Chapter 1: Introduction

1.1 The heat shock response

Throughout the past decade, considerable emphasis has been placed on the physiology, gene regulation and associated molecular biology of the heat shock proteins (hsps), a set of proteins synthesized by all organisms in response to heat. This response, referred to as the heat shock response, is the most highly conserved genetic system known, existing in every organism so far examined, from Archae to eubacteria and fungi, and from plants to invertebrates and vertebrates including humans. A precedent for investigation into the concept of a universal stress response was set in 1962 by Ritossa, who reported that a new set of puffs on the salivary gland polytene chromosomes of *Drosophila buskii* could be induced by a brief heat shock. During ensuing years, it was noted that puffs i) were induced by several other stresses, ii) produced within a few minutes, iii) associated with newly synthesized RNA, iv) found in different tissues from other *Drosophila* species and v) were accompanied by the disappearance of previously active puffs (reviewed by Lindquist, 1986). Molecular analyses of the response were initiated by Tissières *et al.* (1974), who reported that the heat-induction of these puffs effected concordant synthesis of a small number of polypeptides. Subsequent to extensive investigations of the response in *Drosophila* in the period leading to 1980 (reviewed by Ashburner and Bonner, 1979), every organism further examined has been shown to exhibit the ubiquitous heat shock response.

Certain features of the response vary from one organism to the next, such as the temperature of induction, which in each case bears a striking relationship to the organism's growth temperature range (for eg, Deegenaars and Watson, 1997; 1998), many however, are universal. Of major significance is the now well established fact that hsps or closely related proteins are also induced when organisms are subjected to a wide range of environmental stresses other than heat. Among these are ethanol, osmotic shock, sodium arsenite, heavy metals, amino acid analogues, oxidative agents, anaerobiosis, viral infection and abnormal protein synthesis (Lindquist and Craig, 1988; Watson, 1990). Appropriately, the more general term "stress proteins" has been coined to signify this class of proteins. It is generally found that stress proteins are not synthesized exclusively in response to stress, rather, the proteins are produced constitutively in unstressed cells under

physiological conditions (Lindquist and Craig, 1988). Under stressful conditions, increased stress protein synthesis is postulated to confer resistance against intracellular damage and facilitate recovery and survival.

In yeast, intracellular content of the disaccharide trehalose has also been shown to increase in response to various stresses (see section 1.6) and has consequently been implicated in conferral of thermotolerance (Attfield, 1987; De Virgilio *et al.*, 1991; Hottiger *et al.*., 1992). In addition to synthesis of trehalose and hsps, stress elicits a multiplicity of other physiological perturbations including cessation of mitosis and DNA synthesis (Kramhoft and Zeuthen, 1971) damage to nucleoli and nuclear actin filaments, and blockage of the assembly and export of ribosomes from the nucleus (Pelham, 1984; Welch and Suhan, 1985). Generally, molecular effects include repression of many constitutively expressed genes by virtue of changes in RNA processing, mRNA instability, and translation and transcriptional termination (Lindquist, 1980; Morimoto *et al.*, 1990).

1.2 Heat shock protein classification

In the seventeen years following the report by Tissières *et al.* (1974) of enhanced synthesis of several proteins upon subjecting cells to heat, an impressive literature has accumulated that describes a wide variety of events associated with the heat shock response. In fact, the number of publications produced, concerning hsps alone, was reported to exceed 800 per annum (Somero, 1995). Various forms of stress proteins, most of which are produced constitutively, and the wide variety of stresses which induce elevated synthesis of hsps, have added complexity to the nature of the stress response. To more precisely define a heat shock protein, Schlesinger (1986) restricted this designation to those proteins whose synthesis is strongly stimulated by an environmental stress, in particular that resulting from a temperature upshift a few degrees above the normal physiological temperature, and those proteins whose encoding genes contain one or more heat shock consensus sequences in the 5' noncoding region, the heat shock element (HSE) (Pelham, 1982). This sequence serves as the promoter for transcription of the heat shock gene (HSP) and is essential for the heat shock inducibility of transcription from these genes (see section 1.4.1). Within these constraints, the major heat shock proteins were classified into four main groups based on relative molecular weights estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). These groups were 82-90 kDa, the 70 kDa family, the 60 kDa family and the small hsps. The latter consists of a diverse

group of proteins ranging from 14 to 50 kDa. Only a few forms have been found in yeast and human cells including ubiquitin, an 8.5 kDa stress-inducible protein found in all eukaryotes examined, with approximately 30 different forms identified in higher plants (Morimoto *et al.*, 1990). In addition, some larger heat shock proteins of 100-110 kDa with properties that differ from those of other families have been characterized in mammalian and yeast cells (Sanchez and Lindquist, 1990; Watson, 1990; Sanchez *et al.*, 1992).

A marked feature of the heat shock proteins is their high degree of conservation from bacteria to humans. Proteins belonging to the hsp 70 group, for instance, are among the most highly conserved proteins known. For example, hsp 70 exhibits 50 % sequence conservation between *E. coli* and human, with some domains showing 96 % conservation (Schlesinger, 1990). At the amino acid residue level, the hsp 70 from *D. melanogaster* exhibits >70% homology with the human hsp 70 equivalent (Watson, 1990). Furthermore, in eukaryotes, ubiquitin exhibits total amino acid sequence homology in organisms as diverse as humans, fish and insects (Finley *et al.*, 1988) with the yeast ubiquitin 96% homologous to that of animals. The ubiquity of the heat shock response and the striking evolutionary conservation of the stress proteins, coupled with their constitutive expression in unstressed cells, suggests there exists a fundamental role for these proteins in cellular function (Lindquist and Craig, 1988; Watson, 1990; Schlesinger, 1990; Craig and Gross, 1991; Panaretou and Piper, 1992). Moreover, the observation that hsps are also developmentally regulated (Bienz, 1984; Bond and Schlesinger, 1987) further attests to their importance in basic cell physiology.

1.3 Groups of heat shock genes in *Saccharomyces*

1.3.1 *HSP 104*

It has become recognized that hsps of approximately 100 kDa are synthesized in most organisms in response to hyperthermia (Plesofsky-Vig and Brambl, 1993). In *S. cerevisiae*, a 104 kDa stress-inducible nucleolar protein (Lindquist and Craig, 1988) was demonstrated to be required for induced thermotolerance (Sanchez and Lindquist, 1990). Transformation with the wild type gene of mutant cells deficient in *HSP 104*, was found to rescue their heat sensitive phenotype, suggesting a possible role for hsp 104 in cell survival at extreme temperatures. *HSP 104* encodes a 908 amino acid (aa) protein with a predicted relative mass (M_r) 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 proteolysis (Parsell *et al.*, 1991). Hsp 104 synthesis has been shown to increase markedly following heat shock, entry into stationary phase, upon sporulation and during growth on acetate (Sanchez *et al.*, 1992). It has also been implicated in conferral of tolerance to ethanol, arsenite, long term cold storage (Sanchez *et al.*, 1992) as well as temperature-dependent barotolerance (Iwahashi *et al.*, 1997). Investigation of the phenotypes of *hsp 104 / hsp 70* double mutants, suggested a functional relationship between the two proteins. Hsp 70 was found to partially compensate for hsp 104 in thermotolerance acquisition, whereas hsp 104 synthesis compensated for hsp 70 during growth (Sanchez *et al.*, 1993). However, constitutive HSP 104 expression does not regulate the expression of other HSPs (Vogel *et al.*, 1995) nor affect the accumulation of trehalose in exponential cells (Winkler *et al.*, 1991). On the other hand, in stationary phase cells a synergistic effect has been observed between hsp 104 and trehalose. Mutants defective in both hsp 104 and trehalose exhibit heat shock sensitivity whereas single mutants exhibit wild type phenotypes (Elliot *et al.*, 1996). Hsp 104, in cooperation with hsp 70, has also been shown to affect ATP-dependent repair of mRNA splicing subsequent to heat shock (Vogel *et al.*, 1995). Yeast mRNA precursors are particularly susceptible to the latter process, with the exception of most HSPs, which generally lack intervening sequences (Vogel *et al.*, 1990). In addition, using *in vitro* heat-inactivated luciferase assays, Parsell *et al.* (1994) have shown that hsp 104 is functional in disaggregation and resolubilization of heat damaged proteins. A more obscure role for hsp 104 was recently demonstrated in maintenance of protein synthetic fidelity by regulation of inheritance of the extrachromosomal genetic element, *PSI+*, a yeast prion (Lindquist *et al.*, 1995; Schirmer *et al.*, 1996).

1.3.2 *HSP 90* gene family

The *HSP 90* gene family in *S. cerevisiae* constitutes two members exhibiting 97 % sequence homology (Craig, 1992). One gene, *HSC 82*, is constitutively expressed at high levels, exhibiting moderate heat-inducibility. Whereas the other, *HSP 82*, is expressed at low levels under normal physiological conditions, exhibits strong heat-inducibility (Lindquist and Craig, 1988; Borkovich *et al.*, 1989; Craig, 1992) and increased expression upon entry into stationary phase and transfer into sporulation medium (Kurtz and Lindquist, 1984; Borkovich *et al.*, 1989).

HSP 90 protein products, hsp 82 (M_r 81 419) and hsc 82 (M_r 80 885) are highly conserved, exhibiting 50 % aa identity among eukaryotes and in excess of 40 % identity to the *HTPG* gene of *E. coli* (Farrelly and Finkelstein, 1984; Lindquist and Craig, 1988; Borkovich *et al.*, 1989). Experiments in site-directed mutagenesis have indicated that a deficiency in both *HSP 90*s results in cessation of growth at any temperature, suggesting they are essential genes, which is consistent with their abundant synthesis in the cytosol of eukaryotes (Borkovich *et al.*, 1989). While a defect in either *HSP 82* or *HSC 82* alone does not affect growth at 25°C (Borkovich *et al.*, 1989), suggesting related functions, overexpression has been shown to result in decreased thermotolerance and decreased growth at 37.5°C (Cheng *et al.*, 1992).

In vertebrates, hsp 90 has been shown to bind to a variety of proteins, including steroid hormone receptors (Pratt *et al.*, 1992; Picard *et al.*, 1990) and viral tyrosine kinases (Brugge, 1986). Recently, it has been demonstrated that hsp 90 forms a supercomplex with other proteins, including hsp 70 (specifically ssa and sti1p) and DnaJ. This supercomplex facilitates protein folding and renaturation of damaged proteins in the eukaryotic cytosol (Wiech *et al.*, 1992; Chang and Lindquist, 1994; Bose *et al.*, 1996; Schumacher *et al.*, 1996; Yonehara *et al.*, 1996).

1.3.3 *HSP 70* multigene family

In most, if not all, eukaryotes, the heat-inducible *HSP 70* gene is one of a number of related genes. It was believed the family comprised at least eight identifiable genes in *S. cerevisiae*, related to *HSP 70* of higher eukaryotes (Lindquist and Craig, 1988). The structural relationships amongst these genes is complex and nucleotide identities range from about 50-97 % (Craig *et al.*, 1990). Based on structural and genetic analysis, the individual members of the *HSP 70* gene family have been allocated into one of five functional subgroups. To date, ten genes have been coined 'Stress Seventy' (SS) genes and divided into subgroups A, B, C, D and E, yielding SSA, SSB, SSC, SSD and SSE subgroups respectively (Craig, 1989; Mukai *et al.*, 1993).

Hsp 70 proteins exhibit high levels of conservation, with approximately 50 % aa identity across all species characterized in this regard (Parsell and Lindquist, 1994). In addition, the hsp 70s contain well-conserved ATP-binding (Flaherty *et al.*, 1990; McKay, 1991; McKay *et al.*, 1994) and carboxy-terminal substrate domains (Hightower *et al.*, 1994) and have been found to bind diverse substrates, actively facilitating protein folding,

unfolding, assembly and disassembly (Gething and Sambrook, 1992). However, these functions are not carried out independently. For example, in *E. coli*, the hsp 70 homologue, DnaK, functions in cooperation with hsps DnaJ and GrpE (Georgopoulus *et al.*, 1994). Eukaryotic DnaJ homologues include protein products of *SIS1* (Luke *et al.*, 1991) and *YDJ1* (*MAS5*), which are situated in the cytosol (Caplan and Douglas, 1991; Atenico and Yaffe, 1992). *YDJ1* expression in yeast has been shown to be essential for growth at high temperature, is moderately heat shock-inducible and its product, ydj1p, functions in mitochondrial protein import in association with ssa proteins (Atenico and Yaffe, 1992). The *SIS1* product, sis1p, has been demonstrated to interact with ssb1p (Caplan *et al.*, 1992). A function in mitochondrial protein transport, as well as involvement in protein secretion via the ER has also been reported for the yeast DnaJ homologue, scj1p (Blumberg and Silver, 1991). Also, another DnaJ homologue, sec 63, is required for translocation of proteins into the ER (Sadler *et al.*, 1989). Investigations with SEC 63 disruption mutants indicated inhibition of growth at high temperatures and accumulation of secretory precursors in the cellular cytoplasm (Rothblatt *et al.*, 1989).

Subgroup SSA

Subgroup SSA, the most complex, comprises 4 genes, *SSA1*, *SSA2*, *SSA3* and *SSA4*. These genes encode an essential family in the sense that the protein product of at least one must be present in the cellular cytoplasm at relatively high levels for cell survival (Deshaies *et al.*, 1988; Craig, 1989; Craig *et al.*, 1990). While the four *SSA* genes share 84-99 % aa identity (Boorstein *et al.*, 1994; Craig *et al.*, 1994), they exhibit varying patterns of regulation. *SSA1* and *SSA2* are both constitutively expressed, whereas *SSA1* is expressed at higher levels following heat shock and *SSA2* remains about the same. *SSA3* and *SSA4*, constitutively expressed at very low levels, are both induced within minutes of a shift to 37°C (Craig, 1989; Craig *et al.*, 1990). Constitutive *SSA2* expression accounts for 75 % of total *SSA* expression (Sanchez *et al.*, 1993).

Both biochemical and genetic evidence indicates that ssa proteins are involved in transport of proteins across membranes from the cytoplasm into the ER and mitochondria (Deshaies *et al.*, 1988; Craig, 1989; Craig *et al.*, 1990). They have apparent functions in refolding of proteins in cooperation with other hsps such as ssc1p (kar2p) (Sanders *et al.*, 1992; Bush and Meyer, 1996). However, ssa1p and ssa2p do not appear to function in the folding of nascent polypeptides (Bush and Meyer, 1996). *SSA* protein products have also

been shown to have a negative regulatory affect on heat shock induced gene expression (Stone and Craig, 1990; Nelson *et al.*, 1992a; Craig *et al.*, 1994). In addition, members of the ssa subgroup exhibit an association with ubiquitin-dependent protein degredation (Craig *et al.*, 1994). It appears that as a result of their protein-binding ability, they may temporarily protect aberrant proteins from proteolysis (Craig *et al.*, 1994).

Subgroup SSB

The SSB subgroup is composed of two members, *SSB1* and *SSB2*. These two genes are expressed at moderately high levels in logarithmic phase cells, whereas expression ceases following a temperature upshift to 37°C (Craig, 1989). Strains containing mutations in both genes are found to be relatively cold sensitive for growth (Craig and Jacobsen, 1984). This particular phenotype correlates well with the lack of expression of the *SSB1* and *SSB2* genes upon a heat shock and their higher transcript levels during steady-state growth at 23°C (Craig, 1990). *SSB* protein products, ssb1p and ssb2p, which are 99.3 % identical, have been shown to be essential for optimal growth at lower temperatures (Craig, 1989; Craig *et al.*, 1994). The *SSB* protein products appear to be part of a complex, as determined by biochemical fractionation, suggesting they are not soluble components of the cytoplasm (Craig *et al.*, 1990). In fact, the ssb proteins of *S. cerevisiae* have been reported to be nucleolar specific and associated with the snR10 and snR11 small nuclear RNAs (Clark *et al.* 1990). In addition, a role for ssb proteins in assisting passage of nascent polypeptide chains through ribosomes has been suggested, based on their close association with translating ribosomes (Nelson *et al.*, 1992b).

Subgroup SSC

The sole member of the SSC subgroup, *SSC1*, is an essential gene, constitutively expressed at moderate levels with a 10-fold increase in expression upon heat shock (Craig *et al.*, 1989; Craig, 1990). The *SSC1* protein product, ssc1p, is distantly related to other hsp 70s with aa identities ranging from 54.5-49.6 % (Boorstein *et al.*, 1994). It is found to be situated in the mitochondrial matrix (Craig *et al.*, 1987; Craig *et al.*, 1989) and is proposed to function in the translocation of precursors into the matrix, its inactivation resulting in inhibition of mitochondrial protein import (Craig *et al.*, 1990; Kang *et al.*, 1990). In more recent investigations, Nwaka *et al.* (1996) demonstrated that an *ssc1-3* mutant, defective in the ATPase binding domain, was compromised in survival following a

50°C stress, despite a 37°C pre-treatment. The results implied that substrate binding of denatured proteins, and the mitochondrial hsp 70, are essential for survival following stress.

Subgroup SSD

The SSD subgroup consists of an individual gene *SSD1*, sometimes referred to as *KAR2* (Craig, 1989). Gene disruption experiments have shown that *KAR2* is an essential gene (Rose *et al.*, 1989). *KAR2* expression is moderate under normal physiological conditions and increases upon heat shock (Craig, 1989). The *KAR2* gene product, ssd1p, is found in the lumen of the ER and genetic results suggest that it may be directly involved in the translocation of proteins into this organelle (Vogel *et al.*, 1990; Craig *et al.*, 1990). The mammalian homologue of ssd1p, BiP (immunoglobulin heavy chain binding protein) is essentially identical to grp 78, a glucose regulated protein (Vogel *et al.*, 1990). Although not conclusive, data suggests that BiP inhibits transport of misfolded or denatured, glycosylated secretory proteins from the ER to the golgi body (Deshaies *et al.*, 1988; Pelham, 1989).

Subgroup SSE

It has previously been shown that several hsp 70 proteins possess a highly conserved calmodulin-binding domain and this may have an influence on their function (Stevenson and Calderwood, 1990) (see section 1.7). This finding led to the discovery of two additional, essential, members of the *HSP 70* group, *SSE1* and *SSE2*, isolated on the basis of the Ca^{2+}-dependent calmodulin binding properties of their protein products (Mukai *et al.*, 1993). Both genes are moderately expressed at normal growth temperature, however, *SSE1* exhibits slight heat shock-inducibility at 37°C whereas *SSE2* is subject to marked heat shock induction. The protein products, sse1p (M_r 77 408) and sse2p (M_r 77 619), exhibit 76 % aa identity with each other but only 13-28 % with other hsp 70s. Although the function of neither protein has been elucidated, disruption mutants of *SSE1* result in slow growth at any temperature, while disruption of SSE2 has no observable phenotypic effect (Mukai *et al.*, 1993).

1.3.4 *HSP 60*

The hsp 58 isolated from the protozoan *Tetrahymena thermophila* (McMullin and Hallberg, 1988), was found to be induced in response to heat shock and is a member of an immunologically conserved family represented in *E. coli* and mitochondria of plants and animals. The *HSP 60* homologue from *S. cerevisiae* was cloned and sequenced by Reading *et al.* (1989). It was found to be a nuclear gene exhibiting sequence homology to the groEL protein of *E. coli* and the rubisco-binding protein (RBP) of chloroplasts (Ostermann *et al.*, 1989). The *HSP 60* gene codes for a polypeptide of 572 amino acids (Reading *et al.*, 1989), is constitutively expressed and resides in the mitochondrial matrix as an oligomer of 14 subunits (Osterman *et al.*, 1989). *HSP 60* mRNA is induced 2-3-fold above basal levels when cells are heat shocked, in agreement with the heat shock inducibility of its protein product, reported by McMullin and Hallberg (1988). Together with the structurally related groEL and RBP, hsp 60 belongs to a subclass of molecular chaperones termed "chaperonins", which are protein factors assisting in oligomeric protein assembly (Cheng *et al.*, 1989). Osterman *et al.* (1989) have identified hsp 60 as an essential component of the proteinaceous machinery required for refolding of proteins after translocation across the mitochondrial membrane. It 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 hsp 60 and hsp 70 with various proteins at elevated temperature has been postulated to prevent protein aggregation during stress and facilitate recovery via ATP-dependent protein refolding (Martin *et al.*, 1992; Parsell and Lindquist, 1994; Frydmar and Hartl, 1994). Hsp 60, also referred to as 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) associated with the highly conserved cpn 10, which forms a single heptameric ring (Lubben *et al.*, 1990; Hartman *et al.*, 1992; Frydman and Hartl, 1994). This complex structure enables ATP-dependent folding and release of nascent polypeptides and denatured proteins (Parsell and Lindquist, 1994).

A cytosolic hsp 60 homologue to mouse Tcp-1 (tailless complex polypeptide) has also been identified in yeast (Ursic and Culbertson, 1991) and partial sequence similarity has been reported (Ellis, 1990; Gupta, 1990). In eukaryotes, *TCP-1* expression is not heat shock-inducible, in contrast to the *TCP-1* of archae and groEL in eubacteria and mitochondria (Ellis, 1996). Consequently, to distinguish between various members of the

hsp 60s, Ellis (1992, 1996) has suggested a division between groEL found in eubacteria, plastids and mitochondria and the *TCP-1* family found in archae and the eukaryotic cytosol.

1.3.5 *HSP 30*

Hsp 30 was originally isolated from cell membrane fractions and with the exception of hsp 70 (López-Ribot and Chaffin, 1996), is the only other membrane-localized hsp identified to date (Panaretou and Piper, 1992). *HSP 30* expression is induced upon entry into stationary phase, upon nutrient limitation and following exposure to 6 % ethanol (Régnacq and Boucherie, 1993; Piper *et al.*, 1994). It encodes a 332 aa protein (M_r 37 044) and has a putative glycosylation site, three protein kinase C phosphorylation sites and a cAMP-dependent protein kinase site and is also highly hydrophobic (Régnacq and Boucherie, 1993). The total cellular composition of hsp 30 is significantly less than other soluble hsps such as hsps 104, 90, 70 and 26 (Piper *et al.*, 1994). The proposed function for hsp 30 constitutes reduction of plasma membrane damage during and subsequent to heat shock, either by controlling membrane structure or interacting directly with essential membrane proteins such as ATPase (Panaretou and Piper, 1992).

1.3.6 Small HSPs

Stress-induced synthesis of members of the small hsp family is ubiquitous among eukaryotes; however, there is a great diversity in both the numbers of small hsps described for any one species and in their calculated molecular weights. Small hsps from different species exhibit only limited overall homology, whereas certain structural features are common to most (Tuite *et al.*, 1990). Despite considerable heterogeneity, even within species, the small hsps of different organisms are clearly related (Lindquist and Craig, 1988). They have similar hydropathy profiles and small regions of amino acid identity (Rossi and Lindquist, 1989), share the property of forming highly polymeric structures (Lindquist and Craig, 1988) and exhibit significant homology to the eye lens protein α-crystallin (Ingolia and Craig, 1982; de Jong *et al.*, 1993; Plesofsky-Vig *et al.*, 1992). Furthermore, the small hsps share the property of being regulated by both stress and development (Susek and Lindquist, 1990; Tuite *et al.*, 1990; Bentley *et al.*, 1992).

HSP 26

The hsp 26 of *S. cerevisiae* is present in normal, unstressed cells. Synthesis of hsp 26 is inducible by a temperature increase and *HSP 26* transcript accumulates during developmental changes associated with transition from logarithmic to stationary phase growth and upon onset of sporulation (Kurtz *et al.*, 1986). In contrast to other *HSP*s, which are regulated by transcriptional activation, transcription of *HSP 26* appears to be regulated by a mechanism of basal repression during growth at normal temperature and derepression during heat shock (Susek and Lindquist, 1990). The *HSP 26* product is a 213 aa protein (M_r 27 000) with its N-terminal methionine cleaved posttranslationally (Bossier *et al.*, 1989). Rossi and Lindquist (1989) have concluded that the intracellular location of hsp 26 in yeast depends more upon the physiological state of the cell than upon the presence or absence of stress. In heat shocked exponential phase cells, hsp 26 is localized to the nucleus but is re-localized during stationary phase growth. A role for hsp 26 has been sought in sporulation, stationary phase growth, thermotolerance, ethanol tolerance, germination and aerobic and anaerobic growth at various temperatures on different carbon substrates. However, its function remains elusive, as inactivation of the *HSP 26* gene had no apparent affect on performance or survival under these conditions (Kurtz *et al.*, 1986; Petko and Lindquist, 1986; Susek and Lindquist, 1989, Tuite *et al.*, 1990). These observations lead to the suggestion that yeast may possess one or more as yet unidentified small hsps, which could compensate for the lack of hsp 26 function (Petko and Lindquist, 1986; Bossier *et al.*, 1989). More recently, it has been shown that hsp 26 is also induced by osmostress (Varela *et al.*, 1992).

HSP 12

Praekelt and Meacock (1990) reported the isolation of another small heat shock gene from *S. cerevisiae*. *HSP 12* encodes a 14.4 kDa polypeptide with structural similarity but limited sequence identity to other small heat shock proteins. *HSP 12* gene expression was found to be induced several hundred-fold following heat shock and on entry into stationary phase, with constitutive expression below detectable levels in exponential growth. Analysis of *HSP 12* expression in mutants affected in cAMP-dependent protein phosphorylation indicated that the gene is also regulated by cAMP and a disruption of the *HSP 12* coding region had no effect on the ability of cells to acquire thermotolerance (Praekelt and Meacock, 1990). Although a function for hsp 12 has not been demonstrated,

it has been reported to exhibit induction by oxidative stress (Jamieson *et al.*, 1994), ethanol (Piper *et al.*, 1994) and osmostress via the high-osmolarity glycerol pathway (Varela *et al.*, 1992; Varela *et al.*, 1995).

1.3.7 Other *S. cerevisiae* HSPs

From 500 *S. cerevisiae* proteins investigated, Miller *et al.* (1982) reported approximately 80 to be heat shock-inducible, many of which were not further characterized. Moreover, in excess of 2000 of some 6000 genes of the *S. cerevisiae* genomic complement have no assigned function or structural homologues (Dujon, 1996; Hieter *et al.*, 1996). It therefore seems probable that other heat shock-regulated proteins have yet to be identified. Several additional hsps to those already discussed have been characterized to some extent and are listed in Table 1.1.

Ubiquitin

The most highly conserved stress-inducible protein in eukaryotic cells is ubiquitin (reviewed by Watson, 1990). The protein comprises a 76 aa monomeric unit that is encoded by a family of natural gene fusions, either to itself as a polyubiquitin gene or to unrelated aa sequences (Finley *et al.*, 1987). The polyubiquitin gene has heat shock regulatory elements (see section 1.4) and its expression increases 5-7-fold following stress (Schlesinger, 1990). In this context, an important role for ubiquitin in the stress response is the removal of abnormal or denatured proteins (Parag *et al.*, 1987; Grant *et al.*, 1989). Moreover, ubiquitin is found to covalently bind to various acceptor proteins, thereby regulating a number of cellular processes, many of which are related to the stress response (Watson, 1990). These include selective degradation, whereby the ubiquitin system tags specific proteins for proteolysis (Hilt and Wolf, 1992), 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). Recently, over-expression studies have demonstrated that excess levels of this protein elicit increased tolerance to osmostress, ethanol and canavanine (an amino acid analogue) but did not affect thermotolerance. In addition, over-expression was observed to decrease tolerance to cadmium, arsenite and paromycin, which causes accumulation of aberrant proteins (Chen and Piper, 1995).

Table 1.1. HSPs characterized in *S. cerevisiae* other than members in distinct families.

HSP	Properties and notes	References
HSP 150	Secretory glycoprotein induced by heat and nitrogen limitation, which accumulates in ER where it refolds damaged proteins.	Russo *et al.*, 1992a,b Russo *et al.*, 1993 Jämsä *et al.*, 1995
HSP 118	Acidic cAMP regulated glycoprotein that exhibits increased synthesis upon heat shock and sulfur starvation.	Verma *et al.*, 1988a,b
STI1	Encodes a 589 aa protein (M_r 66 246) that activates expression of *SSA4*. Induced in response to canavanine treatment and stationary phase. Required for normal growth at elevated or lowered temperatures.	Nicolet and Craig, 1989
	Sti1p is related to MAP KKK and is part of the *HSP 90* supercomplex.	Chang and Lindquist, 1994 Chang *et al.*, 1997
TIP	Cell wall mannoprotein with possible function in stress protection of cell membrane.	Kowalski *et al.*, 1995 van der Vaart, 1995
	Induced in response to heat and cold shock.	Kondo and Inouye, 1991
TPS1 / TPS2	Encode t-6-p synthase (56 kDa) and t-6-p phosphatase (103 kDa) respectively. *De novo* synthesis and activity increases in response to heat shock.	Hottiger *et al.*, 1987 Neves and François, 1992
MnSOD	Yeast mitochondrial manganese form of superoxide dismutase. Exhibits increased synthesis upon ethanol exposure. Postulated to trap superoxide radicals within mitochondria, limiting their release into the cytosol.	Costa *et al.*, 1993 Piper, 1993
CTT1	Encodes antioxidant cytosolic catalase T. Exhibits negative cAMP regulation and is induced in response to oxidative and osmotic stress.	Belazzi *et al.*, 1991 Wieser *et al.*, 1991 Miralles *et al.*, 1995
DDR2 / DDR48	Encode DNA damage proteins and are induced in response to heat, DNA damaging agents and UV irradiation.	McClanahan and McEntee, 1986
hsp 48 **hsp 35** **phospho-glycerate kinase**	Glycolytic enzymes, enolase (hsp 48), glyceraldehyde-3-phosphate dehydrogenase (hsp 35) and phosphoglycerate kinase, which exhibit heat shock-inducible synthesis.	McAlister and Holland, 1985a,b Iida and Yahara, 1985 Piper *et al.*, 1986 Lindquist and Craig, 1988 Boucherie *et al.*, 1995a
	Enolase also exhibits increased synthesis in response to sulfur starvation and upon entry into stationary phase.	Iida and Yahara, 1985

1.4 Eukaryotic HSP regulation

1.4.1 Heat shock element (HSE)

The regulation of transcription following a heat shock has been studied in a variety of eukaryotes, in particular *S. cerevisiae*, *Drosophila* and mammalian cells. A similar mechanism is responsible for regulation in these diverse organisms (Bienz and Pelham, 1987). All eukaryotic heat shock genes contain within their promoters at least one copy of the heat shock element (HSE), a highly conserved DNA regulatory sequence that is both necessary and sufficient for heat inducible transcriptional regulation (Sorger and Nelson, 1989). HSEs are binding sites for the heat shock transcription factor (HSF) (Sorger and Pelham, 1987). HSEs, like many enhancer sequences, are active at variable distances from the start site of transcription (Craig and Gross, 1991). Generally, HSEs are located upstream of the TATA box in eukaryotic HSPs, often in multiple copies with a consensus sequence of C-GAA-TTC-G (Bienz and Pelham, 1987). The regulation of heat shock genes is somewhat different in animal and yeast cells. In mammalian (HeLa) and *Drosophila* cells, the HSF binds to DNA only after heat shock (Sorger *et al.*, 1987). In *S. cerevisiae*, the HSF is unique in that it binds to DNA under non-stressed or stressed conditions and is necessary for much of the high constitutive expression of several HSPs (Craig and Gross, 1991). Transcriptional activation appears to involve a conformational change in the HSF (Sorger *et al.*, 1987). Increased phosphorylation of the HSF correlates with an increased ability to promote transcription in all eukaryotes thus far examined. Analyses of deletion mutants of HSF suggest that the activity of a constitutive activator is repressed in the absence of heat shock by adjacent regions of the protein (Sorger and Nelson, 1989; Sorger, 1990). Phosphorylation may serve to maintain a conformational change in the HSF, thereby unmasking this activator domain (Craig and Gross, 1991).

1.4.2 Stress response element (STRE)

A HSF-independent stress control element, the stress response element (STRE), has been identified in promoter regions of cytoplasmic catalase T and the DNA damage responsive gene DDR2 (see Table 1.1) (Kobayashi and McEntee, 1990; Wieser *et al.*, 1991; Kobayashi and McEntee, 1993; Marchler *et al.*, 1993). STREs constitute an AGGGG or CCCCT consensus sequence (Kobayashi and McEntee, 1990; Wieser *et al.*, 1991) and have been demonstrated to bind a 140 kDa polypeptide (Kobayashi and

McEntee, 1993). No evidence is available to suggest that this protein be involved in transcriptional regulation (Ruis and Schüller, 1995). However, the high osmolarity glycerol MAP kinase pathway (HOG; Schüller *et al.*, 1994) and the RAS-protein kinase A pathway (Thevelein, 1991; Durnez *et al.*, 1994) have both been implicated in STRE regulation. STRE activation in response to a variety of stresses including osmotic stress, oxidative stress, N_2 starvation (Marchler *et al.*, 1993), ethanol, weak organic acids and low external pH (Schüller *et al.*, 1994) has been reported. Various STRE-like elements have also been identified in promoter regions of HSPs including *SSA3* (Boorstein and Craig, 1990), ubiquitin (Kobayashi and McEntee, 1993; Schüller *et al.*, 1994) and *TPS2* (see section 1.6) (Gounalaki and Thireos, 1994). Stress induced transcriptional activation in *S. cerevisiae* has been recently reviewed by Ruis and Schüller (1995) and Mager and de Kruijff (1995).

1.5 Current awareness concerning functions of hsps

1.5.1 Protein transport (chaperone function)

The scope of the data describing the variety of events in a cell's response to a heat shock ranges from X-ray crystallographic measurements and physical chemical studies on specific hsps to the effects of heat shock gene expression on an organism's ecological niche. Emphasis in this field has focused on the function of various hsps and their role as "molecular chaperones". Laskey *et al.* (1978) to describe the role of a nuclear protein, nucleoplasmin, in assisting the *in vitro* assembly of nucleosomes from isolated histones and DNA originally coined the latter term. Ribulose bisphosphate carboxylase-oxygenase (rubisco) binding protein was also described as a molecular chaperone due to the requirement of rubisco for assembly in chloroplasts (Barraclough and Ellis, 1980; Musgrove *et al.*, 1987). Early investigations led to the suggestion that hsps 70 and 90 were essential for protein assembly and disassembly in the cytosol, nucleus and ER under normal physiological conditions (Pelham, 1986). It was further proposed that during stress, an increase in hsp synthesis facilitated dissolution of protein aggregates, protein refolding and prevention of further aggregation as a result of binding to exposed hydrophobic surfaces. The term "chaperonin" was subsequently coined for GroEL of *E. coli* and the related mitochondrial hsp 60 of eukaryotes, as a consequence of their high sequence conservation to rubisco binding protein (Hemmingsen *et al.*, 1988).

More recently, Hendrick and Hartl (1993) suggested a definition for molecular chaperones to which most hsps (see section 1.3) comply (recently reviewed in Hartl, 1995; Ellis, 1996; Hartl, 1996; Hayes and Dice, 1996; Langer *et al.*, 1996). Specifically, a molecular chaperone may be 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).

1.5.2 Conferral of thermotolerance

Many studies have shown that a pre-shock treatment can render a biological system more resistant to a subsequent stress and that this protective affect is transient (Watson, 1990). Initial studies with *S. cerevisiae* indicated a close relationship between the rate of hsp synthesis and heat shock acquisition of thermotolerance to a challenge of 52°C for 5 min (McAlister and Finkelstein, 1980). However, continuing research over the ensuing seventeen years has not resolved the relationship between hsps and induced thermotolerance. The specific nature of such unequivocal evidence and the controversy surrounding a thermoprotective role for hsps is discussed at length in Chapters 3, 5 and 6 of this thesis. Critical examination of available data highlights a key parameter that is rarely probed with respect to such experiments. Namely, the kinetics of the loss of thermotolerance during recovery of cells at normal growth temperature (Watson, 1990) and this phenomenon is investigated in detail in Chapters 5 and 6 of this work. The likelihood of the involvement of specific hsps in thermotolerance has been made untenable by gene cloning experiments (for eg, hsp 70, Craig *et al.*, 1989; hsp 12, Praekelt and Meacock, 1990). Furthermore, stimulation of stress protein synthesis does not always correlate with acquisition of thermotolerance, which in turn can be induced in the absence of several hsps (Watson, 1990). Nevertheless, some hsps appear necessary but not sufficient alone for thermotolerance, which clearly appears to be important for survival under stress conditions (Schlesinger, 1990). Despite equivocal data relating stress protein synthesis and acquired thermotolerance hsps clearly have important functions in the unstressed cell. These functions may form part of a complex mechanism that facilitates protection and recovery of cells from stress. Given the highly conserved nature of the stress proteins it is inconceivable that the heat shock response is a laboratory artefact (Watson, 1990).

1.5.3 Hsps as immune targets and other medical implications

A role of hsps that has been clearly established is their involvement in immunity. Hsps appear to be major targets of cell-mediated immunity with circulating antibodies and activated T cells having specificity for a number of hsps in diverse organisms (Lindquist and Craig, 1988; Young and Elliot, 1989). Investigations of antigens involved in the immune response to tuberculosis and leprosy bacilli revealed that stress proteins (hsp 70 and hsp 60) are immunodominant targets of both antibody and T cell responses (Young *et al.*, 1988; Shinnick *et al.*, 1988). These observations, in addition to the high conservation and abundance of major stress proteins in other organisms, gave impetus to the idea that stress proteins are likely to be immune targets in many non-viral infections (Young and Elliot, 1989). It has since been reported that a wide variety of infectious agents have been identified as stress proteins. Hsp 60 appears to be the major stress protein antigen recognised by antibodies in bacterial infections while the hsp 70s have been identified as immune targets in all major human parasitic infections including malaria, leishmaniasis, schistosomiasis, filariasis, trypanosomiasis and tuberculosis (Young and Elliot, 1989; Watson, 1990; Cohen and Young, 1991) Furthermore, antigens from a large number of infectious bacterial agents (including *Mycobacterium*, *Shigella*, and *Salmonella*) and pathogenic fungi (including *Aspergillus fumigatis*, *Fonsecae pedrosoi* and *Histoplasma capsulatum*) have been identified as members of hsp families (Maresca and Kobayashi, 1996). Many pathogenic microbes when exposed to a hostile environment experience a stress on entry into their respective host and synthesize hsps as a consequence. The invading microbe also elicits production of hsps by the host to the stress imposed by invasion and intracellular growth of the microbe (Schlesinger, 1990). Antibodies and T cells recognize epitopes on these proteins and destroy cells containing microbes as well as stressed autologous cells to limit the extent of infection and protect the host from disease (Young and Elliot, 1989, Schlesinger, 1990).

Immunological reactions to hsps 60 and 70 have been implicated in neurogenerative diseases, cancer and inflammatory processes associated with pathogens (Garbe, 1992; Maresca and Kobayashi, 1996). The constitutive synthesis and high conservation of hsps raises the possibility of an anti-self stress protein immune response leading to autoimmune disease such as rheumatoid arthritis and multiple sclerosis (Watson, 1990; Georgopoulos and McFarland, 1993). In fact, hsp 60-induced auto-immunity has been illustrated in

animal models and many people inflicted with autoimmune diseases have antibodies that recognize hsps within their sera as well as hsps at the site of immune-mediated tissue damage. However, despite the association of adjuvant arthritis in rats with mycobacterial hsps (van Eden *et al.*, 1988), no direct involvement in human rheumatoid arthritis has been demonstrated for these proteins (Yaung and Feige, 1992; Mollenhauer and Schulmeister, 1992; Gaston and Pearce, 1996).

Another role of hsps is in immunity-antigen presentation. Hsps may assist in several steps including the recognition of aberrant or misfolded proteins, the targeting of antigens to intracellular organelles, membrane translocation of antigens and activation of protein unfolding and partial degredation, resulting in the release of peptides suitable for binding to major histocompatability complex (MHC) molecules and subsequent T cell recognition (Young and Elliot, 1989; Schlesinger, 1990). Hsp deletions may be useful in vaccine production against attenuating pathogens. However, sequence conservation among host and pathogen hsps would prove problematic in such an enterprise (Schoel and Kaufmann, 1996). Further detail on current views concerning roles of stress proteins in the immune response is available in a recent review by van Eden and Young (1996).

1.6 Trehalose

For many years it was generally accepted that the function of the non-reducing disaccharide trehalose (α-D-glucopyranosyl (1-1)-α-D-glucopyranoside) in yeast, was that of a storage form of energy, in a similar manner to glycogen (Thevelein, 1984). The concept that trehalose may function as a stress protectant was initially approached by Keller *et al.* (1982). Intracellular accumulation of trehalose has since been correlated with exposure to heat shock and toxic chemicals (Attfield, 1987), growth on respiratory carbon sources and nutrient starvation (Lillie and Pringle, 1980; Hottiger *et al.*, 1987; Hottiger *et al.*, 1989; Attfield *et al.*, 1992).

In yeast, trehalose is synthesized in a two-step reaction (Cabib and Leloir, 1958). In the first step, trehalose-6-phosphate synthase catalyses the transfer of glucosyl residues from UDP-glucose (UDPG) to glucose-6-phosphate (g-6-p) to form trehalose-6-phosphate (t-6-p). Subsequently, the phosphate group is cleaved from t-6-p by t-6-p phosphatase to form trehalose. T-6-p synthase and t-6-p phosphatase exist in the cell as a complex, encoded by the *TPS1* (or *CIF1*) (Bell *et al.*, 1992) and *TPS2* (De Virgilio *et al.*, 1993)

genes, respectively. *TPS1* and *TPS2* encode 56 and 100 kDa subunits of the complex, respectively, and the third 123 kDa subunit, encoded by *TSL1*, has been proposed to have a regulatory function in addition to some t-6-p phosphatase activity (Vuorio *et al.*, 1993). Heat shock inducibility and transcriptional activation of *TPS1* and *TPS2* were outlined in Table 1.1 and section 1.4.2.

The observation that the accumulation of trehalose correlated with induction of thermotolerance in yeast, led to its proposed function as a stress-protectant (Attfield, 1987; Van Laere, 1989; Wiemken, 1990; De Virgilio, 1994). However, both the mechanism regulating trehalose accumulation and mobilization and its role in thermotolerance have been subject to a controversy that continues. For example, findings that the enzyme neutral trehalase (see below) regulated trehalose concentration (De Virgilio *et al.*, 1991) were disputed by workers who reported its accumulation in response to an increase in the substrates UDPG and g-6-p (Winkler *et al.*, 1991; Ribeiro *et al.*, 1994). In addition, another group claimed that changes in the kinetics of t-6-p synthase / phosphatase activities during heat shock, as opposed to their *de novo* synthesis, was responsible for trehalose accumulation (Neves and François, 1992). Moreover, conflicting reports that cycloheximide does (Ribeiro *et al.*, 1994; Lewis *et al.*, 1995) or does not (De Virgilio *et al.*, 1990; Winkler, 1991; Neves and François, 1992) affect trehalose accumulation, further add to the contention. Despite some observations that trehalose confers significant intrinsic stress tolerance during respiratory growth (De Virgilio *et al.*, 1994; Attfield *et al.*, 1994; van Dijck *et al.*, 1995), several researchers have demonstrated that its accumulation alone is insufficient to account for acquired thermotolerance. For example, Winkler *et al.* (1991) showed that a *hsp 104* mutant, capable of accumulating heat shock-induced trehalose, was unable to acquire thermotolerance. Using a t-6-p synthase mutant, Argüelles (1994) demonstrated that thermotolerance acquisition occurred in the absence of trehalose accumulation, complying with results of more recent work involving a neutral trehalase mutant, which also demonstrated a lack of correlation between trehalose and tolerance (Nwaka, *et al.*, 1995). However, trehalose has been shown to provide *in vitro* protection of proteins and lipids against damage by freeze-thaw, high temperature shift and desiccation (Hottiger *et al.*, 1994). In addition, it has also been implicated in stabilization of isolated proteins including phosphofructokinase, lactate dehydrogenase and the restriction endonuclease *Pst*I (Carpenter *et al.*, 1987; Carpenter, 1993; Sen *et al.*, 1993). More recently, a role for trehalose in barotolerance and resistance to hydrostatic pressure

was also revealed (Fernandes *et al.*, 1997). At a metabolic level, trehalose has been implicated in the regulation of glycolysis and sugar-phosphate/free phosphate balance (Panek and Matoon, 1977; Thevelein and Hohman, 1995). Van Aelst *et al.* (1993) have also suggested a role for the disaccharide as a glucose sensor, based on the observation that growth on glucose was inhibited in a *TPS1* mutant.

In Chapters 5 and 6 of this thesis, an attempt to more clearly discern the significance of trehalose in heat stress protection was made by investigating possible correlation with loss of thermotolerance following return of cells to control temperature and degradation of previously heat shock-induced trehalose. Trehalose is mobilized or hydrolyzed by the enzyme trehalase, three forms of which have been identified in yeast. Neutral trehalase, encoded by the *NTH1* gene, is responsible for intracellular hydrolysis of trehalose and is regulated by temperature, nutrient supply and cAMP-dependent phosphorylation (Londesborough and Varimo, 1984; App and Holzer, 1989; Kopp *et al.*, 1993). A second form, vacuolar acid trehalase, encoded by the *ATH1* gene, is responsible for hydrolysis of extracellular trehalose and is regulated by nutrient supply (Mittenbühler and Holzer, 1988; Destruelle *et al.*, 1994; Destruelle *et al.*, 1995). A third form, nth1p, encoded by the *NTH2* gene, a homologue of *NTH1*, is also regulated by temperature and nutrient availability (Wolfe and Lohan, 1994) but its function in trehalose mobilization remains unknown. Both *NTH1* and *NTH2* genes are heat shock-inducible and have been shown to be essential for recovery of cells from a 50°C heat stress (Nwaka *et al.*, 1995). Other physiological parameters such as spore germination and resumption of growth on ethanol and other nonfermentable carbon sources are also dependent on hydrolysis of trehalose (Souza and Panek, 1968; Thevelein, 1984). For a comprehensive review on trehalose and its role pertaining to thermotolerance, the interested reader is referred to Nwaka and Holzer (1998).

1.7 Other heat shock-inducible factors: a menagerie of metabolic mechanisms

In addition to hsps and trehalose, there exist numerous other factors that play possible roles in the response of yeast cells to hyperthermia (reviewed by Piper, 1993). Briefly, these include intracellular levels of cAMP (Cameron *et al.*, 1988; Piper, 1990; Coote *et al.*, 1992), activation of plasma membrane ATPase (Panaretou and Piper, 1990; Coote *et al.*, 1994), decrease in intracellular pH (Cole and Keenan, 1986; Weitzel *et al.*, 1987; Eraso

and Gancedo, 1987) and the position of the cell in its cycle (Plesset *et al.*, 1987; Barnes *et al.*, 1990; Elliot and Futcher, 1993). A more detailed description is presented in the epilogue of this thesis and Fig. E1.

A further factor possibly contributing to regulation of the stress response, not described above and of particular relevance to theories proposed in Chapters 5 and 6 of this work, is activation of hsps via posttranslational modification. In this regard, some recent findings concerning calmodulin-dependent protein phosphorylation were of particular interst. *S. cerevisiae* contains at least two genes encoding calmodulin-dependent protein kinase II (CaM kinase II), *CMK1* and *CMK2* (Ohya *et al.*, 1991; Pausch *et al.*, 1991) and a single calmodulin (CaM) gene (*CMD1*) that is essential for cell growth (Davis *et al.*, 1986). CaM is a ubiquitous Ca^{2+}-dependent binding protein, which interacts with in excess of 20 different proteins and is a key molecule for signal transduction in eukaryotic cells (Cohen and Klee, 1988). CaM has been shown to bind members of the hsp 70 family (Stevenson and Calderwood, 1990) and hsp 90, inhibiting its binding to F-actin in a Ca^{2+}-dependent manner (Nishida *et al.*, 1986; Minami *et al.*, 1993). It is reported that heat shock induces a rapid increase in the influx of Ca^{2+} in mammalian (Stevenson *et al.*, 1986, 1987) and yeast cells (Kamada *et al.*, 1995). Recently, investigations concerning the effect of mutations in *S. cerevisiae* CaM and CaM kinase II on the induction of thermotolerance indicated that both are required and specifically, *CMK1* may preferentially phosphorylate substrates required for tolerance acquis tion (Iida *et al.*, 1995). Interestingly, yeast cells possess mechanosensitive ion channels that are activated by membrane expansion and capable of importing Ca^{2+} (Gustin *et al.*, 1988). 'Membrane stretch' has subsequently been demonstrated to comprise another component of the *S. cerevisiae* heat shock response (Kamada *et al.*, 1995).

1.8 Thesis aims

The majority of this thesis comprises investigations conducted with two industrial (baking) strains of *S. cerevisiae*. The two strains had been previously characterized in terms of their thermotolerance phenotypes and were reported as exhibiting contrasting, relative thermoresistance (Yres) and thermosensitivity (Ysen) (Gross and Watson, 1992). This presented an unique opportunity to investigate in further detail comparative analyses of the heat shock response using the Yres/Ysen model system, and constitutes the general aim conceived upon initiation of the research project.

Specific aims that arose during the course of experimentation were as follows:

1. To determine the relative contributions of hsps and trehalose to thermotolerance in *S. cerevisiae* strains to contribute towards understanding of the heat shock response.
2. To determine the affect of derepressive metabolism on thermotolerance characteristics of the strains and ascertain the relative contribution of hsps and trehalose under these conditions.
3. To investigate hsp regulation and synthesis and trehalose mobilization during recovery from hyperthermia and determine any correlation with the kinetics of loss of thermotolerance.
4. To investigate whether constitutive or pre-existing hsps have the capacity to convey thermotolerance in the absence of *de novo* protein synthesis, and whether apparent hsp levels affect the extent of tolerance.
5. To identify novel genes induced in the thermotolerance phenotype, in an attempt to contribute to knowledge concerning regulation of the heat shock response, using the novel technique of differential display of reverse transcribed, PCR amplified mRNA.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used were of analytical grade or higher. Specific chemicals and materials were obtained from the manufacturers listed below.

Amersham (Castle Hill, NSW, Australia): Enhanced chemiluminescence (ECL) western blotting detection kit, Hybond N^+ nucleic acid transfer membrane, Hybond C super, Hyperfilm-MP.

Amrad-Biotech (Dupont; Boronia, Victoria, Australia): [α^{32}P]-dATP (~110 TBq mmol^{-1}, 3000 Ci mmol^{-1}), [α^{33}P]-dATP (37-110 TBq mmol^{-1}, 1000-3000 Ci mmol^{-1}).

BDH/Merck Chemicals (Kilsyth, Victoria, Australia): Acrylamide, Ammonium peroxodisulphate (AMPS), Bromophenol blue, Ethanol (95% v/v), Ethidium bromide, β-Mercaptoethanol, NN'-Methylenebisacrylamide, Sodium dodecyl sulphate (SDS), N,N,N'N'-Tetramethylenediamine (TEMED), Xylene cyanol.

Bio 101 (La Jolla, California, USA): Geneclean kit.

Bio-Rad (Sydney, NSW, Australia): Agarose (ultra-pure DNA grade), Cellophane membrane backing, Filter backing paper, Kaleidoscope prestained standards, Silver stain kit, Silver stain plus kit, SDS-PAGE molecular weight standards (low range).

Boehringer Mannheim (Castle Hill, NSW, Australia): Acetic acid test kit (test-combination 148 261), Bovine serum albumin (BSA; endotoxin-free, fatty acid-free, sterile lyophilised), Glucose indicator strips (Diabur-test 5000), Phenylmethylsulphonylfluoride (PMSF), RNase-free DNase.

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

Calbiochem (Alexandria, NSW, Australia): Diethylpyrocarbonante (DEPC).

Davis Gelatine (Botany, NSW, Australia): Technical grade agar.

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

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

Display Systems Biotechnology (Los Angeles, California, USA): Differential Display Kit.

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

Gibco BRL (Melbourne, Victoria, Australia): Superscript RNase H$^-$ reverse transcriptase, *Taq* DNA polymerase (5 units μl^{-1}).

ICN Biomedicals (Seven Hills, NSW, Australia): Agarose (genetic technology grade), Saturated phenol.

ICN Radiochemicals (Seven Hills, NSW, Australia): Trans ^{35}S-Label (75% L-methionine, ^{35}S and 15% L-cysteine, ^{35}S).

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

Progen (Darra, Queensland, Australia): pUC 19/*Hpa*II DNA ladder.

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

Pharmacia (Sydney, NSW, Australia): Ficoll 400, Electrode paper Novablot, Cellophane sheets, Nick Spin Columns.

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

Promega (Annandale, NSW, Australia): Acetylated BSA, Lambda DNA, Recombinant ribonuclease inhibitor (RNasin), Restriction endonucleases and buffers, Herring sperm DNA.

Sigma (Castle Hill, NSW, 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 Yeast and bacterial strains

Two wild type, industrial (baking) strains of *Saccharomyces cerevisiae*, designated Yres and Ysen (Gross and Watson, 1992), were ATCC 38554 (isolated from canned cherries) and a strain from Burns Philp Food and Fermentation, Sydney, Australia (isolated from Dutch yeast product), respectively.

S. cerevisiae strains Klg 102 (a *mal cif1*) and VFP1-8C (α *MAL4^c CIF*) (Ferreira and Panek, 1993) were gifts from Dr A. Panek (Universidade Federal do Rio de Janeiro, Brasil).

E. coli JPA 101, genotype: *sup*E *end*A *hsd*R- ϕ*str*A *svc*B Δ(*lac-pro*) F[*tra*V35 *pro*AB+ *lac*I^q *lac*Z ΔM15] (J.P. Adelman, Genentech, CA, USA), was used for plasmid DNA transformation.

2.1.3 HSP probes

Recombinant plasmids harbouring *HSP 104*, *HSP 70* (SSA1) and *ACT1* inserts were gifts from Drs S. Lindquist (University of Chicago, Chicago, Illinois, USA), E. Craig (University of Wisconsin Medical Center, Madison, Wisconsin, USA) and C. Gancedo (Universidad Autonoma de Madrid, Spain), respectively. To construct probes, restriction endonuclease digests were performed to produce a 1.14 kb *Eco*RI/*Hin*dIII *HSP 104* fragment from pYS104 (Sanchez and Lindquist, 1990), a 1.3 kb *Kpn*I/*Pst*I *SSA1* fragment from CEN30:SSA1vb (Craig and Jacobsen, 1984) and a 1.75 kb *Bam*HI/*Hin*dIII *ACT1* fragment, originally from pYactI (Ng and Abelson, 1980) inserted into pUC18 (construct pJJ16, C. Gancedo).

2.1.4 Anti-hsp antibodies

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 (raised against amino acids 317-706 constituting the C-terminus of *S. cerevisiae* hsp 82) was a gift from P. Piper (University College London, London, UK).

2.2 Methods

2.2.1 General

All media and solutions were prepared with water treated by Millipore Milli-Q filtration (Millipore, Lane Cove, New South Wales, Australia). All media and solutions requiring sterilization were autoclaved at 121°C for 15 min.

2.2.2 Culture conditions

Culture maintenance

Yeast cultures were maintained on YEP agar plates and stored at 4°C. YEP medium contained 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% KH_2PO_4, 0.3% $(NH_4)_2SO_4$ and 2% glucose. *E. coli* JPA 101 was maintained on LM agar plates which consisted of 1% tryptone, 0.5% yeast extract, 10mM NaCl and 10mM $MgSO_4.7H_2O$.

Subcultures were made every 2-4 weeks to maintain viability. Concentrated aliquots of liquid cultures were stored frozen at -70°C in 15% and 50% glycerol for yeast and *E. coli* respectively. Plates were inoculated from frozen stocks every 4-6 months.

Media

YEP and LM media were prepared as 10 x stocks and stored at -20°C until required. Glucose was added to diluted YEP stock subsequent to autoclaving, to avoid generation of H_2O_2, a consequence of heating glucose and phosphate together in culture medium (Woods and Jones, 1986). H_2O_2 imposes a stress on yeast cells (reviewed in Moradras-Ferreira *et al.*, 1996) and as the present studies focus on analyses of hyperthermia, it was important to avoid extraneous stress factors. Ampicillin was added to cooled YEP and LM media to a final concentration of 25 $\mu g\ ml^{-1}$ and 50 $\mu g\ ml^{-1}$, respectively, subsequent to autoclaving.

Solid medium contained 1-1.5% agar, which was added prior to autoclaving. Agar plates were poured immediately on cooling to avoid pH changes arising in media when solidified agar is re-heated (Bridson, 1990).

Starter cultures

Starter cultures were generated by inoculating a loop of streak culture plate into 20 ml of liquid medium in a 50 ml conical flask. Flasks were incubated at 25°C for *S. cerevisiae* and 37°C for *E. coli* in an orbital shaker (Paton Industries, Victor Harbour, South Australia) operating at 180 o.p.m. Generally, yeasts were incubated for 30-40 h while *E. coli* was grown overnight.

Experimental cultures

For some experiments, yeast was grown in YEP supplemented with either 2% (w/v) glucose (YEPG) or 1% (w/v) potassium acetate (YEPA). Most experiments however, were conducted with yeast grown in 0.67% yeast nitrogen base without amino acids (Difco),

0.3% KH_2PO_4 (YNB), supplemented with either 2% glucose (YNBG) or 1% potassium acetate (YNBA).

Experimental yeast cultures were prepared by inoculating the appropriate volume of YEPG, YEPA, YNBG or YNBA in a conical flask with a 0.1 - 2% inoculum of starter culture and grown to the required cell density under the same conditions described for starter cultures. In the case of logarithmic phase cultures, approximately $3x10^6$ – $6x10^6$ cells ml^{-1} corresponded to an optical density at 600 nm of 0.15 – 0.3. Glucose depletion in post-fermentative cultures was assessed using glucose indicator strips (Diabur-Test 5000, Boehringer Mannheim) which were accurate for glucose concentrations below 0.75%.

The use of YNB medium facilitated [^{35}S]-methionine labelling and for the purpose of consistency, all analyses of Yres and Ysen subsequent to Chapter 3 were conducted with cells grown on YNB. In the case of mutant Klg 102 and its derivative control strain VFP1-8C, YEPG grown cells were washed in an equal volume of YNBG and resuspended in 2ml of the same prior to [^{35}S]-methionine labelling.

2.2.3 Growth curves

Cell density and doubling time

Yeast cultures were incubated as described above and sampled at various times throughout the course of incubation. The optical density of the culture was read at 600 nm (OD_{600}) against a reference of the appropriate medium. Viable counts were obtained by spread plating 0.1 ml of decimal dilutions of culture in YEP onto YEPG agar plates. Dilutions were made using as a guideline the estimation that an OD_{600} of 1 corresponds to $3x10^6$ colony forming units ml^{-1} (cfu ml^{-1}) for *S. cerevisiae* (Ausubel *et al.*, 1988). Dilutions were plated in duplicate and an average count of plates containing between 30 and 300 colonies was used to calculate the number of cfu ml^{-1} present. Growth curves were constructed and used to determine the time taken for culture density to double, referred to as doubling time.

Glucose concentration

Accurate determination of glucose content in media was facilitated by use of the Glucose test kit 510 (Sigma Diagnostics). The enzymatic procedure is based on the glucose oxidase-peroxidase reaction.

Ethanol concentration

The ethanol content in cultures was determined by gas chromatography. Samples, taken at required intervals, were frozen until analyzed, when they were thawed and centrifuged. A 1µl aliquot of supernatant was injected onto a Porapak Q column (Alltech Australia, Homebush: glass column length 2m, internal diameter 2mm, mesh range 80/100) in a Packard 427 gas chromatograph (Packard Instrument Company, Downers Grove, USA). The following temperature settings were employed: injector 220°C, oven 190°C, detector 220°C. Sample detection was by flame ionization. Retention time for ethanol was 2-3 min and the response was quantified against an external standard.

Acetate concentration

Acetate content in cultures was determined using an enzymatic test kit (Acetic acid test-combination 148 261, Boehringer Mannheim). The determination is based on the formation of NADH measured by an increase in absorbance at 340 nm. The recovery of an internal standard, using a standard solution supplied with the kit, was found to be 97%. This was indicative that sample solution was relatively free of interfering substances.

2.2.4 Heat treatment and thermotolerance assays

Intrinsic thermotolerance of exponentially growing cells was determined by rapidly raising the temperature from 25°C to 50°C in a 70°C waterbath, with subsequent incubation at the appropriate stress temperature (50, 52 or 55°C) in an oscillating waterbath. Samples were taken at various times during a 60 min time course. Induced thermotolerance and its rate of decay were measured by subjecting 200 ml cultures to a heat shock at 37°C for 30 min. Immediately following heat shock, a 20 ml sample was transferred to a 50 ml flask and exposed to a heat stress time course as described above. The remaining culture was briefly cooled on ice, transferred back to 25°C and sampled for intrinsic thermotolerance at 30, 60, 90 and 240 min following return to the control temperature. Return of cultures to 25°C following sublethal heat treatment, described in Chapter 5, was defined as recovery. For work presented in Chapter 6, re-induction of thermotolerance was determined following return of 200 ml heat shocked cultures (37°C / 30 min) to 25°C. After 30 and 60 min incubation at the control temperature, two 20 ml samples were transferred to 50 ml flasks. One sample was exposed to a heat stress time course to monitor loss of thermotolerance

induced by initial heat shock treatment. The second sample was exposed to a second heat shock and subsequent heat stress time course to assess re-induction of thermotolerance. Similarly, return of cultures to 25°C following heat shock at 37°C was defined as recovery and exposure of recovering cultures to a second heat treatment was termed re-heat shock.

Where required, protein synthesis was inhibited by the addition of 20 μg ml^{-1} cycloheximide, 20-30 min prior to heat treatment. In the case of recovery experiments, 200 ml cultures were centrifuged and washed twice, in the appropriate medium, prior to return to control temperature. The concentration of cycloheximide required to inhibit protein synthesis was determined initially by the level required to arrest cell growth (results not shown) and the efficacy of the procedure confirmed by SDS-PAGE analysis of protein profiles. Samples heat shocked in the presence of cycloheximide showed no protein synthesis by [^{35}S]-methionine labelling while resumption of protein synthesis was noted in washed cultures which had been allowed to recover at 25°C.

Reproducibility

Each experiment was performed a minimum of three times. Small fluctuations in temperature and in particular culture density, were found to have significant affects on absolute tolerance values obtained from one experiment to the next, an observation which has also been previously noted (Sanchez *et al.*, 1993). However, the general shapes of survival curves and the relative differences between strains were consistent and highly reproducible. Tolerance curves for independent experiments performed in parallel for a given strain, in the same water bath, were essentially superimposable. As a consequence of culture variability however, statistical analysis of viability data was not practical or useful and for each case, representative or typical results are shown.

2.2.5 Trehalose determination

Trehalose was extracted from 80 ml of washed cells (5-10 mg dry weight). Duplicate samples of culture were taken during experiments, cells harvested by centrifugation (2500 g for 3 min) and washed twice with chilled distilled water. If trehalose was not to be assayed immediately, cell pellets were frozen until required (-20°C for up to 1 week). Trehalose content from extracts of frozen and unfrozen cells, from the same experiment, was found to be identical.

The extraction procedure adopted was outlined by Lillie and Pringle (1980). Cell pellets were resuspended in 3 ml of 0.5 M trichloroacetic acid (TCA) and placed on ice at 4°C with intermittent agitation during a 90 min incubation. Following centrifugation (2500 g for 3 min), the supernatant was frozen at -20°C and the extraction procedure repeated. Pooled extracts were stored at -20°C (for up to several days) until analyzed.

Assay for trehalose was carried out using the colorimetric method outlined by Stewart (1975), using a reagent constituting 0.5 % (w/v) anthrone and 1 % (w/v) thiourea made up in 66 % H_2SO_4 (Spiro, 1966). Duplicate 1 ml samples of extracts were added to 5 ml of anthrone reagent in 10 ml stoppered glass tubes on ice and subsequently transferred to a boiling water bath for 20 min. The samples were briefly cooled and the optical density (read at 620 nm against a TCA / anthrone reference) compared with a standard curve generated from the optical density of a series of glucose standards prepared at the same time.

When sampling for trehalose, the cells from 80 ml of culture for each experimental condition were washed twice as above and transferred to a pre-weighed 0.45 µm filter (Millipore) to determine dry weight. Filters were dried overnight at 60°C. Trehalose content was expressed as a percentage of the dry weight of cells. All experiments were repeated at least three times and results presented as averages of three typical experiments.

2.2.6 Protein analysis

Radioactive labelling

Protein synthesis in control, heat shocked, recovering and re-heat shocked cells was examined by incorporation of [^{35}S]-methionine into protein. Cultures (60 ml) were pelleted and resuspended in 2 ml of supernatant. [^{35}S]-methionine (100 µCi; *trans* [^{35}S] label, containing 75 % L-[^{35}S]-methionine and 15 % L-[^{35}S]-cysteine, specific activity 1150 Ci mmol^{-1}, ICN Radiochemicals) was added to both 25°C and 37°C test cultures, which were subsequently incubated for 30 min. In the case of recovery, label was added 10 min prior to each designated time point (30, 60, 90 and 240 min) and the sample incubated for 30 min at 25°C. Where required, cycloheximide was added 20-30 min prior to heat treatment as described in 2.2.4. Incorporation of label was terminated by transferring 1.5 ml samples to microfuge tubes containing 150 µl of 100 mg ml^{-1} unlabelled methionine. Cells were

pelleted by centrifugation, washed with 1 ml cold H_2O, pelleted again and the supernatant discarded. Tubes were placed on ice until the extraction was carried out.

Protein extraction

A modification of the general procedure outlined by McAlister *et al.* (1979) was employed to extract proteins. Washed cells, harvested as described above, were resuspended in 100 µl of extraction buffer (50 mM Tris-HCl pH 6.8, 2 % SDS, 2 mM EDTA, 1 % 2-mercaptoethanol and 2 % Ficoll 400) and 15µl of 0.1 M PMSF with an equivalent volume of 0.5 mm glass beads. The suspension was vortexed for 20 s and cooled on ice for 1-2 min. The latter process was repeated 5 times. Finally, cell debris was pelleted by high-speed centrifugation for 2 min in a microfuge, and supernatants, containing extracted proteins, transferred to clean tubes. For each extract, a 10 µl aliquot was taken aside to assay protein content. The bulk of the extract was mixed with an equal volume of sample buffer (0.25 mM Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS and 5 % 2-mercaptoethanol) in addition to 15 µl of 0.1 % bromophenol blue loading dye. Extracts were heated at 95°C for 2 min, dispensed into 20 µl aliquots and stored at -20°C until required.

Protein assay

The protein content of cell extracts was determined using the Coomassie microassay procedure (Pierce), based on the Bradford method (Bradford, 1976). Extract aliquots were diluted 1:100 in water and mixed with 1 ml Coomassie assay reagent in a disposable cuvette. The optical density of the solution was read at 595 nm against a reagent/water blank. The protein concentration was calculated by interpolation from a standard curve of 1-20 µg ml^{-1} BSA samples run at the same time.

Electrophoresis

SDS-polyacrylamide gels were used to separate 10 µg protein samples as well as low range molecular mass standards (Bio-Rad) according to the technique outlined by Laemmli (1970) and Laemmli and Favre (1973). The 4 % stacking gel consisted of 1.3 ml 30 % acrylamide / 0.8 % bisacrylamide, 7.24 ml H_2O, 1.25 ml 1 M Tris-HCl pH 6.8, 100 µl 10 % SDS, 100 µl 10 % AMPS and 10 µl TEMED. The 10 % resolving gel consisted of 13.32 ml 30 % acrylamide / 0.8 % bisacrylamide, 16.1 ml H_2O, 10 ml 1.5 M Tris-HCl pH 8.8, 400 µl 10 % SDS, 140 µl 10 % AMPS and 40 µl TEMED. Electrophoresis was performed at 10°C and 30 mA in a Protean II Slab Cell (Bio Rad) using the discontinuous buffer system of

Laemmli (1970). The gel running buffer contained per litre, 6 g Tris, 28.8 g glycine and 1 g SDS. Generally, the duration of electrophoresis was 6-8 h and was terminated once the bromophenol blue had migrated to the end of the gel.

Gel staining and autoradiography

Following electrophoresis, gels were fixed in a solution of 40 % (v/v) methanol and 10 % (v/v) acetic acid for at least 30 min and subsequently stained with a silver stain kit (Bio-Rad). Stained gels were dried between cellophane in a Bio-Rad model 543 Gel Slab Dryer at 80°C for 1 h. Dried gels were exposed to Hyperfilm-MP (Amersham) or Biomax MR film (Kodak) for 2-5 days at -70°C and developed using Kodak liquid X-ray developer and liquid X-ray fixer.

2.2.7 Western transfer and immunodetection

Unlabelled protein extracts from control, heat shocked, recovering and re-heat shocked cultures, as well as 15 µl kaleidoscope prestained markers (Bio-Rad), were subjected to SDS-PAGE in the manner described above. Following electrophoresis, gels were immersed in continuous transfer buffer (39 mM glycine, 48 mM Tris, 20 % (v/v) methanol and 0.0375 % SDS) for 20 min. Proteins were subsequently transferred to Hybond-C super nitrocellulose (Amersham) using the Novablot system (Pharmacia LKB), at 0.8 mA cm^{-2} of membrane for 1.5 h, according to manufacturers' instructions. Kaleidoscope markers were visible on membranes following transfer and enabled isolation of the portion of membrane containing proteins in the size range of interest (40-140 kDa). Membrane sections, stored overnight in phosphate buffered saline (PBS; 80mM Na_2HPO_4, 20 mM $NaH_2PO_4.H_2O$, 100 mM NaCl), were immersed in a solution of PBS-T (PBS, 0.1 % Tween 20) and 5 % skim milk powder (SMP) for 1 h with gentle agitation. Immuno-cross reaction with anti-hsp antibodies was carried out using the Amersham ECL western blotting detection kit in accordance with manufacturer's instructions. Membranes were rinsed three times in 40 ml PBS-T, incubated with primary antibody in PBS-T SMP, rinsed a further three times in PBS-T and incubated with secondary antibody (mouse or rabbit) in PBS-T SMP at a concentration of 1:1000. Final washes of membranes, prior to detection, were modified to 3 x 5 min in PBS / 0.3% Tween 20, followed by 3 x 5 min in PBS / 0.1% Tween 20. Primary antibodies comprising hsps 104, 70 (Affinity BioReagents), 60 (StressGen) and 90 (gift from Dr. P. Piper, University College, London) were used at dilutions of 1:1000, 1:10 000, 1:1000 and 1:750

respectively. Following incubation with ECL detection reagents, membranes were drained, wrapped in cling-wrap and exposed to autoradiographic film for periods varying between a few seconds to 5 min. It should be noted that anti-hsp 90 also recognizes the cognate *S.cerevisiae* hsc 82, detectable on immuno blots below the more highly heat inducible isoform hsp 82 (hsp 90).

2.2.8 Probe synthesis

Bacterial transformation

Recombinant plasmids harbouring *S. cerevisiae HSP 104* and *HSP 70* inserts, provided as dried pellets, were reconstituted in 50-100 µl TE buffer (10 mM Tris, 1 mM EDTA pH 7.4). Approximately 50-100 ng of plasmid was used for transformation of *E. coli* JPA 101. Transformation procedures performed herein, originally described by Cohen *et al.* (1972), were as outlined in Sambrook *et al.* (1989). Variations to the protocol included use of LM medium rather than LB or SOC and LM agar containing 50 µg ml^{-1} ampicillin prewarmed to 37°C for spread plating of transformed host culture. A control to determine the viability of the host strain, following exposure to $CaCl_2$ treatment, was incorporated by spread plating suitable dilutions of the transformed cells onto LM agar without ampicillin. Non-transformed cells were plated onto LM agar with ampicillin to monitor any revertants. LM agar plates were incubated at 37°C for 24 h.

Ampicillin resistance, conveyed upon host cells by genetic determinants harboured within recombinant plasmids, distinguished presumptive *E. coli* transformants. Several individual colonies were picked from LM / ampicillin plates, inoculated into 5 ml LM medium, containing 100 µg ml^{-1} ampicillin, and incubated overnight at 37°C and 180 opm. Cells were harvested from individual 5 ml cultures and plasmid preparations (see below) performed. To confirm the integrity of putative transformants, restriction endonuclease digests were carried out and analyzed by agarose gel electrophoresis (see below).

Plasmid DNA preparation

The alkaline lysis technique employed for the isolation of plasmid DNA from transformed *E. coli* was based on the method of Birnboim and Doly (1979) and described in Sambrook *et al.* (1989). Plasmid DNA preparations were undertaken in volumes not exceeding 1.5 ml ('mini-preps') and if larger yields were required, the number of small-

scale preparations was increased. Modifications to the procedure included culturing in and harvesting *E. coli* from 2 ml of LM medium rather than LB, and an increase in lysozyme concentration to 2 mg per preparation. In addition, 5 µl of 10 mg ml^{-1} RNase (made up in 10 mM Tris pH 7.5, 15 mM NaCl) was added for 30 min at 37°C prior to phenol / chloroform extraction. Plasmid DNA was precipitated with 2 volumes of 95 % ethanol at -20°C for periods in excess of 30 min.

Restriction endonuclease digests

Plasmid DNA preparations were digested with appropriate restriction enzymes (2 µl plasmid DNA in 10 µl total volume) to validate presumptive transformants. Following validation, digests were repeated in large scale (20-50 µl plasmid DNA in 200-500 µl total volume) in order to isolate and purify sufficient quantities of specified probe fragments. Digests were incubated for 1-2 h at 37°C, with salt and buffer conditions as specified by the manufacturer (Promega). Acetylated BSA (1:10) was added to the reaction mixture to improve stability of restriction enzymes. Where suitable, reactions were terminated by incubation at 70°C for 10 min and samples immediately cooled on ice. Plasmid DNA was precipitated from the reaction mixture by addition of 2 volumes of 95 % ethanol. Following centrifugation at 13 000 rpm for 10 min in a microfuge, plasmid pellets were dried and resuspended in 10 µl TE buffer (10 mM Tris, 1 mM EDTA pH 7.4). The same procedure was employed to produce DNA size markers from *Hin*d III or *Eco* RI / *Hin*d III restriction digests of λ bacteriophage DNA.

Agarose gel electrophoresis

Electrophoretic analysis was carried out in 1 % agarose gels made with 1 x TAE buffer from a 50 x stock solution (242 g Tris, 57.1 ml glacial acetic acid, 100 ml EDTA pH 8.0, per litre). Visualization of nucleic acids was facilitated by incorporation of 50 ng ml^{-1} ethidium bromide into the gel and 1 x TAE electrophoretic buffer. Gels were viewed and photographed under transmitted light on a UV transilluminator (302 nm). Exposures were taken with Polaroid 667 film (ASA 3000) in an MP-4 land camera fitted with a Kodak Wratten (22A) filter (aperture 11, exposure 6 s, development 30-60 s). The addition of 3 µl of tracking buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 30 % glycerol) was made to DNA samples prior to loading of gels. Electrophoresis was generally undertaken at 100 V for a period of 1-1.5 h.

DNA purification

Isolation and purification of DNA fragments from agarose gels, as well as elimination of unincorporated nucleotides from oligo-labelling mixtures (see below), was carried out using Geneclean (BIO 101) and Bresa-Clean (Bresatec) kits. The procedures are based on the method of Vogelstein and Gillespie (1979) and involve use of a silica matrix that binds DNA without binding DNA contaminants. Proteins and small RNAs as well as unincorporated nucleotides do not compete for binding sites on the matrix (from which the DNA is eventually eluted) and are thereby effectively eliminated. The procedures were performed as described by the manufacturers.

Oligo-labelling of HSP DNA probes

Following gel purification, the Gigaprime DNA labelling kit (Bresatec), in conjunction with [α-^{32}P]-dATP (50 µ Ci, specific activity 3000 Ci mmol^{-1}, Amrad Pharmacia), was employed to yield radiolabelled DNA probes with specific activities ranging from 1000-2000 cps. Based on the method described by Feinberg and Vogelstein (1983), the kit comprises random oligo-deoxynucleotides that serve as primers for initiation of DNA synthesis on single-stranded DNA templates, catalyzed by the large Klenow fragment of *E. coli* polymerase I. Three unlabelled dNTPs and [α-^{32}P]-dATP serve as precursors for DNA synthesis. Approximately 60-100 ng of *HSP 104*, *HSP 70* and *ACT1* gel purified restriction fragments were denatured and radiolabelled to generate probes used in subsequent hybridization procedures (see below). Where possible, probe fragments were selected to be within a size range of 1 kb for optimal efficiency of the labelling procedure. Unincorporated nucleotides were removed from the reaction mixture with either the Geneclean or Bresa-Clean process described above.

2.2.9 Total RNA preparation and slot blot hybridization

Total RNA was isolated from 160 ml of control, heat shocked, recovering and re-heat shocked cultures, essentially as described by Ausubel *et al.* (1988). The extraction procedure was carried out at 4°C. Cells were pelleted by centrifugation (3000 rpm for 10 min), resuspended in an equal volume of ice cold DEPC-treated H_2O, re-centrifuged and transferred to clean microfuge tubes in ml RNA buffer (0.5 M NaCl, 200 mM Tris pH 7.5, 10 mM EDTA). Tubes were centrifuged for 5 s at top speed in a microfuge, the supernatant discarded and cells resuspended in 300 µl RNA buffer. An equivalent volume

of chilled, acid-washed glass beads (0.5 mm) and 300 µl 25:24:1 phenol/chloroform/isoamyl alcohol (equilibrated with RNA buffer) were added to the cell suspension. The mixture was vortexed 2 min), centrifuged for 1 min and the supernatant transferred to a clean tube. The latter process was repeated. Three volumes (approximately 600 µl) ice cold 95 % ethanol was added to the pooled supernatants and the tubes were placed at -20°C overnight. Following ethanol precipitation, tubes were centrifuged and RNA pellets were allowed to dry and resuspended in 50 µl H_2O. Yields and purity were assessed spectrophotometrically on the basis that an OD of 1 at 260 nm corresponds to 40 µg ml^{-1} of RNA (Ausubel *et al.*, 1988) and values of 1.8-2.0 for the ratio OD_{260}:OD_{280} were assumed to indicate a pure preparation (Warburg and Christian, 1942). However, in a recent report, Glasel (1995) contested the validity of 260/280 nm absorbance ratios to monitor nucleic acid purities and concluded that the now widely used method is inaccurate. Consequently, verification of total RNA yields and integrity was also conducted electrophoretically in 1 % neutral agarose gels (Promega Notes **28**, December 1990). The procedure involved heat denaturation (65°C for 10 min) of total RNA samples (10 µg), previously combined with 15-20 µl RNA sample buffer (10 ml deionised formamide, 3.5 ml 37 % formaldehyde, 2 ml MOPS buffer) and 2-5 µl RNA loading buffer (50 % glycerol, 1 mM EDTA, 0.4 % bromophenol blue). Final yields were typically 0.5-3.0 µg $µl^{-1}$. Completed RNA preparations were aliquoted into several smaller samples and stored at -70°C until required.

Total RNA samples of 20 µg were made to 20 µl with DEPC-treated H_2O and mixed with 60 µl of a solution containing 50 % formamide, 7 % formaldehyde and 1 x SSC. Following a 15 min incubation at 68°C, samples were cooled on ice and transferred to Hybond-N^+ nylon membrane (Amersham) as outlined in Sambrook *et al.* (1989), using a Schleicher and Schuell slot filtration vacuum manifold. Membranes were subsequently baked at 70°C for 2 h, wrapped in cling wrap and stored at 4°C (up to several days) until required.

The method described by Rose *et al.* (1990) was adopted for hybridization of membranes with radiolabelled *HSP 104*, *HSP 70* and *ACT1* probes (see section 2.2.8). Initially, membranes were immersed in boiling H_2O and allowed to come to room temperature, which facilitated reduction of background hybridization. Subsequently, membranes were placed on 3MM filter paper backing (Reed, 1991) and incubated in 15 ml

of prehybridization solution (7.5 ml deionised formamide, 1.5 ml 100 x Denhardts solution, 3 ml 5 M NaCl, 750 µl 1 M Tris-HCl pH 7.5, 150 µl 10 % SDS, 1.8 ml H_2O and 200 µg ml^{-1} sheared denatured herring sperm DNA (Promega)). This was carried out in sealed plastic bags, in a 42°C water bath for at least 6 h. Following prehybridization, 1 ml hybridization solution (200 µl 100 x Denhardts solution, 100 µl 1 M Tris-HCl pH 7.5, 20 µl 10 % SDS, 650 µl H_2O and 200 µg ml^{-1} sheared denatured herring sperm DNA (Promega)), containing 10 µl radiolabelled, denatured DNA probe (see section 2.2.8), was added to the prehybridization mixture and the membranes further incubated at 42 °C for 16-24 h. Stringency washes involved successive 15-30 min incubations, twice with each of 2 x SSC, 2 x SSC / 0.5 % SDS (65°C) and 0.1 x SSC, with constant agitation.

Membranes were stripped for subsequent rehybridization following guidelines detailed in the Hybond-N^+ instruction booklet (Amersham). Hybridized membranes were exposed to Hyperfilm-MP (Amersham) at -70°C overnight. Solutions, generally treated with 0.1 % DEPC, as well as equipment, were treated to be RNase free as outlined in Sambrook *et al.* (1989).

2.2.10 mRNA differential display reverse transcriptase PCR (DDRT-PCR)

Total RNA preparation

Total RNA was isolated from 160 ml of YNBG, YNBG-heat shocked and YNBA cultures as described in 2.2.9, with the final pellet resuspended in 25 µl 0.1 % DEPC-treated H_2O. Total RNA suspensions were treated with 20 U RNase-free DNase (Boehringer) in the presence of 20 U recombinant ribonuclease inhibitor (RNasin, Promega) and purified with phenol/chloroform (1:1) as outlined in the Differential Display Kit manual (Display Systems Biotechnology). RNA concentrations were determined on the basis of optical density measurements at 260 nm (Ausubel *et al.*, 1988) and verified electrophoretically on 0.8 % agarose 1 x TAE gels. Solutions and equipment were treated to be RNase free as outlined in 2.2.9.

cDNA synthesis

Total RNA, from control (25°C) and heat shocked (37°C, 30 min) cells of repressed Ysen (YNBG), as well as derepressed cells (YNBA) of this strain, was reverse transcribed with SuperScript RNase H^- reverse transcriptase (Gibco BRL) as outlined in the

Differential Display Kit manual (Display Systems Biotechnology). Using anchored, downstream primers of the type $dT_{11}VV$ (V = A, C or G), 9 specific cDNA syntheses were carried out for each cell type of Ysen, resulting in 27 distinct cDNA pools. In each case, first strand cDNA was generated from 300 ng total RNA as template. Volumes of the cDNA master mix suggested by the manufacturer were adjusted to accommodate syntheses from RNA of three cell types (168 µl 5 x first strand cDNA synthesis buffer, 84 µl 0.1 M DTT, 34 µl 500 µM dNTP mix, 21 µl RNasin, 150 µl DEPC-treated H_2O).

PCR amplification and differential display

The mRNA differential display analyses were performed using the Differential Display Kit, strictly as described by the manufacturer. Generally, the protocol followed that originally described by Liang and Pardee (1992), with modifications outlined in Bauer *et al.* (1993). PCR amplification of each cDNA fraction was carried out with the corresponding 3' primer and each of 24 arbitrary, 10-mer 5' primers (see Table 7.1), resulting in 27 x 24 PCR incubations for the complete primary analysis of Ysen. PCR was carried out in the presence of [α-^{33}P]-dATP (1.75 µCi, specific activity 3000 Ci mmol^{-1}, Amrad Pharmacia) and 1 U of *Taq* DNA polymerase (5 units µl^{-1}, Gibco BRL) for 40 cycles (94°C for 30 s; 40°C for 60 s; 72°C for 60 s) followed by additional extensions at 72°C for 5 min, in a GeneAmp 2400 thermocycler (Perkin Elmer). Volumes of the PCR master mix suggested by the manufacturer (Display Systems) were adjusted to enable 27 x 24 PCR reactions from Ysen cDNA (1.5795 ml 10 x PCR buffer, 1.8954 ml 25 mM $MgCl_2$, 63.45 µl 500 µM dNTP mix, 5.7119 ml DEPC-treated H_2O). Nine 1.0278 ml aliquots (one for each downstream primer) of the PCR master mix were dispensed into microfuge tubes and stored at -20°C. When required, one tube was thawed and 8.85 µl [α-^{33}P]-dATP and 16.5 µl *Taq* DNA polymerase were added. From this mixture, 324 µl were transferred to each of three tubes (one for each cell type of Ysen) and supplemented with the appropriate downstream primer (54 µl 25 µM) and cDNA (27 µl). Samples of 15 µl of the pre-mixed components from each of the three tubes were then distributed into 24 PCR tubes (200 µl thin walled, Perkin Elmer) and 5 µl of the 24 different upstream primers (2µM) added. The 24 x 3 tubes were microfuged briefly and PCR carried out as described above. Resulting fragments were resolved on non-denaturing, 6 % polyacrylamide gels (150 ml 38 % acrylamide/2 % N-N-methylenebisacrylamide, 200 ml 5 x TBE, 650 ml

H_2O) as described by the manufacturer (Display Systems). The gels were dried on Whatman paper without fixing and autoradiographed overnight with Biomax MR film (Kodak).

To verify reproducibility of the technique, the analysis was repeated on newly isolated RNA from the three cell types of Ysen, as well as the strain Yres, using only those primer pairs that generated differentially expressed cDNAs in the primary analysis. Volumes of cDNA synthesis and DD-PCR master mix components were adjusted accordingly.

Re-amplification of cDNAs and northern hybridization

The autoradiograms and dried DDRT-PCR gels were oriented with asymmetric needle punctures and cDNA bands of interest were excised with a clean scalpel blade. The cDNA fragments were eluted and re-amplified, using the corresponding primer pair, as described in the Differential Display Kit manual. Following incubation in 100 µl DEPC-treated H_2O for 30 min and 100°C for 10 min, filter paper was discarded and reamplification carried out with the cycle protocol for DDRT-PCR. The reamplification mixture consisted of 26.5 µl eluted DNA, 5.0 µl 10 x PCR buffer, 6.0 µl 25 mM $MgCl_2$, 5.0 µl 500 µM dNTP mix, 2.0 µl 5 µM downstream primer, 5.0 µl 2 µM upstream primer and 0.5 µl *Taq* DNA polymerase. PCR products were run on 2 % agarose 1 x TBE gels against a pUC 19/*Hpa*II DNA ladder (Progen) to estimate their size.

Radioactive probes were generated from re-amplified cDNA fragments and used to screen northern blots of total RNA from the three cell types of both Ysen and Yres, in order to confirm their differential expression. Probes were labeled with [α-^{32}P]-dATP (30 µCi, specific activity 3000 Ci mmol^{-1}, Amrad Pharmacia) using PCR and purification procedures described by the manufacturer (Display Systems). The reaction mixture constituted 1.0 µl reamplified cDNA, 1.0 µl 10 x PCR buffer, 1.0 µl 500 µM dCTP, dGTP, dTTP mix, 3 µl [α-^{32}P]dATP, 2.0 µl 2 µM upstream primer, 2.0 µl 2 µM downstream primer and 0.5 µl *Taq* DNA polymerase. The PCR cycle protocol was the same as for DDRT-PCR but comprised only 15 cycles, followed by 5 min at 72°C. Unincorporated [α-^{32}P]-dATP was removed with pre-packed Sephadex G-50 nick spin columns (Pharmacia).

Total RNA (20 µg), isolated as described above and 0.24 - 9.5 kb RNA size markers (Gibco BRL) were separated on 1 % neutral agarose gels (see section 2.2.9). Transfer to Hybond-N^+ nylon membrane (Amersham) was carried out overnight with 20 x SSC as described in Sambrook *et al.* (1989). Membranes were placed at 70°C for 2 h following transfer and used immediately or stored at 4°C until required. Hybridization with radiolabelled cDNA fragments was performed as described in 2.2.9. Probe fragments generating hybridization signals corresponding to their patterns of differential expression were deemed true positives and excised from the membrane using the autoradiogram for band localisation. Captured cDNAs were subsequently eluted and reamplified, as described above, using the primer set that generated the fragment in DDRT-PCR. In some cases it was necessary to perform a second round of PCR amplification before any product could be visualised on an agarose gel.

Several membranes exhibiting hybridization patterns not corresponding to the original differential display patterns, were stripped of cDNA probes following guidelines detailed in the Hybond-N^+ instruction booklet (Amersham) and rehybridized with *ACT1* to serve as loading controls.

Cloning and sequencing of differentially displayed cDNA fragments

Re-amplified cDNA was blunt-end ligated into the *Sma*I site of pUC18, pre-treated with bacterial alkaline phosphatase, using the SureClone Ligation Kit (Pharmacia) and transformed into *E. coli* JPA 101 as described in 2.2.8. Plasmid preparations from recombinant hosts, selected on the basis of lac Z' inactivation (Sambrook *et al.* 1989), were performed as described in 2.2.8 and correct inserts identified by restriction digestion with *Eco*RI and *Hin*dIII, which flank the cloning site by 34 and 16 bp respectively. Resulting fragments were therefore 50 bp larger than the reamplified PCR products. It should be noted that numerous initial cloning attempts with the pCR-Script Amp SK (+) kit (Stratagene) proved unsuccessful.

Plasmid DNA sequencing was carried out with cycle sequencing using the ABI PRISM Dye Primer Ready Reaction Kit (Perkin Elmer) by the Sydney University and Prince Alfred Macromolecular Analysis Center. Sequences for each clone were obtained on both strands. Homology searches against databases were performed using the BLAST

computer program (Altschul *et al.* 1990) of the National Center for Biotechnology Information.