth arrest or adapt to otherwise lethal levels of ion chelators and zinc (Schnell *et al.*, 1992; Bossier *et al.*, 1993; Wu *et al.*, 1993). Both YAP1 and YAP2 mutants exhibit increased sensitivity to H<sub>2</sub>O<sub>2</sub>, menadione, t-BOOH and increased oxygen pressure as well as a decrease in the expression of SOD, glutathione reductase and glyceraldehyde-6-phosphate dehydrogenase while overexpression results in the converse (Schnell *et al.*, 1992; Kuge and Jone, 1994). Recently, a direct role for YAP1 in transcriptional regulation of the oxidative stress protective genes, GSH1, TRX2 (gene product, thioredoxin), YCF1 (gene product, ABC transporter protein). TPS1 (gene product, T-6-P synthetase) and SSA1 have been identified (Gounalaki and Thireos, 1994; Kuge and Jones, 1994; Wemmie *et al.*, 1994; Wu and Moye-Rowley, 1994; Stephen *et al.*, 1995).

The ACE1 transcription factor was originally identified as a yeast metallothionein CUP1 gene regulator (Thiele, 1988) and subsequently found to also regulate copper induction of CuZnSOD (Carri *et al.*, 1991; Gralla *et al.*, 1991). The MAC1 transcription factor is homologous to ACE1, regulates CTT1 expression and a MAC1 disruption mutant exhibits extreme sensitivity to  $H_2O_2$ , heat, cadmium, zinc and lead (Jungmann *et al.*, 1993). This transcription factor also regulates the expression of FRE1 encoding ferric reductase, which is involved in iron uptake and reduction (Marchler *et al.*, 1993). Regulation of metal irons by ACE1 and MAC1 may aid the prevention of hydroxyl radical generation via the Fenton or Haber Weiss reaction (Jungmann *et al.*, 1993; Kullik and Storz, 1994).

### **1.12** Cold shock response

Microorganisms also respond to a cold shock, a downshift in temperature, with expression of cold shock proteins (csps), specific repression of hsps and continued synthesis of transcriptional and translational proteins (Jones *et al.*, 1987; Jones *et al.*, 1992; Jones and Inouye, 1994). The cold shock response is also induced by exposure to translation inhibitors such as chloramphenicol and tetracycline. It has been proposed that

the state of the ribosome functions as a cold shock response sensor (van Bogelen and Neidhardt, 1990).

In *E. coli*, a cold shock response is elicited when cells grown at 37°C experience a decrease in temperature to 10°C with concomitant synthesis of a family of cold shock proteins CspA, CspB, CspC, CspD and CspE (Jones *et al.*, 1987; Goldstein *et al.*, 1990). The major csp is cspA which displays a 200-fold induction and encodes a 70-amino acid protein exhibiting high amino acid identity (61%) to CspB from *Bacillus subtilis* and 43% sequence similarity to the cold shock domain of eukaryotic Y-box transcription factors (Goldstein *et al.*, 1990; Wistow, 1990; Willimsky *et al.*, 1992; Wolffe *et al.*, 1992). Y-box proteins have been characterized as a highly conserved family of nucleic acid binding proteins (Wolffe, 1993). Proposed functions of cspA are as a cold shock transcriptional activator (La Teana *et al.*, 1991) or as a RNA chaperone (Jones and Inouye, 1994).

Four cold shock proteins have been identified and characterized in S. cerevisiae. A downshift in temperature from 30°C to 1)°C induced the synthesis of a 210-amino acid protein, TIP1 (temperature inducible protein), with a predicted M<sub>r</sub> of 20 7272 (Kondo and Inouye, 1991). However, SDS-PAGE analysis revealed the TIP1 protein as having a molecular mass of 69 kDa and further investigation indicated o-mannosylation posttranslational modification (Kowalski et al., 1995). TIP1 is also induced in response to a heat shock, has no demonstrable function and is not essential for growth (Kondo and Inouye, 1991; Kowalski et al., 1995). Two TIP1 related proteins. TIR1 and TIR2, have also been identified as cold shock inducible proteins exhibiting 49% sequence similarity to TIP1 and 72.2% homology to each other (Kowalaski et al., 1995). TIR1 is the only member of the TIP1 related protein family that is not heat shock inducible (Kowalaski et al., 1995). The TIP1 related protein family have been described as putative membrane proteins containing serine-rich tandemly repeated sequences, N-terminal signal peptides and hydrophobic C-terminal sequences (Kowalaski et al., 1995). Another cold shock protein, NSR1 (nucleolar localization sequence recognition protein), a nucleolin-like 67 kDa protein, was also identified by a downshift in temperature from 30°C to 10°C (Kondo and Inouve, 1992). NSR1 deletion mutants exhibit an inability to grow at low temperatures and have impaired rRNA processing suggesting a function for NSR1 in ribosome biogenesis (Kondo et al., 1992; Kondo and Inouye, 1992).

# **1.13** Yeast from relatively extreme temperature environments

This introduction has focused on the stress response in Saccharomyces cerevisiae as it has the best characterized stress response in yeast. However, studies conducted for this thesis investigated stress responses in yeast from relatively extreme environments. With the exception of work reported by Berg *et al.* (1987) and Julseth and Inniss (1990ab) on psychrotrophic yeast from the Arctic, there is essentially no data concerning the stress response in yeast from extreme temperature environments. At the time of writing this thesis, there were no known published reports concerning the heat shock response, colc. shock oxidative stress response or response in either Antarctic psychrophilic/psychrotrophic yeast or thermophilic enteric yeast besides some preliminary characterization of the heat shock response carried out during the author's honours candidature (Deegenaars, 1991; Deegenaars and Watson, 1992). However, detailed description of the current status of these stress response systems in S. cerevisiae is of particular relevance especially in regard to hsps. As described in sections 1.1 and 1.4, both hsps and the heat shock response are highly conserved in diverse organisms. Intuitively, their presence, and hence function, in yeas from extreme temperature environments would also comprise crucial aspects of metabolism in these organisms. In this respect, yeast in their natural environment often experience temperatures considerably above or below their growth temperature range. As a consequence, research on yeast from extreme temperature environments and their mechanism of adaptation within and outside of their growth range is of interest.

Microorganisms from extreme temperature environments can be divided into three main groups according to their growth temperature ranges; psychrophilic (Gr. *psukhros*, cold), thermophilic (Gr. *thermos*, hot) and mesophilic (Gr. *mesos*, middle). The term psychrotroph has been applied to organisms capable of growth at low temperatures but with optimum temperatures of between 25°C and 30°C. Yeast may be classified on the above basis with respect to their temperature limits for growth (Watson, 1987). An obligate psychrophilic yeast can be defined as having a maximum growth temperature at or below 20°C while a thermophilic yeast has no maximum temperature limit but requires a minimum temperature of 20°C for growth (Watson, 1987). The majority of yeasts are mesophilic and their growth temperature range usually falls between 0°C and 48°C (Stokes, 1971; Watson, 1987).

### 1.13.1 Psychrophiles and psychrotrophs

Yeast have been isolated from Anturctic soil with the majority inhabiting the upper few centimetres and constituting 100% of all fungi in soil samples from several sites (reviewed by Vishniac, 1993). Yeast have also been isolated from the surface layers of ice sheets and snow and appear to be ubiquitous in distribution (reviewed by Abyzov, 1993). Psychrophilic and psychrotrophic yeast in the genus *Leucosporidium* have also been isolated from Antarctic marine waters (Fell *et al.*, 1969; Fell, 1976). Antarctic soil temperatures usually remain quite constant in the range of 5 to 10°C, however increases have been observed for short periods during summer (Russell, 1992; Vishniac, 1993) and lethal temperatures of above 42°C have been recorded on soil surfaces (MacNamara, 1973; Cameron, 1974). Polar sea temperatures are on average 1.8°C below zero and the majority of oceanic water is usually less than 5°C (Russell, 1992). The optimum growth temperatures of psychrophilic and psychrotrophic microorganisms are considerably above the ambient temperatures of their natural environment (Russell, 1992). Psychrophilic and psychrotrophic microorganisms play key roles in the ecology of Antarctica such as in primary biomass production and nutrient cycling (Russell, 1990).

There are few reports in psychrophilic and psychrotrophic microorganisms with respect to the heat shock response. Berg *et al.* (1987) studied the protein synthetic response to heat shock in two Arctic psychrotrophic yeasts, *Trichosporon pullulans* and *Sporobolomyces salmonicolor*. The temperature for maximum heat shock induction was above the maximum temperature for *T. pullulans* but within the physiological range in *S. salmonicolor*. The heat shock proteins synthesized differed according to the temperature used for heat shock but were generally of the range 80-94, 71-78. 56-66 and 33-36 kDa. The protein profile after recovery from anaerobiosis was similar to that shown for heat shock. Julseth and Inniss (1990) extended examination of the heat shock response in *T. pullulans* to include measurement of thermotolerance to a 45°C heat stress for 5 min. Thermotolerance was attained with a pr or 26°C or 29°C heat shock for 45 min. A temperature-dependent pattern of hsp synthesis was also observed in a psychrophilic bacterium and the psychrotroph *Bacillus psychrophilus* (M<sup>e</sup>Callum *et al.*, 1986). At all heat shock temperatures the newly synthesized proteins for the psychrotroph.

On the other side of the spectrum to heat shock is the cold shock response. In their natural environment, psychrophilic and psychrotrophic microorganisms must be able to adapt to sudden decreases in temperature such as during seasonal freeze-thaw cycles (reviewed by Vishniac, 1993). In this respect, the cold shock response was demonstrated in the Arctic, psychrotrophic yeast *T. pullulans* with a down shift in temperature from 21°C to 5°C inducing concomitant synthesis of up to 26 different csps (Julseth and Inniss, 1990b).

### 1.13.2 Thermophiles

It should be emphasized that the term thermophile as applied to prokaryotic microbes (Brock, 1978; Kristjansson, 1989) and fungi (Tansey and Brock, 1972; Tansey and Brock, 1978) is quite different to that for yeast. Nevertheless, within the constraints of the definition for thermophilic yeast described in Chapter 5, all such species have been found to be enteric, inhabiting digestive tracts of wild and domestic animals (Travassos and Cury, 1971; Watson, 1987). There have been no reports on the heat shock response in these highly specialized thermophilic yeast. However, there has been much investigation of the response in archaea reviewed recently by Trent (1996). For example, Jerez (1988) studied the heat shock response in the chemolithotrophic thermoacidophilic archaea Sulfolobus acidocaldarius by shifting cells from 70°C to 85°C. A decrease in the synthesis of several proteins was observed, as well as the induction of presumptive heat shock proteins of 86, 38, 64-66 and 22 kDa. The heat shock response has also been studied in Sulfolobus sp. strain B12, an extreme thermophilic archaea In this organism, a major protein of 55 kDa and two minor proteins of 28 and 35 kDa were synthesized upon heat shock (Trent et al., 1990). A further example constitutes studies of the heat shock response in the genus Halobacterium (Daniels et al., 1984). When challenged with heat shock, from 37°C to 60°C for 3 min, cells from each of the seven Halobacterium strains examined, induced the synthesis of three groups of proteins with apparent molecular weights of 75-105, 44-45 and 21-28 kDa. Trent et al. (1994) also examined acquired thermotolerance and hsp synthesis in thermophiles from the three phylogenetic domains, Bacteria, Archaea and Eucarya. The thermophilic eukaryotic fungus, Thermomyces lanuginosus, acquired thermotolerance to a 58°C heat stress when grown at 50°C and heat shocked for 60 min at 55°C. Hsps were also identified ranging from 31-33, 57-75 and 100-110 kDa.

# 1.14 Thesis aims

The overall aim of the thesis was to characterize the stress response in psychrophilic and psychrotrophic yeast from Antarctica and thermophilic enteric yeast and to examine the contribution of key stress b omolecules in stress tolerance adaptation.

In order to characterize the heat shock and oxidative stress response in the Antarctic, psychrophilic yeast, *Candida psychrophila* and respiratory-competent and respiratory-deficient strains of the thermophilic enteric yeast, *Arxiozyma telluris* it was necessary:

- To optimize temperature regimes and H<sub>2</sub>O<sub>2</sub> concentrations required to induce stress tolerance.
- To identify homology to major hsp families from *S. cerevisiae* by Southern hybridization and western immunoblo<sup>1</sup> analyses.
- To identify *de novo* hsp and psp synthesis using <sup>35</sup>S-methionine labelling and SDS-PAGE.
- To examine trehalose levels during a heat or peroxide shock.

Other aims that arose during the course of experimentation included:

- To utilize the experimental heat shock and oxidative stress conditions identified in *C. psychrophila* to examine stress responses in other Antarctic psychrophilic (*Mrakia frigida*, *M. gelida*, *M. stokesii* and *Leucosporidium antarcticum*) and psychrotrophic (*L. fellii* and *L. scottii*) yeast.
- To examine the influence of growth temperature on stress tolerance in the mesophilic yeast, *S. cerevisiae*.
- To examine the influence of the temperature at which the oxidative stress is applied (incubation temperature) in *S. ccrevisiae*, psychrophilic, psychrotrophic and thermophilic yeast.
- To carry out preliminary investigations of the cold shock response in psychrophilic and thermophilic yeast.

# CHAPTER 2: Materials and methods

# 2.1 Materials

### 2.1.1 Chemicals

All chemicals used were of analytical reagent grade or higher. Specific chemicals and materials were obtained from the following manufacturers:

**Amersham** (Castle Hill, New South Wales, Australia): Enhanced chemiluminescence  $(ECL^{TM})$  western blotting detection kit, Hybond N<sup>+</sup> nucleic acid transfer membrane, Hybond C super, Hyperfilm-MP.

**BDH/Merck Chemicals** (Kilsyth, Victoria, Australia): Acrylamide, Ammonium peroxodisulphate (AMPS), Bromophenol blue, Ethanol (95% v/v). Ethidium bromide, Hydrogen peroxide (30% w/v),  $\beta$ -Mercaptoethanol, NN'-Methylenebisacrylamide. Sodium dodecyl sulphate (SDS), N,N,N'N'-Tetramethylenediamine (TEMED).

**Bio-Rad** (Sydney. New South Wales, Australia): Cellophane membrane backing, Filter paper backing, Kaleidoscope prestained standards, Silver stain kit, SDS-PAGE molecular weight standards (low range), Ultra-pure DNA grade agarose.

**Boehringer Mannheim** (Castle Hill, New South Wales, Australia): Bovine serum albumin (BSA; endotoxin-free, fatty acid-iree, sterile lyophilised), Glucose indicator strips (Diabur-test 5000), Phenylmethylsulphonylfluoride (PMSF), Protoplast-forming enzyme (from *Helix pomatia*).

**Bresatec** (Thebarton, South Australia, Australia): Bresaclean kit, Gigaprime DNA labelling kit.

**Calbiochem** (Alexandria, New South Wales, Australia): 3-(Cyclohexylamino) propanesulfonic acid (CAPS).

Davis Gelatine (Botany, New South Wales, Australia): Technical grade agar.

Difco (Detroit, USA): Yeast nitrogen base without amino acids.

Diploma (Melbourne, Victoria, Australia): Instant skim milk powder.

**Du Pont** (Amrad Pharmacia Biotech, Boronia, Victoria, Australia):  $\alpha^{32}$ P-dATP, Polyscreen PVDF (polyvinylidene difluoride) transfer membrane.

FMC Bioproducts (Rockland, USA): SeaKem GTG (genetic technology grade) agarose.

**ICN Biomedicals** (Seven Hills, New South Wales, Australia): Agarose (genetic technology grade), Saturated phenol.

**ICN Radiochemicals** (Seven Hills, New South Wales, Australia): Trans <sup>35</sup>S-Label (75% L-methionine, <sup>35</sup>S and 15% L-cysteine, <sup>35</sup>S).

**Kodak** (Coburg, Victoria, Australia): Liquid x-ray developer and replenisher, Liquid x-ray fixer and replenisher.

**Oxoid** (West Heidelberg, Victoria, Australia): Bacteriological peptone, Tryptone, Yeast extract.

**Pharmacia Biotech** (Sydney, New South Wales, Australia): Ficoll 400, Electrode paper Novablot, Cellophane sheet.

**Pierce** (Laboratory Supply, Marrickville, New South Wales, Australia): Coomassie protein assay kit.

**Promega** (Annandale, New South Wales, Australia): Acetylated BSA, Lambda DNA, Restriction endonucleases and buffers.

**Sigma** (Castle Hill, New South Wales, Australia): Ampicillin, Anthrone, Cycloheximide, Ethylenediaminetetra-acetic acid (EDTA). Glass beads (425-600 micron diameter), Glucose test kit 510 (Sigma Diagnostics), Lysozyme, L-methionine, Polyvinylpyrrolidone, Ribonuclease A, Tris base.

Whatman (Kent, England): Chromatography paper 3MM paper, Filter paper (grade 1).

### 2.1.2 Strains

Psychrophilic, psychrotrophic, mesophilic and thermophilic yeast strains were used for the analyses of heat shock and oxidative stress responses. Strain description and sources are listed in Table 2.1.

*E. coli* JPA 101, genotype: *supE endA hsd*R-  $\phi$ *strA svc*B  $\Delta$ (*lac-pro*) F[*tra*V35 *pro*AB+ *lac*I<sup>4</sup> *lacZ*  $\Delta$ M15], was used for plasmid DNA transformation.

### 2.1.3 Heat shock protein DNA probes

Recombinant plasmids harbouring HSP inserts were kindly provided as follows: HSP 104 (S. Lindquist, University of Chicago, Illinois, USA), SSA1 and SSC1 (E. Craig, University of Wisconsin Medical Center, Madison, Wisconsin, USA), HSP 60 (R. J. Devenish, Monash University. Melbourne, Victoria, Australia), HSP 30 (H. Boucherie,

Table 2.1.	Experimental	veast strains.
	Dipermental	Jeast bulants.

Strain	Description and Source	References
Psychrophilic yeast		
<b>Candida psychrophila</b> (Torulopsis psychrophila)	Isolated from penguir dung on Ross Island. Antarctica. CBS 5956 <sup>3</sup>	Goto <i>et al.</i> (1969) Yarrow and Meyer (1978)
<b>Mrakia frigida</b> (Candıda frigida, Leucosporidhum frigidum, Vanrija frigida)	Isolated from Antaret c snow and soil. CBS 5270	Sinclair and Stokes (1965) di Menna (1966a) Fell <i>et al.</i> (1969) Yamada and Komagata (1987) Moore (1980)
<b>Mrakia gelida</b> (Candida gelida, Leucosporidium gelidum, Vanrija gelida)	Isolated from Antaret e soil and Greenland soils. CBS 5272	di Menna (1966a.b) Fell <i>et al.</i> (1969) Yamada and Komagata (1987) Moore (1980)
<b>Mrakia stokesii</b> (Leucosporidium stokesii)	Isolated from Antarctic soil and snow. Bouvet Island. CBS 5917	Sinclair and Stokes (1965) Fell <i>et al.</i> (1969) Yamada and Komagata (1987)
Leucosporidium antarcticum	Isolated from Antarctic marine waters near the Antarctic Peninsula and the Indo-Pacific region of the Antarctic. CBS 5942	Fell <i>et al.</i> (1969) Fell (1976)
Psychrotrophic yeast		
Leucosporidium scottii (Candida scottii, Azymocandida scottii, Apiotrichum eucryphiae, Vanrija scottii)	Isolated from Southern Pacific Ocean. Antarctic soil and seawater, soil from Australia, decayed wood and from the Tyrolean Iceman's grass clothing. CBS 5930	Diddens and Lodder (1942) Lodder and Kreger-van Rij (1952) Novák and Zsolt (1961) Fell et al. (1969) Fell (1976) Ramírez and González (1984) Moore (1980) Rollo et al. (1995)
Leucosporidium fellii	Isolated from Antarctic soil and snow. CBS 7287	Giménez-Jurado and van Uden (1989
Mesophilic yeast		
Saccharomyces cerevisiae K7	Saké brewing yeast. A FCC <sup>b</sup> 26422	
Saccharomyces cerevisiae Yres	Isolated from canned cherries. Designated Yres for h gh intrinsic thermotolerance phenotype. A ICC 38554	Put <i>et al.</i> (1976) Gross and Watson (1996)
Thermophilic yeast		
Arxiozyma telluris (Candida pintolopesii, Candida bovina, Candida slooffii, Saccharomyces telluris, Torulopsis bovina)	Isolated from soil, digestive tract of mice, domestic animals, mammals, birds and humans, CBS 1787 respiratory-deficient strain CBS 2760 respiratory-competent strain	van Uden (1952) van Uden and do Carmo-Sousa (1957) van der Walt (1957) van Uden and Vidal-Leira (1970) Mendonça-Hagler and Phaff (1975) van der Walt and Yarrow (1984)

<sup>a</sup>Centraal bureau voor Schimmelcultuur, Delft, The Netherlands. <sup>b</sup>American Type Culture Collection, Rockville, US **1**. University of Bordeaux, Bordeaux, France) and HSP 26 and HSP 12 (P. A. Meacock, Leicester Biocentre, Leicester University, UK).

Table 2.2 lists references for these recombinant plasmids and the restriction endonucleases used to produce suitably sized DNA probes for Southern hybridization analyses.

HSP Probe	Recombinant Plasmid	Restriction endonucleases	DNA probe size (kb)
HSP 104 <sup>a</sup>	pYS104	Eco RI / Hind III	1.14
SSA1 <sup>b</sup>	CEN30:SSA1vb	Kpn I / Pst I	1.30
SSC1 <sup>e</sup>	pMT11:pSSC1H	<i>Hin</i> d III / <i>Sal</i> I	0.90
HSP60 <sup>d</sup>	pRJ28	Hinc II	0.53
HSP 30 <sup>e</sup>	pUC19:HSP30	Hind III / Pst I	1.10
HSP 26 <sup>f</sup>	pUC18:HSP26	Eco RI	0.85
HSP 12 <sup>g</sup>	pUC:HSP12	Eco RI	0.54

**Table 2.2.** Restriction endonucleases required to produce appropriately sized HSP gene probes for Southern hybridization.

<sup>a</sup>Sanchez and Lindquist (1990) <sup>b</sup>Craig and Jacobsen (1984) <sup>c</sup>Craig *et al.* (1987) <sup>d</sup>Reading *et al.* (1989) <sup>e</sup>Récnacq and Boucherie (1993) <sup>f</sup>Bossier *et al.* (1989) <sup>g</sup>Praekelt and Meacock (1990)

#### 2.1.4 Anti-hsp antibodies

Anti-hsp antibodies used in western immunoblot analyses were acquired from the following sources: Anti-hsp 104 polyclonal antibody (PA3-016) and anti-hsp 70 monoclonal antibody (MA3-008) were obtained from Affinity BioReagents (Neshanic Station, New Jersey, USA). Anti-hsp 60 monoclonal antibody (SPA-807) was obtained from StressGen Biotechnologies Corporation (Victoria, British Columbia, Canada). Anti-hsp 90 monoclonal antibody was a kind gift from P. Piper (University College London, London, UK).

# 2.2 Methods

#### 2.2.1 General

Millipore Milli-Q filtration system (Millipore, Lane Cove. New South Wales, Australia) treated water was used to propare all solutions and media. All media and solutions requiring sterilization were autoclaved at 121°C for 15 min.

#### 2.2.2 Maintenance of cultures

#### 2.2.2.1 Liquid and solid media

All yeast strains were grown in YEP medium (0.5% yeast extract. 0.5% bacteriological peptone, 0.3% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub> and 2% glucose). *E. coli* JPA101 was grown in LM medium (1% tryptone, 0.5% yeast extract, 10 mM NaCl. 10 mM MgSO<sub>4</sub>.7H<sub>2</sub>O). YEP and LM media were made up as a 10 x stock and stored at -20°C until required. Glucose was added to the other constituents of YEP media after the stock was diluted and autoclaved, to avoid generation of H<sub>2</sub>O<sub>2</sub> (which itself constitutes a stress) when glucose and phosphate are heated together in culture media (Woods and Jones. 1986). This method was found to be favourable for consistent media concentration and time efficiency. Ampicillin was added to YEP and LM media to a final concentration of 25  $\mu$ g ml<sup>-1</sup> and 50  $\mu$ g ml<sup>-1</sup>, respectively after autoclaving.

For agar plates. 1 to 1.5% agar was added to YEP and LM media before autoclaving. Agar plates were poured immediately on cooling to avoid pH changes to media caused when solidified agar is reheated (Bridson, 1990). All yeast cultures were maintained on YEP agar and stored at 4°C. Subcultures were routinely made every 2 to 4 weeks to maintain viability and for use in starter cultures (2.2.2.4). It was found that this was the best method for short term preservation of psychrophilic, psychrotrophic and thermophilic yeast.

Routine microscopic examination to verify integrity of morphological characteristics was carried out on psychrophilic, mesophilic and thermophilic yeast species.

# 2.2.2.2 Glycerol stocks

For long term storage, concentrated aliquots of yeast aqueous cultures were stored at -70°C in 15% glycerol. *E. coli* JPA101 was similarly stored in 50% glycerol.

### 2.2.2.3 Starter cultures

Starter cultures of all yeast were produced by inoculating a single colony from plate cultures into 20 ml of YEP in a 50 ml conical flask. Flasks were incubated at 15°C for psychrophilic and psychrotrophic yeast, 25°C for *S. cerevisiae* and 35°C for thermophilic yeast in an orbital shaker incubator (Paton Industries, Victor Harbor, South Australia) operating at 180 o.p.m. for 3 to 5 days for psychrophilic and psychrotrophic yeast, 24 to 30 hours for *S. cerevisiae* and overnight for thermophilic yeast. Starter cultures were stored at 4°C and renewed as required.

To obtain yeast cultures for experiments, an inoculum of 0.01% (thermophilic yeast) to 10% (psychrophilic yeast) of starter cultures was used to inoculate YEP media and cultures were grown at their respective temperatures as indicated in Chapters 3 to 8.

#### 2.2.3 Growth curves

All yeast cultures were grown as described above at their respective temperatures (as indicated in Chapters 3 to 8). At various time intervals aliquots were taken. The optical density (OD) of the culture was read at 600 nm ( $OD_{600}$ ) using YEP as a blank. Appropriate dilutions for viable plate counts were estimated according to the optical density reading based on the following formula:

An OD<sub>600</sub> of 1 is approximately equal to 3 x  $10^6$  cfu ml<sup>-1</sup> for *S. cerevisiae* (Ausubel *et al.*, 1988).

Serial dilutions were made in YEP media. The YEP media used was previously acclimated to 15°C, 25°C or 35°C for psychrophilic and psychrotrophic yeast. *S. cerevisiae* and thermophilic yeast, respectively. Appropriate dilutions were spread plated onto YEP agar and incubated for 7 to 14 days at 15°C for psychrophilic and psychrotrophic yeast. 2 days at 25°C for *S. cerevisiae* and overnight at 35°C for thermophilic yeast. Viable counts were made of plates containing 30 to 300 colonies, the colony forming units per ml (cfu ml<sup>-1</sup>) determined and growth curves constructed.

Growth curves were used to calculate the time taken for cultures to double the number of cfu ml<sup>-1</sup> (usually from 5 x  $10^{\circ}$  to 1 x  $10^{7}$  cfu ml<sup>-1</sup>) and was referred to as the culture doubling time.

#### 2.2.4 Glucose determination

Accurate measurements of glucose levels of cultures was determined enzymatically using the Sigma Diagnostics Glucose test kit 510 which is based on the glucose oxidase-peroxidase reaction. To determine when a culture was glucose-depleted, glucose indicator strips (Diabur-Test 5000, Boehringer Mannheim) were used. These strips were accurate for glucose concentrations below 0.75%.

### 2.2.5 Ethanol determination

Ethanol concentrations of cultures was determined by gas chromatography. Samples were taken at various time intervals and frozen until analysed. Prior to analysis, samples were thawed and centrifuged. A 1  $\mu$ l sample of supernatant was injected onto a Porapak Q column (Alltech Australia, Homebush, Australia; glass column length 2 m, internal diameter 2 mm, mesh range 8)/100) on a Packard 427 gas chromatograph (Packard Instrument Company, Downers Grove, USA; injector temperature 220°C, oven temperature 190°C, detector temperature 220°C) with flame ionisation detection. The retention time for ethanol was 2 – 3 min and the response was quantified by an external test sample.

#### 2.2.6 Shock / stress conditions

#### 2.2.6.1 Heat shock / heat stress

Intrinsic thermotolerance was measured by rapidly heating cells grown at 15°C, 25°C or 35°C to the heat stress temperature (as indicated in each Chapter) in a 70°C waterbath and transferring them to an oscillating waterbath set at the heat stress temperature for the duration of the time course (60 to 120 min). Induced thermotolerance was measured by exposing cultures to a heat shock (temperatures as indicated in each Chapter) prior to a heat stress. Subsamples of 0.5 ml were taken at various time intervals, transferred to microfuge tubes and cooled on ice. Samples were diluted in YEP medium (15°C, 25°C or 35°C), spread plated in duplicate onto YEP agar and incubated at 15°C,

25°C or 35°C (as described in section 2.2.3). This was the general protocol utilized to examine intrinsic and induced thermotolerance in psychrophilic, psychrotrophic, mesophilic and thermophilic yeast, however some variations exist as indicated in Chapters 3 and 5.

To examine the effect of inhibition of protein synthesis on induced thermotolerance. 50  $\mu$ g ml<sup>-1</sup> cycloheximice was added 20 min prior to a 37°C heat shock for 15°C and 25°C grown cells of *S. cercvisiae* and prior to a 40°C heat shock for 35°C grown cells of *S. cerevisiae* and *A. telluris* 

### 2.2.6.2 Peroxide shock / peroxide stress

Intrinsic peroxide tolerance was monitored over a 2 h time course by exposing 10 ml cells (washed and resuspended in YEP medium) directly to 1, 2, 5, 10, 50 or 100 mM hydrogen peroxide at 15°C, 25°C or 35°C (as indicated in Chapters 7 and 8). Induced peroxide tolerance was measured by subjecting 10 ml cells (washed and resuspended in YEP medium) to a 0.2 mM hydrogen peroxide shock for 30 min or 3 h (as indicated in Chapters 7 and 8). followed by the addition of a concentration of hydrogen peroxide constituting a peroxide stress (as indicated in Chapters 7 and 8) and return of cultures to 15°C, 25°C or 35°C. At 30, 60 and 120 min time points, 0.5 ml subsamples were diluted in YEP medium (15°C, 25°C or 35°C), spread plated in duplicate and incubated at 15°C, 25°C or 35°C (as described in section 2.2.2).

#### 2.2.6.3 Crosstolerance

Experiments were also carried out as described above (in sections 2.2.6.1 and 2.2.6.3) to determine whether crosstolerance could be induced by a heat shock to a peroxide stress and by a peroxide shock to a heat stress. Experimental conditions including the concentration of hydrogen peroxide, heat shock temperature and duration of shock treatments are described in Chapters 3, 5, 7 and 8.

### 2.2.6.4 Ethanol stress

Intrinsic ethanol tolerance in *C. psychrophila* was monitored over a 4 h time course after addition of 17%, 20% or 23% ethanol. After 1 h, 2 h and 4 h, 0.5 ml subsamples

were diluted in YEP medium (15°C), spread plated in duplicate and incubated at 15°C (as described in section 2.2.3).

### 2.2.6.5 Cold shock

Intrinsic tolerance to a cold shock for *C. psychrophila*, *A. telluris* strains 2760 and 1787 was measured by growing the cultures at 15°C for *C. psychrophila* and 35°C for *A. telluris* and exposing cultures to a 5°C cold shock over a 4 to 6 h time course. Subsamples were taken at various time intervals, diluted in YEP medium, spread plated in duplicate onto YEP agar and incubated at either 15°C or 35°C (as described in section 2.2.3).

#### 2.2.6.6 Viabile plate counts - % Survivors

Viable counts were made of dilution plates containing between 30 and 300 colonies and the cfu ml<sup>-1</sup> determined. Stress tolerance was assessed as the percentage of cfu (% survivors) after the appropriate treatment compared to an unstressed control (100% survivors).

#### 2.2.6.7 Reproducibility of experiments

All stress tolerance experiments were carried out a minimum of three times and the results presented are representative of a typical experiment. It was determined that statistical analysis of stress tolerance results was inappropriate, as minor differences in culture density and small discrepancies in the temperature of incubators at different times were observed to significantly affect the percentage of survivors following a given stress treatment. Other researchers have commented likewise (Sanchez *et al.*, 1993). However, it should be pointed out that the shape of tolerance curves for an individual species and the relative differences between species and strains consistently showed the same trends. Furthermore, if parallel stress tolerance experiments were undertaken for a given strain, in the same water bath, the results were practically superimposable.

#### 2.2.7 Southern hybridization

### 2.2.7.1 Bacterial transformation

Recombinant plasmids harbouring heat shock gene inserts derived from *S. cerevisiae* were provided as dried pellets, which were reconstituted in  $50 - 100 \,\mu$ l of TE buffer (10 mM Tris, 1 mM EDTA pH 7.4). Approximately 40 - 70 ng of each plasmid DNA was used for transformation of *E. coti*.

The bacterial transformation technique used was based on CaCl<sub>2</sub> treatment described by Cohen *et al.* (1972). The procedure is outlined in Sambrook *et al.* (1989). Minor variations included the use of LM media rather than LB or SOC with the *E. coli* strain JPA101, and the use of LM agar plus ampicillin (50  $\mu$ g ml<sup>-1</sup>) for plating out. Prior to plating, all LM agar plates were prewarmed to 37°C. The number of viable *E. coli* cells surviving the CaCl<sub>2</sub> procedure was determined by plating appropriate serial dilution onto LM agar without ampicillin. A control to determine the number of spontaneous reversion mutants was set up by plating out non-trar sformed cells onto LM agar plus ampicillin (50  $\mu$ g ml<sup>-1</sup>). All plates were incubated overnight at 37°C.

### 2.2.7.2 Selection of transformed host cells

Transformants were selected by virtue of the genetic determinants (ampicillin resistance) carried by the plasmid DNA. Selection of transformants was undertaken by plating out onto media containing ampicillin. Numerous transformant colonies were inoculated into 5 ml of LM media plus ampicillin (100  $\mu$ g ml<sup>-1</sup>) and incubated overnight at 37°C. A plasmid DNA preparation of each putative transformant was carried out as outlined in section 2.2.7.3 and subsequently analyzed by restriction enzyme digests (section 2.2.7.6) and agarose gel electrophoresis (section 2.2.2.7).

### 2.2.7.3 Plasmid DNA preparations

All plasmid DNA preparations were performed on a small scale in 1.5 ml microfuge tubes. If large quantities of plasmid DNA were required for a particular protocol the number of small preparations was increased. The alkaline lysis protocol was based on the method of Birnboim and Doly (1979) as described in Sambrook *et al.* (1989) and modified as follows; use of LM media rather than LB, harvesting cells from 2 ml of

culture, an increase in lysozyme concentration (20 $\mu$ l of 100 mg ml<sup>-1</sup>), and the precipitation of DNA with 2 volumes of ethanol overnight at -20°C. Prior to phenol/chloroform extractions and ethanol precipitation, the preparation was treated with 5  $\mu$ l of 10 mg ml<sup>-1</sup> RNase (10 mg ml<sup>-1</sup> in 10 mM Tris pH 7.5, 15 mM NaCl) to remove RNA.

## 2.2.7.4 Genomic DNA preparations

Genomic DNA was isolated from *C. psychrophila, A. telluris* strains 2760 and 1787 and *S. cerevisiae* strain Yres by a modification of the protocol described by Ferbeyre *et al.* (1993). Modifications included using protoplast forming enzyme (2.5 mg ml<sup>-1</sup>) instead of zymolyase-100T, 10 mM Tris-HCl (pH 7 5) instead of citrate phosphate (pH 7.5) in the lysis buffer and DNA was harvested from 40 ml of late-logarithmic phase cultures. To avoid unnecessary shearing of DNA, all pipette tips were cut off prior to autoclaving.

The procedure yielded high molecular weight DNA suitable for Southern blot analysis by completing the spheroplasting and lysis of yeast cells in one step. This protocol was also adopted as it had been used successfully for isolation of genomic DNA from yeast with thicker cell walls than *S. cerevisiae*.

### 2.2.7.5 DNA quantification

Genomic DNA samples (1/50 dilutions in sterile water) were analyzed spectrophotometrically to quantify and assess the purity of preparations. The following formula was used as a guideline:

An absorbance of 1 at 260 nm corresponds to approximately 50  $\mu$ g ml<sup>-1</sup> of doublestranded DNA (Sambrook *et al.*, 1989).

The ratio of the absorbance at 260 nm to the absorbance at 280 nm should lie between 1.8 and 2.0 for a pure preparation uncontaminated by proteins or phenol. A ratio less than 1.8 is indicative of some impurity making quantification less accurate.

### 2.2.7.6 Restriction endonuclease digestion

The restriction enzymes used in digests of both genomic and plasmid DNA were obtained with their appropriate buffers from Promega. The incubation temperature, salt conditions and buffers used were those specified by the supplier. Most reactions were incubated for 1 to 2 h at 37°C. Digests were terminated by incubation at 70°C for 10 min in a heat-block and then chilled on ice. Acetylated BSA was added to the reaction mixture at 1/10 volume to improve stability of restriction enzymes.

Small-scale plasmid digests (approximately 10  $\mu$ l) were carried out in order to distinguish true recombinants. Large-scale plasmid DNA digests (200 – 500  $\mu$ l) were subsequently performed to generate suitable probe fragments from the recombinant clones. Genomic DNA (1  $\mu$ g) digests, for Southerr hybridization analyses (described below), were carried out in a volume of 100  $\mu$ l. Genomic DNA was digested with 4 base (*Sau* 3A) and 6 base (*Eco* RI and *Bam* HI) recognition sequence restriction endonucleases. Samples were precipitated with 2 volumes of 95% ethanol, pelleted by centrifugation at 13000 rpm for 10 min in a microfuge, and resuspended in a small volume (10 to 20  $\mu$ l) of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) for further analysis by gel electrophoresis.

# 2.2.7.7 Agarose gel electrophoresis

All gels were made to a 1% agarose concentration in 1 x TAE buffer from a 50 x stock (242 g Tris, 57.1 ml glacial acetic acid, 100ml 0.5 M EDTA (pH 8.0)  $L^{-1}$ ). Ethidium bromide (final concentration 50 ng ml<sup>-1</sup>) was incorporated directly into the gel and the electrophoretic buffer (1 x TAE) to aid visualization of the DNA. Electrophoresis of agarose gels was carried out at 100V for 1 to 1.5 h. Gels were viewed by illumination from below with UV light (302 nm) using a transilluminator and photographed with Polaroid 667 film (ADA 3000) using a MP-4 land camera fitted with a Kodak Wratten (22A) filter (exposure 6 seconds, aperture 11, development 30 seconds).

Prior to loading samples on the agarose gel. 3  $\mu$ l of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF. 30% glycerol in water) was added to each sample. The dyes contained in the buffer e tabled estimation of the migration of fragments during electrophoresis, and glycerol facilitated loading of DNA samples. Lambda *Eco* RI/*Hind* III digests were loaded as a DNA fragment size marker on each gel.

#### 2.2.7.8 DNA purification (Geneclean, Brest-Clean)

The DNA purification protocols used are described in the BIO 101 Geneclean and Bresatec Bresa-Clean instruction leaflets and are based on the method of Vogelstein and Gillespie (1979) utilizing a silica matrix to preferentially bind DNA. This procedure was employed to purify DNA fragments from agarose gels in order to use them as probes in subsequent hybridization steps. These kits were also used to eliminate unincorporated nucleotides from oligo-labelling reactions (see section 2.2.7.9), on the basis that incorporated nucleotides adhere to the silica matrix.

## 2.2.7.9 Oligo-labelling of DNA probe

The protocol employed for oligo-labelling of DNA is described in the notes accompanying the Breastec Gigaprime DNA labelling kit. The kit is based on the randompriming method described by Feinberg and Vogelstein (1983) and is designed to generate probe lengths of between 100 bp and 1 kb. Restriction endonuclease digests were thus selected to produce heat shock gene probes of less than 1 kb when possible (see Table 2.2). Approximately 60 – 100 ng of DNA was denatured and labelled with  $\alpha^{32}$ P-dATP.

#### 2.2.7.10 Alkaline Southern blotting

The protocol used is based on the procedure developed by Reed (1990) for charged nylon membranes and is described in the Hybond-N<sup>+</sup> membrane booklet from Amersham. The technique differed from the conventional Southern blotting technique in that it involved use of an alkali solution (0.4 M NaOH) rather than a neutral, high ionic strength solvent for the transfer buffer. After transfer of digested genomic DNA from gel to membrane, the DNA was fixed to the membrane by baking at 80°C for 2 h. Digested genomic DNA from *S. cerevisiae* strain Y: es was included as a positive control alongside of digested genomic DNA from *C. psychrophila* and *A. telluris* for hybridization with HSP gene probes

#### 2.2.7.11 Hybridization, stringency washes and autoradiography

The procedure followed for prehybridization and hybridization was essentially as described by Petko and Lindquist (1986). The Southern blots were prehybridized in a heat-sealed plastic bag containing 30 ml of hybridization solution (5 x SSC, 5 x Denhardts, 0.5% SDS, 1 mM EDTA, 30% formanide) for 60 min in a 35°C waterbath. The prehybridization solution was then removed and replaced with 10 ml of prewarmed hybridization solution containing a radioactively labelled, denatured probe (see section 2.2.7.9). The bag was resealed and incubated overnight at 35°C.

On completion of hybridization, the solution was removed and membranes were exposed to  $4 \times 5$  min stringency washes in a solution containing  $5 \times SSC$  and 0.2% SDS at  $35^{\circ}C$ . After the washing procedure, membranes were wrapped in GladWrap, placed in an X-ray cassette and exposed to Hyperfilm-MP at -70°C. The time of exposure was dependent on the intensity of the radioactive signal from the membrane as monitored by a Geiger counter (Neomedix Systems, series 900 mini-monitor). Dupont Cronex intensifying screens were placed behind the autoradiographic film to enhance the intensity of the autoradiographic image. The autoradiograms were developed by immersion in Kodak liquid X-ray developer for 1 min, r. nsed in water, immersed in Kodak liquid X-ray fixer for 1 min and finally rinsed in water.

### 2.2.8 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS - PAGE)

# 2.2.8.1 <sup>35</sup>S-methionine labelling of proteins

Yeast cultures were grown at their respective growth temperatures in YEP medium until they reached a culture density corresponding to an optical density of 0.2 - 0.3 at 600 nm. Prior to <sup>35</sup>S-methionine labelling, 40 ml of culture was washed and resuspended in 2 ml YNB (0.67% yeast nitrogen base, 0.3% KH<sub>2</sub>PO<sub>4</sub> and 2% glucose) medium without amino acids. Trans <sup>35</sup>S-Label (75% L-methionine, <sup>35</sup>S and 15% L-cysteine, <sup>35</sup>S; 100 µCi; specific activity 1150 Ci mmol<sup>-1</sup>) was added to control, heat, cold and peroxide shock samples. Duration and conditions for shock treatments were as outlined above in sections 2.2.6.1 to 2.2.6.5 and in respective Chapters. Samples were then transferred to microfuge tubes containing 150 µl of 100 mg ml<sup>-1</sup> unlabelled methionine.

The above method (Fuge *et al.* 1994) was found to be favourable as compared to growing cultures in YNB, as psychrophilic and thermophilic yeast exhibited poor growth in YNB medium and <sup>35</sup>S-methionine incorporation was poor (Deegenaars, 1991). Furthermore, the thermophilic yeast strain 1787 has special nutrient requirements, including the requirement of choline and methionine for growth (Travassos and Cury, 1971).

### 2.2.8.2 Protein extraction

The method used to extract proteins from unlabelled or <sup>35</sup>S-methionine labelled yeast cultures, suitable for SDS-PAGE or western immunoblot analysis, was based on that

described by M<sup>c</sup>Alister *et al.* (1979). For unlabelled cultures, 40 ml was pelleted by centrifugation and resuspended in remaining supernatant and transferred to a microfuge tube. Cells from unlabelled and labelled cultures were again pelleted by centrifugation and washed with 1 ml dH<sub>2</sub>O. Protein extraction was carried out during six successive vortexing and cooling sessions. An equiva ent volume of 0.5 mm glass beads. 15µl of 0.1 M PMSF and 100 µl of protein extraction buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 2 mM EDTA, 1% 2-Mercaptoethanol. 2% Ficoll 400) was added to the pellet. The mixture was then vortexed for 20 s before being cooled on ice for 2 min. Following extraction, cell debris was removed by centrifugation for 3 min and supernatant containing protein extracts was transferred to a clean microfuge tube. Prior to addition of SDS sample buffer, a 10 µl subsample of supernatant was taken to make a 1:100 dilution for protein determination (see section 2.2.8.3). An equal volume of SDS sample buffer (0.25M Tris-HCL pH 6.8, 10% glycerol, 2% SDS, 5% 2-Mercaptoethanol) along with 15µl of 0.1% bromophenol blue loading dye was added to the remaining supernatant. This sample was then heated at 95°C for 5 min, cooled on ice and stored until recuired at -20°C.

### 2.2.8.3 Protein determination

Protein concentration was determined using a Coomassie protein microassay procedure (Pierce) based on the method described by Bradford (1976) as outlined in the instruction booklet accompanying the protein assay kit. Bovine serum albumin (BSA) (0 –  $20 \ \mu g \ ml^{-1}$ ) was used as a protein standard to enable construction of a standard curve. Protein extracts (1:100 dilution) and BSA standards were taken up in 1 ml of Coomassie protein assay reagent. Protein concentration ( $\mu g \ ml^{-1}$ ) was measured spectrophotometrically at 595 nm against a reagent blank.

#### 2.2.8.4 Polyacrylamide gel electrophoresis

Separation of proteins (10  $\mu$ g) and low range molecular weight standards (Bio-Rad) was achieved by SDS-PAGE essentially as described by Laemmli (1970) and Laemmli and Favre (1973) using a Protean II slab cell (Bio-Rad). Separating gels were made to a concentration of 10% polyacrylamide (13.32 ml of 30% acrylamide/0.8% bisacrylamide, 10 ml of 1.5M Tris-HCl (pH 8.8), 16.10 ml dH<sub>2</sub>O, 400  $\mu$ l 10% SDS, 40 $\mu$ l TEMED. 140 $\mu$ l 10% AMPS) with a 2 cm 4% polyacrylamide stacking gel (1.30 ml of 30%)

acrylamide/0.8% bisacrylamide, 1.25 ml cf 1 M Tris-HCl pH 6.8, 7.24 ml dH<sub>2</sub>O, 100 µl SDS, 10µl TEMED, 100 µl 10% AMPS). Thawed protein samples were loaded onto the gel and electrophoresed at 10°C and 30m A using a Tris/Glycine/SDS (6 g Tris, 28.8 g Glycine, 1 g SDS L<sup>-1</sup>) running buffer. Lcw range molecular weight standards (Bio-Rad) were prepared as described in the manufacturer's instructions and electrophoresed alongside the protein samples for estimation of molecular weights. Electrophoresis generally took 6 – 8 h and was stopped when the bromophenol tracking dye reached the end of the gel.

### 2.2.8.5 Silver staining of polyacrylamide gels

Following electrophoresis, gels were fixed in 40% methanol and 10% acetic acid for 30 min prior to staining with a silver stain kit (Bio-Rad) based on the procedure described by Switzer *et al.* (1979).

### 2.2.8.6 Gel drying and autoradiography

Gels were dried using a Bio-Rad model 543 Gel Slab drier, as described in the manufacturers operating guide, at 80°C for 1 h between cellophane. Dried gels were exposed to Hyperfilm-MP (Amersham) at - 70°C for 5 to 7 days prior to developing as described in section 2.2.7.11.

#### 2.2.9 Western immunoblot analysis

### 2.2.9.1 Transfer of proteins to nitrocellulose

Proteins (10µg) were electrophoresed alongside kaleidoscope prestained markers (15 µl) (Bio-Rad) using SDS-PAGE as described above in section 2.2.8.4. After electrophoresis was terminated, gels were equilibrated in continuous transfer buffer (39 mM Glycine, 48 mM Tris, 0.0375% SDS, 20% Methanol) for at least 20 min. Proteins were transferred to Hybond-C Super nitrocellulose membrane (Amersham) using a 2117-250 Novablot Electrophoresis Transfer kit (AMRAD Pharmacia) as outlined by the manufacturer. Transfer was carried out at 0.8 mA cm<sup>-2</sup> for 90 min. Kaleidoscope markers were visible following transfer and enablec reduction of the size of membrane (resulting in a strip with proteins ranging in molecular weight from 40 kDa to 140 kDa) utilized for

antibody detection. Membranes were stored in phosphate buffered saline (PBS; 80 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O, 100 mM NaCl) overnight at 4°C.

### 2.2.9.2 Enhanced chemiluminescence (ECL) detection of bound antibody

Detection of bound antibody was achieved using the Amersham ECL western blotting detection kit as outlined by the manufacturer. The membrane strip was blocked for 60 min in PBS-T (PBS, 0.1% Tween-20) with 5% skim milk powder (SMP) with shaking at room temperature. The membrane was then washed in 40 ml PBS-T prior to incubation with the primary antibody in PI3S-T and 5% SMP, at a concentration of 1:750 for anti-hsp 90, 1:1000 for anti-hsp 70 and anti-hsp 60 and 1:5000 for anti-hsp 104, for 1 h at room temperature with shaking in a scaled plastic bag. The membrane was washed in 40 ml PBS-T as before and then incubated with the appropriate secondary antibody (mouse or rabbit) in PBS-T and 5% SMP at a concentration of 1:1000 for 1 h at room temperature with shaking in a scaled plastic bag. A modified washing procedure was next employed, with 3 x 5 min washes in PBS and 0.3% Tween 20 followed by 3 x 5 min washed in PBS-T (Lindquist, personal communication). Detection was as described in the accompanying instruction booklet, with exposures on Hyperfilm-MP (Amersham) ranging from a few seconds to 10 min.

### 2.2.9.3 Antibody homology to S. cerevisiae hsps

Anti-hsp 104 polyclonal antibody (PA3-016, Affinity BioReagents) was generated against residues 894-908 from the carboxyl terminal (DDDNEDSMEIDDDLD) of *S. cerevisiae* hsp 104. Anti-hsp 90 monoclonal antibody (P. Piper, University College London) was also raised against amino acids 317-706 constituting the C-terminus of *S. cerevisiae* hsp 82 but also recognises hsc 82 from *S. cerevisiae* and hsp 90 from *C. albicans*. On the other hand, anti-hsp 70 monoclonal antibody (MA3-008, Affinity BioReagents) was generated against amino acids 437-479 of human hsp 70 and reacts with hsp 70 family members from yeast to humans. This amino acid sequence has approximately 90% similarity to *S. cerevisiae* hsp 70, ssa subfamily and ssd1. Anti-hsp 60 monoclonal antibody (SPA-807, StressGen) was raised against residues 383-419 of the human hsp 60 (corresponding to residues 356-393 of the mycobacterial hsp 60 sequence) and recognises hsp 60 from a diverse range of organisms from primates, to bacteria and yeast.

#### 2.2.10 Protein isolation

#### 2.2.10.1 SDS-PAGE

Proteins extracted as described above in section 2.2.8.2. Initially, <sup>35</sup>S-methionine labelled control and heat shock samples from *M. gelida* and *M. stokesii* (30 µg) were separated by SDS-PAGE using a 6% polyacrylamide separating gel (8 ml 30% Acrylamide/0.8% Bisacrylamide, 400 µl 1.5 M Tris-HCl (pH 8.8), 21.38 ml dH<sub>2</sub>O, 400 µl 10% SDS, 80 µl TEMED, 140 µl AMPS) and 4% polyacrylamide stacking gel. The gel was stained with Coomassie brilliant blue for at least 4 h and destained for 4 to 8 h (as described below in section 2.2.10.3). The autoradiogram and stained gel enabled identification of the 110 kDa protein band on a 6% polyacrylamide gel. Subsequently, 15 heat shock protein samples (50 µg) from *M. stokesii* and a Kaleidoscope prestained marker (30 µl) were electrophoresed until the BSA protein marker (approximately 83 kDa) almost ran off the gel.

# 2.2.10.2 Transfer of protein to PVDF membrane

Transfer of proteins to PolyScreen PVDF transfer membrane (Du Pont) was carried out as described above for transfer of proteins to nitrocellulose (section 2.2.9.1) with some modifications. Prior to transfer, the gel was equilibrated in CAPS transfer buffer (100 ml 10 x CAPS stock (22.13 g CAPS L<sup>-1</sup>), 100 ml Methanol, 800 ml dH<sub>2</sub>O) for at least 10 min. The membrane was wetted with 95% ethanol for at least one min, rinsed in dH<sub>2</sub>O and equilibrated in CAPS transfer buffer for 10-<sup>-5</sup> min. Transfer was achieved at 0.8 mA cm<sup>-2</sup> for 1 h.

### 2.2.10.3 Coomassie brilliant blue staining and protein band excision

Immediately proceeding transfer, the membrane was stained with Coomassie Brilliant Blue R250 stain (0.25g in 90 ml of 1:1 (v/v) methanol:dH<sub>2</sub>O, 10 ml glacial acetic acid) for 2-3 min. The membrane was destained by washing in a methanol/acetic acid solution (as above minus the Coomassie E-rilliant Blue R250) and rinsed thoroughly in dH<sub>2</sub>O prior to drying between clean Whatman filter paper. The 110 kDa protein band was excised from 15 lanes, each using a clean disposable scalpel blade. Bands were placed in a microfuge tube, sealed with parafilm and stored at 4°C.

#### 2.2.10.4 Amino acid sequencing

The Australian Proteome Analysis Facility (Macquarie University Centre for Analytical Biochemistry, NSW, Australia) carried out N-terminal amino acid sequencing of 15 amino acids from the isolated 110 kDa hsp. Amino acid sequence homology searches to *S. cerevisiae* protein sequences were obtained using the BLASTP (Altschul *et al.*, 1990) program.

### 2.2.11 Trehalose assay

# 2.2.11.1 Sample preparation

Trehalose was extracted from 80 ml cells based on the method described by Lillie and Pringle (1980). Cells were pelleted by centrifugation (1500g for 2-3 min) and washed twice with 5 ml ice-cold dH<sub>2</sub>O. The washing steps removed traces of glucose or other hexoses from the cells, which would otherwise interfere with the assay. The cell pellet was resuspended in 3 ml 0.5M trichloroacetic acid (TCA) and tubes placed on ice. The tubes were shaken at 15 min intervals for 90 min. The mixture was then centrifuged and the supernatant collected. The pellet was re-extracted with TCA and supernatants pooled and placed on ice for immediate assay or stored at -20°C. Trehalose content of frozen or unfrozen samples was essentially identical (Attfield, personal communication). All extraction steps were performed as rapidly as possible to minimise trehalose degradation.

At the same time as sampling cultures for trehalose, 80 ml culture was transferred to a preweighed 0.45  $\mu$ m filter (Millipore) and dried at 60°C for approximately 24 h to enable determination of dry weight. Trehalose content was then expressed as a percentage of the dry weight (consistently between 5 to 7 mg dry weight) of cells.

### 2.2.11.2 Anthrone assay

The trehalose assay is a colorimetric method based on the method described by Stewart (1975) using the anthrone reagent specified by Spiro (1966). 5 ml of anthrone reagent (0.05% anthrone, 1% thirourea in 66% H<sub>2</sub>SO<sub>4</sub>) was added to 1 ml sample extracts on ice and transferred to a boiling water-bath for 15 min. The mixture was then cooled and the optical density was read at 620 nm and compared to a curve constructed from glucose standards prepared and read spectrophotometrically at the same time. Results presented represent the mean and standard deviation of three separate experiments.