AN ASSESSMENT OF ETHANOL STRESS TOLERANCE FACTORS IN *Saccharomyces cerevisiae*

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

BY

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DECLARATION

I, Ajith Bandara, declare that the PhD thesis entitled "An assessment of ethanol stress tolerance factors in *Saccharomyces cerevisiae*" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Ajith Bandara
August, 2008

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SUMMARY

Ethanol, one of the main end products of sugar catabolism in yeast, can become a significant stress factor as it accumulates in the culture broth during fermentation. Stressful ethanol levels can inhibit yeast growth rate, cell viability, metabolic activity, membrane-associated transport systems and, depending on the ethanol concentration, result in cell death. Ultimately, ethanol toxicity affects the fermentation performance of yeast resulting in lowered fermentation productivity and ethanol yield. To survive and multiply under ethanol stress conditions, yeast cells have developed a set of sensing, signalling and defence mechanisms that enable them to rapidly acclimatise to ethanol toxicity. There is currently only limited knowledge of the complex mechanisms involved in the ethanol stress response of yeast; improving our understanding of these cellular mechanisms could assist in the design of new yeast strains that are more ethanol tolerant and therefore less likely to be compromised during ethanologenic fermentation. It may also lead to the development of strategies and practices that better prepare yeast for fermentation-related stress and improve ethanol productivity and yield. This study was conducted to resolve some of the controversy associated with two ethanol stress tolerance factors in *Saccharomyces cerevisiae,* trehalose and the protein Asr1.

It has been shown that one response by yeast to ethanol stress is an increase in intracellular trehalose levels. There is also some evidence to suggest that trehalose has a role in ethanol tolerance, but to date such evidence is controversial and some of it is circumstantial. For example, a majority of these studies based their findings on correlations developed between intracellular trehalose level and relative ethanol tolerance for a number of different strains; the lack of consensus across such studies is not surprising given the genetic diversity of the strains used within each experiment. Such studies cannot separate the individual role of trehalose from that of other stress response mechanisms, with the influence of such mechanisms on ethanol tolerance likely to vary from strain to strain. In this project, *S. cerevisiae* BY4742 and strains *tsl1*Δ and *nth1*Δ, (containing single gene deletions associated with trehalose metabolism in BY4742) were used to determine if trehalose contributes to cell acclimatisation during ethanol stress. It was found that the influence of trehalose on

S. cerevisiae tolerance to ethanol stress is ethanol-concentration dependent. Intracellular trehalose concentration does not influence *S. cerevisiae* acclimation to, or growth rate during, non-lethal ethanol stress, but it does improve cell survival when subjected to lethal ethanol concentrations; deletion strains with higher trehalose levels always had significantly higher survival rates compared to strains with lower trehalose levels in lethal ethanol conditions. This outcome favours a mechanism for trehalose associated with protecting cytosolic proteins from the damaging and inhibitory effects of ethanol, although a role in protecting membrane integrity cannot be totally discounted.

Very little is known about how cells specifically sense ethanol stress and initiate an appropriate response. Betz *et al.* (2004) were the first group to report that the protein Asr1 shuttles constitutively between the cytoplasm and nucleus, but rapidly and reversibly accumulates in the nucleus in the presence of extracellular alcohol; its response is exclusive to alcohol, making Asr1 a candidate ethanol-specific signalling factor. Its role in the ethanol stress response is still unconfirmed and there is no conclusive evidence on whether Asr1 confers a particular phenotype in *S. cerevisiae* during ethanol stress. The results described in this thesis establish that Asr1 has a positive role in acclimation to ethanol stress by *S. cerevisiae*. Competitive growth experiments demonstrated that cells lacking Asr1 (*asr1*Δ) had a lower growth rate than the wild type strain during long-term incubation in a sub-lethal ethanol concentration. This outcome suggests that Asr1 is likely to have a role in modulating cell growth during ethanol stress. Microarray analysis showed for the first time that Asr1 does play a role in modifying gene expression and that this occurs exclusively in the presence of ethanol stress. Gene expression studies also supported the hypothesis that Asr1 has a cell growth-related role, by showing that 70% of genes expressed at a lower level in *asr1*Δ strains were associated with cell growth and/or maintenance. Another interesting observation was that genes affected by Asr1 are not randomly distributed but are clustered in groups of 2 to 6 on the chromosomes. This novel observation suggests that Asr1 might exert its influence on gene expression by altering local chromatin structure which is not entirely unexpected as Asr1 has a Ring or PHD type finger domain and these domains have previously been implicated in chromatin remodelling and histone modification.

PUBLICATIONS AND PRESENTATIONS

Publications

Bandara, A., S. Fraser, P. Chambers and G. A. Stanley (2008). The ethanol stress-protective effect of trehalose accumulation in *Saccharomyces cerevisiae* is ethanol concentration dependant. (Submitted for publication in Appl. Environ. Microbiol., December 2008).

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Oral presentations

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ABBREVIATIONS

AFM	atomic force microscope
Asr1	Alcohol Sensitive Ring/PHD finger 1 protein
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AWRI	Australian Wine Research Institute
BLAST	Basic local alignment search tool
bp	Base pair
°C	Degree Celsius
cDNA	Complementary DNA
CDRE	Calcineurin-dependant-response element
Ci/mmol	Curies per millimole
CO_2	Carbon dioxide
CP	Crossing point
Cy3	Cyanine dye 3
Cy5	Cyanine dye 5
DAPI	4',6'-diamidino-2-phenylindole
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine 5'-triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DTT	Dithiothreitol
dTTP	Deoxythymidine 5'-triphosphate
dUTP	Deoxyuridine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EUROSCARF	EUROpean Saccharomyces Cerevisiae Archive for Functional analysis)

FDR	False discovery rate
GDP	guanosine triphosphate
GFP	Green fluorescent protein
g/l	Gram per litre
GO	Gene ontology
GTP	guanosine diphosphate
h	Hour
HAT	Histone acetyl transferase
HCl	Hydrochloric Acid
HDAC	histone deacetylase complexes
H_2O_2	hydrogen peroxide
HOG	High-Osmolarity Glycerol
HSE	Heat shock element
HSF	Heat shock factor
Hsp	Heat shock protein
kb	kilobase pair
KCl	Potassium choride
kDa	Kilodaltons
L	Litre
M	Molar
MAPK	Mitogen--Aactivated Protein Kinase
Mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MW	Molecular weight
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate

NaOH	sodium hydroxide
NE	nuclear envelope
NES	nuclear export signals
NLS	nuclear localization signals
nm	nanometer
NPC	nuclear-pore complexes
ORF	open reading frame
PCR	polymerase chain reaction
PDS	postdiauxic shift element
PHD	plant homeodomain
RING	really interesting new gene
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolution per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
SAM	Significance Analysis of Microarrays
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGD	*Saccharomyces* Genome Database
sHSP	small heat shock protein
SSC	sodium chloride-sodium citrate
SS-DNA	salmon sperm DNA
STRE	stress response element
TAP	tandem affinity purification
TIGR	The Institute for Genome Research
TRIS	tris-(hydroxymethy)-aminomethane
UV	ultra violet
V	volt
VU	Victoria University
v/v	volume per volume
YEPD	yeast extract, peptone and D-glucose
YRE	Yap1-response elements

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CHAPTER 1

General Introduction and Literature Review

1.1 Introduction

The unicellular eukaryote yeast *Saccharomyces cerevisiae* has a longstanding history as a domesticated, cultivated organism. Yeasts have been exploited by mankind for thousands of years in mixed microbial populations for traditional fermentations that produce alcoholic beverages and leavened bread (O'Donnell 2002). The first pure yeast culture used in alcoholic beverage production was obtained by Emil Christian Hansen from the Carlsberg Brewery in 1883, and in 1890 Muller-Thurgau (Geisenheim, Germany) isolated a pure culture of wine yeast (Dequin 2001). Since then, the exploitation of yeast in industrial fermentations has expanded beyond traditional brewing, winemaking and breadmaking (de Winde 2003).

In modern times yeast applications include a diverse range of operations including, food and chemical industries, health care, and biological, biomedical and environmental research (Walker 1998). However, ethanol production is likely to remain the premier worldwide biotechnological, yeast-derived commodity for many years to come (Walker 1998). With the continuing threat of warfare in oil producing countries, rising CO_2 levels in the atmosphere due to fossil fuel combustion and finite supplies of crude oil, worldwide interest in the production of ethanol as a renewable and greenhouse-neutral energy source has radically increased.

Cost-effective ethanol production depends on, among other factors, optimal conversion of carbohydrate to ethanol, which in itself depends on improvements in the survival and performance of yeast cells under industrial conditions. During fermentation, yeast cells encounter a range of environmental stress conditions. Modern fermentation technology using high density fermentation substrates results in high osmotic pressure, rapid temperature changes, nutrient limitation and increased ethanol levels. Exposure of yeast to these conditions leads to impaired fermentation, a decline in cell viability and reduced lifespan and

productivity of re-pitched yeast. Yeast cells have however evolved to be more resilient to environmental stresses. Survival and growth of yeast under such stress conditions is achieved through a series of stress responses that depend on a complex network of sensing and signal transduction pathways leading to adaptations in cell cycle, and adjustments in gene expression profiles and cell metabolic activities (Hohmann and Mager 2003).

Ethanol, the main end product of fermentative metabolism in yeast, can become a significant stress factor as it accumulates in the culture broth during fermentation. Generally speaking, *S. cerevisiae* is highly ethanol tolerant, with some strains able to produce up to 20% ethanol in a single batch, albeit over a long period of time. High ethanol concentrations, however, inhibit yeast growth and viability, and affect the fermentation performance of yeast resulting in lowered fermentation productivity and ethanol yield (Norton *et al.*, 1995; Galeote *et al.*, 2001; Aguilera *et al.*, 2006). A better understanding of the cellular consequences of microbial ethanol stress and of the underlying ethanol stress defence mechanisms is crucial for improving the performance of yeast strains under industrial conditions. This study was conducted to investigate the physiological and molecular responses of yeast to ethanol stress.

The focus in this research project was on the roles of the disaccharide trehalose and of the protein Asr1 in yeast defence against ethanol stress. Previous studies on yeast have characterised trehalose as a major carbohydrate reserve compound (Walker 1998). However, recent studies support the theory that trehalose contributes to survival during environmental stress conditions, such as dehydration and heat stress (Thevelein *et al.*, 1984; Wiemken 1990; Gadd *et al.*, 1987). It is suggested that trehalose reduces protein aggregation and maintains polypeptide chains in a folded form, increasing tolerance to heat and dehydration (Thevelein *et al.*, 1984; Wiemken 1990; Singer and Lindquist 1998). Under stress treatments, trehalose is synthesised inside the cell and, at least some, locates to the plasma membrane, where is believed to protect the membrane and stabilise intracellular components against the effects of stress (Herdeiro *et al.*, 2006). There is evidence of trehalose being involved in the survival of yeast cells exposed to severe osmotic stress (Hounsa *et al.*, 1998). Accumulation of trehalose increases yeast stress tolerance to starvation (Lillie and Pringle 1980), freezing (Soto *et al.*, 1999), dehydration (Gadd *et al.*, 1987) suboptimal

temperature (De Virgilio *et al.,* 1994; Eleutherio *et al.,* 1995) and to ethanol (Attfield 1987; Mansure *et al.,* 1994; Kim *et al.,* 1996; Mansure *et al.,* 1997; Soto *et al.,* 1999; Lucero *et al.,* 2000). Conversely, a number of studies advocate that trehalose has no influence on ethanol stress tolerance in yeast (Lewis *et al.,* 1997; Alexandre *et al.,* 1998; Ribeiro *et al.,* 1999; Gomes *et al.,* 2002). Given these conflicting conclusions, the role of trehalose in conferring ethanol tolerance in yeast is still subject to scientific debate. As such, it is an area requiring more work and is explored further in the research described in this thesis.

In 2004 Betz *et al.* (2004) identified a novel yeast Ring/PHD finger protein that specifically responds to alcohol, but not to other environmental stresses; it is called the Alcohol Sensitive Ring/PHD finger 1 protein (Asr1). The authors observed that the protein Asr1 constitutively shuttles between the yeast nucleus and cytoplasm and rapidly accumulates in the cell nucleus in response to extracellular alcohol exposure. Nuclear localisation of Asr1 is unique to alcohol stress, not being observed in the presence of osmotic, oxidative or heat stress, or during starvation. The removal of ethanol containing medium resulted in a rapid dissipation of Asr1 accumulation in the nucleus and its relocation to the cytoplasm, suggesting that the ethanol induced nuclear accumulation of Asr1 is rapid and reversible. Furthermore, Betz *et al.* (2004) reported the requirement of Asr1 for tolerance to alcohol by comparing the wild type strain with *asr1*Δ (*ASR1* gene deletion) strain, although the evidence for this is less than convincing. In 2006, Izawa *et al.* (2006) reconfirmed the alcohol-responsive changes in the intracellular localization of Asr1, however, the authors could not detect any influence of Asr1 on the growth of yeast in response to ethanol stress, concluding that Asr1 is not required for tolerance to alcohol stress. This observation cast doubt on the suggestion that Asr1 plays a role in the ethanol tolerance of yeast. The role of Asr1 in the yeast response to ethanol stress is also an objective of the research described in this thesis.

1.2 Yeast responses to environmental stress

As already discussed, during fermentation yeasts are exposed to a range of stresses such as high ethanol concentration, osmotic pressure, high and low temperature,

oxidative stress, starvation, extreme pH and dehydration (Attfield *et al.,* 1997; Hohmann and Mager 1997; Mager and Hohmann 1997; Barber *et al.,* 2001; Hohmann 2002). This can result in stress-induced denaturation of proteins, disordering of membranes, DNA damage and metabolic disturbance, to which the cell responds by initiating changes in gene transcription, and translational and post-translational modifications of stress-associated proteins (Mager and Moradas-Ferreira 1993; Piper 1993; Siderius and Mager 1997).

Transcriptome-based changes in yeast may result in biochemical and physiological changes to the yeast biochemical profile, including increased synthesis of osmolytes (e.g. glycerol) and heat shock proteins (HSPs), trehalose accumulation, increased molecular chaperone activity, enhanced radical oxygen scavenging, changes in redox state, increased proton pumping activity, adjustments in carbon/nitrogen balance and altered ion and water uptake and changes in plasma membrane composition (Piper 1993; De Virgilio *et al.,* 1994; Buchner 1996; Parrou *et al.,* 1997; Jamieson 1998; Kempf and Bremer 1998; Swan and Watson 1999; Estruch 2000; Yale and Bohnert 2001). These stress-related changes not only initiate the repair of macromolecular damage caused by stress, but also establish a more tolerant state that helps to protect the cell from further damage. Central to these responses are the sensing and signaling pathways that initiate the necessary changes in gene expression.

1.2.1 General response of yeast to environmental stress

Comparative analysis of yeast genomic responses to various stress conditions, including temperature shock, chemical stress and nutrient depletion, has recognized that similar groups of genes (around 14% of all yeast genes) alter their gene expression profile in response to stress (Gasch *et al.,* 2000; Causton *et al.,* 2001; Chen *et al.,* 2003). Based on gene expression patterns, it has been found that around 600 genes have lower transcript levels and around 300 genes have higher transcript levels in response to all stress-causing transitions (reviewed in Gasch and Werner-Washburne 2002). This led to the suggestion that yeast use a general stress response mechanism to protect themselves from environmental insult. In support of this, a number of studies have reported that heat shock genes are not only induced by heat,

but also by other stress conditions (Kurtz *et al.,* 1986; Werner-Washburne *et al.,* 1989; Kobayashi and McEntee 1990; Susek and Lindquist 1990).

The general stress response is primarily controlled by the transcription factors Msn2 and Msn4 (Msn2/4), which bind to Stress Response Elements (STREs) in the promoters of general stress response-associated genes (Marchler *et al.,* 1993; Martinez-Pastor *et al.,* 1996; Schmitt and McEntee 1996). Gene expression analysis has shown that Msn2/4 are responsible for expression of most general stress response genes in yeast subjected to heat stress (Tregger *et al.,* 1998). Furthermore, a number of studies, using *MSN2* and *MSN4* deletions, have identified increased sensitivity of such cells to various stress conditions, supporting the supposition of common Msn2/4 activity under different kinds of stress (Martinez-Pastor *et al.,* 1996; Schmitt and McEntee 1996; Boy-Marcotte *et al.,* 1998; Moskvina *et al.,* 1998). Recent studies have found that Msn2/4 localizes to the cytoplasm under ambient conditions, however, upon stress exposure, they are found in the nucleus and bind to STREs of general stress responsive genes (Aguilera *et al.,* 2006).

1.2.2 Specific stress responses

Global gene expression studies have provided insight into stress responses that are unique to particular stressors. The heat shock response is the most extensively studied stress response in yeast and heat shock protein (HSP) genes are among the best characterised stress response genes. The heat shock response is governed by the activation of a specific heat-shock transcription factor (Hsf1) that binds to a specific conserved promoter sequence, the heat shock element (HSE) (Morimoto 1993; Chatterjee *et al.,* 2000; Grably *et al.,* 2002) (Figure1.1). Most HSP genes have an HSE in their promoter and are regulated by Hsf1; some HSPs (*HSP12, HSP26 and HSP104*) also contain STREs in their promoter regions and are induced by Msn2/4 and Hsf1 during heat stress (Amoros and Estruch 2001). Although *HSP26* and *HSP104* are primarily controlled by Hsf1 in response to heat shock, other stresses such as starvation, oxidative stress or osmotic stress can activate these genes but via transcription factors Msn2/4 (Tregger *et al.,* 1998; Amoros and Estruch 2001).

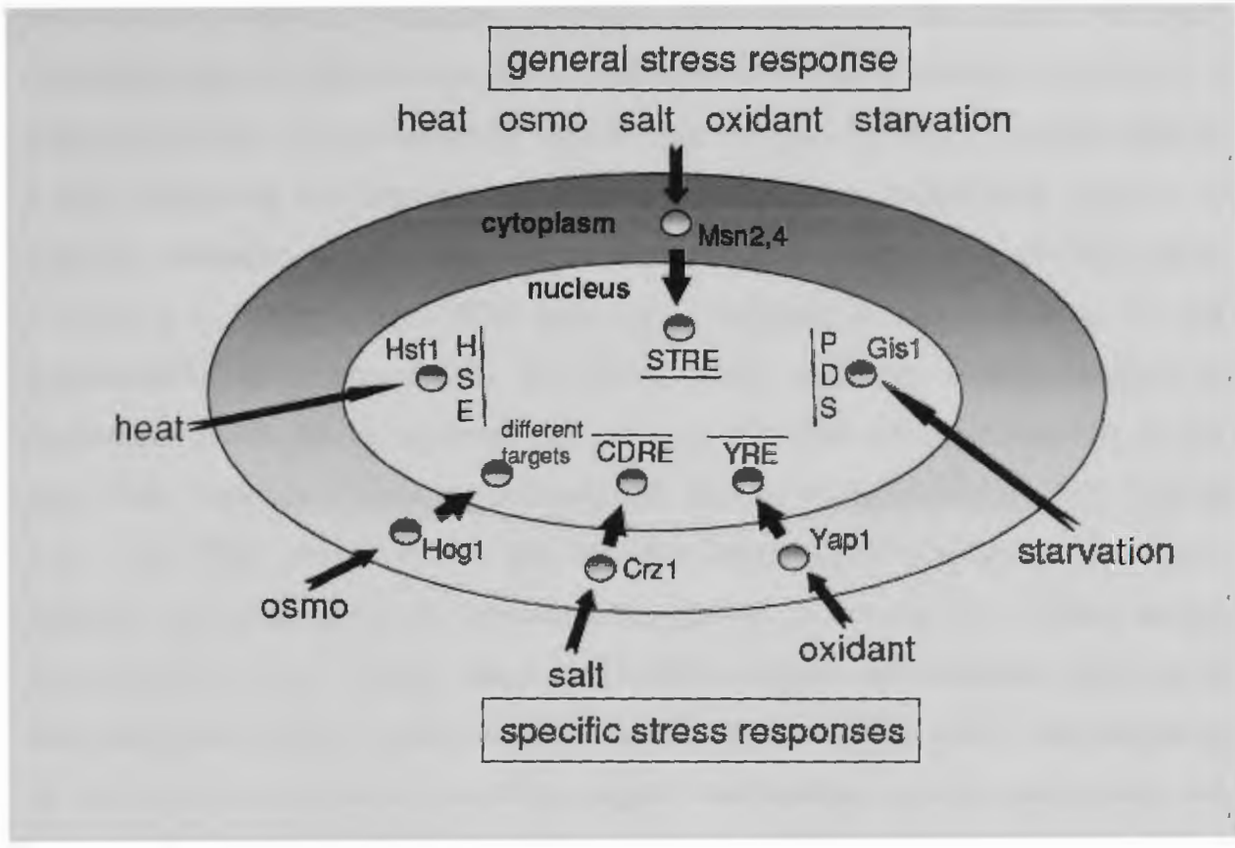


Figure 1.1: Best-established stress response mechanisms in *Saccharomyces cerevisiae*. The general stress response is triggered by a variety of sub-optimal conditions. It involves transfer of the Msn2/4 transcription factors into the nucleus, where they induce transcription by binding to the stress-response elements (STRE) in the promoters of target genes. In the specific stress responses, heat shock factor Hsf1 activates heat shock-induced transcription through the heat shock element (HSE), whereas in the osmostress response the Hog1 protein migrates to the nucleus, where it interacts with several transcription factors to activate transcription of osmostress-induced genes. Salt stress triggers entry of the Crz1 transcription factor into the nucleus, where it interacts with the calcineurin-dependant-response element (CDRE) in target gene promoters. Oxidative stress specifically causes accumulation of the Yap1 transcription factors in the nucleus, where it induces transcription of antioxidant genes through interaction with Yap1-response elements (YRE). Nutrient starvation activates the Gis1 transcription factor, which induces stationary-phase genes through interaction with the postdiauxic shift element (PDS). Reprinted by permission from Springer, Heidelberg: Biodiversity and ecophysiology of yeast (Tanghe *et al.*, 2006), copyright (2006).

Studies on the specific response of yeast to osmotic pressure have provided considerable insight on signalling pathways associated with this stress. The high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway has a major role in the acclimatisation of yeast to high external osmolarity. Sudden shifts to a high osmolarity environment stimulate accumulation of intracellular glycerol in yeast by increasing glycerol uptake and biosynthesis, and decreasing glycerol efflux (Tamas *et al.*, 1999). The HOG pathway is initiated by the activation of cell membrane-bound receptors Sln1 and Sho1, which sense the external osmolarity (Hohmann 2002). These upstream osmosensors stimulate different branches of the Ste11/Ssk1-dependant signaling pathway that lead to the activation of Pbs2 (Figure 1.2). Then Pbs2 phosphorylates and activates Hog1 which modulates osmostress-induced gene expression by controlling the activity of several transcription factors including Hot1, Sko1, Smp1, Msn1 and Msn2/4 (Tamas and Hohmann 2003; Saito and Tatebayashi 2004). These osmostress-induced genes are involved in the activation of general stress responses including signal transduction, protein production, and storage carbohydrate and transient cell cycle arrest in G1 (Hohmann 2002; Escoté *et al.*, 2004). Expression of Glycerol-3-phosphate dehydrogenase (*GDP1*) is induced by transcription factor Hot1 in response to osmotic shock, but in response to heat stress *GDP1* expression appears to be regulated by Msn2/4 (Rep *et al.*, 2000).

Transcription factor Yap1, in association with Skn7, regulates oxidative stress responses in *S. cerevisiae* (Lee *et al.*, 1999). Yap1 binds to its recognition element YRE and confers the ability to tolerate oxidants by activating the expression of genes encoding many antioxidant enzymes and components of the cellular thiol-reducing pathways (Godon *et al.*, 1998). Skn7 assists in the activation of around 15 Yap1 target proteins in response to H_2O_2 and t-butyl hydroperoxide (Lee *et al.*, 1999). General transcription factors Msn2/4 co-regulate a few of the antioxidant enzymes and a number of the heat-shock proteins in response to H_2O_2 (Hasan *et al.*, 2002). Most of these genes are induced by Yap1 in response to H_2O_2 but are activated by Msn2/4 under heat shock stress (Gasch *et al.*, 2000).

Generally in yeast, decreased availability of one substrate in the growth medium induces specific metabolic pathways required for the utilization of an alternative

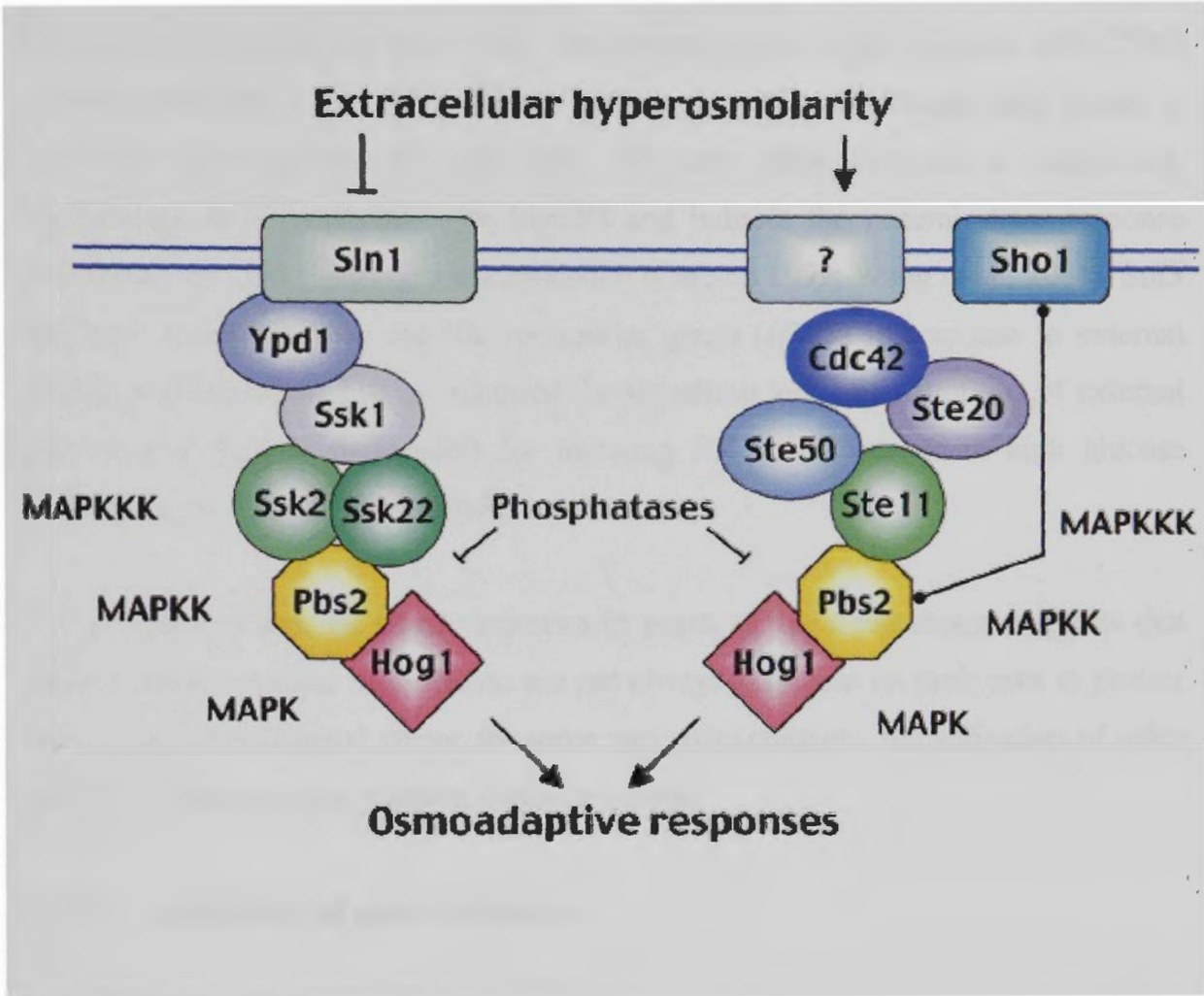


Figure 1.2: Schematic diagram of the yeast HOG mitogen-activated protein (MAP) kinase pathway. Pbs2 integrates signals from two major independent upstream osmosensing mechanisms. Under osmostress, activated Pbs2 activates the Hog1 MAPK, which induces a set of osmoadaptive responses. Reprinted by permission from Macmillan Publishers Ltd: EMBO reports (de Nadal *et al.,* 2002), copyright (2002).

substrate. Eventually, when one or more essential nutrients are depleted, cells enter the stationary phase where cell cycle completion and arrest occur at G1 phase.

Stationary phase cells accumulate storage carbohydrate glycogen and trehalose in response to nutrient depletion (Winderickx *et al.,* 2003). Nutrient depletion induces activation of transcription factor Gis1 that interacts with the post-diauxic shift (PDS) element (Pedruzzi *et al.,* 2000). Under carbon starvation conditions, Snf1 kinase is stimulated and mediates the depression of many genes involved in respiration. Furthermore Snf1 associates with Msn2/4 and induces the general stress response mechanism including glycogen accumulation (Carlson 1999; Wang *et al.,* 2001). Snf3 and Rgt2 induce glucose specific transporter genes (*HXTs*) in response to external glucose concentration. Snf3 is required for signalling low concentrations of external glucose and Rgt2 is responsible for inducing *HXT1* in response to high glucose concentrations (Geladé *et al.,* 2003).

The presence of specific stress responses in yeast, as described above, suggests that general stress response mechanisms are not always sufficient on their own to protect cells from environmental stress; for some particular stressors, the activation of other specific stress regulatory systems is also required.

1.2.3 Acquisition of stress tolerance

An intrinsic aspect of the yeast stress responses is acquisition of stress tolerance, where cells can more effectively withstand severe stress conditions. Pre-exposure of yeast to a sub-lethal level of stressing agent may stimulate an adaptive response, resulting in transient resistance to higher levels of the same stress compared to cells without pre-exposure. The acquisition of tolerance to formerly lethal stress levels has been linked to the activation of specific stress response mechanisms during pre-exposure to the non-lethal stress. In the case of heat stress, the acquisition of thermo-tolerance has been observed in yeast when exposed to transient sub-lethal temperatures, ranging between 37°C and 45°C. Increasing the magnitude of the pre-stress heat shock not only induced greater thermo-tolerance in the cell, but also a quicker response (Coote *et al.,* 1991). For example, yeast are able to survive a

previously lethal temperature of 51°C when cells are preconditioned by exposure to a mild heat shock at 37°C for 20 minutes (Plesset *et al.*, 1982). A one hour incubation of yeast cells at 37°C conferred higher resistance in a subsequence exposure to a 55°C heat shock (Sanchez and Lindquist 1990; Gross and Watson 1998).

This pre-exposure effect has also been observed in stress conditions other than heat shock. A transient pre-treatment of yeast cells with 0.7 M NaCl increased salt tolerance in a subsequent exposure to 1.4 M NaCl (Trollmo *et al.*, 1988; Varela *et al.*, 1992). Davies *et al.* (1995) showed that the growth of yeast cells was arrested when exposed to 0.8 mM H_2O_2 (oxidative stress), however, when the cells were pre-treated by exposure to 0.4 mM H_2O_2 for 45 min, they had acquired tolerance to the subsequent 0.8 mM H_2O_2 stress, i.e. the pre-treated cells grew and divided at a 15-30% faster rate than the non-pretreated cells (Davies *et al.*, 1995).

Vriesekoop and Pamment (2005) investigated the effect of pre-treatment of yeast with mild ethanol stress on the yeast ethanol stress response. The yeast culture with a pre-treated inoculum showed a 70% reduction in the lag period compared to the culture with non-pre-treated inoculum in exposure to higher ethanol concentrations (Vriesekoop and Pamment, 2005).

In general it is recognised that mild stress conditions can trigger cellular responses that prepare cells for coping better with severe stress. Such investigations suggest that yeast and other microorganisms have an inherent ability to improve their stress tolerance provided that the appropriate external and internal triggers are activated. A better understanding of these built-in molecular processes that underpin, and are a part of, the yeast stress response is important for the development of strategies to improve yeast stress tolerance.

1.2.4 Cross-stress protection and adaptive stress responses

The previous section described the use of pre-treatment to increase yeast stress tolerance to a particular stress. Another way of increasing cell tolerance to stress is by cross-stress protection. Cross-stress protection is where exposure of yeast to a mild

dose of stress results in the acquisition of resistance against a subsequent treatment with a higher level of another type of stress. This phenomenon of cross-protection is thought to occur as a consequence of the general stress response mechanism which is activated in response to mild stress conditions (Lewis *et al.,* 1995; Chen *et al.,* 2003). For example, heat shock response studies have shown that midstream temperature shock renders yeast not only more resistant to a higher dose of temperature shock, but also to other stressors such as ethanol (Watson and Cavicchioli 1983; Costa *et al.,* 1993), a high salt concentration (Lewis *et al.,* 1995), oxidative stress (Jamieson 1992; Flattery-O'Brien *et al.,* 1993; Steels *et al.,* 1994), and radiation exposure (Mitchel and Morrison 1982).

Steels *et al.* (1994) investigated the relationship between yeast tolerance to heat and oxidative stress, and found that a mild heat shock induced tolerance to what would otherwise be lethal temperature and H_2O_2 stresses. Similarly, pre-treatment of yeast 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 greater thermotolerance (Plesset *et al.,* 1982; Coote *et al.*, 1991).

Genome-wide gene expression studies have provided a possible explanation for the phenomenon of cross protection. Comparative analyses of transcriptional responses in various stress conditions have identified very similar gene expression profiles in yeast during such stress (Gasch *et al.,* 2000; Causton *et al.,* 2001). These studies found that around 14% (900 genes) of the yeast genome was similarly altered in gene expression when responding to a stressful environment. The transcripts of around 600 genes are decreased in abundance with the products of such genes being involved in growth related process, mRNA metabolism and protein synthesis. The transcripts are increased for the other 300 genes and their products are mainly involved in protein folding and turnover, ROS detoxification, DNA damage repair, cell wall modification, energy metabolism and production of protective proteins and storage carbohydrates. The existence of cross protection led to the speculation that stress conditions require a general stress response mechanism involving cell functions such as cellular protection, energy metabolism and production of protective proteins (HSPs) or storage carbohydrates (eg trehalose). Production of trehalose and heat shock proteins (HSPs)

is one of the main responses associated with cross-protection (Soto *et al.,* 1999; Trott and Morano 2003).

Although cross protection recognises commonality in the yeast stress response, there is a level of exclusivity. For example, the acquisition of higher osmotic stress tolerance does not occur following a mild heat shock (Trollmo *et al.,* 1988; Varela *et al.,* 1992). Pre-treatment of yeast with a low concentration of H_2O_2 (0.1 mM, 60 min) induced higher protection against a formerly lethal H_2O_2 concentration, but did not evoke resistance to heat stress (Steels *et al.,* 1994). 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).

The fact that cross-stress protection is not universal suggests that while a portion of the stress response is common and may be shared (ie, leading to cross protection), there are also stress-specific responses, which must be related to the specific type of damage imposed on the cell by a particular stress as described above (Section 1.2.2). Studies on *S. cerevisiae* suggest that specific adaptive responses rely primarily on the increased synthesis of specialised stress proteins and/or organic solutes such as glycerol (Piper 1993).

1.2.5 Heat shock proteins as molecular chaperones

One of the most well studied and fundamental cell stress responses is activated by temperature stress, and is known as the heat shock response. This response is complex and can involve metabolic remodelling, growth termination and changes in gene transcription (Trott and Morano 2003). The heat shock gene expression program is exceptionally conserved and leads to the key end result of rapid synthesis of heat shock proteins (HSPs) (Lindquist and Craig 1988; Craig *et al.,* 1993; Hartl 1996). Heat Shock Protein genes are among the best characterised stress response genes (Mager and Moradas-Ferreira 1993).

HSPs are categorised into five groups based on their molecular mass and degree of amino acid homology, which includes families of small HSPs, Hsp60, Hsp70, Hsp90

and Hsp100. The small HSP (sHSP) family is grouped together by their low monomeric molecular masses (12-43 kDa) and contain proteins such as Hsp12, Hsp26 Hsp30, Hsp40 and Hsp48 (reviewed in Haslbeck 2002; Burnie *et al.,* 2006). Hsp60 proteins are ~60 kDa in molecular mass and hold a mitochondrial target sequence (Johnson *et al.,* 1989). Hsp70 family of chaperones are ~70 kDa in size and include Ssa1, Ssa2, Ssa3, Ssa4, Sse2, Hsp78 (Werner-Washburne *et al.,* 1987; Mukai *et al.,* 1993). There are two homologue proteins Hsc82 and Hsp82 in Hsp90 family which are ~90 kDa in molecular mass (Borkovich *et al.,* 1989). Hsp104 is a 102 kDa protein belonging to the Hsp100 kDa family (reviewed in Bosl *et al.,* 2006).

Some HSPs are known to protect cell proteins from denaturation during stress. Protein denaturation is a major outcome following stress and molecular chaperone activity is a key stress protection mechanism in yeast cells (Tanghe *et al.,* 2006). Many HSPs function as molecular chaperones, which particularly bind to unfolded or destabilised proteins, protecting them from degradation or aggregation (reviewed in Trott and Morano 2003). Exploring the molecular mechanisms of HSP activity has been the focus of a number of past studies. Defence activities of HSPs in stressed cells include protection of proteins from denaturation, transformation of inactivated proteins to make them functional and promotion of measures responsible for proteolysis of denatured proteins. HSPs activity is also associated with the production of enzymes for DNA replication, reactivation of RNA polymerase, proteolysis of denatured proteins, processing of proteins for export, enhanced flagellar synthesis and repair of damage to DNA by mutagenic agents (reviewed in Barton 2005).

Generally the heat shock response is governed by the heat shock transcription factor (HSF). The HSF binds to a five base pair sequence of heat shock element, nGAAn, in promoters of HSP genes, and activates transcription of these genes in response to heat shock (Chatterjee *et al.,* 2000; Grably *et al.,* 2002). In addition to gene expression initiation by HSF, a parallel pathway has been identified that responds not only to heat shock, but to a variety of other cellular and environmental stress conditions such as starvation, osmotic stress, oxidative stress, changing pH and exposure to ethanol (Carmelo and Sa-Correia 1997; Amoros and Estruch 2001; Quan *et al.,* 2004). This parallel pathway seems to be governed by Msn2/4 via STRE of HSP genes (Tregger

et al., 1998; Amoros and Estruch 2001). This gene expression program is known as the general stress response, and has previously been described in this chapter (Section 1.2.1).

Among the small HSP family, Hsp26 and Hsp42 have been shown to protect proteins from heat denaturation and irreversible aggregation (Gu *et al.,* 1997; Haslbeck *et al.,* 1999; Haslbeck *et al.,* 2004). Hsp26 and Hsp42 activity is found in cells exposed to stress conditions such as heat shock, salt shock, starvation and oxidative stress (Carmelo and Sa-Correia 1997; Amoros and Estruch 2001). These two chaperones suppress the aggregation of one-third of the cytosolic proteins and seem to be regulated in an identical manner by overlapping each other approximately 90% (Haslbeck *et al.,* 2004). For instance, Haslbeck *et al.* (2004) compared the amount of insoluble protein of a wild type and *hsp26*Δ or *hsp42*Δ strains by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). When analyzed after heat shock, both deletion strains displayed a significant increase in insoluble protein compared to the wild type. In the double deletion of both sHSPs, an even higher increase of insoluble protein was observed. Accordingly, both Hsp26 and Hsp42 seem to be necessary to suppress protein aggregation (Haslbeck *et al.,* 2004). In addition, Gu *et al.* (1997) demonstrated that yeast cells exposed to mild heat shock, transiently arrest growth and then resume cell division. Growth arrest is accompanied by transient disorganization of the cytoskeleton. When *hsp42*Δ cells were exposed to mild heat shock, the actin cytoskeleton was found to be more fragile than wild type control cells. These findings suggest that Hsp42 stabilises the cytoskeleton in yeast cells.

The heat-shock protein, Hsp60, is a mitochondrial chaperone that is required for the folding of specific proteins in mitochondria (Cheng *et al.,* 1989; Hallberg *et al.,* 1993; Cabiscol *et al.,* 2002; Kaufman *et al.,* 2003). Hallberg *et al.* (1993) observed Hsp60 to be expressed in yeast cells exposed to heat shock and that it is required for growth at high temperatures. Cabiscol *et al.* (2002), using yeast mutants that overexpressed Hsp60, found that increased Hsp60 levels closely correlated with the ability of the strain to resist oxidative stress.

Both Hsp70 and Hsp104 families have been shown to be involved in cell recovery from stress-induced damage (Parsell and Lindquist 1993; Piper *et al.,* 1997). The Hsp70 family is thought to be associated with the prevention of protein aggregation and refolding of damaged proteins by binding to and protecting newly translated proteins (Hartl 1996; Glover and Lindquist 1998). Furthermore, Hsp70s are engaged in disassembling aggregates of misfolded proteins, and translocating proteins to the mitochondria and ER (Deshaies *et al.,* 1998; Glover and Lindquist 1998; Nishikawa *et al.,* 2001). In addition, Hsp70s also facilitate proteolytic degradation of aberrant proteins to avert the activation of abnormal proteins in cells (reviewed in Bukau and Horwich 1998).

Hsp104 acts with Hsp70 family members and other co-chaperones in refolding and reactivating proteins aggregates (Parsell *et al.,* 1994a; Glover and Lindquist 1998; Abbas-Terki *et al.,* 2001; Lum *et al.,* 2004). A study by Parsell and Lindquist (1993), using a gene deletion strain of *HSP104,* confirmed the importance of this gene in thermotolerance. When wild-type and *hsp104Δ* yeast cells were pre-treated with a mild heat shock and subsequently exposed to a lethal heat shock (50°C), although both strains demonstrated tolerance, this tolerance was compromised in the *hsp104Δ* mutant since cell death occurred at a 1000-fold higher rate than that observed in the wild type (Parsell and Lindquist 1993). Furthermore, elevated thermotolerance has been observed in cells with constitutive *HSP104* expression (Sanchez *et al.,* 1992). Hsp104 is undetectable in unstressed cells but is strongly induced upon exposure to external stress (Parsell *et al.,* 1994b; Lindquist and Kim 1996).

Hsp90 is associated with refolding unfolded proteins back to native status and preventing folded proteins from unfolding and aggregating (reviewed in Picard 2002). In yeast, there are two closely related Hsp90 family proteins, Hsp82 and Hsc82. In cells, Hsp82 is more strongly induced by heat whereas Hsc82 is moderately induced by heat and constitutively expressed at a relatively high level (Borkovich *et al.,* 1989).

The availability of functional HSPs is important if yeast cells are to acquire tolerance against high ethanol concentrations (Plesset *et al.,* 1982; Sanchez *et al.,* 1992). For example, Plesset *et al.* (1982) investigated the effect of heat shock and ethanol stress

on the survival of *S. cerevisiae*. They found that pre-treatment of cells with ethanol or mild heat shock increased cell viability compared to non-pre-treated cells under heat shock. Possibly, in this case, pre-treatment with mild temperature or mild ethanol caused induction of HSPs and this resulted in the acquisition of thermotolerance. Quan *et al.* (2004) observed the accumulation of hsp70 family protein, Ssa4 in the nuclei when cells were treated with ethanol. Upon removal of ethanol stress, Ssa4 relocates to the cytoplasm. This phenomenon suggests that nuclear proteins may be vulnerable to damage by ethanol, and therefore require the nuclear accumulation of heat shock proteins to restore their function (Quan *et al.*, 2004).

An increased amount of global gene expression data of yeasts, grown under different stress conditions, is becoming publicly available. For example, gene array analysis of *S. cerevisiae* grown under various stress conditions has demonstrated the induction of a series of heat shock genes (Gasch *et al.*, 2000; Causton *et al.*, 2001; Yale and Bohnert 2001). In an industrial fermentation environment, particularly during wine fermentation, increased expression of *HSP*s *12, 26, 42, 78,* and *104* and the *HSP70* family member *SSE2* have been observed (Rossignol *et al.*, 2003). Such industrial conditions result in a variety of stress types including osmotic stress, nutrient limitation, pH change and ethanol accumulation. Since there are so many environmental variables and changing conditions over time, it is difficult to determine which stresses are particularly associated with the expression of the above classical heat shock genes. Global gene expression studies in *S. cerevisiae* during ethanol stress, initially by Alexandre *et al.* (2001) and more recently by Chandler *et al.* (2004), found increased expression levels of *HSPs 12*, *26*, *30*, *42*, *70, 78*, *82* and *104* during ethanol stress. The eminent expression of *HSPs* is apparently a common response to most environmental changes.

1.2.6 Trehalose and stress tolerance

1.2.6.1 Overview

Trehalose is a non-reducing disaccharide which is formed by two glucose moieties linked by a α-(1,1) linkage (Figure 1.3) (Zimmermann and Entian 1997; Hohmann

2002). Trehalose is commonly found in many organisms, including yeast, fungi, bacteria, a variety of plants, insects and invertebrates, in which it accumulates significantly during adverse environmental conditions (Clegg 1965; Gaff 1971; Thevelein 1984; Singer and Lindquist 1998; Zentella *et al.*, 1999; Chen *et al.*, 2002; Schluepmann *et al.*, 2004). Principally trehalose serves as a reserve or storage carbohydrate which is considered as a source of energy in times of carbohydrate starvation (Elbein 1974; Lillie and Pringle 1980; Thevelein 1984; Wiemken 1990). It is however also recognised as a cellular protectant involved in stress tolerance (Gadd *et al.*, 1987; Van Laere 1989; Wiemken 1990; Kim *et al.*, 1996; Parrou *et al.*, 1997; Hounsa *et al.*, 1998; Parrou *et al.*, 1999; Rep *et al.*, 2000; Herdeiro *et al.*, 2006).

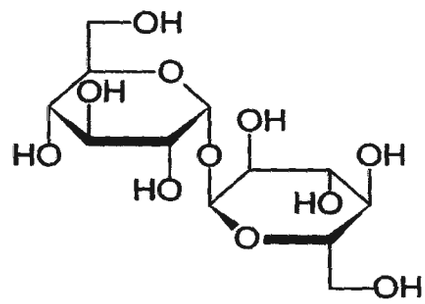


Figure 1.3: Trehalose is a disaccharide consisting of two glucose moeties linked by an α-(1,1) linkage.

Large amounts of trehalose accumulate in *S. cerevisiae* cells during periods of adverse growth conditions such as high temperature (Hottiger *et al.*, 1987; De Virgilio *et al.*, 1994; Eleutherio *et al.*, 1995; Lewis *et al.*, 1995), freezing (Kim *et al.*, 1996), dehydration and desiccation (Gadd *et al.*, 1987; Hottiger *et al.*, 1987; D'Amore *et al.*, 1991; Eleutherio *et al.*, 1993), starvation (Lillie and Pringle 1980), hyperosmotic shock (Hounsa *et al.*, 1998) oxidative stress (Benaroudj *et al.*, 2001; Pereira *et al.*, 2003; Herdeiro *et al.*, 2006) and ethanol stress (Attfield 1987; Mansure *et al.*, 1994; Kim *et al.*, 1996; Mansure *et al.*, 1997; Soto *et al.*, 1999). Trehalose also accumulates when cells are exposed to copper sulfate or hydrogen peroxide and declines rapidly after the stress is removed (Attfield 1987). Trehalose seems to have all the features of a typical storage carbohydrate yet it has been suggested to play a role as a stress protectant, even though the precise role of trehalose is still unclear. Depending on the

environmental conditions, trehalose can represent from less than 1% to more than 25 % of the dry weight of the cell (reviewed in Hohmann 2002). These dramatic and rapid variations in the level of trehalose in response to a variety of environmental changes may reflect a complex regulatory pattern governing the metabolism of this carbohydrate.

1.2.6.2 Trehalose metabolism in yeast

In yeast growing on glucose, trehalose begins to accumulate during diauxic shift and peaks in stationary phase. During stationary phase, as a result of eventual nutritional (glucose) shortage, trehalose is slowly degraded (Lillie and Pringle 1980; Werner-Washburne *et al.,* 1996). Production and degradation of trehalose are regulated by several overlapping mechanisms controlled by enzyme activity and the availability of substrates. In yeast, trehalose is synthesised by a large enzyme complex comprising two enzymes associated with trehalose biosynthesis: trehalose-6-phosphate synthase (Tps1) (Bell *et al.,* 1992) and trehalose-6-phosphate phosphatase (Tps2) (De Virgilio *et al.,* 1993). Tsl1 and Tps3 are probably alternative, regulatory, or stabilizing subunits of the complex (Bell *et al.,* 1998). Trehalose is synthesised from UDP-glucose and glucose-6-phosphate. At first, UDP-glucose and glucose-6-phosphate are converted to trehalose-6-phosphate by trehalose-6-phosphate synthase (Tps1) and then to trehalose by trehalose-6-phosphate phosphatase (Tps2) (Figure 1.4) (Thevelein and Hohmann 1995; Ferreira *et al.,* 1996).

In *S. cerevisiae*, there are several genes that encode subunits of the trehalose synthesis complex, and their expression is induced by stress. These genes have been cloned and further studied. *TPS1* encodes the smallest (56 kDa) subunit responsible for trehalose-6-phosphate synthase (Tps1) activity (Bell *et al.,* 1992), and *TPS2* encodes 100 kDa subunit of trehalose-6-phosphate phosphatase (Tps2). *TSL1* and *TPS3* encode regulatory subunits which are homologues with high sequence similarity (Bell *et al.,* 1998; Hounsa *et al.,* 1998).

During the process of degradation, trehalose is hydrolysed by trehalase into two glucose molecules. Yeast has two trehalase enzymes. The cytosolic neutral trehalase

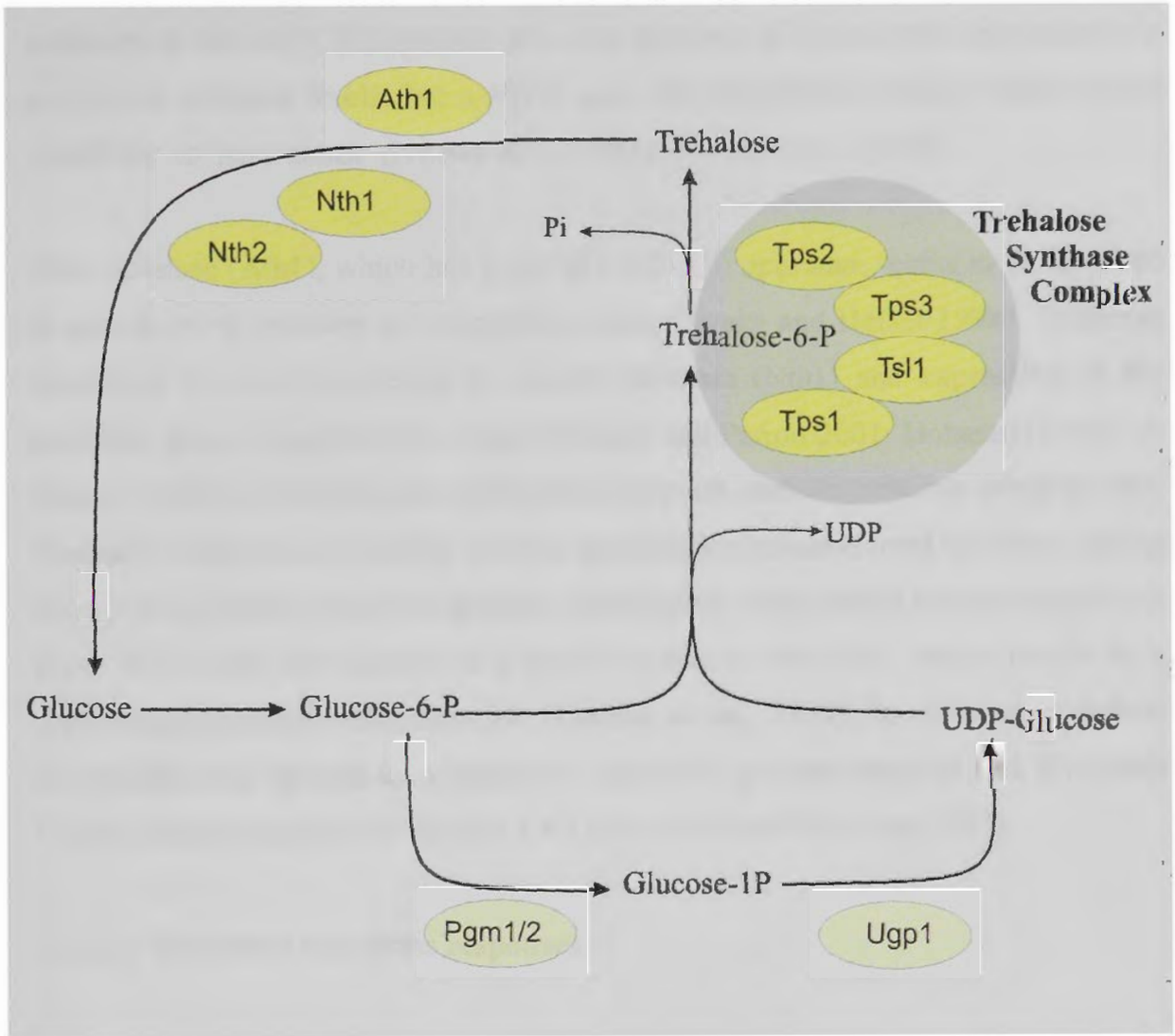


Figure 1.4: Trehalose metabolic pathway in yeast *Saccharomyces cerevisiae*. Trehalose biosynthesis is catalysed by the trehalose synthase complex consisting of four subunits. The trehalose-6-posphate synthase subunit (Tps1) produces trehalose-6-P from UDP-glucose and Glucose-6-P, which is dephosphorylated in trehalose by the trehalose-6-P phosphatase subunit (Tps2). Tps3 and Tsl1 are two regulatory subunits that stabilise the complex. Trehalose is degraded by the neutral (Nth1) or the Acid (Ath1) trehalases. The role of Nth2 in this degradation process has not yet been clarified.

(Nth1), with optimum activity at pH 6.8-7.0, is responsible for the breakdown of the trehalose accumulated in cells (Thevelein *et al.,* 1984). The second enzyme is the neutral trehalase subunit Nth2 encoded by *NTH2*. Nth2 shows 77% sequence similarity to the Nth1. This protein does not have any effect on trehalose hydrolysis activity or trehalose levels, but a *NTH2* gene deletion (*nth2*Δ) strain demonstrated sensitivity to heat shock (Nwaka *et al.,* 1995a; Nwaka *et al.,* 1995b).

Acid trehalase (Ath1), which has a low pH (4.5-5.0) optimum, seems to be involved in growth using trehalose as the carbon source (Nwaka and Holzer 1998). Trehalose hydrolysis is mainly achieved by neutral trehalase (Nth1) and expression of the encoding gene is regulated by stress (Francois and Parrou 2001; Hohmann 2002). A balance of the synthesising and hydrolysing enzymes controls trehalose levels in cells. Neutral trehalase is most active, and the intracellular trehalose level is lowest, during exponential growth of yeast on glucose. On the other hand, neutral trehalase activity is lower when cells are grown on a non-fermentative substrate, which results in a concomitant trehalose accumulation (Hounsa *et al.,* 1998). In addition, trehalose metabolism may operate as a regulatory system in glucose transport and glycolysis which is further explored in Section 1.4.2 (Thevelein and Hohmann 1995).

1.2.6.3 Trehalose and stress responses

Over the past twenty-five years a number of studies have demonstrated a correlation between the intracellular trehalose levels of yeast and its ability to survive in a range of stress conditions (reviewed in Van Laere 1989; Wiemken 1990; Bonini *et al.,* 2004; Aguilera *et al.,* 2006; Crowe *et al.,* 2001). Hounsa *et al.* (1998) demonstrated a relationship between intracellular trehalose levels and resistance to osmotic stress. In particular, the yeast mutant strains *tps1*Δ *tps2*Δ and *tps1*Δ *hxk2*Δ, which are unable to produce trehalose, were more sensitive to severe osmotic stress than the wild type strain, identifying the importance of trehalose in osmotic stress survival (Hounsa *et al.,* 1998). The same study suggested that trehalose does not act as a reserve compound for glycerol synthesis under osmotic stress, suggesting a role other than restoration of cell turgor.

The positive effect of high intracellular trehalose levels has been observed in dehydration resistance of yeast and in re-pitched brewing yeast, where it is known to improve cell viability and increase carbohydrate utilization during the initial stages of fermentation (Guldfeldt and Arneborg 1998). Similarly, a decline in trehalose content has been correlated with a loss of stress resistance (Van Dijck *et al.,* 1995). Soto *et al.* (1999) showed that mutant *Schizosaccharomyces pombe* strains, which were unable to synthesise trehalose, were sensitive to temperature, freeze/thawing, dehydration, sodium chloride, and ethanol stresses. These authors speculated that trehalose is a key determinant in general stress tolerance. Furthermore all genes involved in trehalose metabolism contain one or more copies of STRE in their promoter regions (Parrou *et al.,* 1997) and are induced by general transcription factors Msn2/4 in response to a range of stresses (Zahringer *et al.,* 2000). Post transcriptional regulation of the enzymes in trehalose metabolism is co-induced by internal trehalose-6-phosphate and trehalose levels (Parrou *et al.,* 1997; Zahringer *et al.,* 1998).

Other researchers however have presented evidence suggesting that trehalose may not have a protective role in stress tolerance. Phenotypes of mutants lacking the Nth1, which is responsible for trehalose degradation, accumulate high levels of trehalose, yet their ability to survive extreme heat is reduced (Nwaka *et al.,* 1995a; Nwaka *et al.,* 1995b). This work led to doubts about the role of trehalose in protecting cells from heat stress (reviewed in Nwaka and Holzer 1998). It should be noted however that the *Nth1*Δ potentially could have accumulated excessive intracellular trehalose levels and, as argued by Singer and Lindquist (1998), this may have interfered with other cellular functions including the activities of chaperones that are important for stress tolerance.

It has been suggested that the rapid early accumulation of trehalose, when cells are heat stressed, is needed only for a short time to stabilize proteins in their native state followed by rapid degradation of trehalose, necessary for full recovery from heat stress. The role of trehalose as a protein stabiliser, plasma membrane protector and for protection of yeast cells under stressful conditions is well documented (De Virgilio *et al.,* 1990; Panek and Panek 1990; Omdumeru *et al.,* 1993; Hottiger *et al.,* 1994; Parrou *et al.,* 1997; Guldfeldt and Arneborg 1998; Parrou *et al.,* 1999).

1.2.6.4 Function of trehalose as a stress protectant

The precise role of trehalose in stress tolerance is not entirely clear, although it has been suggested that two unique properties make the disaccharide constructive in protecting cells from environmental insults. The first property is the ability of trehalose to preserve the integrity of cellular membrane during stress (Crowe *et al.*, 1984). The mechanism of this process is known as 'the water replacement hypothesis' which proposes that trehalose can replace water molecules by forming hydrogen bonds with the surface of macromolecules. Using this property trehalose can stabilise the structure of cellular membranes and proteins (Crowe *et al.*, 1984; Crowe *et al.*, 1998; Crowe *et al.*, 2001). High intracellular trehalose levels in yeast improve cell viability against desiccation, even though nuclear magnetic resonance (NMR) experiments on dried yeast demonstrated that only small amounts of trehalose (2-3% of dry mass) is adequate to effect the water replacement hypothesis (Sano *et al.*, 1999). The water replacement hypothesis suggests that trehalose concentrates near the surface of membranes and proteins, protecting them from inactivation and denaturation caused by variety of stress conditions.

The second property is the ability of trehalose to stabilise proteins in their native state. This mechanism is described by 'the vitrification hypothesis' which suggests that trehalose has the ability to form an amorphous glass structure around proteins and membranes, thus reducing structural fluctuations, protein aggregation and free radical diffusion (Sun and Leopold 1997; Crowe *et al.*, 1998; Crowe *et al.*, 2001) under drying conditions. The formation of a glass capsule around the protein protects its native shape and prevents deformation of its structure. Hottiger *et al.* (1994) investigated the thermal stability of purified glucose-6-phosphate dehydrogenase (Glc6PDH) *in vitro*. Trehalose was added at a concentration of 0.5 M to a range of solutions containing glucose-6-P dehydrogenase. These were heat-shocked at a range of temperatures from 40°C to 60°C for eight minutes. After cooling, enzyme activity was measured and compared to glucose-6-P dehydrogenase activities in controls that were treated in the same way but without added trehalose. After a 55°C heat shock, the activity of trehalose-treated enzyme preparations was 60% greater than the controls (Hottiger *et al.*, 1994). This suggests that trehalose increases the thermal

stability of proteins *in vitro*. In addition using two temperature sensitive proteins, Singer and Lindquist (1998) demonstrated that proteins are better able to retain their structure during heat shock in cells that produce trehalose. Importantly, this study suggests that trehalose acts to prevent heat shock-induced protein aggregation until reactivation by a protein chaperone (Singer and Lindquist 1998).

In conclusion, accumulation of trehalose is triggered by various environmental stress conditions. Several studies have demonstrated a close correlation between trehalose levels and tolerance to environmental insults, suggesting that trehalose may act as a cell protectant during stress conditions.

1.3 Ethanol stress in *S. cerevisiae*

1.3.1 Overview

Ethanol produced during fermentation accumulates in the environment of the yeast cell and constitutes a major stress source that negatively impacts on cellular functions. The sensitivity of yeast to ethanol compromises fermentation productivity and yield (Walker 1998). Ethanol stress affects many cell functions including inhibition of metabolism, hindered growth, nutrient intake, plasma membrane ATPase activity and an increased frequency of petite mutations (Mishra 1993; Walker 1998). It is believed that ethanol in high concentrations is disruptive to yeast cell membrane structure resulting in an associated loss of membrane potential. A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzyme activity and these proteins may be denatured (Hallsworth *et al.,* 1998).

Inhibition of yeast growth is one of the major adverse effects caused by increased ethanol concentrations. While low ethanol concentrations are inhibitory to yeast growth and cell division, higher ethanol concentrations result in a reduction in cell viability and increased cell death (Birch and Walker 2000; Marza *et al.,* 2002). Canetta *et al.* (2006) demonstrated the detrimental effect of ethanol toxicity on the physiology of budding yeast, *S. cerevisiae* and fission yeast, *Sc. pombe*. The viability of unstressed control cultures of both strains remained very high (100%) over a period

of one hour. Exposing cells to 10% (v/v) ethanol for a one-hour period resulted in a 92% decrease in cell viability. Exposing both strains to 30% (v/v) ethanol for 10 minutes resulted in a rapid decline in viability to approximately 10%, and after one hour there were no remaining viable strains, confirming the toxicity of high ethanol concentrations (Canetta *et al.*, 2006).

1.3.2 The yeast plasma membrane and ethanol toxicity

The multi-faced adverse effect of ethanol on cellular membranes has been the subject of much debate and speculation in yeast stress response studies. The principal sites of impact of ethanol in yeast cells include the plasma membrane, mitochondrial membrane, nuclear membrane, vacuolar membrane, endoplasmic reticulum (ER) and cytosolic hydrophilic proteins (Walker 1998). It has been recognised that ethanol impacts mainly on structural integrity of membranes and permeability (Rose 1993; Piper 1995; Bisson and Block 2002).

Cell membranes are highly permeable to ethanol. Ethanol has the ability to permeate the cell through the plasma membrane without mediation by specific transporters (Guijarro and lagunas 1984). The presence of ethanol around and in the phospholipid bilayer weakens the water-lattice structure of the membrane and decreases the strength of interactions between fatty acids, and this is thought to promote cell leakage and decrease the integrity of the membrane (Sajbidor *et al.*, 1992). In particular, ethanol molecules are able to penetrate the cytosolic membrane in around 200 ns (Patra *et al.*, 2006). Increased membrane permeability also alters the electrostatic potential, which may affect cell signaling.

Ethanol increases the relative polarity of the hydrophobic regions of the membrane. It thereby increases the membrane's ability to solubilise other polar molecules (Ingram 1986) and perturbs the function of transport proteins in the membrane. This results in decreased nutrient uptake by the cells (Van Uden 1985; Pascual *et al.*, 1988) and increased influx of protons, causing disruption of the proton-motive force (Walker 1998; Ogawa *et al.*, 2000) and intracellular acidification (Alexandre *et al.*, 1998; Walker 1998).

Ethanol-induced passive proton influx is thought to be the trigger for an increase in activity of the plasma membrane H^+-ATPase (Cartwright *et al.,* 1987), the enzyme largely responsible for maintenance of the plasma membrane proton gradient (Serrano 1991). Thus it might be expected that the cell would be able to quickly recover its proton gradient following ethanol stress, but this appears not to happen. This may be connected to Hsp30 synthesis which is also induced during ethanol stress (Alexandre *et al.,* 2001; Chandler *et al.,* 2004); this protein is an inhibitor of the plasma membrane H^+-ATPase (Piper *et al.,* 1997). To date, however, there has been no experimental work to test this in ethanol-stressed cells.

1.3.3 The effect of ethanol on yeast membrane fluidity and transport

It is believed that the major detrimental effect of ethanol on the yeast cell is the increase in plasma membrane leakiness that leads to disruption of cell membrane function. Jones and Greenfield (1987) used passive influx of undissociated acetic acid as an indicator of membrane permeability to observe the effect of ethanol on membrane leakiness for yeast grown in batch and continuous cultures. It was found that increases in ethanol concentration led to increased membrane permeability.

It has also been found, that a significant increase in membrane fluidity of yeast cells occurs upon exposure to stressful levels of ethanol (Lloyd *et al.,* 1993; Piper 1995; Swan and Watson 1997; Marza *et al.,* 2002). Increased membrane fluidity may result in a decrease in structural integrity of cell membrane due to disruption of its permeability barrier (Piper 1995; Marza *et al.,* 2002). These observations were recently confirmed by Canetta *et al.* (2006). Using an atomic force microscope (AFM), the authors investigated the effect of ethanol toxicity on cell surface morphology of *S. cerevisiae* and *Sc. pombe*. Yeasts were subjected to various ethanol concentrations for 10 minute and 1 hour periods, and the surface morphological changes (roughness and shape) were assessed by section analysis of the height images. Exposing both strains to 10% (v/v) ethanol for up to 10 minutes did not change their morphology compared to unstressed cells. After 1 hour exposure to 10% (v/v) ethanol some changes in surface topography and shapes of yeast cells were clearly seen (Figure 1.5). Shapes became irregular and cell shrinkage occurred.

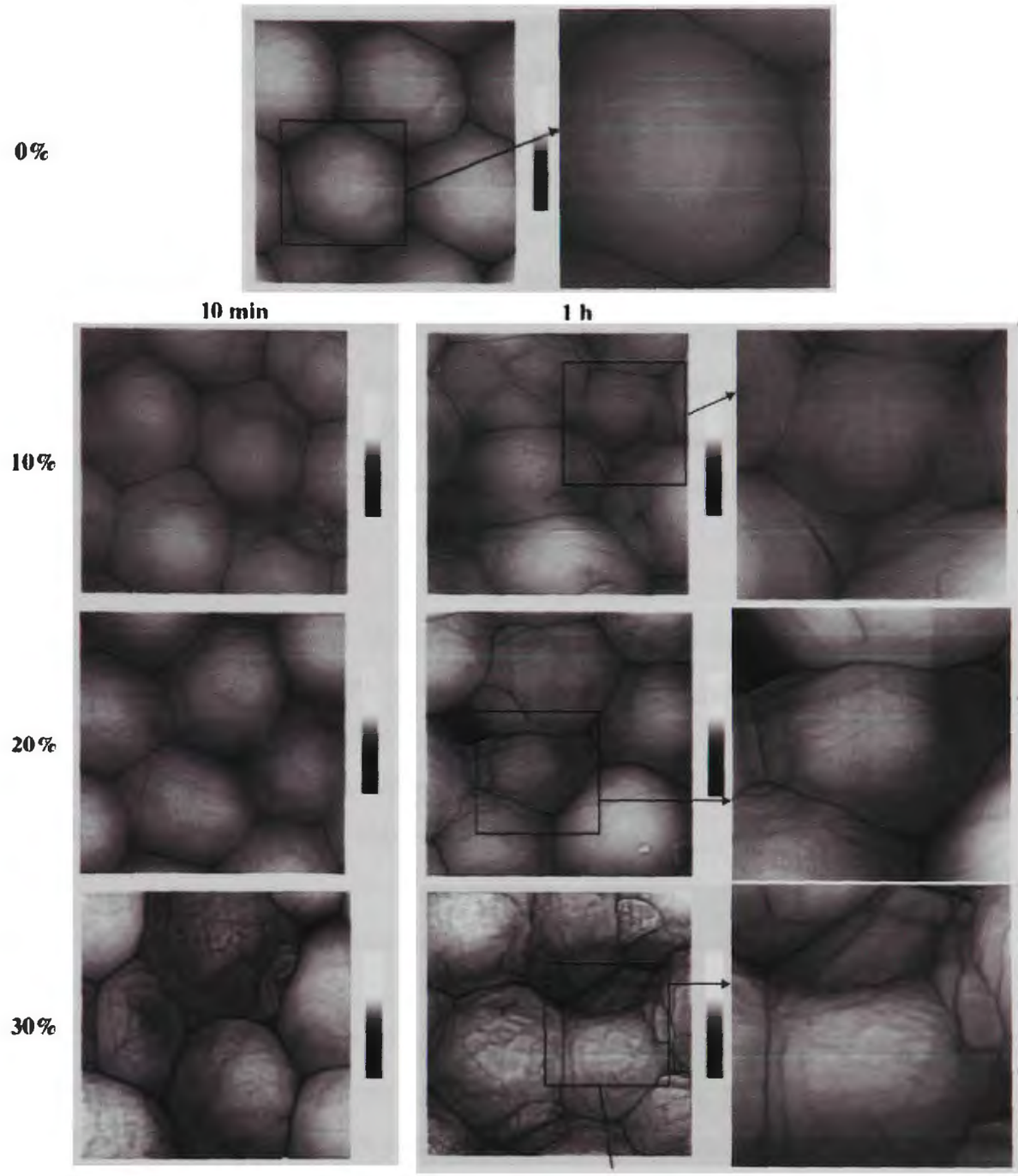


Figure 1.5: Atomic force microscopic images of *Saccharomyces cerevisiae* exposed to 10%, 20%, and 30% (v/v) ethanol concentrations for up to 10 min and 1 h.; Yeast cells not subjected to any ethanol concentration (0%) were used as a control. The high-resolution zoomed images of the zones marked in squares are shown by arrows. Reprinted by permission from Wiley-Blackwell Publishing: FEMS Microbiology Letters (Canetta *et al.*, 2006), copyright (2006).

However, more obvious morphological changes occurred when cells were exposed to 20% (v/v) ethanol concentrations for 1 hour. The ethanol-treated cells were shrunken and rougher than both the unstressed cells and those subjected to low ethanol concentrations. A massive change in both the shape and morphology of cells was observed when the strains were exposed to 30% (v/v) ethanol for 1 h, the cells had almost collapsed. The cell surface roughness increased quickly, while the cell volume rapidly decreased. These findings showed that the effects of ethanol stress on the morphology and physiology of the yeasts were more pronounced for *Sc. Pombe* than *S. cerevisiae* (Canetta *et al.*, 2006). This study confirmed that higher ethanol concentrations increase cell membrane fluidity, eventually leading to disruption of the cell membrane.

The negative impact of ethanol on yeast function extends to the inhibition of various transport systems in the yeast cell such as glucose uptake (Leao and van Uden 1982), maltose transport (Loureiro-Dias and Peinado 1982), and general amino acid permease activity (Leao and van Uden 1984), possibly due to the impact of ethanol on cytoplasmic membrane fluidity, subsequently affecting transport protein activity.

1.4 The response of *S. cerevisiae* to ethanol stress

As described previously in this chapter, ethanol causes a range of inhibitory and disruptive effects to the biochemistry and physiology of yeast. The primary responses of yeast to ethanol stress include changes in plasma membrane composition (ie. an increase in the degree of unsaturation of membrane fatty acids), ergosterol levels in cell membranes, and increased phospholipid biosynthesis, plasma membrane H+ATPase production, and trehalose production. The response of yeast to ethanol assaults, and in particular how they acclimatise to this stress, are discussed in the following sections.

1.4.1 Changes in yeast plasma membrane composition

Changes to the plasma membrane due to ethanol stress have been outlined earlier (Sections 1.3.2 and 1.3.3). Yeast cells have a protective response to these changes

caused by ethanol stress. Overall, resistance to yeast cell membrane disruptions caused by ethanol appears to be mediated by an increase in the proportion of ergosterol, unsaturated fatty acids and phospholipids (Alexandre *et al.,* 1994; Chi and Arneborg 1999; You *et al.,* 2003). In particular, alterations in plasma membrane lipid composition in response to ethanol stress are thought to represent a principal adaptive mechanism. During growth in ethanol, *S. cerevisiae* elevates the level of lipids enriched in mono-unsaturated fatty acids such as $C_{18:1}$ residues (oleic acid) to compensate for a decrease in saturated fatty acids as $C_{16:0}$ residues (palmitic acid) (Thomas and Rose 1979; Beavan *et al.,* 1982; Ghareib *et al.,* 1988; Swan and Watson 1999; You *et al.,* 2003; Aguilera *et al.,* 2006). Sajbidor and Grego (1992) observed an increase in the proportion of $C_{18:1}$ residues relative to $C_{16:1}$ residues in *S. cerevisiae* strain CCY supplemented with up to 15% (v/v) ethanol. This appears to be due largely to a decline in the level of $C_{16:1}$ residues in all phospholipids tested thus there may have been no net synthesis of oleic acid ($C_{18:1}$) but a remodeling of the $C_{16:1}$ residues (Sajbidor and Grego 1992). These findings suggest that ethanol exposure results in an increase in the proportion of longer unsaturated fatty acids.

A number of studies have shown that the most ethanol resistant yeast cells have membranes enriched with mono-unsaturated fatty acids such as oleic acid ($C_{18:1}$) (Swan and Watson 1999; You *et al.,* 2003; Aguilera *et al.,* 2006), followed in order by cells enriched with linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids (Swan and Watson 1999). These changes in membrane lipids are believed to improve ethanol tolerance by lowering membrane leakage. You *et al.* (2003) examined the effect of different unsaturated fatty acid compositions of *S. cerevisiae* on the growth-inhibiting effect 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 knockout (*ole1*Δ) and by supplementing the growth medium of the same strain with synthetic monosaturated 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 effect of ethanol in growing yeast cells (You *et al.,* 2003). Recently, Aguilera *et al.* (2006) established a relationship between ethanol tolerance and the lipid composition of the plasma membrane in five different wine yeast strains that were sampled at various stages of

alcoholic fermentation. Based on their results, the strains that exhibited the highest amount of mono-unsaturated fatty acids and ergosterol in the plasma membrane were also the most ethanol tolerant (Aguilera *et al.,* 2006).

In addition to ethanol-induced changes in fatty acid profile in the plasma membrane, changes in membrane sterol profile appear to play a prominent role in yeast ethanol tolerance. In particular, overall sterol profile is modulated in favour of ergosterol accumulation (Thomas *et al.,* 1978; Walker-Caprioglio *et al.,* 1990; del Castillo 1992; Alexandre *et al.,* 1994; Aguilera *et al.,* 2006). Walker-Caprioglio *et al.*, (1990) observed that yeast cells increased their ergosterol composition from 41% (in the absence of ethanol) to 80% of total sterol content when grown in the presence of 6% (v/v) ethanol, but the total sterol content decreased. Similarly, de Castillo (1992) demonstrated that yeast strains with the highest ergosterol contents are the most ethanol resistant. Evidence suggests that a higher ergosterol/phospholipid ratio in cell membranes increases yeast ethanol tolerance, protecting the cell membrane against the damaging effects of ethanol (Alexandre *et al.,* 1994).

Chi and Arnebory (1999) compared the lipid composition of membranes from yeast strains with differing levels of ethanol-tolerance. The more ethanol-tolerant strains had a higher ergosterol/phospholipid ratio, a higher level of phosphatidylcholine, a lower level of phosphatidylethanolamine, a higher incorporation of long-chain fatty acids and a slightly higher level of unsaturated fatty acids relative to total phospholipid composition (Chi and Arneborg 1999). Recently Aguilera *et al.*, (2006) demonstrated that increased concentrations of ergosterol, long chain fatty acids and unsaturated fatty acids correlated with increased ethanol tolerance, however, the mechanisms by which ergosterol and long chain fatty acids affect ethanol tolerance of yeast cells was not discussed (Aguilera *et al.,* 2006).

In yeast grown anaerobically, the levels of sterols in the membrane are substantially reduced with a concomitant increase of their squalene precursor, as compared to cells grown under aerobic conditions, since under anaerobic conditions yeast cells are unable to synthesize unsaturated fatty acids due to the oxygen requirement of the yeast desaturase enzyme (Paltauf *et al.,* 1992; Walker 1998). Similarly many of the

enzymes involved in sterol synthesis, in particular the conversion of squalene to ergosterol, require oxygen (Paltauf *et al.,* 1992). Therefore, under anaerobic conditions unsaturated lipids must be imported into the cell from the growth medium. Thomas *et al.* (1978) used lipid- and ergosterol-supplemented media to enrich the plasma membrane of anaerobically grown *S. cerevisiae.* In this study, they found that lipid-supplemented cultures had increased tolerance to ethanol when the supplementation included a greater level of unsaturated fatty acids at the expense of saturated fatty acids. Similarly, cultures enriched with ergosterol were significantly more resistant to the toxic effects of ethanol than cells enriched with other sterols (Thomas *et al.,* 1978). Furthermore, in anaerobic cultures the membrane lipid composition of *S. cerevisiae* was modulated towards a higher level of $C_{18:1}$ fatty acid residues with a corresponding decrease in palmitic acid residues ($C_{16:0}$) upon exposure to ethanol, similar to aerobic conditions (Alexandre *et al.,* 1993). Overall membrane sterol profile was also modulated towards greater unsaturation in favour of ergosterol accumulation.

In summary, the exposure of yeast cells to ethanol induces changes in membrane composition that presumably increases membrane integrity. An increase in fatty acid chain length increases the hydrophobicity of the membrane bilayer and this may prevent ethanol accumulation within the membrane and/or help to restore transmembrane gradients and conserve the function of transmembrane proteins.

1.4.2 Trehalose and ethanol tolerance

The role of trehalose in the yeast stress response and as a stress protectant for various stressors was discussed earlier (Section 1.2.6). Although trehalose has been shown to have a role in many types of stress, its role in ethanol stress tolerance is more controversial. There is some evidence to suggest that trehalose has a role in ethanol tolerance, but to date much of this evidence is circumstantial. For example, Mansure *et al.* (1997) showed that trehalose concentrations during fermentation correlate well with ethanol tolerance. In this study, yeast strains (Labatt 625, Montrachet) that accumulated higher levels of intracellular trehalose during ethanol fermentation also demonstrated better survival rates during ethanol stress compared to yeast strains

(S288C) with lower levels of trehalose accumulation. The same study also found an increase in trehalose-6-phosphate synthase activity during ethanol production suggesting a possible increase in trehalose levels. These findings indicate a correlation between trehalose accumulation and enhanced ethanol stress tolerance. Furthermore, the authors found the process of ethanol-induced leakage of electrolytes from cells appeared to be reversed in the presence of trehalose. It was concluded that trehalose inhibits ethanol-induced leakage of electrolytes from intact cells and from liposomes, suggesting that the compound promotes ethanol tolerance (Mansure *et al.,* 1994; Mansure *et al.,* 1997). Similarly, the work of Kim *et al.* (1996) demonstrated that increased ethanol tolerance is correlated with elevated cellular trehalose content. A mutant strain defective in the vacuolar acid trehalase (Ath1; an enzyme that hydrolyses intracellular trehalose to glucose units) had an increased survival rate relative to the wild type strain when exposed to 18% (v/v) ethanol (Kim *et al.,* 1996). Jung and Park (2005) using a recombinant plasmid that resulted in a low activity of acid trehalase in the recombinant yeast found that the strain had around 1.5-fold better survival compared with the control strain upon exposure to 8% (v/v) ethanol.

In contrast to the above, however, Alexandre *et al.* (1998) showed that trehalose accumulates in yeast cells on exposure to ethanol, but it is not correlated with cell survival under ethanol stress. In this study the viability of a wild-type strain and *pma1-1* mutant strain (the *pma1-1* mutant accumulated more trehalose than the wild-type) was estimated after 60 minutes incubation under 12.5% v/v ethanol stress. For both strains, there was no clear correlation between trehalose content and cell survival under ethanol stress (Alexandre *et al.,* 1998). This study suggests that trehalose may not have a protective role in ethanol stress, or that it has little influence on cell survival. Lewis *et al.* (1995), using 14 different strains of *S. cerevisiae,* studied the relationship of trehalose content to cellular tolerance to heat, H_2O_2, freezing, salt, acetic acid and ethanol stress. Trehalose content did not correlate with tolerance to any of the above stresses except acetic acid. This suggests that the contribution of trehalose to stress tolerance is either small or inconsistent, and that the ability of a yeast strain to accumulate trehalose may not be a reliable indicator of stress tolerance (Lewis *et al.,* 1995). The conflicting conclusions generated from all these studies on the role of trehalose in cellular ethanol tolerance indicate that further studies are

needed to determine whether trehalose does contribute to ethanol tolerance in yeast.

Global gene expression studies on ethanol stress by Alexandre *et al.* (2001) and later by Chandler *et al.* (2004) showed that several trehalose biosynthesis genes (*TPS1,TPS2* and *TSL1*) in *S. cerevisiae* are expressed in response to ethanol stress (Alexandre *et al.*, 2001; Chandler *et al.*, 2004). The expression of all genes associated with trehalose biosynthesis may lead to increased trehalose production which may be associated with its role as a stress protectant and the need for elevated levels of this compound during ethanol stress. However the findings of these studies still have some controversy. Alexandre *et al.* (2001) demonstrated that all trehalose synthesis genes as well as hydrolysis (trehalase) were upregulated suggesting trehalose accumulation is not important for ethanol tolerance. Chandler *et al.* (2004) found that only trehalose synthesis genes were upregulated and trehalase hydrolysis genes remained unaffected leading to intracellular trehalose accumulation. Based on their observation, Chandler *et al.* (2004) suggested that trehalose accumulation is important for the stress response. These studies add to controversy over the role of trehalose and its metabolism in ethanol stress.

Contrary to the above, the expression of nearly all genes involved in trehalose and glycogen metabolism (*PGM2, UGP1, TPS1, TPS2, TSL1* and *GYS2*) appears to be inconsistent with the cellular need for greater carbon input into the glycolytic pathway during ethanol stress, since the trehalose and glycogen pathways draw carbon away from energy-yielding processes (Alexandre *et al.*, 2001; Chandler *et al.*, 2004). On the other hand, this may serve a practical function since it is apparent that ethanol stress slows the glycolytic rate causing an accumulation of phosphorylated glycolytic intermediates. In order to maintain cellular function it may be necessary for the cell to temporarily reduce the amount of phosphorylated glucose intermediates (which in high concentrations can be inhibitory to cell function) via a futile, ATP-consuming cycle via trehalose or glycogen metabolism. Considering the possible involvement of trehalose metabolism in maintaining cellular levels of phosphorylated glycolytic intermediates during ethanol stress, it is possible that the trehalose metabolic cycle (Figure 1.4) plays an important role in yeast survival during stress, rather than, or in addition to, the trehalose molecule.

Trehalose-6 phosphate is an intermediate molecule of the trehalose pathway that may reduce sugar influx into glycolysis by inhibiting the hexokinases; the glucose phosphorylating enzymes (Blazquez *et al.,* 1993; Neves *et al.,* 1995). Disruption of the trehalose-6-phosphate synthase (Tps1) in trehalose synthesis complex prevents trehalose-6-phosphate synthesis. The *TPS1* gene encodes the small subunit of the trehalose-6-phosphate synthase in trehalose synthesis complex (Bell *et al.,* 1992) and disruption of this gene blocks the initial step of trehalose synthesis, ie. trehalose-6-phosphate synthesis. Therefore, *tps1*Δ strains have a growth defect on fermentable carbon sources including glucose (Bell *et al.,* 1992; González *et al.,* 1992; Neves *et al.,* 1995) as a result of aberrant glycolytic flux, suggesting that Tps1 and/or trehalose synthesis metabolism may play a significant role in modulating glycolysis (Thevelein and Hohmann 1995). Under ethanol stress conditions the trehalose pathway is activated leading to the synthesis of trehalose-6 phosphate, which inhibits hexokinases and sugar influx into glucolysis. Furthermore, the subsequent production of trehalose from trehalose-6 phosphate decreases the intracellular accumulation of phosphorylated glycolytic intermediates, reducing their inhibitory effects on cell metabolism and increasing intracellular levels of free phosphate, which may be needed for ATP production. Therefore, it may be that during ethanol stress, the trehalose metabolism, rather than the trehalose molecule, is important for maintaining cell homeostasis and therefore cell survival. More work is needed to better understand whether the trehalose metabolism or molecule, or both, have a role in the yeast ethanol stress response.

1.4.3 Ethanol specific responses

It is generally recognised that the primary target of ethanol in cells, is the plasma membrane, leading to impaired energy-generating systems, organisational integrity and fermentation performance (Jones and Greenfield 1987; Mansure *et al.,* 1997; Alexandre *et al.,* 2001). In particular, increased membrane permeability leads to increased proton influx and the dissipation of the electrochemical gradients across the plasma membrane (Alexandre *et al.,* 2001).

There are numerous cellular changes in yeast that occur in response to the adverse affects of ethanol. For example, yeast increase the synthesis rate of HSPs, accumulate trehalose and undertake changes in plasma membrane composition to compensate for the destabilising effect of ethanol (Plesset *et al.,* 1982; Attfield 1987; Alexandre *et al.,* 1994; Mansure *et al.,* 1994; Piper *et al.,* 1994). Although the response of yeast to ethanol stress is associated with general stress response mechanisms, recent work in this area has identified novel ethanol-specific responses (Betz *et al.,* 2004; Takemura *et al.,* 2004). Takemura *et al.* (2004) observed that ethanol stress, as well as heat shock, causes selective mRNA export. Bulk poly(A)+ mRNA accumulates in the yeast nucleus, whereas mRNA of HSPs is exported under such conditions. These authors found that the nuclear localization of DEAD box protein Rat8, changed rapidly and reversibly in response to ethanol stress. This change correlated strongly with the blocking of bulk poly(A)+ mRNA export caused by ethanol stress. Interestingly, the localization of Rat8 did not change in heat-shocked cells, suggesting that it is an ethanol stress-specific response in yeast. The nuclear localization of Rat8 may contribute to the selective export of mRNA in ethanol-stressed cells, suggesting that there are differences in adaptive response in the export of mRNA to ethanol stress compared to other stressors (Takemura *et al.,* 2004).

In another study, (Betz *et al.,* 2004) identified a novel ethanol-specific transcription regulator, Asr1. Asr1 is a yeast Ring/PHD finger protein that accumulates in the nucleus upon exposure to alcohol. The subcellular localization of this protein is exclusive to alcohol stress; not being observed during other stress conditions such as oxidative, osmotic, nutrient limitation or heat stress (Betz *et al.,* 2004). The authors speculated that the nuclear accumulation of Asr1 in yeast upon exposure to alcohol stress is the result of enhanced nuclear import or inhibition of nuclear export. The authors also suggested that Asr1 might be involved in a complex signal transduction pathway during ethanol stress that enables yeast to acclimatise to ethanol, but this is yet to be tested. These two ethanol specific responses raise the possibility of yeast possessing a signal transduction pathway specific for ethanol.

1.4.4 Asr1 signals alcohol stress in *S. cerevisiae*

1.4.4.1 Overview of Asr1

In *S. cerevisiae*, the Alcohol Sensitive Ring/PHD finger 1 protein (Asr1) was the first reported protein that specifically responds to alcohol, having no response to other environmental stresses (Betz *et al.*, 2004). This novel alcohol-sensitive protein constitutively shuttles between the cytoplasm and nucleus but rapidly and reversibly accumulates in the nucleus under alcohol stress (Betz *et al.*, 2004; Izawa *et al.*, 2006).

The open reading frame YPR093C (*ASR1*) was recognized in a yeast 2-hybrid screen using Nup116 as bait. *ASR1* is situated on chromosome XVI (Figure 1.6) and encodes a Ring/PHD finger protein which is known as the Alcohol Sensitive ring/PHD finger 1 protein (Asr1) (Betz *et al.*, 2004). This protein contains 310 amino acids with a molecular mass of 36 kDa. The N-terminal region of the Asr1 contains two regions with homology to the RING (really interesting new gene) type or PHD (plant homeodomain) type finger domain (Betz *et al.*, 2004). The PHD domain is recognised by seven cysteines and a histidine that are spatially arranged in a Cys4-HisCys3 consensus. The PHD domain resembles the RING domain which is defined by Cys3-HisCys4 consensus (Figure 1.7). These consensus sequences form zinc-binding domains that coordinate two zinc ions. The N-terminal of Asr1 folds into two RING or PHD type finger domains, with a high probability within residues 26-69 and another one with a lower probability in residues 143-189 (Capili *et al.*, 2001; Betz *et al.*, 2004). The C-terminal domain (residues 190-310) of Asr1 does not contain any structural features (Betz *et al.*, 2004). Both PHD and RING fingers are structurally similar to each other (Figure 1.7). They are binuclear, interleaved Zn-chelating domains that share a core treble clef motif with numerous other Zn-binding domains; they differ in most other specific structural and functional features (Aravind *et al.*, 2003). These fingers are involved in protein-protein interaction but to date there is no evidence of being involved in binding to DNA (Ragvin *et al.*, 2004).

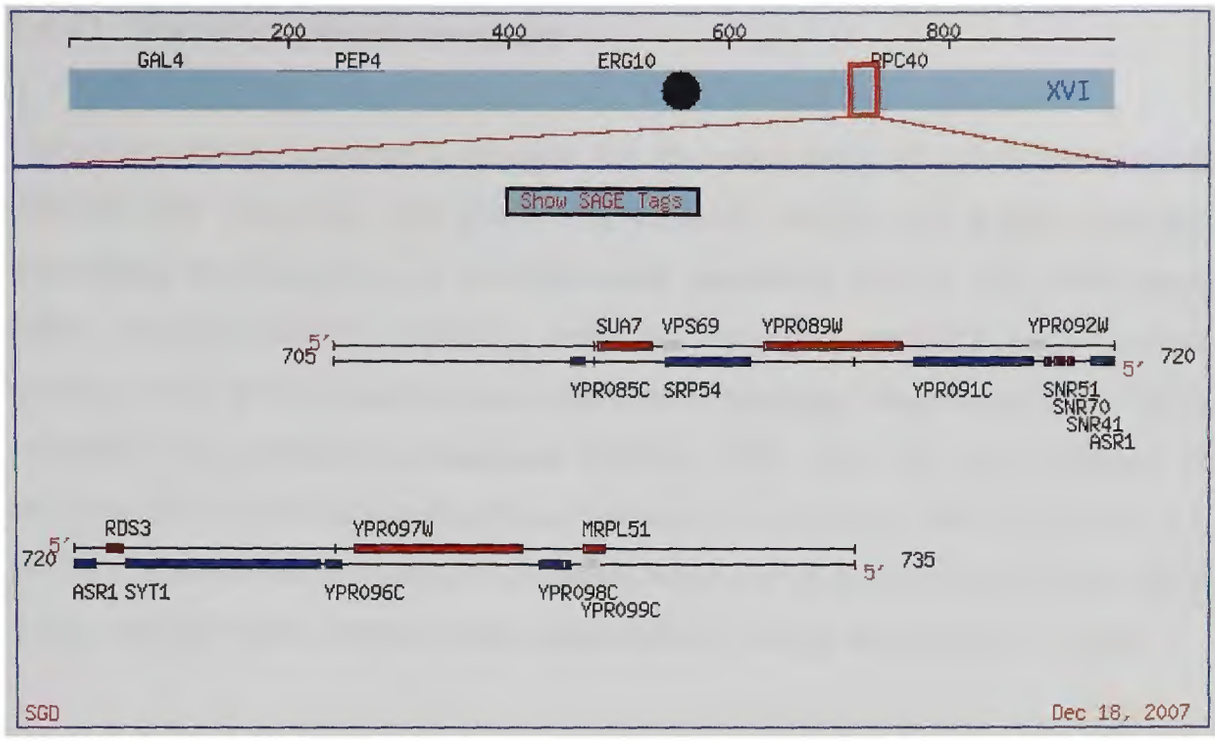


Figure 1.6: Feature map of chromosome XVI of *S. cerevisiae*. Chromosome XVI features that span coordinates 709556 - 730422 bp and represent — Chromosome XVI, — Watson strand ORF and — crick strand ORF (http://db_yeastgenome._org/cgi-bin/locus.pl?locus =YPR093c).

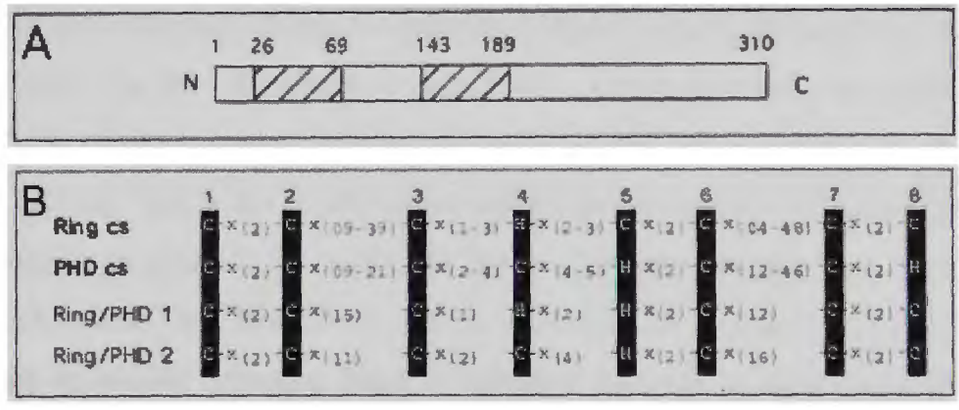


Figure 1.7: Asr1 Ring/PHD finger domains. **A**, Schematic drawing of Asr1 with RING/PHD finger 1 and 2 (*hatched*). **B**, comparison of Asr1 Ring/PHD finger domains 1 and 2 with the consensus sequence (cs) of Ring- and PHD-type finger domains. Reprinted by permission from American Society Biochemistry and Molecular Biology, Inc: Journal of Biological Chemistry (Betz *et al.*, 2004), copyright (2004).

1.4.4.2 Nucleocytoplasmic transport

Nucleocytoplasmic transport is essential for the maintenance of cell function and it involves cell signalling that transforms external stimuli into a cell response, stimulating acclimatisation to environmental conditions (Meyer and Vinkemeier 2004). In all eukaryotes, including yeast, the nucleus is enclosed in the nuclear envelope (NE), which separates nucleoplasm and cytoplasm. Penetration of the NE is controlled via nuclear-pore complexes (NPCs). NPCs allow the free exchange of small molecules, such as water and ions, between the cytoplasm and nucleoplasm, but the NPC restricts the exchange of macromolecules such as proteins with molecular weighs above 40 kDa, between these compartments (Mattaj and Englmeier 1998).

Nucleocytoplasmic transport of proteins occurs via NPCs by an active transport system that is capable of carrying a protein against a concentration gradient. This process occurs in both directions by energy-dependant and carrier-mediated mechanisms (Gorlich and Mattaj 1996). Protein transport of across the NPC is selective and signal-dependent, relying on the identification of nuclear localization signals (NLSs) on proteins imported into the nucleus and nuclear export signals (NESs) on proteins for export from the nucleus. Most NLS and NES are recognized by the β-karyopherin (kap) family of receptor proteins which are known as importins and exportins (Figure 1.8) (Rout *et al.,* 2003). Once importins or exportins are assembled on protein cargo, active transport occurs by Ran-GTPase nucleotide exchange factors, which are highly concentrated in the nucleus. This energy-driven translocation is regulated by a Ran-GTPase gradient between the nucleus and cytoplasm (Macara 2001; Rout *et al.,* 2003). To import NLS-proteins (cargos) into the nucleus, the Ran-GDP importin forms a complex with the protein cargo (importin-cargo). Following nuclear translocation, the importin-cargo complex dissociates by binding of RanGTP to the importin. The reverse reactions take place with formation of a cargo-exportin-RanGTP complex, which is dissociated in the cytoplasm upon GTP hydrolysis (Fries *et al.,* 2007).

A recent study by Fries *et al.* (2007) found that nuclear import of Asr1 is mediated by its C-terminal domain ($Asr1C_{243-280}$). Furthermore, it was demonstrated that the NLS

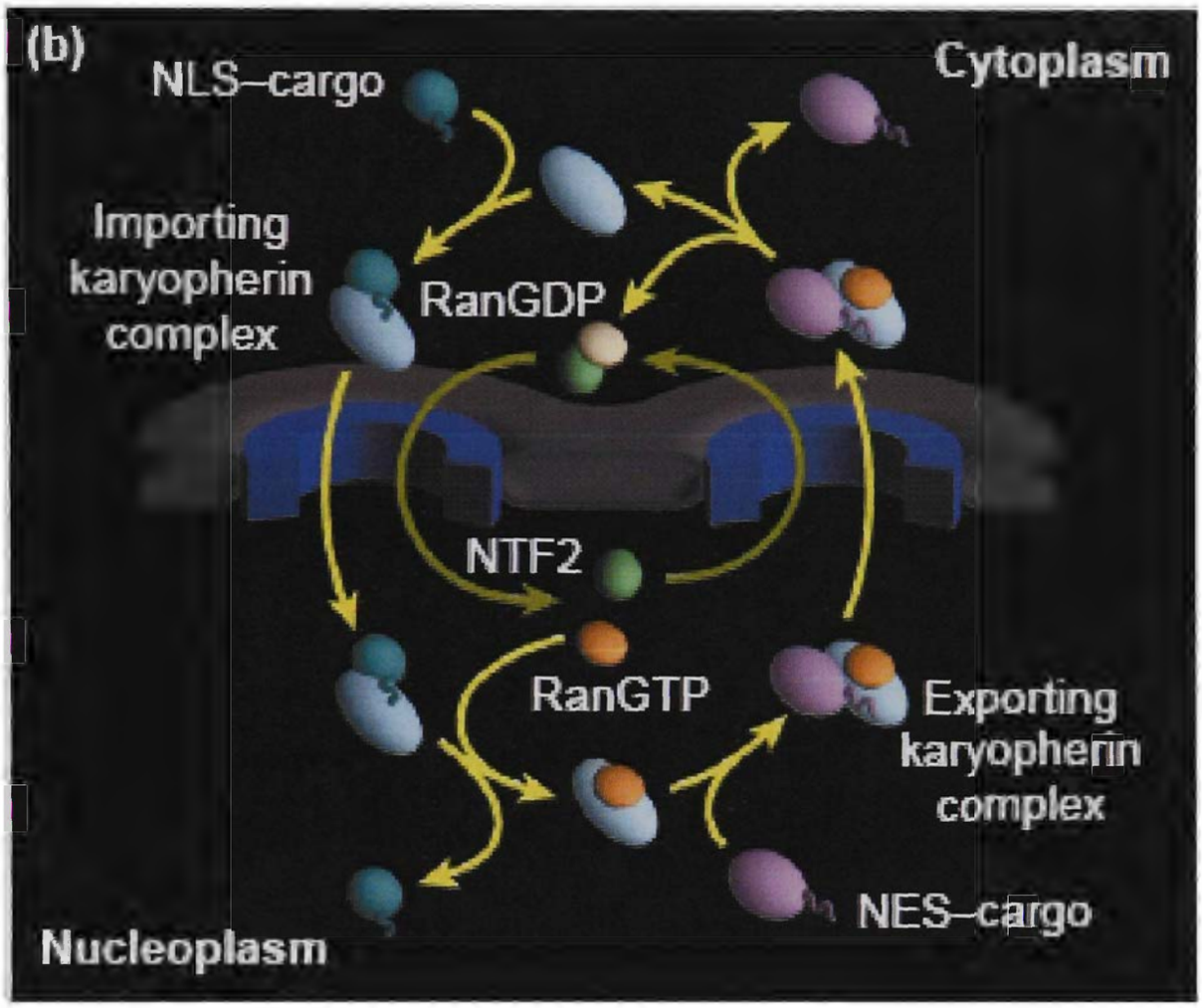


Figure 1.8: The nucleocytoplasmic transport cycle. An importing karyopherin (kap) binds to its NLS-bearing cargo in the cytoplasm and transits the NPC. On the nucleoplasmic side, RanGTP binds to the kap, causing a conformational change that releases the cargo. In the nucleoplasm, exporting kaps bind their cargos in the presence of RanGTP. Once the exporting complexes are on the cytoplasmic side, RanGTP hydrolysis is stimulated by RanGAP, resulting in the release of cargo. RanGDP is then recycled to the nucleoplasm by NTF2 and is reloaded with GTP to begin another cycle. Reprinted by permission from Elsevier Ltd: Trends in Cell Biology (Rout *et al.,* 2003), copyright (2003).

of Asr1 directly interacts with the importins Kapp114, Kap95, Pse1, Kap123 or Kap104 and forms the importin-Asr1 complex. The authors also found that the defined NLS is evolutionary conserved (Fries *et al.,* 2007). Two NES are located in the N-terminal of Ring/PHD finger domain, which are required for nuclear export of Asr1. The first sequence (NES1) is located within residues 125-135 and NES2 is located in residues 145-156. NES-Asr1 is recognized by exportin Xpo1 and forms a trimeric complex with NES-Asr1 and RanGTP (Asr1-Xpo1-RanGTP) (Betz *et al.,* 2004).

A number of transcription factors that respond to environmental stress have been shown to be regulated by a nucleocytoplasmic transport mechanism. For example, the general stress response is controlled by the transcription factors Msn2 and Msn4 (Msn2/4), which are translocated into nucleus in response to environmental stress (Marchler *et al.,* 1993; Martinez-Pastor *et al.,* 1996; Schmitt and McEntee 1996). Also, NES-Yap1 plays a role in its nuclear accumulation in response to oxidative stress (Kuge *et al.,* 1997). In yeast cells exposed to environmental stress, at least one signalling molecule has to be translocated from the cytoplasm to the nucleus in a signal-dependant manner if the cell is to respond to the stress. The nuclear localisation of Asr1 might be a key to understanding the mechanisms responsible for transforming ethanol stress conditions into a cellular response.

1.4.4.3 Asr1 and the ethanol stress response of *S. cerevisiae*

As mentioned previously, subcellular localisation of Asr1 in *S. cerevisiae* during alcohol stress was first reported by Betz *et al.* (2004). These authors observed that Asr1 constitutively shuttles between the nucleus and cytoplasm of *S. cerevisiae* in the absence of stress, however, the protein rapidly accumulates in the nucleus when ethanol is added to the extracellular medium. Removing ethanol from the yeast environment results in the rapid relocation of Asr1 to the cytoplasm, suggesting that the ethanol-induced nuclear accumulation of Asr1 is rapid and reversible (Figure 1.9).

To determine if this phenomenon occurs in response to other stress conditions, Betz *et al.* (2004) investigated the nuclear localisation of Asr1 in yeast subjected to heat

shock (37°C or 42°C), osmotic stress (0.5 M NaCl or 1 M sorbitol), oxidative stress (2 mM H_2O_2) and starvation (nitrogen or stationary phase)(Figure 1.10). These stress conditions did not have any measurable impact on the localisation of Asr1, providing evidence that the phenomenon is specific to alcohol stress (Betz *et al.*, 2004).

Betz *et al.* (2004) investigated the growth response of wild-type and *asr1*Δ strains on medium containing alcohol. Although both the wild-type and *asr1*Δ cells grew normally on medium containing 1% (v/v) ethanol, growth of the disruption strain, *asr1*Δ, was severely compromised on 1% (v/v) butanol. These authors also analysed the growth of wild type and *asr1*Δ strains in medium containing SDS, a detergent that affects membrane fluidity similarly to alcohol. Strain *asr1*Δ had a higher sensitivity to SDS compared to the parent strain. Based on these findings, the authors proposed following:

- The insensitivity of *asr1*Δ cells to 1% ethanol is probably a result of the rather low concentration of ethanol in the medium, being too low to cause stress.

- The sensitivity of *asr1*Δ *cells to* 1% butanol and SDS indicates the importance of Asr1 in stabilising membrane fluidity

- Asr1 is functionally linked to a signalling system that senses change in membrane fluidity and therefore Asr1 represents an highly promising candidate for revealing at least part of the complex signalling that allows the cell to cope with alcohol stress;

- Asr1 is a protein that responds to alcohol stress and is required for tolerance to ethanol.

Following the work of Betz *et al.* (2004), Izawa *et al.* (2006) used a similar method to identify a phenotype associated with Asr1 and ethanol tolerance, but did not find a significant difference in the growth profile of either the wild type or *asr1*Δ strains on solid medium containing ethanol or butanol. These authors did confirm the nuclear localization of Asr1 during ethanol stress, but concluded that Asr1 is not important

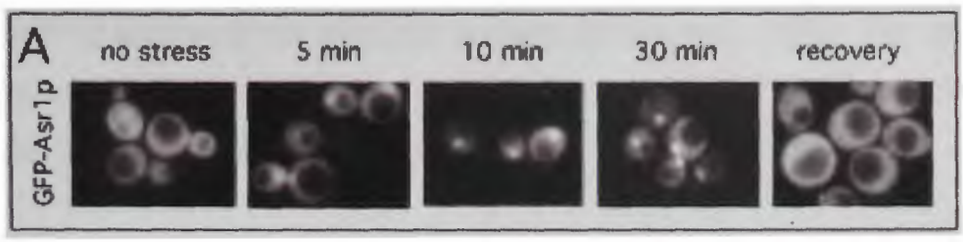


Figure 1.9: Nuclear accumulation of Asr1 in response to ethanol is rapid and reversible. Localisation of GFP-Asr1 expressed in exponentially growing *asr1*Δ cells and exposed to no stress conditions (0% ethanol) or 7.5% ethanol for 5, 10, or 30 min. To analyse the recovery of cytoplasmic location of GFP-Asr1, cells were first exposed to 7.5% ethanol for 10 min. Then, cells were washed twice and placed in fresh medium. After 10 min recovery was analysed by Immunofluorescence Microscopy with DAPI staining. (GFP, green fluorescent protein; DAPI, 4',6'-diamidino-2-phenylindole). Reprinted by permission from American Society Biochemistry and Molecular Biology, Inc: Journal of Biological Chemistry (Betz *et al.,* 2004), copyright (2004).

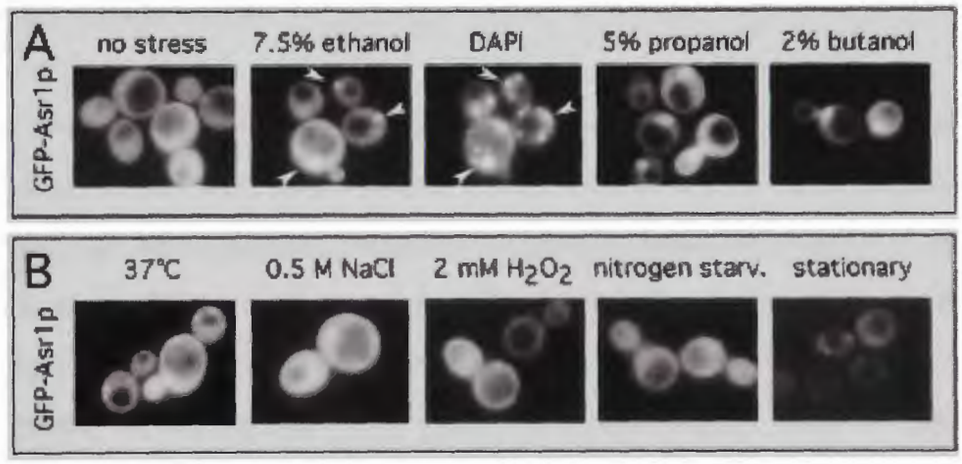


Figure 1.10: Asr1 specifically accumulates in the cell nucleus upon alcohol stress. (A) Localisation of GFP-Asr1 expressed in exponentially growing *asr1*Δ cells before and after exposure to 7.5% ethanol, 5% 2-propanol, and 2% 1-butanol. Nuclei were visualised by DAPI staining. (B) Subcellular localisation of GFP-Asr1 expressed in exponentially growing *asr1*Δ cella following 10 min of heat shock (37°C), osmotic stress (0.5 M NaCl), Oxidative stress (2 mM H_2O_2), nitrogen starvation and during stationary phase. Reprinted by permission from American Society Biochemistry and Molecular Biology, Inc: Journal of Biological Chemistry (Betz *et al.,* 2004), copyright (2004).

nor required for alcohol stress tolerance in yeast (Izawa *et al.,* 2006). The conclusions by Izawa *et al.* (2006) resulted in considerable controversy over the role of Asr1 in the response of *S. cerevisiae* to ethanol stress.

Although both groups independently established that Asr1 rapidly accumulates in the yeast nucleus in response to alcohol stress (Figure 1.10), the significance of Asr1 in the ethanol stress response is subject to conjecture. Betz *et al.* (2004) suggested that Asr1 might play a role in a yeast signalling system responding to alcohol-induced changes in plasma membrane fluidity, based on their observation that growth of *asr1*Δ was compromised in the presence of either 1% butanol or 0.02% SDS. Izawa *et al.* (2006) questioned the involvement of Asr1 in the stress response of yeast alcohol exposure, reporting no significant difference in the growth of a number of *asr1*Δ strains and wild type strains when incubated in the presence of ethanol (5 or 10% v/v), butanol (1 or 2%) or SDS (0.01 or 0.02%). Izawa *et al.* (2006) did not agree with Betz *et al.* (2004) that the accumulation of Asr1 in the nucleus is related to function, suggesting instead that it was due to a failure of the nuclear export machinery under conditions of ethanol stress.

Although both Betz *et al.* (2004) and Izawa *et al.* (2006) did not observe a unique phenotype associated with *asr1*Δ strains during ethanol stress, their method of testing cell growth, was not sufficiently sensitive to identify small differences in growth rate between strains. These authors compared the growth of the wild type and *asr1*Δ strains using a conventional assay for cell growth comprising drip-plates from a dilution series of comparable wild type and *asr1*Δ strains cultures grown under stressed and non-stressed conditions on solid medium. This technique is not however particularly sensitive to subtle differences in growth characteristics. It is possible that a more sensitive growth-measuring technique is required to identify variance in phenotype due to the impact of Asr1 on yeast fitness during ethanol stress. This is important to know since the role of Asr1 in ethanol stress tolerance is questionable if a unique phenotype cannot be associated with the absence or presence of the protein.

1.5 Aims and objectives of this project

The primary objective of this research project is to investigate and characterise the roles of trehalose and Asr1 in the response of *S. cerevisiae* to ethanol stress. The specific were to determine if:

1. Trehalose and/or its metabolism have a role in the survival of ethanol-stressed *S. cerevisiae*.
2. Asr1 has an influence on the physiological response of ethanol-stressed *S. cerevisiae.*
3. Asr1 affects global gene expression profile in *S. cerevisiae* subjected to ethanol stress.

CHAPTER 2

Material and methods

2.1 Materials

2.1.1 Yeast strains

A haploid lab strain, *Saccharomyces cerevisiae* BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and isogenic knockout strains were used for the work described throughout this thesis. Each of these knockouts had one of the genes *TPS1, TPS2, TSL1, NTH1* or *ASR1* replaced by the *kanMX4* module to generate five knockout strains, BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) *tps1*Δ::*kanMX4, tps2*Δ::*kanMX4, tsl1*Δ::*kanMX4, nth1*Δ::*kanMX4* and *asr1*Δ::*kanMX4* (Table 2.1). All of these strains were kindly provided by Australian Wine Research Institute (AWRI).

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. All buffers and solutions were prepared using distilled and de-ionised Milli-Q water (Milli-Q Plus Ultra Pure Water System, Millipore, Billerica, MA, USA). Formulae for all buffers and solutions are provided in Appendix I. Buffers and solutions were stable after they were sterilized by autoclaving at 121°C for 20 minutes or, where indicated, filter sterilized using a 0.22 μm Millipore membrane filter. All glassware and stainless steel components for preparing RNA solutions were baked at 180°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 (0.2%) was 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 I.

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

***S. cerevisiae* Strains**	**Description**	**Genotype**
BY4742	Wild type	BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*)
tps1Δ (BY4742 *tps1Δ::kanMX4*)	Knockout	BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) *tps1Δ::KanMX4*
tps2Δ (BY4742 *tps2Δ::kanMX4*)	Knockout	BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) *tps2Δ::KanMX4*
tsl1Δ (BY4742 *tsl1Δ::kanMX4*)	Knockout	BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) *tsl1Δ::KanMX4*
nth1Δ (BY4742 *nth1Δ::kanMX4*)	Knockout	BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) *nth1Δ::KanMX4*
asr1Δ (BY4742 *asr1Δ::kanMX4*)	Knockout	BY4742 (*MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) *asr1Δ::KanMX4*

All strains were provided by the Australian Wine Research Institute

2.1.3 General equipment used for experimental procedures

Yeast cultures of volumes less than 100 ml were incubated in 500 ml Erlenmeyer flasks. Incubations with more than 100 ml of culture fluid were undertaken in 1 L or 2 L sidearm flasks. All cultures were incubated in orbital-shaker incubators (Innova 4231 refrigerated incubator, New Brunswick Scientific, Edison, New Jersey). A 4054 UV/Visible spectrophotometer (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey) was used for all growth experiments and a DU® 530 UV/visible spectrophotometer (Beckman Instruments, Germany) was used for the molecular work. Centrifugation was carried out either in a Beckman CS-15R swinging rotor centrifuge or an Eppendorf 5415C bench top microfuge (Eppendorf, GmbH, Englesdorf, Germany). A bench vortex mixer (Thermoline Scientific, VIC, Australia) was used for all experiments. PCR experiments 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 connected to Labwork™ analysis software and Digital Graphic printer Up-D890. Gene arrays were scanned using the GenePix-Pro 4000B scanner (Axon Instruments, USA). Real Time PCR experiments were performed in a Roche LightCycler Instrument (Roche Diagnostics, GmbH, Mannheim, Germany).

2.2 Yeast growth

2.2.1 Growth media

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

Defined medium (liquid) contained per 1 L: 20 g D-glucose, 5 g ammonium sulphate and 1.7 g yeast nitrogen base, without amino acids and ammonium sulphate (Difco-

233520). The yeast nitrogen base was prepared according to the manufacturer's instructions as a 10x solution (1.7g 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. L-Leucine was added to final concentration of 100 mg L^{-1} from a filter sterilized stock solution (10 mg ml^{-1}). L-Histidine hydrochloride and uracil were added to final concentration of 20 mg L^{-1} from filter sterilized stock solutions of L-Histidine hydrochloride (10 mg ml^{-1}) and uracil (2 mg ml^{-1}). Lysine was added to a final concentration of 30 mg L^{-1} from filter sterilized stock solutions (10 mg ml^{-1}) (Kaiser *et al.*, 1994).

YEPD medium (solid) comprised per 1 L: 10 g yeast extract, 20 g bacto-peptone, 20 g D-glucose, and 15 g bacto-agar. Components were dissolved in distilled de-ionised water and autoclaved at 121°C for 20 minutes.

YEPD-Geneticin plates, for the selection of knockout strains with an integrated *kanMX4* cassette, comprised YEPD medium with the addition of 200 mg L^{-1} G418 Geneticin (Sigma). G418 was added when the medium had cooled to approximately 55°C and mixed thoroughly before pouring plates.

Glycerol storage medium comprised 2 x YEPD per 1 L: yeast extract (20 g), bacto-peptone (40 g) and glucose (40 g), 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 –20 °C or –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 130 rpm in an orbital-shaker incubator, unless otherwise stated. The culture vessels were Erlenmeyer (500 ml) or sidearm flask (1 or 2 L) with cotton wool plug

stoppers. To harvest cells for RNA extractions, cultures were grown in sidearm flasks, which had working volumes of 2 L.

2.2.2.2 Yeast strain preservation

For long-term storage, yeast cultures were kept in 2 ml vials containing 1 ml of sterile glycerol storage medium at -20°C or -80°C. Strains were initially grown on YEPD plates; yeast was then collected with sterile applicator sticks and suspended in the glycerol storage medium. The caps were tightened and the vials shaken before freezing.

For short-term storage, yeast was streaked on YEPD agar plates and incubated 30°C for 2-3 days then stored at 4°C. YEPD-Geneticin plates were used for the selection and short-term storage of gene knockout strains.

2.2.2.3 Revival of yeast cultures

The yeast cultures were revived by aseptically transferring a small amount of frozen glycerol stocks to a YEPD or YEPD-Geneticin plate using barrier pipette tips, and incubated at 30°C for 2 – 3 days.

2.2.2.4 Inoculum preparation

To prepare inocula for growth curve experiments, a loopful of cells was taken from plates (prepared from stock cultures), and aseptically transferred into 50 ml of fresh defined medium in a 250 ml sterile Erlenmeyer flask plugged with a cotton wool stopper. The yeast cells were incubated overnight at 30°C in an orbital shaker at 130 rpm. Two serial subcultures were made prior to each experiment. The optical density reading at 620 nm (OD_{620}) of these cultures was used to determine the inoculum size required for an initial OD_{620} reading of 0.1 when transferred to fresh medium (parent culture). Parent cultures, containing 100 ml (or 200 ml depending on required inoculum size) of fresh defined medium were inoculated to an initial OD_{620} reading of 0.1 and grown for approximately 8 hours to late exponential phase (OD_{620} of 0.8) at

30°C/130 rpm. Parent cultures for growth experiments were grown in 500 ml Erlenmeyer flasks, plugged with cotton wool stoppers.

Late-exponential phase parent culture cells (OD_{620} of approximately 0.8) were collected by centrifugation at 4,000 rpm (3,313 g) in a swinging rotor centrifuge (Sorvall® RT 7) for 7 minutes. The supernatant was discarded and the cells washed in pre-warmed fresh medium. The temperature was maintained at 30°C during the wash procedure. Transfers between subcultures were performed aseptically in a sterile laminar flow cabinet under a Bunsen burner flame.

2.2.2.5 Ethanol stress experiments (Classical growth and survival experiments)

Prior to the start of each experiment, fresh defined medium was pre-warmed to 30°C to eliminate the effect of temperature shock. Ethanol-stressed cultures were prepared under aseptic conditions immediately prior to the growth experiment. For each ethanol stress experiment, a control culture was prepared using defined medium without added ethanol. Aliquots of a cell suspension of exponential phase parent culture cells (inoculum) were inoculated, to an initial OD_{620} of 0.1 (approximately 3×10^6 cell ml^{-1}), into the control and experimental flasks containing pre-warmed medium. Once inoculated, the cultures were quickly transferred to the shaker incubator and grown under aerobic conditions at 30°C/130 rpm. Samples for optical density and viable plate counts were taken at regular intervals during incubation.

2.2.2.6 Sampling and harvesting cells in ethanol stress experiments

Sampling of cultures was achieved by swiping the sidearm with ethanol (70% v/v), opening the clamped sidearm and removing an appropriate volume via a sterile syringe. The initial 5 ml of culture removed during sampling was discarded before the required volume was collected. Sampling was performed initially at time 0 (inoculation time), and then at regular intervals during the course of the incubation. Samples (around 3 ml) were taken for optical density measurements and viable plate counts.

For extracting RNA and determining intracellular trehalose concentrations, yeast cells were harvested from each culture at specific time intervals after taking each of the above-described samples. Each of these samples comprised 100 ml culture separated into two 50 ml falcon tubes, in which the cells were pelleted by centrifugation for 5 minutes at 4°C in a swing rotor centrifuge (Sorvall® RT 7 Centrifuge) at 4,000 rpm (3,313 g). For RNA extractions, the supernatant was removed and the pelleted cells were frozen in liquid nitrogen and stored at -80 °C until required. For trehalose determination, cells were washed with 0.1 M phosphate buffer (pH 5.9) to remove external glucose, followed by centrifugation. The cell pellets were then frozen in liquid nitrogen and stored at -80°C until needed for trehalose determination.

2.2.2.7 Competition experiments

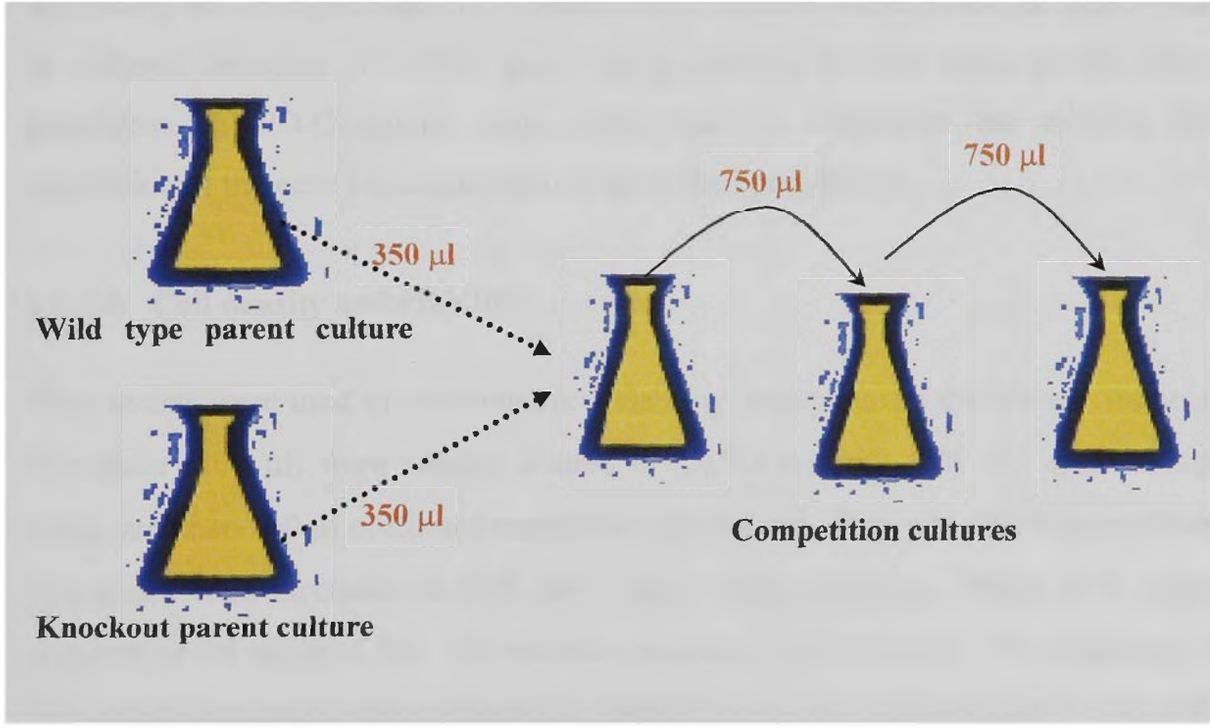


Figure 2.1: Experimental setup for competition experiments. Initially, equal numbers of late exponential phase cells (350 µl of each) were inoculated into 48 ml of defined medium only or defined medium containing 7.5% v/v ethanol. After six generations of growth, mixed populations were serially transferred into fresh medium under similar conditions. All cultures were incubated at 30°C/130 rpm.

Competition experiments were performed in 250 ml Erlenmeyer flasks with 48 ml of defined medium either with or without added ethanol. Ethanol was added by replacing a portion of the defined medium with an appropriate volume of ethanol (This volume of ethanol was very small, such that it would not impact significantly on the medium composition). All the cultures were grown under aerobic conditions at 30°C with continuous agitation at 130 rpm in an orbital-shaker incubator. To create a mixed population, equal cell numbers of wild type (BY4742) and a gene knockout strain (350 µl of each) from late exponential phase parent cultures (OD_{620} of approximately 0.8-0.9) were inoculated into defined medium in the presence or absence of ethanol. All competition cultures were grown for six generations to the late exponential phase to avoid other stresses associated with the stationary phase. At this stage, aliquots of mixed populations (750 µl) were transferred into fresh medium comprising similar conditions (Figure 2.1). Altogether, five to ten rounds of passaging was performed depending on the experiment. After each round, cultures were plated on YEPD with or without Geneticin (G418) to score the population of each strain in the mixed population. YEPD-Geneticin plates were used to determine the relative cell population of the gene knockout strain in the mixed population.

2.2.2.8 Cell density and viability

Plate counts were used to determine cell viability in experimental cultures. Samples of culture (100 µl) were serially diluted in YEPD medium (900 µl) in microfuge tubes, and then 100 µl of diluted sample was spread onto duplicate YEPD agar plates. The plates were incubated at 30°C for 3 days before counting. Plates with colony numbers in the range of 30 – 300 colonies per plate were recorded. The readings for each set of duplicates were averaged and multiplied by the dilution factor to calculate the viable cell population of the culture. Optical density was determined using a spectrophotometer at a wavelength of 620 nm. Samples were diluted appropriately such that the measurements were always between 0.1 and 0.5.

2.3 Trehalose determination

Trehalose concentrations were determined using two enzyme-based assays, essentially as described in Hounsa *et al.* (1998). Initially, trehalose was converted to glucose using trehalase (Sigma) and the resulting glucose was quantified using glucose oxidase/peroxidase (Megazyme). For trehalose determination, cells were harvested as described in Section 2.2.2.6. Frozen cell pellets were thawed on ice. According to viable cell counts, cells were resuspended in 0.25 M Na_2CO_3 solution to obtain a cell density of 1 x 10^8 cells ml^{-1}. Samples of 1 ml were transferred into a boil proof microfuge tube and boiled for 20 minutes to extract intracellular trehalose. After cooling, the samples were centrifuged at 12,000 g using a bench top microfuge (Eppendorf) for three minutes to remove cell debris. Aliquots of 200 µl were neutralised by adding 1 M acetic acid (100 µl) and then mixed with 100 µl buffer T (300 mM sodium acetate and 30 mM $CaCl_2$, pH 5.5). For trehalose quantification, 60 µl of sample or trehalose standard was mixed with trehalase (20 µl) (Sigma) and incubated at 40°C for 1 hour. Pre-existing glucose was determined in a control reaction from which trehalase was omitted. Glucose derived from trehalose was quantified by glucose oxidase and peroxidase assay (Megazyme) as recommended by the supplier. Samples (1 ml) were mixed with chromogen reagent (per 1 L: 1 M potassium dihydrogen orthophosphate, 200 mM para-hydroxibenzoic acid, 0.4% sodium azide, 12000U glucose oxidate, 650U peroxidase and 0.4mM 4-aminoantipyrine) (Megazyme) and incubated at 40°C for 30 minutes. Absorbance at 510 nm was recorded against the reagent blank to determine glucose content.

2.4 Molecular methodology

A list of all buffers, solutions, reagents and a list of suppliers for molecular methods are provided in Appendix I.

2.4.1 Confirmation of gene knockouts in deletion strains

The gene knockout yeast strains used for this work were from the EUROSCARF (EUROpean Saccharomyces Cerevisiae ARchive for Functional analysis) deletion

collection and were kindly provided by the Australian Wine Research Institute (AWRI) as part of an ongoing collaboration. The EUROSCARF deletion strains were created by gene replacement, in which targeted genes are replaced with DNA cassette that confers resistance to both Kanamycin and Geneticin-G418 (Winzeler *et al.*, 1999).

The knockout status of the strains used for this project was confirmed by plating onto medium containing G418 and by PCR.

2.4.1.1 DNA extraction

The method of yeast genomic DNA extraction was essentially as described by Ausubel *et al.* (1997) with minor modifications. Yeast cells were grown overnight in defined medium and harvested from 10 ml of culture as described in Section 2.2.2.6. Frozen cell pellets containing approximately 10^8 cells were thawed on ice. Subsequently, cell pellets were resuspended in 400 µl of DNA extraction buffer (Appendix I) and transferred to a microfuge tube containing approximately 400 µl of chilled, acid-washed 0.4 µm glass beads (Sigma). Phenol/chloroform/isoamyl alcohol (25:24:1) (400 µl) and 20 µl Tris buffer (0.5M, pH 8.0) were added and the mixture vortexed at highest speed for a total of 3 minutes (alternating one minute vortexing with one minute on ice). Samples were centrifuged at 12,000 g in a bench top microfuge (Eppendorf) for five minute in a 4°C cool room to remove proteins and cell debris. The upper aqueous layer was transferred to a new microfuge tube and 10 µl RNase (10 mg ml^{-1}; Promega) enzyme was added and incubated at 37°C for 40 min. Following incubation RNase was removed by a further cycle of phenol extraction. For the DNA precipitation, 1/10 volume (40 µl) of sodium acetate and 1.0 ml chilled 100% ethanol were added into the sample. Then the sample was mixed by inversion and placed on ice for 15 minutes and centrifuged 12,000 g for 5 minutes. The supernatant was discarded and the DNA pellet washed with 500 µl of ice-cold 70% (v/v) ethanol. The sample was centrifuged as described previously, the supernatant removed and the DNA pellet air-dried for 10–15 minutes. The DNA was resuspended in 25 µl of autoclaved double distilled water. DNA was subsequently visualized on a 1% agarose gel (essentially as described in Section 2.4.2.4) and analysed using an

Ultraspec III UV/Vis spectrophotometer (Pharmacia). The absorbance reading at 260 nm allowed for calculation of nucleic acid concentration in the sample and the ratio at 260 nm and 280 nm was used to assess the purity of the DNA.

2.4.1.2 PCR confirmation of gene knockout strains

Gene replacement and orientation of the *KanMx4* module were confirmed by PCR using genomic DNA as the template. The primer pairs to confirm deletion of the specific ORF and insertion of the *KanMx4* cassette were designed using *Saccharomyces cerevisiae* Genome Deletion Project website. http://www-sequence.stanford.edu/group/yeast_deletion_project/Enter_DB.html. Primers were purchased from Gene Works Pty Ltd (Australia).

PCR reactions comprised 23 µl PCR SuperMix (Invitrogen), 1 µl of 10 µM primer mix and 1 µl DNA template. Thermal cycling was performed in the PTC-100 or PTC-200 Peltier Thermal Cycle (MJ Research) with cycling conditions generally consisting of an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation (94°C) for 30 seconds, annealing at primer specific temperatures for 30 seconds and elongation (72°C) for 90 seconds, then final elongation step at 72°C for 5 minutes. Negative controls, containing no genomic DNA, were used in all experiments. The PCR product sizes were confirmed by gel electrophoresis on 1% (w/v) agarose gel (Section 2.4.2.4) and by sequencing (following Section).

2.4.1.3 Sequencing of PCR products

The above PCR products (from Section 2.4.1.2) were purified using a QIAquick® PCR purification kit (QIAGEN) according to the manufactures instructions (see Section 2.4.3.2). Around 8 µl of purified PCR product (50-100 ng) was transfer into PCR tube containing 3 µl of dilution buffer [250 mM Tris HCl (Sigma) pH 9.0, 10 mM $MgCl_2$ (BDH)], 1 µl of forward or reverse primer (3.2 µM), 2 µl of BigDye (Applied Biosystems Australia), and 6 µl double distilled H_2O. The following cycling parameters were used: 44 cycles of 96°C for 30 seconds, 56°C for 15 seconds, and 60°C for 4 min, followed by 5 cycles of 96°C for 30 seconds, 60°C for 4 min. and finally, 25°C for 60 seconds. Following amplification, 3 µl of 3 M sodium acetate and

77 µl of 100% (v/v) ethanol were added to precipitate sample. The sample was mixed by inversion, placed on ice for 15 minutes and centrifuged 12,000 g for 5 minutes. The supernatant was discarded and the precipitated DNA pellet washed with 250 µl of ice-cold 70% (v/v) ethanol. The sample was centrifuged as previously, the supernatant was removed and the DNA pellet air-dried for 10–15 minutes. Finally, samples were sent to Micromon DNA sequencing facility at Monash University for sequencing.

Sequence editing and alignments were conducted using the BioEdit Sequence Alignment editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). The quality of the DNA sequence was assessed by visual analysis of the trace file.

2.4.2 RNA extraction

2.4.2.1 RNase-free procedures and conditions

To ensure extraction of high quality RNA, all chemicals, water, plastic-ware and glassware used for handling RNA were RNase-free. Distilled and de-ionized MilliQ^{TM} water was treated with DEPC (0.1%), mixed well, allowed to stand overnight and autoclaved. Glassware and spatulas were covered in foil and baked at 160°C for at least 12 hours prior to use. Plastic containers, electrophoresis tanks, trays and combs were sprayed with RNase ERASE (ICN) and rinsed with DEPC treated water. Disposable RNase-free plastic ware (pipette tips and microfuge tubes (Eppendorf)) were used for all procedures. Glass beads used for cell disruption were acid washed and rinsed in DEPC-treated water prior to baking at 160°C overnight.

2.4.2.2 Total RNA extraction

Cells from ethanol-stressed and unstressed cultures were stored at -80°C prior to RNA extraction (see Section 2.2.2.6). Frozen cell pellets were thawed on ice and resuspended in an appropriate volume of 1x RNA buffer (Appendix 1) to obtain a cell density of 1x 10^8 cells / 400 µl. Total RNA was extracted using the glass bead extraction method, essentially as described by Ausubel *et al.* (1997) with some minor modifications. Briefly, the cell suspension (400 µl containing 1 x 10^8 cells) was transferred into a microfuge tube containing approximately 400 µl of chilled, acid-

washed 0.45 mm glass beads (Sigma), 400µl phenol/chloroform/isoamyl alcohol (25:24:1) (pH 8.0) and 20 µl Tris buffer (0.5M, pH 8.0). Samples were kept on ice throughout the procedure. The mixture was vortexed for 3 minutes (alternating one minute vortexing with one minute on ice). Samples were centrifuged at 12,000 g in a bench top microfuge (Eppendorf) for five minute at 4°C to remove the glass beads, proteins and cell debris. The upper aqueous layer was transferred to a new microfuge tube and the phenol extraction steps were repeated twice. Finally the RNA was precipitated for 12 hours at -80°C with 3 volumes of chilled 100% ethanol after addition of 25 µl sodium acetate (3.0 M). Following centrifugation (12,000 g for 10 min) the supernatant was removed and the RNA pellets were washed 2–3 times with 500 µl of chilled 75% (v/v) ethanol. The RNA pellets were air-dried at room temperature (10-15 minutes) and resuspended in 25 µl of RNase-free water.

The RNA concentration and purity was assessed from the A_{260} and A_{280} readings determined using a DU® 530 UV/visible spectrophotometer (Beckman Instruments, Germany). RNA was visualized using non-denaturing agarose gel electrophoresis (see Section 2.4.2.4) to assess purity and reproducibility of the extraction procedure. The RNA was stored at -80°C prior to cDNA preparation.

2.4.2.3 DNase Treatment of Total RNA

Total RNA samples were DNase treated to remove any contaminating genomic DNA before cDNA synthesis. This treatment was performed using the Ambion DNA-free kit (Ambion®) according to manufacturer's instructions. Briefly, total RNA (20 µl) was mixed with 2 µl of 10 x DNase Buffer and 3 µl of DNase I enzyme (Ambion®) and then incubated at 37°C for 50 minutes. Following inculabtion, the DNase I enzyme was inactivated by the addition of 4 µl of DNase Inactivation Reagent (Ambion®). Each sample was mixed thoroughly with continued intermittent flicking during the 2 minutes incubation at room temperature. Samples were centrifuged at 12,000 g for 2 minutes to remove the inactivation reagent and the supernatant transferred to a sterile fresh tube. Successful removal of genomic DNA was established by agarose gel electrophoresis and in some cases was confirmed by the

inability to amplify an actin derived PCR product in the absence of reverse transcription.

2.4.2.4 Agarose gel electrophoresis of RNA

Visualization of RNA on non-denaturing gels was carried out before and after DNase treatment to monitor the quality and purity of the RNA preparation, the consistency of RNA extraction and the success of DNase treatment. Electrophoresis of RNA was performed in RNase free 1% non-denaturing agarose gels in 1x TAE buffer containing ethidium bromide (10 mg ml^{-1}). Electrophoresis was conducted at 80 V for 45–60 minutes. Gels were viewed on a UV transilluminator and photographed using a UVP Laboratory Products gel documentation system. RNA samples were stored at -80°C.

2.4.3 Gene expression analysis using DNA microarray

Microarray analysis was performed using microarray slides (MWGSc6Kv4) purchased from the Clive and Vera Ramaciotti Centre for Gene Function Analysis, University of New South Wales. Each microarray representing the ~ 6,528 open reading frames comprising the *S. cerevisiae* genome, as 40 mer oligonucleotide probes spotted on a 25 x 75 mm glass slide.

2.4.3.1 cDNA synthesis for microarray analysis

For microarray analysis the mRNA profile was compared in samples derived from equal numbers of cells (10^8 cells). Complimentary DNA (cDNA) was prepared from wild type or knockout cultures grown under ethanol-stressed or unstressed conditions as described in Section 2.2.2.4 and 2.2.2.5. For each slide 20-25 µg of each DNase-treated total RNA sample was combined (total volume 18.7µl) and mixed with 13.5 µl of **Mix 1** (see Table 2.2). The RNA was denatured by incubation at 65°C for 5 min in a PCR machine followed by quick cooling on ice. After 5 minutes equilibration at 42°C, 5.95 µl of **Mix 2** (containing dNTPs and Aminoallyl-dUTP) and 2 µl of SuperScript™ II RT enzyme (Invitrogen) were added directly to each reaction. After

incubation at 42°C for 2 hrs 15 min, the RNA template was hydrolysed by addition of 4 µl 50 mM EDTA, pH 8.0 and 2 µl 10 M NaOH followed by incubation at 65°C for 20 min. The reaction mixtures were then neutralized with 4 µl 5 M acetic acid.

Table 2.2: Mix 1: Reagents for first strand cDNA synthesis (supplier: Invitrogen)

Number of slides	**1**	**2**	**3**	**4**	**5**
5 X first Strand Buffer (µl)	16.8	32.8	48.8	64.8	80.8
Oligo (dT)12-18 primer, 0.5 µg/µl (µl)	3.15	6.15	9.15	12.15	15.15
DTT, 0.1M (µl)	8.4	16.4	24.	32.4	40.4
Total vol. (µl)	**28.35**	**55.35**	**82.35**	**109.35**	**136.35**

Table 2.3: Mix 2: Reagents for first strand cDNA synthesis (supplier: Invitrogen)

Number of slides	**1**	**2**	**3**	**4**	**5**
dATP,10mM (µl)	2.6	5.2	7.8	10.4	13
dCTP, 10mM (µl)	2.6	5.2	7.8	10.4	13
dGTP, 10 mM (µl)	2.6	5.2	7.8	10.4	13
dTTP, 2.5 mM (µl)	3.4	6.8	10.2	13.6	17
Aminoallyl-dUTP 10 mM (µl)	1.8	3.6	5.4	7.2	9
Total vol. (µl)	**13.0**	**26.0**	**39.0**	**52.0**	**65.0**

2.4.3.2 Purification of cDNA

The cDNA was purified using a QIAquick® PCR purification kit (QIAGEN) according to the manufacturer's instructions. The cDNA was applied to the column after addition of 150 µl PB buffer (QIAGEN). An additional 150 µl PB buffer was used to rinse the tube. Columns were centrifuged at 3,185 g for 1 minute and the flow-through discarded. The cDNA was washed twice by applying 700 µl (70% v/v) ethanol to each column, followed by centrifugation (3,185 g for 1 min). The residual ethanol in columns was removed by centrifugation at 3,185 g for 1 min.

Finally, for cDNA was eluted in 25 µl of DEPC-treated water by incubation at room temperature (5 min.) followed by centrifugation at 3,185 g for 1 min. A second elution of 10 µl was performed to give a final volume of about 35 µl. The cDNA samples were then centrifuged in a Speedivac at a low speed for ~20 min to reduce the volume to 2–5 µl prior to labelling.

2.4.3.3 Preparation of fluorescent dyes for labelling

The fluorescent dyes were used to label cDNA for microarray analysis, were cyanine-3 (Cy3) and cyanine-5 (Cy5) (Amersham). For long term storage, each dye was resuspended in 18 µl DMSO and 2 µl aliquots centrifuged to dryness in the Speedivac and stored at 4°C in the dark until required. Immediately prior to cDNA labelling, Cy3 and Cy5 dyes were resuspended in 2 µl of DMSO.

2.4.3.4 Labelling cDNA probe

The following indirect labelling procedure was used to couple the Cy3 and Cy5 (Amersham) fluorescent dyes to the aminoally-dUTP incorporated in the cDNA. Each cDNA was mixed with 9 µl of $NaHCO_3$ (0.1 M, pH 9) and mixed with 2 µl of either Cy3 or Cy5 dye in DMSO. The samples were mixed thoroughly by repetitive pipetting and the labelling reaction was allowed to proceed for 1 hour 30 minutes at room temperature. The control (wild type) samples were labelled with Cy3 (green), and experimental samples (gene knockout) with Cy5 (red).

Unincorporated dyes were removed using a QIAquick® PCR purification kit (QIAGEN) (Section 2.4.3.2). The fluorescently labelled cDNA samples (35 µl) were concentrated by drying in a Speedivac to a final volume of 10 µl. All labelling and subsequent purification steps were performed in darkness.

2.4.3.5 Washing and blocking microarray slides

Washing and blocking of the microarray slides was required prior to hybridisation. Washes were carried out in a glass slide chamber with gentle agitation according to the following protocol:

- Solution 1 (0.1% Tritron X-100) for 5 min.
- Solution 2 (4.38 mM HCl) for 2 min.
- Repeat wash in Solution 2 for 2 min.
- Solution 3 (100 mM KCl) for 10 min.
- DEPC-treated distilled water for 1 min.

For the blocking of slides, prewarmed blocking buffer (25% Ethyene glycol, 0.01% HCl) was added and agitated gently at 50°C for 30 min. After blocking, the slides were washed with DEPC-treated distilled water and air dried by centrifugation in falcon tubes at 830 g for 10 min at 40°C using a swing out centrifuge rotor (Sorvall® RT 7; GMI, Inc., USA). Dry slides were placed at room temperature in a light blocking slide box until required.

2.4.3.6 Hybridisation

All procedures were performed in darkness to prevent photobleaching of the fluorescent dyes. The Cy3 and Cy5 labelled cDNA samples were mixed thoroughly and the volume reduced from 20µl to 5 µl using a Speedivac at low speed. After addition of 85 µl Hybridisation solution (Table 2.4), the solution incubated at 65°C for 5 min to denature the cDNA and cooled on ice. Finally, an aliquot (approximately 90 µl) of the denatured labelled cDNA mix was loaded onto each microarray slide which was then sealed with a cover-slip, avoiding bubble formation. The microarray slides were placed into a moist hybridisation chamber and incubated at 37°C for 16 hrs in darkness.

Table 2.4: Hybridization solution

Reagents	**Volume (µl)**
***Dig Easy Hyb (filtered)	100
**Yeast tRNA (10 mg/ml)	5
*Salmon Herring Sperm (10 mg/ml)	2.75
Total vol.	**107.75**

Suppliers: *Invitrogen, **Ambion®, ***Roche Diagnostics

2.4.3.7 Microarray slide washing and scanning

After hybridisation, the cover-slip was removed from each slide by immersing gently in 1 x SSC + 0.1% SDS in a 50 ml falcon tube. The slides were then placed in a glass slide chamber and unbound cDNA removed by a series of washes, as described below. After each step, the wash solution was removed and replaced with fresh solution. All solutions for post hybridisation washes were preheated to 50°C before use (see Appendix 1).

- 1 X SSC + SDS for 20 min. This step was repeated 3 times.
- 1 X SSC for 10 min.
- 1 X SSC + Triton X-100 for 10 min.
- 1 X SSC for 10 min.
- 0.5 X SSC for 10 min. This last step was repeated once .

Slides were then air dried by centrifugation at 830g for 10 min at 40°C. Microarrays were scanned using the GenePix-Pro 4000B scanner (Axon Instruments, USA). The photomultiplier tube (PMT) voltage for both the Cy3 excitation wavelength (532 nm) and Cy5 excitation wavelength (635 nm) was adjusted during scanning process as described in the GenePix-Pro6 manual to optimise the intensity of the fluorescence signal of the spots.

2.4.3.8 Microarray data analysis

After scanning, the grid associated with the Genepix Array List (Gal file: MWGSc6kv4_GP.gal) was loaded on to the microarray fluorescent image and features aligned automatically by using GenePix-Pro 6.0. This Gal file automatically provides identification of all features printed on the microarray. For each spot the fluorescence intensity from each dye was quantified using GenePix-Pro 6.0 software (Axon Instruments, USA). In addition to the fluorescent signal from the bound labelled probe, the intensity of a particular feature includes a certain amount of background non-specific fluorescence. In these experiments local background subtraction method was applied to remove background signal. The background value

for a feature was calculated from a region near the feature and has the advantage of tracking background variation across a slide. Contaminated or abnormal features were removed from the dataset by manually "flagging" bad spots. The features with low fluorescent intensity were flagged as not found and automatically removed from the data set.

Data from microarray experiments may be biased for several reasons including differences in dye properties, probe labelling and hybridisation efficiencies. To remove such effects the "lowess normalisation" method was used in data analysis. All statistical analysis of datasets was performed using the TM4 software package from The Institute for Genome Research (TIGR) (Saeed *et al.*, 2003). Significance Analysis of Microarrays (SAM) was used to identify genes with significant differential expression between wild type and gene knockout strains in defined conditions (Tusher *et al.*, 2001). The *Saccharomyces cerevisiae* genome database SGD (http://www.yeastgenome.org) and FunSpec (http://funspec.med.utoronto.ca/) were used to group ORFs according their molecular and biological function.

2.4.4 Confirmation of relative gene expression using Quantitative Real-Time PCR analysis

2.4.4.1 First strand cDNA synthesis for PCR

Quantitative real-time PCR was used to validate the microarray results. The cDNA was prepared using RNA extracted from an equal numbers (10^8 cells) of wild type or *asr1*Δ cells as described in Section 2.4.2.2. For cDNA synthesis 1 µl (1–2 µg) DNase treated total RNA (see Section 2.4.2.3 for DNase treatment) was mixed with 1 µl oligo $(dT)_{12\text{-}18}$ (Invitrogen), 1 µl (10 mM) dNTP (Invitrogen) mix and 12 µl sterile distilled water. RNA was denatured by heating at 65°C for 5 min, followed by quick cooling on ice. The contents were collected by brief centrifugation and 4 µl 5 x first-strand Buffer (Invitrogen), 2 µl 0.1 M dithiothreitol (DTT) (Invitrogen) and 1 µl RNase inhibitor (Invitrogen) were added. Following temperature equilibration at 42°C for 2 min, 1 µl (200 units) reverse transcriptase (SuperScript™ II RT-Invitrogen) was added and the contents gently mixed and then incubated at 42°C for 50 min.

Following cDNA synthesis, the reaction was terminated by heating at 70°C for 15 min. Negative control reactions omitting reverse transcriptase were also prepared for all reactions to test for the presence of genomic DNA contamination.

2.4.4.2 Primer design for Real-Time PCR

Gene-specific primers were designed using the coding sequences available from the *Saccharomyces cerevisiae* genome database (SGD; www.yeastgenome.org). The primers comprised 20–25 nucleotides with 50–60% G+C content, and the target amplicon length was between 200 and 400 bp. The quality of primers was tested to avoid: primer dimer and primer hairpin formation due to complementary sequences using the Primer3 website: (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Finally primers were 'tested' using the BLASTn sequence alignment algorithm for unwanted hybridisation to non-specific target sequences in other coding regions of the yeast genome. Primers were purchased from Gene Works Pty Ltd (Australia).

2.4.4.3 Real Time PCR reactions

For confirmation of relative gene expression, real-time PCR was performed using the Lightcycler (Roche Diagnostics). The cDNA for Real Time PCR reactions was prepared as described in section 2.4.4.1. The optimum $MgCl_2$ concentration for each primer pair was determined empirically prior to quantitative analysis of gene expression. Real-time PCR reactions were performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) in a final volume of 20 µl comprising Taq DNA polymerase, reaction buffer, 0.5 mM each dNTP, 2 to 5 mM $MgCL_2$, SYBR Green I (2 µl), and cDNA template (2 µl of a 1/40 dilution). All PCR protocols included an initial denaturation step of 10 minutes at 96°C before amplification and PCR amplification protocol was run using the following set-up program: 35 cycles of 95°C for 10 seconds, gene-specific annealing temperature for 10 seconds, and 72°C for 15 seconds.

Following amplification the melting curves of each PCR products was analysed by continuous measurement of fluorescence between 68°C and 95°C (ramp speed of 0.1°C/sec). The identity of each PCR amplicon was established by confirmation of the expected Tm and by confirmation of the expected amplicon size using agarose gel electrophoresis (see Section 2.4.2.4). Two negative controls were included, one in which no reverse transcriptase enzyme was added during cDNA synthesis (control for amplification form genomic DNA) and the other in which no template was added (control for non-specific PCR amplification). For real-time PCR analysis, RNA templates were derived from the same cell preparations used for gene array experiments unless otherwise stated.

2.4.4.4 Real-Time PCR data analysis

For quantification a standard curve was established for each gene-specific primer pair using serial dilutions (1/5, 1/20, 1/100, and 1/500) of a single cDNA preparation (standard). These reactions were performed in triplicate. Experimental sample reactions were also prepared in triplicate and the crossing point values analysed using the Fit point method of the Lightcycler Software 3.5 (Roche Diagnostics, NSW, Australia). The crossing point identifies the cycle at which amplification of the product reaches a set level, as defined by the emission of a threshold level of fluorescence. As this level is set to be constant across all samples, the more copies of the template present at the beginning of the reaction, the fewer the number of cycles it takes to reach this defined level of fluorescence. The relative concentration of a gene specific transcript in each cDNA sample was established by comparing cross point values in relation to the standard curve. The fold change in gene expression due to deletion of the *ASR1* gene was established by comparing the level of gene expression in the *asr1*Δ and wild type strain.

CHAPTER 3

The role of trehalose in yeast survival during ethanol stress

3.1 Introduction

Trehalose in yeast has traditionally been studied for its role as a reserve carbohydrate, but it is now believed to also be associated with the protection of cells against environmental stresses, such as starvation, desiccation, dehydration, osmotic and oxidative stress, and extremes in temperature (for reviews see Gadd *et al.,* 1987; Wiemken 1990; Thevelein 1995; Hounsa *et al.,* 1998; Elbein *et al.,* 2003; Herdeiro *et al.,* 2006). Over the past twenty years a number of studies have demonstrated a correlation between intracellular trehalose levels in yeast and survival when exposed to various environmental stresses (Chapter1, Section 1.2.6). There is also some evidence to suggest that this carbohydrate has a role in ethanol tolerance, but to date such evidence is controversial and largely circumstantial (Chapter 1, Section 1.4.2). For example, it has been shown that trehalose synthesis increases in yeast during ethanol stress (Mansure *et al.,* 1997), and several genes associated with trehalose biosynthesis (*TPS1, TPS2* and *TSL1*) are highly expressed in *S. cerevisiae* when exposed to ethanol stress (Alexandre *et al.,* 2001; Chandler *et al.,* 2004). Although such information suggests that trehalose, or its biosynthetic pathway, has a role in the ethanol stress response of *S. cerevisiae*, physiological and biochemical studies in this area have not yet reached a consensus on the role of trehalose in ethanol tolerance of yeast. The purpose of the work described in this chapter was to investigate the importance of trehalose and trehalose metabolism in ethanol stress tolerance of *S. cerevisiae*.

The approach used in this project was to conduct a series of physiological investigations on *Saccharomyces cerevisiae* BY4742 and complementary knockout strains bearing gene deletions in the trehalose synthesis pathway (*tps2*Δ *and tsl1*Δ) and trehalose hydrolysis pathway (*nth1*Δ) (Figure 3.1). Viability profiles of these knockout strains and the wild type were compared in the absence and presence of ethanol stress to determine whether or not trehalose metabolism has a role in the ethanol stress response and ethanol tolerance of *S. cerevisiae*.

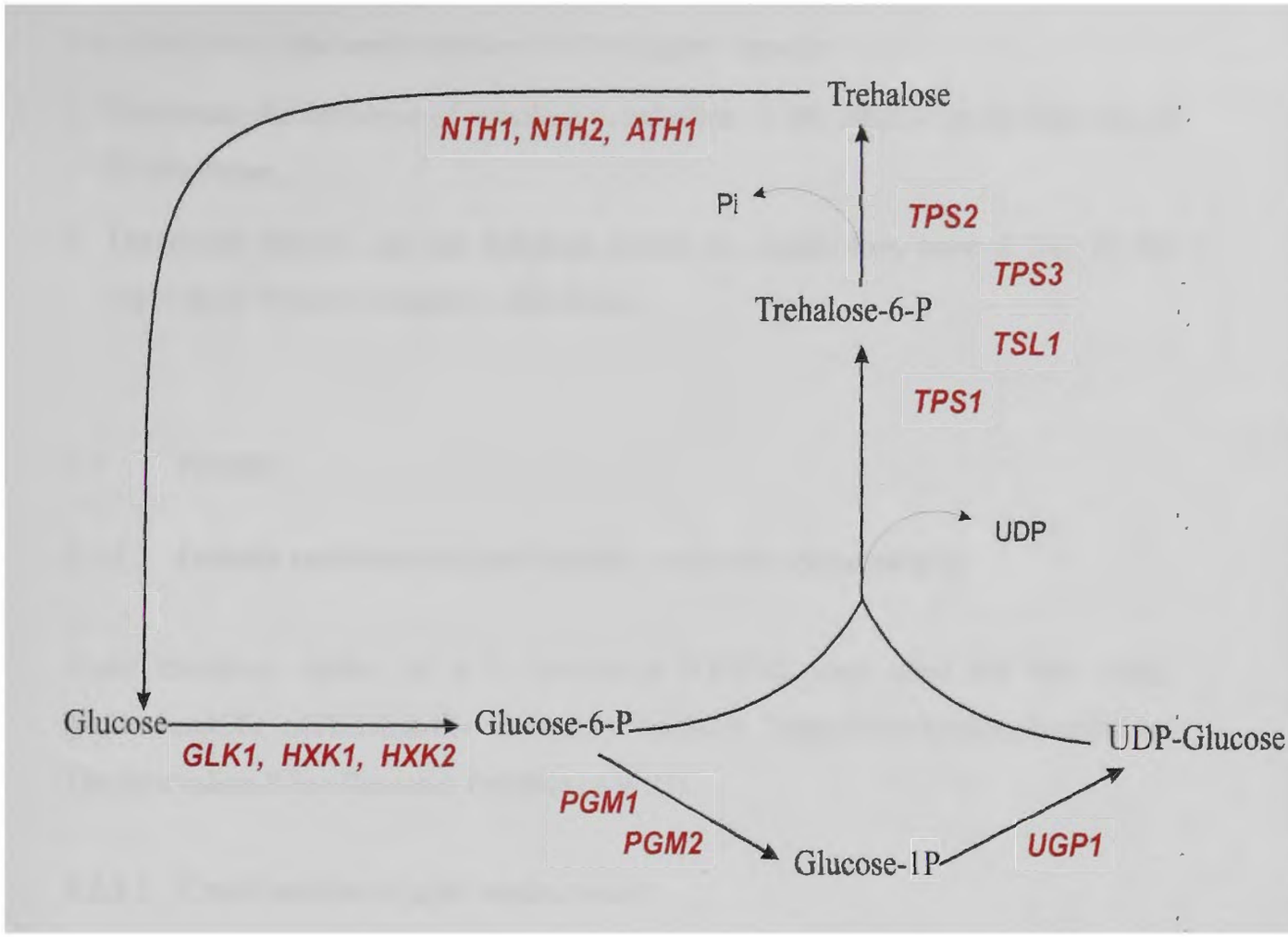


Figure 3.1: Genes involved in the trehalose metabolism of *S. cerevisiae* (shown in italics)

A notable absence in the knockout strains used in this project is strain *tps1*Δ. Previous studies with this strain have shown that it does not grow on glucose due to deregulation of glycolysis (Bell *et al.,* 1992; González *et al.,* 1992; Neves *et al.,* 1995). The *TPS1* gene encodes the small subunit of trehalose-6-phosphate synthase in the trehalose synthesis complex (Bell *et al.,* 1992) and disruption of this gene blocks the initial step of trehalose synthesis involved in trehalose-6-phosphate production. Trehalose-6-phosphate plays an important role in the regulation of the glycolysis, mainly through the inhibition of hexokinase II (Blazquez *et al.,* 1993; Hohmann *et al.,* 1996). Therefore, a *tps1*Δ strain has a growth defect on fermentable carbon sources including glucose (Bell *et al.,* 1992; González *et al.,* 1992; Neves *et al.,* 1995). For this reason, this knockout strain was not used in this project.

The objectives of the work described in this chapter were to:

1. Determine the influence of trehalose metabolism on the ethanol stress response of *S. cerevisiae*,
2. Determine whether or not trehalose and/or its metabolism have a role in the survival of ethanol-stressed *S. cerevisiae*.

3.2 Results

3.2.1 Genetic confirmation and stability of the knockout strains

Three knockout strains of a *S. cerevisiae* BY4742 were used for this work: *tps2*Δ::*kanMX4, tsl1*Δ::*kanMX4* and *nth1*Δ::*kanMX4*. These were kindly provided by The Australian Wine Research Institute (AWRI).

3.2.1.1 Confirmation of gene replacement

The presence of a *kanMX* gene in each of these strains was confirmed by plating onto both YEPD and YEPD with added Geneticin (G418) plates and incubating at 30°C for 3 days. YEPD with added Geneticin did not support the growth of the wild type strain, whereas all of the knockout strains grew on YEPD and YEPD with added Geneticin plates (Table 3.1).

The replacement of the *TPS2, TSL1* and *NTH1* ORFs with the *kanMX4* module was verified by PCR analysis. The presence and position of the *kanMX4* module was tested using a combination of two primers specific to each strain. For example, the forward primer TPS2-A was complementary to the upstream flanking regions of the *TPS2* ORF and the reverse primer TPS2-B was located within the coding region of the *TPS2* ORF of the wild type. The kan-B reverse primer was positioned within the internal sequence of the *kanMX4* module of *tps2*Δ. Details of the primer sequences are given in Appendix I, Table 1. The PCR reaction product was resolved on 1% agarose gel by electrophoresis at 80 V for ~ 60 minutes.

Table 3.1: Viable counts of *S. cerevisiae* BY4742 (wild type) and knockout strains on YEPD and YEPD geneticin (G418) plates after three subcultures in liquid medium for each strain.

Strain	**Medium**	**Viable cell counts (cells/ml)**
BY4742	YEPD	$5.4 \pm 0.2 \times 10^7$
BY4742	YEPD + geneticin	No growth
*tps2*Δ	YEPD	$3.7 \pm 0.2 \times 10^7$
*tps2*Δ	YEPD + geneticin	$3.4 \pm 0.2 \times 10^7$
*tsl1*Δ	YEPD	$5.4 \pm 0.1 \times 10^7$
*tsl1*Δ	YEPD + geneticin	$5.5 \pm 0.2 \times 10^7$
*nth1*Δ	YEPD	$5.3 \pm 0.1 \times 10^7$
*nth1*Δ	YEPD + geneticin	$5.4 \pm 0.08 \times 10^7$

All knockout strains were verified by the following observations:

- No PCR product was observed when primer combination TPS2-A (flanking) with TPS2-B (internal) was used with *tps2*Δ, whereas amplification using wild type DNA as template generated products of the expected size *i.e.* 532 bp. PCR products with sizes 615 bp were generated as predicted from the TPS2-A with kan-B primer combinations for the *tps2*Δ strain. Using the same primer combinations for the wild type did not result in amplification products (Figure 3.2).

- Same as with the primer combination of TSL1-A/TSL1-B, no PCR product was amplified using DNA from *tsl1*Δ cells, whereas amplification using wild type DNA as template generated products of the expected size 912 bp. Using TSL1-A /kan-B primer combination with DNA from *tsl1*Δ cells resulted in a 662 bp PCR product. No PCR product was amplified for the wild type strain (Figure 3.3)

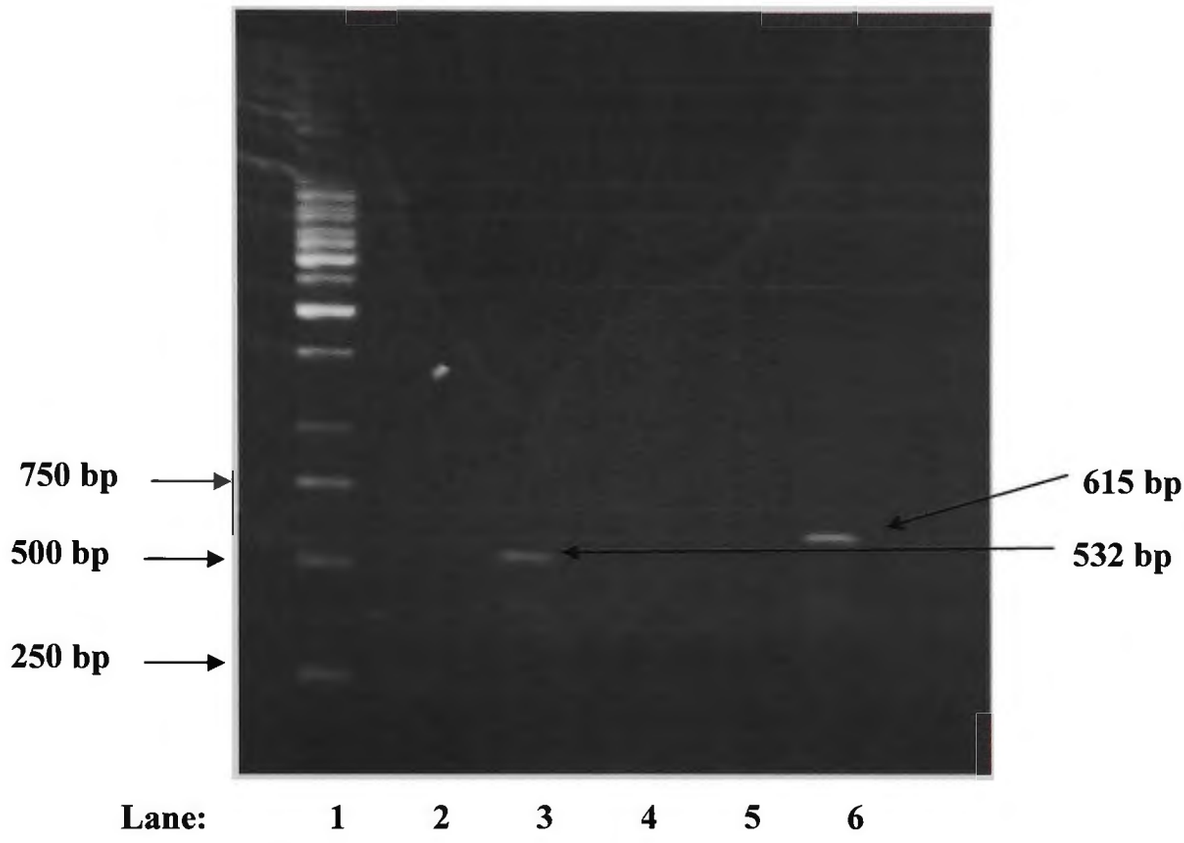


Figure 3.2: Confirmation of the replacement of *TPS2* with *kanMX4* in *S. cerevisiae* BY4742 *tps2*Δ. PCR products were resolved on a 1.5% agarose gel. Lane 1: Marker DNA, Lane 3: A PCR product of 532 bp was amplified from the TPS2-A and TPS2-B primer combination using DNA from wild type strain. Lane 4: No PCR products were generated for primer combinations TPS2-A; kan-B with wild type DNA. Lane 5: No PCR products were amplified for primer combinations TPS2-A; TPS2-B using DNA from *tps2*Δ strain. Lane 6: A product of 615 bp resulted for the primer combinations TPS2-A; kan-B using DNA from *tps2*Δ strain.

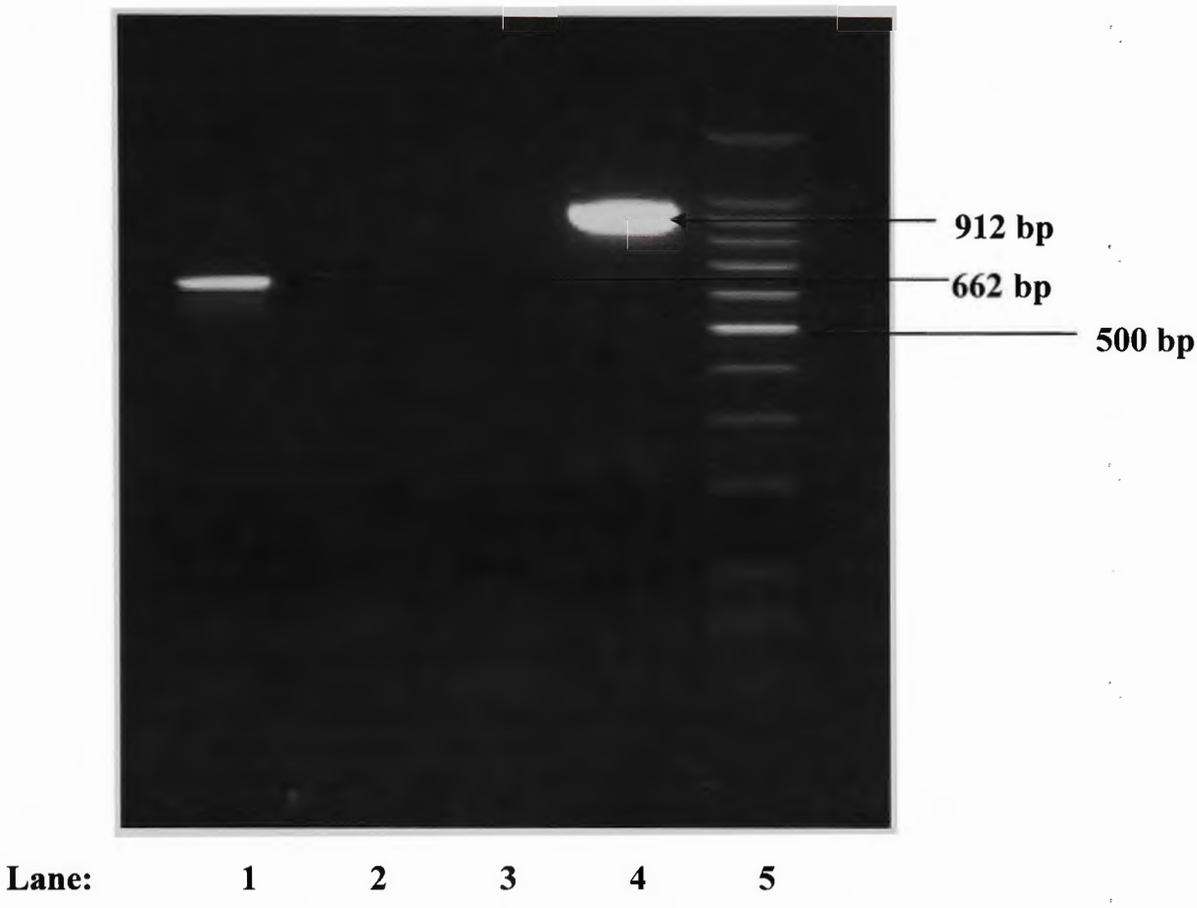


Figure 3.3: Confirmation of the replacement of *TSL1* with *kanMX4* in *S. cerevisiae* BY4742 *tsl1*Δ.. PCR products were resolved on a 1.5% agarose gel. Lane 5: Marker DNA. Lane 4: A PCR product of 912 bp was amplified from the TSL1-A and TSL1-B primer combination using DNA from wild type strain. Lane 3: No PCR products were generated for primer combinations TSL1-A; kan-B with wild type DNA. Lane 2: No PCR products were amplified for primer combinations TSL1-A; TSL1-B using DNA from *tsl1*Δ strain. Lane 1: A product of 662 bp resulted for the primer combinations TSL1-A; kan-B using DNA from *tsl1*Δ strain.

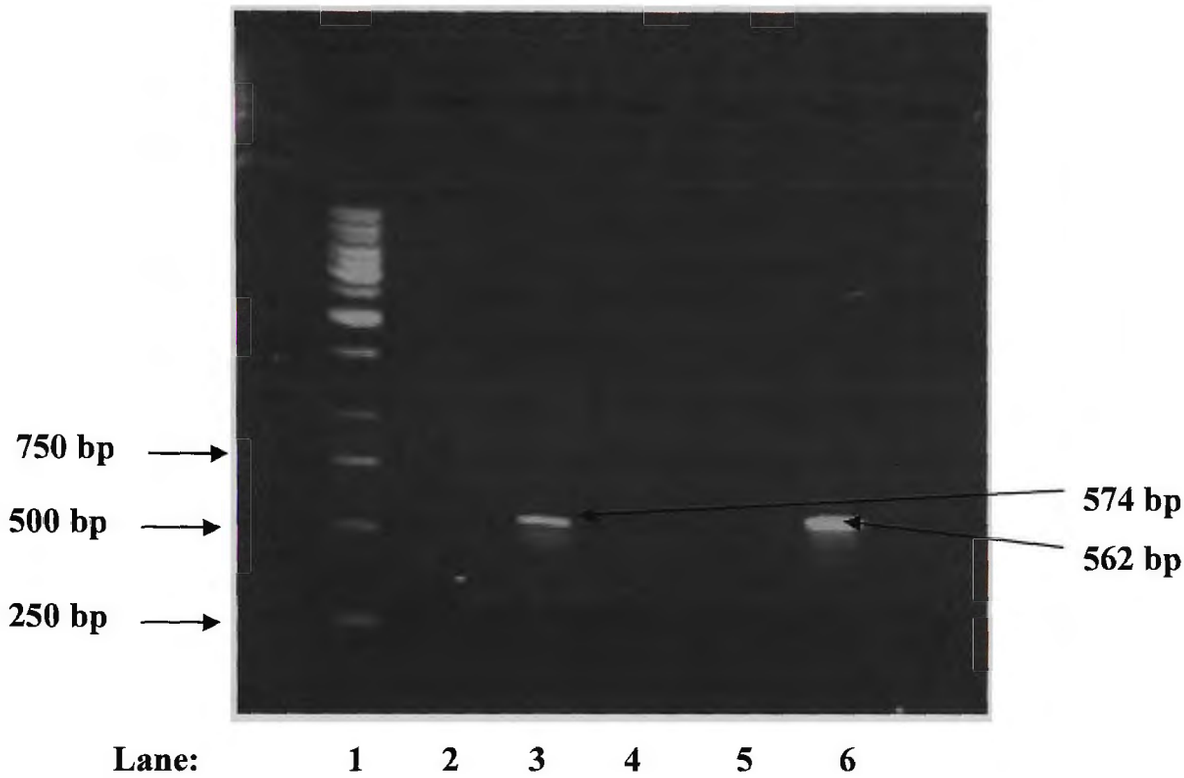


Figure 3.4: Confirmation of the replacement of *NTH1* with *kanMX4* in *S. cerevisiae* BY4742 *nth1*Δ.. PCR products were resolved on a 1.5% agarose gel. Lane 1: Marker DNA. Lane 3: A PCR product of 574 bp was amplified from the NTH1-A and NTH1-B primer combination using DNA from wild type strain. Lane 4: No PCR products were generated for primer combinations NTH1-A; kan-B with wild type DNA. Lane 5: No PCR products were amplified for primer combinations NTH1-A; NTH1-B using DNA from *nth1*Δ strain. Lane 6: A product of 562 bp resulted for the primer combinations NTH1-A; kan-B using DNA from *nth1*Δ strain.

- As shown in Figure 3.4 with primer combination NTH1-A/NTH1-B, no PCR product was amplified using DNA from *nth1*Δ cells, but a 562 bp product was observed for the DNA from wild type cells. A PCR product of 574 bp was generated from the NTH1-A/*kan*-B primer combination for the *nth1*Δ strain and the same primer combination for the wild type did not result in amplification products.

3.2.1.2 Stability of the knockout strains

To test the stability of knockout strains, separate cultures of the wild type (BY4742) and knockout strains were serially subcultured three times in non-selective YEPD medium. The strains were then plated onto both YEPD and YEPD-Geneticin plates and incubated at 30°C for three days. The YEPD-Geneticin plates did not support the growth of wild type cells. After three subcultures of all knockout strains, YEPD and YEPD-Geneticin plates showed equivalent cell numbers (Table 3.1), providing evidence of the stability of *kanMX4* in all knockout strains.

3.2.2 Impact of gene deletions in trehalose metabolism on intracellular trehalose levels in *S. cerevisiae*

Trehalose levels in *S. cerevisiae* wild type and knockout strains were determined at various growth phases according to the method described in Chapter 2, (Section 2.3). The wild type and *nth1*Δ strains accumulated trehalose during late exponential phase. The *nth1*Δ strain, having a disruption in trehalose mobilization, had a trehalose content that was more than two-fold higher than the wild type in late exponential phase (Figure 3.5). Strains *tps2*Δ and *tsl1*Δ, containing disrupted trehalose biosynthesis, did not have detectable amounts of trehalose during the late exponential phase. The intracellular trehalose concentration increased in *tsl1*Δ, wild type and *nth1*Δ cells, when they entered stationary phase and continued to rise with increasing incubation time in stationary phase. The *tsl1*Δ strain, lacking Tsl1 regulatory protein in the trehalose synthase complex, had significantly lower trehalose concentrations than the wild type strain throughout incubation in the stationary phase. The highest trehalose levels were observed in the *nth1*Δ strain at all incubation stages. As reported previously (Ratnakumar and Tunnacliffe 2006), the *tps2*Δ strain had very low

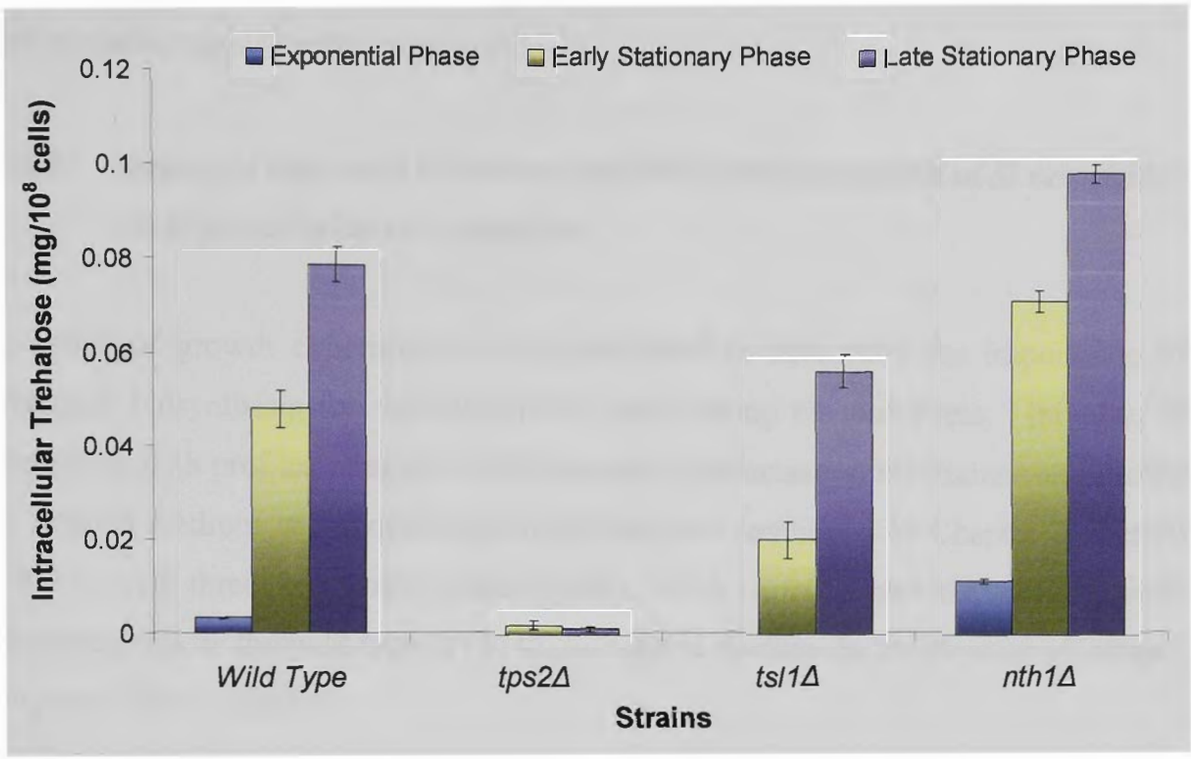


Figure 3.5: Intracellular trehalose concentrations (μg/1x10^8 cells) of wild type (BY4742) and its knockouts (*tps2*Δ, *tsl1*Δ, *nth1*Δ) grown in defined medium at late exponential phase, early stationary phase, and late stationary phase of growth (for growth experiments, see Appendix IV, Figure 5). Cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and error bars indicate standard deviation from the mean. These experiments were repeated twice with reproducible results.

trehalose levels during incubation in the stationary phase, which was not expected given that Tps2 is presumably absent in the cell. It has previously been speculated that trehalose production in such a background might occur via Tps2 homologous proteins or via a different mechanism (Bell *et al.,* 1998). Overall, at all growth stages, strains *tps2*Δ and *tsl1*Δ produced significantly less trehalose than the wild type whereas *nth1*Δ carried significantly more trehalose.

3.2.3 Impact of disrupted trehalose metabolism on the growth of *S. cerevisiae* during non-lethal ethanol stress

A series of growth experiments were performed to determine the importance of trehalose biosynthesis for the survival of yeast during ethanol stress. Initially, to analyse growth profiles of strains under normal circumstances, all strains were grown in defined medium under optimum conditions (see conditions in Chapter 2 Section 2.2.2.1). All three knockout strains (*tps2*Δ, *tsl1*Δ, *nth1*Δ) had the same growth characteristics as the wild type BY4742, in defined medium in the absence of ethanol (Figures 3.6, 3.7 and 3.8).

3.2.3.1 Impact of disrupted trehalose biosynthesis on *S. cerevisiae* growth during non-lethal ethanol stress

These first experiments investigated the impact of metabolic flux restrictions in the trehalose biosynthesis pathway on the ethanol stress response of *S. cerevisiae* in the presence of non-lethal ethanol concentrations (not sufficient to cause death of strains in this study). Late-exponential phase cells were inoculated into fresh medium containing various ethanol concentrations in the range of 0 to 9% (v/v) and their growth profiles were determined. Preliminary experiments identified this ethanol concentration range as being inhibitory, but not lethal, to the growth of the wild type strain, BY4742 (data not shown). The well-documented response of yeast subjected to these conditions is to initially undergo a lag period, the length of which represents the acclimatisation rate of the cells to the stress, and then to commence exponential growth for which the growth rate indicates how well-acclimatised the cells are to the stress (Stanley *et al.,* 1997). These experiments were conducted using BY4742 as the control and strains *tps2*Δ and *tsl1*Δ to determine if a reduction in metabolic flux in the

trehalose pathway and lower intracellular trehalose levels affect the ability of *S. cerevisiae* to acclimatise to, and grow in, non-lethal ethanol concentrations.

S. cerevisiae BY4742 and the *tsl1*Δ strain both had increasingly long lag periods and lower growth rates as ethanol concentrations increased from 6-9 % (v/v) (Figure 3.6). There was no significant difference in the growth profiles of these two strains under each of the conditions used. In the absence of ethanol stress neither strain experienced a detectable lag period and both strains had a specific growth rate of approximately 0.30 h^{-1}. In the presence of 6% (v/v), 7% (v/v) and 8% (v/v) ethanol, both of these strains had lag periods of around 1 hr, 4 hrs and 8 hrs respectively, and specific growth rates of approximately 0.13 h^{-1}, 0.10 h^{-1} and 0.09 h^{-1} respectively (Table 3.2). There was no clear exit from the lag period for either BY4742 or *tsl1*Δ strains after 12 hours of incubation in 9% (v/v) ethanol with the viable cell population remaining constant at around 5 x 10^6 cells ml^{-1} over this time period. The lower metabolic flux in trehalose metabolism and lower intracellular trehalose concentrations apparently had no impact on the ability of the cells to acclimatise to non-lethal ethanol stress nor on their subsequent growth rate in the presence of the stress.

Compared to strain BY4742, the *tps2*Δ strain had considerable difficulties in acclimatising to all ethanol concentrations used and the viable population in *tps2*Δ cultures began to decrease in the absence of added ethanol as the culture entered the late exponential phase (Figure 3.7). This may be related to the impact of not having a fully-functioning trehalose pathway; however, the decline in cell population in the absence of ethanol stress suggests that other factors may be involved. *S. cerevisiae tps2*Δ strains are reported to have viability issues due to intracellular accumulation of trehalose-6-phosphate, especially during exposure to stressors such as heat shock, osmotic stress and nutrition limitation (De Virgiolio, 1993; Hounsa et al., 1998); intracellular accumulation of trehalose-6-phosphate is known to be toxic to yeast (Thevelein, 1995; Gancedo, 2004; Zaragoza, 2002). Given the uncertainty concerning the reasons for the loss of viability in this strain (especially in the absence of ethanol stress), it was decided not to use strain *tps2*Δ for the remainder of the project.

Table 3.2: The effect of ethanol on the growth profiles of *S. cerevisiae* wild type (strain BY4742) and *tsl1*Δ *and nth1*Δ strains (from Figures 3.6 and 3.8).

Ethanol Concentration (v/v)	Strain	Lag Period (h)	Specific Growth Rate (h^{-1})
0%	Wild Type	-	0.298 ± 0.0027
	*tsl11*Δ	-	0.301 ± 0.0037
	*nth1*Δ	-	0.293 ± 0.0088
6%	Wild Type	1.00 ± 0.1	0.133 ± 0.0016
	*tsl1*Δ	1.2 ± 0.2	0.128 ± 0.0033
	*nth1*Δ	1.10 ± 0.1	0.127 ± 0.0016
7%	Wild Type	4.20 ± 0.1	0.103 ± 0.0011
	*tsl1*Δ	4.2 ± 0.2	0.106 ± 0.0023
	*nth1*Δ	4.00 ± 0.1	0.099 ± 0.0030
8%	Wild Type	8.00 ± 0.1	0.090 ± 0.0014
	*asr1*Δ	8.20 ± 0.1	0.084 ± 0.0036
	*nth1*Δ	8.10 ± 0.2	0.087 ± 0.0030
9%	Wild Type	-	-
	*tsl1*Δ	-	-
	*nth1*Δ	-	-

- Not measurable

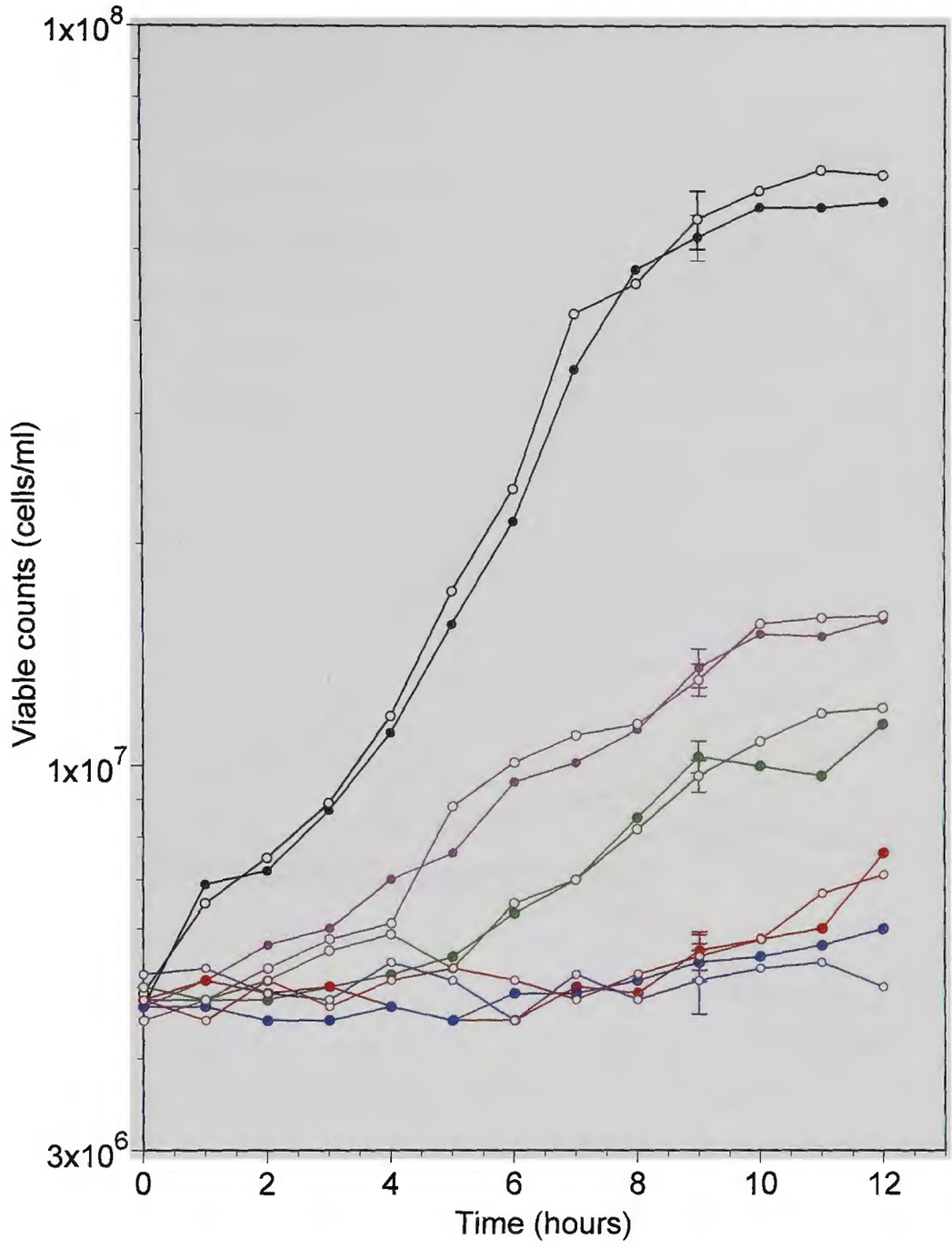


Figure 3.6: The effect of ethanol concentrations on the viable cell populations of *S. cerevisiae* wild type and *tsl1*Δ strains. Cells from a late exponential phase parent culture were washed and inoculated into defined medium only (●, ○) or defined medium containing added ethanol at either 6% v/v (●, ○), 7% v/v (●, ○), 8% v/v (●, ○) or 9% v/v (●, ○). Wild type and *tsl1*Δ cultures are represented by closed and open symbols respectively. The cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for the nine hour time point only. All experiments were repeated at least twice with reproducible results.

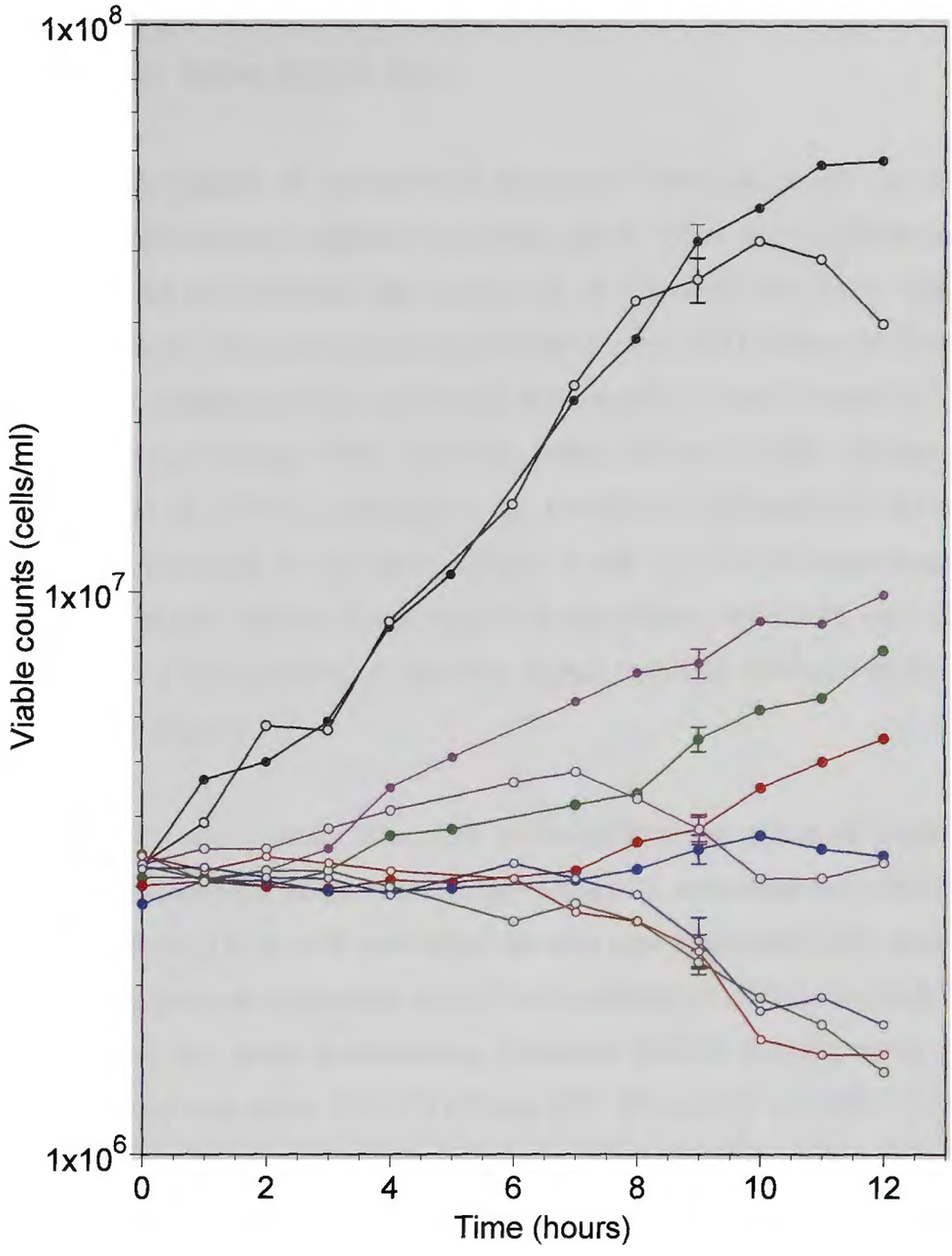


Figure 3.7: The effect of ethanol concentrations on the viable cell populations of *S. cerevisiae* wild type and *tps2*Δ strains. Cells from a late exponential phase parent culture were washed and inoculated into defined medium only (●, ○) or defined medium containing added ethanol at either 6% v/v (●, ○), 7% v/v (●, ○), 8% v/v (●, ○) or 9% v/v (●, ○). Wild type and *tps2*Δ cultures are represented by closed and open symbols respectively. The cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for the nine hour time point only. All experiments were repeated at least twice with reproducible results.

3.2.3.2 Impact of disrupted trehalose mobilization on the growth of *S. cerevisiae* during ethanol stress

Investigating the impact of intracellular trehalose accumulation on the ethanol sensitivity of *S. cerevisiae* is another important aspect of this study. Mobilisation of cytosolic trehalose by hydrolysis into glucose is an important step in the trehalose metabolism of yeast. This part of yeast metabolism is catalysed by three enzymes: two neutral cytosolic trehalases (Nth1 and Nth2), and an acidic vacuolar trehalase (Ath1) (Londesborough and Varimo 1984; Thevelein 1984). Previous studies (Nwaka *et al.,* 1995a; Nwaka *et al.,* 1995b; Zahringer *et al.,* 1998) have indicated that the neutral trehalase, Nth1 (encoded by the gene *NTH1*) is the key enzyme accountable for trehalose mobilisation. Earlier in this chapter is was shown that *nth1*Δ cells lacking neutral trehalase (Nth1) activity accumulate higher cytosolic trehalose levels at all stages of growth (Figure 3.5).

In this study the *nth1*Δ strain was used to investigate the effect of intracellular trehalose accumulation on the growth and lag period of *S. cerevisiae* subjected to non-lethal ethanol stress. The growth profiles of the wild type strain and *nth1*Δ strain were compared in the absence or presence of 6-9% (v/v) ethanol in defined medium. Under these conditions, the strain accumulating trehalose (*nth1*Δ) had the same growth profile as the wild type strain BY4742 (Figure 3.8). The results in Table 3.2 suggest that trehalose accumulation may not have an impact on the ability of yeast to acclimatise to non-lethal ethanol stress, since the wild type and *nth1*Δ strains had the same lag periods and then commenced growth at similar rates when inoculated into medium containing either 6% (v/v), 7% (v/v) or 8% (v/v) ethanol. There was no apparent growth for either strain when incubated in 9% (v/v) ethanol for 12 hours (Figure 3.8).

S. cerevisiae strains *tsl1*Δ and *nth1*Δ responded to non-lethal ethanol stress conditions similarly to the wild type strain, suggesting that trehalose has no significant role in the acclimatisation of *S. cerevisiae* to non-lethal ethanol stress. Given that many studies on the role of trehalose in yeast tolerance to stress use lethal stress conditions, it may be that trehalose has a greater role in *S. cerevisiae* survival in the presence of lethal ethanol concentrations.

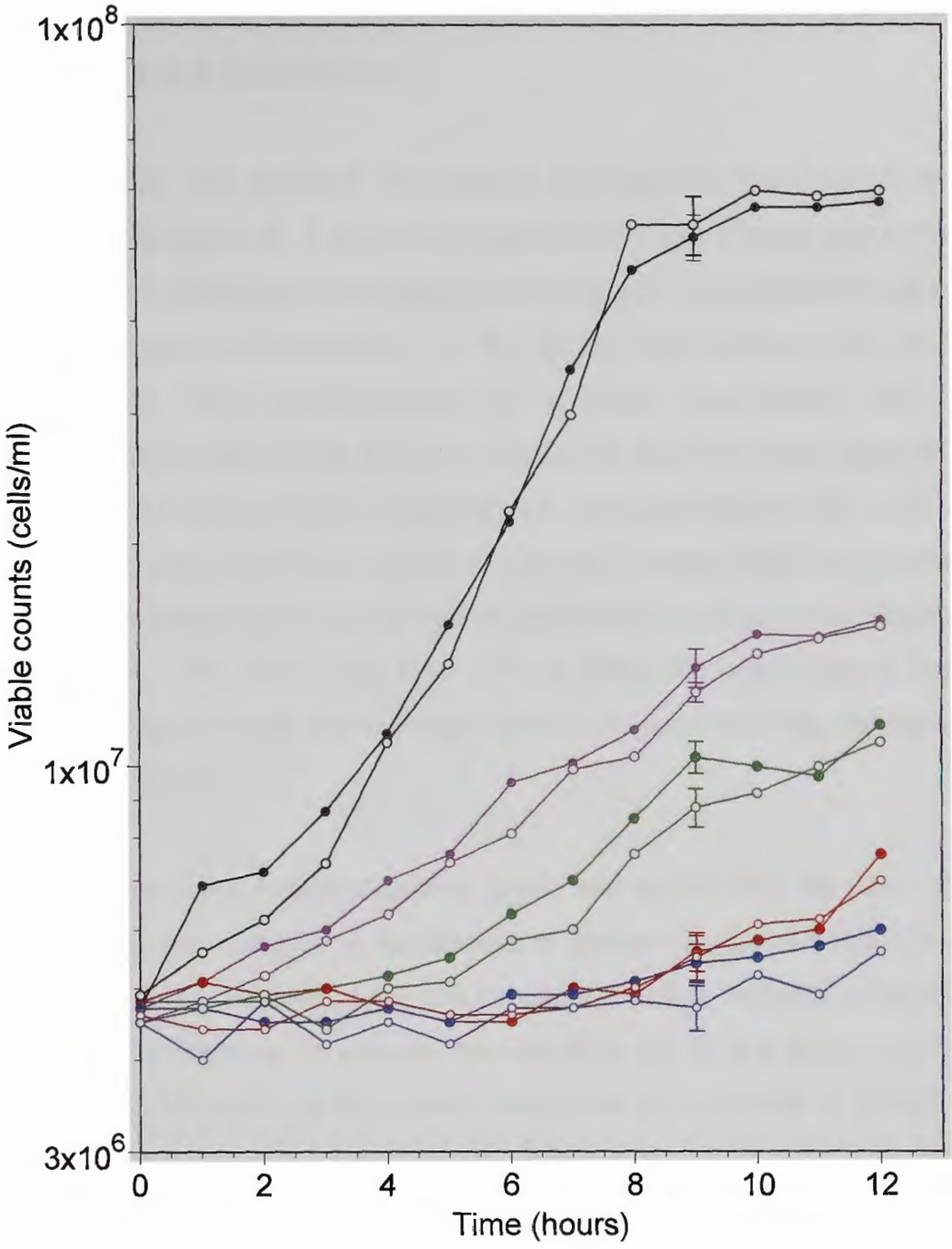


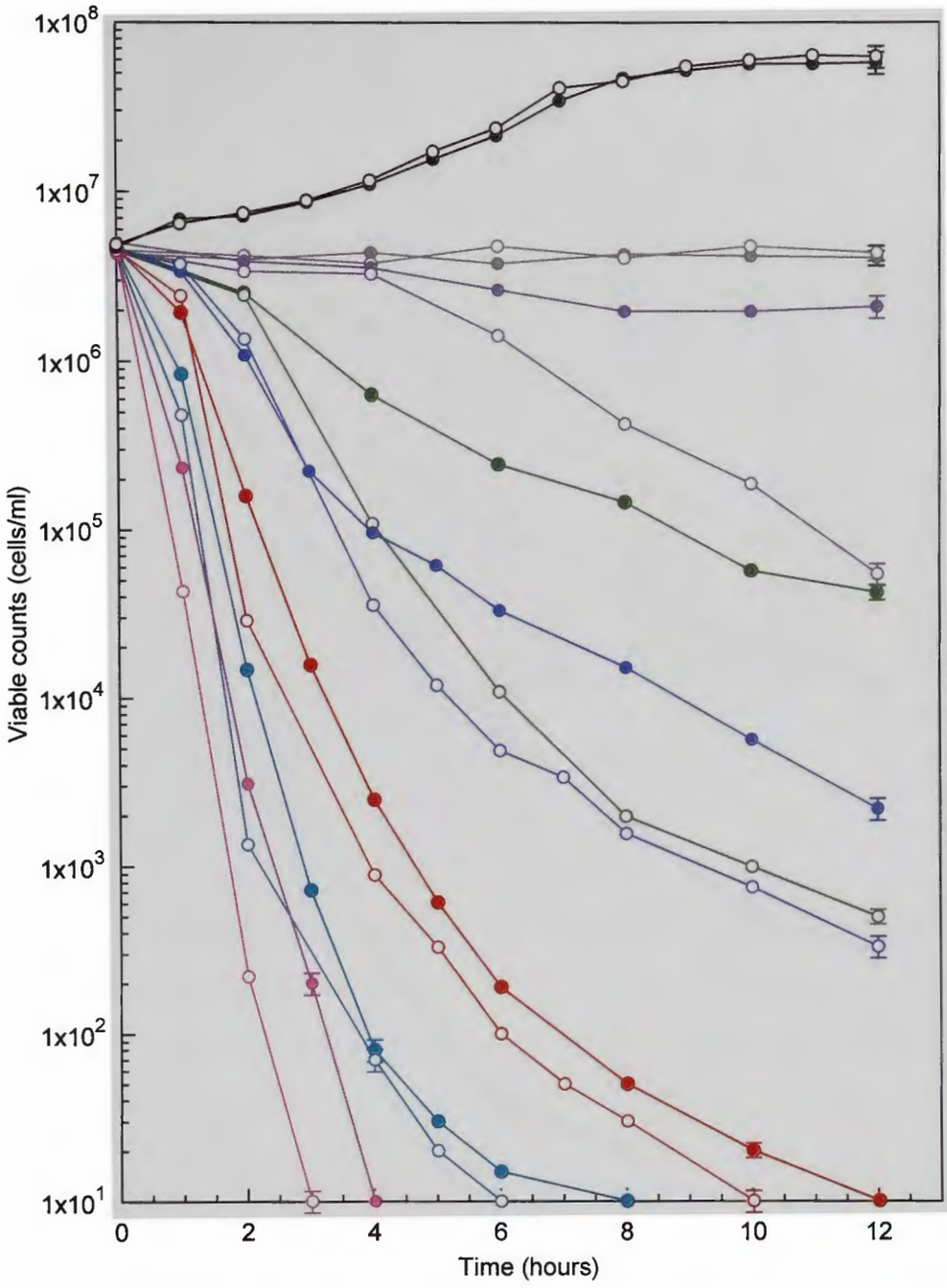
Figure 3.8: The effect of ethanol concentrations on the viable cell populations of *S. cerevisiae* wild type and n*thl1*Δ strains. Cells from a late exponential phase parent culture were washed and inoculated into defined medium only (●, ○) or defined medium containing added ethanol at either 6% v/v (●, ○), 7% v/v (●, ○), 8% v/v (●, ○) or 9% v/v (●, ○). Wild type and *nth1*Δ cultures are represented by closed and open symbols respectively. The cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for the nine hour time point only. All experiments were repeated at least twice with reproducible results.

3.2.4 Impact of disrupted trehalose metabolism on the survival of *S. cerevisiae* during lethal ethanol stress

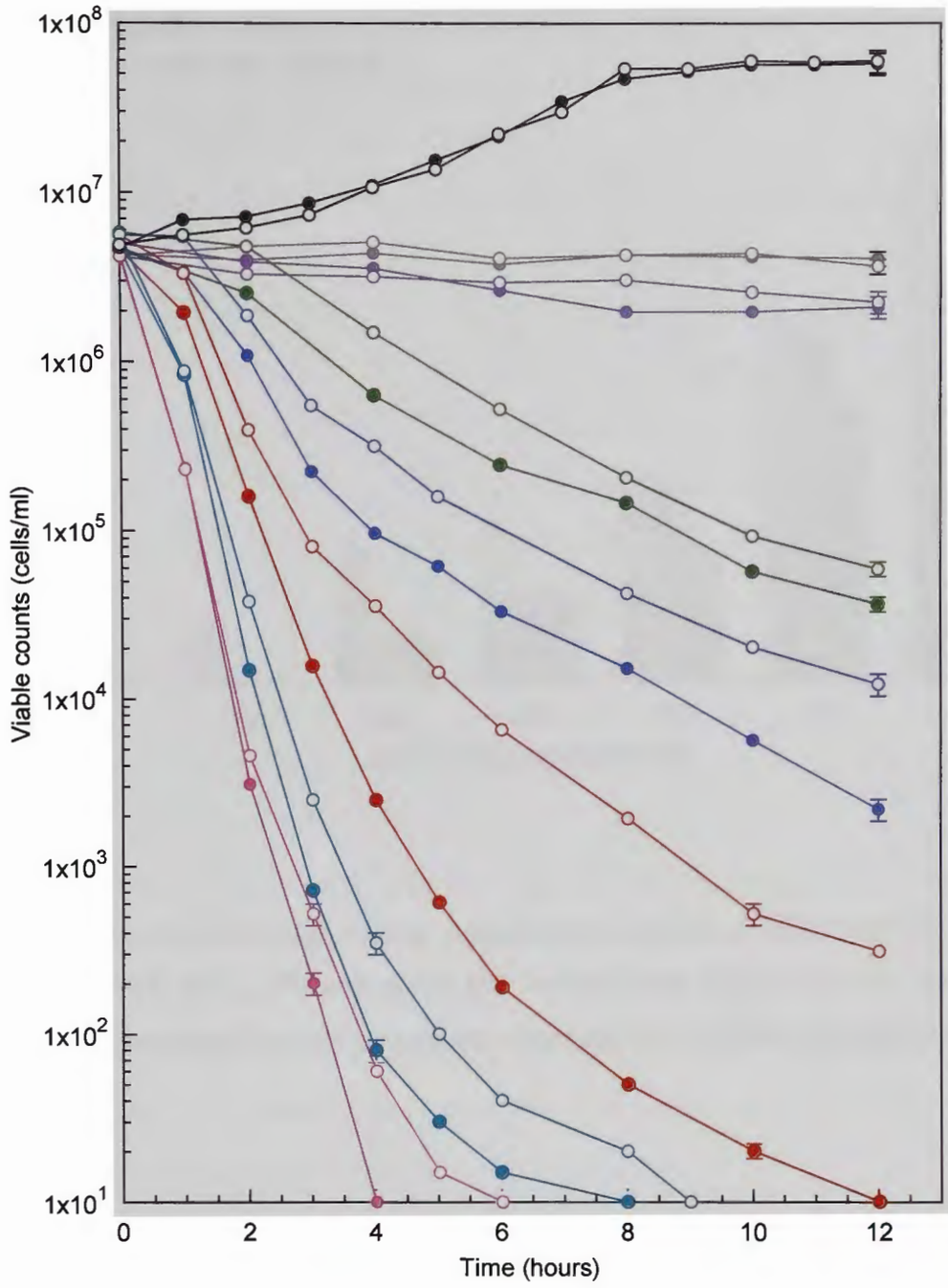
It was speculated that different biochemical and physical mechanisms may be involved in the survival of *S. cerevisiae* exposed to lethal ethanol concentrations, compared to the biochemical mechanisms promoting cell acclimatisation and growth in non-lethal ethanol concentrations. In this section the survival of *S. cerevisiae* knockout strains *tsl1*Δ (compromised in trehalose production) and *nth1*Δ (accumulates intracellular trehalose) were compared to their wild type strain BY4742 in the presence of lethal ethanol concentrations. The experimental plan used in the previous section was modified to study cell survival in lethal ethanol concentrations. Late exponential phase cells were inoculated into defined medium in the absence and presence of 10%, 12%, 14%, 15%, 16%, 17% or 18% (v/v) added ethanol. Samples were taken at regular time intervals and viable cell population was monitored by duplicate plate counts.

As shown before, both knockout strains (*tsl1*Δ and *nth1*Δ) had the same growth characteristics as the wild type in the absence of ethanol (Figures 3.9 and 3.10). The growth rate of all strains (wild type and knockouts) during sublethal ethanol stress declined with an increase in ethanol concentration up to 9% (v/v). An ethanol concentration of 10% (v/v) in the present experiment did not cause a change in the viable cell population over a 12 hour period. When *S. cerevisiae* wild type and *tsl1*Δ strains were subjected to ethanol concentrations higher than 10% (v/v), the viable cell population began to decrease over time. When the death rates of the wild type strain were compared to the trehalose deficient knockout (*tsl1*Δ), the latter was found to be more sensitive than the wild type strain to lethal ethanol conditions ie. The viable cell population of *tsl1*Δ decreased at a substantially higher rate (Figures 3.9 and 3.11). The *tsl1*Δ strain was considerably more sensitive to ethanol than the wild type strain at ethanol concentrations of 12%, 14%, 15% and 18% (v/v).

As reported previously in Hounsa *et al.* (1998), the intracellular trehalose concentration of *nth1*Δ strains grown on various substrates in the exponential or stationary phase, were at least two-fold higher than the wild type strain grown under similar conditions. A similar result was observed earlier in this project when



Figures 3.9: The effect of lethal ethanol concentrations on the viable cell population of *S. cerevisiae* wild type and *tsl1*Δ strains. Cells from a late exponential phase parent culture were washed and inoculated into defined medium only (●, ○) or defined medium containing added ethanol at either 10% v/v (●, ○), 12% v/v (●, ○), 14% v/v (●, ○), or 15% v/v (●, ○), 16% v/v (●, ○), 17% v/v (●, ○) or 18% v/v (●, ○). Wild type and *tsl1*Δ cultures are represented by closed and open symbols respectively. The cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for a single time point only. All experiments were repeated at least twice with reproducible results.



Figures 3.10: The effect of lethal ethanol concentrations on the viable cell population of *S. cerevisiae* wild type and *nth1*Δ strains. Cells from a late exponential phase parent culture were washed and inoculated into defined medium only (●, ○) or defined medium containing added ethanol at either 10% v/v (●, ○), 12% v/v (●, ○), 14% v/v (●, ○), or 15% v/v (●, ○), 16% v/v (●, ○), 17% v/v (●, ○) or 18% v/v (●, ○). Wild type and *nth1*Δ cultures are represented by closed and open symbols respectively. The cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for a single time point only. All experiments were repeated at least twice with reproducible results.

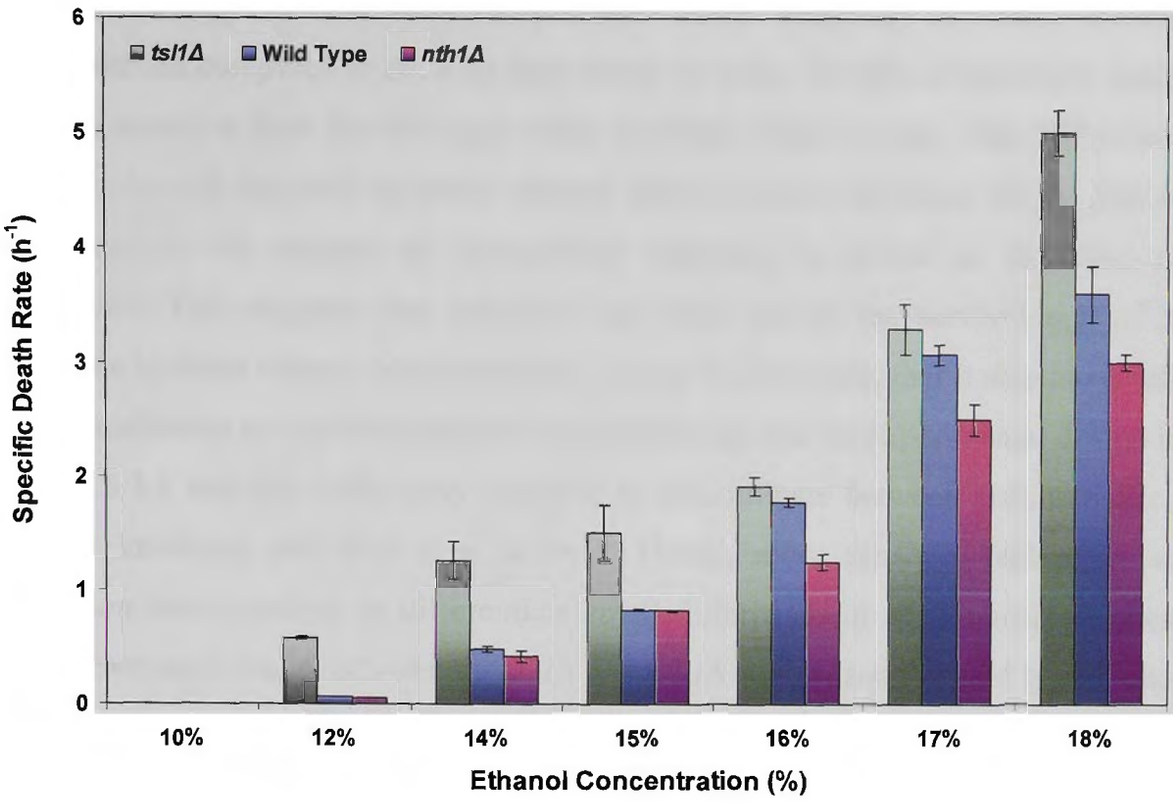


Figure 3.11: The effect of lethal ethanol concentrations on the specific death rate of *tsl1*Δ (■), wild type (BY 4742) (■) and *nth1*Δ (■) strains (from Figures 3.9 and 3.10). Each experiment was conducted in triplicate and error bars indicate standard deviation from the mean.

analysing trehalose levels of the *nth1*Δ strain grown in defined medium up to late exponential phase (Figure 3.5). When the *nth1*Δ strain was exposed to lethal ethanol concentrations, it had a significantly higher tolerance to the lethal stress compared to the wild type strain (Figure 3.10). A considerable difference in viable cell population was observed at ethanol concentrations of 16% 17% and 18% (v/v). The death rate of the *nth1*Δ strain exposed to lethal ethanol concentrations was lower than observed for the wild type and much lower than those measured for the *tsl1*Δ strain (Figure 3.11). No considerable difference in viable cell profiles of either knockout strain was observed when compared with wild type strain in the presence of 10% ethanol during twelve hours of stress, although a longer incubation time might have revealed some differences in phenotype.

Overall, viability of the *tsl1*Δ strain was more sensitive to lethal ethanol concentrations compared to the wild type strain, whereas viability of the *nth1*Δ strain was less sensitive than the wild type strain to lethal ethanol stress. The differences observed in cell survival in lethal ethanol concentrations are most likely due to differences in the amount of intracellular trehalose in strains at the time of inoculation. This suggests that trehalose has some role in the survival rate of *S. cerevisiae* in lethal ethanol concentrations. It may be, however, that it also has a role in acclimatisation to non-lethal ethanol concentrations, but the experimental design in Section 3.2.3 was not sufficiently sensitive to discriminate between acclimatisation rates of knockout and wild type strains. Hence, more sensitive techniques of cultivation were required to differentiate small differences in acclimatisation rates, growth rates and vitality between the *tsl1*Δ and *nth1*Δ strains, and the wild type strain.

3.2.5 Competitive growth experiments

The ability of conventional batch growth experiments to demarcate phenotypes is limited since minor differences in cell vitality may not be detected. Hence, a more sensitive experimental method was needed to determine phenotypic differences between *S. cerevisiae* strains during non-lethal ethanol stress. To meet this need, competitive growth experiments were performed in an attempt to detect marginal differences in growth rates that might exist between wild type and *tsl1*Δ or *nthl1*Δ strains during non-lethal ethanol stress. For example, if the *TSL1* gene in the wild type provides a competitive advantage against the deletion strain (*tsl1*Δ) when grown under conditions of ethanol stress, then a growth competition experiment may detect such a competitive edge. This approach was used by Baganz *et al.* (1998) and Thatcher *et al.* (1998) who found that competition experiments are capable of detecting, and accurately quantifying, growth rate differences that could not be detected using conventional batch growth curves (Baganz *et al.,* 1998; Thatcher *et al.,* 1998).

In this study, competitive growth experiments comprised equal cell populations of wild type and knockout strains incubated together in the same flask containing defined medium with 7% (v/v) added ethanol. A control experiment was designed where the strains were grown in competition, but with no added ethanol. After every

six generations, cells were subcultured into fresh medium and plated onto YEPD plates and YEPD-Geneticin (G418) plates. The relative population (frequency) of each strain in co-culture was determined by viable cell counts. Since both strains can grow on YEPD plates, but only the knockout strain is able to grow in the presence of Geneticin, the cell population of each strain could be determined. The *S. cerevisiae* wild type strain with either the *tsl1*Δ or *nthl1*Δ strain were grown together for multiple generations and the frequencies of each genotype in the population were determined periodically in the presence or absence of added 7% (v/v) ethanol.

In competitive growth experiments, the *tsl1*Δ and wild type strains had similar competitive fitness levels in the presence and absence of added ethanol (Figure 3.12). Similarly, the wild type and *nthl1*Δ strains were indistinguishable in their competitive fitness over 36 generations in the presence or absence of 7% (v/v) (Figure 3.13). This suggests that the presence of intracellular trehalose offers no competitive advantage for *S. cerevisiae* growing in non-lethal (7% v/v) ethanol concentrations. Although trehalose has no detectable role in the growth of *S. cerevisiae* in non-lethal ethanol concentrations, earlier work demonstrates that it has a role in the survival of *S. cerevisiae* when exposed to lethal ethanol concentrations.

3.2.6 Trehalose levels and the survival of *S. cerevisiae* in the presence of lethal ethanol concentrations

It is reasonable to assume that *S. cerevisiae* metabolism is compromised in the presence of lethal ethanol concentrations to the extent that the cell is unlikely to be capable of producing significant amounts of trehalose. The differences observed between the strains in survival rates during exposure to lethal ethanol concentrations are probably due to the differences in the amount of trehalose in the strains at the time of inoculation. To investigate this further, *tsl1*Δ and *nth1*Δ cells harvested from parent cultures at different stages of incubation, were inoculated into medium containing 16% (v/v) ethanol and their cell populations were compared over time.

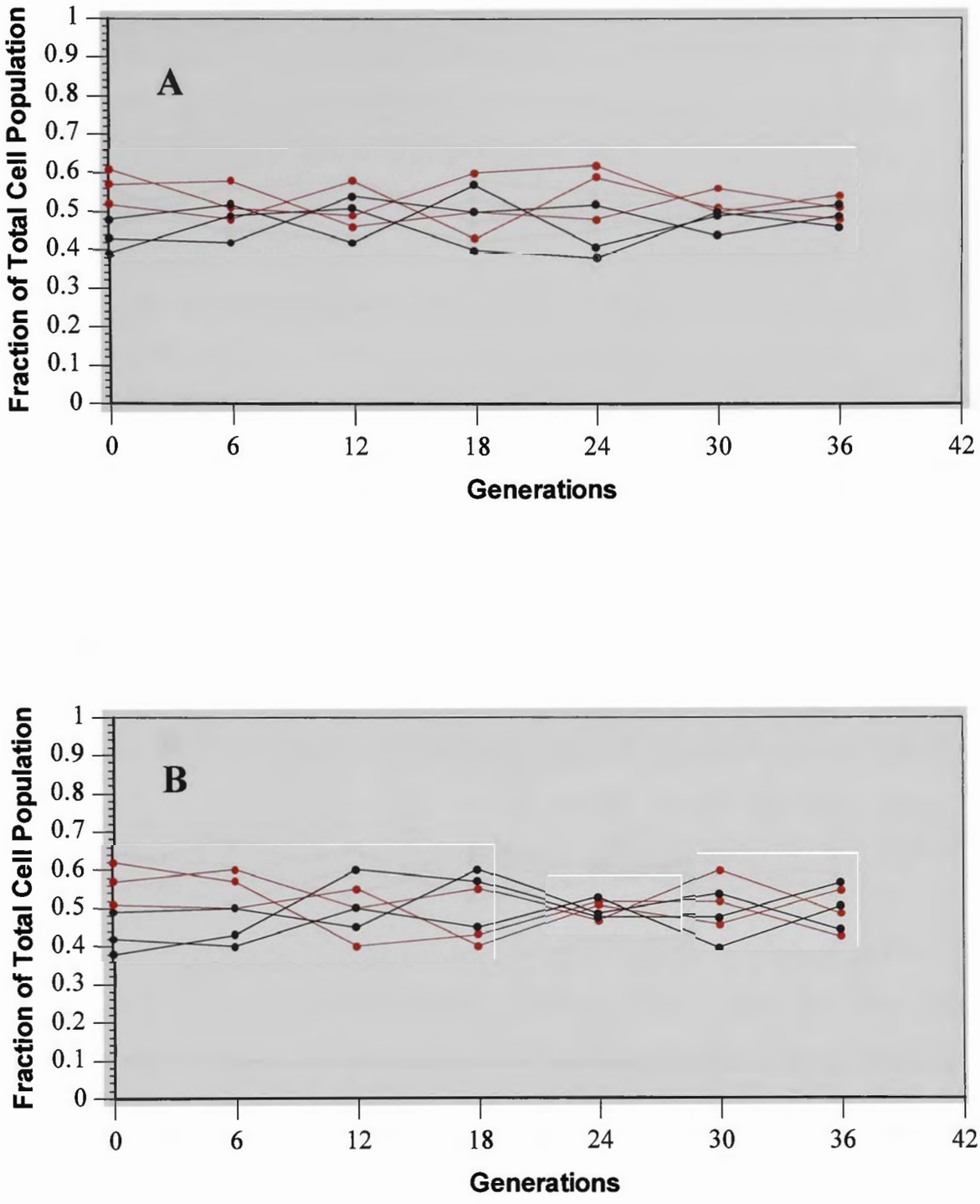
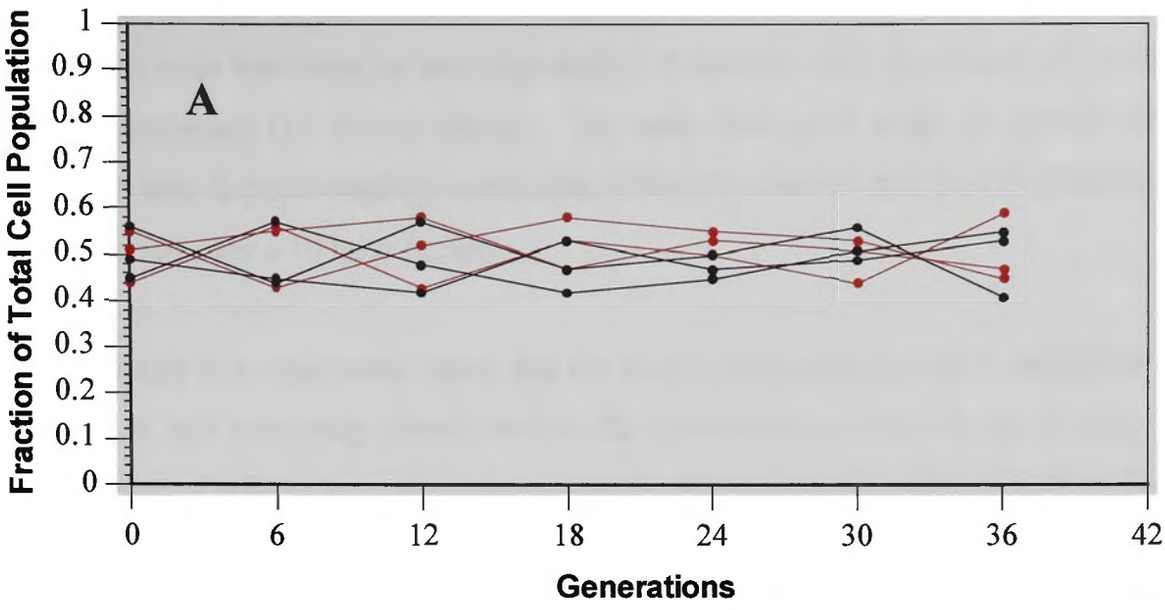


Figure 3.12: The effect of ethanol on viable cell populations in serial co-cultures comprising both wild type (●) and *tsl11*Δ (●) strains. Initially, equal numbers of late exponential phase cells were inoculated into defined medium only (**A**) and defined medium containing 7% v/v ethanol (**B**). After six generations of growth, mixed populations were serially transferred into fresh medium under similar conditions. The cultures were incubated at 30°C/130 rpm. The results of three separate biological replicates which were initiated from three separate patent cultures are presented.



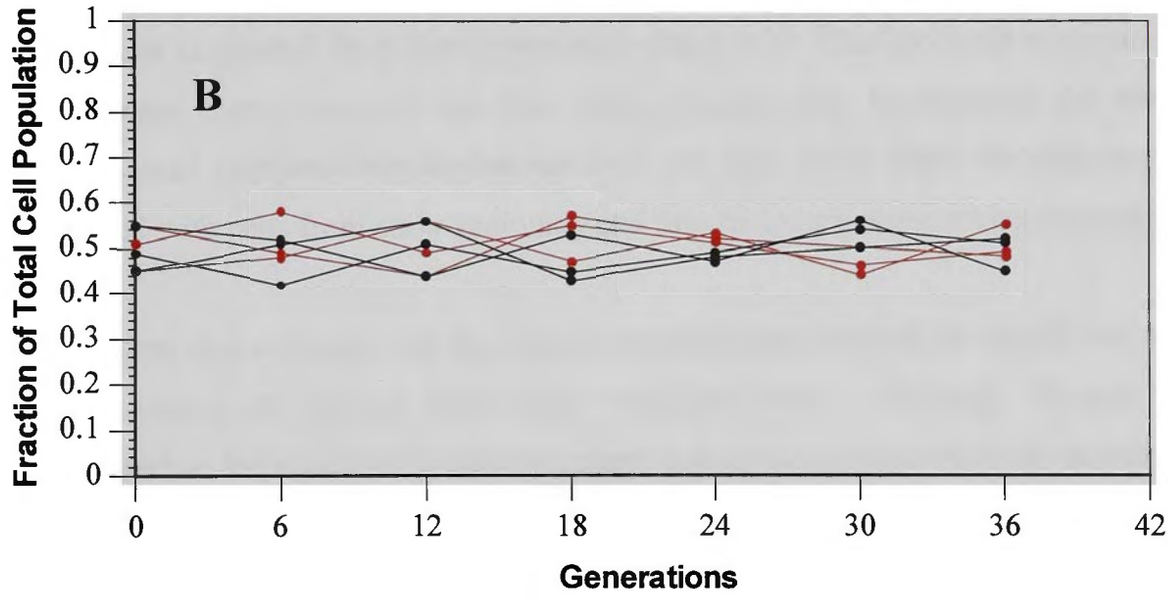


Figure 3.13: The effect of ethanol on viable cell populations in serial co-cultures comprising both wild type (●) and *nth1*Δ (●) strains. Initially, equal numbers of late exponential phase cells were inoculated into defined medium only (**A**) and defined medium containing 7% v/v ethanol (**B**). After six generations of growth, mixed populations were serially transferred into fresh medium under similar conditions. The cultures were incubated at 30°C/130 rpm. The results of three separate biological replicates which were initiated from three separate patent cultures are presented.

The parent cultures of both strains were grown in defined medium and the cells for inoculation were harvested at late exponential (8 hours), early stationary (10 hours) and late stationary (13 hours) phases. The cells from each stage of growth were inoculated into defined medium containing 16% (v/v) ethanol and the cell population was measured over a 10 hour period.

The results of this experiment show that the longer the parent culture is incubated (at least up to late stationary phase) before the inoculum is collected, the higher the survival rate of cells in the lethal ethanol stress culture. Inocula originating from early stationary and late stationary phase parent cultures had an improved survival rate (low death rate) compared to cultures inoculated with cells from late exponential phase parent cultures (higher death rates) (Figures 3.14 and 3.15)

A considerable dissimilarity was observed for survival rates of the two strains where the cultures originated from late exponential phase cells. The knockout strain *nth1*Δ demonstrated higher survival rate than *tsl1*Δ (Figure 3.14). Furthermore the *nth1*Δ strain showed comparatively higher survival rate than *tsl1*Δ when the culture was inoculated with cells from early stationary and late stationary phase parent cultures.

It is evident that cultures with the highest survival rates were those inoculated with cells containing the highest intracellular trehalose levels. Although, for any one strain, survival improved the longer the parent culture was cultivated in the stationary phase, this may not necessarily be due to the higher intracellular trehalose levels in the inoculum; other changes that occur in stationary phase cells, such as the onset of a general stress response in the cell *eg* HSP production, cell membrane changes and the shutdown of growth related pathways.

3.3 Discussion

Past reports on the relationship between trehalose production and tolerance to ethanol stress have been contradictory; some have claimed a positive effect (Kim *et al.,* 1996; Mansure *et al.,* 1997; Pataro *et al.,* 2002; Jung and Park 2005; Vianna *et al.,* 2008) while others advocate that trehalose has no influence on ethanol stress tolerance in yeast (Lewis *et al.,* 1997; Ribeiro *et al.,* 1999; Gomes *et al.,* 2002). The results of this

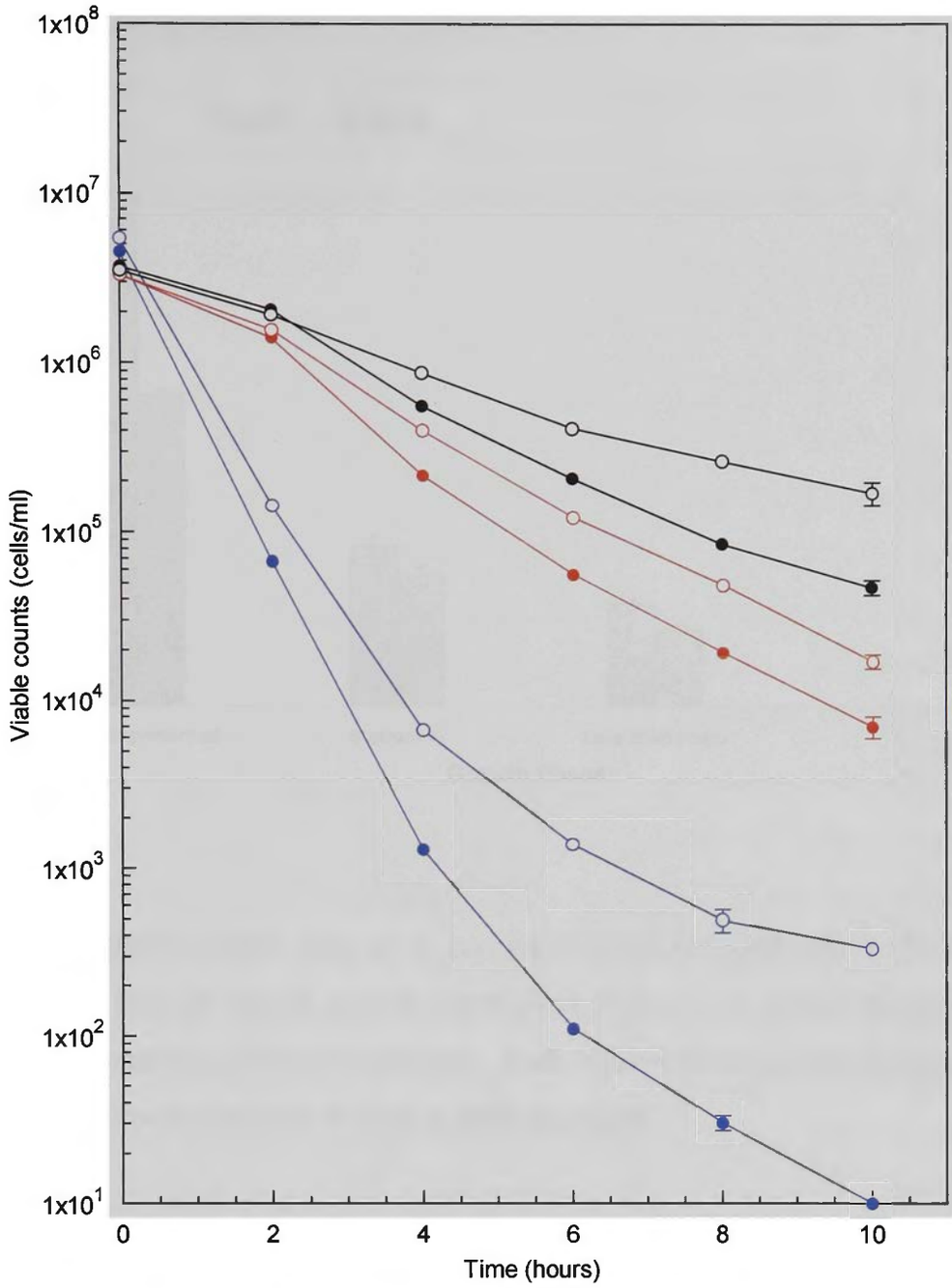


Figure 3.14: The effect of inoculum from different stages of growth on the viable cell populations of *S. cerevisiae tsl1*Δ and *nth1*Δ in presence of 16% ethanol stress. Cells from a late exponential phase (● *tsl1*Δ and ○ *nth1*Δ), early stationary (● *tsl1*Δ and ○ *nth1*Δ) and late stationary (● *tsl1*Δ and ○ *nth1*Δ) phase parent cultures were washed and inoculated into defined medium containing 16% (v/v) ethanol. The cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for a single time point only. All experiments were repeated at least twice with reproducible results.

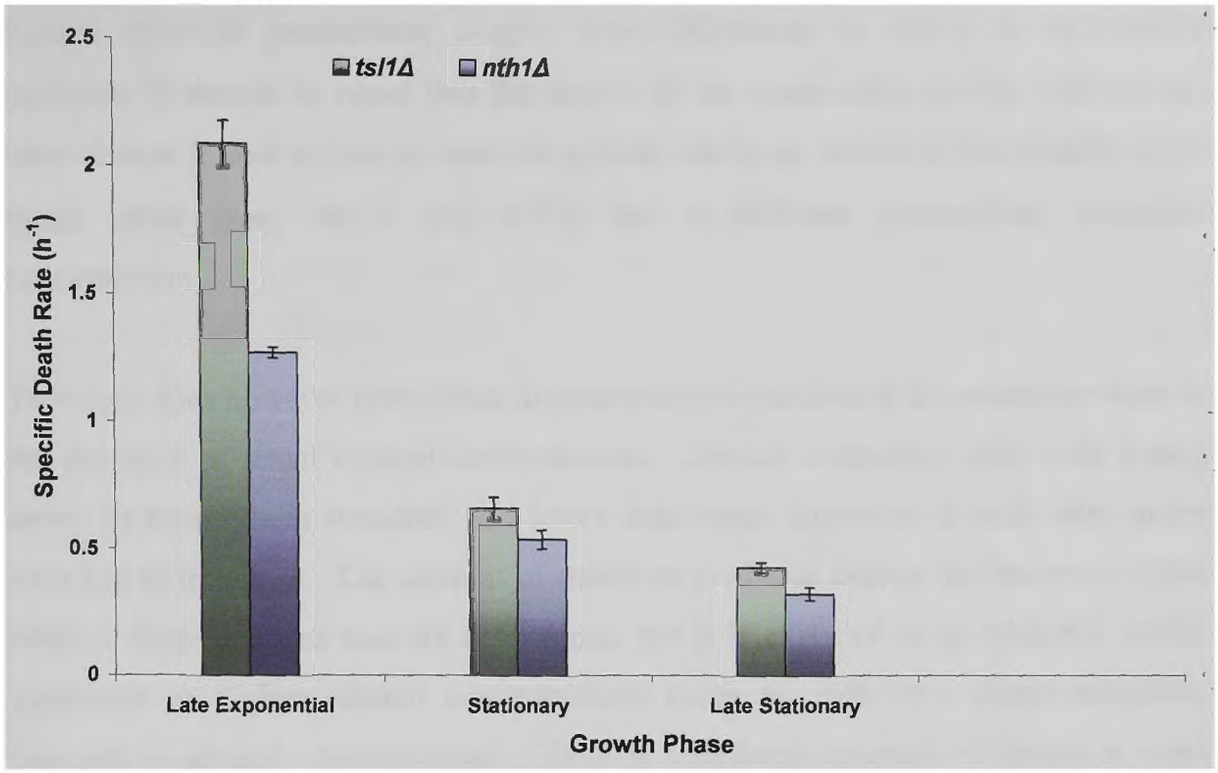


Figure 3.15: Specific death rates of *S. cerevisiae tsl1*Δ ( ) and *nth1*Δ ( ) cultures inoculated with cells at various growth stages (from Figure 3.14). Cells were inoculated into medium containing 16% (v/v) ethanol. Each experiment was conducted in triplicate and error bars indicate standard deviation from the mean.

project go some way to explaining why this controversy exists. The results presented in this chapter demonstrate that although trehalose does have a role in ethanol stress tolerance, its role is only effective when yeast are exposed to lethal ethanol concentrations. Trehalose had no apparent influence on the rate of acclimatisation of *S. cerevisiae* to non-lethal ethanol concentrations nor on the growth rate of cultures that have acclimatised to such concentrations. The absence of competitive advantage or disadvantage in strains that produced either higher or lower amounts of intracellular trehalose during growth in non-lethal ethanol concentrations was particularly convincing. Competitive growth curves are extremely sensitive at detecting small differences in the fitness of competing strains, yet such experiments

could not separate *S. cerevisiae* strains on this basis in the presence or absence of ethanol after 36 generations, despite their differences in ability to accumulate trehalose. It should be noted that the strains in the competitive growth experiments were always in mid to late exponential growth where, as shown in this chapter, each strain (wild type, *nth1*Δ and *tsl1*Δ) has a different intracellular trehalose concentration.

Trehalose does however have a role in promoting the survival of *S. cerevisiae* when in the presence of lethal ethanol concentrations. Inocula containing cells with higher levels of intracellular trehalose had lower death rates compared to cells with lower amounts of trehalose. The amount of trehalose produced during incubation in lethal ethanol concentrations was not determined, but it is expected to be relatively small, especially at higher ethanol concentrations (such as 16% v/v) where metabolic function is severely compromised. Even if significant amounts of trehalose were produced during incubation in lethal ethanol concentrations, higher amounts of trehalose would be expected in strain *nth1*Δ, with lesser amounts produced by the wild type strain and strain *tsl1*Δ in that order, and this was also the order of their survival capacity with strain *nth1*Δ having the highest survival rate and strain *tsl1*Δ having the lowest survival rate. Supporting this outcome was the effect on survival rate of inocula that had been grown to different growth phases. Cells that had been grown to late stationary phase had higher trehalose levels and better survival rates in 16% (v/v) ethanol than late exponential phase cells, noting that other factors will have a role in the survival of stationary phase inocula, such as the switching on of heat shock proteins. One important outcome of the latter experiments is that strain *nth1*Δ always had the highest survival rate compared to strain *tsl1*Δ, with the former always having higher intracellular trehalose concentrations.

The reasons for conflicting findings in past studies on the role of trehalose in ethanol tolerance of yeast can be attributed in part to genetic diversity of the strains used in some of these investigations. A number of studies have examined the relationship between intracellular trehalose levels and ethanol tolerance by comparing natural and industrial yeast strains that have different capacities to accumulate trehalose and also exhibit a range in their level of ethanol tolerance (Lewis *et al.*, 1997; Mansure *et al.*,

1997; Ribeiro *et al.,* 1999; Gomes *et al.,* 2002; Pataro *et al.,* 2002; Vianna *et al.,* 2008). The purpose of these past studies was to determine if there is a correlation across a number of different strains in the amount of trehalose they each accumulate and their relative levels of ethanol tolerance. While some of these studies report a positive correlation between trehalose levels and ethanol tolerance (Mansure *et al.,* 1997; Pataro *et al.,* 2002; Vianna *et al.,* 2008), others did not (Lewis *et al.,* 1997; Ribeiro *et al.,* 1999; Gomes *et al.,* 2002). For example, Lewis *et al.* (1997) compared the trehalose content of fourteen wild-type baking strains of *S. cerevisiae* with their relative tolerance to ethanol (20% v/v for 30 min). Not only did they fail to observe a positive correlation between trehalose and ethanol tolerance, they also concluded that trehalose content did not correlate with strain tolerance to other stressors, including temperature, freezing, H_2O_2 and osmotic shock. The lack of consensus across such studies is not surprising given the diversity in genetic background of the strains used within each experiment. Experiments that compare trehalose levels and corresponding ethanol tolerance across different strains are unable to separate the individual roles of trehalose from that of other stress response mechanisms, such as heat shock protein expression or changes in plasma membrane composition, that assist the cell to cope with the ethanol stress; the influence of such mechanisms on ethanol tolerance are likely to vary from strain to strain.

There are only a few studies that have used closely related *S. cerevisiae* strains to examine the relationship between trehalose and ethanol stress tolerance (Kim *et al.,* 1996; Soto *et al.,* 1999; Pereira *et al.,* 2001; Jung and Park 2005). These studies examined differences between phenotypes of a wild type strain and gene knockouts in the same background (the gene deletion being associated with some aspect of trehalose metabolism) during ethanol stress. All such studies reported a positive correlation between trehalose accumulation and ethanol tolerance, with two of them measuring ethanol tolerance according to strain survival in lethal ethanol concentrations of 18% (v/v) (Kim *et al.,* 1996), 15% (v/v) (Soto *et al.,* 1999) and 10% (v/v) (Pereira *et al.,* 2001), which supports the findings described in this thesis. One of these studies however found that an *S. cerevisiae* strain with decreased *ATH1* expression had higher trehalose levels and growth rate in 8% (v/v) ethanol than the wild type strain, suggesting that a non-lethal ethanol stress was used (Jung and Park 2005). The significance of the results presented by the authors is however debatable

given that very high cell populations were used (around 1×10^8 cfu ml^{-1}) and the purported difference in final cell population after 8 hours of incubation in 8% (v/v) ethanol between the wild type (2×10^8 cfu ml^{-1}) and recombinant strain (3×10^8 cfu ml^{-1}) was very small; even the non-stressed (control) cultures hardly grew at these cell densities (from 1×10^8 cfu ml^{-1} to 3×10^8 cfu ml^{-1} over 8 hours of incubation). The extremely low growth rate of the control cultures suggests that stressors other than ethanol were impacting on cell vitality.

The use of non-lethal and lethal ethanol concentrations, as described in this thesis, has revealed that the influence of trehalose on the ethanol stress tolerance of *S cerevisiae* is ethanol concentration dependant. Although the mechanism by which trehalose protects the cell against the damaging effects of ethanol remains unclear, the outcome of this study sheds light on the proposed roles for trehalose in stress tolerance. The two main proposed roles for trehalose in stress tolerance are: stabilising the plasma membrane and membrane-associated proteins; and the protection of cytoplasmic proteins by assisting in their proper folding and repair, and preventing protein aggregation during stress (Mansure *et al.,* 1994; Singer and Lindquist 1998; Sola-Penna and Meyer-Fernandes 1998; Simola *et al.,* 2000). The ethanol concentration dependence of the trehalose effect on yeast ethanol tolerance suggests that its role in ethanol tolerance is more likely to be associated with the protection of cytosolic proteins rather than mechanisms connected with plasma membrane stability.

One of the main targets of ethanol in yeast is the plasma membrane. The impact of ethanol on plasma membrane integrity is evident even at low ethanol concentrations, causing an increase in plasma membrane permeability and corresponding decreases in transmembrane gradients and transport activities (Juroszek *et al.,* 1987; Pascual *et al.,* 1988; Petrov and Okorokov 1990; Marza *et al.,* 2002; Aguilera *et al.,* 2006). These effects by ethanol on the plasma membrane cause growth lag periods and reduce the specific growth rate of cultures at non-lethal ethanol concentrations as low as 4% (v/v), making it unlikely that the ethanol-stress tolerance role of trehalose is associated with such membrane-related disruptions. Any benefits provided by trehalose on membrane integrity during ethanol stress would have been detected in the competitive growth curve experiments and this was clearly not the case.

The presence of ethanol can impact on cytosolic proteins by interfering with protein structure, causing denaturation and protein aggregation, resulting in the inhibition of protein function. These inhibitory effects by ethanol on protein function in *S. cerevisiae* are known to be inhibitory to cell function only at high ethanol concentrations, usually in the lethal ethanol concentration range of 10% (v/v) or higher (Pascual *et al.,* 1988; Jones 1990; Walker 1998; Hallsworth *et al.,* 1998 ; Sebollela *et al.,* 2004). For example, Sebollela *et al.* (2004) investigated the effect of ethanol on the activity of an important yeast antioxidant enzyme, glutathione reductase (GR). The authors observed that GR activity was inhibited by ethanol in a dose-dependant manner, with approximately 30% inhibition of activity at 10% (v/v) ethanol. The presence of trehalose in the reaction mixture however protected the enzyme resulting in the full recovery of GR activity after the ethanol was removed, even at higher ethanol concentrations of 15% (v/v). The protein stabilising effect of trehalose is described by 'the vitrification hypothesis', which suggests that trehalose has the ability to form an amorphous glass structure around proteins (Sun and Leopold 1997; Crowe *et al.,* 1998; Crowe *et al.,* 2001). The formation of a glass capsule around the protein protects its native shape and prevents structure deformation. The proposed role for trehalose in protecting cytosolic proteins from the damaging effects of ethanol, facilitating cell survival in high ethanol concentrations, is the most likely model to account for the results observed in this thesis.

In conclusion, the results of this study demonstrate a role for trehalose in protecting *S. cerevisiae* against the damaging effects of high ethanol concentrations. This role does not appear to be effective during non-lethal ethanol stress, with higher intracellular trehalose levels providing no competitive advantage for *S. cerevisiae* growing in 7% (v/v) ethanol. The ethanol concentration-dependant nature of the trehalose effect on *S. cerevisiae* ethanol stress tolerance supports a mechanism for trehalose in protecting cytosolic proteins from the damaging effects of ethanol. Although a role for trehalose in protecting membrane stability cannot be discounted, it does not appear to influence cell fitness during growth in non-lethal ethanol concentrations.

CHAPTER 4

Asr1, an ethanol-responsive key element of *Saccharomyces cerevisiae*

4.1 Introduction

The focus of this project was to investigate the molecular consequences of ethanol stress in *S. cerevisiae* in order to obtain a better understanding of the ethanol stress response in yeast. The previous chapter investigated the role of trehalose and its metabolism in the ethanol stress response. As the trehalose work was nearing completion in 2004, a report was released on the discovery of a protein, the Alcohol Sensitive Ring/PHD finger 1 protein (Asr1), in *S. cerevisiae* which is the first reported protein that responds physiologically to ethanol stress; importantly its response is specific to alcohol stress (Betz *et al.,* 2004). This novel alcohol sensitive protein has been investigated by two research groups and both found that Asr1 constitutively shuttles between the cytoplasm and nucleus in the absence of stress, but rapidly and reversibly accumulates in the nucleus under alcohol stress (Betz *et al.,* 2004; Izawa *et al.,* 2006). Although both groups independently established that the protein rapidly accumulates in the nucleus but only in response to alcohol stress, the significance of Asr1 in the ethanol stress response remains in question.

Betz *et al,* (2004) suggested that Asr1 might play a role in a cell signalling system, responding to alcohol-induced changes in plasma membrane fluidity; this suggestion was based on the observation that growth of *S. cerevisiae asr1*Δ cells was compromised in the presence of either 1% butanol or 0.02% SDS. Izawa *et al.* (2006) questioned the involvement of Asr1 in the yeast stress response to alcohol, reporting no difference in the growth profiles of a number of *asr1*Δ strains and wild type strains in response to ethanol (5 or 10%), butanol (1 or 2%) or SDS (0.01 or 0.02%). Izawa *et al.* (2006) also disagreed with the proposal by Betz *et al.* (2004) that accumulation of Asr1 in the nucleus is related to function, suggesting instead that it is a consequence of failure of the nuclear export machinery under conditions of ethanol stress. Izawa *et al.* (2006) therefore cast doubt on the suggestion that Asr1 plays a role, let alone an important role, in ethanol tolerance in yeast.

It is important that the role of Asr1 in the ethanol stress response is elucidated. If it does have a role in signalling ethanol stress in *S. cerevisiae*, then its behaviour potentially impacts on the functioning of the trehalose metabolism which was shown in Chapter 3 to have a role in ethanol stress tolerance. The purpose of the work in this chapter was to determine if Asr1 influences the ethanol stress response of *S. cerevisiae*. One of the limitations of the earlier studies on the impact of Asr1 on the physiological response of ethanol-stressed *S. cerevisiae* is that the techniques used to measure growth response lacked the sensitivity to detect small changes in yeast growth rate during ethanol stress. It was decided to use a more sensitive approach for measuring differences in cell growth rate by performing competition experiments between *S. cerevisiae* wild type and *asr1*Δ strains to determine if *ASR1* influences growth rate (competitive fitness) during ethanol stress. This chapter reports the results of competition experiments comprising 'wild type' versus 'deletion mutant' strains in the presence and absence of ethanol stress to detect marginal fitness differences in ethanol-stress yeast strains due to Asr1 activity (Baganz *et al.*, 1998; Brown and Tuite 1998; Thatcher *et al.*, 1998).

4.2 Results

4.2.1 Genetic confirmation and stability of the *ASR1* knockout strain

The purpose of the work in this chapter was to compare the ethanol tolerance of a yeast strain with and without a functional *ASR1* gene. Therefore this experiment takes advantage of the gene disruption approach (Giaever *et al.*, 2002), where the *ASR1* gene in *S. cerevisiae* is deleted and replaced by the *kanMX4* module. The *kanMX4* module confers resistance to antibiotic Geneticin (G418), it can therefore be used as a marker to differentiate the 'knockout' from its 'wild type'. In this section, genetic confirmation and stability of the *asr1*Δ strain was investigated.

4.2.1.1 PCR confirmation

Initially, Geneticin resistance of the *asr1*Δ strain was confirmed by growing the wild type and *asr1*Δ strain in YEPD medium with added Geneticin. The *asr1*Δ strain was

able to grow in the presence of Geneticin (200mg/l) whereas the wild type strain did not survive, confirming the presence of the *kanMX4* module in the *asr1*Δ strain (Table 4.1).

PCR analysis was used to verify the expected position of the *kanMX4* module in the *asr1*Δ strain. The presence and position of the *kanMX4* module was tested using a combination of *ASR1* and *kanMX4* specific primers: Primers ASR1-A and ASR1-D, complementary to the upstream and downstream flanking regions of the *ASR1* ORF were positioned -320 bp from the start codon and +314 bp from the stop codon of the *ASR1* gene, respectively. Primers ASR1-B and ASR1-C were located within the coding region of the ASR1 ORF. The kan-B and kan-C primers were positioned within the internal sequence of the *kanMX4* module. A diagrammatic representation of the PCR confirmation is shown in Figure 4.1 and details of the primer sequences are given in Appendix I, Table 1.

Deletion of the ASR1 ORF was verified by the following observations:

- No PCR product was observed when primer combinations ASR1-A (flanking) with ASR1-B (internal) or ASR1-C (internal) with ASR1-D (flanking) were used with *asr1*Δ, whereas amplification using wild type DNA as template generated products of the expected size (Figure 4.2).

- The flanking primers (ASR1-A and ASR1-D) generated products of 2151 bp for *asr1*Δ (length of *kanMX4* sequence plus flanking sequences) and 1500 bp for the wild type strain (length of *ASR1* ORF sequence plus flanking sequences) (Figure 4.2).

- PCR products with sizes 555 bp and 938 bp were generated as predicted from the ASR1-A with kan-B, and kan-C with ASR1-D, primer combinations respectively. Using the same primer combinations for the wild type did not result in amplification products due presumably to the absence of the *kanMX4* module (Figure 4.2).

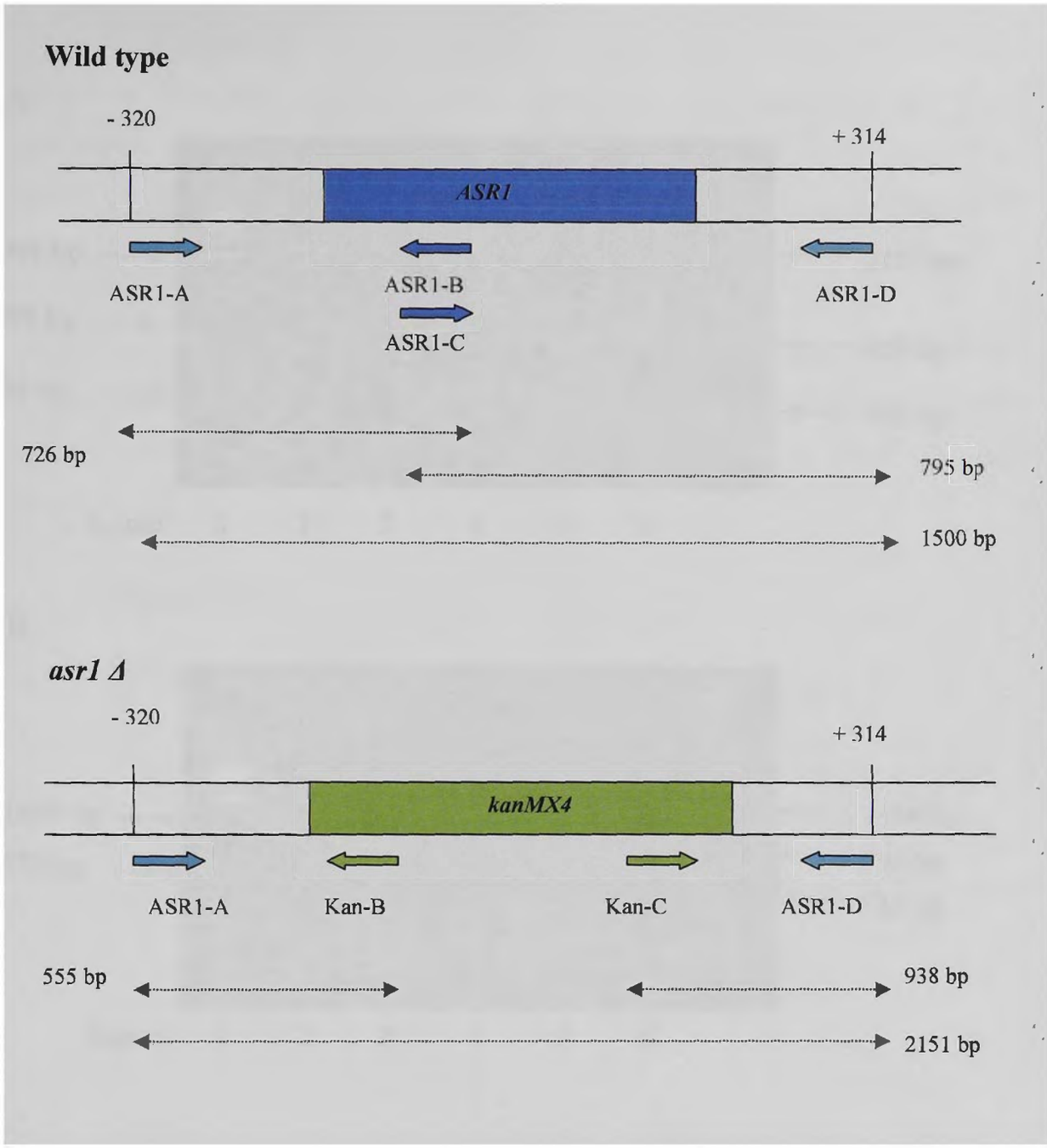


Figure 4.1: Comparison of the *asr1*Δ construct and the BY4742 wild type (not to scale). The positions of PCR primers used for confirmation of the knockout construct are shown with expected product sizes: ASR1-A and ASR1-D - 1500bp (wild type) and 2151bp (*asr1*Δ); ASR1-A and kan-B – 555bp (*asr1*Δ); kan-C and ASR1-D -938 bp (*asr1*Δ); ASR1-A and ASR1-B - 726bp (wild type) ; ASR1-C and ASR1-D - 795bp (wild type).

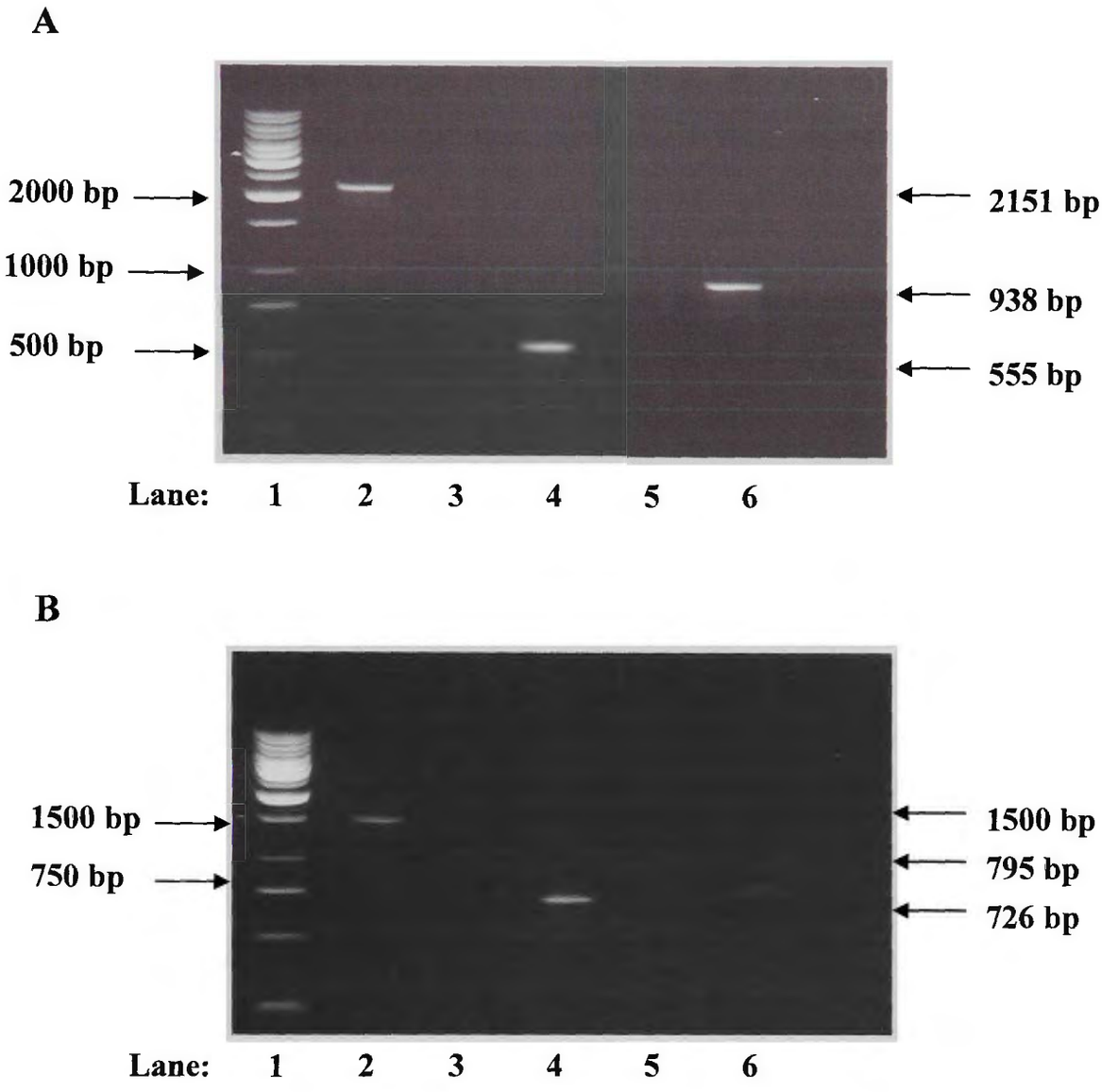


Figure 4.2: PCR conformation of the correct integration of *kanMX4* module in place of the *ASR1* gene. Panel **A** represents the *asr1*Δ strain and panel **B** represents the wild type strain (BY4742). PCR products were resolved on a 1.5% agarose gel. For *asr1*Δ strain, a PCR product of 2151 bp was generated from the ASR1-A and ASR1-D primer combination (lane 2**A**). A product of 1500 bp resulted from the wild type when used the same primer combination (lane 2**B**). For the *asr1*Δ strain for ASR1-A; kan-B primer combination and kan-C; ASR1-D primer combination, 555 bp (lane 4**A**) and 938 bp (lane 6**A**) PCR products were generated. For the wild type the primer combinations ASR1-A; ASR1-B and ASR1-C; ASR1-D gave 726 bp (lane 4**B**) and 795 bp (lane 6**B**) products respectively. With *asr1*Δ strain no PCR products were amplified for primer combinations ASR1-A; ASR1-B and ASR1-C; ASR1-D (lanes 3**A**, 5**A**). Similarly, with the wild type no PCR products were amplified for primer combinations ASR1-A; kan-B and ASR1-D; kan-C (lanes 3**B**, 5**B**).

The first and second observations confirm integration of the kanMX4 module into *asr1*Δ. The third observation verified the correct replacement of the gene with the *kanMX4* module in *asr1*Δ using primers that span the left and right junctions of the deletion module within the genome, producing corresponding PCR products of the correct size.

Sequencing all of the PCR products confirmed the correct integration of the *kanMX4* module into the *ASR1* loci of the *asr1*Δ strain. Sequencing data could be read into the *kanMX4* module in all sequenced fragments indicating the replacement of the *ASR1* gene

4.2.1.2 Stability of the *asr1*Δ strain

To test the stability of the *asr1*Δ strain, separate cultures of the wild type (BY4742) and *asr1*Δ strain were serially subcultured three times in non-selective YEPD medium. The strains were plated on both YEPD and YEPD-Geneticin plates and incubated at 30°C for two days. The YEPD-Geneticin plates did not support the growth of wild type cells. After three subcultures of the knockout strain, YEPD and YEPD-Geneticin plates showed equivalent cell numbers, providing evidence of the stability of *kanMX4* in the *asr1*Δ strain (Table 4.1).

Table 4.1: Growth of the wild type (BY4742) and *asr1*Δ strains in YEPD and YEPD-Geneticin (G418) plates after three subcultures in liquid medium for each strain.

Strain	Viable cell counts (cells/ml)	
	YEPD	YEPD + Geneticin
Wild type BY4742	$7.6 \pm 0.2 \times 10^6$	No growth
*asr1*Δ	$8.0 \pm 0.3 \times 10^6$	$8.4 \pm 0.4 \times 10^6$

4.2.2 Impact of Asr1 on the physiological response of *S. cerevisiae* to ethanol stress

4.2.2.1 Batch growth analysis

To analyse the growth profiles of the wild type and *asr1*Δ strains under ethanol-stressed and non-stressed conditions washed, late exponential-phase cells were inoculated into defined medium at 30°C/130 rpm and the cell population was monitored for a 10 hour period. Both *S. cerevisiae* BY4742 wild type and *asr1*Δ had increasing lag periods and decreasing specific growth rates in the presence of non-lethal ethanol concentrations ranging from 6% to 9% (Figure 4.3; Table 4.2). There was no significant difference between the growth profiles of the *asr1*Δ and wild type strains either in the absence or presence of non-lethal ethanol stress.

Table 4.2: The effect of ethanol on the growth profile of wild type strain and *asr1*Δ strain (from Figure 4.3)

Ethanol Concentration (v/v)	Strain	Lag Period (h)	Specific Growth Rate (h^{-1})
0%	Wild Type	-	0.298 ± 0.0027
	*asr1*Δ	-	0.297 ± 0.0036
6%	Wild Type	2.00 ± 0.05	0.133 ± 0.0016
	*asr1*Δ	2.00 ± 0.10	0.135 ± 0.0018
7%	Wild Type	4.20 ± 0.08	0.103 ± 0.0011
	*asr1*Δ	4.25 ± 0.10	0.102 ± 0.0016
8%	Wild Type	8.00 ± 0.10	0.090 ± 0.0014
	*asr1*Δ	8.10 ± 0.10	0.088 ± 0.0015
9%	Wild Type	-	-
	*asr1*Δ	-	-

- Not measurable

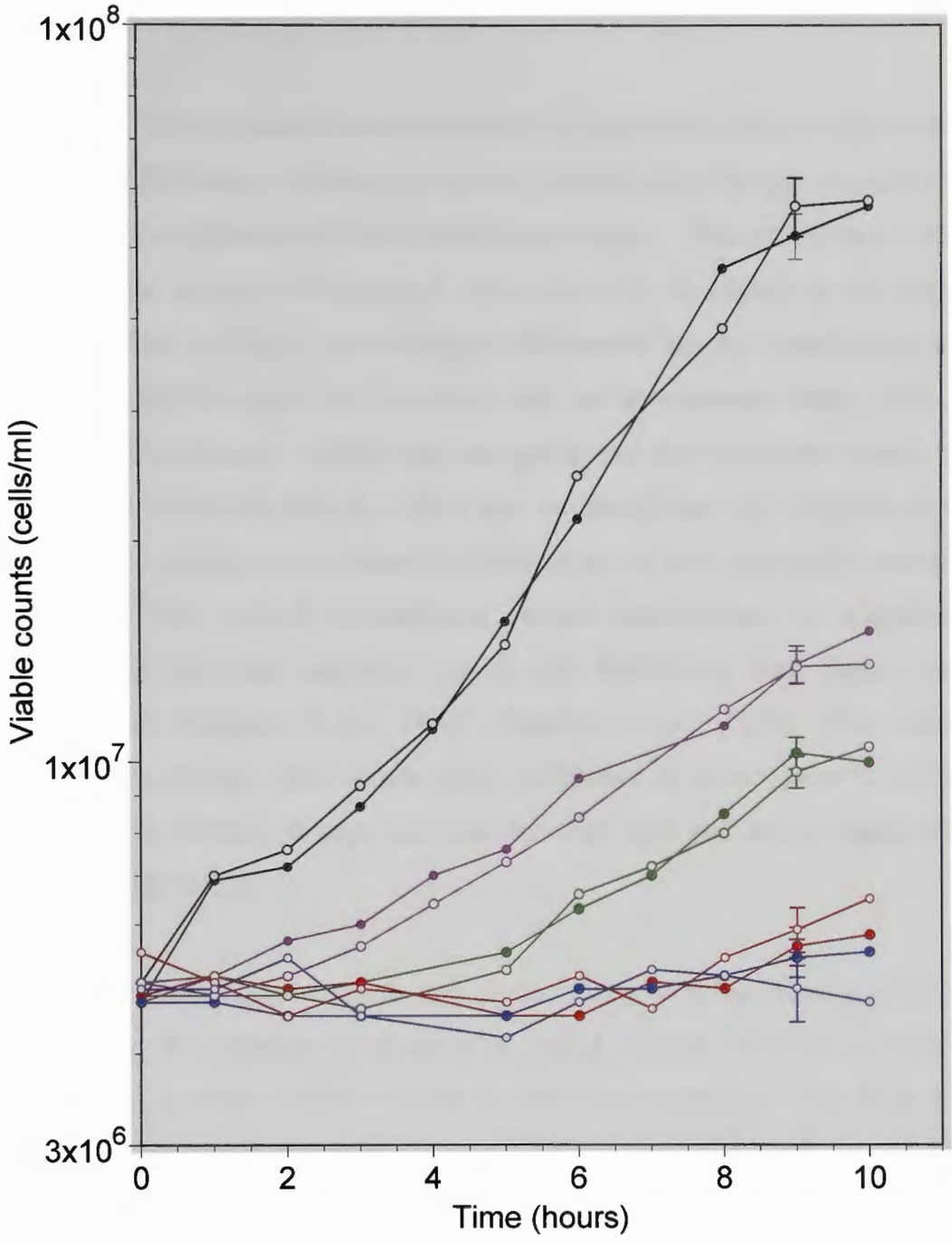


Figure 4.3: The effect of ethanol on the viable cell population of wild type and *asr1*Δ cultures. Washed, late exponential phase cells were inoculated into defined medium only (●, ○) or defined medium containing added ethanol at either 6% v/v (●, ○), 7% v/v (●, ○), 8% v/v (●, ○) or 9% v/v (●, ○). Wild type and *asr1*Δ cultures are represented by closed and open symbols respectively. The cultures were incubated at 30°C/130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for the nine hour time point only. All experiments were repeated at least twice with reproducible results.

4.2.2.2 Competition growth analysis

The batch culture experiments described in the previous section did not identify any significant difference between the growth profiles of wild type and *asr1*Δ strains in the absence or presence of non-lethal ethanol stress. This could mean that Asr1 has no influence on the physiological response of *S. cerevisiae* to non-lethal ethanol stress, or that its effect is too subtle to be detected by the experimental design. The latter is possible given that variation can occur between viable plate counts for samples from the same culture and time point, and the short time periods the cultures spend in exponential growth, which can be insufficient for marginal differences in growth rate to appear as detectable differences in cell population between the two strains. In this respect, competition growth experiments are significantly more sensitive at detecting marginal growth rate differences than single batch growth experiments (Baganz *et al.,* 1998; Thatcher *et al.,* 1998). With this in mind, competitive growth experiments were performed to detect growth rate differences (competitive fitness), if any, between the wild type and *asr1*Δ strains during non-lethal ethanol stress.

Equal cell numbers of wild type and *asr1*Δ strains were incubated together in defined medium in the presence or absence of added ethanol (7.5% v/v). After every six generations, a small volume of the culture was transferred into fresh medium for further incubation *ie* subcultured; samples from the parent culture were plated onto YEPD and YEPD-Geneticin plates. The relative population (relative cell number) of each strain in the co-culture was determined by viable cell counts. As both strains can grow on YEPD plates, but only the knockout strain is able to grow in the presence of Geneticin, the cell population of each strain could be determined. The wild type and *asr1*Δ strains were co-cultured for 60 generations in the presence or absence of ethanol and the relative cell population of each genotype in the subcultures was determined periodically (Section 2.2.2.7).

It shoulc be noted that the *asr1*Δ strain used in this study was created by replacing the *ASR1* gene with a *kanMx4* marker. Studies by Baganz *et al.* (1998) confirmed that the *HO* gene is a neutral site for *kanMX4* replacement and thus the *kanMX4* marker does not have any detectable impact on the growth rates of strains that carry it. This is

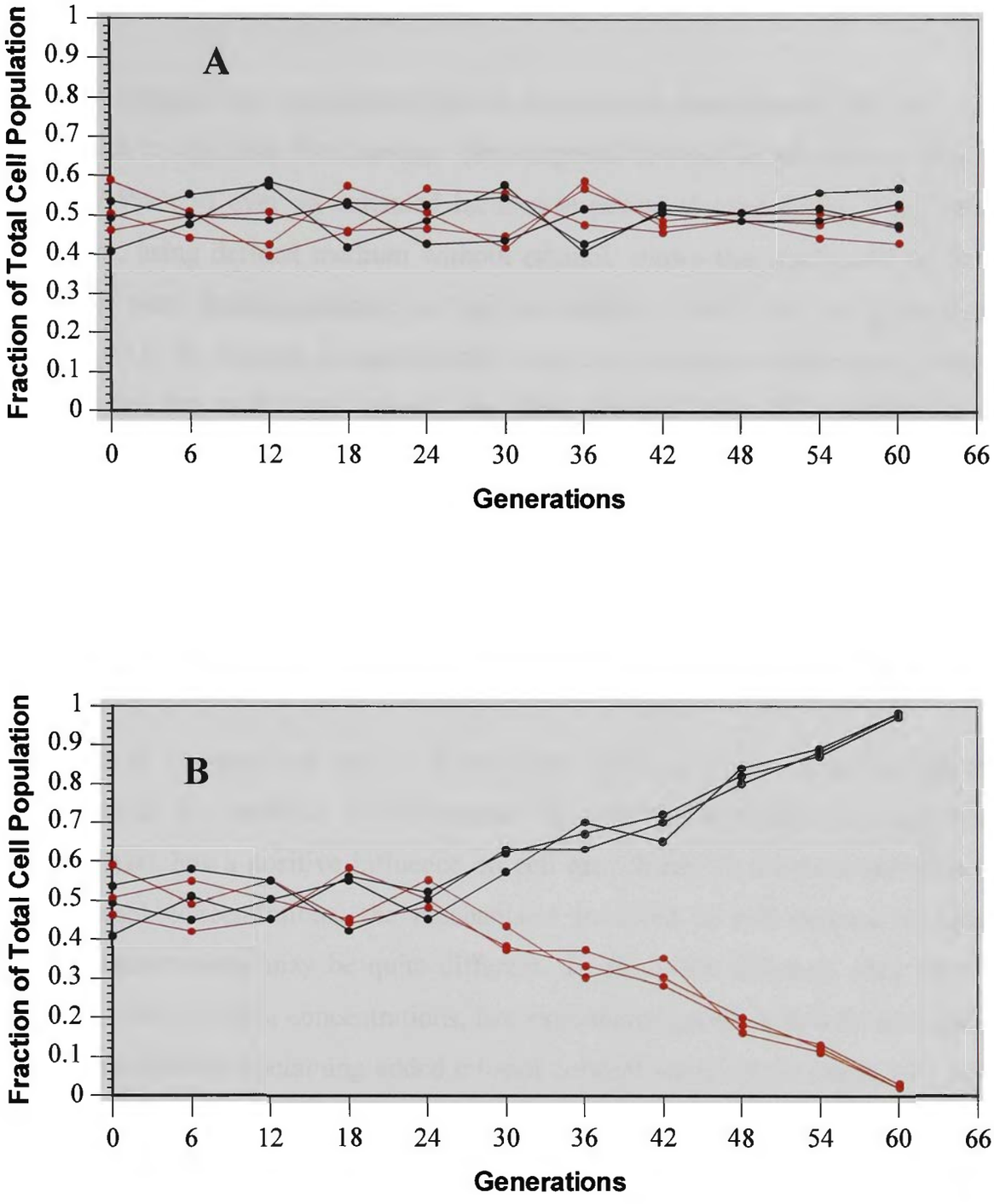


Figure 4.4: The effect of ethanol on viable cell populations in serial co-cultures comprising both wild type (●) and *asr1*Δ (●) strains. Initially, equal numbers of late exponential phase cells were inoculated into defined medium only (**A**) and defined medium containing 7.5% v/v ethanol (**B**). After six generations of growth, mixed populations were serially transferred into fresh medium under similar conditions. The cultures were incubated at 30°C/130 rpm. The results of three separate biological replicates which were initiated from three separate patent cultures are presented.

supported by previous work in this study where *kanMX4* was used as a replacement for the *TSL1* gene (Section 3.2.5) and where no effect of the *kanMx4* marker on growth of the strain was observed in the presence or absence of ethanol.

The above results of the competitive growth experiments demonstrated that the *asr1*Δ strain is at a competitive disadvantage when exposed to a sub-lethal concentration of ethanol (7.5% v/v) over an extended incubation period (Figure 4.4B). The control experiment, using defined medium without ethanol, shows that *asr1*Δ and the wild type strain were indistinguishable in their competitive fitness over 60 generations (Figure 4.4A). In contrast, a significantly lower cell population of the *asr1*Δ strain, compared to the wild type strain, was first detected after 30 generations (5 subcultures) when co-cultured under ethanol stress, with this difference becoming more pronounced over succeeding generations (Figure 4.4B). The *asr1*Δ strain was almost indetectable in the competitive culture after 60 generations.

4.2.2.3 Batch incubation in the presence of lethal ethanol concentrations

The results in the previous section showed that Asr1 does have an impact on the growth rate of *S. cerevisiae* in the presence of a non-lethal ethanol concentration. Although Asr1 has a positive influence on cell growth rate in the presence of non-lethal ethanol concentrations, the mechanisms involved in cell defence at lethal ethanol concentrations may be quite different. To study the effect of Asr1 on cell survival in lethal ethanol concentrations, late exponential phase cells were inoculated into defined medium containing added ethanol concentrations in the range 10 - 18% (v/v). The control cultures were grown in defined medium without added ethanol. Samples were taken regularly and viable cell population was monitored by duplicate viable plate counts.

At an ethanol concentration of 10% (v/v), the viable cell populations of both wild type BY4742 and *asr1*Δ strains were unchanging with no increase or decrease in cell numbers over a 12 hour incubation period (Figures 4.5 and 4.6). The effect of higher ethanol concentrations on both strains was a steady decline in viable cell population over time. The death profiles of the *asr1*Δ strain compared with the wild type show that disruption of *ASR1* gene provided an advantage in survival rate (Figure 4.6).

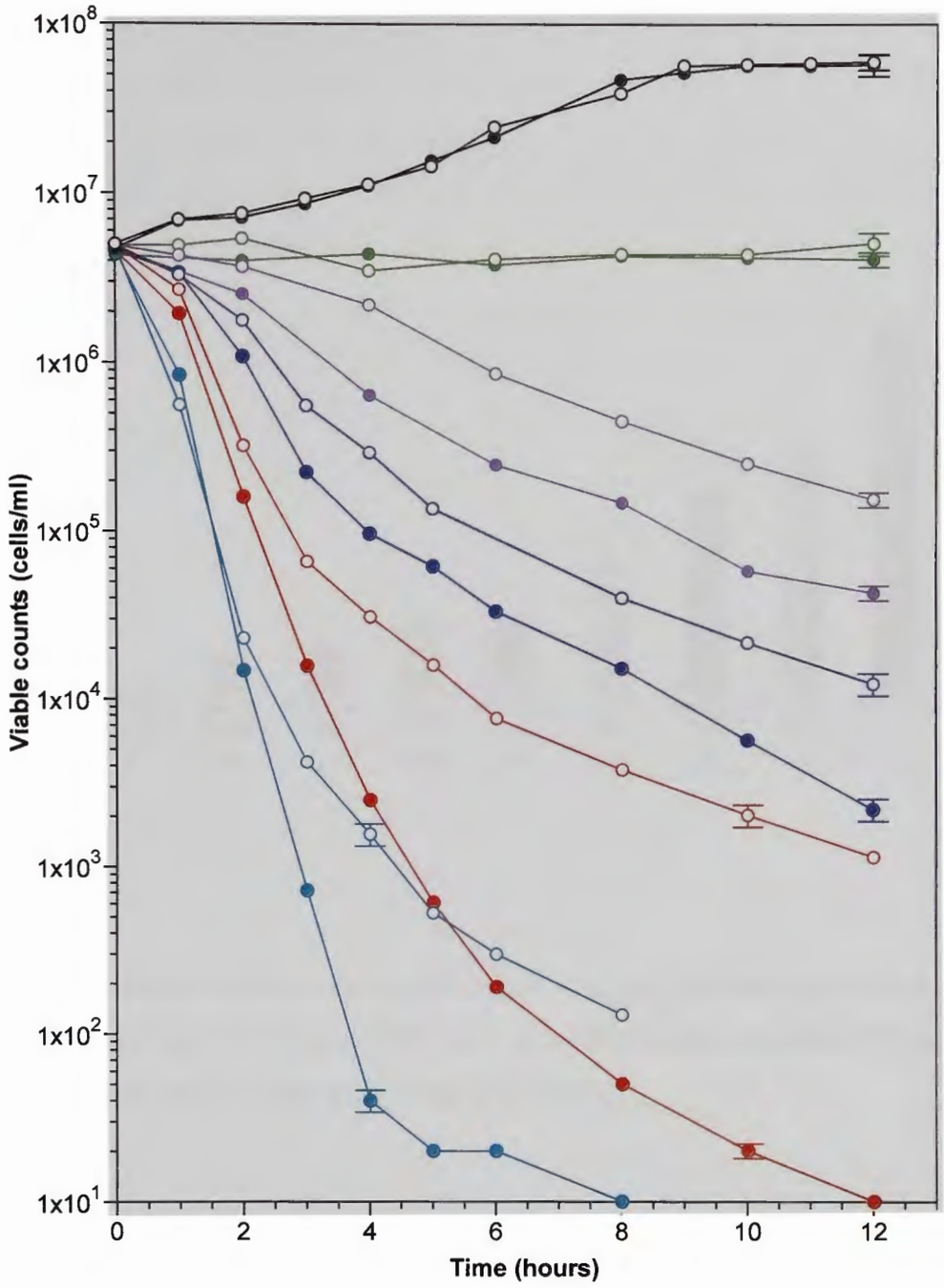


Figure 4.5: The effect of lethal ethanol concentrations on the viable cell populations of wild type and *asr1*Δ. Cells from a late exponential phase parent culture were washed and inoculated in to defined medium only (●, ○) or defined medium containing added ethanol at either 10% v/v (●, ○), 14% v/v (●, ○), 15% v/v (●, ○), 16% v/v (●, ○) or 17% v/v (●, ○). Wild type and *asr1*Δ cultures are represented by closed and open symbols respectively. The cultures were incubated at 30°C in shaking incubator at 130 rpm. Each experiment was conducted in triplicate and representative error bars showing the standard deviation from the mean are shown for a single time point only. All experiments were repeated at least twice with reproducible results.

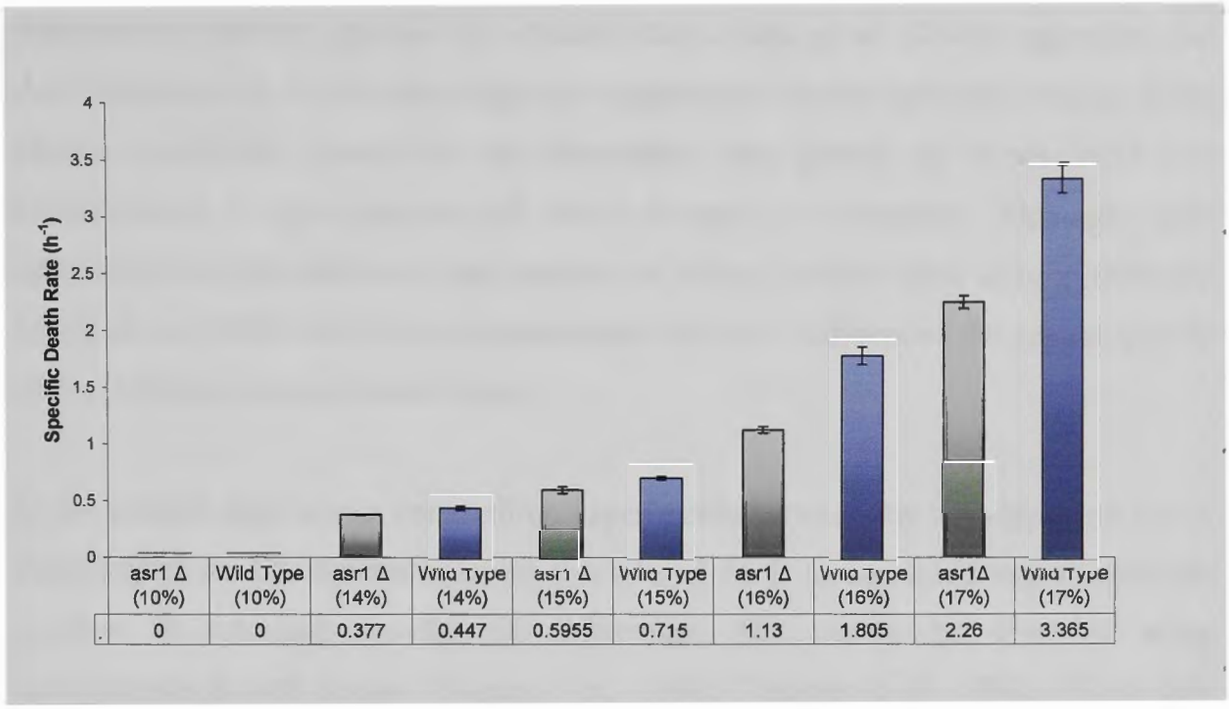


Figure 4.6: The effect of lethal ethanol concentrations on specific death rate of wild type (BY 4742) and *asr1*Δ strains (from Figure 4.5). Each experiment was conducted in triplicate and error bars indicate standard deviation from the mean.

The *asr1*Δ strain had a significantly higher tolerance to lethal ethanol concentrations compared to the wild type in stress conditions comprising 14 – 17 % (v/v) ethanol (Figures 4.5 and 4.6), this effect being most evident in 16% (v/v) ethanol.

4.3 Discussion

During incubation yeast cells must sense and respond to stress factors in the environment. In *S. cerevisiae*, the Alcohol Sensitive Ring/PHD finger 1 protein (Asr1) constitutively shuttles between the cytoplasm and nucleus but rapidly and reversibly

accumulates in the nucleus under alcohol stress (Betz *et al.*, 2004; Izawa *et al.*, 2006). Asr1 accumulates in the nucleus of *S. cerevisiae* specifically in response to alcohol stress, it does not respond to other environmental stresses. On the basis of this ethanol stress specific response it was suggested that Asr1 might be part of a signal transduction pathway specific for ethanol stress. Betz *et al.* (2004) suggested that Asr1 relocation to the nucleus might be triggered by alcohol-induced changes in the plasma membrane, based on the observation that growth of *asr1*Δ cells was compromised in the presence of either butanol or detergent. Although Asr1 accumulates in the nucleus in the presence of ethanol, neither Betz *et al.* (2004) nor Izawa *et al.* (2006) were able to demonstrate that Asr1 influenced the growth profile of *S. cerevisiae* during ethanol stress.

In the present study direct competition experiments between the wild type and *asr1*Δ strains were used to further examine the role of Asr1. Competition experiments are capable of detecting growth rate differences that cannot be observed using conventional growth curves (Baganz *et al.*, 1998; Thatcher *et al.*, 1998). Using this approach, it was shown that Asr1 does increase the growth rate of *S. cerevisiae* under ethanol stress. This influence by Asr1 was subtle with the reduced growth rate of the *asr1*Δ strain only being detectable after 24 generations in competitive culture. Although expression of *ASR1* appears to be non-essential (since growth in the presence of non-lethal ethanol concentrations occurred in its absence), the results demonstrate that *ASR1* does have a positive role in stimulating the growth rate of yeast in the presence of ethanol, as the *asr1*Δ strain was almost totally outgrown by the wild type strain within 60 generations. The role of Asr1 in cell growth rate only appears to be important in the presence of ethanol as no difference was seen in the growth of the wild type and *asr1*Δ strains in the absence of ethanol.

Yeast exposed to adverse conditions display rapid adaptive responses (Hohmann and Mager 1997) that act to improve functionality of the cells under stress conditions. The advantage conferred by Asr1 was only evident in a situation where cells were growing in a non-lethal stress environment. This suggests that Asr1 may have a role in stimulating cell growth and cell cycle progression in the presence of ethanol stress. It has been known for a long time that cell growth and cell cycle progression are intimately correlated with the external environment, although the control mechanisms

are not fully understood (Nakai and Ishikawa 2001; Shapira *et al.*, 2004; Barberis *et al.*, 2007).

The survival advantage conferred by Asr1 was not observed in cells exposed to lethal concentrations of ethanol. To the contrary *asr1*Δ strain exhibited improved survival rates in lethal ethanol concentrations. Lethal concentrations of ethanol present a different challenge to the cell. Whereas yeast exposed to non-lethal adverse conditions have rapid adaptive responses that act to increase the capacity of the cells for growth and proper functioning, in lethal stress conditions these responses are primarily focussed on cell survival with cellular energetics being severely compromised (Ferreira *et al.*, 1997). This result for lethal ethanol stress was not expected however it could be related to the apparent role of Asr1 in stimulating cell division during ethanol stress, which would provide little or no benefit in dying cells. Such a role of Asr1 could have a negative impact on cell energetics by attempting to stimulate cell cycle activity which may be an energy drain in a cell that is already energy compromised by the effects of ethanol. If this were the case, the *asr1*Δ mutants would have more energy available for cell survival mechanisms (as opposed to cell division) than the wild type strains in the presence of lethal ethanol concentrations. Indeed, nucleocytoplasmic transport of Asr1 via nuclear pore complexes (NPCs) is itself an energy dependant process (Mattaj 1998; Lorenz *et al.*, 2000; Rout *et al.*, 2003; Meyer and Vinkemeier 2004) further compromising the energetics of the wild type strain in a lethal ethanol environment.

In conclusion, the results described in this chapter support the hypothesis of Betz *et al.* (2004) that Asr1 has a positive role in the physiological response of *S. cerevisiae* to non-lethal ethanol stress. At a physiological level, deletion of *ASR1* resulted in lower growth rates of *S. cerevisiae* in non-lethal ethanol concentrations yet its absence was an advantage for the survival of cells in lethal ethanol concentrations. This suggests that Asr1 may be involved in stimulating cell growth/cycle regulation in ethanol-stressed cells.

CHAPTER 5

The use of DNA microarrays to characterise the role of Asr1 in acclimatising to ethanol stress

5.1 Introduction

Very little is known about the way in which cells specifically sense ethanol stress and initiate an appropriate response. Asr1 is a protein with unknown function that shuttles constitutively between the cytoplasm and nucleus, but rapidly and reversibly accumulates in the nucleus in the presence of alcohol (Betz *et al.*, 2004; Izawa *et al.*, 2006). Interestingly, the changing sub-cellular localisation of this protein occurs exclusively in response to alcohol, making Asr1 a candidate for an ethanol-specific signalling factor.

Results presented in the previous chapter, established that actively growing cells lacking Asr1, were indeed slightly less tolerant to the effects of ethanol than the wild type strain, supporting the proposal that Asr1 plays a role in conferring ethanol-tolerance. In this chapter the role of Asr1 was investigated at a molecular level, the primary objective being to determine whether it impacts on gene expression at the level of transcription. To this end, a comparison was made between the transcriptomes of wild type and an *ASR1* gene knockout (*asr1*Δ) strains soon after the inoculation of an exponentially growing cell population into fresh media containing a sub-lethal concentration of ethanol.

These studies were performed using microarray technology and the results confirmed using real-time PCR. Compared with other methods, DNA microarray technology allows parallel and comparative analysis of the expression of thousands of different mRNA transcripts (Gasch *et al.*, 2000; Causton *et al.*, 2001). In combination with the complete genomic sequence of *Saccharomyces cerevisiae,* determined in 1996 (Goffeau *et al.*, 1996), microarray technology represents an extraordinary resource to the yeast community, enabling the monitoring of whole genome expression in a single experiment.

The current experiments focused specifically on the difference in gene expression due to the presence or absence of Asr1 rather than the overall response of the cells to ethanol, which has previously been the focus of a number of studies (Alexandre *et al.*, 2001; Chandler *et al.*, 2004). Two sets of microarray experiments were performed in this investigation. Initially gene expression was compared in the wild type strain and the *asr1*Δ strain after 30 minutes growth in the absence of ethanol. Subsequently, gene expression was compared at the same time point in the presence of 7.5% (v/v) ethanol.

5.2 Results

5.2.1 RNA preparations: quality and reproducibility

The key to any successful comparison of gene expression is preparation of high quality template RNA. The traditional approach to normalisation is based on a comparison of mRNA expression in samples represented by an equal amount of total RNA. However, it has previously been shown that levels of mRNA and rRNA are generally reduced in cells exposed to environmental stress, which may lead to inaccuracy in interpreting gene expression data (Mager and Moradas-Ferreira 1993; Emslie 2002; Chandler *et al.*, 2004). To reduce the subsequent risk of generating artificial positive results, RNA was extracted from an equal number of cells (1×10^8) for comparison in the current gene array experiments.

To isolate high quality RNA, phenol: chloroform extraction was performed, followed by ethanol precipitation as described previously in Chapter 2, Section 2.4.2.2. The reproducibility of the extraction procedure was established by performing triplicate RNA extractions from separate yeast cultures. Comparable RNA yields and quality was observed across the triplicate experiments (Figure 5.1). The ratio of absorbance at 260:280 nm was measured to determine purity of the RNA. Ratios of 1.7 or higher were consistently obtained indicating the RNA was essentially free from protein and phenol contamination.

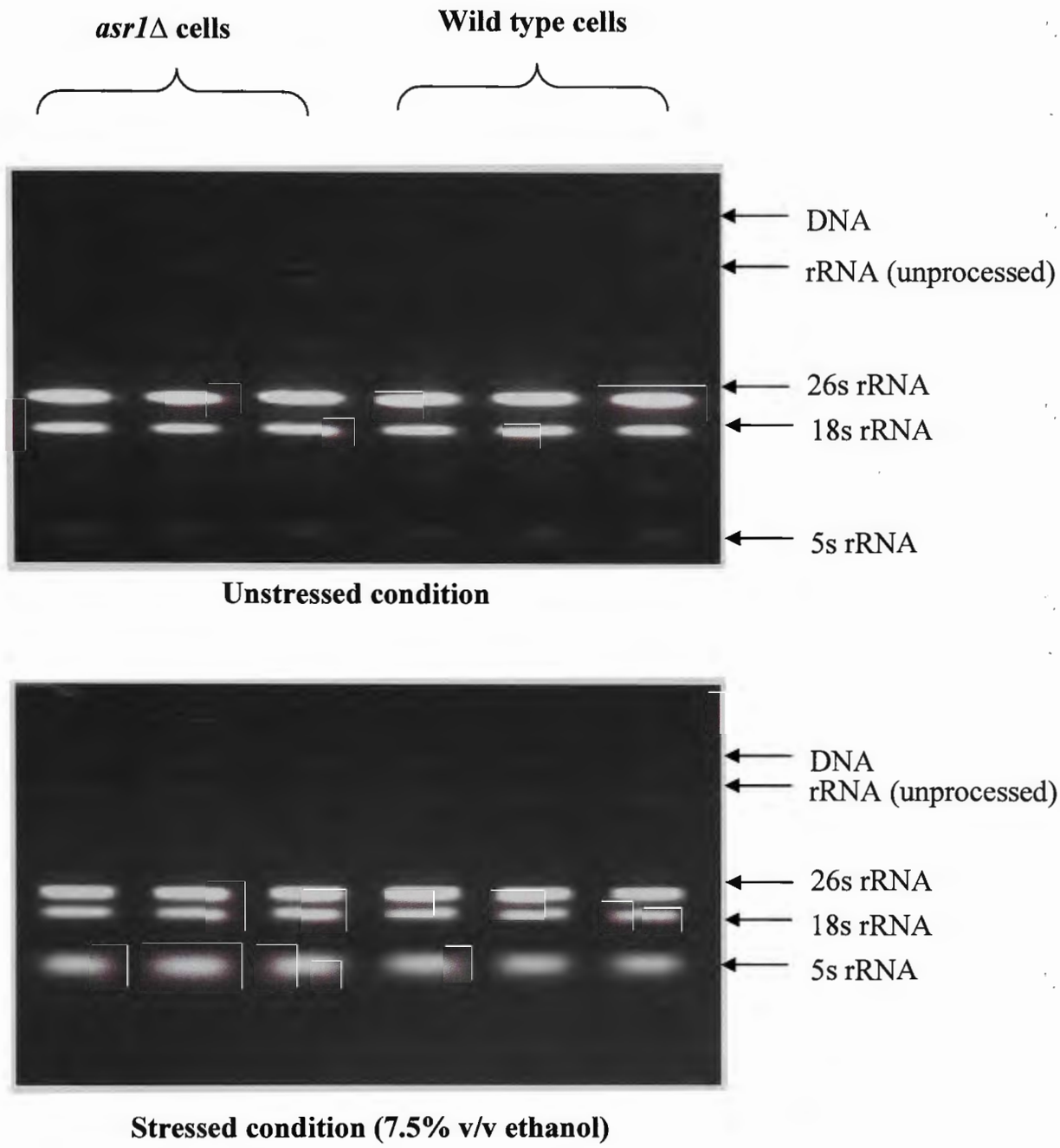


Figure 5.1: Reproducibility of total RNA extractions for microarray analysis. Total RNA was extracted from 1 x 10^8 cells of wild type and *asr1*Δ. Cells were harvested after 30 minute in absence (unstressed) or presence of 7.5% (v/v) ethanol (stressed) cultures. RNA was visualized on ethidium bromide-stained 1% agarose gels. The relatively constant yields of DNA and RNA in the replicates indicate that the RNA isolation method was consistent. The overall quality of the preparations is apparent from the integrity of the bands. This experiment was repeated several times and always gave similar results.

Any residual DNA contamination in RNA samples was removed by digestion with RNase-free DNase (see Chapter 2 Section 2.4.2.3 for more details). This step was considered important, as the RNA was required both for microarray analysis and subsequent real-time PCR validation. To test purity, DNase treated RNA was first resolved on a 1% agarose gel (Figure 5.2**A**) and subsequently tested for its inability to act a template in a PCR reaction using *ACT1* primers. As shown in Figure 5.2, DNase treatment removed visible signs of genomic DNA contamination without causing significant RNA degradation. Moreover, after DNase treatment, amplification of Actin specific PCR products did not occur, indicating that DNase treatment had effectively eliminated residual genomic DNA contamination (Figure 5.2**B**).

The RNA samples selected for microarray analysis are shown in Figure 5.1. These samples were taken from the cultures of wild type or *asr1*Δ strains grown in 30 minutes after inoculation into media containing either 0 or 7.5% (v/v) ethanol. These RNA preparations were considered suitable for gene expression analysis as RNA was high quality and free of genomic DNA.

5.2.2 Microarray analysis

Microarray analysis was performed using oligonucleotide microarray slides (Clive and Vera Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Sydney). Each microarray contained ~ 6,528 open reading frames encoded by the *S. cerevisiae* genome and represented by 40 mer oligonucleotides spotted on 25 x 75 mm glass slide.

In all experiments, RNA was extracted from equal cell numbers (1 x 10^8 cells), converted to cDNA and labelled separately with either Cy3 or Cy5. Wild type cDNA was labelled with Cy3 (green), and *asr1*Δ cDNA was labelled with Cy5 (red). Following hybridization, the microarray slides were scanned using GenePix400B scanner (Axon instrument, USA) and fluorescence of the Cy3 and Cy5 dyes analysed using the Genepix-Pro 6.0 (Axon instrument, USA) software to determine relative gene expression. All subsequent statistical analysis of datasets was performed using the TM4 software package from TIGR (Saeed *et al.*, 2003).

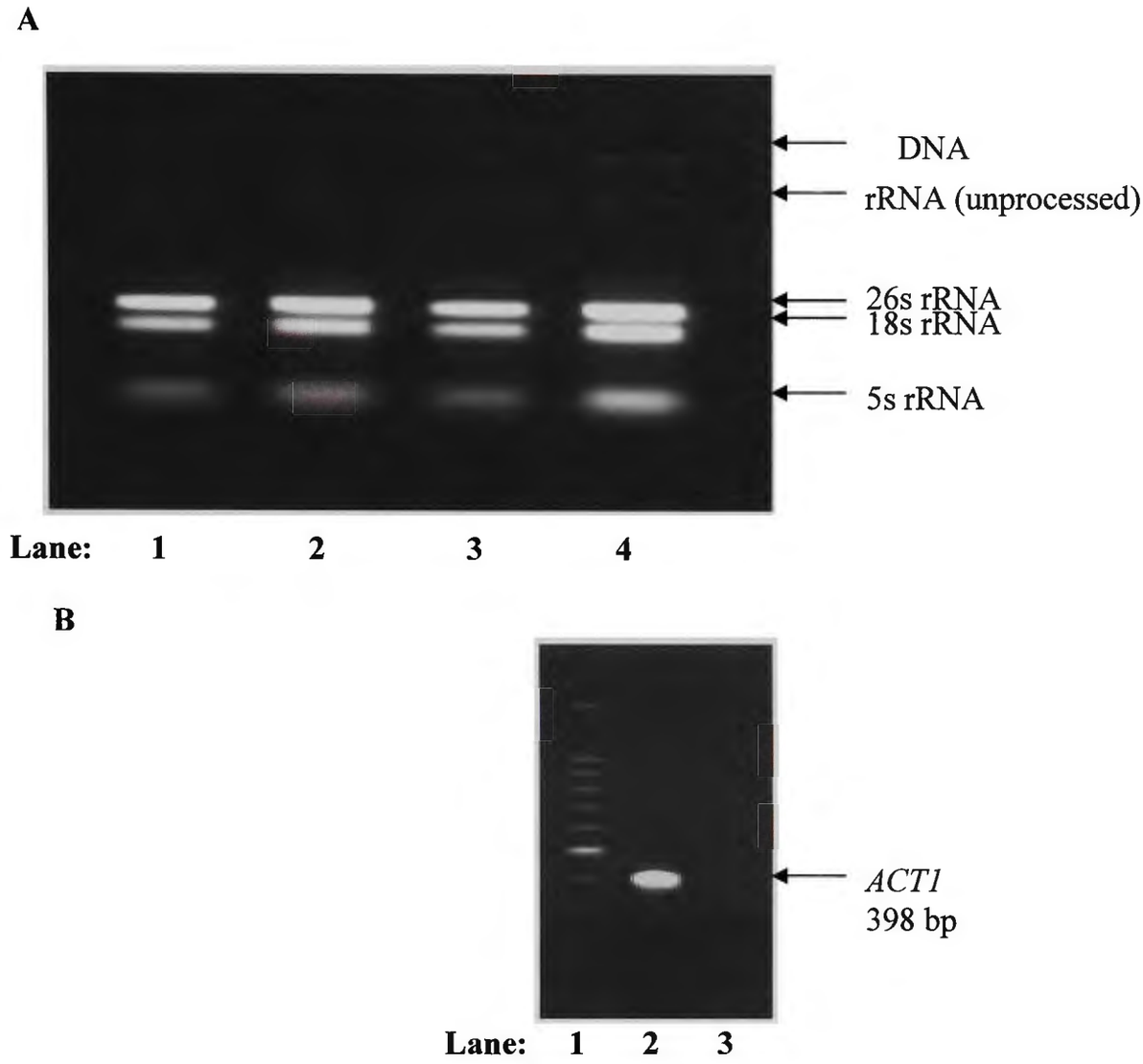


Figure 5.2: DNase treatment of total RNA. **A**, RNA samples were DNase treated and visualised on 1% ethidium bromide-stained agarose gel. DNase treatment was effective in reducing to undetectable level of DNA contamination (lane 1, 2 and 3) comparatively untreated samples in which originally DNA presented (lane 4). **B**, RNA samples were DNase treated and PCR amplification was performed using an *ACT1* primer combination. PCR products were electrophoresed on an ethidium bromide-stained 1% agarose gel. Untreated RNA samples produced the expected 398 bp PCR product derived from genomic DNA (lane 2). No PCR products were obtained after DNase treatment (lane 3).

Gene expression profiles were compared for cells from cultures with 0% (duplicate) and 7.5% (v/v) ethanol (triplicate). Representative microarrays are shown in Figure 5.3. For subsequent data analysis, a number of filtering steps were performed using Genepix-Pro 6.0 software. The fluorescent intensity of a particular feature usually includes a certain amount of background fluorescence in addition to the fluorescent signal from derived specifically from hybridisation to the probe. In these experiments local background subtraction method was applied to remove background from microarray images. Furthermore where the features were sub-standard, they were removed from the dataset by "flagging" as 'Bad' to indicate false expression. Data from microarray experiments may be biased for several reasons including differences in dye properties, probe labelling and hybridisation efficiencies. To remove such biases which are not a part of the biological interpretation, "local regression via lowess normalisation" was used in data analysis.

5.2.3 The impact of Asr1 on gene expression

Following filtering and normalisation, genes with significant differential expression were identified using Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001). SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific *t* tests. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than a threshold are deemed potentially significant. The percentage of such genes identified by chance is the false discovery rate (FDR) .The present study set a false discovery rate (FDR) of 1.8%.

Gene expression was compared in the wild type and *asr1*Δ strains, after 30 minutes growth in the presence and absence of 7.5% ethanol stress. In the absence of ethanol no significant difference was observed in gene expression between the wild type and *asr1*Δ strains. In contrast, SAM identified 140 genes that were expressed at a lower level ($\geq$ 1.5 fold) in the *asr1*Δ strain when compared with the wild type strain, in the presence of 7.5% (v/v) ethanol stress. The mean fold changes and description of gene products in this dataset are listed in Table 5.1.

A **B**

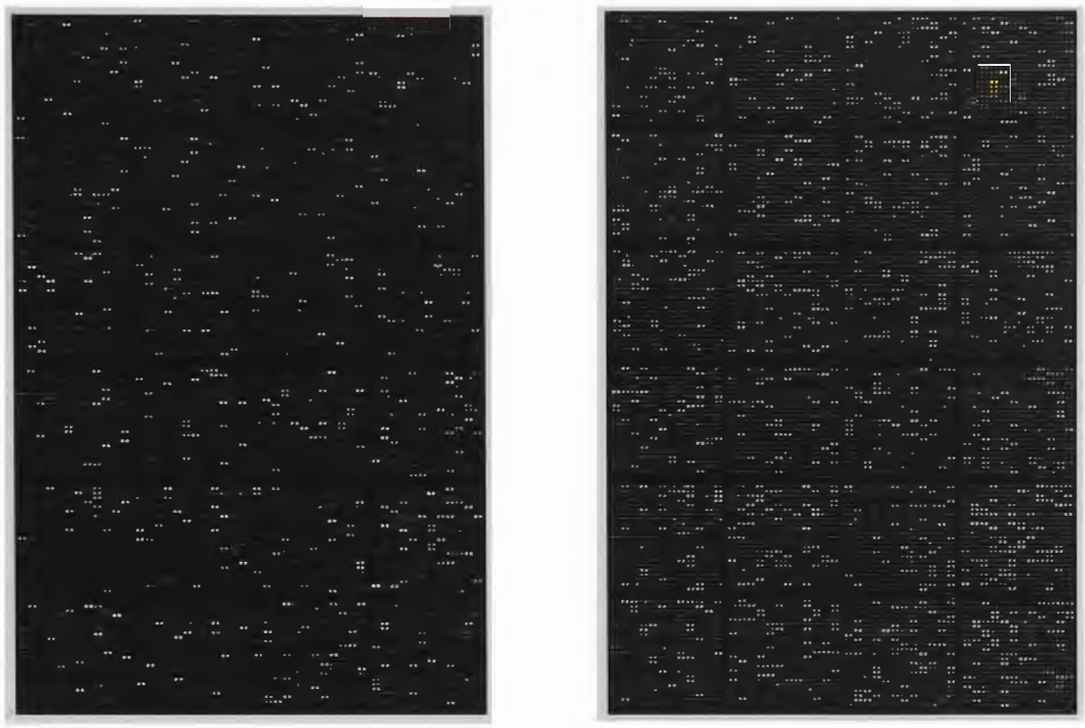


Figure 5.3: Representative microarrays comparing gene expression in *asr1*Δ and wild type strains, 30 minute after inoculation into media containing (**A**) 0% ethanol (non-stressed) or (**B**) 7.5% (v/v) ethanol (stressed). In this ratio image, each feature represents an ORF of *S. cerevisiae*. A red spot indicates that expression of the ORF is higher in *asr1*Δ; a green spot indicates that expression of the ORF is lower in *asr1*Δ than the wild type; a yellow spot means that there is no change in the expression level between *asr1*Δ and wild type.

Table 5.1: Genes expressed at a lower level (≥ 1.5 fold) in the absence of Asr1 following 30 minute ethanol stress (7.5% v/v)

Gene name	Fold repression	Description of gene product[a]
FUN30	-13.3	Chromosome organization and biogenesis
ATS1	-10.8	Alpha Tubulin Suppressor involve in Budding and cell bud growth
PEX28	-10.4	Peroxisome organization and biogenesis
BUD8	-9.8	Cellular bud site selection Pseudohyphal growth
VPS8	-9.7	Vacuolar Protein Sorting
LRP1	-9.3	Telomere maintenance
TRR2	-9.2	ThioRedoxin Reductase
REC102	-7.4	Meiotic recombination
YLR001c	-7.2	Unknown function
LTE1	-7.2	Regulation of exit from mitosis
TEA1	-7.1	Ty enhancer-mediated transcription
FUN26	-7.1	Nucleoside transport
YDR149c	-7.0	Unknown function
FBP1	-6.9	Fructose-bisphosphatase activity
CYC3	-6.5	Holocytochrome-c synthase activity
CLN3	-6.4	G1/S transition of mitotic cell cycle
IMP3	-6.0	Ribosome biogenesis and assembly
YHR105w	-6.0	Unknown function
YLR326w	-5.8	Unknown function
TFC3	-5.8	RNA polymerase III transcription factor activity
SWI5	-5.6	G1-specific transcription in mitotic cell cycle
YOR309c	-5.6	Unknown function
NUP42	-5.6	RNA export from nucleus
RDR1	-5.5	Response to xenobiotic stimulus
CCR4	-5.5	mRNA catabolic process
SUP35	-5.5	mRNA catabolic process, deadenylation-

		dependent decay
CDC24	-5.4	Cell Division Cycle
YDR193w	-5.3	Unknown function
SAE3	-5.2	Meiotic recombination
REF2	-5.2	mRNA processing, snoRNA 3'-end processing
MLS1	-5.2	Malate synthase activity
YKL031w	-5.0	Unknown function
ARP1	-4.9	Establishment of mitotic spindle orientation
SPS4	-4.7	Sporulation
KRE5	-4.7	1,6-beta-glucan biosynthetic process
YGR139w	-4.6	Unknown function
SNU66	-4.6	Nuclear mRNA splicing, via spliceosome
PAT1	-4.5	Chromosome segregation
YAL037w	-4.4	Unknown function
PES4	-4.3	Polymerase Epsilon Suppressor
YKL174c	-4.3	Excretion of putrescine and spermidine
ALA1	-4.3	alanyl-tRNA aminoacylation
YLR352w	-4.2	Unknown function
MSS116	-4.0	RNA splicing
LAS1	-4.0	Budding and cell bud growth
CCE1	-4.0	DNA recombination
YNR021w	-3.9	Unknown function
YBR113w	-3.9	Unknown function
ERP3	-3.8	Secretory pathway
YJL043w	-3.8	Unknown function
YDL091c	-3.8	Unknown function
YFL030w	-3.7	Glycine biosynthetic process
OAR1	-3.7	Aerobic respiration
		Fatty acid metabolic process
YGL045w	-3.7	Unknown function
YLR376c	-3.6	Unknown function
YBR134w	-3.6	Unknown function
SFH1	-3.5	G2/M transition of mitotic cell cycle

YDL176w	-3.4	Unknown function
MKC7	-3.3	Proteolysis
EBP2	-3.3	rRNA processing
OCT1	-3.3	Mitochondrial intermediate peptidase activity
SKI12	-3.2	RNA helicase activity
HSD1	-3.1	Phosphatidic acid biosynthetic process
MEC1	-3.1	Meiotic recombination, Mitosis Entry Checkpoint protein
YHR125w	-3.1	Unknown function
VTS1	-3.1	Regulation of mRNA stability
EKI1	-3.1	Ethanolamine kinase activity
HSN1	-3.1	Unknown function
YFL067w	-3.0	Unknown function
YBR178w	-3.0	Unknown function
MPS3	-3.0	Spindle pole body duplication in nuclear envelope
BIG1	-2.9	Chitin- and beta-glucan-containing cell wall biogenesis
URA3	-2.9	Pyrimidine base biosynthetic process
NMA1	-2.9	NAD metabolic process
TPK3	-2.8	cAMP-dependent protein kinase activity
HAP5	-2.8	Transcriptional activator and global regulator of respiratory gene expression
UTP14	-2.8	rRNA processing
RTR1	-2.8	Transcription from RNA polymerase II promoter
SPA2	-2.8	Actin cytoskeletal organization during polarized growth
SEC7	-2.7	Intra-Golgi transport and ER-to-Golgi transport
SDA1	-2.6	Actin cytoskeleton organization and traversing start control point of mitotic cell cycle
PRP9	-2.6	mRNA processing
SIR2	-2.6	NAD-dependent histone deacetylase activity
RMD8	-2.5	Response to drug

SNF7	-2.5	Late endosome to vacuole transport
YKL115c	-2.5	Unknown function
TRM1	-2.4	tRNA -methyltransferase activity
BUD6	-2.4	Cellular bud site selection
SLX9	-2.4	rRNA processing
SDS22	-2.3	Chromosome segregation
DRS1	-2.3	Ribosome biogenesis and assembly
CCZ1	-2.3	Vacuolar assembly
EFB1	-2.3	Translation elongation factor
IXR1	-2.2	DNA repair
ELM1	-2.2	Cell morphogenesis and pseudohyphal growth
NOC3	-2.2	rRNA processing
PCL7	-2.2	Regulation of glycogen biosynthetic process
MRS2	-2.2	Mitochondrial RNA Splicing
YHR078w	-2.2	Unknown function
DBP2	-2.2	mRNA catabolic process and rRNA processing
SSH4	-2.1	Vesicle-mediated transport
FRE2	-2.1	Ferric reductase and cupric reductase activity
ROM2	-2.1	Rho guanyl-nucleotide exchange factor activity, budding and cell bud growth
HOS2	-2.1	NAD-dependent histone deacetylase activity
PMT2	-2.1	Protein O-MannosylTransferase activity
IRE1	-2.1	Unfolded protein response
YKL050c	-2.1	Unknown function
PRP40	-2.0	Nuclear mRNA splicing, via spliceosome
TRM11	-2.0	tRNA methylation
SMC2	-2.0	Mitotic chromosome condensation
MIF2	-2.0	Mitotic spindle organization and biogenesis in nucleus
TRK2	-2.0	Cellular potassium ion homeostasis
CHS5	-2.0	Cell wall chitin catabolic process and cellular bud site selection
TPC1	-2.0	Thiamin pyrophosphate transport

YHL018w	-2.0	Unknown function
PRP11	-1.9	Spliceosome assembly
UPC2	-1.9	Sterol biosynthetic process
VPS62	-1.9	Protein targeting to vacuole
MSC6	-1.9	Meiotic recombination
RPA34	-1.9	RNA Polymerase A
RRP14	-1.9	Ribosome biogenesis and assembly
RMA1	-1.8	Tetrahydrofolylpolyglutamate synthase activity
ALK1	-1.8	Protein kinase activity during cell cycle
DNL4	-1.8	DNA ligase activity
MTF2	-1.7	mRNA processing
MMS21	-1.7	Chromosomal organization and DNA repair
BUD27	-1.7	Cellular bud site selection
NTR2	-1.7	Nuclear mRNA splicing, via spliceosome
VID22	-1.7	Vacuolar protein catabolic process
ATG18	-1.7	Vacuolar protein processing
RIA1	-1.7	rRNA processing
LST7	-1.7	Golgi to plasma membrane transport
LCP5	-1.7	rRNA processing
YEL033w	-1.6	Unknown function
SPS2	-1.6	Spore wall assembly
KGD2	-1.6	2-oxoglutarate metabolic process
WHI5	-1.6	G1/S transition of mitotic cell cycle
YBR108w	-1.6	Unknown function
SHR5	-1.6	Protein amino acid palmitoylation
FAA2	-1.5	Long-chain-fatty-acid-CoA ligase activity
YFR022w	-1.5	Unknown function

[a] Gene description taken from Saccharomyces Genome Database (SGD) http://www.yeastgenome.org/.

The web-based cluster interpreter FunSpec (http://funspec.med.utoronto.ca) was used to investigate the possible function of these genes with respect to information held in the Gene Ontology (GO) database. The Bonferrerroni correction was applied to compensate for multiple testing over many categories of the databases (Robinson *et al.*, 2003). A *P* value cut-off of 0.01 was used to determine clusters that were enriched using the "guilt-by-association" predictive methodologies.

In comparison with the GO database, the following categories were significantly over-represented in the group of genes expressed at a lower level in the *asr1*Δ: Cell growth and/or maintenance (GO: 0008151), Metabolism (GO: 0008152), and nucleobase nucleoside nucleotide and nucleic acid metabolism (GO: 0006139) (Table5.2).

On exposure to 7.5% (v/v) ethanol stress for 30 minutes, only 9 genes were expressed at significantly higher levels (≥ 1.5 fold) in absence of Asr1 compared with the wild type strains. Of these, four genes encode proteins of unknown function. The most highly expressed gene, *RDS3* is involved in xenobiotic responses. The remaining four genes, *PRM6*, *NUD1*, *SET2*, and *PAC11* are associated respectively with pheromone responses, mitosis, histone methyltransferase activity, and nuclear migration (Table 5.3).

5.2.4 Verification of array results using real-time-PCR analysis

Quantitative real-time PCR was performed to validate microarray results. Seven genes were selected including 5 genes found to be expressed at a lower level in the *asr1*Δ, one gene expressed at a higher level and one expressed at a similar level in both strains according to the microarray analysis (Table 5.4). Relative expression levels of these genes were compared for wild type versus *asr1*Δ cells exposed to 7.5% (v/v) ethanol after 30 minutes. All cDNAs were prepared independently and used pools of RNA derived from the three stressed or non-stressed growth experiments for microarray analysis. Gene specific primers were designed for the seven candidate genes using *Saccharomyces cerevisiae* genome database (SGD: www.yeastgenome.org). The primer sequences, their melting temperatures and product sizes are shown in the Table 5.5.

Table 5.2: Functional categories of genes, that were expressed at a lower level (≥ 1.5 fold) in the absence of Asr1 following 30 minute ethanol stress (7.5% v/v)

Category	P value[a]	Genes[b]
Cell growth and/or maintenance	3.75×10^{-11}	*TFC3 VPS8 EFB1 FUN30 ATS1 CCR4 FUN26 PMT2 LTE1 CYC3 CLN3 CDC24 CCZ1 MEC1 PAT1 ERP3 PRP9 SIR2 PRP11 MTF2 TRM1 MKC7 SWI5 EKI1 KGD2 SEC7 SUP35 NUP42 MSS116 REF2 UPC2 SPS2 MMS21 URA3 FAA2 LCP5 BUD27 AUT10 PES4 SMC2 HOS2 LST7 TPC1 VPS62 SDA1 IRE1 SAE3 BIG1 TRR2 ARP1 IMP3 PCL7 SMC3 RPA34 CCE1 PRP40 IXR1 ELM1 OAR1 MIF2 TPK3 EBP2 SDS22 FRE2 TRK2 LAS1 DRS1 SOF1 SPA2 NOC3 SNF7 BUD6 SFH1 NMA1 REC102 CHS5 BUD8 ROM2 VID22 FBP1 SKI2 UTP14 MLS1 RIA1 ARG1 ITR2 SHR5 DNL4 WHI5 SNU66 SPS4 MRS2 ALA1 KRE5 TEA1 MSC6 HAP5 VTS1*
Metabolism	5.99×10^{-6}	*TFC3 EFB1 CCR4 PMT2 CYC3 CDC24 MEC1 PAT1 ERP3 PRP9 SIR2 PRP11 MTF2 TRM1 MKC7 SWI5 EKI1 KGD2 SEC7 SUP35 NUP42 MSS116 REF2 UPC2 MMS21 URA3 FAA2 LCP5 AUT10 PES4 HOS2 LST7 VPS62 IRE1 SAE3 BIG1 TRR2 IMP3 PCL7 RPA34 CCE1 PRP40 IXR1 ELM1 OAR1 TPK3 EBP2 SDS22 DRS1 SOF1 NOC3 SFH1 NMA1 REC102 CHS5 VID22 FBP1 SKI2 UTP14 MLS1 ARG1 SHR5 DNL4 SNU66 SPS4 MRS2 ALA1 KRE5 TEA1 MSC6 HAP5 VTS1*
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	7.16×10^{-6}	*TFC3 CCR4 MEC1 PRP9 SIR2 PRP11 MTF2 TRM1 SWI5 NUP42 MSS116 REF2 MMS21 URA3 LCP5 PES4 HOS2 SAE3 IMP3 RPA34 CCE1 PRP40 IXR1 EBP2 DRS1 SOF1 NOC3 SFH1 REC102 SKI2 UTP14 DNL4 SNU66 SPS4 MRS2 TEA1 MSC6 HAP5*

[a] Gene functions were identified by addressing the GOC databases with the FunSpec statistical evaluation program.
[b] Probability of the functional set occurring as a chance event.

Table 5.3: Genes expressed at a higher level (≥ 1.5 fold) in the absence of Asr1 following 30 minute ethanol stress (7.5% v/v)

Gene name	Fold repression	Description of gene product[a]
RDS3	+5.6	Response to xenobiotic stimulus
PRM6	+2.7	Response to pheromone
NUD1	+2.6	Exit from mitosis
YNL114c	+2.2	Unknown function
YJL215c	+2.1	Unknown function
YML047w-A	+2.0	Unknown function
SET2	+2.0	Histone methyltransferase activity
PAC11	+1.9	Nuclear migration, microtubule-mediated
YDR526c	+1.7	Unknown function

[a] Gene description taken from Saccharomyces Genome Database (SGD) http:// www.yeastgenome.org/.

Table 5.4: Gene expression level determinations using microarray analysis and relative quantitative Real Time PCR for selected transcripts prepared from wild type and *asr1*Δ strains following 30 minute ethanol stress (7.5% v/v)

Gene Name	Microarray fold alteration	Real Time PCR fold alteration
VPS8	-10	-10
FUN26	-7	-5
CLN3	-6	-13
SWI5	-6	-9
CDC24	-5	-7
ACT1	1	1
RDS3	+6	+8

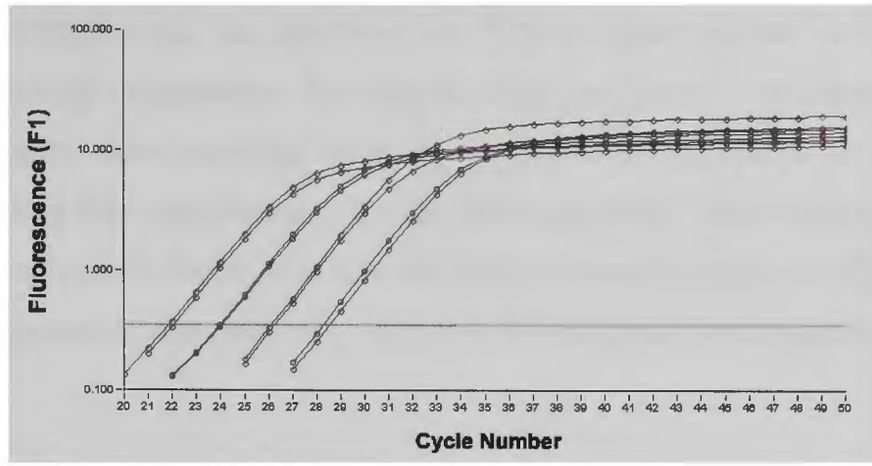
Fold change is given as the mean (n=3) in a single experiment

Table 5.5: Specific primers designed for Real-Time PCR analysis

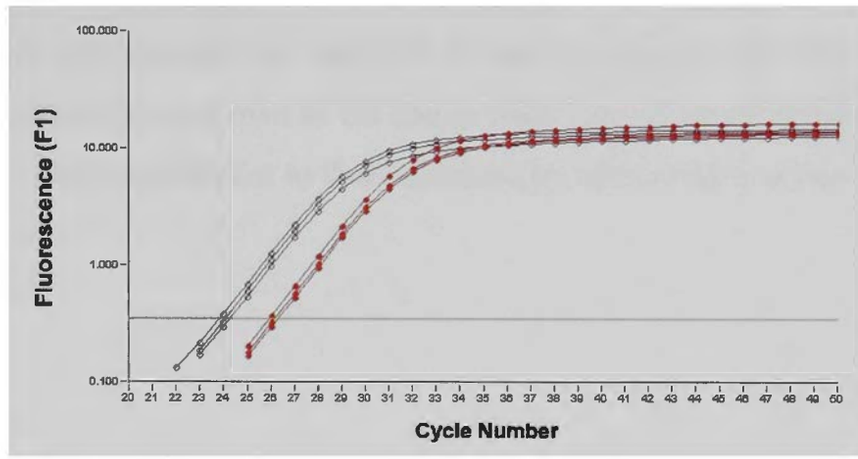
Gene name	Primer sequences	PCR Product size (bp)	Tm of PCR product (°C)
VPS8	5' TGGAGAAGCAGAATTGGTAA	241	79.48
	5' AAGCGACTCCAGAGCTATTT		
FUN26	5' GAATGTGGAAGACGGACATA	260	87.23
	5' ACGCAAATACAGGAAAAACA		
CLN3	5' ACAAGTATTCCTCGCGGTTCA	299	83.33
	5' CAAAGGGGCAGAAAGGACAA		
SWI5	5' TTTGGAGTGAAGGGCGTAAT	232	81.13
	5'TTTGGAGTGAAGGGCGTAAT		
CDC24	5' ATTGCGAGAAGTATCAACGA	281	78.85
	5' GCTGAGGTTGAGGATTTCTT		
ACT1	5' AGGTATCATGGTCGGTATGG	398	82.61
	5' CGTGAGGTAGAGAGAAACCA		
RDS3	5' AAAATGTGATGGCAAATGTC	217	72.1
	5' TCTATTGCTGCCAAGGTTTA		

To calculate the relative levels of gene expression in the *asr1*Δ versus wild type cells by Real-Time PCR, it was necessary to determine the crossing points (CPs) for each gene-specific transcript (Figure 5.4). The CP is defined as the point at which fluorescence rises above a defined level in all PCR reactions. Based on the CP, the relative expression of each candidate gene was calculated, in relation to a standard curve derived using a five fold dilution series of a cDNA pool (designated the 'standard') as the template. A representative example of this approach is shown in Figure 5.4 which shows real-time PCR analysis the *FUN26* expression. For comparison, cDNA derived from an equal number of cells (*asr1*Δ versus wild type cells) was diluted 1:40 and used as a template for amplification of a *FUN26* specific product.

A



B



C

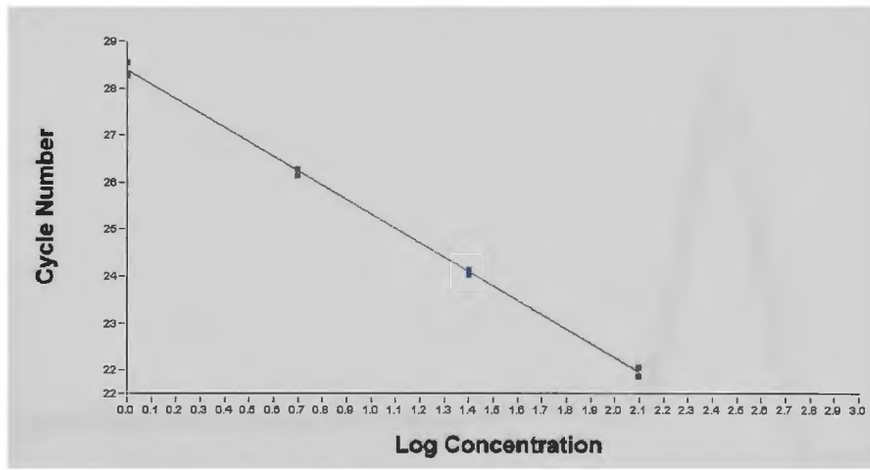


Figure 5.4 Quantitative Real-time PCR analysis of *FUN26* mRNA expression in *asr1*Δ and wild type strain (BY4742) in the presence of 7.5% (v/v) ethanol (SYBR Green 1 detection): **(A)** Logarithmic plot of florescence data during amplification of a dilution series of a cDNA pool, designated as standard (1:4, 1:20, 1:100 and 1:500) and **(B)** wild type (black) and *asr1* Δ (red); **(C)** Standard curve derived from amplification of cDNA standard dilutions showing logarithm of concentration versus crossing point (CP). The threshold for determination of crossing point is shown in green.

For each gene specific PCR product, the expected product size was confirmed by agarose gel electrophoresis. In addition, all PCR products melted at a single characteristic melting temperature. No amplification products of the characteristic melting temperature were amplified from any negative control reactions (RT step omitted), indicating that amplification did not reflect genomic DNA contamination. Melting curve analysis of the *FUN26* specific PCR products is shown in Figure 5.5 and melting temperatures and products sizes of all RT-PCR products are shown above in Table 5.5.

This approach was used to confirm observed differences in gene expression between the wild type and *asr1*Δ strains for each of the selected genes after 30 minutes exposure to 7.5% ethanol. As shown in the above table 5.4, values obtained by real-time PCR analysis were very similar to those obtained by microarray analysis for each of the seven genes.

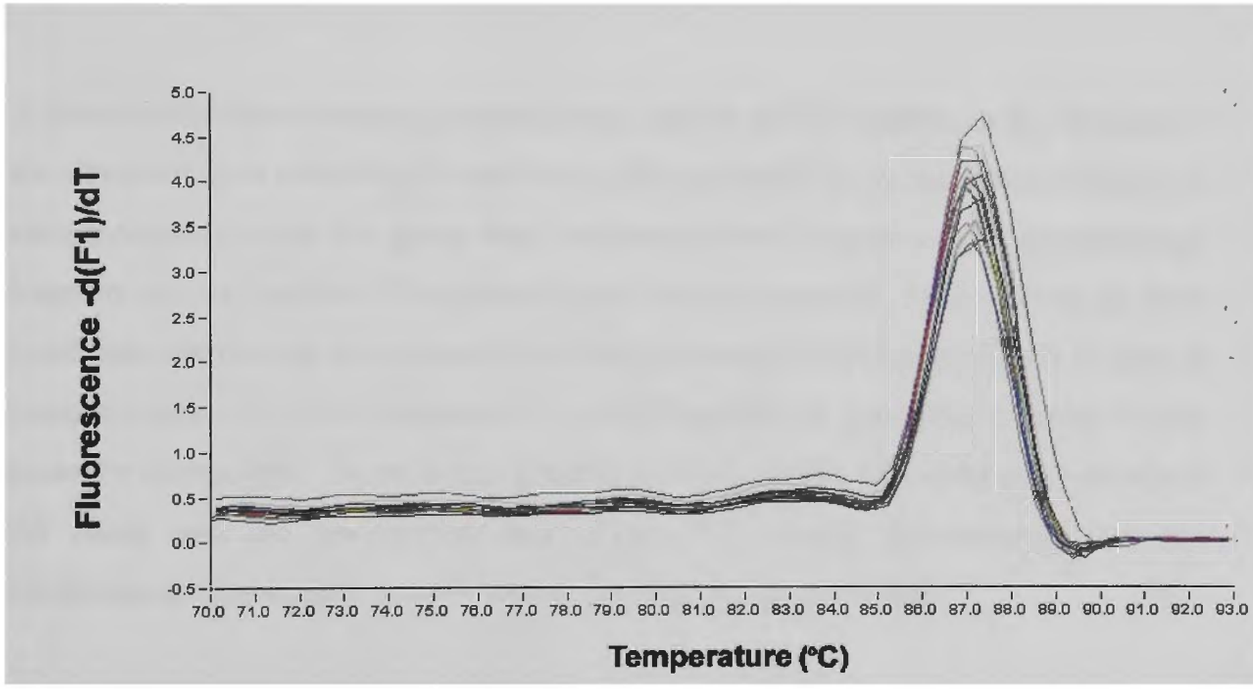


Figure 5.5: Melting curve analysis of *FUN26* real-time PCR products derived from the cDNA standard dilutions and sample cDNAs (*asr1*Δ and wild type strain). All amplification products melted with a Tm of 87 °C

5.2.5 Non-random chromosomal distribution of differentially expressed genes

Further inspection revealed that genes affected by *ASR1* deletion were non-randomly distributed across the genome. In the set of genes expressed at a lower level in the *asr1*Δ under ethanol stress, there were a disproportionate number of adjacent gene pairs and groups of neighbouring genes showing a similar expression profile. The size of these groups varied from 2-6 genes (Figure 5.6). In total 17 groups of co-expressed genes were detected, including 7 pairs of genes and 10 groups of three or more genes. The largest group comprising six genes was located in chromosome I and a second group with four neighbouring genes was found in chromosome XV. The five genes expressed a lower level in the *asr1*Δ, whose expression profile was confirmed by real-time PCR, were located in 4 separate cluster groups, as shown in Figure 5.6

As shown in Figure 5.6, co-expression of neighbouring genes within a cluster was independent of their orientation. Genes exhibited divergent (←→), tandem (←← or →→), or convergent (→←) orientation with represent to their upstream promoter sequences.

A repetitive random sampling approach was used to test the statistical significance of the observed gene clustering in relation to physical location on the chromosome. For each independent test, the genes were randomized with respect to their chromosomal location and the number of regulated gene clusters recorded. From a total of 1000 repetitions, there were no instances in which the randomized data produced at least as many clusters as in the chromosomally ordered experiment, providing p-values for the presence of regulated chromosomal gene clusters of <0.001. The comparison between the actual and the randomized data (Figure 5.7) clearly demonstrated that the clustering of neighboring genes was not occurring by chance alone.

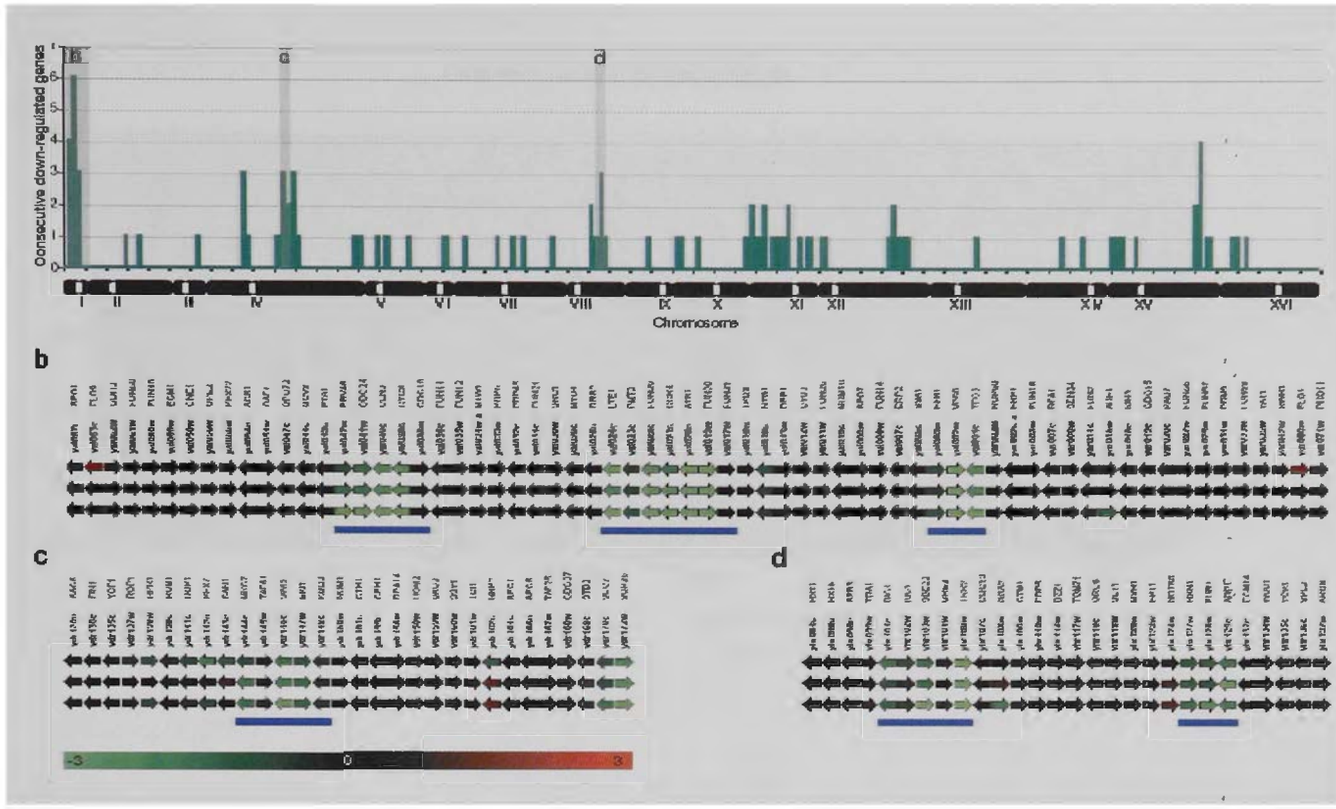


Figure 5.6: Distribution of neighbouring gene clusters over the yeast chromosomes. A significant number of genes expressed at a lower level in the *asr1*Δ after 30 minutes exposure to 7.5% ethanol stress were located in neighbouring clusters on the chromosomes. (a) Histogram demonstrating the number of genes clustered as groups of 2-6 genes on each chromosome. (**b-d**) Maps showing the physical location, gene expression and orientation of Asr1 regulated genes in selected gene clusters on chromosome I (**b**), chromosome IV (**c**) and chromosome VIII (**d**). Arrows show the orientation of the gene with respect to up-stream promoter region. Gene expression is shown as the $\log_2$ fold change in the *asr1*Δ strain relative to the wild type strain, according to the colour scale. Groups of co-expressed genes have been underlined in blue. (For enlarge version of Figure 5.6 and 5.6 (**b**), see Appendix III, Figure 3 and 4).

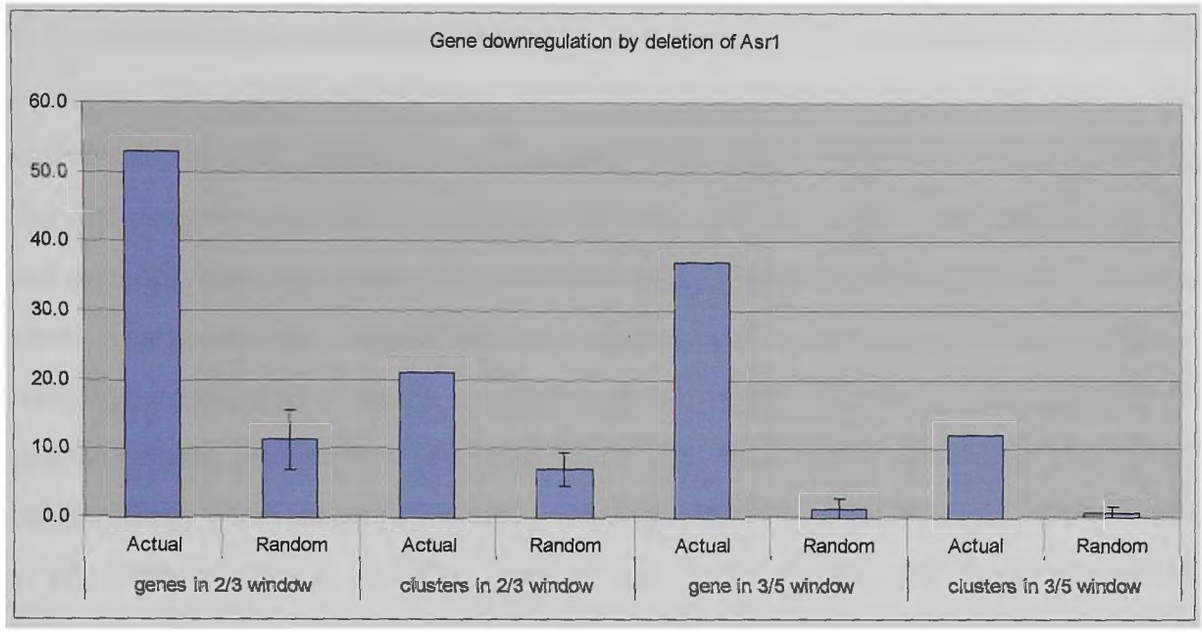


Figure 5.7: Histogram demonstrating significant levels of gene clustering according chromosomal order within the set of genes expressed at a lower level in the *asr1*Δ strain during ethanol stress. The graph shows the actual number of observed clusters with either 2/3 or 3/5 genes showing co-expression; the total number of genes in the 2/3 or 3/5 gene cluster windows and corresponding values predicted by random assortment of chromosomal order ($p = 0.001$). Error bars indicate standard deviation.

5.3 Discussion

Results from the previous chapter demonstrated that, when yeast cells are ethanol-stressed, *ASR1* gives a selective advantage compared to its knockout counterpart. Because Asr1 locates to the nucleus when cells are exposed to alcohol and it has features that suggest it may have a role in regulating gene expression, it was decided to test the impact of deleting this gene on the transcriptome of yeast. More specifically, the aim of the study was to compare the transcriptomes of wild type and

knockout (*asr1*Δ) strains of *S. cerevisiae* BY4742 in the presence and absence of ethanol.

In the presence of sub-lethal concentration of ethanol (7.5%), a number of genes were expressed at a significantly lower level following deletion of the *ASR1* gene. This difference was only detected in cells compromised by ethanol stress implying firstly that differences were directly related to the function of the *ASR1* gene product (Asr1), and secondly that Asr1 plays a role specifically related to the presence of ethanol stress. The results also suggest that the nuclear role of Asr1 is related to transcription, a finding which is not entirely unexpected given recent evidence regarding predicted protein-protein interactions involving Asr1. The most recent genome-wide screens using affinity purification followed by mass spectrometry (Gavin *et al.*, 2006; Krogan *et al.*, 2006; Collins *et al.*, 2007) predict that Asr1 interacts with Rpo21 and Rbb9, both of which are subunits of RNA polymerase II. Interactions with other subunits of RNA polymerase II (Rpb3, Rpb9 and Rpb11) were suggested by Gavin *et al.* (2006) but were not predicted by Collins *et al.* (2007) who imposed more comprehensive selection criteria for protein complex identification. Interestingly all genes that showed a statistically significant difference in expression, were expressed at a lower level in the absence of Asr1 indicating that Asr1 plays a role that in some way promotes rather than hinders expression of affected genes.

Of the 140 or so genes that were expressed differently in the *asr1*Δ background, few have previously been implicated in the ethanol stress response reported by Alexandre *et al.* (2001) and Chandler *et al.* (2004). Both groups identified genes that change in response to ethanol stress early after exposure to ethanol (30 minutes and 1 hr respectively). These genes are likely to be involved primarily in the initial 'survival' response. The finding that the majority of these genes were unaffected by the presence of *ASR1* suggests that Asr1 is not involved in the initial protective response. This agrees well with the finding that Asr1 confers no advantage to cells when challenged with higher concentrations of ethanol approaching lethality. It is also in accord with physiological data obtained in the current study which showed no difference in growth over short term exposure to ethanol shock, but a clear competitive advantage, due to the presence of Asr1, over the long term.

Clustering differentially regulated genes according to common molecular function, biological process or cellular location, can provide insight into the biological relevance of the data. When considering the relationship of the set of genes expressed at a lower level in the absence of Asr1, cluster analysis revealed that 70% of these genes were in some way related to cell growth and/or maintenance. This is interesting, given our previous observation that Asr1 only confers an advantage in cells that are actively growing. It supports the idea that Asr1 plays a role in the promotion or maintenance of adequate growth in response to the external conditions, rather than immediate survival of cells under threat. It is however, important to recognise that the current study represents a snapshot of the ethanol stress response taken only 30 minutes after ethanol exposure and a more far-reaching temporal study of expression profiles is required to fully comprehend the role of Asr1.

Growth and cell cycle progression are profoundly influenced by environmental conditions. Yeast cells vary the duration of G1 phase in their cell cycle principally by controlling passage through START and, in this context, it is interesting that a key regulator of the G1/S transition, cyclin Cln3, is expressed at a much lower level in the *asr1*Δ strain. Recent studies by Berberis *et al.* (2007) support earlier reports that Cln3 plays an important role in controlling cell cycle progression. Several studies have shown that levels of Cln3 are affected by growth conditions including glucose availability. This is consistent with the proposal of Chandler *et al.* (2004) that ethanol-stressed cells are in a state of pseudo-starvation where nutrients such as glucose are present in the growth medium but are not accessible to the cell. It is possible that Asr1 plays a role in modulating cell cycle progression despite reduced energy levels available to the cell, ensuring that growth and cell division are minimally affected by the detrimental affects of ethanol. There is however no direct evidence for this at present.

One of the most interesting observations is that the arrangement of Asr1-affected genes along the chromosomes of ethanol-stressed yeast cells is not random. Groups of two or more neighbouring genes exhibiting similar expression profiles appeared in the dataset more frequently than might be expected by chance alone. The phenomenon of non-random positioning of similarly expressed genes on chromosomes, was first observed in *Drosophila melanogaster* in 1991 (Hager and Miller 1991). Cho *et al.*

(1998) was the first to show physical clustering of co-expressed genes in *S. cerevisiae*; 25% of genes with cell-cycle dependant expression patterns were directly adjacent to genes induced in the same phase of the cell cycle. Subsequently a number of studies have shown that co-expressed genes are frequently located in close proximity, in a wide range of species including *Arabidopsis thaliana* (Williams and Bowles 2004; Ren *et al.*, 2005; Schmid *et al.*, 2005; Zhan *et al.*, 2006) *D. melanogaster* (Spellman and Rubin 2002; Kalmykova *et al.*, 2005) *Caenorhabditis elegans* (Lercher *et al.*, 2003) and *S. cerevisiae* (Cohen *et al.*, 2000; Kruglyak and Tang 2000; Lercher and Hurst 2006).

Kruglyak and Tang (2005) suggested that a common regulatory system might be responsible for co-expression of genes. In this situation pairs of genes can be transcribed on the same strand (unidirectional) with the regulatory system located upstream of both genes. Alternatively, pairs of similarly expressed genes might be transcribed away from each other on opposite strands (divergent) with the regulatory system located between the genes (intergenic region) (Cohen *et al.*, 2000; Kruglyak and Tang 2000). However, this is clearly not the explanation for the co-expressed genes observed in the current study, as co-regulated genes showed a range of orientations with respect to the promoter regions.

A more likely explanation for this observation is that co-expression of neighbouring genes reflects localised changes in the structure of the chromatin. Chromatin within the nucleus is generally inaccessible to the transcription machinery until specific histone modifying proteins and chromatin remodelling complexes initiate the opening or closing of higher order chromatin structure. Opening of chromatin for one gene can result in the opening of neighbouring chromatin, allowing access by transcription factors to promoter regions of neighbouring genes (Roy *et al.*, 2002). This has been proposed as an explanation for the co-localisation of similarly expressed genes observed in a number of studies (Gerasimova and Corces 2001; Spellman and Rubin 2002; Kalmykova *et al.*, 2005; Ma *et al.*, 2005). It is therefore a plausible explanation for prevalence of gene pairs and physically associated groups of genes affected by Asr1 observed in the present study.

Although Asr1 is not a known component of a chromatin -remodelling complex in yeast, a role in histone modification would not be entirely unexpected given the predicted structure of this protein. The N-terminal half of this protein folds into two Really Interesting New Gene (Ring)- or plant homeodomain (PHD)-type finger domains. As in all eukaryotes, the PHD/Ring finger domains of Asr1 are arranged around two zinc ions coordinated by cysteine residues (C) and a histidine (H), in a consensus sequences C4HC3 (Betz *et al.,* 2004). Although each of the two domains in Asr1 show some homology with both the RING finger and the PHD finger domains, it is not possible to categorize them with certainty on the basis of sequence data alone. Therefore it is important to consider the roles played by each of these zinc finger domains, when considering the possible roles for Asr1. These domains are generally associated with protein-protein interactions, are found predominantly in association with nuclear proteins and frequently involved in chromatin remodelling or histone modification.

More than 300 PHD domain proteins are found in eukaryotes and most of these localize to the nucleus and are involved in transcription (Pascual *et al.,* 2000; Capili *et al.,* 2001). The PHD domains of ING family proteins (ING_{PHD}) for example, have been shown to interact with histone acetyl transferase (HAT) and histone deacetylase (HDAC) complexes (Feng *et al.,* 2002). Interestingly a number of proteins appear to be recruited to specific chromatin modifications, such as histone methylation signals, via PHD domains (Pascual *et al.,* 2000; Capili *et al.,* 2001; Feng *et al.,* 2002; Gozani *et al.,* 2003; Ragvin *et al.,* 2004; Zhou and Grummt 2005; Li *et al.,* 2006; Peña *et al.,* 2006; Shi *et al.,* 2006; Wysocka *et al.,* 2006; Li *et al.,* 2007) . Thus it is possible Asr1 plays a role in histone acetylation under ethanol stress and possibly in the recruitment of an unidentified histone acetylation complex to chromatin. However it should be pointed out that to date, no known protein interactions have been predicted for Asr1 with components of a known histone-acetylation complex or indeed with histone proteins, although the latter interaction may well be dependant on the correct posttranslational modification such as methylation.

The motifs in Asr1 also bear a close resemblance to the RING finger domains. RING finger domains are generally found in proteins that function as an E3 ubiquitin ligase, the enzyme which directs the transfer of ubiquitin to target proteins for subsequent

destruction via the ubiquitin pathway (Ohta *et al.,* 1999; Joazeiro and Wa 2000; Yeh *et al.,* 2001; Zheng *et al.,* 2002; Hwang *et al.,* 2003; Wood *et al.,* 2003). Thus, it is possible that Asr1 plays a role in regulating the turnover of nuclear proteins by targeting components of the ubiquitin complex to target nuclear proteins. Ubiquitation plays a key role in the turnover of transcriptional factors, cell cycle regulators and indeed RNA polymerase II itself as exemplified by the studies of during removal of stalled elongation complexes from the DNA (Zheng *et al.,* 2002; Hwang *et al.,* 2003; Wood *et al.,* 2003; Bray *et al.,* 2005). It might also be relevant that Krogan *et al.* (2006) using affinity purification of protein complexes, followed by mass spectrometry predicted an interaction between Asr1 and two proteins involved in ubiquitination - Mdm30 which has been shown to promote ubiquitin-mediated degradation of the transcription factor Gal4 in some strains and YDR131c, a substrate adaptor subunit that recruits substrates to a core ubiquitination complex

However, more recently it has been recognized that the importance of ubiqitination lies well beyond its classical association with protein degradation and also rests in its ability to signal the changes in chromatin that are required not only for the recruitment of RNA polymerase II to the promoter region but also for transcriptional elongation. This is currently an area of great interest and one that fits well with our present observations that suggest a possible role for Asr1 in affecting localised changes in chromatin structure.

In conclusion, Asr1 has significant role in improving the growth of *S. cerevisiae* during ethanol stress. However, this study suggests that Asr1 is unlikely to be involved in instantaneous response to ethanol shock. Over long term exposure to ethanol stress, yeast cells demonstrated competitive advantage due to the presence of Asr1, suggesting that Asr1 might be associated with cell acclimatisation to ethanol stress. This hypothesis is supported by cluster analysis of genes expressed at a lower level in absence of Asr1. The cluster analysis highlights that a significant number of genes were associated with cell growth and/or maintenance. This occurrence clearly demonstrates the role of Asr1 in moderating growth in response to external ethanol as opposed to the immediate survival of cells under ethanol shock.

Another interesting observation was that co-expressed neighbouring genes are clustered in groups of 2-6 along the chromosomes in absence of Asr1 following ethanol stress. This phenomenon suggests that Asr1 associates with chromatin remodelling and or histone modification in response to ethanol. This is not entirely unexpected for the Asr1 which has Ring or PHD type finger domain. These domains are frequently involved in chromatin remodelling or histone modification, association with protein-protein interaction. This provides some evidence that Asr1 may play a role in chromatin remodelling or histone modification.

CHAPTER 6

Conclusions and future directions

The primary objective of this study was to investigate and characterise the roles of trehalose and Asr1 in the response of *S. cerevisiae* to ethanol stress. Previous studies gave conflicting data on whether or not trehalose provides protection against the inhibitory effects of ethanol, and, although Asr1 has characteristics one might expect of an ethanol-stress response protein, a specific phenotype has not been assigned to this protein. This project used a number of physiological-based approaches in an attempt to identify ethanol-stress tolerance phenotypes associated with trehalose metabolism and Asr1 production.

Trehalose was found to have a protective role when *S. cerevisiae* was exposed to ethanol. However, this protection was effective only in reducing lethality when cells were exposed to ethanol at high concentration; trehalose appeared not to have a role in tolerance to sub-lethal, inhibitory concentrations of ethanol.

Following growth in conventional batch cultures did not provide a means of identifying a phenotype for Asr1 in *S. cerevisiae*. However, competitive growth cultures showed that wild type cells (containing *ASR1* gene) had a clear selective advantage over *asr1*Δ cells. Consistent with this, gene expression studies suggested that Asr1 has a role in promoting cell growth during ethanol stress, Based on the evidence provided in this thesis, trehalose and Asr1 are considered to be ethanol-stress tolerance factors in *S. cerevisiae*.

6.1 Conclusions

6.1.1 Trehalose and ethanol stress in *S. cerevisiae*

- Trehalose had no influence on the acclimation rate or growth rate of *S. cerevisiae* in the presence of non-lethal ethanol concentrations; higher

intracellular trehalose concentrations provided no competitive growth advantage, during exposure to non-lethal ethanol stress.

- Although trehalose does not play a role in cell acclimatisation to sub-lethal ethanol stress, it does have a role in promoting survival of *S. cerevisiae* cultures containing lethal ethanol concentrations. Inocula containing high intracellular trehalose concentrations had greater survival rates than cells with lower trehalose levels when inoculated into medium containing lethal ethanol concentrations. The ethanol concentration dependence of the role of trehalose in the response of *S. cerevisiae* to ethanol stress has not previously been observed.

- The survival rate of *S. cerevisiae* in lethal ethanol concentrations increases with increasing age of the inoculum. For example, late stationary phase cells had better survival rates than early stationary phase cells which, in turn, survived better than late exponential phase cells when inoculated into medium containing lethal concentrations of ethanol. Although other stress survival factors undoubtedly played a role, it was found that intracellular trehalose concentrations increased with increasing cell age and that, regardless of inoculum age, cells containing more trehalose survived longest.

- Previous studies that used diverse *S. cerevisiae* strains to explore the relationship between intracellular trehalose concentrations and ethanol tolerance have led to conflicting data. Such studies do not account for the influence of other stress response factors, such as Hsp's and differences in membrane composition, which are likely to vary from strain to strain. The few studies that used closely related *S. cerevisiae* strains to examine the relationship between trehalose and ethanol stress tolerance are consistent with the finding in this project that trehalose has a role in promoting cell survival during exposure to lethal ethanol concentrations,

- The ethanol concentration dependence of the trehalose effect on yeast ethanol-tolerance suggests that the role of this disaccharide during ethanol stress is

likely to be associated with the protection of cytosolic proteins from ethanol damage, although the present study cannot totally discount a role for trehalose in protecting membrane stability.

6.1.2 Asr1 and ethanol stress in *S. cerevisiae*

- In the absence of ethanol there was no difference between the growth of wild type and *asr1*Δ strains. However, competition experiments demonstrated that deletion of *ASR1* resulted in increased sensitivity to non-lethal ethanol concentration. This supports the hypothesis of Betz *et al.* (2004) that Asr1 has a positive role in the physiological response of *S. cerevisiae* to non-lethal ethanol stress.

- This study also suggests that, while Asr1 is unlikely to be involved in an instantaneous response to ethanol shock, it confers a competitive advantage over long term exposure to ethanol stress. This protein might be associated with cell acclimatisation to ethanol, having a role in stimulating cell growth or cell cycle progression in the presence of ethanol stress.

- In contrast, the absence of Asr1 was an advantage for the survival of cells exposed to lethal ethanol concentrations. This result may be linked to the putative role of Asr1 in stimulating cell growth during ethanol stress. The energy demand of cell growth might impact negatively on cells exposed to lethal ethanol stress. In this circumstance, the absence of Asr1 may lead to less depletion of energy reserves, making more available for cellular functions associated with survival.

- Gene expression analysis showed that a number of genes were expressed at significantly lower levels in *asr1*Δ strain in the presence of ethanol. This response was only detected in cells exposed to ethanol, providing further evidence that the role of Asr1 ethanol specific. Genes that were expressed at lower levels in absence of Asr1 were associated with cell growth and/or maintenance, metabolism, nucleobase, nucleoside, nucleotide and nucleic acid metabolism. A significant number (70%) of genes were related to cell growth

associate processes. Specifically, one of the key regulators of the G1/S transition, the cyclin Cln3 was expressed at a much lower level in the strain lacking Asr1. These findings are consistent with the role of Asr1 in modulating cell growth in response to external ethanol.

- Another interesting observation of this study was that genes affected by ASR1 are clustered in groups of 2 to 6 along the chromosomes. This phenomenon suggests that Asr1 exerts its effect by influencing chromatin remodelling and/or histone modification. This is not entirely unexpected as Asr1 has a Ring finger or PHD type domain and these domains have previously been implicated in chromatin remodelling and histone modification.

6.2 Future directions

The findings of this project provide evidence that trehalose and Asr1 have a positive role in ethanol tolerance of *S. cerevisiae*. The conclusive results provide some insight into the environmental conditions under which these cell components have a significant influence on the physiological response of *S. cerevisiae* to ethanol stress. Gene expression studies provided some evidence on how Asr1 impacts on the gene expression profile of *S. cerevisiae* during ethanol stress, leading to speculation on the role of Asr1 and how this relates to cell function. As with most discoveries, although the results and conclusions described in this thesis resolve some important issues, they also raise new research questions that require further investigation. The following future research directions are suggested in order to gain further disclosure into roles of trehalose and Asr1 in the ethanol tolerance of *S. cerevisiae*.

- Results presented in this thesis provide some explanation for the conflicting conclusions that appear in the literature on whether or not trehalose has a role in protecting ethanol-stressed cells. Controversy also exists for previous studies on the roles of trehalose in *S. cerevisiae* subjected to other types of stress, such as temperature, oxidative and osmotic stress. It is suggested that the lethal stress dependence of the trehalose effect in *S. cerevisiae* is explored for other types of stress.

- It was speculated in this thesis that the main role for trehalose during ethanol stress is likely to be associated with the protection of cytosolic proteins from denaturation and aggregation. This could be further explored by conducting *in vitro* assays on the activities of key enzymes in the central metabolism of *S. cerevisiae*, such as phosphofructokinase, glyceraldehyde 3 phosphate dehyrogenase and pyruvate decarboxylase, in the presence and absence of various ethanol and trehalose concentrations.

- With respect to the above suggestion, further work in this area could explore the extent of protein aggregation in *S. cerevisiae* wild type and knockout (*tsl1*Δ and *nth1*Δ) strains following exposure to a range of ethanol concentrations. Differential centrifugation could be used to determine the relative proportion of aggregated proteins compared to native cellular proteins in *S. cerevisiae* cells (Chen *et al.*, 2002).

- The role of trehalose in promoting the survival of *S. cerevisiae* during lethal ethanol stress is likely to be advantageous for increasing ethanol yield in fermentations involving high sugar concentrations. The fermentation of nutrient-rich media containing high glucose concentrations is usually halted by the toxicity of the accumulated ethanol, even though sugar remains in the medium. Under these circumstances, ethanol productivity and yield is negatively affected by the toxicity of ethanol. Higher intracellular trehalose levels during the later stages of fermentation may help protect the cell from the inhibitory effects of accumulated ethanol and facilitate the further utilization of any remaining sugar, increasing overall ethanol yield and productivity. This could be explored by conducting batch fermentations with *S. cerevisiae* wild type, *tsl1*Δ and *nth1*Δ strains in defined, nutrient-rich medium containing high glucose concentrations to determine if fermentations with strains containing higher trehalose levels have higher ethanol productivities and yields.

This PhD thesis clearly demonstrates, for the first time, that Asr1 has a role in stimulating growth of *S. cerevisiae* during adaptation to ethanol stress. Gene

expression analysis supported this, providing evidence of lower expression for some cell cycle regulated genes in the absence of Asr1 during ethanol stress. In addition, it was found that some neighbouring gene clusters are co-expressed leading to the hypothesis that Asr1 plays role in chromatin remodelling or histone modification. These findings provide some avenues to pursue in future research.

- It would be informative to extend the gene expression analysis performed for this thesis, by using the same experimental conditions, but with more sample points analysed during the experiment. Samples taken at more frequent time points would provide a more detailed picture of the role of Asr1 in the transient adaptation of yeast during ethanol stress.

- It is important to understand how Asr1 interacts with other proteins within the cell, particularly during ethanol stress. Tandem affinity purification (TAP) following by mass spectrometry, together with yeast two-hybrid analysis, could be used to analyse protein-protein interactions involving Asr1.

- One novel possibility raised by this work is that Asr1 exerts an effect on gene expression by influencing overall chromatin structure. This might be explored using chromatin immuno-precipitation techniques to follow post-translational histone modifications in the wild type and *asr1*Δ strains during ethanol stress.

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APPENDICES

Appendix I

Yeast growth media

Defined medium (liquid) contained per litre: 20 g D-glucose, 5 g ammonium sulphate and 1.7 g yeast nitrogen base, without amino acids (Difco) and ammonium sulphate. The yeast nitrogen base was prepared according to the manufacturer's instructions as a 10x solution (1.7g 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.

YEPD medium (solid) comprised per litre: 10 g yeast extract, 20 g bacto-peptone, 20 g D-glucose, and 15 g bacto-agar. Components were dissolved in distilled de-ionised water (ddH_2O) and autoclaved at 121°C for 20 minutes.

Glycerol storage medium comprised 2 x YEPD per litre: yeast extract (20 g), bacto-peptone (40 g) and glucose (40 g), with the addition of 15% (v/v) glycerol. The dry components were dissolved in ddH_2O and autoclaved at 121 °C for 20 minutes. This medium was used for the storage of all yeast strains at –20 °C or –80°C.

Buffers and solutions

A number of buffers and solutions were supplied with kits or enzymes for use in trehalose determination, PCR reactions, sequencing of PCR products, DNase treatment, cDNA synthesis and purification, and microarray analysis. In addition, the following buffers and solutions were prepared:

Amino acid stock solutions: 10 mg l^{-1} stocks solutions of L-Leucine, L-Histidine Lysine hydrochloride and 2 mg ml^{-1} stocks solution of Uracil were prepared by dissolving the amino acids separately in sterile distilled de-ionised water (ddH_2O). All stocks solutions were autoclaved and the Leucine, Histidine and Lysine solutions

stored at 4°C. The Uracil solutions was stored at room temperature and shaken well prior to use. Uracil and Histidine were used at final concentration of 10 mg ml^{-1}; Leucine and Lysine at final concentration of 100 and 30 mg ml^{-1} respectively (Kaiser *et al.*, 1994).

Blocking buffer (for microarray slides): 25% Ethyene glycol and 0.01% HCl were mixed together.

Buffered phenol: was prepared using Special Grade phenol (Wako Pure Chemical Industries limited), 5x RNA buffer and 0.1% (w/v) 8- hydroxyquinoline. Equal volumes of phenol and 5x RNA 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 1 x RNA buffer. The procedure was repeated using 1x RNA buffer until the aqueous top phase was at pH 7.5 when tested with pH paper. The buffered phenol was stored at 4°C.

Buffer T (300 mM Sodium Acetate, 30 mM $CaCl_2 \cdot 2H_2O$): 12.3 g of Sodium Acetate and 2.21 g of $CaCl_2 \cdot 2H_2O$ were dissolve in 475 ml of ddH_2O. This solution was adjusted to pH 5.5 and made up to final volume of 500 ml with ddH_2O, sterilised with a 0.22 μm filter and stored at room temperature.

Chloroform/ Isoamyl alcohol (25:24): Chloroform (25 ml) and isoamyl alcohol (24 ml) added together and mixed well.

DEPC water: 0.1% DEPC and ddH_2O were mixed well and allowed to stand overnight. Then, the mixture was autoclaved.

DNA extraction buffer: 0.5 M NaCl, 200 mM Tris base and 10 mM EDTA were dissolved in ddH_2O and made up to final volume of 1L. The pH was adjusted to 7.5 with HCl. The solution was autoclaved.

EDTA 0.5 M: was prepared by dissolving 186.1 g EDTA in 800 ml of ddH_2O. The solution was dissolved with gentle heating for several hours. The solution was cooled

and adjusted to pH 8.0 with NaOH; made up to final volume of 1 L with ddH_2O and autoclaved.

Ethidium Bromide (10 mg l^{-1}): 0.5 g ethidium bromide (Sigma) was dissolved in 50 ml of ddH_2O by stirring for 2 hours with magnetic stirrer. The stock solution was stored in a baked lightproof glass bottle at 4°C.

Geneticin: A stock solution of 100 mg ml-1 was prepared by adding 1 ml of sterile ddH_2O to 100 mg Geneticin G418 in a sterile bottle. The stock solution was dissolved and stored at 4°C.

Gel loading buffer (6 x): 0.2% (w/v) bromophenol blue, 20% (w/v) Ficoll and 10 mM EDTA were dissolved in ddH_2O water and filter sterilized into a sterile glass bottle. The solution was stored at 4°C.

RNA buffer (5x): 2.5 M NaCl, 1 M Tris base and 50 mM EDTA were dissolved in DEPC treated water and made up to final volume of 1L. 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 buffer (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%: 10 g of sodium dodecyl sulphate (SDS) (BioRad) was dissolved in 100 ml of ddH_2O by heating to 68°C until the SDS was completely dissolved. The solution was filter sterilized through a 0.22 µm filter into a sterile baked glass bottle and stored at room temperature.

3M Sodium acetate: 40.8 g of sodium acetate-$3H_2O$ (Ajax Chemicals) was dissolved in a small amount of DEPC treated water in baked glassware. The pH was adjusted to 5.2 with dilute glacial acetic acid and made up to 100 ml with DEPC water. The prepared solution was filter sterilized through a 0.22 µm filter into a baked glass bottle.

Solution 1 (0.1% Tritron X-100): 1 g of Tritron X-100 was dissolved in 1 L of filtered DEPC treated water.

Solution 2 (4.38 mM HCl): 0.38 ml of concentrated HCL (36%, 11.64 M) was added into 1 L of DEPC treated water. The solution was filter sterilized through a 0.22 μm filter into a baked glass bottle.

Solution 3 (100 mM KCl): 7.4551 g of KCL was added into 1 L of DEPC treated water. The solution was filter sterilized through a 0.22 μm filter into a baked glass bottle.

SSC (20x): 175.3 g NaCl and 88.2 g tri-sodium citrate were dissolved in 1 L of 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 1l of DEPC treated water. pH was adjusted to approximately 7.2 with glacial acetic acid prior to autoclaving.

TBE buffer (10 x): Tris base (0.89 M), 0.89 M boric acid and 20 mM EDTA (pH 8) were dissolved in ddH_2O water and autoclaved.

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.

1M Tris: 121.1 g Tris base was dissolved in ddH_2O. The pH was adjusted to 7.5 with glacial acetic acid made up to 1 L. The solution was autoclaved.

Enzymes, molecular biology kits, reagent and molecular weight markers

Enzymes: SuperMix (Invitrogen), DNase I enzyme (Ambion®), SuperScript™ II RT (Invitrogen), RNase A (Epicentre Technologies), Trehalase (Sigma), and Glucose oxidase/peroxidase (Megazyme).

Molecular biology kits: Ambion DNA-free kit (Ambion®) LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics), QIAquick® PCR purification kit (QIAGEN) and Megazyme Glucose Test Kit (Megazyme).

Reagents: BigDye (Applied Biosystems Australia), RNase ERASE (ICN), Dig Easy Hyb (Roche Diagnostics), Yeast tRNA (Ambion®), Salmon Herring Sperm (Invitrogen), cyanine-3 (Cy3) (Amersham) and cyanine-5 (Cy5) (Amersham)

Molecular Weight Markers: 100 bp DNA ladder (Promega), GeneRuler 100 bp DNA Ladder Plus (MBI Fermentas) GeneRuler 1 kb DNA Ladder Plus (MBI Fermentas).

Oligonucleotide primers

Table 1: Sequences of the oligonucleotide primers used for PCR confirmation of *tps2*Δ, *tsl1*Δ and *nth1*Δ strains (*Saccharomyces* Genome Deletion Project).

Primer	Sequence
TPS2-A	5'-ACAATCTCGATTCTCATTTTCTTTG-3'
TPS2-B	5'-TGGTTTTTCTTTCGCTTCTCTAGTA-3'
TSL1-A	5'-CCAGATAGAAATTTCGAGAAAAGC-3'
TSL1-B	5'-CACTAACAATGACTTGTTGACGTTC-3'
NTH1-A	5'-GACTGGTTCACAAGGTTATCAATATG-3'
NTH1-B	5'-AAACGACGATTACCTTGACTACTTG-3'
ASR1-A	5'-CAATACAGGCTTAATCGAGAAACAT-3'
ASR1-B	5'-CAATACAGGCTTAATCGAGAAACAT-3'
ASR1-C	5'-ATGTTTCTCGATTAAGCCTGTATTG-3'
ASR1-D	5'-CACAGAAAGAAAATGGAAAAAGAAA-3'
Kan-B	5'-CTGCAGCGAGGAGCCGTAAT-3'
Kan-C	5'-TGATTTTGATGACGAGCGTAAT-3'

Appendix II

Calculation of lag period and growth rate

Growth rates (**Gr**) were calculated mathematically using natural log (**ln**) formula during exponential phase of growth.

$$\mathbf{Gr = ln\ (2) / DT}$$

Where, **DT** is doubling time of the population and the natural log of 2 is 0.69. An example method of calculation and corresponding results are shown in Figure 1. and Table 1.

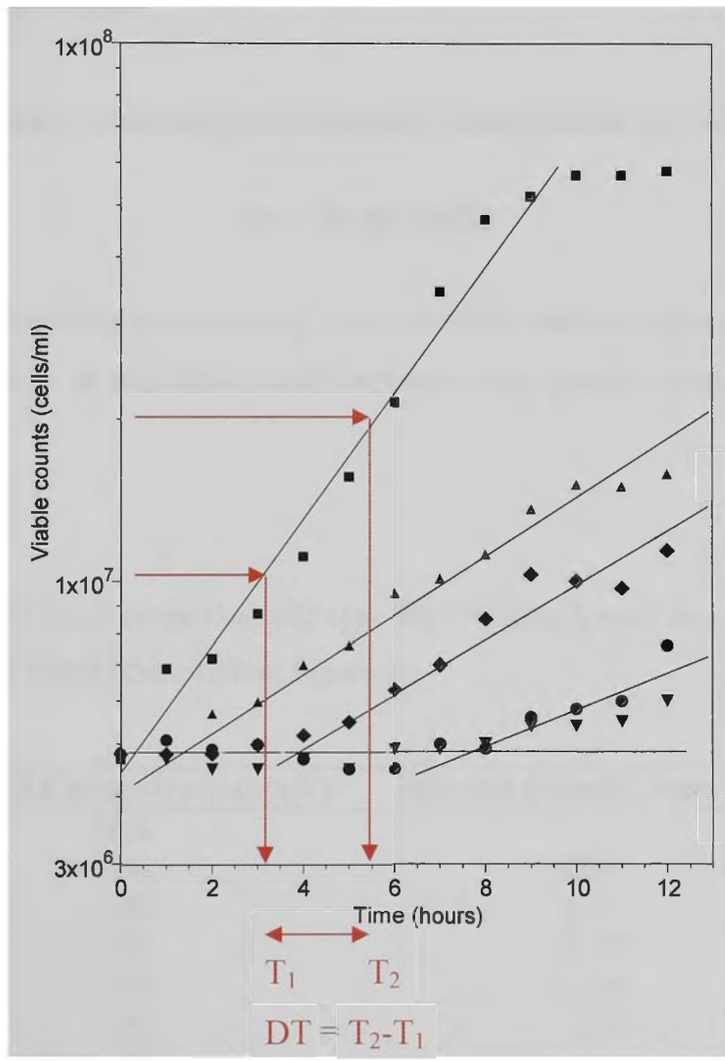

Figure 1: Determination of lag period, doubling time and specific growth rate for *S. cerevisiae* wild type BY4742 grown in defined medium only (■) or defined medium containing added ethanol at either 6% v/v (▲), 7% v/v (♦), 8% v/v (●) or 9% v/v (▼).

Table 1: Lag period, doubling time and specific growth rate of *S. cerevisiae* wild type BY4742 grown in defined medium only or defined medium containing added ethanol (From Figure 1)

Ethanol Concentration (v/v)	Lag period (h)	Doubling time (h)	Specific Growth Rate (h^{-1})
0%	-	2.33	2.96
6%	1.00	5.16	0.134
7%	4.2	6.7	0.103
8%	8.00	7.58	0.91
9%	-	-	-

- Non detectable

Calculation of death rate

Death rates (Dr) were calculated mathematically using natural log (**ln**) formula.

$$\mathbf{Dr = ln\ (2) / DDT}$$

Where, **DDT** is doubling death time of the population and the natural log of 2 is 0.69. An example method of calculation and corresponding results are shown in Figure 2. and Table 2.

Table 2: Death rate for *S. cerevisiae* wild type BY4742 which were inoculated into defined medium containing added ethanol (from Figure 2)

Ethanol Concentration (v/v)	Specific Growth Rate (h^{-1})
10%	-
12%	0.061
14%	0.45
15%	0.72
16%	1.86
17%	3.28
18%	3.8

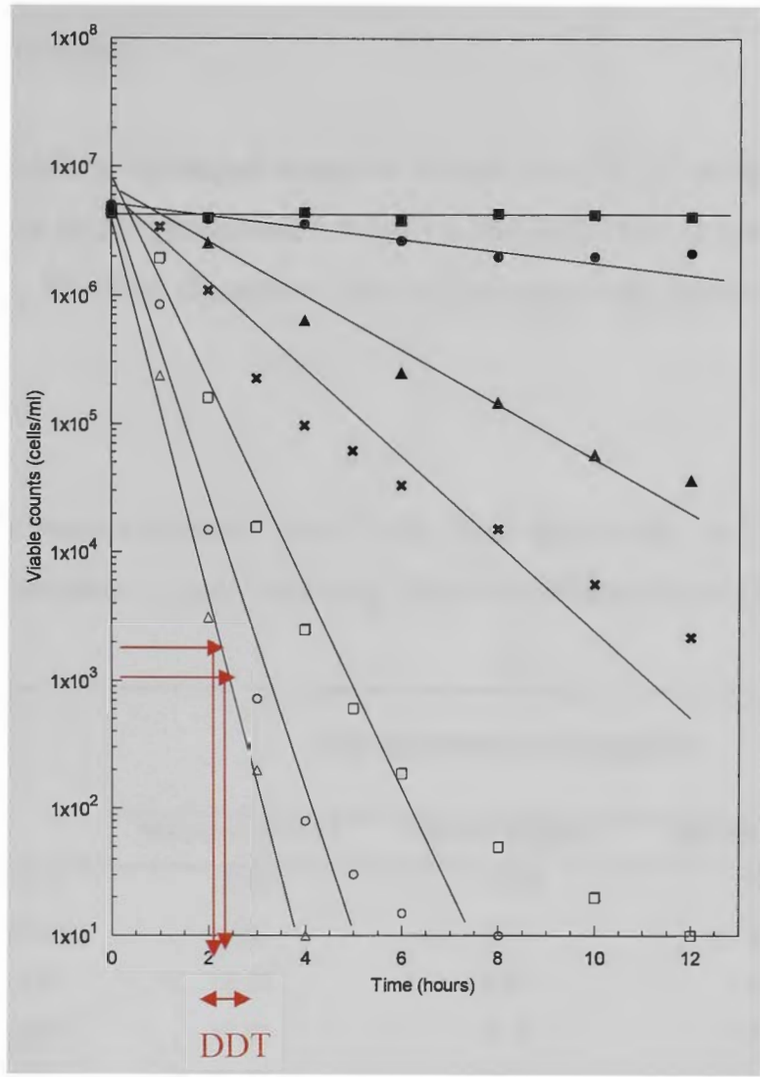

Figure 2: Determination of death rate for *S. cerevisiae* wild type BY4742 which were inoculated into defined medium containing added ethanol at either 10% v/v (■), 12% v/v (●), 14% v/v (▲), or 15% v/v (x), 16% v/v (□), 17% v/v (○) or 18% v/v (Δ).

Appendix III

Gene expression analysis

On exposure to 7.5% (v/v) ethanol stress for 30 minutes, 140 genes were expressed at significantly lower levels (more than 1.5 fold) in absence of Asr1 compared with the wild type strains. The fold changes of those genes across the three microarrays are listed in Table 3.

Table 3: Genes were expressed at a lower level (more than 1.5 fold) and fold repression in the absence of Asr1 following 30 minute ethanol stress (7.5% v/v)

ORF	Gene Name	Fold Repression in microarrays		
		Microarray No 1	Microarray No 1	Microarray No 1
yal019w	*FUN30*	17.46	11.90	11.45
yal020c	*ATS1*	15.01	9.54	8.93
yhr150w	*PEX28*	18.24	8.93	6.98
ylr353w	*BUD8*	17.91	8.70	5.99
yal002w	*VPS8*	13.95	7.96	8.27
yhr081w	-	12.13	7.50	8.72
yhr106w	*TRR2*	15.20	6.94	7.37
ylr329w	*REC102*	8.88	6.69	6.93
ylr001c	-	9.30	6.34	6.44
yal024c	*LTE1*	11.00	4.66	7.16
yor337w	*TEA1*	9.08	8.08	4.84
ydr149c	-	8.47	9.63	4.33
yal022c	*FUN26*	10.16	6.52	5.31
ylr377c	*FBP1*	7.06	9.82	4.63
yal039c	*CYC3*	7.57	5.85	6.15
yal040c	*CLN3*	8.82	5.39	5.44
yhr148w	*IMP3*	5.30	11.56	3.46
yhr105w	-	8.54	6.20	3.90
ylr326w	-	7.26	3.52	7.73
yal001c	*TFC3*	7.91	4.84	5.03

ydr146c	*SWI5*	9.93	4.53	3.89
yor309c	-	4.35	4.39	9.08
ydr192c	*NUP42*	8.21	4.80	4.40
yor380w	-	6.67	7.11	3.57
yal021c	*CCR4*	7.01	6.01	3.94
ydr172w	*SUP35*	6.93	5.25	4.52
yal041w	*CDC24*	10.31	4.05	3.67
ydr193w	-	7.49	6.21	3.12
yhr079c-a	*SAE3*	8.84	4.72	3.44
ydr195w	*REF2*	6.78	4.53	4.61
ynl117w	*MLS1*	6.24	5.48	4.14
ykl031w	-	6.38	4.49	4.29
yhr129c	*ARP1*	4.22	7.40	3.81
yor313c	*SPS4*	4.79	6.78	3.31
yor336w	*KRE5*	6.97	4.73	3.12
ygr139w	-	3.51	6.66	4.26
yor308c	*SNU66*	5.21	5.09	3.74
ycr077c	*PAT1*	4.96	3.87	4.67
yal037w	*yal037w*	4.10	4.30	4.78
yfr023w	*PES4*	4.90	3.40	4.91
ykl174c	*ykl174c*	4.78	3.91	4.25
yor335c	*ALA1*	4.88	4.40	3.68
ylr352w	*ylr352w*	4.76	3.58	4.25
ydr194c	*MSS116*	4.93	4.10	3.25
ykr063c	*LAS1*	3.21	5.83	3.51
ykl011c	*CCE1*	4.94	3.40	3.89
ynr021w	-	4.40	4.06	3.35
ybr113w	-	3.70	4.25	3.66
ydl018c	*ERP3*	5.49	2.57	4.02
yjl043w	-	4.61	3.18	3.82
ydl091c	-	6.19	2.85	3.14
yfl030w	-	4.25	2.80	4.44
ykl055c	*OAR1*	4.45	4.66	2.51
ygl045w	-	4.01	4.40	2.89
ylr376c	-	3.85	4.46	2.88
ybr134w	*ybr134w*	5.52	2.96	2.78
ylr321c	*SFH1*	4.05	2.55	4.27
ydl176w	*ydl176w*	4.19	2.43	4.02
ydr144c	*MKC7*	3.93	4.16	2.33
ykl172w	*ykl172w*	3.01	4.84	2.41

ylr398c	*SKI2*	4.55	2.74	2.64
yor311c	*yor311c*	3.20	3.14	3.01
ybr136w	*MEC1*	3.37	3.66	2.45
yhr125w	*yhr125w*	2.96	3.67	2.74
yor359w	*VTS1*	4.09	3.12	2.31
ydr147w	*EKI1*	3.18	3.53	2.58
yhr127w	*HSN1*	3.49	2.94	2.79
yfl067w	*yfl067w*	2.77	2.98	3.34
ybr178w	*ybr178w*	2.80	2.45	4.01
yjl019w	*MPS3*	2.65	3.00	3.22
yhr101c	*BIG1*	2.94	2.30	3.73
yel021w	*URA3*	4.01	2.30	2.63
ylr328w	*NMA1*	3.53	2.67	2.47
ykl166c	*TPK3*	3.30	2.89	2.41
yor358w	*HAP5*	3.20	3.46	2.07
yml093w	*UTP14*	2.92	3.06	2.43
yer139c	*yer139c*	3.20	2.87	2.34
yll021w	*SPA2*	2.61	2.55	3.17
ydr170c	*SEC7*	3.57	2.24	2.41
ygr245c	*SDA1*	2.70	3.07	2.05
ydl030w	*PRP9*	3.13	1.88	2.87
ydl042c	*SIR2*	2.89	2.13	2.71
yfr048w	*RMD8*	2.96	2.31	2.42
ylr025w	*SNF7*	2.21	2.15	3.38
ykl115c	*ykl115c*	2.31	3.29	2.03
ydr120c	*TRM1*	2.03	3.15	2.26
ylr319c	*BUD6*	2.25	2.67	2.34
ygr081c	*SLX9*	2.34	2.54	2.23
ykl193c	*SDS22*	2.51	2.45	2.07
yll008w	*DRS1*	2.07	2.62	2.20
ybr131w	*CCZ1*	2.10	1.98	2.81
yal003w	*EFB1*	2.58	1.84	2.38
ykl032c	*IXR1*	2.54	2.22	1.98
ykl048c	*ELM1*	2.05	2.57	2.08
ylr002c	*NOC3*	1.95	2.13	2.58
yil050w	*PCL7*	2.29	2.24	2.00
yor334w	*MRS2*	2.36	2.21	1.97
yhr078w	*yhr078w*	2.27	2.24	1.96
ynl112w	*DBP2*	1.91	2.49	2.09
ykl124w	*SSH4*	2.22	1.90	2.30

ykl220c	*FRE2*	1.85	2.33	2.16
ylr371w	*ROM2*	2.41	1.68	2.29
ygl194c	*HOS2*	2.40	1.94	1.95
yal023c	*PMT2*	2.53	2.01	1.77
yhr079c	*IRE1*	2.35	1.73	2.18
ykl050c	*ykl050c*	1.90	2.01	2.31
ykl012w	*PRP40*	1.85	1.92	2.31
yol124c	*yol124c*	2.02	2.24	1.81
yfr031c	*SMC2*	2.15	1.69	2.19
ykl089w	*MIF2*	2.24	2.04	1.70
ykr050w	*TRK2*	2.21	1.98	1.77
ylr330w	*CHS5*	1.66	2.28	2.03
ygr096w	*TPC1*	2.24	1.79	1.90
yhl018w	*yhl018w*	2.11	1.73	2.09
ydl043c	*PRP11*	2.27	1.83	1.79
ydr213w	*UPC2*	1.83	2.18	1.84
ygr141w	*VPS62*	1.88	1.69	2.12
yor354c	*MSC6*	2.05	1.72	1.83
yjl148w	*RPA34*	1.81	1.85	1.94
ykl082c	*RRP14*	1.99	1.58	2.01
ykl132c	*RMA1*	1.96	1.68	1.80
ygl021w	*ALK1*	1.89	1.60	1.90
yor005c	*DNL4*	1.87	1.81	1.60
ydl044c	*MTF2*	1.59	1.75	1.91
yel019c	*MMS21*	1.52	1.77	1.88
yfl023w	*BUD27*	1.76	1.54	1.82
ykr022c	*ykr022c*	1.49	1.80	1.82
ylr373c	*VID22*	1.71	1.52	1.82
yfr021w	*ATG18*	1.78	1.66	1.58
ynl163c	*ynl163c*	1.84	1.59	1.58
ygr057c	*LST7*	1.75	1.64	1.60
yer127w	*LCP5*	1.59	1.82	1.55
yel033w	*yel033w*	1.81	1.58	1.55
ydr522c	*SPS2*	1.72	1.57	1.54
ydr148c	*KGD2*	1.62	1.50	1.64
yor083w	*WHI5*	1.69	1.52	1.53
ybr108w	*ybr108w*	1.61	1.43	1.66
yol110w	*SHR5*	1.60	1.54	1.52
yer015w	*FAA2*	1.58	1.42	1.58
yfr022w	*yfr022w*	1.56	1.56	1.41

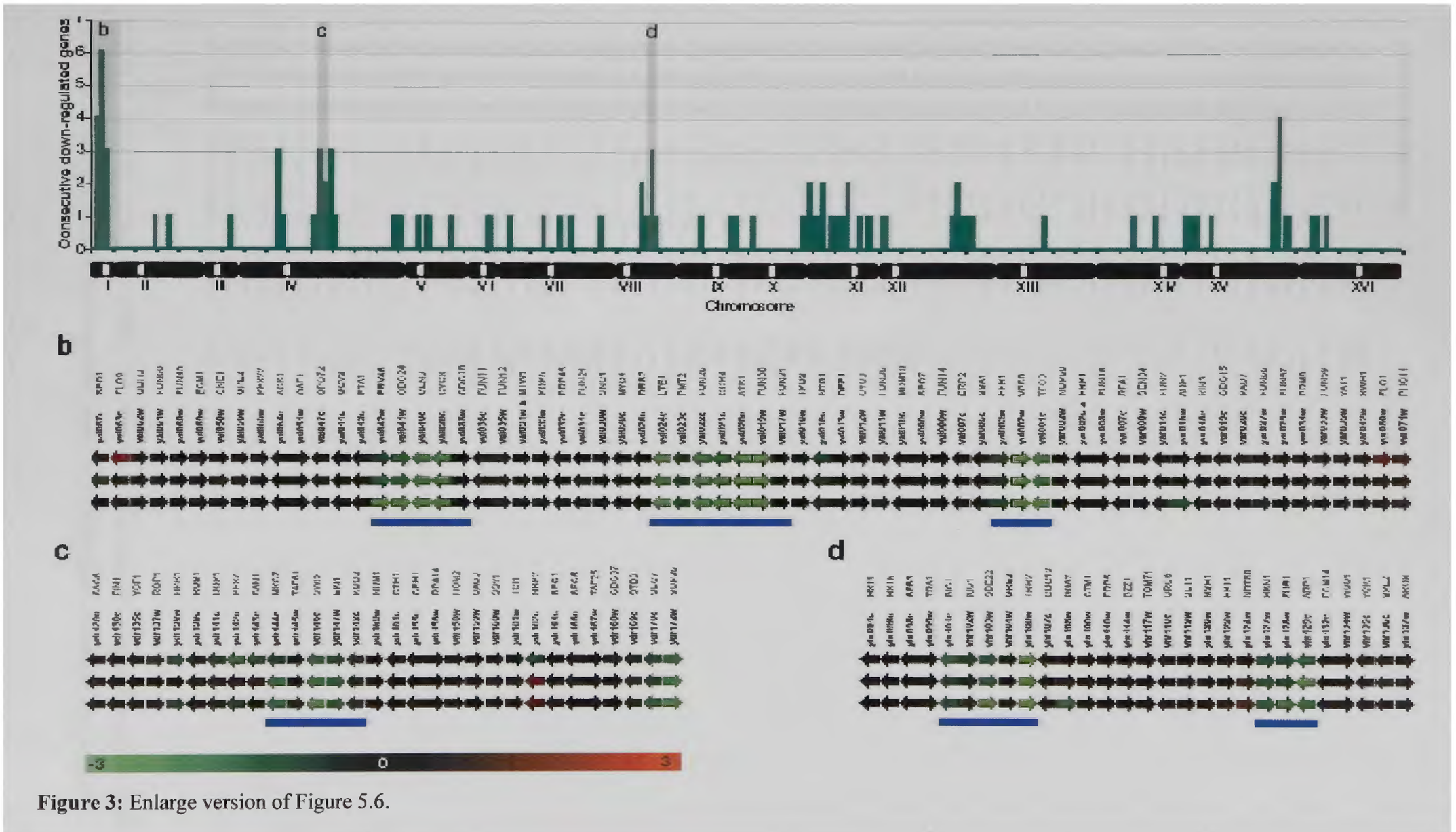

Figure 3: Enlarge version of Figure 5.6.

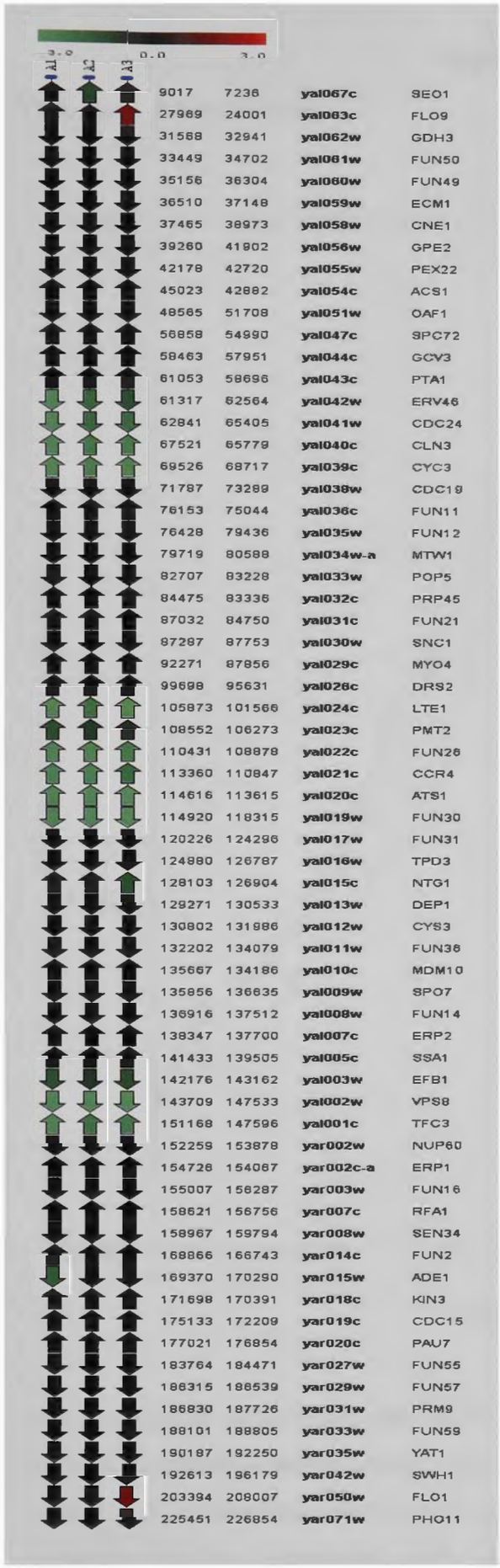

Figure 4: Enlarge version of Figure 5.6 **(b)**. Maps showing the physical location, gene expression and orientation of Asr1 regulated genes in selected gene clusters on chromosome I

Appendix IV

Trehalose determination

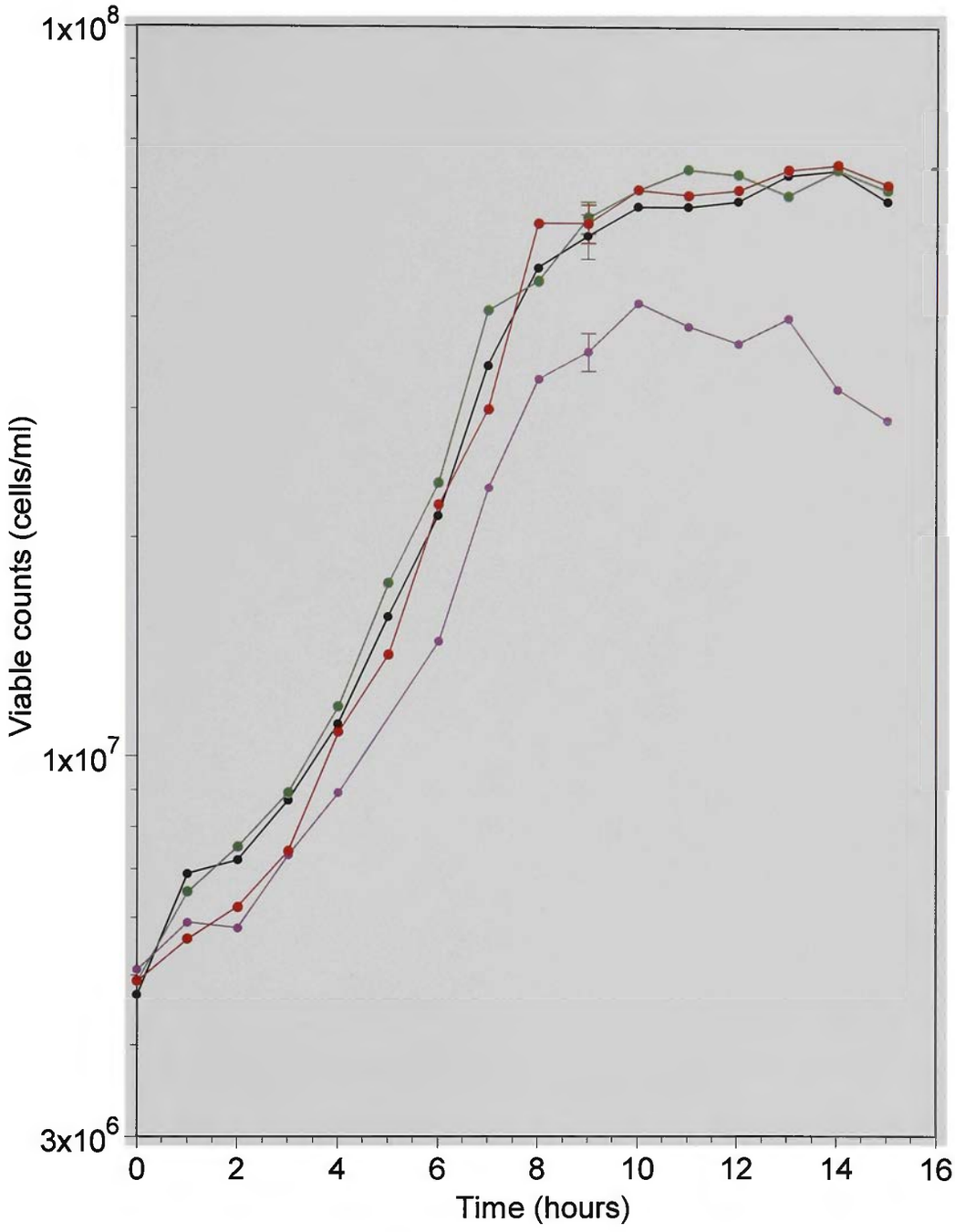

Figure 5 Growth of wild type (●), *tps2*Δ (●), *tsl1*Δ (●) and *nth1*Δ (●) strains in defined medium for trehalose determination. Cells from a late exponential phase parent culture were washed and inoculated into defined medium and incubated at 30°C/130 rpm. Yeast cells were extracted from late exponential, early stationary and late stationary phase for trehalose determination. Each experiment was conducted in triplicate and error bars indicate standard deviation from the mean. All experiments were repeated at least twice with reproducible results