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THE EFFECT OF OSMOTIC STRESS AND HSPI2 ON PASSIVE
AND ACTIVE TRANSPORT IN THE YEAST

Saccharomyces

cerevisiae

by

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A thesis submitted for the degree of

Master of Science

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PREFACE

This dissertation was supervised initially under the late Professor George G. Lindsey and completed under Associate Professor Wolf Brandt. I hereby declare that this dissertation, submitted for the degree of Master of Science in Molecular and Cell Biology at the University of Cape Town, is the result of my own investigations, apart from the referenced work of others.

Signed by candidate

Signature removed

Leela Shanti Kleinsmidt
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March 2010

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is used extensively in many important industrial applications that often expose these organisms to a range of stresses. Consequently, a considerable amount of research has focused on understanding the nature of stress response in yeast and the development of more stress resistant yeast. The stress protein Hsp12 has since garnered interest, as it is upregulated in response to a wide variety of environmental stresses. Using an Hsp12-GFP construct, we confirmed that Hsp12 was induced in response to the osmolytes mannitol and salt. In the first part of this thesis, we used the non-fluorescent probe fluorescein diacetate to measure membrane permeability after osmotic shock on cells in which the ABC transporters have been inactivated. Intracellular esterases rapidly convert FDA into fluorescein and accumulation of fluorescein inside the cell was used as a measure of FDA diffusion. We also used a fluorophore rhodamine 6G that is commonly used to measure passive diffusion into yeast cells. Fluorescein is a substrate for the ABC transporter Pdr12, which is induced in cells subjected to weak acid stress. Contrary to previous research we have shown that cells grown in normal conditions also show ATP-dependent efflux of fluorescein. RT-PCR confirmed that *PDR12* was only expressed in cells subjected to weak acid stress, which suggests that our lab strains may constitutively express another ABC transporter. After osmotic shock, the *hsp12* cells displayed reduced diffusion rates, which suggest that Hsp12 may maintain the integrity of the plasma membrane thereby reducing permeability. In the second part of the thesis, we investigated the effect of osmotic stress on the activity of ABC transporters. We found that after osmotic shock wild-type cells displayed enhanced rates of fluorescein efflux whereas the opposite was observed for *hsp12* cells, which suggest that Hsp12 modulates the activity of ABC transporters during stress. We have also found that *hsp12* cells may possess compensatory mechanisms that confer a phenotype similar to that of stressed wild-type cells and therefore mask the effect of the *HSP12* deletional mutation.

ABBREVIATIONS

α	Alpha
	Beta
	Delta – indicates gene deletion
	Lambda
$^{\circ}\text{C}$	Degrees Celsius
	Micro, indicates 10^{-6}
l	Microlitre (10^{-3} ml)
g	Microgram (10^{-6} g)
A	Adenine
ABC	ATP binding cassette
AFM	Atomic force microscopy
ATP	Adenosine triphosphate
AU	Arbitrary units
bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine
CO_2	Carbon dioxide
cAMP	3',5'-cyclic-adenosine-monophosphate
cDNA	Complementary DNA
CIP	Cell integrity pathway
cm	Centimeter
Cys	Cysteine
Da	Dalton ($\text{g}\cdot\text{mol}^{-1}$)
DEPC	Diethylpyrocarbonate
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate

DOG	2-Deoxy-D-glucose
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium bromide
EtOH	98 % Technical ethanol
F	Fluorescence
FDA	Fluorescein diacetate
G	Guanine
g	Gram
g	Standard gravitational acceleration (9.8 m.s ⁻²)
GFP	Green fluorescent protein
GPD1	Glycerol-3-phosphate dehydrogenase
GPP2	Glycerol-3-phosphate phosphatase
GSRP	General stress response pathway
h	Hours
H	Histone
H ₂ O	Water
HCl	Hydrogen chloride
His	Histidine
HOG Pathway	High-osmolarity glycerol pathway
HSE	Heat shock element
HSF	Heat shock transcription factor
HSP	Heat shock protein
<i>HSP12</i>	Notation signifying gene
Hsp12	Notation signifying protein
kDa	Kilodalton (10 ³ Da)
KO	<i>hsp12 URA3 S. cerevisiae</i> strain
l	Litre
LEA	Late embryogenesis abundant

m	Metre
ml	Millilitre (10^{-3} l)
mm	Millimetre (10^{-3} m)
mM	Millimolar (10^{-3} M)
M	Molar (mol.l^{-1})
MAP Kinase Pathway	Mitogen-activated kinase pathway
MDR	Multidrug resistance
MgCl ₂	Magnesium chloride
min	Minutes
MW	Molecular weight in daltons
mRNA	Messenger RNA
Na-acetate	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBD	Nucleotide binding domain
ng	nanogram (10^{-9} g)
nm	nanometer (10^{-9} m)
OD _{600nm}	Optical density at 600nm
ODU	Optical density units at 600nm/ml
Oligo dT	Oligonucleotide deoxythymine
ORF	Open reading frame
Osmolyte	Organic compound which affects osmosis akin to an osmoticum
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
pH	Hydrogen ion concentration
pH _{in}	Intracellular pH
pI	Isoelectric point
PDR	Pleiotropic drug resistance

PDREs	Pleiotropic drug resistance elements
pmol	picomole (10^{-12} mol)
PKA	Protein kinase A
R6G	Rhodamine-6G
RNA	Ribonucleic acid
RNAse	Ribonuclease
RT	Room temperature (~ 23°C)
RTPCR	Reverse transcriptase polymerase chain reaction
s	Second
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SAB	Sample application buffer
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
STREs	Stress response elements
T	Thymine
T6P	Trehalose-6-phosphate
Taq	<i>Thermophilus aquaticus</i>
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TMS	Transmembrane spanning domain
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	4-octylphenol polyethoxylate
U	Unit of enzyme activity
UK	United Kingdom
USA	United States of America
UV	Ultra violet
V	Volt
v/v	volume / volume (1 ml / 100 ml)

Vis	Visible
w/v	weight / volume (1 g / 100 ml)
WAREs	Weak acid response elements
WT	Wild-type <i>S. cerevisiae</i> strain (W303)
YPD	Yeast extract peptone dextrose (glucose) growth medium

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CHAPTER ONE

LITERATURE REVIEW

1.1 YEAST STRESS

The budding yeast *Saccharomyces cerevisiae* is a unicellular organism and can survive extremely variable and hostile environments. Consequently, it must respond quickly and adapt its growth under these conditions, which can range from variations in osmotic, heat and oxidative stress to nutrient availability. Adaptive strategies employed by yeast in response to mild stress not only allow yeast to resume growth but they are then equipped to withstand more severe forms of the same stress or any new stresses they may experience, by what is referred to as cross protection (Helmut and Kuchler, 2006; Prasad and Panwar, 2004). Such cross protection increases the complexity of how this organism responds to stress and highlights the interconnectedness of stress response pathways in yeast. An important aspect of the stress response occurs in the cell wall and plasma membrane, the barriers between the organism and its external environment, and involves alterations in both the chemical and physical properties in response to stress (Heinisch and Rodicio, 2009).

1.1.2 The General Stress Response

Yeast cells respond to a range of stresses by the production of various proteins that have protective functions (Estruch, 2000). Deletion studies involving genes induced by multiple stresses identified a common cis-acting element in the promoter of these genes (Helmut and Kuchler, 2006). This element, the Stress Response Element (STRE), has an AGGGG consensus sequence and can be found in varying amounts or orientations in the promoter (Helmut and Kuchler, 2006; Prasad and Panwar, 2004). Msn2 and Msn4, two structurally homologous members of the Cys₂His2 Zinc finger family of transcription factors, are master regulators that mediate the general stress response in *S. cerevisiae* (Helmut and Kuchler, 2006; Prasad and Panwar, 2004). In response to stress, the proteins Msn2/Msn4 are translocated from the cytoplasm to the nucleus where they bind to STREs in the promoters of general stress response associated genes (Burke and Ardehali, 2007). A main component of the general stress

response is the Mitogen Activated Protein (MAP) kinase cascades, which are activated in response to a wide variety of environmental stresses (Cowan and Chun-An, 2006). The MAP kinase cascades are made up of a family of protein kinases and five different cascades have been characterised in yeast (Helmut and Kuchler, 2006; Prasad and Panwar, 2004). In *S. cerevisiae* the MAP kinase pathway most characterised is activated in response to changes in osmolarity and is known as the High-Osmolarity Glycerol (HOG) mitogen-activated protein kinase pathway (HOG pathway) (Belle and Andre, 2001).

1.1.3 The HOG Pathway

One of the universal responses to osmotic stress is the synthesis and accumulation of compounds known as compatible solutes. These compounds act to balance the osmotic differences brought about by osmotic stress agents (Bohnert and Shen, 1999). In *S. cerevisiae*, an increase in external osmolarity leads to the synthesis of the compatible solute glycerol, which acts to increase the intracellular osmolarity (Prasad and Panwar, 2004). The two important genes involved in glycerol synthesis are glycerol-3-phosphate dehydrogenase (*GPM*) (Prasad et al., 2002) and glycerol-3-phosphate phosphatase (*GPP2*) (Frelet and Klein, 2006), both of which are under the control of the HOG pathway (Bidder and Reiehm, 1970). Synthesis of glycerol is accompanied by a decrease in glycerol permeability, which is mediated by the plasma membrane aquaporin protein Fpsl (Riordan and Ling, 1979; Hofmann, 1997). During osmotic stress, Fpsl closes and once osmotic equilibrium is re-established, it reopens and the glycerol is passively exported from the cell (Bidder and Reiehm, 1970; Carlsen et al., 1979).

The HOG pathway MAP kinase cascade comprises three kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) (Gustin, 1998). A stress signal is transduced through the step-wise phosphorylation of each kinase (Prasad and Panwar, 2004). The membrane proteins Sln1 and Sho1 act as sensors for the osmotic stress signal (Ueda et al., 1986; Bodor et al., 2005) however each initiate two independent pathways (Decottignies and Goffeau, 1997) which both culminate in the activation of the MAPK Hog1 (Prasad and Panwar

,2004).

The first step in the HOG pathway is the autophosphorylation of the histidine kinase

In the unphosphorylated state Ssk1 is bound to the MAPKKK Ssk2 which renders Ssk2 inactive. Phosphorylation releases Ssk1 from Ssk2, after which Ssk2 in turn phosphorylates the MAPKK Pbs2. Phosphorylation of two serine or threonine residues in the Pbs2 catalytic domain activates the kinase which in turn phosphorylates the final kinase in the cascade, the MAPK Hog1 (Prasad and Panwar, 2004) (Figure 1.1). Hog1 activation is followed by nuclear accumulation after which transcription of osmotic stress related genes is initiated. The mechanism by which these genes are upregulated is unknown although the role of Hog1 in the regulation of glycerol synthesis genes and enzymes has been established (Prasad et al., 2002; Bielder and Reiehm, 1970; Riordan and Ling, 1979).

The downstream targets of the second branch of the HOG pathway are identical to those of the Sln1 cascade however, the conditions that lead to Shol activation are unclear. Shol recruits Ste11 (MAPKKK) in a phosphorylation independent manner after which Ste11 and Pbs2 form a complex that undergoes phosphorylation. Pbs2 then phosphorylates Hog 1, as in the final step of the Shol branch (Prasad and Panwar, 2004) (Figure 1.1).

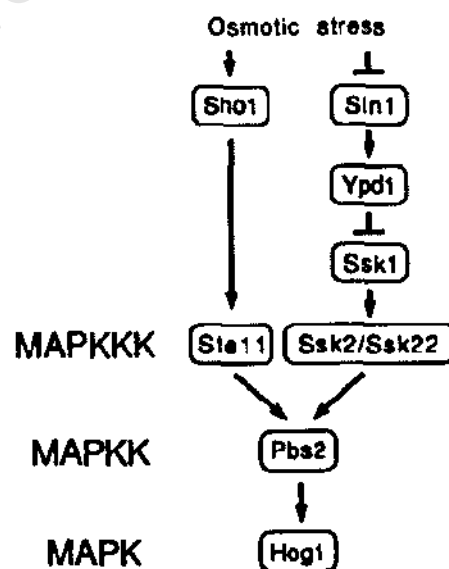


Figure 1.1: The yeast HOG pathway (Takekawa et al., 1997).

1.1.4 The Heat Shock Response

One of the most common stresses experienced by many organisms including yeast, bacteria, and plants is exposure to elevated temperatures. The universal response to high temperatures involves changes in gene expression and the synthesis of various proteins. One of the most ubiquitous set of proteins synthesised under these conditions have been termed the "Heat Shock Proteins" or "HSPs" (Praekelt and Meacock, 1990; Mukhopadhyay et al., 2003). Further investigation lead to the discovery that HSPs are not only synthesised in response to heat but also stresses such as osmotic, anoxia, starvation, DNA damage and protein misfolding (Praekelt and Meacock, 1990; Mukhopadhyay et al., 2003). The HSPs are mainly classified into two classes based on molecular weight. The first class represents the large HSPs and comprises proteins between 70-100 kDa and the second class constitutes the small HSPs that are between 17-30 kDa (Praekelt and Meacock, 1990). The precise function of these proteins during stress still remains unclear, although most of the evidence points to a role as molecular chaperones which act to prevent protein aggregation and misfolding during stress (Buchner and Jakob, 1994; Mtwisha et al., 1998). Research suggests that the large HSPs are mainly involved in normal functions and less important for thermotolerance since gene inactivation has no effect on the ability of *S. cerevisiae* to withstand short exposure to high temperatures (Praekelt and Meacock, 1990; Yamamoto et al., 2008).

1.1.5 The Small HSPs

A lot of interest and research has been directed towards the small HSPs because of the distinct correlation between the accumulation of these proteins and the acquisition of thermotolerance (Praekelt and Meacock, 1990). Research has shown that in *S. cerevisiae* the small HSPs are not only synthesised after heat shock but also at certain stages of development, most importantly on entry into the stationary phase when nutrients are limiting. Further analysis of these proteins has been hampered by the lack of sequence similarity between the small HSPs of different species (Praekelt and Meacock, 1990).

1.2 THE LEA PROTEINS

In the final stage of development, all plant seeds undergo as much as 90% water loss as part of the drying down process (King and Roberts, 1973). During this stage of development a distinct group of proteins accumulate and have been termed the Late Embryogenesis Abundant (LEA) proteins (Mtwisha et al., 1998). In addition, LEA proteins have been shown to accumulate in the vegetative tissues of plants during water deficit stress (Farrant et al., 1996; Mtwisha et al., 1998.) The LEA proteins are divided into six groups based on conserved amino acid sequence motifs, five of which are involved in desiccation tolerance in the seed embryo (Sales et al., 2000). A distinct characteristic of the LEA proteins is their solubility at elevated temperatures, a feature that is supported by the highly hydrophilic amino acid content of these proteins and consequent absence of a defined tertiary structure (Mtwisha et al., 1998; Grelet et al., 2005).

The function of the LEA proteins during water deficit stress still remains unclear however their hydrophilicity and lack of an ordered tertiary structure (Grelet et al., 2005) suggests possible mechanisms by which they exert a protective function. As water replacement molecules, the LEA proteins may aid membrane stabilisation or contribute to vitrification, the formation of a glassy state in the cytoplasm (Berjak, 2006 and references therein; Grelet et al., 2005). Another possible function may involve a role in ion sequestration during desiccation, by binding excess salt ions reducing their concentration inside the cell (Dure, 1993).

1.3 HSP12

The appearance of the LEA proteins during water deficit stress in plants and the synthesis of HSPs in response to heat stress in yeast are two sets of proteins both associated with a decrease in water activity inside the cell (Mtwisha et al., 1998). Extensive work on large HSPs such as Hsp70 and Hsp90 has linked their function to the ATP-dependent refolding of denatured proteins (Mtwisha et al., 1998). Studies on the small heat shock protein Hsp26, one of the first characterised HSPs, shifted interest towards other small HSPs and their function since inactivation of Hsp26 had no effect on yeast functioning during stationary phase and thermotolerance (Prackelt and Meacock, 1990). The small 12 kDa heat shock protein Hsp12 has since garnered particular interest and has been identified as an important component of

the yeast stress response. During exponential growth at 30°C, very low levels of Hsp12 transcripts are detectable and then increase 100-fold on entry into the stationary phase (Praekelt and Meacock, 1990). Induction of Hsp12 occurs in response to a broad range of stresses including osmotic, ethanol, oxidative, heat, nutrient limitation, non-fermentable carbon-sources, mechanical, agents affecting cell wall integrity and pH extremes (Praekelt and Meacock, 1990; Groot et al, 2000; Karreman and Lindsey 2005).

1.3.1 The *HSP12* Gene Sequence

The *HSP12* gene (Figure 1.2) is a single copy gene of 1275 base pairs (bps) yielding an mRNA product of approximately 560 nucleotides. The promoter of the gene (Figure 1.3) contains many features common to other yeast promoters that include two TATA boxes, a pyrimidine-rich (CT) block, an A-rich 5'untranslated region and a high codon bias index representative of highly expressed genes (Praekelt and Meacock, 1990). Another feature of the *HSP12* gene is the hexanucleotide sequence GGAAAA that occurs seven times of which five are in tandem all upstream of the promoter (Varela et al., 1995). The coding region is followed by two motifs of unknown function with similarities to the eukaryotic polyadenylation signal AATAAA (Praekelt and Meacock, 1990). The promoter of the *HSP12* gene contains a number of putative stress response elements. The CCCCT stress response motif is found twelve in total, with five repeats and seven in different orientations. Studies show that this motif may be significant in the expression of Hsp12 in response to heat shock and diauxic shift. More specifically, the Stel and Stre2 motifs of the CCCCT class show involvement in gene activation during salt stress, which leads to *HSP12* induction (Varela et al., 1995). Upstream of the promoter are two possible heat shock elements (HSEs) although their deletion has no effect on *HSP12* activation (Praekelt and Meacock, 1990). The protein Mdj 1 is a heat shock protein associated with the mitochondria and involved in protein folding. The MDJ/gene is situated upstream of the *HSP12* gene between the genes *SUF9* and *CDC4* (Rowley et al., 1994; Praekelt and Meacock, 1990).

1.3.2 *HSP12* Gene Regulation

The yeast Heat Shock Transcription Factor 1 (HSF1) is a transcriptional regulator involved in a broad range of stresses and initially associated with the heat shock response (Hashikawa and

The central regions of HSFI contain regulatory domains for DNA binding and trimerisation as well as the repression element CE2. The N - and C - termini represent two transcription activation domains and interaction with the central region renders Hsfl inactive under normal growth condition. Conformational changes and posttranslational modifications in response to certain stresses may also induce Hsfl (Hashikawa and Sakurai, 2004).

```

GATCCCACTAACGCCAGCCGAAAATGGAAAAAAGGGTTCGGTGATGTGTGGGTGCCAGC
TGGCGGTAGCAATGACGACGTGTTGACGGGCCCTTGGCTCTTGGGACAAGGACTAGAAGC
CAAAGCCAGAGGCGGTAAAAATAGCAAGA████████████████████GGCATCTGTTAAGGGCA
TATGTTGCAACTTGCAGGGCGCGGCACAAAATAACATAGAAACGTAGTAAAGAGGGGAAA
AGGAAAAGGAAAAGGAAAAGGAAAGGAAAAAACCATTGACGTAGAAATTGAAAGAAGGA
AAGGTATACGCAAGCATTATAACAACCCACAAACACAGACCAGAAGCACTCTAGACGGAG
AGTAACTAGATCTACAGCCCCTGGAAAAATCGTTTGGTCAACTTTGAGGTTCCGGTTCGTCC
CCCTCTTGATCT████████████████████TTTCTCTAAATCTATATTAAAACGTATAAATAGGACGGTG
AATTGCGTTCTACTTCCTCAATTGCGTTTGATCTTATTTAATCTCTCTCTAAATATATAGA
AAAAAAAACCATCTGATTATTCGATAATCTCAAACAAACAACCTCAAAACAAAAAAAATA

```

████████ Repeated Hexamer motif

████████ STRE 5,4,3,2,1

████████ Heat shock Element

████████ TATA box

Figure 1.3: The promoter region of the *HSPI2* gene showing the important regulatory elements. Only 606 bps upstream have been shown since this is the minimum region required for proper induction (Varela et al., 199S). The STRE 3 element overlaps with the hexamer motif at the GG.

There are two predominate pathways that lead to *HSP12* upregulation. The Cell Integrity Pathway (CIP) responds to stresses that affect the state of the cell wall and its assembly. The second and most recognised is the General Stress Response Pathway (GSRP) (Karreman et al., 2007). During osmotic stress one of the main branches of the GSRP, the HOG pathway regulates Hsp12 expression (Varela et al., 1995). The presence of σ^H stress response elements (core sequence CCCCT/AGGGG) upstream and close to the initiation start site signifies the importance of *HSP12* during stress (Karreman and Lindsey, 2005).

1.3.3 The PICA Pathway

The switch from stressful to non-stressful conditions, for example from growth in the absence of glucose to fermentative growth, requires the repression of stress responsive genes such as *HSP12* (Groot et al., 2000).

HSP12 expression is negatively regulated by the Protein Kinase A (PKA) pathway (Praekelt and Meacock, 1990). PKA constitutes three catalytic subunits TPK1, TPK2, TPK3 and a regulatory subunit BCY1 (Varela et al., 1995; Groot et al., 2000). Cyclic AMP (cAMP), a derivative of ATP, acts as a second messenger involved in signal transduction and works by activating PKA (Estruch, 2000). Mutant studies show a correlation between a drop in the cAMP levels inside the cell and an increase in the Hsp12 levels. As yeast cells enter the stationary phase cAMP levels drop, which triggers *HSP12* induction (Praekelt and Meacock, 1990). *HSP12* regulation is mediated through PICA, which regulates the cAMP levels inside the cell (Praekelt and Meacock, 1990; Groot et al., 2000). In the switch to fermentative growth, glucose acts as the primary signal for the pathway that leads to *HSP12* repression. This signal is detected by a yet unknown factor and leads to the activation of adenylate cyclase, followed by an increase in cAMP. cAMP binds to the regulatory subunits of PICA releasing its catalytic subunits and activating the protein (Groot et al., 2000).

1.3.4 Hsp12 Protein Sequence

The primary sequence of Hsp12 is rich in hydrophilic and charged amino acids such as aspartate, glutamate, lysine, alanine and glycine, which together comprise 66 % of the total amino acid composition (Figure 1.4A and B). The distribution of the amino acid side chains

in Hsp12 yields a negative hydropathy plot (Figure 1.4C) indicative of highly hydrophilic proteins and also common to other small HSPs. Hsp12 has a hydropathy value of - 0.79, whereas myoglobin a more globular protein has a value of more than one, indicative of more hydrophobic proteins (Mtwisha et al., 1998). Hsp12 shows only limited homology with heat shock proteins from other organisms and no homology with two of the best characterised small heat shock proteins Hsp26 and α -crystallin (Praekelt and Meacock, 1990; Orlandi et al., 1996).

A.

```

+1
ATGTCTGACGCAGGTAGAAAAGGATTTCGGTGAAAAAGCTTCTGAAGCTTTG
M S D A G R K G F G E K A S E A L

AAGCCAGACTCTCAAAAGTCATACGCTGAACAAGGTAAGGAATACATCACTGACAAGGCC
K P D S Q K S Y A E Q G K E Y I T D K A

GACAAGGTCGCTGGTAAGGTTCAACCAGAAGACAACAAGGGTGTCTTCCAAGGTGTCCAC
D K V A G K V Q P E D N K G V F Q G V H

GACTCTGCCGAAAAAGGCAAGGATAACGCTGAAGGTCAAGGTGAATCTTTGGCAGACCAA
D S A E K D K D N A E G Q G E S L A D Q

GCTAGAGATTACATGGGAGCCGCCAAGTCCAAGTTGAACGATGCCGTGGAATATGTTTCC
A R D Y M G A A K S K L N D A V E Y V S

GGTCGTGTCCACGGTGAAGAAGACCCAACCAAGAAG
G R V H G E E D P T K K

```

B.

Amino acid	Residues	mol/mol %
Alanine (A)	13	11.93
Cysteine (C)	0	0.00
Aspartic acid (D)	11	10.09
Glutamic acid (E)	11	10.09
Phenylalanine (F)	2	1.83
Glycine (G)	13	11.93
Histidine (H)	2	1.83
Isoleucine (I)	1	0.92
Lysine (K)	15	13.76
Leucine (L)	3	2.75
Methionine (M)	2	1.83
Asparagine (N)	3	2.75
Proline (P)	3	2.75
Glutamine (Q)	6	5.50
Arginine (R)	3	2.75
Serine (S)	8	7.34
Threonine (T)	2	1.83
Valine (V)	7	6.42
Tryptophan (W)	0	0.00
Tyrosine (Y)	4	3.67

C.

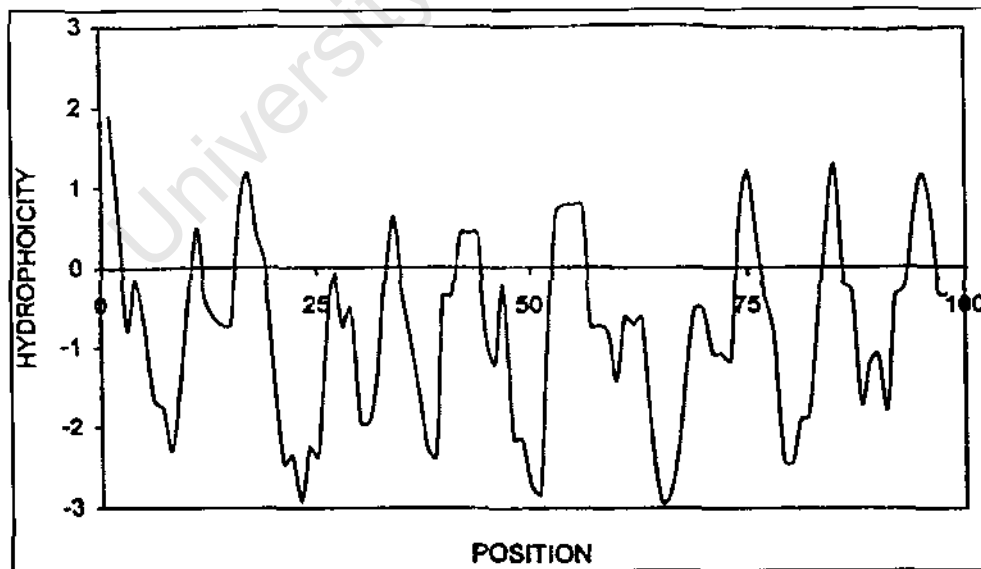


Figure 1.4: Amino acid sequence of the ORF (A), amino acid composition (B) and hydrophobicity plot (C) of Hsp12. Hydrophobicity was plotted according to the method of Kyte and Doolittle (1982) using a 4 amino acid window (Mtwisha et al., 1998).

1.3.5 *HSP12* Knockout

The *HSP12* knockout contains a *URA3* gene insertion at the only Styl site in the *HSP12* gene. Analysis of stationary phase proteins in which Hsp12 is usually highly abundant, showed no Hsp12 present in the knockout. Low levels of a truncated version of the Hsp12 RNA transcript are only present in the knockout after a heat shock treatment. In addition analysis of the protein complement of the knockout strain show additional proteins present during exponential growth which are only present in the wild-type after heat shock and in early stationary phase, suggesting other mechanisms in the knockout which compensate for the absence of Hsp12 (Praekelt and Meacock, 1990).

1.3.6 Hsp12 is LEA-like

There are a number of similarities that suggest that Hsp12 could be considered a LEA-like protein (Mtwisha et al., 1998):

1. Significant increases in Hsp12 levels only after heat shock not during growth at high temperatures.
2. Hsp12 accumulates on entry into stationary phase and in response to osmotic stress. Stationary phase is analogous to seed maturation in that during this stage of growth yeast cells are preparing to dry-down and assume a dormant stage.
3. Hsp12 is highly hydrophilic, which may allow it to act as a water-replacement molecule during water deficit stress.
4. It assumes a random coil conformation in solution and lacks many hydrophobic residues preventing precipitation at high temperatures.
5. The composition and distribution of amino acids is similar to that of the wheat group 1 LEA Em, which exhibits osmoprotectant capabilities when expressed in *S. cerevisiae* (Swire-Clark and Marcotte, 1999).

1.4 TREHALOSE

In *S. cerevisiae*, one of the first lines of defence after heat shock and osmotic stress is the synthesis of the compatible solute trehalose, which is a disaccharide composed of two glucose monomers (α -D-glucopyranosyl- α -D-glucopyranoside) (Leslie et al., 1994) (Figure 1.5). During exponential growth, its levels are barely detectable but in response to osmotic stress trehalose levels can represent up to 20 % of the dry weight of the cell (Conlin and Nelson, 2007; Singer and Lindquist, 1998). Synthesis of the HSPs appears to follow the synthesis of trehalose. Trehalose contributes to thermotolerance as a water replacement molecule that functions to stabilise proteins in their native conformation thereby preventing protein misfolding as well as prevent aggregation of misfolded proteins (Sales et al., 2000; Conlin and Nelson, 2007). High levels of trehalose can also have an inhibitory effect since it prevents the refolding of misfolded proteins by the HSPs. However, *S. cerevisiae* is equipped with the means to synthesise neutral enzymes known as trehalases, which degrade intracellular trehalose (Conlin and Nelson, 2007).

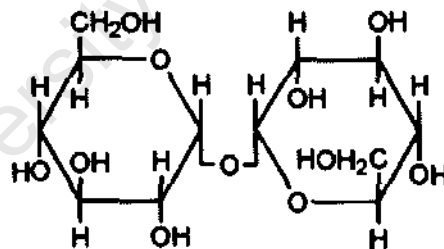


Figure 1.5: The structure of trehalose (Wingler, 2002).

1.5 HSP12 IN THE PLASMA MEMBRANE

The plasma membrane is one of the main sites of interaction between the cell and its environment. Immunocytochemical analysis of wild-type stationary phase yeast, which contains elevated levels of Hsp12, revealed association of Hsp12 with the plasma membrane as well as in the space between the wall and the membrane (Figure 1.6). Immunocytochemistry reveals that the addition of osmolytes increases the amount of Hsp12, which seems to appear on the cytoplasmic side of the membrane. This is not considered artifactual since loss of plasma membrane integrity during preparation would generate labelling throughout the cytoplasm since Hsp12 is highly soluble (Sales et al., 2000).

Dehydration will have a detrimental effect on the yeast cell unless it is able to compensate for the loss of water. Plasma membrane stability is of paramount importance in the yeast cells desiccation tolerance (Hoekstra et al., 2001). Artificial membrane systems using liposomes have showed that the presence of either trehalose or Hsp12 on both sides of the membrane prevents leakage of the liposome contents upon rehydration after drying. It has been suggested that this may be attributed to an increase in membrane stability attributed to the presence of Hsp12 and trehalose. More globular proteins such as myoglobin and cytochrome C, similar in size to trehalose and Hsp12, fail to protect the liposomes during these experiments (Sales et al., 2000).

1.5.1 Hsp12 and the Plasma Membrane Interaction

The presence of Hsp12 near the plasma membrane may provide further insight into Hsp12 at both a molecular and structural level. Altering the composition of liposomes to alter their overall charge and incorporating Hsp12 shows that Hsp12 must interact with the plasma membrane in an electrostatic charge dependent manner. At neutral pH, Hsp12 only protects positively charged liposomes containing stearylamine and since Hsp12 has an isoelectric point of 4.9 it will have an overall negative charge at this pH. As the pH is dropped membrane stability is decreased with a further decrease below the isoelectric point. The suggested mode of interaction between Hsp12 and the membrane involves the negatively charged amino acids interacting with the stearylamine groups and the positively charged amino acids interacting with the phospholipids head groups. Hydrogen bonding between Hsp12 and membrane

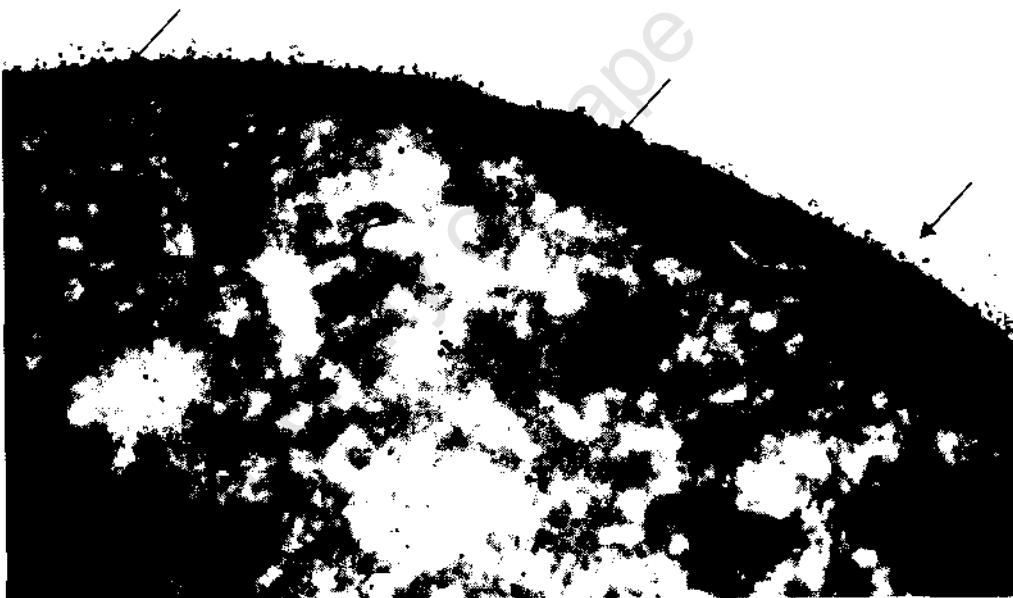
A.**B.**

Figure 1.6: Electron micrographs of immunogold-labelled yeast probed with rabbit anti-Hsp12 antiserum. (A) Immunocytochemical analyses showed gold particles present on the exterior side of the plasma membrane facing the cell wall (see arrows) (Sales et al., 2000). (B) Cryosectioning revealed gold particles mainly in the cell wall with some in the plasma membrane and cytoplasm (see arrows) (Motshwene et al., 2004).

proteins as well as glycolipids together with additional binding to surrounding water molecules would help maintain structural integrity and membrane hydration during desiccation. Further support for a protective function of Hsp12 in the membrane is its lack of tertiary structure that allows interaction with larger sections of the membrane, which would not be the case for more globular proteins such as myoglobin and cytochrome C, as was shown in the liposome studies (Sales et al., 2000).

1.6 HSP12 IN THE YEAST CELL WALL

The yeast cell wall comprises three layers, an outer mannoprotein layer involved in cell signalling and two glucan layers of predominately uncharged sugar molecules (Figure 1.7A). The outer glucan layer is unstructured and composed of sugar molecules in the -1,6 conformation, whereas the second and most essential layer in the wall is an organised insoluble triple helical arrangement of -1,3-linked D-glucose polymers. The polymers within the -1,3 layer hydrogen bond with one another and their charged residues form ionic bonds and thereby provide the cell wall with strength and flexibility (Karreman et al., 2005).

Advances in immunocytochemistry in the form of cryosectioning allow better preservation of ultrastructure during sample preparation. Re-analysis of the location of Hsp12 using cryosectioning newly revealed the majority of immuno-gold particles in the cell wall with some additional labelling found close to the plasma membrane and in the cytoplasm (Figure 1.6B) (Motshwene et al., 2004).

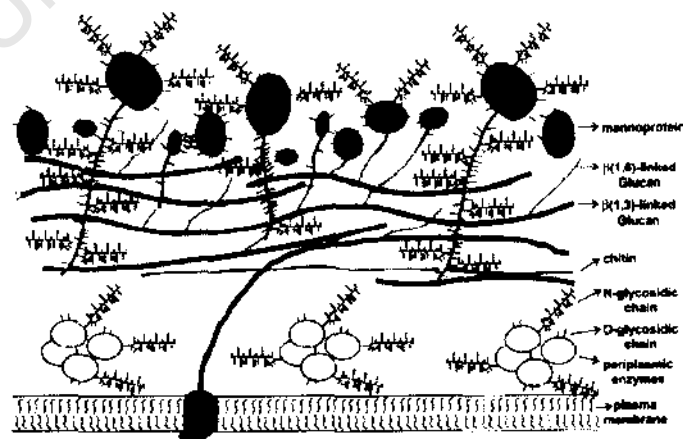


Figure 1.7: A schematic representation of the cell wall layers in *S. cerevisiae* (Shreuder et al., 1996).

1.6.1 Hsp12-Cell Wall Interaction

Alkaline extraction has previously been used to isolate cell wall proteins from *S. cerevisiae* on the premise that hydrolysis of the O-linked covalent attachments (glycolytic linkages) will release these proteins (Motshwene et al., 2003). This non-lethal treatment on whole yeast cells using 0.6 M sodium hydroxide released two proteins one of which was identified by western blotting as Hsp12. The possibility of artifactual attachment of Hsp12 to the cell wall externally from the cytoplasm of lysed cells seems unlikely since neither mechanical damage nor cell lysis released significant quantities of cytosolic proteins into the medium (Motshwene et al., 2004). Studies involving trehalose-6-phosphatase deletion mutants (T6P) suggest a possible protective function for Hsp12 in the cell wall. These T6P mutants are more prone to cytoskeletal damage after being subjected to hydrostatic pressure stress. However when these mutants are subjected to a heat shock treatment, which upregulates Hsp12, cytoskeletal deformations are prevented during pressure stress (Motshwene et al., 2004).

1.6.2 Hsp12 and Cell Wall Flexibility

During osmotic stress loss of water may promote increased hydrogen bonding between adjacent polysaccharides in the cell wall, increasing the rigidity and thus hamper cell volume changes which generally occur during water deficit stress (Motshwene et al., 2004). It has been suggested that the presence of Hsp12 in the cell wall may act to diminish carbohydrate hydrogen bonding and thus maintain cell wall flexibility during water stress (Karreman et al., 2005). Volume determination studies have shown that Hsp12 allows quicker changes in cell volume in response to changes in osmolarity. In studies using an agarose matrix as a model of the -1,3-glucan layer of the yeast cell wall, incorporation of osmolytes increases the gel strength thus decreasing the flexibility of the agarose. Incorporation of Hsp12 into an agarose matrix containing lyotropic solutes restores its gel strength to that of agarose alone, akin to the synthesis of Hsp12 in high salt environments. The suggested mode of action of Hsp12 has been likened to that of plasticizers in plastic polymers, whereby the presence of Hsp12 would disrupt glucan-glucan hydrogen bonding and thereby promote hydrogen bonding between the protein and the polysaccharide, which would in turn enhance cell wall flexibility (Karreman et al., 2005).

Atomic force microscopy (AFM) can be used to monitor changes in the yeast cell wall at a molecular level. AFM studies together with soft particle analysis appear to support the involvement of Hsp12 in cell wall flexibility since knockout cell walls show more compactness and rigidity. However, differences in permeability, measured using soft particle analysis highlight the possibility that Hsp12 may affect diffusion, which could also explain differences in the rate of cell volume change between the wild type and knockout during osmotic stress. Characterization of the yeast cell wall using infrared spectroscopy has identified similar chemical and protein compositions in both the wild-type and knockout strains. Notably is the higher polysaccharide to protein ratio as well as the level of -1,3-glucans in wild-type strain. Yeast mutants lacking glucan synthase are shown to increase cell wall chitin, which appears to stiffen the cell wall (Ram et al., 1998). A possible consequence of the *HSP12* mutation is a decrease in the cell wall glucans, mannans and phosphopeptidomannans and it has been suggested that the knockout might compensate with an increase in chitin, which may therefore lead to a stiffer cell wall (Karreman et al., 2007).

1.7 ABC TRANSPORTERS

The ABC (ATP Binding Cassette) transporters are a large superfamily of proteins (3000 members) (Helmut and Kuchler, 2006) that were first discovered two decades ago as specialised nutrient transporters in bacteria (Prasad and Panwar, 2004). Although most abundant in bacteria this diverse family of proteins is now found throughout nature from prokaryotes to eukaryotes (Burke and Ardehali, 2007). The ABC transporters are embedded in the plasma or intracellular membranes (Burke and Ardehali, 2007) and couple ATP hydrolysis with the transport of substances into or out of cells and organelles (Cowan and Chun-An, 2006). Although most commonly associated with drug extrusion, their abundance and wide substrate specificity has suggested a broader, possible physiological role (Prasad et al., 2002).

1.7.1 Structure

In general, ABC transporters consist of two homologous halves with each half containing a nucleotide binding domain (NBD) and a transmembrane spanning domain (TMS) (Figure 1.8A and B) (Prasad and Panwar, 2004). The arrangement of the transporter can take on either

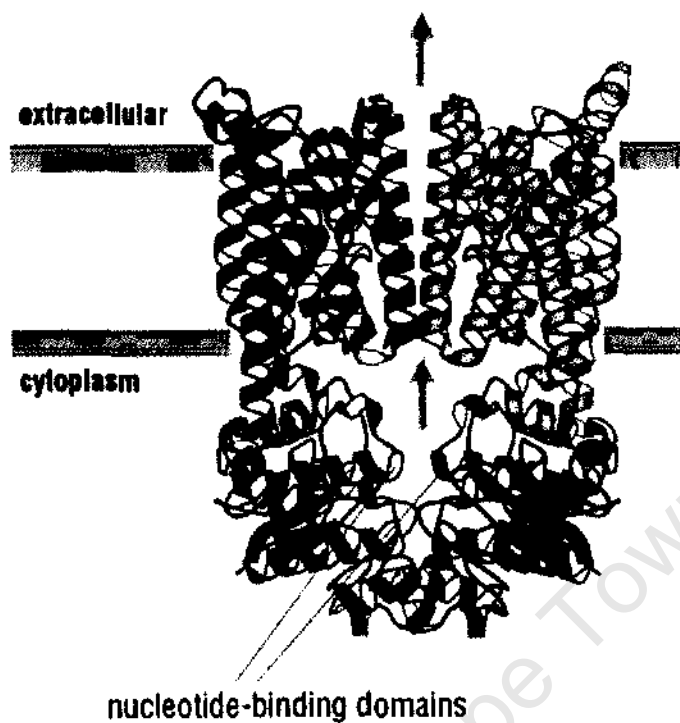
one of two orientations: the forward (TMS₆-NBD)₂ or reverse (NBD₂-TMS)₆ (Helmut and Kuchler, 2006).

The NBD is situated on the cytosolic side of the membrane (Prasad and Panwar, 2004) and comprises 250 residues (Helmut and Kuchler, 2006). There are three highly conserved and characteristic sequences of this domain, which include the Walker A and B motifs and the ABC signature motif or C-loop (Helmut and Kuchler, 2006). The Walker A and B motifs of the NBD represent the sites for ATP binding and hydrolysis as mutational studies showed loss of ATP-driven efflux (Frelet and Klein, 2006). The ABC signature motif comprised of amino acids LSGGQ is situated between the walker A and B motifs. Although conserved, a specific function for this motif is still unknown (Helmut and Kuchler, 2006; Frelet and Klein, 2006). The TMS is a hydrophobic domain comprised of six alpha helical stretches that span the membrane (Prasad and Panwar, 2004). It has been suggested that this domain may be responsible for determining the substrate specificity of the ABC transporter (Helmut and Kuchler, 2006).

1.7.2 Mammalian ABC Transporters

Interest in ABC transporters was heightened by the discovery of the involvement of an ABC transporter in the resistance of cancer cells to chemotherapeutic drugs (Prasad and Panwar, 2004). This resistance termed multidrug resistance (MDR) (Bielder and Reichm, 1970) involves the overexpression of the membrane glycoprotein P-glycoprotein (P-gp) (Riordan and Ling, 1979; Carlsen et al., 1979), and is encoded by the gene *MDR1* (Ueda et al., 1986). P-gp has since become one of the best characterised ABC transporters (Prasad and Panwar, 2004) and has been shown to efflux a wide variety of drugs and xenobiotics (Bodor et al. 2007).

A.



B.

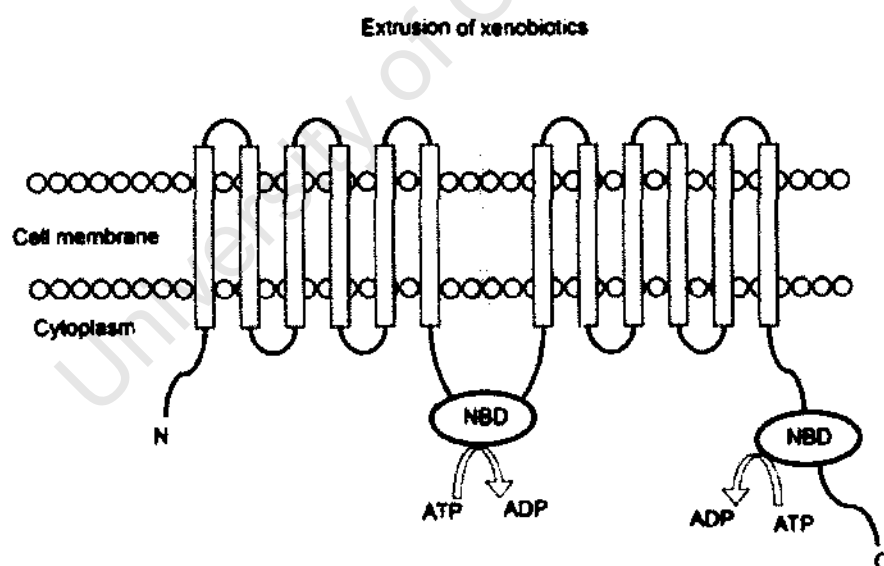


Figure 1.8: (A) Crystal structure of an ABC transporter (Petsko and Ringe, 2007) and (B) Structural features of ABC transporters showing the six membrane spanning regions with two identical halves containing two NBDs with conserved sequences (Lin et al., 2006).

1.8 YEAST ABC TRANSPORTERS

The yeast genome sequencing project has identified at least 29 yeast ABC transporters (Decottignies and Goffeau, 1997) with putative functions ranging from the transport of a vast array of xenobiotics and cellular detoxification to maintenance of membrane lipid homeostasis as well as possible involvement in stress responses (Helmut and Kuchler, 2006; Belle and Andre, 2001). As is the case for mammalian ABC transporters, yeast ABC transporters are most well known for their role in drug extrusion collectively termed Pleiotropic Drug Resistance (PDR) (Helmut and Kuchler, 2006).

1.8.1 Pleiotropic Drug Resistance (PDR)

PDR in *S. cerevisiae* and MDR in humans (Nourani et al., 1997) are closely related. Both phenotypes are due to ABC transporters that show extensive sequence homology (Decottignies and Goffeau, 1997). ABC transporters in yeast are divided into six subfamilies, with PDR being the largest and containing most of the drug transporters (Prasad and Panwar, 2004). Genes of the PDR network also enable tolerance to a wide range of compounds including antifungals, herbicides and heavy metals (Prasad and Panwar, 2004; Frelet and Klein, 2006).

Two genes, *PDR1* and *PDR3*, act as master regulators of the PDR network (Helmut and Kuchler, 2006) and are members of the Zn(11)2Cys6 family of transcription regulators (Prasad et al., 2002) that bind to their target promoters as either homo- or heterodimers at cis-acting PDR responsive elements (PDREs) (Helmut and Kuchler, 2006). Three of the most well known targets of Pdr1/Pdr3 regulation are *PDR5*, *SNQ2* (sensitivity to 4-Nitroquinoline) and *YORI* (yeast oligomycin resistance) all of which are homologues of human ABC transporters (Prasad and Panwar, 2004).

1.8.2 Pdr12

PDR12, although a homologue of *PDR5* and *SNQ2* (46% and 37% sequence similarity respectively), differs from most known members of the PDR network in that it is not involved in drug efflux (Piper et al., 1998). This 170 kDa protein has been identified as the most

abundant protein in the plasma membrane of acid adapted cells (Piper et al., 1998). Substrates for Pdr1 2 are generally lipophilic carboxylate anions of chain lengths C1-7 (Holyoak et al. 1999). The most common class of inducers are weak organic acid food preservatives such as sorbate and benzoate (Piper et al., 2001). In the food industry the use of weak acid preservatives to inhibit microbial growth has become common practice. A major setback is the ability of certain organisms including *S. cerevisiae* to overcome the otherwise inhibitory effects of these preservatives (Papadimitriou et al., 2007). In the presence of these weak acids, yeast experience an extended lag phase after which they resume normal growth (Piper et al., 1998). Sorbate concentrations between as low as 0.45 - 0.9 mM yield significant Pdr12 upregulation (Papadimitriou et al., 2007) and yeast growth in the presence of sorbic acid has been attributed to the extrusion of these acids via the Pdr12 protein (Piper et al., 1998).

The mode of action of these preservatives lies in the pH dependent equilibrium of their undissociated and dissociated states. Sorbate, the most effective inducer of Pdr12, has a pKa of 4.76 and at pH 4.5 75 % of sorbate will exist in the undissociated form. At an acidic pH the undissociated sorbate can easily diffuse across the hydrophobic cell membrane and into the cell where it encounters a higher pH. Inside the cell the sorbate dissociates releasing protons and sorbate anions, which cannot diffuse across the plasma membrane and acidify the cytoplasm. Intracellular acidification, which will affect metabolic functions, leads to Pdr1 2 overexpression and in an energy dependant mechanism the sorbate anions are pumped out of the cell (Piper et al., 1998). Most aspects of the signal transduction pathway from acidification to Pdr1 2 expression remain unknown.

1.83 *PDR12* Regulation

The weak acid stress response is a novel response distinct from the well-known heat shock and osmotic stress responses (Piper et al., 1998; Piper et al., 2001). The application of sorbate stress leads to the rapid induction of many genes related to the general stress response however these genes are not required for adaptation to weak acid stress (Schuller et al., 2004). The genes required for adaptation to sorbate stress are independent of both the Pdr1/3 and Msn2/4 transcription factor master regulators (Piper et al., 1998). *PDR12* is the only gene both highly induced by sorbate and essential for the stress response (Schuller et al., 2004).

The weak acid stress response is mediated by a novel protein Wan (weak acid response protein) which is a member of the Zn₂Cys₆ zinc finger family of transcription factors. Wan is composed of 944 residues and recognises a 48 base pair cis-acting Weak Acid Response Element (WARE) in the *PDR12* promoter. The mechanism of Wan mediated *PDR12* activation is unknown (Kren et al., 2003).

1.8.4 Fluorescein Diacetate

A useful tool to study the activity of the Pdr12 transporter involves the use of Fluorescein Diacetate (3',6'-diacetylfluorescein) (FDA) since its fluorescent product is a substrate for Pdr12 (Holyoak et al. 1999). FDA, commonly used to measure cell viability, is a non-polar colourless molecule that diffuses easily into the cell where it is hydrolysed by non-specific esterases to form fluorescein (Figure 1.9) (Breeuwer et al., 199S). Cell viability assays involving FDA work on the premise that since only live cells have intact plasma membranes the active esterases within these cells will convert the FDA to the polar fluorescent fluorescein, which will therefore accumulate and remain trapped within the cells (Clarke et al., 2001). Fluorescent intensity is dependent on three factors: diffusion of FDA into the cell, the intracellular esterase activity and the efflux of the fluorescent product (Breeuwer et al., 199S). Fluorescein acts as a substrate for Pdr12 and provides a functional system which can be used to investigate the behaviour of this ABC transporter *in vivo*. Yeast cells can be de-energised to render the Pdr12 pump inactive and thus accumulation of fluorescein inside the cell can be monitored. The addition of an energy source will re-activate Pdr12 and the concomitant efflux of fluorescein will follow (Holyoak et al. 1999).

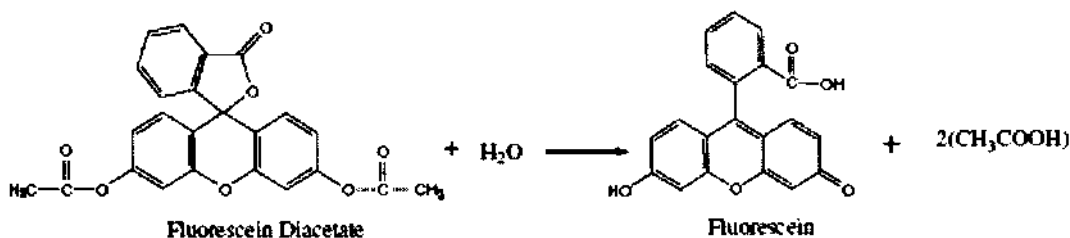
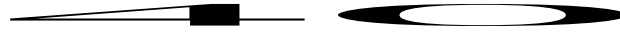


Figure 1.9: Hydrolysis of fluorescein Diacetate produces the fluorescent product fluorescein (Green et al., 2006).



It has previously been suggested that efflux of fluorescein was caused by a reduction in the internal pH (pH_i) as a consequence of adding glucose to the de-energised cells. This reduction in pH_i would shift equilibrium of the preservative anions inside the cell towards the associated form and therefore the acid would flow out of the cell down the concentration gradient. However, studies have shown that growth in the presence of inhibiting concentrations of sorbic acid (0.9 mM) as well as the addition of glucose, produces no significant changes in the measured internal pH and therefore changes in intracellular fluorescence and efflux of fluorescein can only be attributed Pdr12 (Holyoak et al., 1999).

RESEARCH QUESTION

Saccharomyces cerevisiae is considered an industrial organism since it is widely used in the fermentation and brewing industries. In the industrial setting yeast are subjected to a range of stressful conditions including osmotic, temperature, mechanical and ethanol (Karreman and Lindsey 2005). These stresses produce changes in the yeast at a molecular level that ultimately affects the product. Most important in industry is the optimisation of all processes using the most efficient means at the lowest cost. An understanding of the molecular responses in yeast will help us to manipulate them and thus enhance productivity. In

S. cerevisiae the protein Hsp12 is of particular interest because it is expressed in response to multiple stresses and is used as stress marker in environmental toxin screens (Teixeira et al., 2005) and during fermentation (Perez-Torrado et al., 2005). Previous studies have identified Hsp12 predominately in cell wall and surrounding the plasma membrane during stress (Sales et al., 2000; Motshwene et al., 2004) and suggest a possible role as a plasticizer in the cell wall (Karreman et al., 2007). We chose to investigate the effect of osmotic stress and Hsp12 on yeast cell permeability and the ABC transporter Pdr12.

CHAPTER 2

MATERIALS AND METHODS

2.1 Yeast Strains and Culture Conditions

The wild-type strain of the yeast *Saccharomyces cerevisiae* was of the W303 haploid form (*a/α, ade2-1/ade2-1, trp1-1/trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, can^r1-100/CAN*) and the $\Delta hsp12$ yeast mutant strain (*a/α, ade2-1/ade2-1, trp1-1/trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/URA3, can^r1-100/CAN, hsp12::URA3*) were both a gift from Dr P. Meacock at the Department of Genetics, University of Leicester, Leicester, U.K.

Yeast cells were cultured in YPD media (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) together with 2S I of 1 mg/ml ampicillin stock (1 1/m1) for sterility. For experiments that required Pdr12 induction, the pH of YPD was adjusted to 4.3 with FIG and potassium sorbate (final concentration 0.4S mM) was added to the media before inoculation. Yeast were grown at 30°C with continuous agitation and harvested at mid-exponential phase (1.4 ODU for the wild-type and 1.8 ODU for *hsp12*) (Figure 3.1). Yeast growth was monitored by measuring the absorbance at 600nm on a DUS30 Life Science UV/Vis spectrophotometer where one optical density unit (1 ODU) represents 2×10^7 cells tut'.

2.2 Yeast Genomic DNA Extraction

A volume (10mIs) of yeast culture was harvested and centrifuged at $12\ 100 \times g$ for S min at 4°C in a Beckman J2-21 centrifuge. The supernatant was discarded and the cell pellet was resuspended in S00 I of distilled water (dH2O) and transferred into 2ml Eppendorf tubes. The tubes were spun in a bench top centrifuge ($10\ 000 \times g$, 20 s, 4°C) after which the supernatant was discarded and 300 I of breaking buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8.0) was added. The samples

were vortexed to resuspend the pellet and 200 μ l of acid washed glass beads (0.5 mm diameter) as well as 200 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) were added. The Eppendorf tubes were sealed and vortexed in the fume hood at maximum speed for 4 min to rip apart the cells. TE buffer (10 mM Tris, 10 mM EDTA pH 8.0) (200 μ l) was added and the samples were briefly vortexed and bench top centrifuged (10 000 \times g, 5 min, 4°C). The top layer containing DNA was carefully transferred into clean 2ml Eppendorf tubes taking care not to disrupt the middle protein layer. In order to remove RNases, 30 μ l of RNase A (1 mg/ml) was added and the samples were briefly vortexed and incubated at 37 °C for 10 min. 3 M Na-acetate pH 5.2 was added at 1/10 the sample volume together with two volumes of ice-cold 100% ethanol (EtOH) and the tubes were gently inverted to mix. The samples were stored at -70°C for 1.5 h to precipitate the DNA. The samples were centrifuged for 15 min at 4°C and the supernatants discarded. The pellets were washed with ice-cold 70% EtOH and vacuum dried to remove all the liquid. The DNA pellet was resuspended in 50 μ l 10 mM Tris pH 8.0 and stored at -20°C. The quantity and the purity of the DNA were determined using a NanoDrop ND-1000 spectrophotometer for a peak at 260nm.

2.3 Polymerase Chain Reaction (PCR)

All PCR reactions were performed in a Bioer XP Thermal Cycler (Version 1.2). Taq polymerase, Taq polymerase reaction buffer and MgCl₂ were obtained from Promega (USA). Nucleotides (dNTPs) were purchased from Bioline (USA). The following primers designed by Dr. Rob Karreman (PhD, Molecular and Cell biology, University of Cape town, 2006) were used to amplify *HSP12*: forward primer: 5'-CAC-TGA-CAA-GGC-CGA-CAA-3' and reverse primer: 5'-CCA-TGT-AAT-CTC-TAG-CTT-GGT-CTC-3'. The reaction mixture had a 50 μ l total volume and contained the following components: 5 μ l 10X Reaction buffer, 2 μ l 6.25 mM dNTP stock, 30 pmol forward and reverse primers (200 pmol stock), 0.5 μ l 1 U Taq polymerase, 4 μ l 2 mM MgCl₂ (25 mM stock), 1 μ l DNA template and distilled water (dH₂O). The PCR program was as follows: 95°C (5 min), 30 cycles of 94°C (30 minutes), 62°C (30 minutes), 72°C (32 minutes) followed by 72°C (5 min) and finally 25°C (1 second).

2.4 Agarose gel electrophoresis

An agarose solution (1 %) was prepared in a 50 ml volume with TBE (0.09 M Tris-Cl, 0.09 M boric acid, 0.002 M ethylenediaminetetra-acetic acid (EDTA) pH 8.0). In order to visualize DNA within the gel, 2 μ l of 10 mg/ml ethidium bromide (EtBr) was added to the agarose solution prior to casting the gel. Samples were prepared for loading by mixing each with DNA sample application buffer (SAB)(0.25 % (w/v) bromophenol blue, 40 % (w/v) sucrose and 20 mM EDTA pH 8.0), applied to the gel and run at a constant voltage of 100 V for approximately 45 min. The standards used for size comparison were the phage DNA digest (D-1501, Promega) with PstI and a 100 base pair DNA ladder (O'Gene Ruler, Fermentas). The DNA bands in the gels were visualized under ultraviolet light.

2.5 Automated plate reader growth curves

Growth curves of yeast cells grown in low pH YPD with and without sorbic acid were generated using the KC4 (Kineticcalc for windows, Version 3.4) program. Aliquots of yeast starter culture were diluted with YPD to OD 0.2 in a 1 ml total volume of which 200 μ l was loaded onto a sterile ELISA plate together with ampicillin (1 μ l/ml). The plates were placed into a Bio Tek PowerWave XS reader and KC4 was programmed to monitor growth for 15 h without shaking and record readings every hour. Data was imported into Excel and the growth curves were manually constructed.

2.6 Yeast RNA extraction

Cultures of both the wild-type and $\Delta hsp12$ were grown overnight. At the mid-exponential phase for both strains, 2 ml of yeast cells were transferred to Eppendorf tubes, harvested by centrifugation at 10 000 \times g for 2 min and washed twice with sterile dH₂O. RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The pelleted cells were resuspended in 1 ml of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5 % sarcosyl (N-lauroylsarcosine), 0.1

M 2-mercaptoethanol pH 7.0) together with 200 μ l of acid washed glass beads (0.5 mm diameter). The tubes were sealed with parafilm and the cells were ruptured by vortexing for 5 min at 4°C after which the cellular debris was removed by centrifugation at 10 000 \times g for 2 min. The supernatants were transferred to clean 2 ml Eppendorfs to which 0.13 ml 1.5 M Na-acetate pH 4.0, 1 ml Tris-saturated phenol pH 4.0 and 0.2 ml chloroform-isoamyl alcohol (49:1) was added. The tubes were vortexed for 10 seconds and incubated on ice for 15 min. To isolate the RNA, the samples were centrifuged at 12 000 rpm for 20 min at 4°C, the top aqueous layer was transferred to a new 2 ml Eppendorf and to precipitate the RNA, 1 ml isopropanol was added and they were incubated at -80°C for at least 1 h. The RNA was pelleted by centrifugation as before and 300 μ l Solution D and 1 ml isopropanol was added the tubes again incubated at -80°C for a further 1 h. The RNA was again pelleted as before and washed once with 70 % EtOH and vacuum dried for 15 min. The RNA was resuspended in 40 μ l diethylpyrocarbonate (DEPC)-treated water.

2.7 Reverse Transcriptase PCR

2.7.1 Complementary DNA (cDNA) synthesis

Equal concentrations of RNA (ng/ μ l) from wild-type and Δ *hsp12* strains were transferred into sterile PCR tubes with 1 μ l oligo(dT)15 primer (500 μ g/ml) and dH₂O to a final volume of 12.5 μ l. The tubes were spun briefly in a microfuge (10 000 \times g), incubated at 72°C for 2 min and placed on ice for another 2 min. The final components of the reaction, 4 μ l 10X M-MuLV Reverse Transcriptase buffer, 2 μ l (40 U/ml) RNASE I (Rnase inhibitor, Promega, USA), 2 μ l dNTPs, 2 μ l M-MuLV reverse transcriptase enzyme (M-0253S, New England Biolabs, USA) and dH₂O to a final volume of 25 μ l, were added to the tubes and cDNA synthesis was initiated with an incubation at 42°C for 1 h followed by a 10 min incubation at 70°C to terminate the reaction. The cDNA was quantified by spectrophotometry on a NanoDrop ND-1000 spectrophotometer at 260nm and stored at -20°C.

2.7.2 Primers and PCR

The primers used to amplify *PDR12* were according to Piper et al., 1998: forward primer: 5'-CGA-CTG-ACG- AAT-TCA-TTG-AGA-AAG-3' and reverse primer: 5'-CAT-TTC-ACC-GAA-TTC-AAC-GAC-ACC-3'. The components for the PCR reaction were as follows: 50 pmol (2 μ l) forward primer, 50 pmol (2 μ l) reverse primer, 25 μ l PCR master mix (0.05units/ μ l Taq DNA polymerase (recombinant) (in PCR buffer), 4 mM MgCl₂, 0,4 mM dNTPs), 1 μ l cDNA and 20 μ l dH₂O. The PCR program comprised: 95°C (5 min), 30 cycles of 95°C (35 seconds), 56°C (35 seconds), 72°C (35 seconds) and finally 72°C (5 min).

2.8 Extraction of Hsp12 from yeast cell walls

Yeast cells from mid-exponential phase cultures before and after osmotic shock were pelleted by centrifugation (12 100 x g, 5 min, 4°C) and washed twice with PBS. The mass of the pellet was determined and the pellet resuspended in 1 μ l ice-cold 0.6 M NaOH/mg cells followed by incubation on ice for 30 min. The samples were centrifuged at 12 100 x g for 10 min at 4°C and the supernatant containing cell wall proteins was stored at - 20°C. Samples were neutralised for SDS-PAGE.

2.9 Protein concentration

Protein concentrations in cell lysates were quantified using the Bradford method for protein determination (Bradford, 1976). The amount of protein in each sample was determined by using a standard curve constructed with different concentrations of bovine serum albumin (BSA, Boehringer Mannheim) ranging from 0 – 8 μ g/ml.

2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Extracted cell wall proteins were analyzed by separation according to size using SDS-PAGE, as described by Laemmli, 1970. The composition of the separating and stacking gel were 20 % and 6.7 % respectively. Chicken erythrocyte histones (1 mg/ml) were used as a standard for size comparison with approximate molecular weights (kDa) of: H1: 22.5; H5: 20.6; H3: 15.4; H2B: 13.7; H2A:14.0 and H4: 11.2. Samples were mixed with sample application buffer (20 % 0.375 M Tris-HCl (v/v), 20 % SDS (10 %) (v/v), 20 % glycerol (v/v), 5 % 2-mercaptoethanol (v/v), 35 % dH₂O (v/v) pH 6.8) and electrophoresed in the gel overnight at 4°C at a constant voltage of 100 V. Proteins were visualized by staining the gels with Coomassie brilliant blue for 1 h and destained with an ethanol-acetic acid solution (25 % technical ethanol and 7 % acetic acid (v/v)).

2.11 Densitometry

The relative increase in Hsp12 synthesis in yeast cell wall extracts was determined by densitometry using the ImageJ software (Rasband, 1997; Abramoff et al., 2004). This program calculates the ratio of Hsp12 band pixel intensities relative to that of normalised control bands. Control bands were normalised relative to each other to account for possible unequal loading.

2.12 Hsp12-GFP fluorescence stress assays

In order to monitor stress induced Hsp12 expression a wild-type strain transformed with the pYES2-HSP12-GFP2 plasmid (Hsp12-Green fluorescent protein (GFP) construct) (pYH12G2) (Dr. R. Karreman, PhD, Molecular and Cell Biology, University of Cape Town, 2006) was grown to early exponential phase (0.4 ODU). The cells were stressed with either 800 mM Mannitol or 400 mM NaCl that was added directly to the growing culture. At various time points (0, 30, 60, 90 min) after the addition of the stressing agent, 500 μ l of the culture was transferred into a sterile 1.5 ml Eppendorf tube and the cells were pelleted by centrifugation in a microfuge (10 000 \times g, 5 sec). The cell pellets were

immediately resuspended in 1 ml phosphate buffered saline (50 mM phosphate, 150 mM NaCl pH 7.4) and the fluorescence was measured using an Aminco SPF-500 fluorimeter (American Instrument Company, USA) at an excitation wavelength of 450 nm and an emission wavelength of 507 nm (Cormack et al., 1996). Samples were normalised by measuring the OD₆₀₀ of each sample using a DU530 Life Science UV/Vis spectrophotometer. Fluorescence readings for the wild-type strain without the construct were also measured at each time point for comparison.

2.13 Hsp12-GFP fluorescence microscopy

GFP yeast cells (pYH12G2) were stressed as above and only samples at time zero and 60 min were collected for visualisation under the fluorescent microscope. A sample of the lab strain unstressed was also collected and viewed for comparison. Fluorescence and phase contrast microscopy was performed using an Axiovert 200-m microscope (Zeiss, USA). The cells were viewed using a 40 x objective and fluorescence was visualised using a blue DM510 Dichroic mirror, excitation filter 450-490nm. Images were captured using a Nikon camera attached to the microscope and processed using the software Axiovision AC version AxioVs40AC V4.4.0.0.

2.14 Fluorescein diacetate (FDA) assay

The method for monitoring fluorescein influx and efflux in yeast was adapted from Hasworth et al., (1991) and Holyoak et al., (1999). Yeast cells grown to mid-exponential phase were pelleted by centrifugation (12 100 x g, 5 min, 4°C) and washed twice with 25 ml 10 mM Tris-HCl pH 7.0. The pellet was resuspended in 40 ml of the same buffer and 20 ml transferred into a sterile McCartney bottle for the assay. Samples were normalized with the OD of the 40 ml cell suspension. The cells were de-energised by the addition of 200 μ l of 1 M 2-Deoxy-D-glucose (DOG) (10 mM) (Grade III, D6134-G, Sigma) and left to incubate for 3 h at room temperature (RT) after which a 1 ml aliquot was removed to represent the background fluorescence. The cells were incubated with FDA (17 mM in acetone) to a final concentration of 10 μ M and accumulation was monitored for 30 min

during which 1 ml aliquots were collected at the following time points: 0 (immediately after the addition of FDA), 1, 5, 10, 15, 20, and 30 min. Each sample was immediately centrifuged (10 000 x g, 1min) and an aliquot of the supernatant was set aside and the pellet resuspended in 1 ml 10 mM Tris-HCl pH 7.0. Efflux of FDA was initiated with the addition of 120 μ l of 500 mM glucose (5 mM) and monitored for another 30 min during which samples were collected, as previously, at time points 1, 5, 10, 15, 20, and 30 min. The fluorescence of both the supernatant and the pellet at each time point was measured using an Aminco SPF-500 fluorimeter (American Instrument Company, USA) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

The rates of fluorescein accumulation and efflux were determined by curve fitting using LAB Fit Curve Fitting Software -V7.2.37 (1999-2007) and examples of typical fits are shown in Figure 2.12. It was assumed that the rate of fluorescein accumulation in the cells was linear over the first 30 min and therefore the initial rate was calculated using the following function:

$$Y = A \cdot X + B$$

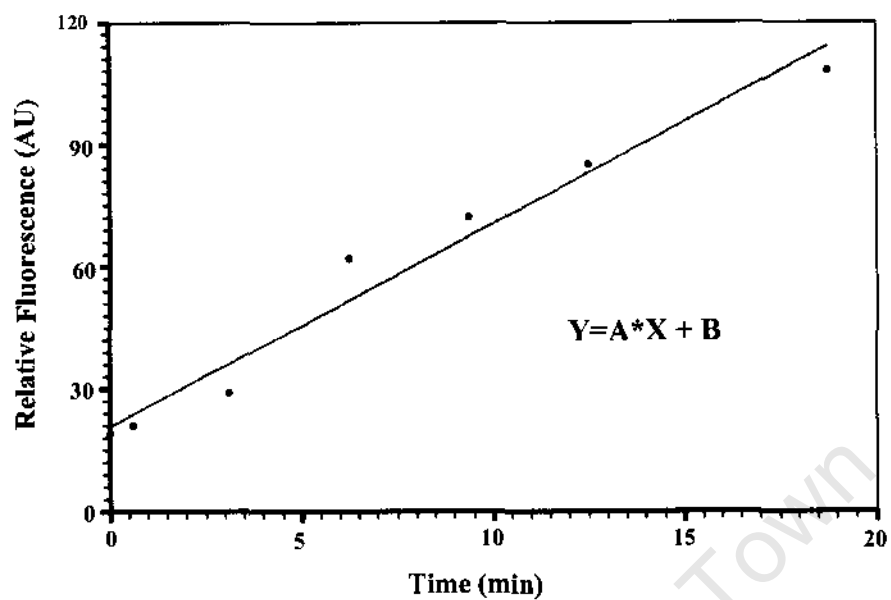
where **A** represents the change in fluorescein per unit time (**F/min**), and **Y** is the fluorescence at time **X**.

The rate of fluorescein efflux was fitted to a first order exponential curve using the function:

$$Y = A - B / \exp(C \cdot X)$$

where **A** is the initial fluorescence, **B** the difference between the initial and final background fluorescence and **C** the first order rate constant (*k*) of fluorescein efflux per minute(min^{-1}) and **X** the time in minutes.

A.



B.

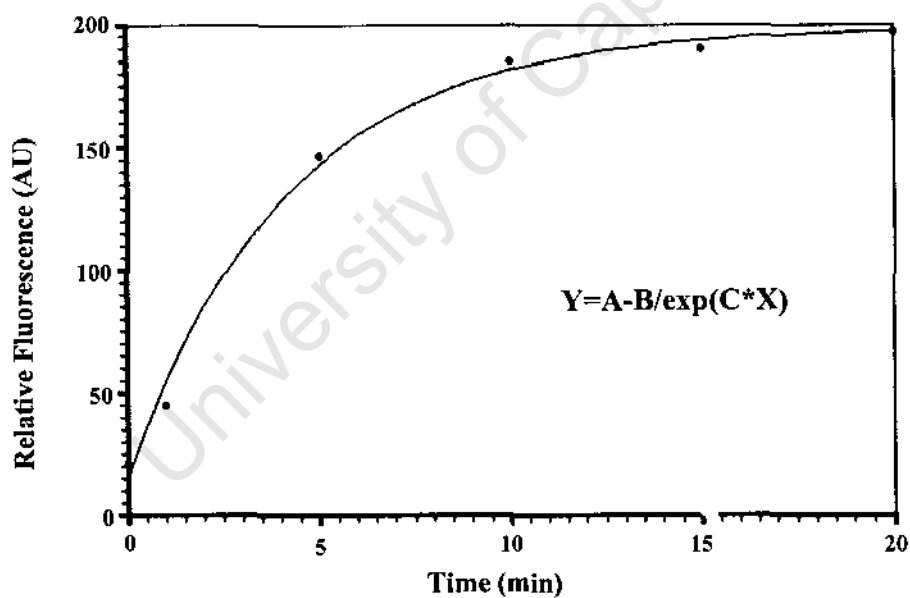


Figure 2.12: Examples of the curve fitting that was used to determine fluorescein accumulation (A) and efflux (B). A: A straight line function was used to determine the initial rate of fluorescein accumulation, B: A first order exponential curve was used to determine the rate of fluorescein efflux.

2.15 Enzyme assay for esterase activity

In order to measure the activity of the esterases inside yeast cells that hydrolyse FDA the following method based on that of Breeuwer et al., (1999) was applied. Exponentially growing yeast cells were washed with PBS and resuspended in the same volume as that which was spun down. The cell suspension was placed together with glass beads (0.5 mm diameter) in a metal container and the cells were ruptured using a ball mill. Samples were ball milled for approximately 1 min in 10 sec intervals with 10 sec breaks and were kept during the ball milling process using liquid CO_2 . The degree of yeast cell lysis was assessed microscopically followed by additional ball milling until no whole cells were visible. The cell debris was removed by centrifugation ($12\ 100 \times g$, 5 min) and the supernatant re-centrifuged to ensure removal of all residues. The assay was performed at 40°C and the cell extract was diluted with buffer and pre-incubated for 3 min, after which a 1 ml aliquot was abstracted to represent the sample background. Fluorescein diacetate was added to a final concentration of 10 μM and 1 ml aliquots were removed at various time points (0, 5, 10, 20, 40 and 60 min). For normalisation purposes, the protein concentration of the cell extract was determined according to the Bradford method (Bradford, 1976) and the fluorescein fluorescence of the samples was measured as before.

2.16 Rhodamine-6-G diffusion assay

The method for measuring rhodamine-6G (R6G) diffusion in yeast was adapted from Hazel et al., (1999). Yeast cells from exponentially growing cultures were collected by centrifugation ($12\ 100 \times g$, 5 min, 4°C) and washed twice with 10 mM Tris-HCl pH 7.0. The cells were subsequently resuspended in 20 ml of the buffer, the OD determined for normalisation purposes, and incubated with 200 μl of 1 M DOG (10 mM) for 30 min at RT to de-energise the cells. A 1 ml aliquot of the cell suspension to represent background fluorescence was pelleted and the fluorescence determined. R6G (14 mM in methanol) was then added to the remaining cell suspension to a final concentration of 10 μM . An aliquot of 1 ml was taken at various time points (0, 5, 10, 20, 40 and 60 min) and immediately centrifuged, the supernatant removed and the fluorescence of the cell pellet

determined. R6G fluorescence was measured at an excitation wavelength of 529 nm and an emission wavelength of 553 nm using an Aminco SPF-S00 fluorimeter (American Instrument Company, USA) (van den Hazel et al., 1999).

The initial rate of rhodamine diffusion was determined using curve fitting (LAB Fit Curve Fitting Software -V7.2.37 (1999-2007)) with the following function:

$$Y = A \cdot X + B$$

where A represents the change in fluorescence per unit time (F/min), and Y is the fluorescence at time X.

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CHAPTER THREE

RESULTS and DISCUSSION

3.1 Yeast growth curves

Growth curves (Figure 3.1) were determined in order to establish the growth patterns of the yeast strains to be used in the experiments. The wild-type strain reached the exponential phase after 5 h and mid-exponential phase after 10 h. The *hsp12* strain had a similar early growth pattern also reaching mid-exponential phase at about 10 h but with a lower final cell density at the stationary phase. However, the possibility that the *hsp12* cells possess altered light scattering properties due to their increased cell clumping phenotype (Karreman and Lindsey, 2007) could not be ruled out.

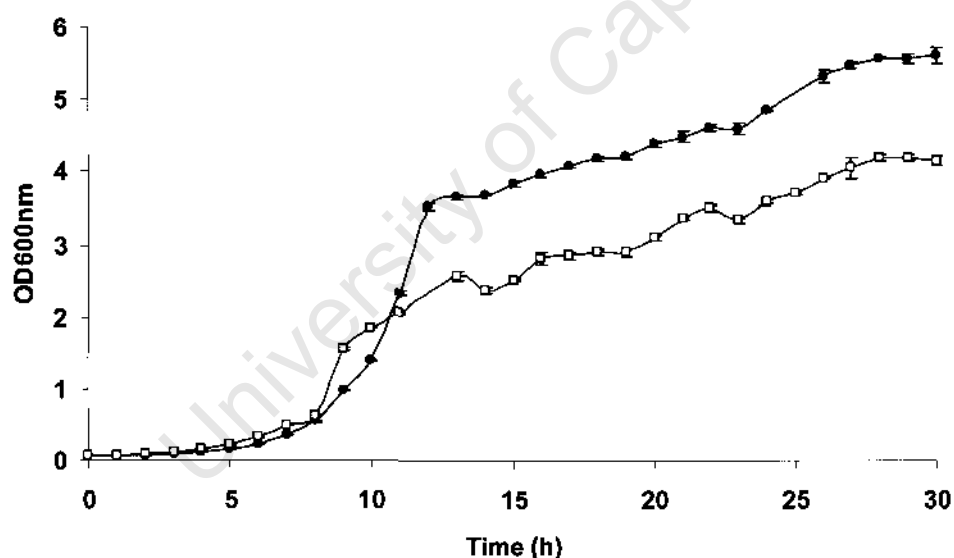


Figure 3.1: Growth pattern for the wild-type (●) and the *hsp12* (□) in YPD at 30°C. Absorbance (OD) readings were recorded at 600 nm every 1 h. Data represented are the mean (\pm SD) of three independent experiments. Error bars not visible are within the data symbols.

3.2 PCR for the *HSP12* gene

In order to verify that the knockout strain did not contain the *HSP12* gene, PCR with *HSP12* primers was carried out using DNA extracted from both strains. The knockout strain was previously constructed by insertion of a 1.1 kb *Hind* III fragment containing a *URA3* gene inserted at the unique *Sty*I site (Praekelt and Meacock, 1990). Agarose gel electrophoresis of the PCR products showed the presence of a 149 bp product in the wild-type indicating the presence of the *HSP12* gene (Figure 3.2; Lane 3). A 1.2 kb product, which is indicative of disruption of the *HSP12* locus in the knockout strain, was visible in lane 4 (Figure 3.2). To rule out any cross contamination from the reagents a PCR without template was run and produced no products as shown in figure 3.2 (Lane 5).

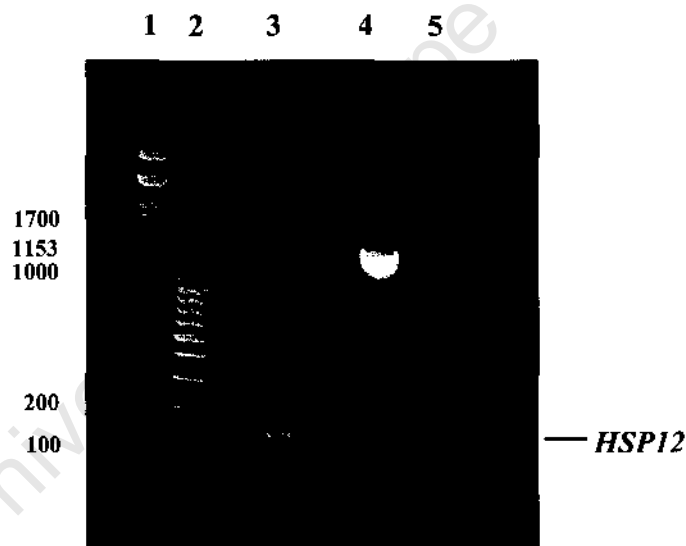


Figure 3.2: PCR to confirm the presence and absence of *HSP12* in the wild-type and knockout (*hsp12*) strains. Lane 1: Lambda DNA Pst I Digest (pst) marker with approximate sizes; 2:100 bp DNA marker with approximate sizes; 3: wild-type; 4: *hsp12*; 5: Control (no template).

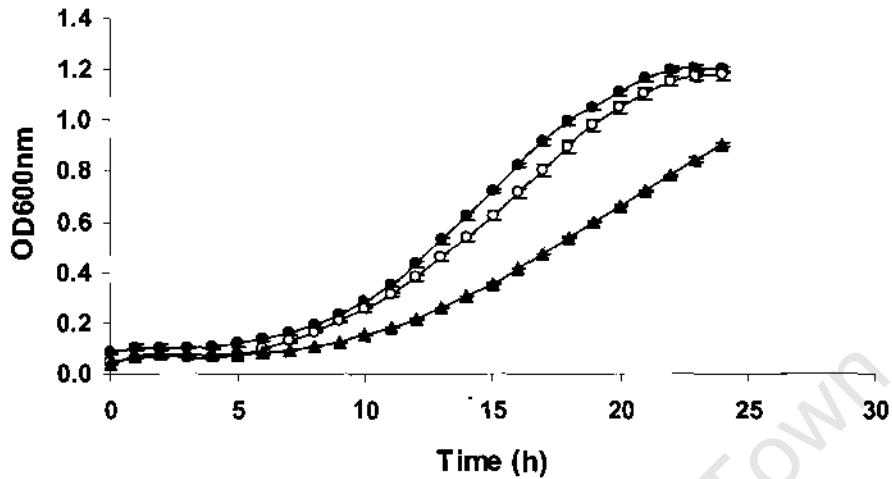
3.3 Yeast growth behaviour during weak acid stress

We investigated the effects of yeast growth in the presence of pH 4.5 YPD with and without sorbic acid. Growth of yeast in low pH YPD with sorbic acid leads to induction of the ABC transporter Pdr12 (Holyoak et al., 1999). In standard YPD (pH 6.3) sorbic acid is almost completely dissociated and cannot diffuse into the cell and therefore is non-toxic. However, at pH 4.3 a large fraction of the acid exists in the undissociated (protonated) form allowing it to diffuse across the membrane and inhibit the growth of the yeast (Piper et al., 1998). We compared the growth patterns of the wild-type and *hsp12* strains used in our experiments. In both cases growth in pH 4.3 YPD alone had no significant effect on the rate when compared to the control (pH 6.3 YPD) (Figure 3.3 A and B). The addition of 0.45 mM sorbic acid to pH 4.3 YPD reduced the growth rate for both strains which included an extended lag phase (Figure 3.3 A and B). In all experiments that require Pdr12 induction, wild-type and *hsp12* cells were subjected to weak acid stress by growth at pH 4.3 and 0.45 mM sorbate.

3.4 RT-PCR for Pdr12 expression

In order to confirm that the selected growth conditions (pH 4.3 YPD with 0.45 mM sorbic acid) did in fact induce *PDR12*, reverse-transcriptase PCR for the presence of Pdr12 mRNA was carried out. We used the *ACTN1* gene as a positive control to indicate successful cDNA synthesis and in all cases it yielded the expected 100 bp band after PCR (Figure 3.4 A and B (lanes 2 and 3)). As a negative control, cDNA synthesis was carried out without the reverse transcriptase enzyme. After PCR no bands were visible except those representing the primer species (Figure 3.4A and B (lanes 4 and 5)). PCR for the *PDR12* gene, performed with cDNA synthesised from both the wild-type and *hsp12* strains grown in standard YPD, confirmed the absence of *PDR12* expression under these conditions by the absence of a 500 bp band which corresponds to the expected *PDR12* mRNA product (Figure 3.3 A and B (lane 6)). The presence of *PDR12* mRNA was visible in wild-type and *hsp12* yeast grown in pH 4.3 YPD with 0.45 mM sorbate (Figure 3.3 A and B (lane 7)).

A.



B.

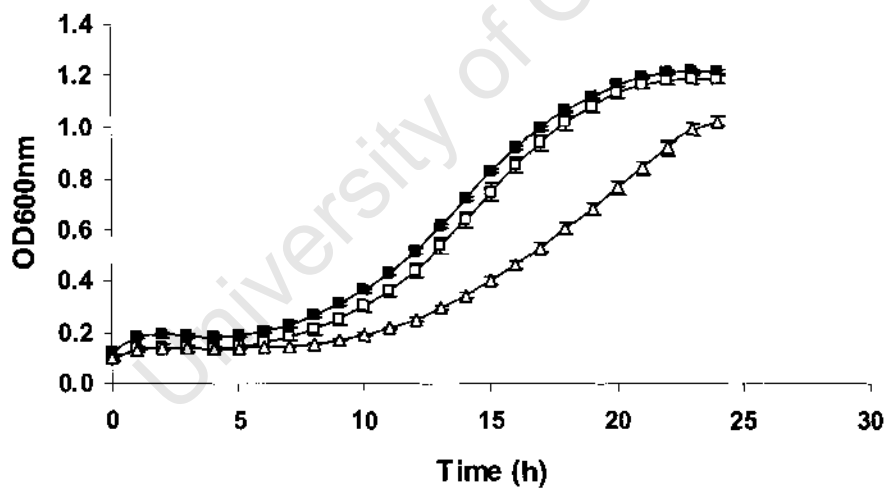
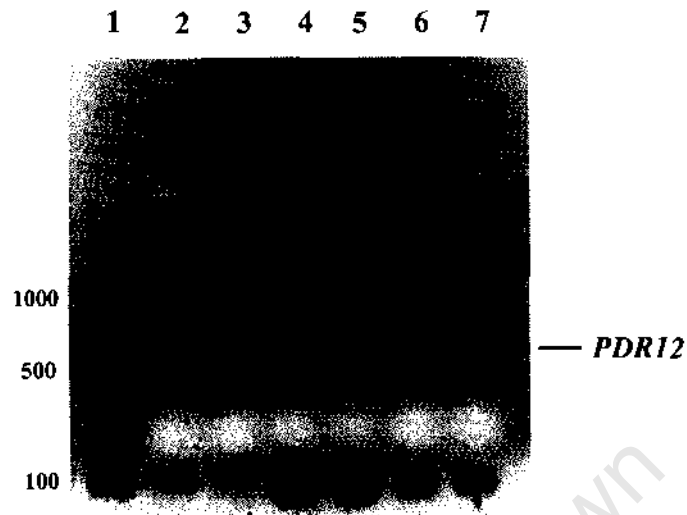


Figure 3.3: Automated growth curves of wild-type (A) and *hsp12* (B) strains comparing growth patterns in response to pH and weak acid stress. A: wild-type strain grown in standard YPD (pH 6.3) (●), pH 4.3 YPD (○) and pH 4.3 YPD with 0.45 mM sorbate (▲), B: *hsp12* strain grown in standard YPD (●), pH 4.3 YPD (□) and pH 4.3 YPD with 0.45 mM sorbate (△). Data represented are the mean (\pm SD) of three independent experiments. Error bars not visible are within the data symbols.

A.



B.

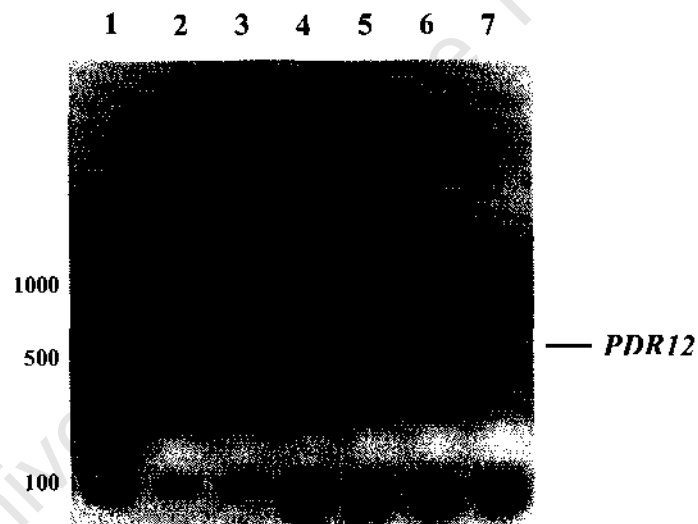


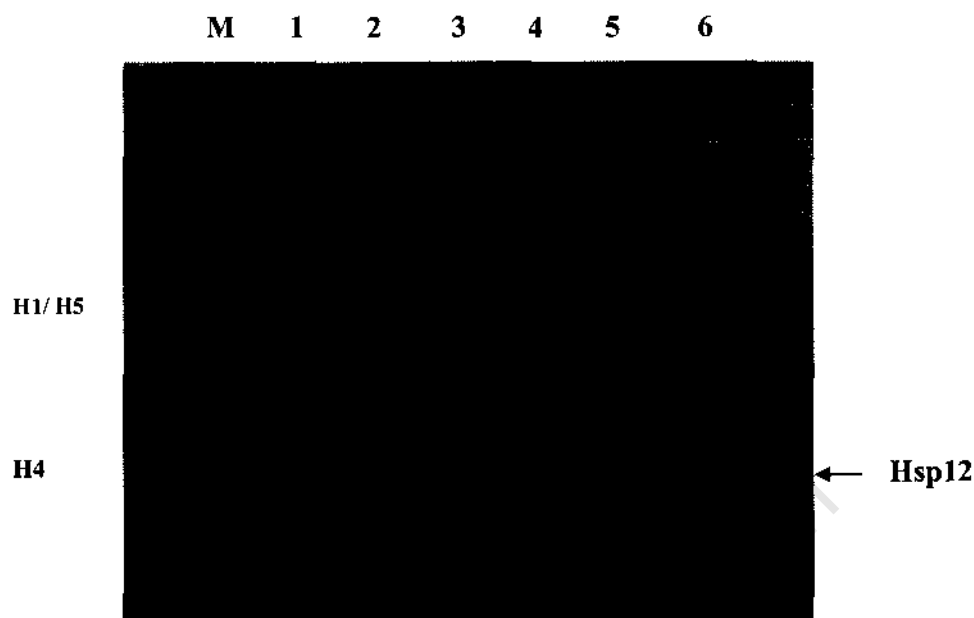
Figure 3.4: PCR products showing *PDR12* expression in the wild-type (A) and *hsp12* (B) strains after growth at 30 °C in standard YPD and pH 4.3 YPD with 0.45 mM sorbate. Lane 1 A and 1 B: 100 bp DNA marker with approximate sizes; 2 A and 2 B: positive control (*ACTN1*: 100bp) for the wild-type and *hsp12* cells grown in normal YPD respectively; 3 A and 3 B: positive control (*ACTN1*:100bp) for the wild-type and *hsp12* grown in pH 4.3 YPD with 0.45 mM sorbate respectively; 4 A and 4 B: negative control for the wild-type and *hsp12* cells grown in standard YPD respectively; 5 A and 5 B: negative control (minus reverse transcriptase) for the wild-type and *hsp12* grown in pH 4.3 YPD with 0.45 mM sorbate respectively; 6 A and 6 B: cDNA from the wild-type and *hsp12* cells grown in standard YPD respectively; 7 A and 7 B: cDNA from the wild-type and *hsp12* cells grown in pH 4.3 YPD with 0.45 mM sorbate respectively.

Note: Additional bands in lanes 4 - 7 represent *PDR12* primer dimer species.

3.5 Cell wall protein extraction

Previously it has been shown that NaOH solutions can be used to extract proteins from intact yeast cell walls without compromising their viability (Motshwene et al., 2004). Hsp12 was shown localised to the cell wall (Motshwene et al., 2004) and was extracted from the cell wall using 0.6 M NaOH. Since Hsp12 is induced in response to osmotic stress (Varela et al., 1995), we used SDS-PAGE and compared the levels of Hsp12 in cell wall extracts of yeast grown in standard YPD and expressing Pdr12, after mannitol and salt stress treatments. This extraction method released virtually the same set of proteins from each sample (Figure 3.5A). However, salt stressed samples lacked a high molecular weight band visible in the other samples (Figure 3.5: Lanes 3, 6) which may be indicative of slightly different cell wall protein expression profiles in response to the different stresses. All cell wall extracts show the presence of a band with molecular weight of approximately 11 kDa and based on previous studies (Mtwisha et al., 1998) corresponds to Hsp12 (Figure 3.4 A). Densitometry was used to estimate the amounts Hsp12 relative to the total protein content in each sample (Figure 3.5B). Both salt and mannitol stress treatments increased the levels of Hsp12 in the cell walls of yeast grown in normal YPD and those expressing Pdr12 (Figure 3.5 A and B). The untreated cells expressing Pdr12 had higher basal levels of Hsp12 (Figure 3.5A, lane 4; 3.5B) which was not unexpected since weak acid stress treatment has been shown to upregulate several stress genes, however it has been shown that many of these genes including *HSP12* are not required for that particular stress (Schuller et al., 2004).

A.



B.

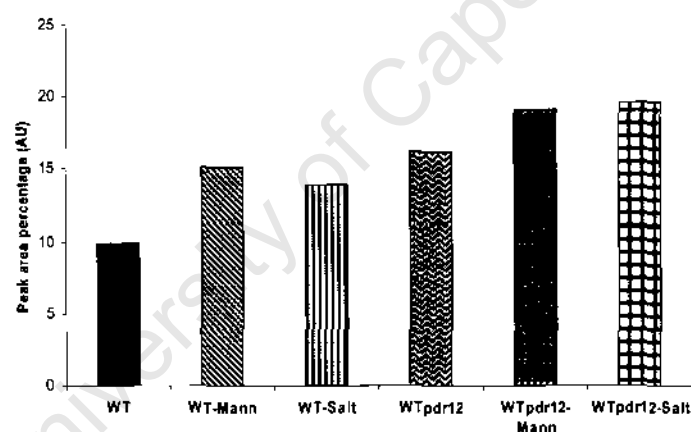
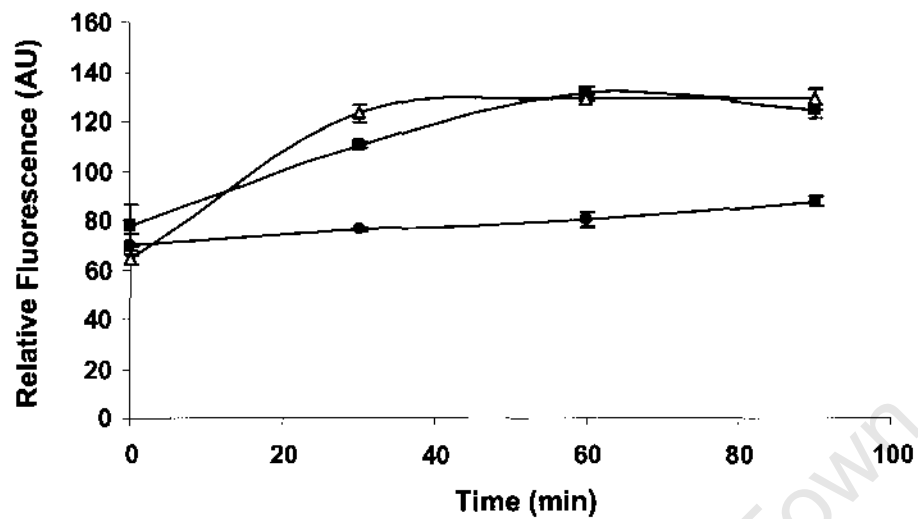


Figure 3.5: (A) SDS-PAGE of proteins extracted from the cell walls of whole yeast cells of the wild-type strain with and without *PDR12* induction, using 0.6 M NaOH: Marker lane M: Chicken erythrocyte histones with molecular masses (kDa): H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0 and H4, 11.2, were used as molecular weight markers; 1: wild-type grown in standard YPD; 2: wild-type grown in standard YPD and stressed with 400 mM mannitol; 3: wild-type grown in standard YPD and stressed with 200 mM salt; 4: wild-type grown in pH 4.3 YPD with sorbate; 5: wild-type grown in pH 4.3 YPD with sorbate and stressed with 400 mM mannitol; 6: wild-type grown in pH 4.3 YPD with sorbate and stressed with 200 mM salt. (B) The amount of Hsp12 relative to the total protein composition of each sample present in the cell wall extracts, loaded equally and electrophoresed in gel A: The relative Hsp12 content was determined using densitometry.

3.6 GFP stress assays

Certain genes such as *HSP12* can be used as an indicator of stress during industrial processes such as brewing. Unfortunately, upregulation of these genes does not always signify protein synthesis. An Hsp12-Green fluorescent protein (GFP) construct containing a stress promoter was previously created since fluorescent Hsp12 would directly indicate protein synthesis in response to stress (Karreman and Lindsey, 2004). The *GFP2* gene was fused to the *HSP12* gene so that the *HSP12* promoter drove expression of GFP2. It has been shown that conditions that upregulate Hsp12 produce fluorescence in yeast containing the *HSP12-GFP2* plasmid. The growth rate and morphology of the transformants are unchanged from that of the normal wild-type without the plasmid (Karreman and Lindsey, 2004). In order to determine the time course of Hsp12 expression for our experimental conditions, changes in GFP fluorescence in response to osmolytes known to upregulate Hsp12 was monitored. The relative fluorescence of samples removed at various time points after the addition of the stressing agent was determined and plotted as shown in Figure 3.6. The fluorescence of the unstressed cells harbouring the pYES-HSP12-GFP2 plasmid (GFP-yeast) grown in standard YPD (non-adapted cells) and pH 4.3 YPD with sorbate to induce PDR12 expression (weak acid stress; adapted cells), remained unchanged throughout the chosen time course and was used to establish a baseline. The addition of 400 mM NaCl and 800 mM mannitol produced similar changes in fluorescence over time for both cells grown in standard YPD and those subjected to weak acid stress. The adapted cells showed a 33% higher fluorescence compared to the non-adapted cells prior to osmotic shock (at time zero) (Figure 3.6 A) which could be attributed to induction of Hsp12 during weak acid exposure. This correlates with the SDS-PAGE analysis of the cell wall extracts which also showed higher levels of Hsp12 in weak acid stressed cells prior to osmotic shock (Figure 3.5). After 30 min of osmotic stress, the fluorescence increase relative to that at time zero was approximately 66 % and 42% for the non-adapted and adapted cells respectively (Figure 3.6A and B). In both cases, maximum fluorescence was observed after 60 min (Figure 3.6). Based on these assays we chose stress treatments of 40 min with NaCl and mannitol for all experiments.

A.



B.

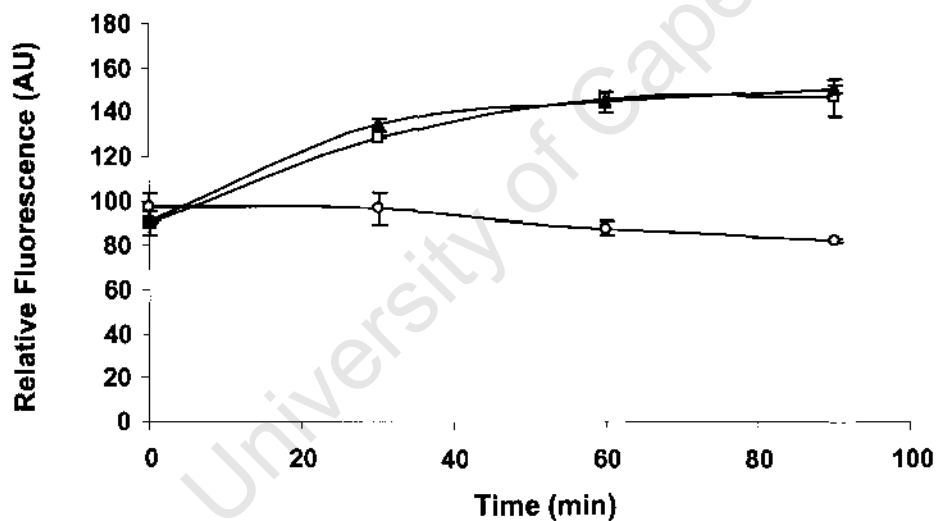


Figure 3.6: Exponentially growing yeast cells in standard YPD (A) and pH 4.3 YPD with 0.45 mM sorbate (B) were stressed with the addition of 800 mM mannitol and 400 mM NaCl after which the fluorescence was monitored at various times. A: Control (YPD only) (●); 800 mM mannitol (■) and 400 mM NaCl (△). B: Control (pH 4.3 YPD with sorbate) (○); 800 mM mannitol (□) and 400 mM NaCl (▲).

3.7 GFP fluorescence Images

Fluorescence images of yeast cells harbouring the pYES2-HSP12-GFP2 plasmid were obtained to visualise changes in GFP fluorescence in response to mannitol stress. Phase contrast and the corresponding fluorescence images were obtained for unstressed cells (before the addition of mannitol) and 40 min after the addition of 800 mM mannitol. Both the yeast cells grown in standard YPD and those expressing Pdr12 displayed no fluorescence before the addition of mannitol (Figure 3.7.1 and 2B). In both cases brightly fluorescent cells were visible 40 min after the addition of mannitol (Figure 3.7.1 and 2D). The presence of relatively few fluorescent cells indicates that many of the cells had lost the GFP plasmid however, this does not alter the finding that Hsp12 has been induced by stress.

1.

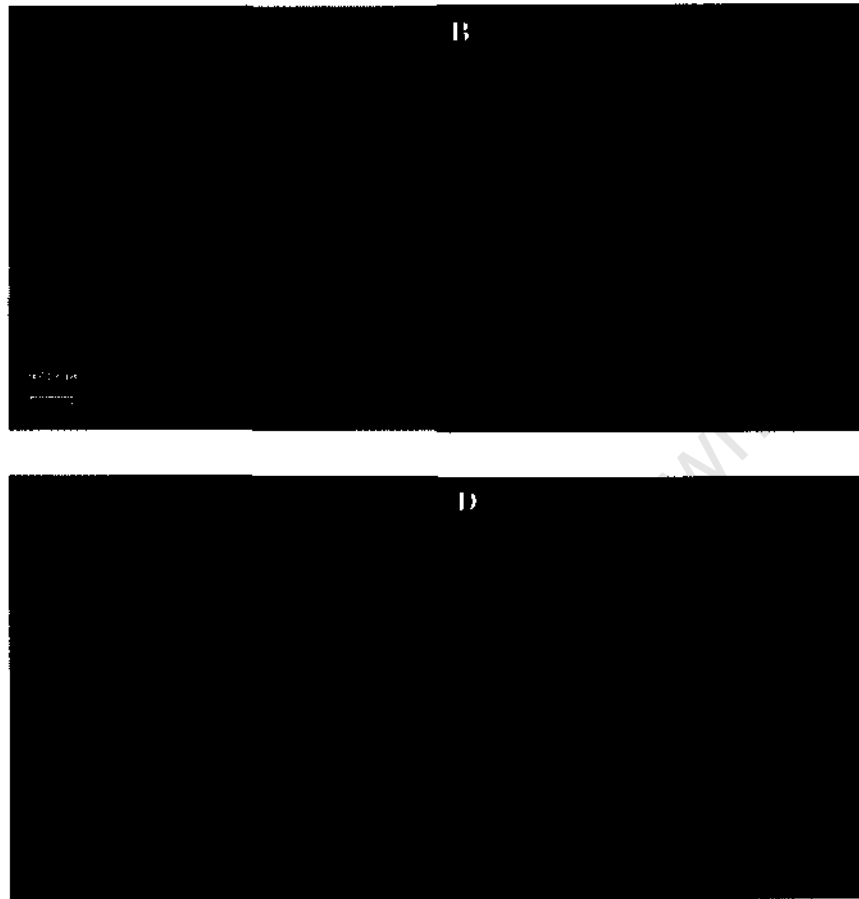


Figure 3.7.1: Phase contrast (A, C) and fluorescent (B, D) images of GFP-wild-type cells grown in standard YPD before the addition of 800 mM mannitol (A and B) and 40 min after the addition of mannitol (C and D). Scale bar represents 0.01mm in all cases.

2.

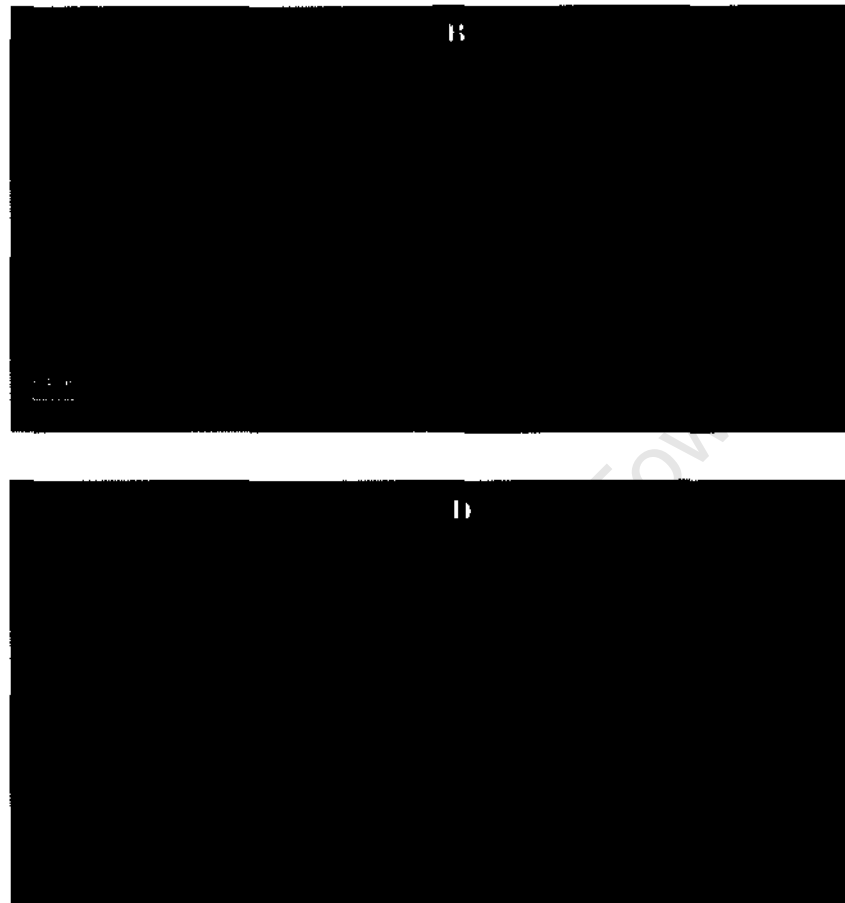


Figure 3.7.2: Phase contrast (A, C) and fluorescent (B, D) images of weak acid stressed GFP-wild-type cells immediately before the addition of 800 mM mannitol (A and B) and 40 min after the addition of mannitol (C and D) to the medium. Scale bar represents 0.01mm in all cases.

3.8 Fluorescein diacetate assay

The lipophilic compound fluorescein diacetate (FDA) diffuses easily into yeast cells where it is rapidly hydrolysed by intracellular esterases yielding a fluorescent product fluorescein, which is polar and remains trapped inside the cell (Holyoak et al., 1999). Fluorescein can be actively extruded from the cell by the ABC transporter Pdr12 that is expressed in the plasma membrane of yeast cells that have been exposed to weak acid preservative stress (Holyoak et al., 1999). The activity of Pdr12 can be regulated by withholding an energy source and reactivated by the addition of glucose (Holyoak et al., 1999). Growth of yeast cells in standard YPD (pH 6.4) with sorbic acid induces very low levels of Pdr12 expression (Piper et al., 1998). Strong expression of Pdr12 can be achieved if yeast cells are grown in low pH YPD (4.3) with the preservative sorbic acid (Holyoak et al., 1999). Low pH YPD allows optimal diffusion of protonated sorbate into the cell followed by deprotonation due to the higher intracellular pH followed by the accumulation of toxic sorbate anions which generate the Pdr12 stress response (Piper et al., 1998). The plasma membrane is one of the primary sites affected by osmotic stress (Guyot et al., 2006). The stress response protein Hsp12 is rapidly induced in response to osmotic stress (Varela et al., 1995) and is located in close proximity to the plasma membrane (Sales et al., 2000). We therefore investigated whether osmotic stress affects passive diffusion of FDA into de-energised cells. In addition, we investigated whether stress and Hsp12 modulate the stress inducible – ABC transporter Pdr12 by measuring the energy mediated efflux of fluorescein after mannitol and salt stress.

In determining the rates of fluorescein accumulation and efflux, slightly different kinetic parameters were used. The rate of fluorescein accumulation is directly related to rate of FDA diffusion into the cell and probably follows first order kinetics. Since we did not follow fluorescein accumulation to equilibrium, we used the initial rate as an indicator of membrane permeability (initial velocities are extensively used in enzyme kinetics). The rate of intracellular fluorescein reduction is ATP dependent and the rate of efflux was substantially larger and reached equilibrium within 30 minutes. The kinetics of fluorescein efflux also appears to obey the first order kinetic rate law and the data fitted

the expected exponential curve. Examples of how the data were fitted in both cases are given in the materials and methods (Figure 2.12). The rates of FDA influx and fluorescein efflux cannot be directly compared since they are different parameters i.e. the initial rate (F/min) and the first order kinetic rate constant (k , sec^{-1}) respectively.

According to the results shown in Figures 3.8.1 and 3.8.2, yeast cells grown in standard YPD (non-adapted cells) followed by de-energisation with 2-deoxyglucose, accumulated fluorescein passively. First we monitored diffusion of FDA into the ATP-depleted non-adapted cells. Under all conditions insignificant leakage of fluorescein was observed while the cells were de-energised (Figure 3.8.1 B, D, F, and 3.8.2H, and J). Overall, only *hsp12* cells pre-stressed with mannitol showed a drastic increase (65 %) in the rate of fluorescein accumulation when compared to the unstressed *hsp12* cells (Table 3.1). The rate of fluorescein accumulation was 14 % higher in mannitol stressed wild-type yeast cells when compared to unstressed cells (Figure 3.8.1A; Table 3.1) however, the salt treatment marginally reduced the rate of FDA diffusion (Figure 3.8.1C; Table 3.1). Unstressed *hsp12* cells displayed a 10 % higher rate of fluorescein accumulation than the unstressed wild-type cells (Figure 3.8.1E; Table 3.1). Results from the non-adapted cells seem to indicate that osmotic stress generally has a small effect on the diffusion rate. Although FDA diffusion was enhanced in both the wild-type and *hsp12* cells after the mannitol treatment, the 4-fold greater rate increase in the *hsp12* cells (15 to 65 %) suggests that the presence of Hsp12 reduces permeability after stress. Previous studies have also shown that *hsp12* cells displayed enhanced permeability when compared to wild-type cells during oxidative stress suggesting that Hsp12 maintained the integrity of the plasma membrane in the wild-type cells (Shamrock, 2007).

Diffusion of FDA was then monitored in weak acid adapted cells expressing high levels of Pdr12 (adapted cells) (Figure 3.8.3). As before, cells were de-energised and loaded with FDA after which insignificant leakage of fluorescein was also observed under all conditions (Figure 3.8.2B, D, F, and 3.8.3H, and J). In addition all adapted cells showed an approximately 40 % lower diffusion rate when compared to the non-adapted cells (Tables 3.1 and 3.2). The effect of the osmolytes on the diffusion rate of adapted

Cells grown in standard YPD

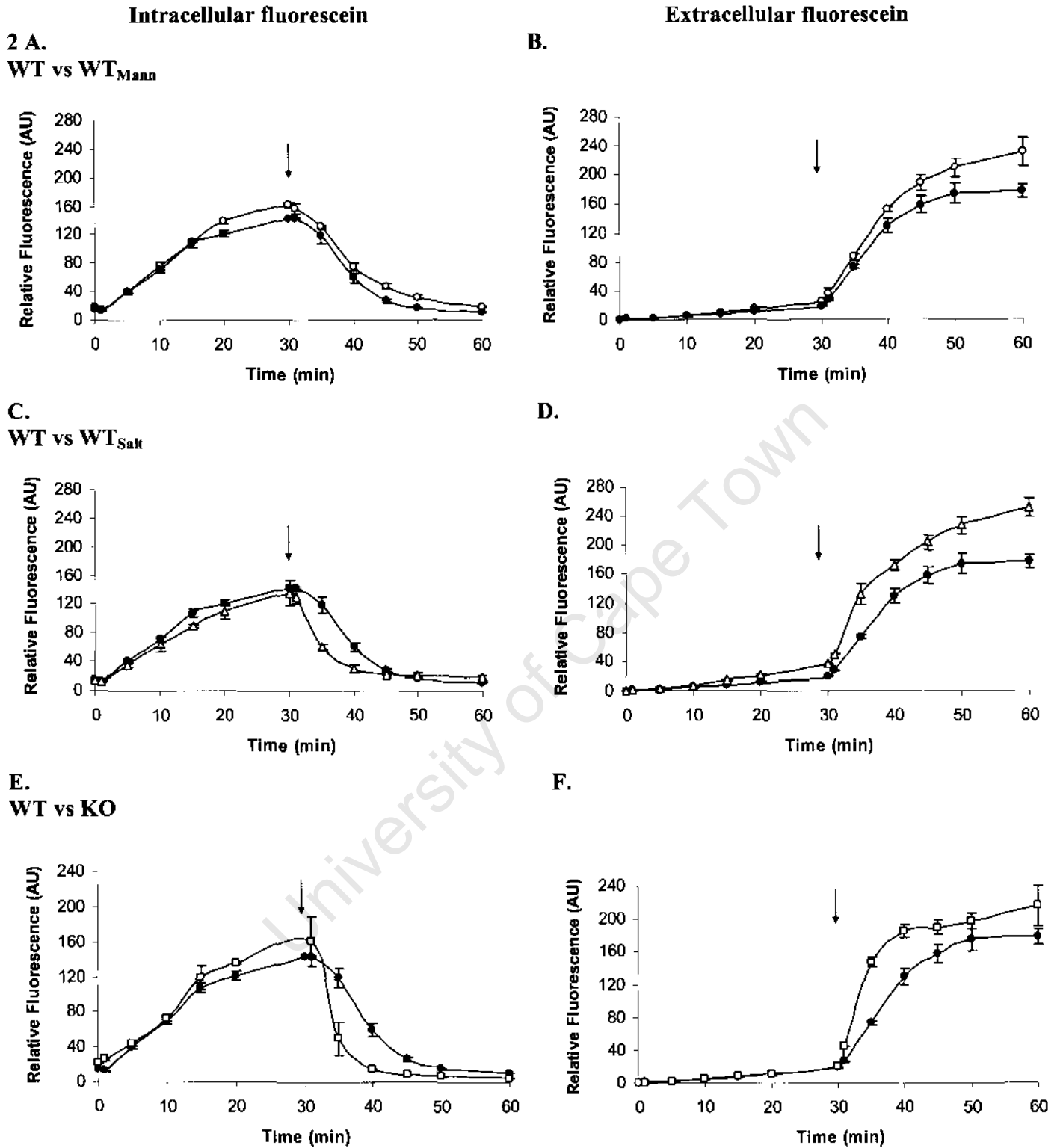


Figure 3.8.1: Fluorescein accumulation and efflux from non-adapted wild-type cells stressed with either 800 mM mannitol or 400 mM NaCl and from non-adapted *hsp12* cells: wild-type (●) and 800 mM mannitol (○) (A and B), wild-type (●) and 400 mM NaCl (□) (C and D), wild-type (●) and *hsp12* (□) (E and F). Intracellular fluorescence and medium fluorescence were monitored simultaneously. Efflux of fluorescein was initiated with the addition of 5 mM glucose (↓). Each datum point represents the mean and standard deviation of three independent experiments. Error bars not visible are within the data symbols.

Cells grown in standard YPD

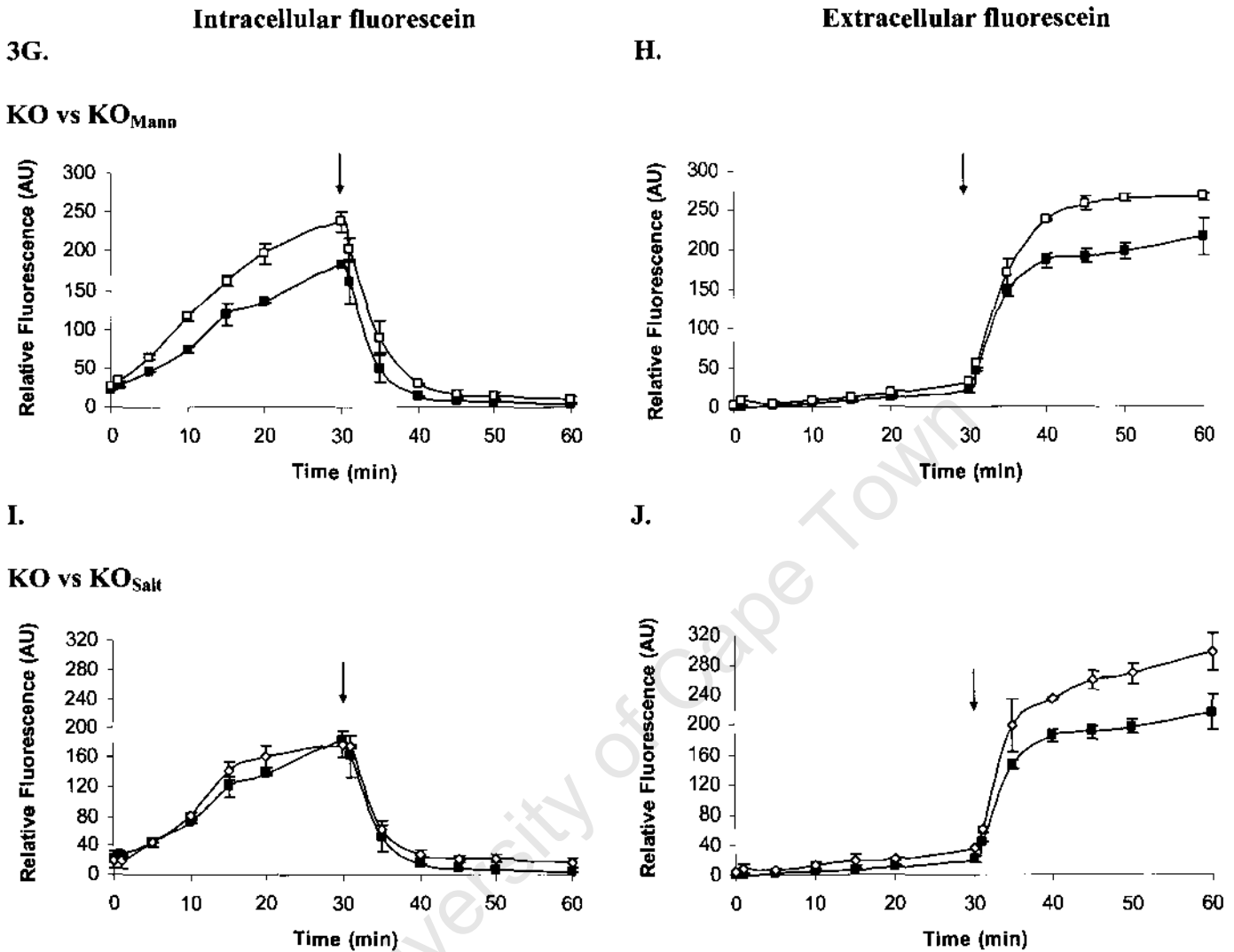


Figure: 3.8.2: Fluorescein accumulation and efflux from non-adapted *hsp12* cells stressed with either 800 mM mannitol or 400 mM NaCl: *hsp12* (•) and 800 mM mannitol (□) (A and B), *hsp12* (•) and 400 mM NaCl (◇). Intracellular fluorescence and medium fluorescence were monitored simultaneously. Efflux of fluorescein was initiated with the addition of 5 mM glucose (↓). Each datum point represents the mean and standard deviation of three independent experiments. Error bars not visible are within the data symbols.

cells was more prominent (Table 3.2). Adapted wild-type cells stressed with either mannitol or salt, both showed a significantly greater rate of fluorescein accumulation, 37 and 27 % respectively, when compared to the unstressed adapted wild-type cells (Figure 3.8.2A and C; Table 3.2). The rate of fluorescein accumulation in adapted *hsp12* cells prior to osmotic shock was 24 % lower than in unstressed adapted wild-type cells (Figure 3.8.2E and F; Table 3.2). Both osmotic stress treatments marginally enhanced the rate of fluorescein accumulation in *hsp12* cells (Figure 3.8.3G and I; Table 3.2). The non-adapted wild-type and *hsp12* cells displayed overall higher rates of FDA diffusion, 33 and 54 % respectively, when compared to those expressing Pdr12 (Figure 3.8.5; Table 3.3).

Table 3.1: A comparison of the rates of fluorescein accumulation and efflux in unstressed and stressed wild-type (WT) and *hsp12* (KO) cells grown in standard YPD (non-adapted cells).

Strains	Influx rate ** (F/min)	Difference (%)	Efflux rate *** $k(\text{min}^{-1})$	Difference (%)
*WT	4.680		0.0708	
WT _{Mann}	5.313	+ 14	0.0771	+ 8
*WT	4.680		0.0708	
WT _{Salt}	4.337	- 7	0.1110	+ 57
*WT	4.680		0.0708	
KO	5.112	+ 10	0.2390	+ 138
*KO	5.112		0.2390	
KO _{Mann}	8.765	+ 65	0.1810	- 24
*KO	5.112		0.2390	
KO _{Salt}	5.371	+ 5	0.1820	- 24

Note: Subscript denotes stress treatment with either 800 mM mannitol (mann) or 400 mM salt (salt).

* Denotes reference strain used for rate comparisons.

** F/min: Initial rate of fluorescence per minute

***k: First order exponential rate constant with the units min^{-1}

Cells were grown in pH 4.3 YPD with sorbic acid to induce Pdr12 expression

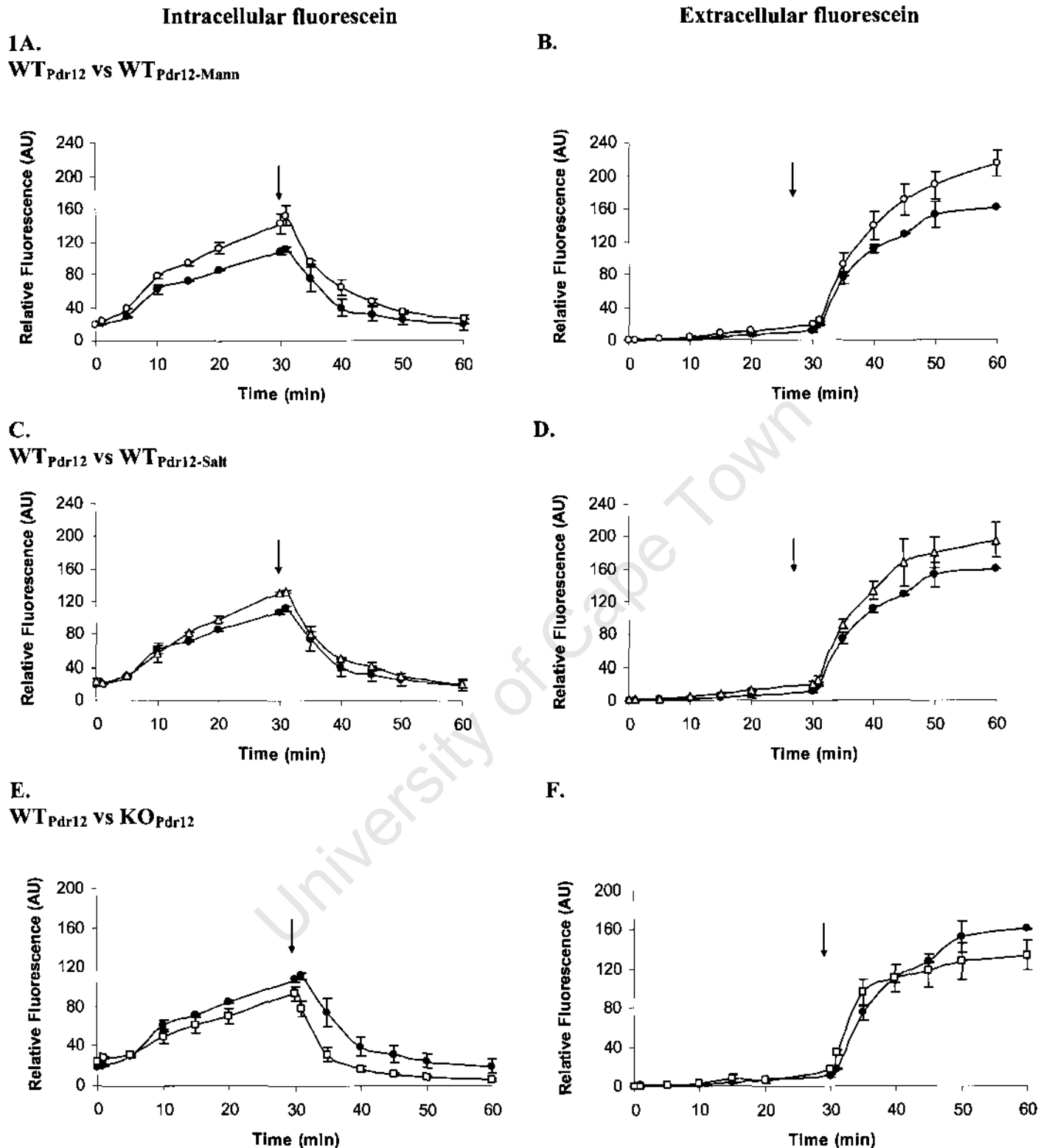


Figure 3.8.3: Fluorescein accumulation and efflux from adapted wild-type cells stressed with either either 800 mM mannitol or 400 mM NaCl and from non-adapted *hsp12* cells: wild-type (●) and 800 mM mannitol (○) (A and B), wild-type (●) and 400 mM NaCl (□) (C and D), wild-type (●) and *hsp12* (□) (E and F). Intracellular fluorescence and medium fluorescence were monitored simultaneously. Efflux of fluorescein was initiated with the addition of 5 mM glucose (↓). Each datum point represents the mean and standard deviation of three independent experiments. Error bars not visible are within the data symbols.

Cells grown in pH 4.3 YPD with sorbic acid to induce Pdr12 expression

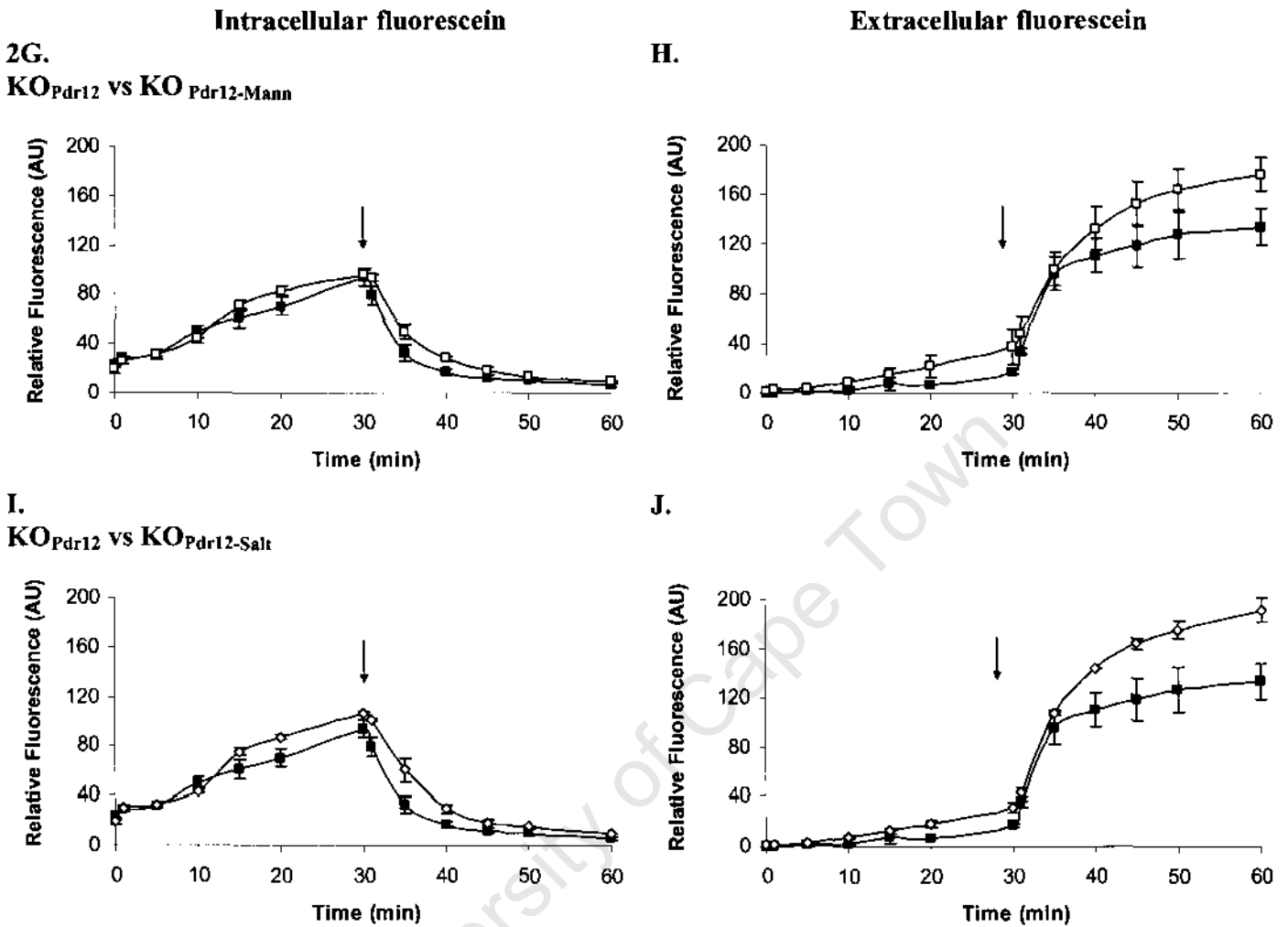


Figure: 3.8.4: Fluorescein accumulation and efflux from adapted *hsp12* cells stressed with either 800 mM mannitol or 400 mM NaCl: *hsp12* (•) and 800 mM mannitol (□) (A and B), *hsp12* (•) and 400 mM NaCl (◇) (C and D). Intracellular fluorescence and medium fluorescence were monitored simultaneously. Efflux of fluorescein was initiated with the addition of 5 mM glucose (↓). Each datum point represents the mean and standard deviation of three independent experiments. Error bars not visible are within the data symbols.

Table 3.2: A comparison of the rates of fluorescein accumulation and efflux in unstressed and stressed wild-type (WT) and *hsp12* (KO) cells expressing Pdr12 (adapted cells).

Strains	Influx rate ** (F/min)	Difference (%)	Efflux rate *** <i>k</i> (min ⁻¹)	Difference (%)
*WT _{Pdr12}	3.116		0.1022	
WT _{Pdr12-Mann}	4.282	+ 37	0.0866	- 15
*WT _{Pdr12}	3.116		0.1022	
WT _{Pdr12-Salt}	3.824	+ 23	0.1006	- 2
*WT _{Pdr12}	3.116		0.1022	
KO _{Pdr12}	2.363	- 24	0.1597	+ 56
*KO _{Pdr12}	2.363		0.1597	
KO _{Pdr12-Mann}	2.710	+ 15	0.1120	- 30
*KO _{Pdr12}	2.363		0.1597	
KO _{Pdr12-Salt}	2.880	+ 22	0.1120	- 30

Note: Subscript denotes pdr12 expression and stress treatment with either 800 mM mannitol (Mann) or 400 mM salt (Salt).

* Denotes reference strain used for rate comparisons.

** **F/min:** Initial rate of fluorescence per minute

*****k:** First order Exponential rate constant with units min⁻¹

Interpretation on the effect of osmotic stress on adapted cells is complicated by the possibility that these cells may have undergone morphological changes as part of the adaptation process. In addition, acid stress, which induces Pdr12 in yeast cells, also induces *HSP12* (Schuller et al., 2004). We hypothesise that the overall lower rates of diffusion into adapted cells could be attributed to a thicker cell wall, a possible adaptive change to the weak acid stress. Thicker cell walls are also a characteristic of stationary phase cells, which are more stress tolerant (Park et al., 1997). The absence of Hsp12 in the knockout strain could result in compensatory protective mechanisms employed by these cells during the sorbic acid adaptation process. Therefore, under these conditions adapted *hsp12* cells would be better suited to cope with the osmotic stress treatments than adapted wild-type cells. The reduced rate of FDA diffusion into adapted *hsp12* cells when compared to adapted wild-type cells prior to osmotic shock, as well as the reduced effect of the osmolytes on adapted *hsp12* cell permeability when compared to

adapted wild-type cell permeability (Figure 3.8.3 and 4; Table 3.2), supports our hypothesis that the adaptive mechanisms associated with weak acid stress masked the effect of the *HSP12* deletional mutation.

We then investigated the effect of salt and mannitol on fluorescein efflux. Results in Figures 3.8.1 and 3.8.2 show that non-adapted cells also displayed ATP-dependent efflux of fluorescein. Our RT-PCR results showed that no Pdr12 mRNA was produced when cells were grown in standard YPD (Figure 3.4). Therefore, we suggest that fluorescein may be a substrate for another ABC transporter that may be constitutively expressed in our lab strain. After the addition of glucose to non-adapted cells, only the salt stressed wild-type cells showed a significant increase in fluorescein efflux with a 57 % greater rate than the unstressed wild-type cells (Figure 3.8.1B and D; Table 3.1). The rate of fluorescein efflux from unstressed *hsp12* cells was 138 % higher than the rate from unstressed wild-type cells (Figure 3.8.1F; Table 3.1). However, both stress treatments reduced the rate of fluorescein efflux from *hsp12* cells by 24 % after the addition of glucose (Figure 3.8.2H and J; Table 3.1). Analysis of fluorescein efflux from adapted cells after the addition of glucose, showed no significant difference in the rates of fluorescein efflux from wild-type cells before and after osmotic shock (Figure 3.8.3B and D; Table 3.2). The unstressed *hsp12* cells showed a 56 % higher rate of fluorescein efflux into the medium when compared to unstressed wild-type cells (Figure 3.8.3F; Table 3.2). Both stress treatments reduced fluorescein efflux by 30 % from *hsp12* cells (Figure 3.8.4H and J; Table 3.2). Adapted wild-type cells displayed a 31 % higher rate efflux when compared to non-adapted wild-type cells (Figure 3.8.5; Table 3.3) which suggests that the 30 % increase is due to the Pdr12 pump. However adapted *hsp12* cells displayed overall lower rates of efflux (10 %) than non-adapted *hsp12* cells (Figure 3.8.5; Table 3.3) which could be attributed to additional morphological changes that may have occurred during weak acid adaptation as a consequence of the Hsp12 mutation, which may have adversely affected the functioning of Pdr12 (Figure 3.8.5; Table 3.3).

Non-adapted versus adapted cells

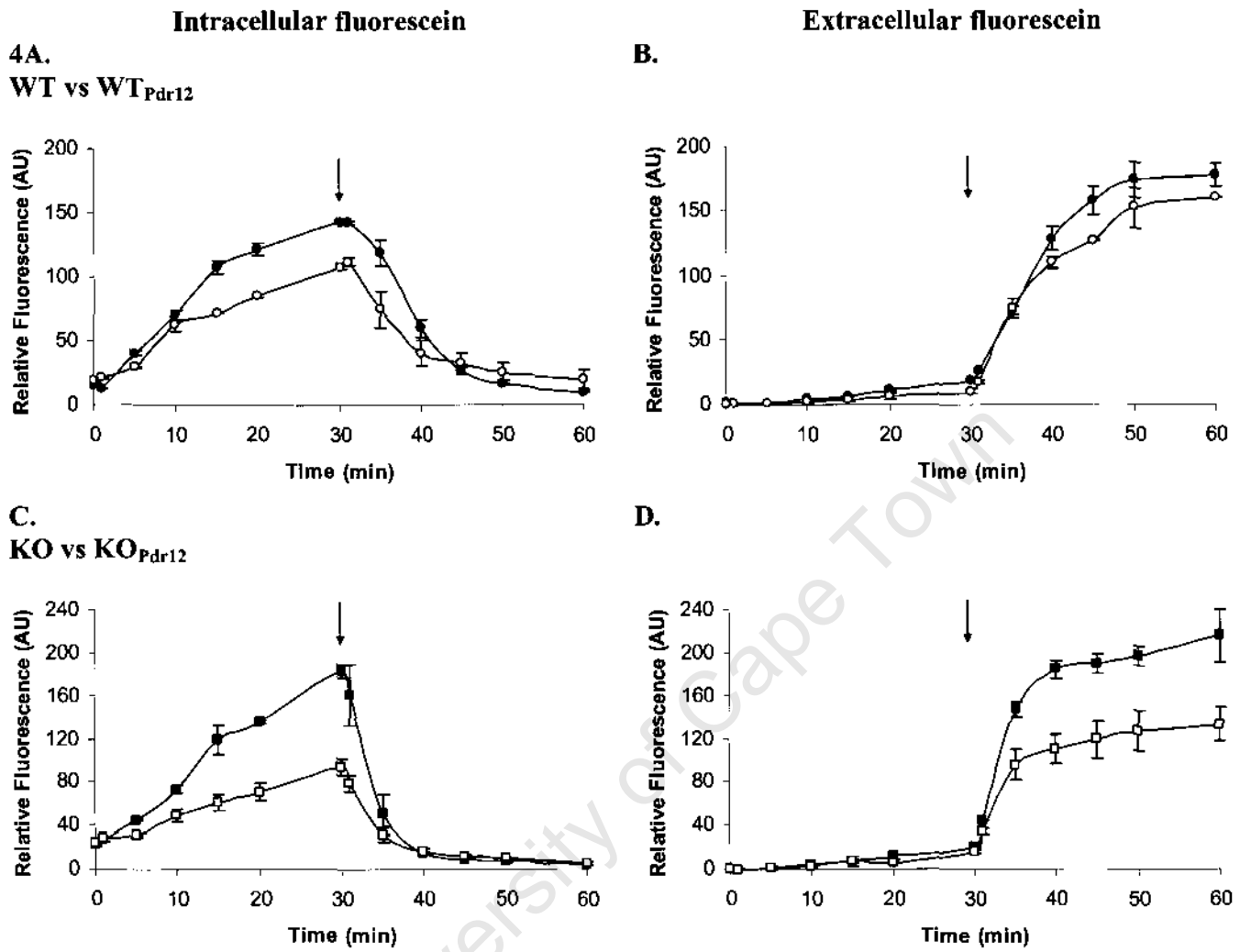


Figure: 3.8.5: A comparison of fluorescein accumulation and efflux between non-adapted and adapted wild-type and *hsp12* cells: non-adapted wild-type (●) and adapted wild-type (○)(A and B); non-adapted *hsp12* (●) and adapted *hsp12* (□)(C and D). Intracellular fluorescence and medium fluorescence were monitored simultaneously. Efflux of fluorescein was initiated with the addition of 5 mM glucose (↓). Each datum point represents the mean and standard deviation of three independent experiments. Error bars not visible are within the data symbols.

Table 3.3: A comparison of the rates of fluorescein accumulation and efflux between non-adapted and adapted wild-type (WT) and *hsp12* (KO).

Strains	Influx rate ** (F/min)	Difference (%)	Efflux rate *** <i>k</i>	Difference (%)
*WT	4.680		0.0708	
WT _{Pdr12}	3.116	- 33	0.1022	+ 31
*KO	5.112		0.2390	
KO _{Pdr12}	2.363	- 54	0.2135	- 11

Note: Subscript denotes strains expressing Pdr12.

* Denotes reference strain used for rate comparisons.

** F/min: Initial rate of fluorescence per minute

****k*: First order exponential rate constant with units min⁻¹

The patterns in the rates of fluorescein efflux from non-adapted and adapted cells correlate closely. We hypothesise that, prior to stress, the lack of Hsp12 and other possible morphological changes in the mutant enhances the efficiency of the pump. However, during stress the protective mechanisms provided by Hsp12 would be absent in *hsp12* cells and therefore they may have incurred damage to the membrane reducing the activity of the pump. The maintenance or enhanced activity of the pump in wild-type cells after the osmotic stress suggests that Hsp12 acts either indirectly by protecting the membrane, which in turn protects the pump, or directly on the pump enhancing its functioning during stress.

Only certain conditions for the non-adapted and adapted cells were selected for further experiments.

3.7 Enzyme assay for esterase activity

Esterases are found in most eukaryotic organisms including yeast and act through the hydrolysis of the ester bond between a carboxylic acid and an alcohol (Lomolino et al., 2001). FDA has been widely used for the determination of yeast intracellular esterase activity (Guldfeldt et al., 1998). Since FDA is cleaved by intracellular esterases the enzyme activity is one factor that would determine the rate of FDA conversion to fluorescein. Enzyme assays were carried out in order to determine whether differences in the rates of intracellular fluorescein accumulation were attributed to varying esterase activities. The initial rates of cleavage of FDA were determined by monitoring the fluorescence of the products as a function of time. The enzymatic hydrolysis of FDA yields a monosubstituted intermediate fluorescein monoacetate (FMA), which is in turn converted into fluorescein (Figure 3.9.1) (Hofmann and Sernetz, 1983). In general enzyme activity usually follows hyperbolic kinetics according to Michaelis-Menton (Michaelis and Menton, 1993). However the presence of the weak fluorescing intermediate (FMA) during FDA hydrolysis consequently reduces the initial rate of fluorescein accumulation which in turn lowers the initial fluorescence producing the observed sigmoidal curve (Birrell et al., 2003) (Figure 3.9.2A and B).

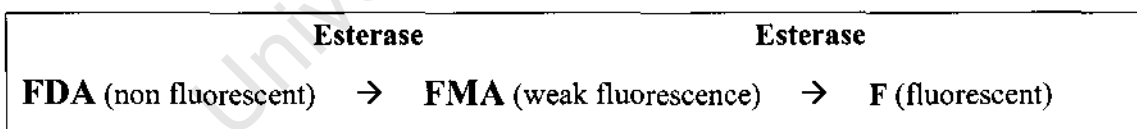
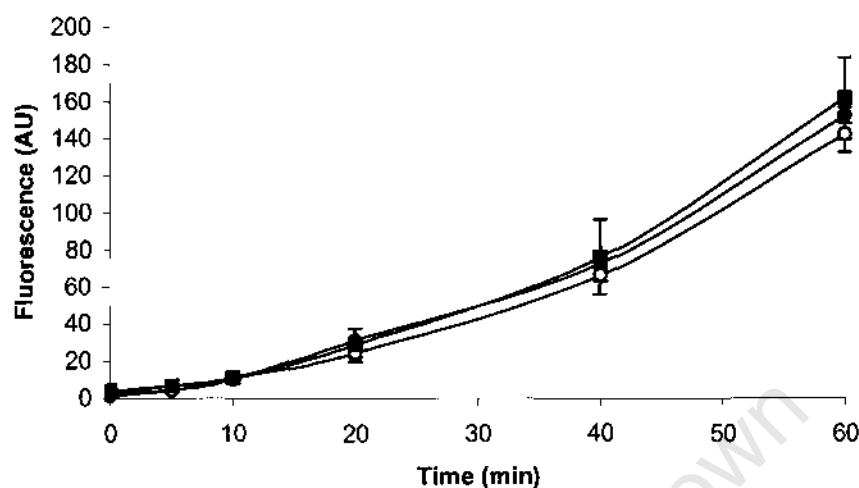


Figure 3.9.1: Step-wise conversion of the non-fluorescent fluorescein diacetate (FDA) to the fluorescent fluorescein (F) by intracellular esterases with the formation of the intermediate fluorescein monoacetate (FMA)

A.



B.

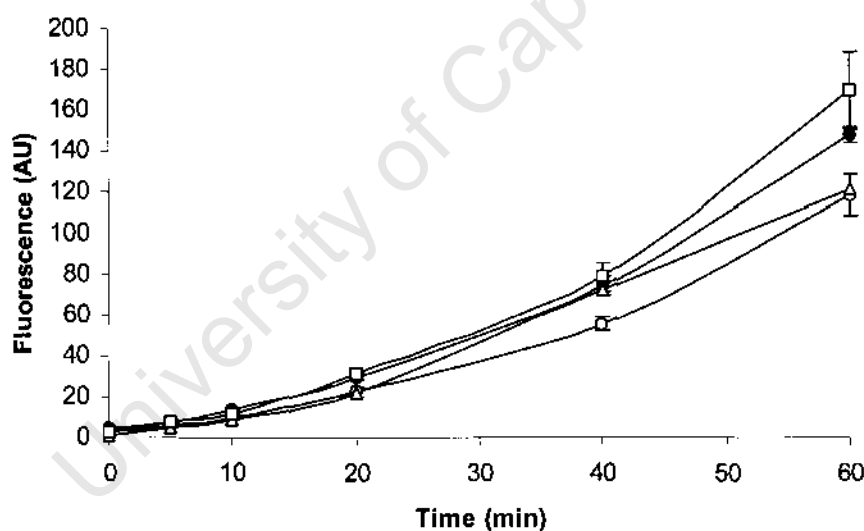


Figure 3.9.2: The esterase activity of (A) non-adapted and (B) adapted cells. A: Wild-type (●), *hsp12* (○) and *hsp12* stressed with 800 mM mannitol (◼). **B:** Wild-type (●), *hsp12*(○), wild-type stressed with 800 mM mannitol (△) and wild-type cells stressed with 400 mM salt (◻). Each datum point represents the mean and standard deviation of three independent experiments. Error bars not visible are within the data symbols.

A comparison of the enzyme activity from the non-adapted yeast cell extracts of unstressed wild-type, *hsp12* and mannitol stressed *hsp12* cells, showed no significant differences in esterase activity (Figure 3.9.2B).

We then compared the deacetylase activity in adapted cells. The unstressed wild-type cells and the mannitol and salt stressed wild-type cells, displayed the same levels of esterase activity. However, in adapted yeast the unstressed *hsp12* cells exhibited approximately 40 % lower activity when compared to all other conditions (Figure 3.9.2A and B).

Since accumulation of fluorescein was only monitored for 30 min in the FDA assay, a comparison of the rates observed under the various stress conditions used, showed no significant differences. In addition, the rate of enzyme activity is expected to be much faster since the enzyme concentration would be much higher inside the cell as opposed to the several hundred-fold dilution of the esterase extract. Therefore, we have concluded that the hydrolysis of FDA to fluorescein would be substantially faster when compared to the diffusion rate and would thus not have been the rate-limiting step in the intracellular accumulation of fluorescein under the different conditions.

4. Diffusion assay

In order to verify whether different rates of passive transport could account for the different rates of intracellular fluorescein accumulation, we used the fluorophore Rhodamine 6G (R6G) to measure passive diffusion into the yeast cells. Unlike FDA, R6G is fluorescent, undergoes no chemical change, and is widely used to measure passive diffusion. R6G is a cationic lipophilic molecule that is positively charged at pH 7 and diffuses easily into yeast cells (Gear., 1974; Figure 4.1). Since R6G is also a substrate for the ABC transporter Pdr5 (Kolaczowski et al., 1996), cells were first depleted of energy to inhibit active transport before being incubated with R6G. The rate of R6G accumulation inside the cells was then measured by the appearance of fluorescence inside the cells as function of time (see Materials and Methods and Figure 4.2). From the rate curves, the initial velocity of passive diffusion of the rhodamine into yeast cells was calculated as shown in Materials and Methods (Tables 4.2, 4.3 and 4.3).

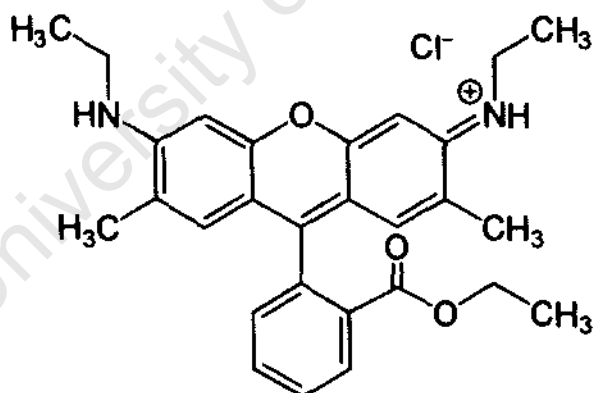


Figure: 4.1: Molecular structure of rhodamine 6G (chloride form) (Wikipedia, 2009).

A comparison of the rates of R6G diffusion in non-adapted yeast cells show that prior to osmotic shock $\Delta hsp12$ cells displayed the highest rate of diffusion with a 63 % higher rate than in wild-type cells (Figure 4.2A; Table 4.1) which supports our previous suggestion that Hsp12 reduces plasma membrane permeability. However, the mannitol stress treatment reduced the diffusion rate into the $\Delta hsp12$ cells by 75 % (Figure 4.2A; Table 4.1). Non-adapted cells displayed overall higher rates of diffusion than adapted cells, with a five and two times greater rate for the wild-type and $\Delta hsp12$ cells respectively (Table 4.3). This is in keeping with the FDA data, which also showed overall greater rates of fluorescein accumulation in non-adapted cells (Figure 3.8.5, Table 3.3). However, in the FDA assay, mannitol stressed non-adapted $\Delta hsp12$ cells displayed a 65 % enhanced rate of fluorescein accumulation that does not correlate with the reduced rate of R6G diffusion observed in $\Delta hsp12$ cells after mannitol stress. It has been suggested that in response to osmotic stress the $\Delta hsp12$ cells increase the levels of chitin in the cell wall as a compensatory response to the lack of Hsp12, which stiffens the wall (Karreman et al., 2007). We suggest that the stiffer and thicker cell wall of $\Delta hsp12$ cells may impede the movement of R6G and promote interactions between the positively charged R6G and the negatively charged mannoproteins in the cell wall (Odani et al., 1997). FDA, which is uncharged, would pass more easily through the cell wall of $\Delta hsp12$ cells and diffusion would be enhanced across the cell membrane of the $\Delta hsp12$ cells which is more permeable after stress.

Table 4.1: A comparison between the rates of rhodamine 6-G diffusion and fluorescein accumulation into non-adapted wild-type (WT) and $\Delta hsp12$ (KO) cells.

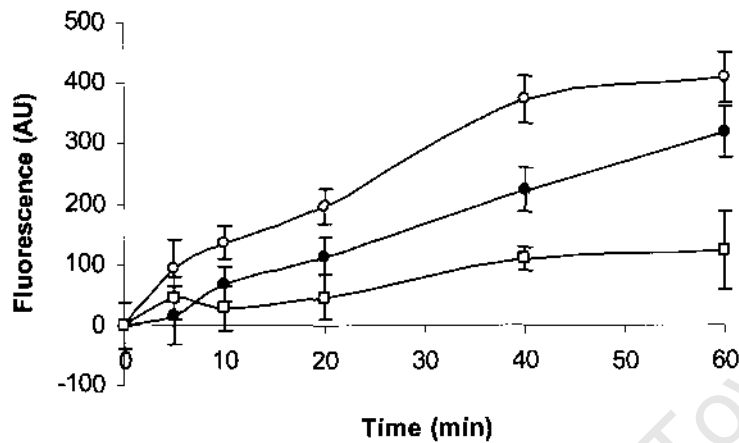
Strains	R6G Influx rate ** (F/min)	Difference (%)	FDA Influx rate ** (F/min)	Difference (%)
*WT	5.339		4.680	
KO	8.670	+ 63	5.112	+ 10
*KO	8.670		5.112	
KO _{Mann}	2.151	- 75	8.765	+ 65

Note: Subscript denotes stress treatment with 800 mM mannitol (mann).

* Denotes reference strain used for rate comparisons.

** F/min: Initial rate of fluorescence per minute

A.



B.

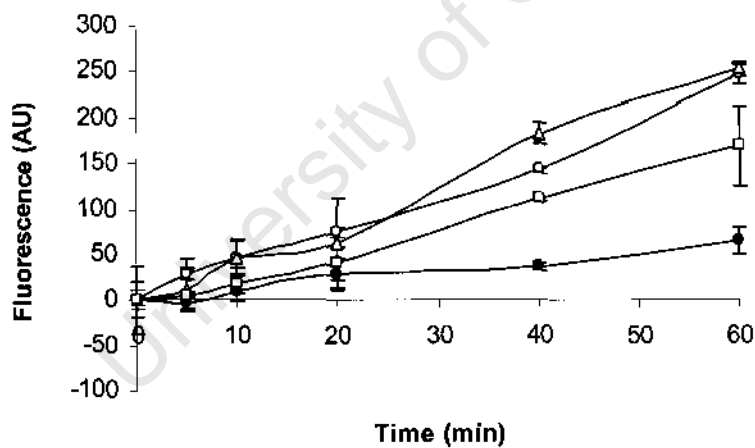


Figure 4.2: Passive diffusion of rhodamine 6G into yeast cells grown in standard YPD (non-adapted) and (A) in pH 4.3 YPD with sorbic acid (adapted) (B). A: wild-type (●), $\Delta hsp12$ (○), $\Delta hsp12$ stressed with 800 mM mannitol (□) B: wild-type (●), $\Delta hsp12$ (○), wild-type stressed with 800 mM mannitol (▲) and wild-type stressed with 400 mM salt (□). Each datum point represents the mean and standard deviation of three independent experiments. Error bars not visible are within the data symbols.

Table 4.2: A comparison between the rates of rhodamine 6-G diffusion and fluorescein accumulation into adapted wild-type (WT) and $\Delta hsp12$ (KO) cells.

Strains	R6G Influx rate ** (F/min)	Difference (%)	FDA Influx rate ** (F/min)	Difference (%)
*WT _{Pdr12}	1.027		3.116	
WT _{Pdr12-Mann}	4.340	+ 323	4.282	+ 37
*WT _{Pdr12}	1.027		3.116	
WT _{Pdr12-Salt}	2.932	+ 185	3.824	+ 23
*WT _{Pdr12}	1.027		3.116	
KO _{Pdr12}	3.918	+ 281	2.363	- 24

Note: Subscript denotes pdr12 expression and stress treatment with 800 mM mannitol (mann).

* Denotes reference strain used for rate comparisons.

** **F/min:** Initial rate of fluorescence per minute

Both stress treatments enhanced the rate of R6G diffusion into adapted wild-type cells, which was also the case for fluorescein accumulation (Table 4.2.2). The rates of R6G diffusion into mannitol and salt stressed adapted wild-type cells was 323 and 185 % higher respectively, when compared to unstressed wild-type cells (Figure 4.2B; Table 4.2.2). Hsp12 has been shown to increase the flexibility of the cell wall in response to osmotic stress and suggested to enhance its permeability (Karreman et al., 2007). This is supported by the enhanced rate of R6G diffusion observed in mannitol and salt stressed wild-type cells. Prior to osmotic shock adapted $\Delta hsp12$ cells displayed a 281 % greater rate of R6G diffusion than adapted wild-type cells which does not correspond with the FDA data for adapted cells (Figure 4.2B; Table 4.2.2). As stated previously the compensatory changes in the $\Delta hsp12$ cells as a result of the *HSP12* mutation together with the adaptive changes associated with growth in weak acid makes it difficult to interpret the effects this may have on the cell and consequently interactions with rhodamine and rhodamine diffusion.

Table 4.3: A comparison of the rates of rhodamine 6-G diffusion into non-adapted and adapted wild-type (WT) and $\Delta hsp12$ (KO) cells.

Strains	Influx rate ** (F/min)	Difference (%)
*WT	5.339	
WT _{pdr12}	1.027	- 81
*KO	8.670	
KO _{pdr12}	3.918	- 55

Subscript denotes cells grown in pH 4.5 YPD with sorbic acid and expressing pdr12 (adapted cells).

* Denotes reference strain used for rate comparisons.

** F/min: Initial rate of fluorescence per minute

University of Cape Town

CHAPTER 4

GENERAL DISCUSSION

Previously it has been shown that Hsp12 is upregulated in response to osmotic stress. This was confirmed using an Hsp12-GFP construct which showed that GFP fluorescence rapidly increased after the addition of mannitol and salt, osmolytes previously shown to upregulate Hsp12 (Mtwisha et al., 1998). Since Hsp12 has been found close to the plasma membrane and in the yeast cell wall in response to osmotic stress (Motshwene et al., 2004), we investigated whether permeability was changed in cells subjected to osmotic stress. We also investigated whether osmotic stress affects the ABC (ATP Binding Cassette) transporter Pdr12, which is situated in the plasma membrane (Holyoak et al., 1999). Since Pdr12 is induced by weak acid stress experiments were performed on cells that had been exposed to low pH-sorbate YPD media (Holyoak et al., 1999). Consequently all experiments were carried out using two different growth conditions in which wild-type and *hsp12* cells were grown in standard YPD (non-adapted cells) and pH 4.3 YPD with sorbic acid (adapted cells) Results from both growth conditions were compared independently as well as together.

A problem arose when interpreting the data from cells adapted to growth on sorbate because exposure to any stress including weak acid stress generates a global stress response in which many genes unrelated to that particular stress response are upregulated (Schuller et al., 2004). However, the global stress response usually occurs early during the stress exposure after which adapted cells display a different expression profile containing only essential genes (Schuller et al., 2004). Regulation of *PDR12* is controlled by the transcription factor War1 (Kren et al., 2003) and *PDR12* is the only gene upregulated by weak acid stress and essential for adaptation to growth under these conditions (Schuller et al., 2004). *HSP12* is induced by weak acid stress but regulated by the general stress response transcription factor Msn2/4 and *HSP12* is not required for sorbic acid resistance (Schuller et al., 2004). In our investigation adapted (weak acid stressed) wild-type cells already contained higher levels of Hsp12 prior to the exposure to

osmolytes. Similarly experiments with the GFP-Hsp12 showed that the fluorescence at time zero was 33 % higher in adapted cells indicating expression of Hsp12 during growth in sorbic acid. However after the addition of osmolytes the levels of GFP-Hsp12 fluorescence in non-adapted and adapted cells reached the same levels after 30 min.

Osmotic stress acts primarily at the plasma membrane of the cell during which rapid changes in cell volume due to the rapid exit and re-entry of water compromises the integrity of the plasma membrane (Guyot et al., 2006). There are two proposed mechanisms for cell damage after osmotic shock both of which lead to cell death (Mille et al., 2005). In the first case, the outflow of water from yeast cells in hypertonic media causes the plasma membrane to pull away from the cell wall, which creates invaginations in the plasma membrane. Upon rehydration, the decreased area of the plasma membrane and rigid cell wall prevents cell volume increase, which could then lead to permeabilisation and rupture of the plasma membrane (Gervais et al., 2003; Mille et al., 2005). In the second mechanism, the enhanced permeability of the plasma membrane after osmotic stress may be attributed to lipid phase transitions in the membrane (Lawaczeck et al., 1988; Beney and Gervais, 2001). Such phase transitions occur in localised areas due to the compositional heterogeneity of membranes and result in increased permeability and leakage of intracellular contents (Beney and Gervais, 2001; Mille et al., 2005). Previously it has been shown that Hsp12 protects membrane integrity during desiccation stress in vitro possibly by binding free water and intercalating between the phospholipids preventing membrane fusion (Sales et al., 2000). We investigated whether osmotic stress and Hsp12 affects yeast cell permeability using fluorescein diacetate (FDA) and rhodamine 6G (R6G). Non-adapted wild-type cells before and after the mannitol treatment were much less permeable to fluorescein diacetate than *hsp12* cells which suggested that the presence of Hsp12 may have maintained the integrity of the plasma membrane in wild-type cells during osmotic stress. Measurements of diffusion using R6G verified the higher permeability of non-adapted *hsp12* cells when compared to non-adapted wild-type cells prior to osmotic shock however, mannitol stress then reduced the permeability of R6G into the $\Delta hsp12$ cells. Based on previous research that suggests that in response to osmotic stress *hsp12* cells may compensate for the lack of

Hsp12 by increasing the level of chitin in the cell wall creating a more compact cell wall (Karreman et al., 2007). We suggest that influx of R6G is impeded into these cells after stress because of enhanced interactions between the positively charged R6G and the negatively charged cell wall components promoted by a less flexible and thicker cell wall of $\Delta hsp12$ cells. As a result, we could not see changes in diffusion of R6G across the plasma membrane, which may have been compromised during osmotic shock as a result of the lack of protection provided by Hsp12.

Overall, permeability of adapted cells for FDA and R6G was reduced which may be a consequence of morphological changes associated with adaptation to growth in weak acids. Adapted cells are generally more stress tolerant and may have thicker cell walls that would reduce permeability of substances into the cell (Park et al., 1997). Osmotic stress enhanced the rates of diffusion of both FDA and R6G into adapted wild-type cells. Permeability of adapted *hsp12* cells to FDA was also enhanced after stress however it was 38 % lower when compared to stressed wild-type cells. We have suggested that during the adaptation to growth in weak acids *hsp12* cells would undergo additional compensatory changes associated with the lack of Hsp12 and therefore be more stress tolerant than the adapted wild-type cells to osmotic shock. Also the protection exerted by Hsp12 in the wild-type cells may have reached a maximum during adaptation to weak acids and therefore the additional osmotic treatments were more detrimental to the integrity of the plasma membrane in adapted wild-type cells which showed higher permeability. Adapted *hsp12* cells prior to osmotic shock were also less permeable than wild-type cells to FDA, which supports our previous suggestion.

Next, we investigated the effect of osmotic stress on Pdr12-mediated efflux of fluorescein from adapted cells. We have found that contrary to published results (Holyoak et al. 1999) which showed no fluorescein efflux from cells grown in standard YPD (non-adapted cells), our non-adapted cells show ATP-dependent efflux of fluorescein which suggested the presence of another ABC transporter constitutively expressed in our lab strain. This was confirmed by RT-PCR, which showed no expression of Pdr12 in cells grown in standard YPD media. This will allow investigation into the effect of osmotic

stress and Hsp12 on the activity of the constitutively expressed transporter as well as the weak acid induced Pdr12 transporter

It has been suggested that the state of the membrane may affect the functioning of membrane transporters (Hohmann and Mager, 2003). We expected that cells expressing Pdr12 (adapted cells) would display overall higher rates of efflux when compared to non-adapted cells which was the case for the wild-type. However, adapted *hsp12* cells showed reduced efflux from Pdr12 when compared to efflux from the constitutively expressed ABC transporter in non-adapted *hsp12* cells. This suggested that the adaptive changes to weak acid stress together with the compensatory changes associated with the *HSP12* mutation might have adversely affected the functioning or expression of Pdr12 in adapted *hsp12* cells. Furthermore, both transporters (Pdr12 and the constitutively expressed ABC transporter) responded in a similar fashion to osmotic stress. Osmotic stress either had no effect or slightly enhanced the rate of efflux from wild-type cells which suggested that Hsp12 modulates the activity of transporters which could possibly help control cell volume changes during osmotic stress. Prior to osmotic shock *hsp12* cells displayed enhanced efflux when compared to wild-type cells which may compensate for their higher permeability. However, the stress treatments greatly reduced the rate of efflux from *hsp12* cells. It has been shown that although *hsp12* cells are more stress tolerant prior to heat shock, attributed to compensatory mechanisms, after heat shock treatment loss of viability is higher in *hsp12* cells when compared to the wild-type which has been attributed to the inability to upregulate Hsp12 during the subsequent stress (Pacheco et al., 2009). Therefore, we have suggested that the compensatory mechanisms in the *hsp12* cells enhance the activity of the membrane transporters prior to stress however, the absence of Hsp12 during osmotic shock adversely affects the pump either through loss of membrane integrity or through the loss of direct interactions between Hsp12 and the pump.

There are certain factors of our experimental conditions that may have affected the overall outcome of our experiments and will need to be investigated further. One of these factors is the use of 2-deoxyglucose, a non-metabolisable analogue of glucose (Barban

and Schulze, 1961), to inactivate the ABC transporters. Deoxyglucose enters glycolysis, is converted into deoxyglucose-6-phosphate by hexokinase, and blocks metabolism. This inhibition is reversible by the addition of glucose (Barban and Schulze, 1961; Svoboda et al., 1969). However it is fully understood how deoxyglucose affects the overall state of the cell and the effect on protein synthesis. Indications are that deoxyglucose inhibits cell growth by sequestering uridine nucleotides required for polysaccharide synthesis (Heredia et al., 1964; Biely and Bauer, 1967) and deoxyglucose also shown to induce lysis at certain sites of the cell wall (Megnet, 1965; Johnson, 1968). Nevertheless, this method of de-energisation is widely used despite these shortcomings. Another important factor is the weak acid stress treatment that was required to upregulated Pdr12. The results seemed to indicate possible adaptive changes in the cells however further analysis of the cell morphology will need to be carried out. Immuno-blotting could be used to determine the levels of Pdr12 and Hsp12 in adapted cells as well as to confirm the absence of Pdr12 in cells grown in standard YPD. In the future we will need to characterise the constitutively expressed ABC transporter in our lab strain.

CHAPTER 5

CONCLUSIONS

In the first part of this thesis an investigation into the effect of osmotic shock on diffusion into wild-type and *hsp12* cells have shown that the presence of Hsp12 may help maintain the integrity of the plasma membrane thereby reducing permeability after osmotic shock. Membrane fluidity changes associated with osmotic stress is another factor connected to membrane permeabilisation. Further studies could involve using fluorescence polarisation to measure changes in membrane fluidity in the wild-type and *hsp12* strain after osmotic shock treatments since Hsp12 is also upregulated in response to heat shock and ethanol stress both of which directly affect membrane fluidity.

In the second part of this thesis, we investigated the effect of osmotic stress on fluorescein efflux by the ABC transporter Pdr12. Previously it was shown that only cells subjected to weak acid stress show Pdr12-mediated efflux of fluorescein (Holyoak et al., 1999). However, we have shown Pdr12-independent efflux of fluorescein from the wild-type and *hsp12* strains grown under normal conditions, which suggest the presence of a constitutively expressed ABC transporter in our strains. This would allow a more direct investigation into the effect of osmotic stress and Hsp12 on monocarboxylic ABC transporters since this model does not require an additional stress treatment that is required for upregulation of Pdr12. Nevertheless, both the non-adapted and adapted cells displayed similar patterns of fluorescein efflux. Hsp12 appears to modulate the activity of the transporters in stressed wild-type cells, which displayed enhanced efflux. The reduced rates of efflux from the *hsp12* cells after osmotic shock, provides further support of a modulatory role for Hsp12.

Problems with interpretation of data associated with weak acid adapted cells may have been because of physical changes in the cells associated with adaptation to growth under those conditions. In addition, application of weak acid stress generates a global stress response, which includes upregulation of *HSP12* (Schuller et al., 2004). Taken together

these factors may affect how adapted cells respond to osmotic stress and possibly mask the effect of Hsp12.

The use of a knockout strain in our experiments was intended to provide a straightforward method to determine the function Hsp12, however in some cases we found certain contradictory results. Previous research has suggested that the *hsp12* strain employs compensatory mechanisms that enhances the stress tolerance of these cells and therefore mask the *HSP12* deletional mutation (Shamrock and Lindsey, 2008). We have provided further evidence for this with the data that showed enhanced rates of efflux from *hsp12* cells when compared to wild-type cells prior to stress treatments. These signified compensatory mechanisms in *hsp12* cells that provide a similar function to Hsp12 in modulating the activity of the transporters, however after the application of stress, *hsp12* cells display reduced transporter activity possibly linked to the absence of Hsp12.

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