DEFINING THE ETHANOL-STRESS RESPONSE IN *Saccharomyces cerevisiae*

by

MEREDITH CHANDLER

2004

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

DEFINING THE ETHANOL-STRESS RESPONSE IN *Saccharomyces cerevisiae*

by

MEREDITH CHANDLER

2004

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

In
Victoria University
School of Molecular Sciences
Australia

DECLARATION

I hereby certify that the work embodied in this thesis is my own unless otherwise stated and has not been submitted previously, in whole or in part in respect to any other academic award.

MEREDITH CHANDLER

SUMMARY

Industrial yeast performance is often compromised during alcoholic fermentations due to bi-product inhibition. Ethanol is arguably the product with the greatest impact on yeast performance, acting as a potent chemical stress on yeast cells. This stress eventually inhibits yeast growth and reduces cell viability, therefore limiting alcohol concentrations in the final product and increasing fermentation turnover times. The reduced cell growth rate and viability, as well as an increased growth lag period, are characteristic signs of cell stress. This is often accompanied at a molecular level by the induction of stress response genes. While there have been several investigations into the effects of ethanol on yeast, few have focused on the underlying genetic mechanisms that enable yeast cells to tolerate and adapt to this stress. This thesis used differential display and gene array technologies to determine, at a molecular genetics level, how yeast cells adapt to sub-lethal concentrations of ethanol. Such information is of fundamental importance to the development of yeast strains and strategies for the improvement of yeast performance in fermentation.

Initial experiments focused on developing a model in which yeast cells undergo a clear adaptation phase when placed in ethanol-containing medium. To this end the effect of ethanol on *Saccharomyces cerevisiae* PMY1.1 was investigated to identify a concentration of ethanol of sufficient magnitude to induce a lag phase adaptation period followed by exponential growth (i.e. recovery). Between 5% and 7% (v/v) ethanol proved to be suitable for this, inducing adaptation phases of sufficient duration to permit sampling at several time points.

Using time course differential display, three genes were found to be up-regulated during adaptation to ethanol stress. However, while differential display is a powerful tool for identifying novel genes with altered expression profiles in cells exposed to ethanol stress, it is time consuming and problems associated with false positive results were encumbering.

To further define the ethanol stress response of *S. cerevisiae*, yeast gene arrays were used to study changes in gene expression at two time intervals, one and three hours,

during the adaptation to ethanol stress. Results from these arrays clearly demonstrated a transient change in expression of many genes but once adapted to the presence of ethanol the gene expression profile was similar to that of unstressed cells. Early in response to ethanol stress, genes associated with energy metabolism were up-regulated and a large number of genes associated with protein synthesis were down-regulated, remaining down-regulated throughout the lag period. Analysis of promoter sequences of up-regulated genes demonstrated the central role of STRE's, and to a lesser extent HSE's, in the cellular response to ethanol stress.

The physiological importance of two highly up-regulated ethanol stress response genes, *HSP26* and *ALD4*, were chosen for further investigation, requiring gene knockouts for each to be constructed in a *S. cerevisiae* PMY1.1 background. Performance of the two knockouts was tested under several ethanol stress conditions but both appeared to perform as well as the parent strain. Thus, a phenotype for these genes, under ethanol stress conditions was not defined. It should be noted however that the only parameters tested were adaptation rate and growth rate so it cannot be concluded that these genes have no influence on adaptation or tolerance to ethanol stress.

PUBLICATIONS AND PRESENTATIONS

Oral presentation:

M. Chandler, G. Stanley, P. Rogers, P. Chambers, (2002). A genomic approach to defining the ethanol stress response in yeast. Yeast: Products and Discovery, CSIRO Melbourne.

Publication:

M. Chandler, G. Stanley, P. Rogers, P. Chambers, (2003). A genomic approach to defining the ethanol stress response in *Saccharomyces cerevisiae* (in submission).

Poster presentations:

M. Chandler, G. Stanley, P. Rogers, P. Chambers, (2002). The ethanol stress response in yeast. 9th International Symposium on the Genetics of Industrial Micro-organisms. Korea.

M. Chandler, G. Stanley, P. Rogers, P. Chambers, (2002). Analysing the ethanol stress response in *Saccharomyces cerevisiae* using gene arrays. 23rd Annual Conference on the Organization and Expression of the Genome, Lorne, Victoria.

I. Dawes, R. Day, D. Duan, F. Roddick, G. Stanley, P. Chambers, M. Chandler, V. Higgins, A. Lentini, and P. Rogers, (2002). Brewing yeast, gene expression and beer quality. American Society of Brewing Chemists Annual Meeting, Tucson, Arizona.

M. Chandler, G. Stanley, P. Rogers and P. Chambers, (2001). Profiling ethanol stress response genes in *Saccharomyces cerevisiae*. XXth International Conference on Yeast Genetics and Molecular Biology, Prague, Czech Republic.

M. Chandler, G. Stanley, P. Rogers, P. Chambers, (2000). Identification and characterisation of novel yeast genes up regulated in response to ethanol stress. Yeast: Products and Discovery, South Stradbroke Island, Queensland.

ACKNOWLEDGMENTS

Firstly, I would like to thank to my two supervisors, Dr Paul Chambers and Dr Grant Stanley, for their invaluable supervision, guidance and encouragement throughout the course of this research. I would also like to thank Dr Peter Rogers for his involvement in the project.

Thank-you also to Dr Dianne Emslie for her valuable help and advise at the beginning of this project. Additionally, I am grateful to my fellow post-grad students and the technical staff in the School of Molecular Science at Victoria University, Werribee campus, for their friendly advice and the making of an enjoyable work place.

I am especially thankful to all my family and friends for their endless support and encouragement throughout this work. In particular I would like to thank my parents for their continuous support in anything I choose to pursue. A special thanks also goes to Heath Merlo for his constant belief in me and unlimited support in so many different ways.

Without the valuable contribution of all these people this work could not have been completed. This project was financially supported by an Australian Postgraduate Industry Award and Carlton and United Breweries.

ABBREVIATIONS

ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BLAST	Basic local alignment search tool
bp	base pair
cDNA	complementary DNA
BSA	bovine serum albumin
Δald4	PMY1.1*Δald4*::kanMX4
Δhsp26	PMY1.1*Δhsp26*::kanMX4
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
MIPS	Munich Information Centre for Protein Sequences
ml	millilitre
MOPS	3-Morpholinopropanesulphonic acid
mRNA	messenger RNA
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RSAT	regulatory sequence analysis tools
RT-PCR	reverse transcription polymerase chain reaction
TEMED	N,N,N,N-tetramethylenediamine
SDS	sodium dodecyl sulphate
SGD	*Saccharomyces* genome database
SS-DNA	salmon sperm DNA
STRE	stress response element
TRIS	Tris-(hydroxymethy)-aminomethane
V	volt
v/v	volume per volume
w/v	weight per volume
YMGV	Yeast Microarray Global Viewer

TABLE OF CONTENTS

FIGURES

TABLES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* has been used in the production of food and beverages since the neolithic age and it is almost certainly mankind's oldest 'domesticated' microorganism. The art of producing beer and wine developed through several independent discoveries where fermented products arose from exposing cereal extracts and fruit juice to the air. The explanations for these fermentations were not available until the nineteenth century when yeasts were recognized to convert fermentable sugars to ethanol and carbon dioxide. Lack of knowledge did not, however, impede the production of alcoholic beverages from Egyptian and Babylonian civilizations, some 4300 years ago. Details of brewing are well illustrated, and during Greek, and later Roman, domination of the Mediterranean wine became an important item of international commerce (Hough, 1985). While beer and wine were valuable commodities and undoubtedly gave rise to a welcome alcoholic euphoria, other advantages included the rendering of poor quality water as safe, due to the low pH and high alcohol content, and also these beverages provided a rich source of B vitamins and protein (Hardwick, 1995).

At present, fermentations producing high percentage alcohol are commercially desirable in the brewing industry as well as for alternative fuel production. The productivity of high alcohol fermentations on an industrial scale, however, depends on many factors, especially the ability of yeasts to tolerate a wide range of physiological stresses associated with large-scale operations.

1.1.1 Yeast stress associated with industrial fermentation

The role of *S. cerevisiae* in fermentation was first accepted in 1876 when Louis Pasteur proved that fermentation was due to living cells (Hardwick, 1995). The first pure *S. cerevisiae* yeast culture used in alcoholic beverage production was obtained by Emil Christian Hansen from the Carlsberg Brewery in 1883. A pure culture of wine yeast was subsequently obtained by Muller-Thurgau from Geisenheim (Germany) in 1890 (from Dequin, 2001). Prior to this, fermentations were considered spontaneous; yeasts were unknowingly transferred from brew to brew in fermentation vessels or from remnants of previous brews saved for use in starting the next brew. Brewing yeasts have in fact adapted over thousands of years from serial re-pitching (re-using) and this selection has led to the evolution of adaptive mechanisms to tolerate many of the physiological stresses encountered during brewing and industrial fermentations.

A stress condition is usually considered any environmental factor that has an adverse affect of cell growth. During the brewing process, yeast cells encounter a variety of environmental stresses. Over recent years, most brewing related research has focused on the ability of yeasts to withstand stressful conditions, especially in relation to high gravity brewing. Modern brewing technology uses high density fermentation substrates in very large, high pressure vessels, resulting in: high osmotic pressure and increased levels of produced ethanol (Stewart *et al.*, 1988); nutrient limitation, especially concerning dissolved oxygen and assimilable nitrogen (Casey *et al.*, 1984); and increased viscosity and carbon dioxide concentration (Pakova *et al.*, 2000). Brewing yeasts are also subject to rapid temperature changes upon removal from fermentation vessels, cold stress when stored for extended periods at low temperatures and acid stress when washed to eliminate contaminating microorganisms prior to re-pitching. Exposure of yeast to these stressing conditions leads to a general decline in cellular viability and vitality and affects the life span and productivity of re-pitched yeast (Cunningham and Stewart, 2000).

The strains of *S. cerevisiae* that ferment sugars from grape juice in wine making are also exposed to numerous stressful conditions. These conditions are similar to those encountered during brewing such as high sugar concentrations, temperature extremes and ethanol stress. In addition, wine fermentations often become nitrogen limited depending on the nitrogen sources present in must (Ivorra *et al.*, 1999). This is exacerbated by the effect of increasing ethanol concentrations during fermentations, which have been shown to negatively effect yeast nitrogen uptake resulting in nitrogen starvation (Boulton *et al.*, 1996).

Even though ethanol is a major metabolic product of fermentation, at high concentrations it inhibits fermentation and growth, and acts as a potent chemical stress on yeast cells. The effect of ethanol on yeast and their adaptive responses is of distinct commercial significance.

1.2 THE YEAST STRESS RESPONSE

Many environmental stresses induce an adaptive stress response in yeast. This stress response is a reprogramming of cellular activities to ensure survival, to protect essential cell components and permit a resumption of cellular activities during the recovery period (Birch and Walker, 2000). *S. cerevisiae* exhibits characteristic adaptive stress responses to a number of stress conditions, the common ones being ethanol stress, osmotic stress, oxidative stress, high pressure stress, temperature stress and nutrient starvation (for reviews see Mager and Hohmann, 1997; Attfield *et al.*, 1997; Mager and Moradas-Ferriera, 1993). The effect of stress on yeast generally leads to a decline in cell growth and metabolic activity.

1.2.1 The induction of general stress responsive genes

To cope with the potentially deleterious effects of stress, yeast cells (like cells of all species) have developed rapid molecular responses to repair damage and protect against further damage caused by ongoing exposure to the same or other forms of stress. These responses include changes in gene transcription, changes in translational

and post-translational modifications of stress-associated enzymes. Such responses are triggered, at least in part, by stress-induced denaturation of proteins, disordering of membranes, DNA damage and metabolic disturbances (Mager and Moradas-Ferreira, 1993; Piper, 1993; Siderius and Mager, 1997). Stress responsive genes that are part of the general stress response machinery of yeast are presumed to encode proteins with functions that are necessary to cope with damage under various stress conditions. Table 1.1 lists the stress responsive proteins of yeast, including their function and cellular localization. Some of the stress response genes encoding these proteins will be discussed below.

As the heat shock response is the most extensively studied stress response in yeast, heat shock protein (HSP) genes are among the best-characterised stress response genes (Mager and Moradas-Ferreira, 1993). The spectrum of HSPs induced upon a stress challenge is highly conserved across bacteria, fungi, plants and animals (Lindquist and Craig, 1988). HSP expression also occurs when cells encounter a heat shock and other types of stress including exposure to ethanol (Plesset *et al.*, 1982) and hydrogen peroxide (Collinson and Dawes, 1992). Many HSPs are also expressed constitutively at low levels suggesting they have fundamental roles in the cell. HSPs are known to play essential roles in the synthesis, transport, translocation, proteolysis and proper folding of proteins under both normal and stressful conditions (Santoro *et al.*, 1998). Under stress conditions they are considered to play important roles as chaperones (in particular Hsp70) which have been implicated in the repair of damaged proteins generated by exposure to stress. A putative protease function of Hsp104 also suggests it could be involved in disaggregation of damaged proteins (Parsell *et al.*, 1994). The roles of HSPs in stress tolerance are further described in Section 1.2.3.

Other general stress responsive genes include the polyubiquitin gene, *UBI4* (Finley *et al.*, 1987), and the DNA damage response gene, *DDR2* (Kobayashi and McEntee, 1993). *UBI4* is involved in the nonlyosomal proteolysis of proteins and it enables the cell to rid damaged proteins that may accumulate to toxic levels (Jentch *et al.*, 1990). *DDR2* is induced either by DNA damage or heat shock, possibly to avoid the adverse effects of stress on cellular DNA. In addition, the gene encoding catalase T, *CTT1*,

Table 1.1 Stress proteins of yeast (adapted from Mager and Moradas-Ferreira, 1993)

Stress response proteins	Cellular localization	Function
Hsp150	Secretory	Cell wall glycoprotein
Hsp104	Nucleolus	Stress tolerance
Hsp83	Cytosol/nucleus	Chaperone
Hsp70		
Ssa1	Cytosol/nucleus	Chaperone
Ssa2	Cytosol	Chaperone
Ssa3	Cytosol	Chaperone
Ssa4	Cytosol/nucleus	Chaperone
Ssb1	Unknown	Chaperone
Ssb2	Unknown	Chaperone
Ssc1	Mitochondria	Chaperone
Ssd1 (Kar2)	ER	Chaperone
Sse1	Cytosol	Chaperone
Ssa2	Cytosol	Chaperone
Hsp60	Mitochondria	Chaperone
Hsp30	Plasma membrane	Chaperone
Hsp26	Cytosol/nucleus	Chaperone
Hsp78	Mitochondria	Chaperone
Ddr2	Unknown	Unknown
Ubi4 (Ubiquitin)	Cytosol	Protein degradation
Enolase (Hsp48)	Cytosol	Glycolysis
Glyceraldehyde 3-phosphate dehydrogenase	Cytosol	Glycolysis
Phosphoglycerate kinase	Cytosol	Glycolysis
Catalase T	Cytosol	Antioxidant defence

prevents some of the damaging effects of stress, most likely in avoiding the harmful effects of reactive oxygen intermediates (Davidson *et al.*, 1996). *CTT1* is induced in *S. cerevisiae* under heat shock, osmotic shock and oxidative stress (Piper, 1993; Schuller *et al.*, 1994; Davidson *et al.*, 1996).

1.2.2 Pre-stressing and cross protection

An intrinsic aspect of the stress response of yeast cells is that of acquired stress resistance, where cells can withstand a severe stress condition more effectively when they have been previously exposed to a mild form of stress. For instance, the pretreatment of yeast cells at mildly elevated temperatures leads to the attainment of tolerance against a more severe heat shock. Yeast cells grown at 23°C develop enhanced tolerance to a lethal temperature of 51°C following prior incubation at 37°C for 20 minutes (Plesset *et al.*, 1982). This induction of thermotolerance has been observed in cells incubated at a series of sub-lethal temperatures, ranging between 37°C and 45°C (Coote *et al.*, 1991). Within this range of temperatures, a higher pre-stress heat shock not only produced a greater thermotolerance response, but also a quicker response. With a higher pre-stress temperature, the time of thermotolerance could be reduced (Coote *et al.*, 1991). Thermotolerance develops rapidly in yeast upon a temperature shift from 23°C to 37°C reaching a maximum at 2 hours (Piper, 1997).

This pre-exposure effect is also true for stresses other than temperature. A short pretreatment of cells with 0.7 M NaCl leads to an increase in the number of surviving cells when they are subsequently exposed to 1.4 M NaCl (Trollmo *et al.*, 1988; Varela *et al.*, 1992). The same is also true for oxidative stress, where cells pre-treated with 0.4 mM H_2O_2 survive a challenge of peroxide at concentrations of 0.8 mM H_2O_2. Under these conditions, cells that were challenged without the pretreatment exhibited a 90% loss in viability (Davies *et al.*, 1995). Thus, mild stress conditions may trigger the relevant cellular responses that prepare cells to cope with severe stress.

Yeast cells exposed to mild stress can develop tolerance not only to higher levels of the same stress, but also to stress caused by other agents. For example, a brief heat shock treatment not only increases thermotolerance but also results in cross protection to other stresses such as ethanol (Watson and Cavicchioli, 1983; Costa *et al.*, 1993), high salt concentration and freezing (Lewis *et al.*, 1995). Similarly, the pretreatment of cells with a mild osmotic shock conferred increased resistance to heat shock (Trollmo *et al.*, 1988; Varela *et al.*, 1992) and the exposure of yeast to ethanol, sorbic acid or low external pH induced higher thermotolerance (Plesset *et al.*, 1982; Coote *et al.*, 1991). This phenomenon is known as cross-protection and suggests at least some commonality in the cellular responses to different forms of stress.

Although the effects of cross protection suggest a shared response, this is not always true as in some situations the acquisition of stress tolerance does not occur. For example, a mild heat shock does not result in increased osmotolerance (Trollmo *et al.*, 1988; Varela *et al.*, 1992). Also, while the treatment of cells with H_2O_2 did not evoke resistance to the superoxide-generating drug, menadione, treating cells with menadione did induce resistance to H_2O_2 (Jamieson, 1992). Thus, while parts of the stress response of yeast cells may be shared and lead to certain levels of cross protection, there are also stress-specific responses which are presumably necessary for survival under complex and variable adverse conditions. Additionally, Lewis *et al.* (1995) found that salt-shocked cells acquire increased tolerance to freezing and heat stress, and although the treatment of these cells with the protein synthesis inhibitor cycloheximide only reduced heat and salt tolerance, the previously acquired tolerance to freezing was completely lost. Therefore, protein synthesis seems to be required for freeze tolerance but it is not essential for some tolerance to heat and salt.

1.2.3 The role of HSPs in stress tolerance

The functions of many HSPs have been studied in detail, especially in relation to thermotolerance (see reviews by Parsell and Lindquist, 1993; Piper, 1993; Mager and Moradas-Ferreira, 1993). Two particular HSPs, Hsp70 and Hsp104, are the most interesting in regard to stress tolerance. The cytosolic Hsp70 family has been found to

be involved in two processes that relate directly to thermotolerance: the prevention of protein aggregation and the refolding of proteins damaged during heat shock (Piper, 1997). The latter role is related to their function as chaperones (Craig *et al.*, 1994). In *S. cerevisiae* there are around 10 genes related to Hsp70 (Estruch, 2000).

In regard to Hsp104, when wild type and *Δhsp104* mutant cells are grown at 25°C and given a mild heat shock (30 minutes at 37°C) before exposure to 50°C, thermotolerance is induced in both strains. However, this tolerance was very transient in the *Δhsp104* mutant since cells began to die at 100-1000 times the rate of the wild type (Parsell and Lindquist, 1993). In addition, cells with constitutive *HSP104* expression were found to have elevated thermotolerance in the absence of a pre-stress (Sanchez *et al.*, 1992). The acquirement of tolerance against high ethanol concentrations (and to a lesser degree arsenite) is similarly dependant on a functional *HSP104* gene (Sanchez *et al.*, 1992). Hsp104 is suggested to rescue heat-inactivated proteins directly from insoluble aggregates, which is not a function of other chaperones (Parsell *et al.*, 1994). There is also evidence that Hsp70 and Hsp104 may have complementary roles: *SSA* gene products (of the Hsp70 family) assume an important role in tolerance to extreme temperatures in the absence of Hsp104 while, in cells with low levels of Hsp70, Hsp104 assumes an important role in growth at normal temperatures (Sanchez *et al.*, 1993).

The protective functions of many HSPs in yeast are still unknown. The small HSPs (Hsp12 and Hsp26) are without any demonstrable function, even though both of these proteins are induced by many stresses (Praekelt and Meacock, 1990; Mager and Moradas-Ferreira, 1993). The heat shock induced Hsp150, a secretary glycoprotein, also has an unknown function.

The hypothesis that the role of HSPs is to provide tolerance to heat and other forms of stress has been the object of some controversy since characterization of phenotypes associated with mutations in some HSPs reveal that they are required at any temperature. Smith and Yaffe (1991) reported a yeast strain containing a mutant allele for the heat shock factor, *hsf1-m3*, for which the acquisition of thermotolerance was not affected even though the mutant is defective in the induction of HSPs. De Virgilio

et al. (1991) reported a mutant yeast strain unable to synthesize proteins during heat shock, that nonetheless acquired thermotolerance, albeit to a lesser degree than the wild type strain. This suggests that mechanisms other than those requiring HSPs are involved in thermotolerance. Hall (1983) also reported thermotolerance was induced by heat shock in the absence of *de novo* protein synthesis, suggesting that high levels of HSPs may not be required for thermotolerance.

As well as the induction of stress response genes, a number of other changes have been observed in stressed yeast cells. For example, trehalose synthesis has been observed in heat-shocked yeast cells (Hottiger *et al.*, 1987) and glycerol has been shown to accumulate during osmotic shock when yeast cells are exposed to 300 mM NaCl for 45 minutes (Lewis *et al.*, 1995). Thus, the accumulation of trehalose and glycerol may contribute to cellular stress protection.

1.2.4 The role of trehalose in stress protection

Accumulated trehalose was originally thought to serve as a storage carbohydrate (Lillie and Pringle, 1980, Thevelein, 1984), however, more recently it has been suggested that trehalose functions as a cellular protectant, being involved in stress tolerance (Kim *et al.* 1996; Van Leare, 1989). Large amounts of trehalose accumulate in *S. cerevisiae* cells during periods of adverse growth conditions such as high temperature (Hottiger *et al.*, 1987; Lewis *et al.*, 1995), freezing (Kim *et al.*, 1996), dehydration and dessication (Gadd *et al.*, 1987; D'Amore *et al.*, 1991), starvation (Lillie and Pringle, 1980), hyperosmotic shock (Hounsa *et al.*, 1998) and ethanol shock (Attfield, 1987; Kim *et al.*, 1996). This carbohydrate also accumulates when cells are exposed to copper sulphate or hydrogen peroxide and declines rapidly after the stress is removed (Attfield, 1987). A decline in trehalose concentration has also been correlated with a loss of stress resistance. Van Dijck *et al.* (1995) showed the addition of glucose to stationary phase cells resulted in the mobilization of trehalose and a subsequent decline in stress resistance. Similarly, a high trehalose content in re-pitched yeast in brewing fermentations improved cell viability, increased

carbohydrate utilization and increased the production of isoamyl alcohol and isobutanol during the initial stages of fermentation (Guldfelt and Arneborg, 1998).

The precise role of trehalose in stress tolerance is however unknown. It has been suggested that it acts to stabilize proteins in their native state and preserve the integrity of cellular membranes during stress (Crowe *et al.*, 1984; Colaco *et al.*, 1994). Hottiger *et al.* (1994) found that trehalose increases the thermal stability of proteins and reduces heat induced protein aggregates *in vitro*. Trehalose has also been found to be more effective than other sugars in stabilizing the tertiary structure and activity of enzymes when heated at 50°C (Sola-Penna and Meyer-Fernandez, 1998). Trehalose accumulation on both sides of cellular membranes is thought to stabilize membrane structures by reducing permeability, thereby protecting them against ethanol-induced water stress (Hallsworth *et al.*, 1998). Consistent with this, the inhibition of endocytosis that has been shown to occur in the presence of ethanol is also reduced by trehalose accumulation (Lucero *et al.*, 2000).

Mutation of the *TPS1* gene, which encodes a subunit of trehalose synthase, renders the yeast cell unable to produce trehalose and makes it sensitive to heat (De Virgilio *et al.*, 1994). However, phenotypes of mutants lacking the *NTH1* gene, which is responsible for trehalose degradation, accumulate high levels of trehalose yet their ability to survive extreme heat is impaired (Nwaka *et al.*, 1995 a; Nwaka *et al.*, 1995 b). This and other work led to doubts as to whether trehalose does protect cells from heat stress (reviewed by Nwaka and Holzer, 1998). Hazell *et al.* (1995) suggested it is not trehalose itself, but the Tps1 protein that is important for stress resistance by stimulating induction of HSP genes. Another explanation is that trehalose protects cells at mildly elevated temperatures and HSP genes are induced to provide protection if the stress becomes more severe (Winkler *et al.*, 1991; Nwaka *et al.*, 1994). Singer and Lindquist (1998 a) report that under moderate heat-shock conditions, mutations of *TPS1* do not effect HSP induction, yet these cells show greatly diminished tolerance to extreme heat. The authors suggest trehalose acts directly during heat shock to stabilize proteins in their native state. Proteins that do unfold are bound by HSPs, which suppress their aggregation and promote their proper re-folding. Trehalose also stabilizes these substrates and reduces aggregation when the proper protein-repair

machinery is overwhelmed. However, as the stabilization of denatured proteins by trehalose can interfere with their subsequent reactivation, the disaccharide is rapidly degraded after heat shock so as not to impede HSPs in resolving heat-induced damage to proteins swiftly (Singer and Lindquist, 1998 b).

1.3 THE RESPONSE OF *S. cerevisiae* TO ETHANOL

1.3.1 The physiological effect of ethanol on *S. cerevisiae*

The productivity of industrial yeast fermentations is compromised by the accumulation of ethanol in the culture medium. High ethanol concentrations act as a potent chemical stress, inhibiting fermentation, cell growth and viability. These inhibitory effects have been topics of extensive research (reviewed by D'Amore *et al.*, 1990; Ingram and Buttke, 1984; Casey and Ingledew, 1986; Jones, 1990). Table 1.2 summarizes some of the principal inhibitory affects of ethanol on yeast cells and the possible target sites of ethanol are illustrated in Figure 1.1. The physiological impacts of ethanol on yeast cells are complex.

1.3.1.1 The effect of ethanol on cellular membrane composition

A predominant effect of ethanol exposure on yeast cells is the disruption of membrane structure and function. Ethanol causes changes in the lipid composition of the plasma membrane, inducing alterations to the lipid and fatty acid composition resulting in increased membrane fluidity (Jones and Greenfield, 1987; Ingram, 1986; Sajbidor, 1997) and a consequential decreased membrane structural integrity. Ethanol also affects hydrophobic proteins of the mitochondrial membrane, nuclear membrane, vacuolar membrane and endoplasmic reticulum (Sajbidor, 1997).

The characteristic changes in membranes exposed to ethanol have been well studied (see reviews by D'Amore *et al.*, 1990; Rose, 1993; Sajbidor, 1997) and it is now established that the phospholipid and sterol composition of cellular membranes

Table 1.2 The effects of ethanol on yeast cell physiology (adapted from Walker, 1998).

Physiological function	Effect of ethanol
Cell viability and growth	- General inhibition of cell growth, viability and division - Decrease in cell volume - Enhancement of thermal death
Metabolism and macromolecular biosynthesis	- Denaturation of intracellular proteins - Lower rate of RNA and protein accumulation - Enhancement of petite mutation - Induction of stress response proteins - Increase in oxygen free radicals - Induced synthesis of cytochrome P450
Membrane structure and function	- Altered fatty acid and sterol composition - Increased ionic permeability - Inhibition of H^+-ATPase and dissipation of proton motive force - Passive re-entry of protons and lowering of cytoplasmic pH - Hyperpolarisation of plasma membrane - Inhibition of nutrient uptake - Induced lypolysis of cellular phospholipids

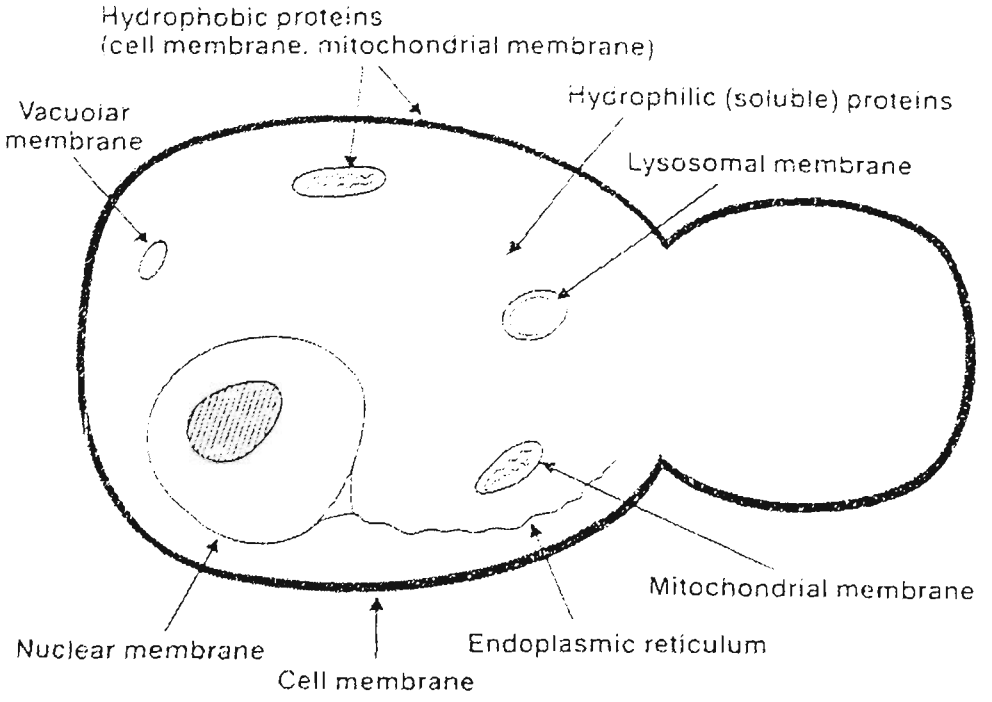


Figure 1.1: Possible target sites of ethanol in yeast (from D'Amore *et al.*, 1990)

influence ethanol tolerance; this adaptive response will be further discussed in section 1.4.1.

In a study of *S. cerevisiae* NCYC 431 cultures grown in the presence of increasing ethanol concentrations (from 3.5-9% (v/v) added ethanol), Beaven *et al.* (1982) reported a dose-dependant increase in the content of mono-unsaturated fatty acyl residues (primarily oleic acid, C18:1) in the plasma membrane accompanied by a concomitant decrease in the proportion of saturated (16:0) fatty acyl residues. Alexandre *et al.* (1993) observed that the membrane lipids of a *S. cerevisiae* wine strain were adjusted towards a higher level of oleic (18:1) fatty acid residues with a corresponding decrease in palmitic acid (16:0) under aerobic growth in the presence of 10% (v/v) ethanol. Sajbidor and Grego (1992) noted an increase in C18:1 fatty acyl residues associated with a decrease in the proportion of C16:1 fatty acyl residues, instead of C16:0, in *S. cerevisiae* CCY cultures grown in the presence of 15% (v/v) ethanol. These changes in lipid composition are viewed as an adaptation to maintain membrane integrity, however they do impact on membrane fluidity as will be discussed in Section 1.3.1.2.

Yeast cells are unable to synthesize unsaturated fatty acids under anaerobic conditions as the yeast desaturase enzyme has an oxygen requirement (Walker, 1998). Under anaerobic growth conditions unsaturated fatty acids must be transported into the cell from the growth medium. Thomas *et al.* (1978) used supplemented media to enrich the plasma membrane of anaerobically grown *S. cerevisiae* NCYC 366 with exogenously added unsaturated fatty acids and sterols. In doing so they found that lipid supplemented cultures show increased tolerance to ethanol when the supplementation included a greater level of unsaturated fatty acids at the expense of saturated fatty acids. You *et al.* (2003) examined the effects of different unsaturated fatty acid compositions of *S. cerevisiae* on the growth-inhibiting effects of ethanol. The authors altered the unsaturated fatty acid composition of yeast cells in a uniform genetic background by genetic complementation of a desaturase deficient *ole1* knockout and by supplementing the growth medium of the same strain with synthetic monounsaturated fatty acids. The unsaturated fatty acid composition was found to be a significant determinant in ethanol tolerance and oleic acid was the most efficacious

unsaturated fatty acid in overcoming the toxic effects of ethanol in growing yeast cells.

In addition to ethanol-induced changes in fatty acids in the plasma membrane there is also a change in membrane sterols. Walker-Caprioglio *et al.* (1990) found a pronounced alteration in the type of sterols synthesized when *S. cerevisiae* X2180-1A cultures were exposed to ethanol. Ergosterol was found to account for 41% of total sterol content in cells grown in the absence of ethanol. However, when 6% ethanol was added to the growth medium, ergosterol was found to account for over 80% of the total sterol content; the total amount of sterols decreased slightly in ethanol. The decreased level of sterol, together with the type of sterol present in the cell, increased the membrane fluidity (Walker-Caprioglio *et al.*, 1990). An increase in the unsaturation of membrane sterols, modified towards ergosterol, in cells exposed to ethanol was also reported by Alexandre *et al.* (1993). Ergosterol levels increased from 40% in unstressed cells to 60% in 10% (v/v) ethanol stressed cells. In contrast, however, Novotny *et al.* (1992) observed an accumulation of Δ 5-7 sterols during growth of *S. cerevisiae* in the presence of ethanol and Del Castillo (1992) found a decrease in the ergosterol content when ethanol in the medium was increased to 6% (v/v), followed by a marked increase at the highest ethanol concentration.

When anaerobically grown *S. cerevisiae* cultures were supplemented with a variety of sterols, modifications to the membrane sterol composition were observed (Thomas *et al.*, 1978). When these cultures were exposed to 10% ethanol, cells enriched with ergosterol were significantly more resistant to the toxic effects of ethanol than cells enriched with other sterols.

In summary of the above work, the exposure of yeast cells to ethanol induces changes in membrane composition that result in increased length and unsaturation of membrane lipids that presumably increases membrane fluidity. The reason for the increased chain length is perplexing since this would normally decrease membrane fluidity, however, an increase in fatty acid chain length also increases the hydrophobicity of the membrane bilayer and this may prevent ethanol accumulation within the membrane. An increase in hydrophobicity may also reduce the passive

diffusion of small ions and protons across the cellular membrane. On the other hand, the induced changes in membrane lipids may lead to an increased amount of ethanol that could be trapped within the cell membranes, thereby limiting its entry into the cell.

1.3.1.2 The effect of ethanol on membrane fluidity

Numerous studies report a fluidizing effect of ethanol on yeast membranes (Alexandre *et al.*, 1994; Jones and Greenfield, 1987; Walker-Caprioglio *et al.*, 1990; Lloyd *et al.*, 1993; Mishra and Prasad, 1989). The alteration in lipid composition during growth in the presence of ethanol has been correlated with a resultant increase in membrane fluidity (i.e. the rate of lateral motion of molecules in the membrane) (Beaven *et al.*, 1982; Alexandre *et al.*, 1994).

Jones and Greenfield (1987) measured the passive influx of undissociated acetic acid into the yeast cytoplasm to observe the effect of up to 25% (v/v) ethanol on membrane fluidity and permeability. In batch cultures, an ethanol-induced increase in membrane fluidity was correlated to increased membrane permeability, due to changes in membrane composition, following step increases in ethanol concentration. In comparison, continuous exposure of cells to 15% (v/v) ethanol eventually made membranes less fluid and thus less permeable. The authors speculated that increased membrane fluidity is an adaptation to ethanol that may increase stability but it may not necessarily be important in ethanol tolerance.

The fluidity of sub-cellular membrane fractions was measured following up to 9% (v/v) ethanol stress, by electron spin resonance spectroscopy (ESR) (Lloyd *et al.*, 1993). Microsomal fractions from cells grown in medium containing 5% (v/v) ethanol showed a significant increase in fluidity. By contrast, mitochondria prepared from yeasts grown in medium containing 7% and 9% (v/v) ethanol showed similar overall fluidity to membranes from cells grown without ethanol but a change in the temperature dependency behaviour suggested membrane alterations had occurred. Lloyd *et al.* (1993) proposed the effects of ethanol to be membrane associated, but not involving mitochondria.

Swan and Watson (1997), using fluorescence anisotropy to measure membrane fluidity of stress-sensitive and stress-resistant sake strains of *S. cerevisiae*, found that membrane fluidity increased slightly in all strains following one hour of exposure to 17% (v/v) ethanol. The observed increase in fluidity was accompanied with a small increase in cell survival; however membrane fluidity was found not to correlate with stress tolerance. Alexandre *et al.* (1994) proposed an increase in membrane fluidity, in *S. cerevisiae* adapted to ethanol, was correlated with a decrease in sterol: phospholipid and sterol: protein ratios and an increase in unsaturation index. Gille *et al.* (1993), on the other hand, reported that fluidization of the plasma membranes in *S. cerevisiae* and *Schizosaccharomyces pombe* takes place during the mere aeration of the cell culture in the absence of substrates. The presence of 200 mM ethanol only slightly increased the extent of the aeration-induced fluidization.

Curtain *et al.* (1985), using ESR, compared the effect of ethanol on the plasma membrane of protoplasts from two strains of *Saccharomyces* to the effect of ethanol on phospholipid vesicles. The spin probes used were distearoyl phosphatidylcholine with a stable nitroxide free radical attached to either the 5- or 16-position of one of the fatty acid chains. Increasing ethanol concentrations were found to have a much stronger fluidizing effect on the plasma membranes of the yeast protoplasts than on the protein-less membrane vesicles. In addition, it was found that the fluidity effect was more pronounced closer to the membrane surface.

To test whether changes in phospholipids alter membrane fluidity and ethanol tolerance, Mishra and Prasad (1988) used L-alanine uptake and H^+ efflux as measures of ethanol tolerance in *S. cerevisiae* cultures unable to synthesize lipids. Cultures were supplemented with different phospholipids and exposed to 12% ethanol for 10 minutes before transport measurements were taken. It was found that cells enriched with phosphatidylserine, rather than phosphatidylcholine and phosphatidylethanolamine, exhibited a greater tolerance to ethanol presumably resulting from the altered charge of membrane phospholipids polar head groups rather than changes in membrane fluidity. It is possible that in the short term changes in phospolipid head group composition rather than membrane fluidity render the yeast more tolerant to

ethanol but longer term adaptation is likely to require changes in fatty acid and/or sterol composition.

1.3.1.3 The effect of ethanol on membrane function

Associated with an ethanol-induced disruption to membrane structure, there is an increase in passive proton influx into the cell. This proton influx is thought to activate the plasma membrane H^+-ATPase (Cartwright *et al.*, 1986; Alexandre *et al.*, 1993) which is largely responsible for maintenance of the plasma membrane proton gradient. This gradient drives the uptake of nutrients and is implicated in intracellular pH homeostasis (Monteiro and Sa-Correira, 1998). However, the activity of the H^+-ATPase is down-regulated by its negative regulator, the plasma membrane Hsp30 (Piper *et al.* 1994), that reduces the V_{max} of the plasma membrane H^+-ATPase in heat shocked cells (Braley and Piper, 1997). Although the initial response to heat shock and ethanol stress is the activation of H^+-ATPase, Hsp30 (produced in response to ethanol stress) is thought to down-regulate this pump perhaps in an attempt to conserve energy (Alexandre *et al.*, 2001). Whether Hsp30 regulates the H^+ATPase in ethanol stressed cells is not known.

A decrease in intracellular pH, due to the passive uptake of protons resulting from membrane disruption, is thought by some to be particularly important in inducing a stressed state in yeast cells (Leao and Van Uden, 1984a; Cartwright *et al.*, 1987). In a study by Imai and Ohno (1995), measurement of yeast intracellular pH, by a fluorescence microscope image processor, demonstrated that intracellular pH varies with growth phase even when the external pH remains unchanged. Intracellular pH varied from 5.7 during the lag phase, to 6.8 during exponential growth, and pH 5.5 during stationary phase. The involvement of the plasma membrane H^+-ATPase enzyme was however not tested in this study. A further study by Fernanda-Rosa and Sa-Correia (1996) measured intracellular pH by determining the relative distribution of [2-^{14}C]-propionic acid between the cytoplasm and the extracellular medium. The authors showed intracellular acidification did not account for ethanol-induced inhibition of yeast growth since growth was inhibited by ethanol concentrations (3-6% v/v) that did not lead to a decrease of intracellular pH. However, studies relying

on measurements of intracellular pH of yeast cells are controversial because some methods for intracellular pH determination are not precise (Cartwright *et al.*, 1987).

Ethanol has been found to inhibit membrane transport systems for glucose (Leao and van Uden, 1982), maltose (Loureiro-Dias and Peinado, 1982) and ammonium (Leao and van Uden, 1983) in *S. cerevisiae*. The inhibitory effects are thought to be due to interactions between ethanol and transport systems within the hydrophobic regions of the plasma membrane; ethanol is a non-competitive inhibitor of glycine, alanine, phenylalanine, tyrosine and tryptophan transport across the cytoplasmic membrane (Leao and van Uden, 1984b). A threshold concentration of ethanol was required before the transport of amino acids was affected but at higher ethanol levels inhibition of amino acid transport was greater than that of glucose. This suggests that the disruption of amino acid transport is not a factor in ethanol inhibition of growth at low ethanol concentrations, however at higher ethanol concentrations it may be of importance.

1.3.1.4 The effect of ethanol on organelles

The accumulation of ethanol during fermentation and the ability of ethanol to diffuse across cellular membranes suggest it is likely that all cellular membranes and organelles are possible targets of ethanol-induced damage. The target sites of ethanol in yeast cells are illustrated in Figure 1.1.

The mitochondrion is a likely target for ethanol-induced damage (van Uden, 1984; Aguilera and Benitez, 1985). The presence of functional mitochondria is thought to be essential for tolerance to ethanol stress. When functional mitochondria were transferred to a petite (respiration-deficient) strain, an increase in cell survival in the presence of ethanol was observed (Aguilera and Benitez, 1985). Costa *et al.* (1993) demonstrated the importance of the mitochondrial manganese dependant superoxide dismutase (MnSOD) in the acquisition of ethanol tolerance. Ethanol stress (8% v/v) increased MnSOD activity by 50-150%, and this activity was thought to be responsible for the resistance of *S. cerevisiae* aBR10 to 14% ethanol exposure. In contrast, the cytoplasmic CuZnSOD did not increase in activity following ethanol

stress, suggesting it may only have a minor role in ethanol tolerance (Costa *et al.*, 1993). In addition, a MnSOD deficient strain demonstrated ethanol sensitivity whereas a CuZnSOD deficient strain showed no impairment in the acquisition of ethanol tolerance. MnSOD was subsequently found to be essential for tolerance; viable plate counts demonstrated that, unlike the parent strain, a MnSOD deficient strain, in diauxic and post-diauxic growth, became nonviable after 30 minutes of exposure to 20% (v/v) (Costa *et al.*, 1997). MnSOD was presumed to inactivate reactive oxygen species generated in the mitochondria during respiratory growth on ethanol (Costa *et al.*, 1997).

The yeast vacuole is also targeted under conditions of ethanol-stress. Using a lipophilic dye (FM 4-64), Meaden *et al.* (1999) showed vacuolar morphology altered in the presence of ethanol. Exposure of yeast to 6% (v/v) ethanol resulted in the formation of large single vacuoles rather than the usual segregated structures typical of cells growing under optimal conditions. Loureiro-Dias and Santos (1990) studied the effect of ethanol on yeast vacuolar membranes. While ethanol increased acidification of the cytoplasm, the pH of the vacuole remained unchanged. The passive influx of protons that occurs across the plasma membrane in the presence of ethanol seemed not to occur across the vacuolar membrane.

1.3.2 Relationship between ethanol stress and heat stress

Several of the changes induced in yeast by exposure to stressful ethanol levels are identical to those caused by heat stress (for review see Piper, 1995); heat shock and ethanol stress are suggested to exhibit a 'functional overlap' in yeast cells (Piper, 1995).

The effects of ethanol on yeast cells become much more severe with an increase in temperature, the optimum and maximum temperatures of growth becoming significantly lower in the presence of ethanol levels above 3% (v/v), while thermal death on exposure to extreme temperatures is enhanced (van Uden, 1984). As discussed previously (Section 1.2.2) increased thermal tolerance can be attained by

pre-exposing cells to non-lethal ethanol concentrations. Similarly, a sub-lethal heat shock leads to the subsequent acquisition of increased ethanol tolerance (Costa *et al.*, 1993; Watson and Cavicchioli, 1983). However, when cells are concurrently heat-stressed and ethanol-stressed they lose viability quicker than if exposed to each stress separately.

Aguliera and Benitez (1986) isolated 21 monogenic ethanol-sensitive mutants in *S. cerevisiae*. One-third of these ethanol-sensitive mutants were also temperature sensitive. This result suggests that many of the genes involved in the mechanisms for mediating ethanol tolerance in *S. cerevisiae* are also required for tolerance to heat.

Both heat and ethanol are observed to cause membrane disordering and protein denaturation (Casey and Ingledew, 1986; Piper, 1993) as well as an inhibition of glycolysis and enhanced induction of petites (Neves and Francois, 1992; Leao and van Uden, 1982; Leao and van Uden, 1984a). Both stresses also increase the permeability of the plasma membrane, resulting in an increased passive proton influx that acts to dissipate the membrane electrochemical potential. This is reflected by a decline in intracellular pH that is observed following both ethanol addition (Leao and van Uden, 1984a) and heat shock (Coote *et al.*, 1991) to yeast cells.

Both sub-lethal heat shock (Coote *et al.*, 1991) and ethanol exposure (Cartwright *et al.*, 1987; Rosa and Sa-Correia, 1991) stimulate the activity of the plasma membrane H^+-ATPase, the enzyme responsible for maintaining the proton gradient across the plasma membrane. The increase in H^+-ATPase activity causes an enhanced proton efflux that counteracts the dissipation of proton motive force, resulting from a stress-induced increase in membrane permeability (Piper, 1995). Consistent with these findings, Panaretou and Piper (1990) describe mutations that alter plasma membrane ATPase activity that also increase tolerance to ethanol and heat stress.

Both heat and ethanol also induce synthesis of HSPs in yeast (Plessest *et al.*, 1982). The induction of Hsp30, a highly hydrophobic integral membrane protein, is induced to similar levels by heat shock and exposure to 6% (v/v) ethanol (Piper *et al.*, 1994) and Sanchez *et al.* (1992) showed that respiring cells that constitutively express

HSP104 were more resistant to heat shock and high ethanol than a Δ*hsp104* deletion mutant.

1.3.3 Relationship between ethanol and oxidative stress

Aerobic organisms have to maintain a cellular redox balance in the face of oxidative conditions. Oxidative stress is induced in aerobic organisms due to the production of reactive oxygen species (ROS) that cause DNA lesions, lipid peroxidation, and damage to proteins, carbohydrates and other cellular components. The superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH) are the most important ROS produced by cells. These ROS are frequently formed as a result of normal cellular metabolism, such as respiration and β-oxidation of fatty acids, however they are produced at higher levels as a result of ethanol or chemical stresses (Jamieson, 1998; Costa *et al.*, 1993). Yeast cells have evolved adaptive responses to neutralize such ROS in defense of oxidative damage (for review see Jamieson, 1998).

A number of enzymes are induced to inactivate ROS. These include the cytoplasmic superoxide dismutase (CuZnSOD; encoded by the *SOD1* gene), the mitochondrial superoxide dismutase (MnSOD; encoded by the *SOD2* gene) (both previously mentioned in section 1.3.1.4), cytochrome c peroxidase (*CCP*), and cytoplasmic catalase T (encoded by the *CTT1* gene). Pereira *et al.* (2001), using *S. cerevisiae* strains containing specific *Δsod* deletions, verified that the Sodp isoforms play different roles in the process of acquisition of tolerance to oxidative stresses. MnSOD was involved in the acquisition of tolerance to ethanol and heat stress, while a deficiency in CuZnSOD was found to be beneficial for protection against to ethanol and heat stress. The authors suggested MnSOD could prevent the reaction of high levels of O_2^-, produced during ethanol stress, with biomolecules inside the mitochondria. This would also prevent diffusion of O_2^- to the cytosol, thereby protecting lipids and proteins from oxidative damage. The importance of MnSOD in ethanol stress tolerance is also covered in Section 1.3.4. The damage ROS can inflict is also reflected by an increase in ethanol tolerance of respiratory deficient petites,

cells that have lost ROS production by the respiratory chain (Costa *et al.*, 1993; Moradas-Ferreira *et al.*, 1996).

Gille *et al.* (1993) found intra- and extra-cellular catalase activity was greater in *S. cerevisiae* grown on ethanol rather than glucose. It was also found that many antioxidant genes were glucose-repressed. The authors suggested that extra-cellular catalase acted to protect against ethanol-induced cellular damage by oxidation outside the cell. Some studies also report the accumulation of cytochrome P_{450} enzyme activity in yeast exposed to ethanol (Mishra, 1993; Piper, 1995). Cytochrome P_{450}, encoded by *ERG11,* is involved in the oxidation of endogenous and exogenous substrates. It is involved in ergosterol synthesis in the endoplasmic reticulum and is able to oxidatively detoxify chemicals such as ethanol.

1.3.4 The molecular response of *S. cerevisiae* to ethanol

From a molecular point of view, information concerning the genes involved in ethanol tolerance in yeast is rather patchy. While high gravity fermentations to generate high alcohol products are commonplace in the beer and wine industries (Casey & Ingledew, 1986), surprisingly few studies have focused on the under-lying genetic response to ethanol stress. The exposure of yeast cells to ethanol results in the synthesis of a set of HSPs including Hsp104 (Piper *et al.*, 1994; Sanchez *et al.*, 1992), Hsp26, Hsp30, Hsp70, Hsp82 (Piper *et al.*, 1994) and Hsp12 (Prakelt and Meacock, 1990; Varela *et al.*, 1995). However, of these HSPs, only Hsp104 (Glover and Lindquist, 1998) and Hsp12 (Sales *et al.*, 2000) appear to influence yeast tolerance to ethanol. An increase in ethanol tolerance has been correlated with an increased expression of Hsp104 (Glover and Lindquist, 1998; Sanchez *et al.*, 1992). When cells constitutively expressing *HSP104* were exposed to 20% (v/v) ethanol, a higher percentage of the population survived compared with an *HSP104* knockout (Sanchez *et al.*, 1992). Hsp104 acts to remodel proteins directly from insoluble aggregates (Parsell *et al.*, 1994). *HSP12* was shown to confer increased integrity of the liposomal membrane in the presence of ethanol (Sales *et al.*, 2000). Incubation in 12% (v/v) ethanol resulted in the complete inhibition of a *Δhsp12* knockout strain, whereas the

wild-type strain exhibited a growth rate of 75% relative to growth in the absence of ethanol, therefore *HSP12* was suggested to be important in ethanol tolerance (Sales *et al.*, 2000).

In attempts to identify novel genes involved in the genetic response to ethanol stress, several studies have been undertaken using mutant strains. Aguilera and Benitez (1986) suggested a large number of genes were involved in ethanol tolerance/sensitivity as 21 monogenic ethanol-sensitive mutants were selected in the *S. cerevisiae* strain S288C. These mutants were found not to be altered in the glycolytic pathway since when maintained on glucose they could produce as much ethanol as the wild type. They were also not mutated in the lipid biosynthesis pathway, as when grown in the absence or presence of ethanol, their concentrations of fatty acids and ergosterol were similar to those of the wild type under the same conditions. Growth sensitivity to ethanol was therefore considered not to be related to carbohydrate or lipid metabolism.

Inoue *et al.* (2000) isolated 10 ethanol-sensitive mutants from the sake yeast, *S. cerevisiae* SY-32. Unlike the parent strain, all 10 mutants were unable to grow in the presence of 7% (v/v) ethanol. One of these ethanol-sensitive strains was found to contain a point mutation in the gene *ERG6*, encoding the S-adenosylmethionine: delta 24-sterol-C-methyltransferase. This mutant had a reduced ability to synthesize ergosterol suggesting that *S. cerevisiae* requires *ERG6* to grow in the presence of ethanol. This is consistent with observations on ergosterol synthesis following ethanol stress as discussed in Section 1.3.1.1.

Takahashi *et al.* (2001) identified five ethanol-sensitive mutants in *S. cerevisiae* YPH499 using transposon mutagenesis. These mutants were able to grow normally in rich medium, however unlike the parent strain, they were unable to grow in the same medium containing 6% (v/v) ethanol. Sequence analysis revealed that the transposon had inserted in the coding regions of the genes *BEM2, PAT1, ROM2, VPS34* and *ADA2*. Further analysis using deletion mutants for these genes confirmed the deletants, like the transposon generated mutants, were ethanol sensitive. Four of the deletion mutants (*Δbem2, Δpat1, Δrom2* and *Δvps34*) showed temperature sensitivity

as well as ethanol sensitivity. Overall, these five genes were considered important for growth under ethanol stress in the strain studied.

Ogawa *et al.* (2000a) compared gene expression profiles of an ethanol-tolerant mutant with its parent sake yeast to identify genes involved in ethanol tolerance. Several genes, including *GPD1, CTT1, SPI1, HSP12, CYC7* and *HOR7* were highly expressed in the ethanol-tolerant mutant and not in the parent strain. The authors also suggested that catalase, and enzymes involved in glycerol and trehalose synthesis may have protective roles in yeast cells under ethanol stress, as there was an increased expression of the genes encoding these products in the ethanol-tolerant mutant. However, the up-regulation of these genes in the ethanol-tolerant mutant does not necessarily mean the gene product has a function in cellular adaptation to the ethanol.

Other ethanol stress responsive genes include *YGP1, DIP5* and *YAT2*, which were identified by Emslie (2002) in *S. cerevisiae* PMY1.1 using time-course differential display. These three genes were up-regulated in response to 5% (v/v) ethanol stress during the lag phase adaptation period. The *SOD2* gene, encoding MnSOD, has also been identified as important in ethanol stressed yeast by Costa *et al.* (1997).

Alexandre *et al.* (2001) used microarray analysis to study gene expression in ethanol-shocked *S. cerevisiae.* The authors added ethanol (7% v/v) to a mid-exponential phase culture, then after 30 minutes sampled the yeast for gene expression analysis. This work identified a large number of up-regulated genes during the ethanol shock, with many of these genes associated with energy metabolism, ionic homeostasis, heat protection, antioxidant defence and trehalose synthesis. It is important to note however that the data presented by Alexandre *et al.* (2001) is not consistent with their raw data presented on the Yeast Microarray Global Viewer website (http://www.transcriptome.ens.fr/ymgv/). It has since been acknowledged to have some errors (B. Blondin, Personal Communication).

Although many genes such as the ones mentioned above, have been shown to respond to ethanol stress, the pleiotropic effects of ethanol suggest that a large number of genes involved in this stress response are yet to be discovered. While Alexandre *et*

al.'s (2001) analysis of global changes in gene expression following exposure to ethanol stress attempted to identify all genes with significantly changed expression in response to ethanol stress, the data from this work is questionable. This is further followed up in Chapter 5 of this thesis. Additionally, the long-term adaptation response to this stress and the stress signalling pathways are yet to be identified.

1.4 THE TOLERANCE OF *S. cerevisiae* TO ETHANOL

While in the previous section many of the effects of ethanol on *S. cerevisiae* have been described, the precise roles of the factors involved in the tolerance of *S. cerevisiae* to ethanol remain to be defined. The following sections will focus on this with reference to earlier sections as appropriate.

There are a number of issues associated with studying ethanol tolerance of yeast. Firstly, as discussed above, ethanol has a number of complex inhibitory effects on a yeast cell. Secondly, ethanol added exogenously to *S. cerevisiae* is less toxic than endogenous ethanol produced by the yeast (Thomas and Rose, 1979; Beaven *et al.*, 1982; D'Amore *et al.*, 1990). Thus, the determination of ethanol tolerance by exogenous ethanol addition does not truly reflect the tolerance of the yeast under fermentation conditions. In addition, a number of factors such as plasma membrane composition and the influence of trehalose and other metabolites can affect or influence the ethanol tolerance of yeasts.

1.4.1 Plasma membrane composition and ethanol tolerance

Changes to the plasma membrane in response to ethanol stress have been outlined earlier, in Section 1.3.1.1 and some of these changes have been shown to promote ethanol tolerance. According to Beaven *et al.* (1982), high ethanol tolerance is correlated with high levels of mono-unsaturated fatty acyl residues in the plasma membrane. With the addition of 3% and 6% (v/v) ethanol to *S. cerevisiae* cultures, cell viability was unaffected but the fatty acid composition of the membrane was

adjusted towards longer chain mono-unsaturated fatty acids. These were suggested to be adaptive changes by the cells to increase their ethanol tolerance.

Thomas *et al.* (1978) studied the lipid and plasma membrane composition in anaerobically grown yeast supplemented with a range of sterols and fatty acids to determine the effects of membrane lipid composition on ethanol tolerance. Longer and more unsaturated fatty acids and sterols (ergosterol and stigmasterol rather than cholesterol and campesterol) were found to be present in ethanol tolerant yeast cultures. Alexandre *et al.* (1994) used gas chromatography to measure the sterol composition of *S. cerevisiae* grown in the presence of 10% (v/v) ethanol. An increase in ergosterol, accompanied by a decrease in zymosterol, suggests ethanol tolerance in yeast is correlated with increasing ergosterol levels. Furthermore, Swan and Watson (1998) demonstrated that an ergosterol supplemented, 17% (v/v) ethanol-stressed *S. cerevisiae* sterol auxotroph had improved ethanol tolerance, as measured by cell viabilities. When the same strain was grown in 17% (v/v) ethanol supplemented with oleic acid (C18:1), linoleic acid (C18:2) or linolenic acid (C18:3), ethanol tolerance was greater for the former (Swan and Watson, 1999).

Mishra and Prasad (1989) found that an unsaturated fatty acid auxotroph of *S. cerevisiae* had increased ethanol tolerance when supplemented with certain unsaturated fatty acids. Using L-alanine uptake, proton efflux and fermentation rates as a measure of ethanol tolerance, the authors observed that the cells acquired a greater ethanol tolerance when supplementation was with polyunsaturated, rather than saturated fatty acids. Further, it was demonstrated that the cells became more resistant to ethanol as the degree of unsaturation and the supplementation of fatty acids increased.

While it has been demonstrated that changes in membrane composition influence ethanol tolerance, these changes may actually be adaptive responses to ethanol induced membrane damage. Some of the findings also appear to be contradictory. It is clear from the work of Curtain *et al.* (1985), and others (see Section 1.3.1.2), that ethanol increases the fluidity of cellular membranes, and this is thought to be a major factor in disrupting membrane structure leading to many of the deleterious effects of

ethanol. However, the cellular response to this appears to be, in part, to make phospholipids with unsaturated fatty acids that would contribute to increased fluidity. Why does the yeast cell do this? Presumably, part of the change in membrane structure is also associated with increased levels of unsaturated sterols, which are thought to trap ethanol in the membrane preventing its entry into the cell (Thomas *et al.*, 1978). The nett effect of change in lipid composition ultimately reduces membrane fluidity, however, there is insufficient information in the literature on this issue therefore it is difficult to provide rational explanations on why the changes take place.

1.4.2 Trehalose and ethanol tolerance

While the precise role of trehalose in ethanol stressed yeast cells is unclear, its intracellular accumulation is thought to be correlated with increased ethanol tolerance. Mansure *et al.* (1994) used several yeast strains with differences in their trehalose metabolism to test their ability to survive in the presence of 10% (v/v) ethanol. A positive correlation was observed between increased cell viability and increased trehalose concentration. Further, the authors found ethanol induced the leakage of electrolytes from cells, but the presence of trehalose reversed the effect (Mansure *et al.*, 1994). Similarly, the work of Kim *et al.* (1996) suggests increased ethanol tolerance is correlated with elevated cellular trehalose content. A mutant strain defective in the vacuolar acid trehalase (*ATH1*) had an increased survival rate relative to the wild-type strain when exposed to 18% (v/v) ethanol (Kim *et al.*, 1996).

D'Amore *et al.* (1991) demonstrated that trehalose accumulation enhanced cell survival in 5% (v/v) ethanol beer and under high substrate conditions. Mutant *S. cerevisiae* strains defective in trehalose synthesis or transport were used to determine the importance of trehalose in cell survival. Trehalose levels increased following exposure to 10% (v/v) ethanol in the trehalose transport mutants, though not for the trehalose synthesis mutants or the wild type strain. Trehalose content correlated to cell survival, the level of trehalose increasing after 24 hours exposure to 10% (v/v) ethanol

for trehalose transport mutants but not for trehalose synthesis mutants or the wild type strain.

In contrast to the above, however, Alexandre *et al.* (1998) suggested trehalose is not associated with cell survival under conditions of 10% (v/v) ethanol stress. Although trehalose did accumulate in *S. cerevisiae* on exposure to ethanol, it was not correlated to cell survival, thus from the data presented, the protective role of trehalose in ethanol stressed cells was suggested to be of minor importance.

1.4.3 Magnesium and ethanol tolerance

Magnesium ions have been implicated in playing an ameliorating role against the detrimental effects of ethanol toxicity (Birch and Walker, 2000). Although this is not an adaptive response to ethanol stress, magnesium ions interact with membrane phospholipids (Petrov and Okorokov, 1990) resulting in stabilization of the membrane bilayer. The associated decrease in proton and anion permeability of the plasma membrane is thought to increase ethanol tolerance (Walker, 1998).

Birch and Walker (2000) compared cultures of a wine strain of *S. cerevisiae* grown in 20 mM and 2 mM magnesium for ethanol tolerance. They found that long term exposure to 10% ethanol resulted in absolute cell death for the culture in 2 mM magnesium, however viability of cells in 20 mM magnesium remained at >40% viability. The viability of the same wine strain when incubated in the presence of increasing ethanol concentrations up to 20% (v/v) for one hour showed a significant improvement in viability when 50 mM magnesium was added. This effect was also observed when cells were preconditioned with elevated levels of magnesium (Birch and Walker, 2000). Pronounced topological damage was also observed in scanning election micrographs, upon exposure of yeast cells to 10% (v/v) ethanol (Birch and Walker, 2000). Elevated levels of magnesium however reduced the extent of cell wall damage following the ethanol stress.

Magnesium was found to have a protective effect against the action of ethanol in the work of Ciesarova *et al.* (1996). Yeast growth with or without 10% (v/v) ethanol addition was stimulated by magnesium more than calcium during fermentation. The supplementation of fermentations with 0.5 mM magnesium was shown to reduce the required time for conversion of 20% glucose into ethanol by one third and result in no loss of ethanol yield (Dombek and Ingram, 1986). The authors also showed that magnesium reduces the inhibitory effect of ethanol accumulation on yeast growth and fermentation.

1.4.4 Acetaldehyde and ethanol tolerance

Acetaldehyde at low concentrations has been shown to ameliorate the effects of ethanol stress. Acetaldehyde is a known inhibitor of a wide range of metabolic activities and is thought to be more toxic than ethanol (Jones, 1988, 1990), however, a growth stimulatory effect has been observed following the addition of small amounts of acetaldehyde to ethanol-stressed cultures (Stanley *et al.*, 1997; Walker-Caprioglio and Parks, 1987). When *S. cerevisiae* was grown in complex media containing ethanol under aerobic conditions, the resulting lag phase was reduced by the addition of acetaldehyde (Walker-Caprioglio and Parks, 1987). A similar effect was observed by Stanley *et al.* (1997) where the lag time was reduced by acetaldehyde addition to ethanol-shocked (4% v/v) *S. cerevisiae* cultures grown in complex medium. An initial acetaldehyde concentration of 0.046 g L^{-1} increased the cellular adaptation rate by 61% and also increased the exponential growth rate by 58% relative to the control. Acetaldehyde concentrations of between 0.046 g L^{-1} and 0.5 g L^{-1} were demonstrated to be stimulatory to ethanol stressed yeast (Stanley *et al.*, 1997).

Stanely *et al.* (1997) proposed a model to explain the stimulatory effects of acetaldehyde. In the presence of an ethanol stress, the cell membrane becomes more leaky to acetaldehyde therefore less endogenously produced acetaldehyde is available intracellularly for reduction to ethanol resulting in a lower rate of NAD^+ regeneration and an NADH/NAD^+ ratio imbalance. This redox imbalance could potentially limit glycolytic flux and the overall energetics of the cell. Acetaldehyde added exogenously

to the medium would diffuse into the cells and restore the redox balance consequently stimulating the rate of energy production.

1.5 REGULATION OF THE YEAST STRESS RESPONSE

Yeast cells sense and respond to stress. The cellular machinery to respond to stress conditions involves the rapid synthesis of protective molecules and the activation of signal transduction pathways which induce secondary events. These secondary events include the activation of enzymes and the transcription of genes encoding factors with protective functions (Hohmann and Mager, 1997). The regulation of the yeast stress response has been well reviewed of late (see Estruch, 2000; Mager and De Kruijff, 1995; Ruis and Schuller, 1995; Hohmann and Mager, 1997; Dawes, 1999; see table 1.3).

The regulatory factors involved in controlling yeast stress responses are complex and regulatory pathways for many genes involved in specific stress situations are now defined; it appears that most stresses induce a specific response with some overlap. There are presumably still regulatory pathways that remain to be elucidated. A simplified summary of the current understanding of the regulation of the yeast stress response is given in Figure 1.2. At least three positive transcriptional elements are activated by stress: the heat shock response element (HSE), the stress response element (STRE) and the AP-1 responsive element (ARE). These elements appear to have overlapping but separable functions (Ruis and Schuller, 1995).

When subjected to stress conditions, which cause the accumulation of abnormal or denatured proteins, yeast cells respond by synthesizing HSPs. Two distinct regulatory elements are involved in the induction of Hsp genes, an HSE and a STRE. HSEs are promoter-binding sites for heat shock factor (HSF; encoded by a single-copy essential gene, *HSF1*) and are composed of at least three copies of the repeating element nGAAn, arranged in alternating orientations (Sorger, 1991). The number of 5 bp units within a functional HSE can vary though usually it ranges from three to six (Mager and De Kruijff, 1995). Deviations in the consensus (both in the sequence and/or in the distance between modules) may be tolerated, but they can influence the affinity of the

Hsf1p and, thereby the level of transcriptional activation (Santoro *et al.*, 1998; Bonner *et al.*, 1994). HSF, the positive regulator of HSE-containing genes, is thought to be negatively regulated by Hsp70 (Craig and Gross, 1991). Hsp70 is suggested to serve as a cellular thermometer, in heat-shocked cells, regulating the modulation of HSF activity and the expression of HSP genes. In this model Hsp70 interacts directly with HSF under non-stress conditions maintaining the transcription factor in low-active form. Upon heat shock, there would be an increase in the concentration of misfolded proteins, the Hsp70 substrates, leading to a depletion of free Hsp70. As the Hsp70 pool is reduced, HSF would be released in an active form. The response would be self-limiting, as the overproduction of Hsps would restore the free pool of Hsp70 (Craig and Gross, 1991; Estruch, 2000).

The STRE was originally identified as a HSF-independent heat stress control element but it is now recognized that STREs can mediate transcription induced by other forms of stress, such as nutrient starvation, osmotic stress, oxidative stress, weak acids, heat shock and ethanol (Marchler *et al.*, 1993; Schuller *et al.*, 1994). STREs are present in the promoter regions of various stress-induced genes including *CTT1, DDR2, UBI4, HSP104* and *HSP12* (Moskvina *et al.*, 1998). The core consensus sequence AGGGG (or CCCCT) is functional in both orientations. To date, STRE sequences have been identified in many genes and computer searches of the entire yeast genome predict 186 potential STRE regulated genes (Moskvina *et al.*, 1998; Treger *et al.*, 1998). A single copy of a STRE is sufficient to activate a gene in a stress situation, however two or more copies provide a greater effect on stress-induced gene expression (Kobayashi and McEntee, 1993). The presence of a STRE-like sequence in a gene promoter does not confirm the functionality of this element (Estruch, 2000).

The transcription factors Msn2p and Msn4p are zinc finger proteins that recognize and bind to STREs both *in vitro* and *in vivo* (Martinez-Pastor *et al.*, 1996). These

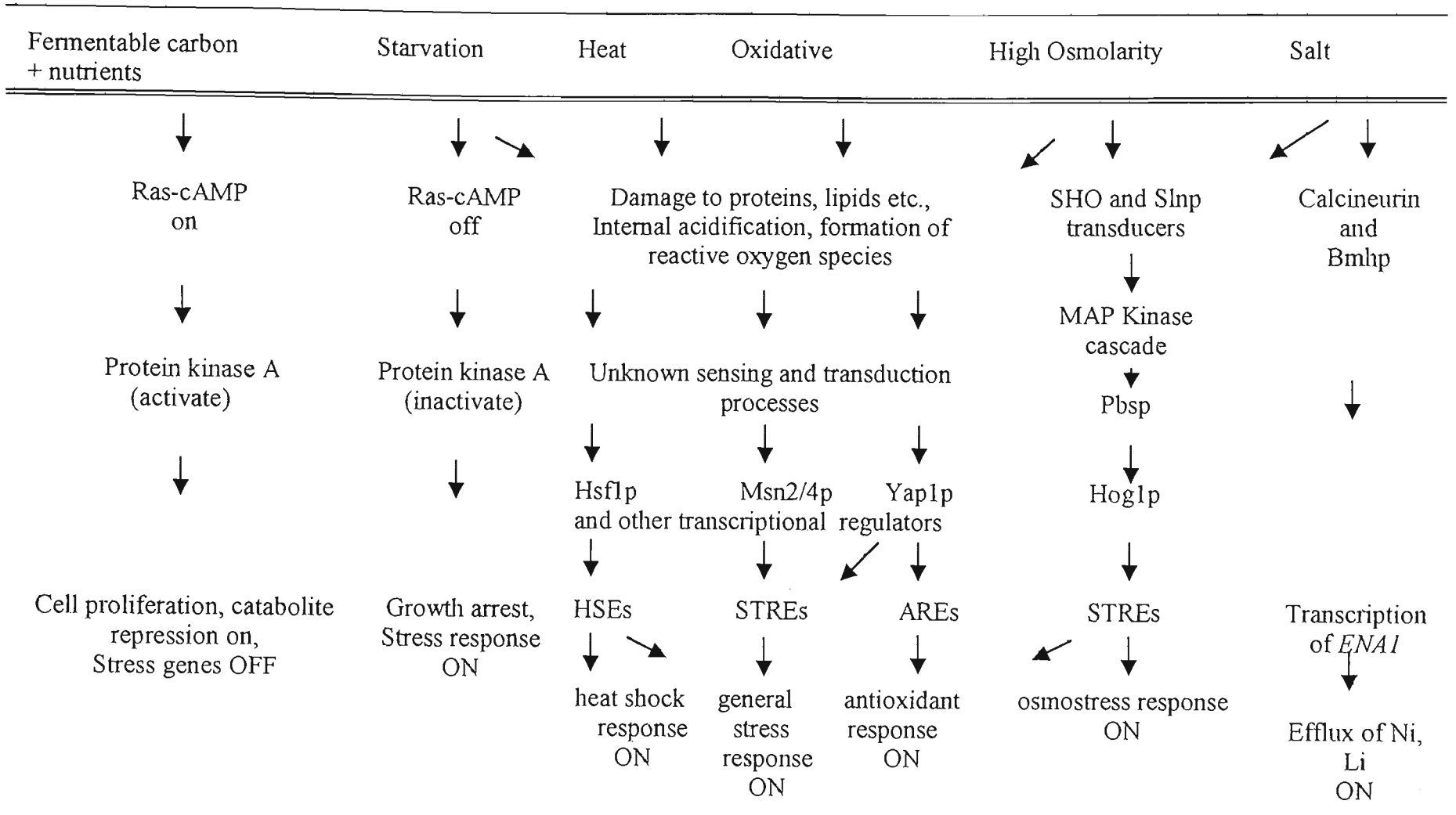


Figure 1.2: A simplified summary of the regulation of the yeast stress response (adapted from Attfield *et al.*, 1997)

authors found that the induction of a STRE-controlled reporter gene was reduced in a *msn2 msn4* mutant exposed to 7% (v/v) ethanol, heat shock, low pH and sorbic acid. Further, the expression of the STRE regulated genes, *CTT1* and *HSP12*, was also lower in the *msn2 msn4* mutant exposed to 7% ethanol, suggesting that at least some genes induced in response to ethanol stress are STRE regulated (Martinez-Pastor *et al.*, 1996).

Two signalling pathways acting through STREs in the yeast stress response have been identified. The first is the HOG (high osmolarity glycerol) pathway, a mitogen-activated protein (MAP) kinase pathway involved in the protection of cells against increases in external osmolarity (Toone and Jones, 1998). The first components of this pathway are the two membrane osmosensors, Sho1p and Sln1p. Under osmotic stress, phosphorylation of Sln1p is inhibited and this kinase activates the MAP kinase pathway where activated Pbs2p phosphorylates Hog1p and increases its nuclear accumulation. The activation of Pbs2p by hyperosmolarity can also be produced by the interaction with Sho1p (Maeda *et al.*, 1995). This accumulation of Hog1p is transient and coincides with the osmotic induction of a variety of genes, suggesting a role for Hog1p in gene expression (Estrush, 2000). Several transcription factors, including Msn2/4p, Hot1p and Msn1p, are suggested to be regulated by Hog1p. Both Hot1p and Msn2p are responsible for the bulk of the Msn2/4p-independent osmotic stress activation of several genes (Rep *et al.*, 1999).

The other mechanism of signalling acting through a STRE is the cAMP-protein kinase A pathway, which negatively regulates STREs (Marchler *et al.*, 1993) and the nuclear location of Msn2/4p in response to various stresses. The first indication of the involvement of the cAMP-protein kinase A pathway in the yeast stress response came from *S. cerevisiae* mutants displaying high protein kinase A activity and sensitivity to heat shock and starvation. Conversely mutants with low protein kinase A activity showed opposite characteristics (Piper, 1993). The activity of the cAMP-protein kinase A is essential for growth and has been implicated in many cellular processes (Thevelein, 1994). Therefore, in stress situations protein kinase A acts to repress STRE-mediated transcription, providing a link between positive control of cell growth and negative control of stress responses (Ruis, 1997).

The overlap between the STRE and HSE regulons has to date proven to be small. However, several STRE-containing genes also contain HSE regulatory sequences in their promoters. Using strains carrying mutations in both regulatory systems, Treger *et al.* (1998) demonstrated that several stress-induced genes are controlled redundantly by both Msn2/4p and Hsf1p after heat shock. Amoros and Estruch (2001) studied the contribution of Msn2/4p and Hsf1p in the transcriptional activation of two yeast HSP genes when induced under different stress conditions; the two genes *HSP26* and *HSP104*, have both STREs and HSEs in their promoters. The authors demonstrated that the relative contributions of Msn2/4p and Hsf1p are different depending on the gene and the stress condition. It is clear from this and numerous other studies that the control of yeast gene expression is more complex than simply being regulated by one type of promoter element.

ARE's of yeast cells activate the expression of a number of genes under oxidative stress conditions by binding transcription factors homologous to mammalian AP-1 (Yap1p, Yap2p). Yap1p is the best-characterised transcription factor, binding to the APE consensus sequence TGACTCA (Estruch, 2000). Regulation of Yap1p takes place at the level of sub cellular localisation; under normal conditions Yap1p is restricted to the cytoplasm, but it becomes nuclear in response to oxidative stress (reviewed by Toone and Jones, 1999). Transcriptional activation requires two different segments of Yap1p that function differently depending on the agent used to generate the oxidative stress. This suggests that the mechanism underlying the response of Yap1p to different oxidants may not be the same (Wemmie *et al.*, 1997). The Yap1p protein appears to have some interaction with the regulation of STRE activity. Varela *et al.* (1995) demonstrated expression of a STRE-controlled reporter gene was diminished in a *yap1* mutant, however, Yap1p does not bind to STREs therefore this effect was probably indirect.

In spite of the number and variety of proteins induced in response to stress, the numbers of transcriptional regulatory systems are small. Of the three major transcriptional activation systems, the transcription factor Yap1p is specific to oxidative stress. Hsf1p is more specific to stress situations which cause an accumulation of abnormal proteins, thereby being important in the heat shock response, though it also activates the metallothionein gene, *CUP1*, in response to

oxidative stress and glucose starvation (Tamai *et al.*, 1994; Liu and Thiele, 1996). The Msn2/4p transcriptional elements, controlling STRE induction, are activated by different stress conditions (as indicated in Figure 1.3). A major question is how these pathways sense environmental stress and how information is transmitted to activate transcription factors. In relation to STRE, further work is required to determine whether a variety of stress conditions that induce transcription via this element converge into a unique stress signal or if different signals are able to activate Msn2p and Msn4p. While at least two signal transduction pathways converge on the STRE, the HOG cascade and the cAMP- protein kinase A pathway, more complex regulatory mechanisms may yet be identified.

1.6 MOLECULAR ANALYSIS OF THE ETHANOL STRESS RESPONSE

While there have been several studies to characterise the genomic responses of yeast to stress (see Table 1.3), this review of the literature highlights the research effort devoted to the ethanol stress response of *S. cerevisiae*. Nevertheless it highlights the lack of information on the ethanol-stress response at the molecular level. Whilst the physiological effect of ethanol on the yeast cells appears to be primarily focussed at the cell membrane and cell wall, ethanol also inhibits metabolic processes. Considering the pleiotropic effects of ethanol can be critical to cell survival, the ethanol-induction and regulation of only a minority of genes is known. Only Hsp12 and Hsp104 have been shown to influence ethanol tolerance positively. Moskvina *et al.* (1998) demonstrated that STRE-containing ethanol-stress genes *HSP104, HSP78, HSP42, SSA4* and *YNL077w*, to be regulated independently of Msn2p and Msn4p, probably by Hsf1p, as all these genes possesses HSEs and their induction was observed in *msn2 msn4* mutants. The induction of *HSP12* in cells exposed to 7% ethanol-stress has been reported to be under the control of Msn2/4p (Martinez-Pastor *et al.*, 1996). Additionally, Alexandre *et al.* (2001) reported the induction of 194 genes following a 30 minute ethanol shock, 20 of which contained putative STRE elements.

Apart from this information, the regulatory elements of induced ethanol-stress genes and putative signal transduction pathways involved in ethanol-stress signalling remain

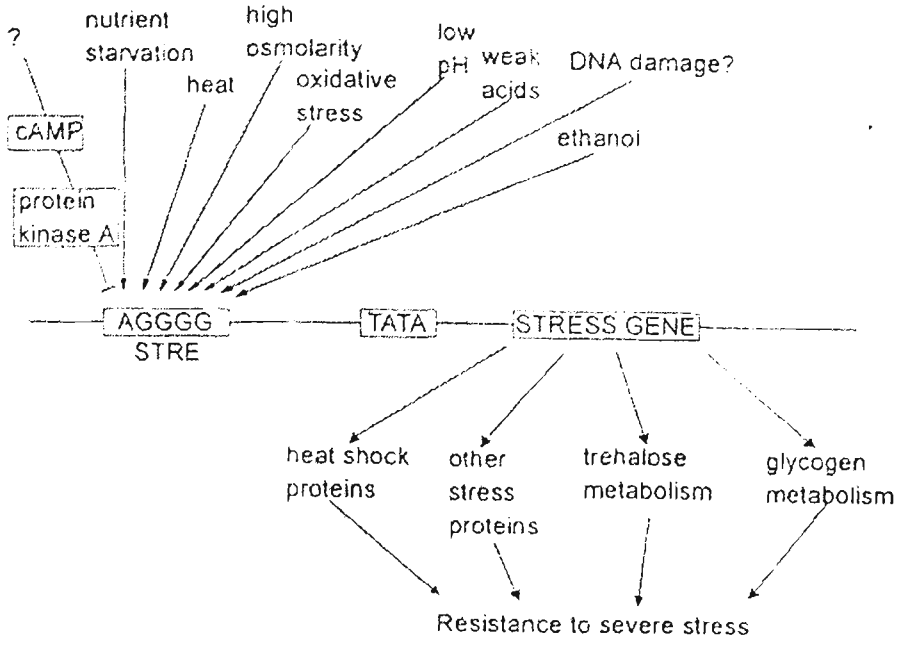


Figure 1.3: Factors controlling STREs and effects triggered by STRE activation (from Ruis and Schuller (1995)).

to be elucidated. An investigation into the gene expression of yeast cells exposed to an ethanol stress was therefore warranted and the identification and characterisation of genes involved in yeast adaptation to ethanol stress was the focus of the research project on which this thesis was based.

1.7 AIMS

The overall aim of this project was to define the molecular response of *S. cerevisiae* to ethanol stress.

The specific aims were to:

1. Determine ethanol-stressing conditions that are inhibitory but non-lethal to the growth of a lab strain (PMY1.1) of *S. cerevisiae*. This stress should be sufficient to induce a period of adaptation for a defined time period in the stressed cells followed by exponential growth.

2. Use differential display procedures to identify novel ethanol-stress response genes from ethanol-stressed cultures.

3. Develop and use gene array procedures to characterise the adaptation of *S. cerevisiae* to ethanol-stressed cultures.

4. Confirm and validate the identified ethanol stress response genes and determine the influence of selected genes on cell physiology during ethanol stress.

Table 1.3: Genomic expression studies characterizing wild-type yeast responses to stress (adapted from Gasch and Warner-Washburne, 2002).

Environment	References
Heat shock	Gasch *et al.* (2000); Causton *et al.* (2001)
Ethanol shock	Alexandre *et al.* (2001)
pH extremes	
Acid	Causton *et al.* (2001); Kapteyn *et al.* (2001); de Nobel *et al.* (2001)
Alkali	Causton *et al.* (2001); Lamb *et al.* (2001)
Oxidative and reductive stress	
Hydrogen peroxide	Gasch *et al.* (2000); Causton *et al.* (2001)
Menadione	Gasch *et al.* (2000)
Diamide	Gasch *et al.* (2000)
Cadmium	Momose and Iwahashi (2001)
DTT	Gasch *et al.* (2000); Travers *et al.* (2000)
Hyper-osmotic shock	
Sorbitol	Gasch *et al.* (2000); Causton *et al.* (2001); Rep *et al.* (2000)
Potassium/sodium chloride	Posas *et al.* (2000); Rep *et al.* (2000); Causton *et al.* (2001); Yale and Bohnert (2001)
Starvation	
Entry into stationary phase	DeRisi *et al.* (1997); Gasch *et al.* (2000)
Amino acid starvation	Gasch *et al.* (2000); Jia *et al.* (2000); Natarajan *et al.* (2001)
Nitrogen starvation	Gasch *et al.* (2000)
Phosphate starvation	Ogawa *et al.* (2000b)
Zinc starvation	Lyons *et al.* (2000)
Copper starvation	Gross *et al.* (2000)
Respiration	
Petite mutants	Travern *et al.* (2001)
Non-fermentable carbon sources	Kuhn *et al.* (2001)
Anaerobic growth	Kwast *et al.* (2002)
Sporulation	Chu *et al.* (1998); Primig *et al.* (2000)
Drug treatments	Bammert and Fostel (2000); Hughes *et al.* (2000)
DNA damaging agents	

	Alkylating agents	Jelinsky *et al.* (2000); Gasch *et al.* (2001); Natarajan *et al.* (2001)
	Ionizing radiation	Gasch *et al.* (2001); De Sanctis *et al.* (2001)
Others		
	Nutritional up-shift	Brejning *et al.* (2003)
	Cryoresistance	Tanghe *et al.* (2000)
	Brewing	James *et al.* (2003)

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Yeast strains

A haploid lab strain, *Saccharomyces cerevisiae* PMY1.1 (*MAT***a** *leu2, ura3, his4*), was used for the work described throughout this thesis. PMY1.1 was originally from the laboratory of Dr Peter Piper, University College, London, UK. Two genes, *HSP26* and *ALD4*, were removed from this strain and replaced by the kanMX4 module to generate two knockout strains, PMY1.1 (*MAT***a** *leu2, ura3, his4*) *Δhsp26*::kanMX4 and PMY1.1 (*MAT*a *leu2, ura3, his4*) *Δald4*::kanMX4 (see Section 2.3.8 and Table 2.1).

2.1.2 General buffers and solutions

Analytical grade chemicals were used to prepare all buffers and solutions unless otherwise stated. Chemicals used were supplied by BDH (UK) or Sigma (USA) unless otherwise stated. Distilled and de-ionised Milli-Q water (Milli-Q Plus Ultra Pure Water System, Millipore, Billerica, MA, USA) or where indicated distilled water (Milli RO Ultra Pure Water System, Millipore, Billerica, MA, USA) was used.

Recipes for all buffers and solutions are provided in Appendix 1. Buffers and solutions were sterilized by autoclaving at 121°C for 20 minutes or, where indicated, filter sterilized using a 0.22 μm or 0.45 μm Millipore membrane filter. All glassware and stainless steel components for preparing RNA solutions were baked at 160°C for at least 12 hours. Buffers for RNA work were prepared in RNase-free glassware using diethyl pyrocarbonate (DEPC) treated water. DEPC-treated water (0.1%) was

S. cerevisiae STRAIN	DESCRIPTION	GENOTYPE	SOURCE
PMY1.1	Wild type	PMY1.1 (*MATa leu2, ura3, his4*)	P.Piper*
PMY1.1*Δhsp26*::kanMX4	Knockout	PMY1.1 (*MATa leu2, ura3, his4*) *ΔHSP26*::KanMX4	constructed in this work
PMY1.1*Δald4*::kanMX4	Knockout	PMY1.1 (*MATa leu2, ura3, his4*) Δ*ALD4*::KanMX4	constructed in this work

* Dr. Peter Piper, University College, London, UK.

Table 2.1: *S. cerevisiae* strains used in the work described in this thesis.

prepared with distilled and de-ionized Milli-Q water. A list of all enzymes, molecular weight markers, molecular biology kits and a list of suppliers are also provided in Appendix 1.

2.1.3 General equipment used for experimental procedures

Yeast cultures of volumes less then one litre were incubated in Erlenmeyer flasks in an orbital-shaker incubator (Innova 4231 refrigerated incubator, New Brunswick Scientific, Edison, New Jersey). Incubations requiring one or more litres of culture were undertaken in 2 litre Braun Biostat® fermenters (B. Braun, Melsungen, Germany). The spectrophotometer used for all experimental analysis was the LKB Ultraspec Plus, 4054 UV/Visible spectrophotometer (Pharmacia). Centrifugation was carried out either in a Beckman CS-15R swinging rotor centrifuge or an Eppendorf 5415C bench top microfuge (Eppendorf, GmbH, Englesdorf, Germany). The scintillation counter used was the Wallac 1410 Liquid Scintillation Counter, Pharmacia. PCRs were performed in a PTC-100 programmable thermocycler with a heated lid (MJ Research Inc., Waltham, MA, USA). Gels were photographed with a UVP Laboratory Products gel documentation system (Upland, CA, USA). Gene arrays and Southern blots were scanned using the Fujifilm FLA 3000 phosphor image analyser (Fuji Photo Film Co. LTD, Kangawa, Japan).

2.2 YEAST GROWTH METHODOLOGY

2.2.1 Growth media

Yeast cultures were grown in a defined medium or nutrient rich YEPD medium. Most media and culture vessels were autoclaved at 121°C for 20 minutes. The glucose component of the medium was always autoclaved separately. Where indicated other components were filter sterilized using a 0.22 μm Millipore membrane filter. All water used in growth media was distilled and de-ionised Milli-Q water.

Defined medium contained per litre: 20 g D-glucose, 5 g ammonium sulphate and 1.7 g yeast nitrogen base, without amino acids and ammonium sulphate (Difco). The yeast nitrogen base was prepared according to the manufacturer's instructions as a 10 x solution (1.7 g nitrogen base in 100 ml sterile water). This solution was filter sterilized using a 0.22 μm filter prior to adding to 900 ml of autoclaved glucose and ammonium sulphate. Leucine was added to a final concentration of 100 μg ml^{-1} from a filter sterilized 20 mg ml^{-1} stock solution. Histidine and uracil were added to a final concentration of 20 μg ml^{-1} from filter sterilized 20 mg ml^{-1} stock solutions (Kaiser *et al.*, 1994).

YEPD medium comprised of per litre: 10 g yeast extract, 20 g bacto-peptone, and 20 g D-glucose. Components were dissolved in distilled de-ionised water and autoclaved at 121°C for 20 minutes. For solid YEPD media, bacto-agar was added to a final concentration of 15 g l^{-1} prior to autoclaving.

YEPD Geneticin plates for the selection of knockout strains with an integrated KanMX4 cassette were composed of YEPD medium with the addition of per litre: 15 g bacto-agar, and 200 mg G418 Geneticin (Sigma). G418 was added when the media had cooled to approximately 55°C.

Glycerol storage medium was composed of 2 x YEPD (40 g) glucose, 20 g yeast extract and 40 g bacto-peptone, with the addition of 15% (v/v) glycerol. The dry components were dissolved in distilled and de-ionised water and autoclaved at 121°C for 20 minutes. This medium was used for the storage of all yeast strains at –80°C.

2.2.2 Yeast cultivation

2.2.2.1 Standard culture conditions

Yeast cultures were grown under aerobic conditions in defined medium at 30°C and shaken at 160 rpm in an orbital-shaker incubator, unless otherwise stated. The culture vessels were Erlenmeyer flasks with wool plug stoppers. The culture working volume

was always 1/5 of the flask capacity. To grow cultures of one litre volume for the harvesting of cells across a time course, two Braun Biostat® fermenters were used. The fermenters had working volumes of 2 litres. Temperature was measured using a thermocoupler inserted in a stainless steel well in the lid of the vessel. The temperature was controlled automatically at 30°C by the circulation of heated water through the vessels cooling jacket. The culture was agitated at 200 rpm. No supplementary aeration of the cultures was required and pH was not controlled in these experiments since the buffering capacity of the media maintained a constant pH.

2.2.2.2 Growth of yeast on plates

Yeast was streaked on YEPD agar plates for short-term storage. Cultures were also spread on YEPD agar plates for the determination of viable cell populations using colony counts. YEPD Geneticin plates were used for the selection and short-term storage of gene knockout strains.

2.2.2.3 Yeast storage

For long-term storage, yeast cells were kept in glycerol storage medium at –80°C. Transfers from glycerol stocks were undertaken aseptically using barrier pipette tips.

2.2.2.4 Inoculum preparation and experimental cultures

Prior to growth experiments, yeast cells were streaked on YEPD plates and incubated at 30°C for two days. A loopful of cells was aseptically transferred into 20 ml of defined medium in a 100 ml Erlenmeyer flask plugged with a cotton wool stopper. Flasks were incubated overnight at 30°C at 160 rpm. Two serial subcultures, in 250 ml conical flasks containing 50 ml of medium, were made prior to each experiment. The OD_{620} reading of these cultures was used to determine the inoculum size required for an initial OD_{620} reading of 0.1 when transferred to the fresh medium. Transfers between subcultures were performed in a sterile laminar flow cabinet. Parent cultures,

containing 100 ml (or 200 ml depending on required inoculum size), of defined media were inoculated to an OD_{620} reading of 0.1 and grown for approximately 8 hours under standard conditions to late exponential phase (OD_{620} of 1.0).

Growth experiments were conducted in 500 ml conical flasks containing 100 ml of medium and plugged with wool stoppers, unless otherwise stated. Late-exponential phase parent culture cells (OD_{620} of 1.0), grown in defined medium, were collected by centrifugation at 2000 g for 3 minutes. The supernatant was discarded and the cells washed in pre-warmed fresh defined medium. The temperature was maintained at 30°C during the wash procedure and transfers were performed aseptically in a laminar flow cabinet.

Aliquots of the cell suspension were then inoculated, to an OD_{620} of 0.1 (approximately 2 x 10^6 cell ml^{-1}), into pre-warmed defined medium in the absence (unstressed control) and presence (stressed) of ethanol. For ethanol-stressed cultures, a portion of the growth medium was removed prior to the growth experiment and ethanol was added to the medium at the appropriate concentration immediately prior to inoculation. Once inoculated, the cultures were quickly transferred to the shaker incubator and grown under aerobic conditions at 30°C and 160 rpm. Samples were taken at regular time intervals for optical density measurements and viable plate counts.

2.2.2.5 Pre-stress conditions

Ethanol pre-stress experiments were conducted in 500 ml conical flasks containing 100 ml of defined medium and plugged with wool stoppers. Parent cultures were prepared from both PMY1.1*Δhsp26*::kanMX4 and PMY1.1 as described in Section 2.2.2.4. Washed late exponential phase parent cells, from both PMY1.1 *Δhsp26*::kanMX4 and PMY1.1 wild type, were split into two groups, washed and resuspended in pre-warmed fresh defined medium. One portion of cells, from each of PMY1.1 and PMY1.1*Δhsp26*::kanMX4, were inoculated to an OD_{620} of 0.1 into pre-warmed defined medium in the presence and absence of 5% (v/v) ethanol. These cultures were placed at 30°C and 160 rpm in an orbital shaker incubator. The other

portion of cells, from each of PMY1.1 and PMY1.1*Δhsp26*::kanMX4, were incubated in the presence 5% (v/v) ethanol (added to the resuspension medium) at 30°C and 160 rpm in an orbital shaker incubator for one hour to serve as a pre-stress. Following the incubation, these pre-stressed cells were inoculated to an OD_{620} of 0.1 into fresh pre-warmed medium in the presence and absence of 5% (v/v) ethanol. Samples were taken at regular time intervals and cell numbers monitored by duplicate plate counts.

2.2.2.6 Low inoculum size experiments

Low inoculum size experiments were conducted in 500 ml conical flasks, plugged with wool stoppers, containing 100 ml of defined medium. Parent cultures were prepared from PMY1.1 and PMY1.1*Δald4*::kanMX4 strains as essentially as described in Section 2.2.2.4. Three flasks for each strain were inoculated to approximately 10^6 cells ml^{-1}, 10^5 cells ml^{-1} or 10^4 cells ml^{-1} in no ethanol and 5% (v/v) ethanol stress containing medium. To achieve the correct inoculum sizes, late exponential phase parent cells from PMY1.1 and PMY1.1*Δald4*::kanMX4 were prepared for an inoculum OD_{620} of 0.1, then diluted 1 in 10, or 1 in 100 for the 10^5 - and 10^4 cells ml^1 cultures, respectively. All cultures were incubated at 30°C and 160 rpm in an orbital shaker incubator. Cells were harvested at regular time intervals and plate counts were used to measure cell populations.

2.2.2.7 Harvesting cells from ethanol stress experiments

Two Braun Biostat® fermenters were used to grow control and ethanol-stressed yeast cultures from which to harvest cells. The fermenters had total volumes of two litres and each contained one litre of pre-warmed defined medium. Immediately prior to the growth experiment, a portion of medium was removed aseptically from one fermenter and this medium was replaced with an appropriate volume of ethanol immediately prior to inoculation. Inoculum cells at late exponential phase (OD_{620} reading of 1.0) were washed as described above (Section 2.2.2.4) and inoculated into the fermenters to an OD_{620} of 0.1 (approximately 2×10^6 cell ml^{-1}). Sampling of the cultures was achieved by opening a clamped sampling line and removing an adequate volume via a

sterile syringe. An initial 5 ml of culture was discarded from the sampling line before the required volume collected. Cells from each culture were harvested initially at time 0 (inoculation time), and then at hourly intervals during the course of the incubation. Samples were also taken for optical density measurements and viable plate counts. Aliquots of 150 ml were harvested, collected by centrifugation at 4°C and 2000 g for 5 minutes. Using the optical density reading at the time of harvest and an appropriate calibration curve (generated from data obtained in previous experiments, Section 3.2.2), the number of viable cells per ml^{-1} was estimated for the stressed and control (no ethanol) cultures. Cells were resuspended to a concentration of 10^8 cells ml^{-1} in DEPC treated water on ice, pelleted in 1.0 ml aliquots and frozen in liquid nitrogen. Cell pellets were stored at –80°C prior to RNA isolation.

2.2.2.8 Cell viability and population determination

Cell viability was measured by plate counts. Samples of 100 µl aliquots of cell culture, serially diluted in 900 µl of YEPD medium in microfuge tubes, were spread onto duplicate YEPD agar plates. Plates were incubated at 30°C for 2 days and counted immediately. Plates with cell counts in the range of 30-300 cells per plate were counted. The readings for each set of duplicates were averaged and multiplied by the dilution factor to give the viable cell population of the culture.

2.3 MOLECULAR METHODS

2.3.1 RNA extraction

2.3.1.1 RNase-free procedures

For all RNA work, all equipment, including chemicals, water and glassware, were made RNase-free. Glassware and spatulas were covered in foil and baked at 160°C for at least 12 hours prior to use. Electrophoresis tanks and combs were sprayed with RNase away (Molecular Bio-Products) and washed in DEPC-treated water. Distilled and de-ionized Milli-Q water was DEPC-treated overnight and autoclaved. All microfuge tubes were purchased RNase free, autoclaved, and only used for RNA associated procedures. RNase-free barrier tips were also used for all RNA procedures. Glass beads used in RNA extractions were acid washed, rinsed in DEPC-treated water and baked.

2.3.1.2 Total RNA extraction from *S. cerevisiae*

Total RNA was extracted from *S. cerevisiae* using the glass bead extraction method, essentially as described by Ausubel *et al.*, (1997). Pellets of approximately 10^8 cells ml^{-1} were resuspended in 300 µl (1 x) RNA buffer and added to approximately 300 µl of chilled, acid-washed 0.55 mm glass beads (Sigma). Samples were kept on ice throughout the procedure. The mixture was vortexed for a total of 3 minutes (alternating one minute vortexing with one minute on ice). Samples were centrifuged at 10,000 g in a microfuge for one minute to pellet cell debris and the upper phase transferred to a new tube. An equal volume of phenol/choloroform/isoamyl alcohol (25:24:1) was added and the mixture vortexed for 20 seconds then centrifuged for two minutes at 10,000 g to separate the precipitated protein phase. The upper phase was removed to a new microfuge tube and 3 volumes of chilled 100% ethanol added to precipitate the RNA. These solutions were mixed and the RNA allowed to precipitate for 2 hours at -80°C. Following centrifugation at 4°C and 10,000 g for 2 minutes, the

supernatant was removed and the RNA pellet washed with 100 µl of chilled 70% ethanol, re-centrifuged for 2 minutes at 10,000 g and the supernatant removed. The RNA pellets were air-dried and resuspended in 25 µl of RNase-free water. To test purity, RNA gel loading solution (1 µl) was added to 1.0 µl of extracted RNA and 4 µl of RNase free water and the RNA was resolved on a 0.8% agarose gel (Section 2.3.1.4). RNA samples were stored at –80°C.

2.3.1.3 DNase treatment of RNA

Total yeast RNA was DNase treated to remove any contaminating DNA for differential display and gene array analysis. DNase treatment was performed in a 50 µl reaction volume. Total yeast RNA (24 µl) was mixed with 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 200 units of RNase free DNase (Roche), and 20 units of RNasin (Promega) according to the Differential Display Kit Manual (Display Systems). Components were mixed and incubated at 37°C for 25 minutes. Following incubation, 5 µl of 2 M sodium acetate (pH 4) was added followed by 50 µl of water-saturated phenol and 10 µl of chloroform/isoamyl alcohol (49:1). The sample was mixed by gentle inversion, cooled on ice for 10 minutes and centrifuged at 10,000 g for 5 minutes in a microfuge. The upper phase was carefully removed and transferred to a new microfuge tube to which 15 µl of 3 M sodium acetate (pH 5) and 1.0 ml of ice-cold 100% ethanol were added to precipitate the RNA. The contents of the tube were gently mixed and precipitated at –80°C for 10 minutes, followed by a 4°C centrifugation for 10 minutes at 10,000 g. The supernatant was removed and the RNA pellet washed with 100 µl chilled 70% ethanol and centrifuged for 2 minutes at 10,000 g. Again the supernatant was removed and the RNA pellet air-dried for 10-15 minutes. RNase-free water (12 µl) was added to resuspend the pellet and the mixture was left at room temperature for 10 minutes. To test purity, RNA gel loading buffer (1.0 µl) was added to 1.0 µl of DNase treated RNA and 4 µl of RNase free water and the RNA was resolved on a 0.8% agarose gel (Section 2.3.1.4). DNase treated RNA samples were stored at –80°C.

2.3.1.4 Agarose gel electrophoresis of RNA

RNA was visualised to test for purity and consistency of extraction across different time points. Electrophoresis of RNA was performed in RNase free 0.8% non-denaturing agarose gels in 1 x TAE buffer. Ethidium bromide solution was added to a final concentration of 0.5 μg ml^{-1}. RNA samples (1.0 μl) were mixed with 1.0 μl of 6 x RNA loading buffer and 4 μl of RNase free water and loaded onto the gel. Electrophoresis was conducted at 80 V typically for 45-60 minutes. Gels were viewed on a UV transilluminator and photographed using a UVP Laboratory Products gel documentation system.

2.3.2 Differential display

Differential display methodology was essentially as described in the Display Systems Differential Display™ Kit manual. The PCR thermocycler used throughout was an MJ Research Inc. PTC-100 programmable thermocycler with a heated lid. The thermocycler was preheated to the desired temperature before placing tubes in the block.

2.3.2.1 First strand cDNA synthesis

Five downstream $T_{11}VV$ (V= A, C or G) anchored primers (Table 4.1) targeting the 3' Poly A tract of mRNAs were used to prepare cDNAs as described in the Display Systems Differential Display™ Kit manual. cDNAs were generated from RNA derived from equal numbers (10^8) of stressed and control cells harvested over a time course at one, three and five hours. First strand cDNA synthesis was performed in 30 μl reaction volumes. Firstly, 25 μM downstream primer (Operon), approximately 1-3 μl (200-300 ng) RNA and DEPC-treated water were heated to 70°C for 10 minutes. 5 x First Strand Buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$], 10 mM DTT, 20 μM dNTPs and 30 units of Recombinant RNasin® Ribonuclease Inhibitor (Promega) were then added and the samples incubated for 2 minutes at room temperature. Superscript II™ RNase H-Reverse Transcriptase (270 units; GibcoBRL)

was subsequently added and the reaction mixes incubated for 1 hour at 42°C. Reactions were inactivated at 70°C for 15 minutes. Negative controls prepared for random templates were prepared at the same time. These controls contained all reagents except the Superscript II™ RNase H-Reverse Transcriptase (GibcoBRL), which was replaced with water. Negative controls were amplified on separate differential display gels.

2.3.2.2 Differential display procedure

PCR reactions were prepared essentially as described in the Display Systems Differential Display™ Kit Protocol 1. Reaction mixes were prepared for each downstream primer and contained PCR buffer, 3 mM $MgCl_2$, 2 μM dNTPs, 2.5 μM downstream primer, 1 unit Amplitaq® DNA Polymerase (Perkin Elmer) and 1 μCi (10 μCi/μl) α-^{33} P dATP (Perkin Elmer). Reaction mixes were prepared concurrently on ice in PCR tubes to a final volume of 20 μl. The appropriate upstream primers (Table 4.1) (0.5 μM final concentration) and cDNA templates (1.0 μl) were aliquoted prior to the addition of all other reagents. The PCR amplification protocol consisted of 40 cycles of 30 seconds at 94°C, 60 seconds at 40°C, 60 seconds at 72°C, and then a further 5 minutes at 72°C.

Products of differential display reactions were resolved on 6% acrylamide non-denaturing gels in a Poker Face II SE1600 Sequencing Gel System (Hoefer Scientific Instruments). Gels were prepared as follows: glass sequencing plates were soaked in 10% NaOH and then washed and wiped with 70% ethanol. The front plate was pre-treated by wiping with 1.0 ml of Repel Silane™ (Amersham Biosciences) and the back plate was similarly treated with 3 μl of Bind-Silane™ (Amersham Biosciences) mixed with 1.0 ml of 95% ethanol/0.5% acetic acid. The two plates were separated with 0.2 mm spacers, sealed with duct tape and clamped together. Gels were prepared with 7.5 ml of 40% acrylamide (19:1 acrylamide and N-N-methylene bisacrylamide), 5 ml 10 x TBE and 37.5 ml distilled and de-ionized water filtered through a 0.22 μm filter into a side arm flask. The acrylamide mix was degassed for 20 minutes. Following this, 40 μl of TEMED and 300 μl 10% (w/v) ammonium persulphate was

added to the acrylamide mix. This was immediately poured between the glass plates, using a 50 ml syringe. The gel comb (12 well) was inserted, clamped in place and the gel allowed to set for 2 hours.

The gel was loaded in the sequencing apparatus and TBE buffer added to the top and bottom reservoirs. The comb was removed and the wells flushed with buffer using a syringe to remove air bubbles. Differential display reactions were prepared by mixing a 5 µl aliquot of PCR product with 2 µl of differential display gel loading buffer and loading a 5 µl aliquot into the wells of the gel using a micropipette. The gel was subsequently run at 25 watts (Bio-Rad Power Pac 3000) for about 2 hours, or until the bromophenol blue of the loading dye reached the bottom of the gel.

The gel was then disassembled from the sequencing apparatus and the glass plates carefully separated. The gel remained bound to the Bind-Silane™ pre-treated glass plate and was allowed to dry, marked for orientation with Indian ink spiked with a small amount of radioactive isotope, and placed in an X-ray cassette with Kodak BioMax™ MS scientific imaging film placed on top. The film was exposed to the gel overnight at room temperature. Film was developed in total darkness for 2 minutes in Agfa-Gevaert X-ray developer (1:6 dilution), fixed for 2 minutes in total darkness and then a further 8 minutes in Agfa-Gevaer X-ray fixing solution (1:4 dilution), then washed in running water for 10 minutes. Following washing the film was air-dried.

2.3.2.3 Band excision and re-amplification

Bands of interest in differential display gels were identified in the developed autoradiograph and located in the dried gels, by orientation of radioactive ink spots. The interesting bands were moistened with water and removed to tubes containing 20 µl of sterile distilled and de-ionised water. The DNA was allowed to diffuse from the gel bands and 1-2 µl aliquots of DNA solution were PCR amplified using the original differential display primer pairs and PCR conditions. The PCR products were separated on 2% agarose gels (Section 2.3.4.4) to estimate the band sizes and stabbed gel bands were used as a template for re-amplification with a modified upstream primer (Table 4.1). The extended upstream primers had an overall G+C content of

60% and carried ten additional nucleotides at their 5' ends to provide a target for sequencing primers. PCR conditions for re-amplification with extended primers were as described above (Section 2.3.4.2) except that 20 μM dNTPs, 2.8 μM downstream and 1.0 μM modified upstream primer were used.

2.3.2.4 Gel electrophoresis of PCR products

A 50 ml 2% agarose gel (unless otherwise stated) was prepared by heating agarose with 1 x TAE buffer in a microwave set on high for 1 minute 30 seconds. Ethidium bromide was added to a concentration of 0.5 μg ml^{-1} and the gel poured. PCR products (5 μl) were mixed with 1.0 μl of 6 x gel loading buffer prior to loading. Electrophoresis was conducted at 80 V typically for 45-60 minutes.

2.3.2.5 Sequencing of differential display bands

Re-amplified PCR products were sized with a 100 bp DNA ladder (Promega) on a 2% agarose gel. PCR products were excised from the gel and purified using a Qiagen™ gel extraction kit according to the manufacturers instructions. The PCR products were sequenced directly from the modified upstream primer (see section 2.3.4.3) using ABI Prism Dye Terminator Cycle Sequencing Kit. Approximately 50 ng of purified PCR product and 5-10 picomoles of modified upstream primer were added to 8 μl of Terminator Ready Reaction Mix (Applied Biosystems) and made to 20 μl with sterile distilled and de-ionized water. The sequencing reaction consisted of 26 cycles of 30 seconds at 96°C, 15 seconds at 50°C and 4 minutes at 60°C in a PTC-100 Programmable Thermal Controller. PCR products were ethanol precipitated with 2 μl of 3M sodium acetate and 50 μl of ice cold 100% ethanol. The samples were mixed well by vortexing, placed on ice for 10 minutes and centrifuged at 10,000 g for 20 minutes. The pellet was washed with 70% ethanol and air dried for 20 minutes.

Electrophoresis of sequencing reaction products was performed by Micromon, Monash University Microbiology Department, Clayton, Victoria.

2.3.2.6 Identification of genes from differential display

Sequencing data of approximately 150-250 bp was obtained from the above sequencing reactions. This data was used to identify genes using the BLASTn sequence alignment algorithm (Altschul *et al.*, 1990) in alignment with the *S. cerevisiae* genome using the *Saccharomyces* Genome Database (www.yeastgenome.org).

2.3.3 Northern analysis

2.3.3.1 Design of oligonucleotide probes for Northern analysis

Gene specific oligonucleotide probes (Table 4.3) were designed to be complementary to mRNAs of the putative ethanol-stress induced genes identified by differential display. These probes, comprising 30-35 nucleotides and approximately 50% G+C content, were tested for complementarity to other yeast RNA and DNA sequences in the *Saccharomyces* genome database (www.yeastgenome.org) using the BLASTn sequence alignment algorithm (Altschul *et al.*, 1990). The expected threshold value was raised to reveal a greater number of possible matches. Probes were only selected if it was deemed they would not hybridise to a transcript other than the one of interest. Probes were purchased from Invitrogen Custom Primers.

2.3.3.2 Formaldehyde Gel Electrophoresis

RNA was prepared from equal cell numbers of control and ethanol stressed lag phase cells over a time course. Denaturing formaldehyde gels were used to separate RNA species of different molecular weights. Gels were prepared with 0.72 g (1.2% w/v) DNA grade agarose and 51 ml of RNase free water. This solution was microwaved on high power for 1.0 minute, cooled slightly and 6 ml 10 x MOPS buffer and 3 ml formaldehyde (pH 4-5) added. The gel was poured into an RNase free gel tray and allowed to cool for 1-2 hours.

RNA samples (5.5 µl) (Section 2.3.1.2) were denatured at 65°C in a total volume of 20µl containing 1.0 µl 1 x MOPS, 3.5 µl formaldehyde and 10 µl formamide. A RNA marker (4 µl) (Promega) was prepared in the same way. Formaldehyde-gel loading buffer (5 µl) and 1.0 µl of ethidium bromide (100 µg ml^{-1}) were added to each tube, mixed well, spun down, and loaded to the prepared gel. Electrophoresis was carried out in an RNase-free gel tank in 1 x MOPS buffer at 80 V typically for 2-2.5 hours. Gels viewed on a UV transilluminator and photographed using a UVP Laboratory Products gel documentation system.

2.3.3.3 Capillary blotting of RNA

Denatured RNA was transferred by capillary action onto a positively charged nylon membrane (Hybond N^+; Amersham Biosciences) for Northern hybridisation. The denaturing gel was washed for 10 minutes in 10 x SSC while the blot was prepared. A glass dish, filled with 300 ml of 10 x SSC, was set up with a glass bridge across the dish. A long piece of blotting paper (Watman 3M filter paper) was wetted in 10 x SSC and placed over the bridge with both ends in the 10 x SSC buffer to act as a wick. Three pieces of blotting paper were cut to the same size as the gel, wet in 10 x SSC, and placed on top of the paper bridge. The denaturing gel was then placed face up on the blotting paper. The surface of the gel was rolled with a sterile glass pipette with firm downward pressure to remove air bubbles. The nylon membrane (Hybond N^+) was placed on top of the gel followed by three dry pieces of blotting paper again cut to the same size as the gel. Paper towels cut to a similar size as the gel were stacked on top of the blotting paper to a height of 7-10 cm. Plastic wrap was used to seal around the gel to minimize evaporation. A weight of around 0.5 kg was placed on a glass sheet on top of the stack and the blot was left to transfer overnight.

Following the overnight blot, the stack was disassembled and the membrane rinsed in 4 x SSC to remove any agarose. To ensure that complete transfer of RNA was achieved, the gel was stained with 0.1 mg ml^{-1} ethidium bromide for 20 minutes and viewed on a UV transilluminator. The membrane was air-dried and the RNA cross-linked on a UV transilluminator for 3 minutes. The RNA marker was cut from the membrane, stained with methylene blue and de-stained in DEPC water. The

membrane was stored in a sealed hybridisation bag until required for Northern hybridisation.

2.3.3.4 Northern hybridisation

Pre-hybridisation: The membrane was placed in a perspex box containing 30 ml of DIG Easy Hyb hybridisation solution (Roche). The perspex box was placed in a XTRON HI 2002 hybridisation oven (Bartelt Instruments) at 40°C (unless otherwise stated) for 2-4 hours with the rocking platform set at 11 rpm.

Probe preparation: Gene specific oligonucleotide probes (Tables 4.3 and 5.5) were 5' end labelled with γ ^{32}P-dATP (Perkin Elmer) and T4 polynucleotide kinase (MBI Fermantas). The method is essentially as described in Sambrook *et al.* (1989).

One µl of gene specific oligonucleotide probe (10 pmol μl^{-1}) was transferred to a new microfuge tube with 20 units 10 x T4 PNK buffer A (MBI Fermantas) and 20-200 µCi γ ^{32}P-dATP (3000Ci/mmol). T4 PNK (10 units; MBI Fermantas) was added and sterile distilled and de-ionized water to achieve a total volume of 20 µl. The reaction volume was centrifuged for 5 seconds at 10,000 g in a microfuge and incubated at 37°C for 45 minutes in a heating block. The reaction was subsequently inactivated at 68°C for 10 minutes and placed on ice. The entire probe reaction volume was added to the pre-hybridisation mixture and placed in the hybridisation oven at the optimum temperature overnight.

Hybridisation: Hybridisations were undertaken in a XTRON HI 2002 hybridisation oven (Bartelt Instruments) at 40°C (unless otherwise stated), overnight, with the rocking platform set at 11 rpm. The following day the hybridisation solution was poured off and an equal volume of a low stringency wash solution (4 x SSC with 0.1% SDS) was added. The membrane was incubated at room temperature for 2 minutes and the wash was repeated. Further washes were carried out under more stringent conditions of lower salt and/or longer time to further reduce background

adherence of the probe to the membrane and remove non-specific hybridisation products. These conditions were (unless otherwise stated):

2 minutes at room temperature in 4 x SSC + 0.1% SDS (2 washes)

2 minutes at room temperature in 2 x SSC + 0.1% SDS (3 washes)

When background levels of radioactivity were reduced the membrane was allowed to dry, placed in a hybridisation bag and exposed to Kodak BIO-MAX™ MS scientific x-ray film.

Autoradiography: Autoradiography was performed with a single intensifying screen at -80°C from 24-60 hours depending on the strength of the signal. After the required exposure time the cassette was removed from the freezer and allowed to warm to room temperature prior to development of the film. The film was developed manually, allowing 2 minutes in developer and 5 minutes in fixer (as described in Section 2.3.4.2). Finally the film was washed extensively under running tap water and air-dried.

Stripping blots for re-probing: Following autoradiography the blots were stripped and re-probed with a constitutively expressed actin (*ACT1*) gene specific oligonucleotide probe. Blots were added to 100 ml of boiling 1 x SSC and 0.5% SDS for 1 minute to facilitate stripping. Membranes were then screened for any background radioactivity. If not complete the stripping procedure was repeated until no radioactivity could be detected. Membranes were then air dried and stored in a hybridisation bag at -20°C before re-probing.

Band sizing: Bands generated from the Northern blots were sized to ensure they matched the expected size of the gene of interest. Bands were sized using a correlation curve of the log of the molecular weight RNA markers (Promega) versus the distance migrated on a semilog plot.

2.3.3.5 Cerenkov counts to determine probe labelling efficiency

To determine the labelling efficiency of Northern probes, 0.5 µl aliquots of the probe reaction were removed after incubation with the T4 Polynucleotide Kinase. This aliquot was added to 4.5 µl 0.2M EDTA and mixed. To determine the amount of labelled oligonucleotide, 0.5 µl of the above sample was pipetted onto four Whatman DE81 ion exchange 2.3 cm circular filters. Two filters were washed twice in 0.5 M sodium phosphate buffer, rinsed in 100% ethanol, dried and placed in scintillation vials. The remaining two unwashed filters were also placed in scintillation vials. In this method, unwashed oligonucleotides were washed away and those incorporated into the probe remained bound to the ion exchange paper. Cerenkov counts were performed in the tritium isotope window of a Wallace 1410 liquid scintillation counter. The percentage of incorporation was calculated by dividing the average counts of the washed filters by the average counts of the unwashed filters and dividing by 100.

2.3.4 Gene array analysis

Gene array methodology is essentially as described in the ResGen™ Technical Handbook GF100 (Research Genetics). Total RNA from equal cell numbers (10^8 cells) of unstressed and 5% (v/v) ethanol-stressed lag phase cells, harvested at one and three hours post inoculation, was extracted (Section 2.3.1.2) and DNase treated (Section 2.3.1.3). The gene arrays used were the Yeast Index Gene Filters supplied by Research Genetics. New Gene Filters were rinsed in boiling 0.5% SDS prior to use to rid the membranes of any residuals.

2.3.4.1 Gene array pre-hybridisation

Yeast Index Gene Filter membranes (Research Genetics) were placed in a hybridisation roller tube (35 x 150 mm) with the DNA side facing the interior of the tube. MicroHyb solution (5 ml; Research Genetics) was added to the roller tube containing the membranes. The MicroHyb solution was rolled around to saturate the

membranes. Poly dA (5 μg; Research Genetics) was added, to block the membranes, and the roller tube vortexed to mix. Any air bubbles between the membranes and the tube were removed with forceps and the membranes placed so they were not overlapping. The membranes were pre-hybridised for 3-5 hours at 42°C in a hybridisation roller oven with rotation set at approximately 10 rpm.

2.3.4.2 Probe synthesis and labelling

Complementary DNA (cDNA) was prepared from RNA extracted from control and ethanol-stressed cells, harvested at one and three hours, to serve as a probe. First strand cDNA synthesis was performed in 30 μl reaction volumes. Total RNA from 10^7 cells (approximately 1 μg) and 5 μg oligo(dT) were mixed in 8 μl of sterile distilled and de-ionized water, heated to 70°C, then chilled on ice for 2 minutes. First Strand Buffer (5 x; Gibco BRL Life Technologies), 20 μM dNTP mix (only dATP, dGTP and dTTP), 10 mM DTT, 300 U Superscript II reverse transcriptase (Gibco BRL Life Technologies) and [α-^{33}P] dCTP (100 μCi, 3000 Ci/mmol; Perkin Elmer) were added, mixed well and spun down. The mixture was incubated at 37°C for 90 minutes.

The cDNA probe was purified by passage through a sterile Bio-Spin 6 chromatography column (Bio-Rad). Firstly, the probe reaction volume was brought up to 100 μl with DEPC water and Bio-spin 6 column was prepared for use by centrifugation at 1000 g in a microfuge for 5 minutes. Column packing buffer was removed and the column placed into a new microfuge tube. The entire probe volume was loaded onto the Bio-Spin 6 column and centrifuged as before at 3200 rpm for 5 minutes. The purified probe was collected, heated to 100°C in a boiling water bath for 3 minutes, and chilled on ice for a further 2 minutes.

2.3.4.3 Gene array hybridisation

The purified and denatured cDNA probe was pipetted into the pre-hybridisation mixture, avoiding the membranes. The roller tube was closed, vortexed thoroughly and allowed to hybridise overnight at 42°C at approximately 10 rpm.

Following an overnight hybridisation, the hybridisation solution was discarded and replaced with 30 ml of wash solution. Membranes were washed twice in 2 x SSC, 1% SDS at 50°C for 20 minutes. These washes were performed in the hybridisation roller tube at 50°C and 14 rpm. A third wash in 100 ml of 0.5 x SSC, 1% SDS was performed in a plastic container at room temperature. The membranes were not allowed to overlap or adhere to the side of the container during the wash.

To prevent drying after washing the membranes were placed on a piece of filter paper which had been moistened with sterile distilled and de-ionized water and wrapped in plastic cling wrap. All air bubbles were removed from between the plastic wrap and the filters.

2.3.4.4 Analysis of gene filters

Hybridised Gene Filters were placed in a cassette and carefully aligned with a BAS-MS 2340 phosphor-imaging screen (Fijifilm). Gene Filters were exposed to the phosphor-imaging screen for 48 hours to generate optimal signal intensities. Following this exposure, the phosphor-imaging screen was scanned using a FLA 3000 phosphor image analyser (Fujifilm) to obtain digital images, which were subsequently analysed using ArrayGauge™ software (version 1.3, Fujifilm). All spot intensities were normalised to the intensity of genomic DNA control spots. Comparisons of spot intensities for each time interval were calculated relative to the no-stress control.

2.3.4.5 Stripping gene filters for re-use

The gene filter membranes were stripped after analysis to allow their reuse. Membranes were placed into separate 500 ml solutions of boiling 0.5% SDS, covered, and agitated briskly for 1 hour. Following this, both membranes were checked with a Geiger counter, placed on moistened filter paper and covered with plastic cling wrap. Again the filters were placed in a cassette, exposed to a phosphor-imaging screen for 48 hours, and scanned with a phosphor image analyser. The hybridisation intensity of the images was checked to make sure the stripping process was efficient.

Following stripping, membranes were stored moist at 4°C until their next use. Gene filters were successfully stripped and reused a maximum of 6 times.

2.3.4.6 Northern blot confirmation of array analysis

A selection of genes identified as up-regulated in gene array analysis were confirmed by Northern analysis. Gene specific oligonucleotide probes (Table 5.5) were designed to the genes of interest as described in Section 2.3.5.1. Northern analysis is described in Section 2.3.5.

2.3.5 Promoter analysis

Promoter analysis was undertaken on all up-regulated genes identified from the gene arrays. A region spanning 800 bp upstream of the translation start sites of up-regulated genes was recovered using the Regulatory Sequence Analysis Tools (RSAT) database (http://rsat.ulb.ac.be/rsat/). Upstream sequences were then searched for specific sequence motifs using RSAT.

Unknown sequence motifs, possibly common to both the up-regulated or down-regulated genes were searched in regions 800 bp upstream of the transcriptional start site using the MEME algorithm (http://meme.sdsc.edu/meme/website/meme.ht-ml; Bailey & Elkan, 1994).

2.3.6 Construction of knockout strains

Two knockout strains were constructed according to the PCR-based gene replacement protocol of Wach *et al.* (1994). This protocol used the KanMX4 module, conferring geneticin resistance, to replace the yeast gene of interest. A diagrammatical representation of the gene replacement is described in Figure 2.1.

2.3.6.1 Amplification of the KanMX4 module from the pFA6-KanMX4 plasmid

Pairs of PCR primers, with regions flanking a gene for removal, were designed for amplification of the kanMX4 module from the plasmid pFA6a-kanMX4. Each pair of primers contained at the 5'end approximately 40 bases matching to the flanking regions of the target gene of interest and at the 3' end approximately 20 bases matching to the flanking regions of the kanMX4 module as published in Wach *et al.* (1994) (Table 6.1). The 5' region of the upstream primer was complementary to the non-coding strand of the gene ORF immediately downstream of the start codon and the 5' region of the downstream primer was complementary to the coding strand of the gene ORF 3' region that included the stop codon. The 3' region of the upstream primer was complementary to the upstream flanking region of the non-coding stand of the kanMX4 module and the 3' region of the downstream primer was complementary to the coding strand of the downstream flanking region of the kanMX4 module. Primers were purchased from Invitrogen Custom Primers.

The upstream and downstream primers flanking the gene to be replaced were used for PCR amplification of the KanMX4 module from the pFA-KanMX4 plasmid. The amplification of the kanMX4 module was performed in a reaction volume of 25 µl, containing 0.5 µM of each genes primer pair, 200 µM dNTPs, 10 x PCR buffer (Invitrogen), 1.5 mM MgCl, 20 ng pFA6a-kanMX4 plasmid DNA, 2.5 units Platinum Taq (Invitrogen), and sterile distilled and deionized water. The PCR amplification protocol consisted of 2 minutes at 92°C, followed by 28 cycles of 30 seconds at 92°C, 30 seconds at 55°C and 90 seconds at 72°C, and then 4 minutes at 72°C. PCR product sizes were confirmed and quantified by electrophoresis on a 0.8% agarose gel (Section 2.3.4.4).

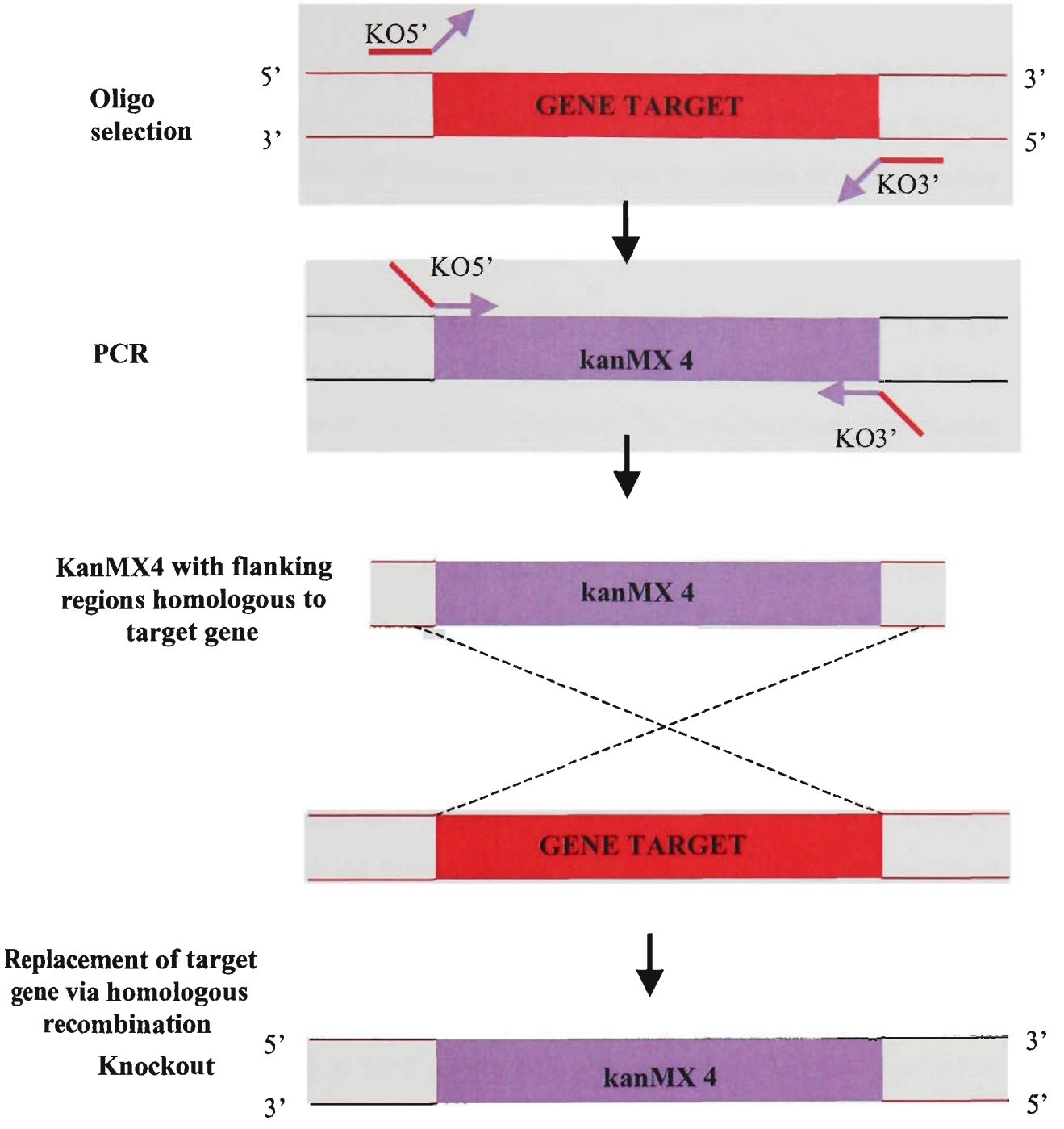


Figure 2.1: Strategy for the replacement of a target yeast gene ORF with the kanMX4 marker. The 5' region of the oligos KO5' and KO3' (shown in pink) contain a stretch of about 40 bases matching the flanking regions of the target gene, whereas the 3' regions (shown in the purple) match the kanMX4 cassette. These oligos were used to PCR amplify the kanMX4 cassette with flanking regions homologous to the target gene. PCR products were used to transform the wild type, where replacement of the target gene occurred via homologous recombination to generate a knockout with a selectable marker.

2.3.6.2 Yeast Transformation

PCR products from the above were used to transform the yeast strain PMY1.1 by homologous recombination using the lithium acetate method of Geitz and Schiestl (1995). Cells from an overnight culture were inoculated to a density of approximately 5 x 10^6 cells ml^{-1} in YEPD media and incubated aerobically at 30°C and 160 rpm. Cells were
incubated until two doublings had occurred (approximately 2 x 10^7 cells ml^{-1}). A 500 µl aliquot of the culture was removed for plating as a cell viability control and 50 ml of the remaining cells were collected and prepared for transformation. This culture was centrifuged at 2000 g at room temperature for 5 minutes, the growth medium removed and the cells washed with 12.5 ml of sterile water and centrifuged as previously. The water was then removed and the cell pellets resuspended in 500 µl of 100 mM lithium acetate. The cells were transferred to a microfuge tube and centrifuged at 10,000 g for 15 seconds. The lithium acetate solution was removed and the cells resuspended in a further 500 µl of 100 mM lithium acetate.

The transformation reaction was assembled by adding, in order, to 50 µl of prepared cells: 240 µl PEG, 36 µl 1M lithium acetate, 5 µl (10 mg ml^{-1}) ss-DNA and 50 µl (approximately 2.5 µg) kanMX4 PCR product. The reaction mix was vortexed vigorously for 1 minute. A transformation control was prepared in the same manner though sterile water was added instead of the PCR product. The transformation reactions were incubated at 30°C and 25 rpm for 30 minutes then heat shocked at 42°C in a heating block for 20 minutes. The transformation mixture was subsequently removed following a 15 second centrifugation at low speed and the cells gently resuspended in 1.0 ml of sterile water. Aliquots of 200 µl, 100 µl and 20 µl of cells were plated onto YEPD Geneticin plates for selection of transformants.

2.3.6.3 Confirmation of gene replacement via colony PCR

Gene replacement and orientation of the kanMX4 module were confirmed by colony PCR, using 3 primers (Table 6.2). A primer complementary to the non-coding strand of the deleted ORF, approximately 200 bases upstream of the start codon, and a

primer complementary to the coding strand flanking the deleted ORF, approximately 180 bases downstream of the stop codon were used, along with third primer targeting the non-coding strand of the kanMX module (Wach *et al.* 1994). Primers were purchased from Invitrogen Custom Primers.

A small amount of cells, collected from a G418 resistant transformed colony, was selected with a pipette tip. Cells were smeared into a PCR tube and microwaved on high for 1.0 minute and immediately placed on ice. The cells were resuspended in a 20 µl reaction volume containing 0.5 µM of each positioning primer, 200 µM dNTPs, 10 x PCR buffer (Invitrogen), 1.5 mM MgCl, 2.5 units Platinum Taq (Invitrogen) and sterile water. The PCR amplification protocol consisted of 2 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 90 seconds at 72°C. The entire reaction was loaded onto a 1.5% agarose gel and the resulting bands were sized, gel purified and sequenced as previously described in Section 2.3.4.5.

2.3.7 Southern analysis

To confirm the correct and single integration of the KanMX4 module, replacing the target gene, Southern analyses were undertaken. Restriction enzymes were selected, by reference to a published gene sequence, which would digest yeast chromosomal DNA upstream and downstream of a gene of interest and not within the KanMX4 module or the target gene. A single band representing a replaced gene ORF would be generated in the knockout strains.

2.3.7.1 Yeast DNA isolation

The method of yeast genomic DNA isolation is essentially as described by Ausubel *et al.* (1996) with slight modifications. Yeast cells were grown overnight in 10 ml of YEPD medium and centrifuged at 2000 g for 5 minutes. The supernatant was discarded and the cell pellet washed by resuspension in 0.5 ml of water. The resuspended cells were transferred to a microfuge tube and briefly centrifuged at 1000 g in a microfuge. Again the supernatant was discarded.

Cell pellets were resuspended in 200 µl of DNA breaking buffer (Appendix 1) and added to approximately 300 µl of chilled, acid-washed 0.55 mm glass beads (Sigma). Phenol/chloroform/isoamyl alcohol (25:24:1) (200 µl) was added to precipitate proteins and the sample vortexed at highest speed for three minutes. TE buffer (200 µl) was added and the sample vortexed briefly and centrifuged for 5 minutes at 10,000 g. The aqueous layer was transferred to a new microfuge tube and 1.0 ml chilled 100% ethanol added. The sample was mixed by inversion and placed on ice for 15 minutes. The supernatant was removed following a three-minute centrifuge at 10,000 g and 500 µl of ice cold 70% ethanol added, mixed, and centrifuged as previously. Again the supernatant was removed and the DNA pellet air-dried for 10-15 minutes. The pellet was resuspended in 300 µl of TE buffer and 3 µl of RNase A (10 mg ml^{-1}; Promega) added. Following a 30-minute incubation at 37°C, the DNA was re-extracted by vortexing for 2 minutes with 300 µl of phenol/chloroform/isoamyl alcohol (25:24:1). The sample was centrifuged at 10,000 g for 5 minutes and 260 µl of the aqueous layer transferred to new microfuge tube. The aqueous layer was precipitated with 10 µl of 10M ammonium acetate and 1.0 ml of chilled 100% ethanol, mixed well by inversion, placed on ice for 15 minutes and centrifuged at 10,000 g for 5 minutes. The supernatant was discarded and the DNA pellet washed with 500 µl of ice cold 70% ethanol. The sample was centrifuged as previously, the supernatant removed and the DNA pellet air dried for 15 minutes. The DNA was resuspended in 50 µl of TE buffer, vortexed, and left in the fridge overnight. DNA was subsequently visualized on a 0.8% agarose gel (essentially as described in Section 2.3.4.4), and the concentration determined by spectrophotometric analysis (2.3.9.2).

2.3.7.2 Spectrophotometric analysis of DNA quality and quantity

DNA concentration was determined using the method described by Sambrook *et al.* (1989). DNA samples (10 µl) were added to sterile distilled water (990 µl). The optical density (OD) of the diluted DNA samples were measured at 260 and 280nm. Sterile distilled water was used as a blank. The reading at 260 nm allowed for

calculation of nucleic acid concentration in the sample: an OD of one corresponded to 50 μg ml^{-1} double stranded DNA. Pure preparations of DNA has an OD260/OD280 ratio value of 1.8. If contaminated with protein or phenol the OD260/OD280 value is less 1.8, in such cases the DNA sample was again phenol extracted ethanol precipitated as above.

2.3.7.3 Restriction enzyme digestion of DNA

DNA sequences of chromosomes on which the genes of interest lay, as well as the kanMX4 module, were analysed by the Yeast Genome Restriction Analysis program (www.yeastgenome.org) to search for the sites of 6-base cutter restriction enzymes. The selected enzymes did not cut within the gene of interest or within the inserted kanMX4 module but on the chromosomal regions both upstream and downstream of the two genes. Approximately 3 μg of genomic DNA from knockout and wild type *S. cerevisiae* strains were digested in 20 μl reactions using appropriate restriction endonucleases with manufacturers buffers and following manufacturers instructions. The same enzymes were used for parent and knockout strains.

2.3.7.4 Gel electrophoresis of digested DNA

Separation of digested DNA (Section 2.3.9.3) was performed on a 1% agarose gel in 1 x TAE buffer. Ethidium bromide solution (10 mg ml^{-1}) was added to a final concentration of 0.5 μg ml^{-1}. Digested DNA samples (45 μl) were loaded after the addition of 6 x gel loading buffer. An λ *Hind*III/*Eco*RI DNA size marker was also run alongside the DNA samples. Electrophoresis was conducted at 50 V, to avoid smearing of DNA samples, for 6-7 hours or until the bromophenol blue of the gel loading buffer was near the base of the gel. The gels were viewed on a UV transilluminator and photographed using a UVP Laboratory Products gel documentation system. The DNA size marker was excised from the gel and photographed separately alongside a ruler for later sizing of DNA bands.

2.3.7.5 Southern blotting

The methodology for Southern blotting is essentially as described in the Hybond-N+ handbook (Amersham Biosciences) with slight modifications.

The neutral agarose gel containing the separated DNA samples was submerged in denaturation buffer and incubated for 30 minutes with gentle agitation. Following this, the gel was ready for capillary transfer. A glass dish, filled with approximately 300 ml of denaturation buffer (Appendix 1), was set up with a glass bridge across the dish. A long piece of blotting paper was wetted in the denaturation buffer and placed over the bridge with both ends in the denaturation buffer acting as wicks. Three pieces of blotting paper were cut to the same size as the gel, wetted in denaturation buffer, and placed on top of the bridge. The gel was then placed face up on the blotting paper. The surface of the gel was rolled with a glass pipette with firm downward pressure to remove air bubbles. The nylon membrane (Hybond-N+; Amersham Biosciences) was placed on top of the gel followed by three dry pieces of blotting paper again cut to the same size as the gel. Paper towel cut to a similar size as the gel was stacked on top of the blotting paper to a height of 7-10 cm. Plastic wrap was used to seal around the gel to minimize evaporation. A weight of around 0.5 kg was placed on a glass sheet on top of the stack and the blot was left to transfer overnight.

Following blotting, the stack was disassembled and the membrane marked with pencil to allow identification of the wells. The membrane was then air-dried and the DNA cross-linked to the surface by UV irradiation for 3 minutes. Blots were stored in a sealed hybridisation bag for later hybridisation.

2.3.7.6 Probe preparation

The kanMX4 coding region was amplified as a DNA probe, to hybridise to the kanMX4 module present in the knockout strains. A pair of PCR primers (Table 6.3), complementary to regions at the 5' and 3' end of the coding sequence of the kanMX4 module, was used to amplify a cDNA probe fragment (810 bases) from the pFA6-

kanMX4 plasmid. The amplification of the kanMX4 coding region was performed in a reaction volume of 20 µl, containing 0.5 µM of each primer, KanCR-F and KanCR-R, 200 µM dNTPs, 10 x PCR buffer (Invitrogen), 1.5 mM MgCl, 20 ng pFA6a-kanMX4 plasmid DNA, 2.5 units Platinum Taq (Invitrogen), and sterile water. The PCR amplification protocol consisted of 2 minutes at 92°C, followed by 28 cycles of 30 seconds at 92°C, 30 seconds at 55°C and 90 seconds at 72°C, and then 4 minutes at 72°C. The size of the PCR product was confirmed by electrophoresis on a 0.8% agarose gel (Section 2.3.1.3). The band was then excised from the gel and placed in a microfuge tube for purification.

Purification of the probe DNA fragment from the agarose gel was undertaken using the Concert gel extraction kit supplied by Gibco BRL. The excised agarose gel slice was weighed and 30 µl of Gel Solubilization buffer (L1) added per 10 mg of gel. Samples were incubated at 50°C for 15 minutes or until the gel fragment dissolved. The sample was pipetted into a spin column sitting in a collection tube and centrifuged at 10,000 g for one minute in a microfuge. The flow through was discarded, the column placed back in the collection tube and a further 500 µl of Gel Solubilization buffer (L1) added to the column. The sample was centrifuged for a further one-minute and the flowthrough discarded. To the cartridge, 700 µl of wash buffer (L2) was added and the column left at room temperature for 5 minutes. Following this incubation the column was centrifuged for one minute at 10,000 g, the flow through discarded, and the centrifugation step repeated. For elution of the purified DNA, the column was placed in a clean 1.5 ml microfuge tube and 50 µl of warm TE buffer added. The column was allowed to stand at room temperature for one minute and then centrifuged at 10,000 g for two minutes.

The approximate DNA concentration of the purified product was determined on a 1.5% agarose gel using a quantitative 100 bp DNA ladder (Promega). The intensity of DNA band was compared to the marker DNA for an estimate of concentration. The products were ready for radiolabelling for use as a hybridisation probe.

2.3.7.7 Probe labelling

DNA probes to be used in the Southern hybridisations were labelled using the Ready To Go DNA labelling bead kit supplied by Amersham Biosciences. DNA (50 ng) was denatured at 95-100°C on a heating block for 3 minutes then placed on ice for 2 minutes. To the denatured DNA, 5 µl of α^{32}P dCTP (50 µCi at 3000 Ci/mmol) and sterile water to a total volume of 50 µl were added. The sample was mixed well and incubated at 37°C for 15 minutes.

The labelled DNA probe was purified, using the ProbeQuant™ G-50 Microcolumns (Amersham Biosciences) to remove any unincorporated labelled nucleotides. The labelled probe was added to a prepared G-50 column, placed in a microfuge collection tube and the sample was centrifuged at 1000 g in a microfuge for 2 minutes. The eluted product served as the purified hybridisation probe.

2.3.7.8 Southern hybridisation

Pre-hybridisation: Southern blots were pre-wetted in 2 X SSC and placed in a hybridisation roller tube with 15 ml of DIG-Easy Hyb hybridisation solution (Roche). All air bubbles between the roller tube and blot were removed and the tube placed in a XTRON HI 2002 (Bartelt Instruments) hybridisation oven at 50°C and 12 rpm for 3 hours.

Hybridisation: The labelled KanMX4 probe was denatured in a boiling water bath for 3 minutes and the entire reaction mix was added to the pre-hybridisation solution. The roller tube was vortexed and placed in the hybridisation oven, again at 50°C and 12 rpm, for approximately 16 hours.

Washes: The following day the hybridisation solution was poured off, the membrane removed from the roller tube and placed into a perspex box containing 50 ml of 2 X SSC, 0.1% SDS. The membrane was gently agitated at room temperature for 5 minutes. The wash solution was removed and the step repeated, though the wash time

was extended to 15 minutes. Again the wash solution was removed, and a third wash was carried out in 50 ml of 0.1 X SSC, 0.1% SDS at 65°C, with gentle rocking, for 10-20 minutes depending on the level of background radiation. When background levels of radioactivity were reduced significantly the membrane was allowed to dry, placed in a hybridisation bag and exposed to a BAS-MS 2340 phosphor imaging plate (Fujifilm).

Analysis of imaging: Probed Southern blots were placed in a cassette and carefully aligned with a phosphor-imaging screen (Fijifilm). Blots were exposed to the phosphor-imaging screen for approximately 24 hours to generate optimal signal intensities. Following this exposure, the phosphor-imaging screen was scanned using a FLA 3000 phosphor image analyser (Fujifilm) to obtain digital images, which were subsequently analysed using Image Gauge™ software (version 1.3, Fujifilm).

Sizing of the bands: Bands generated on the blots were sized by measuring their distance against the DNA molecular weight marker, photographed with a ruler.

CHAPTER 3

THE EFFECTS OF ETHANOL STRESS ON THE GROWTH OF *S. cerevisiae* PMY1.1

3.1 INTRODUCTION

Ethanol is a major metabolic product of *S. cerevisiae* fermentations that accumulates and acts as a potent chemical stress on yeast cells. Increasing concentrations of ethanol are initially inhibitory and latterly lethal to yeast (Walker, 1998). The affect of ethanol on yeast populations has been to decrease growth rate (Jones and Greenfield, 1987; Kalmokoff and Ingledew, 1985), cell viability (Brown *et al.*, 1981) and fermentation ability (Casey *et al.*, 1984). The accumulation of ethanol during fermentation is a major reason for slow and incomplete industrial fermentations, leading to lower ethanol yields and ultimately lower productivity (Ingram, 1986).

If the underlying reasons for the inhibitory and lethal effects of ethanol on yeast vitality could be determined, then this information could be used to modify either the environment or genetic make-up of yeast strains to improve their tolerance to ethanol, thereby potentially improving ethanol yields and productivity during fermentation. Although a considerable amount of research has examined ethanol tolerance from a physio-chemical perspective, relatively few studies have focussed on gene expression in yeast when challenged with an ethanol stress (Alexandre *et al.*, 2001; Piper *et al.*, 1994; Emslie, 2002).

It is the purpose of this project to study global gene expression in *S. cerevisiae* during periods of ethanol stress. Genes with substantially altered expression levels during ethanol stress could potentially identify metabolic and structural aspects of the cell that are the most critical in cell survival and adaptation to the stressful environment. It is important in this study that the ethanol stress is non-lethal so that energy production and transcription is not completely shut down, yet it must be of sufficient magnitude to incite a stress and subsequent period of adaptation by the cells to their

changed environment. At a physiological level, such adaptation periods in yeast cultures are recognised by a growth lag phase in which cell division is temporarily halted until the cells have made sufficient structural and metabolic changes for growth to commence.

With this in mind, a physiological investigation into the growth response of *S. cerevisiae* to various ethanol concentrations was required, before any gene expression studies, to determine environmental conditions that would induce a non-lethal, ethanol-induced lag period in the experimental cultures. A lag period of around three to six hours would make it logistically possible for a representative number of samples to be taken during the adaptation period. Therefore, the aims of this chapter were to:

1. develop an experimental methodology for inducing a reproducible and clearly defined growth lag period in *S. cerevisiae* PMY1.1 when challenged by an ethanol stress,
2. examine the inhibitory effect of different ethanol concentrations on the growth and viability of *S. cerevisiae* PMY1.1,
3. determine a non-lethal ethanol concentration that will cause a three to six hour adaptation period in *S. cerevisiae* PMY1.1 prior to commencing exponential growth.

3.2 THE PHYSIOLOGICAL RESPONSE OF *S. cerevisiae* PMY1.1 TO ETHANOL STRESS

3.2.1 The experimental model

It was necessary at the beginning of this project to design an experimental model that permitted an unambiguous study of ethanol stress on gene expression in *S. cerevisiae*. In this context, it was important that yeast cells sampled during incubation in the presence of ethanol had acquired differences in gene expression that could only be

attributable to the ethanol stress, i.e. not compromised by other influences. This was achieved by:

(i). Inoculating the experimental cultures with yeast in the late exponential phase (all inocula being prepared from the same parent culture) instead of inocula from overnight cultures that are usually in the stationary phase. This avoided the complication of studying gene expression in cells that are already in a stressed, non-growing state (stationary phase), where such a cell state may mask the affect of ethanol on gene expression.

(ii). Washing the inoculum prior to inoculation to prevent the carry over of byproducts from the parent culture that could influence the adaptation by the yeast to the ethanol.

(iii). Inoculating fresh medium ensuring all experimental cultures have the same initial cell population and are exposed to the same defined chemical and physical environment. The exception being the experimental cultures that contained the appropriate amount of ethanol in the fresh medium prior to inoculation.

By carrying out the above steps during preparation and inoculation of the cultures, it was possible to induce a clearly definable lag period in the culture containing added ethanol compared to the control culture without added ethanol. Under such circumstances the lag period was largely a consequence of the ethanol in the culture since all other conditions were the same. This lag phase represents a period of adaptation by the cells to their changed environment prior to exponential growth. Cells inoculated into a fresh medium that contains a non-lethal, but stressful, concentration of ethanol would in theory need to adapt differently (express different genes) to cells from the same parent culture inoculated into fresh medium without ethanol (control). Ethanol-stressed cultures and unstressed cultures would both express genes needed for adaptation to the new growth medium, but only the ethanol-stressed cultures would be expected to express genes specifically related the presence of ethanol. The premise being that these differentially expressed genes play a specific role in cell adaptation to the ethanol stress. In this regard, the lag phase produced

under the above conditions was considered to be a suitable period during the cultivation to study the molecular response of yeast to ethanol stress.

3.2.2 The affect of ethanol on the lag period of *S. cerevisiae* PMY1.1

The laboratory strain *S. cerevisiae* PMY1.1 was used throughout this project. This strain has previously been used to investigate the yeast stress response both in the laboratory of Dr Peter Piper, Department of Biochemistry and Molecular Biology, University College, London, England and also by Dianne Emslie at Victoria University. Preliminary experiment profiling the growth of PMY1.1 was undertaken with cells from overnight cultures inoculated into defined medium (without the addition of ethanol) to an initial OD_{620} of around 0.1. The growth of these cultures was monitored by OD_{620} readings and duplicate plate counts at hourly intervals over a 12 hour period. From this experiment, all future parent cultures were deemed to be in late exponential phase when their OD_{620} was around 1.0, after approximately 8 hours of growth (Figure 3.1).

To determine the most suitable ethanol concentration for the stress experiments in this project, the growth of PMY1.1 was investigated when in the presence of a range of ethanol concentrations. The ideal concentration of ethanol being that which induced a non-lethal lag period of up to five or six hours, with the cells recovering from the stress sufficiently to commence exponential growth. The lag period needed to be of sufficient length to allow multiple time point sampling during the adaptation phase. Sampling and sample preparation for subsequent molecular analysis took approximately 40 minutes for each time point; therefore samples were taken at hourly intervals.

The ethanol stress response of PMY1.1 was characterised in defined medium. The growth conditions are described in greater detail in Chapter 2 (Section 2.2). Late exponential phase parent cells, grown in defined medium, were washed and inoculated into fresh defined medium containing 3%, 5% and 7% (v/v) added ethanol; growth was monitored and compared to a control culture without ethanol addition

(Figure 3.2). Samples were taken at hourly intervals and the growth monitored by optical density measurements and duplicate plate counts. Figure 3.2 (a) shows optical density measurements during incubation and Figure 3.2 (b) shows the viable cell population. Comments in this chapter on the physiology of the yeast cells are primarily based on the viable cell populations since they represent cell numbers (compared to biomass levels as represented by optical density measurements), providing a definitive representation of when the lag period ends and cell division commences. The lag periods, doubling times and growth rates (Table 3.1) were calculated from Figure 1, Appendix 2. Cultures subject to a 3% (v/v) ethanol stress did not have a sufficiently long lag phase for multiple sampling of cells during the ethanol adaptation period. Cultures exposed to 5% and 7% (v/v) ethanol stress had longer lag phases of approximately 3.5 and 5.6 hours, respectively. The latter ethanol concentrations fulfilled the requirement of inciting a lag period of sufficient duration to allow multiple hourly sampling. The growth rates for the 3%, 5% and 7% (v/v) ethanol-stressed cultures were proportionally lower than the specific growth rates of the control culture by 81%, 69% and 55%, respectively.

The above experiment was repeated to determine the reproducibility of the lag phases generated by a 5% and 7% (v/v) ethanol stress. A culture containing 3% (v/v) ethanol was not used, but was instead replaced by a culture containing 10% (v/v) ethanol (Figure 3.3). The purpose of the 10% culture was to investigate the upper limit of cell recovery from ethanol stress over a 12 hour period. Optical density measurements were taken at half hourly intervals throughout the experiment to monitor growth and samples were removed for duplicate viable counts every hour. The lag periods, doubling times and growth rates (Table 3.1) were calculated from Figure 2, Appendix 2. The lag periods observed for cultures grown in the presence of 5% and 7% (v/v) ethanol were 3.1 and 5.5 hours, which are similar to those observed in the previous experiment (Figure 3.2). The specific growth rates for the 5% and 7% (v/v) ethanol stressed cultures were again around 70% and 55% lower than the specific growth rate of the unstressed control culture. In the presence 10% (v/v) ethanol, the

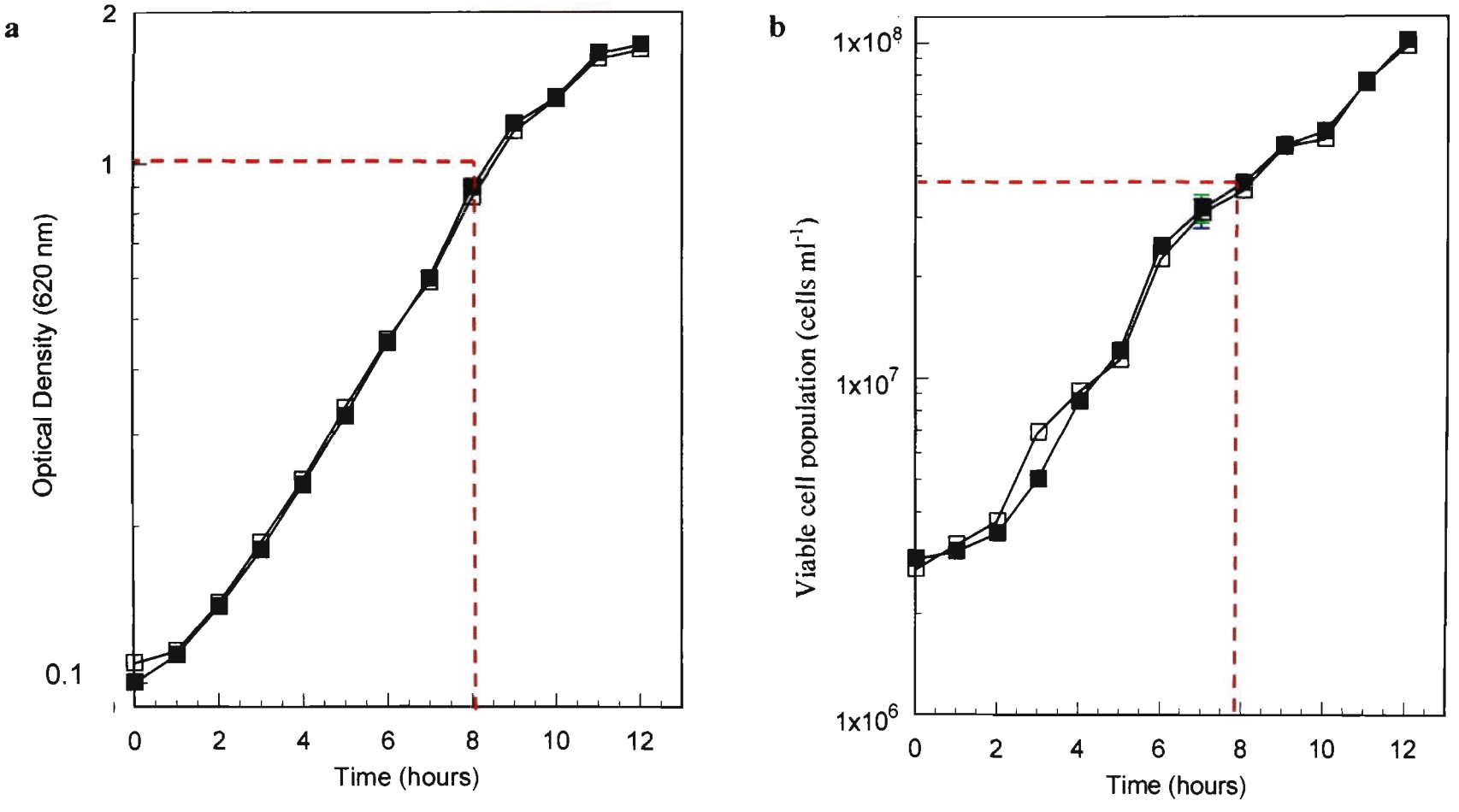


Figure 3.1. The growth of *S. cerevisiae* PMY1.1 in defined medium. Cells from late exponential phase parent cultures were washed and inoculated into defined medium. Duplicate cultures were incubated aerobically at 30°C and 160 rpm. (a) represents growth monitored by optical density (620 nm) and (b) represents viable plate counts. At an OD_{620} of around 1.0 cells are in late exponential phase (- - -).

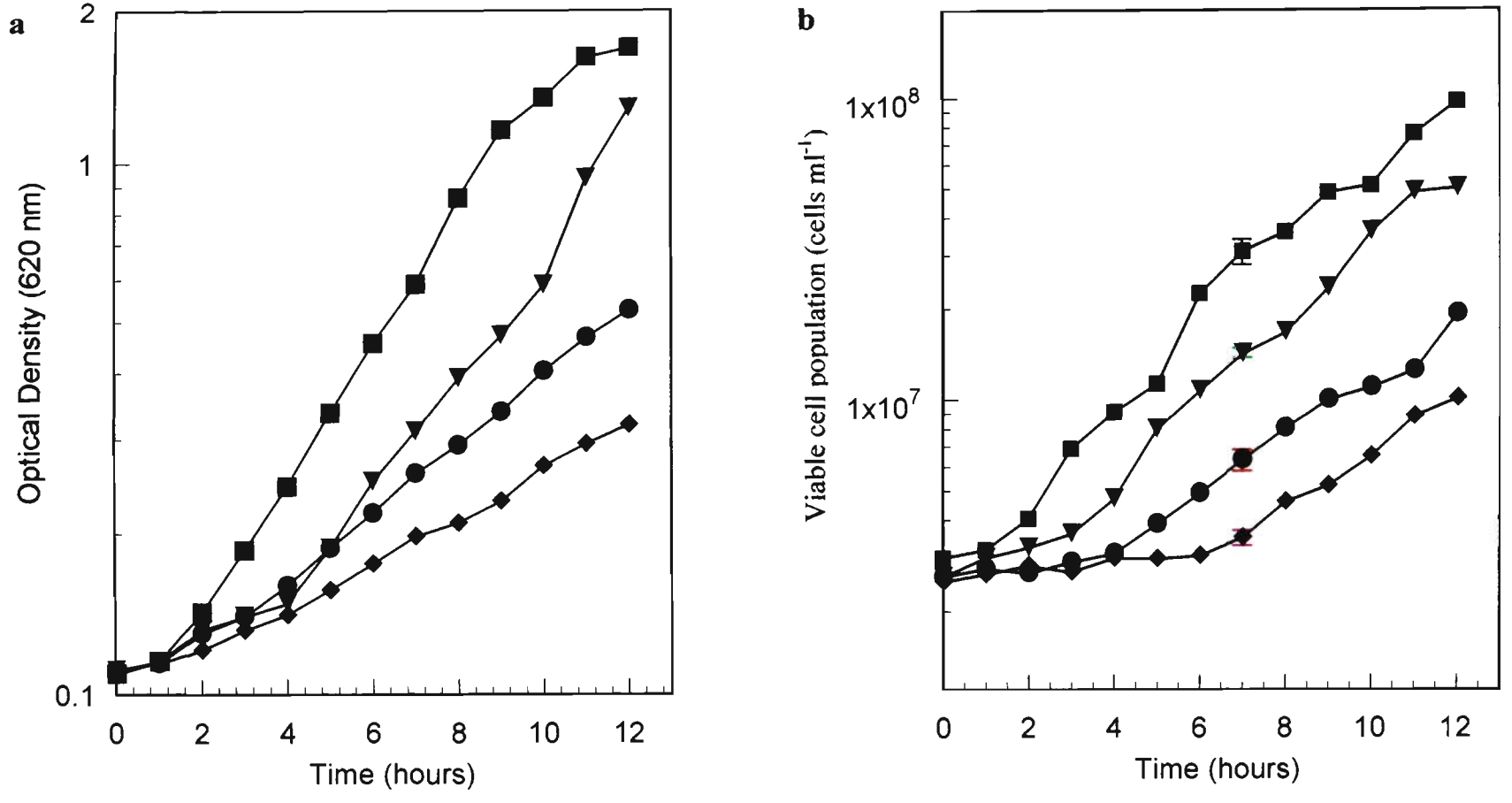


Figure 3.2. The affect of ethanol on *S. cerevisiae* PMY1.1 in defined medium. Cells from late exponential phase parent cultures were washed and inoculated into defined medium only (■) or defined medium containing 3% (▼), 5% (●) or 7% (♦) (v/v) added ethanol. The cultures were incubated aerobically at 30°C and 160 rpm. Panel (a) represents growth monitored by optical density (620 nm) and panel (b) represents viable plate counts.

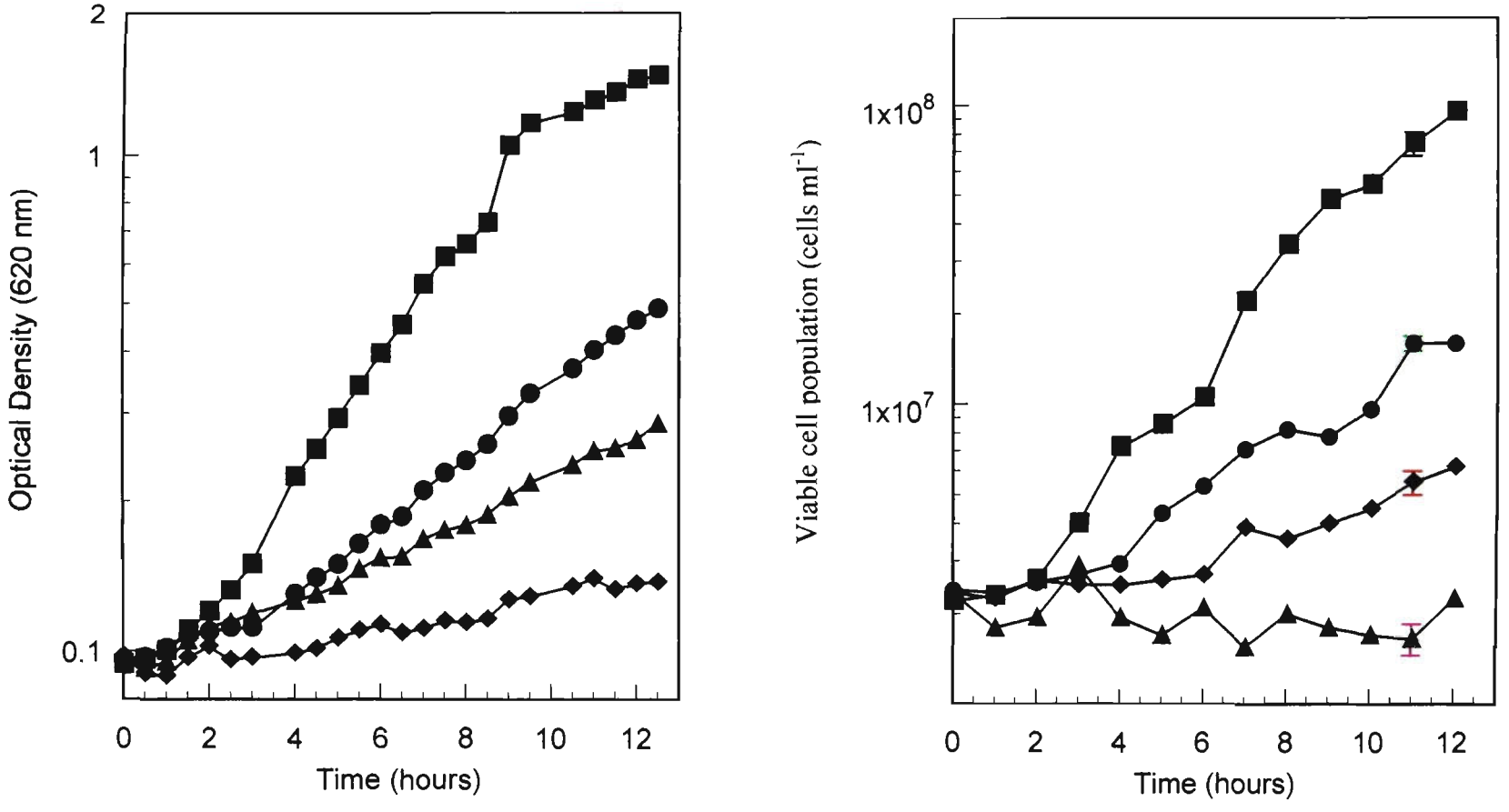


Figure 3.3. The affect of ethanol on the growth of *S. cerevisiae* PMY1.1 in defined medium. Cells from an overnight parent culture were washed and inoculated into defined medium only (◀) or defined medium containing 5% v/v (●), 7% v/v (♦) or 10% (▲) added ethanol. The cultures were incubated aerobically at 30°C and 160 rpm. Panel (a) represents growth monitored by optical density (620 nm) and panel (b) represents viable plate counts.

Table 3.1 The effect of ethanol concentration on the lag period, doubling time and specific growth rate of *S. cerevisiae* PMY1.1 in defined medium.

FIGURE	ETHANOL CONCENTRATION (v/v)	LAG PERIOD (h)	DOUBLING TIME (h)	SPECIFIC GROWTH RATE (h^{-1})	GROWTH RATE RELATIVE TO UNSTRESSED CONTROL (%)
3.1	0%	N/A	2.0	0.35	N/A
3.2	0%	0.5	2.1	0.33	N/A
	3%	1.5	2.5	0.27	84.8%
	5%	3.5	3.0	0.23	69.6%
	7%	5.5	3.8	0.18	54.5%
3.3	0%	0.6	2.0	0.34	N/A
	5%	3.1	3.0	0.23	65.7%
	7%	5.5	3.8	0.18	51.4%
	10%	N/A	N/A	N/A	N/A

cells did not recover from the stress over the 12 hour period of the experiment, however, the viable cell population only marginally decreased over this period.

A step change in ethanol concentration of 5% or 7% (v/v) resulted in a lag period of approximately 3.3 and 5.5 hours, respectively, which was sufficient to allow for multiple time point sampling during the adaptation of PMY1.1 to the ethanol stress. Following the adaptation period, the yeast cells recovered from the stress situation to resume growth at a growth rate around 70% (5% v/v ethanol) or 55% (7% v/v ethanol) of the specific growth rate of unstressed cultures.

3.3 DISCUSSION

The purpose of this chapter was to assess the ethanol stress response of PMY1.1. The assessment of ethanol stress response was based on the lag periods and specific growth rates of PMY1.1 over increasing non-lethal ethanol concentrations.

3.3.1 Factors affecting the adaptation of *S. cerevisiae* to ethanol

An inhibitory effect of ethanol on the growth of *S. cerevisiae* has been well described by a number of authors (see reviews by Ingram and Buttke, 1984; Casey and Ingledew, 1986; D'Amore *et al.*, 1990; Jones, 1990). It is well documented that ethanol affects yeast cell growth rate and viability, however, there are few reports of its influence on the length of time cultures need for adaptation to the presence of non-lethal ethanol concentrations i.e. the ethanol induced lag period. Walker-Caprioglio *et al.* (1985) examined the affect of ethanol concentration on the length of the lag period in *S. cerevisiae*. When *S. cerevisiae* X2180-1A was inoculated into nutrient rich ethanol-containing medium (ranging between 1% and 8% v/v) cultures showed increased lag periods and decreased growth rates with increasing ethanol concentration. The use of a defined medium resulted in even longer lag periods. When ranges of initial populations, 1×10^3 to 1×10^6 cells ml^{-1}, were used a decrease in lag time was seen with an increase in inoculum size. It was speculated that growth-enhancing substances were contributing to the medium along with the inoculum

(Walker-Caprioglio *et al.*, 1985). The inoculum size data presented by the authors, however, was not accurate in determining the lag period and time of entry into exponential phase of low cell population cultures. The authors used a Klett-Summerson photoelectric colorimeter, rather than plate counts or a coulter counter, to measure cell populations. The Klett-Summerson photoelectric colorimeter proved to be insensitive at low cell populations (below 1 x 10^6 cells ml^{-1}) and the authors were required to predict growth rates and lag periods below 1 x 10^6 cells ml^{-1} from data obtained at higher cell populations.

Stanley (1993) also demonstrated that the lag period is reduced by experimental variables, such as an increase in initial cell population and when cells are grown in complex rich medium in comparison to defined medium. When washed late exponential phase *S. cerevisiae* X2180-1A were inoculated at 5 x 10^4 cells ml^{-1} into 4% (v/v) ethanol-containing complex medium, a lag period of 3.6 hours was observed. When the initial cell population was increased to 5 x 10^6 cells ml^{-1} the lag period was reduced to 2.6 hours. When the same cell populations were inoculated into defined medium containing 4% (v/v) added ethanol, the lag periods were around 4.86 and 3.4 hours respectively (Stanley, 1993; Stanley *et al.*, 1997). A coulter counter was used to determine cell populations though duplicate plate counts were also performed when low cell populations (1 x 10^4 cell ml^{-1} or less) were used. The inocula used in the experiments of Stanley (1993) and Stanley *et al.* (1997) were washed to remove any pre-conditioned parent medium that may have been stimulatory to the ethanol-stress effect. Given this inoculum preparation, the lag reducing effects due to an increase in initial cell population were suggested to be due to secreted growth activating factors that interact with the cells and influence their adaptation to stress, i.e. the higher the cell population, the greater the accumulation of secreted cell products. For this reason, the inocula in all growth experiments in this thesis were washed in pre-warmed medium so a true representation of the effects of ethanol stress on yeast cells could be observed.

Based on the results of Stanley *et al.* (1997) and Walker-Caprioglio *et al.* (1985) initial cell populations of approximately 2 x 10^6 cells ml^{-1} were used in this study. This population allowed the induction of a measurable lag period and the cell population was in a range to monitor cell densities during growth with a

spectrophotometer. In addition, as approximately 1 x 10^8 cells were required from each sampling time point (for molecular analysis) a harvested sample size of 50-100 ml could be removed at each hour. A lower initial cell population (i.e. 2 x 10^5 cells ml^{-1}) would have required an impractical sample volume at each time point.

Late exponential phase inocula were used in preference to overnight-grown, usually stationary phase, cells in this study. As considerable amounts of ethanol are produced during stationary phase, stationary phase inocula would be intrinsically more ethanol tolerant than exponentially growing cells. Stationary phase yeast cells have been demonstrated to be more tolerant to stresses, including heat and chemical mutagens (Parry *et al.*, 1976), freezing (Lewis *et al.*, 1993) and ethanol (Warner-Washbourne *et al.*, 1996). Distinct changes in gene expression also occur during stationary phase. The up-regulation of stress responsive genes, including five HSP genes was identified by Riou *et al.* (1997) in a stationary phase wine yeast strain. Previous analyses of these genes demonstrated that they are specifically induced in stationary phase or as a response to stress (Warner-Washbourne *et al.*, 1996). In this regard, inocula for this project used exponential phase cells to avoid complications from 'pre-stressing' the inocula and the subsequent effects on adaptation to ethanol.

3.3.2 The affect of ethanol on the growth of *S. cerevisiae* PMY1.1

High ethanol concentrations inhibit yeast cell growth and viability, acting as a potent chemical stress. Although *S. cerevisiae* is relatively ethanol tolerant, being able to grow at concentrations ranging between 7-13% (v/v) ethanol (Casey and Ingledew, 1986), its ability to grow at the higher end of these ethanol concentrations is strain dependant. Ingram and Buttke (1984) suggest that ethanol resistance in a yeast strain is determined, in part, by its genetic composition.

A number of comparative studies on ethanol tolerance in different *S. cerevisiae* strains have been documented in the literature. In a comparative study of 16 wine and laboratory *S. cerevisiae* strains, Carrasco *et al.* (2001) found only one wine strain would grow on glucose in the presence of 15% (v/v) ethanol, and laboratory strains were more sensitive to ethanol than many (but not all) commercial strains. Unaldi *et*

al. (2002) analysed the ethanol tolerance of 34 wild-type yeast strains by measuring cell populations after 48 hours ethanol exposure. The authors found only 6 strains with the ability to tolerate ethanol up to 9% (v/v) in concentration. The viability of the majority of these strains decreased when exposed to 6% (v/v) ethanol. Ivorra *et al.* (1999) monitored cell survival rates, in the presence of 10% (v/v) ethanol, to compare the ethanol tolerance of four yeast strains grown in defined medium. One strain, LYCC 047, showed a 25% reduction in cellular viability following two hours of ethanol exposure. The other three strains continued growing, however, at an overall reduced rate. By comparison, Sajbidor and Grego (1992) demonstrated that 15% (v/v) ethanol was required before a decline was observed in the viable population of *S. cerevisiae* CCY.

In this project, ethanol at low concentrations (3% v/v) did not have a strong inhibitory effect on the growth of PMY1.1 (Figure 3.2). On the other hand, cells inoculated into medium containing 10% (v/v) ethanol did not grow during the 12-hour sampling period, although the viable cell population remained constant during this time (Figure 3.3). This is similar to Holzberg *et al.* (1967), who found a threshold ethanol concentration of 2.7% (v/v) below which no ethanol-induced inhibition of growth was observed. Above this concentration, inhibition of growth increased in a concentration-dependant manner up to 9% (v/v) ethanol, at which concentration the strain failed to grow. Beaven *et al.* (1982), using rich medium, found little inhibition of growth when *S. cerevisiae* NCYC 431 was exposed to 3% (v/v) ethanol, however, exposure to 9% (v/v) ethanol considerably reduced cell viability.

S. cerevisiae HSc, used in ethanol tolerance studies by Lloyd *et al.* (1993), was reported to have an above average level of ethanol tolerance. When HSc was inoculated at an OD_{600} of approximately 0.01 in nutrient rich medium containing ethanol concentrations of 0%, 2.5%, and 5% (v/v), respective doubling times of 1.4, 1.5 and 2.2 hours were observed. The corresponding lag periods were approximately 3, 10 and 15 hours. When incubated in 10% (v/v) ethanol HSc failed to grow, however, the cells remained viable for 30 hours. Comparison between PMY1.1 (used in this study) and HSc requires caution due to the different medium and initial cell populations used in the two experiments. The doubling times for PMY1.1 in defined medium containing 0% and 5% (v/v) ethanol were 2 and 3 hours, respectively (Table

3.1). These slower doubling times are however expected in a defined medium. The lag time for PMY1.1 in defined medium containing 5% (v/v) ethanol was around 3.5 hours; for HSc in rich medium, the lag time was 15 hours. PMY1.1 therefore was able to adapt to the presence of ethanol more rapidly than HSc, though this observation is possibly due to the higher inoculum size (OD_{620} of 0.1) used with PMY1.1. A reduction in lag phase has been observed in ethanol-shocked *S. cerevisiae* cells when high inoculum sizes were employed (Stanley *et al.*, 1997; Walker-Caprioglio *et al.*, 1985). The affect of inoculum size on the adaptation of PMY1.1 to ethanol stress was discussed previously in Section 3.3.1 of this chapter.

A study of the effects of ethanol on the specific growth rate of *S. cerevisiae* IGC 3507 III, grown aerobically in defined medium at 30°C, was undertaken by Fernanda-Rosa and Sa-Correira (1996). The specific growth rates for IGC 3507 III grown in 5% and 7% (v/v) ethanol were 77% and 61% relative to the control (0% ethanol). In comparison, the specific growth rates of PMY1.1 in 5% and 7% (v/v) ethanol were around 69% and 55% relative to the control (Table 3.1). Therefore, IGC 3507 III was able to tolerate the ethanol stress better than PMY1.1, however the times taken to adapt to the ethanol stress situation (i.e. the lag period) were not shown.

Emslie (2002) measured growth rates and lag periods of PMY1.1 to compare its ethanol tolerance in rich medium with one other laboratory strain, *S. cerevisiae* SUB61. PMY1.1 was found to have a higher level of ethanol tolerance when grown in the presence of 4% (v/v) ethanol. Few ethanol stress studies have been undertaken in defined medium, in which it is generally recognized that cells are less tolerant to ethanol stress. Despite this, it appears that PMY1.1 has a comparable level of ethanol tolerance to the *Saccharomyces* strains cited in the literature. A meaningful comparison between PMY1.1 and other *S. cerevisiae* strains is difficult to make due to the different experimental approaches and environmental conditions used in the various studies documented, all of which influence the ability of the yeast to adapt to, and tolerate, the presence of ethanol in its environment.

3.3.3 Investigating the ethanol stress response

The ethanol-induced lag phase described in this chapter is a period for observing the adaptive changes induced in yeast exposed to ethanol. The purpose of this chapter was to select a concentration of ethanol of sufficient magnitude to induce a lag phase adaptation period of 3-6 hours prior to the cells commencing exponential growth. The stress could not be too severe so as to avoid critically damaging effects on molecular and metabolic events occurring within the cell.

Both 5% and 7% (v/v) ethanol satisfied the above criteria, with respective lag periods of approximately 3.3 and 5.5 hours. The choice of 7% (v/v) ethanol stress for molecular analysis was selected as this allowed for multiple time point analysis throughout the adaptation to ethanol.

CHAPTER 4

THE IDENTIFICATION OF ETHANOL STRESS RESPONSE GENES IN *S. cerevisiae* USING DIFFERENTIAL DISPLAY

4.1 INTRODUCTION

At the commencement of work for this thesis, very little was known of the molecular basis of the ethanol stress response in *S. cerevisiae*, particularly at the level of transcription. A major aim of this thesis was to begin to address this shortfall by identifying genes that are up-regulated in yeast during adaptation to ethanol stress. There are several techniques that can be used to this end but the two that are most likely to deliver results over a reasonable timescale are undoubtedly differential display and gene array analysis. When work for this chapter was started only the former of these approaches was available to most laboratories, including our lab at Victoria University of Technology and thus was the method of choice at that time.

Differential display allows the comparison of transcript profiles between test and control cells or tissues. The method makes use of sets of arbitrary DNA primers to PCR amplify cDNA populations derived from mRNAs of different cell types or the one cell-type under different environmental conditions (Liang and Pardee, 1992). Amplified cDNAs are separated by polyacrylamide gel electrophoresis (PAGE) on DNA sequencing gels and the expression profiles in test and control samples are compared. cDNA bands representing differential expression can then be isolated from the gel and genes can be identified by sequencing. Although differential display was originally devised for the identification and isolation of genes expressed under designated conditions in mammalian cells, its widespread use has found application in studies on a variety of metabolic processes in organisms as diverse as *Leishmania chagasi* (Lewis *et al.*, 1996) and *Arabidopsis thaliana* (Callard *et al.*, 1996). Differential display has also proven to be a powerful method for detecting novel genes with differential expression in *S. cerevisiae* under different environmental conditions; successful applications have identified novel genes involved in nutrient limitation (Crawels *et al.*, 1997), thermotolerance (Gross and Watson, 1998), osmotic stress

(Garray-Arroyo and Covarrubias, 1999), isooctane tolerance (Miura *et al.*, 2000), cold stress (Rodriguez-Vargas *et al.*, 2002), and ethanol stress (Emslie, 2002).

A partial differential display had previously been undertaken in our laboratory to identify genes up-regulated in *S. cerevisiae* during adaptation to ethanol stress. This work revealed new insights into the molecular mechanisms involved in the ethanol stress response (Emslie, 2002). For this work, a modified differential display protocol, based on the original methods of Liang and Pardee (1992), was developed that reduced the potential of generating false positive results; results in which genes appear to be up-regulated in differential display but cannot be confirmed using other methods. The method was also streamlined, enabling rapid processing of results (Emslie, 2002). These modifications will be described in the results and discussion sections of this chapter.

For work described in this chapter, differential display was used to identify genes in *S. cerevisiae* that were up-regulated in response to ethanol stress during a lag phase adaptation period. The concentration of ethanol used to stress yeast cells for this work, based on results from the previous chapter, was 7% (v/v) ethanol. Gene expression was analysed over three time points during the adaptation to 7% (v/v) ethanol stress to identify novel ethanol stress response genes.

The general aim of this chapter was to use differential display to identify up-regulated ethanol-stress response genes in *S. cerevisiae* during its adaptation to ethanol stress.

4.2 ETHANOL-STRESS CONDITIONS

The growth curves described in the previous chapter demonstrate the affect of ethanol stress on *Saccharomyces cerevisiae*. The experimental model, whereby growth was profiled with increasing ethanol concentrations, established 7% (v/v) ethanol to be an appropriate level of stress for future molecular experiments. In response to 7% (v/v) ethanol stress an adaptation (or lag) phase of 5.5 hours was induced, with the cells subsequently overcoming the stress situation to resume exponential growth at a rate

that was approximately 55% of the control. The lag phase of 5.5 hours allowed for multiple time point sampling of cells during adaptation to ethanol.

4.3 DIFFERENTIAL DISPLAY

4.3.1 Ethanol stress and the level of cellular RNA

The traditional approach to differential display uses equal quantities of RNA prepared from test and control cells, as template for cDNA production. This is usually achieved by measuring the absorbance of each RNA sample at 260 nm, determining its concentration and preparing test and control samples of equal concentration. This approach was, however, considered to be unsuitable for experiments on ethanol-stress. It was consistently found in this work (Figure 4.1), and in the work of Emslie (2002), that the amount of total RNA in cells exposed to 5% (v/v) or more ethanol stress is reduced in comparison to unstressed cells. A similar observation was also reported in Mager and Moradas-Ferreira (1993) for heat-stressed cells. Thus, if RNA concentrations were equalized for ethanol-stressed and control cells, the concentration of mRNAs in the stress sample would be artificially boosted compared to the unstressed control (see, for example, Figure 4.1). It was therefore decided, based on the methods of Emslie (2002), that differential display experiments would compare mRNA populations from equal cell numbers rather than equalizing the amounts of RNA. Samples of total RNA were isolated from equal numbers of cells (approximately 10^8) from control (0% ethanol) and stressed (7% v/v ethanol) lag phase cultures over a time course of one, three and five hours.

One complication with using equal cell numbers rather than equalizing for RNA concentration from stress and control cells in differential display is that the efficiency of extraction may vary across samples, leading to variation in the yields of RNA, thus generating spurious results. In this context it was important to validate the method of RNA extraction by showing that it delivers similar yields across preparations. This was done by testing RNA isolations from two series of cell pellets, each consisting of three samples of 10^8 harvested cells. Following RNA isolation and subsequent DNase

treatment little variation in RNA concentration or integrity was apparent (Figure 4.2). Thus the RNA extraction protocol used for these experiments gave reproducible yields.

4.3.2 Modifications to the differential display protocol

Two other modifications to the traditional Liang and Pardee (1992) protocol were introduced by Emslie (2002) to further minimize the risk of generating spurious data; an additional acid phenol extraction step was included to reduce the risk of carrying over and amplifying DNA from RNA preparations, and differential expression was followed over a time course rather than relying on a single time point. Both of these modifications were used in the work described in this chapter and will be discussed further in Section 4.4.

4.3.3 The identification of ethanol-stress response genes

In this study, differential display was used to identify genes with up-regulated transcripts in response to ethanol stress during the lag phase adaptation period. A Display Systems Differential Display^{TM} Kit was used for this work; primers associated with the kit were from Operon Technologies Inc.

An exhaustive differential display (to detect most changes in gene expression under a given set of conditions) requires the use of 9 downstream and 24 upstream primers amounting to 216 primer pair reactions. This was not feasible in the timescale of this project, so a partial differential display was undertaken with the aim of confidently identifying some genes that are clearly up-regulated in response to ethanol stress. This differential display analysis used 5 randomly chosen downstream anchor primers to generate 5 subset cDNA populations from both the control and stressed RNA samples over the time course. The cDNAs were then PCR amplified using the appropriate

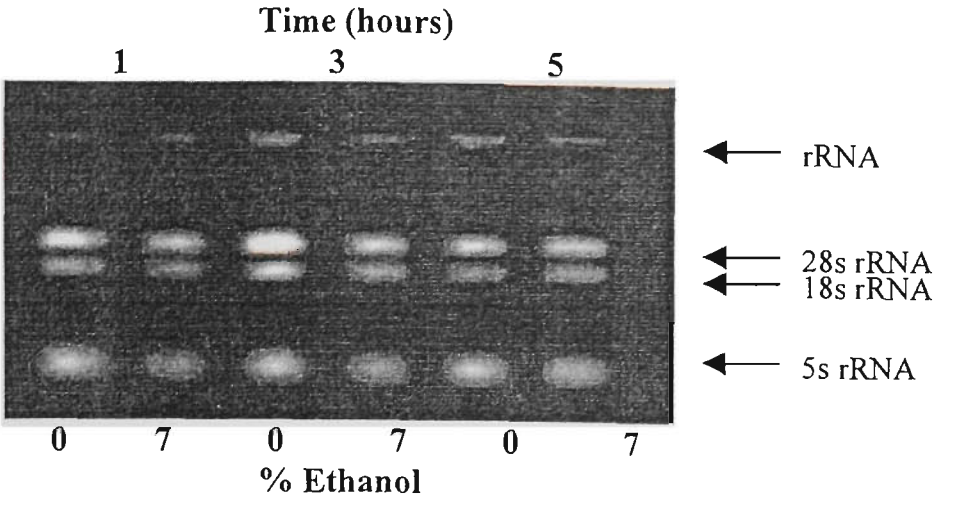


Figure 4.1: RNA preparations derived from 10^8 cells. Cells were harvested from unstressed control (0% ethanol) and stressed (7% v/v added ethanol) cultures over a time course, sampling at one, three and five hours during the lag phase adaptation period to ethanol stress. RNA samples were DNase treated to remove any contaminating DNA and run on a 0.8% agarose gel for one hour. Note that the amount of RNA in the stressed samples is reduced in comparison to the controls.

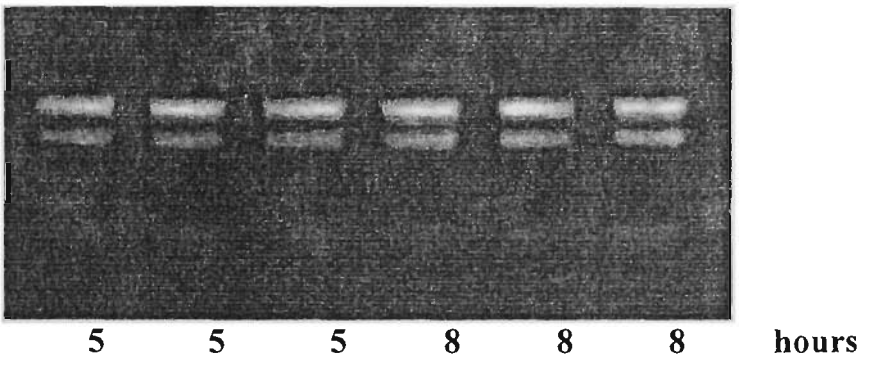


Figure 4.2. Demonstration of the reliable RNA isolation methodology. RNA preparations taken from 10^8 cells from exponential phase cultures grown for either 5 hours or 8 hours to show the consistency of the RNA isolation method for work described in this thesis. RNA was isolated from triplicate cell pellets from the two time intervals. The RNA was subsequently DNase treated and resuspended to a volume of 25 µl. Samples of 4 µl were visualized on a 0.8% agarose gel.

cDNA template and 3' downstream anchor primer, along with random combinations of 10 different arbitrary upstream 10 mer 5' primers. In total, 20 combinations of primers were used on the control and stress cDNA templates in this procedure (Table 4.1).

cDNAs generated from the above were then resolved by PAGE, generating approximately 30 to 60 bands in each lane of the gel. When banding patterns of the control and stress samples were compared, most bands were common to both and of similar intensity, however, some bands showed differential expression under ethanol stress (Figures 4.3 a-e). Both up- and down-regulated genes were evident in the ethanol stressed samples but only genes that were clearly up-regulated in the stress samples across at least two time points were chosen as putative ethanol stress-response genes for further analysis. From the 20 random primer combinations, 7 cDNA bands were selected for further study.

Negative controls, in which reverse transcriptase was left out of the reaction for cDNA generation, were also routinely included in this analysis. There were no visible products in the lanes representing these control cDNA preparations (data not shown). This indicated that genomic DNA was not being PCR amplified and that the bands observed represented differentially expressed mRNAs.

Bands representing genes up-regulated under ethanol stress were then analysed to determine their identity. To precisely excise bands representing putative up-regulated genes for sequencing and identification, differential display gels were dried on PAGE glass plates and aligned with the band of interest on the autoradiographs. To confirm the band of interest was correctly excised, a second film was exposed to the gel following excision. Analysis of excised differential display bands was undertaken by re-amplifying the bands with a modified upstream differential display primer. The modified primers carried an extra 10 bp sequence to enable direct PCR sequencing rather than using the traditional approach of cloning differential display products (Wang and Feuerstein, 1995). PCR re-amplification of differential display bands allowed the separation of target bands from other amplification products so sequencing was performed only on the product of interest. Sequencing of all bands

Table 4.1. Differential Display primer combinations.

Downstream	Upstream
D9: 5'-TTTTTTTTTTTGG	U5: 5'-GGAACCAATC U6: 5'-AAACTCCGTC U10: 5'-GGTACTAAGG U12: 5'-CTGCTTGATG
D8: 5'-TTTTTTTTTTTGC	U12: 5'-CTGCTTGATG U22: 5'-GATCGCATTG U17: 5'-GATCTGACAC U 6: 5'-AAACTCCGTC
D2: 5'-TTTTTTTTTTTAC	U6: 5'-AAACTCCGTC U10: 5'-GGTACTAAGG U15: 5'-GATCCAGTAC U5: 5'-GGAACCAATC
D5: 5'-TTTTTTTTTTTCC	U1: 5'-TACAACGAGG U2: 5'-TGGATTGGTC U24: 5'-GATCATGGTC U15: 5'-GATCCAGTAC
D4: 5'-TTTTTTTTTTTCA	U24: 5'-GATCATGGTC U10: 5'-GGTACTAAGG U6: 5'-AAACTCCGTC U2: 5'-TGGATTGGTC

Extended upstream primers

U5-EXT: 5'- TGC CAA GCT AGG AAC CAA TC

U12-EXT: 5'- AAT CCT TGC GCT GCT TGA TG

U1-EXT: 5'- CGA CTA GCC TTA CAA CGA GG

U24-EXT: 5'- CAT GCA GTC AGA TCA TGG TC

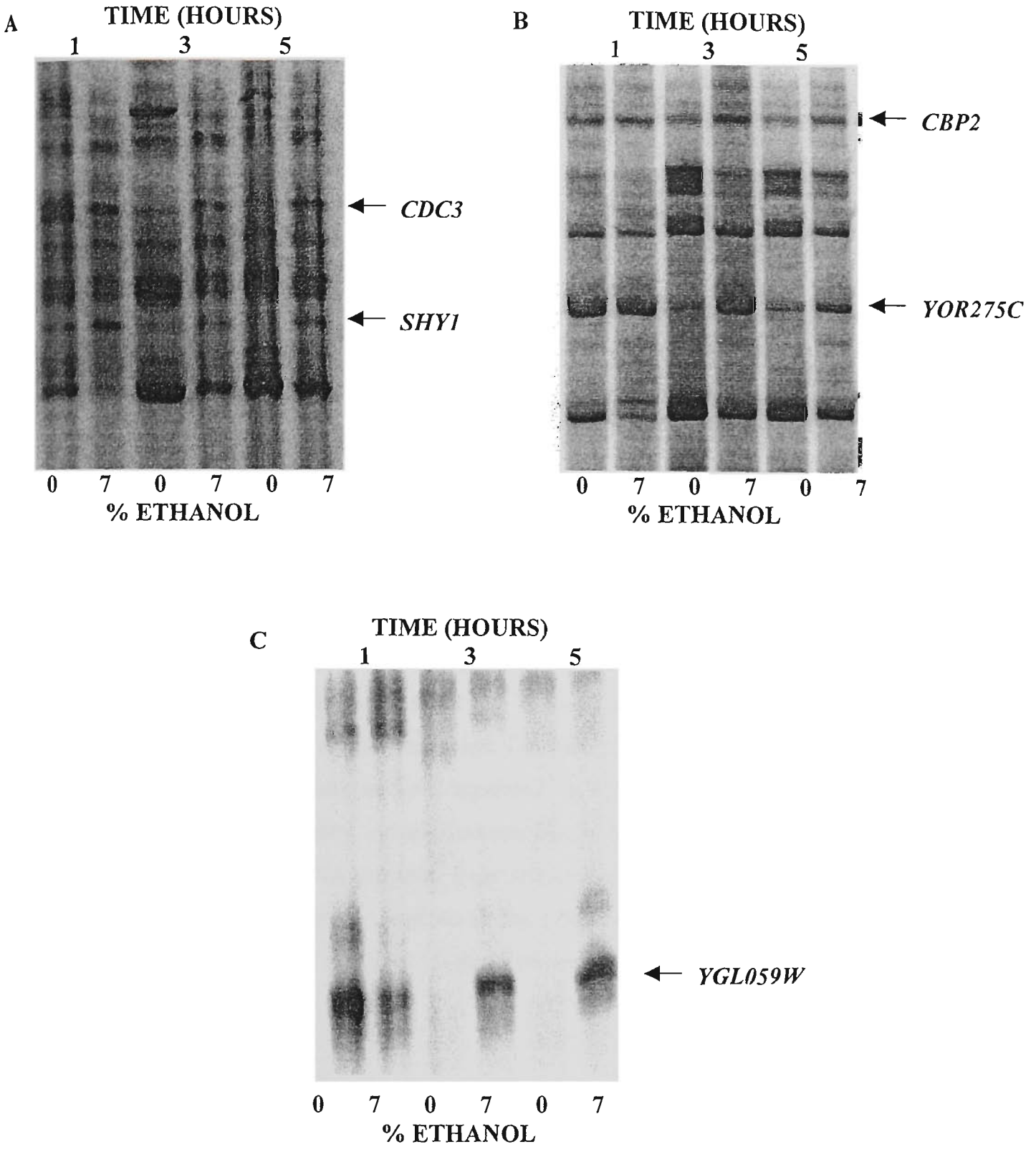


Figure 4.3 (see legend on following page)

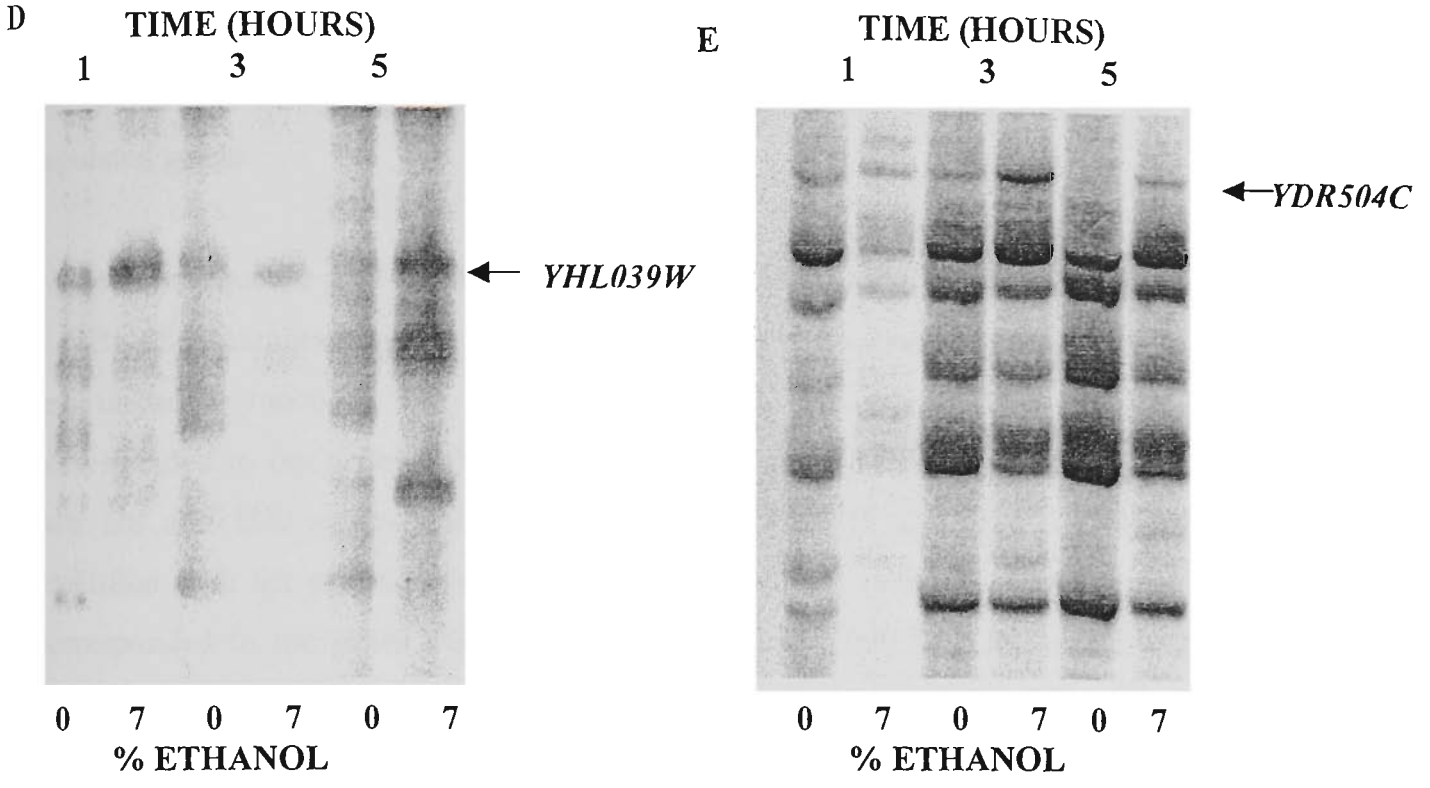


Figure 4.3. Time course differential display to identify genes up-regulated by ethanol stress. Samples are from unstressed control (0% ethanol) and stressed (7% v/v added ethanol) cultures harvested at one, three and five hours post-inoculation. Products from differential display reactions were separated on 6% polyacrylamide gels and visualized after autoradiography. Arrows indicate bands that were more intense in at least two lanes carrying cDNA prepared from stressed cultures. The identity of the band, determined by sequencing, is given in the labels associated with the arrows (Autoradiographs A, B and C are given on the previous page).

across the time-course also gave increased confidence in the identification of up-regulated bands. Sequence data was analysed using the BLAST algorithm (Altschul *et al.*, 1990) in SGD (www.yeastgenome.org), enabling the identification of up-regulated genes.

Nucleotide sequence data for each of the seven selected cDNAs is presented in Figure 4.4. BLAST searches revealed sequence homologies to *S. cerevisiae* genes of known and unknown function. The bands amplified with the primer pair D9 and U5 corresponded to the genes *CDC3* and *SHY1*. The bands amplified with the primers pair D8 and U12 corresponded to the genes *YRO275C* and *CBP2*. The bands amplified with the primer combinations D2 and U5, D5 and U1 and D4 and U24 corresponded to the genes *YGL059W*, *YHL039W* and *YDR504C*, respectively. The gene functions of the putative ethanol stress genes are shown in (Table 4.2).

4.3.4 Confirmation of differential expression of the differential display genes

While the differential display method is convenient for detecting genes that are differentially expressed under different conditions, it is known to generate false positives at high frequency (Liang, 1996). Northern blot analyses were therefore performed to test the validity of the above differential display results. For validation experiments, growth conditions and RNA isolation were undertaken as described for the differential display work. Oligonucleotide probes were designed to target sequences of the seven putative ethanol stress induced genes in Northern blots of total RNA from both control and 7% (v/v) ethanol-stressed samples at one, three and five hour time points (Table 4.3). Blots were probed with the ethanol-stress gene specific oligonucleotide probe, stripped and re-probed with the actin (*ACT1*) specific control probe, which hybridized to the 1.2 Kb *ACT1* transcript. Expression patterns of the putative ethanol-stress response genes was compared to the *ACT1* expression, which was relatively constant across the time course but was consistently lower in stressed cultures (see bottom panels in Figures 4.5 and 4.6).

A

```
D9-U5:      1 AACCAATCAAGACAGGTAAGAGCCCAAATGATGATTNTCGAAGAGAAAGATCCTTTGGCA 60
              |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
SHY1: 717507 AACCAATCAAGACAGGTAAGAGCCCAAATGATGATTCTCGAAGAGAAAGATCCTTTGGCA 717566

D9-U5:     61 AGAAANTTGTTCTGGGTCTGATGTTCGCGATGCCAATAATANCCTTCTATTTGGGAACTT 120
              ||||| |||||||||||||||||||||||||||||||||||| ||||||||||||||||||
SHY1: 717567  AGAAAATTGTTCTGGGTCTGATGTTCGCGATGCCAATAATATCCTTCTATTTGGGAACTT 717626

D9-U5:    121 GGNAAGTAAGGAGATTGAAGTGGAAAACCAAGCTGATTGCGGCATNCGAAACTAAACTTA 180
              || ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
SHY1: 717627  GGCAAGTAAGGAGATTGAAGTGGAAAACCAAGCTGATTGCGGCATGCGAAACTAAACTTA 717686

D9-U5:    181 CTTATGAACCAATACCACTTCCTAAGTCATTTACACCTGACATGTGCGAGGATTGGG 237
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SHY1: 717687  CTTATGAACCAATACCACTTCCTAAGTCATTTACACCTGACATGTGCGAGGATTGGG 717743
```

B

```
D9-U5:    304 ATTGCATAANCTTCAGGTCCANGGNCTTTAAGTAATGTCCTGTAGGTTCAATGAAATAAA 245
              ||||||||| |||||||||| || ||||||||||||||||||||||||||||||||||||
CDC3:  763318 ATTGCATAAACTTCAGGTCCAGGGGCTTTAAGTAATGTCCTGTAGGTTCAATGAAATAAA 763377

D9-U5:    244 GACATGCATGGATTCTTTTATCGTTTATTGAATGTCTGTTGANTTTGTTCTCCGCATCCA 185
              |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
CDC3:  763378 GACATGCATGGATTCTTTTATCGTTTATTGAATGTCTGTTGATTTTGTTCTCCGCATCCA 763437

D9-U5:    184 AGTATTGATCAAAACGAGAGTCAATCTCCTTAATGATCGGGTCCCATGATTTTTGATCAT 125
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CDC3:  763438 AGTATTGATCAAAACGAGAGTCAATCTCCTTAATGATCGGGTCCCATGATTTTTGATCAT 763497

D9-U5:    124 TATTTAAAAAAATCACCAAATCCTTCAGTATCGATCACGTTCAAATTCANTTTAACACCAT 65
              |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
CDC3:  763498 TATTTAAAAAAATCACCAAATCCTTCAGTATCGATCACGTTCAAATTCAGTTTAACACCAT 763557

D9-U5:     64 TTTCTTCAATAACCGACTCATATGACTTAATTTNGACCTTGTGCCTTTGTNCTTGTGATT 5
              |||||||||||||||||||||||||||||||||| ||||||||||||||| |||||||||
CDC3 : 763558 TTTCTTCAATAACCGACTCATATGACTTAATTTTGACCTTGTGCCTTTGTTCTTGTGATT 763617

Query:      4 GGTT 1
              ||||
Sbjct: 763618 GGTT 763621
```

C

```
D8-U12:     167 CGNCTCGTCCTATCNATATAATCATTTCTTTCCTGTATGATCATCTTAATCTTATTCGTT 108
                || |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
YOR275C: 839517 CGCCTCGTCCTATCAATATAATCATTTCTTTCCTGTATGATCATCTTAATCTTATTCGTT 839576

D8-U12:     107 AGAGGATCATTAGTTTCCGGTAACCTGATCTCTGATCCAATCAAATTTTNATCGATGCTT 48
                |||||||||||||| ||||||||||||||||||||||||||||||||||| ||||||||||
YOR275C: 839577 AGAGGATCATTAGATTCCGGTAACCTGATCTCTGATCCAATCAAATTTTTATCGATGCTT 839636

D8-U12:      47 TGAAATAGTTCATTTGTTTGTTTGTCAATCNCACTACCTTCATCAAG 1
                |||||||||||||||||||||||||||||| ||||||||||||||||
YOR275C: 839637 TGAAATAGTTCATTTGTTTGTTTGTCAATCGCACTACCTTCATCAAG 839683
```

D

```
D8-U12:   261 CTTNCGTTCATTCAAGGTGCAAAGTGGCGAAGCTATCTTGGTCTTCAATTTGTCTACCTC 202
              ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CBP2:   24766 CTTTCGTTCATTCAAGGTGCAAAGTGGCGAAGCTATCTTGGTCTTCAATTTGTCTACCTC 24825

D8-U12:   201 AGTAACGAAAAATTCTCTATGTTTATCTACAAAGTAAGGGAAAATACCAATTCCATCGAA 142
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CBP2:   24826 AGTAACGAAAAATTCTCTATGTTTATCTACAAAGTAAGGGAAAATACCAATTCCATCGAA 24885

D8-U12:   141 ATCCCCAATCATCGCCTCATCGGAAACCTGGAAAAGAGGCAAGGGCTTTGCATTAGCTGG 82
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CBP2:   24886 TTCCCCAATCATCGCCTCATCGGAAACCTGGAAAAGAGGCAAGGGCTTTGCATTAGCTGG 24945
```

Figure 4.4 (continued overleaf)

```
D8-U12:     81 GTTTACAAACATGGGTACCTCACCATTATTTTGTTCCAAGAGTCTATCGTAAGTGCTCTT 22
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CBP2:    24946 GTTTACAAACATGGGTACCTCACCATTATTTTGTTCCAAGAGTCTATCGTAAGTGCTCTT 25005

D8-U12:     21 CTTAACCCNGATTCCCCGACA 1
               |||||||| ||||||||||||
CBP2:    25006 CTTAACCCCGATTCCCCGACA 25026
```

E

```
D2-U5:         1 CTAGAACCAATGAGTTATCNTAGCATCATAAATGGGCACATCAAATATGAAACTCCCCTA 60
                 |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
YGL059W: 393456 CTAGAACCAATGAGTTATCCTAGCATCATAAATGGGCACATCAAATATGAAACTCCCCTA 393515

D2-U5:        61 ATTGAATTGTTAAAGCGGTCTTTTAGATACAAGCTTGGGATTGGGTTAGCCATGTGTAAA 120
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
YGL059W: 393516 ATTGAATTGTTAAAGCGGTCTTTTAGATACAAGCTTGGGATTGGGTTAGCCATGTGTAAA 393575

D2-U5:       121 GTGTATGCTGAATATTGGAACGGCGACCTTTCATTGCATTCAATGCCTGGATATGGTACC 180
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
YGL059W: 393576 GTGTATGCTGAATATTGGAACGGCGACCTTTCATTGCATTCAATGCCTGGATATGGTACC 393635

D2-U5:       181 GATGTTGTATTAAAATTANGCAACTTGATG 210
                 |||||||||||||||||| ||||||||||
YGL059W: 393636 GATGTTGTATTAAAATTAGGCAACTTGATG 393665
```

F

```
D5-U1:         1 AGACAGTTCATTGTGGCAGATACTGCGATTGATAGATTGGTTTGGATCAGGAAATCAAAT 60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
YHL039W: 23430 AGACAGTTCATTGTGGCAGATACTGCGATTGATAGATTGGTTTGGATCAGGAAATCAAAT 23489

D5-U1:        61 AAAGAGCCTATATTTCTAATGAAGAAGGCATACGACTTACAGATTTAG 108
                 ||||||||||||||||||||||||||||||||||||||||||||||||
YHL039W: 23490 AAAGAGCCTATATTTCTAATGAAGAAGGCATACGACTTACAGATTTAG 23537
```

G

```
D4-U24:      130 AATGAACATTTTCTTCTATTTCTCCCTGTGAAGTTTTCATATAGAAAAAAACATAAGGGA 71
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
YDR504C: 1456391 AATGAACATTTTCTTCTATTTCTCCCTGTGAAGTTTTCATATAGAAAAAAACATAAGGGA 1456450

D4-U24:       70 AAGAAAATATTGATAATAGAAAGATAGTACCAAATTGAATTGGCAGGAAAATAGGTAAAC 11
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
YDR504C: 1456451 AAGAAAATATTGATAATAGAAAGATAGTACCAAATTGAATTGGCAGGAAAATAGGTAAAC 1456510

D4-U24:       10 AACGAACAGA 1
                 ||||||||||
YDR504C: 1456511 AACGAACAGA 1456520
```

Figure 4.4. Nucleotide sequence homology of the seven differentially regulated cDNAs identified using differential display. cDNA sequences were compared to *S. cerevisiae* genes using the BLAST algorithm in SGD; *SHY1* (A), *CDC3* (B), *YOR275C* (C), *CBP2* (D), *YGL059W* (E), *YHL039W* (F) and *YDR504C* (G). Positions of homology to each ORF are shown in bp and identical residues are indicated by "|".

Table 4.2. Gene functions of the differentially expressed putative ethanol-stress genes

Gene/ ORF	Primer pair	Gene Function[a]	Length of gene
CDC3	D9/U5	Cytokinesis and cell wall organization	1563bp
SHY1	D9/U5	Involved in respiration	1170 bp
YOR275C	D8/U12	Unknown function	1986 bp
CBP2	D8/U12	RNA splicing	1893 bp
YGL059W	D2/U5	Protein kinase activity, unknown function	1476 bp
YHL039W	D5/U1	Unknown function	1758 bp
YDR504C	D4/U24	Unknown function	348 bp

[a] Gene functions are listed according to the SGD and MIPS (http://mips.gsf.de) databases

Table 4.3. Gene specific oligonucleotide probes for Northern analysis

Primer name	Sequence
CDC3:	GGA TAG GAA CGG CCT CTC ACT TGA TTA CC
SHY1:	TCA ACA GCT TGC GTT CCG TTT ACA GCA GTC
YOR275C:	GCA GAA AGA CCG TCG ATT GAG ATA TCG GGC
CBP2:	CTT AAA CGC TTG CTT ACA GCG AGG GAA CTC
YGL059W:	GTT GGC TGT GTA TAC GAG GTG TTC CTC CAT
YHL039W:	CTT CGT TCT GAT TGG GC GGT CTG TAC CG
YDR504C:	GTG ACC ATG AAA CCA TGA TTG GGA GAC G
ACT1:	CGG TTT GCA TTT CTT GTT CGA AGT CCA AGG CGA CG

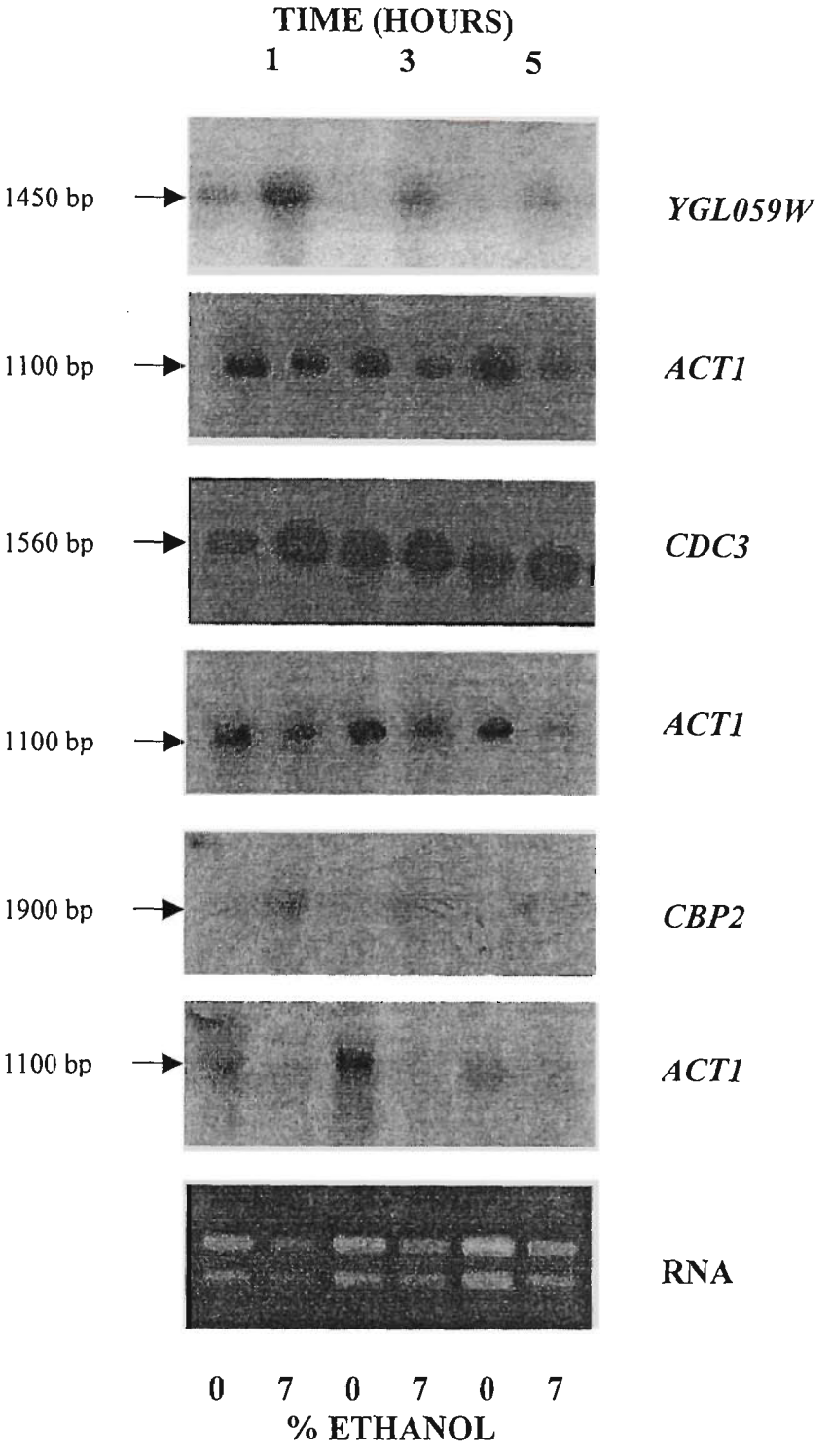


Figure 4.5. Northern analysis of *YGL059W, CDC3* and *CBP2* expression in ethanol-stressed and control cultures. RNA for Northern analysis was prepared from equal numbers of unstressed (0% ethanol) and stressed (7% v/v added ethanol) cells harvested at one, three and five hour intervals post-inoculation. Northern blots were probed with gene-specific oligonucleotide probes, stripped and re-probed with an *ACT1*-specific oligonucleotide probe. Up-regulation of *YGL059W, CDC3* and *CBP2* is observed in ethanol-stressed cells, confirming the differential display results. Representative RNA profile was used in the *CBP2* Northern. Similar RNA profiles were obtained from all RNA extractions for Northern analysis.

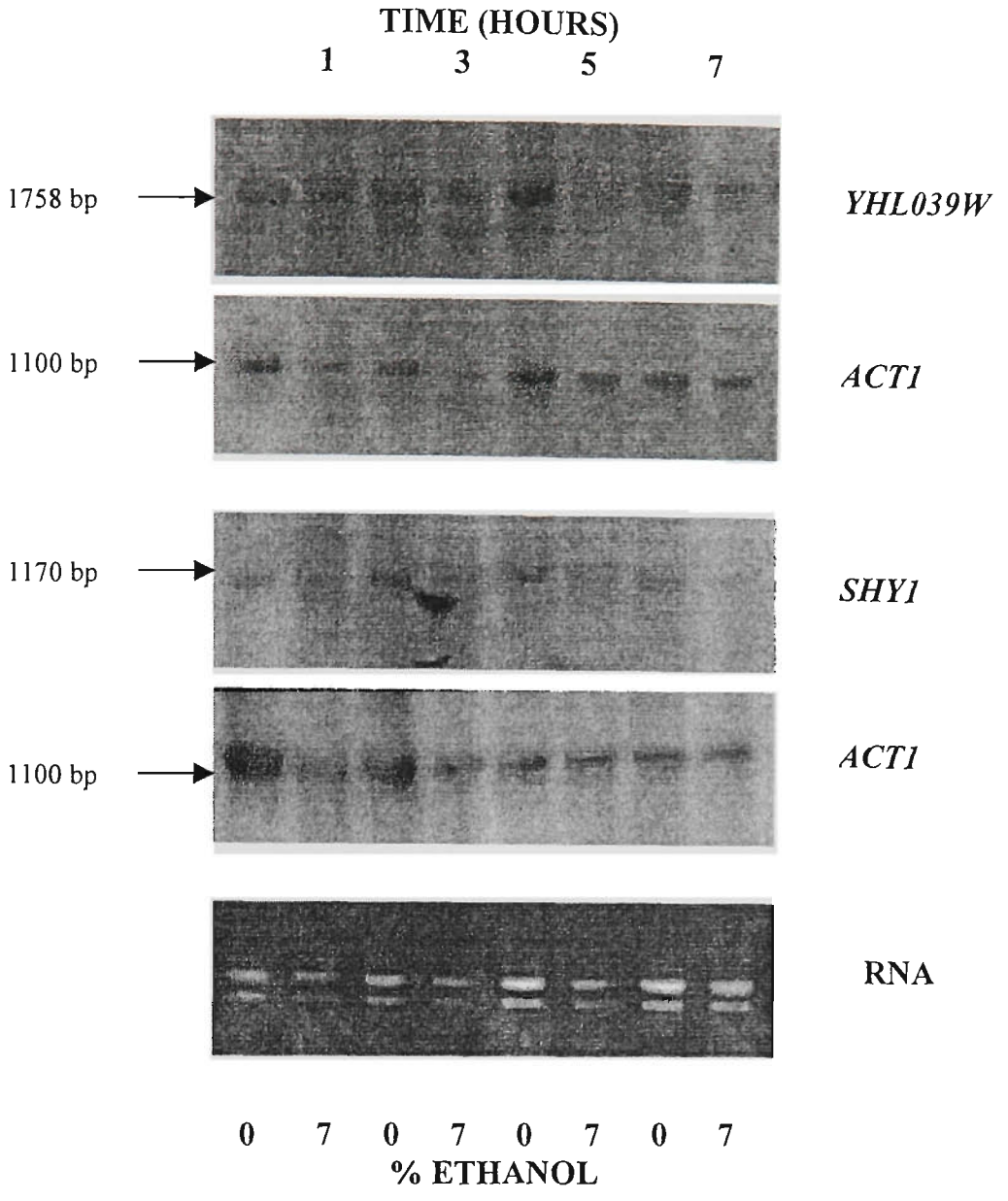


Figure 4.6. Northern analysis of *YHL039W* and *SHY1* expression in ethanol-stressed and control cultures. RNA for Northern analysis was prepared from equal numbers of unstressed control (no ethanol) and stressed (7% v/v added ethanol) cells harvested at one, three, five and seven hour intervals post-inoculation. Northern blots were probed with gene-specific oligonucleotide probes, stripped and re-probed with an *ACT1*-specific oligonucleotide probe. The expression of *YHL039W* and *SHY1* showed no great difference between the ethanol-stressed and control cells. Representative RNA profile was used in the *YHL039W* Northern. Similar RNA profiles were obtained from all RNA extractions for Northern analysis.

Confirmation of differential expression was obtained for only three of the seven putative ethanol-stress induced genes. These genes, *YGL059W, CDC3* and *CBP2* generated transcripts of expected sizes, being 1.5 Kb, 1.6 Kb and 1.9 Kb, respectively, and their up-regulation was confirmed for at least one time point by stronger signals for the ethanol-stressed samples relative to the control (no ethanol) samples (Figure 4.5). The Northern analysis was also successful in detecting two other transcripts, *YHL039W* and *SHY1*, of the expected sizes, 1.8 Kb and 1.2 Kb respectively. However, there was no difference in expression between the ethanol-stress and control samples, signifying *YHL039W* and *SHY1* were probably false positives generated from the differential display (Figure 4.6). The two other genes, *YOR275C* and *YDR504C*, could not be detected by Northern analysis. Stringencies of the wash conditions were lowered and the blots repeated, however, no hybridization signals could be generated.

4.4 DISCUSSION

Differential display was used to analyze gene expression, in the presence and absence of ethanol, in *S. cerevisiae* to identify genes up-regulated specifically in response to ethanol stress. The approach to this work used a modified differential display protocol developed by Emslie, (2002), based on the original method of Liang and Pardee (1992). The original protocol was modified by Emslie (2002) in an attempt to minimize the generation of false positive data and to simplify the overall processing of results.

False positives are results generated that cannot be subsequently validated and it has been reported that they can constitute more than 70% of all differential display data (Liang *et al.*, 1993; Nishio *et al.* 1994; Wan *et al.*, 1996). The technique developed by Emslie (2002) introduced three modifications to the traditional Liang and Pardee (1992) method to deal with this: equalizing cell number from control and test cultures instead of equalizing RNA concentration, adding an extra acid phenol extraction step to minimize the risk of carrying over contaminating DNA into reverse transcription reactions, and analyzing differential gene expression over a time-course rather than relying on a single time point.

RNA was isolated from equal cell numbers (approximately 10^8 cells) of control and ethanol-stressed cultures rather than equalizing RNA concentrations. This was undertaken as it was found by Emslie (2002) that the amount of RNA in ethanol-stressed cells was consistently reduced when compared to unstressed cells. It is clear from data presented in this chapter that this issue is pertinent to the work described here (see the bottom panels in figures 4.5 and 4.6). Equalizing total RNA concentrations from control and ethanol-stressed cells would thus artificially have boosted mRNA levels in stressed cultures, favoring the generation of false positive data.

DNA carryover in RNA samples has been identified as another major cause of spurious results in differential display, acting as a template in PCR reactions (Liang, 1996; Ivanova and Ivanov, 2002). An additional acid phenol precipitation was therefore also included in the RNA extraction method to remove any contaminating chromosomal DNA.

The analysis of gene expression profiles across a time-course was also aimed at minimizing the generation of spurious results; altered expression profiles across more than one time interval gives increased confidence in the validity of the result observed (Sompraynac *et al.*, 1995; Emslie, 2002). One can be more confident that bands up-regulated at two or more time intervals sampled during the experiment are ethanol stress-responsive genes.

4.4.1 Ethanol stress response genes identified from differential display

Seven putative ethanol-stress response genes were identified using the Emslie (2002) modified approach to differential display. Verification of these putative genes by Northern blot analysis only confirmed the up-regulation of three of these; *CDC3*, which encodes a septin protein involved in cytokinesis and cell wall organization (Longtine *et al.*, 1996; Field and Kellogg, 1999); *CBP2*, which is involved in RNA splicing (Lewin *et al.*, 1995); and *YGL059W*, encoding a protein kinase of unknown function (Tatusov *et al.*, 2000). The four other genes were not confirmed as ethanol-stress induced. For two of these genes, *YHL039W* and *SHY1*, Northern analysis

indicated the same levels of transcript were present in the stress and control cultures and thus represented false positives. However, the remaining two genes, *YOR275C* and *YDR504C*, were not detected in Northerns and thus could not be validated as real or dismissed as false positives. It is possible that these genes were differentially expressed but produce low abundance or rare mRNAs that cannot be detected by Northern analysis (Rodriguez-Vargas *et al.*, 2002; Wan *et al.*, 1996; Liang *et al.*, 1993). Further investigations are required to test this possibility. A more sensitive detection method such as Real-Time PCR may have provided better confirmation of the differential display genes, however this technology was not available at the time that this work was undertaken.

4.4.2 The generation of false positive results

While modifications to the differential display protocol were introduced to minimize the generation of false positives, it is clear that they did not prevent this problem. Previous analysis of the yeast ethanol stress response using this modified differential display protocol confirmed three positive ethanol stress response genes from a total of four sequenced differential display bands; only one of the band apparently up-regulated in differential display proved to be a false positive (Emslie, 2002). In the work described here, two of the seven putative up-regulated genes were false and two remain to be validated.

It has been suggested that some of the problems intrinsic to differential display, including the generation of false positive data, arise due to the low stringency PCR conditions required by the protocol (Bauer *et al.*, 1993). The rate of false positives has been reported to vary between experiments by some authors, with this being partly due to the sensitivity of the PCR and the susceptibility to variations in the quality of reagents and templates used (Liang, 1996). In addition, the use of differential display primers with two-base anchors, as those used in this work, have since been found to be sub-optimal, contributing to high instances of false positives. The recent development of one-base anchor primers has shown to improve the reliability of differential display (Liang, 2002).

4.4.3 Future analysis of the ethanol stress response in *S. cerevisiae*

While differential display is a powerful tool for identifying genes with altered expression profiles resulting from ethanol stress, the approach is very time consuming and the problems associated with generating false positives were challenging. However, at the time of performing Northern analysis to validate the differential display data a phosphorimage analyser became available at Victoria University, enabling the use of gene array technology to analyze global gene expression in a single experiment. Therefore, it was decided to leave the differential display at this point and continue with gene arrays to analyse the adaptation of *S. cerevisiae* to ethanol stress.

4.4.4 Concluding statement

The work described in this chapter identified, for the first time three genes, *YGL059W*, *CDC3* and *CBP2*, that were up-regulated under ethanol stress. However, further analysis of these genes was not pursued as the focus of ethanol-stress studies shifted to using gene arrays. It was anticipated that array analysis would further verify that the above three genes are up-regulated under ethanol stress. It would also test whether the apparent up-regulation of *YOR275C* and *YDR504C* (neither of which were detected by Northern analysis) were 'real' or not and identify other important ethanol stress-responsive genes.

CHAPTER 5

A GENOMIC APPROACH TO DEFINING THE ETHANOL STRESS RESPONSE IN *S. cerevisiae*

5.1 INTRODUCTION

The differential display analysis, described in the previous chapter, provided a means of analysing transcriptional changes associated with an adaptation to ethanol stress in yeast. However at the time of performing this work, the laboratory at VU purchased a phosphor-image analyser and yeast gene arrays became commercially available, making gene array analysis accessible. In light of this, it was decided to switch to this more powerful technique to analyse the ethanol stress response in *S. cerevisiae*.

The major advantage of using gene array technology over other approaches to studying changes in gene expression is that it allows the simultaneous analysis of essentially every gene in the genome at a given moment, providing a glimpse of genomic expression programs (Gasch *et al.*, 2000). The types of arrays commonly used in yeast research include DNA microarrays on glass slides or chips and filter arrays, also termed macro-arrays or gene arrays. In each case arrays consist of reproducible patterns of 'target' gene sequences spotted onto a solid support, being a chip, glass slide or nylon membrane. These target genes are either spotted as oligonucleotides or PCR products. To compare gene expression patterns of test and control cells, labelled mRNA (or, most commonly, cDNA made from this) is then hybridised to the array. The mRNA or cDNA to be probed is labelled either flourescently (usually with Cy5 or Cy3) for glass slides and chip arrays or radioactively (usually with ^{33}P) for the gene arrays. In more recent time whole proteome microarrays have been employed in yeast research (Michaud *et al.*, 2003). Whatever the experimental protocol employed, all array analyses have in common that they produce massive amounts of data (Burgess, 2001). This data can be analysed to identify expression patterns and variations that correlate with cellular physiology and function. Yeast Index Gene Filters (Research Genetics) were used in this study to

analyse global changes in gene expression following ethanol stress. These gene arrays comprise of two nylon membranes over which 6144 PCR amplified yeast open reading frames are spotted.

With the advent of array technology, *S. cerevisiae* gene expression has been studied under a wide variety of conditions, furthering our understanding of the regulation, coordination and extent of the different aspects of cellular responses to sub-optimal conditions. The effects of a wide variety of stresses and environmental changes, including heat shock (Gasch *et al.*, 2000; Causton *et al.*, 2001), ethanol shock (Alexandre *et al.*, 2001), pH extremes (Causton *et al.*, 2001; Kapteyn *et al.*, 2001; Lamb *et al.*, 2001), oxidative and reductive stress (Gasch *et al.*, 2000; Causton *et al.*, 2001; Travers *et al.*, 2000; Momose & Iwahashi, 2001); hyper-osmotic stress (Gasch *et al.*, 2000; Rep *et al.*, 2000; Posas *et al.*, 2000; Causton *et al.*, 2001; de Nobel *et al.*, 2001; Yale & Bohnert, 2001), starvation (DeRisi *et al.*, 1997; Gasch *et al.*, 2000; Jia *et al.*, 2000; Ogawa *et al.*, 2000a; Lyons *et al.*, 2000; Gross *et al.*, 2000; Natarajan *et al.*, 2001) and DNA damaging agents (Jelinsky & Samson, 1999; Gasch *et al.*, 2001; Natarajan *et al.*, 2001; De Sanctis *et al.*, 2001) have all been investigated.

While there have been several molecular investigations into the effects of ethanol on yeast, few have focused on the underlying genetic mechanisms that enable yeast cells to tolerate and adapt to this stress. One recent study, by Alexandre *et al.* (2001), used microarray analysis to study gene expression in ethanol shocked *S. cerevisiae.* The authors added ethanol to a mid-exponential phase culture, then sampled the yeast for gene expression analysis following a 30-minute exposure. This work identified a large number of up-regulated genes during the ethanol shock, many of which were associated with energy metabolism, ionic homeostasis, heat protection, antioxidant defense or trehalose synthesis. Although these findings provided broad new insights into the global response of *S. cerevisiae* to ethanol stress, especially in aspects of metabolism and stress protection mechanisms, no follow-up work has been published. Additionally the data in this publication could not be reconciled with the raw data on which it was based (lodged at http://www.transcriptome.ens.fr/ymgv/). It was subsequently acknowledged by the authors that there were some errors in their data that had not been taken into account (B. Blondin, personal communication).

To obtain a greater understanding of the adaptation of *S. cerevisiae* to ethanol stress the work of Alexandre *et al.* (2001) has been extended in this thesis by following gene expression over two time points, encompassing the yeast adaptation period to ethanol, enabling early and late genetic responses to be aligned with physiological responses. In addition, the work described used washed exponential phase cells inoculated into fresh defined medium and fresh defined medium containing added ethanol, to remove the influence of culture by-products on adaptation. This was in contrast to Alexandre *et al.* (2001), who inoculated unwashed mid-exponential phase cells into no ethanol and ethanol-containing medium. Furthermore, the traditional approach of equalizing RNA concentrations for gene array analysis was considered to be unsuitable in this experimental setting; as discussed in the previous chapter it has been found that the amount of total RNA in ethanol stressed cells is greatly reduced in comparison to unstressed cells (Figure 4.1). Equalized cell numbers, rather than equalized RNA concentrations were used for all gene array and Northern analyses so that levels of RNA from the stressed culture were not artificially boosted as would happen if the test and control culture samples were equalized for RNA content.

The aims of this chapter were:

1. To study the transcriptional response of *S. cerevisiae* to ethanol stress.

2. To compare early and late gene expression profiles during the lag phase adaptation period to the ethanol stress.

3. To gain an understanding of cellular adaptation mechanisms that enable yeast cells to adapt to ethanol stress.

5.2 CONDITIONS FOR INDUCING ETHANOL STRESS

Experiments on ethanol stress described in the previous chapter utilized 7% (v/v) ethanol, which induced a lag phase of around 5.5 hours. However, it was subsequently found that 7% (v/v) ethanol stress was too inhibitory at times, extending the lag phase

to greater than 5.5 hours[1] (data not shown). In light of this, a decrease in ethanol concentration to 5% (v/v) was introduced based on findings reported in Chapter 3. Thus late exponential phase PMY1.1 cells were washed and inoculated to an OD_{620} of 0.1 into defined medium and defined medium with the addition of 5% (v/v) ethanol. In these conditions the unstressed control culture had no detectable lag period, whereas 5% (v/v) ethanol-containing medium induced a lag phase adaptation period of 3 hours followed by exponential growth, with a specific growth rate 75% that of the unstressed control (Figure 5.1). The culture growth profiles of Figure 5.1 were as previously observed in Chapter 3 for cells under 5% (v/v) ethanol stress.

5.3 GLOBAL GENE EXPRESSION DURING ADAPTATION TO ETHANOL STRESS

5.3.1 Gene array analysis

Global gene expression during adaptation to ethanol stress was studied using Yeast Index Gene Filters (Research Genetics) that carry PCR-amplified open reading frames (ORFs) for 6144 yeast genes[2] spotted onto two nylon membranes. Gene array analyses were performed on RNA preparations from equal cell numbers of stressed and control cultures at two-time points; one and three hours post-inoculation which correspond to early and late stages in the adaptation to ethanol stress (see Figure 5.1).

Total RNA extracted from an equivalent of 10^8 cells, from one and three hour time points, from stress and control cultures was converted to ^{33}P-labeled single-stranded cDNA, and this was subsequently hybridized to the Gene Filters. To test reproducibility, hybridizations were performed twice with cDNAs from the same culture using different Gene Filters. Genes were regarded as up- or down-regulated

[1] It is important to note that the lag phase in growth curves of the ethanol stressed yeast used in experiments described in the previous chapter was 5.5 hours.

[2] At the time of undertaking this work 6144 genes was the estimated yeast gene count, however it is now estimated to stand at 5651 genes (Salzberg, 2003).

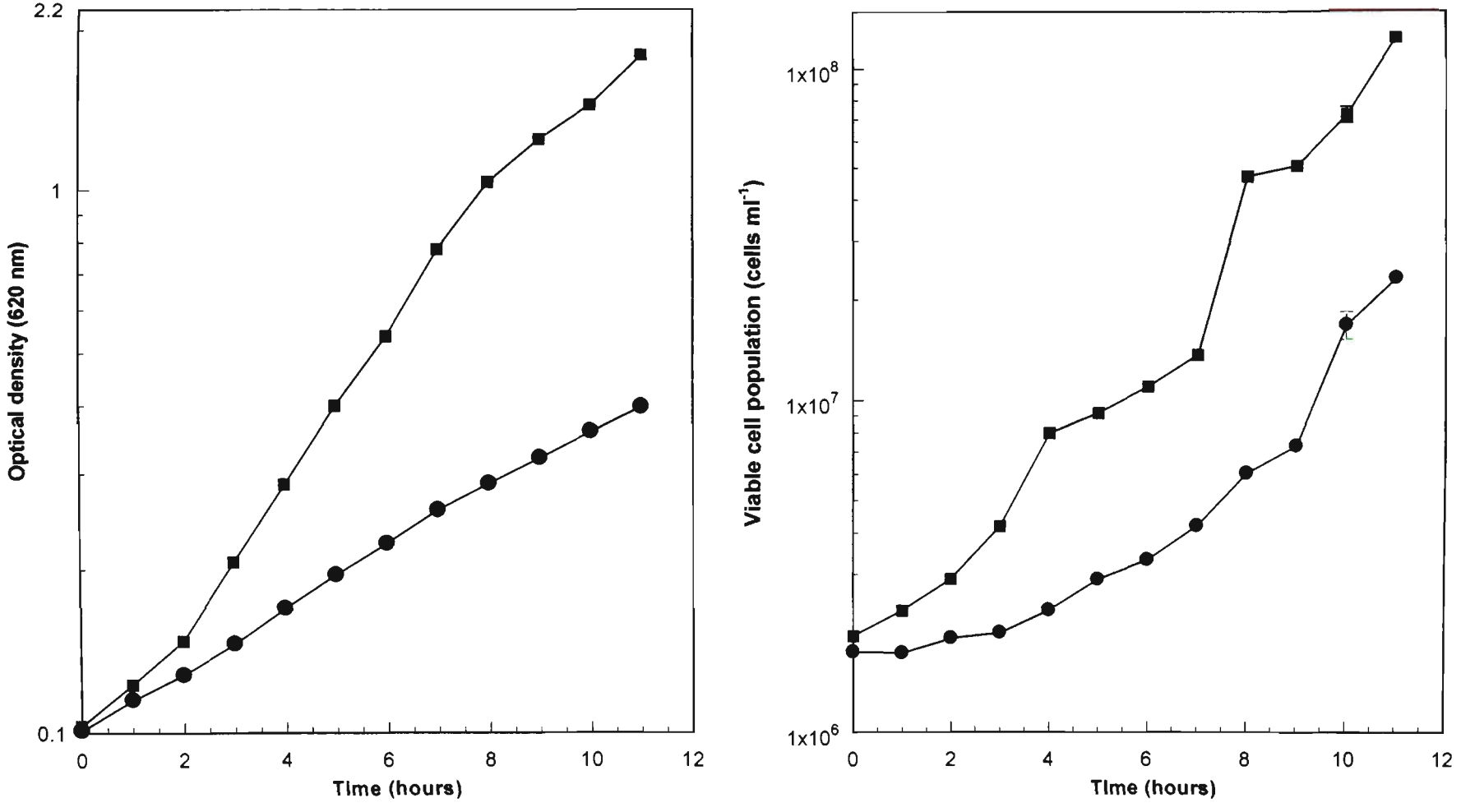


Figure 5.1. Effect of 5% (v/v) ethanol stress on the growth of *S. cerevisiae* PMY1.1. Cells from a late exponential phase culture were washed and inoculated into defined medium only (■) or defined medium containing 5% v/v (●) added ethanol. The cultures were incubated at 30°C/160 rpm.

when the difference in expression level between stressed and control cultures was reproducibly greater than three-fold, as adopted by other workers in the field (e.g. Rep *et al.*, 2000).

The probed Gene Filters were analysed using the ArrayGauge™ software (Fujifilm; version 1.21). Examples of expression profiles for control and stress cultures can be seen in Figure 5.2. All spot intensities were normalised to the intensity of genomic DNA control spots on the filters. Determining absolute changes in gene expression by normalising against a constantly expressed gene, such as *ACT1*, could not be undertaken due to its down-regulation in the stress samples; *ACT1* was down-regulated 1.2- and 1.7-fold at the one hour and three hour time points, respectively (data not shown). Comparisons of spot intensities for each time interval were calculated relative to the no-stress control.

Overall, gene array analysis revealed 94% of the ORFs represented on the Gene Filters showed no significant difference in expression levels between the stressed and control cultures following one hour of growth. One hundred genes were highly up-regulated during the initial ethanol stress whilst 271 genes were down-regulated in the same culture (these genes are listed in Tables 5.1 and 5.3). The magnitude of these differences in gene expression between stress and control cultures was however greatly reduced at the three-hour time point. Following three hours of ethanol stress, 98% of the ORFs represented on the Gene Filters showed no significant difference in expression levels between stress and control cultures, with only 14 genes up-regulated and 101 genes down-regulated (See Tables 5.2 and 5.4)

5.3.2 Up-regulated genes following one hour of ethanol stress

The 100 genes up-regulated by ethanol stress at the one hour time point were clustered into six functional classes: general stress response, energy utilization, transport, cell surface interactions, lipid metabolism, miscellaneous and genes of unknown function (Table 5.1). Descriptions of the gene products and cellular roles were taken from the SGD (http://www.yeastgenome.org) and MIPS (http://mips.gsf.de) databases.

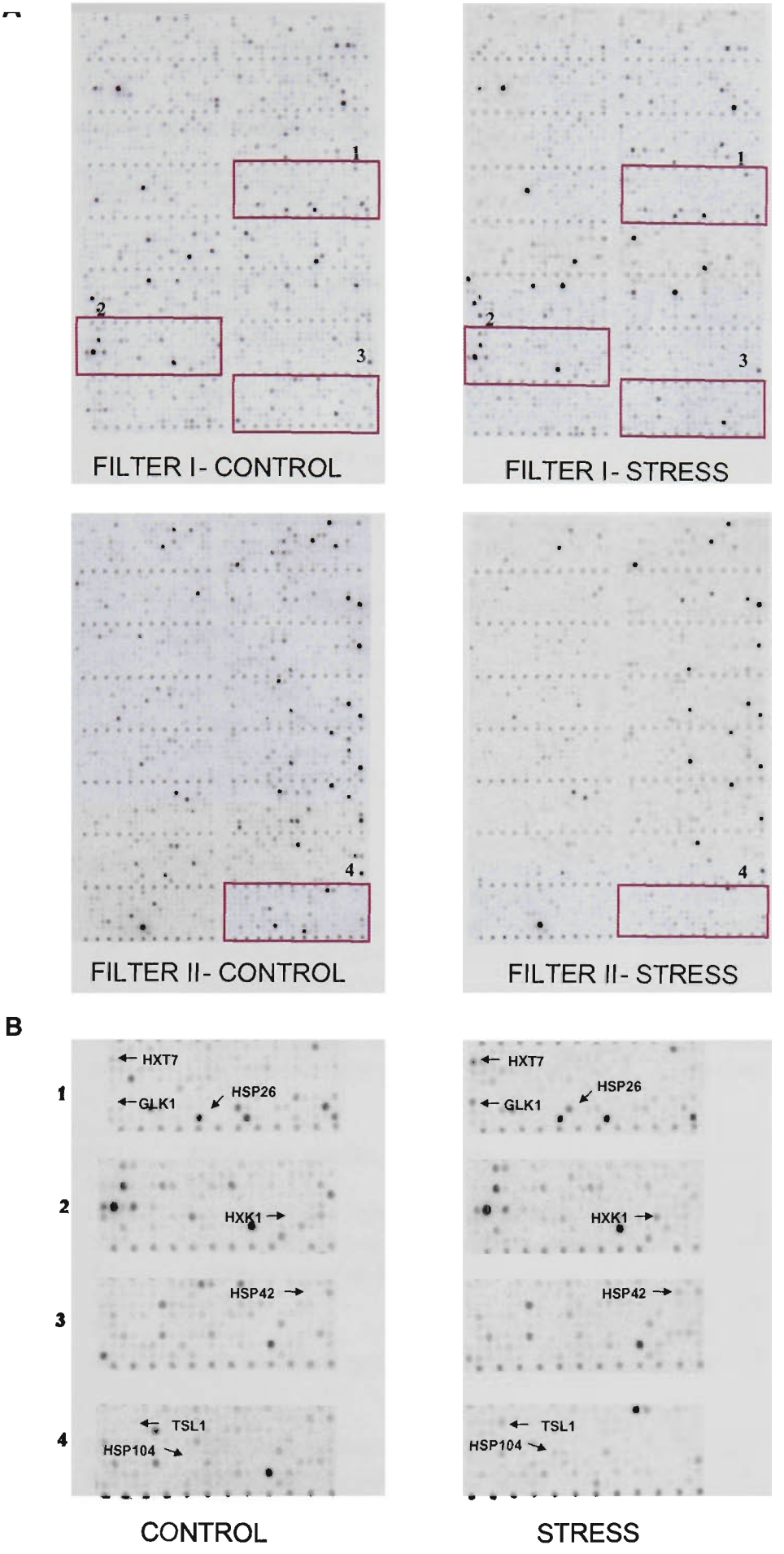


Figure 5.2. Expression profiles for control and ethanol-stressed cells from the Yeast Gene Filters. (A) Comparative expression profiles between control and stressed samples one hour following inoculation. (B) Enlarged sections of gene arrays as indicated by boxes in Panel A. Arrows indicate random genes that were more highly expressed in the stressed samples compared to the control.

Table 5.1
Genes up-regulated following one hour ethanol stress (5% v/v)

Gene name	Fold induction	Gene description	Putative transcription factors
Stress			
HSP26†#	67.8	Small HSP of unknown function	Hsf1p; Msn2/4p
YRO2	22.8	Homolog to *HSP30* heat shock protein Yro1p	Hsf1p; Msn2/4p
HSP42†#	12.9	HSP with similarity to Hsp26p	Hsf1p; Msn2/4p
HSP30†	10.9	HSP	None identified
HSP104†#	8.9	HSP	Hsf1p; Msn2/4p
HSP12†#	8.8	HSP	Msn2/4p
DDR2#	8.8	DNA Damage Responsive	Msn2/4p
SSE2	7.9	Chaperone of the *HSP70* family	Hsf1p; Msn2/4p; Yap1p
CTT1†#	7.2	Cytoplasmic catalase T	Msn2/4p
HOR7†#	5.5	Hyperosmolarity-responsive gene	Hsf1p; Msn2/4p
HSP78†#	5.3	HSP	Hsf1p; Msn2/4p
SOD2	5.2	Manganese-containing superoxide dismutase	Msn2/4p; Yap1p
GRX4	5.2	Glutaredoxin	Hsf1p
UBI4#	5.0	Ubiquitin, cytoplasmic	Hsf1p; Msn2/4p
GRX1	4.7	Glutaredoxin	Msn2/4p
TTR1#	4.0	Glutaredoxin, glutathione reductase	Hsf1p; Msn2/4p
GRE3†	3.5	Aldose reductase	Hsf1p; Msn2/4p
SSA4†#	3.2	HSP of the *HSP70* family, cytosolic	Hsf1p; Msn2/4p
Energy			
Trehalose, glycerol, glycogen metabolism			
TSL1†#	26.4	Subunit of trehalose synthase complex	Hsf1p; Msn2/4p
TPS1†#	11.5	Trehalose-6-phosphate synthase	Msn2/4p
TPS2†#	9.5	Trehalose-6-phosphate phosphatase	Msn2/4p
PGM2†#	6.4	Phosphoglucomutase	Hsf1p; Msn2/4p
UGP1†#	8.7	Uridinephosphoglucose pyrophosphorylase	Msn2/4p
DAK1†	3.4	Dihydroxyacetone kinase	None identified
GSY2#	6.5	UDP-glucose-starch glucosyltransferase	Msn2/4p
Glycolytic pathway			
GLK1†#	50.4	Aldohexose specific glucokinase	Msn2/4p
HXK1†#	34.9	Hexokinase I	Msn2/4p
TDH1†#	18.5	Glyceraldehyde-3-phosphate dehydrogenase 1	Msn2/4p
TDH3#	5.5	Glyceraldehyde-3-phosphate dehydrogenase 3	Msn2/4p
PGK1	7.5	3-phosphoglycerate kinase	Hsf1p; Msn2/4p
ENO1	5.0	Enolase I	Msn2/4p
ENO2	7.0	Enolase	Hsf1p
PDX1#	3.8	Pyruvate dehydrogenase complex	Msn2/4p
Others			
ALD4†#	28.8	Aldehyde dehydrogenase (NAD+)	Hsf1p; Msn2/4p
ADH5	8.7	Alcohol dehydrogenase isoenzyme V	Msn2/4p; Yap1p
PYC1†	5.8	Pyruvate carboxylase	Msn2/4p
CIT2†#	10.7	Citrate synthase, peroxisomal	Msn2/4p
SDH4	4.4	Succinate dehydrogenase membrane subunit	Hsf1p; Msn2/4p
YEL047C	3.7	Fumarate reductase (NADH)	Msn2/4p
MCR1†#	7.3	NADH-cytochrome b5 reductase	Msn2/4p
COX15	6.0	Cytochrome oxidase assembly factor	None identified

Gene name	Fold induction	Gene description	Putative transcription factors
Cell surface			
SPI1#	23.5	Similarity to Sed1 glycoprotein	Hsf1p; Msn2/4p
TIP1	7.3	Mannoprotein of the cell wall	None identified
SED1	5.2	Cell surface glycoprotein	Msn2/4p
YGP1†#	4.0	Secreted glycoprotein	Hsf1p; Msn2/4p
Lipid metabolism			
ETH1	5.4	Alcohol acyl transferase	Hsf1p
OPI3	6.5	Phospholipid-N-methyltransferase	Hsf1p; Msn2/4p
FAA1†#	4.6	Long chain fatty acyl:CoA synthetase	Msn2/4p
Amino acid metabolism			
ARG4	9.6	Argininosuccinate lyase	Msn2/4p; Yap1p
SER3	4.4	3-phosphoglycerate dehydrogenase	Hsf1p; Msn2/4p
UGA1	4.2	4-aminobutyrate aminotransferase	Hsf1p
Transport			
HXT7#	8.0	High affinity hexose transporter	Hsf1p; Msn2/4p
HXT6#	7.5	High affinity hexose transporter	Msn2/4p
AGP1#	12.3	Amino acid permease, amino acid transport	Msn2/4p
PTR2#	10.6	Transport of small peptides into the cell	Msn2/4p
MEP2	4.7	Ammonia transport protein, plasma membrane	None identified
DIP5	4.8	Dicarboxylic amino acid permease, transport	Msn2/4p
Miscellaneous			
PHD1#	11.1	Transcription factor, signal transduction	Hsf1p; Msn2/4p
YHR211W	9.1	Flocculin, similar to flocculation protein Flo1p	None identified
LAP4#	10.6	Vacuolar aminopeptidase *ysc1*	Msn2/4p
YAT2	5.7	Carnitine acetyltransferase	Hsf1p; Yap1p
HHT2#	5.7	Histone H3, chromatin assembly	Hsf1p;Msn2/4p
GSP2#	4.7	GTP binding protein	Hsf1p; Msn2/4p; Yap1p
PCL5	3.9	*PHO85* cyclin	Msn2/4p
PCT1	3.7	Phosphorylcholine transferase	Msn2/4p
Genes with unknown functions			
YCL042W†	54.4	Unknown function	Msn2/4p
YBR214W#	23.1	Unknown function	Msn2/4p
YGL037C†#	13.2	Unknown function	Hsf1p; Msn2/4p
YDR516C†#	12.5	Unknown function	Msn2/4p
YGR161C†#	10.8	Unknown function	Msn2/4p
YHL021C†#	10.5	Unknown function	Msn2/4p
YMR195W#	10.0	Unknown function	Msn2/4p
YKL044W#	9.4	Unknown function	Hsf1p; Msn2/4p
YER053C†	8.9	Unknown function	None identified
YGL117W	8.0	Unknown function	Yap1p
YDR533C	7.6	Unknown function	Msn2/4p
YER067W#	7.5	Unknown function	Hsf1p; Msn2/4p
YBR139W†#	7.5	Unknown function	Hsf1p; Msn2/4p
YJL016W†	7.1	Unknown function	Hsf1p
SSU1#	6.8	Unknown function, sensitive to sulphite	Msn2/4p
YKL151C†#	6.3	Unknown function	Msn2/4p
YOL119C	6.1	Unknown function	None identified
YOR285W#	5.7	Unknown function	Msn2/4p

Gene name	Fold induction	Gene description	Putative transcription factors
YDR133C#	5.6	Unknown function	Msn2/4p
YBL064C	5.5	Unknown function	Msn2/4p
MPM1#	5.3	Unknown function	Hsf1p; Msn2/4p
YBR287W#	5.4	Unknown function	Msn2/4p
YCR013C	5.3	Unknown function	None identified
YGR146C	5.0	Unknown function	None identified
COS8†	4.9	Unknown function	Msn2/4p
TOS1	4.5	Unknown function	None identified
YFL066C	4.4	Unknown function	None identified
YNL134C†#	4.3	Unknown function	Msn2/4p
YGL059W	4.1	Unknown function	Yap1p
COS6†	4.1	Unknown function	Hsf1p; Msn2/4p
YOR247W	4.1	Unknown function	None identified
COS7	3.8	Unknown function	Msn2/4p
ODH7	3.9	Unknown function	None identified
YHL050C	3.6	Unknown function	None identified
YBR147W	3.3	Unknown function	Msn2/4p; Yap1p

† Genes previously found to be up-regulated following a 30 minute ethanol shock (Alexandre *et al.*, 2001)
# Genes with multiple STRE elements in their promoter regions

Table 5.2

Genes up-regulated following three hours ethanol stress (5% v/v)

Gene name	Fold induction	Gene description	Putative transcription factors
Stress			
*YRO2**	4.5	Homolog to *HSP30* heat shock protein Yro1p	Hsf1p; Msn2/4p
Mitochondrial functions			
ALD4†#*	3.6	Aldehyde dehydrogenase (NAD+)	Hsf1p; Msn2/4p
Amino acid metabolism			
*ARG4**	3.4	Argininosuccinate lyase, arginine biosynthesis	Msn2/4p; Yap1p
YLR089C	3.3	Alanine aminotransferase	Msn2/4p
Vacuolar biogenesis and function			
CPS1	3.2	Vacuolar carboxypeptidase *yscS*	None identified
LAP4#*	3.0	Vacuolar aminopeptidase *ysc1*	Msn2/4p
Miscellaneous			
*PCL5**	4.5	*PHO85* cyclin	Msn2/4p
CUP1	3.7	Copper-binding metallothionein	None identified
DLD3	3.1	D-lactate dehydrogenase, lactate metabolism	Hsf1p
SSU1#*	3.0	Translation initiation factor 3 (eIF3)	Msn2/4p
FET3	3.0	Ferro-O2-oxidoreductase	None identified
Gene with unknown functions			
*YGL117W**	3.9	Unknown function	None identified
SNZ1	3.1	Unknown function	None identified
FIT2	3.1	Unknown function	Hsf1p/ Msn2/4p

† Genes previously found to be up-regulated following a 30 minute ethanol shock (Alexandre *et al.*, 2001)
* Genes also induced at one hour ethanol stress
# Genes with multiple STRE elements in their promoter regions

Table 5.3
Genes down-regulated following one hour ethanol stress (5%)

Gene name	Fold Repression	Gene Description
Ribosomal proteins		
RPL9A	13.8	Ribosomal protein L9A
RPL32	10.4	Ribosomal protein L32
RPL16A†	9.8	Ribosomal protein L16A
RPL15A	9.4	Ribosomal protein L15A
RPS8B†	9.3	Ribosomal protein S8B
RPS17B†	9.3	Ribosomal protein S17BB
RPL20B†	9.0	Ribosomal protein L20B
RPS11B	8.7	Ribosomal protein S11B
YST1†	8.4	Ribosomal protein S0A
RPS26A†	8.4	Ribosomal protein S26A
RPS1A†	8.3	Ribosomal protein S1A
RPS16B†	8.3	Ribosomal protein S16B
RPL31A†	8.1	Ribosomal protein L31A
RPL24A†	8.1	Ribosomal protein L24A
RPS11A	8.0	Ribosomal protein S11A
RPL17B†	8.0	Ribosomal protein L17B
RPS18A†	8.0	Ribosomal protein S18A
RPL25†	7.9	Ribosomal protein L25
RPS7A†	7.9	Ribosomal protein S7A
RPL1B†	7.8	Ribosomal protein L1B
RPL35B	7.8	Ribosomal protein L35B
RPL40B	7.8	Ribosomal protein L40B
RPL11B†	7.8	Ribosomal protein L11B
RPS18B†	7.8	Ribosomal protein S18B
RPL17A†	7.7	Ribosomal protein L17A
RPL24B	7.6	Ribosomal protein L24B
RPS1B†	7.3	Ribosomal protein S1B
RPL21A	7.3	Ribosomal protein L21A
RPL21B	7.2	Ribosomal protein L21B
RPS10A†	7.2	Ribosomal protein S10A
RPL19A	7.2	Ribosomal protein L19A
RPS8A	7.1	Ribosomal protein S8A
RPS23A	7.1	Ribosomal protein S23A
RPS5	7.0	Ribosomal protein S5
RPS17A	7.0	Ribosomal protein S17A
RPL13A	6.9	Ribosomal protein L13A
RPS19A	6.9	Ribosomal protein S19A
RPL12B†	6.8	Ribosomal protein L12B
RPS28A†	6.7	Ribosomal protein S28A
RPL38†	6.7	Ribosomal protein L38
RPL39	6.6	Ribosomal protein L39
RPL27B†	6.5	Ribosomal protein L27B
RPL27A†	6.5	Ribosomal protein L27A
RPL33B	6.5	Ribosomal protein L33B
RPL18A†	6.5	Ribosomal protein L18A
RPS0B†	6.4	Ribosomal protein S0B
RPL34B†	6.4	Ribosomal protein L34B
RPS25A	6.4	Ribosomal protein S25A
RPS4A	6.3	Ribosomal protein S4A

Gene name	Fold Repression	Gene Description
RPS21B†	6.1	Ribosomal protein S21B
RPS2	6.0	Ribosomal protein S2
RPL34A†	6.0	Ribosomal protein L34A
RPL8B†	5.9	Ribosomal protein L8B
RPL35A†	5.8	Ribosomal protein L35A
RPS20	5.8	Ribosomal protein S20
RPL1A	5.7	Ribosomal protein L1A
RPL15B	5.7	Ribosomal protein L15B
RPS31	5.6	Ribosomal protein S31
RPS9B	5.5	Ribosomal protein S9B
RPL37B†	5.4	Ribosomal protein L37B
RPP0	5.3	Ribosomal protein P0
RPL22A†	5.2	Ribosomal protein L22A
RPS15†	5.2	Ribosomal protein S15
RPP2A	5.1	Ribosomal protein P2A
RPS24B†	5.0	Ribosomal protein S24B
RPL5	5.0	Ribosomal protein L5
RPL42B†	5.0	Ribosomal protein L42B
RPL7A	4.9	Ribosomal protein L7A
RPS16A	4.8	Ribosomal protein S16A
RPL19B†	4.8	Ribosomal protein L19B
RPS29B†	4.8	Ribosomal protein S29B
RPS14A	4.7	Ribosomal protein S14A
RPS26B†	4.6	Ribosomal protein S26B
RPL20A	4.5	Ribosomal protein L20A
RPL2B	4.5	Ribosomal protein L2B
RPL8A	4.5	Ribosomal protein L8A
RPS13	4.4	Ribosomal protein S13
RPL23B†	4.3	Ribosomal protein L23B
RPL14A†	4.3	Ribosomal Protein L14A
RPL14B†	4.2	Ribosomal protein L14B
RPL2A†	4.1	Ribosomal protein L2A
RPL10	4.1	Ribosomal protein L10
RPL30	3.9	Ribosomal protein L30
RPL42A†	3.9	Ribosomal protein L42A
RPS6A	3.9	Ribosomal protein S6A
RPS22A†	3.8	Ribosomal protein S22A
RPL7B	3.8	Ribosomal protein L7B
RPL6A†	3.8	Ribosomal protein L6A
RPS29A	3.6	Ribosomal protein S29A
RPL26A†	3.6	Ribosomal protein L26A
RPS4B	3.5	Ribosomal protein S4B
RPL33A†	3.5	Ribosomal protein L33A
RPL13B	3.4	Ribosomal protein L13B
RPL3	3.3	Ribosomal protein L3
RPP1B	3.3	Ribosomal protein P1B
RPS27A†	3.3	Ribosomal protein S27A
RPS24A†	3.1	Ribosomal protein S24A
RPL40A	3.1	Ribosomal protein L40A

Gene name	Fold Repression	Gene Description
Protein synthesis, translocation, modification, degradation and complex assembly		
EFB1	15.4	Translation elongation factor
SSZ1	10.4	Protein chaperone *HSP70* family
SSB1	10.2	Protein chaperone *HSP70* family
KAP123	8.8	Protein carrier activity
KAR4	8.2	Transcription factor
YLA1†	7.8	tRNA processing
YGR103W†	7.5	60S ribosomal subunit, biogenesis
VAS1	6.1	valine-tRNA ligase
TRM82†	6.0	Transfer RNA methyltransferase
SSB2	5.5	Heat shock protein of *HSP70* family
YEF3	5.1	Translation elongation factor 3 (EF-3)
CDC33†	4.9	Translation initiation factor eIF4E
HMT1†	4.7	Arginine methyltransferase
TIF2	4.7	Translation initiation factor eIF4A
THS1	4.6	Threonine-tRNA ligase
MAK21†	4.4	60s ribosome biogenesis
EFT2	4.4	Translation elongation factor 2 (EF-2)
CIC1	4.2	Protein binding activity, bridging
TRA1	4.2	Regulation of transcription from Pol II promoter
GRS1	4.1	glycine-tRNA ligase
GCD2	4.0	Translation initiation factor eIF2B subunit
ZUO1	4.0	Protein folding
SOL3	4.0	tRNA processing
YCL059C†	4.0	Ribosome biogenesis
BRX1†	3.9	5S RNA binding activity
PPT1†	3.8	Protein serine/threonine phosphatase activity
SUI3	3.8	Translation initiation factor eIF-2 beta subunit
TIF1	3.7	Translation initiation factor eIF4A
YIR012W	3.6	60S ribosomal subunit assembly
YOR056C†	3.5	Protein complex assembly
SES1	3.5	Serine-tRNA ligase
KRS1	3.4	Lysine-tRNA ligase
YGL105W	3.4	tRNA binding activity
SUP35	3.4	Translation termination factor eRF3
ARX1	3.4	Ribosomal large subunit
GCD6	3.4	Translation initiation factor eIF-2B epsilon subunit
SRO9	3.2	Associates with translating ribosomes
CNS1	3.1	Component of the *Hsp90* chaperone complex
SXM1	3.1	Protein carrier activity
YDR429C	3.1	Translation initiation factor 3 p33 subunit
EFT1	3.1	Translation elongation factor 2 (EF-2)
RNA synthesis, processing/modification, splicing and turnover		
NOP13†	8.1	Nucleolar Protein 13
SIK1†	6.3	Processing of 20S pre-rRNA
HCA4	5.5	ATP dependent RNA helicase activity
CBF5†	5.3	rRNA modification
NHP2	5.1	rRNA modification
SNU13	4.5	Processing of 20S pre-rRNA
DBP5	4.5	RNA helicase
NOP5	4.4	Processing of 20S pre-rRNA

Gene name	Fold Repression	Gene Description
NOP1	4.2	rRNA modification
RPA34†	4.0	RNA polymerase I subunit
TEF4	3.9	Translation elongation factor EF-1gamma
NOP15†	3.8	Ribosome biogenesis
EGD1	3.8	Pol II transcribed genes regulator
EPB2†	3.7	rRNA processing
NOP2	3.4	RNA methyltransferase activity
UTP4	3.4	Processing of 20S pre-rRNA
YDL148C†	3.4	Processing of 20S pre-rRNA
GSP1	3.3	rRNA processing
UTP6†	3.2	Processing of 20S pre-rRNA
ERB1	3.2	rRNA processing
YJL050W	3.2	RNA helicase
RRP8	3.1	Required for processing pre-ribosomal RNA
PAB1	3.1	Poly(A) binding protein
RPB8	3.1	RNA polymerase subunit
Amino acid metabolism/biosynthesis		
LYS9†	23.4	Lysine biosynthesis
LEU1	12.2	Isopropylmalate isomerase
YIL094C	11.3	Isocitrate dehydrogenase
AAH1†	9.9	Adenine catabolism
SAM1†	8.1	S-adenosylmethionine synthetase
MET6†	8.0	Methionine metabolism
MET3	7.6	Sulfate adenylyltransferase activity
MET25	5.7	Methionine metabolism
MET10	5.6	Sulfur amino acid biosynthesis
CYS3	5.6	Sulfur amino acid metabolism
ARO2	5.4	Aromatic amino acid biosynthesis
SRM1	5.4	Acetolactate synthase
THR4	4.9	Threonine synthase
SHM2	4.9	Serine hydroxymethyltransferase
SER1	4.0	Phosphoserine transaminase
ILV5	3.9	Branched-chain amino acid biosynthesis
TWT1	3.8	Branched-chain amino acid transaminase
SAM2†	3.6	Methionine biosynthesis regulation
HOM2	3.1	Threonine and methionine biosynthesis
MET14	3.0	Adenylylsulfate kinase
Nucleotide metabolism		
URA7†	8.9	Pyrimidine biosynthesis
ADE17	8.1	Biosynthesis of purine nucleotides
URA1	5.3	Pyrimidine base biosynthesis
FUR1	4.5	Regulation of the pyrimidine salvage pathway
YLR432W	3.8	Biosynthesis of purine nucleotides
GUA1	3.8	Biosynthesis of purine nucleotides
HPT1†	3.7	Purine nucleotide biosynthesis
URA4	3.6	Pyrimidine nucleotide biosynthesis
GUK1†	3.6	Guanylate kinase
YJR105W	3.3	Purine base metabolism
URA5†	3.1	Pyrimidine base biosynthesis

Gene name	Fold Repression	Gene Description
Hexose transporters		
HXT2	18.5	Moderate affinity hexose transporter-2
HXT1	6.6	Low-affinity hexose (glucose) transporter
Small molecule transporters		
CTP1	6.5	Mitochondrial citrate transport
OAC1	3.7	Oxaloacetate transport protein
PHO3	3.5	Acid phosphatase
ARF1	3.4	Implicated in intracellular protein transport
PDR5	3.4	Multidrug resistance transporter
NTF2	3.3	Nuclear transport factor
YDR091C	3.2	Putative ATP-binding cassette transporter activity
PMA1	3.0	Proton transport, pH regulation
Cell wall organization		
UTR2	4.0	Cell wall organization and biogenesis
Lipid metabolism		
ERG25	5.0	C4 sterol methyl oxidase; synthesis of ergosterol
OLE1	4.3	Stearoyl-CoA desaturase; unsaturated fatty acid synthesis
ERG3†	3.9	C5 sterol desaturase; synthesis of ergosterol
Cell cycle		
HSL7	5.1	G2/M transition of mitotic cell cycle
CDC33†	4.9	Regulation of cell cycle
YBR158W	4.8	Involved in exit from mitosis
SAP4	3.6	G1/S transition of mitotic cell cycle
FAR1	3.4	Cell cycle arrest protein
Cytoskeleton		
YTM1	4.3	Microtubule-associated protein
CCT6	3.8	Cytoskeleton organization and biogenesis
Cellular response to stress		
ATC1	3.6	Involved in protein cation homeostasis
TRR1	3.1	Thioredoxin reductase, regulation of redox homeostasis
Mating		
SAG1	41.6	Cell adhesion receptor activity
MF(ALPHA)2	26.6	Alpha mating factor
AGA1	13.7	Cell adhesion receptor activity
STE3	7.3	a-factor mating receptor
MF(ALPHA)1	5.9	Alpha mating factor
FUS1	5.0	Cell-surface protein required for cell fusion
FIG2	3.5	GPI-anchored cell wall protein (putative)
Carbohydrate metabolism		
ALD6	14.2	Aldehyde dehydrogenase
Miscellaneous		
MPT4	5.6	Nuclear telomere cap complex
GCV1	4.6	Glycine metabolism
YKL029C	3.8	Mitochondrial malic enzyme
QCR9	3.8	Cytochrome c oxidoreductase complex

Gene name	Fold Repression	Gene Description
NSP1	3.8	Nuclear pore complex subunit
RKI1†	3.6	Ribose-5-phosphate isomerase activity
Unknowns		
YDL228C	8.8	Unknown
YOL109W	8.2	Unknown
YLL044W†	7.6	Unknown
YMR116C†	7.2	Unknown
YBL109W	6.5	Unknown
YDR442W	6.3	Unknown
YER006W†	6.3	Unknown
YGL131C	6.3	Unknown
YLR062C	6.0	Unknown
YML056C	5.7	Unknown
YNL174W†	5.6	Unknown
YLR339C	5.6	Unknown
YPL197C	5.5	Unknown
YDR361C	5.5	Unknown
YLR302C	5.0	Unknown
YMR290C	4.8	Unknown
YDR496C†	4.6	Unknown
YDR544C	4.6	Unknown
YJL188C	4.6	Unknown
YPL142C	4.6	Unknown
YLR196W†	4.3	Unknown
YKL056C	4.3	Unknown
YPR044C	4.3	Unknown
YNL338W	4.2	Unknown
YEL001C	4.2	Unknown
YDR346C	4.1	Unknown
YGL102C†	4.1	Unknown
YJL200C	4.0	Unknown
YJR115W	3.9	Unknown
YJL069C	3.7	Unknown
YAL036C†	3.7	Unknown
YGL139W	3.6	Unknown
YDR545W	3.6	Unknown
YLR076C†	3.6	Unknown
SNZ1	3.5	Unknown
YGR079W	3.3	Unknown
YOR051C†	3.3	Unknown
YCR016W	3.3	Unknown
YOR271C	3.3	Unknown
YKR071C	3.2	Unknown
YDR458C	3.2	Unknown
YIL127C†	3.1	Unknown
YGL072C	3.1	Unknown

† Genes previously found to be repressed following a 30 minute ethanol shock (Alexandre *et al.*, 2001)

Table 5.4
Genes down-regulated following three hours ethanol stress (5%)

Gene	Fold Repression	Gene description
Ribosomal proteins		
*RPL40A**	6.4	Ribosomal protein L40A
RPS27B	5.9	Ribosomal protein S27B
*RPL20B**	5.8	Ribosomal protein L20B
*RPS18A**	5.2	Ribosomal protein S18A
RPS7A†**	4.9	Ribosomal protein S7A
RPS16B[a]**	4.7	Ribosomal protein S16B
*RPL32**	4.7	Ribosomal protein L32
RPS0A†**	4.6	Ribosomal protein S0A
RPL25†**	4.6	Ribosomal protein L25
RPS26A†**	4.5	Ribosomal protein S26A
*RPL13A**	4.4	Ribosomal protein L13A
*RPL20A**	4.3	Ribosomal protein L20A
*RPS17A**	4.2	Ribosomal protein S17A
RPL11B†**	4.1	Ribosomal protein L11B
RPS22A†**	4.1	Ribosomal protein S22A
RPL2A†**	4.0	Ribosomal protein L2A
RPL16A†**	4.0	Ribosomal protein L16A
RPL33A†**	4.0	Ribosomal protein L33A
RPL37A†	4.0	Ribosomal protein L37A
*RPL21A**	3.9	Ribosomal protein L21A
*RPL24A**	3.9	Ribosomal protein L24A
RPS26B†**	3.8	Ribosomal protein S26B
RPS29B†**	3.6	Ribosomal protein S29B
RPS15†**	3.6	Ribosomal protein S15
*RPL39**	3.6	Ribosomal protein L39
RPS28A†**	3.6	Ribosomal protein S28A
RPL43A†**	3.5	Ribosomal protein L43A
*RPS18B**	3.5	Ribosomal protein S18B
*RPS9B**	3.5	Ribosomal protein S9B
*RPL40B**	3.5	Ribosomal protein L40B
*RPS8A**	3.4	Ribosomal protein S8A
*RPL32**	3.4	Ribosomal protein L32
*RPL5**	3.4	Ribosomal protein L5
RPL8B†**	3.4	Ribosomal protein L8B
*RPS31**	3.4	Ribosomal protein S31
RPL18A†**	3.3	Ribosomal protein L18A
RPL35A†**	3.2	Ribosomal protein L35A
RPS8B†**	3.2	Ribosomal protein S8B
RPS18B†**	3.2	Ribosomal protein S18B
*RPS4A**	3.2	Ribosomal protein S4A
*RPS5**	3.2	Ribosomal protein S5
*RPL22A**	3.2	Ribosomal Protein L22A
*RPL24B**	3.1	Ribosomal protein L24B
RPL41B†**	3.1	Ribosomal protein L41B
RPL15A†**	3.1	Ribosomal protein L15A
*RPL13A**	3.1	Ribosomal protein L13A
*RPS16A**	3.1	Ribosomal protein S16A
RPS1B†**	3.0	Ribosomal protein S1B
RPL17B†**	3.0	Ribosomal protein L17B

Gene	Fold Repression	Gene description
Protein synthesis, translocation, modification, degredation and complex assembly		
*SOL3**	8.2	tRNA processing
MIG2	7.5	RNA polymerase II transcription factor
TOM1	4.7	Ubiquitin-protein ligase activity
EGD2	4.4	GAL4 enhancer protein
SUA7	3.7	Transcription factor
PNO1†	3.2	Protein complex assembly
CPH1	3.1	Cyclophilin peptidyl-prolyl cis-trans isomerase
*SSB2**	3.1	Heat shock protein of HSP70 family
Transport		
*HXT2**	7.6	High affinity hexose transporter-2
*PDR5**	5.9	Multidrug resistance transporter
*HXT1**	4.9	Low-affinity hexose (glucose) transporter
ITR1	4.5	Myo-inositol transporter
ZRT2	3.9	Low-affinity zinc transport protein
HNM1	3.8	Choline transport protein
Amino acid metabolism		
GAP1	8.2	General amino acid permease
*TWT1**	3.7	Branched-chain amino acid transaminase
*LEU1**	3.4	Isopropylmalate isomerase
GDH1	3.4	Glutamate dehydrogenase
Nucleotide metabolism		
*YJR105W**	6.2	Purine base metabolism
*FUR1**	3.1	Regulation of the pyrimidine salvage pathway
RNR2	3.1	Small subunit of ribonucleotide reductase
Mating		
AGA1	5.4	Cell adhesion receptor activity
*MF(ALPHA)2**	4.4	Alpha mating factor
SAG1	3.9	Cell adhesion receptor activity
Cell wall		
*UTR2**	5.2	Cell wall organization and biogenesis
Cytoskeleton		
ABP140	3.8	Actin filament binding protein
NDC1	3.5	Structural constituent of cytoskeleton
STM1	3.3	Telomere maintenance
Carbohydrate metabolism		
PDC1	10.1	Pyruvate decarboxylase
*ALD6**	8.8	Aldehyde dehydrogenase
PDC5	7.7	Pyruvate decarboxylase
TDH3	4.2	Glyceraldehyde-3-phosphate dehydrogenase 3
GND1	3.9	6-phosphogluconate dehydrogenase
Lipid metabolism		
*OLE1**	19.7	Stearoyl-CoA desaturase activity
ERG2	3.4	Sterol biosynthesis

Gene	Fold Repression	Gene description
Miscellaneous		
RIB4	4.4	Vitamin B2 biosynthesis
HRP1	3.6	mRNA cleavage factor complex
Unknown		
YDL038C	6.7	Unknown
YDR417C	4.4	Unknown
*YBL109W**	4.0	Unknown
*YJL188C**	4.0	Unknown
RTN1	3.8	Unknown
YNR021W	3.8	Unknown
YOR263C	3.6	Unknown
*YMR116C†**	3.6	Unknown
*YLR062C**	3.5	Unknown
*YGL102C†**	3.3	Unknown
YCR013C	3.2	Unknown
YKR075C	3.2	Unknown
*YOL109W**	3.1	Unknown

† Genes previously found to be repressed following a 30 minute ethanol shock (Alexandre *et al.*, 2001)
* Genes also down-regulated following one hour ethanol stress

Of the stress response genes that were up-regulated following one hour of stress, six encoded heat shock proteins (HSPs). These HSPs included *HSP26*, *HSP42*, *HSP30*, *HSP104*, *HSP12* and *HSP78*. The induction of these HSP genes has been reported previously for yeast cells under several stress conditions including ethanol stress (Piper *et al.*, 1994; Piper, 1995; Alexandre *et al.*, 2001). It was also found that *SSA4* and *SSE2*, which belong to a group of genes that encode members of the *HSP70* family, were up-regulated by ethanol stress. In addition the ethanol-induced expression of *YRO2*, a homolog of the *HSP30* (*YRO1*) gene was established. Several oxidative-stress response genes including *CTT1, SOD2, GRX1, GRX4* and *TTR1* were also up-regulated, as were genes known to be involved in osmotic stress responses, including *HOR7* and *GRE3*.

Many genes involved in energy utilization were up-regulated in response to ethanol stress (Figure 5.3). Transcript levels of many glycolytic and TCA cycle genes were highly up-regulated as were the genes involved in trehalose and glycogen metabolism. Two genes encoding plasma membrane high affinity hexose transporters, *HXT6* and *HXT7*, showed an increase in expression, as did two genes encoding glucose-phosphorylating enzymes, *GLK1* and *HXK1*. The level of expression of *GLK1* and *HXK1* increased 50.4-fold and 34.9-fold respectively. Interestingly, *HXT6* and *HXT7* are usually expressed under conditions of low glucose (Luyten *et al.*, 2002; Ye *et al.*, 2001; Reifenberger *et al.*, 1997) and this will be discussed later (see section 5.4.1).

The trehalose metabolism genes, *TPS1, TPS2, TSL1, UGP1* and *PGM2* were all up-regulated at the one hour time point. This is consistent with the findings of other groups who report trehalose accumulation during ethanol stress (Mansure *et al.*, 1994; Mansure *et al.*, 1997). In contrast to other ethanol exposure studies (Alexandre *et al.*, 2001; Ogawa *et al.*, 2000 a), it was found that the neutral trehalose gene *NTH1*, involved in trehalose degradation was not up-regulated, at least not above the experimental cut-off of three-fold. Up-regulation of the glycogen synthase gene, *GSY2* was also noted. Only one gene involved in glycerol metabolism was up-regulated. This gene, *DAK1*, was up-regulated 3.4-fold, and is involved in glycerol catabolism. In contrast to the findings of Alexandre *et al.* (2001) no glycerol synthase genes were found to be up-regulated following one hour of ethanol stress.

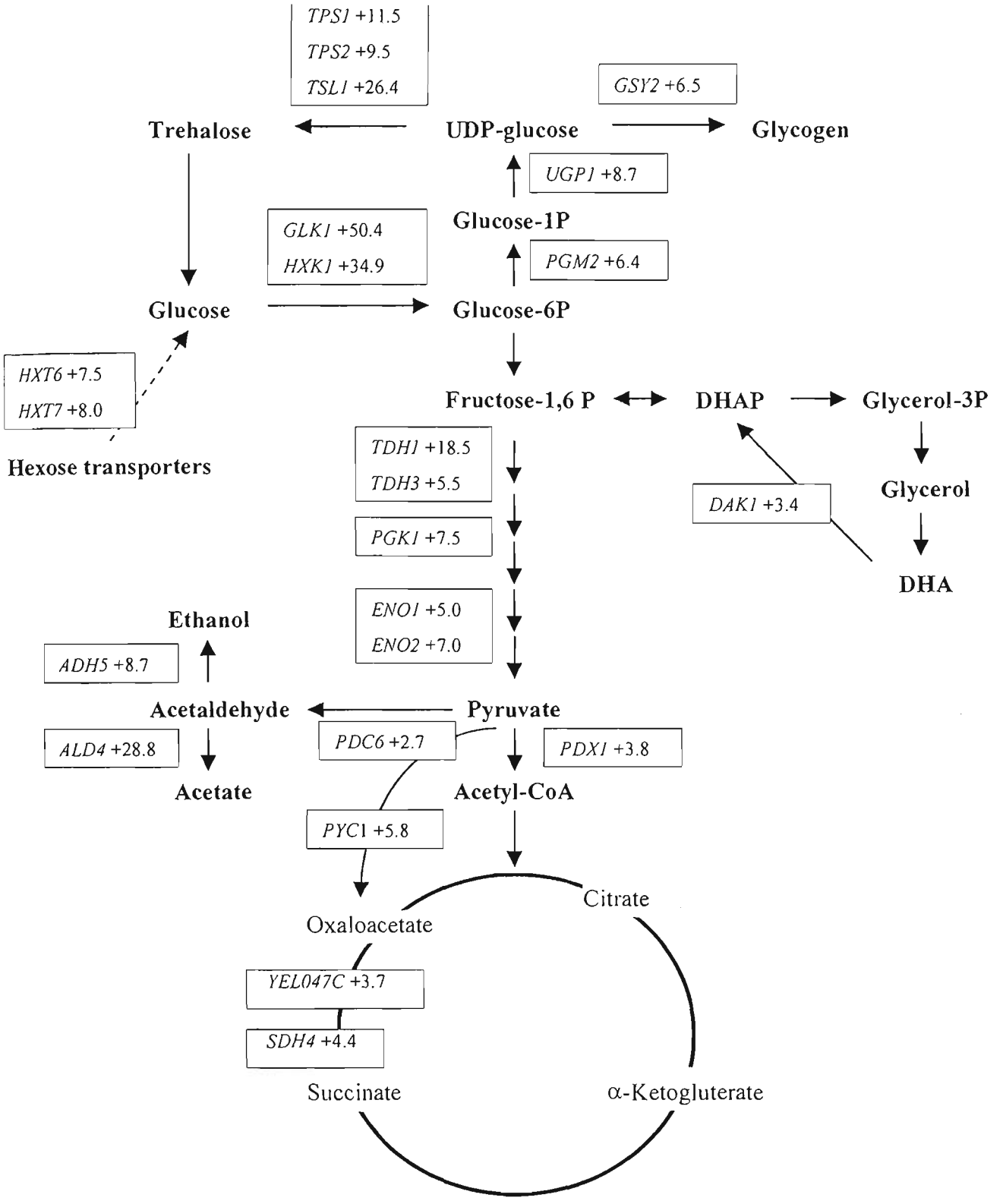


Figure 5.3. Level of ethanol-induced increases in transcript levels for genes involved in trehalose and glycogen biosynthesis, glycolysis and the citric acid cycle following one hour of ethanol (5% v/v) stress. Gene names are given in boxes with their level of up-regulation.

Several genes involved in cell surface interactions and lipid metabolism were up-regulated after one hour of ethanol stress. These include the abundant cell surface glycoprotein gene (*SED1*), a cell wall mannoprotein gene (*TIP1*), and a secreted glycoprotein gene (*YGP1*). *SPI1*, a gene that encodes a protein with 62% identity to Sed1p (Puig & Perez-Ortin, 2000), showed a 23.5-fold higher level of expression at this time point. The lipid metabolism genes *OPI3*, *FAA1* and *EHT1* were up-regulated, as was *HSP12*; interestingly Hsp12p has been shown to confer increased integrity on the cell membrane during ethanol-induced stress (Sales *et al.*, 2000).

Few genes associated with nitrogen metabolism had increased expression greater than three fold after one hour of ethanol stress. *MEP2*, an ammonium phosphate transporter and *DIP5*, a dicarboxylic amino acid permease, involved in ammonia transport across the plasma membrane were induced. *ARG4*, *SER3* and *UGA1* associated with amino acid metabolism were similarly up-regulated, however genes associated with nitrogen regulation were not up-regulated to the same extent as carbon regulation genes.

Thirty-five genes encoding proteins of unknown function were up-regulated following one-hour ethanol stress. Of these, 12 have previously been associated with a 30-minute ethanol shock (Alexandre *et al.*, 2001). Two genes, *YCL042W* and *YBR214W* were very highly up-regulated, at 54.4-fold and 23.1-fold respectively. *YBR214W* has not previously been noted as up-regulated in response to ethanol stress.

5.3.3 Down-regulated genes following one hour ethanol stress

The 273 genes down-regulated more than three-fold following one hour ethanol stress were also grouped into functional classes. These down-regulated genes were involved in protein synthesis, amino acid metabolism, nucleotide metabolism, transport, cell cycle, lipid metabolism and mating among others (Table 5.3).

The majority of down-regulated genes encoded components of the machinery for protein synthesis; this group represented 59% of all down-regulated genes. Ninety-eight genes were associated with ribosomal proteins, 40 with protein synthesis and 24

with RNA synthesis and processing. The down-regulation of these genes is reflected in the growth arrest following ethanol stress.

Perhaps the most noteworthy of the other classes of down-regulated genes are those associated with transport. Eight genes associated with small molecule transport were down-regulated, as were two hexose transporters: only one transporter gene, *HMT1*, was identified as repressed in response to ethanol shock in the work of Alexandre *et al.* (2001). The two hexose transporter genes, *HXT1* and *HXT2*, were down-regulated, 6.6-fold and 18.5-fold respectively. *HXT1* and *HXT2* have not previously been shown to be down-regulated under conditions of ethanol stress.

In addition, 20 genes associated with amino acid metabolism and 11 genes associated with nucleotide metabolism were also down-regulated. Three genes associated with lipid metabolism were down-regulated, including two ergosterol biosynthesis genes, *ERG25* and *ERG3*.

Seven genes associated with cell mating were repressed following one hour of ethanol stress. Five of these gene products are associated with cell fusion, promoting cell-to-cell contact to facilitate mating. Two genes *SAG1* and *AGA1* were strongly repressed 41.5- and 13.7-fold respectively. The other three genes were associated with pheromone production. Of these *MF(ALPHA)2* was strongly repressed 26.6-fold. A general ethanol-stress induced down-regulation of genes associated with cell mating is apparent from these results.

The down-regulation of five genes involved in cell cycle control resulted from one-hour ethanol stress. *CDC33* is involved in cell cycle regulation in addition to playing a role in protein synthesis (Brenner *et al.*, 1988). Two genes, *CCT6* and *YTM1*, associated with cytoskeleton organization were also down-regulated. Interestingly, the cytosolic aldehyde dehydrogenase, *ALD6*, was down-regulated 14.2-fold at this stage of ethanol stress where the mitochondrial aldehyde dehydrogenase, *ALD4*, was coordinately up-regulated 28.8- fold.

Forty-three genes encoding proteins with unknown functions were down-regulated following one-hour ethanol stress. Of these genes, 11 were previously identified as down-regulated following a 30 minute ethanol shock (Alexandre *et al.*, 2001). None

of the unknown down-regulated genes were very strongly repressed. The levels of repression ranged from 3.1-fold to 8.8-fold.

5.3.4 Up-regulated genes following three hours of ethanol stress

Following three hours exposure to ethanol stress, only 14 genes were up-regulated three fold or greater compared to the control (Table 5.2). Seven of these genes were also up-regulated following one hour stress, the other 7 being associated only with the three hour time point. Of the 7 genes up-regulated under ethanol stress at both one and three hours, expression levels relative to the unstressed control were much lower than at the three hour time point.

Genes up-regulated following three hours of ethanol stress were clustered into the following six functional classes: stress, mitochondrial functions, vacuolar functions, amino acid metabolism and genes of miscellaneous and unknown function. Only one stress response gene, *YRO2*, was up-regulated under ethanol stress at both the one and three hour time points. The size of the difference in expression levels over the three hours, however, decreased from 22.8 fold to 4.5 fold, and this decrease was due solely to a decrease in the expression of the *YRO2* gene in the stressed culture and not an increase in its expression in the control culture (data not shown). The aldehyde dehydrogenase gene, *ALD4*, was the only gene associated with fermentative pathways that remained up-regulated after three hours exposure to ethanol stress. However, the relative level of expression had declined considerably compared to the one-hour time point (from 28.8 fold to 3.6 fold) and again this was not due to increased expression *ALD4* in the control.

5.3.5 Down-regulated genes following three hours ethanol stress

One hundred and one genes were down-regulated greater than three-fold following three hours ethanol stress. The down-regulated genes at three hours were generally involved in protein synthesis, transport, amino acid metabolism, nucleotide metabolism, mating, carbohydrate metabolism, the cytoskeleton, the cell wall and lipid metabolism (Table 5.4).

As with one hour ethanol stress, the majority of down-regulated genes at this time point encoded components of the machinery for protein synthesis; this group represented 56% of all down-regulated genes. Forty-nine repressed genes encoded ribosomal proteins and all of these genes, except *RPS27B* and *RPL37A*, were also repressed following one hour ethanol stress. Eight genes involved with protein synthesis were down-regulated; only two of these *SOL3* and *SSB2* were also down-regulated at one hour ethanol stress. Interestingly, no genes associated with RNA synthesis and processing were down-regulated at three hours.

Of the other genes down-regulated at this time point, six were associated with transport and two of these, *HXT1* and *HXT2* were also repressed at one hour ethanol stress. However, the level of difference in expression between stress and control cultures had diminished considerably from 18.5-fold at the one hour time point to 7.6-fold at three hours for *HXT2*. One other plasma membrane-associated transport gene, *PDR5*, was down-regulated at both the one and three hour time points, however, the level of repression of *PDR5* increased from 3.4-fold at one hour ethanol stress to 5.9-fold following three hours ethanol stress.

Four genes associated with amino acid metabolism and three genes associated with nucleotide metabolism were also down-regulated. Two of the amino acid metabolism genes, *LEU1* and *TWT1*, were also down-regulated following one hour ethanol stress. The level of down-regulation of *TWT1* remained the same across the two time points whereas the difference between stress and control for *LEU2* had declined from 12.2-fold to 3.4-fold. *GAP1* and *GDH1* were newly down-regulated following three hours of stress. The nucleotide metabolism gene, *FUR1,* remained down-regulated at roughly the same level across the two time points sampled. Overall, the number of down-regulated amino acid and nucleotide metabolism genes declined from one to three hours of ethanol stress.

The mating genes *MF(ALPHA)2, SAG1* and *AGA1* were down-regulated following three hours ethanol stress. These genes were also down-regulated following one hour ethanol stress, however, their early high levels of down-regulation were reduced to approximately 4- and 5-fold at three hours ethanol stress.

Five genes associated with carbohydrate metabolism were down-regulated after three hours ethanol stress, and only one of these genes (*ALD6*) was also down-regulated at the one hour time point. One of the other four genes, *TDH3* was up-regulated 5.5-fold at the one hour time point and down-regulated 4.2-fold following three hours of stress.

Two genes involved in lipid metabolism and cell wall organization, *OLE1* and *UTR2*, were down-regulated (19.7-fold and 5.2-fold respectively) after three hours ethanol stress. The level of down-regulation for *OLE1* increased over the two time intervals, from 4.3-fold at one-hour to 19.7-fold following three hours of ethanol-stress. The level of down-regulation for *UTR2* was similar across the time intervals.

Thirteen genes encoding proteins with unknown functions were down-regulated following three hours ethanol stress. Of these, six were also down-regulated after one hour ethanol stress. Two of these six genes, *YGL102C* and *YMR116C*, were previously reported to be down-regulated following a 30 minute ethanol shock (Alexandre *et al.*, 2001).

5.3.6 Validation of gene array results by Northern blotting

To validate the methodology and results for the gene array experiments nine genes that were identified as up-regulated under ethanol stress were selected from global array data for confirmation via Northern blot analysis. These candidate genes were chosen as they cover a wide range of differences (5-fold to 67-fold) in expression levels between stress and control conditions. For Northern verification experiments, growth conditions and RNA isolation were performed as described for the gene array analysis.

Oligonucleotide probes (Table 5.5) were designed to gene specific target sequences within the ORFs of the nine genes to be screened. Blots of total RNA from both 5% ethanol-stressed and control samples over a time-course of one, two and three hours were prepared. Figure 5.4 shows the mRNA levels of the nine candidate genes, compared to an actin control. Down-regulation of the actin gene at all time points in the stressed cells is evidence that the observed up-regulation of genes in response to ethanol stress are not artefacts of RNA yield. The up-regulation of the nine candidate

genes analysed here serves to validate the findings of the array analysis. The expression levels of the nine genes were higher in ethanol stressed cells following one-hour ethanol exposure in comparison to control cells. After three hours of exposure to ethanol the expression level of some of these genes was similar to the control and all were significantly reduced compared to the one hour time point.

5.3.7 Promoter analysis of ethanol-stress induced genes

Analysis of array data using the RSAT database (http://rsat.ulb.ac.be/rsat/) enabled the identification of known sequence motifs in regions 800 bp upstream of up-regulated gene start sites. Given the number of HSP genes up-regulated under ethanol stress, many of which are known to have HSE sequences in their promoters, a search was undertaken for genes containing these elements. Overall, 29 of the 100 genes up-regulated at one hour ethanol stress (Table 5.1) and 4 of the 14 genes up-regulated at three hours ethanol stress (Table 5.2) contained putative HSEs indicated by the presence of its cognate transcription factor (Hsf1p). This shows a likely importance of this promoter element in the activation of genes up-regulated during ethanol stress.

Hsf1p is however not the only regulatory factor involved in the induction of HSPs. Several HSP genes are also induced as part of the so-called general stress response (Moskvina *et al.*, 1998) in which the two transcription factors Msn2p and Msn4p (abbreviated to Msn2/4p) bind to specific STRE elements (Marchler *et al.*, 1993; Martinez Pastor *et al.* 1996). Analysis of the data using the RSAT database to identify putative STRE elements revealed the consensus sequence (AGGGG), in both orientations (Marchler *et al.*, 1993), in 52 of the 100 genes that were up-regulated after one hour ethanol stress and in 3 of the 14 genes up-regulated after three hours ethanol stress (Tables 5.1 and 5.2). This indicates a strong contribution of the Msn2/4p transcription factors in gene induction during ethanol stress.

Table 5.5: Gene specific oligonucleotide probes for Northern analysis

Name	Sequence
HSP26:	CTC TGG GAT CAT AAA GAG CGC CAG CAT AG
GLK1:	CTT CAT AAA GGC CAG TGT ACG ACG TGC TAG
ADH5:	GGA TAT AGT GAC CCA TTG GCC TGG TAT CAC
ALD4:	CGT TCG CCA TGT TAA TGA CTT CGT CGG CAG
DIP5:	CGG TAC CTG TAC CTA TAA GCA GAC CTG TAC CT
MEP2:	CCG TTA CCT CTA GTG TTG TGT GAG AAA GC
LAP4:	GGA TCC AGT GAT AAA GTG ATT CCG ACA TTA GGC
PTR2:	CCA AAC TTT GTA GAA CAG CAG CCC ATG TCA TG
YER053C:	GCA CAA CAT GAT ATC AGC GAG GAA TTC AGC G
ACT1:	CGG TTT GCA TTT CTT GTT CGA AGT CCA AGG CGA CG

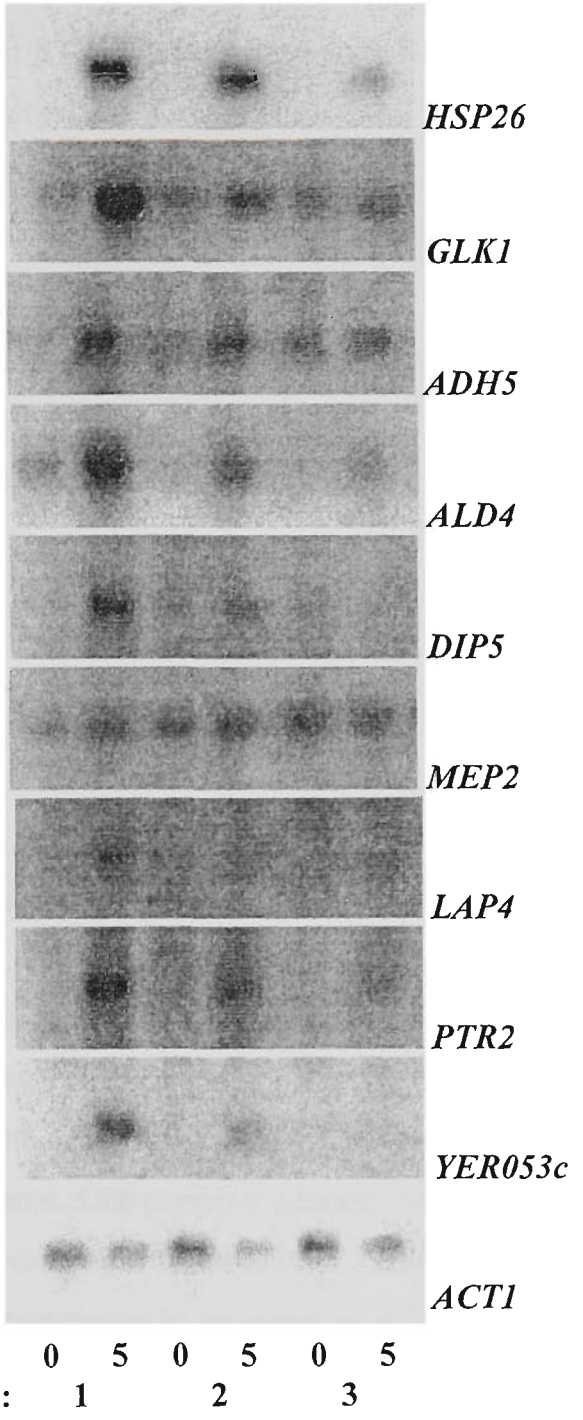


Figure 5.4. Northern blot analysis of genes previously identified as up-regulated under ethanol stress in gene array experiments. Yeast cells from a mid-exponential phase culture were inoculated into defined media in the presence and absence of 5% (v/v) ethanol. Equal numbers of cells from control and stress cultures were harvested for RNA extraction at 1, 2 and 3 hours post-inoculation. Transcript levels were detected with gene specific probes. The *ACT1* gene (bottom panel) was used as a control.

Apart from the HSE and STRE there are other promoter elements in yeast that are responsive to environmental stresses. One is the AP-1 response element with a consensus of TGACTCA (Estruch, 2000) that binds the Yap1 protein homologue. Sequence analysis identified nine up-regulated genes with this putative binding site.

Promoter analysis using the MEME algorithm (Bailey and Elkan, 1994) to identify novel and conserved motifs in upstream regions of all up-regulated genes revealed only consensus sequences relating to HSEs and STREs. Analysis using the MEME algorithm of all down-regulated genes also revealed no novel conserved regulatory sequences.

5.4 DISCUSSION

The overall aim of the work described in this chapter was to investigate changes in gene expression during an ethanol-stress induced lag phase to achieve a better understanding of the molecular events that take place during adaptation to this stress. While there have been several investigations into the affects of ethanol stress on yeast few have focused on the underlying genetic mechanisms that enable the cell to tolerate and adapt to ethanol. One previous genome wide analysis of the ethanol stress response of yeast, performed by Alexandre *et al.* (2001), identified a large number of genes with differential expression in response to a 30-minute exposure to 7% (v/v) ethanol. As can be seen from the results in Tables 5.1 and 5.2 there are many differences between results presented in this thesis and those published by Alexandre *et al.* (2001). In some cases this may be due to inconsistencies between the results reported in Alexandre *et al.* (2001) and the corresponding raw data presented at the Yeast Microarray Global Viewer (YMGV) website (http://www.transcripto-me.ens.fr/ymgv/). For example, in the Alexandre *et al.* (2001) paper *HSP26* is reported to be 12-fold up-regulated during ethanol stress yet the raw data for this gene shows it was up-regulated in one and repressed in two independent microarray experiments. This data is however now acknowledged to have some errors (B.

Blondin, personal communication)[3]. Other possible reasons for the differences between these results and the findings of Alexandre *et al.* (2001) include variation in strain background and the use of different methodologies.

The work described in this chapter tests and extends the study of Alexandre *et al.* (2001). Where the previous study focused on a single time point, 'early' in the response to 7% (v/v) ethanol stress, the analysis of global gene expression at two time points, one ('early') and three ('late') hours following inoculation into medium containing 5% (v/v) ethanol, was undertaken in this work. This covers a period during which cells are in growth arrest and are adapting to their environment. After three hours exposure to ethanol the cells entered exponential growth and thus had sufficiently adapted to the ethanol for growth to commence. Analyzing global gene expression at these two time points has enabled distinctions to be drawn between genes that are transiently up- or down-regulated and genes that are up- or down-regulated for a longer period.

Generally results from array experiments are normalized to all open reading frames or control genomic DNA spots on the slides, chips or filters (relative quantification) or to one single constantly expressed gene (absolute quantification) (Brejning *et al.*, 2003). Results from the gene array experiments described in this thesis were normalized to control genomic DNA spots (relative quantification). It was not appropriate in this analysis to perform absolute quantification and normalize to a single 'constantly' expressed gene such as *ACT1*, as undertaken by other authors (i.e. Brejning *et al.*, 2003). As previously mentioned (see Section 5.3.1), ethanol-stress led to decreased transcript levels of *ACT1* (repressed 1.2- and 1.7-fold at one and three hours respectively). Thus, normalizing in such a way would have artificially increased the numbers of genes apparently up-regulated in response to ethanol stress, potentially generating false positive results.

[3] I sought clarification from the corresponding author of Alexandre *et al.* (2001) (B. Blondin) on the discrepancy between results reported in their paper and their raw data lodged at the YMGV website. The following is a copy of the reply that I received: *"We actually discovered that there is some errors in the data provided by Alexandre. Some unreliable measurements failed to be discarded from Alexandre data. Since to my knowledge YMGV displays the results of the 3 raw measurements, these have to be taken into consideration".*

5.4.1 The 'early' response to ethanol stress

Of the 100 genes up-regulated early in response to ethanol stress were found to be associated with cellular stress responses, energy utilisation, transport, cell surface interactions and lipid metabolism. Of these, 37 were also identified as ethanol-stress induced in the work of Alexandre *et al.* (2001), including genes associated with energy utilisation: *GLK1, HXK1, TDH1, ALD4, CIT2, MCR1* and *PYC1*. However unlike Alexandre *et al.* (2001), up-regulation of the high affinity hexose transporter genes, *HXT6* and *HXT7*, usually expressed under conditions of low glucose (Luyten *et al.*, 2002; Ye *et al.*, 2001; Reifenberger *et al.*, 1997), was observed. This finding is consistent with what is known about ethanol-induced physico-chemical changes to the plasma membrane that compromise the transport of nutrients across the plasma membrane (Leao & Van Uden, 1982; Salmon *et al.*, 1993). It is proposed here that cells under ethanol stress are likely to be in a pseudo-starvation state where nutrients, such as glucose, are present in the growth medium but are not accessible to the cell. A plausible cellular response to this would be the observed up-regulation of genes associated with hexose transport and central metabolism.

Nearly all genes associated with trehalose and glycogen metabolism (*PGM2, UGP1, TPS1, TPS2, TSL1 and GYS2*) were up-regulated in this study. This seems to be inconsistent with the need for greater carbon input into the glycolytic pathway since the pathways that these genes are associated with draw carbon away from energy-yielding processes. It has been suggested, however, that trehalose metabolism regulates glycolysis since mutations in the gene for a trehalose synthase subunit, Tps1p, have been shown to result in aberrant glycolytic flux, suggesting that Tps1p and/or trehalose synthesis may play a role in modulating glycolysis (Gonzalez *et al.*, 1992; Thevelein and Hohmann, 1995). Alternatively, the increased production of trehalose may also be associated with its role as a stress protectant and the need for elevated levels during times of stress. In support of the latter, it was found that the neutral trehalase gene, *NTH1*, involved in trehalose degradation, was not significantly up-regulated and this is in contrast to findings of Alexandre *et al.* (2001). The accumulation of glycogen is usually associated with building carbon reserves, which are known to be critical to cell survival especially during starvation (Francois & Parrou, 2001).

A number of recognized stress response genes had elevated expression levels at the early stage of adaptation to ethanol stress. Among these genes a group of highly responsive HSPs (*HSP26, HSP42, HSP30, HSP104, HSP78* and *HSP12*) were up-regulated. The expression of these genes is consistent with previous ethanol stress studies (Piper *et al.*, 1994; Piper, 1995; Alexandre *et al.*, 2001). The ethanol-mediated induction of *SSE2* and *SSA4*, members of the HSP70 family, suggests that one of the effects of ethanol on the cell is to cause protein unfolding (Parsell and Lindquist, 1993). The induction of other stress response genes common to oxidative and osmotic stress was also observed. These genes possibly help the cell avoid the damaging effects of reactive oxygen species and water stress generated from ethanol exposure (Costa *et al.*, 1993; Hallsworth, 1998).

Several genes associated with the cell surface and lipid metabolism were up-regulated early in the response to ethanol stress. As the cell surface and membrane lipids are known 'targets' of ethanol (reviewed by Sajbidor (1997) and covered in Chapter 1 Section 1.3.1 of this thesis), this confirms, at a genetic level, what has been known for some time at the cellular level. Alexandre *et al.* (2001) found no up-regulation of genes associated with cell surface functions or lipid metabolism, however, they did identify up-regulated genes involved in ionic homeostasis and protein targeting. Remembering that Alexandre *et al.* (2001) analysed the mRNA profile of ethanol-stressed yeast at an even earlier time point than the first time point studied for this thesis, some of these differences in results may reflect ongoing changes in gene expression over the time course of adaptation to stress but, as pointed out previously, there is also doubt as to the validity of the results presented by Alexandre *et al.* (2001).

Perhaps the most striking feature of the 'early' response to ethanol stress is the down-regulation of genes associated with protein synthesis; 161 of the 273 genes that were down-regulated early in response to ethanol stress were associated with protein synthesis, ribosome biogenesis, RNA synthesis and processing, amino acid metabolism or nucleotide metabolism. This is typical of cells undergoing growth arrest as a result of encountering physiological stress (Gasch *et al.*, 2000) and a reduced expression of genes encoding ribosomal proteins has previously been linked with cellular growth arrest (Warner, 1999). As synthesis of ribosomes requires

substantial energy it is not surprising that transcript levels of ribosomal protein genes are reduced. A large number of down-regulated genes involved in protein synthesis and RNA metabolism following ethanol shock was also noted by Alexandre *et al.* (2001) and in a number of other microarray experiments following environmental stresses (Eisen *et al.*, 1998; Gasch *et al.*, 2000; Causton *et al.*, 2001). The down-regulation of genes encoding products involved in amino acid and nucleotide metabolism may also reflect a decreased need for amino acids and nucleotides by the cells as the rate of transcription and translation decreases.

Down-regulation of two low to medium affinity hexose transporters, *HXT1* and *HXT2*, coincided with up-regulation of the high affinity hexose transporters, *HXT6* and *HXT7* in response to ethanol stress. Interestingly it has been shown by other workers that the low affinity hexose transporter, *HXT1*, is induced in the presence of high glucose and repressed when glucose is scarce (Tomas-Cobos & Sanz, 2002) adding support to the argument that, during ethanol stress, yeast cells are in a pseudo-starvation state.

Several genes involved in cell cycle (*HSL7, CDC33, YBR158W, SAP4, FAR1*) were down-regulated early in the ethanol stress response. It is clear from viable counts for cultures in 5% (v/v) ethanol that, at the one hour time point, cells are not dividing (see Figure 5.1). From the optical density curve, at one-hour ethanol stress, the cells are however growing, presumably increasing in size and perhaps producing buds. The significance of this uncoupling of growth from cell division should be explored in future studies.

5.4.2 The 'late' response to ethanol stress

Overall, as the lag phase adaptation period was drawing to a close, the magnitude of the differences in gene expression levels between the control and stress cultures decreased considerably, with few genes still being up-regulated late in the lag period. This finding was unexpected given that the ethanol, and hence the stress, was still present. It could be speculated from this finding that the response in yeast resulting from sudden ethanol exposure is mostly focused on cellular modification (e.g. change in metabolism, cell structures, etc) rather than 'detoxification' of the ethanol, since the latter would presumably require elevated expression of requisite genes until the

ethanol concentration had been considerably reduced. It is clear from other studies in the field that, in experiments such as those described here there is no significant drop in ethanol concentration over a time course of only three hours (see, for example Stanley *et al.*, 1997).

The number of down-regulated genes following three hours ethanol stress was also reduced compared to the one-hour time point. As with the 'early' response to ethanol stress, the repressed genes at three hours were primarily associated with protein synthesis; the repression of many ribosomal protein genes was sustained throughout the ethanol-induced lag phase. Forty-nine ribosomal protein genes remaining down-regulated and of these all bar four were also repressed at one-hour ethanol stress. This continued down-regulation of genes associated with anabolic functions is consistent with what was observed at a physiological level; even after recovery from ethanol stress the cells have a slower growth rate than in the control culture and this would be expected to be reflected in anabolic processes. Several amino acid and nucleotide metabolism genes also remained repressed later in the lag, as was the situation early on. The RNA synthesis genes down-regulated at the 'early' time point were, however, not down-regulated late in the ethanol stress response.

Interestingly the cytosolic aldehyde dehydrogenase, *ALD6,* which converts acetaldehyde to acetate was repressed both early and late in the adaptation to ethanol stress while the mitochondrial aldehyde dehydrogenase, *ALD4*, was up-regulated at both time points. Assuming the proposed pseudo-starvation state of ethanol stressed cells to be correct, the pyruvate to acetyl CoA reaction following glycolysis would presumably be starved of substrate. Thus, to facilitate ongoing delivery of carbon to mitochondria for access to the TCA cycle, ethanol stressed cells may be attempting to oxidize available carbon from cytosolic acetaldehyde (which readily diffuses across the mitochondrial membrane), thus providing a carbon source for the TCA cycle. Repression of *ALD6* would facilitate this by minimizing conversion of acetaldehyde to acetate in the cytosol.

In comparison to the early response to ethanol stress, genes involved in cell cycle were not down-regulated at three hours ethanol stress. This correlates with the physiology of the culture; at three hours, stressed cells were about to divide and

initiate exponential growth (see Figure 5.1). For this to occur they must have sufficiently adapted to the ethanol-stress for growth to commence.

5.4.3 Transcriptional regulation of ethanol-stress induced genes

The expression of genes in ethanol-stressed cells appears to be regulated by two main signal transduction pathways, the HSE-mediated pathway and the general stress response pathway (reviewed by Estruch, 2000 and Mager & Kruijff, 1995). These pathways were described in Chapter 1 (see Section 1.5). The majority (i.e. 52) of up-regulated ethanol-stress genes were found to contain STRE sequence motifs, which are potential binding sites for stress-inducible Msn2/4p transcription factors. While a single copy of this element in a promoter can induce expression in a cell under stress, two or more copies have a greater effect (Kobayashi & McEntee, 1993). The promoter analysis undertaken in this study searched for the presence of the STRE consensus sequence and thus detected promoters with single and multiple copies of this motif. Of the 77 STRE-containing genes, 28 contained two or more elements (Table 5.1 and 5.2).

Of the genes up-regulated following one hour of ethanol stress, 35% contained a potential Hsf1p binding site, indicating possible gene regulation by the HSE-mediated pathway. In this analysis promoters of up-regulated genes were screened for at least three copies of the 5'-nGAAn-3' consensus repeat of HSEs, in alternating orientations as described in Estruch (2000), also allowing a one base pair substitution. It is possible however that some HSE-containing genes were missed as HSEs can display gaps of up to 5 bp between modules without affecting the binding of Hsf1p (Mager & Moradas-Ferreira, 1993).

The AP-1 response element, which binds the Yap1p transcription factor and is found in promoters of genes that are up-regulated during oxidative stress, was found in only 9 of the up-regulated genes in this study and most of these genes also had STRE and/or HSE, suggesting that Yap1p plays a minor, if any, role in regulating gene expression during ethanol stress (Tables 5.1 and 5.2).

Up-regulation of several genes, including *DAK1*, *COX15*, and *MEP2*, that had none of the three regulatory sequence elements discussed above suggested that other signal transduction pathways may be involved in ethanol stress signaling. Further analysis of the promoter regions of ethanol-responsive genes did not identify any other (novel or already identified) conserved sequence motifs. Thus it would seem that STREs and HSEs are the most important regulatory elements in genes that are up-regulated by the stress associated with exposure to ethanol.

5.4.4 The transient nature of the ethanol stress response

A common feature of genomic expression responses to environmental change is their transient nature (Gasch *et al.*, 2000; Rep *et al.*, 1999; Causton *et al.*, 2001; Yale and Bohnert, 2001; Brejning *et al.*, 2003). The period of change in gene expression represents an adaptation, during which time the cell adjusts its internal system to function in the new environmental conditions (Gasch & Werner-Washburne, 2002). Results presented in this chapter concur with this finding. With the reduction in gene expression changes at three hours ethanol stress, especially in numbers of up-regulated genes, the majority of the adaptive changes necessary for growth in the presence of ethanol appear to have occurred prior to this time point. Either demand for the products of the 'early' ethanol stress response genes no longer exists or the cell may still require these products but sustaining their levels requires fewer transcripts than was the case in the early stages.

It is also possible that some of the genes up-regulated early in the adaptation phase are expressed coincidentally, their expression level being elevated due to general changes in the cell brought on by the stress and associated cell damage, but for which they have no ameliorating role. Further studies with deletion and over-expression strains may help to resolve this.

5.4.5 Comparison of gene expression between an ethanol-induced lag phase and a 'typical' lag phase

In a recent study of transcriptional changes during the lag phase of *S. cerevisiae* (Brejning *et al.*, 2003), a very different pattern of gene expression was observed in

comparison to that of an ethanol stress-induced lag phase reported in this thesis and in work by Alexandre *et al.* (2001). The Brejning *et al.* (2003) study analysed a typical lag phase, where late respiratory phase yeast cells were inoculated into fresh complete medium thereby inducing a three hour lag period.

The functional classes of genes up-regulated during the nutrient up-shift lag period were the same as those found to be repressed during the ethanol stress-induced lag period in this thesis. For example, many genes involved in the cellular protein synthesis machinery were induced upon nutrient up-shift and repressed during ethanol-stress. Of the protein synthesis genes induced after one-hour nutrient up-shift, 49% of these were repressed following one-hour ethanol stress. Other functional groups showing this effect included genes encoding proteins involved in amino acid and nucleotide metabolism, transport, mating and the cell cycle. Functional classes of genes up-regulated during the ethanol stress lag and repressed during the nutrient up-shift lag, included those involved in energy utilization, stress responses and lipid metabolism. One common feature of the two lag phases, however, was the transient nature of the molecular response. As observed with the ethanol stress-induced lag period, a higher number of gene expression changes were observed early in the nutrient up-shift lag phase with differences leveling out late in the lag phase as the cells initiated growth. Brejning *et al.* (2003) found 211 genes induced 20 minutes into the lag, 99 induced after one hour and only 16 induced after three hours.

It is clear from the results of Brejning *et al.* (2003) that the experimental model used for the work described in this thesis was not simply mimicking a typical lag phase. The changes in gene expression during a typical lag are almost the inverse of those associated with an ethanol stress-induced lag. Thus the results presented in this thesis clearly do not reflect the general changes associated by an induced lag period.

5.4.6 Confirmation of ethanol stress-response genes identified in differential display with gene array data

Differential display analysis, described in Chapter 4 of this thesis, identified seven putative ethanol stress response genes when *S. cerevisiae* was grown in the presence

of 7% (v/v) added ethanol. Of these seven genes, three (*YGL059W, CDC3* and *CBP2*) were confirmed by Northern analysis. Two other putative ethanol stress-responsive genes (*YOR275C* and *YDR504C*) could not be further confirmed by Northern analysis and another two genes (*SHY1* and *YHL039W*) were found to be false positives. The lack of confirmation of *YOR275C* and *YDR504C* by Northern analysis in Chapter 4 of this thesis may have been associated with the very low absolute level of expression of these genes. In the gene array data, following one and three hours ethanol stress, the absolute level of gene expression for *YOR275C* and *YDR504C* was very low (hardly detected), possibly explaining why they could not be detected in the Northern analysis. The investigation of the adaptation of *S. cerevisiae* to 5% (v/v) ethanol stress, using gene arrays as described in this Chapter, further verified the up-regulation of *YGL059W*. *YGL059W* was up-regulated 4.1-fold following one hour of ethanol stress. The further verification of the ethanol stress-response genes *CDC3* and *CBP2* was not achieved in the array analysis as the up-regulation of these genes was below the designated 3-fold experimental cut-off. Differences in the up-regulation of genes identified using differential display and array analysis can be attributed to the different levels of ethanol stress used in the two experimental settings. The up-regulation of *YGL059W* in cells under both 5% and 7% (v/v) ethanol stress further confirms it as an ethanol stress responsive gene.

5.4.7 Concluding remarks

The work described in this chapter provides information that is important in developing our understanding of how yeast cells adapt to ethanol stress. The results clearly show for the first time that there is a transient up-regulation of many genes associated with a range of cellular functions, but once adapted to the presence of ethanol in the growth medium, the gene expression profile is more similar to that of an unstressed cell. The grouping of genes according to function showed many genes with similar cellular roles were either up- or down-regulated. Genes involved mainly in energy metabolism and stress responses were induced early in response to ethanol stress, but were not sustained throughout the adaptation phase. A large number of genes associated with the protein synthesis machinery were repressed during ethanol stress, with many of these genes remaining repressed throughout the lag. The work also demonstrates for the first time the central role of STREs and, to a lesser extent,

HSEs, in the cellular response to ethanol stress in yeast cells. This study has provided a picture of the global transcriptional changes occurring during the lag phase induced by ethanol stress and the approach has resulted in the detection of novel genes not previously linked to ethanol stress.

On completion of the array work it was decided to investigate the roles of two genes that were highly up-regulated early in the adaptation to ethanol stress, *HSP26* and *ALD4*. To investigate the phenotype of these two genes, knockout strains were constructed in a PMY1.1 background and the performance of these strains tested under ethanol stress conditions. This work is described in the following chapter.

CHAPTER 6

PHENOTYPE OF YEAST GENE KNOCKOUT STRAINS

6.1 INTRODUCTION

The previous chapter described the analysis of global gene expression in *S. cerevisiae* during adaptation to ethanol stress, revealing a large number of genes with up- or down-regulated levels of transcription. While this information provides insight into the dynamics of the transcriptome during ethanol stress, the role of the products of genes affected by ethanol stress remains unknown. It is reasonable to assume that in a stressful environment, when the energy status of the cell is low and there is an overall decrease in transcription, up-regulated genes would be supplying a demand for proteins that have some role in overcoming the disruptive effects of the stress. It is this premise on which the experiments in this chapter are based. The purpose of the work described in this chapter was to examine the roles of genes with elevated expression in the ethanol stress adaptation process. While it was not possible to rigorously test all of the up-regulated genes within the timeframe of this project, it was possible to closely examine the role of two genes in the adaptation of *S. cerevisiae* PMY1.1 to ethanol stress.

Selecting two genes from a total of more than 100 candidate genes required careful consideration. The two genes chosen, *HSP26* and *ALD4*, were selected in part due to their very high levels of expression during the first hour of ethanol stress (67.8-fold and 28.8-fold up-regulated in ethanol-stressed cultures respectively) and because of their perceived roles in cellular adaptation to the ethanol.

6.1.1 *HSP26*

Hsp26, a small heat shock protein of *S. cerevisiae*, accumulates intracellularly in response to a variety of cellular stresses. The expression of *HSP26* increases substantially when cells are exposed to osmotic stress (Blomberg, 1997), H_2O_2

(Godon *et al.*, 1998), heat shock (Roth *et al.*, 1998), low pH (Carmelo and Sa-Correia, 1997), acetaldehyde stress (Aranda *et al.*, 2002), sorbic acid (de Nobel *et al.*, 2001), ethanol stress (Plesset *et al.*, 1982; Gropper and Rensing, 1993; Alexandre *et al.*, 2001) and when the cell enters stationary phase (Dickson and Brown, 1998).

The cellular role and function of Hsp26 remains undetermined. *HSP26* gene deletion and disruption mutations have previously been shown to have no detectable effect on the viability of cells held in stationary phase for extended periods, growth patterns during osmotic stress, dessication tolerance, thermotolerance, sporulation, germination or ethanol tolerance (Petko and Lindquist, 1986). One study has, however, demonstrated sensitivity of a *HSP26* deletion strain to sorbic acid indicating that *HSP26* confers resistance to the inhibitory effects of this compound (de Nobel *et al.*, 2001).

Although *HSP26* is induced by ethanol stress in *S. cerevisiae* (Table 5.1 of this thesis; Alexandre *et al.*, 2001; Gropper and Rensing, 1993; Plesset *et al.*, 1982) the function of the gene product remains unclear. Two previous studies have attempted to determine a physiological role for the *HSP26* gene product in cells grown in the presence of ethanol using gene deletion and disruption mutations. Petko and Lindquist (1986) tested the ability of mutant and wild type strains to acquire ethanol tolerance. Cells were grown aerobically to mid-exponential phase in acetate medium, centrifuged and resuspended in acetate medium with the addition of 8%, 15% and 25% (v/v) ethanol. Cells were exposed to each ethanol concentration for two hours and the percentage of surviving cells determined by cell counts. In the presence of ethanol, no detectable difference was observed in viable cell populations between the wild type and mutant strains. The lack of a discernable mutant phenotype was accredited to; 1) a possible subtle effect that was not identified in the experiment undertaken, 2) a possible overlapping function with other Hsp's, or 3) Hsp26 having no cellular function. Sharma *et al.* (2001) used transposon mutagenesis to create a *HSP26* disruption mutant, as well as a *HSP26* gene knockout, to study the 'fitness' of Hsp26 mutants in a mixed population during ethanol fermentation. All Hsp26 mutants were shown to decrease in cell number during fermentation. The author speculated that the wild type *HSP26* gene is necessary for survival during ethanol production. In this study, however, ethanol was not the only product of cell metabolism accumulating in the culture broth. Other compounds such as organic acids, other

alcohols, dissolved CO_2 and acetaldehyde would be produced and potentially impact on the 'fitness' of the tested strains. Therefore it could not be concluded that ethanol *per se*, rather than some other metabolic product, caused the loss of competitiveness by the deletion and disruption strains.

The above studies demonstrate the level of interest in the role of Hsp26 during ethanol stress but such studies have so far failed to determine a role for Hsp26 in the physiology of ethanol-stressed cells. The work of Petko and Lindquist (1986), described above, focussed on ethanol tolerance in acetate-respiring *S. cerevisiae* cells from which it is difficult to draw conclusions about glucose fermenting cells, in which the energetics and metabolic processes are quite different. In the work of Sharma *et al.* (2002) cells were grown on glucose but they did not provide definitive evidence that ethanol, and not some other metabolic product or environmental influence, was the reason for greater sensitivity of the knockout strains to their environment. Consequently, the debate on the role of Hsp26 in ethanol tolerance of *S. cerevisiae* is not yet resolved. The lack of conclusive evidence on the role of Hsp26 in the adaptation of *S. cerevisiae* to ethanol stress, combined with the very high level of expression of *HSP26* (67.8-fold; Table 5.1) in ethanol-stressed cells, suggests that further studies on the role of this gene in ethanol stressed cells is warranted.

6.1.2 *ALD4*

The aldehyde dehydrogenase (ALD) genes, of which there are five, have recently been recognised as having important roles in the metabolism and detoxifying systems in yeasts. These enzymes are critical in the utilization of ethanol through their conversion of acetaldehyde to acetyl-CoA and in the metabolism of toxic aldehydes, that accumulate under stress conditions (i.e. lipid peroxidation), to less reactive forms (Navarro-Avino *et al.*, 1999). Of the ALD genes, the main cytosolic ALD, *ALD6*, (which uses NAD^+ as a co-factor), is activated by Mg^{2+} and is not glucose repressed (Dickinson, 1996; Meaden *et al.*, 1997). The major mitochondrial ALD, *ALD4*, uses NAD^+ and $NADP^+$ as co-factors, is activated by K^+ and thiols and is highly glucose repressed (Jacobson and Berfonsky, 1974).

Expression of the mitochondrial *ALD4* gene in *S. cerevisiae* is increased during diauxic shift (De Risi *et al.*, 1997), acetaldehyde stress (Aranda and del Olmo, 2003), exposure to ethanol (Tables 5.1 and 5.2 of this thesis; Aranda and del Olmo, 2003; Alexandre *et al.*, 2001), salt stress (Posas *et al.*, 2000; Yale and Bonnert, 2001) and osmotic shock (Rep *et al.*, 2000). To date, however, there has been no attempt to define the role of *ALD4* in the cellular response to ethanol stress.

Studies with *ALD4* deletion mutants have confirmed a role for the *ALD4* gene product in ethanol metabolism. Ald4 is involved in the oxidation of acetaldehyde to acetate for biosynthetic purposes during growth on ethanol (Boubekeur *et al.*, 2001). Initially, however, studies with *ALD4* knockouts led to contradictory reports on the growth of knockout strains and consequently the role of the gene product during stress. Inconsistencies in the growth of these knockout strains were attributed to different levels of enzyme activity in the various *S. cerevisiae* strains (Boubekeur *et al.*, 2001; Aranda and del Olmo, 2003). In general, the cytosolic product of *ALD6* is considered to be involved in the formation of acetate from glucose while the main role of the mitochondrial *ALD4* gene is thought to take precedence during growth on ethanol (Wang *et al.*, 1998; Saigal *et al.*, 1991). Another suggested role for *ALD4* is in cellular detoxification processes. Since the accumulation of acetaldehyde is toxic to cells (Jones, 1989), the increased expression of *ALD4* in the presence of ethanol may reflect a cellular detoxification mechanism in reducing intracellular acetaldehyde concentration (Remize *et al.*, 2000; Boubekeur *et al.*, 2001).

The cellular location of Ald4 and its position in the ethanol respiratory pathway are good reasons to further explore the influence of *ALD4* expression on the adaptation rate of *S. cerevisiae* to ethanol stress. When this is coupled to its significantly increased expression level (28.8-fold; Table 5.1) during ethanol stress in the presence of high glucose concentrations, which repress its expression under non ethanol stress conditions (Jacobson and Bernofsky, 1974), then there are considerable grounds on which to speculate that *ALD4* has a key role in the ethanol stress response. Given that there are no studies published to date that examine the role of *ALD4* expression in the ethanol stress response, it was decided to construct an *ALD4* knockout strain in *S. cerevisiae* and study its phenotype in the presence and absence of ethanol stress.

6.1.3 Aims

The overall aims of this chapter were to:

1. construct gene knockout strains for *HSP26* and *ALD4* from *S. cerevisiae* PMY1.1,

2. profile the physiological response of these knockout strains during adaptation to ethanol stress,

3. determine if *HSP26* and *ALD4* have significant roles in the physiological response of *S. cerevisiae* PMY1.1 to ethanol stress.

6.2 CONSTRUCTION OF GENE KNOCKOUT STRAINS

Gene disruption is a powerful method to study and verify the function of a gene product. Transformations with PCR-generated DNA fragments for gene disruption in *S. cerevisiae* were first described by McElver and Weber (1992) and Bauldin *et al.* (1993). Several groups have since published modifications of this method and gene disruption has become a standard technique used in many yeast laboratories. For the purpose of this study we have used the pFA6-kanMX4 plasmid, which belongs to a series of marker plasmids, designed for PCR-based gene disruption in fungi by homologous recombination (Wach *et al.*, 1994; Steiner *et al.*, 1995). The selection marker, kanMX4, is a hybrid of the ORF of the kanamycin resistance gene *kan*^{r} from the transposon Tn903 (Oka *et al.*, 1981) flanked by promotor and terminator sequences of the strongly expressed *TEF* gene of *Ashyba gossypii* (Wach *et al.*, 1994; Steiner and Philippsen, 1994). The kanMX4 marker is also important as it lacks homology to yeast DNA decreasing the incidence of false positives (Wach *et al.*, 1998). When transformed with the kanMX4 module *S. cerevisiae* acquires resistance to the drug geneticin (G418).

Targeted disruptions of the *S. cerevisiae* genome with the kanMX4 module, bearing PCR-generated short flanking regions (of around 40 bp) homologous to the target gene, have been successful in many laboratories. Short flanking homology of only 40 base pairs takes advantage of the high levels of recombination in yeast and allows the

precise replacement of an open reading frame with the selectable marker. Relatively few geneticin resistant transformants carrying incorrectly targeted kanMX4 modules have been reported using this method, however, successful targeting of the short flanking homology PCR products depends on perfect homology between the short ends of the transforming DNA and the target locus. Previous work in our laboratory at Victoria University has shown it to be effective and successful for creating gene knockouts in *S. cerevisiae* PMY1.1 (Emslie, 2002). The plasmid containing the kanMX4 module was kindly donated by Dr. Paul Vaughan, Division of Molecular Science, CSIRO, Parkville, Australia.

6.2.1 Construction of *HSP26* and *ALD4* gene knockout strains using the kanMX4 module

The two target genes of interest, *HSP26* and *ALD4*, were replaced with the kanMX4 marker module, designed for generating yeast gene knockouts by Wach *et al.* (1994). This knockout strategy aimed to replace the target gene ORF with the kanMX4 module, as shown in Figure 2.1. PCR primers (of 40 bases) were designed to target the flanking regions of the ORF of interest with overhangs of sequence complementary to the flanking regions of the kanMX4 module (Wach *et al.*, 1994). The kanMX4 marker module was PCR amplified from the pFA6-kanMX4 plasmid using the primers HSP26-KO5' and HSP26-KO3' for the *HSP26* knockout (Table 6.1). The primer pair ALD4-KO5' and ALD4-KO3' were used in the amplification of the kanMX4 module from the pFA6-kanMX4 plasmid for the *ALD4* knockout (Table 6.1). The PCRs generated a kanMX4 module flanked by short sequences (40 bases) homologous to the target gene. PCR products from the two amplifications were resolved on 1% agarose gels producing the expected bands of approximately 1600 bp (Figure 6.1). The PCR products were transformed into *S. cerevisiae* strain PMY1.1 using the lithium acetate method of Gietz and Schiestl (1995). Cells were plated on YEPD geneticin plates for selection of positive colonies (i.e. colonies carrying the integrated kanMX4 cassette). Plates were incubated at 30°C for three days after which single geneticin resistant colonies were picked and re-streaked onto fresh YEPD geneticin plates.

It should be noted that the constructed knockout strains, PMY1.1*Δhsp26*::kanMX4 and PMY1.1*Δald4*::kanMX4, are abbreviated to Δ*hsp26* and Δ*ald4* throughout the remainder of this thesis.

6.2.2 Confirmation and stability of the *Δhsp26* and *Δald4* knockout strains

6.2.2.1 PCR confirmation

The replacement of *HSP26* and *ALD4* with the kanMX4 module in the transformed strains was verified by 1) colony PCR and 2) southern blotting. A positive geneticin resistant colony was selected from each *HSP26* and *ALD4* knockout strain. The presence and position of the kanMX4 module was tested using a combination of three primers specific to each strain. The primers complementary to the upstream and downstream flanking regions of the *HSP26* ORF; HSP26-PF (-231) and HSP26-PR (+175) were used in combination, as was the KanMX-PF primer, complementary to an internal sequence of the kanMX4 module, and the HSP26-PR (+175) primer (Table 6.2). To confirm a positive *ALD4* knockout the primers complementary to the upstream and downstream flanking regions of the *ALD4* ORF: ALD4-PF (-242) and ALD4-PR (+185) were used in combination, as were the KanMX-PF and ALD4-PR (+185) primers (Table 6.2). A diagrammatic representation of the PCR confirmation is shown in Figures 6.2 and 6.3.

Colony PCR using transformed cells as a source template generated products of expected sizes when resolved on a 1.2% agarose gel. A product of 2029 bp (length of kanMX4 sequence plus flanking sequences) was generated from the HSP26-PF (-231) and HSP26-PR (+175) primer combination. The kanMX-PF and HSP26-PR (+175) primer combination generated a product of 343 bp indicating the integration of the kanMX4 module into *Δhsp26*. Using the same primer combinations and PMY1.1 wild type cells only one band of 1051 bp (length of the *HSP26* ORF plus flanking sequence) was evident from the HSP26-PF (-231) and HSP26-PR (+175) primer combination. The smaller fragment was not evident due to the absence of the kanMX4 module (Figure 6.4). A PCR product of 2052 bp (length of kanMX4 sequence plus flanking sequences) was generated from colony PCR with the ALD4- PF (-242) and

Primer	Sequence
HSP26-KO5'	5'-TCATTTAACAGTCCATTTTTTGATTTCTTTGACAAC ATCCAGCTGAAGCTTCGTACGC-3'
HSP26-KO3'	5'-TTAGTTACCCCACGATTCTTGAGAAGAAACCTCAA TCGCATAGGCCACTAGTGAATCTC-3'
ALD4-KO5'	5'-TCAGTAGATCTACGCTCTGCTTAAAGACGTCTGCAT CCTCCAGCTGAAGCTTCGTACGC-3'
ALD4-KO3'	5'- CCTTACTCGTCCAATTTGGCACGGACCGCTTTAACT TGCATAGGCCACTAGTGGATCTC-3'

Table 6.1: Sequences of oligonucleotide primers used to amplify the kanMX4 module. Underlined characters correspond to the sequences complementary to the flanking regions of the kanMX4 module (Wach *et al.*, 1994); standard characters represent sequences matching the target gene.

Primer	Sequence
HSP26-PF (-231)	5'-GATGTCCTTGCGGATCTATG-3'
HSP26-PR (+175)	5'-AACGGTCATATATCGAAGCC-3'
ALD4-PF (-242)	5'-CTTCCGTCCACAGGTATCTT-3'
ALD4-PR (+185)	5'-AACGGAATCGTAACGCAAT-3'
kanMX-PF	5'-TCGACATCATCTGCCCAGAT-3'

Table 6.2: Sequences of the oligonucleotide primers used for PCR confirmation of gene replacement. HSP26-PF was complementary to a region 231 bases upstream of the knockout module, HSP26-PR was complementary to a region 175 bases downstream of the knockout module. ALD4-PF was complementary to a region 242 bases upstream of the knockout module, ALD4-PR was complementary to a region 185 bases downstream of the knockout module. The kanMX-PF primer was complementary to a region within the kanMX4 module (Wach *et al.*, 1994).

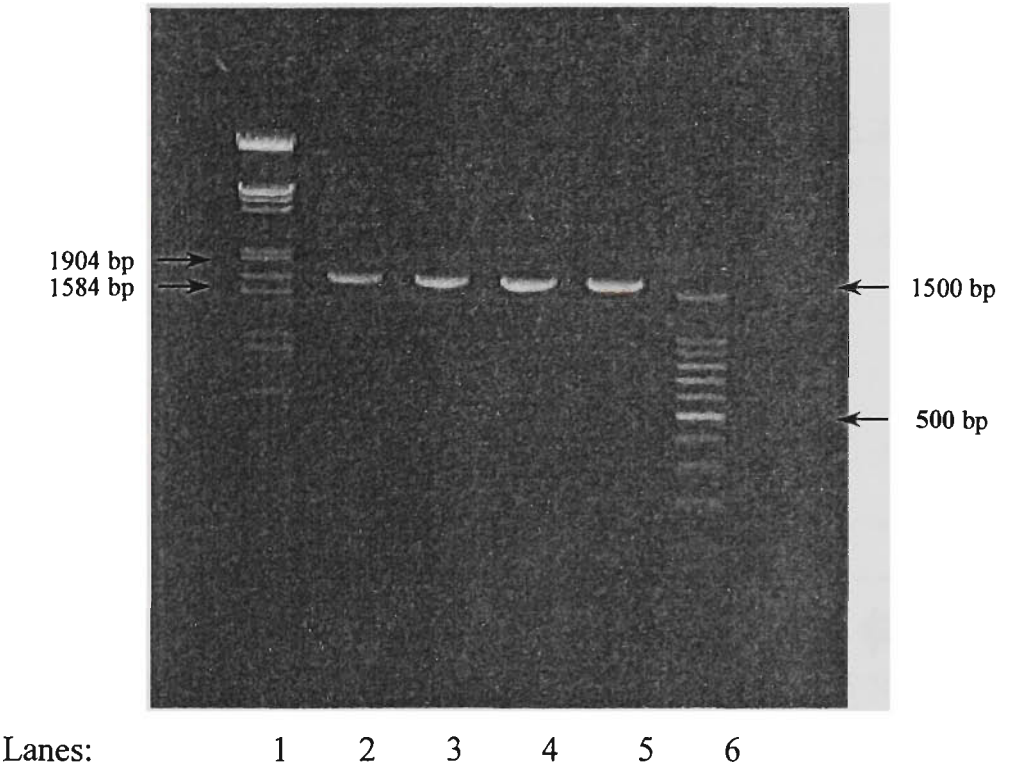


Figure 6.1: PCR amplification of the kanMX4 module from the pFA6-kanMX4 plasmid. The kanMX4 module was PCR amplified with the primers: HSP26-KO5'and HSP26-KO3' for generation of the *HSP26* knockout, and ALD4-KO5' and ALD4-KO3' for generation of the *ALD4* knockout. The PCR generated the kanMX4 module with flanking regions specific to the target genes, *HSP26* or *ALD4*. Products from the two amplifications were resolved on a 1% agarose gel where lane 1 corresponds to a Lambda DNA/*Eco*RI+*Hind*III size marker (MBI Fermentas), lanes 2 and 3 represent the 1622 bp kanMX4 module flanked with short sequences (40 bases) homologous to the *HSP26* gene and lanes 4 and 5 represent the 1624 bp kanMX4 module flanked with short sequences homologous to the *ALD4* gene. Lane 6 corresponds to a 100 bp quantitative DNA ladder (Promega).

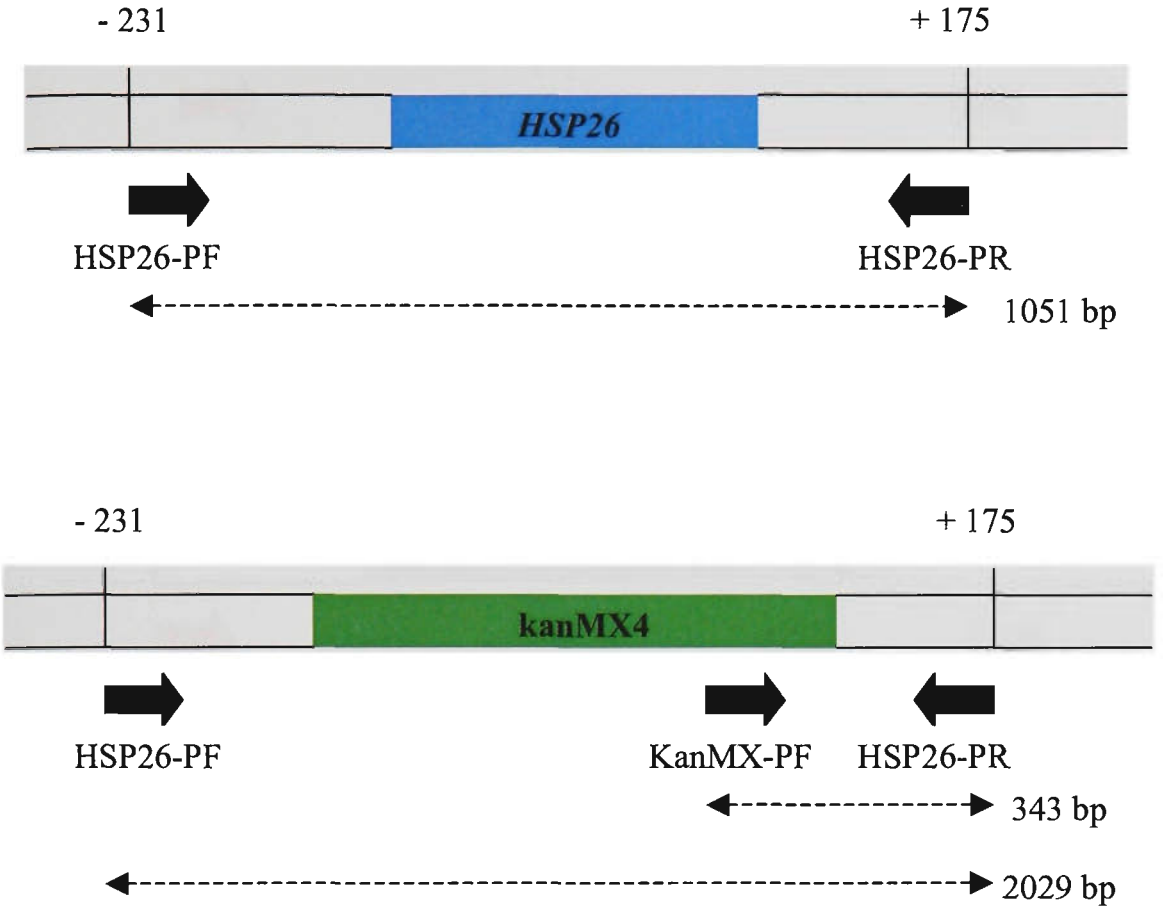


Figure 6.2: Diagram representing the *Δhsp26* knockout construct in comparison to the PMY1.1 wild type (not to scale). PCR positioning primers for confirmation of the knockout construct are listed with expected product sizes. The size of PCR products from the positioning primers HSP26-PF and HSP26-PR are 1051 bp (wildtype) and 2029 bp (*Δhsp26*). The size of the PCR product from positioning primers kanMX-PF and HSP26-PR is 343 bp.

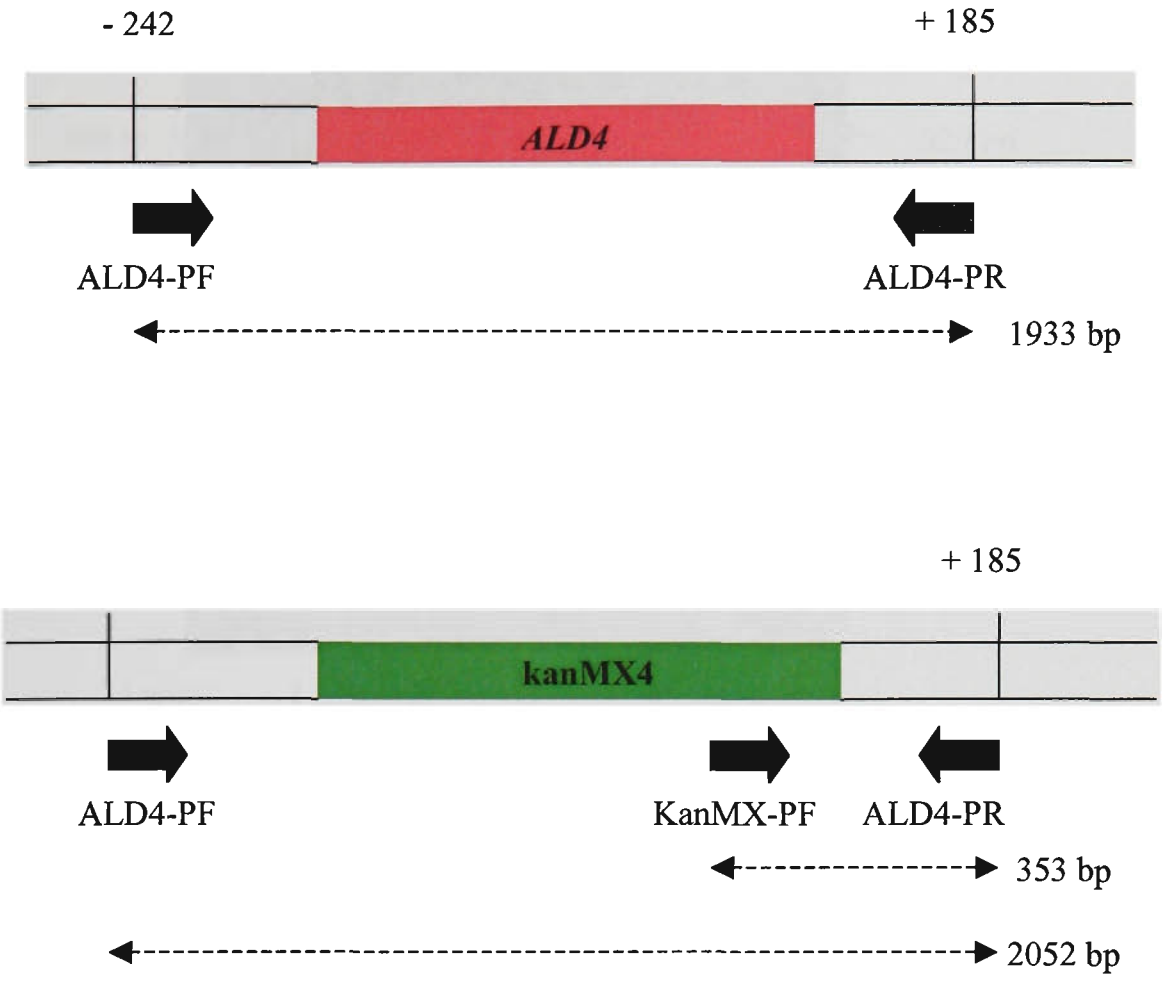


Figure 6.3: Diagram representing the *Δald4* knockout construct in comparison to the PMY1.1 wild type (not to scale). PCR positioning primers for confirmation of the knockout construct are listed with expected product sizes. The size of PCR products from the positioning primers ALD4-PF and ALD4 are 1933 bp (wild type) and 2052 bp (*Δald4*). The size of the PCR product from positioning primers KanMX-PF and ALD4-PR is 353 bp.

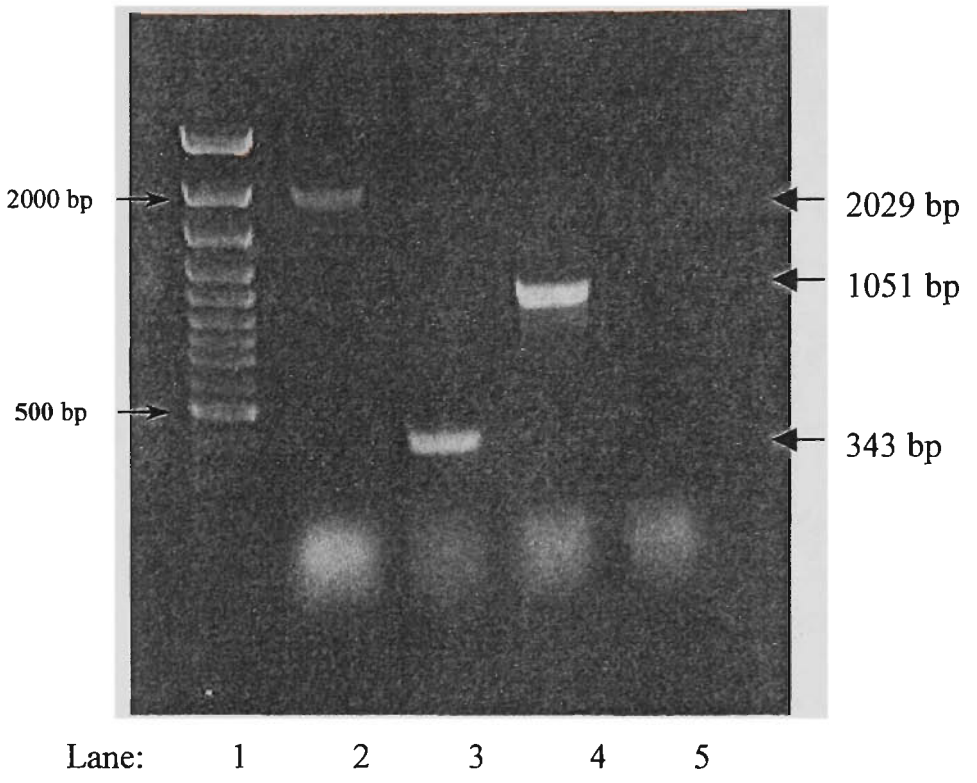


Figure 6.4: PCR confirmation of the correct integration of the kanMX4 module in place of the *HSP26* gene. A PCR product of 2029 bp was generated from the HSP26-PF and HSP26-PR primer combination (lane 2), as was a product of 343 bp from the KanMX-PF and HSP26-PR primer combination (lane 3) when using *Δhsp26* cells as a template. Using the same primer combinations and PMY1.1 wild type cells only one band of 1051 bp was evident (lane 4). No product was amplified from the KanMX-PF and HSP26-PR primer combination (lane 5). A size standard, Generuler^{TM} 100 bp DNA ladder Plus (MBI Fermentas), was run in lane 1.

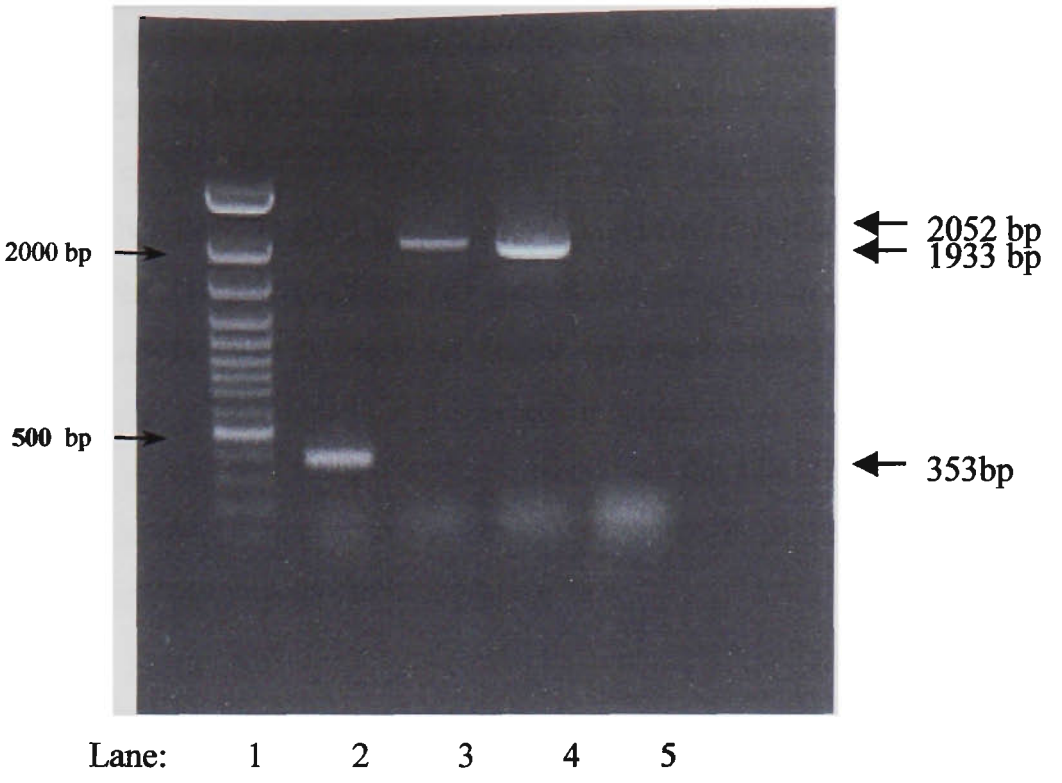


Figure 6.5: PCR confirmation of the correct integration of the kanMX4 module in place of the *ALD4* gene. A PCR product of 2052 bp was generated from the ALD4-PF and ALD4-PR primer combination (lane 2), as was a product of 353 bp from the KanMX-PF and ALD4-PR primer combination (lane 3) when using *Δald4* cells as a template. Using the same primer combinations and PMY1.1 wild type cells only one band of 1933 bp was evident (lane 4). No product was amplified from the KanMX-PF and ALD4-PR primer combination (lane 5). A size standard, Generuler™ 100 bp DNA ladder Plus (MBI Fermentas), was run in lane 1.

ALD4-PR (+185) primers. A second band of 353 bp was also generated with the primer combinations KanMX-PF and ALD4-PR (+185) indicating the integration of the kanMX4 module in *Δald4*. Using the same primer combinations and PMY1.1 wild type cells only one band of 1933 bp (length of the *ALD4* ORF plus flanking sequence) was evident from the ALD4-PF (-242) and ALD4-PR (+185) primer combination. The smaller fragment was not evident due to the absence of the kanMX4 module (Figure 6.5). Sequencing of all PCR products confirmed the correct integration of the kanMX4 module into the *HSP26* and *ALD4* loci of the relevant knockout strains. Sequence data could be read into the kanMX4 module in all sequenced fragments indicating the correct replacement of the two genes.

6.2.2.2 Confirmation via Southern blotting

The replacement of the *HSP26* and *ALD4* genes with the kanMX4 module and its incorporation into the genome as a single copy in the knockout strains was further confirmed by Southern blotting. Genomic DNA from the knockout strains and the PMY1.1 wild type was digested in separate reactions with three, six-base cutting, restriction endonucleases. Restriction enzymes were chosen to target regions upstream and downstream of the gene of interest. In addition the enzymes did not cut within the gene of interest or within the inserted kanMX4 module. Three different enzymes were selected to give confidence in the validity of the results. Digested DNA (Figures 6.6 a and b) was blotted to a membrane and probed with a labelled cDNA probe (810 bases) homologous to a region within the kanMX4 module. The probe was PCR amplified from the pFA6-kanMX4 plasmid using the KanCR-F and KanCR-R primers (Table 6.3) complementary to regions 5' and 3' of the kanMX4 module coding sequence.

Southern analysis of *Δhsp26* confirmed the single integration of the kanMX4 module in place of the *HSP26* gene. Three single bands of approximately 3.8 Kb, 9.2 Kb and 7.8 Kb were generated from DNA digests with NdeI, BclI and AclI, respectively, when probed with the kanMX probe, confirming the integration of the kanMX4 module in the position of the *HSP26* gene. No bands were generated from the PMY1.1 wild type strain (Figure 6.7). Sizes of the three bands observed were anticipated from the restriction enzyme cut sites as represented in Figure 6.10 a.

Southern analysis of *Δald4* was expected to show fragment sizes of approximately 9.9 Kb, 5.5 Kb and 3.1 Kb from the NdeI, BanI, and AclI DNA digests respectively. These fragments were produced, however, other bands were also generated in each digest (Figure 6.8). This indicated that whilst the kanMX4 module had replaced the *ALD4* gene it may have integrated elsewhere in the genome. This potentially 'double' mutant was not used in any further studies.

A second attempt at constructing an *ALD4* knockout strain was more successful. The construction steps were repeated, including the confirmation PCRs and the sequencing of products generated. The final confirmation step of Southern analysis was repeated but, because of problems with availability, some different restriction enzymes were used. Three single bands of approximately 5.5 Kb, 11.6 Kb and 5.55 Kb were generated from DNA digests with Ban1, AflII and Kpn1, respectively, when probed with the kanMX probe. No bands were generated from the PMY1.1 wild type strain (Figure 6.9). The three single bands from each digest were of the expected size as anticipated from the position of the restriction enzyme cut sites (Figure 6.10 b). The three single bands confirmed the correct and single integration of the kanMX4 module in place of the *ALD4* gene in the new *Δald4* strain.

6.2.3 Stability of the knockout strains

To test the stability of the knockout mutants, cultures of the PMY1.1, *Δhsp26* and *Δald4* strains were sub-cultured three times in non-selective YEPD media. Serial dilutions of the strains were plated onto both YEPD and YEPD Geneticin plates and incubated at 30°C for 2 days. The YEPD Geneticin plates did not support the growth of PMY1.1 wild type cells whereas viable cell counts for the knockout strains were very similar on YEPD as on YEPD Geneticin plates (Table 6.4). This confirmed the kanMX4 module was not lost from the knockout strains and they could be regarded as stable constructs.

Table 6.3: Oligonucleotides used to amplify part of the coding region of the KanMX4 module to create an 810 bp cDNA probe for Southern blotting.

Primer	Sequence
KanCR-F	5'-TGGGTAAGGAAAAGACTCACG-3'
KanCR-R	5'-ACTCATCGAGCATCAAATGA -3'

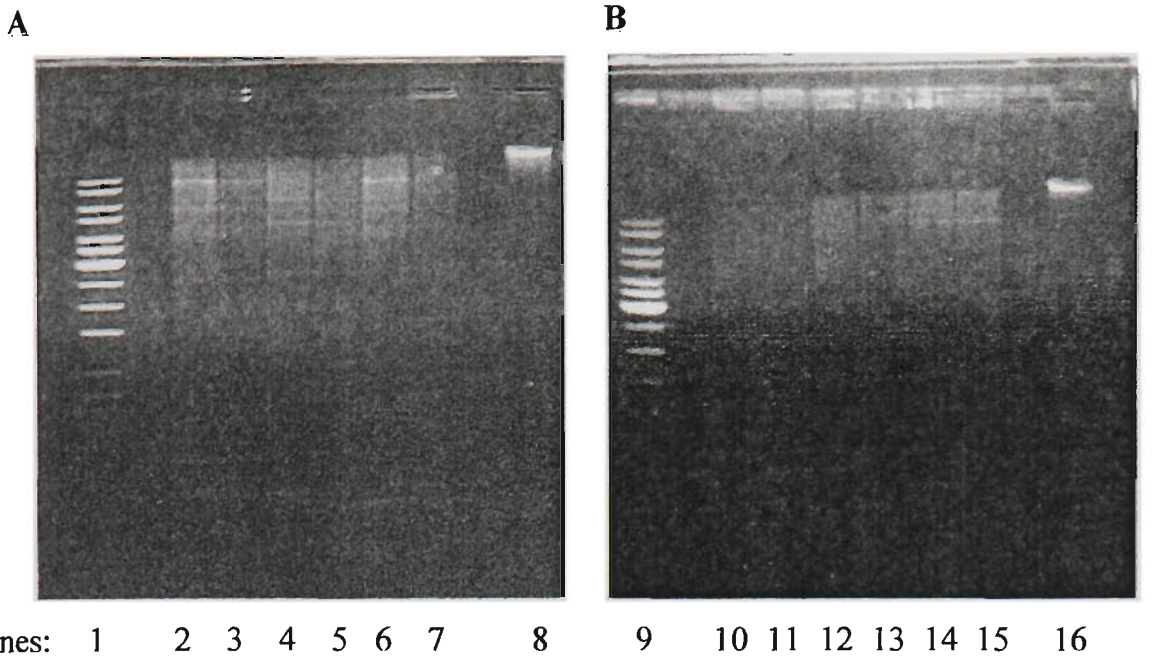


Figure 6.6: Genomic DNA from PMY1.1 wild type and *Δhsp26* and *Δald4* strains. Approximately 3 μg of genomic DNA was digested with restriction enzymes and separated on 1.0% agarose gels. **A)** represents digests of PMY1.1 wild type and *Δhsp26* DNA. Lanes 2 and 3 represent NdeI digests of wild type and knockout DNA respectively. Lanes 4 and 5 represent BclI digests and lanes 6 and 7 represent AclI digests, for wild type and knockout DNA respectively. **B)** represents digests of PMY1.1 wild type and *Δald4* DNA. Lanes 10 and 11 represent Ban1 digests of wild type and knockout DNA respectively. Lanes 12 and 13 represent AflII digests and lanes 14 and 15 represent KpnI digests, for wild type and knockout DNA respectively. A size standard, Generuler^{TM} 1kb DNA ladder (MBI Fermentas), was run in lanes 1 and 9. Lanes 8 and 16 represents approximately 3 μg of undigested genomic DNA as a control.

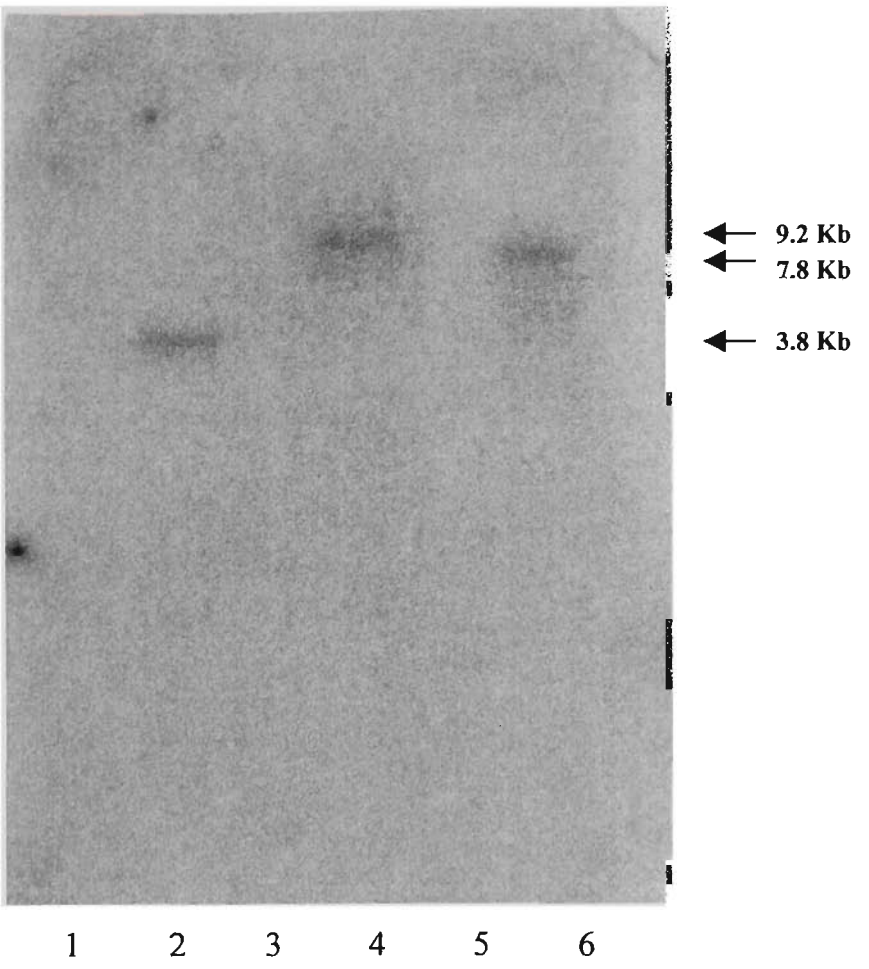


Figure 6.7: Southern analysis for confirmation of the *HSP26* knockout. Digested DNA from PMY1.1 wild type and *Δhsp26* cells was blotted and probed with a labelled cDNA probe homologous to the kanMX4 module. Lanes 2, 4 and 6 represent *Δhsp26* DNA, containing the kanMX4 module, from restriction digests with the enzymes NdeI, BclI, and AclI, respectively. Band sizes of approximately 3.8 Kb, 9.2 Kb and 7.8 Kb were calculated from the size standard on the restriction digest gel (Figure 6.6 a). Lanes 1, 3 and 5 represent PMY1.1 wild type DNA from restriction digests with the enzymes NdeI, BclI and AclI, respectively.

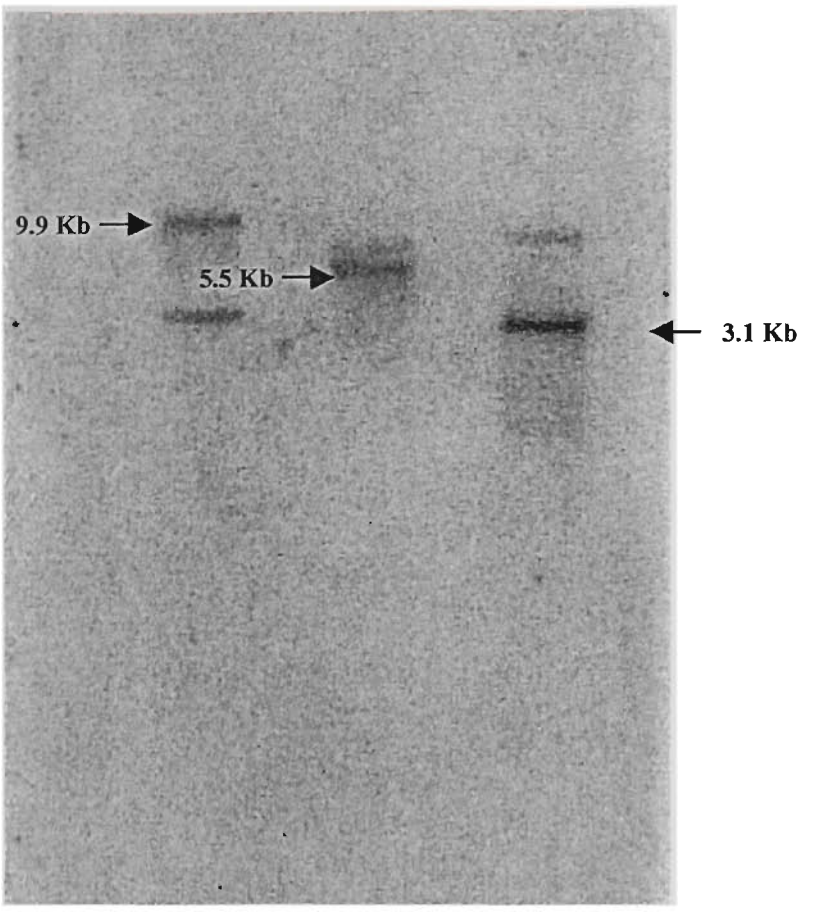


Figure 6.8: Initial Southern analysis for confirmation of the *ALD4* knockout. Digested DNA from PMY1.1 wild type and *Δald4* cells was blotted and probed with a labelled cDNA probe homologous to the kanMX4 module. Lanes 2, 4 and 6 represent *Δald4* DNA, containing the kanMX4 module, from restriction digests with the enzymes NdeI, BanI and AclI, respectively. Two bands were observed in each digest from *Δald4* indicating the double integration of the kanMX4 module. The expected band sizes of approximately 9.9 Kb, 5.5 Kb and 3.1 Kb for each respective digest were observed along with an additional unexpected band. Sizes were calculated from the size standard on the restriction digest gel (Figure not shown). Lanes 1, 3 and 5 represent PMY1.1 wild type DNA from restriction digests with the enzymes NdeI, BanI and AclI, respectively.

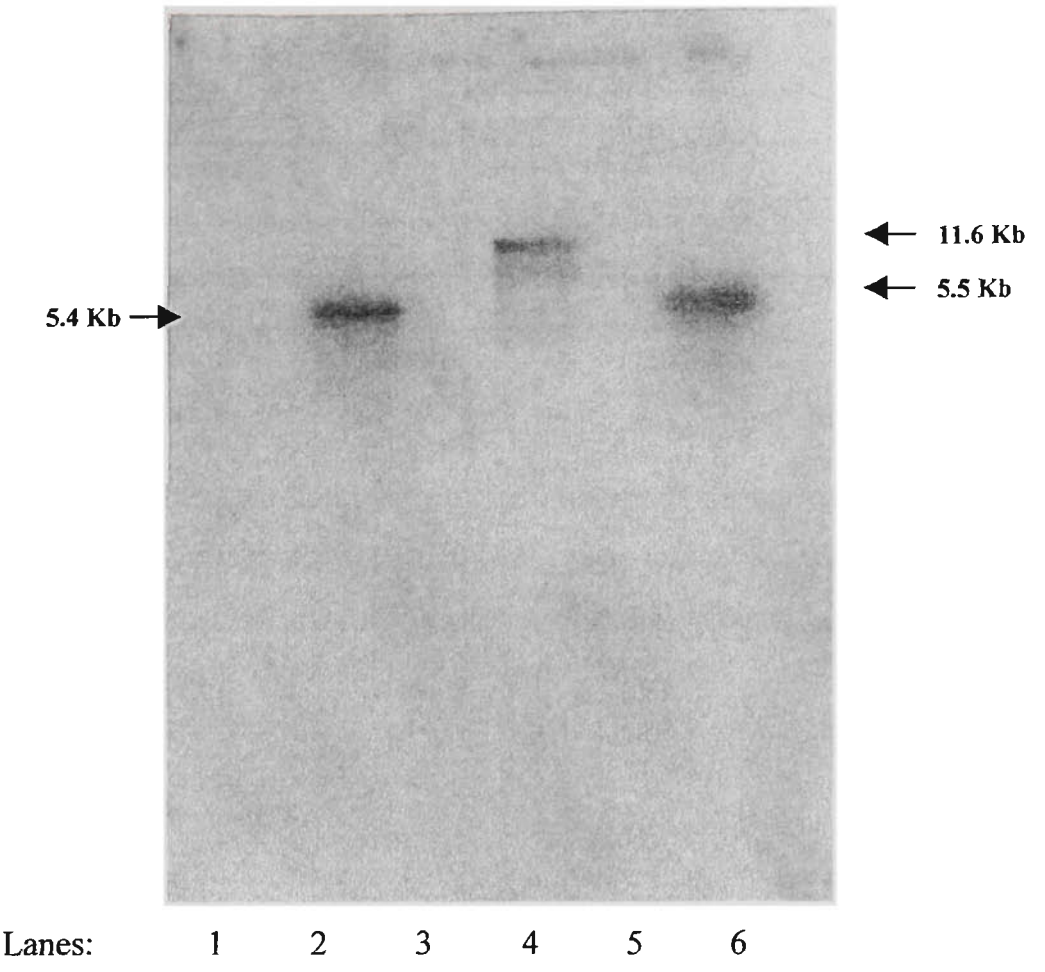


Figure 6.9: Southern analysis for confirmation of the *ALD4* knockout. Digested DNA from PMY1.1 wildtype and *Δald4* cells was blotted and probed with a labelled cDNA probe homologous to the kanMX4 module. Lanes 2, 4 and 5 represent *Δald4* DNA, containing the kanMX4 module, from restriction digests with the enzymes BanI, AflII and KpnI, respectively. Band sizes of approximately 5.4 Kb, 11.6 Kb and 5.5 Kb were calculated from the size standard on the restriction digest gel (Figure 6.6 b). Lanes 1, 3 and 5 represent PMY1.1 wild type DNA from restriction digests with the enzymes BanI, AflII and KpnI, respectively.

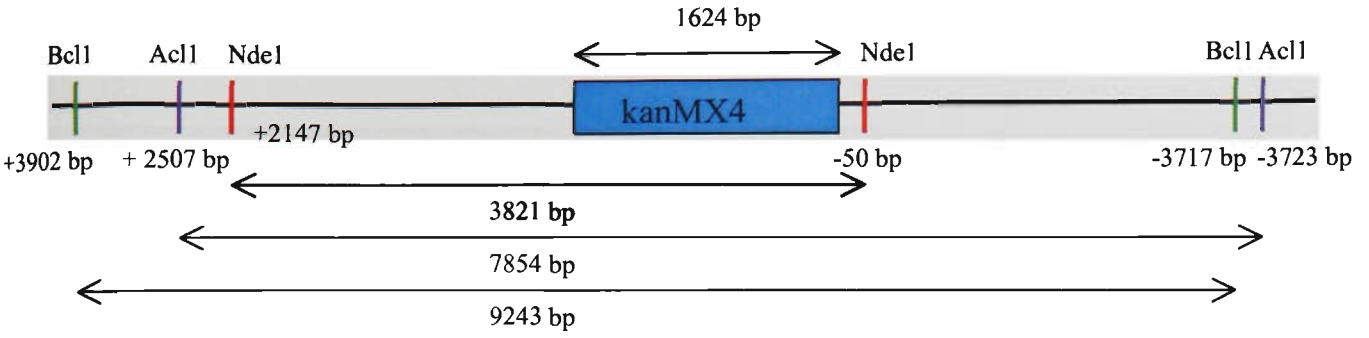


Figure 6.10 a: Diagram (not to scale) for a region of chromosome II showing regions upstream and downstream of the *HSP26* gene which has been replaced with the kanMX4 module. Cut sites for restriction endonucleases and expected fragment lengths represented.

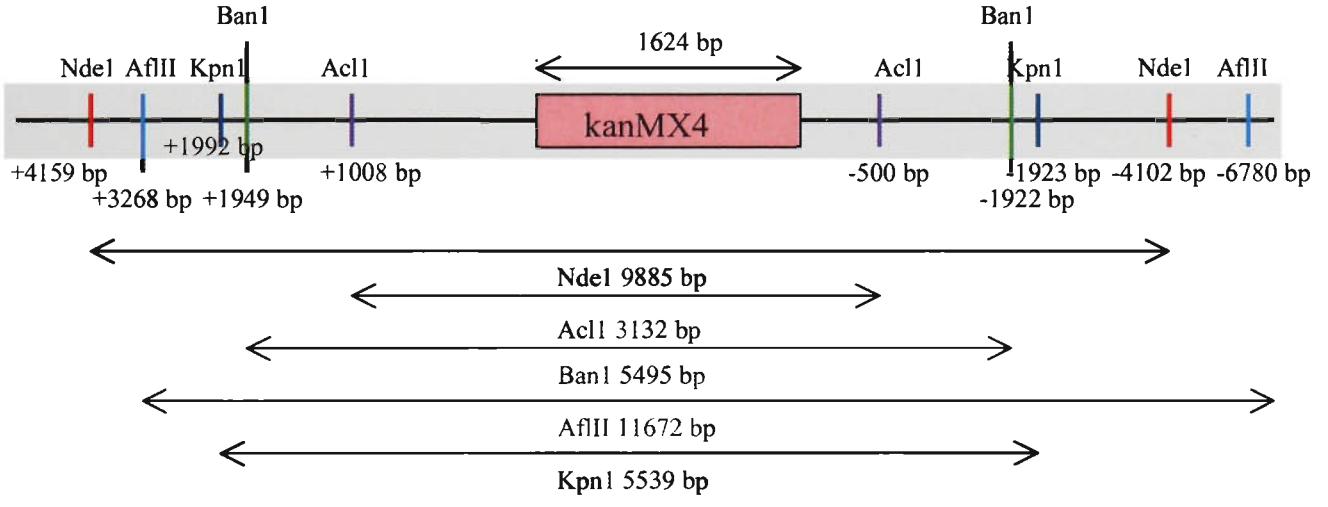


Figure 6.10 b: Diagram (not to scale) for a region of chromosome XV showing regions upstream and downstream of the *ALD4* gene which has been replaced with the kanMX4 module. Cut sites for restriction endonucleases and expected fragment lengths represented.

Table 6.4: Stability of the *Δhsp26* and *Δald4* knockout strains.

STRAIN	MEDIUM	VIABLE CELL COUNTS (cells ml^{-1})
Δhsp26	YEPD	8.6×10^7
Δhsp26	YEPD + geneticin	9.2×10^7
PMY1.1	YEPD	7.8×10^7
PMY1.1	YEPD + geneticin	no growth
Δald4	YEPD	8.1×10^7
Δald4	YEPD + geneticin	8.6×10^7
PMY1.1	YEPD	7.8×10^7
PMY1.1	YEPD + geneticin	no growth

6.3 PHYSIOLOGY OF *Δhsp26* AND *Δald4* DURING ETHANOL STRESS

6.3.1 Growth profiles of *Δhsp26* and *Δald4* in the presence of non-lethal ethanol concentrations

The knockout strains, *Δhsp26* and *Δald4* were grown in defined medium in the presence and absence of added ethanol. Growth profiles of the two knockout strains were compared to the wild type PMY1.1 parent strain. The experimental design was based on growth studies described in Chapter 2 (Section 2.2) and Chapter 3 (Section 3.2). Late exponential phase cells were inoculated into fresh defined medium in the absence and presence of 5%, 7% or 10% (v/v) ethanol. Samples were taken at regular time intervals and growth was monitored by optical density measurements (OD_{620}) and duplicate plate counts.

No discernable differences were observed in the growth profiles of *Δhsp26* (Figure 6.11) and *Δald4* (Figure 6.12) when compared to the growth profiles of the PMY1.1 wild type strain, in the presence and absence of ethanol. In the absence of ethanol stress, all three strains commenced exponential growth within one hour post-inoculation and their growth rates were not significantly different (Table 6.5). Lag periods of between 3-4 hours were observed for all three strains in 5% (v/v) ethanol-stressed cultures, and 5-6 hours in 7% (v/v) ethanol-stressed cultures. There was no significant difference between the exponential growth rates of the parent strain and the knockout strain in the presence of either 5% or 7% (v/v) ethanol (Table 6.5). At 10% (v/v) ethanol stress, none of the strains commenced exponential growth during the 11-hour sampling period, even though there was no loss in cell viability. Optical density growth curves are not shown as the viable cell count growth curves more accurately reflect viable cell populations and cell division.

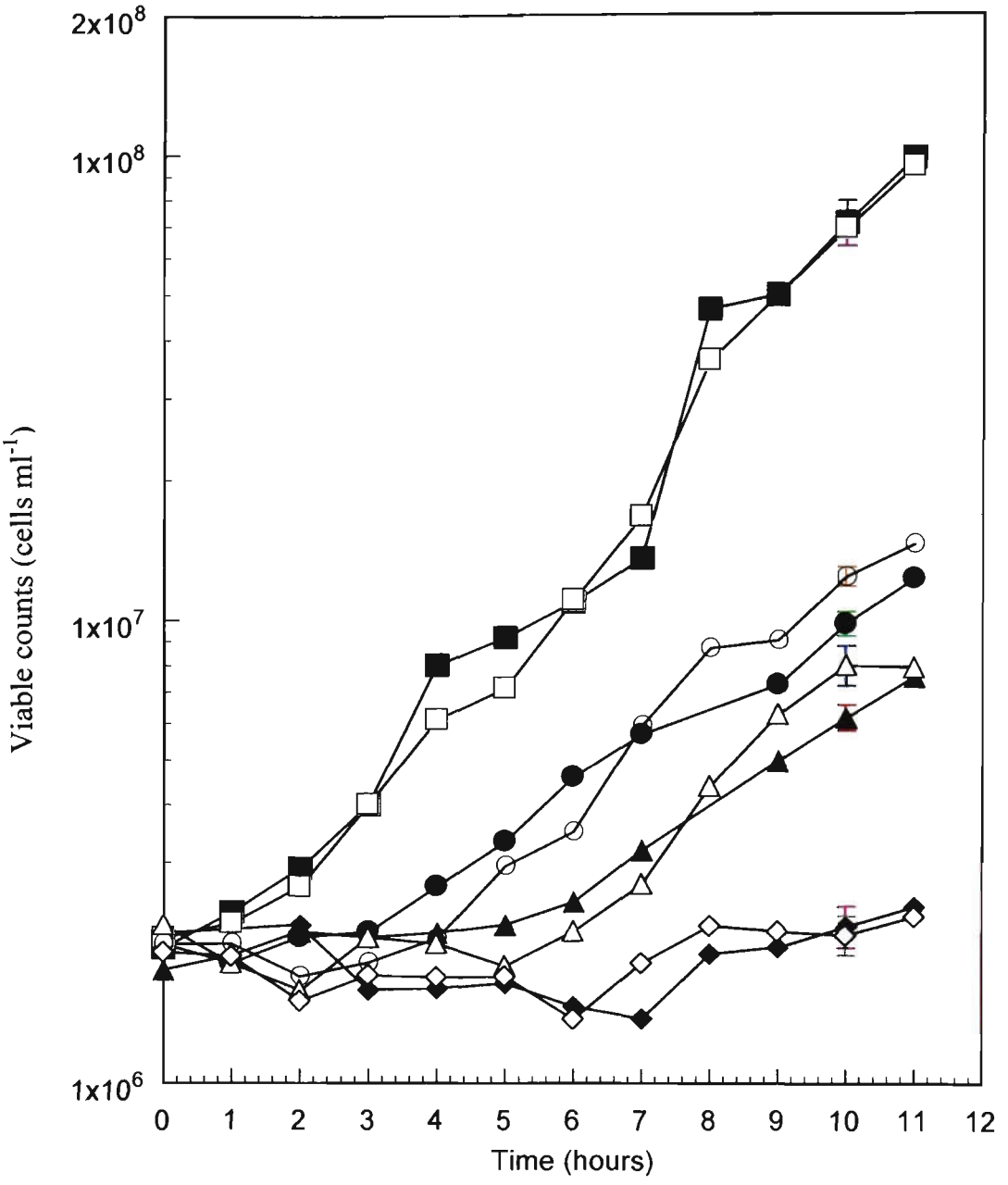


Figure 6.11: Effect of ethanol concentration on the viable cell population of PMY1.1 and *Δhsp26*. Cells from a late exponential phase parent culture were centrifuged, washed and inoculated into defined medium only (■) PMY1.1, (□) *Δhsp26*, or defined medium containing either 5% (v/v) ethanol (●) PMY1.1, (○) *Δhsp26*; 7% (v/v) ethanol (▲) PMY1.1, (Δ) *Δhsp26*; or 10% (v/v) ethanol (♦) PMY1.1, (◇) *Δhsp26*. The cultures were incubated at 30°C and 160 rpm. Error bars are shown for a representative time point in each line series.

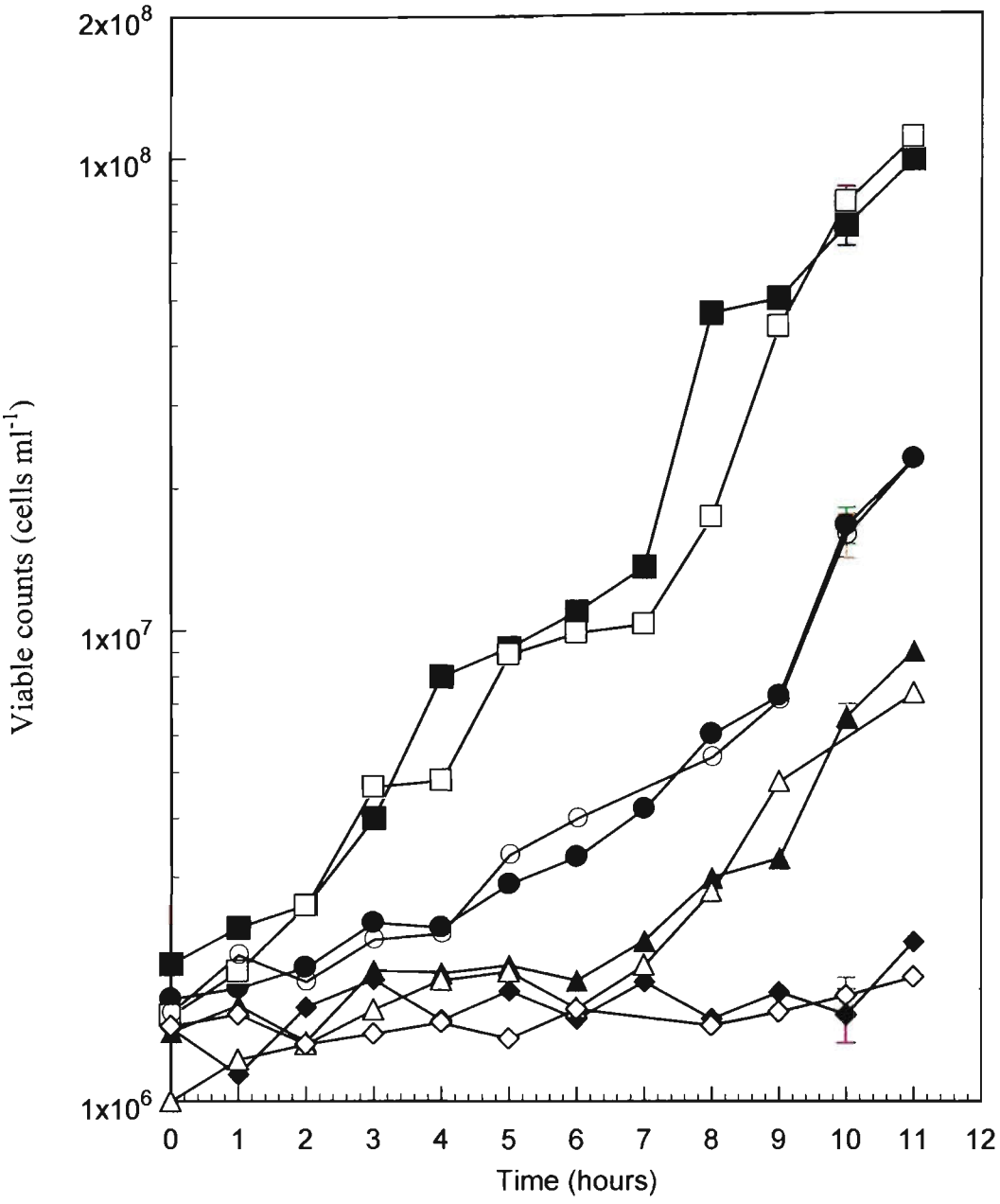


Figure 6.12: Effect of ethanol concentration on the viable cell population of PMY1.1 and *Δald4*. Cells from a late exponential phase parent culture were centrifuged, washed and inoculated into defined medium only (■) PMY1.1, (□) *Δald4*, or defined medium containing either 5% (v/v) ethanol (●) PMY1.1, (○) *Δald4*; 7% (v/v) ethanol (▲) PMY1.1, (Δ) *Δald4*; or 10% (v/v) ethanol (♦) PMY1.1, (◇) *Δald4*. The cultures were incubated at 30°C and 160 rpm. Error bars are shown for a representative time point in each line series.

6.3.2 Viable population profile of *Δhsp26* and *Δald4* under lethal ethanol concentrations

Ethanol tolerance and cell viability of the *Δhsp26* and *Δald4* strains were compared to the PMY1.1 wild type in the presence of lethal ethanol concentrations. It was speculated that different biochemical mechanisms may be involved in yeast tolerance and survival in lethal ethanol concentrations, compared to the biochemical mechanisms promoting cell adaptation and growth in non-lethal ethanol concentrations, as studied in the previous section. The experimental plan in the previous section was modified slightly to study cell survival in high ethanol concentrations. Late exponential phase cells were inoculated into defined medium in the absence and presence of 10%, 11%, 12%, 13% or 14% (v/v) added ethanol. Samples were taken at regular time intervals and viable cell population was monitored by duplicate plate counts.

No discernable difference in viable cell population profiles of *Δhsp26* (Figure 6.13) and *Δald4* (Figure 6.14) was observed when compared with the viable cell population of the PMY1.1 wild type, in the absence or presence of lethal ethanol concentrations. In the absence of ethanol stress all three strains commenced exponential growth within one hour post-inoculation and their growth rates were not significantly different from each other (Table 6.5). For all three strains, cell viability did not significantly change at 10% (v/v) ethanol over 9 hours, while at 11% (v/v) ethanol, cell viability began to decline slowly following 3 hours of exposure to ethanol. At ethanol concentrations of 12% (v/v) and above, cell populations declined more rapidly, with exposure to 14% (v/v) ethanol being considerably lethal to all strains. The overall ethanol tolerance and rate of cell death in *Δhsp26* and *Δald4* was not notably different to the wild type strain over the ethanol concentration range of 10-14% (v/v).

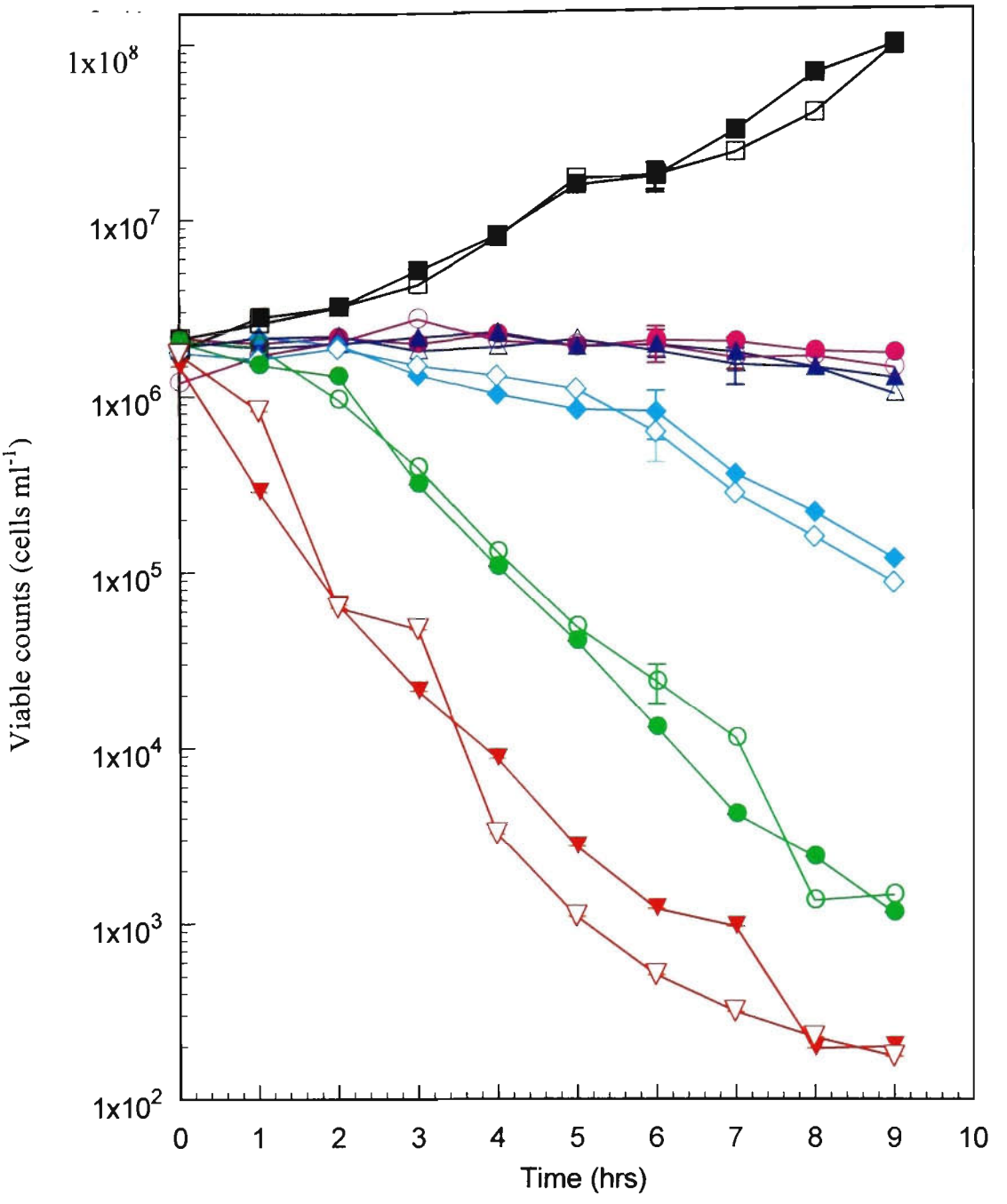


Figure 6.13: The effect of increasing ethanol concentrations on the viable cell populations of PMY1.1 and *Δhsp26*. Cells from a late exponential phase parent culture were washed and inoculated onto defined medium only (■) PMY1.1, (□) *Δhsp26*, or defined medium containing 10% (v/v) ethanol (●) PMY1.1, (○) *Δhsp26*; 11% (v/v) ethanol (▲) PMY1.1, (Δ) *Δhsp26*; 12% (v/v) ethanol (♦) PMY1.1, (◇) *Δhsp26*; 13% (v/v) ethanol (●) PMY1.1, (○) *Δhsp26*; and 14% (v/v) ethanol (▼) PMY1.1, (▽) *Δhsp26*. The cultures were incubated at 30°C and 160 rpm. Error bars are shown for a representative time point in each line series. Some error bars maybe smaller than the corresponding symbol.

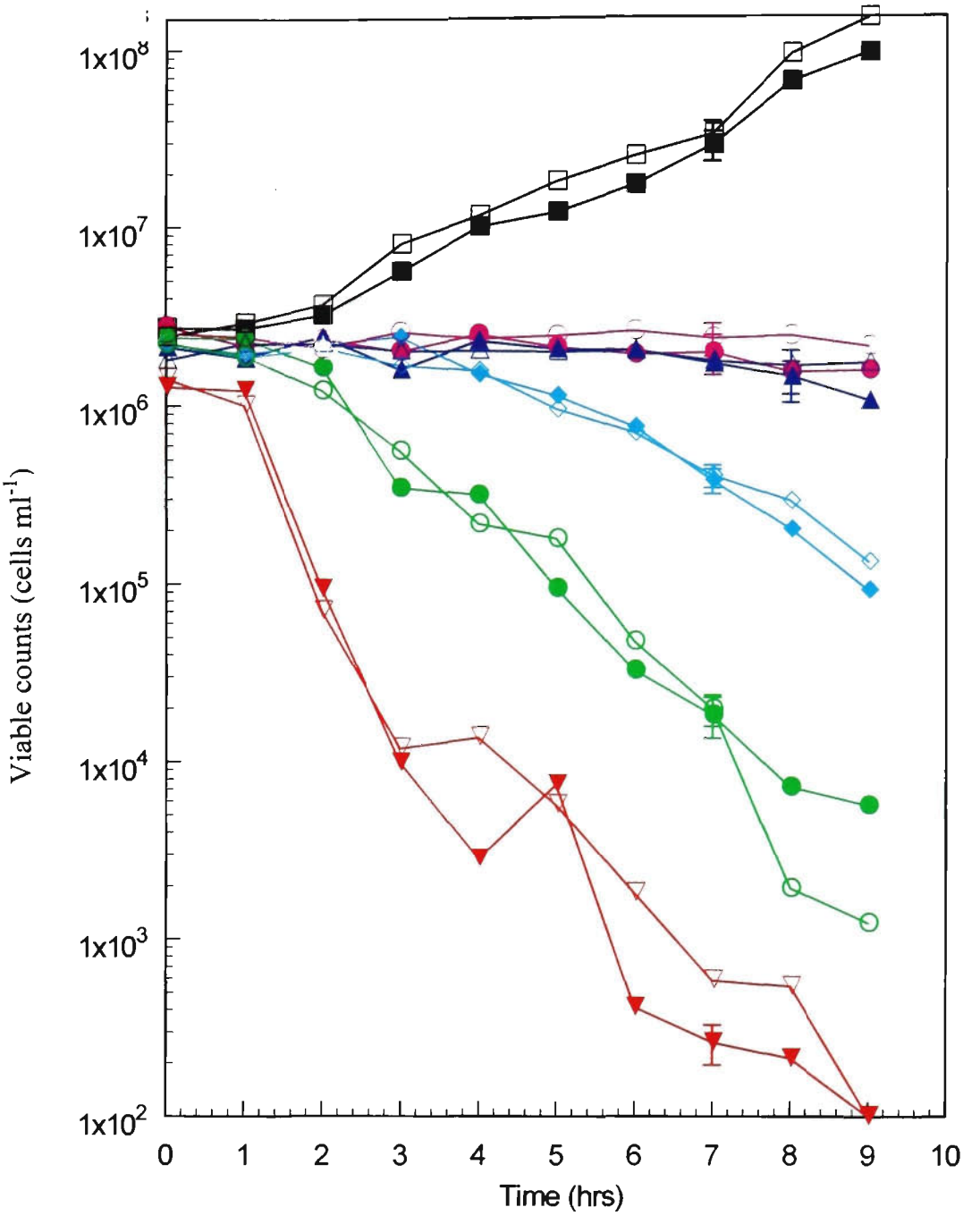


Figure 6.14: The effect of increasing ethanol concentrations on the viable cell populations of PMY1.1 and *Δald4*. Cells from a late exponential phase parent culture were washed and inoculated onto defined medium only (■) PMY1.1, (□) *Δald4*, or defined medium containing 10% (v/v) ethanol (●) PMY1.1, (○) *Δald4*; 11% (v/v) ethanol (▲) PMY1.1, (Δ) *Δald4*; 12% (v/v) ethanol (◆) PMY1.1, (◇) *Δald4*; 13% (v/v) ethanol (●) PMY1.1, (○) *Δald4;* and 14% (v/v) ethanol (▼) PMY1.1, (▽) *Δald4*. The cultures were incubated at 30°C and 160 rpm. Error bars are shown for a representative time point in each line series. Some error bars maybe smaller than the corresponding symbol.

6.4 STUDIES INTO A SPECIFIC ROLE FOR *HSP26* AND *ALD4* DURING THE ADAPTATION OF *S. cerevisiae* TO ETHANOL STRESS

The results described in the previous two sections showed no apparent difference in the ability of *Δhsp26* and *Δald4* to adapt and grow in the presence of ethanol, or survive high ethanol concentrations, compared to the wild type strain. It was decided to undertake additional physiological studies to test the phenotype of each knockout strain during ethanol stress, but under conditions designed to test a specific hypothetical role for the products of *HSP26* and *ALD4*.

6.4.1 Ethanol prestressing; a specific role for *HSP26* in the adaptation of *S. cerevisiae* to ethanol stress

Sub-lethal heat and ethanol exposure induce a similar stress response in *S. cerevisiae*. Such responses are characterised by the induction of HSPs, proteins requiring heat stress caused by temperatures greater than 35°C or ethanol concentrations greater than 4-6% (v/v) for strong induction (Piper, 1995). Pre-exposure of cells to a heat shock leads to the acquisition of thermotolerance and ethanol tolerance, however, ethanol pre-exposure actually hypersensitises cells to heat (van Uden, 1984; Piper, 1995). Ethanol pre-exposure has been demonstrated to induce the tolerance of yeast cells to subsequent and lethal concentrations of ethanol (Costa *et al.*, 1993).

The induction of *HSP26* results from exposure to both heat shock and ethanol stress (Haslebeck *et al.*, 1999; Piper *et al.*, 1994; Alexandre *et al.*, 2001). The generally accepted mechanism to explain the effects of ethanol or temperature pre-exposure on cell adaptation to a subsequent stress is that the pre-exposure induces an early stress response, thereby better preparing the cell for the subsequent stress compared to the absence of pre-exposure. Hsp26 has been previously been recognised as a major component in cells pre-exposed (Piper *et al.*, 1994). In this case, it is possible that the biggest contribution to stress tolerance by Hsp26 is apparent only when the cell has relatively high concentrations of the protein prior to stress exposure. The *HSP26* knockout experiments described in the previous section may not have detected a unique phenotype during ethanol stress because the cellular Hsp26 concentrations in the parent strain were no greater than in the knockout when inoculated into ethanol

containing medium. This needed further investigation. It was decided to compare the phenotype of the *HSP26* knockout with that of the parent following an ethanol pre-stress. A one hour exposure to 5% (v/v) ethanol containing medium was thought to be an appropriate pre-stress protocol, as this time frame and ethanol percentage have been shown to considerably increase the expression of *HSP26* (Table 5.1). The premise for these experiments is that when both strains are inoculated after the pre-stress, the parent PMY1.1 strain will have a significantly higher Hsp26 concentration compared to the *HSP26* knockout strain, *Δhsp26*.

Δhsp26 was tested for its growth response following a 5% (v/v) ethanol pre-stress for one hour. The growth profile of pre-stressed *Δhsp26* was compared to prestressed PMY1.1 wild type in the presence of 5% (v/v) ethanol, based on ethanol pre-stress methods as described in Chapter 2 (Section 2.2.2.5). Samples from control and pre-stress cultures were taken at hourly intervals and viable cell populations monitored by duplicate plate counts.

No prestressed and non-prestressed inocula showed any discernable lag period when added to fresh medium without added ethanol; the prestressed cells not showing any apparent side effects from the prestress. When inoculated into 5% (v/v) ethanol containing medium (with no pre-stress), the *Δhsp26* knockout and the PMY1.1 wild type strains both had lagged for three-hours before exponential growth commenced (Figure 6.15). This was consistent with lag periods observed in previous 5% (v/v) ethanol stress experiments (Section 6.3). Following the one-hour, 5% (v/v) ethanol pre-stress, the *Δhsp26* knockout and PMY1.1 wild type strains both had a lag period of approximately two hours when inoculated into fresh 5% (v/v) ethanol containing medium; this was one hour shorter than the lag period observed with these strains in the absence of pre-stressing. The one-hour reduction in lag period of the pre-stress strains corresponded to the one hour pre-exposure to 5% (v/v) ethanol. The growth rates of similarly treated *Δhsp26* knockout and PMY1.1 wild type strains were not dramatically different (Table 6.5).

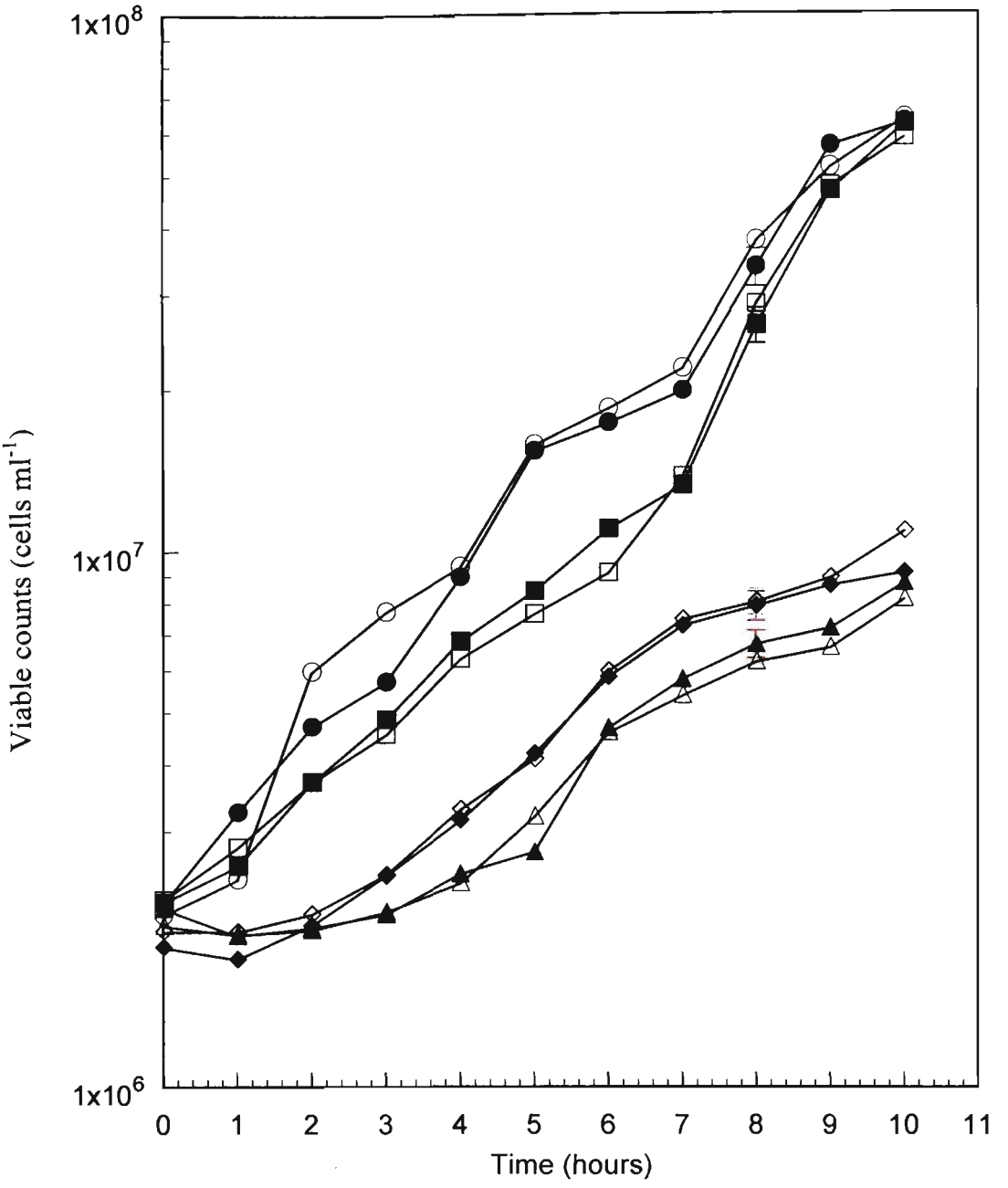


Figure 6.15: The effect of an ethanol pre-stress on the adaptation rate of *S. cerevisiae* strains PMY1.1 and *Δhsp26* subjected to a 5% (v/v) ethanol stress. Cells from late exponential phase parent cultures were centrifuged, washed and divided into two. One portion was inoculated into defined medium only(■) PMY1.1, (□) *Δhsp26,* or defined medium containing 5% (v/v) added ethanol(▲) PMY1.1, (Δ) *Δhsp26.* The other portion was subjected to a one hour 5% (v/v) ethanol pre-stress before inoculation into defined medium only (●) PMY1.1, (○) *Δhsp26* or defined medium with the addition of 5% (v/v) added ethanol (♦) PMY1.1 (◇) *Δhsp26.* All incubations were conducted at 30°C and at 160 rpm. Error bars are shown for a representative time point in each line series.

6.4.2 Acetaldehyde accumulation; a specific role for *ALD4* in the adaptation of *S. cerevisiae* to ethanol stress

Evidence that small quantities of extracellular acetaldehyde may play a stimulatory role in the adaptation of yeast to ethanol stress was first reported by Walker-Caprioglio and Parks (1987). The lag periods observed when *S. cerevisiae* was inoculated into medium containing 4% (v/v) ethanol, and incubated under aerobic conditions, were greatly reduced by the addition of small quantities of acetaldehyde (Walker-Caprioglio *et al.*, 1987; Stanley *et al.*, 1997). A similar reduction in lag phase due to a 4% (v/v) ethanol stress was also observed when high inoculum sizes, rather than acetaldehyde addition, were used (Walker-Caprioglio *et al.*, 1985; Stanley *et al.*, 1997). The relationship between increasing inoculum size and decreasing lag period was considered to be due, in part, to high inoculum size subcultures containing higher lag-reducing quantities of endogenously produced acetaldehyde which accumulated in the extracellular medium i.e. the higher the cell population, the greater the rate of acetaldehyde accumulation (Walker-Caprioglio *et al.*, 1987; Stanley *et al.*, 1997).

Acetaldehyde is now generally accepted to have a stimulatory role in the adaptation of *S. cerevisiae* to ethanol stress. In this context, and that of prior work (Stanley *et al.*, 1997), it is believed that the amount of endogenous acetaldehyde produced by *S. cerevisiae* during ethanol stress has some influence on the time it takes for the cell to adapt to the ethanol and commence growth (i.e. the higher the acetaldehyde production rate, the faster the adaptation rate). In *S. cerevisiae*, acetaldehyde is produced by the decarboxylation of pyruvate. It is then either reduced by alcohol dehydrogenase to ethanol, or oxidized by aldehyde dehydrogenase to acetate (Figure 5.3). It is plausible that *S. cerevisiae Δald4* might have a changed acetaldehyde accumulation rate, given that it no longer had a functional Ald4 enzyme to oxidise the acetaldehyde, and consequently its adaptation rate to ethanol stress could also be affected. Although earlier experiments found no detectable difference in lag period between *Δald4* and the parent strain during ethanol stress (Figure 6.12), such experiments were performed at relatively high inoculum sizes (approximately 2×10^6 cells ml^{-1}) where subtle changes in acetaldehyde production rate are potentially masked by the relatively large amounts of acetaldehyde excreted into the culture (Stanley *et al.*, 1997). To test this further, it was decided to investigate the adaptation

rate of *Δald4* to ethanol stress at lower cell populations, where potentially small changes in specific acetaldehyde production rates may exert an influence on the stress adaptation process.

The effect of inoculum size on the length of lag phase and growth of PMY1.1 wild type and *Δald4* knockout strains in the presence and absence of ethanol stress (5% v/v) was examined (Figure 6.16). Plate counts were used to measure cell populations during the growth for the entire population range examined (3×10^4 to 3×10^6 cells ml^{-1}). This initial cell population range was chosen since it had previously been shown that cell populations below 10^4 cells ml^{-1} had lag periods that were no longer dependent on the biomass level (Stanley *et al.*, 1997). The inocula for this experiment were washed in fresh medium to remove effects resulting from carry over of different amounts of parent culture medium, as described in Section 2.2.2.

No discernable inoculum size effect on the length of lag period was demonstrated in cultures inoculated into medium without added ethanol. Soon after inoculation, all cultures commenced exponential growth, however the growth rates of the higher cell population cultures (10^6 cells ml^{-1}) were lower than that of the lower cell population cultures (Figure 6.16). An inoculum size effect on the ethanol-induced lag period was, however, seen in cultures inoculated into medium containing ethanol. Ethanol induced lag periods decreased with increasing inoculum size. Cultures with lower initial cell populations of approximately 2×10^4 and 2×10^5 cells ml^{-1} showed extended ethanol-induced adaptation periods. Cultures inoculated at 2×10^4 cells ml^{-1} did not recover from the ethanol stress over the course of 13 hours, while cultures inoculated at 2×10^5 cells ml^{-1} began to slowly grow at around 5 hours post inoculation. Cultures inoculated to a population of 2×10^6 cells ml^{-1} recovered from the ethanol stress following a 3 hour lag phase/adaptation period. The exponential growth rates after the lag period were lower at lower cell populations (Table 6.5). There was no significant difference in growth profiles between wild type and *Δald4* strain, both in the presence and absence of ethanol and at different cell populations.

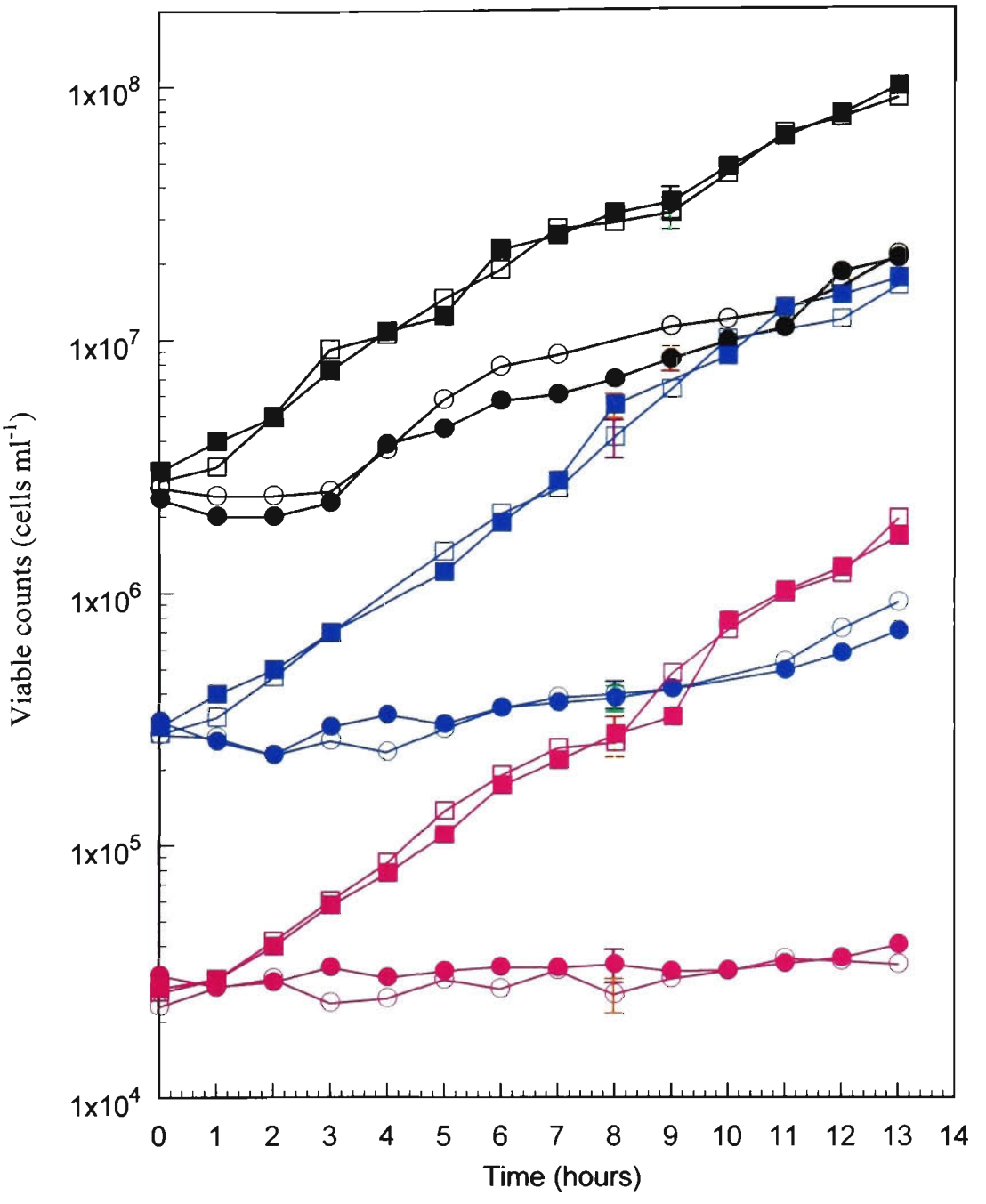


Figure 6.16: The effect of inoculum size on the length of lag phase and growth rate of *S. cerevisiae* strains PMY1.1 and *Δald4* in the presence of an ethanol stress. Cells from late exponential phase parent cultures were centrifuged, washed, and inoculated to the appropriate initial cell population in defined media only, (■) PMY1.1 wild type and (□) *Δald4*, or defined media containing added ethanol (5% v/v) (●) PMY1.1 wild type and (○) *Δald4* knockout. The cultures were incubated at 30°C and 160 rpm. Error bars are shown for a representative time point in each line series. Some error bars may be smaller than the corresponding symbol.

Table 6.5: Summary of the effect of ethanol on the lag period, doubling time and specific growth rate of the *S. cerevisiae* strains PMY1.1, *Δhsp26* and *Δald4*.

ETHANOL (%)	FIGURE	STRAIN	LAG PERIOD (H)	DOUBLING TIME (H)	GROWTH RATE (H^{-1})
0%	6.11	PMY1.1	0	2	0.34
		Δhsp26	0	2	0.34
	6.12	PMY1.1	0	2	0.34
		Δald4	0	2	0.34
	6.13	PMY1.1	0	1.9	0.36
		Δhsp26	0	1.9	0.36
	6.14	PMY1.1	0	1.9	0.36
		Δald4	0	1.9	0.36
	6.15	PMY1.1 (prestress)	0	2	0.34
		PMY1.1 (no prestress)	0	2	0.34
	6.16	PMY1.1 (10^6)	0	2.2	0.31
		Δald4 (10^6)	0	2.2	0.31
		PMY1.1 (10^5)	0	2	0.34
		Δald4 (10^5)	0	2	0.34
		PMY1.1 (10^4)	0	2	0.34
		Δald4 (10^4)	0	2	0.34
5%	6.11	PMY1.1	3	3	0.23
		Δhsp26	3	3	0.23
	6.12	PMY1.1	4	2.5	0.27
		Δald4	4	2.5	0.27
	6.15	*Δhsp26* (prestress)	3	3	0.23
		Δhsp26 (prestress)	2	3	0.23
	6.16	PMY1.1 (10^6)	3	3	0.23
		Δald4 (10^6)	3	3	0.23
		PMY1.1 (10^5)	2	2	0.34
		Δald4 (10^5)	2	2	0.34
		PMY1.1 (10^4)	N/D	N/D	N/D
		Δald4 (10^4)	N/D	N/D	N/D
7%	6.11	PMY1.1	5	3.8	0.18
		Δhsp26	5	3.8	0.18
	6.12	PMY1.1	6	2	0.34
		Δald4	6	2	0.34
10%	6.11	PMY1.1	N/D	N/D	N/D
		Δhsp26	N/D	N/D	N/D
	6.12	PMY1.1	N/D	N/D	N/D
		Δald4	N/D	N/D	N/D

6.5 DISCUSSION

6.5.1 Construction of gene knockout strains

This chapter described the construction of Δ*hsp26* and Δ*ald4* gene knockout strains using the PCR-based gene deletion methodology of Wach *et al.* (1994). The gene deletion modules were generated directly by PCR of the kanMX4 marker module using short flanking homology primers that contained approximately 40 bp of flanking sequence of the relevant *HSP26* or *ALD4* ORF. Direct transformation of the PCR product into the PMY1.1 wild type haploid yeast strain produced many geneticin resistant transformants.

While gene deletion using the PCR-based methodology of Wach *et al.* (1994) is a recognised method for creating gene specific deletions, it is not without some obstacles. In the construction of an *ALD4* knockout strain, a single geneticin resistant transformant colony was selected and the successful integration of the kanMX4 module into colony isolates appeared confirmed via colony PCR. Southern blotting to further confirm the correct and single integration of the kanMX4 module revealed the module had integrated in place of the *ALD4* ORF, however it had probably also integrated elsewhere in the genome rendering the knockout unusable for the physiology experiments. Overall the number of reported incorrectly targeted kanMX4 modules in geneticin resistant transformants are few. Wach *et al.* (1998) report the successful replacement of 20 different ORFs with the kanMX4 module. Of 350 geneticin-resistant transformants tested, six were reported to carry incorrectly targeted kanMX4 modules. Using the same gene disruption technique, Watson (2001) showed 95% (149/156 colonies) of geneticin resistant transformant colonies could be classified as true transformants. The selection of transformant colonies appears to be based upon the chance of correct kanMX4 module integration. Southern analysis of selected transformants is crucial to indicate the single integration of the kanMX4 marker module into the targeted site. In this work, the selection of a different geneticin resistant transformant colony confirmed the positive and correct integration

of the kanMX4 module in place of the *ALD4* ORF. Confirmation of the *HSP26* knockout strain was more straightforward, with the selected colony proving positive.

6.5.2 The *HSP26* knockout phenotype

A unique phenotype for *Δhsp26* was not observed in the ethanol stress experiments described in this chapter. Deletion of the *HSP26* gene did not result in a significant difference in viable population profile compared to the wild type strain, either when incubated in the presence of lethal or non-lethal ethanol concentrations or without added ethanol. In addition, pre-adaptation of the knockout and wild type strains to 5% (v/v) ethanol and a subsequent exposure to an ethanol stress did also not result in any discernable difference in the growth profile of the two strains.

Other studies to determine a role for Hsp26 have been undertaken by Susek and Lindquist (1989), Petko and Lindquist (1986) and de Nobel *et al.* (2001). To uncover a function of Hsp26 in thermotolerance, Susek and Lindquist (1989) expressed the *HSP26* gene in the absence of heat shock. The premise was if Hsp26 plays a role in thermotolerance, its constitutive expression might provide the cells constitutive thermotolerance. It was, however, found that only a very minor increase in thermotolerance (after 10 to 13 minutes of exposure to 50°C) was achieved by *HSP26* over expression. Experiments with *HSP26* deletion strains during ethanol stress have been undertaken in one other study. Petko and Lindquist (1986) examined the survival of *Δhsp26* deletion and disruption mutants compared to the wild type strain when subjected to high ethanol concentrations. The ability of log phase cells (approximately 2×10^6 cells ml^{-1}) grown in an acetate medium to survive 8%, 15% and 25% (v/v) added ethanol for two hours was based on examination of viable cell populations. No detectable effects on the ability of the mutant and control strains to withstand the toxic effects of ethanol were observed. While this study provides some insight into the tolerance of a *HSP26* mutant to ethanol stress, the growth profile of the mutants in comparison to the wild type was not made clear as results were obtained from only one time point. Examination of population profiles over a series of time points might have revealed differences in rate of cell death. Although the results of this chapter support those observed by Petko and Lindquist (1986), it must be remembered that

their experiments were undertaken in an acetate medium where the metabolism will be different to that of cells grown on glucose, as used in this study (Section 6.1.2).

A demonstratable function for *HSP26* has, however, been suggested by de Nobel *et al.* (2001). A *HSP26* knockout strain was found to be sensitive to sorbic acid. At pH 4.5, growth of the *Δhsp26* mutant was identical to that of the wild type but after addition of 1.8 mM sorbic acid inhibition of growth was much greater in the mutant than the wild type. A similar effect was also observed by spotting serial dilutions of wild type and *Δhsp26* mutant cultures on YEPD plates (pH 4.5) containing 0.9 mM sorbic acid. The role of *HSP26* during adaptation to sorbic acid was suggested to confer resistance to the inhibitory effects of the compound.

The inability to detect a unique phenotype using a *HSP26* knockout under ethanol stress could be due to one or more other genes compensating or masking the role of Hsp26. Petko and Lindquist (1986) postulated that another small HSP might compensate for the loss of Hsp26 in deletion strains. Several attempts to identify such a protein proved unsuccessful because: i) no other protein of the size was strongly induced by the same stresses which induced *HSP26*, ii) no other protein was noticeably overexpressed in compensation for the loss of *HSP26* in the Δ*hsp26* strain, and iii) no other gene was detected by a low stringency hybridisation with a *HSP26* probe (Petko and Lindquist, 1986). It is possible, however, that another protein may have escaped the above criteria and could compensate for the loss of Hsp26 in cells under ethanol stress. Many of the genes up-regulated in the gene array data given in Chapter 5 (Section 5.3.2) of this thesis could well have compensatory roles for Hsp26. Given the number of these up-regulated genes, and the large number with unknown functions, it is feasible that a compensatory gene (or number of genes) may replace the loss of function of Hsp26.

Susek and Lindquist (1989) speculated that small HSP genes represent ancient viruses or selfish DNA elements that provide no important physiological advantage to the cell. This speculation is supported by the amino acid relatedness of Hsp26 to a known viral coat protein and to yeast nucleases and maturases. It is also supported by the fact that small HSPs from a majority of eukaryotes form cytoplasmic particles, which are

reminiscent of viral particles. Even if such an origin is true for small HSPs, it would not exclude these proteins from having important cellular functions during the course of evolution (Susek and Lindquist, 1989).

6.5.3 The *ALD4* knockout phenotype

A unique phenotype for *Δald4* was not established from the ethanol stress experiments described in this chapter. Deletion of the *ALD4* gene did not result in a significant difference in cell population profile compared to the wild type strain, either when incubated in the presence of non-lethal ethanol concentration, or without added ethanol. The exposure to high ethanol concentrations also did not result in any difference in death rate between the two strains. In addition, different inoculum sizes had no effect on the rate of adaptation to ethanol stress between *ALD4* knockout and wild type strains; cell populations for the two strains were similar over the time course.

A unique phenotype for an *ALD4* knockout strain has not previously been described in a stress situation. *ALD4* knockout mutants have however been used to establish a gene function for *ALD4*. Remize *et al.* (2000) and Boubekeur *et al.* (1999, 2001) used *ALD4* knockout strains to identify a role for *ALD4* in the oxidation of acetaldehyde to acetate for biosynthetic purposes. In addition to this role it was also suggested that *ALD4* may play a role in cellular detoxification (Remize *et al.*, 2000; Boubekeur *et al.*, 2001). The regulation of acetaldehyde accumulation via *ALD4*, and other aldehyde dehydrogenases, was suggested to control the accumulation of acetaldehyde, which could be considered toxic to the cells (Boubekeur *et al.*, 2001).

The induction of *ALD4* (3.3–fold) in *S. cerevisiae* following a 7% (v/v) ethanol-shock was first reported by Alexandre *et al.* (2001) and then a very strong increase in *ALD4* expression (28.8-fold) was identified in the gene array studies described in Chapter 5 (Section 5.3.2) in this thesis. Recently, Aranda and del Olmo (2003) also described the importance of the aldehyde dehydrogenase genes, especially *ALD4*, in an industrial flor yeast strain exposed to 12% (v/v) ethanol stress and acetaldehyde stress (1.0 g l^{-1}). *ALD4* expression strongly increased (approximately 18-fold) when the

yeast was exposed to the ethanol and acetaldehyde stresses. The authors proposed that *ALD4* plays an important role in oxidising acetaldehyde to acetate in the industrial flor yeast strain (responsible for the biological aging of sherry wines) when high ethanol concentrations, and high levels of produced acetaldehyde, result in growth inhibitory conditions.

Design of the inoculum size experiment used to identify a unique *Δald4* phenotype was based upon both a hypothesised role for Ald4 in cellular redox balance and the recognised stimulatory role of acetaldehyde in cellular adaptation to ethanol stress. The role of the Ald4 gene product in the breakdown of acetaldehyde to acetate (Figure 5.3), and the high up-regulation of the *ALD4* gene during ethanol stress inferred an important connection between acetaldehyde and the gene product in the ethanol stress response. Several authors have noted an effect of inoculum size on ethanol stressed yeast cells (Walker-Caprioglio *et al.*, 1985; Walker-Caprioglio and Parks, 1987; Stanley, 1993; Stanley *et al.*, 1997). Walker-Caprioglio *et al.* (1985) found that in medium containing 4% (v/v) ethanol, the lag period decreased as the inoculum size increased in the range of 10^3 to 10^6 cells ml^{-1}. Walker-Caprioglio *et al.* (1985) proposed that lag times should be independent of inoculum size unless a growth-enhancing substance was being contributed to the medium by the yeast, with cultures containing a higher initial cell number achieving a higher concentration of the metabolite required for growth stimulation in a shorter time period.

Stanley (1993) extended the work of Walker-Caprioglio *et al.* (1985) and proposed that a 4% (v/v) ethanol-induced lag phase is dependent on inoculum size above initial cell populations of 10^5 cell ml^{-1}. Below this cell density, the lag period was of a constant length (around 5 hours) and independent of the inoculum size. This indicates that the cellular adaptation to ethanol is an entirely internal process, but the rate of adaptation can be influenced by excreted metabolic products; the latter only affecting adaptation rates when above a certain threshold concentration in the extracellular medium. Stanley *et al.* (1997) attributed lag-reducing effects to the accumulation of a metabolite in the extracellular pool enabling the culture to adapt and recommence growth; where below 10^5 cells ml^{-1} the metabolite is either not produced or is not present in sufficient concentration to affect the lag phase. Walker-Caprioglio and

Parks (1987) and Stanley *et al.* (1997) proposed the lag-reducing effects to be due, in part, to the excretion of acetaldehyde. In contrast to its inhibitory role, Stanley *et al.* (1997) observed that in small concentrations acetaldehyde is strongly stimulatory to yeast growth during ethanol stress. The mechanisms behind the lag reducing effects of acetaldehyde on ethanol stressed cultures are unclear, however, it was suggested that acetaldehyde may have a general role in the yeast cell, perhaps as a signal of cell stresses, or it may serve to restore the redox balance (NAD^+/NADH ratio) in ethanol stressed cells. This inoculum size effect and the ability of acetaldehyde to accelerate the adaptation of *S. cerevisiae* subjected to ethanol shock (Walker-Caprioglio *et al.*, 1987; Stanley, 1993) provided an important link in defining a purpose of the Ald4 gene product in the ethanol stress response. With low cell populations, accumulated acetaldehyde is transported to the extracellular environment, where it stimulates stress adaptation above a threshold concentration.

The inoculum size experiments with *Δald4* were designed with the findings of Stanley *et al.* (1997) in mind. The effect of deleting the *ALD4* gene on the extracellular accumulation of acetaldehyde in ethanol stressed cultures (and therefore the adaptation rate) was unknown, but it was assumed that such an effect might not have been noticeable at high inoculum levels because of the relatively high amounts of acetaldehyde accumulating in the extracellular medium. Although the results described in this chapter confirmed the lag reducing effect of increasing inoculum size on the adaptation rate, *Δald4* responded to the ethanol stress in a similar way to the parent culture. Given the results generated from this work, it appears that the *ALD4* product on its own is not a key element in the adaptation of *S. cerevisiae* to ethanol stress; at least not under the conditions used in this project.

The failure to detect a unique phenotype for *ALD4* using the *ALD4* gene knockout may also be due to a compensatory effect from other genes, as suggested for *HSP26* (Section 6.5.2). Recent growth studies of gene knockout strains encoding the main ALD genes (*ALD4* and *ALD6*) suggest that the mitochondrial and cytosolic enzymes may (at least partially) compensate for each other (Boubekeur *et al.*, 1999, 2001; Remize *et al.*, 2000). The repression of *ALD6* during the ethanol stress response in the gene array data (Table 5.4) led to the assumption that *ALD4* (up-regulated both early

and late in the ethanol stress response) was of principal importance in the oxidation of acetaldehyde to acetate as a carbon source for the TCA cycle (due to the mitochondrial location of *ALD4*) in ethanol stressed cells. The deletion of *ALD4* may be compensated for by *ALD6,* or genes for other aldehyde dehydrogenases, although the cytosolic location of Ald6 would mean that the produced acetate would not be located inside the mitochondria and therefore could not feed into the TCA cycle. Northern analysis of the *Δald4* strain using *ALD6* and other ALD gene probes may identify the changes expression of these genes in the knockout strains to compensate for the *ALD4* deletion.

6.5.4 Further work on the roles of *HSP26* and *ALD4* in the ethanol-stress response

Using simple growth experiments to determine phenotype is limited since small differences in cell vitality may not be detected in the absence of an appropriate selection pressure. To further detect phenotypes for *HSP26* and *ALD4,* more sensitive and diverse methods of determining phenotype should be used. For example, if the genes of interest in the wild type provide a competitive advantage against the deletion strain when grown under conditions of ethanol stress, then a growth competition experiment may detect such a competitive edge. This approach has been used, for example, by Thatcher *et al.* (1998). The experiment would require starting with initially equal cell populations of the respective knockout and wild type together in rich and minimal medium in the presence and absence of a 5% (v/v) ethanol stress. Cells could be sub-cultured into fresh medium every 24 hours, or cultivated in a chemostat and plated onto YEPD and YEPD Geneticin plates. The viable cell populations could then be determined since both strains would grow on YEPD and only the knockout could grow in the presence of geneticin. This technique would be sufficiently sensitive to discriminate between slight differences in ethanol tolerance between the two strains; the more ethanol tolerant strain eventually comprising a majority of the total cell population.

The screening of knockout strains for all genes up-regulated in response to ethanol stress from the gene array data (Chapter 5) may reveal single genes of importance in

the adaptation to ethanol stress. The availability of the yeast gene knockout collection provides an opportunity to screen a large number of knockout strains for their growth profile in the presence and absence of ethanol. Due to time constraints and the limited availability of the yeast knockout collection, it was not possible to undertake such a study in the time frame of this project.

The results of this chapter suggest that *HSP26* or *ALD4* alone do not significantly influence the adaptive response of *S. cerevisiae* to ethanol stress. The specific roles of these genes in the ethanol stress response are yet to be determined, noting that the adaptation rate and growth rate were the only parameters tested in the work described here. It is possible that *HSP26* and *ALD4* may have a marginal influence on yeast adaptation to ethanol, or in their absence their role may be compensated by other genes. A more comprehensive analysis is required to confirm or disprove this.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

A molecular analysis of the ethanol stress response of *S. cerevisiae* was successfully conducted using both differential display and gene array analysis. Both techniques identified changes in gene expression in response to the ethanol stress imposed on the yeast cells. Differential display was the method of choice for gene expression analysis when this research commenced, however gene array technology became available during the project and was subsequently adopted. The use of gene arrays allowed the simultaneous analysis of essentially every gene in the yeast genome providing a 'snap shot' of genomic expression profiles. This provided a comprehensive analysis of the transcriptome when the yeast cells were adapting to the ethanol stress. It should be noted, however, that differential display is still a widely used technique for the identification of differences in gene expression in species where the genome is not available on a microarray.

Phenotype analysis was subsequently undertaken in an attempt to define a phenotype for two of the genes up-regulated under ethanol stress. The approach used here was to generate knockouts of the two genes and use these in studies on aspects of physiology under ethanol stress.

7.1 CONCLUSIONS

The effect of ethanol stress on yeast cells has been described in the literature however the mechanisms underlying the cellular adaptation to this stress are not clearly understood. The work described in this thesis adds considerably to what was previously known of the ethanol-stress response.

Seven putative ethanol stress response genes were identified using differential display. Three of these genes, *YGL059W*, *CDC3* and *CBP2*, were validated as 'real' ethanol stress response genes. Of the remaining four genes, *SHY1* and *YHL039W* were found to be false positives, showing the same level of transcript in stress and control

cultures. *YOR275C* and *YDR504C* however could not be detected by Northern analysis and thus could not be validated as real or dismissed as false positives. Later gene array analysis revealed the expression of the two genes did not change significantly between control and ethanol-stress cultures.

Global gene expression using gene arrays showed for the first time the transient up- and down-regulation of many genes in response to ethanol stress. A large number of up-regulated genes 'early' in the cellular adaptation to ethanol were associated with the cellular stress response, energy utilisation, transport, cell surface interactions and lipid metabolism. The up-regulation of genes associated with energy utilisation suggested a 'pseudo-starvation' state was induced in the yeast cell when in the presence of ethanol. Genes associated with central metabolism and hexose transport were up-regulated early in response to ethanol stress suggesting a cellular demand for a high carbon flux possibly to increase ATP generation needed for cellular growth and recovery. Additionally, an up-regulation of genes involved in trehalose and glycogen metabolism was observed which initially seemed inconsistent with the need for greater carbon input into glycolysis as this draws carbon away from energy yielding processes. However, as mentioned in Chapter 5 (Section 5.3.2), an increase in trehalose maybe associated with its role as a stress protectant.

The up-regulation of a number of stress response genes early in the response to ethanol exposure is typical of stress responses. In fact many of the stress response genes were the same as those identified in work on other stresses, including osmotic and oxidative stress. Presumably these genes are important in helping the cell avoid the damaging effects of reactive oxygen species and water stress associated with ethanol stress.

The number of genes down-regulated early in the adaptation to ethanol stress included a large number of genes associated with protein synthesis. This response is typical of cells undergoing a growth arrest. As the synthesis of ribosomes requires substantial energy it is not surprising that transcript levels of ribosomes are repressed. This response also correlates with the reduced amounts of rRNA in ethanol stressed cells.

The magnitude of difference in gene expression between control and stress cultures decreased considerably as the lag phase was drawing to a close. For example, few genes remained up-regulated relative to the control, despite the fact that ethanol and hence the stress was still present in the growth medium. It is speculated that the cellular response of yeast, resulting from sudden ethanol exposure is mostly focussed on cellular modification of metabolism and cellular structures.

Genes that were up-regulated in ethanol stress had regulatory elements in their promoters that are controlled by two signal transduction pathways; the general stress response pathway and the HSE-mediated pathway. The majority of up-regulated genes contained one or more STRE sequence motifs, binding sites for the Msn2/4p transcription factor. A proportion of genes, mainly including the stress response genes, contained HSE motifs, binding sites for the Hsf1p transcription factor. Other signal transduction pathways were also involved in the ethanol stress response. The AP-1 response element, binding the Yap1p transcription factor, was found to be of minor importance in the ethanol stress response as sequence motifs were evident only in few genes. In addition, other signal transduction pathways may be involved in the ethanol stress response as promotor regions of several up-regulated genes contained no known regulatory elements. Searching of the promoter regions of up- and down-regulated genes revealed no novel regulatory elements.

Knockout strains, *Δhsp26* and *Δald4*, behaved the same as the parent they were derived from under ethanol stress. There were no observable difference in viable cell population profiles between the knockout and wild type strains in the presence of lethal and non-lethal ethanol concentrations. In addition, the pre-adaptation of *Δhsp26* with an ethanol pre-stress (i.e. to induce differences in cellular HSP levels prior to the ethanol stress experiment) did not result in any discernable difference in growth profile between it and the wild type. The effect of levels of excreted metabolites, such as acetaldehyde, that are known to influence adaptation rate to ethanol was tested for the ald4 knockout by inoculating the cultures with different initial cell populations. Differences in inoculum size had no notable effect on the rate of adaptation of *Δald4* to ethanol when compared to control cells. The inability to detect unique phenotypes for *Δhsp26* and *Δald4* may be due to one or other genes masking the roles of the

representative gene products, or hsp26 and ald4 alone may not significantly influence the adaptive response of *S. cerevisiae* to ethanol stress. Specific roles are yet to be determined for *HSP26* and *ALD4* when yeast cells are under ethanol stress. Adaptation rate and growth rate were the only parameters to be tested.

7.2 RECOMMENDED FUTURE EXPERIMENTAL STUDIES

1. To further define the ethanol-stress response, gene array analysis could be undertaken using the same ethanol-stressing conditions described in this thesis, but with more samples analysed during the experiment. Samples taken at earlier and more frequent time points, post-inoculation, (i.e. every 30 minutes) would provide a more detailed picture of the transient of adaptation and further enable specific genes or gene groups to be targeted for knockout and/or overexpression studies. Samples taken after three hours would enable the identification of genes that are important for ongoing tolerance to ethanol stress.

2. The collection of *S. cerevisiae* gene knockout strains could be screened for their growth profiles in the presence of ethanol enabling the identification of individual gene deletions that are important for growth in the presence of ethanol.

3. In addition to screening gene knockout strains, double knockouts could be constructed to eliminate some of the possibilities of a compensatory effect by other genes. For example, the screening of an *Δald4Δald6* knockout strain under ethanol stressing conditions could further elucidate if Ald6 compensates for Ald4 during the cells adaptation to ethanol.

4. Evident from the array data was the large number of up-regulated genes. Many of these genes are up-regulated following exposure to a range of stresses and perhaps play little or no part in the adaptation to ethanol stress. To pinpoint the key genes, chemically generated ethanol tolerant mutants could be raised from the parent strain used here and then subjected to the same ethanol stressing procedure. This might help identify genes that are important in adapting to ethanol stress. Array analysis could

be conducted using these mutants under ethanol stress and the data compared to the results described in this thesis.

Gene libraries could also be constructed from these mutants to enable the isolation of genes that confer increased ethanol tolerance.

5. A proteomic analysis of ethanol stressed yeast would complement and extend the work described here; transcriptome data alone is not sufficient to determine what the protein profile of a cell is at a given time. Proteomic data has the added advantage that it enables the characterisation of protein modifications; thus proteins that are modified in response to ethanol stress could be identified using this approach.

6. Genes isolated from array data that are shown to confer cellular protection against the effects of ethanol could potentially be used to construct recombinant yeast strains with a higher tolerance to ethanol stress. In addition, these genes could be used as molecular probes to screen collections of industrial yeast strains with for potentially greater tolerance to ethanol.

7. Cross tolerance has been observed and reported for *S. cerevisiae* in the literature (see Section 1.2.2). For example, prior short term exposure of yeast to heat shock improves subsequent tolerance of the cells to ethanol stress. This approach could be used to help identify genes by conducting a series of pre-stress protocols. (i.e. osmotic stress, heat stress, oxidative stress, etc.) and inoculating the cells into an ethanol stress environment. Microarray analysis of the various prestressed cells prior to, and after, inoculation into an ethanol stress may identify a common set of up-regulated genes that could have an important role in ethanol tolerance.

APPENDIX 1

BUFFERS AND SOLUTIONS

Amino acid and uracil stock solutions: 20 mg/ml stock solutions of leucine, histidine and uracil were prepared by dissolving the amino acids separately in sterile distilled de-ionized water. All stock solutions were autoclaved and the leucine and histidine solutions stored at 4°C. The uracil solution was stored at room temperature and shaken well prior to use. Uracil and histidine were used at a final concentration of 20 μg/ml and leucine at a final concentration of 100 μg/ml (Kaiser *et al.*, 1994).

Ammonium Acetate (10 M): Ammonium acetate was dissolved with gentle heating in sterile distilled and de-ionized water. The solution was sterilized by passage through a 0.45 μm filter.

Acid phenol (water buffered phenol): was prepared using Special Grade phenol (Wako Pure Chemical Industries limited), DEPC-treated water and 0.1% (w/v) 8-hydroxyquinoline. Equal volumes of phenol and DEPC water were mixed with the 8-hydroxyquinoline in a brown (light proof) baked bottle with a stirring bar for 10 minutes. The phases were allowed to separate; the aqueous top phase removed and replaced with an equal volume of DEPC treated water. The procedure was repeated until the water phase was at pH 5.0 when tested with pH paper. The acid phenol was stored, covered by a layer of DEPC water, at 4°C.

Buffered phenol (for RNA isolation): was prepared using Special Grade phenol (Wako Pure Chemical Industries limited), RNA buffer (see below) and 0.1% (w/v) 8-hydroxyquinoline. Equal volumes of phenol and RNA buffer (5 x) were mixed with the 8-hydroxyquinoline in a brown (light proof) baked bottle with a stirring bar for 10 minutes. The phases were allowed to separate, the aqueous top phase removed and replaced with an equal volume of 1 x RNA buffer. The procedure was repeated using 1 x RNA buffer until the aqueous top phase was at pH 7.5 when tested with pH paper. The buffered phenol was stored, covered by a layer of 1 x RNA buffer, at 4°C.

Buffered phenol (for DNA isolation): was prepared using Special Grade phenol (Wako Pure Chemical Industries limited), 50 mM Tris.Cl buffer (see below) and 0.1% (w/v) 8-hydroxyquinoline. Equal volumes of phenol and 50 mM Tris.Cl buffer were mixed with the 8-hydroxyquinoline in a brown (light proof) baked bottle with a stirring bar for 10 minutes. The phases were allowed to separate, the aqueous top phase removed and replaced with an equal volume of 50 mM Tris.Cl buffer. The procedure was repeated using 50 mM Tris.Cl buffer until the aqueous top phase was at pH 8.0 when tested with pH paper. The buffered phenol was stored, covered by a layer of 50 mM Tris.Cl buffer, at 4°C.

Chloroform/ Isoamyl alcohol (49:1): Chloroform (49 ml) and isoamyl alcohol (1.0 ml) added together and mixed well.

Denaturation Buffer: NaCl (87.66 g) and NaOH (20 g) were mixed and dissolved in distilled de-ionized water. The solution was made up to a 1.0 L volume.

DEPC water: 0.1% DEPC and distilled de-ionized water were mixed well, allowed to stand overnight, and autoclaved.

Differential Display Loading Buffer: 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol, 20% (w/v) Ficoll and 10 mM EDTA were dissolved in distilled de-ionized water and filter sterilized into a sterile bottle.

DNA breaking buffer: 2% (v/v) Triton X, 1% SDS, 1 mM EDTA, 100 mM NaCl and 10 mM Tris.Cl (pH 8.0) were mixed. The solution was filter sterilized into a baked glass bottle.

EDTA 0.5 M: was prepared by dissolving 186.1 g EDTA in 800 ml of distilled de-ionized water. The solution was dissolved with gentle heating for several hours. The solution was cooled, the pH adjusted to 8.0 with NaOH and the volume adjusted to 1 litre. The solution was autoclaved.

Ethidium Bromide: for non-denaturing RNA and DNA gels was prepared as a 10 mg/ml stock solution by dissolving ethidium bromide with distilled de-ionized water. The stock solution was stored in a baked lightproof glass bottle at 4°C. Ethidium bromide was added to a cooled agarose gel at a final concentration of 1 $\mu g\ ml^{-1}$.

Ethidium bromide for denaturing formaldehyde gels was prepared as a 1 mg/ml stock and added to the samples prior to leading at a concentration of 0.03 $\mu g\ \mu l^{-1}$. This stock solution was also stored at 4°C in a baked lightproof glass bottle.

First strand buffer (5 x): 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ supplied by Gibco BRL.

Formaldehyde gel-loading buffer: Glycerol (50%), 1 mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol were dissolved in distilled de-ionized water and filter sterilized into a sterile bottle.

Geneticin: A stock solution of 100 mg ml^{-1} was prepared by adding 1 ml of sterile distilled de-ionized water to 100 mg Geneticin G418 in a sterile bottle. The stock solution was dissolved and stored at 4°C. The solution was used at a final concentration of 0.2 mg ml^{-1}.

Gel loading buffer (6 x): 0.2% (w/v) bromophenol blue, 20% (w/v) Ficoll and 10 mM EDTA were dissolved in distilled de-ionized water and filter sterilized into a sterile glass bottle. The solution was stored at 4°C.

Lithium Acetate (1 M): was prepared by mixing lithium acetate (169 mg/ 100ml) with distilled de-ionized water to a final volume of 100 ml. This solution was autoclaved in a baked bottle and stored at 4 °C.

25 mM $MgCl_2$: (Perkin Elmer or Invitrogen): used in PCR reactions and supplied with the enzyme, Amplitaq™ DNA Polymerase or Platinum Taq DNA Polymerase.

Methylene Blue stain: Methylene Blue (0.04% w/v) was dissolved in 0.5 M sodium acetate. The pH was adjusted to 5.2 with glacial acetic acid and the solution was filter sterilized through a 0.22 μm filter into a baked bottle.

MOPS buffer (10x): 0.2 M MOPS (morpholinopropansulphonic acid), 80 mM sodium acetate anhydrous and 100 mM EDTA were dissolved in DEPC-treated water. The pH was adjusted to 7.0 with 2 M NaOH prior to autoclaving in light safe bottles.

PCR buffer (10x): 10 mM Tris-HCl (pH 8.3), 50 mM KCl. PCR buffer was supplied with the Taq DNA polymerase enzymes, Amplitaq^{TM} (Perkin Elmer) or Platinum Taq (Invitrogen).

Phosphate buffer: contained NaH_2PO_4 (0.4 M). The pH of the solution was adjusted to 6.8 with 2 M NaOH prior to autoclaving.

Polyethylene glycol (PEG) 50%: Polyethylene glycol 8000 was dissolved in distilled de-ionized water to a concentration of 0.5 g/ml and filter sterilized through a 0.45 μm filter. The solution was always made fresh prior to use.

RNA buffer (1 x): 0.5 M NaCl, 200 mM Tris (pH 7.5) and 10 mM EDTA were dissolved in DEPC-treated water to a final volume of 1 litre. The solution was autoclaved

RNA lysis buffer (5 x): 2.5 M NaCl, 1 M Tris base and 50 mM EDTA were dissolved in distilled water. The pH was adjusted to 7.5 with HCl and the buffer filter sterilized through a 0.22 μm filter into a baked glass bottle.

RNA gel loading solution (6 x): 0.2% (w/v) bromophenol blue, 20% (w/v) Ficoll and 10 mM EDTA were dissolved in DEPC treated water and filter sterilized into a baked glass bottle. The solution was stored at 4°C.

SDS 10%: Sodium dodecyl sulphate was dissolved in distilled de-ionized water by heating to 68°C. The solution was filter sterilized through a 0.45 μm filter into a sterile baked glass bottle.

2M Sodium acetate: Sodium acetate was dissolved in DEPC treated water. The pH was adjusted to 4.0 with glacial acetic acid prior to autoclaving.

3M Sodium acetate: Sodium acetate was dissolved in a small amount of DEPC treated water in baked glassware. The pH was adjusted to 5.3 with dilute glacial acetic acid and the solution filter sterilized through a 0.22 μm filter into a baked glass bottle.

SSC (20 x): NaCl (175.3 g) and tri-sodium citrate (88.2 g) were dissolved in DEPC treated water. The pH was adjusted to 7.0 with 10M NaOH prior to autoclaving.

TAE buffer (10 x): Tris base (400 mM), 200 mM Sodium acetate, 20 mM EDTA (pH 8) were dissolved in DEPC treated water. pH was adjusted to approximately 7.2 with glacial acetic acid prior to autoclaving. The 10 x stock solution was diluted with DEPC treated water prior to use with RNA.

TBE buffer (10 x): Tris base (0.89 M), 0.89 M boric acid and 20 mM EDTA (pH 8). The 10 x stock was autoclaved and diluted with distilled de-ionized water prior to use.

TE buffer: contained 10 mM Tris.Cl (pH 7.4) and 1 mM EDTA (pH 8.0). The solution adjusted to pH 8.0 with HCl and was autoclaved.

Tris 1M: Tris base (121.1 g) was dissolved in distilled de-ionized water. The pH was adjusted to 7.5 with glacial acetic acid, the volume adjusted to 1 L and the solution autoclaved.

X-ray developer

350 ml developer concentrate (Agfa-Gevaert)

2 litres dH_2O

X-ray film fixer bath

500 ml fixer concentrate (Agfa-Gevaert)

2 litres dH_2O

ENZYMES, MOLECULAR WEIGHT MARKERS AND MOLECULAR BIOLOGY KITS

Enzymes: RNase-free DNase (Roche), Amplitaq (Perkin Elmer), Platinum Taq (Invitrogen), RNase A (Epicentre Technologies), T4 Polynucleotide kinase (Amersham Pharmacia Biotech), Superscript ^{TM} II RNase H^- Reverse Transcriptase (Gibco BRL Life Technologies), Recombinant RNasin Ribonuclease Inhibitor (Promega), ABI Prism Cycle Sequencing.

Restriction Endonucleases: *Bcl*I (Promega), NdeI (New England Biolabs), AclI (New England Biolabs), *Ban*I (New England Biolabs), AflII (New England Biolabs), *Kpn*I (New England Biolabs).

Molecular Weight Markers: RNA markers, 0.28-6.58 kb (Promega), 100 bp DNA ladder (Promega), GeneRuler 1 kb DNA Ladder (MBI Fermentas), Lambda DNA/*Eco*RI+*Hin*dIII Marker (MBI Fermentas), GeneRuler 100 bp DNA Ladder Plus (MBI Fermentas).

Molecular Biology Kits: Differential Display^{TM} Kit (Display Systems), Qiagen^{TM} Gel Extraction Kit (Qiagen), Concert Gel Extraction Kit (Gibco BRL Life Technologies), Ready to go DNA Labelling Beads (-dCTP) (Amersham Biosciences), ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

LIST OF SUPPLIERS

Applied Biosystems (Foster City, CA, USA)

Amersham Biosciences (Little Chalfont, Buckinghamshire, UK)

Bartelt Instruments Pty Ltd (Heidelberg, Victoria, Australia)

Beckmam Instruments (GmbH, Munchen, Germany)

Bio-Rad Laboratories (Hercules, CA, USA)

B.Braun Biotech International (Melsungen, Germany)

Bresatech Pty Ltd (Adelaide, South Australia)

Epicenter Technologies (Maddison, USA)

Invitrogen Corporation (Carlsbad, California, USA)

New England Biolabs, Inc. (Beverly, MA, USA)

Operon Technologies, Inc. (Alameda, CA, USA)

PerkinElmer (Wellesley, MA, USA)

Promega Corporation (Maddison, USA)

Progen Industried Limited (Darra, Queensland, Australia)

Qiagen Pty Ltd (Clifton Hill, Victoria, Australia)

Roche Diagnostics (GmbH, Mannheim, Germany)

Sigma-Aldrich Corporation (St Louis, Missouri, USA)

APPENDIX 2

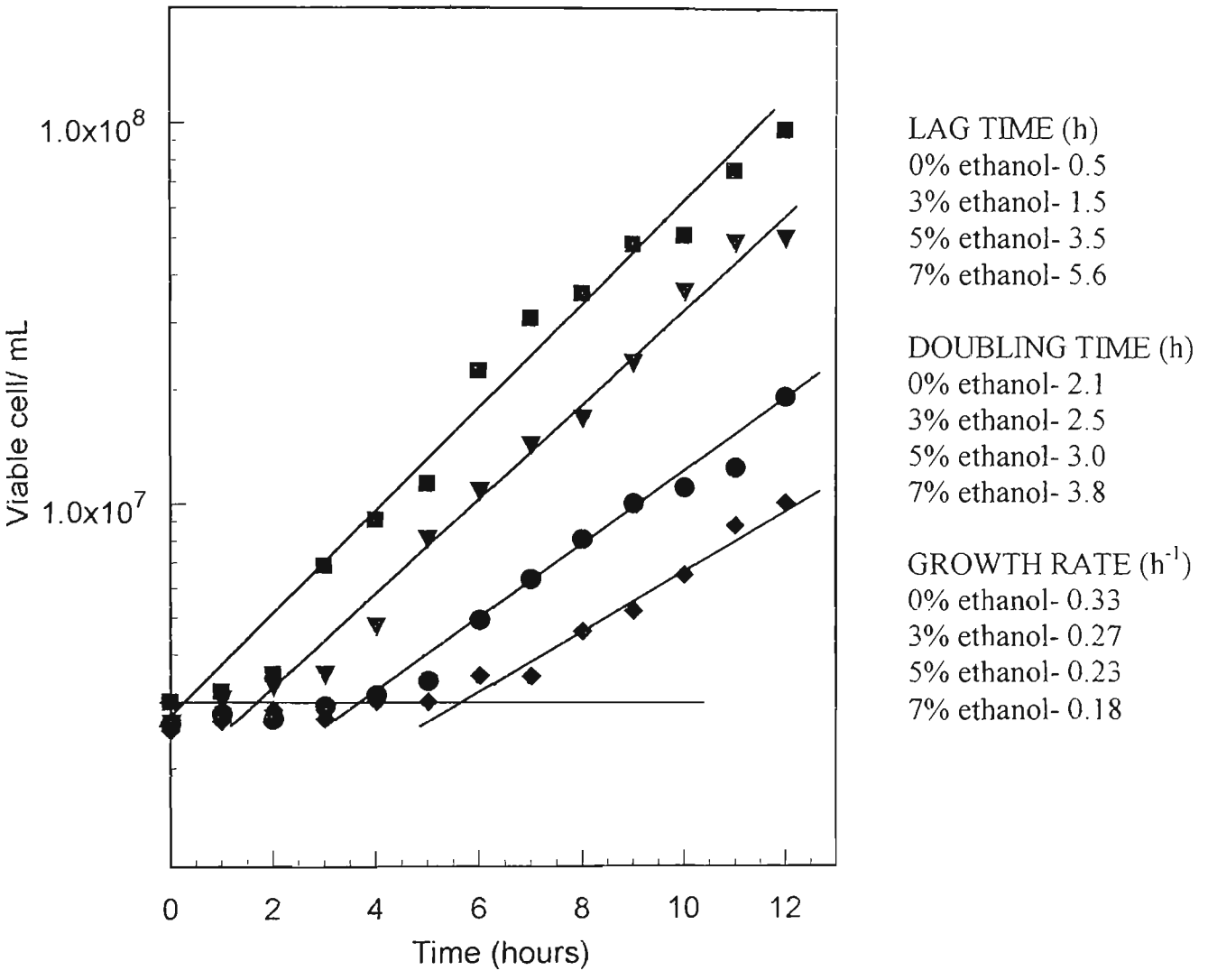
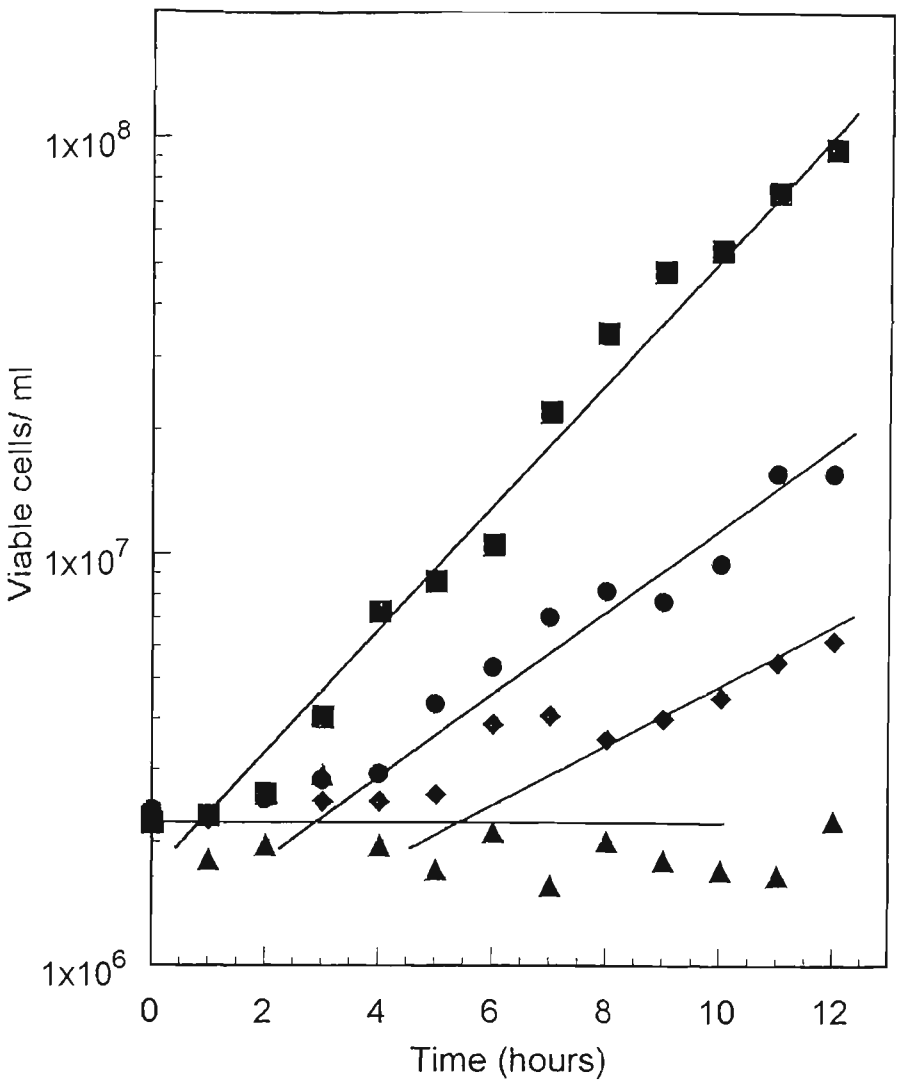


Figure 1- Determination of lag period, doubling time and growth rate for *S. cerevisiae* PMY1.1 grown in defined medium (■) and defined medium with the addition of 3% (▼), 5% (•) and 7% (♦) (v/v) added ethanol.



LAG TIME (h)
0% ethanol- 0.6
5% ethanol- 3.1
7% ethanol- 5.5
10% ethanol-N/A

DOUBLING TIME (h)
0% ethanol- 2.0
5% ethanol- 3.0
7% ethanol- 3.8
10% ethanol-N/A

GROWTH RATE (h^{-1})
0% ethanol- 0.34
5% ethanol- 0.23
7% ethanol- 0.18
10% ethanol- N/A

Figure 2- Determination of lag period, doubling time and growth rate for *S. cerevisiae* PMY1.1 grown in defined medium (■) and defined medium with the addition of 5% (•), 7% (♦) and 10% (▲) (v/v) added ethanol.

REFERENCES

Aguilera, A. & Benitez, T. (1985). Role of mitochondria in ethanol tolerance of *Saccharomyces cerevisiae*. *Archives of Microbiology* **142**, 389-392.

Aguilera, A. & Benitez, T. (1986). Ethanol-sensitive mutants of *Saccharomyces cerevisiae*. *Archives of Microbiology* **143**, 337-344.

Alexandre, H., Ansanay-Galeote, V., Dequin, S. & Blondin, B. (2001). Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. *FEBS Letters* **498**, 98-103.

Alexandre, H., Plourde, L., Charpentier, C. & Francois, J. (1998). Lack of correlation between trehalose accumulation, cell viability and intracellular acidification as indicated by various stresses in *Saccharomyces cerevisiae*. *Microbiology* **144**, 1103-1111.

Alexandre, H., Rousseaux, I. & Charpentier, C. (1993). Ethanol adaptation mechanisms in *Saccharomyces cerevisiae*. *Biotechnology and Applied Biochemistry* **20**, 173-183.

Alexandre, H., Rousseaux, I. & Charpentier, C. (1994). Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and *Kloeckera apiculata*. *FEMS Microbiology Letters* **124**, 17-22.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Amoros, M. & Estruch, F. (2001). Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes *HSP26* and *HSP104* in a gene- and stress type-dependant manner. *Molecular Microbiology* **36**, 1523-1532.

Ananthan, J., Goldberb, A. L. & Voellmy, R. (1985). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of stress genes. *Science* **232**, 522-524.

Aranda, A. & del Olmo, M. (2003). Response to acetaldehyde stress in the yeast *Saccharomyces cerevisiae* involves a strain-dependant regulation of several *ALD* genes and is mediated by the general stress response pathway. *Yeast* **20**, 747-759.

Aranda, A., Querol, A. & del Olmo, M. (2002). Correlation between acetaldehyde and ethanol resistance and expression of *HSP* genes in yeast strains isolated during the biological aging of sherry wines. *Archives of Microbiology* **177**, 304-312.

Attfield, P. (1987). Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat shock response. *FEBS Letters* **225**, 259-263.

Attfield, P., Myers, D. & Hazell, B. (1997). The importance of stress tolerance to bakers yeast. *Australasian Biotechnology* 7, 149-154.

Attfield, P. V. (1997). Stress Tolerance: The key to effective strains of industrial baker's yeast. *Nature Biotechnology* **15**, 1351-1357.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & K.Struhl (1997). In *Current Protocols in Molecular Biology*: John Wiley & Sons Inc.

Bader, G. H., Heilbut, A., Andrews, B., Tyers, M., Hughes, T. & Boone, C. (2003). Functional genomics and proteomics: charting a multidimensional map of the yeast cell. *Trends in Cell Biology* **13**, 344-356.

Bailey, T. L. & Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In *Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology*, pp. 28-36. Menlo Park, California: AAAI Press.

Bammert, G. F. & Fostel, J. M. (2000). Genome-wide expression patterns in *Saccharomyces cerevisiae*: comparison of drug treatments and genetic alteration affecting biosynthesis of ergosterol. *Antimicrobial Agents and Chemotherapy* **44**, 1255-1265.

Bauer, D., Muller, H., Reich, J., Riedel, H., Ahrenkiel, V. & Warthoe, P. (1993). Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Research* **21**, 4272-4280.

Bauldin, A., Ozier, K. O., Denouel, A., Lacroute, F. & Cullin, C. (1993). A simple and effective method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **21**, 3329-3330.

Beaven, M., Charpentier, C. & Rose, A. H. (1982). Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. *Journal of General Microbiology* **128**, 1447-1455.

Birch, R. M. & Walker, G. M. (2000). Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology* **26**, 678-687.

Blomberg, A. (1997). Osmoresponsive proteins and functional assessment strategies in *Saccharomyces cerevisiae*. *Electrophoresis* **18**, 1429-1440.

Bonner, J. J., Ballou, C. & Fackenthal, D. L. (1994). Interactions between DNA bound trimers of the yeast heat shock factor. *Molecular and Cellular Biology* **14**, 501-508.

Boubekeur, S., Bunoust, O., Camougrand, N., Castroviejo, M., Rigoulet, M. & Guerins, B. (1999). A mitochondrial pyruvate dehydrogenase bypass in the yeast *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **274**, 21044-21048.

Boubekeur, S., Camougrand, N., Bunoust, O., Rigoulet, M. & Guerin, B. (2001). Participation of acetaldehyde dehydrogenases in ethanol and pyruvate metabolism of the yeast *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **268**, 5057-5065.

Boulton, R., Singleton, V., Bisson, L. & R.E, Kunkee. (1996). Yeast and biochemistry of ethanol fermentation. In *Principles and practices of Wine Making*, pp. 153-181. New York: Chapman & Hall.

Braley, R. & Piper, P. W. (1997). The C-terminus of the plasma membrane H^+-ATPase is essential for the regulation of this enzyme by the heat shock protein, HSP30, but not for stress activation. *FEBS Letters* **418**, 123-186.

Brejning, J., Jespersen, L. & Arneborg, N. (2003). Genome-wide transcriptional changes during the lag phase of *Saccharomyces cerevisiae*. *Archives of Microbiology* **179**, 278-294.

Brenner, C., Nakayama, N., Goebl, M., Tanaka, K., Toh-e, A. & Matsumoto, K. (1988). *CDC33* encodes mRNA cap-binding protein eIF-4E of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **8**, 35568-9.

Brown, S. W., Oliver, S. G., Harrison, D. E. & Righelato, R. C. (1981). Ethanol inhibition of yeast growth and fermentation: differences in the magnitude and complexity of the effect. *European Journal of Applied Microbiology and Biotechnology* **11**, 151.

Burgess, J. (2001). Gene expression studies using microarrays. *Clinical and Experimental Pharmacology and Physiology* **28**, 321-328.

Callard, D., Axelos, M. & Mazzolini, L. (1996). Novel molecular markers for late phases of the growth cycle of *Arabidopsis thaliana* cell-suspension cultures are expressed during organ senescence. *Plant Physiology* **112**, 705-715.

Carmelo, V. & Sa-Correia, I. (1997). *HSP26* gene transcription is strongly induced during *Saccharomyces cerevisiae* growth at low pH. *FEMS Microbiology Letters* **149**, 85-88.

Carrasco, P., Querol, A. & del Olmo, M. (2001). Analysis of stress resistance of commercial wine yeast strains. *Archives of Microbiology* **175**, 450-457.

Cartwright, C. P., Juroszek, J., Beaven, M., Ruby, F. M., De Morais, S. M. F. & Rose, A. H. (1986). Ethanol dissipates the proton-motive force across the plasma membrane of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **132**, 369-377.

Cartwright, C. P., Veazey, F. J. & Rose, A. H. (1987). Effect of ethanol on activity of the plasma membrane ATPase in, and the accumulation of glycine by, *Saccharomyces cerevisiae*. *Journal of General Microbiology* **133**, 857-865.

Casey, G. P. & Ingledew, W. M. (1986). Ethanol tolerance in yeasts. *CRC Critical Reviews in Microbiology* **13**, 219-280.

Casey, G. P., Ingledew, W. M. & Magnus, C. A. (1984). High-gravity brewing - Effects of nutrition on yeast composition, fermentative ability and alcohol production. *Applied and Environmental Microbiology* **48**, 639-646.

Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S. & Young, R. A. (2001). Remodelling the yeast genome expression in response to environmental changes. *Molecular and Cellular Biology* **12**, 323-337.

Chu, S., DeRisi, J. L., Eisen, M. B., Mulholland, J., Botstein, D., Brown, P. O. & Herskowitz, I. (1998). The transcriptional program of sporulation in budding yeast. *Science* **282**, 699-705.

Ciesarova, Z., Smogrovicova, D. & Domeny, Z. (1996). Enhancement of yeast ethanol tolerance by calcium and magnesium. *Folia Microbiol* **41**, 485-488.

Colaco, C. A. L. S., Smith, C. J. S., Sen, S., Roser, D. H., Newman, Y., Ring, S. & Roser, B. J. (1994). Chemistry of protein stabilisation by trehalose. In *Formulation and Delivery of Proteins and Peptides*, pp. 222-240. Edited by J. L. Cleland & R. Langer. Washington DC: American Chemical Society.

Collinson, L. P. & Dawes, I. (1992). Inducability of the response of yeast cells to peroxide stress. *Journal of General Microbiology* **138**, 329-335.

Coote, P. J., Cole, M. B. & Jones, M. V. (1991). Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH. *Journal of General Microbiology* **137**, 1701-1708.

Costa, V., Amorim, M. A., Reis, E., Quintanilha, A. & Moradas-Ferreira, P. (1997). Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. *Microbiology* **143**, 1649-1656.

Costa, V., Reis, E., Quintanilha, A. & Moradas-Ferreira, P. (1993). Acquisition of ethanol tolerance in *Saccharomyces cerevisiae*: The key role of the mitochondrial superoxide dismutase. *Archives of Biochemistry and Biophysics* **300**, 608-614.

Craig, E. A., Baxter, B. K., Becker, J., Halliday, J. & Zigelhoffer, T. (1994). Cytosolic hsp70 of *Saccharomyces cerevisiae*: roles in protein synthesis, protein translocation, proteolysis, and regulation. In *The Biology of Heat Shock Proteins and Molecular Chaperones*, pp. 31-52. Edited by R. I. Morimoto. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press.

Craig, E. A. & Gross, C. A. (1991). Is hsp70 the cellular thermometer? *Trends in Biochemical Science* **16**, 135-140.

Crawels, M., Winderickx, J., Winde, J. D. & Thevelein, J. (1997). Identification of genes with nutrient-controlled expression by PCR-mapping in the yeast *Saccharomyces cerevisiae*. *Yeast* **13**, 973-984.

Crowe, J. H., Crowe, L. M. & Chapman, D. (1984). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **223**, 701-703.

Cunningham, S. & Stewart, G. (2000). Acid washing and serial re-pitching a brewing ale strain of *S. cerevisiae* in high gravity wort and the role of wort oxygenation conditions. *Journal of the Institute of Brewing* **106**, 389-402.

Curtain, C. C., Atwell, J. L., Looney, F. D. & Zajac-Cade, A. (1985). Membrane mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*: an electron spin resonance study. *Procedings of the Convention of the Institute of Brewing* **18**, 235.

D'Amore, T., Crumplen, R. & Stewart, G. (1991). The involvement of trehalose in yeast stress tolerance. *Journal of Industrial Microbiology* 7, 191-196.

D'Amore, T., Panchel, C., Russel, I. & Stewart, G. G. (1990). A study of ethanol tolerance in yeast. *Critical Reviews in Biotechnology* **9**, 287-304.

Davidson, J. F., Whyte, B., Bissinger, P. H. & Schiestl, R. H. (1996). Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Science* **93**, 5116-5121.

Davies, J. M., Lowry, C. V. & Davies, K. J. A. (1995). Transient adaptation to oxidative stress in yeast. *Archives of Biochemistry and Biophysics* **317**, 1-6.

Dawes, I. (1999). Stress Responses. In *Molecular Physiology of Saccharomyces cerevisiae*. Edited by J. R. Dickinson & M. Schweizer. London: Taylor & Francis Ltd.

Del Castillo, L. (1992). Lipid content of *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance. *Applied Microbiology and Biotechnology* **37**, 647-651.

de Nobel, H., Lawrie, L., Brul, S., Klis, F., Davis, M., Alloush, H. & Coote, P. J. (2001). Parallel and comparative analysis of the proteome and transcriptome of sorbic acid-stressed *Saccharomyces cerevisiae*. *Yeast* **18**, 1413-1428.

De Risi, J., Tylet, L. & Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**, 680-686.

De Sanctis, V., Bertozzi, C., Costanzo, G., Di Mauro, E. & Negri, R. (2001). Cell cycle arrest determines the intensity of the global transcriptional response of *Saccharomyces cerevisiae* to ionizing radiation. *Radiation Research* **156**, 379-387.

De Virgillio, C., Hottiger, T., Dominguez, J., Boller, T. & Wiemkem, A. (1994). The role of trehalose synthesis for the acquisition of thermotolerance in yeast: Genetic evidence trehalose is a thermoprotectant. *European Journal of Biochemistry* **219**, 179-186.

De Virgillio, C., Piper, P. W., Boller, T. & Wiemkem, A. (1991). Aquisition of thermotolerance in *Saccharomyces cerevisiae* without heat shock protein hsp104 and in the absence of protein synthesis. *FEBS Letters* **288**, 86-90.

Dequin, S. (2001). The potential of genetic engineering for improving brewing, wine-making and baking yeasts. *Archives of Microbiology and Biotechnology* **56**, 577-588.

Dickinson, F. M. (1996). The purification and some properties of Mg^{2+} activated cytosolic aldehyde dehydrogenase of *S. cerevisiae*. *The Biochemical Journal* **315**, 393-399.

Dickson, L. M. & Brown, A. L. P. (1998). mRNA translation in yeast during entry into stationary phase. *Molecular and General Genetics* **258**, 282-293.

Dombek, K. M. & Ingram, L. (1986). Magnesium limitation and its role in the apparent toxicity of ethanol during yeast fermentation. *Applied and Environmental Microbiology* **52**, 975-981.

Eisen, M., Spellman, P., Brown, P. & Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences USA* **95**, 14863-14868.

Emslie, D. (2002). Molecular basis of the ethanol-stress response in the yeast *Saccharomyces cerevisiae*. Ph.D Thesis. In *School of Life Sciences and Technology*. Melbourne: Victoria University of Technology.

Estruch, F. (2000). Stress-controlled transcription factors, stress induced genes and stress tolerance in budding yeast. *FEMS Microbiology Reviews* **24**, 469-486.

Ferea, T. L., Botstein, D., Brown, P. O. & Rosenzweig, R. F. (1999). Systematic changes in gene expression patterns following adaptive evolution in yeast. *Proceedings of the National Academy of Sciences* **96**, 9721-9726.

Fernanda-Rosa, M. & Sa-Correia, I. (1996). Intracellular acidification does not account for inhibition of *Saccharomyces cerevisiae* growth in the presence of ethanol. *FEMS Microbiology Letters* **135**, 271-274.

Field, C. M. & Kellogg, D. (1999). Septins: cytoskeleon polymers or signalling GTPases. *Trends in Cell Biology* **9**, 387-394.

Finley, D., Ozkaynak, E. & Varshavsky, A. (1987). The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. *Cell* **48**, 1035-1040.

Francois, J. & Parrou, J. L. (2001). Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **25**, 125-145.

Gadd, G. M., Chalmers, K. & Reed, R. H. (1987). The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. *FEMS Microbiology Letters* **48**, 249-254.

Garray-Arroyo, A. & Covarrubias, A. (1999). Three genes whose expression is induced by stress in *Saccharomyces cerevisiae*. *Yeast* **15**, 879-892.

Gasch, A. P., Huang, M. X., Metzner, S., Botstein, D., Elledge, S. J. & Brown, P. O. (2001). Genomic expression responses to DNA damaging agents and the regulatory role of yeast *ATR* homolog Mec1p. *Molecular Biology of the Cell* **12.** 2987-3003.

Gasch, A. P., Spellmann, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D. & Brown, P. O. (2000). Genomic expression programs in response of yeast cells to environmental changes. *Molecular Biology of the Cell* **11**, 4241-4257.

Gasch, A. P. & Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. *Functional and Integrative Genomics* **2**, 181-192.

Gietz, R. D. & Schiestl, R. H. (1995). Transforming Yeast with DNA. *Methods in Molecular and Cellular Biology* **5**, 225-269.

Gille, G. K., Sigler, K. & Hofer, M. (1993). Response of catalase activity and membrane fluidity of aerobically grown *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to aeration and the presence of substrates. *Journal of General Microbiology* **139**, 1627-1634.

Glover, J. R. & Lindquist, S. (1998). *HSP104*, *HSP70* and *HSP40* - a novel chaperone system that rescues previously aggregated proteins. *Cell* **94**, 73-82.

Godon, C., Lagniel, G., Lee, J., Buhler, J., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M. B. & Labarre, J. (1998). The H_2O_2 stimulon in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **273**, 22480-22489.

Gonzalez, M., Stucka, R., Blazquez, M.A., Feldmann, H. & Gancedo, C. (1992). Molecular cloning of *CIF1*, a yeast gene necessary for growth on glucose. *Yeast* **8**, 183-192.

Grayhack, E. J. & Phizicky, E. M. (2001). Genomic analysis of biochemical function. *Current Opinions in Chemical Biology* **5**, 34-39.

Gropper, T. & Rensing, L. (1993). Inhibitors of proteases and other stressors induce low molecular weight heat shock proteins in *Saccharomyces cerevisiae*. *Experimental Mycology* **17**, 46-54.

Gross, C., Kelleher, M., Iyer, V., Brown, P. O. & Winge, D. R. (2000). Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. *The Journal of Biological Chemistry* **275**, 32310-32316.

Gross, C. & Watson, K. (1998). Application of mRNA differential display to investigate gene expression in thermotolerant cells of *Saccharomyces cerevisiae*. *Yeast* **14**, 431-442.

Guijarro, J. M. & Lagunas, R. (1984). *Saccharomyces cerevisiae* does not accumulate ethanol against a concentration gradient. *Journal of Bacteriology* **160**, 874.

Guldfeldt, L. U. & Arneborg, N. (1998). The effect of yeast trehalose content at pitching on fermentation performance during brewing fermentations. *Journal of the Institute of Brewing* **104**, 37-39.

Hall, B. G. (1983). Yeast thermotolerance does not require protein synthesis. *Journal of Bacteriology* **156**, 1363-1365.

Hallsworth, J. E., Nomura, Y. & Iwahara, M. (1998). Ethanol induced water stress and fungal growth. *Journal of Fermentation and Bioengineering* **86**, 451-456.

Hardwick, W. A. (1995). *Handbook of Brewing*. New York: Marcel Dekker, Inc.

Haslebeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H., Chen, S., Saibil, H. & Buchner, J. (1999). Hsp26: a temperature regulated molecular chaperone. *The EMBO Journal* **18**, 6744-6751.

Hazell, B. W., Nevalainen, H. & Attfield, P. V. (1995). Evidence that the *Saccharomyces cerevisiae CIF1* (*GGs1/TPS1*) gene modulates heat shock response positively. *FEBS Letters* **377**, 457-460.

Hohmann, S. & Mager, W. H. (1997). *Yeast Stress Responses*. Austin, Texas: R.G. Landes Company.

Holzberg, I., Finn, R. K. & Steinkraus, K. H. (1967). A kinetic study of the alcohol fermentation of grape juice. *Biotechnology and Bioengineering* **9**, 413-427.

Hottiger, T., Boller, T. & Wiemkem, A. (1987). Rapid changes of heat and desiccation tolerance correlated with changes of trehalose content in *Saccharomyces cerevisiae* cell subject to temperature shifts. *FEBS Letters* **220**, 113-115.

Hottiger, T., De Virgillio, C., Hall, M. N., Boller, T. & Wiemkem, A. (1994). The role of trehalose synthesis for the acquisition of thermotolerance in yeast II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*. *European Journal of Biochemistry* **219**, 187-193.

Hough, J. S. (1985). *The Biotechnology of Malting and Brewing*. Cambridge: Cambridge University Press.

Hounsa, C., Brandt, E. V., Thevelein, J. M., Hohmann, S. & Prior, B. A. (1998). Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. *Microbiology* **144**, 671-680.

Hughes, T. R., Marton, M. J., Jones, A. R., Roberts, C., Stoughton, R., Armour, C. D., Bennet, H. A., Coffey, E., Dai, H., He, Y. D., Kidd, M. J., King, M. A., Meyer, M. R., Slade, D., Lum, P. Y., Stepaniants, S. B., Shoemaker, D. D., Gachotte, D., Chakraburtty, K., Simon, J. R., Bard, M. & Friend, S. H. (2000). Functional discovery via a compendium of expression profiles. *Cell* **102**, 109-126.

Imai, T. & Ohno, T. (1995). The relationship between viability and intracellular pH in the yeast *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **61**, 3604-3606.

Ingram, L. & Buttke, T. M. (1984). Effects of alcohols on micro-organisms. *Advances in Microbial Physiology* **25**, 253-300.

Ingram, L. O. (1986). Microbial tolerance to alcohols: role of the cell membrane. *Trends in Biotechnology* **4**, 40-44.

Inoue, T., Iefugi, H., Fugii, T., Soga, H. & Satoh, K. (2000). Cloning and characterisation of a gene complementing the mutation of an ethanol-sensitive mutant of *sake* yeast. *Bioscience Biotechnology and Biochemistry* **64**, 229-236.

Ivanova, A. V. & Ivanov, S. V. (2002). Differential display analysis of gene expression in yeast. *Cellular and Molecular Life Science* **59**, 1241-1245.

Ivorra, C., Perez-Ortin, J. E. & Olmo, M. (1999). An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study. *Biotechnology and Bioengineering* **64**, 698-708.

Jacobson, M. K. & Berfonsky, C. (1974). Mitochondrial acetaldehyde dehydrogenase from *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **350**, 277-291.

James, T. C., Campbell, S., Donnelly, D. & Bond, U. (2003). Transcription profile of brewery yeast under fermentation conditions. *Journal of Applied Microbiology* **94**, 432-448.

Jamieson, D. J. (1992). *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. *Journal of Bacteriology* **174**, 6678-6681.

Jamieson, D. J. (1998). Oxidative stress response of the yeast *Saccharomyces cerevisiae*. *Yeast* **14**, 1511-1522.

Jelinsky, S. & Samson, L. (1999). Global response of *Saccharomyces cerevisiae* to an alkylating agent. *Proceedings of the National Academy of Sciences* **96**, 1486-1491.

Jelinsky, S. A., Estep, P., Church, G. M. & Samson, L. D. (2000). Regulatory networks revealed by transcriptional profiling of damages *Saccharomyces cerevisiae* cells: Rpn4 links base excision repair with proteosomes. *Molecular and Cellular Biology* **20**, 8157-8167.

Jentch, S., Seufert, W., Sommer, T. & Reins, H. A. (1990). Ubiquitin-conjugating enzymes: novel regulators of eukaryotic cells. *Trends in Biochemical Science* **15**, 195-198.

Jia, M. H., Larossa, R. A., Lee, J. M., Rafalski, A., Derose, E., Gonye, G. & Xue, Z. (2000). Global expression profiling of yeast treated with an inhibitor of amino acid biosynthesis, sulfometuron methyl. *Physiological Genomics* **3**, 83-92.

Jones, R. P. (1988). Intracellular ethanol accumulation and exit from yeast and other cells. *FEMS Microbiology Reviews* **4**, 239-258.

Jones, R. P. (1989). Biological principals for the effects of ethanol. *Enzyme and Microbial Technology* **11**, 130-153.

Jones, R. P. (1990). Roles for replicative deactivation in yeast-ethanol fermentation. *CRC Critical Reviews in Biotechnology* **10**, 205-222.

Jones, R. P. & Greenfield (1987). Ethanol and the fluidity of the yeast plasma membrane. *Yeast* **3**, 223-232.

Kaiser, C. S., Michaelis, S. & Mitchell, A. (1994). *Methods in yeast genetics- A Cold Springs Harbour Laboratory Course Manual.* New York: Cold Spring Harbour Laboratory Press.

Kalmokoff, M. & Ingledew, W. M. (1985). Evaluation of ethanol tolerance in related *Saccharomyces* strains. *Journal of the American Society of Brewing Chemists* **43**, 189.

Kapteyn, J. C., ter Riet, B., Vink, E., S, B., De Nobel, H., Van Den Ende, H. & Klis, F. (2001). Low external pH induces HOG1 dependent changes in the organisation of the *Saccharomyces cerevisiae* cell wall. *Molecular Microbiology* **39**, 469-479.

Kim, J., Alizadeh, P., Harding, T., Hefner-Gravink, A. & Klionsky, D. J. (1996). Disruption of the yeast *ATH1* gene confers better survival after dehydration, freezing and ethanol shock: potential commercial applications. *Applied and Environmental Microbiology* **62**, 1563-1569.

Kobayashi, N. & McEntee, K. (1993). Identification of *cis* and *trans* components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **13**, 248-256.

Kuhn, K. M., De Risi, J. L., Brown, P. O. & Sarnow, P. (2001). Global and specific translational regulation in the genomic response of *Saccharomyces cerevisiae* to a rapid transfer from a fermentable to a non-fermentable carbon source. *Molecular and Cellular Biology* **21**, 916-927.

Kurtz, S., Rossi, J., Petko, L. & Lindquist, S. (1986). A ancient development induction: heat shock proteins induced in sporulation and oogenesis. *Science* **231**, 1154-1157.

Kwast, K. E., Lai, L. C., Menda, N., James, D. T., Aref, S. & Burke, P. V. (2002). Genomic analysis of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response. *Journal of Bacteriology* **184**, 250-265.

Lamb, T. M., Xu, W., Diamond, A. & Mitchell, A. P. (2001). Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the RIM101 pathway. *The Journal of Biological Chemistry* **276**, 1850-1856.

Leao, C. & Van Uden, N. (1982). Effects of ethanol and other alkanols on the glucose transport system of *Saccharomyces cerevisiae*. *Biotechnology Letters* **4**, 721-724.

Leao, C. & van Uden, N. (1983). Effects of ethanol and other alkanols on the ammonium transport system of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **25**, 2085.

Leao, C. & Van Uden, N. (1984). Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **774**, 43-48.

Leao, C. & van Uden, N. (1984 a). Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **774**, 43-48.

Leao, C. & van Uden, N. (1984 b). Effects of ethanol and other alkanols on the general amino acid permease of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **26**, 403-405.

Lewin, A. S., Thomas, J. & Tirupati, H. K. (1995). Co-transcriptional splicing of a group I intron is facilitated by CBP2 protein. *Molecular and Cellular Biology* **15**, 6971-6978.

Lewis, C. S., Heard, P. L. & Chaudhuri, G. (1996). Differential expression of novel mRNAs in heat shocked *Leishmania* cells. *Medical Science Resources* **24**, 511-513.

Lewis, J. G., Learmonth, R. P. & Watson, K. (1993). Role of growth phase and ethanol in freeze-thaw stress resistance of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **59**, 1065-1071.

Lewis, J. G., Learmonth, R. P. & Watson, K. (1995). Induction of heat, freezing and salt tolerance by heat and salt shock in *Saccharomyces cerevisiae*. *Microbiology* **141**, 687-694.

Liang, P. (1996). Analysis of messenger RNA by differential display. In *A laboratory guide to RNA: Isolation, Analysis and Synthesis*, pp. 223-236. Edited by P. A. Kreig: Wiley-Liss Inc.

Liang, P. (2002). A decade of Differential Display. *BioTechniques* **33**, 338-346.

Liang, P., Averboukh, L., Keyomarsi, R., Sager, R. & Pardee, A. B. (1992). Differential Display and cloning messenger RNAs from human breast cancer versus mammary epithelial cells. *Cancer Research* **52**, 6966-6968.

Liang, P., Averboukh, L. & Pardee, A. B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimisation. *Nucleic Acids Research* **21**, 3269-3275.

Liang, P. & Pardee, A. B. (1992). Differential Display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.

Lillie, S. H. & Pringle, J. R. (1980). Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. *Journal of Bacteriology* **143**, 1384-1394.

Lindquist, S. L. & Craig, E. A. (1988). The heat-shock proteins. *Annual Reviews in Genetics* **22**, 631-677.

Lindquist, S. L. & Kim, G. (1996). Heat-shock protein 104 expression is sufficient for thermotolerance. *Proceedings of the National Academy of Science* **93**, 5301-5306.

Liu, X. & Thiele, D. (1996). Oxidative stress induced heat shock factor phosphorylation and HSF-dependant activation of yeast metallohionein gene transcription. *Genes and Development* **10**, 592-603.

Lloyd, D., Morrell, S., Carlsen, H. N., Degn, H., James, P. E. & Rowlands, C. C. (1993). Effects of growth with ethanol on fermentation and membrane fluidity of *Saccharomyces cerevisiae*. *Yeast* **9**, 825-833.

Longtine, M. S., DeMarini, D. J., Valencik, M. L., Al-Awar, O. S., Fares, H., DeVirgilio, C. & Pringle, J. R. (1996). The septins: roles in cytokinesis and other processes. *Current Opinions in Cell Biology* **8**, 106-119.

Loureiro-Dias, M. C. & Peinado, J. M. (1982). Effect of ethanol and other alkanols on the maltose transport system of *Saccharomyces cerevisiae*. *Biotechnology Letters* **4**, 721-724.

Loureiro-Dias, M. C., and Santos, H. (1990). Effects of ethanol on *Saccharomyces cerevisiae* as monitored by in vivo ^{31}P and ^{13}C nuclear magnetic resonance. Archives of Microbiology. **153,** 384-391.

Lucero, P., Penalver, E., Moreno, E. & Lagunas, R. (1997). Moderate concentrations of ethanol inhibit endocytosis of the yeast maltose transporter. *Applied and Environmental Microbiology* **63**, 3831-3836.

Lucero, P., Penalver, E., Moreno, E. & Lagunas, R. (2000). Internal trehalose protects endocytosis from inhibition by ethanol in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **66**, 4456-4461.

Luyten, K., Riou, C. & Blondin, B. (2002). The hexose transporters of *Saccharomyces cerevisiae* play different roles during enological fermentation. *Yeast* **19**, 713-726.

Lyons, T. J., Gasch, A. P., Gaither, L. A., Botstein, D., Brown, P. O. & Eide, D. J. (2000). Genome-wide characterisation of the Zap1p zinc-responsive regulon in yeast. *Proceedings of the National Academy of Science* **97**, 7957-7962.

Maeda, T., Takekawa, M. & Saito, H. (1995). Activation of yeast *PBS2* MAPKK by MAPKKK or by binding of an SH3-containing osmosensor. *Science* **269**, 554-558.

Mager, W. H. & De Kruijff, A. J. J. (1995). Stress-Induced transcriptional activation. *Microbiological Reviews* **59**, 506-531.

Mager, W. H. & Hohmann, S. (1997). Stress response mechanisms in the yeast *Saccharomyces cerevisiae*. In *Yeast Stress Responses*, pp. 1-5. Edited by S. Hohmann & W. H. Mager. Austin, Texas, U.S.A.: R.G. Landes Company.

Mager, W. H. & Kruijff, A. J. (1995). Stress induced transcriptional activation. *Microbiology Reviews* **59**, 506-531.

Mager, W. H. & Moradas-Ferreira, P. (1993). Stress response of yeast. *Journal of Biochemistry* **290**, 1-13.

Mansure, J. J., Panek, A. D., Crowe, J. M. & Crowe, J. H. (1994). Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochimica et Biophysica Acta* **1191**, 309-316.

Mansure, J. J., Souza, R. C. & Panek, A. D. (1997). Trehalose metabolism in *Saccharomyces cerevisiae* during alcoholic fermentation. *Biotechnology Letters* **19**, 1201-1203.

Marchler, G., Schuller, C., Adam, G. & Ruis, H. (1993). A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO Journal.* **12**, 1997-2003.

Martinez, P., Valcarcel, M. J., Perez, L. & Benitez, T. (1998). Metabolism of *Saccharomyces cerevisiae* flor yeasts during fermentation and biological aging of fino sherry: by-products and aroma compounds. *American Journal of Enology and Viticulture* **49**, 240-250.

Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (S.T.R.E). *EMBO Journal.* **15**, 2227-2235.

Mc Elver, J. & Weber, S. (1992). Flag N-terminal epitope overexpression of bacterial alkaline phosphatase and flag C-terminal epitope tagging by PCR one-step targeted integration. *Yeast* **8 (Suppl.)**, S627.

Meaden, P. G., Arneborg, N., Guldfeldt, L., Siegumfeldt, H. & Jakobsen, M. (1999). Endocytosis and vacuolar morphology in *Saccharomyces cerevisiae*are altered in response to ethanol stress or heat shock. *Yeast* **15**, 1211-1222.

Meaden, P. G., Dickinson, F. M., Mifsud, A., Tessier, W., Westwater, J., Bussey, H. & Midgley, M. (1997). The *ALD6* gene of *Saccharomyces cerevisiae* encodes a Mg^{2+}-activated acetaldehyde dehydrogenase. *Yeast* **13**, 1319-1327.

Michaud, GA., Salcius, M., Zhou, F., Bangham, R., Bonin, J., Guo, H., Snyder, M., Predki, P.F. & Schweitzer, B.I. (2003). Analysing antibody specificity with whole proteome microarrays. *Nature Biotechnology* **10**, 1509-12.

Mishra, P. (1993). Tolerance of fungi to ethanol. In *Stress Tolerance of Fungi.* Edited by D. H. Jennings. New York, NY: Marcel Dekker.

Mishra, P. & Prasad, R. (1988). Role of phospholipid head groups in ethanol tolerance of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **134**, 3205-3211.

Mishra, P. & Prasad, R. (1989). Relationship between ethanol tolerance and fatty acyl compostion of *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **30**, 294-298.

Miura, S., Zou, W., Ueda, M. & Tanaka, A. (2000). Screening of genes involved in isooctane tolerance in *Saccharomyces cerevisiae* by using mRNA differential display. *Applied and Environmental Microbiology* **66**, 4883-4889.

Momose, Y. & Iwahashi, H. (2001). Bioassay of cadmium using DNA microarray: genome wide expression patterns of *Saccharomyces cerevisiae* response to cadmium. *Environmental Toxicology and Chemistry* **20**, 2353-2360.

Monteiro, G. A. & Sa-Correia, I. (1998). *In vivo* activation of yeast plasma membrane H^+-ATPase by ethanol: effect on the kinetic parameters and involvement of the carboxyl-terminus regulatory domain. *Biochimica et Biophysica Acta* **1370**, 310-316.

Moradas-Ferreira, P., Costa, V., Piper, P. W. & Mager, W. H. (1996). The molecular defences against reactive oxygen species in yeast. *Molecular Microbiology* **19**, 651-658.

Moskvina, E., Schuller, C., Maurer, C. T., Mager, W. H. & Ruis, H. (1998). A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. *Yeast* **14**, 1041-1050.

Natarajan, K., Meyer, M. R., Jackson, M. J., Slade, D., Roberts, C., Hinnebusch, A. G. & Marton, M. J. (2001). Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Molecular and Cellular Biology* **21**, 4347-4368.

Navarro-Avino, J., Prasad, R., Miralles, V., Benito, R. M. & Serrano, R. (1999). A proposal for the nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterisation of the stress-inducible *ALD2* and *ALD3* genes. *Yeast* **15**, 829-842.

Neves, M.-J. & Francois, J. (1992). On the mechanisms by which a heat shock induces trehalose accumulation in *Saccharomyces cerevisiae*. *Biochemistry Journal* **288**, 559-564.

Nisho, Y., Aiello, L. & King, G. (1994). Glucose-induced genes in bovine aortic smooth muscle cells identified by differential display. *FASEB Journal.* **8**, 103-106.

Nobel, H. d., Lawrie, L., Brul, S., Klis, F., Davis, M., Alloush, H. & Coote, P. (2001). Parallel and comparative analysis of the proteome and transcriptome of sorbic acis-stressed *Saccharomyces cerevisiae*. *Yeast* **18**, 1413-1428.

Novotny, C., Flieger, M., Panos, J. & Karst, F. (1992). Effect of 5,7-unsaturated sterols on ethanol tolerance in *Saccharomyces cerevisiae*. *Biotechnology and Applied Biochemistry* **15**, 314-320.

Nwaka, S. & Holzer, H. (1998). Molecular biology of trehalose and the trehalases in the yeast *Saccharomyces cerevisiae*. *Progress in Nucleic Acid Research and Molecular Biology* **58**, 198-237.

Nwaka, S., Kopp, M., Burgert, M., Deuchler, I., Kienle, I. & Holzer, H. (1994). Is thermotolerance of yeast dependant on trehalose accumulation? *FEBS Letters* **344**, 225-228.

Nwaka, S., Kopp, M. & Holzer, H. (1995 a). Expression and function of the trehalse genes *NTH1* and *YBR0106* in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **270**, 10193-10198.

Nwaka, S., Mechler, B., Destruelle, M. & Holzer, H. (1995 b). Phenotypic features of trehalase mutants in *Saccharomyces cerevisiae*. *FEBS Letters* **360**, 286-290.

Ogawa, Y., Nitta, A., Uchiyama, H., Imamura, T., Shimoi, H. & Ito, K. (2000a). Tolerance mechanisms of the ethanol tolerant mutant of sake yeast. *Journal of Bioscience and Engineering* **90**, 313 - 320.

Ogawa, N., De Risi, J. L. & Brown, P. O. (2000b). New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. *Molecular and Cellular Biology* **11**.

Oka, A., Sugisaki, H. & Takanami, M. (1981). Nucleotide sequence of the kanamycin resistance transposon Tn903. *Journal of Molecular Biology* **147**, 217-226.

Pakova, J., Smogrovicova, D., Domeny, Z. & Bafrncova, P. (2000). Very high gravity wort fermentation by immobilised yeast. *Biotechnology Letters* **22**, 1173-1177.

Panaretou, B. & Piper, P. W. (1990). Plasma-membrane ATPase action affects several stress tolerances of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as well as the extent and duration of the heat shock response. *Journal of General Microbiology* **136**, 1763-1770.

Parry, J. M., Davis, P. J. & Evans, W. E. (1976). The effects of cell age upon the lethal effects of chemical and physical mutagens in the yeast *Saccharomyces cerevisiae*. *Molecular and General Genetics* **146**, 27-35.

Parsell, D. A., Kowal, A. S., Singer, M. A. & Lindquist, S. L. (1994). Protein disaggregation mediated by heat-shock protein Hsp104. *Nature* **372**, 475-478.

Parsell, D. A. & Lindquist, S. L. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Reviews in Genetics* **27**, 437-496.

Parsell, D. A., Sanchez, Y. & Lindquist, S. (1991). Hsp104 is a highly conserved protein with two essential nucleotide-binding sites. *Nature* **353**, 270-273.

Pereira, M. D., Eleutherio, E. C. A. & Panek, A. D. (2001). Acquisition of tolerance against oxidative damage in *Saccharomyces cerevisiae*. *BMC Microbiology* **1**, 11.

Petko, L. & Lindquist, S. (1986). Hsp26 is not required for growth at high temperatures, nor for thermotolerance, spore development, or germination. *Cell* **45**, 885-894.

Petrov, V. V. & Okorokov, L. A. (1990). Increase of the anion and proton permeability of *Saccharomyces cerevisiae* plasmalemma by n-alcohols as a possible cause of its de-energisation. *Yeast* **6**, 311-318.

Piper, P. W. (1993). Molecular events associated with acquisition of heat tolerance by yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **11**, 339-356.

Piper, P. W. (1995). The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiology Letters* **134**, 121-127.

Piper, P. W. (1997). The yeast heat shock response. In *Yeast Stress Responses*, pp. 75-99. Edited by S. Hohmann & W. H. Mager. Austin, Texas, USA: R.G. Landes Company.

Piper, P. W., Talreja, K., Panaretou, B., Modres-Ferreira, P., Byrne, K., Praekelt, U. M., Meacock, P., Recnacq, M. & Boucherie, H. (1994). Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma membrane

Hsp30, by ethanol levels above a critical threshold. *Microbiology* **140**, 3031-3038.

Pirt, S. J. (1975). Principles of microbe and cell cultivation. pp. 194-198. Oxford: Blackwell Scientific Publications.

Plesset, J., Palm, C. & McLauchlin, C. S. (1982). Induction of heat-shock proteins and thermotolerance by ethanol in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications* **108**, 1340-1345.

Posas, F., Chambers, J. R., Heyman, J. A., Hoeffler, J. P., de Nadal, E. & Arino, J. (2000). The transcriptional response of yeast to saline stress. *The Journal of Biological Chemistry* **275**, 17249-17255.

Praekelt, U. M. & Meacock, P. (1990). *HSP12*, a new small heat shock protein gene of *Saccharomyces cerevisiae*: Analysis of structure regulation and function. *Molecular and General Genetics* **223**, 97-106.

Primig, M., Williams, R. M., Winzeler, E. A., Trevadze, G. G., Conway, A. R., Hwang, S. Y., Davis, R. W. & Esposito, R. E. (2000). The core meiotic transcriptome in budding yeast. *Nature Genetics* **26**, 415-423.

Puig, D. & Perez-Ortin, J. E. (2000). Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. *Yeast* **16**, 139-148.

Reifenberger, E., Boles, E. & Ciriacy, M. (1997). Kinetic characterisation of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. *European Journal of Biochemistry* **245**, 324-333.

Remize, F., Andrieu, E. & Dequin, S. (2000). Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae I*: Role of the cytosolic Mg^{2+} and mitochondrial K^{+} acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. *Applied and Environmental Microbiology* **66**, 3151-3159.

Rep, M., Krantz, M., Thevelein, J. M. & Hohmann, S. (2000). The transcriprional response of *Saccharomyces cerevisiae* to osmotic shock. *The Journal of Biological Chemistry* **275**, 8290-8300.

Rep, M., Reiser, V., Gartner, U., Thevelein, J. M., Hohmann, S., Ammerer, G. & Ruis, H. (1999). Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p. *Molecular and Cellular Biology* **19**, 5474-5485.

Riou, C., Nicaud, J., Barre, P. & Gaillardin, C. (1997). Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. *Yeast* **13**, 903-915.

Rodriguez-Vargas, S., Estruch, F. & Randez-Gil, F. (2002). Gene expression analysis of cold and freeze stress in bakers yeast. *Applied and Environmental Microbiology* **68**, 3204-3030.

Rosa, M. F. & Sa-Correia, I. (1991). *In vivo* activation by ethanol of plasma membrane ATPase of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **57**, 830-835.

Rose, A. H. (1993). Composition of the envelope layers of *Saccharomyces cerevisiae* in relation to flocculation and ethanol tolerance. *Journal of Applied Bacteriology Symposium Supplement* **74**, 110S-118S.

Roth, F. P., Hughes, J. D., Estep, P. W. & Church, G. M. (1998). Finding DNA regulatory motifs within unaligned non-coding sequences clustered by whole genome mRNA quantification. *Nature Biotechnology* **16**, 939-945.

Ruis, H. (1997). Yeast stress responses: Achievements, goals and a look beyond yeast. In *Yeast Stress Responses*, pp. 231-274. Edited by S. Hohmann & W. H. Mager. Berlin: Springer.

Ruis, H. & Schuller, C. (1995). Stress signalling in yeast. *BioEssays* **17**, 959-965.

Saigal, D., Cunningham, S. J., Farres, J. & Weiner, H. (1991). Molecular cloning of the mitochondrial aldehyde dehydrogenase gene of *Saccharomyces cerevisiae* by genetic complementation. *Journal of Bacteriology* **173**, 3199-3208.

Sajbidor, J. (1997). Effect of some environmental factors on the content and composition of microbial membrane lipids. *Critical Reviews in Biotechnology* **17**, 87-103.

Sajbidor, J. & Grego, J. (1992). Fatty acid alterations in *Saccharomyces cerevisiae* exposed to ethanol stress. *FEMS Microbiology Letters* **93**, 13-16.

Sales, K., Brandt, W., Rumbak, E. & Lindsey, G. (2000). The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. *Biochimica et Biophysica Acta* **1463**, 267-278.

Salmon, J. M., Vincent, O., Mauricio, J. C., Bely, M. & Barre, P. (1993). Sugar transport inhibition and apparent loss of activity in *Saccharomyces cerevisiae* as a major limiting factor of enological fermentations. *American Journal of Enology and Viticulture* **44**, 56-64.

Salzberg, S. L. (2003). Yeast rises again. *Nature* **423**, 233-234.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Sanchez, Y. & Lindquist, S. L. (1990). *HSP104* is required for increased thermotolerance. *Science* **248**, 1112-1115.

Sanchez, Y., Parsell, D. A., Taulien, J., Vogel, J. C., Craig, E. A. & Lindquist, S. (1993). Genetic evidence for a functional relationship between Hsp104 and Hsp70. *Journal of Bacteriology* **175**, 6484-6489.

Sanchez, Y., Taulien, J., Borkovich, A. & Lindquist, S. L. (1992). Hsp104 is required for tolerance to many forms of stress. *The EMBO Journal* **11**, 2352-2364.

Santoro, N., Johansson, N. & Thiele, D. (1998). Heat shock element architecture is an important determinant in the temperature and transactivation domain requirements for heat shock transcription factor. *Molecular and Cellular Biology* **18**, 6340-6352.

Schuller, C., Brewster, J. L., Alexander, M. R., Gustin, M. C. & Ruis, H. (1994). The HOG pathway controls osmotic regulation of transcription via the stress-response element (STRE) of the *Saccharomyces cerevisiae CTT1* gene. *The EMBO Journal* **13**, 4382-4389.

Serrano, R. (1991). Transport across the yeast vacuolar and plasma membranes. In *Molecular biology of the yeast Saccharomyces cerevisiae: Genome Dynamics, Protein Synthesis and Energetics*, pp. 523-585. Edited by J. N. Strathern, E. W. Jones & J. R. Broach. Cold Springs Harbour, N.Y.: Cold Springs Harbour Laboratory Press.

Sharma, V. M., Chopra, R., Ghosh, I. & Ganesan, K. (2001). Quantitative target display: a method to screen yeast mutants conferring quantitative phenotypes by 'mutant DNA fingerprints'. *Nucleic Acids Research* **29**, E86-86.

Siderius, M. & Mager, W. H. (1997). General stress response: in search of a common denominator. In *Yeast Stress Responses*, pp. 213-229. Edited by S. Hohmann & W. H. Mager. Austin, Texas, U.S.A.: R.G. Landes Company.

Singer, M. A. & Lindquist, S. L. (1998a). Multiple effects of trehalose on protein folding in vitro and in vivo. *Molecular Cell* **1**, 639-648.

Singer, M. A. & Lindquist, S. (1998b). Thermotolerance in *Saccharomyces cerevisiae*: the yin and yang of trehalose. *TIBTECH* **16**, 460-468.

Sive, H. L. & St John, T. (1988). A simple subtractive hybridization technique employing photoactive biotin and phenol extraction. *Nucleic Acids Research* **16**, 10937-10941.

Smith, B. J. & Yaffe, M. P. (1991). Uncoupling thermotolerance from the induction of heat shock proteins. *Proccedings of the National Academy of Science* **88**, 11091-11094.

Smolka, M., Zhou, H. & Aebersold, R. (2002). Quantitative protein profiling using two-dimentional gel electrophoresis, isotope-coded affinity tag labelling and mass spectrometry. *Molecular and Cellular Proteomics* **1**, 19-29.

Sola-Penna, M. & Meyer-Fernandez, J. R. (1998). Solubilization against thermal inactivation promoted by sugars on enzyme structure and function: Why is trehalose more effective than other sugars. *Archives of Biochemistry and Biophysics* **360**, 10-14.

Sompraynac, L., Jane, S., Burn, T. C. & Tenen, D. G. (1995). Overcoming limitations of the mRNA differential display technique. *Nucleic Acids Research* **23**, 4738-4739.

Sorger, P. K. (1991). Heat shock factor and heat shock response. *Cell* **65**, 363-366.

Stanley, G. A. (1993). Acetaldehyde effects in *Saccharomyces cerevisiae*. Ph.D. thesis: University of Melbourne.

Stanley, G. A., Hobley, T. J. & Pamment, N. B. (1997). Effect of acetaldehyde on *Saccharomyces cerevisiae* and *Zymomona mobilis* subjected to environmental shocks. *Biotechnology and Bioengineering* **53**, 71-78.

Steiner, S. & Philippsen, P. (1994). Sequence and promoter analysis of the highly expressed *TEF* gene of the filamentous fungus *Ashbya gossypii*. *Molecular and General Genetics* **242**, 263-271.

Steiner, S., Wendland, J., Wright, M. C. & Philippsen, P. (1995). Homologous recombination as the main mechanism for DNA integration and cause of rearrangements in the filamentous ascomycete *Ashbya gossypii*. *Genetics* **140**, 973-987.

Stewart, G. G., D'Amore, T., Panchal, C. J. & Russel, I. (1988). Factors that influence the ethanol tolerance of brewers yeast strains during high gravity wort fermentations. *Technical Quarterly MBAA* **25**, 47-53.

Susek, R. E. & Lindquist, S. (1989). hsp26 of *Saccharomyces cerevisiae* is related to the superfamily of small heat shock proteins but is without a demonstratable function. *Molecular and Cellular Biology* **9**, 5265-5271.

Swan, T. M. & Watson, K. (1997). Membrane fatty acid composition and membrane fluidity as parameters of stress tolerance in yeast. *Canadian Journal of Microbiology* **43**, 70-77.

Swan, T. M. & Watson, K. (1998). Stress tolerance in a yeast sterol auxotroph: a role of ergosterol, heat shock proteins and trehalose. *FEMS Microbiology Letters* **169**, 191-197.

Swan, T. M. & Watson, K. (1999). Stress tolerance in a yeast mutant: membrane lipids influence tolerance to heat and ethanol independently of heat shock proteins and trehalose. *Canadian Journal of Microbiology* **45**, 472-479.

Takahashi, T., Shimoi, H. & Ito, K. (2001). Identification of genes required for growth under ethanol stress using transposon mutagenesis in *Saccharomyces cerevisiae*. *Molecular Genetics and Genomics* **265**, 1112-1119.

Tamai, K. T., Liu, X., Silar, P., Sosinowski, T. & Thiele, D. (1994). Heat shock transcription factor activates yeast metallothionein gene expression in response to heat and glucose starvation via distinct signalling pathways. *Molecular and Cellular*

Biology **14**, 8155-8165.

Tanghe, A., Teunissen, A., Dijck, P. V. & Thevelein, J. M. (2000). Identification of genes responsible for improved cryoresistance in fermenting yeast cells. *International Journal of Food Microbiology* **55**, 259-262.

Tatusov, R. L., Galperin, M. Y., Natale, P. A. & Koonin, E. V. (2000). The COG database: a tool for genome scale analysis of protein functions and evolution. *Nucleic Acids Research* **28**, 33-36.

Tessier, W. D., Meaden, P. G., Dickinson, F. M. & Midgley, M. (1998). Identification and disruption of the gene encoding the K^+-activated acetaldehyde dehydrogenase of *Saccharomyces cerevisiae*. *FEMS Microbiology Letters* **164**, 29-34.

Thatcher, J., Shaw, J. & Dickinson, W. (1998). Marginal fitness contributions of nonessential genes in yeast. *Proceedings of the National Academy of Sciences USA* **95**, 253-257.

Thevelein, J. M. (1984). Regulation of trehalose metabolism in fungi. *Microbiology Reviews* **48**, 42-59.

Thevelein, J. M. (1994). Signal transduction in yeast. *Yeast* **10**, 1753-1790.

Thevelein, J. M. & Hohmann, S. (1995). Trehalose synthase: guard to the gate of glycolysis in yeast. *Trends in Biochemical Science* **20**, 3-10.

Thomas, D. S., Hossack, A. J. & Rose, A. H. (1978). Plasma membrane lipid composition and ethanol tolerance. *Archives of Microbiology* **117**, 239-245.

Thomas, D. S & Rose, A. H. (1979). Inhibitory effects of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. *Archives of Microbiology.* **122,** 19-55.

Tomas-Cobos, L. & Sanz, P. (2002). Active Snf1 protein kinase inhibits expression of the *Saccharomyces cerevisiae HXT1* glucose transporter gene. *Biochemistry Journal* **368**, 657-663.

Toone, W. M. & Jones, N. (1998). Stress-activated signalling pathways in yeast. *Genes to Cells* **3**, 485-498.

Toone, W. M. & Jones, N. (1999). AP-1 transcription factors in yeast. *Current Opinion in Genetic Developments* **9**, 55-61.

Travern, A., Wong, J. M. S., Xu, D. M., Sopta, M. & Ingles, C. J. (2001). Interorganellar communication: altered nuclear gene expression profiles in a yeast mitochondrial DNA mutant. *Journal of Biological Chemistry* **276**, 4020-4027.

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S. & Walter, P. (2000). Functional and genomic analysis reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101**, 249-258.

Treger, J. M., Schmitt, A. P., Simon, J. R. & McEntee, K. (1998). Transcriptional factor mutations reveal regulatory complexities of heat shock factor and newly identified stress genes in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry* **273**, 26875-26879.

Trollmo, C., Andre, L., Blomberg, A. & Adler, L. (1988). Physiological overlap between osmotolerance and thermotolerance in *Saccharomyces cerevisiae*. *FEMS Microbiology Letters* **56**, 321-326.

Unaldi, M. N., Arikan, B. & Coral, G. (2002). Isolation of alcohol tolerant, osmotolerant and thermotolerant yeast strains and improvement of their alcohol tolerance by UV mutagenesis. *Acta Microbiologica Polonica* **15**, 115-120.

Van Dijck, P., Colavizza, D., Smet, P. & Thevelein, J. M. (1995). Differential importance of trehalose in stress resistance in fermenting and non-fermenting *Saccharomyces cerevisiae* cells. *Applied and Environmental Microbiology* **61**, 109-115.

van Leare, A. (1989). Trehalose, reserve and/or stress metabolite? *FEMS Microbiology Reviews* **63**, 201-210.

van Uden, N. (1984). Effects of ethanol on the temperature relations of viability and growth in yeast. *CRC Critical Reviews in Biotechnology* **1**, 263-272.

Varela, J. C. S., Praekelt, U. M., Meacock, P., Planta, R. J. & Mager, W. H. (1995). The *Saccharomyces cerevisiae HSP12* geneis activated by the high osmolarity glycerol pathway and negatively regulated by protein kinase A. *Molecular and Cellular Biology* **15**, 6232-6245.

Varela, J. C. S., van Beekvelt, C. A., Planta, R. J. & Mager, W. H. (1992). Osmo-stress induced changes in yeast gene expression. *Molecular Microbiology* **6**, 2183-2190.

Wach, A., Brachat, A., Poehlmann, R. & Philippsen, P. (1994). New heterologous modules for classical or PCR based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**, 1793-1808.

Wach, A., Brachat, A., Rebischung, C., Steiner, S., Pokorni, K., te Heesen, S. & Philippsen, P. (1998). PCR-based gene targeting in *Saccharomyces cerevisiae*. *Methods in Microbiology* **26**, 67-81.

Walker, G. M. (1994). The role of magnesium in biotechnology. *Critical Reviews in Biotechnology* **14**, 311-354.

Walker, G. M. (1998). *Yeast Physiology and Biotechnology*. West Sussex, England: John Wiley and Sons Ltd.

Walker-Caprioglio, H. M., Casey, W. M. & Parks, L. W. (1990). *Saccharomyces cerevisiae* and membrane sterol modifications in response to growth in the presence of ethanol. *Applied and Environmental Microbiology* **56**, 2853-2857.

Walker-Caprioglio, H. M. & Parks, L. W. (1987). Autoconditioning factor relieves ethanol-induced growth inhibition of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **53**, 33-35.

Walker-Caprioglio, H. M., Rodriguez, R. & Parks, L. W. (1985). Recovery of *Saccharomyces cerevisiae* from ethanol-induced growth inhibition. *Applied and Environmental Microbiology* **50**, 685-689.

Wan, J., Sharp, S., Poirier, G., Wagaman, P., Chambers, J., Pyati, J., Hom, Y., Galindo, J., Huvar, A., Peterson, P., Jackson, M. & Erlander, M. (1996). Cloning differentially expressed mRNAs. *Nature Biotechnology* **14**, 1685-1691.

Wang, X. & Feuerstein, G. Z. (1995). Direct sequencing of DNA isolated from mRNA Differential Display. *BioTechniques* **18**, 480-453.

Wang, X., Mann, C. J., Bai, Y., Ni, L. & Weiner, H. (1998). Molecular cloning, characterisation, and potential roles of cytosolic and mitochondrial aldehyde dehydrogenases in ethanol metabolism in *Saccharomyces cereviasiae*. *Journal of Bacteriology* **180**, 822-830.

Warner, J. R. (1999). The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Science* **24**, 437-440.

Watson, K. & Cavicchioli, R. (1983). Acquisition of ethanol tolerance in yeast cells by heat shock. *Biotechnology Letters* **5**, 683-688.

Watson, M. (2001). Disruption and basic phenotype analysis of six novel genes from the right arm of chromosome XII of *Saccharomyces cerevisiae*. *Yeast* **18**, 473-480.

Wemmie, J. A., Steggerda, S. M. & Moye-Rowley, W. S. (1997). The *Saccharomyces cerevisiae* AP-1 protein discriminates between oxidative stress elicitated by the oxidants H_2O_2 and diamide. *Journal of Biological Chemistry* **272**, 7908-7914.

Werner-Washburne, M., Braun, E. L., Crawford, M. E. & Peck, V. M. (1996). Stationary phase in *Saccharomyces cerevisiae*. *Molecular Microbiology* **19**, 1159-1166.

Winkler, K., Kienle, I., Burgert, M. & Wagner, M. (1991). Metabolic regulation of trehalose content of vegetative yeast. *FEBS Letters* **291**, 260-272.

Yale, J. & Bohnert, H. J. (2001). Transcript expression in *Saccharomyces cerevisiae* at high salinity. *The Journal of Biological Chemistry* **276**, 15996-16007.