STRESS INDUCED CHANGES AT THE YEAST PLASMA MEMBRANE

Thesis submitted for the degree of doctor of philosophy

By

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I WANDERED LONELY AS A CLOUD

I wandered lonely as a cloud
That floats on high o' er vales and hills,
When all at once I saw a crowd, a host of golden daffodils;
Beside the lake, beneath the trees,
Fluttering and dancing in the breeze.

..............For oft, when on my couch I lie
In vacant or in pensive mood,
They flash upon that inward eye
Which is the bliss of solitude;
And then my heart with pleasure fill
And dances with the daffodils.

WORDSWORTH
ABSTRACT

In most organisms a mild sub-lethal stress increases the capacity for survival during a subsequent lethal stress. The mechanisms that implement this acquisition of tolerance have been subject to numerous studies. Best characterised is the induction of heat shock proteins (hsp) at elevated temperatures. Very little is known about heat shock associated events occurring at the plasma membrane. This is surprising bearing in mind that the first component of the cell to encounter certain types of environmental stress may be the cell envelope.

Work described in this thesis has identified several changes to yeast plasma membrane proteins with ethanol stress and weak acid stress. Two prominent changes to the protein composition of the S. cerevisiae plasma membrane were seen with 6% and 8% ethanol stress; (i) a marked decrease in levels of the H⁺-ATPase and (ii) the acquisition of hsp30. These are the same two changes that result from a sub-lethal heat shock. Weak acid stress was found to induce a protein of approx. 150kDa at the plasma membrane in both S. cerevisiae and Z. bailii. Protein sequencing identified this protein in S. cerevisiae as the product of the SNQ2 gene. Pulse labelling studies have shown this protein to be specifically induced by sorbic acid and not appreciably glycosylated. Salt and heat stress were found to induce this protein at the level of transcription but it is not seen by SDS-PAGE analysis of purified plasma membranes from cells exposed to these stresses.

A mutant disrupted in the HSP30 gene was generated in a protease deficient genetic background and used in comparative physiological studies with the wild type parent in order to establish a phenotype for loss of the heat shock protein. Thermotolerance and membrane permeability studies were carried out with the wild type and hsp30 strains but no significant difference between these strains was apparent. However the hsp30 mutant took longer to adapt to growth in the presence of sorbic acid.
ACKNOWLEDGMENTS

I would firstly like to thank Peter Piper for his understanding and patience with me. His guidance throughout this study has been inestimable. I am indebted to him for all the time and support he has given me.

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Finally, I would like to thank my family and friends for their financial and emotional support throughout. My parents in particular showed great patience with me for which I am very grateful. I dedicate this work to my parents and Harish, my brother.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>bis-acrylamide</td>
<td>N,N'-methylene bisacrylamide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(cyclohexylamino)-1-propanesulphonic acid</td>
</tr>
<tr>
<td>CCP</td>
<td>carbonyl cyanide m-chlorophenylhydrazone</td>
</tr>
<tr>
<td>Ci(μCi)</td>
<td>curies (microcuries)</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilboestrol</td>
</tr>
<tr>
<td>dH₂O</td>
<td>deionised plus 1 x distilled water</td>
</tr>
<tr>
<td>DNAase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>Fig</td>
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<td>h</td>
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<tr>
<td>HEPES</td>
<td>(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])</td>
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<tr>
<td>HSC</td>
<td>heat shock cognate</td>
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<tr>
<td>HSE</td>
<td>heat shock element</td>
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<tr>
<td>HSF</td>
<td>heat shock transcription factor</td>
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<tr>
<td>HSP</td>
<td>heat shock protein</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>Kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodaltons</td>
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<tr>
<td>KHZ</td>
<td>kilohertz</td>
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<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
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<tr>
<td>NEPHGE</td>
<td>non-equilibrium pH gradient gel electrophoresis</td>
</tr>
<tr>
<td>NP40</td>
<td>nonidet P40</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino terminal</td>
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<tr>
<td>ONPG</td>
<td>O-nitrophenyl-β-D-galactoside</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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PUBLICATION

Publication arising from work presented in this thesis:

(see pocket inside back cover)

CHAPTER 1

1. INTRODUCTION

1.1 Stress and the plasma membrane

Major environmental changes cause stress, damage or death. Not surprisingly organisms have evolved a variety of adjustments that help buffer the physiological impact of environmental change. These adjustments have been studied from diverse perspectives ranging from the gross changes in protein synthesis to the morphological and physiological alterations elicited by stress. Table 1.1 shows a variety of agents causing stress.

The plasma membrane delimits the cell, physically separating the cytoplasm from the surrounding cellular environment. Diverse organisms can adapt to changes in their environmental temperature by altering the lipid composition of their membranes. In general, the higher the growth temperature the more unsaturated the membrane fatty acid composition (Houslay and Stanley, 1982). This acts to offset the increase in membrane fluidity that occurs on temperature up shift. It is generally accepted that an increase in membrane fluidity will have two effects. Firstly, there will be a partial dissipation of ionic gradients that exist across membranes leading, for example, to altered intracellular pH and ionic composition. Secondly, the activities of membrane bound enzymes will be affected.

Even though different forms of stress must have marked effects on the activity or regulation of a number of key plasma membrane proteins, there are only a few examples in the literature
of such events. Among them is the four fold decrease in activity of $\text{Na}^+ / \text{K}^+$ ATPase when HeLa cells are shifted from 37°C to 45°C (Burdon and Cutmore, 1982). In addition, there is an overall decrease in the ability of ligands to bind to their receptors, as in the case of epidermal growth factor binding to its membrane receptor (Welch, 1990).

It is well established that specific stress proteins exist within different compartments of the eukaryotic cell, such as the endoplasmic reticulum, golgi body, mitochondrion, nucleus and nucleolus (Sambrook et al., 1989). Surprisingly, bearing in mind that the plasma membrane is damaged during stress, there have been very few studies on eukaryotic stress proteins associating with this membrane. A single heat shock protein, hsp30, has been found to be associated with the yeast plasma membrane under the stress conditions of heat shock and starvation (Panaretou and Piper, 1992). Beyond this there have been no other reports of stress proteins associating with this membrane in yeast. A 30kDa heat shock protein is associated with this membrane in soybean roots (LaFayette and Travis, 1990) and a 60kDa osmostress-induced protein in the alga Dunaliella (Fisher et al., 1994). It is nevertheless conceivable that cells target proteins with a damage limitation function to the plasma membrane as part of several inducible protective responses.
Table 1.1  Inducers of the heat shock response

<table>
<thead>
<tr>
<th>Inducing agent or treatment</th>
<th>Proposed effect</th>
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<tr>
<td>Heat shock</td>
<td>Increasing intracellular levels of denatured or aberrant protein.</td>
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<tr>
<td>Sodium arsenite</td>
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<td>Cadmium</td>
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<tr>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
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<tr>
<td>Amino acid analogues</td>
<td></td>
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<tr>
<td>Various heavy metals</td>
<td></td>
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<tr>
<td>Iodoacetamide</td>
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<tr>
<td>Return from anoxia</td>
<td>Oxygen toxicity, free radical fragmentation of proteins.</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
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<td>Superoxide ions</td>
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<tr>
<td>Other free radicals</td>
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<tr>
<td>Antimycin</td>
<td>Disturbing the processes involved in energy metabolism by inhibiting oxidative phosphorylation, or dissipating the ionic gradients that exist across membranes.</td>
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<td>Rotenone</td>
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<tr>
<td>Oligomycin</td>
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<tr>
<td>Azide</td>
<td></td>
</tr>
<tr>
<td>Dinitrophenol</td>
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Complied from data reviewed in Ashburner and Bonner, 1979; Ananthan et al., 1986; Burdon, 1986 and Lindquist, 1986.
1.2 Stress proteins

The induction of heat shock proteins (hsp) was first observed in *Drosophila buskii* by Ritossa who reported that when larvae raised at 25°C were exposed to 32°C several new puffs appeared on the giant salivary gland chromosomes, representing regions of high transcriptional activity. At the same time puffs active prior to heat shock treatment regressed (Ritossa, 1962). The heat shock genes from *Drosophila* were among the first eukaryotic genes to be cloned (Livek *et al.*, 1978; Craig *et al.*, 1979; Voellmy *et al.*, 1981). A typical heat shock response was reported in chicken fibroblasts (Kelley and Schlesinger, 1978), *E. coli* (Lemaux *et al.*, 1978), yeast (McAlister and Finkelstein, 1980), plants (Barnett *et al.*, 1980) and in many other organisms.

Although the early data suggested the universal nature of the heat shock response, differences were shown to exist in the way *Drosophila* and yeast achieve the fast changes to protein synthesis (Lindquist, 1981). In *Drosophila* a sudden heat shock induces a translational control mechanism, that specifically represses the translation of pre-existing mRNAs while stimulating translation of mRNAs encoding hsp. In yeast such translational control of mRNAs does not occur.

The spectrum of hsp synthesized in yeast upon a stress challenge is similar to that produced in other cells. Several families can be distinguished which are designated according to their average molecular mass (Table 1.2): hsp100 (in yeast hsp104), hsp90 (in yeast hsp83), hsp70, hsp60 (the chaperonin or groEL-family) and small size hsp (in yeast hsp26 and hsp12). An
overview of some of these hsps follows. For detailed reviews see Schlesinger, 1990; Hightower, 1991; Parsell and Lindquist, 1993; Mager et al., 1993.

1.2.1 Hsp70

Hsp70 proteins belong to the most highly conserved proteins in the cell. A 40-60% identity exists between hsp70 proteins found in higher eukaryotes and *E. coli* hsp70 (dnaK). Among eukaryotes this percentage is even higher (reviewed by Lindquist, 1986; Lindquist and Craig, 1988). The genes encoding hsp70 in *S. cerevisiae* constitute a multigene family, its eight members showing amino acid sequence identities ranging from 50-97% (Ingolia et al., 1982; Craig et al., 1990). They are subdivided into four families, SSA, SSB, SSC and SSD. The SSA subfamily is indispensable for growth and encodes the Ssa1p, Ssa2p, Ssa3p, and Ssa4p proteins, which are localized in the cytoplasm (Slater and Craig, 1987; Boorstein and Craig, 1990). Mutations in *SSBl* and *SSB2* result in a cold-sensitive phenotype, the cellular localization of the corresponding proteins being unknown (Craig et al., 1990). The protein encoded by *SSCl* is mitochondrial (Craig et al., 1987), whereas the *SSD1* (better known as *KAR2*) gene product occurs in the endoplasmic reticulum (Norminton et al., 1989). Both genes are essential for growth.
### Table 1.2 Heat shock proteins of yeast

(modified from table in Moradas-Ferreira & Mager, 1993)

<table>
<thead>
<tr>
<th>Designation</th>
<th>Cellular localisation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP150</td>
<td>(Secretory)</td>
<td>Unknown</td>
</tr>
<tr>
<td>HSP104</td>
<td>Nucle(ol)us</td>
<td>Stress tolerance</td>
</tr>
<tr>
<td>HSP90</td>
<td>Cytosol/nucleus</td>
<td>Chaperone</td>
</tr>
<tr>
<td>HSP70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSA1</td>
<td>Cytosol</td>
<td>Chaperone</td>
</tr>
<tr>
<td>SSA2</td>
<td>Cytosol</td>
<td>Chaperone</td>
</tr>
<tr>
<td>SSA3</td>
<td>Cytosol</td>
<td>Chaperone</td>
</tr>
<tr>
<td>SSA4</td>
<td>Cytosol</td>
<td>Chaperone</td>
</tr>
<tr>
<td>SSB1</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>SSB2</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>SSC1</td>
<td>Mitochondria</td>
<td>Chaperone</td>
</tr>
<tr>
<td>SSD1 (KAR2)</td>
<td>Endoplasmic reticulum</td>
<td>Chaperone</td>
</tr>
<tr>
<td>HSP60</td>
<td>Mitochondria</td>
<td>Chaperone</td>
</tr>
<tr>
<td>HSP30</td>
<td>Plasma membrane</td>
<td>Unknown</td>
</tr>
<tr>
<td>HSP26</td>
<td>Cytosol/nucleus</td>
<td>Unknown</td>
</tr>
<tr>
<td>HSP12</td>
<td>Cytosol</td>
<td>Unknown</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>Cytosol</td>
<td>Protein degradation</td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enolase</td>
<td>Cytosol</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>Cytosol</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>Cytosol</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>Catalase</td>
<td>Cytosol</td>
<td>Antioxidant defense</td>
</tr>
</tbody>
</table>
Sequence similarity between hsp70 proteins extends over the entire protein, but particularly conserved regions are present in the N-terminal part. In this region an ATP binding site is present and a weak ATPase activity (Chapell et al., 1987). This shared property of hsp70 proteins is consistent with the current view that hsp70s, are important chaperones present in all cell compartments, that associate specifically with newly synthesized polypeptides. The first hsp70 whose cellular function was established is the mammalian uncoating enzyme, involved in ATP-dependent release of clathrin from coated vesicles (Rothman, 1989). Hsp70s have also been implicated in the nuclear transport of proteins (Shi and Thomas, 1992), mitochondrial protein import and protein secretion (Gething and Sambrook, 1992). Recent studies (Ohba, 1994) showed that SSBl suppressed the defects caused by a proteosome mutation in S. cerevisiae, thus facilitating intracellular protein degradation.

It is likely that hsp70 proteins induced upon stress exposure perform functions similar to those under normal growth conditions. During stress, the cellular concentration of potential substrates, e.g. denatured proteins, is likely to increase, thus depleting the free pool of hsp70 and generating the need for an increase in the level of these proteins. Hsp70 has therefore been considered as the cellular thermometer (DiDomenico et al., 1982; Craig and Gross, 1991).

Recent studies of protein translocation suggest that certain functions of hsp70 chaperones involve more than mere binding to polypeptide substrates. It is suggested that hsp70
chaperones are force generating proteins and that this capacity has been harnessed by the cell to catalyse a variety of important reactions (reviewed by Glick, 1995).

1.2.2 Hsp60

Hsp60 proteins fulfill cellular functions that, apparently, are similar to those of hsp70. Hsp60 in yeast has been identified as a mitochondrial protein showing homology to the *E. coli* groEL protein (Cheng *et al.*, 1989). groEL encodes a protein involved in bacteriophage head assembly (reviewed by Zeilstra-Ryall *et al.*, 1991). In mutants of *S. cerevisiae* defective in the constitutive expression of hsp60, incompletely processed proteins imported into the mitochondrial matrix appear to accumulate (Cheng *et al.*, 1989). Hsp60 therefore belongs to the proteins that facilitate post translational assembly of polypeptides inside an organelle, commonly called "chaperonins" (Ellis and Vies, 1991). Proteins imported into the mitochondria do not fold spontaneously but need hsp60 function for proper folding (Ostermann *et al.*, 1989; Koll *et al.*, 1992).

1.2.3 Hsp83

Yeast hsp83 belongs to the family of hsp90 proteins, which also possess chaperone-like properties (Gething and Sambrook, 1992). In eukaryotes, hsp90 is abundantly present in the cytoplasm, but a small fraction translocates to the nucleus upon heat shock (Schlesinger, 1990). Hsp90s have been found to interact with a variety of cellular proteins, including glucocorticoid receptors, several kinases and cytoskeleton proteins actin and tubulin may
hold these proteins in an inactive state until their function is required. Complex formation between hsp90 and the steroid receptors has been studied in particular detail (Hunt, 1989).

*S. cerevisiae* contains two genes encoding hsp90: *HSP83* and *HSC83* (Borokovich, 1989). *HSC83* (heat shock cognate) is a constitutively expressed gene and is only weakly induced upon stress exposure. *HSP83* is expressed at a much lower basal level and is strongly activated upon heat treatment. Notably, expression of this gene is also induced when cells enter stationary phase (Kurtz et al., 1986).

### 1.2.4 Hsp104

*HSP104* has been isolated by Sanchez and Lindquist (1990) and sequenced by Parsell et al., (1991). Hsp104 protein is not detectable during normal growth on fermentable carbon sources, but is constitutively synthesized in respiring cells (Sanchez et al., 1992) and strongly induced following heat shock. Expression of this protein is also activated when cells enter stationary phase or are induced to sporulate. Hsp104 displays a striking similarity to the highly conserved ClpA/ClpB protein family, first identified in *E. coli* (Parsell et al., 1991). Like in other eukaryotes, it may be a nucleolar protein to which an important function in the acquisition of stress tolerance has been assigned. Two putative sites showing homology with a nucleotide consensus binding site have been identified in hsp104. By site-directed mutagenesis these sites were shown to be essential for the stress protective function of this protein (Parsell et al., 1991). It has been suggested that changes in the relative concentrations of ATP and ADP affect the oligomerization and ATPase activity of *HSP104*,
thereby regulating its function in stress tolerance (Parsell et al., 1994). Hsp104 has also been found to regulate the development and magnitude of induced radiation resistance as well as induced thermotolerance after cellular stress (Boreham et al., 1994). Recent studies have highlighted a functional relationship between hsp104 and hsp70 (Sanchez et al., 1993).

1.2.5 Small hsps: hsp26 and hsp12

Among the heat shock proteins induced in response to heat shock in all organisms, the low molecular weight hsps are the most abundant and unique in plants (review Yeh et al., 1994). Yeast cells contain two small hsps: hsp12 and hsp26. The small hsps form a very diverse group which, nevertheless, display conserved structural elements (Lindquist and Craig, 1988; Silva et al., 1994), and share the ability to form highly polymeric structures referred to as heat shock granules, HSGs (Tuite et al., 1990). They show a notable and significant sequence similarity to the eye lens protein α-crystallin, in particular a highly conserved hydrophobic domain located at the C-terminus of these proteins (Tuite et al., 1990).

A universal property of the small hsps may also be their developmental regulation. Both yeast genes belonging to this group, HSP26 and HSP12 (Praekelt and Meacock, 1990) show, apart from a very strong stress-induction, dramatically increased expression following transition of cells to the stationary phase and upon induction of sporulation. Inhibitors of proteases have also been found to induce small hsps below 26kDa (Gropper et al., 1993). The cellular role of these proteins, however, has yet to be elucidated.
INTRODUCTION

1.2.6 Ubiquitin

Polyubiquitin, a protein encoded by the *UBI4* gene in yeast, is generally considered as a heat shock protein since it displays a strongly enhanced rate of synthesis under stress conditions (Finley *et al.*, 1987). Ubiquitin is a very highly conserved, 76 amino acid peptide found in all eukaryotic cells. It acts by becoming covalently attached to the free amino groups of target proteins through its C-terminal glycine, an attachment catalysed by the ubiquitin ligases (Jentsch *et al.*, 1990; Stadtman, 1990; Finley and Chau 1991; Rechsteiner, 1991). This ubiquitination is thought to be primarily a means of targeting proteins for cytoplasmic degradation, those proteins that are abnormal or short-lived becoming extensively polyubiquitinated immediately prior to degradation by the multicatalytic proteosome complex (Jentsch *et al.*, 1990; Finley and Chau, 1991; Rechsteiner, 1991). In a few cases ubiquitination of a protein appears not to cause its degradation, since histones H2A and H2B, actin and integral membrane receptors are ubiquitinated apparently as part of their normal cellular functioning (Monia *et al.*, 1990).

Ubiquitin genes have been isolated from several organisms. By far the most well characterised of these are the four genes in yeast. *UBI1, UBI2* and *UBI3* code hybrid proteins in which ubiquitin is fused to unrelated amino acid sequences. The fourth gene, *UBI4* contains five consecutive ubiquitin coding repeats.

*UBI4* is expressed at much higher levels under conditions of heat shock, starvation and α/α diploid sporulation and during treatments with cadmium or DNA damaging alkylating agents
Genes for two ubiquitin conjugating enzymes *UBC4* and *UBC5* are heat shock inducible (Seufert and Jentch, 1990). Inactivation of *UBI4* in yeast cells results in a considerably reduced resistance to starvation, higher cell mortality at the highest temperatures of growth, greater sensitivity to amino acid analogues and alkylating agents, and a block to the sporulation of *a/α* *ubi4/ubi4* diploid cells (Finley et al., 1987; Tanaka et al., 1988; Treger et al., 1988; Fraser et al., 1991). These are all treatments or conditions which probably impose on the cell a need for greatly enhanced intracellular protein turnover. It is thought that *UBI4* induction under these situations provides the higher ubiquitin levels needed for this increased protein degradation. Recent studies have focused on investigating the significance of ubiquitin-ubiquitin linkages. Finley *et al.*, (1994) found that multiubiquitin chains on target proteins consisting of Lys-48 linkages to the C-terminus of the adjacent ubiquitin play a critical role in protein degradation *in vivo*.

### 1.3 Regulation of heat shock gene transcription (in eukaryotes)

The study of how the heat shock response is regulated has provided a great deal of information about the molecular mechanisms involved in mounting a transient global response to an environmental stimulus. It is apparent that the response is orchestrated in such a way as to suit the specific requirements of different organisms. In *Drosophila*, regulation is exerted on both transcription and translation (Lindquist, 1981). This is not surprising given that mRNAs in *Drosophila* have half-lives of between 6-9 hours. To effect rapid changes in protein synthesis the cells must therefore block translation of pre-existing mRNAs. *E. coli*
does not have this problem, consequently the response is controlled almost entirely at the level of gene transcription (Yamamori and Yura, 1980).

Eukaryotic hsp genes contain one or more repeats of the heat shock response element (HSE) in their promoter region. The HSE is necessary and sufficient for heat shock-dependent transcription and is highly conserved (reviewed by Morimoto et al., 1992). It serves as a binding site for a specific heat-shock regulated transcription factor, HSF (Perisic et al., 1989). The HSF gene has been cloned from various organisms (Werner-Washburne et al., 1989). The HSF acts as a multimer and in most organisms binds to the HSE only upon heat shock (Lis et al., 1993; Sorger, 1991; Sorger and Pelham, 1987). The *S. cerevisiae* HSF (yHSF) binds DNA before and after heat shock (Sorger et al., 1987) and is phosphorylated in response to heat shock (Sorger and Pelham, 1988). The role of this phosphorylation has not been established. Little is known about the signal transduction pathway that senses the stress signal and leads to activation of HSF in yeasts or in higher eukaryotes. Recent studies have found that not only in mammals, but also in *S. cerevisiae*, the RAS pathway controls the transcription of heat shock genes via a mechanism not involving the heat shock transcription factor (Engelberg et al., 1994).

In *S. cerevisiae* the HSF gene is essential for viability (Sorger and Pelham, 1988). This is thought to be due to the need for basal levels of hsp70s during growth at normal temperatures. There are several lines of evidence for this, HSF binding sites in the promoter of one of the hsp70 genes mediates 80% of non heat shock (basal) activity (Park and Craig, 1989). Also,
overexpressing HSF in the absence of heat shock results in a four fold increase of the level of a major species of hsp70 (Sorger and Pelham, 1988). The sustained basal and transient heat shock inducible activities of HSF appear to be mediated by physically separable regions of the protein (Sorger, 1990).

Evidence has also been presented that transcriptional expression of hsp genes in human cells is activated by stress denaturation of proteins and that this activation is inhibited when the concentration of nascent polypeptides is reduced (Baler et al., 1992). This suggests that, at least at the level of regulation of hsp genes, the cells are capable of integrating changes in the concentrations of stress-denatured and of not-yet-folded nascent polypeptides. The factor(s) involved in these regulatory events is postulated to be normally associated with nascent polypeptides and may be identical with hsp70. HSF and hsp70 have been found to interact with some specificity (Baler et al., 1992).

1.4 The effects of heat stress on yeast

The heat shock response not only elicits changes in gene expression but also biochemical and physiological changes within the cell. Investigating the protein synthesis independent biochemical and physiological changes caused by stress may yield important information about the mechanisms that confer stress tolerance to cells.

Targeted HSP gene disruption has shown only a minority of the hsps of *S. cerevisiae* exerting appreciable effects on thermotolerance, even though rather more of these proteins are
essential for proliferation at the highest temperatures of growth. Also, heat shock can induce substantial increases in thermotolerance even in the absence of protein synthesis, doubtless as a result of physiological changes that occur independently of \textit{de novo} hsp synthesis. Furthermore, while both thermotolerance acquisition and the heat shock response can be triggered by a number of chemical agents (Mager, 1993), not all chemical inducers of thermotolerance are strong inducers of HSPs. Figure 1.1 summarizes the yeast response to heat stress.

\subsection{1.4.1 Protective effects of moderate heat shock}

As discussed above, cell survival during a severe heat stress can be enhanced when heat shock proteins are induced prior to the severe heat treatment. The increase in survival at severe temperatures after pre-treatment is referred to as survival thermotolerance. It has also been shown previously that mild treatment allows RNA splicing in cells subjected to a severe heat treatment, now referred to as splicing thermotolerance (reviewed in Grabowski \textit{et al.}, 1985; Padgett \textit{et al.}, 1986).

Correl and Riordan (1994) showed that while induction of the stress proteins by exposure to chemicals induces the acquisition of survival thermotolerance, they do not induce the acquisition of splicing thermotolerance. This suggests the possibility of different pathways for these separate aspects of thermotolerance. They also showed (1992) that pre-mRNA synthesized at the severe temperatures in splicing thermotolerant cells, although protected in
splicing competent complexes, is not actually processed to mature mRNAs until the cells are returned to their normal temperature.

1.4.2 Morphological effects

The simplest way of obtaining an overall view of cellular damage is by visualizing the cell. The effects of hyperthermia on the ultrastructure of mammalian cells has been thoroughly investigated (reviewed by Welch, 1990) and similar studies have been performed on various plant cells (Fransolet et al., Mansfield et al., 1988). The most characteristic feature of heat shocked cells from both plants and animals is the aggregation and vacuolation of the nucleolus due to the loss of the nucleolar granular component (Welch and Suhan, 1985). Recent studies have found that in S. cerevisiae following heat shock there is a contraction of the nucleolus, formation of electron dense particles in mitochondria and formation of heat shock granules in the cytoplasm (Webster and Watson, 1993). When cells were observed during a recovery period after heat stress it was found that nucleolar ultrastructure was regained more rapidly in cells that were previously heat shocked compared to cells that were stressed directly with no prior heat shock (Webster and Watson, 1993).
The primary signal sensing protein damage is probably the lack of free, uncomplexed HSP70, as all the available HSP70 binds to the accumulated heat-damaged protein (Craig and Gross, 1991). The same signal may also trigger the trehalose induction with heat shock, since trehalose is also known to be under negative regulation by heat shock proteins during the recovery from heat stress (Hottiger et al., 1992). The increase in plasma membrane ATPase action is rapid and therefore, at least initially, reflects the pHi decline rather than any ATPase activation due to the slower increase in cAMP. Proton extrusion from the cell resulting from this enhanced ATPase action probably helps to counteract the effects of the stress-induced pHi decline (Coote et al., 1991; Panaretou and Piper, 1990).
1.4.3 Physiological effects

In addition to displaying induction of hspS, many cells accumulate a large cytoplasmic pool of trehalose (α-D-Glucopyranosyl-β-glucopyranoside) during heat shock. This trehalose is thought to act primarily as a stress protectant rather than a storage carbohydrate, its levels generally showing a good correlative relationship with thermotolerance (Wiemken, 1990). This trehalose is mobilized very rapidly during a subsequent temperature shift down. This mobilization resembles hsp synthesis in being regulated by the levels of certain hsps [notably hsp70 (Hottiger et al., 1992)]. Heat shock also causes an increase in intracellular glucose, together with a partial inhibition of glycolysis (Neves et al., 1992). Conversion of this glucose to trehalose may be one way of preventing potential toxicity problems that may result when glucose is no longer totally phosphorylated to glucose-6-phosphate immediately upon entry to the yeast cell. Heat also causes perturbations to intracellular ion levels and pH (Coote et al., 1991), these being attributable in part to an enhanced permeability of membranes. Two important enzyme activities affected by these ion and pH changes are the plasma membrane ATPase and cyclic AMP-dependent protein kinase A (cAMP-PK). Heat shock also causes a transient arrest in the G1 phase of the cell cycle (Plesset et al., 1987). Also, biophysical studies show that it leads to alterations to the physical states of both membranes and intracellular water (Komatsu et al., 1991; Iwahashi et al., 1991; Obuchi et al., 1992).
1.5 Ethanol stress

The increasing ethanol during batch fermentation of *S. cerevisiae* on high sugar substrates acts to cause reductions in the specific growth rate, the fermentation rate and cell viability (Van Uden, 1984). These effects become much more severe with increasing temperature, the optimum and maximum temperatures of growth becoming appreciably depressed in the presence of ethanol levels above 3%, while thermal death on exposure to extreme temperatures is enhanced (Van Uden, 1984). These adverse influences of ethanol on the growth, viability and fermentation of *S. cerevisiae* have been largely explained in terms of its effects on membrane-associated processes (Leao and Van Uden, 1984, 1985; Jones, 1989; Rose, 1993). Not only are these toxic effects influenced by the lipid composition of yeast cell membranes but growth in the presence of ethanol causes changes to membrane lipids which are possibly part of an adaptive response [reviewed in Rose (1993), Lloyd et al., (1993)]. The concentration of ethanol that totally inhibits *S. cerevisiae* growth is also medium and strain-dependent (Rose, 1993).

Several of the changes induced in yeast by exposure to stressful ethanol levels are identical to those caused by a heat stress. It is not surprising therefore that ethanol acts in a synergistic way to increase the damage caused by heat (Van Uden, 1984, Aguilera and Benitez, 1989). Both heat and ethanol cause membrane disordering and protein denaturation (reviewed in Casey and Ingledew, 1986; Piper, 1993), besides an inhibition of glycolysis and an enhanced induction of petites (Neves and Francois, 1992; Leao and Van Uden, 1982; Van Uden, 1984). Ethanol also inhibits glucose transport (Leao Van Uden, 1982; 1985). Both stresses increase
the permeability of the plasma membrane, resulting in an increased passive proton influx that acts to dissipate the electrochemical potential gradient that the cell maintains at this membrane. This is reflected in the intracellular pH decline that is observed both with ethanol addition (Leao and Van Uden, 1984) and with heat shock (Coote et al., 1991; Coote, 1993). Ethanol and heat stress will adversely affect those vital functions for which a plasma membrane electrochemical gradient is essential, processes such as nutrient uptake, the maintenance of potassium balance, and the regulation of intracellular pH (Serrano, 1991).

Both sublethal heat shock (Coote et al., 1991) and addition of ethanol (Cartwright et al., 1987; Rosa and Sa-Correia, 1991) dramatically stimulate the activity of plasma membrane ATPase, the enzyme largely responsible for maintaining the proton gradient across the plasma membrane (Serrano, 1991). This increase in ATPase activity causes an enhanced proton efflux that counteracts the dissipation of proton motive force resulting from the stress-induced increase in membrane permeability (Figure 1.1). It is not surprising therefore that mutations that alter plasma membrane ATPase activity also influence cellular tolerances of both ethanol and heat (Panaretou and Piper, 1990; Coote, 1993). Heat and ethanol also induce heat shock proteins in yeast (Plesset et al., 1982) and other organisms (Nover, 1991). The trigger for their induction is probably the cytoplasmic accumulation of aberrant or partially denatured protein (Ananthan et al., 1985). Thus ethanol may be an inducer of hsp through its destabilisation of the hydrophobic interactions of protein structure, this leading to the association of hsp chaperones with these destabilised proteins and the concomitant activation of heat shock genes (reviewed in Mager and Moradas-Ferreira, 1993).
1.6 Weak acid preservatives

Weak acid preservatives are an important means of limiting microbial growth in various types of pharmaceutical, cosmetic and food products, as well as in other specialised areas. Sorbate and benzoate are in extensive use as food preservatives because they inhibit the growth of fungi and bacteria. Phenotypically acquired resistance to such lipophilic acids is well documented in yeasts (Warth 1977, 1988). Warth has proposed that it results from induction in adapted cells of the ability to catalyse an energy dependent extrusion of the acids. While the mechanism of the inhibitory action of these acids remains poorly understood it has been clearly established that both their penetration into cells and their inhibitory action increase with acidification of the medium (see below), being essentially proportional to the concentration of the undissociated acid (Russell, 1991). Nonetheless it remains unclear whether the acid must enter the cell to exert its activity or if the inhibition of growth is due to membrane disruptive effects of the acid.

Preservatives are also lipophilic molecules, their accumulation in membranes, disrupting membrane structure thus interfering with substrate transport and oxidative phosphorylation (Free and Levin, 1978). This may account for their effectiveness in preventing the growth of many sensitive bacteria and yeasts under some conditions, but it is not likely as a cause of growth inhibition under conditions such as anaerobic fermentation and nutrient rich foods (Warth, 1986). The anti-microbial effect of weak acids is greatly increased by lowering the pH of the suspending medium, being less effective as pH increases (Macris, 1975; Eklund, 1983). At low external pH (<5.0) weak acids exist substantially in an undissociated
molecular form (Beauchat, 1981). Eklund (1983) found that the inhibitory action of the undissociated acid was 10-600 times greater than that of the dissociated acid, the undissociated form being uncharged and therefore readily penetrating the cell. Nevertheless, it was found that the dissociated acid caused more than 50% of the growth inhibition at pH levels above 6. Macris (1975) noted a fast uptake rate of benzoic acid in *S. cerevisiae*, reaching saturation in about 2 min and then remaining constant at this level. Cheng and Piper (1994) found that sorbate inhibited the heat shock induction of heatotolerance in low pH cultures of *S. cerevisiae* but acted as a chemical inducer of heatotolerance in the absence of heat shock in high pH cultures. They also found that it blocks the heat shock response in low pH cultures of *S. cerevisiae* (Cheng and Piper, 1994).

Kreb's *et al.*, (1983) reported that 2mM benzoate lowers the internal pH of yeast cells leading to a drop in CO₂ production and lowering inhibition of 6-phosphofructo-1-kinase (a major regulatory enzyme of glycolysis) and of ATP levels. They proposed that these effects be accounted for in terms of intracellular acidification, irrespective of the chemical nature of the acid employed. Francois *et al.*, (1986) reported that benzoate, sorbate and salicylate inhibit 6-phosphofructo-2-kinase even at pH 7.5. Burlini *et al.*, (1993) investigated the metabolic effects of benzoate and sorbate in yeast at neutral pH in order to detect only those effects directly related to their structural properties. They concluded that a major metabolic effect provided by benzoate and sorbate at neutral pH is prevention of glucose-induced switch of metabolism from a gluconeogenic to a glycolytic state, the effect being marginally pH dependent.
1.7 Plasma membrane $H^+$-ATPase

The plasma membrane $H^+$-ATPase of *S. cerevisiae* is a member of the E1-E2 or P-type family of cation-transporting ATPases found in bacteria, fungi, plant and animal cells (Goffeau and Green, 1990; Serrano, 1989). This *PMA1* gene encoded ATPase is an abundantly expressed polypeptide, constituting 7-10% of plasma membrane protein (Rao et al., unpublished results) and hydrolysing as much as 25-40% of total cellular ATP (Gradman et al., 1978).

It's chief role is to generate a large electrochemical proton motive force that drives the energy-dependent uptake of sugars, amino acids, nucleosides and other ions (reviewed by Serrano, 1988). In addition, by pumping protons out of the cell, it helps to regulate intracellular pH (Eraso and Gancedo, 1987). Direct evidence for the essential physiological role of the ATPase first came from the finding that disruption of the *PMA1* gene created a null mutant that was unable to grow (Serrano et al., 1986).

Different environmental factors modulate the yeast plasma membrane $H^+$-ATPase (Serrano, 1983; Tuduri et al., 1985; Lentzen et al., 1987; Eraso and Gancedo, 1987; Rosa and Sa-Correia, 1991; Benito et al., 1992; Amigo et al., 1993) and glucose metabolism is one of the most important regulatory factors. Glucose regulation takes place at two levels. At the post transcriptional level, glucose metabolism induces activation of ATPase activity (Serrano, 1991). The activation of the ATPase by glucose is based on the modulation of an inhibitory interaction of the carboxyl terminus with the active site of the enzyme (Portillo et al., 1991)
and is mediated by a ser/thr phosphorylation of the enzyme (Chang and Slayman, 1991). At the transcriptional level, glucose increases the synthesis of PMA1 mRNA (Rao et al., 1993) due to a TUF/RAPI/GRF1 transcription factor control over the PMA1 gene (Capieux et al., 1989). Mutants have been isolated in a gene APA1 which causes a decrease in the level of ATPase (Garcia-Arranz, et al., 1994). Deletion of gene APA1 does not affect the expression of other TUF-regulated genes, thus providing evidence for a new pathway that controls PMA1 gene expression.

The necessity for increased plasma membrane ATPase action, and for general restoration of homeostasis, probably represents one of the major energy demands imposed by heat stress (Piper and Panaretou, 1992). It is not surprising therefore, that plasma membrane ATPase is an important influence over the thermotolerance of S. cerevisiae and S. pombe, as well as tolerances to other stresses (Piper and Panaretou, 1990). Also, inhibition of this ATPase using diethylstilbestrol hypersensitizes yeast to thermal death (Coote et al., 1991). Plasma membrane ATPase action is also vital for the ability of S. cerevisiae cells to synthesize hsp70 in response to heat shock. A reduction in this ATPase activity reduces hsp induction with heat shock (Piper and Panaretou, 1990), while total inhibition of this ATPase by diethylstilbestrol treatment has the almost immediate effect of rendering cells incapable of any induction of hsp70 by heat shock, even though these cells still retain a capacity for protein synthesis (Cheng and Piper, 1994).
There has been great controversy over the pathway triggering phosphorylation of ATPase. The amino acid sequence of the *PMA1* gene product does not show a consensus sequence for cAMP-dependent protein phosphorylation (Serrano, 1983). However, there are consensus sites for other protein kinases (e.g., Ca$^{2+}$/calmodulin-dependent protein kinase) and the protein was also demonstrated to be phosphorylated both *in vivo* and *in vitro* (McDonough *et al.*, 1982; Yanagita *et al.*, 1987; Kolarov *et al.*, 1988), including phosphorylation by an endogenous plasma membrane associated kinase. Moreover, two C-terminal amino acid residues that form part of a potential phosphorylation site for Ca$^{2+}$/calmodulin-dependent protein kinase have been identified as important for glucose-induced activation of the H$^+$/ATPase (Portillo *et al.*, 1991). Recent studies by Brandao *et al.*, (1994) support a model in which glucose activates plasma membrane H$^+$/ATPase through activation of a phosphatidylinositol-type signaling pathway, utilising both protein kinase C and one or more Ca$^{2+}$/calmodulin-dependent protein kinases for phosphorylation of the enzyme.

### 1.8 Aims of this study

Earlier studies in this laboratory had discovered that the induction of the heat shock response causes a single heat shock protein (hsp30) to become associated with the plasma membrane (Panaretou and Piper, 1992). It is not found in other cell fractions, either before or after heat shock, and is therefore a heat shock protein that is targeted to the plasma membrane. The gene (*HSP30*) was cloned and sequenced by Recnacq and Boucherie (1993). It has also been sequenced as ORF YCR21 by a group led by H. Feldman as part of the yeast chromosome III
sequencing project (Oliver et al., 1992). At the start of this project the aims were to further characterise the induction of hsp30 and to try to find a role for this protein by:

(1) Identifying conditions other than heat shock and starvation that cause hsp30 induction (Chapter 3).

(2) Construction of strains isogenic but for a HSP30 gene inactivation leading to investigation of whether there are differences between the mutant bearing this mutation and its parent (Chapter 4).

In addition, since the mechanism of acquired resistance to weak acid stress had never been investigated at the molecular level, changes in plasma membrane protein composition, elicited by weak acid stress of S. cerevisiae and Zygosaccharomyces bailii were also investigated. Hsp30 was found to be one of two membrane proteins induced by this stress. The other protein was identified as the product of the SNQ2 gene from peptide sequence information. Also SNQ2 was shown to be stress-inducible (Chapter 5).
CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

Standard reagents: AR grade supplied by Sigma and BDH.

Microbiological media: Supplied by Difco. Auxotrophic requirements supplied by Sigma.

Electrophoresis reagents: Acrylamide and Bis-acrylamide (Electran grade) and ammonium persulphate supplied by BDH. TEMED supplied by Sigma.

Molecular weight markers: i) 29-205 KD ii) 14.3-66 KD (prestained) all supplied by Sigma.

Enzymes: Protease V8 from Staphylococcus aureus supplied by ICN immunobiologicals. RNAase A type IIIA supplied by Sigma.

Protease inhibitors: PMSF, TLCK, TPCK and Pepstatin A all supplied by Sigma.

Transfer membranes: Genescreen (0.2μ pore size) supplied by Dupont (NEN research products). PVDF supplied by Millipore (0.4μ pore size).

Immunological reagents: Biotinylated goat anti-rabbit, IgG, streptavidin-horse-radish-peroxidase conjugate and 4CN all from Bethesda Research Laboratories Life Technologies, Inc (BRL).

Reagents for protein assay: Dye regent concentrate and BSA standard supplied by BIO-Rad.
Radiolabelled amino acids: L4, 5-3H(N)-Leucine (60 Ci/mmol; 1μCi/μl) supplied by Dupont (NEN research products). Adenosine 5'-[γ-32P] triphosphate (3,000 Ci/mmol; 10μCi/ul) supplied by Amersham.

Other reagents: Cycloheximide and Coomassie blue R-250 were supplied by Sigma. SDS (biochemical grade) was supplied by Boehringer Mannheim. Ecoscint A was supplied by National Diagnostics.
2.2 Yeast strains

Table 2.1 Yeast strains used in this study.
(all strains are *S. cerevisiae* unless otherwise stated)

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT3</td>
<td>as above plus <em>hsp30::URA3</em>.</td>
<td>This study</td>
</tr>
<tr>
<td>YPH98 (<em>snq2</em>)</td>
<td>as above plus <em>snq2::leu</em></td>
<td>Gift of J. Servos</td>
</tr>
<tr>
<td>X21801B</td>
<td>a, <em>leu2</em>, <em>ura3</em></td>
<td>Gift of H. Boucherie</td>
</tr>
<tr>
<td><em>Hsp30::URA3</em></td>
<td>a, <em>leu2</em>, <em>ura3</em>, <em>hsp30::URA3</em></td>
<td>Gift of H. Boucherie</td>
</tr>
<tr>
<td>NCYC 563 (<em>Z. bailii</em>)</td>
<td>Prototrophic</td>
<td>NCYC Norwich</td>
</tr>
</tbody>
</table>

2.3 *E. coli* strains

*E. coli* strain JM101 (*lac pro thi supE Ftra D36 proAB lacZ M15*) was employed for plasmid DNA manipulations and transformations.

2.4 Plasmids

All the plasmids used are listed and were derived from the yeast :*E. coli* shuttle vectors pMA3a (*Kingsman et al.*, 1985) or pUC19-HSP30 (*Regnacq and Boucherie*, 1993).
2.5 Growth media and culture conditions

(All percentages are w/v)

Yeast cultures were grown in rich media (YEPD) or minimal media plus glucose (MMG):

YEPD: 1% yeast extract, 2% bactopeptone, 2% glucose

MMG: 0.67% yeast nitrogen base (without amino acids), 2% glucose

Plus one or more of the following auxotrophic requirements where appropriate; L-leucine (30mg/ml), L-tryptophan (20mg/ml), L-histidine (20mg/ml) and uracil 20mg/ml).

For solid media 2% agar was added.

In liquid media cells were grown at 28°C with rapid agitation in an orbital shaker. Yeast strains were maintained in frozen stocks in 2 x YEPD plus 15% glycerol at -70°C not exceeding volume 1/4 of the flask volume.

2.6 E. coli

Competent E. coli cells were prepared as described in Hanahan (1983) and E. coli transformation was as described in Cohen et al., (1972).

E. coli were cultured in 2 x TY (1.6% bactopeptone, 1% yeast extract, 0.5 % NaCl) at 37°C.

Plates for plasmid selection were 2 x TY plus ampicillin at a final concentration of 100mg/ml.
2.7 Monitoring cell growth

Yeast cell growth in liquid media was monitored by either taking OD550 readings after appropriate dilution of the culture to provide conditions that would fall within the limits of the Beer-Lambert law, or counting cells by using an improved Neubauer haemocytometer (Hawksley).

2.8 Yeast transformation

Yeast transformation with plasmid DNA was achieved by using the method by Geitz (1994).

2.9 Assaying for stress tolerance

All stress tolerance experiments were done on cells in exponential growth (0.5-1x10^7 cells/ml) at 28°C in YEPD medium. Before each experiment *S. cerevisiae* cultures were briefly sonicated for five seconds at 5u, followed by 5 seconds rest (x5), a treatment which was just sufficient for no cell aggregates to be seen by light microscopy (a microprobe [tuned to 23KHZ] of an MSE Soniprep Ultrasonic Disintegrator was used). The stress was then applied and cells were then spread on YEPD plates using dilutions designed to give approximately 300 cells per plate. In all of the stress tolerance experiments killing was measured from the colonies (including petites) on YEPD plates maintained 2-3 days at 28°C.
2.9.1 Assaying for tolerance to ultraviolet irradiation

Cells were diluted appropriately in YEPD and 0.15ml aliquots were spread on YEPD plates. Immediately after plating, lids were removed from the petri dishes and the exposed surfaces were irradiated by an ultraviolet light source (2.6x10^5 ergs/sec/cm^2) for various times.

2.9.2 Assaying for tolerance to high ethanol and NaCl concentrations

To cells appropriately diluted in YEPD were added appropriate concentrations of absolute ethanol or NaCl. Aliquots were removed immediately (for the zero time point) and then at subsequent intervals thereafter; these being diluted 100-1,000 fold prior to plating on YEPD plates.

2.9.3 Assaying for tolerance to nitrogen starvation

Cells were appropriately diluted in nitrogen-free MM medium and aliquots were removed over a period of time and plated.

2.9.4 Assaying for tolerance to freeze-thaw

Cells were appropriately diluted and after each freeze-thaw cycle aliquots were removed and plated. Cells were frozen at -20°C.

2.10 Measurement of thermotolerance

Samples (10mls) of late exponential phase *S. cerevisiae* were harvested, washed and resuspended in fresh broth, and allowed to resuscitate at 25°C for 30 min. The cells were then
subjected to various treatments to study their effects on the induction of thermotolerance (either 6% ethanol, 9mM sorbic acid or 42°C for 40 min) in a shaking water bath. After incubation, a 50μl sample of culture was inoculated into 950μl of preheated fresh broth in a modified thermocouple block calibrator DB40-L (Techne, Cambridge) (Coote et al., 1991). The preheated broth was held at different lethal temperatures over various time periods. Samples were removed at various times throughout a heating profile and immediately inoculated into 5ml of fresh broth. Cells were resuscitated, serially diluted and plated.

2.11 Measurement of acid efflux

Glucose-induced acid efflux has been shown by two methods to be an accurate reflection of membrane-ATPase activity \textit{in vivo} (Serrano, 1980; Cid et al., 1987).

Late-exponential cultures were harvested, washed and resuspended in sterile distilled water (40-50mg wet wt./ ml) and 15ml samples placed in a magnetically stirred water jacketed vessel at 25°C. A Corning ion analyser 255 pH meter was used to monitor the suspension pH, which was adjusted to 4.5 with HCl. The system was calibrated using 50μl aliquots of HCl and NaOH (0.01M). Glucose (0.1%) was added to initiate proton pumping. Acid efflux was monitored for 5 mins before the system was recalibrated and adjusted to pH 4.5. The rate of acid efflux was determined as described by Cole and Keenan (1987) and expressed in molar equivalents of HCl. To measure the rate of acid efflux after sublethal heat stress, separate cell suspensions were incubated in a water bath at temperatures between 5°C and 52°C for 30 min.
Prior to acid efflux measurements, the temperatures of stressed samples were allowed to equilibrate to 25°C in the water jacketed vessel.

2.12 Measurement of cellular fluorescence

Cellular fluorescence of cells incubated at different temperatures was carried out as described by Coote et al., (1994).

2.13 Procedures for protein analysis

2.13.1 Pulse Labeling

1-2x10^8 cells from exponential YEPD cultures were collected by centrifugation (3, 000 x g for five minutes), resuspended in 10ml MMG medium, recentrifuged, resuspended again in 10ml MMG medium and incubated for 20 mins at 25°C prior to heat shock. During this 20 min incubation the cultures were subdivided into 1ml aliquots in glass tubes. To heat shock the cell aliquots were shifted to the appropriate temperatures and, at variable times after this upshift, were labelled with [3H]-Leucine (30μCi/ml). A control aliquot was pulse labelled for 15 mins at 28°C without any heat shock. Incorporation of radiolabelled amino acid was stopped after the appropriate amount of time by rapidly chilling the aliquots on ice, transferring them to 1.5ml eppendorfs and pelleting the cells by a 30s spin at high speed in a microcentrifuge (ca. 5,500xg). The supernatant was discarded and the pellets were rapidly frozen in dry ice and stored at -70°C.
2.13.2 Extraction of total cell protein

The labelled cell pellets were thawed on ice followed by addition of two pellet volumes of acid washed glass beads (BDH, 40 mesh). Sufficient protein extraction buffer to just cover the pellet and glass beads was added (ca. 150μl). Protein extraction buffer was: 500mM Tris/HCl pH 8.0, 2% SDS, 2mM EDTA, with the following added just before use, 2.5% β-mercaptoethanol, 0.5mg/ml RNAase, 1mM PMSF, 1mM TPCK, 0.5mM TLCK and 2μg/ml pepstatin A. Protein was extracted by vortexing for 30s followed by chilling on ice for 30s (x8). Glass beads and unbroken cells were pelleted by a 10s spin at high speed in a microcentrifuge (5,500xg). The supernatants were transferred to precooled eppendorfs and stored at -70°C.

2.13.3 Measuring radioactivity incorporated into cellular protein

Protein from a 20μl aliquot of each sample was precipitated by addition to 5ml of a solution containing 5% TCA and 0.1% bactopeptone. After 15 minutes on ice, the precipitated protein was filtered by suction onto glass fibre filters (Whatman GF/C, 2.5cm diameter). The filters were washed once with 5ml of cold 5% TCA 0.1% bactopeptone and twice with cold absolute ethanol. Filters were dried at 80°C for 15 minutes and, once cool, were placed into 5ml scintillation vials and covered with 4ml Ecoscint A scintillation fluid. Radioactivity precipitated onto the filters was counted by a Packard Tri-carb Liquid Scintillation Analyzer using the appropriate channel and gating settings for the relevant radioisotope.
2.13.4 Protein assay

Protein concentrations were determined by the dye binding assay demonstrated by Bradford (1976), using the BioRad Protein Assay Kit and BSA as standard. Both the standard and microassay procedures were as described in the manufacturer's instructions.

2.13.5 Subcellular fractionation

Plasma membranes were isolated as described by Panaretou and Piper (1992).

2.13.6 Separation of proteins by gel electrophoresis (SDS-PAGE)

Proteins were fractionated by size using the discontinuous electrophoresis system as described by Laemmli (1970). Gels were cast between two glass plates and run on a Studier type slab gel apparatus as described in Hammes and Rickwood (1990).

2.13.7 Analysis of gels following electrophoresis

Upon completion of electrophoresis gel plates were separated and stacking gels discarded. The resolving gels were analysed by one of the following ways:

2.13.8 Direct staining of gels

Abundant proteins (1μg or more) were detected by staining with Coomassie blue R-250. Gels were incubated for 4 hours at room temperature in a solution composed of 0.05% Coomassie blue, 50% methanol and 10% acetic acid. Destaining was implemented by successive incubations at room temperature with gentle agitation in solutions composed of 5% methanol
and 7.5% acetic acid. To visualise proteins that were present in low amounts (10^3 g or more) the neutral silver stain procedure as described by Harlow and Lane (1988) was used.

2.13.9 Autoradiography/fluorography

Gels were dried onto Whatman 3MM paper under vacuum on a BIO-RAD Laboratories gel dryer, as described by Harlow and Lane (1988). Dried gels were placed in direct contact with Fuji RX film followed by exposure in light proof cassettes for the appropriate length of time at -70°C. Film was developed according to manufacturers instructions. ^32P labelled proteins were easily detected by this method. If required, sensitivity could be increased by placing a calcium tungstate intensifying screen against the side of the X-ray film not in contact with the dried gel.

Gels on which proteins labelled with ^3H had been separated were visualised by fluorography. Gels were soaked in 1M sodium salicylate (pH 5-7) for 20 mins prior to drying (Chamberlain, 1979). Dried gels were then used to expose X-ray film as described above. Typically, 150,000 c.p.m. of total cellular protein labelled with ^3H could be visualised by an overnight or three day exposure, respectively.

2.13.10 Transfer of proteins from gels to membranes

Separated proteins from a gel were blotted onto thin support matrices by electrophoretic transfer (Western blotting). A nylon blotting membrane was used (Genescreen due to its superior protein binding capacity and its physical strength). Also, nylon membranes do not
change size during subsequent processing steps. The membrane was prepared for blotting according to manufacturer's instructions and electrophoretic elution was achieved by complete immersion of a gel membrane sandwich in a buffer tank with steel plate electrodes followed by electrophoresis at 50V, as described by Harlow and Lane (1988). Efficiency of transfer was determined by staining the gel with Coomassie blue, also, prestained markers were run on gels to serve as internal markers for transfer and molecular weight.

The transfer buffer is typically of low ionic strength (25mM Tris, 150mM glycine [pH 8.3] and contains 20% methanol to minimise swelling of the gel as blotting progresses. This was found to be unsuitable for the transfer of plasma membrane proteins for which the best results were achieved using a buffer with a high pH and a low concentration of methanol (35mM CAPS-NaOH pH 10, 5% methanol) and blotting for five hours at 50V.

2.13.11 Detection of specific proteins by immunostaining

Specific proteins immobilised on nylon membranes were visualised by probing with the appropriate anti-sera followed by incubation with the various components of the BRL streptavidin IgG detection systems. The procedure described below is a combination of the methods reported by Maniatis et al., (1990) and the BRL Products for Immunodetection Applications guide. All incubations were carried out at room temperature with gentle agitation on a platform shaker. Solutions containing immunological reagents or chromogenic substrate were freshly prepared. Also incubations carried out in the presence of primary antibody, goat anti-rabbit IgG and peroxidase were performed in heat sealable bags in a final
Blots were then washed in TBS followed by a one hour incubation with streptavidin Horseradish peroxidase.
volume of 0.1ml per cm² of membrane. Preliminary experiments to determine optimal
dilutions of primary antibody were carried out; quantities of immunodetection reagents used
were as recommended by individual product profiles.

After electrophoretic transfer remaining protein binding sites on the blot were blocked by
incubation for one hour in a blocking solution composed of 5% (w/v) non-fat dried milk in
Tris buffered saline (TBS: 50mM Tris- HCl pH 7.5, 150mM NaCl) followed by incubation for
2 hours with blocking solution plus an appropriate amount of the antibody. This was followed
by a one hour incubation in blocking solution plus the appropriate quantity of biotinylated
goat anti-rabbit IgG. Blots were then washed as described, followed by a one hour incubation
with TBS containing 0.01% H₂O₂, and 0.06% 4CN. Within 5-10 minutes, polypeptides to
which the primary antibody had bound appeared blue against the white background of the
blot. The stained blots were washed for 10 mins in 3 changes of ddH₂O and stored in the
dark.

2.13.12 Origin of anti-sera used in this work described in this thesis

Polyclonal antisera were raised in rabbits as described by Harlow and Lane, (1988).
Essentially, this involved subcutaneous injection of polyacrylamide gel fragments from
multiple 1D SDS gel fractionations of hsp30-Gst fusion protein. The E. coli containing the
vector for overexpression of this fusion protein was kindly given to us by H. Boucherie. After
IPTG induction the fusion protein was isolated and identified on a SDS gel. Antiserum
crossreacting with hsp30 was prepared by the Immunology Dept. at Unilever Research, Bedford.

**2.13.13 Detection of glycoconjugates**

Glycoproteins immobilised on nylon membranes were detected by their binding to the plant lectin concanavalin A. The procedure used was a combination of methods devised by Hawkes (1982) and is described below. All incubations were carried out at room temperature with gentle agitation on a platform shaker. Solutions containing concanavalin A, peroxidase and chromogenic substrate were all freshly prepared.

After electrophoretic transfer remaining protein binding sites on the blot were blocked by incubation for two hours in solution A (composed of 2.5% (w/v) BSA in TBS). The blot was then placed in a heat sealable bag into which was introduced concanavalin A (50µg/ml) in solution A(0.1ml/cm²) of blot). The bag was sealed leaving as few air bubbles as possible. After a 30min incubation the blot was washed for 15 minutes in 3 changes of TBS followed by a 30 minute incubation with horseradish peroxidase (50µg/ml) in TBS, again in a heat sealable plastic bag. The blot was then washed as before. Bound peroxidase was detected by the addition of 0.01% H₂O₂ and 0.06% 4CN in TBS. Within 1-5 minutes the concanavalin A binding polypeptides appeared blue against the white background of the blot. The stained blots were washed in ddH₂O and stored in the dark.
2.13.14 Peptide mapping by limited proteolysis

This technique which is especially suitable for analysis of a protein band in a gel slice, involved partial enzymatic proteolysis in the presence of SDS followed by analysis of the cleavage products by SDS-PAGE. The pattern of peptide fragments produced is highly characteristic of the protein substrate and the proteolytic enzyme used, and is highly reproducible. The procedure described below is modified from Cleveland et al., 1977.

After SDS-PAGE using 1mm thick gels, proteins were visualised by staining with Coomassie blue R-250. However to avoid possible acid hydrolysis, staining and destaining conditions were not the same as those described in section 2.13. Instead proteins were visualised as quickly as possible by staining for 1 hour in 0.1% (w/v) Coomassie blue R-250 in methanol: acetic acid: ddH₂O (5:1:4 v/v/v) followed by rapid destaining (45 minutes) in methanol: acetic acid: ddH₂O (5:1:4v/v/v). Even under these mild staining conditions there was significant protein hydrolysis at room temperature (typically, the 110kDa plasma membrane H⁺-ATPase was cleaved into 3 fragments), this probably being due to the peptide bond between aspartate and proline being particularly susceptible to acid hydrolysis (Matsudaira, 1990). This problem was eliminated by carrying out these staining and destaining steps at 4°C. Bands from SDS-PAGE gels stained with Coomassie blue were cut out with a scalpel blade and then soaked for 30 minutes with occasional swirling in 10 ml of solution A: 125mM Tris-HCl pH6.8, 0.1% SDS and 1mM EDTA. At this point gel slices could be stored at -20°C.
A second 1mm thick SDS gel was prepared as described in section 2.13 but with a longer than usual stacking gel (5cm) which contained 1mM EDTA; and that was twice as long as usual. Also, the final concentration of polyacrylamide in the resolving gel was 15%. The sample wells were filled with solution A. The bands from the first SDS gel were conveniently digested, without prior elution, by placing them in the sample wells of this second SDS gel followed by over laying each slice with 20μl of solution A containing 10% glycerol, 0.1% bromophenol blue and an appropriate concentration of protease. Electrophoresis was performed in the normal manner with the exception that the current was turned off for 30 minutes when the bromophenol blue dye neared the bottom of the stacking gel. Bearing in mind that each gel slice contained only 1-2μg of protein, the pattern of peptide fragments generated on the second gel was visualised by silver staining.

Of the 10 or so proteases that are commonly used for peptide mapping (such as trypsin and chymotrypsin), endoproteinase from staphylococcus aureus V8 (protease V8) is probably one of the most ideal and was used for the experiments in chapter 5. It has the additional advantage that the extent of proteolysis can be controlled by manipulation of the digestion buffer as well as by the concentration of the protease. In phosphate buffer protease V8 cleaves peptide bonds on the carboxy terminus of either aspartate or glutamate, whereas in phosphate free buffer the enzyme acts specifically at glutamoyl bonds (Bond, 1989). Phosphate free conditions were used here to promote limited proteolysis. Protease V8 from the manufacturer's was resuspended in 0.125M Tris-HCl pH6.8 to give a final concentration of 5mg/ml (2.6 units/μl) and stored at -20°C. According to the product profile supplied by
manufacturers, 1 unit is that amount of enzyme required to cause a change in extinction coefficient at 280nm of 0.001 absorbance units/minute under conditions of the assay.

2.13.15 Limited N-terminal sequence analysis

Polypeptides which were to be sequenced were prepared by blotting them onto PVDF membrane. However, several modifications to the usual SDS-PAGE and Western blotting were found to be required to minimise N-terminal blocking. These were collated from a variety of published procedures (Matsudaira, 1987; Flannery et al., 1989 and manufacturers instructions for use of PVDF) and are described below.

SDS PAGE gels were cast as described in section 2.13 except that resolving gels were allowed to polymerise completely by letting the gel stand for 4 days at room temperature prior to use. Gels were pre-electrophoresed for 2 hours at 8mA using the standard electrode buffer plus 50µM reduced glutathione at the cathode and 100µM thioglycolic acid. Samples were loaded and electrophoresis performed as usual.

Electrotransfer to PVDF was performed using the standard Western blotting protocol described in section 2.13 with the following modifications: i) PVDF was prepared by immersion in 100% methanol until the membrane became translucent, then equilibrated in transfer buffer (see section 2.13) for 5 minutes in 0.05% (w/v) Coomassie blue dissolved in 50% (v/v) methanol, followed by destaining for 10-15 minutes in 50% methanol.
The stained blot was rinsed in ddH$_2$O, air dried and stored at -20°C. The appropriate band was cut from the membrane with a clean scalpel blade and trimmed to give a 2x4 mm segment. A limited N-terminal sequence was determined using repeated automated cycles of the Edman degradation using an applied Biosystems 470A GAS Phase Protein Sequencer equipped with an on-line phenylthiohydantoin amino acid analyser (this was carried out by Dr. Brian Coles, Molecular Toxicology Unit).

It was important to determine whether proteins that could not be sequenced were naturally N-terminally blocked or blocked due to electrophoresis or Western blotting procedures. For this reason 10 µg of rat glutathione transferase 3-3 (donated by B.Coles) was treated as described above. This protein is not blocked \textit{in vivo} and after being treated as above gave a yield of (90%) the expected N-terminal sequence.

2.14 Recombinant DNA techniques

2.14.1 Restriction enzyme digests

Routinely, 1-10 µg of DNA was digested with 10U of enzyme in 10 µl of 1x buffer for 2 hrs at 37°C. Restriction buffers, prepared as 10 x stocks, were as recommended by BCL.

2.14.2 Gel electrophoresis of DNA

DNA fragments were separated by electrophoresis on agarose gels containing 0.1 µg/ml ethidium bromide (EtBr). Gels were viewed using a short wave UV transilluminator and photographed using Polaroid type 55 positive/negative film. DNA fragments were separated
on 0.8% agarose gels in 0.5 x TBE buffer (90mM Tris base, 90mM boric acid, 2mM EDTA), run at a voltage of 5-20v/cm.

2.14.3 Recovery of DNA fragments from agarose gels
DNA fragments were recovered from agarose gels by procedure of Young et al., (1985).

2.14.4 Ligation and alkaline phosphatase treatment of plasmid DNA
Ligations were carried out in 10μl ligation mix (50mM Tris-HCl, 10mM MgCl₂, 10mM DTT, 1mM spermidine, 1mM ATP, 100g/ml BSA) at 15°C overnight. Routinely 100ng of vector DNA was ligated with a four fold excess of fragment DNA, using 0.1U of T4 DNA ligase. Where appropriate the vector DNA was pre-treated with Boehringer calf intestinal phosphatase (CIP) to prevent self ligation. The DNA was dissolved in CIP buffer (50mM Tris-HCl pH 9.0, 1mM MgCl₂, 0.1mM ZnCl₂, 10mM spermidine). 0.01U of CIP was added for each pM of DNA termini and the mix incubated at 37°C for 1hr. CIP was inactivated by heating 2min at 65°C, followed by recovery of DNA by phenol extraction.

2.14.5 Large scale purification of plasmid DNA
Large scale purification of plasmid DNA was prepared as described in Sambrook et al., (1989). The cells were then harvested and resuspended in 8ml ice-cold 10% sucrose, 50mM Tris-HCl (pH 8.0). Lysozyme was then added at a final concentration of 2mg/ml and the cells were incubated for 5min on ice, before the addition of 1.6ml of 0.25M EDTA. After a further 15min on ice, 6.4ml of 2% Triton X100, 60mM EDTA, 50mM Tris-HCl (pH 8.0) was added
to the cells which were then centrifuged in a Sorvall SS34 rotor at 20,000rpm for 30 min. The lysate was decanted and weighed. Caesium chloride (CsCl) was added to a final concentration of 75.4\mu g/g lysate. The lysate was transferred to a Beckman ultraclear tube and was centrifuged in a Beckman 70Ti fixed angle rotor at 40,000rpm for 36hrs at 15°C. The plasmid DNA was collected via a syringe and 1.1mm needle. EtBr was removed by repeated extractions with isopropanol saturated with CsCl. The DNA was then precipitated with isopropanol.

2.14.6 In vitro labelling of DNA probes

DNA probes were prepared by random prime labelling as described in the instructions by Amersham.

2.15 Procedures for nucleic acid analysis

2.15.1 Isolation of total cellular yeast nucleic acid

Yeast total nucleic acid was isolated by a method based upon that described by Sprague et al., (1983) and modified by Chaleff and Tatchell (1985).

30ml of yeast liquid culture were harvested at 4°C in a corex tube and washed with ice cold sterile ddH2O. 1-2 cell volumes of glass beads (BDH 40 mesh) were added to the pellet, followed by 3ml RNA extraction buffer (10mM Tris-HCL pH 7.5, 10mM EDTA, 1% SDS) plus 3ml phenol. After vortexing for 5min, the two phases were then separated by centrifugation. The aqueous phase was transferred to a fresh tube with 1.2M ammonium
acetate and precipitated with 2 volumes ethanol. Nucleic acid was pelleted by centrifugation, the pellets washed with cold 70% ethanol and then dried under vacuum before resuspension in TE. DNA was isolated from total nucleic acid by incubation with 2μg DNAase-free pancreatic RNAase per 100μg nucleic acid for 1hr at 37°C. Following phenol/chloroform extraction, the DNA was recovered by ethanol precipitation.

2.15.2 Determination of nucleic acid concentrations
The nucleic acid was diluted 1:200 in ddH₂O and the absorbance at A₂₆₀ was read spectrophotometrically. Nucleic acid concentrations were determined assuming 1 A₂₆₀ unit (1cm light path)=approximately 50μg/ml DNA or 40μg/ml RNA.

2.15.3 Southern transfer
Following electrophoresis agarose gels were blotted as described in Sambrook et al., (1989). The membrane used was Genescreen Hybridisation Transfer Membrane (NEN Research products). Capillary blotting was carried out for 16-20hrs using 20xSSC (3M sodium chloride, 0.3M sodium citrate).

2.15.4 Northern transfer
4.5μl RNA (10-20μg) was denatured in 2μl 5xNorthern running buffer (0.2M MOPS, 50mM sodium acetate, 5mM EDTA), 3.5μl formaldehyde, 10μl formamide at 55°C for 15min. The RNA was then loaded onto denaturing 1.1% agarose gel containing 1x Northern running buffer, 2.2M formaldehyde and electrophoreses at 60V for 4hr. The gel was then blotted onto
Genescreen hybridisation Transfer Membrane as described in Sambrook *et al.*, (1989) for 16-20hr using 20 x SSC.

### 2.15.5 Hybridisation of membrane bound nucleic acid to radio-labelled probes

Membranes were dried at 80°C for 2-4 hr and then pre-hybridised in a sealed bag containing 50% formaldehyde, 0.2 % polyvinylpyrrolidone, 0.02% BSA, 0.2 % ficoll, 50mM Tris-HCL pH 7.5, 1M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulphate, 100μg/ml denatured calf thymus DNA. Pre-hybridisation took place at 42°C for a minimum of 6hrs after which the membrane was transferred to a fresh bag and hybridised with the relevant nick translated probe in pre-hybridisation buffer without NaCl.

Blots were washed at room temperature for 15 min with 2xSSC, followed by a high stringency wash at 65°C in 2xSSC, 1% SDS for 15 min and a final low stringency wash at room temperature in 0.1% SSC for 15 min. All washes were carried out in duplicate with constant agitation.

### 2.15.6 Autoradiography and quantification of hybridisation signal

Membranes were exposed to Fuji X-ray film at -70°C in an autographic cassette (Protex). RNA-DNA hybridisation signals were quantified by aligning membranes with the corresponding autoradiography, then cutting out the region corresponding to each band plus a non hybridising region of the blot of a similar size prior to counting in Ecoscint (*Piper et al.*, 1988).
2.16 Glucose assay

Glucose in yeast culture supernatants was determined using the GLU-cinet Glucose assay kit (Technicon). 1ml of culture was harvested by centrifugation. 20, 10 or 5 µl of supernatant was added to 2.5ml of Glu-cinet reagent and assayed according to the manufacturer's instructions. Volumes used depended on the stage of growth at which the sample was taken in order to stay within the limits of the assay.

2.17 Electron microscopy of membranes

Plasma membranes were isolated from unstressed and stressed cultures as described in section 2.13. Fresh plasma membranes were always used. Transmission electronmicroscopy of the membranes was carried out by the Dept. of Crystallography, Birkbeck College, London.

2.18 A general assay for plasma membrane associated kinase activities

Plasma membranes were purified from yeast subjected to various conditions. Kinase activity associated with these membrane preparations was investigated using a modification of the procedure described by Kolarov et al., (1988). Purified plasma membranes (50µg of protein) were incubated for 10mins at 30°C in 50mM MES-KOH (pH 6.0), 6mM MgCl2 and 15µM Na2ATP mixed with 1µCi of 3, 000Ci/mmol [γ32P]-ATP. The final reaction volume was 30µl. Reactions were started by the addition of ATP and stopped by adding an equal volume of Laemmli gel protein sample buffer. Samples were submitted to SDS-PAGE and phosphorylated proteins were visualised by autoradiography.
3. INVESTIGATION OF ALTERED PLASMA MEMBRANE PROTEIN COMPOSITION IN S. CEREVISIAE SUBJECTED TO DIFFERENT STRESSES

3.1 Introduction

The lipid composition of yeast cells and their membranes exhibits great variation and can be easily manipulated by feeding cultures different phospholipids and sterols. Therefore yeasts are an ideal model for studying the properties and functions of lipids in eukaryotic cell membranes and have been used extensively for this purpose (Prasad and Rose, 1985). There have been numerous investigations of the effects of stress (especially ethanol stress) on yeast membrane lipids (reviewed in Rose, 1993 and Lloyd et al., 1993), but no study of the effects of ethanol stress on membrane proteins has been done, and only one study of the effects of heat shock on the plasma membrane proteins of S. cerevisiae has been done (Panaretou and Piper, 1992). Investigations into the proteins associated with the plasma membrane have generally been limited to finding marker polypeptides with which to assess purity of membrane fractions (Robertson et al., 1980) or to study the membrane assembly in relation to secretory processes (Tschoop et al., 1984). At the start of this study it was thought worthwhile therefore to investigate effects of stresses other than heat shock on the plasma membrane proteins of yeast.
Investigations in this laboratory by Dr. B. Panaretou revealed a specific heat shock protein (hsp30) associated with the plasma membrane. This he showed was induced by heat shock and entry to stationary phase (Panaretou and Piper, 1992). This study therefore began with the investigation into whether other stresses which might induce this novel heat shock protein. Ethanol stress on yeast cells was investigated initially.

With the discovery of hsp30, as a first step towards elucidating the function of this membrane protein, efforts were aimed at creating a $HSP30$ gene disruption mutant. This gene had been sequenced by a group in France and by the group of H. Feldman as part of the sequencing of yeast chromosome III. The $HSP30$ gene was provided to us by the former group, together with a hsp30::URA3 strain that they had constructed. However for this study it was necessary to construct an additional hsp30 mutant in a multiple protease deficient genetic background since B. Panaretou had previously shown that it was necessary to use protease deficient cells in order to isolate pure plasma membranes from stressed yeast with intact proteins (Panaretou and Piper, 1992). Also, since the $HSP30$ gene disruption vector used by the French group was no longer available, it was necessary to reconstruct a vector for $HSP30$ disruption in a protease deficient strain.
3.2 Results

3.3 Influence of ethanol stress on plasma membrane $H^+$-ATPase levels of *S. cerevisiae*

A one litre culture of the protease deficient *S. cerevisiae* strain BJ2168 was grown overnight in YEPD to mid-exponential phase at 28°C. Aliquots of the culture were then either left at 28°C, stressed by the addition of 6% or 8% (v/v) alcohol for 40 mins, or stressed by heat shock at 40°C for 40min (HS fraction). Plasma membranes were then isolated from these cultures as described in section 2.13.5, the proteins of these plasma membranes being resolved on a 15% gel and visualised by staining with Coomassie blue [Fig. 3.1(a)]. It can be clearly seen that the levels of $H^+$-ATPase, the most abundant plasma membrane protein, decline in both ethanol stressed fractions. This decline is also seen in the HS fraction, as noted previously by Panaretou and Piper (1992). The extent of $H^+$-ATPase decline with ethanol is comparable to that seen in the HS fraction.

3.4 Induction of hsp30 by ethanol stress in *S. cerevisiae*

The plasma membrane proteins analysed in 3.3 were resolved on an identical 15% gel and blotted for Western blot analysis of hsp30 levels as described in Chapter 2. The blot was probed for hsp30 using anti-hsp30 antiserum by M. Regnacq at the University of Bordeaux. Hsp30 was detected in both 6% and 8% ethanol stressed fractions; greater induction being seen in the 6% fraction. [Fig. 3.1(b)]. Fig 3.1 therefore shows that ethanol produces the same two major changes to plasma membrane protein composition as a heat shock, a decline in $H^+$-ATPase level and acquisition of hsp30.
3.5 Induction of hsp30 by weak acid stress

One litre cultures of *S. cerevisiae* (BJ2168) were grown overnight at 28°C in pH4.5 YEPD to mid-exponential phase; transferred to pH4.5 minimal medium and then pulse labelled using [H\(^3\)]-Leucine in the presence and absence of 0.75mM sorbic acid for 4 hours. Plasma membrane proteins were isolated from these cultures, resolved on a 9% gel and visualised using autoradiography.

From Fig. 5.8(a) it can be clearly seen that sorbic acid treatment at pH4.5 leads to the induction of two plasma membrane proteins; one of approximately 150kDa [denoted as S, Fig. 5.8(a)] and the other of 30kDa. The smaller protein is hsp30 since it is absent in the *hsp30::URA3* mutant generated in work described in this chapter [Fig. 5.8(a) and C. Ortiz-Calderon, unpublished]. The induction of the larger protein will be discussed in Chapter 5 together with other investigative work carried out to identify this protein.
Figure 3.1(a) Decline in levels of H⁺-ATPase due to ethanol stress.
Plasma membranes were isolated from *S. cerevisiae* (BJ2168) and resolved on a 15% gel and visualised by staining with Coomassie blue. 20μg of total protein was fractionated in each lane.
1- exponentially growing cells
2- cells heat shocked for 40 min at 40°C
3- cells treated with 6% ethanol, 40 min, 28°C
4- cells treated with 8% ethanol, 40 min, 28°C

Figure 3.1(b) Induction of hsp30 by ethanol stress.
An identical gel fractionation of the samples, blotted and probed for hsp30 using an anti-hsp30 antiserum. Lanes 1-4 as above.
3.6 Construction of a protease deficient strain disrupted in *HSP30*

pUC19-HSP30 (Regnacq and Boucherie, 1993) was cleaved with PstI and BamH1, the 2kB DNA fragment containing the entire *HSP30* gene being ligated to pSP46 cleaved also with PstI and BamH1. pSP46 is the same as pSP64 (Melton, 1984) except that the HindIII restriction site in the polylinker of the latter plasmid is a BgIII site in pSP46. This ligation resulted in plasmid pSPHSP30 shown in Fig. 3.2.

The 1.1 kb BamH1 *URA3* fragment was BamH1 linker from Ydp-11 and ligated into the unique HindIII site of pSPHSP30 to give pSPHSP30::URA3 (Fig. 3.2). *S. cerevisiae*, BJ2168 was then transformed to uracil prototrophy using BamHI-PstI-cleaved pSPHSP30::URA3 and then transformants selected using selective media plates lacking uracil. Three transformants (KT3, KT5 and KT10) were grown up together with the BJ2168 parent, their genomic DNA prepared, and these DNAs restricted with Xbal, HindIII and PstI. Southern blots were then probed with sequences of the 2.0kb Pst-BamHI fragment of pUC19-*HSP30* and the 1.1 Kb *URA3* fragment from YDP11 [Fig. 3.3(b)]. The hybridisation showed that KT3 and KT5 contained a single *URA3* gene fragment inserted into HSP30 coding sequences in the orientation shown in Fig 3.3(b). KT10 appeared to contain multiple integration events [Fig. 3.3(a)]. KT3 was the strain used as a hsp30::URA3 mutant in all subsequent work.

The Xba digests with both probes (*HSP30* & *URA3*) clearly show that the *HSP30* gene was successfully disrupted. The HindIII digest with the *HSP30* probe shows a number of bands in lane 1 (corresponding to the wildtype strain). This cannot be explained since the wildtype *HSP30* gene only displays a single HindIII site.
Figure 3.2 construction of vector pSPHSP30::URA3 used to disrupt HSP30.
The genomic DNA samples were from the wild type strain (lane 1), transformant KT3 (lane 2), transformant KT5 (lane 3) and transformant KT5 (lane 4). They were restricted with Hind III, PST1 and Xba as shown and probed with \textit{HSP30} (the top row) and \textit{URA3} (the bottom row).
Figure 3.3(b) Map of URA3 integration into HSP30. URA3 gene fragment insertion present in strains KT3 and KT5 [Fig. 3.3(a)]. The upper diagram shows the restriction map of PstI and HindIII sites at the HSP30 locus on Chromosome III.
3.7 Isolation of plasma membranes from a strain disrupted in the \textit{HSP30} gene.

One litre cultures of \textit{S. cerevisiae} BJ2168 and KT3 were grown overnight in YEPD to mid-exponential phase at 28°C, then heat-shocked at 40°C for 40 mins. Plasma membrane proteins were isolated from these cultures as described in section 2.13.5. Plasma membrane proteins were resolved on a 15\% gel and visualised by staining with Coomassie blue as described in section 2.13.8. Fig. 3.4 shows loss of the hsp30 protein in the KT3 strain, clearly confirming that there are no other highly-expressed genes for this protein in the genome. Also of interest is the decline in the level of H\textsuperscript{+}-ATPase in the HS fraction of this strain (Fig 3.2). That this decline still occurs in the mutant strain indicates that it is not a function of hsp30 protein (investigated in greater detail in section 3.9).
Figure 3.4 Isolation of plasma membrane proteins from wt & *hsp30* cells.
Plasma membranes were isolated from *S. cerevisiae* (BJ2168 and KT3/hsp30::URA3), and their proteins resolved on a 15% gel. 50\(\mu\)g of total protein was fractionated in each lane and proteins were visualised by staining with Coomassie blue.

(-) unshocked, (+) heat-shocked 40°C, 40 mins.
3.8 In vitro phosphorylation of hsp30

Hsp30 is phosphorylated in vitro, being the only major difference in phosphoproteins labelled when plasma membranes of unstressed and heat shocked cells are incubated with (γ-<sup>32</sup>P) ATP (B. Panaretou, PhD dissertation, 1992).

One litre cultures of <i>S. cerevisiae</i> BJ2168 and KT3 cultures were grown overnight in YEPD to mid-exponential phase at 28°C, then heat-shocked at 40°C for 40mins. Cells were harvested, plasma membranes isolated as described in section 2.13.5. and these purified membrane samples then incubated with [γ<sup>32</sup>P]-ATP as described in section 2.18. At the end of the reaction, phosphorylated proteins were resolved on a 15% gel, after which the gel was dried and phosphorylated proteins were visualised using autoradiography (section 2.13.9). As shown in Fig. 3.5, phosphorylated proteins in membranes from heat shocked cells of the mutant lack hsp30, whereas there is no difference in the two strains prior to heat shock and hsp30 induction. There seems to be an overall reduction in kinasing activity with the HS fraction of the mutant strain (Fig. 3.5) which might be due to the enhanced ATPase activity of this membrane fraction discovered much later by other workers in our group and discussed later.
Figure 3.5  

*In vitro* protein phosphorylation of plasma membranes.

Plasma membranes from stressed and heat-shocked *S. cerevisiae* (BJ2168 & KT3) were incubated with \([\gamma^{32}\text{P}]\)-ATP, labelled proteins were then resolved on a 15% gel and visualised by autoradiography. (-) unstressed, (+) HS 40°C, 40 mins. 50μg of total protein was fractionated in each lane.
3.9 Investigation into decline of levels of H⁺-ATPase during heat-shock.

Since hsp30 induction by heat shock or ethanol is associated with a decline in H⁺-ATPase levels (Fig. 3.1) we considered the possibility that hsp30 is responsible for turnover of much of the H⁺-ATPase. If this is so, KT3 cells should be defective in H⁺-ATPase loss during heat shock.

One litre cultures of *S. cerevisiae* (BJ2168 and KT3) were grown overnight in YEPD to mid-exponential phase at 28°C at which point they were heat shocked at 40°C for different time periods. After heat-shock the cultures were harvested, plasma membranes isolated and plasma membrane proteins were resolved on a 15% gel [Figs. 3.6(a) and (b)]. Levels of H⁺-ATPase the major plasma membrane protein were seen to rapidly decline over the first 20min of heat shock (possibly more dramatically in the mutant strain) but then to stabilise and not decline further with more prolonged heat stress. Noticeably in the wild type strain the level of H⁺-ATPase appeared to rise slightly after 40mins of HS, an observation not made with the mutant strain. This could perhaps indicate a role for hsp30 but was not investigated further. However Fig. 3.6 (b) clearly shows the major decline in H⁺-ATPase is not due to hsp30 since this decline occurs even when this protein is absent. Turnover of plasma membrane proteins is thought to occur via endocytosis of regions of the membrane. Possibly the rapid decline in H⁺-ATPase with stress represents a sudden increase in endocytosis. If so, it should be absent in endocytosis mutants (not tested in this study). Consistent with endocytosis being involved is my finding that the H⁺-ATPase decline with heat shock is not inhibited by cycloheximide (data not shown).
Figure 3.6(a) Decline in levels of $H^+$-ATPase with heat-shock in *S. cerevisiae*, BJ2168. Proteins were resolved on a 15% gel (20μg of total membrane protein was fractionated in each lane) and were stained with Coomassie blue. (A) Plasma membranes from unstressed cells. B-E plasma membranes isolated from cells heat-shocked at 40°C for: (B) 10 min; (C) 20 min; (D) 30 min and (E) 40 min.
Figure 3.6(b) Decline in levels of H\(^+\)-ATPase with heat-shock in *S. cerevisiae* KT3.
Proteins were resolved on a 15% gel (20\(\mu\)g of total protein was fractionated in each lane) and were visualised by staining with Coomassie blue.
(A) Plasma membranes from unstressed cells.
B-E plasma membranes isolated from cells heat-shocked at 40\(^\circ\)C for: (B) 10 min; (C) 20 min; (D) 30 min and (E) 40 min.
3.10 Electron microscopy of plasma membranes from unstressed and stressed cultures

Membranes consist primarily of molecules containing carbon, hydrogen, oxygen and nitrogen with low electron scattering power which cannot be studied by simply mounting whole membranes on a grid. Due to this lack of contrast, the various components, such as proteins and lipids, cannot be sufficiently differentiated in the electron micrograph. However, greater specimen contrast can be obtained by negative staining to reveal important information about overall size and shape of proteins, their orientation in macromolecular assemblies and the interaction with other proteins or molecules (Bremer et al., 1992).

Plasma membranes from unstressed and heat shocked *S. cerevisiae* (BJ2168) were treated as described in section 2.17. and viewed using Transmission Electron Microscopy. [Figs. 3.7(a) and (b)].

The electron micrographs show membrane vesicles but no significant difference between the unstressed and heat-shocked fractions. Further studies using electron microscopy were not continued with for this reason.
Figure 3.7(a) Electron micrograph of unstressed cell plasma membranes.

Fresh plasma membranes from unstressed cultures of *S. cerevisiae* (BJ2168) were negative stained with 2% uranyl acetate and photographed at 25K and 80KV on a 1200EX transmission electron microscope.
Figure 3.7(b) Electron micrograph of heat-shocked cell plasma membranes.
Fresh plasma membranes from heat-shocked cultures of *S. cerevisiae* (BJ2168).
Imaged as described in Fig. 3.7(a).
3.11 Preparation of hsp30 antisera

A vector for expression of a hsp30-GST fusion protein (pGEX19) in E.coli was kindly provided by H.Boucherie. This was transformed into E. coli and the fusion protein was isolated as described in the Pharmacia instruction manual. (IPTG was used to induce the fusion protein for 8 hours). Both insoluble and soluble fractions were fractionated on a 15% gel and visualised by Coomassie staining. The fusion protein can clearly be seen overexpressed as a 43kDa protein in the induced insoluble fraction (Fig. 3.8).

The 43kDa protein band (estimated as 30μg total protein) was excised from the gel and prepared for injection into the rabbit as described in Harlow and Lane (1988). A test bleed was taken 10 days after injection into the rabbit in order to establish if an immune response had been elicited. Unstressed and HS membranes were dot blotted and probed using the test bleed antisera. It was shown that an immune response had been elicited and therefore further test bleeds were taken. Anti-sera from the second bleed was used to probe western blots of unstressed and heat shocked plasma membranes. However a signal corresponding to hsp30 was not seen in the HS fraction. Further work with this anti-serum was not continued with.
Figure 3.8  Isolation of hsp30-GST fusion protein.

Both soluble and insoluble fractions from *E. coli* were fractionated on a 15% gel and visualised with Coomassie stain (-)-uninduced fractions, (+) IPTG induced for 8 hours.
3.12 Discussion

Ethanol stress and plasma membranes

Membranes, in particular the plasma membrane, are important targets in the interaction of the yeast cell with ethanol (Ingram and Buttke, 1984). Damage to the plasma membrane is thought to be a major cause of ethanol toxicity (see Introduction). While the effects of ethanol on the lipids of this membrane have been the subject of considerable study, there have been no investigations of whether ethanol influences plasma membrane protein composition. At least one plasma membrane protein (plasma membrane H⁺-ATPase) influences both heat and ethanol tolerance (Panaretou and Piper, 1990). Both heat stress (Coote et al., 1987; Coote, 1993) and ethanol (Cartwright et al., 1987; Rosa and Sa-Correia, 1991) cause stimulation of this H⁺-ATPase activity, the resulting enhancement of catalysed proton efflux from the cell possibly helping to counteract the depolarisation of the plasma membrane resulting from the stress induced increases in membrane permeability.

Several of the changes induced by exposure to stressful ethanol levels are identical to those caused by a heat stress. For example both stresses will increase membrane permeability and cause protein denaturation. It is not surprising therefore that ethanol acts in a synergistic way to increase the damage caused by heat (Van Uden, 1984). The similarities of cellular responses to both stresses was seen in this study of plasma membrane proteins from ethanol stressed cultures. While it is well established that cells alter the lipid composition of their membranes in response to heat and ethanol (Ghareib et al., 1988) this is the first demonstration that they also rapidly alter the protein composition of the plasma membrane.
There was a decline in levels of plasma membrane ATPase in cultures treated with 6 and 8% ethanol stress [Fig 3.1(a)] and an induction of hsp30 [Fig.3.1(b)], identical changes to those observed with heat shock (Panaretou and Piper, 1992). Hsp30 was induced to similar levels by heat shock and treatment with 6% ethanol, although its induction was appreciably less with 8% ethanol [Fig. 3.1(b)]. It is shown later that hsp30 is also induced by weak acids [Fig. 5.8(a)]. hsp30 is therefore not simply a heat shock protein but a stress protein induced by stress agents other than heat shock.

The ATPase loss with heat shock appears to reflect protein turnover in the absence of de novo synthesis since it still occurs in cells subjected to these stresses in the presence of cycloheximide (Talreja, unpublished results). This progressive and relatively rapid loss of ATPase from the plasma membrane with heat or ethanol stress contrasts with the stimulation of the residual fraction of this enzyme with these stresses (Coote et al., 1991; Coote et al., 1994 and Rose and Sa-Correia, 1992). Both factors almost certainly influence the capacity of the cells for sustained maintenance of homeostasis during the stress. Using a hsp30 mutant later studies (Chapter 4) determined whether the acquisition of hsp30, contributes significantly to ethanol or heat tolerance.

There is a critical threshold (4%) for induction of heat-shock proteins by ethanol. Maximal induction is seen at higher ethanol levels which are different for individual heat-shock proteins and heat shock promoters (Piper et al., 1994). Increasing ethanol levels from 4 to 10% increases the synthesis of hsp104 and hsp70 in BJ2168 (Piper et al., 1994). However hsp30
levels with this strain are seen to decline with increasing ethanol concentration. This suggests that the level of induction of different heat shock genes is regulated by factors that sense different sublethal ethanol concentrations.

Besides hsp60, several physiological changes resulting from heat or ethanol exposure are also important in determining cellular tolerance to these stresses (Mager and Moradas-Ferreira, 1993; Piper 1993; Parsell and Lindquist, 1993). The rapid loss of plasma membrane H⁺-ATPase protein with ethanol [Fig. 3.1(a)] or heat stress (Panaretou and Piper, 1992) contrasts with the ethanol stimulation of the activity of this enzyme (Rosa and Sa-Correia, 1991). Both these influences on the ATPase will affect its role in the maintenance of homeostasis (Serrano, 1991) and should therefore have important affects on tolerance to prolonged exposure to ethanol.

**Decline in levels of H⁺-ATPase during heat shock**

Results in this chapter show a decline in the level of H⁺-ATPase protein levels with heat shock in both the hsp30 mutant and parent strain [Figs. 3.6(a) and (b)]. The drop in the level of ATPase over the first 20 min of HS in both strains is identical. This shows that hsp30 is not necessary for ATPase decline. However by 40 minutes of heat shock the levels appear to recover to a greater extent in the wt strain than in the hsp30 mutant. This observation suggests that ATPase protein levels may be being partially restored by a stabilising affect of hsp30. However the effect is small and would have to be duplicated many times to be proven (this experiment was actually only performed once). With the mutant the ATPase activity in
purified membranes does not drop but is actually activated by heat shock (Piper et al., submitted), even though protein levels of the enzyme are reduced [Fig. 3.1(a)]. Possibly lowering of ATPase protein levels is to compensate for stimulation of this enzyme by stress, preventing total depletion of cellular ATP by an enzyme that consumes 15-50% of all the ATP generated by the cell (Serrano, 1989).

The crucial physiological role of plasma membrane H⁺-ATPase and the fact that it is a major consumer of ATP, requires its strict regulation. The carboxyl terminus of this ATPase may act as an autoinhibitory domain, several regulatory residues of this carboxyl terminus have been identified using site-directed mutagenesis (Portillo et al., 1991). Evidence has now been obtained that hsp30 downregulates the activity of H⁺-ATPase (Piper et al., submitted). How this operates is unknown, but hsp30 could be interacting with the carboxyl terminus, inhibiting phosphorylation of residue Ser-819, (Eraso and Portillo, 1994) and thus preventing an increase in utilisation of cellular ATP by ATPase.

**Electron microscopy of plasma membranes**

In order to understand how cells are protected against a given stress it is necessary to know what damage a cell experiences during that stress. The effects of hypothermia on the ultrastructure of mammalian cells have been investigated by several different groups (reviewed by Welch, 1990) and similar studies have been performed on various plant cells (Fransolet et al., 1979; Neumann et al., 1984; Mansfield et al., 1988). The most characteristic feature of heat shocked cells from both plants and animals is the aggregation and vacuolation of the
nucleolus due to the loss of the nucleolar granular component (Fransolet et al., 1979; Welch and Suhan, 1985).

There are surprisingly few reports on temperature induced ultrastructural changes in mesophilic yeasts. Leakage of intracellular components, as observed by scanning electron microscope, has been reported by Harada et al., (1976) in a temperature sensitive mutant of \textit{C. pseudotropicalis} at the non-permissive temperature of 40°C. To date the only ultrastructural studies of yeast cells subjected to heat shock have looked at changes in nucleolus or granulation of the cytoplasm following heat treatment (Rossi and Lindquist, 1989; Webster and Watson, 1993). A distinct feature of heat-shocked cells was the formation of electron dense granules in the mitochondria.

Differences between unshocked and heat-shocked plasma membranes were investigated to see whether this might indicate a role for hsp30. It is conceivable that a hsp targeted to the membrane under conditions of heat stress which cause disordering of the membrane, may somehow be involved in altering the ultrastructure of the membrane thereby limiting the damage caused by heat. This damage limitation role of hsp30 could perhaps then be compared to that of other hsps such as hsp70 which acts as a chaperone protein preventing excessive protein aggregation in heat shocked cells (Parsell and Lindquist, 1993). Very little difference in ultrastructure was apparent between unshocked and heat shocked membrane fractions [Figs. 3.7(a) and (b)]. Therefore electron microscopy of the mutant strain was not continued.
In vitro phosphorylation of plasma membrane proteins.

There are various reports of in vitro phosphorylation of plasma membrane proteins from both S. cerevisiae (McDonough and Mahler, 1982; Kolarov et al., 1988) and Sch. pombe (Amory and Goffeau, 1982). Heat shock results in at least a 50% decline in levels of ATPase protein (Fig. 3.1; Panaretou and Piper, 1992). However phosphorylation studies suggest that there is equal phosphorylation of the ATPase in both heat shocked and non-stressed membranes (although at no point was it conclusively demonstrated that the 100kDa phosphoprotein is indeed the ATPase) (Fig. 3.5). This indicates that the ATPase polypeptide from heat shocked cells may be phosphorylated in vitro to a greater extent than the polypeptide from the unstressed cells. This could be due to increased activity of the appropriate kinase, or activity of additional kinases distinct from those acting in membranes from unstressed cells.

There is also a diffuse phosphoprotein band of 50kDa (Fig. 3.5). Other accounts of in vitro phosphorylation of membrane proteins invariably report the existence of a prominent phosphoprotein of this molecular weight (e.g. McDonough and Mahler, 1982, Amory and Goffeau, 1982). One report identifies it as the regulatory subunit of cAMP dependent protein kinase (Behrens and Mazon, 1988).

This apparent enhanced phosphorylation of ATPase in membranes from heat shocked cells might be linked to a number of cellular processes activated by heat shock or the stress-induced activation of the ATPase (Coote et al., 1994; Rosa and Sa-Correia, 1991). One of the ways by which extracellular signals are transduced into intracellular events is activation of
phospholipase C. There are numerous examples of this in mammalian cells (Nishizuka, 1988) and yeast (Kaibuchi et al., 1986). Phospholipase C hydrolyses plasma membrane inositolphospholipids; the initial products being diacylglycerol (Dag) and inositol triphosphate (IP$_3$). Water soluble IP$_3$ diffuses into the cytosol and releases Ca$^{2+}$ from internal stores. Dag remains in the membrane and activates protein kinase C. In mammalian cells heat shock is one of the signals that causes this. Given the evolutionary conservation of heat inducible phenomena and the fact yeast has the phosphoinositide signaling pathway (Patton and Lester, 1992; Thevelein, 1995) and protein kinase C (Hunter, 1991), it is possible that release of Dag and IP$_3$ occurs in yeast during heat shock. Protein kinase C could then activate pathways leading to phosphorylation of H$^+$-ATPase at the regulatory C-terminal domain.
CHAPTER 4

4. INVESTIGATION INTO THE PHENOTYPE OF HSP30 LOSS

4.1 Introduction

Determination of whether hsp30 has any specific function can be investigated by detailed physiological analysis of isogenic hsp30 and wildtype strains, such as KT3 and BJ2168. It may well be that it does not have an easily identifiable role, as is the case for the hsp26 heat shock protein of yeast (Petko and Lindquist, 1986). However, since it is specifically targeted to the plasma membrane, it is conceivable that it might exert a damage limitation function at the plasma membrane. Hsp30 is induced by heat shock, stationary phase, ethanol and weak acid stress. It accumulates in the membrane at comparatively high levels; it's levels in isolated plasma membranes from heat shocked or stationary cells being approximately equimolar to the levels of the much larger 100-kDa H⁺-ATPase (Panaretou and Piper, 1992). Conceptually, hsp30 may influence membrane order or might act to protect key membrane proteins. Targets for such protection may include the plasma membrane H⁺-ATPase, an enzyme which the cell possibly cannot replenish readily if inactivated in the stressed state (Panaretou and Piper, 1992).

It is postulated that some of the hsps, specifically hsp70, may play a role in protecting cells from thermal or other environmental stresses (for review see Lindquist and Craig, 1988). Li et al., (1991) found that the constitutive expression of hsp70 conferred heat resistance to rat cells. In S. cerevisiae, mutations in the highly conserved HSP104 gene specifically and
severely impair induced thermotolerance (Sanchez and Lindquist, 1990). They do not abolish it. Thus although hsp104 plays a pivotal role in thermotolerance, other inducible factors must also be important.

An initial step towards investigating the function of hsp30 was the comparison of stress tolerances of WT and hsp30 mutant strains. This would identify if hsp30 is involved in stress adaptation, as is the case with hsp70 and hsp104 (Moradas-Ferreira and Mager, 1993). Cell inactivation was measured in BJ2168 and KT3 (hsp30 mutant) cells exposed to potentially lethal levels of: UV light, NaCl, H$_2$O$_2$ and ethanol stress. Survival was also measured during nitrogen starvation.

Cells given a mild, sublethal heat stress show the development of induced or acquired thermotolerance, manifested as increased capacity for survival during subsequent exposure to higher, potentially lethal temperatures (reviewed in Piper, 1993). However a mild heat shock is only one of a number of preconditioning treatments known to induce thermotolerance. Certain chemical agents, osmotic dehydration and nutritional status have effects on yeast thermotolerance which are just as dramatic (reviewed in Piper, 1993).

Pre-conditioning treatments investigated were: 9mM sorbic acid, 6% ethanol stress and heat. The latter two pre-conditioning treatments are known to induce hsp30 (Fig. 3.1), while Piper and Cheng (1994) showed the induction of hsp30 mRNA by 9mM sorbic acid by northern analysis.
H⁺-ATPase is vital in nutrient uptake, maintenance of potassium balance and the regulation of intracellular pH. It has been shown that H⁺-ATPase levels decline with heat shock in cells that are simultaneously showing hsp30 (Chapter 3; Panaretou and Piper, 1992). With this observation, it was of interest to investigate whether hsp30 had any influence over the activity of H⁺-ATPase during changes in temperature. Total acid efflux by intact cells is a good measure of ATPase activity in vivo in yeast (Serrano, 1980; Cid et al., 1987); therefore changes in rate of acid efflux were investigated over a range of temperatures in both wildtype and hsp30 strains. More detailed studies of net proton flux during heat stress were also carried out. If the WT strain showed greater acid efflux than the hsp30 mutant, it would clearly implicate hsp30 influencing H⁺-ATPase under the stress of high temperatures. Certain stresses (e.g. heat shock) will permeabilise membranes thereby causing pronounced disturbance to transmembrane ion gradients. Evidence was therefore sought for a role of hsp30 influencing membrane permeability of S. cerevisiae using fluorescence microscopy to measure membrane permeability changes during temperature stress.

Finally, preliminary investigations were carried out into weak acid sensitivity in both the WT and hsp30 strains, since Piper and Cheng (1994) found that hsp30 is induced by sorbic acid at the level of transcription. Detailed studies investigating weak acid stress are described in Chapter 5.
4.2 Results

All experiments following were performed at least twice unless otherwise stated; error bars where shown being SD.

4.3 Comparison of stress tolerances

The survival of WT and \textit{hsp30} cultures in exponential growth subjected to different stresses were measured as described in section 2.9. Results for UV light and 750 mM sorbic acid stress are shown in Figs. 4.1(a) and (b). 2.5M NaCl, 0.25 mM H$_2$O$_2$, freeze thaw and nitrogen starvation were also investigated (not shown). Stress tolerance was investigated using mid-exponential cells in the cases of UV light, 2.5M NaCl and 0.25 mM H$_2$O$_2$. In the other cases stationary phase cultures were used as \textit{hsp30} is induced during this phase of growth. With all stresses investigated no appreciable differences in stress tolerance between the two strains were observed. This indicates that \textit{hsp30} has no major impact on several of the stress tolerances of \textit{S. cerevisiae}. 

Figures 4.1(a-b)  Effect of stress on stress tolerance of WT and hsp30 cells.

(a)  UV light (2.6x10^5 ergs/sec/cm^2)

(b) 750mM sorbic acid stress (pH4.5)
4.4 Influence of hsp30 on the rate of net proton flux

Glucose addition to yeast cells causes a rapid stimulation of proton pumping by the H⁺-ATPase. The change in rate of net proton flux by intact hsp30 and WT cells subjected to heat shock was carried out as described in Coote et al., 1994. Results show an initial increase in acid efflux over the first 3 minutes in both strains which was greater in the WT than the hsp30 strain (Fig. 4.2). However the unstressed WT strain showed greater initial peak in acid efflux compared to the unstressed hsp30 strain.

4.5 Influence of hsp30 on the rate of glucose induced acid-efflux.

The rate of this acid efflux was measured as described in section 2.11 and in Cole and Keenan (1987) and expressed in molar equivalents of HCl. To measure the rate of acid efflux after thermal stress, separate cell suspensions were incubated in a water bath at temperatures between 5°C and 50°C for 30min. Prior to acid efflux measurements, the temperatures of stressed samples were allowed to equilibrate to 25°C in the water jacketed vessel. Results show the WT strain consistently showing a greater acid efflux rate compared to the mutant over the whole spectrum of temperatures investigated (Fig. 4.3).
Figure 4.2  Effect of prior sub-lethal incubation (42°C for 30 min, denoted as PRE) on the rate of glucose-induced acid efflux on both WT and hsp30 cells.
Figure 4.3  Effect of incubation temperature (30 min in sterile distilled water) on the acid efflux rate of both WT and *hsp30* cells.
4.6 Influence of hsp30 on membrane permeability

Membrane permeability during heating was measured as described in Coote et al., 1994 for both WT and hsp30 cell suspensions. The fluorescent nuclear stain ethidium bromide was used (Arkhammer et al., 1990). For each time point at least 20 individual cells were assayed. The results (Figs. 4.4 and 4.5) show no major differences in membrane permeability associated with loss of hsp30.
Figure 4.4(a) Effect of heat on membrane permeability on WT cells (measured by fluorescence microscopy). Membrane permeability was measured at 25°C, 42°C, 52°C and 52°C after a prior incubation at 42°C for 40 min (denoted as INDU).
Figure 4.4(b) Effect of heat on membrane permeability on hsp30 cells (measured by fluorescence microscopy). Membrane permeability was measured at 25°C, 42°C, 52°C and 52°C after prior incubation at 42°C for 40 min (denoted as indu).
Figure 4.5(a-d) Comparison of membrane permeability of WT and hsp30 strains at a range of different temperatures (25°C, 42°C, 52°C; and 42°C for 40 mins followed by 52°C denoted as INDU).

(a)

(b)
Cellular fluorescence
Average cellular pixel intensity

Time (min)

Average cellular pixel intensity

Time (min)
4.7 Investigation into whether hsp30 is involved in induced thermotolerance

Both WT and hsp30 strains were pre-incubated with either 9mM sorbic acid at 25°C, 6% ethanol at 25°C or heat shocked at 42°C for 40 min before they were shifted to a lethal temperature of 52°C. During exposure to 52°C samples were aliquoted and thermotolerance determined as described in section 2.10.

Results show a slight reduction in heat-induced thermotolerance in the hsp30 mutant [(Fig. 4.6(a)] but this is not seen throughout the whole time period. No significant differences in the thermotolerance of the two strains were apparent after pre-incubation with 6% ethanol or 9mM sorbic acid [Figs. 4.6(b) and (c)]. Neither of the latter two stresses caused large inductions of thermotolerance.
Figure 4.6(a) Effect of absence or presence of pre-incubation at 42°C for 40 min (PRE) on thermotolerance. Data from a representative experiment.
Figure 4.6(b) Effect of absence or presence of pre-incubation with 6% ethanol for 40 minutes at 28°C on thermotolerance. Data from a representative experiment.
Figure 4.6(c) Effect of absence or presence of pre-incubation with 9mM sorbic acid for 40 minutes at 28°C on thermotolerance. Data from a representative experiment.
4.8 Influence of hsp30 on weak acid sensitivity

Cell suspensions of both BJ2168 and KT3 were evenly spread onto the surface of two separate pH4.5 YEPD agar plates. 10μl of 0.3M, 0.6M and 0.9M potassium sorbate pH7 were then added to three paper discs placed on the surface of the agar. Growth of both strains in the presence of sorbic acid was monitored closely. An halo effect due to sorbate inhibition of growth was seen to appear within one day at 28°C. Initially the halo of growth inhibition was larger for KT3 cells (Fig. 4.7) but this effect was less evident and eventually by 3 days the halos of growth inhibition were the same for both strains. For this reason careful monitoring of the plates was required.

There was a larger halo of growth inhibition initially seen with KT3 (Fig. 4.7). Actually a secondary halo is seen with KT3 which is not seen with BJ2168. The fact that this halo difference is seen to diminish over 2-3 days at 28°C indicates that the hsp30 mutation extends the time needed for cells to adapt to growth in the presence of weak acids (i.e. increases lag phase as opposed to inhibiting growth altogether). This was later confirmed by C. Holyoak comparing growth profiles of the two strains; KT3 shows a greater lag phase than BJ2168 in the presence of sorbic acid [Fig. 4.8(b)]. Hsp30 is not required for adaptation to growth in the presence of sorbic acid at pH4.5, but assists this adaptation since wild-type cells adapt more readily (Fig. 4.7). This work therefore identified a clear phenotype associated with loss of hsp30 (Figs. 4.7 and 4.8), extension of the time needed for adaptation to the stress of exposure to non-growth inhibitory levels of weak acids.
Figure 4.7 Legend opposite
Opposite Legend

**Figure 4.7** Plate assays of sensitivity of BJ2168 and KT3 to sorbic acid
The plate was photographed after one day growth at 28°C.

The following data (Figure 4.8) was obtained by C.Holyoak, Unilever Research Laboratories.

**Figure 4.8** Growth curve of BJ2168 and KT3 cultured (a) pH4.5 YPD or (b) in 1.8mM sorbic acid. (BJ2168, thick line, KT3 thin line). Culture ATP shown as BJ2168 (filled symbols) and KT3 (open symbols).
4.9 Discussion

The stress response in *S. cerevisiae* is characterised by simultaneous acquisition of increased thermotolerance and expression of heat-shock proteins. A role for hsp104 in thermotolerance and ethanol tolerance acquisition has been clearly demonstrated (Parsell and Lindquist, 1993). Other hsps (notably hsp70) have been implicated in cellular recovery following heat stress (Lindquist and Craig, 1988) while others such as hsp12 and 26, have no known function. This chapter compares the stress tolerances of isogenic hsp30-deficient and wildtype cells, a study conducted to see whether hsp30 contributes to the stress tolerances of yeast.

Comparing BJ2168 and KT3 cultures in vegetative growth we could find no effect of hsp30 inactivation on thermotolerance, irrespective of whether or not the cells had been heat shocked (40min, 42°C), incubated with 6% ethanol or incubated with 9mM sorbic acid, three conditions known to induce hsp30 in the BJ2168 strain [Figs. 4.6(a-c)]. We were also unable to identify any difference in the survival of vegetative cells of these two strains, measured by subsequent colony formation on plates, following short-term exposure to: 15% (v/v) ethanol; 500mM or 750mM potassium sorbate at pH4.5 (Fig 4.1), 2.5M NaCl and a single freeze thaw cycle (not shown).

Hsp30 is also induced at stationary phase (Panaretou and Piper, 1992; Regnacq and Boucherie, 1993). We therefore investigated the survival of BJ2168 and KT3 cells transferred to nitrogen free minimal medium and maintained at 28°C over 14 days (not shown). There was no difference in the survival of these two strains over this time period. There were also
no differences in the thermotolerance or freeze thaw tolerance of YPD cultures of BJ2168 and KT3 that had entered stationary phase. It appears therefore that hsp30 protein has no major impact on several of the stress tolerances of *S. cerevisiae* cells.

It was found however that there is a phenotype associated with the loss of hsp30 (Fig. 4.7; also data obtained independently by C. Holyoak, Fig. 4.8). Cells lacking this protein take longer to adapt to stresses (such as osmostress and weak acid treatment) that perturb homeostasis and for which restoring homeostasis will entail considerable expenditure of ATP. This can be seen on agar plates; the effect of the *hsp30* mutation in slowing adaptation to weak acid being sometimes apparent as a secondary halo of growth inhibition in agar diffusion tests (Fig. 4.7).

The phenotype of the *hsp30* deletion mutant in extending lag phase before growth resumes is also seen after cells are inoculated into medium containing 1M NaCl, a degree of osmostress that slows growth but is not completely inhibitory (C. Holyoak, unpublished results). Also it has been found that cells deleted for *hsp30* scored on plates containing 100mM lithium acetate took 2-3 times longer to resume growth than its wildtype counterpart (Piper *et al*., unpublished results). Intracellular Li⁺ is toxic and must exit the cell by systems usually employed for ATP-dependent Na⁺ extrusion (notably the pump encoded by *ENAl*; Haro *et al*., 1993).
When cells are suddenly exposed to these stresses of weak organic acid, ethanol or osmostress, a higher ATP usage is necessary simply to attain the levels of homeostasis that will allow subsequent growth. This is apparent from the dramatic reduction in ATP levels in late-exponential cultures due to weak acid, evident from WT cells that have resumed growth after inoculation into growth medium containing sorbic acid as compared to BJ2168 cells inoculated into the same medium lacking weak acid (Fig. 4.8). Although sorbic acid causes a considerable extension to lag phase in wildtype cells, \( hsp30 \) mutant cells take even longer (approx. 10h) to adapt to growth in the presence of weak acid. This is presumably because they are less efficient at attaining the level of homeostasis that will support growth. C. Calderon has obtained evidence that hsp30 is a negative regulator of plasma membrane ATPase. The lack of this hsp30-induced down regulation of plasma membrane ATPase in sorbate adapting \( hsp30 \) cells should further enhance ATP consumption. This is consistent with the increased lag period before growth commences and the 2 to 3-fold decreased ATP levels in adapted cells of the mutant, indicative of a higher ATP usage (Fig. 4.8).

Previous studies have shown that sub-lethal heat stress results in the disruption of the proton gradient across the plasma membrane of \( S. \) cerevisiae (Weitzel et al., 1987). Other studies have implicated a role for \( \text{H}^+ \text{-ATPase} \) in heat resistance of yeast (Coote et al., 1991 and 1994; Panaretou and Piper, 1990). However the precise role of the membrane ATPase proton gradient and maintenance of pHi homeostasis in stress-induced thermotolerance in \( S. \) cerevisiae has yet to be determined. An investigation into whether hsp30 modulates the role of ATPase in heat stressed cells was investigated using rates of acid efflux. Comparison of
acid efflux rates of WT and cells deleted for \textit{hsp30} showed an initial large peak in acid efflux which was greater in the WT than the deleted strain (Fig. 4.2). However the unstressed WT strain showed a greater initial peak in acid efflux compared to the unstressed \textit{hsp30} strain. This is rather anomalous as one would expect, if \textit{hsp30} is a negative regulator of the ATPase, greater efflux rates in \textit{hsp30} cells. This same pattern of acid efflux rate is observed with the prestressed strains, the WT again showing greater acid efflux. Stress-induced stimulation of efflux could be a mechanism to restore pHi homeostasis after the large drop in pHi which occurs upon exposure to heat (Weitzel et al., 1987). However it is counteracted by stress-induced impermeability of the membrane to protons. This may be the reason no significant overall difference in rate was observed between unstressed and prestressed cells. The enhanced ability for catalysed extrusion with sublethal heat stress may however help restore homeostasis. The change in acid efflux rate with varying temperature shows the WT strain consistently showing a greater acid efflux rate compared to the mutant. However induction of \textit{hsp30} has so far only been shown to occur at 40°C-42°C, while the difference in efflux rates is seen over the whole spectrum of temperatures investigated (Fig. 4.3). Therefore this difference cannot at present unambiguously be attributable to \textit{hsp30} (without further experimentation).

\textit{Hsp30} could possibly affect the integrity of the membrane during heating. Membrane permeability studies showed at the temperatures investigated, no real difference in cellular fluorescence between the WT and \textit{hsp30} strains. In both strains sub-lethal heat stress resulted in protection against the large increase in membrane permeability occurring at 52°C,
protective mechanism afforded to both strains with or without the induction of hsp30 [Figs. 4.5(a-d)]. Consequently the hsp30 protein appears not to be the protective mechanism reducing plasma membrane permeability.
5.1 Introduction

Sorbate and benzoate are in extensive use as food preservatives because they inhibit the growth of fungi and bacteria. It has been clearly established the penetration of these acids into cells and growth inhibition both increase with medium acidification, being essentially proportional to the concentration of the undissociated acid (Russell, 1991). Following entry to the cells of low pH cultures in the undissociated state, sorbic and benzoic acids dissociate in the higher pH of the cytosol to cause proton release and intracellular accumulation of the anionic form of the acid. If these protons are not extruded by increased H⁺-ATPase action, or absorbed by the buffering capacity of the cell, an intracellular acidification will result. This drop in pH is thought to be the major cause of growth inhibition.

The regulation of cytoplasmic pH is of vital importance to all organisms, and a variety of methods have evolved by which intracellular pH can be maintained within a narrow physiological range. Different species have evolved different resistance mechanisms to changing intracellular pH. *S. cerevisiae* tolerates lower levels of sorbic acid than *Zygosaccharomyces bailii*, the latter being a major food spoilage yeast since it is tolerant to preservative concentrations in excess of those legally permitted in foods. Gene induction is considered to be involved in this acquired or induced protection but the mechanisms of resistance remain poorly understood. There is, as yet, no coherent model of the physiological,
biochemical and molecular events associated with adaptation to weak acid preservatives. One aim of this project was to investigate changes to the yeast plasma membrane proteins, if any, caused by exposure of yeast cells to sorbic acid. Sorbic acid was chosen for these experiments since it is not only a common food preservative, but also not metabolised by yeast (P. Coote, unpublished), unlike certain other weak organic acids such as acetate or propionate. Sorbic acid levels in the culture medium are therefore essentially unchanged during batch fermentation, therefore providing a continuous weak acid stress.

Initial studies looked at long term adaptation of cells to sorbic acid. This long term exposure should give rise to changes in gene and thus protein expression. Very little genetic manipulation has been done with Z. bailii and thus mutants of this are few. Also, a protease deficient strain in Z. bailii is not available, leading to the potential problem that results obtained might be due to proteolytic artefacts. For these reasons initial investigations employed S. cerevisiae (BJ2168). They revealed that a new high molecular weight protein associated with the plasma membrane when cells were adapting to sorbic acid. These experiments were repeated with Z. bailii to see whether a similar response could be elicited in this acid tolerant yeast. A plasma membrane protein of similar size was found also to be induced in Z. bailii suggesting that this protein may be an important aspect of adaptation to weak acids. Identification of this protein in S. cerevisiae was therefore initiated. Sequencing of this protein proved difficult due to N-terminal blocking, but was eventually solved, the protein being identified as the SNQ2 product, an ATP binding cassette (ABC) transporter.
protein previously shown to confer resistance to the DNA damaging agent 4-nitroquinoline-1-oxide (NQO) (Servos et al., 1992).

Pulse labelling of plasma membranes showed Snq2 protein to be induced by sorbic acid but not by heat shock although northern blot analysis of RNA indicated $SNQ2$ mRNA was heat-inducible.

Finally investigations focused on attempting to answer whether Snq2 protein is involved in weak acid adaptation, by comparing the tolerance of snq2 mutant and wt cells to different concentrations of sorbic acid. The snq2 mutant was no less tolerant of weak acids than an isogenic wild-type, suggesting that if Snq2 protein is a weak acid pump, its action is futile in conferring resistance to weak acids.
5.2 Results

5.3 Sorbic acid stress and plasma membrane proteins

To determine if weak acid adaptation affects plasma membrane protein composition, one litre cultures of *S. cerevisiae*, BJ2168 and KT3 were grown overnight at 28°C in pH 4.5 YEPD containing either no sorbic acid or 0.75mM sorbic acid and harvested when at mid-exponential phase. Plasma membrane proteins were then isolated from these cultures, their proteins being resolved on a 9% gel and visualised by staining with Coomassie blue (Fig 5.1). A large plasma membrane protein band of Mv approx. 150kDa was induced by sorbic acid in both strains (denoted by S in Fig. 5.1), staining to about half the level of H⁺-ATPase. Hsp30 is also induced by sorbate [Fig. 5.8(a)] but stains too weakly in Fig. 5.1 to be seen clearly.
Figure 5.1  Isolation of plasma membranes from sorbic acid stressed cultures.
Plasma membrane proteins were isolated from *S. cerevisiae* (BJ2168 and KT3) adapted to growth in the presence of 0.75 mM sorbate. 50 μg of total protein was fractionated in each lane. Proteins were resolved on a 9% gel and visualised by staining with Coomassie blue.
5.4 Phosphorylation of protein S

One litre cultures of *S. cerevisiae* BJ2168 and KT3 were grown overnight at pH4.5 YEPD containing zero, 0.5mM or 0.75mM sorbic acid (to mid-exponential phase at 28°C). Cultures were then harvested and their plasma membranes isolated. Plasma membranes were then incubated with \([\gamma^{32}P]\text{-ATP}\) as described in Chapter 2, and their *in vitro* phosphorylated proteins then separated on a 9% gel and visualised using autoradiography. It can be clearly seen that a protein comigrating with protein S is phosphorylated. It is more intensely labelled in cultures grown in 0.75mM as compared with 0.5mM sorbic acid probably due to greater induction of protein S by 0.75mM sorbic acid (Fig. 5.2). Hsp30 is also phosphorylated under these conditions (Fig. 3.5) but would have been run off the gel in Fig. 5.2.
**Figure 5.2**  *In vitro* phosphorylation of plasma membrane proteins from sorbic acid adapted cultures.

Plasma membranes from *S. cerevisiae* BJ2168 and KT3, were incubated with $[^{32}P]$-ATP, their proteins resolved on a 9% gel and exposed to film for visualisation of phosphorylated proteins. 50µg of total protein was fractionated in each lane.
5.5 Investigation into induction times of protein S

One litre cultures of *S. cerevisiae* BJ2168 were grown overnight in pH4.5 YEPD with and without 0.75mM sorbic acid to mid-logarithmic, late-logarithmic and stationary phases of growth. Cultures were harvested at mid-logarithmic and stationary phase, and plasma membranes then isolated. Cultures in mid-logarithmic growth were also stressed with 0.75mM sorbic acid for the shorter period of 4 hours at 28°C. Plasma membrane proteins were resolved on a 9% gel and visualised by staining with Coomassie blue (Fig 5.3). Protein S appears to be induced after 4 hours of sorbic acid treatment but not in late logarithmic and stationary phases of growth irrespective of whether sorbic acid is present.
Figure 5.3  Induction of protein S from logarithmic phase to stationary phase.
Plasma membranes were isolated from *S. cerevisiae* (BJ2168). Proteins were resolved on a 9% gel. 20μg of total protein was fractionated in each lane. Proteins were visualised by staining with Coomassie blue.
1-4hr induction with 0.75mM sorbic acid
2-mid-log phase, 20h with 0.75mM sorbic acid
3-late-log phase, 20h with 0.75mM sorbic acid
4-unstressed, stationary phase
5-stationary phase, 20h with 0.75mM sorbic acid
5.6 Investigation into induction of protein S by other forms of stress

One litre cultures of *S. cerevisiae* (BJ2168) were grown overnight in pH4.5 YEPD to the mid-logarithmic phase of growth after which cultures were heat-shocked at 40°C for 2 hours or treated with 6% ethanol for 2 hours. Plasma membranes were then isolated from these stressed cultures. Plasma membrane proteins were resolved on a 9% gel and visualised by staining with Coomassie blue (Fig. 5.4). Protein S was not found to be strongly induced by heat shock or ethanol stress at a low pH. Protein S thus appears to be specifically induced by sorbic acid and not merely by growth at low pH.
Figure 5.4  Isolation of plasma membranes from low pH cultures (pH4.5).
Plasma membrane proteins were isolated from *S.cerevisiae* (BJ2168).
Proteins were resolved on a 9% gel. 50µg of total protein were fractionated in each lane.
Proteins were visualised by staining with Coomassie blue.
1- 0.75mM sorbic acid stress
2- Molecular weight markers
3- unstressed
4- HS, 2hrs at 40°C
5- 6% ethanol stress, 2 hours at 28°C.
5.7 Membrane stress proteins in Z. bailii

One litre cultures of Z. bailii (strain 563) were grown overnight in pH4.5 YEPD containing zero, 0.75mM or 4mM sorbic acid to mid-exponential phase at 28°C. Plasma membrane proteins were isolated from these cultures, resolved on a 9% gel and visualised by staining with Coomassie blue. A protein apparently of the same size as protein S is induced in Z. bailii (Fig. 5.5). It is also seen with growth of Z. bailii at a higher concentration of sorbic acid (4mM) than permits growth of S. cerevisiae since the latter yeast only grows when sorbic acid levels in the same media are less than 2-3mM.
### Figure 5.5  Isolation of plasma membranes from *Z. bailii*.

Plasma membranes from *Z. bailii* cultures growing in the presence of 0.75 mM sorbic acid were resolved on a 9% gel. 20μg of total protein was fractionated in each lane. Proteins were visualised by staining with Coomassie blue.

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5.8 Induction of plasma membrane heat shock protein in Z. bailii

One litre cultures of Z. bailii were grown overnight in YEPD to mid-exponential phase and heat-shocked at 35°C for 2 hours. Previous work in the laboratory by I. Seymour had shown that 35°C is the optimum temperature for induction of cytosolic heat shock proteins in Z. bailii. A long heat shock was employed, since protein S is a large membrane protein and H^+-ATPase, another large membrane protein needs >1h for its synthesis in S. cerevisiae (Slayman and Chang, 1989). During the period of heat shock cultures were pulse labelled with tritiated leucine. After pulse labelling was complete, cultures were harvested and plasma membranes were isolated, resolved on a 15% gel and visualised using autoradiography (Fig 5.6). It was found that a protein of the same size as in S. cerevisiae, hsp30 was induced at 35°C in Z. bailii. Proteins were also induced in the 150kDa size range, but Fig. 5.6 is not conclusive as to whether protein S is heat-induced.
Figure 5.6  *In vivo* ([³H]-leucine) pulse labelling of proteins of the plasma membrane fraction of *Z.bailii*.

Proteins were resolved on a 15% gel.
1-unshocked
2-heat shocked, 35°C for 2 hours
5.9 Protein sequencing of protein S

During long term adaptation with 0.75mM sorbic acid protein S becomes associated with the plasma membrane. Peptide sequencing of this protein from *S. cerevisiae* would identify whether it has already been cloned and whether a null mutant is available in order to establish its function in weak acid adaptation. A clone of the corresponding gene would allow: i) production of the protein in large quantities for structural studies; and ii) use of the cloned DNA itself to probe the genomes of other yeasts in order to ascertain whether the gene is conserved in evolution. The DNA sequence may also shed light on the function of the protein by virtue of homology with the sequences of genes with known functions in the data banks. More than one third of sequences present in genomes are related to entries in the current data bank (Clothia, 1992).

In addition, the quantity of protein within cells can be modulated. In yeast overexpression this can be achieved by introducing extra copies of the gene on a multicopy vector; and the protein can be modified *in vivo* by gene transplacements using genes altered by site-directed mutagenesis, exploiting the high frequency of homologous recombination. In addition, if the gene is non-essential there is the additional prospect of deleting it. Determination of the function of protein S could be revealed by detailed biochemical and physiological analysis of a strain disrupted in the gene, or replacing the wild type gene with *in vitro* mutagenised alleles which have been modified at a region of interest. Before this can be done, however, protein S must first be identified.
5.10 Protein S is N-terminally modified

Resolution of S on a 1D 9% SDS-PAGE was exploited in an attempt to obtain a limited N-terminal sequence of the polypeptide. Polypeptides which are to be sequenced in a gas phase sequencer may be easily prepared by blotting the polypeptide band directly onto polyvinylidene difluoride (PVDF) membrane. The membrane is stained and the band cut out and sequenced directly. Several precautions to the usual SDS-PAGE and blotting methods are required to minimise N-terminal modification, as described in Chapter 2.

Plasma membrane proteins from 0.75mM sorbic acid adapted mid-exponential phase pH4.5 YPD cultures were resolved using a 9% gel and blotted onto PVDF. Protein S was easily visualised using amido black stain. It was estimated from the degree of staining that there was at least 3μg of the protein present per band, constituting at least 10pm of protein S. Up to 2 such bands were loaded into a sequencing cartridge thereby providing more than enough material for sequencing. However no sequence was obtained (not shown) which suggested that the protein did not possess a free N-terminus. A sample of glutathione transferase 3-3, subjected to the same procedure, sequenced satisfactorily. This protein is not blocked in vivo (B. Coles personal communication) and gave a high yield (90%) of the detected N-terminal sequence (not shown). This strongly suggests that protein S is naturally N terminally modified and that this modification was not due to the SDS-PAGE or Western blotting procedures employed.
5.11 Internal sequencing of proteins

Due to the N-terminus of protein S being blocked, it was necessary to generate internal sequences of the protein. Internal sequences are obtained either by re-eluting the bound protein, followed by cleavage, or by *in situ* cleavage of the immobilised proteins.

5.11.1 Digestion of material on PVDF membrane

Protein S was blotted onto PVDF and the band excised. The membrane was then digested with approximately 0.2μg of lysylendopeptidase in 1% octylglucoside, 200mM Tris pH 9 at 37°C overnight. The supernatant expected to contain the peptides generated from the digest was loaded onto a reverse phase column. No peptides were found to be present in the supernatant.

Possible explanations for this lack of digestion were: (i) Lack of activity of the protease; (ii) The protease being inactive on the blot because of the way it was presented to the protein or (iii) Extensive glycosylation of the protein.

The activity of the protease was checked using a control protein (GST 3-3) and found to be reasonable (not shown). This suggested that the protein was not cleaved on the PVDF membrane because of limited access of protease to the protein.

5.11.2 Digestion of material in gel

Protein S was excised from a 9% gel and the gel band treated with the protease as described in 5.11.1. (Note: This endopeptidase is active in 0.1% SDS). Again no peptides appeared to be generated from the digestion.
The problem therefore seemed to lie, in part, from inability to release the protein from the PVDF matrix. To check whether the protein was actually being released from the gel, prior to proteolysis the supernatant from each fraction was run on a 9% gel and the proteins visualised using silver staining. Protein S was found to be present but only recovered in low yield, approximately 5% (not shown).

5.12 Protein S and glycosylation studies

Membrane proteins which are anchored to the plasma membrane are difficult to analyse if they possess significant amounts of carbohydrate. This may interfere with peptide mapping in two ways: Firstly it will shield the peptide bonds from proteolysis; and secondly its inherent microheterogeneity may cause glycosylated peptides to migrate as multiple species on the peptide map. This problem was attacked by attempting to deglycosylate protein S prior to peptide mapping.

5.12.1 Lectin Blot

Firstly it was established whether protein S was glycosylated by probing a blot with concanavalin A (con A) as described in Chapter 2. Yeast protein glycosylation involves extensive mannose addition to extracellular and cell wall proteins; mannosyl groups that are capable of reacting with ConA. Membrane proteins from 0.75mM sorbic acid adapted cultures were resolved on a 9% gel and blotted onto nitrocellulose. A control glycoprotein, invertase (Sigma) was also run. Results from this con A blot showed a positive signal in the region of protein S (not shown). A sharp band was not observed and therefore it can only be
said that protein S is suspected to be glycosylated. Subsequent efforts were aimed at removing any glycans present in protein S.

The type and extent of glycosylation can also be assessed by removing glycosyl groups and observing the effects on the apparent size of the protein. For example, apparent protein size can be decreased by treatment of the protein with trifluoromethanesulphonic acid or N-glycosidase F. Also yeast cultures can be treated with tunicamycin, although this can cause incompletely glycosylated proteins to accumulate in the endoplasmic reticulum and not arrive at their final cellular destination (Mizuanga and Noguchi, 1982).

5.12.2 Chemical removal of glycans

The chemical deglycosylation procedure removes all N & O-linked sugars. The supernatant from the gel digest was dialysed against water (i.e. overnight at 4°C using a semipermeable membrane). It was then freeze dried thoroughly and the desiccate incubated in trifluoromethane sulphonic acid at -40°C. This chemically removes the glycans. The resultant mixture was then dialysed overnight then incubated in 200mM Tris pH6.8 with 1μg of V8 glutamylendopeptidase from Staphylococcus aureus at 37°C overnight. The supernatant was loaded onto a reverse phase column for detection of peptide fragments. There were no differences found between the non-protease-treated (control) and protease treated samples. This could be because of the high protein loss during this extensive procedure. For this reason enzymatic removal of glycans was used, this being less harsh.
5.12.3 Enzymatic removal of glycans

This procedure will not remove all of the carbohydrate in the case of highly glycosylated samples. There are neither specific glycosidases that remove O-linked mano-oligosaccharide chains nor inhibitors of O-mannosylation. N-glycosidase F will remove all of the N-linked sugars, concomitantly converting the asparagine residue to aspartic acid. Membranes from 0.75mM sorbic acid adapted cultures were treated with N-glycosidase F, according to the protocol provided by Oxford Glycosystems, and their proteins resolved on a 9% gel. A control glycoprotein, invertase was also treated with the enzyme. Fig. 5.7 indicates that protein S did not shift in apparent molecular weight on enzyme treatment indicating that it is not appreciably N-glycosylated. Invertase did change in molecular weight, its apparent size decreasing from 100kDa to 66kDa (Fig 5.7), thereby proving that the N-glycosidase F was active under the conditions used.
Figure 5.7  Response of protein S and invertase to treatment with N-glycosidase F.
Proteins were resolved on a 9% gel. 20μg of total protein was fractionated in each lane.
Proteins were visualised by staining with Coomassie blue.
1- Untreated plasma membrane proteins from 0.75mM sorbic acid stressed cultures
2-Plasma membrane proteins from 0.75mM sorbic acid stressed cultures treated with N-glycosidase F.
3-Untreated invertase.
4-Invertase treated with N-glycosidase F.
5.12.4 Inhibition of N-glycosylation using tunicamycin

Tunicamycin inhibits N-glycosylation in vivo by blocking the synthesis of the lipid-linked precursor oligosaccharide (Hanson & Lester, 1982). Cultures of S. cerevisiae strain BJ2168 & Z. bailii (strain 563) were pulse labelled at 28°C in the presence of both tunicamycin (10µg/ml) and 0.75mM sorbic acid or sorbic acid alone and their plasma membranes were isolated. Fig. 5.8 shows that in the presence of tunicamycin, the mobility of protein S is unaffected, both in S. cerevisiae [Fig. 5.8(a)] and in Z. bailii [Fig 5.8(b)]. This suggests that protein S is not appreciably N-glycosylated. Also pulse labelling conclusively showed that this protein is specifically sorbic acid induced in both yeast species.

Fig. 5.8(a) shows hsp30 is specifically induced by sorbic acid in S. cerevisiae.
Figure 5.8(a) Effects of tunicamycin on \textit{in vivo} (\textsuperscript{3}H-leucine) pulse labelling of proteins of the plasma membrane fraction of \textit{S. cerevisiae} adapted to growth in the presence of \textbf{sorbic acid}. Plasma membrane proteins were resolved on a 12\% gel and visualised using autoradiography.

1- unstr\textsuperscript{2}ssed cells.
2- 0.75mM sorbic acid for 4 hours at 28\degree C.
3- treated with tunicamycin (10\,\mu g/ml) and 0.75mM sorbic acid for 4 hours.
4- molecular weight markers.
Figure 5.8(b) Effects of tunicamycin on in vivo ([^3]H-leucine) pulse labelling of proteins of the plasma membrane fraction of Z. bailii adapted to growth in the presence of sorbic acid. Plasma membrane proteins were resolved on a 9% gel.

1- unstressed cells
2- 0.75mM sorbic acid for 4 hours at 28°C
3- treated with tunicamycin (10μg/ml) and 0.75mM sorbic acid for 4 hours
5.13 Identification of protein S

After a great deal of investigative work, identification of protein S was achieved by Dr J.Hsuan, Ludwig Institute. This was achieved by treatment of the protein S band with lysylendopeptidase and separation of the fragments using HPLC. Sequences of 3 proteins was found to be present in the protein S sample analysed. The 3 proteins were the 110kDa major glycoprotein of the yeast plasma membrane Gas1, H⁺-ATPase (100kDa) and Snq2. Gas1 is an integral membrane protein, the expression of which is significantly increased in growth arrested cells (Del Sal et al., 1992). The yeast gene SNQ2 confers hyper resistance to the mutagens 4-nitroquinoline-N-oxide (4NQO) and triazoquinol (Servos et al., 1993). Out of the several fragments sequenced, most showed sequences of Snq2 (Fig. 5.9). The presence of Gas1 and H⁺-ATPase sequences (neither of which are stress inducible) probably indicates that the protein S sample was not completely pure but contaminated by the two most abundant large plasma membrane proteins.
Figure 5.9 Sequence of protein S identifies it as the product of the \textit{SNQ2} gene. The sequence of Snq2 is shown. Peptides identified with protein S lysylendopeptidase digest are underlined, with differences in the determined sequences as compared to the published snq2 sequence being given above the Snq2 sequence.
5.14 Investigation into induction of Snq2 by sorbic acid

Since protein S was identified as primarily being substantially Snq2, it had to be established conclusively that Snq2 was in fact being induced by sorbic acid. We therefore obtained the snq2 deletion mutant and its parent strain from M.Brendel, Germany. With these strains we proceeded to isolate plasma membranes from cultures treated with sorbic acid. One litre cultures of \textit{S. cerevisiae SNQ2} and \textit{snq2} were grown in pH4.5 YEPD with the addition of 0.75mM sorbic acid for 4 hours at 28°C. Plasma membranes were isolated from these cultures and their proteins resolved on a 9% gel and visualised by staining with Coomassie blue (not shown). This gel showed in both wt and \textit{snq2} strain samples the presence of numerous low molecular weight protein bands, indicating extensive proteolysis. This is not surprising since these strains are not protease deficient. However plasma membranes from these fractions were incubated with [\gamma-P^{32}]-ATP (in order to determine if any protein S was sorbate induced in the \textit{snq2} strain), resolved on a 9% gel and visualised by autoradiography. Labelling of band S was distinctly less, although possibly not totally abolished, in the \textit{snq2} mutant (Fig. 5.10).
Figure 5.10 Phosphorylation of plasma membrane proteins isolated from a strain deleted in SNQ2. Plasma membrane proteins were resolved using a 9% gel and visualised using autoradiography. 50μg of total protein was fractionated in each lane.

1- unstressed, BJ2168
2- 0.75mM sorbic acid stressed BJ2168
3- 0.75mM sorbic acid, 4 hours, YPH98, snq2
4- 0.75mM sorbic acid, 4 hours, YPH98, SNQ2
5.15 Induction of **SNQ2** as shown by Northern blot analysis

Plasma membrane isolation from wt and *snq2* strains did not show that Snq2 protein was being induced by sorbic acid and it was therefore necessary to carry out Northern blot analysis of the two strains.

Cultures of BJ2168 (pH 4.5) were heat shocked (40 min, 40°C), treated with 0.5M or 1M NaCl at 28°C or 9mM sorbic acid for 40 min at 28°C. Total RNA was isolated and Northern blotted. The blot was probed with **SNQ2** sequenced as prepared by PCR (a 1kb fragment was used). Results show a single transcript response with all three stresses investigated thus conclusively showing that **SNQ2** is stress inducible; in particular it is specifically sorbic acid induced and not merely induced by low pH (Fig. 5.11).
Figure 5.11 Northern blot analysis of BJ2168 treated with a range of stresses at pH4.5. Blots were probed with \textit{SNQ2}.

1- untreated, 2- 9mM sorbic acid (28°C, 40min), 3- 1M NaCl (28°C, 40min), 4- 0.5M NaCl (28°C, 40min) and 5- heat shock (40min, 40°C).
5.16 Comparison of weak acid sensitivity of $SNQ2$ & $snq2$ strains

Both wt and $snq2$ strains were grown to stationary phase and spread onto minimal media agar plates, pH4.5 (plus supplements). Wells were bored into the plates into which 50µl of a range of concentrations of sorbic acid were added (0.1M, 0.5M and 0.15M). Growth of both strains was observed closely and zones of growth inhibition noted. The following bar chart shows that the wild type strain was more sensitive to sorbic acid as opposed to the $snq2$ mutant strain as greater zones of growth inhibition were observed with the wt strain. If Snq2 was contributing to weak acid resistance then the wild type should have been more resistant. This will be discussed later.

![Bar chart showing comparison of growth inhibition zones for wt and snq2 strains.]  

Figure 5.12 Difference in sensitivity to sorbic acid between wt and $snq2$ strains.
5.16 Discussion

Resistance to multiple cytotoxic compounds is an acquired property in many species from bacteria to man. Major determinants of multidrug resistance in eukaryotic cells are ATP-binding cassette (ABC) transport proteins driving an energy-dependent export of drugs and toxic substrates across the cell membrane. All drug resistance conferring proteins identified so far can be classified within two superfamilies; the major facilitators superfamily and the ATP binding cassette superfamily (ABC). Yeast ABC proteins include the α mating factor transporter STE6 (Mcgrath and Varsharvsky, 1989), the multi-drug transporters PDR5 (Balzi et al., 1994; Bissinger and Kuchler, 1994; Hirata et al., 1994) and Snq2 (Servos et al., 1993), the yeast cadmium factor YCF (Szczypka et al., 1994), the oligomycin resistance factor YOR1, and several newly discovered genes of totally unknown function like PDR10 and PDR11 (Decottignes et al., 1995). With the exception of STE6, the normal physiological substrates of the yeast ABC transporters remain unknown. PDR5 encodes a putative ATP dependent plasma membrane protein resembling the mammalian transporter for drug efflux, (the P-glycoprotein, encoded by MDR1) and confers resistance to several unrelated drugs in mammalian cells, including the protein synthesis inhibitor cycloheximide, the acetolactate synthase inhibitor sulfomethuron methyl and several antitumour drugs. The ABC transporter that most resembles PDR5 is the SNQ2 gene product.

It was demonstrated that weak acid stress induces two plasma membrane proteins, Snq2 and hsp30 [Figs. 5.1 and 5.8(a)]. These are the first yeast genes shown to be weak acid inducible and are encoded by SNQ2 and HSP30 genes respectively. Cells deleted in HSP30 are found
to have an extended lag phase in the presence of sorbic acid (Chapter 4) implicating hsp30 in the adaptation to weak acid stress. HSP30 is therefore the first gene shown to be both induced by weak acids and contributing to weak acid adaptation. It was also established by this study that Snq2 protein is specifically weak acid induced as opposed to being induced by low pH (Fig. 5.4). It is also induced by heat shock being the first ABC transporter protein to be shown to be stress inducible. Instead all other ABC transporters of yeast are under PDR1 and PDR3 transcriptional regulator control (Katzmann et al., 1994). This is of particular importance as it shows (1) a specific cellular response to weak acid stress, never shown before and (2) that one of the genes determining multi-drug resistance in yeast is a stress gene.

Snq2 protein significantly resembles products of the white and brown loci of Drosophila melanogaster (Pepling and Mount, 1990; Dreesen et al., 1988). Both Drosophila proteins are thought to function as pigment transport proteins in the eye and other tissues of Drosophila (Riordan et al., 1989). Similarity with the Snq2 protein was highest in the ATP binding domains of the above-mentioned transport proteins, the product of the yeast STE6 gene (Mcgrath and Varsharvsky, 1989) and the polypeptides encoded by the human MDR1 (Chen et al., 1986) and Cf loci (Riordan et al., 1989). From its amino acid homologies with known ATP-dependent transporters, from the hyper-resistance to 4-nitroquinoline-N-oxide that the multi-copy SNQ2 gene confers on yeast, and the hypersensitivity to the same chemicals in the snq2 mutant, it has been suggested that SNQ2 encodes an export permease (Servos et al., 1993). However, since proteins with similar amino acid homologies exist that may have other, non-transporter properties, e.g. protein Yef3 (yeast elongation factor 3) (Sandbaken et al.,
1990) and *E. coli* repair enzyme UvrA (Doolittle *et al*., 1986) there may be still other physiological roles for the Snq2 protein in a yeast cell.

The induction of a similar-sized protein under weak acid stress in the weak acid tolerant yeast *Z. bailii* suggested discovery of a transport protein which may be involved in weak acid adaptation. The tolerance mechanism of *Z. bailii* to sorbic acid has not been discovered but has been suggested by many to involve an inducible weak acid export pump mechanism (Warth, 1977), which transports the sorbate anion out of the cell, thus preventing a high intracellular build-up. We find that *snq2* mutant (*S. cerevisiae*) cells are not more sensitive to weak acid at pH 4.5, but instead slightly more resistant to weak acid than wild-type cells (Fig. 5.12). Therefore Snq2 protein is possibly catalysing an extrusion of weak acid that is actually futile since this acid can so readily traverse the cell membrane at pH4.5. Not only may its action be futile, but it may also be using ATP that could be used in more productive ways of maintaining homeostasis such as proton extrusion by the plasma membrane H⁺-ATPase. Future studies can investigate whether the *snq2* mutant might be more resistant simply because it is not wasting ATP in futile weak acid pumping.

The regulation of yeast stress inducible genes is thought to be via the HSE or STRE promoter elements. The HSE is not weak acid inducible (Cheng and Piper, 1994). Therefore transcription of *SNQ2* by heat shock, salt stress and sorbic acid (Fig 5.11) may be due to STRE activity. Indeed the STRE element has been shown to be weak acid inducible under certain conditions (I. Seymour, unpublished results). The *YDR1* and *SNQ2* genes are induced
even by drugs for which no drug resistances are conferred by these genes (Hirata et al., 1994). Whether the induction of these genes by various drugs and heat shock are mediated by a common mechanism, or is the result of different response mechanisms has yet to be determined. Balzi et al., (1994) have shown the interaction of \textit{PDR5} (\textit{YDR1}) and the other \textit{PDR} genes (\textit{PDR1}, 3, 4 and 7) and most recent studies have shown that the level of \textit{SNQ2} transcript is controlled by both \textit{PDR1} and \textit{PDR3} (Decottignies et al., 1995).

Both \textit{SNQ2} mRNA and protein were found to be induced by weak acids. We could not however demonstrate Snq2 protein induction by heat shock (Fig. 5.4). The transcription of \textit{SNQ2} by heat shock but not its translation can be explained by analogy to the transcription/translation of H\textsuperscript{+}-ATPase. Upon heat shock the transcription of all genes ceases apart from that of heat shock genes and H\textsuperscript{+}-ATPase (Garcia-Arranz et al., 1994). However the major control of H\textsuperscript{+}-ATPase levels is not exerted at the level of transcription but occurs by posttranscriptional controls (Garcia-Arranz et al., 1994). The ATPase mRNA is not efficiently translated during heat shock (stress) (Panaretou and Piper, 1992) but may only become translated and inserted into the plasma membrane after the stress ceases as an adaptive response to aid in stress recovery (Fig. 3.6). The induction of \textit{SNQ2} mRNA with heat shock may therefore be providing mRNA that can be translated during a period of recovery. With sorbic acid stress this is not seen, perhaps because the heat shock-induced changes to mRNA selection by the protein synthetic apparatus have not been induced. This idea is re-inforced by the observation that \textit{SNQ2} mRNA is translated within 4 hours of induction (Fig. 5.3), although induction of \textit{SNQ2} decreases sorbic acid resistance (Fig 5.12), it
is probable that the true physiological role of stress-induction of SNQ2 is not weak acid resistance.

Several proteins in S. cerevisiae are quite stable showing half life values 70h (Gancedo et al., 1982). However, the plasma membrane proteins in this organism generally display shorter half-lives. Sugar transport systems have a half-life of about 3h (Gancedo et al., 1982), and the yeast plasma membrane ATPase has been found to have a half-life of about 11h in the presence of glucose (Benito et al., 1991). However the half life of ATPase during the first 40 min of heat shock is even lower [Figs. 3.6(a) and (b); Panaretou, PhD dissertation, 1993]. The Snq2 protein contains a so-called PEST region, a domain often present in short-lived proteins of a half life less than 2h (Rechsteiner et al., 1987) and consisting mainly of proline (P), glutamic acid (E), serine (S) and threonine (T) (Rogers et al., 1986). The fact that all plasma membrane proteins so far examined show low half life values suggests that a low stability could be a general characteristic of these proteins.

The expression of the mammalian MDR1 gene is inducible by heat shock and arsenite. The promoter of the human MDR1 gene is a target of the c-Has-ras-1 oncogene and the p53 tumour suppressor gene products, both of which are associated with tumour progression (Chin et al., 1992). The results from a variety of experiments suggest that the regulation of MDR gene expression is mediated by the interaction of several transcription factors with different regulatory cis-elements MDR gene promoters (Chin et al., 1990; Higgens, 1993; Yu
et al., 1993). Genetic analysis of the yeast drug resistance system may provide valuable information for an understanding of the mammalian MDR phenomenon.
CHAPTER 6
6. DISCUSSION

The yeast *S. cerevisiae* presents several advantages as an experimental system for studying the stress responses of a eukaryote. Many genes of stress protection have now been cloned in this organism and loss of function mutants analysed. Most major heat shock proteins of this species have now been subject to detailed analysis, some being apparently nonessential for stress protection whilst others having clear chaperone functions (Mager and Moradas-Ferreira, 1993; Parsell and Lindquist, 1993). Also different gene promoter elements responsive to stress have been identified (Schuller et al., 1994) and stress-responsive signal transduction pathways that trigger the activation of these promoter sequences are beginning to be unraveled (Thevelein, 1995).

Heat shock is known to activate plasma membrane ATPase activity in intact yeast cells, as shown by proton extrusion measurements (Coote et al., 1994). Other conditions that lower intracellular pH e.g. growth under acid conditions (Eraso and Gancedo, 1987); ethanol (Cartwright et al., 1987; Rosa and Sa-Correia, 1991) or weak organic acid treatment (Viegas and Sa-Correia, 1991) also stimulate plasma membrane ATPase activity. This enhanced ATPase-driven proton efflux helps to counteract the decrease in intracellular pH that result from these stresses. At first sight, therefore, it is surprising that protein levels of plasma membrane ATPase decline during these stresses (Fig. 3.1; Panaretou and Piper, 1992). However it has to be remembered that this ATPase is normally not fully active even though it
hydrolyses much of the ATP generated by the cell (Serrano, 1991) such that the maximal ATPase activity of this ATPase over a long period of stress might therefore be unsustainable. Such is the case during extended exposure to a nonmetabolisable weak acid, when ATP levels are dramatically reduced even in dividing cultures (Fig. 4.8), almost certainly due to the high demand for ATP hydrolysis in maintaining homeostasis.

Plasma membrane ATPase decline and hsp30 induction are the two major changes to yeast plasma membrane protein composition with sublethal heat or ethanol stress (Fig. 3.1; Panaretou and Piper, 1992; Piper et al., 1994). While it has long been known that cells alter the lipid composition of their membranes in response to ethanol, these are the first demonstrations that they also rapidly alter the protein composition of the plasma membrane. This reduction in plasma membrane ATPase protein levels is not the result of HSP30 expression since it is also seen in heat shocked hsp30 cells (Fig. 3.6). A consequence of this reduction in ATPase protein levels is that the specific activity measurements of this ATPase in isolated membranes from heat shocked and sorbic acid treated wild type cells (measured relative total membrane protein content) can disguise an actual ATPase activation with heat shock (Panaretou, PhD thesis; Piper et al., submitted).

Plasma membrane ATPase activity influences both heat and ethanol tolerance (Panaretou and Piper, 1990; Coote et al., 1994). It is also important in adaptation to growth in presence of weak acids (Cole and Keenan, 1987; Coote et al., 1995), heat stress (Coote et al., 1991; 1994) and ethanol (Cartwright et al., 1987; Rosa and Sa-Correia, 1991). All these stresses
cause stimulation of plasma membrane ATPase activity, this enhanced ATPase-driven proton efflux possibly helping to counteract the decrease in intracellular pH that results from the stress-induced increases in membrane permeability. Stress responses generally have a modulation system whereby the response, once induced is subsequently downregulated. Hsp30 may be part of this downregulation, acting to reduce the stimulation of plasma membrane ATPase with the above stresses, probably so that cellular ATP levels do not become completely depleted (Piper et al., submitted).

Cell survival during a severe heat stress can be enhanced when heat shock proteins are induced prior to a severe heat treatment. Induction can be accomplished either by heat or by chemical treatment e.g. cadmium (Courgeon et al., 1984), arsenite (Koninkx, 1976) and with recovery from anoxia (Koninkx, 1976). In addition to inducing the stress related proteins, these non heat treatments also increase cell survival upon subsequent exposure to severe heat shock (Li, 1983). Ecdysone induces the small heat shock genes (hsp22, hsp23, hsp26 and hsp27) in Drosophila cells and increases survival rates when these cells are subsequently exposed to severe heat stress (Berger, 1983). Sorbic acid and ethanol (chemical inducers) have been found to induce hsp30. However there was no real difference in thermotolerance, even after prior incubation at 42°C, between WT and hsp30 mutant cells (Chapter 4). Prior to this it had been reported that sorbate has no effect on the thermotolerance of S. cerevisiae, but shifts the Arrhenius plots and T_max of growth to lower temperatures (Van Uden, 1984). It is difficult to assess this study as the conditions, notably pH of growth were not reported. Coote et al., (1991) showed thermotolerance increasing during a 9mM sorbic acid treatment,
both at pH4.5 and at pH6.0. However a partial thermotolerance increase was also seen in cells exposed to pH4.5 in the absence of sorbate. Sorbic acid inhibits the normal induction of the major heat shock proteins in low pH cultures by heat shock, hence inhibiting thermotolerance induction (Cheng and Piper, 1994). However, when the culture pH was above pH5.5 sorbate acted as a powerful chemical inducer of thermotolerance in the absence of heat shock. Sorbate can also act as a chemical inducer of heat shock proteins. The literature thus highlights the strong influence that medium pH exerts over the effects of sorbate on thermotolerance.

Sorbic and benzoic acids are in extensive use as food preservatives. It has been clearly established that the penetration of these acids into cells and growth inhibition both increasing with medium acidification, being essentially proportional to the concentration of undissociated acid (for literature see Russell, 1991). Following entry to the cells of low pH cultures as undissociated forms, sorbic and benzoic acids dissociate into the higher pH of the cytosol to cause intracellular acidification. More osmotolerant yeasts, notably Z. bailii, are important causative agents of the spoilage of low pH foods and beverages of high sugar content (Thomas and Davenport, 1985). They are able to undergo an adaptation that allows growth in the presence of preservative concentrations in excess of those permitted legally in foodstuffs (Warth 1978, 1988; Cole and Keenan, 1986, 1987). This study shows that this adaptation involves specific gene induction; a stress response that we term the response to weak acids. This is shown both by S. cerevisiae (Fig. 5.1) and Z. bailii (Fig. 5.5).
The mutant showed 4-5 times greater activity than the wildtype strain.
Heat stress has been shown to affect the activity of plasma membrane ATPase by observing acid efflux rates (Coote et al., 1991; 1994). Coote et al., (1994) showed that although membrane ATPase is essential for basal heat resistance, thermotolerance induced by prior exposure to stress is largely conferred by a mechanism that is independent of this enzyme. Hsp30 was found not to be involved in heat induced thermotolerance, but has been suggested to be a regulator of the ATPase. The activities of ATPase, measured from rates of acid efflux by intact cells in this study (Figs. 4.2 and 4.3) did not clearly establish this relationship between hsp30 and ATPase.

Studies by Coote et al., (1994) have concluded that increased ATPase activity confers greater thermotolerance to the cell. Studies have also shown a 4-5 fold activation of ATPase in purified plasma membranes from heat-shocked cells of the hsp30 mutant as compared to only 1-5 fold activation in the wt (Piper et al., submitted). According to the literature one would expect the mutant strain to have shown increased thermotolerance on exposure to sub-lethal heat stress. However this was not the case and affords greater investigation.

Resistance to multiple cytotoxic compounds is an acquired property in many species from bacteria to man. The major molecular determinants mediating multi-drug resistance are transport proteins driving the traffic of drugs and physiological substrates across the cell membrane. This study has identified the ATP-dependent export permease Snq2 (as well as hsp30) being induced by sorbic acid in S. cerevisiae [Fig. 5.1 and 5.8(a)] and a similar-sized protein induced under the same conditions in Z. bailii (Figs. 5.5 and 5.6). Since it appears not
to assist weak acid adaptation (Fig. 5.12) its stress induction must fulfill another physiological purpose. Disruption of the \textit{SNQ2} gene has been shown to markedly lengthen the lag phase, during which stationary cells undergo metabolic and physiological changes before starting exponential phase (Decottignies \textit{et al.}, 1995). However the exponential growth rate was not affected. It is possible that Snq2 may be extruding intracellular cytotoxic metabolites accumulated during stationary maintenance.

It has been proposed that the adaptation of \textit{Z. bailii} to growth in the presence of weak acids involves induction of an energy-requiring system for extrusion of the acid (Warth, 1978 1988). This study identified a possible contender for this pump. However, any active extrusion of the acid by Snq2 protein is probably futile as the acid can so readily traverse the membrane at pH4.5. Therefore increased plasma membrane-ATPase-catalysed proton extrusion, a decreased protoplast volume and possibly increased intracellular buffering may contribute to maintaining the pHi of \textit{Z. bailii} cells that have adapted to growth in the presence of weak acids (Cole and Keenan, 1987). In addition the \textit{PFK1} of \textit{Z. bailii} shows less dramatic inhibition in response to pHi depression (Cole, 1987) as compared to the \textit{PFK1} of \textit{S. cerevisiae} (Krebb's \textit{et al.}, 1983; Francois \textit{et al.}, 1986). This may better equip \textit{Z. bailii} to maintain glycolytic flux in the presence of pHi-depressing amounts of weak acids.

If, as predicted, PDR5 and Snq2 are drug efflux pumps, one might expect a certain substrate specificity for each pump. Hirata \textit{et al.}, (1994) have shown that multiple copies of \textit{SNQ2} gene
confer resistance to 4-nitroquinoline-N-oxide, but not cycloheximide, while the converse is true for PDR5 gene overexpression.

The major findings from this study can be summarized as:

(1) Proof that ethanol stress elicits the same two changes to the plasma membrane as heat stress; decline in levels of H^+-ATPase and induction of hsp30 (Chapter 3).

(2) Proof that hsp30 is not a major determinant of acquired or induced thermotolerance, membrane permeability or rates of acid efflux under heat stress (Chapter 4).

(3) Demonstration that hsp30 is involved in weak acid adaptation; the first yeast gene to be identified as involved in this process (Chapter 4).

(4) The finding that weak acid stress induces an export permease, Snq2 protein; that the deletion mutant (snq2) is more resistant to weak acid stress than the wildtype and that the SNQ2 gene is stress-inducible (Chapter 5).

This study has highlighted certain areas for further investigation as follows:

(1) Further investigation into the decay of levels of ATPase during ethanol stress, in order to determine how the cell controls ATPase levels and ATPase activity to ethanol stress and to heat shock. If ATPase loss is by the same mechanism with ethanol stress as with heat stress, then greater parallels could be established between the stress reactive mechanisms of both heat and ethanol stress. Comparison of ATPase activity during ethanol stress and heat stress in specific ATPase mutants could identify the mechanism of activation of ATPase by these stresses.
(2) Use of the 2-hybrid system to investigate whether the hydrophilic domain of hsp30 is interacting directly with the C-terminus of H⁺-ATPase.

(3) Use of co-immunoprecipitation to investigate the interaction of hsp30 with other membrane proteins.

(4) Protein sequencing of the protein suspected to be protein S in Z. bailii could conclusively show if this is a homologue of Snq2 protein; indicating if there is a parallel response to weak acid stress in both Z. bailii and S. cerevisiae.

(5) Nothing is known about the SNQ2 gene apart from its control by PDR1 and PDR3 (Decottignies et al., 1995). Identification of the promoter sequence in SNQ2 gene which is induced by weak acid, heat and salt stress would show if this is a STRE element.

(6) Investigation into whether Snq2 protein acts to pump out the sorbate anion by monitoring radioactive labelled anion fluxes in snq2 and wild type cells. Radiolabelled benzoate could be used as it is commercially available; but the ready re-entry to the cell by diffusion across the membrane could present technical problems.

(7) Investigation into the differences in growth between the wt and snq2 disruption strain in the presence of weak acid stress. This would identify whether in the presence of sorbic acid the snq2 mutation increases the lag phase or alters the growth profile thus showing if weak acid stress demands the presence of an efflux pump at all times.

(8) The Snq2 protein has not been biochemically characterised apart from it having been shown to exhibit nucleoside triphosphatase activity (Decottignies et al., 1995). Monitoring levels of intracellular ATP in the wt and snq2 strains could be used to determine whether increased utilisation of ATP causes the increased sensitivity to sorbic acid in the wt strain.
The elucidation of the yeast weak acid resistance mechanism would have great benefit for producing less-preserved and therefore more natural food. Any method of control of preservative resistant organisms is therefore an area of great commercial interest. In addition this study has shown that an understanding of stress resistance in yeast may provide valuable information for an understanding of the mammalian MDR phenomenon.
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Induction of major heat-shock proteins of Saccharomyces cerevisiae, including plasma membrane Hsp30, by ethanol levels above a critical threshold

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Many of the changes induced in yeast by sublethal yet stressful amounts of ethanol are the same as those resulting from sublethal heat stress. They include an inhibition of fermentation, increased induction of petites and stimulation of plasma membrane ATPase activity. Ethanol, at concentrations (4–10%, v/v) that affect growth and fermentation rates, is also a potent inducer of heat-shock proteins including those members of the Hsp70 protein family induced by heat shock. This induction occurs above a threshold level of about 4% ethanol, although different heat-shock proteins and heat-shock gene promoters are optimally induced at different higher ethanol levels. In addition, ethanol (6–8%) causes the same two major changes to integral plasma-membrane protein composition that result from a sublethal heat stress, reduction in levels of the plasma membrane ATPase protein and acquisition of the plasma membrane heat-shock protein Hsp30.

Keywords: Saccharomyces cerevisiae, stress response, ethanol, heat-shock proteins, plasma-membrane proteins

INTRODUCTION

The increasing ethanol level during batch fermentation of Saccharomyces cerevisiae on high sugar substrates acts to cause reductions in the specific growth rate, the fermentation rate and cell viability (see van Uden, 1984a, for literature). These effects become much more severe with increasing temperature, the optimum and maximum temperatures of growth becoming appreciably depressed in the presence of ethanol levels above about 3% (v/v), while thermal death on exposure to extreme temperatures is enhanced (van Uden, 1984a, b). These adverse influences of ethanol on the growth, viability and fermentation of S. cerevisiae have been largely explained in terms of its effects on membrane-associated processes (Leao & van Uden, 1984, 1985; Jones, 1989; Rose, 1993). Not only are these toxic effects influenced by the lipid composition of yeast cell membranes but growth in the presence of ethanol causes changes to membrane lipids which are possibly part of an adaptive response (reviewed in Rose, 1993, and Lloyd et al., 1993). The concentration of ethanol that totally inhibits S. cerevisiae growth is also remarkably medium- and strain-dependent (Rose, 1993).

Several of the changes induced in yeast by exposure to stressful ethanol levels are identical to those caused by a heat stress. It is not surprising therefore that ethanol acts in a synergistic way to increase the damage caused by heat (van Uden, 1984a, b; Aguilera & Benitez, 1989). Both heat and ethanol cause membrane disordering and protein denaturation (Casey & Ingledew, 1986; Piper, 1993), besides an inhibition of glycolysis and an enhanced induction of petites (Neves & Francois, 1992; Leao & van Uden, 1982; van Uden, 1984a, b). Ethanol also inhibits glucose transport (Leao & van Uden, 1982, 1985). Both stresses increase the permeability of the plasma membrane, resulting in an increased passive proton influx that acts to dissipate the electrochemical potential gradient that the cell maintains at this membrane. This is reflected in the decline in intracellular pH that is observed both with ethanol addition (Leao & van Uden, 1984) and with heat shock (Coote et al., 1991; Coote, 1993). Ethanol and heat stress will adversely affect those vital functions for which a plasma membrane electrochemical gradient is essential, processes such as nutrient uptake, the main-
these same proteins by heat shock, or if perhaps only a shock genes (Mager & Moradas-Ferreira, 1993). The trigger for their induction is probably the disturbed proteins and the concomitant activation of heat-shock genes (Ananthan

the cytoplasmic accumulation of aberrant or partially denatured protein (Ananthan et al., 1985). Thus ethanol may be an inducer of Hsps through its destabilization of the hydrophobic interactions of protein structure which leads to the association of Hsp chaperones with these destabilized proteins and the concomitant activation of heat-shock genes (Mager & Moradas-Ferreira, 1993). While it is clear that major Hsps are ethanol-inducible in yeast (Plesset et al., 1982), it is not obvious from published data if this induction is comparable with the induction of these same proteins by heat shock, or if perhaps only a subset of yeast Hsps are strongly ethanol-inducible. This report collates experiments from a number of laboratories that clarify the ethanol induction of the major S. cerevisiae Hsps and the range of ethanol concentrations most effective in this induction. It also reports the first study of the major effects of ethanol on plasma-membrane protein composition.

**METHODS**

Yeast strains, culture and conditions of ethanol and heat stress. The strains used for this study are listed in Table 1. Cultures were grown with vigorous shaking at 25 °C in standard defined (SD) pH 6.8 medium, with 2% (w/v) glucose as initial carbon source, plus necessary amino acid supplements (Sherman et al., 1983). They were used for ethanol stress experiments when in mid-exponential phase (5 × 10⁴ cells ml⁻¹), by which stage the medium ethanol levels produced by fermentation were less than 0.3%. To heat shock cells, cultures were shifted to 39 °C for 40 min prior to harvesting, this being the optimum temperature for induction of heat-shock element sequences (Kirk & Piper, 1991a, and references cited therein). To ethanol stress cultures the stated amount of additional ethanol was added to cultures and incubation continued at 25 °C.

**Pulse-labelling of proteins.** Pulse-labelling of proteins with [³H]leucine was as described by Cheng & Piper (1994). For efficient uptake of labelled amino acids in the presence of 4–10% ethanol it is essential that the pH of the medium is greater than 6.0 as ethanol strongly inhibits uptake of labelled amino acids in cultures of lower pH (unpublished observations).

**Protein analysis.** Samples of total cell protein were isolated as described by Piper et al. (1988). Protein determinations were performed using the Bio-Rad Protein Assay Kit and bovine serum albumin as standard. Protein samples were prepared for gel electrophoresis by incubation at 37 °C in SDS-protein gel sample buffer, then analysed on one-dimensional 12.5% or 7%
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Fig. 1. Autoradiographs of two 12.5% SDS-gels showing the proteins labelled in S. cerevisiae BJ2168 cells during heat and ethanol stress. Cells were pulse-labelled for 40 min at 25 °C, in the absence of ethanol (lanes 1, 5); at 25 °C with 2% (lane 7), 4% (lane 8), 6% (lane 2), 8% (lanes 3, 9) or 10% ethanol (lane 4); or immediately after heat shock to 40 °C (lane 6). Molecular mass marker positions are indicated on the left, and major Hsps on the right of each autoradiograph.

(w/v) SDS-polyacrylamide gels. Gel staining with Coomassie brilliant blue, detection of labelled proteins by fluorography and blotting of proteins onto nitrocellulose membrane was according to standard procedures (Piper et al., 1988; Panaretou & Piper, 1992).

Plasma membrane purification and protein analysis. Purification of plasma membranes, assessment of the purity of membrane preparations and analysis of plasma-membrane proteins on 12.5% gels was as described by Panaretou & Piper (1992). Blotted plasma-membrane proteins were analysed for Hsp30 as described by Rencaneq & Boucherie (1993).

Northern blotting of RNA. Total cell RNA was prepared, Northern blotted onto Hybond N membranes, and probed to HSP26, HSP12 and ACT1 gene probes as described by Piper et al. (1988).

lacZ expression measurements. β-Galactosidase activity, measured as in Kirk & Piper (1991a, b), is presented as fold-induction relative to the low constant level of β-galactosidase in uninduced cultures at 25 °C.

RESULTS

A threshold concentration of approximately 4% ethanol is required for appreciable Hsp induction, subsets of these proteins showing optimal induction at different higher ethanol levels

The addition of 4–10% ethanol to exponentially growing yeast cultures causes strong induction of a few specific proteins. From 1D gel analyses Plesset et al. (1982) found that many of these were the same as proteins induced by heat shock (see also Fig. 1). However there has been no systematic study showing if each major yeast Hsp is inducible to the same extent by ethanol as by heat shock, or indeed whether all Hsps are optimally induced at the same ethanol concentration. Most heat-shock genes of S. cerevisiae have now been characterized (Mager & Moradas-Ferreira, 1993; Parsell & Lindquist, 1993) and the availability of several strains with targeted inactivation of these genes facilitates comparison of the ethanol and heat inducibility of most individual Hsps.

We have observed that there is a threshold concentration for appreciable Hsp induction by adding ethanol to vegetative yeast cultures. Appreciable induction started to become detectable by protein pulse-labelling with the addition of approximately 4% ethanol (Fig. 1, lane 8), negligible induction being observed at lower ethanol levels. We also found that different Hsps were optimally induced at different levels of ethanol above 4%; also, while some Hsps appear to be induced as strongly by ethanol as by heat shock, others were not. The induction of Hsp104, Hsp70 and Hsp26 progressively increased as the ethanol addition increased from 4 to 10% (Fig. 1, lanes 1–4). At 8% ethanol (Fig. 1, lanes 3 and 9), Hsp104 and Hsp70 are induced almost as strongly as with heat shock, but this is not the case for Hsp82 and Hsp26 (Fig. 1, lanes 6 and 9).

One of the proteins induced most strongly by ethanol is Hsp104, an important determinant of ethanol tolerance (Sanchez et al., 1992) and a homologue of the cipA and
cph proteases of E. coli (Parsell et al., 1991). The form of the Hsp90 protein which is most strongly heat-inducible (Hsp82) was observed to be induced by ethanol to a lesser degree than by heat shock (Fig. 1). S. cerevisiae has two functionally equivalent Hsp90 genes (HSC82 and HSP82; Borkovich et al., 1989). HSC82 displays a high constitutive expression that increases only slightly after heat shock, whereas HSP82 displays much lower basal expression yet is strongly activated by heat shock. HSC82 therefore contributes most of the Hsp90 present during normal vegetative growth, whereas HSP82 expression is mainly responsible for the increase in Hsp90 protein with heat shock (Borkovich et al., 1989). The product of HSP82 (M, 81419), distinguishable from that of HSC82 (M, 80885) by its slightly slower mobility on 1D gels of stress-induced proteins, is the Hsp90 form induced by ethanol (Fig. 1, lanes 6 and 9; also data not shown).

**Induction of small heat shock proteins by ethanol**

A recent study has confirmed that several small Hsps are induced by ethanol (Gropper & Rensing, 1993) although loss of at least one of these proteins (Hsp26) does not affect ethanol tolerance (Petko & Lindquist, 1986; Susko & Lindquist, 1989). Northern analysis of the mRNA of strain BJ2168 probed for **Hsp26** gene transcripts indicated that Hsp26 mRNA, undetectable with addition of 2% ethanol, is barely detectable after addition of 4% ethanol (Fig. 2b). However it is much more strongly induced with further increase in ethanol concentration, eventually (at 10% ethanol) becoming as strongly induced by ethanol as by heat shock (Fig. 2b). Protein pulse-labelling in this strain also shows the increasing Hsp26 induction with increase in ethanol (Fig. 1, lanes 1–4).

We also analysed the ethanol inducibility of the heat-inducible **HSP12** (Praekelt & Meacock, 1990) and **UBI4** (Finley et al., 1987) promoters using strains containing the appropriate promoter–**lacZ** fusions (Tables 1, 2). An HSP12 promoter–**lacZ** fusion carried on an episomal centromeric vector [PMY3(pUP41a); Table 1] was optimally induced by 4–6% ethanol. Its induction rapidly decreased to uninduced levels at higher ethanol concentrations (Table 2). This maximal 12-fold induction of the HSP12 promoter–**lacZ** fusion by ethanol compares to an optimal 61-fold induction of the same sequence by heat shock (Table 2) and 20-fold induction by methanol (U. M. Praekelt and P. Meacock, unpublished results). Northern blotting (Fig. 2c) provided data broadly in agreement with these **lacZ** expression measurements, the Hsp12 mRNA of PMY3(pUP41a) displaying optimal induction at 6% ethanol and declining at higher ethanol levels. The 10% ethanol addition that caused a strong Hsp26 mRNA induction in BJ2168 resulted in no Hsp12 mRNA induction in PMY3(pUP41a) (Fig. 2).

The integrated UBI4 promoter–**lacZ** fusion of strain PMY501 (Table 1) showed optimal 5.2-fold induction at 4–6% ethanol (Table 2). This induction was more sustained at higher ethanol levels as compared to that of the HSP12 promoter–**lacZ** fusion (Table 2) and was similar to the maximal four- to fivefold inducibility of the same UBI4 promoter–**lacZ** fusion by methanol or heat shock (Kirk, 1993).

**The Hsp70 genes induced by ethanol are the same as those induced with heat shock**

Ethanol causes strong induction of proteins that co-migrate on 1D gels with the heat-induced Hsp70 (Fig. 1). The Hsp70 family proteins of S. cerevisiae are encoded by a multigene family comprising no less than 8–9 genes, three of which (**SSA1, SSA3** and **SSA4**) are induced by heat shock (Craig & Jacobsen, 1984; Werner-Washburne et al., 1987, 1989). To establish whether the Hsp70 proteins induced by ethanol are the same as those induced by heat shock, proteins induced by ethanol in strains with one, two or three disruptions in Hsp70-family proteins (Table 1) were investigated by in vivo pulse-labelling at 25 °C in the absence and presence of 6% ethanol. Labelled proteins with an apparent *M*₁ greater than 40000 were then analysed on 1D 7% SDS gels (Fig. 3). This gel system was...
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Table 2. Ethanol inducibility of HSP12 or UBI4 promoter-lacZ fusions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Induction over 25 °C basal level (-fold)</th>
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<tr>
<td></td>
<td>PMY3(pUP41a) (HSP12-lacZ) PMY501 (UBI4-lacZ)</td>
</tr>
<tr>
<td>Ethanol:</td>
<td></td>
</tr>
<tr>
<td>4%, 40 min</td>
<td>12</td>
</tr>
<tr>
<td>6%, 40 min</td>
<td>12</td>
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<tr>
<td>8%, 40 min</td>
<td>3</td>
</tr>
<tr>
<td>10%, 40 min</td>
<td>0</td>
</tr>
<tr>
<td>Heat shock:</td>
<td>25-39 °C, 40 min</td>
</tr>
<tr>
<td></td>
<td>61</td>
</tr>
</tbody>
</table>

Strain: MW109 MW127 MW122 MW121
6% ethanol: - + - + - +

- Hsp104
- Hsp82
- Hsp70

Strain: MW109 MW115 MW123 MW116
6% ethanol: - + - + - +

- Hsp104
- Hsp82
- Hsp70

Fig. 3. Analysis on a 7% gel of the proteins with greater than 40000 labelled in a 40 min pulse-labelling of the Hsp70 gene disruption strains listed in Table 1, in the absence (−) and presence (+) of 6% ethanol.

Ethanol causes the two major changes to plasma-membrane protein composition also seen with heat shock, a rapid decline in plasma-membrane ATPase levels and acquisition of Hsp30

Damage to the plasma membrane is thought to be a major cause of ethanol toxicity (see Introduction). While the effects of ethanol on the lipids of this membrane have been the subject of considerable study (see Introduction), there has been no investigation of whether ethanol influences plasma-membrane protein composition. At least one activity of this membrane (plasma membrane ATPase) influences both heat and ethanol tolerance (Panaretou & Piper, 1990). Both heat stress (Coote et al., 1991; Coote, 1993) and ethanol (Cartwright et al., 1987; Rosa & Sacorreia, 1991) cause stimulation of this ATPase activity, the resulting enhancement of catalysed proton efflux from the cell helping to counteract the depolarization of the plasma membrane resulting from the stress-induced increases in membrane permeability (see Introduction).

With heat shock a single integral membrane Hsp (Hsp30) is targeted to the plasma membrane (Panaretou & Piper, 1992; Rénaud & Boucherie, 1993). Also, levels of the proton-pumping plasma-membrane ATPase decline (Panaretou & Piper, 1992). Since Hsp30 is a membrane protein its total cellular abundance is considerably less than that of major soluble Hsps such as Hsp104, Hsp82, Hsp70 or Hsp26. It is therefore not prominent on gels of total cell heat- or ethanol-induced proteins (Fig. 1), its analysis requiring the preparation of membrane fractions.

Brief treatment of BJ2168 cells with 6 or 8% ethanol resulted in a reduction in levels of the most abundant plasma-membrane protein, plasma-membrane ATPase (Fig. 4a) and induction of Hsp30 (Fig. 4b). These are the same two changes to plasma-membrane protein composition previously shown to result from sublethal heat shock of this same strain (Panaretou & Piper, 1992). Hsp30 was induced to similar levels by heat shock and treatment with 6% ethanol, although its induction was
Fig. 4. Major changes to the protein composition of the plasma membranes of BJ2168 cells following a 40 min heat shock at 40 °C or a 40 min ethanol treatment at 25 °C. (a) Coomassie-brilliant-blue-stained gel of plasma-membrane protein samples; (b) an identical gel fractionation of the same samples, blotted and probed for Hsp30 using an anti-Hsp30 antiserum. Lanes: 1, exponentially growing cells; 2, cells heat shocked for 40 min at 40 °C; 3, cells treated with 6% ethanol; 4, cells treated with 8% ethanol. Thirty micrograms of total plasma membrane protein was loaded in each gel lane.

appreciably less with 8% ethanol (Fig. 4b). The ATPase loss with heat shock appears to reflect protein turnover in the absence of de novo synthesis since it still occurs in cells subjected to these stresses in the presence of cycloheximide (K. Talreja & P. W. Piper, unpublished results). This progressive and relatively rapid loss of ATPase from the plasma membrane with heat or ethanol stress almost certainly influences the capacity of the cells for sustained maintenance of homeostasis during the stress. Using the appropriate mutants future studies can determine whether this reduction in plasma-membrane ATPase, or the acquisition of Hsp30, contribute significantly to ethanol or heat tolerance.

DISCUSSION

The results described here provide further evidence of the strong similarity between the cellular responses to ethanol and to heat shock. The forms of Hsp90 and Hsp70 induced by ethanol are shown to be the same as those induced by heat (Figs 1 and 3). Also these two stresses are shown to have similar effects on the levels of two major integral plasma-membrane proteins, the ATPase and Hsp30 (Fig. 4). While it has long been known that cells alter the lipid composition of their membranes in response to ethanol, this is the first demonstration that they also rapidly alter the protein composition of the plasma membrane.

There is a critical threshold (4%) for induction of heat-shock proteins by ethanol (Figs 1 and 2). Maximal induction is seen at higher ethanol levels which are different for individual heat-shock proteins and heat-shock promoters (Figs 1, 2 and 4 and Table 2). Increasing ethanol levels from 4 to 10% increases the synthesis of Hsp104, Hsp70 and Hsp26 in strain BJ2168 (Figs 1, 2b). However Hsp30 induction declines in this strain as ethanol levels increase above 6% (Fig. 4), as does Hsp12 in strain PMY3(pUP41a) (Fig. 2c, Table 2). This suggests that the level of induction of different heat shock genes is regulated by factor(s) that sense different sublethal ethanol concentrations.

The effects of alkanols on cell inactivation relate to their lipid solubility, indicating that they act in a non-specific way to disorder the membrane lipids (Leao & van Uden, 1982; Van Uden, 1984a). Methanol is the least toxic, inactivating cells only above 18-20%, even though the heat-shock element sequences of yeast promoters are induced maximally at about 10% methanol (Kirk & Piper, 1991b). Ethanol is cytotoxic at lower levels (van Uden, 1984a), inducing most Hsps maximally at 4-10% (Fig. 1). Further increases in the length of the aliphatic chain and lipophilic character of the alcohol increase its cytotoxicity, presumably due to the increased membrane solubility (Jones, 1989). They also increase the potential for induction of heat-shock protein (Gropper & Rensing, 1993), presumably due to an increased capacity for the alcohol to destabilize the hydrophobic interactions that maintain protein conformations.

In vegetative cells tolerances of potentially lethal heat and ethanol are highest in cells with low cAMP-dependent protein kinase activity, increasing as cultures progress from exponential to early-stationary phase (Casey & Ingledew, 1986; Iida, 1988; Piper, 1993). Increased ethanol tolerance is inducible, frequently occurring under the same conditions as those leading to increased thermo-tolerance (Plesset et al., 1982; Watson, 1990; Sanchez et al., 1992; Costa et al., 1993). With the close parallels between the physiological states leading to ethanol...
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tolerance and thermotolerance in yeast it is not surprising that determinants of thermotolerance are also important influences on ethanol tolerance. Thus loss of Hsp104 has been shown to reduce both ethanol tolerance, tolerance to heat, and heat induced tolerance to ethanol (Parsell et al., 1991; DeVirgilio et al., 1991; Sanchez et al., 1992). Besides Hsps, several physiological changes resulting from heat or ethanol exposure are also important in determining cellular tolerances to these stresses (Mager & Moradas-Ferreira, 1993; Piper, 1993; Parsell & Lindquist, 1993). The rapid loss of plasma membrane ATPase protein with ethanol stress (Fig. 4a) contrasts with the ethanol stimulation of the activity of this enzyme (see Introduction). Both these influences on the ATPase will affect its role in maintenance of homeostasis (Serrano, 1991) and should therefore have important effects on tolerance to prolonged exposure to ethanol. Other factors that influence homeostasis will also influence tolerance to ethanol and heat. Thus cells survive heat stress best at pH 6–7 (Cheng & Piper, 1994), when their extracellular pH approximates to intracellular pH. The same probably applies to exposure to alkanols and other agents that increase membrane permeability.

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