





**THE MOLECULAR BASIS OF THE  
ETHANOL-STRESS RESPONSE IN THE  
YEAST *Saccharomyces cerevisiae***

by

**DIANNE EMSLIE**

**2002**

A thesis submitted for the degree of  
**DOCTOR OF PHILOSOPHY**

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The molecular basis of the  
ethanol-stress response in  
the yeast *Saccharomyces*

# DECLARATION

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



**DIANNE EMSLIE**

# ABSTRACT

For thousands of years, brewers and winemakers have used the yeast *Saccharomyces cerevisiae* to produce alcoholic beverages by fermentation of carbohydrates in grains, but recent large-scale industrial processes create an environment in which the microorganisms are stressed and unable to perform satisfactorily. Ethanol stress is one of the major physiological stresses encountered in industrial fermentations and is therefore the principal focus of investigations described in this thesis.

The presence of ethanol in fermenters inhibits yeast growth and fermentation capacity and limits the number of times yeast cells can be repitched. At a physiological level, ethanol-stressed cells exhibit numerous ethanol-induced changes in cell wall and cell membrane composition and structure, reduced activity of some cellular nutrient importers and increased activity of the plasma membrane proton pump. However, very little is known about the response of yeast cells to ethanol stress at the molecular level.

In this project, the gene expression of *S. cerevisiae* cells stressed in the presence of 5% (v/v) ethanol was compared to that of unstressed cells grown in the absence of added ethanol, using the comparative expression analysis technique, Differential Display. The traditional approach to Differential Display involves comparing mRNA profiles from control and test cells (or tissues) using equal quantities of RNA from both. However, in work described in this thesis it was found that the amount of RNA in ethanol-stressed cells was reduced compared to unstressed cells. Therefore the gene expression of stressed and unstressed cultures was compared by equalising samples according to cell number rather than by amount of RNA. This and several other modifications to the standard Differential Display procedure, including performing the analysis over a time-course, were aimed at increasing the efficiency of the method and reducing the possibility of generating false-positive results.

The performance of Time-course Differential Display resulted in the identification of several putative ethanol stress-induced genes, *YGPI*, *DIP5*, *MNN4* and *YER024w* (*YAT2*); *YGPI*, *DIP5* and *YER024w* were subsequently confirmed as ethanol stress-induced genes using Northern and/or RT-PCR analysis. To further investigate the phenotype of *YGPI*, knockout and over-expression strains were constructed and the growth profiles of these strains compared to the wildtype and rescue strain. Growth experiments failed to detect the *YGPI* phenotype, however, a bioinformatics approach involving an extensive analysis of published data relating to the expression of *YGPI*, *DIP5* and *YER024w* has enabled a model of the effects of ethanol stress on cellular metabolism to be proposed, including hypothetical roles for the above three genes.

# PUBLICATIONS AND PRESENTATIONS

The work described in this thesis has been presented at conferences in Australia and overseas.

## Oral presentations

- D. Emslie, G. Stanley, P. Chambers, (2000), A functional genomics approach to studying the ethanol stress response in *Saccharomyces cerevisiae*. Yeast: Products and Discovery, South Stradbroke Island, Queensland (oral presentation and poster).
- Emslie D., Fraser S., Stanley G., Chambers P., (1998), Characterizing Genes Associated with Ethanol Stress in the Yeast *Saccharomyces cerevisiae* Using Time-course Differential Display, 9<sup>th</sup> International Specialized Symposium on Yeasts, Braga, Portugal (oral presentation and poster).
- D. Emslie, G. Stanley, P. Chambers, (2001), Using differential display to identify ethanol stress-response genes in *Saccharomyces cerevisiae*, The Heart Research Institute, Camperdown, Sydney, NSW (oral presentation).

## Publication

- Emslie, Dianne; Stanley, Grant; Fraser, Sarah and Chambers, Paul (2000), Time-course differential display to identify ethanol stress-induced genes in *Saccharomyces cerevisiae*, The Secomb Papers, Victoria University Library.

## Poster presentations

- D. Emslie, G. Stanley, S. Fraser, P. Chambers, (1999), Time-course differential display to identify ethanol stress-induced genes in *Saccharomyces cerevisiae*, XIX International Conference on Yeast Genetics and Molecular Biology, Rimini, Italy.
- Emslie D., Fraser S., Stanley G., Chambers P., (1998), Characterizing Genes Associated with Ethanol Stress in the Yeast *Saccharomyces cerevisiae* Using Time-course Differential Display, Australian Society for Biochemistry and Molecular Biology Combined Conference, Adelaide, South Australia.
- Dianne Emslie, Grant Stanley, Sarah Fraser, Paul Chambers, (1998), Characterizing Genes Associated with Ethanol Stress in the Yeast *Saccharomyces cerevisiae*, 20<sup>th</sup> Annual Conference on the Organization and Expression of the Genome, Lorne, Victoria.

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# ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BLASTn	nucleotide BLAST search
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetriacetate
gDNA	genomic DNA
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
LTR	long terminal repeat
mDNA	mitochondrial DNA
MIPS	Munich Information Centre for Protein Sequences
mL	millilitre
MOPS	morpholinopropansulfonic acid
mRNA	messenger RNA
NCI	nitrogen catabolite inhibition
NCR	nitrogen catabolite repression
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDS	post diauxic shift element
PEG	polyethyleneglycol
RNA	ribonucleic acid
Rnase	ribonuclease
RNP	ribonuclear protein
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
SAGE	serial analysis of genome expression
SDS	sodium dodecyl sulfate
SGD	<i>Saccharomyces</i> Genome Database
SS-DNA	salmon sperm DNA
STRE	stress response element
TBS	tris buffered saline
TBST	tris buffered saline Tween 20 <sup>®</sup>
TRIS	tris-(hydroxymethyl)-aminomethane
V	volt
v/v	volume per volume
w/v	weight per volume

# TABLE OF CONTENTS

TITLE PAGE.....	i
DECLARATION.....	ii
ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	iv
ABBREVIATIONS.....	v
TABLE OF CONTENTS.....	vi
FIGURES.....	xiv
TABLES.....	xix
CHAPTER 1 .....	1
INTRODUCTION AND LITERATURE REVIEW .....	1
1.1    INTRODUCTION .....	1
1.1.1    Historical perspective.....	1
1.1.2    Yeast stress in modern brewing and winemaking.....	2
1.2    THE YEAST STRESS RESPONSE - OVERVIEW .....	3
1.2.1    Induction of stress response genes.....	3
1.2.2    Stress tolerance and cross-tolerance .....	5
1.2.3    The accumulation of trehalose and its role in the stress response .....	8
1.2.4    Metabolic stress: stress due to nutrient limitation.....	9
1.3    THE RESPONSE OF <i>S. cerevisiae</i> TO ETHANOL STRESS .....	10
1.3.1    The physiological response of <i>S. cerevisiae</i> to ethanol stress .....	10
1.3.2    The effects of ethanol on membrane transporters.....	12
1.3.3    The effect of ethanol on membrane composition .....	12
1.3.4    The effect of ethanol on membrane fluidity .....	15
1.3.5    The effect of ethanol on organelles and organellar membranes .....	16
1.3.6    The relationship between ethanol and oxidative stress.....	18
1.3.7    The genetic response of <i>S. cerevisiae</i> to ethanol stress .....	19
1.4    FACTORS INCREASING <i>S. cerevisiae</i> TOLERANCE TO ETHANOL ..	22
1.4.1    The influence of trehalose on ethanol tolerance .....	23
1.4.2    The effect of magnesium on ethanol tolerance.....	24

1.4.3	The effect of small amounts of acetaldehyde on the lag time of ethanol-stressed <i>S. cerevisiae</i> cultures.....	25
1.4.4	The influence of membrane composition on ethanol tolerance .....	25
1.4.5	The influence of assimilable nitrogen compounds on ethanol tolerance	27
1.4.6	Cell immobilization .....	27
1.5	REGULATION OF THE YEAST STRESS RESPONSE.....	29
1.5	TECHNIQUES FOR GENE EXPRESSION ANALYSIS: DIFFERENTIAL DISPLAY .....	35
1.6	AIMS OF RESEARCH.....	38
CHAPTER 2 .....		39
MATERIALS AND METHODS.....		39
2.1	MATERIALS.....	39
2.1.1	Microorganisms and plasmids .....	39
2.1.1.1	<i>Saccharomyces cerevisiae</i> .....	39
2.1.1.2	<i>Escherichia coli</i> .....	41
2.1.1.3	Plasmids .....	41
2.1.2	Solutions and buffers .....	43
2.2	MICROBIOLOGICAL METHODS.....	43
2.2.1	Growth media.....	43
2.2.1.1	Yeast growth media .....	43
2.2.1.2	<i>E. coli</i> growth media.....	45
2.2.2	Growth of yeast.....	46
2.2.2.1	Standard culture conditions.....	46
2.2.2.2	Growth conditions for yeast strains carrying a Genestorm™ vector.....	46
2.2.2.3	Growth of yeast on plates .....	46
2.2.2.4	Storage of yeast.....	46
2.2.2.5	Yeast inoculum preparation .....	47
2.2.2.6	Growth of yeast during ethanol stress.....	47
2.2.2.7	Cell harvesting and storage in ethanol stress experiments.....	47
2.2.2.8	Cell density and viability determination.....	48
2.2.3	Growth of <i>E. coli</i> .....	49
2.2.3.1	<i>E. coli</i> growth conditions .....	49

2.3	MOLECULAR METHODS .....	49
2.3.1	RNA isolation .....	49
2.3.1.1	Standard RNase-free procedures and conditions .....	49
2.3.1.2	RNA isolation .....	50
2.3.2	DNase treatment of RNA and acid phenol extraction .....	50
2.3.2.1	Non-denaturing RNA gel electrophoresis.....	51
2.3.3	Differential Display (Display Systems) .....	51
2.3.3.1	cDNA synthesis .....	52
2.3.3.2	Differential Display reactions .....	52
2.3.3.3	Differential Display gels .....	53
	Gel preparation.....	53
	Sample preparation .....	54
	Running gel.....	54
	Autoradiography .....	54
2.3.3.4	Band excision and re-amplification with modified primers .....	54
2.3.3.5	Sequencing of re-amplified gel bands.....	55
2.3.3.6	Identification of genes associated with Differential Display bands.....	56
2.3.4	Northern analysis .....	56
2.3.4.1	Denaturing gel electrophoresis .....	56
	Denaturing RNA gel preparation .....	56
	Sample preparation .....	56
	Electrophoresis.....	56
2.3.4.2	Northern blotting.....	57
2.3.4.3	Design of probes for Northern analysis .....	57
2.3.4.4	Labeling probes.....	58
2.3.4.5	Determining labeling efficiency by Cerenkov counts .....	58
2.3.4.6	Northern hybridization.....	59
2.3.4.7	Autoradiography of probed Northern blots.....	59
2.3.4.8	Sizing hybridization bands.....	59
2.3.5	RT-PCR analysis.....	60
2.3.5.1	cDNA synthesis .....	60
2.3.5.2	RT-PCR primer design .....	60
2.3.5.3	RT-PCR reactions .....	60
2.3.5.4	Gel electrophoresis of RT-PCR products .....	61

2.3.5.5 Sequencing RT-PCR products .....	61
2.3.6 Gene replacement.....	63
2.3.6.1 Primer design for gene replacement .....	63
2.3.6.2 PCR-amplification of the <i>kanMX</i> module from pFA6- <i>kanMX4</i> .....	63
2.3.6.3 Yeast transformation.....	64
2.3.6.4 Design of positioning primers to confirm gene replacement.....	65
2.3.7 Confirmation of gene replacement using colony PCR .....	65
2.3.8 Southern analysis .....	66
2.3.8.1 DNA isolation for Southern analysis .....	66
2.3.8.2 Restriction enzyme digest of DNA.....	66
2.3.8.3 Gel electrophoresis and Southern blotting.....	67
2.3.8.4 Probe preparation .....	67
2.3.8.5 Southern hybridization.....	68
2.3.8.6 Southern Hybridization detection, autoradiography and sizing bands ....	68
2.3.9 Plasmid transformation of knockout and wildtype yeast strains .....	69
2.3.10 Plasmid transformation of <i>E. coli</i> .....	69
2.3.11 Western analysis .....	70
2.3.11.1 Protein preparation.....	70
2.3.11.2 Determination of protein concentration using the Lowry assay .....	70
2.3.11.3 Protein de-glycosylation .....	71
2.3.11.4 SDS-PAGE protein gel analysis .....	71
2.3.11.5 Electrophoretic transfer and Western blotting .....	71
2.3.11.6 Detection of target protein .....	72
CHAPTER 3 .....	73
YEAST GROWTH DURING ETHANOL STRESS .....	73
3.1 INTRODUCTION .....	73
3.2 ETHANOL STRESS CONDITIONS AND STRAIN SELECTION .....	74
3.2.1 Selection of yeast strains.....	74
3.2.1.1 Growth of <i>S. cerevisiae</i> strains PMY1.1 and SUB61 .....	74
3.2.1.2 Effect of ethanol on the growth rate and doubling time of strains.....	75
PMY1.1 and SUB61 .....	75
3.2.2 Effect of ethanol concentration on the lag period of strain PMY1.1 ...	75
3.2.3 The relationship between optical density and viability of cells during lag phase	84

3.3	DISCUSSION .....	85
3.3.1	Effect of ethanol on the growth and metabolism of <i>S. cerevisiae</i> .....	85
3.3.2	Yeast adaptation to ethanol.....	88
3.3.3	Experimental systems used to examine the response of <i>S. cerevisiae</i> to ethanol stress.....	90
CHAPTER 4 .....		91
IDENTIFICATION AND PRELIMINARY CHARACTERISATION OF ETHANOL-STRESS RESPONSE GENES USING DIFFERENTIAL DISPLAY .....		91
4.1	INTRODUCTION .....	91
4.2	RNA PREPARATION.....	95
4.2.1	The effect of ethanol stress on the level of cellular rRNA .....	95
4.3	THE IDENTIFICATION OF PUTATIVE ETHANOL-STRESS RESPONSE GENES USING DIFFERENTIAL DISPLAY .....	98
4.3.1	<i>DIP5</i> : a putative ethanol-stress response gene.....	99
4.3.2	<i>YGP1</i> : a putative ethanol-stress response gene.....	104
4.3.3	<i>YER024w</i> : a putative ethanol-stress response gene .....	107
4.3.4	<i>MNN4</i> : a putative ethanol-stress response gene.....	110
4.3.5	Putative ethanol-stress-induced Ty elements.....	114
4.4	NORTHERN AND RT-PCR ANALYSES TO VALIDATE DATA FROM DIFFERENTIAL DISPLAY EXPERIMENTS.....	118
4.4.1	Is <i>DIP5</i> a real ethanol-stress response gene?.....	118
4.4.2	Is <i>YGP1</i> a real ethanol-stress response gene? .....	121
4.4.3	Is <i>YER024w</i> a real ethanol-stress response gene?.....	123
4.4.4	Is <i>MNN4</i> a real ethanol-stress response gene?.....	123
4.4.5	Ty elements in the <i>S. cerevisiae</i> ethanol-stress response.....	125
4.5	DISCUSSION OF RESULTS .....	125
4.5.1	Reduction in RNA level due to ethanol stress .....	125
4.5.2	Modified Differential Display.....	127
4.5.3	Streamlining validation procedures for differentially expressed genes	130
4.5.4	Ethanol-stress response gene transcripts.....	131
4.5.5	The putative ethanol-stress response gene <i>MNN4</i> .....	131
4.5.6	Ty elements.....	132

4.5.7	The influence of mRNA stability and copy number on the interpretation of the Differential Display results .....	133
4.5.8	Prior phenotypic studies of <i>DIP5</i> , <i>YGP1</i> and <i>YER024w</i> .....	136
CHAPTER 5 .....		138
ANALYSIS OF PHYSIOLOGICAL ROLE (S) OF <i>YGP1</i> .....		138
5.1	INTRODUCTION .....	138
5.2	CONSTRUCTION OF A <i>YGP1</i> KNOCKOUT USING <i>kanMX</i> MODULE REPLACEMENT .....	139
5.2.1	Selection, validation and stability of the <i>YGP1</i> knockout strain .....	139
5.2.2	Southern analysis of PMY1.1 and PMY1.1 $\Delta$ <i>YGP1</i> to confirm replacement of <i>YGP1</i> with the <i>kanMX</i> cassette .....	145
5.2.3	Characterising the <i>YGP1</i> phenotype during ethanol stress .....	148
5.3	THE <i>YGP1</i> RESCUE FOR CONTROLLED EXPRESSION OF <i>YGP1</i> IN ETHANOL-STRESSING CONDITIONS.....	148
5.3.1	Selection, validation and growth of pYES2 transformants.....	148
5.3.2	Confirmation of expression of gp37 by Western analysis .....	155
5.3.3	Growth of plasmid-bearing strains during ethanol stress .....	155
5.4	DISCUSSION.....	159
5.4.1	The <i>YGP1</i> phenotype .....	159
5.4.2	Further experiments that may reveal the role of <i>YGP1</i> in the ethanol-stress response.....	160
CHAPTER 6 .....		163
THE POSSIBLE ROLES OF <i>YGP1</i> , <i>DIP5</i> AND <i>YER024w</i> IN THE YEAST ETHANOL-STRESS RESPONSE .....		163
6.1	INTRODUCTION .....	163
6.2	A PUTATIVE PHENOTYPE FOR <i>YGP1</i> .....	164
6.2.1	Predictions regarding <i>YGP1</i> regulation and function .....	164
6.2.2	<i>YGP1</i> expression.....	165
6.2.2.1	Cell surface .....	165
6.2.2.2	Diauxic shift.....	167
6.2.2.3	Nutrient limitation or slow growth?.....	168
6.2.2.4	General nutrient limitation? .....	169
6.2.2.5	Stationary phase .....	170

6.2.3	<i>YGP1</i> transcript stability .....	170
6.3	REGULATION OF <i>YGP1</i> .....	172
6.3.1	The evolution in <i>YGP1</i> expression during carbon limitation.....	172
6.3.2	What is known about the <i>YGP1</i> promoter?.....	172
6.3.2.1	Putative stationary phase elements .....	173
6.3.2.2	Msn2p and Msn4p-dependent STRE regulation.....	173
6.3.2.3	The transcription factor Mcm1p and cell cycle regulation .....	175
6.3.2.4	Nitrogen catabolite repression and GATAA elements .....	176
6.3.2.5	The PDS (post diauxic shift element) and HSE (heat shock element) ..	176
6.3.3	<i>YGP1</i> regulation: experimental evidence.....	177
6.3.3.1	<i>YGP1</i> expression in the multidrug resistance response .....	177
6.3.3.2	<i>YGP1</i> regulation by the yeast cell wall integrity signaling pathway .....	179
6.3.3.3	<i>MSN2/MSN4</i> -dependent expression in response to heat shock and oxidative stress.....	179
6.3.3.4	Is <i>YGP1</i> expression <i>CDC28</i> -dependent? .....	180
6.3.3.5	The rye (defective for the regulation of <i>YGP1</i> expression) mutants .....	181
6.3.3.6	Response of <i>S. cerevisiae</i> to short-term ethanol stress .....	181
6.3.4	A possible role for <i>YGP1</i> in yeast cells .....	182
6.3.5	The nutrient limitation effect of ethanol and role of <i>YGP1</i> in the ethanol-stress response.....	183
6.4	THE POSSIBLE ROLE OF <i>DIP5</i> IN THE ETHANOL-STRESS RESPONSE.....	184
6.4.1	What is known about <i>DIP5</i> from the literature?.....	184
6.4.2	What is known about <i>DIP5</i> expression?.....	185
6.4.3	<i>DIP5</i> regulation.....	188
6.4.4	What role does <i>DIP5</i> play in the ethanol-stress response? .....	189
6.5	THE POSSIBLE ROLE OF <i>YER024w</i> ( <i>YAT2</i> ) IN THE ETHANOL- STRESS RESPONSE .....	190
6.5.1	What is known about <i>YER024w</i> ( <i>YAT2</i> ) function, expression and regulation?.....	190
6.5.2	What role does <i>YAT2</i> play in the ethanol-stress response?.....	193
CHAPTER 7 .....		199
CONCLUDING REMARKS AND FUTURE STUDIES .....		199
7.1	CONCLUSIONS.....	199

7.2	FUTURE EXPERIMENTAL WORK .....	201
7.2.1	Experiments to clarify the roles of ethanol-stress response genes.....	201
7.2.1.1	Further physiological experiments to determine the <i>YGP1</i> .....	201
	phenotype.....	201
7.2.1.2	Experiments to investigate the <i>DIP5</i> and <i>YAT2</i> phenotypes .....	202
7.2.2	Further characterisation of the <i>S. cerevisiae</i> ethanol-stress response	202
APPENDIX I	.....	204
	SOLUTIONS AND BUFFERS .....	204
	ENZYMES, MOLECULAR WEIGHT STANDARDS AND MOLECULAR	
	BIOLOGY KITS.....	210
	SUPPLIERS.....	211
APPENDIX II	.....	213
	PRIMERS AND PROBES.....	213
APPENDIX III	.....	216
APPENDIX IV	.....	221
REFERENCES	.....	228

# FIGURES

	PAGE
<b>Figure 1.1:</b>	
Diagram of stress response pathways in <i>S. cerevisiae</i> .	30
<b>Figure 2.1:</b>	
Diagrammatic view of plasmid pYES2/GS.	42
<b>Figure 2.2:</b>	
Diagram showing YGP1 knockout strategy using the <i>kanMX</i> module.	62
<b>Figure 3.1</b>	
Growth of <i>S. cerevisiae</i> strains PMY1.1 and SUB61 in YEPD medium.	76
<b>Figure 3.2</b>	
Effect of 4% (v/v) ethanol on the growth of <i>S. cerevisiae</i> strains PMY1.1 and SUB61.	77
<b>Figure 3.3</b>	
Effect of 2-10% (v/v) ethanol on the growth of <i>S. cerevisiae</i> PMY1.1.	80
<b>Figure 3.4</b>	
Effect of 3-7% (v/v) ethanol on the growth of <i>S. cerevisiae</i> PMY1.1.	81
<b>Figure 3.5</b>	
Effect of 5 and 10% (v/v) ethanol on the growth of <i>S. cerevisiae</i> PMY1.1.	82
<b>Figure 3.6</b>	
Relationship between optical density and viable counts when <i>S. cerevisiae</i> PMY1.1 is grown in the presence or absence of 5% (v/v) ethanol.	86
<b>Figure 4.1</b>	
Differential Display flow chart.	92
<b>Figure 4.2</b>	
RNA level is reduced in ethanol-stressed cells.	96
<b>Figure 4.3</b>	
RNA level and integrity is reduced in unstressed cells early in the time course. RNA level and integrity is not affected appreciably by the addition of 2% or 3% ethanol.	96

**Figure 4.4**

Triplicate DNase-treated RNA isolations from  $10^8$  cells show consistency of the RNA isolation method. 97

**Figure 4.5**

The relatively constant amount of DNA in RNA preparations indicates that the extraction of nucleic acids was independent of ethanol stress and culture incubation time. 97

**Figure 4.6**

Identification of the differentially expressed Differential Display band representing the *DIP5* gene. 101

**Figure 4.7**

*DIP5* gene sequence and Differential Display band alignment. 103

**Figure 4.8**

Identification of the differentially expressed Differential Display band representing the *YGP1* gene. 105

**Figure 4.9**

*YGP1* gene sequence and Differential Display band alignment. 106

**Figure 4.10**

Identification of the differentially expressed Differential Display band representing the *YER024w* ORF. 108

**Figure 4.11**

*YER024w* ORF sequence and Differential Display band alignment. 109

**Figure 4.12**

Identification of the differentially expressed Differential Display band representing the *MNN4* gene. 111

**Figure 4.13**

*MNN4* gene sequence and Differential Display band alignment. 112

**Figure 4.14**

Identification of the differentially expressed Differential Display band related to a Ty2 element. 115

**Figure 4.15**

Diagram representing the relationship of the differentially expressed band to a Ty2 element and *YGR161c* on *S. cerevisiae* chromosome VII. 116

	PAGE
<b>Figure 4.16</b>	
Identification of the differentially expressed Differential Display band related to a Ty1 element.	117
<b>Figure 4.17</b>	
Validation of <i>DIP5</i> as an ethanol stress-response gene by Northern and RT-PCR analyses.	119
<b>Figure 4.18</b>	
Validation of <i>YGPI</i> as an ethanol stress-response gene by Northern and RT-PCR analyses.	122
<b>Figure 4.19</b>	
Validation of <i>YER024w</i> as an ethanol stress-response gene by RT-PCR analysis.	124
<b>Figure 4.20</b>	
<i>MNN4</i> could not be confirmed as an ethanol stress-response gene using RT-PCR.	124
<b>Figure 5.1:</b>	
Ethidium bromide-stained gel of PCR-amplified <i>kanM</i> module.	141
<b>Figure 5.2:</b>	
Diagram depicting the PMY1.1 <i>YGPI</i> knockout construct in comparison to the PMY1.1 wildtype with positioning primer sites and sizes of expected PCR products.	143
<b>Figure 5.3:</b>	
Colony PCR of transformant colonies using positioning primers.	144
<b>Figure 5.4:</b>	
Colony PCR performed on a transformant colony and wildtype colonies as controls using flanking positioning primers.	144
<b>Figure 5.5:</b>	
PCR products from DNA isolated from PMY1.1 <i>YGPI</i> knockout and PMY1.1 wildtype using primers flanking the <i>YGPI</i> ORF.	144
<b>Figure 5.6:</b>	
Genomic DNA from PMY1/1 wildtype and PMY1.1 <i>YGPI</i> knockout digested with restriction enzymes and resolved on duplicate 1% agarose gels.	147

**Figure 5.7:**

Southern blots from restriction digests of PMY1.1 wildtype and PMY1.1 *YGP1* knockout probed with a *YGP1*-specific probe and a *kanMX*-specific probe.

147

**Figure 5.8a:**

Growth of PMY1.1 wildtype and PMY1.1 *YGP1* knockout in the presence of 0%, 7% and 12% (v/v) ethanol.

149

**Figure 5.8b:**

Growth of PMY1.1 wildtype and PMY1.1 *YGP1* knockout in the presence of 0%, 5% and 10% (v/v) ethanol.

150

**Figure 5.9:**

Death curves of *S. cerevisiae* strains PMY1.1 wildtype and PMY1.1 *YGP1* knockout in the presence of a lethal ethanol concentration.

151

**Figure 5.10:**

Colony PCR to confirm the presence of pYES2/*YGP1* in PMY1.1 *YGP1::kanMX* pYES2/*YGP1* (rescue strain).

153

**Figure 5.11:**

Growth of PMY1.1 wildtype, PMY1.1 *YGP1* knockout and PMY1.1 wildtype and knockout transformed with pYES2/*YGP1* under non-inducing conditions.

154

**Figure 5.12:**

Western analysis of PMY1.1 *YGP1::kanMX* pYES2/*YGP1* (rescue strain) and PMY1.1 *YGP1::kanMX* pYES2/GS (rescue control strain) for identification of gp37.

156

**Figure 5.13:**

Growth of PMY1.1 *YGP1* pYES2/*YGP1* rescue strain in 4% raffinose minimal medium and in 4% raffinose 2% galactose minimal medium.

157

**Figure 5.14:**

Growth of PMY1.1 *YGP1* pYES2/*YGP1* and rescue control strain PMY1.1 *YGP1* pYES2/GS (plasmid without insert) under inducing conditions in the presence and absence of 5% (v/v) ethanol.

158

	PAGE
<b>Figure 6.1:</b> The 1000 bp promoter region of <i>YGP1</i> indicating putative regulatory elements.	174
<b>Figure 6.2:</b> Cellular location of the proteins involved in carnitine-dependent metabolic activities.	192
<b>Figure 6.3:</b> Diagram showing metabolic pathways essential for ethanol utilization.	194
<b>Figure 6.4:</b> Diagram representing the increasing contribution of ethanol to carbon metabolism in carbon-limited cultures.	196

# TABLES

<b>Table 1.1:</b>	PAGE
<i>S. cerevisiae</i> Heat Shock Proteins.	4
<b>Table 1.2:</b>	
<i>S. cerevisiae</i> genes expressed in response to ethanol stress.	20
<b>Table 1.3:</b>	
Consensus binding sites for stress-related promoter motifs in <i>S. cerevisiae</i> .	31
<b>Table 2.1:</b>	
<i>S. cerevisiae</i> strains used for the work described in this thesis	40
<b>Table 3.1</b>	
Effect of 4% (v/v) exogenous ethanol on the specific growth rates and generation time of <i>S. cerevisiae</i> strains PMY1.1 and SUB61 grown in YEPD medium.	78
<b>Table 3.2</b>	
Effect of 2-8% (v/v) exogenous ethanol on the lag period, specific growth rate and generation time of <i>S. cerevisiae</i> strain PMY1.1 grown in YEPD medium.	83
<b>Table 4.1</b>	
Summary of Differential Display experiments to identify ethanol stress-response genes and their validation.	100
<b>Table 4.2</b>	
The expression level of ethanol stress-response genes <i>DIP5</i> , <i>YGP1</i> and <i>YER024w</i> , putative ethanol stress-response gene <i>MNN4</i> and control gene <i>ACT1</i> .	135
<b>Table 4.3</b>	
The abundance of mRNA species and classes in a typical mammalian cell.	135
<b>Table 5.1:</b>	
PCR primers for replacement of <i>YGP1</i> ORF with <i>kanMX</i> module and primers to determine correct insertion.	140
<b>Table 5.2:</b>	
Stability of the $\Delta YGP1$ mutation in PMY1.1.	146
<b>Table 6.1:</b>	
YGP1 induction and signaling pathways.	178

# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

### 1.1 INTRODUCTION

#### 1.1.1 Historical perspective

The yeast *Saccharomyces cerevisiae*, used to brew beer by fermenting the sugars in wheat and barley, was probably the first “domesticated” microorganism. It has even been suggested that the effects of the alcohol produced by wild yeasts in the “first brews” convinced our hunter-gatherer ancestors to give up their nomadic lifestyle and settle down as grain producers (Protz 1998). Archaeological evidence suggests that beer-making was already a sophisticated and important industry in ancient Egypt, since a large beer-brewing complex was found beneath the Sun Temple of Queen Nefertiti at Tell el Armana, circa 1550-1100 BC (Protz 1998). At this stage beer may have been brewed primarily for ritual purposes by powerful ruling groups, but by medieval times, or even earlier, the advanced technology had spread to the cooler regions of northern Europe and the British Isles where monks and housewives brewed beer in their kitchens.

As well as its valuable alcohol content, beer was probably an important source of vitamins and calories and, in industrialized Europe, was likely to have been more potable than the water supply. The keeping quality of beer depends in part on its alcohol content; the higher the alcohol content the longer it keeps, and fermentations producing high percentage alcohol are commercially desirable in present day brewing processes as well as for fuel alcohol production. However, the productivity of fermentations performed on an industrial scale is compromised by many factors including the inability of yeast to tolerate a range of physiological stresses associated with large-scale operations.

### 1.1.2 Yeast stress in modern brewing and winemaking

The role of yeast in brewing was not recognized until relatively recently. In the past yeasts were simply transferred unknowingly from brew to brew in the cracks of wooden fermentation vessels or else a little of the previous brew was saved to be used as a starter for the next. Brewing yeasts have thus been selected over hundreds or thousands of years by continuous re-pitching (the re-use of yeast as starter for the next brew) and presumably this selection has led to the acquisition of adaptations to stresses encountered during the brewing process.

Much of the recent brewing-related yeast research has focused on the ability of brewing yeasts to withstand stresses related to the high volume processing conditions of modern brewing technology. For example, high-gravity brewing utilizes high-density substrates in fermentation vessels that are several storeys high, thus yeast cells are subjected to high osmotic and hydrostatic pressures as well as high concentrations of ethanol and other fermentation products. Brewing yeasts are also subjected to rapid temperature changes and shear forces as they pass through heat exchangers, cold stress when stored at 4°C and acid stress when acid washed to kill contaminating microorganisms prior to re-pitching. Such stressful environmental conditions for re-pitched yeast presumably contribute to a loss of cell viability and vitality (ability to perform in fermentations) and possibly to a reduction in the number of times the organisms can be re-pitched (pers.com. Hawthorne (1996)).

Yeasts that ferment the sugars in grapes in the winemaking industry are similarly exposed to high concentrations of sugar substrates and in addition are prone to become nutrient limited, particularly for nitrogen, as the wine fermentation progresses. There is some evidence to suggest that the cumulative effect of these various stresses during ethanol production may be more deleterious to cells than each stress imposed individually. For example the toxic effect of ethanol on yeast cells is enhanced at elevated temperature (van Uden 1984). Ethanol stress is considered to be one of the most important chemical stresses in industrial *S. cerevisiae* fermentations and is usually present concurrently with other stresses. The effects of ethanol on yeast

cells and the adaptive response of the cells to ethanol stress are therefore of huge commercial significance.

## 1.2 THE YEAST STRESS RESPONSE - OVERVIEW

*S. cerevisiae* strains used for brewing, baking and wine making are intrinsically tolerant to a range of extreme conditions and, in addition, brewing and winemaking strains are tolerant to a level of ethanol that is toxic to many competing microorganisms. *S. cerevisiae* exhibits characteristic adaptive responses to a variety of stress conditions, the principal ones being: ethanol stress, oxidative stress, osmotic stress (salt and carbohydrate), pressure stress (atmospheric), temperature stress and nutrient depletion (for reviews see Hohmann and Mager (1997); Mager and Moradas Ferreira (1993); Welch (1993)). Exposure of yeast cells to such stresses decreases their metabolic rate and, depending on the severity of the stress, may stop growth, eventually leading to loss of viability.

### 1.2.1 Induction of stress response genes

At a molecular level, cells exposed to stress cease the transcription of numerous genes but typically increase synthesis of several of the well-characterized stress proteins (also called heat-shock proteins (hsp)) that play a protective role in stress adaptation. Changes in protein profiles in *S. cerevisiae* following a heat shock were first reported by Miller *et al.* (1979) and reviewed by Craig (1992). The cellular location and function of *S. cerevisiae* heat shock (stress) proteins are presented in Table 1.1. The heat shock response is rapid but transitory and highly conserved across all species of bacteria, fungi, plants and animals (see reviews by Lindquist (1991); Watson (1990)). The heat shock response is the most extensively studied stress response in yeast but other stresses such as exposure to ethanol (Plesset *et al.* 1982) and hydrogen peroxide (Collinson and Dawes 1992) have also been investigated at protein level and have been found to generate specific and characteristic stress protein profiles.

Many stress-induced proteins are expressed constitutively at low levels suggesting they may have fundamental roles in the cell. This is likely since most hsp are molecular chaperones, refolding proteins denatured by heat or preventing misfolding

STRESS PROTEIN	CELLULAR LOCATION	FUNCTION	REFERENCE
Hsp150	cell wall	cell wall structure	(Russo <i>et al.</i> 1993)
Hsp104	nucleolus	stress tolerance	(Glover & Lindquist 1998; Sanchez <i>et al.</i> 1992)
Hsp83/82/90	cytosol/nucleus	chaperone	(Lindquist 1991)
Hsp70:			
Ssa1	cytosol	chaperone	(Slater & Craig 1989a)
Ssa2	cytosol	chaperone	(Slater & Craig 1989a)
Ssa3	cytosol	chaperone	(Boorstein & Craig 1990b; Ivorra <i>et al.</i> 1999)
Ssa4	cytosol	chaperone	(Boorstein & Craig 1990a; Werner-Washburne <i>et al.</i> 1989)
Ssb1	unknown	unknown	(Craig <i>et al.</i> 1990; Slater & Craig 1989b)
Ssb2	unknown	unknown	(Craig, Kang & Boorstein 1990)
ssc1	mitochondria	chaperone	(Craig <i>et al.</i> 1987)
Ssd1(Kar2)	ER	chaperone	(Rose <i>et al.</i> 1989; Vogel <i>et al.</i> 1990)
Sse1	cytoplasm	cochaperone	(Mukai <i>et al.</i> 1993)
Sse2	cytoplasm	protein folding	(Mukai <i>et al.</i> 1993)
Ssq1	mitochondria	chaperone	(Schilke <i>et al.</i> 1996)
Hsp60	mitochondria	chaperone	(Cheng <i>et al.</i> 1989)
Hsp30	plasma membrane	regulates H <sup>+</sup> ATPase	(Panaretou & Piper 1992; Regnacq & Boucherie 1993)
Hsp26	cytosol/nucleus	perinuclear	(Petko & Lindquist 1986)
Hsp12	membrane	membrane protection	(Ivorra, Perez-Ortin & Del Olmo 1999; Praekelt & Meacock 1990)
Ddr2			(McClanahan & McEntee 1986)
Ubiquitin	cytosol	protein degradation	(Finley <i>et al.</i> 1987)
Enolase (Hsp48)	cytosol	glycolysis	(Iida & Yahara 1985)
Glyceraldehyde- 3-phosphate dehydrogenase (Hsp35)	cytosol	glycolysis	(Craig 1992)
Phosphoglycerate kinase	cytosol	glycolysis	(Piper <i>et al.</i> 1986)
Catalase T	cytosol	antioxidative defence	(Hortner <i>et al.</i> 1982)

**Table 1.1:** *S. cerevisiae* Heat Shock Proteins

of nascent proteins (reviewed by Gething and Sambrook (1992)). There are several families of stress proteins in *S. cerevisiae* with principal roles in polypeptide folding, protein translocation, resolubilization of denatured proteins, maintenance of membrane integrity and possibly gene regulation. They have been implicated in all major growth processes in yeast such as cell division, DNA synthesis, transcription and translation, protein processing and membrane function (see review by Mager and Moradas Ferreira (1993)).

### 1.2.2 Stress tolerance and cross-tolerance

As well as enabling cells to tolerate sub-lethal stress conditions, the induction of stress proteins following a sub-lethal stress has been implicated in the elevated survival rate of cells exposed to a potentially lethal stress. For example yeast cells grown at 23°C develop tolerance to a lethal temperature of 51°C following incubation for 20 minutes at 37°C (Plesset, Palm and McLaughlin 1982). However, a brief temperature shock not only increases thermotolerance but also induces cross-tolerance to other stressors such as ethanol (Watson and Cavicchioli 1983), high salt concentration and freezing (Lewis *et al.* 1995). Heat shocked cells treated with the protein synthesis inhibitor cycloheximide, maintain their induced salt tolerance and thermotolerance, although at a reduced level, but tolerance to freezing is lost, suggesting that *de novo* protein synthesis is required for freeze-tolerance and for maximal salt and thermotolerance (Lewis, Learmonth and Watson 1995).

Stress tolerance may be induced by a variety of stress challenges apart from heat shock. For example, yeast cells conditioned by osmotic dehydration consisting of exposure to a non-lethal osmotic stress, not only become osmo-tolerant but also acquire thermotolerance (Trollmo *et al.* 1988). In addition to thermotolerance and osmotolerance, Lewis, Learmonth and Watson (1995) found that salt-shocked cells acquire increased tolerance to freezing although treatment with cycloheximide was found to reduce heat and salt tolerance, and freezing tolerance was absent. Thus, protein synthesis again seems to be required for freeze tolerance but is not essential for tolerance to heat and salt. Therefore hsp's are not wholly responsible for stress tolerance induction under these conditions.

Thermotolerance may also be acquired by pre-exposure of cells to a non-lethal ethanol concentration. Yeast cells incubated with up to 9% (v/v) ethanol acquire thermotolerance and this correlates to the production of a set of proteins that appear to be identical to those produced by heat shock (Plesset, Palm and McLaughlin 1982). In the reverse situation, a sub-lethal heat shock leads to a subsequent increase in ethanol tolerance (Costa *et al.* 1993; Watson and Cavicchioli 1983). However, when cells are concurrently heat-stressed and ethanol-stressed they lose viability at a greater rate than if exposed to each separately (van Uden 1984). The reason for this effect is unknown but there seems to be a connection between the yeast response to heat shock and ethanol stress since pre-exposure to alcohols lowers the temperature required for maximum activation of promoters with a heat shock element (Curran and Khalawan 1994). When *S. cerevisiae* is exposed to a heat shock, genes containing a heat shock element (HSE) in their promoters are activated (Kobayashi and McEntee 1993). Between 33°C and 37°C, exponentially growing cells had a 3-5 fold higher level of heat shock element (HSE) -controlled  $\beta$ -galactosidase induction in the presence of 2.6% ethanol than cells not exposed to ethanol suggesting a synergistic relationship between heat and ethanol-stress responses (Curran and Khalawan 1994).

The temperature of industrial fermentations frequently becomes elevated leading to “stuck” fermentations, loss of cell viability and a reduced final ethanol concentration (Casey and Ingledew 1986). One possible explanation for these effects is that increased temperature reduces ethanol tolerance. Odumeru *et al.* (1992) grew a *Saccharomyces uvarum* lager brewing strain under high gravity wort conditions and tested its heat and ethanol tolerance (5 minute at 40°C or 45°C or 15 minutes incubation with 15% ethanol) at intervals during fermentation. As the ethanol concentration of the wort increased the cells became less ethanol and heat shock tolerant as measured by decreasing viability. However when the cells were washed prior to viability testing the decrease in viability was not observed. The authors suggested that the removal of ethanol by washing increased the resistance of the cells to heat shock and ethanol shock but it is possible that other media components also influenced stress tolerance.

The role of hsps in the development of thermotolerance is complex and experimental results and conclusions have often been conflicting. At some temperatures the presence of specific hsps has actually been correlated with loss of thermotolerance (see reviews by Parsell and Lindquist (1993); Piper (1993); Singer and Lindquist (1998); Watson (1990)). Some discrepancies in results can be partially attributed to variations in experimental procedures and the different *S. cerevisiae* strains used. Another reason may be that the kinetics of stress protein synthesis/degradation and their relationship to stress tolerance have been given little consideration (Watson 1990). Analysis of heat shock protein function by gene disruptions in *S. cerevisiae* has revealed that, although numerous hsps are induced by stress, these proteins do not necessarily influence thermotolerance appreciably (Piper 1993). Whilst there is some evidence to show that expression of certain hsps leads to thermotolerance and tolerance to other stresses (e.g. Hsp104 (Sanchez *et al.* 1992)), the experimental temperature of the sub-lethal heat shock and subsequent stress may influence the cellular stress response. For example, the *S. cerevisiae* Hsp70 proteins are not essential for tolerance to extreme temperatures (50°C) either with or without pre-induction by heat shock (Werner-Washburne *et al.* 1987) and mutational analysis of Hsp90 indicates that it is not required for tolerance to extreme temperatures (Borkovitch *et al.* 1989). Moreover, over-expression of Hsp90 led to a strain-specific reduction in thermotolerance at high temperature (39°C) and a reduction in growth rate at 37.5°C (Cheng *et al.* 1992). In addition, thermotolerance is induced by heat shock in the absence of *de novo* protein synthesis, suggesting that high levels of hsps may not be required for thermotolerance (Hall 1983). However, this result may be partly explained by the model in which Hsp 70 is constitutively present in cells bound in an inactive form to the heat shock factor (HSF) at the site of the heat shock element (HSE) until it is released upon exposure to stress.

From the work described here it appears that, whilst our knowledge of the stress response in yeasts is rudimentary, many yeast stress responses share common features and it is likely that, despite conflicting evidence, some stress-response proteins are required for adaptation to stress. However, as well as inducing the synthesis of stress proteins, stress has been observed to cause other changes in the cell. For example, yeast cells exposed to a salt shock of 300 mM NaCl for 45 minutes accumulate

glycerol (Lewis, Learmonth and Watson 1995) and heat-shocked cells accumulate trehalose (Hottiger *et al.* 1987) thus the accumulation of trehalose and glycerol may also contribute to stress tolerance.

### **1.2.3 The accumulation of trehalose and its role in the stress response**

Piper (1993) suggested that physiological changes such as pH decline and the accumulation of trehalose accompanying heat stress might be more important than hsps in the development of thermotolerance. Trehalose is thought to act principally as a reserve carbohydrate but also as a stress protectant (Wiemken 1990). Trehalose is a non-reducing disaccharide that generally accumulates in cells during periods of adverse growth conditions such as starvation, heat shock, hyperosmotic shock, freeze-drying and desiccation (reviewed in Piper (1993); Van Laere (1989)). It also accumulates when cells are exposed to ethanol, copper sulphate or hydrogen peroxide but declines rapidly after the stress is removed (Attfield 1987). There is also a rapid breakdown of trehalose, releasing glucose, following the addition of nutrients (including glucose itself) to stationary phase cells (Wiemken 1990). The loss of accumulated trehalose may signify loss of stress tolerance since van Dijck *et al.* (1995) found that the breakdown of trehalose that occurs when sugar is added to bakers yeast correlates with a decline in stress tolerance.

The evidence for trehalose as a stress protectant and as a factor in stress tolerance is strong. Trehalose accumulation has been implicated in osmotolerance (Hounsa *et al.* 1998), dehydration tolerance and ethanol tolerance (Eleutherio *et al.* 1995), heat and desiccation tolerance (Hottiger, Boller and Wiemken 1987), and tolerance to dehydration, freezing and ethanol shock (Kim *et al.* 1996). The accumulation of trehalose in the presence of ethanol was also correlated to acetic acid tolerance and, interestingly, endogenous ethanol induced a higher level of trehalose and a higher level of acetic acid tolerance than added ethanol (Arneborg *et al.* 1997).

The importance of trehalose in induced stress tolerance has been argued by Coote *et al.* (1992) since trehalose accumulation in late exponential phase cells was observed to be correlated with stress-induced thermotolerance. However, the addition of protein synthesis inhibitors reduced the level of trehalose therefore *de novo* trehalose

synthesis was thought to be responsible for the acquired thermotolerance. The author proposed that the increased level of stress-induced trehalose is due to the activity of trehalose-6-phosphate synthase encoded by *TPS1*. This is likely since disruption of *TPS1* prevents cells synthesizing trehalose and severely compromises their thermotolerance (De Virgilio *et al.* 1993).

The role of trehalose in stress tolerance however is unknown. It has been proposed that trehalose acts as a membrane protectant, an energy reserve or a compatible solute. Hottiger *et al.* (1994) found that trehalose increases thermal stability of proteins and reduces heat induced protein aggregates *in vitro* and it was more effective than other sugars in stabilizing the tertiary structure and activity of enzymes when heated at 50°C (Sola-Penna and Meyer-Fernandes 1998). How trehalose behaves *in vivo* however, is unclear although it is known to stabilize cell membranes (Crowe *et al.* 1988) and its content in yeast cells is inversely correlated to heat-induced membrane fluidity as determined by whole cell NMR (Iwahashi *et al.* 1995). Trehalose accumulation on both sides of the plasma membrane is thought to stabilise cell membranes by reducing membrane permeability thus protecting cell membranes against ethanol-induced water stress (Hallsworth *et al.* 1998). In line with this membrane stabilization, the inhibition of endocytosis that occurs in the presence of ethanol is reduced by trehalose accumulation (Lucero *et al.* 2000).

#### **1.2.4 Metabolic stress: stress due to nutrient limitation**

Many researchers have found that nutrient limitation or nutrient starvation leads to induction of a stress tolerant state that is very similar to a general cellular stress response (see review by de Winde *et al.* (1997)). Limitation of an essential nutrient causes yeast cells to arrest in G1 of the cell cycle whilst they reprogram their metabolic response to the limitation, whereas starvation for one or more nutrients causes cells to enter stationary phase or G0. Stationary phase cells are required to re-enter the cell cycle at G1 where nutrient sensing occurs but progression through G1 will not occur without sufficient resources to complete the cycle, thus growth phase and nutrient limitation are linked.

Carbon is the most common limiting nutrient in yeast fermentations but yeast cells may also experience nitrogen, phosphate or sulphate limitation. Respiring cells are more stress tolerant than fermentative cells and cells grown on fermentable carbon sources such as glucose are stress sensitive in exponential growth but acquire stress resistance at diauxic shift when they switch to growth on ethanol, a non-fermentable carbon source, and when approaching stationary phase. In diauxic and post diauxic cultures glucose-repressed genes are de-repressed, including the heat shock proteins *HSP12*, *SSA3*, *CTT1*, *DDR2* and *UBI4* and cells accumulate glycogen and trehalose. The characteristics of yeast cells that enter stationary phase when starved for one or more nutrients are very similar to those of cells growing exponentially on a non-fermentable carbon source such as ethanol or glycerol. Stationary phase cells accumulate elevated levels of glycogen and trehalose and stress protein expression increases whilst most protein synthesis is repressed. Stress resistance, particularly thermotolerance, is increased and the cells become resistant to cell wall lysis (see reviews by de Winde, Thevelein and Winderickx (1997); Fuge and Werner-Washburne (1997); Werner-Washburne *et al.* (1993)).

Interestingly, there appears to be a difference between the starvation-induced arrest brought about by slow depletion of resources and the nutrient limitation that occurs when cells are directly shifted to a low nutrient medium. This is illustrated by the expression of two genes upregulated in stationary phase, *SNZ1* (Braun *et al.* 1996) and *YGP1* (Destruelle *et al.* 1994). Both are induced at low glucose levels during stationary phase and diauxic shift respectively but not when transferred directly to low glucose growth media.

### **1.3 THE RESPONSE OF *S. cerevisiae* TO ETHANOL STRESS**

#### **1.3.1 The physiological response of *S. cerevisiae* to ethanol stress**

The inhibitory effect of ethanol on yeast fermentation performance, growth and metabolism has been the topic of considerable research (see reviews by Casey and Ingledew (1986); D'Amore *et al.* (1990); Ingram and Buttke (1984); Jones (1989); Mishra (1993)). At a physiological level, ethanol causes disruption of membrane structures and transport systems, it induces alterations in lipid and fatty acyl

composition (reviewed by Sajbidor (1997) leading to increased membrane fluidity, permeability and passive proton influx (Leao and Van Uden 1984b). There is an associated loss of membrane potential (Juroszek *et al.* 1987) and leakage of electrolytes (Mansure *et al.* 1994), amino acids and ribose-containing compounds from the cell (Salgueiro *et al.* 1988).

In the presence of ethanol, passive proton influx is thought to be the trigger for an increase in activity of the plasma membrane H<sup>+</sup>-ATPase (Cartwright *et al.* 1987) the enzyme largely responsible for maintenance of the plasma membrane proton gradient (Serrano 1991). However, activity of the plasma membrane H<sup>+</sup>ATPase complex is downregulated via its negative regulator, the plasma membrane heat shock protein Hsp30 (Piper *et al.* 1997) that reduces the V<sub>max</sub> of the plasma membrane H<sup>+</sup>ATPase in heat-shocked cells (Braley and Piper 1997). Thus, although the immediate response to heat shock or ethanol is activation of the plasma membrane H<sup>+</sup>ATPase, Hsp30 is thought to down-regulate the proton pump in response to prolonged stress to conserve ATP, but whether Hsp30 regulates the H<sup>+</sup>ATPase in ethanol-stressed cells is not known. Endocytosis is also an energy-requiring plasma membrane-associated activity that is inhibited by ethanol. Internalization of substances by endocytosis is delayed in the presence of 6% ethanol (Meaden *et al.* 1999) and endocytosis of the maltose transporter and *GAP1*, the general amino acid permease, are inhibited by 2-6% ethanol (Lucero *et al.* 1997).

At higher than physiological levels *in vitro*, ethanol denatures proteins and inhibits glycolytic enzyme activity (see references in review by Casey and Ingledew (1986)). It is unlikely however, that the inhibitory effect of ethanol is due to a build-up of intracellular ethanol since ethanol diffuses freely across the plasma membrane, but a decrease in intracellular pH is thought by some to have an inhibitory effect on cells exposed to ethanol. This observed reduction in intracellular pH following exposure to ethanol is controversial however because of the unreliability of some of the methods used for measuring this parameter, the different strains used and phase of growth when tested. A fluorescence microscopic image processor was used by Imai and Ohno (1995) to measure the intracellular pH of a growing culture of the *S. cerevisiae* brewing strain K1084. This work demonstrated that intracellular pH varies with growth phase even when the external pH remained unchanged. Intracellular pH

values were pH 5.7 during lag phase, pH 6.8 during exponential growth and pH 5.5 during stationary phase but the involvement of the plasma membrane H<sup>+</sup>ATPase in pH maintenance was not tested in these experiments.

Loureiro-Dias and Santos (1990) used a *S. cerevisiae* petite strain to simulate the absence of respiration during rapid glucose fermentation to investigate, by *in vivo* <sup>31</sup>P and <sup>13</sup>C nuclear magnetic resonance, the effect of ethanol on intracellular pH and metabolism. Contrary to suggestions that the reduction in intracellular pH following ethanol exposure is due to increased permeability of the plasma membrane and passive proton influx (Cartwright, Veasey and Rose 1987), the lower pH was found to be due instead to the conversion of ethanol via acetaldehyde to acetic acid. Thus the inhibitory effect of ethanol may be due to a build-up of acetate during respiratory metabolism rather than the presence of ethanol itself.

### **1.3.2 The effects of ethanol on membrane transporters**

Ethanol has been shown to have an inhibitory effect on plasma membrane transporters and this is thought to be due to interactions between ethanol and the lipids surrounding the transporters (Leao and Van Uden 1982; Loureiro-Dias and Peinado 1982). The maltose transporter, the general amino acid permease *GAP1* (Loureiro-Dias and Peinado 1982) and ammonium ion uptake in general (Leao and Van Uden 1984a) are non-competitively inhibited by ethanol. In addition, members of the glucose transport system, such as the D-xylose transporter, are inhibited in the presence of 10% ethanol (Leao and Van Uden 1984b). Because these transporters were non-competitively inhibited by ethanol, the authors proposed that changes in the lipid surrounds of the proteins due to interactions with ethanol were the most probable cause of the inhibition.

### **1.3.3 The effect of ethanol on membrane composition**

Many yeast physiologists and biochemists consider that ethanol exerts its toxic effects principally on cell membranes, therefore changes in membrane characteristics in the presence of ethanol have been studied extensively. It is well established that the phospholipid and sterol composition of cell membranes influences ethanol tolerance

(see reviews by D'Amore *et al.* (1990); Mishra (1993); Rose (1993); Sajbidor (1997)) and this aspect of the yeast adaptive response to ethanol stress will be discussed in section 1.4.4. A common trend in the reported work is that exposure to ethanol leads to increased fatty acid length and an increased proportion of unsaturated fatty acids and sterols in the cell membrane.

In anaerobic *S. cerevisiae* NCYC 431 cultures supplemented with 3.5 - 9% (v/v) ethanol Beaven *et al.* (1982) demonstrated that the proportion of mono-unsaturated fatty acids increased, particularly 18:1 residues (oleic acid), whilst the proportion of saturated fatty acids decreased, compared to cultures without ethanol. Similarly, Sajbidor and Grego (1992) observed an increase in the proportion of 18:1 residues relative to 16:1 residues in anaerobic cultures of *S. cerevisiae* strain CCY supplemented with up to 15% (v/v) ethanol. This appears to be largely due to a decline in the level of 16:1 residues in all phospholipids tested (phosphatidylcholine and phosphatidylethanolamine and phosphatidylinositol) thus there may have been no net synthesis of oleic acid (18:1) but a remodeling of the 16:1 residues.

Yeast cells are unable to synthesize unsaturated fatty acids under anaerobic conditions since the yeast desaturase enzyme has an oxygen requirement (Walker 1998b). Similarly many of the enzymes involved in sterol synthesis, in particular the conversion of squalene to ergosterol, require oxygen (Paltauf *et al.* 1992). Therefore, in anaerobic experiments unsaturated lipids must be imported into the cell from the growth medium. Under aerobic conditions however, in the presence of 10% (v/v) ethanol, membrane lipids of the *S. cerevisiae* wine strain 3079 were also modulated towards a higher level of 18:1 fatty acid residues with a corresponding decrease in palmitic acid residues (16:0) in a similar way to anaerobic cultures. As well as the increase in unsaturation of fatty acids, sterols were also modulated towards greater unsaturation in favour of ergosterol (Alexandre *et al.* 1993).

The uptake of nutrients in the presence of ethanol has also been shown to be influenced by membrane composition. Thomas and Rose (1979) examined the influence of membrane lipid components on the uptake of nutrients in the presence of 4.5 or 6% (v/v) ethanol. Cells were grown anaerobically with ergosterol and either monounsaturated oleic or polyunsaturated linoleic acid supplements and buffered cell

suspensions were incubated with radiolabeled nutrients prior to addition of ethanol and measurement of nutrient uptake. The addition of ethanol was accompanied by an initial loss of glucose, glucosamine and lysine from cells that was thought to constitute loss of sugars and amino acids from the cell wall. In addition, the growth rate was immediately reduced, particularly in cultures containing oleic (18:1) rather than linoleic (18:2) acid. Similarly, the accumulation rate within cells of labeled solutes glucose, glucosamine, lysine and arginine was reduced following ethanol addition, more so in oleic acid-supplemented cultures than polyunsaturated linoleic acid-supplemented cultures although phosphate uptake was unchanged in either enriched culture. The increased capacity for solute uptake of cells enriched with polyunsaturated fatty acid residues, compared to monounsaturated residues, was thought to be due to increased membrane fluidity (the rate of lateral motion of molecules). This would presumably compensate for a decrease in fluidity due to penetration of ethanol within the phospholipid bilayer and would thus prevent ethanol from inhibiting solute transporters.

In summary, the work of Alexandre, Rousseaux and Charpentier (1993); Beaven, Charpentier and Rose (1982); Sajbidor and Grego (1992) shows that exposure of yeast cells to ethanol induces changes in membrane composition that result in increased length and unsaturation of membrane lipids that presumably increase membrane fluidity. The reason for increased length is puzzling since this would normally decrease fluidity but an increase in fatty acid chain length also increases the hydrophobicity of the bilayer and therefore may prevent ethanol accumulation within the bilayer. An increase in hydrophobicity may also influence the passage of small ions and protons across cellular membranes. On the other hand the combined changes in membrane lipids may lead to an increase in the amount of ethanol that could be trapped within cell membranes, thus limiting its entry into the cell and/or membrane bound organelles. Another explanation may be that either an increase in fatty acid length or an increase in unsaturation is an immediate deleterious response to the presence of ethanol that is followed by an adaptive response opposed to the first reaction in order to maintain membrane stability.

### 1.3.4 The effect of ethanol on membrane fluidity

The increased fluidity (rate of lateral motion of molecules) of cell membranes has been inferred from the increase in membrane lipid unsaturation when cells are exposed to high ethanol concentrations, but is there a relationship between membrane fluidity and membrane permeability? Jones and Greenfield (1987) measured the passive influx of undissociated acetic acid to model the effect of ethanol concentrations up to 25% (v/v) on membrane permeability. In batch cultures, increased membrane fluidity following step changes in ethanol concentration was correlated to membrane permeability. However, the membranes of cells acclimated to ethanol by continuous culture in the presence of 6.5% (v/v) ethanol and subsequently exposed to a higher concentration of ethanol were less fluid, thus less permeable, than the cells in batch cultures. Furthermore, prolonged exposure to ethanol led to increased lysis of cells from the batch cultures compared to those from continuous cultures. The authors proposed that increased membrane fluidity is an adaptation to ethanol that may increase membrane stability but not necessarily influence ethanol tolerance.

The effect of ethanol (7-10% v/v) on membrane fluidity has also been investigated using electron spin resonance spectroscopy (ESR). Membrane fluidity increased in cell suspensions of microsomal fractions incubated with 9% (v/v) ethanol (Lloyd *et al.* 1993). Using fluorescence anisotropy to measure the membrane fluidity of a sake strain, a stress-sensitive strain and stress-resistant strain of *S. cerevisiae*, Swan and Watson (1997) found that membrane fluidity increased slightly in all strains following treatment for 60 minutes with 17% ethanol. Whilst the increased fluidity was accompanied by a minor increase in cell survival, fluidity did not correlate with stress tolerance given the intrinsic stress tolerance of the two ethanol tolerant strains used.

To test whether changes in phospholipid head group would alter membrane fluidity and ethanol tolerance, Mishra and Prasad (1988) used L-alanine uptake and the ethanol-induced reversal of H<sup>+</sup> efflux as indices of ethanol tolerance in cultures of a yeast mutant unable to synthesize lipids. Cells from cultures supplemented with different phospholipids were exposed to 12% (v/v) ethanol for 10 minutes prior to the transport measurements. The authors found that phosphatidylserine (PS) enrichment

of ethanol-stressed cells rather than membrane fluidity led to increased ethanol tolerance. There was also less inhibition of fermentation in the presence of 12 % ethanol when cultures were enriched with PS compared to phosphatidylcholine and phosphatidylethanolamine, that was comparable to the wildtype suggesting that enrichment with PS increases ethanol tolerance. Therefore, in the short term it appears that phospholipid head group composition rather than fluidity *per se* renders yeast membranes more resistant to ethanol but long-term adaptation is likely to require changes in membrane lipid length and unsaturation.

### **1.3.5 The effect of ethanol on organelles and organellar membranes**

Since ethanol diffuses freely across cell membranes, it is likely that all cellular membranes suffer from similar ethanol-induced structural alterations as the plasma membrane but there are few reports published in this area.

The fluidity of mitochondrially-enriched fractions of extracts from cells grown in the presence of 9% (v/v) ethanol was examined using electron spin resonance spectroscopy (ESR) (Lloyd *et al.* 1993). These subcellular membrane fractions did not demonstrate increased fluidity compared to membranes from cells grown without ethanol but the authors noted changes in temperature dependency behavior suggesting that changes in lipid composition may have occurred. In contrast, microsomal membrane fractions from cells grown with 9% ethanol were highly fluid even at 5°C suggesting that ethanol has a greater effect on membranes from the endoplasmic reticulum than mitochondrial membranes. Mitochondria are involved in cell survival in the presence of ethanol since transferral of mitochondria from an ethanol tolerant wine yeast to a laboratory strain has been observed to increase the survival of the recipient strain in the presence of ethanol (Aguilera and Benitez 1989).

Since ethanol is a powerful inducer of respiratory-deficient mutants, it is thought to be the major cause of the high diversity in mitochondrial DNA (mtDNA) found in the *Saccharomyces* Flor yeasts that proliferate at the surface of sherry wine containing over 15% (v/v) ethanol (Ibeas and Jimenez 1997). DNA analysis of these Flor yeasts revealed that ethanol-induced Flor yeast petites completely lack mtDNA (i.e. they are

*rho*<sup>0</sup>). Protoplast fusion of a Flor yeast mitochondrial variant (*rho*<sup>-</sup>) with a laboratory *rho*<sup>-</sup> strain conferred ethanol tolerance to the recipient laboratory strain suggesting that under ethanol stress there is a selection pressure in favour of the *rho*<sup>-</sup> genotype in Flor yeasts because of its ability to confer ethanol tolerance. Because of the high level of polymorphism in mtDNA in Flor yeasts mitochondrially-encoded genes are probably not involved in ethanol tolerance. Reactive oxygen species (ROS), generated in the mitochondria of cells during respiratory growth, damage lipids, proteins and DNA thus a cell without functional oxidative phosphorylation may have an adaptive advantage. Thus, the *rho*<sup>-</sup> phenotype with its enhanced ethanol tolerance may constitute an adaptive response to ethanol stress.

Increased activity of the manganese-dependent mitochondrial superoxide dismutase, MnSOD, by 50-150% following exposure to 8% ethanol was thought to be responsible for the resistance of *S. cerevisiae* strain aBR10 to 14% ethanol (Costa *et al.* 1993). This was in contrast to the cytoplasmic CuZnSOD that did not increase in activity following heat or ethanol stress and therefore appears to play only a modest role in ethanol tolerance. In addition, a MnSOD<sup>-</sup> strain exhibited ethanol sensitivity but in a CuZnSOD<sup>-</sup> strain there was a four-fold increase in MnSOD activity indicating that it was able to compensate for loss of CuZnSOD. MnSOD was subsequently found to be essential for the tolerance of cells to 14 or 20% ethanol during diauxic phase and post diauxic phase, as measured by plate counts, presumably to inactivate ROS produced in mitochondria by the electron transport chain during respiratory growth on ethanol (Costa *et al.* 1997).

Loureiro-Dias and Santos (1990) investigated the effect of ethanol on yeast vacuolar membranes. The addition of ethanol led to a decrease in intracellular pH as measured by *in vivo* <sup>31</sup>P nuclear magnetic resonance whereas the pH of the vacuole remained unchanged. This finding suggests that the passive influx of protons that occurs across the plasma membrane in the presence of ethanol does not occur across vacuolar membranes. The vacuolar membrane H<sup>+</sup>ATPase was not activated by ethanol, unlike the ethanol-induced activation of the plasma membrane H<sup>+</sup>ATPase observed by Cartwright, Veasey and Rose (1987). Despite these findings, the morphology of the vacuole was altered in cells in the presence of 6% ethanol (Meaden *et al.* 1999). Large vacuoles formed instead of the more fragmented structures usually present

suggesting that ethanol may influence the intracellular compartmentalization of ions and polyphosphates.

### 1.3.6 The relationship between ethanol and oxidative stress

Under aerobic growth conditions yeast cells are exposed to oxidative stress due to the production of partially reduced forms of molecular oxygen. Reactive oxygen species (ROS) such as the hydroxyl radical ( $\text{OH}^\cdot$ ), the superoxide anion ( $\text{O}_2^-$ ) and  $\text{H}_2\text{O}_2$  are highly reactive and damaging to cellular components causing DNA lesions, lipid peroxidation, oxidation of proteins and causing perturbations to the cellular redox balance. Lipid peroxidation is thought to impair the structural integrity of cell membranes by generating shorter fatty acid chains thus increasing membrane fluidity (Moradas-Ferreira *et al.* 1996). Yeast cells have evolved adaptive responses as a defence against the above reactive oxidants (see reviews by Jamieson (1998); Moradas-Ferreira *et al.* (1996)). Cellular defences include a number of enzymes that can inactivate ROS such as the cytoplasmic superoxide dismutase (CuZnSOD), the mitochondrial superoxide dismutase (MnSOD), cytochrome *c* peroxidase (CCP) and cytoplasmic catalase T (*CTT1*). In brewing, the role of dissolved oxygen and developed mitochondria are important for the biosynthesis of unsaturated fatty acids and ergosterol (Casey *et al.* 1984; O'Connor-Cox *et al.* 1996). However, the mitochondrial respiratory chain is a major source of the  $\text{O}_2^-$  radical therefore, compared to fermentative growth, respiration of non-fermentable substrates such as ethanol, causes cells to express high levels of molecules involved in antioxidant defence. In addition, the production of ATP from respiration would be expected to be more sensitive to ethanol than ATP production via glycolysis.

The treatment of cells with low concentrations of  $\text{H}_2\text{O}_2$  or superoxide-generating menadione triggers adaptive responses that protect cells against subsequent challenges with high levels of these oxidants (Collinson and Dawes 1992). Thus stress tolerance can be induced by the expression of the antioxidant defence genes. Heat, osmotic stress and ethanol all induce antioxidant defences (Costa *et al.* 1993; Steels *et al.* 1994, Marchler *et al.* 1993). Using respiratory deficient mutants as controls, Costa *et al.* (1997) showed that ethanol toxicity correlates with the production of ROS in the

mitochondria and that the mitochondrial superoxide dismutase, MnSOD, is essential for ethanol tolerance in diauxic and post-diauxic-phase cells.

Many anti-oxidant genes are glucose-repressed therefore both intracellular and extracellular catalase activity in an aerated *S. cerevisiae* distillery strain was greater with ethanol as substrate than with glucose (Gille *et al.* 1993). The authors suggested that the extracellular catalase acted as a protection against the damaging effects of ethanol by oxidizing the ethanol outside the cell.

Cytochrome P-450, an enzyme catalysing the oxidation of endogenous and exogenous substrates in *S. cerevisiae*, accumulates to a high level when yeast grows fermentatively on glucose and is also present when ethanol is added to low glucose cultures. Cytochrome P-450 encoded by *ERG11* is required for the C-14 demethylation of lanosterol during ergosterol synthesis in the endoplasmic reticulum and is able to oxidatively detoxify toxic chemicals such as benzo (a) pyrene.

### **1.3.7 The genetic response of *S. cerevisiae* to ethanol stress**

At a genetic as well as a physiological level the ethanol-stress response appears to have many features in common with the heat shock response (Piper 1995). *S. cerevisiae* genes expressed in response to ethanol stress are listed in Table 1.2. Yeast cells exposed to ethanol synthesize a suite of heat shock proteins including Hsp104 (Piper *et al.* 1994) (Sanchez *et al.* 1992), Hsp26, Hsp30, Hsp70, Hsp82 (Piper *et al.* 1994) and Hsp12 (Praekelt and Meacock 1990; Varela *et al.* 1995). However, of these hsps only Hsp104 (Glover and Lindquist 1998) and Hsp12 (Sales *et al.* 2000) influence yeast tolerance to ethanol. An increase in ethanol tolerance has been correlated with the increased expression of Hsp104 (Parsell *et al.* 1991; Sanchez *et al.* 1992). Hsp104 acts as a remodelling agent in the disaggregation of denatured proteins (Glover and Lindquist 1998). When cells expressing Hsp104 induced by heat-treatment were exposed to 20% ethanol, a higher percentage of the population survived than for non-treated cells or the *HSP104* knockout (Sanchez *et al.* 1992). Hsp12 is a membrane-associated protein that can protect liposomal membrane integrity against desiccation and ethanol (Sales *et al.* 2000). These authors also found

GENE	CELLULAR LOCATION	FUNCTION	REFERENCE
<i>HSP12</i>	membrane	protects membrane integrity	(Ivorra <i>et al.</i> 1999; Moskvina <i>et al.</i> 1998; Sales <i>et al.</i> 2000)
<i>HSP26</i>	cytoplasm	protein folding	(Piper <i>et al.</i> 1994)
<i>HSP30</i>	membrane	negative regulator plasma membrane H <sup>+</sup> ATPase	(Panaretou and Piper 1992; Piper <i>et al.</i> 1997; Piper <i>et al.</i> 1994; Seymour and Piper 1999)
<i>HSP70(SSA3)</i>	cytosol	prevents protein aggregation	(Piper <i>et al.</i> 1994)
<i>HSP82/83/90</i>	cytoplasm	protein folding	(Piper <i>et al.</i> 1994)
<i>HSP104</i>	cytoplasm	disaggregates denatured proteins	(Piper <i>et al.</i> 1994)
<i>MnSOD (SOD2)</i>	mitochondrion	antioxidant defence	(Costa <i>et al.</i> 1997)
Cytochrome P-450 ( <i>ERG11</i> ) <i>YGP1</i>	endoplasmic reticulum extracellular	C-14 demethylation of lanosterol unknown	(Aoyama <i>et al.</i> 1984; Paltauf <i>et al.</i> 1992) this project
<i>DIP5</i>	integral membrane	amino acid permease	this project
<i>YER024w(YAT2)</i>	unknown	carnitine acetyl transferase	this project

**Table 1.2:** *S. cerevisiae* genes expressed in response to ethanol stress

that the *HSP12* knockout had a reduced growth rate over 24 hours in 10% ethanol compared to the wildtype.

As well as the induction by ethanol of several heat shock proteins and some genes involved in anti-oxidant defences (section 1.3.6), the induction of cytochrome P-450 synthesis by ethanol has been reported by a number of workers cited in Mishra (1993). Blatiak *et al.* (1987) observed accumulation of the enzyme in late exponential phase cells that correlated to an increased concentration of endogenous ethanol and a decreased concentration of glucose. The authors suggested that a cytochrome P-450 isoenzyme might play a role in ethanol tolerance by oxidative removal of ethanol from the endoplasmic reticulum. This may be an important role in light of the severe effects of ethanol on ER membranes (Lloyd *et al.* 1993). However, under strict fermentative conditions (5-20% w/v glucose) *S. cerevisiae* accumulates large amounts of cytochrome P-450, apparently larger than is required for its role in sterol biosynthesis (Aoyama *et al.* 1984). This enzyme or its isoenzyme may therefore convert ethanol to acetaldehyde by an alternative route to the alcohol dehydrogenase route in a similar way to the free-radical mediated mechanism of cytochrome P-450 present in the mammalian liver.

There is a reduction in the level of expression of many genes following both heat shock and ethanol shock that may be partially explained by the blockage of most mRNAs from leaving the nucleus following a heat shock or ethanol stress (Saavedro *et al.* 1996). However, transport of mRNAs of the hsps *SSA4* and *SSA1* was not blocked following a heat shock (42°C) or ethanol stress (10%) suggesting that there is a selective export of mRNA in response to these stresses in yeast. Rip1p, a nuclear protein associated with nuclear pore complexes, is specifically required for export of heat shock mRNAs following stress (Saavedro *et al.* 1997). Another protein involved in nuclear export of mRNA, the mRNA binding protein Npl3p that shuttles between the cytoplasm and nucleus, is still exported under stress conditions such as exposure to 10% ethanol (Saavedro *et al.* 1997). However, UV-cross-linking experiments revealed that Npl3p is bound to mRNA under normal conditions but not following stresses such as heat shock, high salt or ethanol. The authors proposed that Npl3p is a component of a ribonuclear protein (RNP) complex and its uncoupling from the

complex renders the RNP complex export-incompetent and it therefore remains in the nucleus. These results shed some light on the mechanisms involved in the selective expression of hsps following stress.

Piper (1995) has suggested that interaction of ethanol with proteins may occur at high ethanol concentrations, thus exposing hydrophobic amino acids and leading to binding of chaperones such as Hsp70. Reduced chaperone level is thought to be a signal for activation of heat shock genes (reviewed in Parsell and Lindquist (1993); Piper (1993) therefore ethanol may induce heat shock protein synthesis by depleting the “free” chaperone pool within cells.

The cell membrane, its inter-membrane proteins and cell wall are likely to be the initial site of ethanol stress signaling but little is known about the way this might occur. In mammalian systems the enzyme Phospholipase C, linked to phosphoinositide-linked signaling at the cell surface is involved in ethanol-induced changes and development of ethanol tolerance. Thus the cell surface is a possible site of stress signaling in *S. cerevisiae* as proposed by Hoek and Taraschi (1988).

#### **1.4 FACTORS INCREASING *S. cerevisiae* TOLERANCE TO ETHANOL**

In the previous section the known responses of yeast cells exposed to ethanol were described but how many of these responses influence the ability of yeast cells to withstand the deleterious effects of ethanol; which ethanol-stress responses confer ethanol tolerance? What are the adaptive changes that have been demonstrated to increase ethanol tolerance, or are necessary for cell survival in the presence of ethanol? Pre-exposure of cells to a non-lethal ethanol concentration can confer tolerance to a subsequent lethal ethanol concentration although the level of resistance to high ethanol concentrations is strain specific. For example, *S. cerevisiae* aBR10 cells developed resistance to an ethanol concentration of 14% (v/v) by pre-exposure to 8% (v/v) ethanol for 30 minutes (Costa *et al.* 1993).

Stress tolerance is also growth phase-specific, with *S. cerevisiae* being more stress tolerant and resistant to lytic enzymes in stationary phase. Ethanol tolerance has not been reported as a feature of this stage of growth although Costa *et al.* (1997) found

that cells increased in ethanol tolerance in post-diauxic phase provided MnSOD was present. Elliott and Futcher (1993) demonstrated that slow growth was responsible for increased stress tolerance rather than growth phase, but slow growth *per se* is probably not the direct cause of stress tolerance since nutrient limitation, the degree of glucose de-repression, respiratory growth and the Ras-cAMP pathway are also involved. Whether any of these factors are involved in ethanol tolerance is not known, but of the numerous stress proteins, only Hsp104 (Piper 1995; Sanchez *et al.* 1992) and Hsp12 (Sales *et al.* 2000) and possibly Hsp70 have been demonstrated to contribute to ethanol tolerance.

#### **1.4.1 The influence of trehalose on ethanol tolerance**

Whether or not accumulation of intracellular trehalose occurs as an adaptive response that impacts on yeast ethanol tolerance is still the subject of debate. However, in the presence of a stressful level of ethanol the proteins required for mobilization of trehalose are synthesized (see the review by Nwaka and Holzer (1998)). The amount of trehalose that accumulates in cells depends in part on a balance between the synthesis of trehalose and its hydrolysis by trehalases. Thus, disruption of *ATH1* encoding the vacuolar acid trehalase, leads to increased tolerance to ethanol shock inferring that accumulated trehalose helps cells tolerate this stress (Kim *et al.* 1996).

Although the precise role of trehalose during ethanol stress is unclear, its accumulation during fermentation has been correlated with ethanol tolerance by Mansure *et al.* (1997). *S. cerevisiae* brewing strains and strain S228C were grown in molasses (equivalent to 200 g L<sup>-1</sup> sucrose) and the trehalose level was measured after 3 and 6 hours growth and cell viability determined by plate counts. In the presence of such high substrate concentration, the level of intracellular trehalose and the percentage of surviving cells increased over the period in all strains, but more so in the brewing strains. At the same time the activity of trehalose-6-phosphate synthase increased approximately two-fold. D'Amore *et al.* (1991) also found that trehalose accumulation enhanced cell survival under high substrate conditions as well as cell survival in 5% ethanol beer. These authors used mutant strains of *S. cerevisiae* defective in trehalose synthesis or transport to investigate the importance of trehalose accumulation on cell survival. Trehalose content correlated to survival, the level of

trehalose increasing after 24 hours exposure to 10% (v/v) ethanol for trehalose transport mutants but not for trehalose synthesis mutants or the wildtype strain. In contrast, other workers (Alexandre *et al.* 1998; Coote *et al.* 1992) have shown that although cells accumulate trehalose on exposure to ethanol, trehalose is not correlated to cell survival, thus from the data presented, it appears that the level of ethanol protection afforded by trehalose may be minor.

#### **1.4.2 The effect of magnesium on ethanol tolerance**

Although not an adaptive response to ethanol stress, the presence of magnesium reduces the inhibitory effect of ethanol on growth and fermentation rate (Dombek and Ingram 1986) and partially relieves the loss of viability associated with rapid ethanol accumulation (Dasari *et al.* 1990). An essential inorganic ion involved in growth, cell division and enzyme activity (Walker 1994), magnesium decreases the permeability of the plasma membrane to protons and anions through interactions with membrane phospholipids (Petrov and Okorokov 1990) thus it is proposed to increase yeast ethanol tolerance by stabilizing cell membranes (Walker 1998a). Elevation of the magnesium concentration in ethanol-stressed cultures reduces the amount of magnesium ion leakage that occurs when cells are ethanol- or heat-stressed and affords the cells a level of stress protection (Walker 1998a). Birch and Walker (2000) demonstrated that the addition of 20 mM magnesium to a wine yeast in the presence of 10% (v/v) ethanol leads to a reduction in cell mortality compared to the addition of 2 mM magnesium. When the same wine yeast was incubated for 60 minutes in the presence of ethanol at concentrations up to 20% (v/v), both the concurrent addition of 50 mM magnesium and preconditioning with 50 mM magnesium increased the level of cell viability, particularly for ethanol concentrations above 5% (v/v). Using a scanning electron microscope Birch and Walker (2000) also demonstrated that cell surface perturbations caused by a 10% (v/v) ethanol stress were reduced in cultures containing 20 mM magnesium. Cells exposed to ethanol have a pitted surface that does not appear to be as cohesive as control cells or cells grown with elevated magnesium levels. The majority of enzymes involved in phospholipid synthesis require  $Mg^{2+}$  as a cofactor (Paltauf, Kohlwien and Henry 1992) therefore magnesium is necessary for synthesis of lipids for cell membrane repairs and adaptive responses.

### **1.4.3 The effect of small amounts of acetaldehyde on the lag time of ethanol-stressed *S. cerevisiae* cultures**

The growth inhibitory effect of ethanol is also reduced by the addition of small amounts of acetaldehyde to ethanol-stressed cultures. Compared to unstressed cells, cells exposed to ethanol take longer to achieve the size required for cell division and thus cells exposed to ethanol exhibit a longer lag period than unstressed cultures. The addition of between 0.005 and 0.05 g L<sup>-1</sup> acetaldehyde to exponential phase *S. cerevisiae* cells inoculated into complex medium containing 4% (v/v) ethanol, stimulates cellular adaptation to ethanol stress. Addition of these small amounts of acetaldehyde reduces the ethanol-induced lag period by as much as 68% and also increases the growth rate (Stanley *et al.* 1997). Presumably the ethanol-stressed cells were able to achieve division size earlier in the presence of a low concentration of acetaldehyde than in the presence of ethanol alone.

Although the lag reducing effect of acetaldehyde is not strictly related to ethanol tolerance of cells but rather influences the rate of yeast adaptation to ethanol stress, it has been included in this section since it impacts on yeast fermentative performance. Higher concentrations of acetaldehyde than those used by Stanley, Hoble and Pamment (1997) however have a toxic effect on yeast cells. Acetaldehyde is the first metabolite produced in the breakdown of ethanol but is more toxic to cells than ethanol itself and is rapidly transformed into acetate. Chromosomal DNA of *S. cerevisiae* cells grown with 37.5 g L<sup>-1</sup> acetaldehyde acquires double and single strand breaks and similarly, DNA damage occurs in *S. cerevisiae* cells when ethanol is catabolised and this is thought to be principally caused by acetaldehyde (Ristow *et al.* 1995).

### **1.4.4 The influence of membrane composition on ethanol tolerance**

The changes in membrane composition that occur in cells grown in the presence of ethanol have already been detailed in an earlier section however some of these changes have also been shown to have a positive influence on ethanol tolerance. Beaven, Charpentier and Rose (1982) demonstrated that cell viability was unaffected by the addition of 3% or 6% (v/v) ethanol to yeast cultures when the fatty acid

composition of the cells was modulated towards longer chain length mono-unsaturated residues suggesting that these were adaptive changes that increase ethanol tolerance.

Thomas *et al.* (1978) examined the lipid and plasma membrane composition of anaerobically grown cells supplemented with a range of sterols and fatty acids and determined their ethanol tolerance. These authors found that longer and more unsaturated fatty acid residues and sterols (ergosterol and stigmasterol rather than cholesterol and campesterol) were present in the most ethanol tolerant cultures and postulated that the  $\Delta^{22}$  unsaturated alkyl chain of ergosterol and stigmasterol created a more effective barrier to ethanol entering the cell. In addition, ergosterol supplementation of an ethanol-stressed (17% v/v) *S. cerevisiae* sterol auxotroph, improved its ethanol tolerance as measured by cell viability (Swan and Watson 1998). These authors also demonstrated that, when the same sterol auxotrophic strain was grown in an aerobic lipid-supplemented culture with 17% (v/v) ethanol, tolerance to ethanol was greatest in cultures supplemented with oleic acid (C<sub>18:1</sub>) compared to linoleic (C<sub>18:2</sub>) or linolenic (C<sub>18:3</sub>) acids and the wildtype (Swan and Watson 1999). It should be noted however that *S. cerevisiae* is unable to synthesize the polyunsaturated linoleic and linolenic fatty acids (Paltauf, Kohlwien and Henry 1992).

Mishra and Prasad (1988) also observed that ethanol tolerance could be improved by changes to membrane lipids. In a *S. cerevisiae* mutant requiring the addition of phospholipid bases for growth, the effect of supplementation with various phospholipid bases was determined in cell suspensions containing 12% (v/v) ethanol. Cultures enriched with phosphatidylserine (PS) were more ethanol tolerant as measured by the ability of the cells to ferment and take up amino acids (L-alanine). Cultures enriched in PS also reversed the proton efflux caused by the increased activity of the plasma membrane H<sup>+</sup>ATPase in the presence of ethanol although the phospholipid head group did not affect membrane fluidity.

Thus phospholipid head group, fatty acid chain length and degree of unsaturation have been demonstrated to have a bearing on ethanol tolerance. How these membrane changes influence ethanol tolerance is unclear but sterols with unsaturated side chains

are thought to trap ethanol in the membrane preventing its entry into the cell (Thomas, Hossack and Rose 1978). However, which of these responses to ethanol are adaptive and which are deleterious remains to be determined. It is possible that some membrane changes may actually constitute membrane damage caused by ethanol.

#### **1.4.5 The influence of assimilable nitrogen compounds on ethanol tolerance**

In an industrial setting, ethanol tolerance in yeast is dramatically influenced by nutrients in the wort, particularly unsaturated lipids and assimilable nitrogen. Compared to addition of nutrients at the commencement of fermentation, the stepped addition of glucose and nutrients to a culture of the industrial yeast strain 1400, increased the maximum ethanol yield from 9.5% to 14.3% (w/v) (D'Amore *et al.* 1990). However, there was no increase in ethanol concentration when the glucose was added without additional nutrients suggesting that nutrient limitation is an important factor in fermentative performance and ethanol tolerance. Nutritional deficiency was found to have a greater inhibitory effect on fermentation and the production of high levels of ethanol than ethanol itself (Casey, Magnus and Ingledew 1984).

#### **1.4.6 Cell immobilization**

Immobilization of *S. cerevisiae* cells to an artificial solid surface for fermentation has been investigated as a method of increasing ethanol production whilst making the process continuous and has recently been shown to increase ethanol tolerance (Jirku 1999; Norton *et al.* 1995).

Jirku *et al.* (1980) demonstrated that cells could still grow and divide on a solid substrate and that secretion of extracellular macromolecules was not impeded. Immobilized cells were covalently linked to the surface of modified hydroxy-alkyl methacrylate Separon H-100 E gel beads and cell division proceeded without separation of daughter buds or appreciable loss of daughter cells into the growth medium. The formation of chain-like filaments of elongated cells indicated that polarization of budding occurs under these conditions. Norton, Watson and D'Amore

(1995) investigated the survival of brewer's yeast cells immobilized on a carrageenan gel matrix following ethanol shock (4.5% v/v for 1 hour or 18% v/v for 2 hours). Under both stress conditions, cells entrapped in the gel matrix exhibited a much higher survival rate than cells released from the gel beads prior to ethanol shock or ethanol-shocked freely growing cells, particularly for exponential phase cells compared to stationary phase cells. Norton, Watson and D'Amore (1995) also found that the percentage of respiratory deficient petites induced by an 18% ethanol shock was only 1.3% for immobilized cells compared to 1% for unstressed cells and 9.9% for ethanol-stressed freely growing cells. This is a clear indication that immobilized cells are more ethanol tolerant than free-growing cells suggesting that cell wall components that help cells adhere to each other and foreign surfaces may be involved in ethanol tolerance.

Investigation of cell wall polysaccharides showed that immobilized cells have a higher glucan and mannan cell wall content compared to free cells (Jirku 1991). Glucans contribute to cell rigidity and mannans contribute to cell wall porosity (Klis 1994). In addition, the mannoprotein profile of immobilized baker's yeast cells was the same as for free cells that had been ethanol-stressed by growth in the presence of 6.5% (v/v) ethanol for four hours (Parascandola *et al.* 1997). This suggests that cell wall-associated proteins, usually induced by ethanol stress, may play a role in ethanol tolerance of immobilised cells and the high level of glycosylation suggests that interactions between cells may be protective against ethanol stress.

At cell membrane level the fatty acid profiles of these cells was similar, but freely growing cells were relatively rich in unsaturated fatty acids, palmitoleic acid (16:1) and oleic acid (18:1) and immobilized cells had a higher proportion of saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0) probably reflecting the slightly more anaerobic environment of the entrapped cells (Norton, Watson and D'Amore 1995). However the fatty acid composition of the cells was not correlated to ethanol tolerance since released cells with the same fatty acids as entrapped cells were not ethanol tolerant. The authors suggest that the high cell density of immobilised cells creates a protective microenvironment for the cells that leads to increased ethanol tolerance.

The plasma membrane composition of yeast in relation to ethanol tolerance was also investigated by measuring the leakage of UV-absorbing substances from immobilized cells exposed to 5, 10 and 15% (v/v) ethanol (Jirku 1999). There was less leakage of UV-absorbing substances from immobilized cells than freely growing cells that corresponded to an increase in saturated fatty acids and ergosterol accompanied by a reduction in unsaturated fatty acids, increases in the level of phospholipids phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine but a decrease in phosphatidylinositol. In summary, immobilization of yeast cells on an artificial matrix leads to changes in cell wall and plasma membrane composition that appears to enhance their ethanol tolerance, possibly as a result of high cell density and interactions between cells.

## 1.5 REGULATION OF THE YEAST STRESS RESPONSE

There is an extensive literature related to regulation of genes involved in the yeast stress response (see reviews by Dawes (1999); Estruch (2000); Hohmann and Mager (1997); Mager and De Kruijff (1995)). In this section therefore, only an overview of general stress regulation will be provided with a focus on regulatory elements that may be involved in the yeast ethanol-stress response.

The regulatory factors involved in the yeast stress response are complex and, although there are some common mechanisms, most stresses also induce a specific response that regulates genes in pathways required for the response to that particular stress. An overview of current knowledge on stress regulation is presented in simplified form in a diagram taken from Attfield *et al.* (1997) (Figure 1.1). The principle *S. cerevisiae* stress response transcription factors, their consensus binding sites and some of the genes they regulate are summarized in Table 1.3 (Dawes 1999). Regulatory elements included are the heat shock element (HSE), stress response element (STRE), haem activated transcription factor binding sites (HAP1-5), AP-1 responsive elements (AREs). These regulatory elements appear to have overlapping but separate functions (Attfield, Myers and Hazell 1997).

Genes containing HSE promoter sequences are expressed under moderate stress conditions when abnormally folded proteins accumulate. The products of these genes,

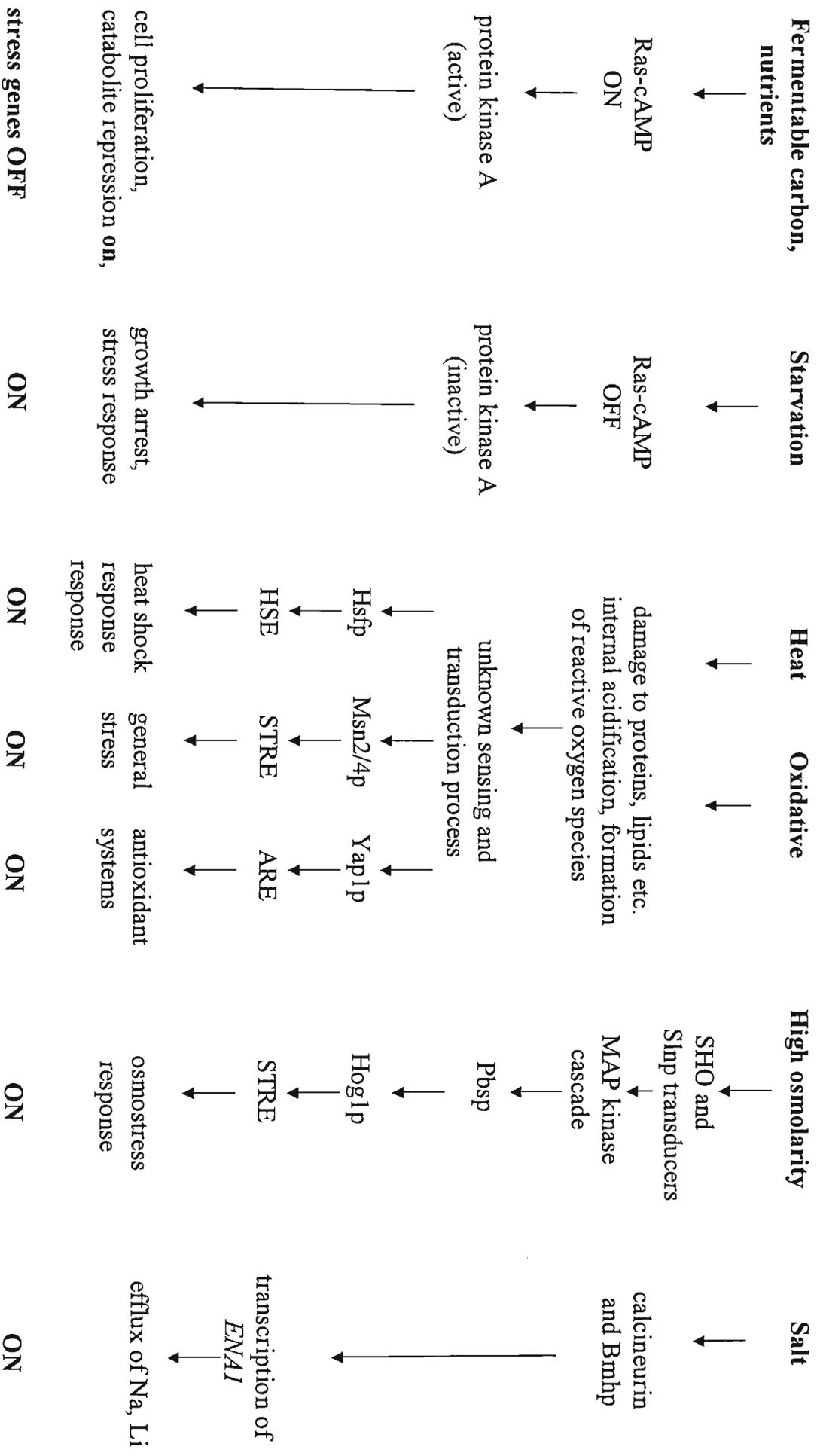


Figure 1.1: Diagram of stress response pathways in *S. cerevisiae* from Attfield *et al.* (1997).

Control site*	Consensus motif	Genes regulated
HSE ( <i>HSP1</i> )	nGAAnTTTCnnGAAn (multimers of nGAAn)	<i>SSA1, SSA3, HSP82, UBI4</i>
STRE, TRS** ( <i>MSN2, MSN4</i> )	AAGGGG	<i>CTT1, DDR2, HSP12, HSP26, HSP104, GSY2, UBI4, GPD1, HAL1, ENA1, GAC1, TPS1, TPS2, SOD2</i>
HAP2,3,4,5 ( <i>HAP2-5</i> )	TNATTGGC/T	<i>CYC1, HEM1, LPD1, KGD1, KGD2, SOD2, ASN1, ASN2, GDH1, CIT1, COX4, COX5a, COX5b</i>
HAP1 ( <i>HAP1</i> )	CGGN <sub>3</sub> TANCCGN <sub>3</sub> TA	<i>CYC1, CYC7, CTT1, CTA1, SOD2</i>
yAP-1 and other yAPs ( <i>YAP1, YAP2, YAP3?, YAP5?</i> )	TTA(G/C)TAA	<i>GSH1, TRX2, YCF1, GLR1, SSA1, PDR5, SNQ2, ATR1</i>
ACE1 ( <i>ACE1</i> )	TTTTGCTG	<i>CUP1, SOD1, CRS5</i>
MAC1 ( <i>MAC1</i> )	?	<i>CTT1, FRE1</i>

\* Transcription factors that bind to sites in parenthesis

\*\* Kobayashi (1993)

**Table 1.3:** Consensus binding sites for stress-related promoter motifs in *S. cerevisiae* taken from Dawes (1999).

such as the molecular chaperones, have functions in normal growth as well as under stress. Heat shock factor (HSF), the positive regulator of HSE-containing genes, is thought to be negatively regulated by Hsp70 during normal growth (Boorstein and Craig 1990). The model proposes that sequestering of Hsp70 to denatured proteins during stress releases the HSF for activation of stress response genes (Mager and Moradas Ferreira 1993). Genes with STREs are required for survival during and after severe stress and are responsive to a variety of stress conditions. Genes regulated by STREs are induced by heat shock (Kobayashi and McEntee 1993), nutrient starvation, osmotic stress, oxidative stress (Marchler *et al.* 1993), low pH, weak acids and ethanol (Schuller *et al.* 1994) and are involved in trehalose and glycogen metabolism as well as other cell rescue functions. Genes with AREs such as *YAP1*, are induced by oxidative stress and in response to the presence of heavy metal ions (Mager and De Kruijff 1995; Ruis and Schuller 1995).

The STRE (CCCCT or AGGGG) is present in the promoters of numerous stress response genes including *UBI4*, *HSP12*, *DDR2* and *CTT1*. Two homologous and functionally redundant zinc finger transcription factors, Msn2p and Msn4p are required for STRE activation (Martinez-Pastor *et al.* 1996). These authors found that induction of a STRE-driven *lac Z* reporter gene was severely reduced in a *msn2 msn4* mutant exposed to 7% ethanol and expression of *CTT1* and *HSP12* was also lower in mutant cells exposed to 7% ethanol compared to the wildtype. This suggests that, for some genes at least, the response to ethanol stress is mediated by STRE. An increased general stress tolerance was observed in cells over-expressing Msn2p or Msn4p but the double mutant was sensitive to heat, oxidative, osmotic stress and carbon source starvation. However, this stress sensitivity was only apparent under severe acute stress not mild stress.

The pleiotropic nature of Msn2/Msn4 transcriptional activation suggests that more than one stress response pathway may be involved and that these activators may interact with other regulators. For instance, HOG pathway-dependent induction of the osmotic stress response occurred in the *Msn2 Msn4* mutant (Martinez-Pastor *et al.* 1996) and a decrease in plasma membrane H<sup>+</sup> ATPase activity triggered Msn2p translocation to the nucleus and STRE-mediated transcription (Moskvina *et al.* 1999).

The Ras-cAMP pathway negatively regulates STREs (Marchler *et al.* 1993) and nuclear localization of Msn2p and Msn4p in response to various stresses. The treatment of cells with 7.5% ethanol was shown to be inversely correlated to cAMP levels and protein kinase A (PKA) activity (Gorner *et al.* 1998). The activity of the cAMP-dependent PKA is essential for growth and has been implicated in many cellular processes (see review by Thevelein (1994)). PKA-deficient cells arrest in G1, accumulate the glucose storage molecules trehalose and glycogen and are resistant to heat and oxidative stress, but cells with elevated PKA activity are highly stress-sensitive. Cells with too little PKA activity are unable to progress through Start and are locked into G0. The primary trigger for induction of cAMP-dependent PKA is a fermentable sugar such as glucose but a complete growth medium is required for growth since limiting nitrogen, phosphate or sulphate can also arrest cells in G1. Therefore there is a co-relationship between the Ras-cAMP pathway and the main glucose repression pathway as well as the fermentable-growth-medium-induced pathway proposed by Thevelein and Hohmann (1995).

Since stress causes cells to transiently arrest in G1 and mutations inactivating cAMP production or PKA give rise to cessation of growth and arrest of cells in G0 there is an interplay between growth control and stress control. The cAMP-dependent protein kinases are important mediators of growth, whereas cyclin-dependent kinases are mediators of division. Thus mutations inactivating cyclin-dependent kinase *cdc28* arrest the cell division cycle in G1 while growth continues (Mager and De Kruijff 1995).

As well as PKA, two other protein kinases, Gcn2 and protein kinase C, have a role in gene regulation in response to stress. Starvation for a single amino acid leads to Gcn2 indirectly stimulating expression of amino acid biosynthetic genes by derepression of *GCN4* mRNA expression. The Gcn4 trans-activator protein then activates synthesis of the amino acid biosynthetic genes (see review by Hoekstra *et al.* (1991); Mager and De Kruijff (1995); Struhl (1989); Svetlov and Cooper (1995)). Gcn4p may therefore be described as a stress-responsive transcription factor.

Protein kinase C activates a MAP kinase cascade involved in cell wall remodelling. This pathway is also involved in nutrient sensing and its activity appears to be

mediated by the cyclin-dependent cell cycle protein kinase Cdc28p. Loss of Pkc1p function causes a cell lysis defect, sensitivity to low osmolarity, reduced cell wall thickness and lysis of bud tips (Stark 1999). Pkc1p may also negatively regulate some stress responses. Whilst investigating a link between the secretory pathway and transcription of rRNA and ribosomal protein genes, Nierras and Warner (1999) observed that transduction of a stress signal at the plasma membrane by protein kinase C led to severe repression of transcription of both rRNA and ribosomal protein genes. However, deletion of *PKC1* relieved the repression of rRNA genes and ribosomal protein genes that occurs in response to a secretory pathway defect (Nieras and Warner 1999).

Transcription of genes expressed upon nitrogen limitation is under negative regulation by the TOR signaling pathway. Expression of nitrogen limitation genes is prevented due to promotion of the association of the GATA transcription factor Gln3p, positive regulator of nitrogen catabolic genes, with the cytoplasmic protein Ure2p. The phosphatidylinositol class kinases Tor1p and Tor2p, similarly inhibit expression of stress genes by stimulating the binding of the STRE-binding transcription factors Msn2p and Msn4p to the cytoplasmic protein Bmh2. Thus the TOR signaling pathway controls nutrient metabolism by sequestering several transcription factors (Beck and Hall 1999). An industrial wine strain lacking *URE2* function was able to utilize nonpreferred nitrogen sources such as proline that would normally be prevented by nitrogen catabolite repression in the presence of a preferred nitrogen source such as ammonium, glutamate or glutamine (Salmon and Barre 1998).

This summary of the regulation of the yeast stress response describes the different regulatory pathways that are activated by stress and has noted the known regulatory proteins involved in the ethanol stress response. There have been few reports of genes induced by ethanol stress but the STRE-binding transcription factors Msn2p and Msn4p increased the expression of ethanol-stress response gene, *HSP12* in cells exposed to 7% ethanol. These transcription factors play a prominent role in regulation of the response to severe stress but are negatively regulated by the Ras-cAMP pathway. Nuclear localization of transcription factors Msn2p and Msn4p was prevented by cAMP and the activity of PKA in cells treated with 7.5% ethanol (Gorner *et al.* 1998).

This review of the literature not only highlights the research effort devoted to this topic but it also highlights the lack of information available on the ethanol-stress response at a molecular level. Whilst the physiological effect of ethanol on yeast cells appears to be primarily focused at the cell membrane and cell wall, ethanol also inhibits metabolic processes and can increase the sensitivity of cells to heat.

Considering that the pleiotropic effects of ethanol can be critical to cell survival, there are very few genes known to be ethanol-stress-induced and, although several heat shock proteins are induced by ethanol stress, only Hsp12 and Hsp104 are known to influence ethanol tolerance positively. An investigation of the gene expression of cells exposed to ethanol stress was therefore warranted and the identification of genes involved in yeast adaptation to ethanol stress was the focus of the research project on which this thesis is based.

## **1.5 TECHNIQUES FOR GENE EXPRESSION ANALYSIS: DIFFERENTIAL DISPLAY**

It is clear from the above that changes in gene expression are a key part of induced tolerance to ethanol stress therefore a technique such as Differential Display that enables the identification of genes induced under specific conditions would provide the analytical capability necessary to identify such genes.

Differential Display, developed by Liang and Pardee (1992), is a PCR-based method of comparing the transcriptional profiles of two or more cell populations to resolve and clone individual differentially expressed mRNAs from a pair of mammalian cell populations. It involves creating a set of cDNAs that represent the mRNA population of a cell (or tissue), then resolving these cDNAs using PAGE. Bands appearing in the experimental samples but not in the controls are then cloned and sequenced and identified from known sequences in sequence databases. Using Differential Display, Liang and Pardee (1992) compared PCR products derived from quiescent murine cells to those from cells undergoing division and were able to identify cell cycle-regulated transcripts with as few as 30 copies per cell. Later papers from the same authors described the use of Differential Display to identify oncogenes and tumour suppressor genes in breast cancer and normal human mammary epithelial cells (Liang *et al.*

1992) and to characterize the involvement of *ras* and p53 in oncogenesis in rat embryo fibroblasts (Liang *et al.* 1994). Differential Display has since been successfully performed using cells, tissues and even organs from many eukaryote species.

At the time of writing there were close to 2000 Differential Display publications listed in Current Contents but relatively few associated with yeast (Appleyard *et al.* 1995; Crauwels *et al.* 1997; Garay-Arroyo and Covarrubias 1999; Gross and Watson 1998; Nag and Axelrod 1998; Shen and Green 1998). Garay-Arroyo and Covarrubias (1999) were the first to report the use of Differential Display to investigate a yeast stress response, identifying three genes, GRE1/2/3, involved in the *S. cerevisiae* osmotic stress response.

There are several alternative methods of determining comparative gene expression profiles but at the time of publication of the Liang and Pardee Differential Display protocol only subtractive hybridization (Sive and St John 1988) and SDS-PAGE analysis were available. Representational Difference Analysis (RDA) (Lisitsyn *et al.* 1993) that kinetically enriches target sequences by PCR-amplification prior to hybridization, is a later development and improvement on the technique of subtractive hybridization. RDA was further developed for gene expression analysis by Hubank and Schatz (1994), but the method is technically complex and only two samples can be compared at any time point.

Recent methods have been developed in which the gene expression of whole genomes can be determined rather than just the detection of differentially expressed genes. One example of this technology is Serial Analysis of Gene Expression (SAGE) (Velculescu *et al.* 1995) whereby an ORF-specific sequence tag is generated from each mRNA, the tags are concatenated and sequenced as a single molecule. The expression pattern of a population of transcripts can be quantitatively evaluated and identified using the tag sequencing data. When SAGE was used for characterization of the *S. cerevisiae* transcriptome (Velculescu *et al.* 1995) approximately 20,000 SAGE tags were generated from each of three growth states: log phase, S phase-arrested and G2/M phase arrested. Of a total 60,633 tags, 56,291 (93%) precisely matched the yeast genome, 88 tags matched the mitochondrial genome and 91

matched the 2-micron plasmid. Transcript expression per gene was found to vary from 0.3 to over 200 copies per cell. Combined with the *Saccharomyces* Genome Database (SGD) this is a powerful yet sensitive method of analyzing yeast gene expression although it is technically complex.

The most advanced technology, gene-array by photolithographical synthesis of gene-specific oligonucleotides on microchips (Fodor *et al.* 1991) or, the similar cDNA microarray by which PCR-amplified cDNAs are spotted onto glass for probing with labelled transcription products (Chee *et al.* 1996; Schena *et al.* 1995) is presently the method of choice. Quantitative expression data is obtained for all *S. cerevisiae* ORFs using gene array methods and if applied to a time-course the information is extremely valuable as in the investigation of genes regulated by the cell cycle (Spellman *et al.* 1998). There have been several important publications using the fully sequenced yeast genome data as a basis for microarray experiments but the first and most frequently cited is the analysis of gene expression during the diauxic shift by De Risi *et al.* (1997). Gene arrays of yeast cDNAs on proprietary membrane filters are less expensive and are also a powerful method for gene expression analysis with several publications citing their use. There has only been one publication to date on the application of gene array technology to ethanol stress studies (Alexandre *et al.* 2001). This paper reported that a large number of genes were up-regulated but the work awaits confirmation. This paper is described further in chapter 6 of this thesis in the context of work described here.

Yeast microarray and SAGE techniques were not available at the commencement of this work and, for the experiments described here, the Differential Display approach was used because it is a less technically demanding, less expensive and more versatile than RDA for detecting novel genes.

## 1.6 AIMS OF RESEARCH

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

The specific aims were to:

1. Determine ethanol-stressing conditions that are non-lethal but inhibitory to yeast growth. This stress should also be such that the stressed cells are able to adapt to the stress within a defined period of time prior to exponential growth.
2. Develop efficient RNA isolation and Differential Display procedures suitable for yeast cultures grown under the conditions defined in 1. Using these procedures and growth conditions, identify ethanol-stress induced *S. cerevisiae* genes.
3. Confirm that the identified *S. cerevisiae* genes are valid ethanol-stress response genes and characterise them by physiological and bioinformatics means.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 MATERIALS

##### 2.1.1 Microorganisms and plasmids

###### 2.1.1.1 *Saccharomyces cerevisiae*

*S. cerevisiae* PMY1.1 (*MAT $\alpha$  leu2, ura3, his4*) was used for ethanol stress and Differential Display work. PMY1.1 was originally from the laboratory of Dr Peter Piper, University College, London, UK and constructs derived from this parent strain for the work described in this thesis are listed in Table 2.1. The *YGP1* gene was removed from the wildtype and replaced by the *kanMX* module to generate PMY1.1 (*MAT $\alpha$  leu2, ura3, his4*) *YGP1::kanMX*. The knockout strain was transformed with a Genestorm™ plasmid encoding the *YGP1* open reading frame (pYES2/*YGP1*) to generate the rescue strain PMY1.1 (*MAT $\alpha$  leu2, ura3, his4*) *YGP1::kanMX* pYES2/*YGP1*. A rescue control strain was also generated by transforming the knockout with the Genestorm™ pYES2 plasmid without the *YGP1* insert, PMY1.1 (*MAT $\alpha$  leu2, ura3, his4*) *YGP1::kanMX* pYES2/GS.

The *S. cerevisiae* yeast strain SUB61 (*MAT $\alpha$  leu2, ura3, his3, lys2, trp1*) was obtained from the same source as strain PMY1.1 and was used in initial experiments described in Chapter 3.

PARENT STRAIN	DESCRIPTION	GENOTYPE	SOURCE
<i>S. cerevisiae</i> PMY1.1	wildtype	PMY1.1 ( <i>MATa leu2, ura3, his4</i> )	P. Piper*
	knockout	PMY1.1 ( <i>MATa leu2, ura3, his4</i> ) <i>YGP1::kanMX</i>	constructed for this work
	rescue	PMY1.1 ( <i>MATa leu2, ura3, his4</i> ) <i>YGP1::kanMXp YESS2/YGP1</i>	as above
	rescue control	PMY1.1 ( <i>MATa leu2, ura3, his4</i> ) <i>YGP1::kanMXp YESS2/GS</i>	as above
<i>S. cerevisiae</i> SUB61	wildtype	SUB61 ( <i>MAT<math>\alpha</math> leu2, ura3, his3, lys2, trp1</i> )	P. Piper*

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

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

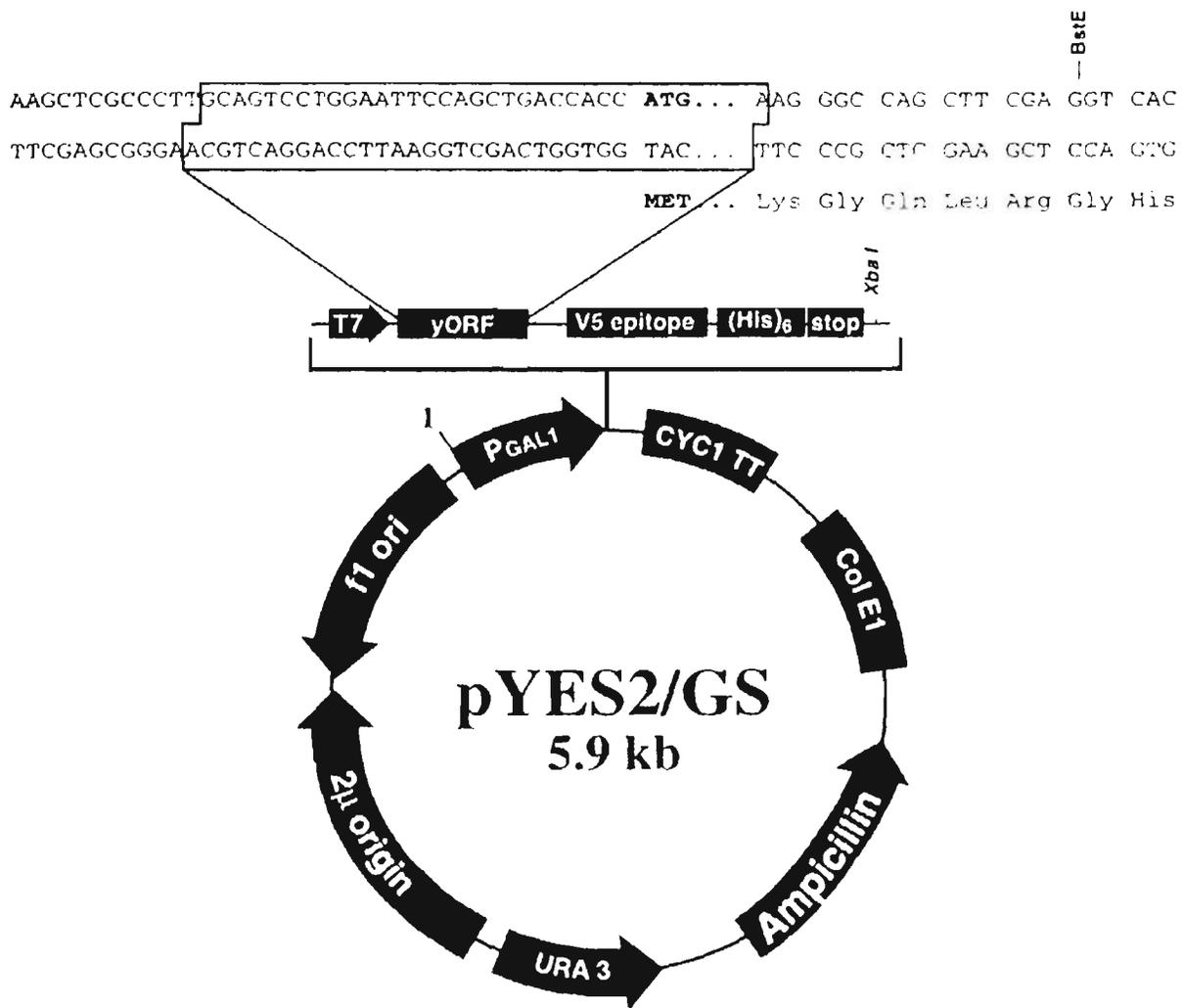
### 2.1.1.2 *Escherichia coli*

The *E. coli* competent cells used for cloning work were One Shot™ TOP10F' Competent Cells (Invitrogen) supplied by Bresatec Pty Ltd, South Australia. One Shot™ TOP10F' have the genotype F' {lacI<sup>q</sup> Tn10 (Tet<sup>R</sup>)} *mcrA* (*mrr-hsd RMS-mcrBC*)  $\Phi$ 80*lacZ* M15 *lac* X74 *deoR* *rec* A1 *araD*139 (*ara-leu*) 7697 *galU* *galK* *rpsL* *endA* *nupG*.

### 2.1.1.3 Plasmids

The plasmid pFA6-*kanMX* containing the *kanMX* module cloned in *PmeI* from the laboratory of Dr Peter Philippsen (Wach *et al.* 1994) was kindly supplied by Dr Paul Vaughan, Division of Molecular Science, CSIRO, Parkville, Australia. The *kanMX* module contains the *E. coli* *kan*' reading frame fused to translational and transcriptional control sequences of the *TEF* gene of the filamentous fungus *Ashbya gossypii* conferring geneticin (G418) resistance in yeast. The plasmid contains the *amp*' open reading frame for ampicillin resistance in *E. coli* and SP<sub>6</sub> and T<sub>7</sub> primer binding sites flanking the multiple cloning site.

The plasmid pYES2/GS (no insert) was used as a rescue control (Figure 2.1). This plasmid is a high-copy number 5.9 kb GeneStorm™ expression vector with a yeast ORF cloning site, *GAL1* promoter, T7 promoter/priming site, V5 epitope, polyhistidine region and *CYC1* transcription termination signal. On the opposite strand it contains a pMB1 (pUC) origin, Ampicillin resistance gene, *URA3* gene, 2 micron origin and *f1* origin. pYES2/GS was kindly supplied by Dr Hiromi Kimura, Invitrogen Corporation, San Diego, California. The plasmid used for rescue of the knockout strain was the GeneStorm™ pYES2/GS vector described above with the *YGP1* ORF, *YNL160w* insert (1065 bp) (Invitrogen) supplied by Bresatec Pty Ltd, Adelaide, South Australia.



**Figure 2.1:** Diagrammatic description of the plasmid pYES2/GS (Invitrogen). Features include: *GAL1* promoter, T7 promoter/priming site, yORF cloning site, V5 epitope, Polyhistidine region, *CYC1* transcription termination signal, PUC origin, Ampicillin resistance gene, *URA3* gene, 2 micron origin and f1 origin.

## **2.1.2 Solutions and buffers**

Analytical grade chemicals were used to prepare buffers and solutions unless otherwise stated. Chemicals were supplied by Sigma (USA) or BDH (UK). Water was distilled (Milli-RO Water Purification System) or, where indicated, distilled and de-ionized (Milli-Q Ultrapure Water System). Chemicals were dissolved in water in glass containers, brought to the desired pH value using a HI 8418 pH meter (Hanna Instruments). Buffers and solutions used in DNA and RNA work were either sterilized by autoclaving at 121°C for 20 minutes or, when indicated, sterilized by filtering through a 0.22 µm or 0.45 µm Millipore® membrane filter. Buffers for RNA work were prepared with RNase-free utensils, chemicals and DEPC-treated water or, where indicated, prepared solutions were treated with DEPC prior to autoclaving in baked bottles. Glassware and stainless steel utensils for RNA work were baked in an incubator set at 160°C overnight. DEPC-treated water (0.1% v/v) was prepared with distilled and de-ionized water. Buffers were stored at room temperature unless otherwise stated. Solutions and buffers, enzymes, molecular weight standards, suppliers and molecular biology kits are listed in Appendix I.

## **2.2 MICROBIOLOGICAL METHODS**

### **2.2.1 Growth media**

#### **2.2.1.1 Yeast growth media**

Most media and culture vessels were autoclaved at 121°C for 20 minutes. However, some media or media components were filter sterilized through a 0.22 µm Millipore® membrane filter as indicated. Growth media was purchased from Oxoid unless otherwise stated. All water used in growth media was distilled, de-ionised MilliQ™ water.

**YEPD** contained 1% (w/v) yeast extract, 2% (w/v) bactopectone and 2% (w/v) D-glucose dissolved in distilled de-ionised water and autoclaved. YEPD agar plates contained 1.5% (w/v) bacteriological agar added to YEPD prior to autoclaving.

**YEPD Geneticin plates** for selection of strains with an integrated *kanMX* cassette consisted of YEPD medium with 1.5% bacteriological agar plus 200 mg L<sup>-1</sup> G418 (Geneticin) (Sigma) added when the medium had cooled to approximately 50°C.

**Glycerol storage medium** contained 2% (w/v) yeast extract, 4% (w/v) bactopectone, 4% (w/v) D-glucose and 15% (v/v) glycerol dissolved in distilled de-ionized water and autoclaved. Two-time concentrated YEPD with 15% glycerol was prepared for the storage of wildtype and knockout strains at -80°C.

**Minimal medium 2% glucose:** Difco yeast nitrogen base (without amino acids and ammonium sulphate) was prepared according to the manufacturers instructions as a 10 x solution containing 1.7 g nitrogen base made up to 100 mL with sterile water. It was filter sterilized using a 0.22 µm filter prior to adding to 900 mL of autoclaved media comprising 2% (w/v) D-glucose (final concentration) and 0.5% (w/v) ammonium sulphate (final concentration). Leucine and histidine were added to a final concentration of 100 mg L<sup>-1</sup> and 20 mg L<sup>-1</sup> respectively from autoclaved 20 mg mL<sup>-1</sup> stock solutions. Uracil was added to a final concentration of 20 mg L<sup>-1</sup> from an autoclaved 20 mg mL<sup>-1</sup> stock solution as required (Kaiser *et al.* 1994).

**Minimal medium 4% raffinose** was prepared as above for minimal medium 2% glucose except that filter sterilised 2 x raffinose was added to a final concentration of 4% (w/v) instead of glucose.

**Minimal medium 1% raffinose 2% galactose** was prepared by adding filter sterilized 10 x Yeast Nitrogen Base (without amino acids and ammonium sulphate) (Difco) and a filter sterilized solution of 5 x raffinose and galactose to autoclaved 0.5% (w/v) (final concentration) ammonium sulphate. The 5 x raffinose and galactose solution required for 1 L of medium was prepared by dissolving with gentle heat 10 g raffinose and 20 g galactose in 200 mL de-ionised water. Amino acids were added as previously described.

**Minimal medium 2% raffinose 4% galactose** was prepared in the same way as minimal medium 1% raffinose 2% galactose, except that a filter sterilized 2 x solution

of raffinose and galactose (20 g raffinose, 40 g galactose dissolved in 500 mL water) was added to achieve a final concentration of 2% (w/v) raffinose and 4% (w/v) galactose.

**Minimal media agar plates** were prepared from the above medium with the addition of 1.5% (w/v) bacteriological agar. Amino acids were spread on dry plates as follows: 50  $\mu\text{L}$  of 20  $\text{mg mL}^{-1}$  stock solutions of leucine and histidine were spread on each plate to give final concentrations of approximately 40  $\mu\text{g mL}^{-1}$ . Uracil was added to a final concentration of 16  $\mu\text{g mL}^{-1}$  when required (Kaiser, Michaelis and Mitchell 1994).

**Minimal medium/glycerol storage medium** was prepared for storage of yeast strains carrying a Genestorm™ vector. The medium was prepared by combining aliquots of stock solutions as follows:

100  $\mu\text{L}$  of filter sterilized 10 x glucose/ammonium sulphate stock solution containing 10 g D-glucose and 2.5 g ammonium sulphate made up to 50 mL with distilled de-ionized water.

100  $\mu\text{L}$  of filter sterilized 10 x yeast nitrogen base stock solution (Difco)

5  $\mu\text{L}$  filter sterilised leucine 20  $\text{mg mL}^{-1}$  stock solution

1  $\mu\text{L}$  filter sterilised histidine 20  $\text{mg mL}^{-1}$  stock solution

500  $\mu\text{L}$  autoclaved glycerol

Sterile water was added to a total volume of 1 mL.

#### 2.2.1.2 *E. coli* growth media

**Luria Bertani broth:** 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl was dissolved in distilled de-ionised water and autoclaved. For selection of plasmid-carrying bacteria, ampicillin was added to the cooled autoclaved medium to a final concentration of 100  $\mu\text{g mL}^{-1}$  from a filter sterilized 1  $\text{mg mL}^{-1}$  stock solution stored at  $-20^\circ\text{C}$ . LB agar plates were prepared by adding 1.5% (w/v) bacteriological agar to the above medium prior to autoclaving.

**LB Amp plates** were prepared by adding ampicillin to a final concentration of 150  $\mu\text{g mL}^{-1}$  from a 75  $\text{mg mL}^{-1}$  stock solution to cooled autoclaved LB medium containing 1.5% (w/v) bacteriological agar.

## **2.2.2 Growth of yeast**

### **2.2.2.1 Standard culture conditions**

Unless otherwise stated, yeast liquid cultures were grown under aerobic conditions at 30°C in an orbital shaker-incubator (Innova 4230 refrigerated incubator, New Brunswick Scientific, Edison, New Jersey) at 200 rpm. The standard culture vessels were Erlenmeyer flasks with cotton stoppers covered with aluminium foil and the culture volume was 1/5 of the flask capacity.

### **2.2.2.2 Growth conditions for yeast strains carrying a Genestorm™ vector**

Minimal medium as described above was used for the growth of yeast strains carrying the pYES2/GS Genestorm™ vector. The carbon source was either 4% (w/v) raffinose or 4% (w/v) raffinose with 2% (w/v) galactose for gene induction. Uracil was omitted for plasmid maintenance. Standard culture conditions were used.

### **2.2.2.3 Growth of yeast on plates**

Yeast colonies grown on agar streak plates for short term storage and spread plates for viability determination by colony counts were incubated in a standing incubator at 28°C for 48 hours then stored at 4°C. YEPD plates were used for all viability determinations. YEPD Geneticin plates were used for selection and storage of the PMY1.1 *YGP1* knockout.

### **2.2.2.4 Storage of yeast**

For long-term storage yeast colonies were scraped from agar plates and suspended in glycerol storage medium and stored at -80°C. Yeast cells containing plasmids were

stored in minimal medium/glycerol storage medium at  $-80^{\circ}\text{C}$ . All cell transfers were carried out using aseptic technique in proximity to a bunsen flame using Gilson™ pipettes with barrier tips or sterile glass pipettes.

#### **2.2.2.5 Yeast inoculum preparation**

In preparation for yeast growth experiments, a loopful of cells taken from glycerol stocks, was used to inoculate 5 mL of YEPD medium in loosely sealed McCartney bottles. The yeast cells were grown overnight at  $30^{\circ}\text{C}$  at standard growth conditions. The  $\text{OD}_{620}$  reading of these cultures was used to determine the inoculum size required for an  $\text{OD}_{620}$  reading of 0.1 when transferred to 25 mL of fresh medium. After 6 hours growth at  $30^{\circ}\text{C}$  under standard conditions they were transferred to 50 mL of fresh culture medium, to an  $\text{OD}_{620}$  of 0.1, for overnight growth at  $30^{\circ}\text{C}$  under standard conditions. These overnight parent cultures were used to inoculate 50 mL or 100 mL of fresh medium for growth experiments. Transfers were performed using unwashed cells under aseptic conditions unless stated otherwise.

#### **2.2.2.6 Growth of yeast during ethanol stress**

Ethanol-stressed cultures were prepared by removing a portion of the YEPD medium under aseptic conditions immediately prior to the growth experiment and replacing it with an equal appropriate volume of ethanol. For each ethanol stress experiment a control culture was prepared using the same medium without added ethanol. Ethanol stress and control culture vessels were then inoculated from overnight parent cultures to an  $\text{OD}_{620}$  of 0.1, placed in a rotary incubator and incubated at  $30^{\circ}\text{C}/200\text{rpm}$ . Samples for optical density and viability by plate counts were taken at regular intervals during incubation. Growth experiments in defined medium were prepared as above along with any auxotrophic requirements.

#### **2.2.2.7 Cell harvesting and storage in ethanol stress experiments**

Beginning at time 0 (inoculation time) and then at regular intervals, whole cultures of ethanol-stressed and unstressed control cultures were harvested. After obtaining an

optical density value at 620 nm for each culture, the cultures were transferred to Falcon tubes and cells pelleted by centrifugation for 5 minutes at 4°C in a Beckman CS-15R swing rotor centrifuge at 4800 rpm (2000 x g). The supernatant was poured off and the pelleted cells were placed on ice. Using the optical density reading of the culture at harvest and the appropriate calibration curve (Section 3.4) the number of viable cells mL<sup>-1</sup> in each stressed or unstressed (control) culture was calculated and the cells were resuspended in DEPC treated water to a cell density of 10<sup>8</sup> viable cells mL<sup>-1</sup>. Aliquots of 1 mL were distributed to microfuge tubes and the cells pelleted by centrifuging in an Eppendorf 5415C bench microfuge at 14,000 rpm (16000 x g) for 4 minutes. The water was removed using a micropipette and the pellets were snap-frozen by dropping the tubes into liquid nitrogen. They were stored at -80°C prior to RNA isolation.

#### **2.2.2.8 Cell density and viability determination**

Cell viability was determined by plate counts made by spreading 100 µL aliquots of cell culture serially diluted in 900 µL sterile water in microfuge tubes onto duplicate YEPD plates. After incubation at 28°C for 48 hours, plates with 30 to 300 colonies were counted and the duplicates averaged and multiplied by the dilution factor to obtain a cell population in the original cultures.

Cell viability, as measured by plate counts, was lower in ethanol-stressed cultures than in unstressed control cultures therefore calibration curves relating optical density to viable cell counts were prepared from data generated from both ethanol-stressed and unstressed cultures during lag phase growth. However, it should be noted that cell viability was close to 100% after inoculation into medium containing up to 5% (v/v) ethanol. The calibration curves enabled optical density readings to be used to estimate the number of viable cells mL<sup>-1</sup> in each growing culture. The number of viable cells in harvested ethanol-stressed and unstressed cultures was calculated from the optical density readings with reference to the calibration curves, and then the cells were pelleted and re-suspended on ice in sterile DEPC water to a cell density of 10<sup>8</sup> viable cells mL<sup>-1</sup>.

### **2.2.3 Growth of *E. coli***

#### **2.2.3.1 *E. coli* growth conditions**

*E. coli* was grown in Luria Bertani broth in sterile Falcon tubes at 37°C in a shaker incubator at 100 rpm or on LB plates in a standing incubator at 37°C. *E. coli* was grown on LB plates or LB Ampicillin plates for selection and short term storage of plasmid carrying strains.

## **2.3 MOLECULAR METHODS**

### **2.3.1 RNA isolation**

#### **2.3.1.1 Standard RNase-free procedures and conditions**

All chemicals, water, plastic-ware and glassware used for RNA preparation were RNase-free. Foil covered glassware, spatulas etc were baked at 160°C overnight and electrophoresis tanks, trays and combs were sprayed with RNase Away (Molecular Bio-Products) and rinsed with DEPC treated water. Glass beads were acid washed prior to baking at 160°C overnight. Distilled and de-ionized MilliQ™ water was treated with DEPC (0.1%), mixed well, allowed to stand overnight then autoclaved. Plastic-ware such as microfuge tubes was purchased RNase-free and autoclaved prior to use. RNase-free barrier tips were used for all pipetting and freshly opened chemicals were handled with RNase-free implements. An MT19DL Deluxe bench vortex mixer (Chiltern Scientific) set at the maximum vibration speed and a 5415C bench microfuge (Eppendorf) were used for all experiments.

### 2.3.1.2 RNA isolation

Cells from ethanol-stressed and unstressed cultures were stored as pellets of  $10^8$  cells in microfuge tubes at  $-80^{\circ}\text{C}$  (see section 2.2.2.8). Cell pellets were thawed on ice and RNA isolation was carried out essentially as described by Ausubel *et al.* (1997). Thawed pellets were re-suspended in 300  $\mu\text{L}$  (1 x) RNA lysis buffer, added to 300  $\mu\text{L}$  of acid washed, baked 0.4  $\mu\text{m}$  glass beads chilled on ice in microfuge tubes and vortexed for 2 minutes. The upper phase was removed to fresh tubes after pelleting the cell debris by microfuging for 1 minute at 14,000 rpm. Protein was precipitated by the addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), vortexing for 20 seconds and microfuging for 2 minutes at 14,000 rpm. The top phase was carefully removed to fresh tubes and the RNA was ethanol precipitated from the top phase using 3 volumes of ice cold 100% ethanol and stored at  $-70^{\circ}\text{C}$  for 2-12 hours or on dry ice for 2-10 minutes. RNA pellets were microfuged for 2 minutes at  $4^{\circ}\text{C}$ , washed once with ice cold 70% ethanol and microfuged 1 minute at  $4^{\circ}\text{C}$ . The RNA pellets were air-dried at room temperature and re-suspended in 25  $\mu\text{L}$  RNase-free water. RNA gel loading solution was added to 1  $\mu\text{L}$  of RNA solution for resolution on a 0.8% agarose non-denaturing gel (section 2.3.2.1).

### 2.3.2 DNase treatment of RNA and acid phenol extraction

The following rigorous approach was used for RNA preparations for Differential Display, Northern analysis and RT-PCR experiments, to remove contaminating DNA from RNA solutions. DNA was first digested with RNase-free DNase (Boehringer-Mannheim) in 50  $\mu\text{L}$  reactions as outlined in the Differential Display Kit Manual (Display Systems). The RNA solution prepared above (section 2.3.1.2) was incubated at  $37^{\circ}\text{C}$  for 25 minutes with 200 units RNase-free DNase (Boehringer-Mannheim), 50 mM Tris pH7.5, 10 mM  $\text{MgCl}_2$  and 20 units Recombinant RNasin® Ribonuclease Inhibitor (Promega) made up to 50  $\mu\text{L}$ . The RNA was then acid phenol washed to remove any remaining undigested DNA fragments that might act as template or primer in PCR reactions. This was achieved by adding 1/10 volume of 2 M sodium acetate (pH4), 1 volume of water saturated phenol (pH5) and 1/5 volume of chloroform/isoamyl alcohol (49:1). The mixture was transferred to 0.2 mL microfuge

tubes and placed on ice for 10 minutes. Tubes were then microfuged for 5 minutes at 14,000 rpm. The aqueous phase was carefully removed to fresh tubes and 1 mL of ice cold 100% ethanol and 15  $\mu\text{L}$  of 3 M sodium acetate (pH5) was added prior to storage at  $-80^{\circ}\text{C}$  for 10 minutes. Precipitated RNA was pelleted by microfuging the tubes for 10 minutes at 14,000 rpm and the pellets were washed with ice cold 75% ethanol and air-dried. RNA to be used for Northern analysis was re-suspended in 12  $\mu\text{L}$  RNase-free water and RNA for differential display was re-suspended in 25  $\mu\text{L}$  RNase-free water. A 1  $\mu\text{L}$  aliquot diluted with 4  $\mu\text{L}$  RNase-free water and 1  $\mu\text{L}$  of (6 x) RNA sample loading buffer was visualized on a 0.8% agarose non-denaturing gel (section 2.3.2.1).

### **2.3.2.1 Non-denaturing RNA gel electrophoresis**

Visualization of RNA on non-denaturing gels was carried out before and after DNase treatment to monitor the quality of the RNA preparation, the consistency of RNA preparation from different time-points in the same experiment and to estimate RNA concentration. RNase-free, 0.8% agarose, non-denaturing TAE gels were prepared by microwaving 0.8% (w/v) agarose and 1 x TAE in an RNase-free flask. After cooling slightly, ethidium bromide was added to a final concentration of  $0.1 \mu\text{g mL}^{-1}$  and the gel poured and allowed to set for at least 20 minutes prior to electrophoresis in 1x TAE buffer at 80 V for 20-45 minutes.

### **2.3.3 Differential Display (DisplaySystems )**

An MJ Research Inc. PTC-100 Programmable Thermal Controller PTC-100 thermocycler with heated lid was used for all PCR-amplification and for incubations where indicated. Unless stated otherwise, the thermocycler was pre-heated to the denaturing temperature of the PCR program prior to placing tubes in the block. Primers used for Differential Display are listed in Table 1, Appendix II.

### 2.3.3.1 cDNA synthesis

RNA for cDNA synthesis was isolated from equal cell numbers ( $10^8$  cells) derived from cultures grown under ethanol-stressed or unstressed conditions and harvested at 3 or 4 points over a time-course (6-8 RNA templates). cDNA synthesis from each RNA template was initiated by individual downstream primers. Nine individual T<sub>11</sub>NM anchored downstream primers (Table 1 Appendix II) targeting the 3' Poly A tract of mRNAs were used to prepare cDNAs from an estimated 200-300 ng of RNA template essentially as described in the Differential Display Kit manual (DisplaySystems). First strand synthesis was performed in 30  $\mu$ L reactions that contained First Strand Buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>] (Gibco BRL), 10 mM DTT, 20  $\mu$ M dNTPs, 30 units Recombinant RNasin<sup>®</sup> Ribonuclease Inhibitor (Promega), 270 units Superscript<sup>™</sup>II RNase H<sup>-</sup> Reverse Transcriptase (Gibco BRL), 2.5  $\mu$ M downstream primer (Operon), 1-3  $\mu$ L (200-300 ng) RNA template and DEPC-treated water. The downstream primer, RNA and 9  $\mu$ L of RNase-free water were first placed in 0.2 mL microfuge tubes, incubated for 10 minutes at 70°C and placed on ice. A reaction mix comprising First Strand Buffer, DTT, dNTPs, Recombinant RNasin and DEPC-treated water was prepared and 16.5  $\mu$ L of the mix was added to each tube, mixed and incubated for 2 minutes at room temperature. Finally 1.35  $\mu$ L Superscript II RNase H<sup>-</sup> Reverse Transcriptase (GibcoBRL) was added and the tubes were incubated at 42°C for 1 hour after which tubes were immediately placed on ice and stored at -80°C until templates were required for Differential Display reactions. Negative control reactions for several templates chosen at random were prepared at the same time. These controls contained all reagents except Superscript II RNase H<sup>-</sup> Reverse Transcriptase (GibcoBRL) which was replaced with water. The controls were PCR-amplified and separated on Differential Display gels to identify any bands derived from DNA rather than RNA.

### 2.3.3.2 Differential Display reactions

PCR reactions were prepared essentially as described in the Display Systems Differential Display<sup>™</sup> Kit Protocol 1 (DisplaySystems). Reaction mixes were prepared for each downstream primer and contained PCR buffer [10 mM Tris-HCl

(pH 8.3), 50 mM KCL], 3 mM MgCl<sub>2</sub>, 2 μM dNTPs, 2.3 μM downstream primer, 1 unit Amplitaq<sup>®</sup> DNA Polymerase (Perkin Elmer) and 1 μCi α-<sup>33</sup>P dATP (Bresatec). The above reaction mixes were prepared simultaneously on ice and dispensed to each well in a PCR microtitre plate (Robbins Scientific) to a final volume of 20 μL. The appropriate upstream primer (0.5 μM final concentration) and cDNA template (1 μL) were dispensed to each well prior to the addition of the above reaction mix. PCR amplification was carried out under conditions recommended in the Display Systems Differential Display<sup>™</sup> Kit Protocol 1 (DisplaySystems ) as follows:

PCR amplification protocol: 

30 sec 94°C	}	40 cycles
60 sec 40°C		
60 sec 72°C		
5 min 72°C		

### 2.3.3.3 Differential Display gels

Differential Display reactions were separated on 6% SDS poly-acrylamide non-denaturing gels using a Poker Face II SE1600 Sequencing Gel System (Hoefer Scientific Instruments). Glass sequencing plates were cut and supplied by a local glazier.

#### Gel preparation

Glass sequencing plates were cleaned by soaking overnight in 10% (w/v) NaOH, washed with detergent, rinsed with distilled de-ionized water then wiped dry with 70% ethanol. The long front plate was wiped quickly and evenly with 1 mL of Repel-Silane<sup>™</sup> on a wad of laboratory tissues and the short back plate was treated similarly with 3 μL Bind-Silane<sup>™</sup> mixed with 1 mL solution of 95% ethanol/0.5% acetic acid. The plates were taped together with lightweight electrical tape using 0.2 mm teflon spacers and clamped with bulldog clips. Gels were prepared by mixing 7.5 mL of 40% acrylamide (acrylamide and N-N-methylene bisacrylamide 19:1), 5 mL 10 x TBE buffer and 37.5 mL distilled de-ionized water and filtering through a 0.22 μm filter into a side arm flask prior to de-gassing for 20 minutes. TEMED<sup>™</sup> (40 μL) and 300 μL 10% (w/v) ammonium persulphate were added and the gel poured

immediately between prepared sequencing plates using a 50 mL disposable syringe. The gel comb (sharks-tooth or normal) was inserted and the gels were allowed to set for 1-2 hours prior to loading samples.

### **Sample preparation**

Samples from Differential Display reactions were prepared according to the Differential Display Manual (DisplaySystems ) by mixing 5  $\mu$ L of sample with 2 $\mu$ L of Differential Display sample loading buffer. A 3-5  $\mu$ L aliquot of this mixture was loaded into the wells of the gel using a micropipette.

### **Running gel**

The gel was assembled in the sequencing apparatus and TBE buffer added to the bottom and top reservoirs. The comb was removed and the wells were washed out with buffer using a syringe. The samples were loaded into the wells and the gel apparatus connected to the power pack (Bio-Rad Power Pac 3000) and run with limiting power of 25 watts for 1.5-2 hours, until the bromphenol blue component of the loading buffer reached the bottom of the gel.

### **Autoradiography**

After disassembly and separation of the glass plates the gels were air-dried onto the Bind-Silane<sup>TM</sup> pre-treated back glass plate, marked for orientation with India ink containing a small amount of radioactive isotope and placed in X-ray cassettes. Kodak BioMax<sup>TM</sup> MS scientific imaging film was directly exposed to the gel overnight at room temperature. Films were developed in total darkness for 2 minutes in Agfa-Gevaert Manual X-ray Developer at the recommended dilution (1:6), fixed for 2 minutes in darkness and 10 minutes total in Agfa-Gevaert Manual X-ray Fixing Bath at the recommended dilution (1:4) then washed for 20 minutes in running water and airdried.

#### **2.3.3.4 Band excision and re-amplification with modified primers**

Bands identified by autoradiography as being up-regulated in ethanol-stressed samples were located by aligning the gel with the autoradiograph using the India ink marks for orientation. The bands were moistened with sterile water and removed with a sterile scalpel blade to tubes containing 20  $\mu$ L sterile H<sub>2</sub>O. DNA was allowed to diffuse from the gel bands and a 1-2  $\mu$ L aliquot of DNA solution was used as template

in PCR reactions with the original Differential Display primer pairs. The PCR products were separated on a 2% agarose gel and a stabbed gel band was then used as template for re-amplification with a modified upstream primer (Table 2 Appendix II). The modified upstream primers had an overall G+C content of 60% and were synthesized with ten additional nucleotides at the 5' end to enable them to be used as sequencing primers after band re-amplification. PCR conditions for the second re-amplification were as described above except that 20  $\mu$ M dNTPs, 28  $\mu$ M downstream primers and 1  $\mu$ M modified upstream primers were used.

### 2.3.3.5 Sequencing of re-amplified gel bands

Re-amplified gel bands were either gel purified (Bandpure™) or purified from PCR products (Wizard™) using the manufacturers instructions. After sizing and quantifying by comparison with a 100 bp DNA Ladder (Promega) on a 2% agarose gel the PCR products were sequenced directly from the modified upstream primer using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit. Approximately 50 ng of purified PCR product and 5-10 picomoles of modified upstream primer were added to 8  $\mu$ L of Terminator Ready Reaction Mix and made up to 20  $\mu$ L with sterile water. Sequencing reactions were PCR-amplified as follows:

30 sec 96°C	}	26 cycles
15 sec 50°C		
4 min 60°C		

Sequencing reaction PCR products were ethanol precipitated by addition of 2  $\mu$ L of 3 M sodium acetate and 50  $\mu$ L absolute ethanol. Tubes were vortexed briefly, placed on ice for 10 minutes and microfuged for 20 minutes at 14,000 rpm. Pellets were washed with 70% ethanol and dried in a standing incubator at 37°C for 20 minutes. Electrophoresis of sequencing reactions was performed by the Monash University Microbiology Department, Clayton, Victoria.

### 2.3.3.6 Identification of genes associated with Differential Display bands

Approximately 150-250 bp of sequence data obtained as described in section 2.3.3.5 was used to identify genes using the BLASTn (Altschul *et al.* 1990) or FASTA (Pearson and Lipman 1988) algorithms by alignment with the entire *S. cerevisiae* genome using the *Saccharomyces* Genome Database (SGD).

## 2.3.4 Northern analysis

### 2.3.4.1 Denaturing gel electrophoresis

RNA was prepared from equivalent numbers of ethanol-stressed and unstressed lag phase cells harvested over a time-course and RNA was resolved on a 5% formaldehyde 1.2% agarose denaturing gel.

#### Denaturing RNA gel preparation

All electrophoresis tanks, plates and combs were pre-treated to remove RNases and all solutions and plastic-ware were RNase-free (see section 2.3.1.1). To prepare a 60 mL RNA denaturing gel, 0.72 g (1.2% w/v) DNA grade agarose and 51 mL DEPC water was microwaved on High for 1 minute 10 seconds. The solution was cooled slightly prior to the addition of 6 mL 10 x MOPS buffer and 3 mL formaldehyde (pH4-5) and poured into a prepared gel tray. The gel was allowed to set for approximately 1 hour prior to loading samples.

#### Sample preparation

Samples were prepared in microfuge tubes as follows: 5.5  $\mu\text{L}$  RNA (section 2.3.1.2), 1  $\mu\text{L}$  10 x MOPS buffer, 3.5  $\mu\text{L}$  formaldehyde (pH3-5) and 10  $\mu\text{L}$  formamide were mixed and heated to 65°C for 10 minutes in a heating block and placed on ice. RNA markers (4  $\mu\text{L}$ ) (Promega) were prepared in the same way. Prior to loading, 1  $\mu\text{L}$  (0.1 mg mL<sup>-1</sup>) ethidium bromide and 5  $\mu\text{L}$  6 x formaldehyde gel loading solution was added to each tube, mixed and spun down.

#### Electrophoresis

Samples were loaded onto a gel in an electrophoresis tank containing 1 x MOPS buffer and electrophoresed at 80 V for approximately 2 hours and photographed on a UV transilluminator prior to blotting.

#### 2.3.4.2 Northern blotting

After electrophoresis RNA was transferred overnight by capillary action onto a positively charged nylon membrane (Boehringer Mannheim) using the standard procedure described in Sambrook *et al.* (1989). The gel was placed in 10 x SSC whilst the blot was prepared. A filter paper wick for capillary flow was placed on a perspex bridge over a tray of 10 x SSC with both ends in the buffer. Three sheets of Whatman 3M filter paper cut to the size of the gel were pre-wet with buffer and placed on the paper wick. The gel was placed on top of the filter papers face up and covered with a nylon membrane cut to the same size and handled with baked forceps. The membrane was immediately wet with buffer and air bubbles between paper and membrane removed by rolling gently with a baked glass pipette. Three more sheets of wet filter paper were placed on top of the membrane and covered with a stack of paper towels. The edge of the gel was sealed with plastic wrap that also covered the tray of buffer to prevent evaporation. A perspex sheet was placed on top of the paper towels and weighted with two full 1 L Schott bottles. The RNA transfer was allowed to proceed overnight. The blot was disassembled, the membrane rinsed briefly in 4 x SSC and air-dried on plastic wrap. To ensure that complete transfer of RNA had taken place, the gel was stained with 0.1 mg mL<sup>-1</sup> ethidium bromide for 30 minutes and viewed on a UV transilluminator. The membrane was placed face down on plastic wrap over a UV transilluminator and UV cross-linked for 2 minutes. The RNA markers were cut from the membrane, stained with methylene blue, de-stained with DEPC water and stored damp in a sealed hybridization bag. The blot was stored in a sealed hybridization bag at room temperature until required for Northern hybridization.

#### 2.3.4.3 Design of probes for Northern analysis

Gene specific oligonucleotide probes (Table 3 Appendix II) comprising 30-35 nucleotides with approximately 50% G+C content were designed and tested for complementarity to other yeast RNA and DNA sequences in the *Saccharomyces* Genome Database (SGD) using the BLASTn local alignment sequence search tool (Altschul *et al.* 1990). BLASTn default parameters were used for alignments although all filters were removed and the expected threshold value was raised to 100

so that a greater number of matches would be revealed than with the default settings. Low homology matches were then identified and their complementarity to the probe investigated to determine if the probe would be likely to anneal to their transcripts. Probes were selected only when it was judged they would not anneal to any transcript other than the one of interest.

#### **2.3.4.4 Labeling probes**

Probes designed and tested for high specificity as described in section 2.3.4.3 were purchased from Pacific Oligos Pty. Ltd. and end-labeled with  $\gamma$ - $^{32}\text{P}$ -ATP (Bresatec) using T4 Polynucleotide Kinase (Amersham Pharmacia Biotech unless otherwise stated) and the procedure described in Sambrook, Fritsch and Maniatis (1989). Reactions of 20  $\mu\text{L}$  total volume contained 10 pmol oligonucleotide, One-Phor-All buffer [10 mM Tris acetate, 10 mM magnesium acetate, 5 mM potassium acetate] (Amersham Pharmacia Biotech), 20-200  $\mu\text{Ci}$   $\gamma$ - $^{32}\text{P}$ -ATP (4000 Ci  $\text{mmol}^{-1}$ ), 10-20 units T4 Polynucleotide Kinase (Amersham Pharmacia Biotech) and sterile distilled de-ionized water and incubated in a heating block at 37°C for 45 minutes. The enzyme was de-activated by incubation at 68°C for 10 minutes and the probe was used immediately for Northern hybridization or stored at -20°C overnight.

#### **2.3.4.5 Determining labeling efficiency by Cerenkov counts**

For determination of labeling efficiency, 0.5  $\mu\text{L}$  aliquots of each labeling reaction were removed after incubation with T4 Polynucleotide Kinase, added to 4.5  $\mu\text{L}$  0.2 M EDTA and stored at -20°C. To determine the proportion of label that had become incorporated with the oligonucleotides, 0.5  $\mu\text{L}$  of the thawed samples were pipetted onto quadruplicate 2.3 cm circular Whatman DE81 ion exchange paper and allowed to dry on laboratory tissues. Duplicate filters were washed six times in 0.5 M sodium phosphate buffer, rinsed in 100% ethanol, dried and placed in scintillation vials for Cerenkov counting using the tritium isotope window of a Wallac 1410 liquid scintillation counter. Duplicate unwashed filters were also placed in scintillation vials and the counts read. Using this method the oligonucleotides remained bound to the ion exchange paper whilst unincorporated nuclides were washed away. The

percentage incorporation of the label was calculated by dividing the average of the counts obtained from washed duplicate filters by the average readings from the unwashed duplicate filters and multiplying by 100.

#### **2.3.4.6 Northern hybridization**

Northern hybridizations were carried out in a Bartelt Instruments XTRON HI 2002 hybridization oven at 42°C (unless otherwise stated) with the rocking platform set at 11. Northern blots were pre-hybridized in DIG Easy Hyb (Boehringer Mannheim) in the hybridization oven at 42°C for at least 1 hour. The labeled probe was added and hybridized with the blot overnight at 42°C (unless otherwise stated). After hybridization the probe solution was removed and the blots washed in 4 x SSC, 2 x SSC and 1 x SSC (if necessary) to leave a count of between 5 and 50 cpm on the blot as detected using a geiger counter.

#### **2.3.4.7 Autoradiography of probed Northern blots**

The air-dried blots were placed in hybridization bags or covered with polythene cling wrap and placed in an X-ray cassette for exposure of Kodak BioMax™ MS scientific imaging film. The film was exposed overnight at -80°C. and developed as described in section 2.3.3.3 After autoradiography the membranes were stripped and re-probed with an oligonucleotide probe for the constitutive *ACT1* (actin) transcript (Table 3 Appendix II) as an expression control.

#### **2.3.4.8 Sizing hybridization bands**

Bands on autoradiographs of probed Northern blots were sized using a standard curve prepared by plotting the log of the molecular weight of the RNA markers (Promega) versus the distance migrated on a semilog plot.

## **2.3.5 RT-PCR analysis**

### **2.3.5.1 cDNA synthesis**

cDNA was synthesized from RNA according to the instructions of the manufacturers of SuperScript<sup>TM</sup>II RNase H<sup>-</sup> Reverse Transcriptase (GibcoBRL) in 20  $\mu$ L reactions containing: First Strand Buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>], 10 mM DTT, 500  $\mu$ M dNTPs, 2  $\mu$ M Oligo dT(18) (Advanced Biotechnologies), 20 units Recombinant Rnasin<sup>®</sup> Ribonuclease Inhibitor (Promega), 2  $\mu$ L RNA (approximately 1-2  $\mu$ g), 200 units SuperScript<sup>TM</sup>II RNase H<sup>-</sup> Reverse Transcriptase (GibcoBRL) and DEPC-treated water. Negative controls for each RNA sample were prepared concurrently omitting SuperScript<sup>TM</sup>II RNase H<sup>-</sup> Reverse Transcriptase. Negative controls were PCR-amplified using the same primers, PCR conditions and number of PCR cycles as the experimental cDNA templates.

### **2.3.5.2 RT-PCR primer design**

Gene-specific RT-PCR primers 20 nucleotides long and with 50% G+C content (Table 4 Appendix II) were designed to generate a PCR product between 200-500 bp and were tested for complementarity to other DNA sequences in the SGD as described in section 2.3.4.3 for Northern probes. The upstream primer was designed to complement the non-coding strand and the downstream primer was designed to complement the coding strand.

### **2.3.5.3 RT-PCR reactions**

RT-PCR reaction mixes were prepared containing PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 2.5 units (5 U  $\mu$ L<sup>-1</sup>) Amplitaq<sup>®</sup> DNA Polymerase (Perkin Elmer). A 25  $\mu$ L aliquot of the reaction mix was added to 0.2 mL tubes containing 0.4  $\mu$ M each primer (Table 4 Appendix II) and 1  $\mu$ L of cDNA (prepared as described in section 2.3.5.3). PCR reactions were placed immediately in a pre-heated block for 3 minutes denaturation at 95°C followed by PCR cycles as follows:

3 min 95°C	}	18-40 cycles
30 sec 95°C		
30 sec 60°C		
45 sec 72°C		

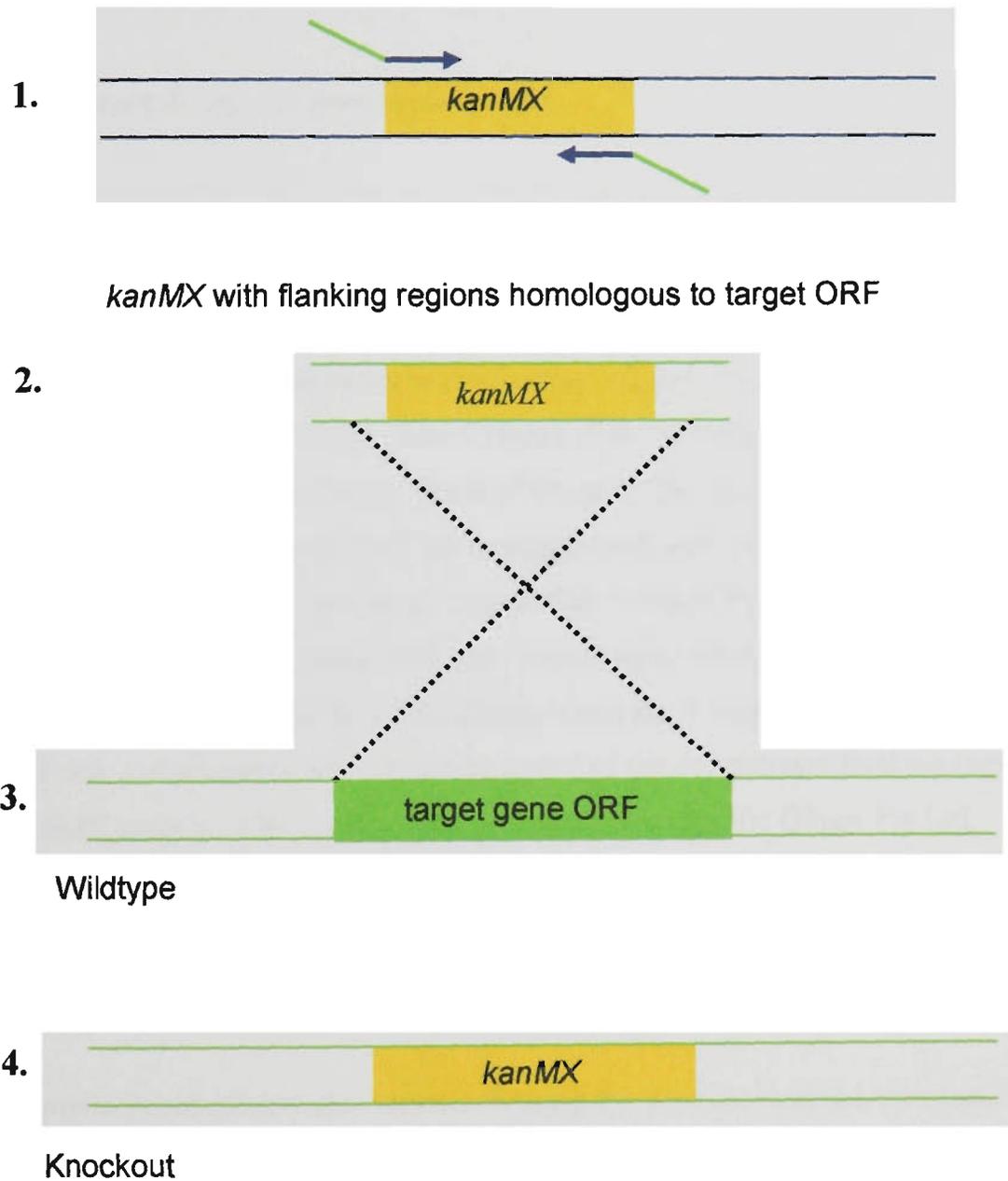
PCR reactions were cycled between 18 and 40 times depending on the level of expression of the target i.e. cDNA originating from abundant mRNAs required fewer PCR cycles to be visible on an ethidium bromide gel. However, it was important that reactions were not "over-amplified" to the point where PCR products had reached saturation, thus obscuring differences between samples. In order to determine the optimal number of cycles, an aliquot of each PCR reaction was removed for gel electrophoresis after around 20 cycles. After removal of the aliquot, the tubes were returned to the thermocycler for a further 2-4 cycles and another aliquot removed. This procedure was repeated until 40 cycles were completed. Each aliquot was stored on ice and all stored PCR products were prepared for gel electrophoresis at the same time.

#### **2.3.5.4 Gel electrophoresis of RT-PCR products**

A 100 mL 1.5% agarose gel was prepared by heating agarose with 1 x TBE buffer in a microwave oven set on High for 1 minute 20 seconds. Ethidium bromide to a final concentration of 0.1 µg mL<sup>-1</sup> was added prior to pouring. RT-PCR gel loading solution was added to all PCR products and they were loaded onto a 16 well gel. The samples that were cycled the greatest number of times were loaded first and the remainder loaded sequentially at 4 -20 minute intervals.

#### **2.3.5.5 Sequencing RT-PCR products**

To verify that the RT-PCR bands were derived from the appropriate gene, the PCR products were purified (Wizard) and sequenced as described previously in section 2.3.3.5 and identified using the BLASTn algorithm (Altschul *et al.* 1990) in SGD.



**Figure 2.2:** Strategy for replacement of a yeast gene ORF with the *kanMX4* marker. 1. The *kanMX* module was PCR-amplified using complementary primers that were tailed with sequences complementary to the flanking regions of the gene for removal. 2. PCR products were used for transformation of the wildtype and 3. replacement of the target gene occurred via homologous recombination to generate a knockout with a selectable marker, 4.

### 2.3.6 Gene replacement

A knockout strain was constructed using the *kanMX* module, conferring geneticin resistance according to the PCR-based protocol of Wach *et al.* (1994). The strategy for this gene replacement method is described diagrammatically in Figure 2.2.

#### 2.3.6.1 Primer design for gene replacement

Two primers of approximately 60 nucleotides were designed with 5' regions (approximately 40 nucleotides) complementary to the 5' and 3' regions of the ORF to be replaced (Table 5 Appendix II). The 3' regions of the primers (approximately 20 nucleotides) were complementary to the flanking regions of the *kanMX* module as published in Wach *et al.* (1994). The 5' region of the upstream primer was complementary to the non-coding strand of the gene ORF immediately downstream of the start codon and the 5' region of the downstream primer was complementary to the coding strand of the gene ORF in a 3' region that included the termination codon. The 3' region of the upstream primer was complementary to the upstream flanking region of the non-coding strand of the *kanMX* module and the 3' region of the downstream primer was complementary to the coding strand of the downstream flanking region of the *kanMX* module. The primers were purchased from Pacific Oligos Pty Ltd, Adelaide, South Australia.

#### 2.3.6.2 PCR-amplification of the *kanMX* module from pFA6-*kanMX4*

The upstream and downstream primers flanking the gene for removal were used for PCR amplification of the *kanMX* module from the pFA6-*kanMX4* plasmid.

Amplification of the *kanMX* module was carried out in 25  $\mu$ L volumes containing PCR buffer (Perkin Elmer), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 0.5  $\mu$ M each primer, approximately 20 ng plasmid DNA (pFA6-*kanMX4*), sterile water and 2.5 units Amplitaq (Perkin Elmer) added after the first denaturation step.

PCR amplification protocol: 2 min 92°C  
30 sec 92°C }  
30 sec 55°C } 28 cycles  
90 sec 72°C }  
4 min 72°C

The size of the PCR product was confirmed and quantified by electrophoresis on a 0.8% agarose gel.

### 2.3.6.3 Yeast transformation

Approximately 2.5 µg of the PCR product was used to transform yeast strain PMY1.1 by homologous recombination using the lithium acetate method of Gietz and Schiestl (1995). Cells from an overnight culture were inoculated to a cell density of  $5 \times 10^6$  cells mL<sup>-1</sup> (determined by haemocytometer counts) into YEPD medium and incubated aerobically at 30°C and 200 rpm until two doublings had occurred (approximately  $2 \times 10^7$  cells mL<sup>-1</sup>). A 500 µL aliquot of the culture was removed for plating as a cell viability control and the remainder divided into two 50 mL tubes and prepared for transformation. Cultures were centrifuged at 4800 rpm (2000 x g) at room temperature for 5 minutes, growth medium removed and the cells washed with 12.5 mL sterile water and centrifuged as previously. The water was removed and the cell pellets were resuspended in 0.5 mL of 100 mM lithium acetate, transferred to microfuge tubes and microfuged at 14,000 rpm for 15 seconds. The lithium acetate solution was removed and cells were resuspended in 500 µL of 100 mM lithium acetate. The transformation reaction was assembled by adding in order, to 50 µL of prepared cells: 240 µL PEG, 36 µL 1 M lithium acetate, 5 µL (10 mg mL<sup>-1</sup>) SS-DNA, 50 µL *kanMX* PCR product (approximately 2.5 µg) and vortexed vigorously for 1 minute. A transformation control was prepared in the same manner but sterile water was added instead of PCR products. Transformation reactions were incubated for 30 minutes at 30°C/25 rpm and heat shocked in a heating block at 42°C for 20 minutes. The transformation mixture was removed after microfuging the reactions for 15 seconds at low speed and the cells were gently resuspended in 1 mL sterile water and

plated in 200  $\mu$ L, 100  $\mu$ L and 20  $\mu$ L aliquots onto YEPD Geneticin plates for selection of transformants.

#### 2.3.6.4 Design of positioning primers to confirm gene replacement

Primers were designed for PCR reactions to target the flanking regions of the gene ORF that was replaced, to confirm the replacement ORF by the *kanMX* module (Table 6 Appendix II). The upstream primer was complementary to the non-coding strand of the ORF, approximately 100 nucleotides upstream of the start codon. The downstream primer was complementary to the coding strand, flanking the ORF approximately 100 nucleotides downstream of the termination codon. A primer targeting the non-coding strand of the *kanMX* module (Wach *et al.* 1994) and the above positioning primers were purchased from Pacific Oligos Pty Ltd, Adelaide, South Australia.

#### 2.3.7 Confirmation of gene replacement using colony PCR

Gene replacement and orientation of the *kanMX* module was verified by colony PCR using 3 primers targeting the 5' and 3' flanking regions of the replaced gene ORF and the *kanMX* module (Table 6 Appendix II). A small amount of cells from a selected colony carrying the *kanMX* gene replacement module was removed using a pipette tip and smeared into the bottom of a microfuge tube, microwaved on High for 1 minute and immediately placed on ice. The cells were resuspended in the following 20  $\mu$ L reaction mix on ice: PCR buffer (Perkin Elmer), 2 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 0.5  $\mu$ M of each positioning primer and 2.5 units Amplitaq<sup>®</sup> DNA Polymerase (Perkin Elmer) and sterile deionized MilliQ<sup>™</sup> water. PCR amplification was carried out under the following conditions: 4 min 94°C

60 sec 94°C	} 30 cycles
30 sec 55 °C	
90 sec 72°C	

The entire reaction was loaded onto a 1.5% agarose gel containing  $0.1 \mu\text{g mL}^{-1}$  ethidium bromide and the resulting gel bands were sized, gel purified and sequenced as previously described in section 2.3.3.5.

### **2.3.8 Southern analysis**

Southern analysis was used to confirm replacement of the target gene by the *kanMX* module in gene replacement constructs. By reference to the published sequence, restriction enzymes were selected that would digest yeast DNA in such a way as to generate a single band representing the replaced gene ORF in the control and a single band of a different size representing the *kanMX* module in the knockout construct.

#### **2.3.8.1 DNA isolation for Southern analysis**

Approximately 3  $\mu\text{g}$  of genomic DNA from the wildtype and knockout was prepared using the MasterPure™ Yeast DNA Purification Kit (Epicentre Technologies) following the manufacturers instructions. DNA was RNase-treated by the addition of DNase-free RNase to a concentration of  $10 \mu\text{g mL}^{-1}$  incubated at  $37^\circ\text{C}$  for 20 minutes and precipitated following the addition of  $\frac{1}{4}$  volume 10 M ammonium acetate and 1 volume of isopropanol and incubation at  $-80^\circ\text{C}$  overnight. Tubes were microfuged for 20 minutes at 14,000 rpm, pellets were washed with ice-cold 70% ethanol, microfuged 6 minutes at 14,000 rpm, air-dried at room temperature and resuspended in 30  $\mu\text{L}$  sterile distilled de-ionized water.

#### **2.3.8.2 Restriction enzyme digest of DNA**

Approximately 1-3  $\mu\text{g}$  of RNase-treated DNA from knockout and wildtype was digested in 20  $\mu\text{L}$  reactions using restriction enzymes with manufacturers buffers for 1 hour at  $37^\circ\text{C}$  followed by enzyme denaturation at  $65^\circ\text{C}$  for 10 minutes. For both wildtype and knockout the same enzymes were used in two single and one double digest, each enzyme or combination of enzymes was expected to cut out the wildtype target ORF or the *kanMX* module as a single band.

### 2.3.8.3 Gel electrophoresis and Southern blotting

Digest products from section 2.3.8.2 were resolved alongside a Promega 100 bp Ladder and a  $\lambda$ *HindIII/EcoRI* DNA marker on a 1% agarose TAE gel. After denaturation in 0.5 N NaOH and 1.5 M NaCl and neutralization in 0.5 M Tris-HCl and 3 M NaCl the DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) using the capillary transfer method described in Sambrook, Fritsch and Maniatis (1989). The blots were UV cross-linked, whilst damp, by 3 minutes exposure on a UV transilluminator, rinsed briefly in sterile water, airdried, sealed in a hybridization bag and stored at 4°C. Prior to placing the blots in sealed bags, the markers were cut from the blots and stained separately with methylene blue marker stain and stored moist in sealed hybridization bags at room temperature for use in sizing Southern bands following hybridisation.

### 2.3.8.4 Probe preparation

Duplicate blots were probed with either a probe specific to the target gene or a *kanMX*-specific probe. Probes for the target gene were prepared from purified DIG High Prime labeled RT-PCR products (section 2.3.5). The PCR products were cut from a 1% agarose TAE gel with a sterile scalpel blade and gel purified (Bandpure). Approximately 800 ng of DNA was random primed using 4  $\mu$ L DIG-High Prime mix (Boehringer Mannheim) in a 16  $\mu$ L final volume. DIG-High Prime mix contains random hexamers, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile-digoxigenin-11-dUTP 1 U  $\mu$ L<sup>-1</sup> labeling-grade Klenow enzyme, 5 x reaction buffer in 50% (v/v) glycerol. Reactions were denatured at 95°C for 10 minutes then incubated at 37°C overnight. The products were purified (Wizard) and the labeling efficiency tested using DIG colour detection quantification test strips.

The *kanMX* probe was prepared by PCR amplifying the *kanMX* fragment from the plasmid pFA6-*kanMX4* using DIG-labeled dUTP (Boehringer Mannheim). PCR reactions contained an appropriate volume of PCR buffer (10 x) (Perkin Elmer), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dATP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTPs, 130  $\mu$ M dTTP, 70  $\mu$ M Digoxigenin-11-dUTP, 0.5  $\mu$ M gene replacement primers (section 2.3.6.1),

approximately 40 ng plasmid DNA and sterile water made up to 50 $\mu$ L. The reactions were denatured at 92°C for 2 minutes, 5 units of AmpliTaq<sup>®</sup> DNA Polymerase (Perkin Elmer) were added to each tube and the reactions were PCR-amplified as follows:

30 sec 92°C	}	28 cycles
30 sec 55°C		
90 sec 72°C		
4 min 72°C		

The PCR products were used directly for probing Southern blots.

### **2.3.8.5 Southern hybridization**

The blot was pre-hybridized for 2 hours at 38°C in 25 mL of DIG Easy Hyb (Boehringer Mannheim) in a sealed hybridization bag placed flat in a hybridization oven with rocking platform set at 10. Approximately 1.5 ng of labeled probe was denatured at 98°C for 10 minutes and added to the blot in 25 mL fresh DIG Easy Hyb in a hybridization bag and hybridized overnight under the same conditions as for the pre-hybridization. The blot was washed twice for 5 minutes in 2 x SSC with 0.1% (v/v) SDS then twice for 15 minutes in 0.5 x SSC with 0.1% (v/v) SDS at room temperature.

### **2.3.8.6 Southern Hybridization detection, autoradiography and sizing bands**

Hybridization was detected by first equilibrating the blot for 2 minutes in DIG washing buffer in a sterile container on a gently rocking platform, then blocking in DIG blocking buffer for 30 minutes. The blot was then exposed to DIG Anti-Digoxigenin-AP antibody in DIG antibody solution for 30 minutes, washed twice for 15 minutes in DIG washing buffer then equilibrated in DIG detection buffer for 2 minutes. The drained blot was covered with DIG CSPD<sup>®</sup> diluted 1:50 in 1 mL DIG detection buffer and sealed in a hybridization bag after air bubbles had been removed by wiping the bag gently with a laboratory tissue. The blot was incubated for 15 minutes at 37°C then placed in an X-ray cassette for exposure of Kodak BioMax

Scientific Imaging film for 20 seconds to 2 hours at room temperature and developed as described in section 2.3.3.3. The probes were later stripped from the blots by a brief wash in sterile water followed by two 20 minute washes at 37°C in 0.2 M NaOH with 0.1% (v/v) SDS. Blots were re-probed under the same conditions using the second DIG labeled probe. A semi log standard curve of the log molecular weight of the molecular weight marker versus the mobility of the marker bands, was used to determine the Southern hybridisation band sizes.

### **2.3.9 Plasmid transformation of knockout and wildtype yeast strains**

Approximately 500 ng of plasmid DNA from the GeneStorm™ Expression Vector pYES2/GS (Invitrogen) containing a yeast gene ORF was transformed into the yeast PMY1.1 knockout strain using the Gietz and Schiestl (1995) lithium acetate protocol. This strain had a single gene replaced with the *kanMX* marker gene. A similar amount of plasmid DNA from the GeneStorm™ Expression Vector pYES2/GS (Invitrogen), without insert, was also transformed into the PMY1.1 knockout as a control strain. Transformants were selected for their ability to grow without uracil on 1.5% agarose minimal media plates composed of yeast nitrogen base without amino acids and ammonium sulfate, 5% (w/v) ammonium sulfate, 2% (w/v) D-glucose, 40 mg mL<sup>-1</sup> leucine and histidine. The transformations were confirmed by colony PCR (section 2.3.7) using four primers targeting both the ORF of the replaced gene and *kanMX* marker (Table 6 Appendix II). The PCR products were separated on a 1% agarose gel and the bands were sized, removed for sequencing as described in section 2.3.3.5 and verified using the BLASTn algorithm of Altschul *et al.* (1990) in SGD.

### **2.3.10 Plasmid transformation of *E. coli***

Cloning of vectors used for work described in this thesis was performed as follows: plasmids were transformed into *E. coli* One Shot™ TOP10F' competent cells (Invitrogen) using the suppliers instructions and controls. To 50 µL of gently mixed competent cells thawed on ice in microfuge tubes, 2 µL of 0.5 M β-mercaptoethanol and 2.5 - 20 ng (1-5 µL) of plasmid DNA were added and the tubes gently tapped to mix. The cells were stored on ice for 30 minutes then incubated at 42°C for exactly

30 seconds and replaced on ice for 2 minutes. Room temperature SOC medium (50  $\mu\text{L}$ ) (Invitrogen) was added to the cells and the resultant cell suspension was incubated in microfuge tubes secured and lying on their sides in on orbital shaker at 37°C/225 rpm for 1 hour then placed on ice. Aliquots of 50  $\mu\text{L}$  and 100  $\mu\text{L}$  of the transformation mixtures were spread on LB-Amp plates and incubated at 37°C overnight. Single colonies of transformants growing in the presence of ampicillin were transferred to 2 mL of Luria Bertani broth containing ampicillin (150  $\mu\text{g mL}^{-1}$ ) and incubated at 37°C/225 rpm overnight.

### **2.3.11 Western analysis**

#### **2.3.11.1 Protein preparation**

Cells from 50 mL yeast cultures were pelleted in Falcon tubes by centrifugation at 4800 rpm for 4 minutes, supernatants were removed and the pellets stored at -20°C until used for protein analysis. Cell pellets were resuspended in 300  $\mu\text{L}$  0.1 M NaOAC with 300  $\mu\text{L}$  of 0.4  $\mu\text{m}$  glass beads and vortexed vigorously for 4 x 1 minute with 1 minute on ice following each vortex step. Cells were then pelleted and the supernatants transferred to fresh tubes.

#### **2.3.11.2 Determination of protein concentration using the Lowry assay**

The amount of protein in 100  $\mu\text{L}$  of the supernatant in section 3.3.11.1 was determined by Lowry assay (Lowry *et al.* 1951). A standard curve was drawn following assay of BSA standards prepared from 0.1% stocks in 0, 10, 20, 30, 40 and 50  $\mu\text{g}$  amounts in microfuge tubes to a total volume of 250  $\mu\text{L}$ . Four-fold serial dilutions of the protein samples were assayed in a total volume of 250  $\mu\text{L}$  at the same time.

### **2.3.11.3 Protein de-glycosylation**

Prior to separation by electrophoresis, approximately 300-500  $\mu\text{g}$  of protein was denatured at 95°C for 5 minutes with 0.27 M NaOAC, 0.5% SDS and 0.5%  $\beta$ -mercaptoethanol and de-glycosylated by incubation in a 37°C waterbath for 40 hours with 60 units of Endoglycosidase H (Boehringer-Mannheim).

### **2.3.11.4 SDS-PAGE protein gel analysis**

SDS PAGE mini gels (Bio-Rad Mini-Protean II), were prepared in pairs using the discontinuous system of Laemmli (1970). Resolving gels of 12% acrylamide were prepared by mixing 3.8 mL water, 4 mL 30% acrylamide stock (29.1% (w/v) acrylamide, 0.9% (w/v) N-N-methylenebis-acrylamide) (BioRad), 2 mL 1.875 M Tris-HCL (pH 6.8), 50  $\mu\text{L}$  10% (w/v) ammonium persulphate, 150  $\mu\text{L}$  10% (w/v) SDS and 4  $\mu\text{L}$  TEMED™. The gel solution was quickly poured between the assembled glass plates and the top of the gel was covered with N-butanol and water (1:1). Gels were allowed to set for 1 hour and the water and N-butanol was poured off. Stacking gels of 5% acrylamide were prepared by mixing 4.26 mL water, 0.66 mL 30% acrylamide (BioRad), 0.5 mL 1.25 M Tris-HCl (pH6.8), 20  $\mu\text{L}$  10% (w/v) ammonium persulphate, 50  $\mu\text{L}$  10% (w/v) SDS and 5  $\mu\text{L}$  TEMED™ and were pipetted onto the top of the resolving gel. An 8 well comb was immediately placed in the gel and the gel covered with plastic wrap and set overnight at room temperature. Protein samples (10  $\mu\text{L}$  or approximately 20  $\mu\text{g}$  of protein) combined with 10  $\mu\text{L}$  of loading buffer were denatured for 5 minutes in microfuge tubes in boiling water and loaded onto the gels, accompanied by BioRad prestained broad range markers (28,400-212,000 Dalton). The proteins were resolved by electrophoresis in a mini gel apparatus (BioRad) containing PAGE electrode buffer, at 15 mA for 1 hour 45 minutes.

### **2.3.11.5 Electrophoretic transfer and Western blotting**

After electrophoresis PAGE gels were removed from the glass plates and the stacking gels discarded. Each gel was assembled in a transfer sandwich composed of 3 sheets

of 3M filter paper pre-wet with transfer buffer and cut to the size of the gel, the gel (face down), nitrocellulose membrane (BioRad Trans-Blot Transfer Medium) pre-wet with buffer and another 3 sheets of pre-wet 3M filter paper. Two assembled sandwiches were clamped into the transfer apparatus containing transfer buffer and the proteins were electrophoretically transferred to the nitrocellulose membrane overnight. The transfer apparatus was run at 15 V and 100 mA at 4°C. The transfer sandwiches were disassembled and the membranes removed and blocked by incubating in TBS buffer with 1% (w/v) gelatin on a rocking platform for 1 hour at room temperature. The blots were washed twice for 5 minutes in TBST and incubated in blocking buffer with 4 µL (1:5000) of Anti-V5-HRP Antibody (Invitrogen) on a rocking platform for 2 hours at room temperature. The blots were washed twice for 5 minutes in TBST and once for 5 minutes in TBS on a rocking platform prior to detection.

#### **2.3.11.6 Detection of target protein**

The target protein was detected using a Super Signal<sup>®</sup> West Pico detection kit (Pierce). Blots were incubated in Super Signal<sup>®</sup> West Pico substrate working solution for 5 minutes, removed and placed in plastic wrap. After approximately 20 seconds – 2 hours the signal could be detected by exposure of BioMax MS<sup>™</sup> (Kodak) scientific imaging film for 20 – 40 seconds. The film was developed as described in section 2.3.3.3.

# CHAPTER 3

## YEAST GROWTH DURING ETHANOL STRESS

### 3.1 INTRODUCTION

The production and accumulation of ethanol by *S. cerevisiae* during beer and wine fermentations has an inhibitory effect on its growth and metabolism which eventually halts further ethanol production. To better understand the mechanisms of ethanol inhibition, the objectives of this project were to subject a *S. cerevisiae* laboratory strain to a non-lethal ethanol stress and identify genes that are specifically up-regulated during the period of adaptation to the stress. It was important in these experiments that the ethanol stress was non-lethal so that it did not completely inhibit energy production and transcription, yet it needed to be sufficiently high to incite a period of adaptation by the cells to their changed environment. At a physiological level, such adaptation periods in yeast cultures are recognized by a growth lag phase in which cell division is temporarily halted until the cells make sufficient structural and metabolic changes for growth to commence. Therefore, before studying gene expression in yeast during ethanol stress, a physiological investigation into the growth response of the cells to various levels of ethanol was required to determine a set of environmental conditions that would induce a non-lethal, ethanol-induced lag period in the experimental cultures. A lag period of around three to five hours would make it logistically possible for a representative number of samples to be taken during the adaptation period.

With the above criteria in mind, the aims of this chapter were to:

1. choose a suitable *S. cerevisiae* strain i.e. a haploid laboratory strain that grows well in rich medium, is robust and intrinsically ethanol tolerant,
2. develop an experimental protocol for inducing a reproducible and clearly defined growth lag period in yeast caused by ethanol stress,
3. determine an ethanol concentration that is non-lethal to the yeast cells, yet causes sufficient stress to induce a three to five hour lag period in growth.

## 3.2 ETHANOL STRESS CONDITIONS AND STRAIN SELECTION

Traditionally, the lag phase is recognized as the non-growth period that occurs immediately following the inoculation of cells into fresh medium, representing a period of adaptation by the cells to their changed environment prior to growth. Cells inoculated into a fresh nutrient-rich growth medium that contains a non-lethal, but stressful, concentration of added ethanol would in theory need to adapt differently (e.g. express different genes) to cells from the same parent culture inoculated into fresh nutrient-rich medium without ethanol (control). Ethanol-stressed cultures and unstressed cultures would both express genes needed for adaptation to the new growth medium, but only the ethanol-stressed cultures would need to express genes specifically in response to the presence of ethanol. The premise being that these differentially expressed genes have a role in the adaptation by the cells to the ethanol stress.

Based on the above principles, an experimental protocol was developed for the physiological experiments in this project to generate ethanol-stressed and unstressed yeast cells. In general, ethanol-stressed yeast cells were generated in batch cultures using cells from unstressed overnight cultures that were inoculated into fresh medium containing a non-lethal amount of ethanol. Yeast cells from the same overnight parent culture were also inoculated into fresh medium without added ethanol, comprising the control culture. In this context, experimental cultures were deemed to be ethanol-stressed when they exhibited an extended lag period compared to unstressed control cultures.

### 3.2.1 Selection of yeast strains

#### 3.2.1.1 Growth of *S. cerevisiae* strains PMY1.1 and SUB61

Two laboratory strains of *S. cerevisiae* were considered for this project, PMY1.1 and SUB61. These strains had previously been used to investigate the role of ubiquitin in the yeast stress response by Dr Yaping Chen in the laboratory of Dr Peter Piper, Department of Biochemistry and Molecular Biology, University College, London, England. A series of tests were conducted on the two strains to compare

their growth profiles in the absence and presence of ethanol. Cells from overnight cultures of the two strains were inoculated into YEPD medium to an OD<sub>620</sub> of around 0.05 and incubated at 30°C/ 200 rpm. The growth of PMY1.1 and SUB61 cultures was monitored by OD<sub>620</sub> readings and plate counts at regular intervals until the cultures entered the stationary phase (Figure 3.1). Both strains grew similarly without a measurable lag period under these conditions.

### **3.2.1.2 Effect of ethanol on the growth rate and doubling time of strains**

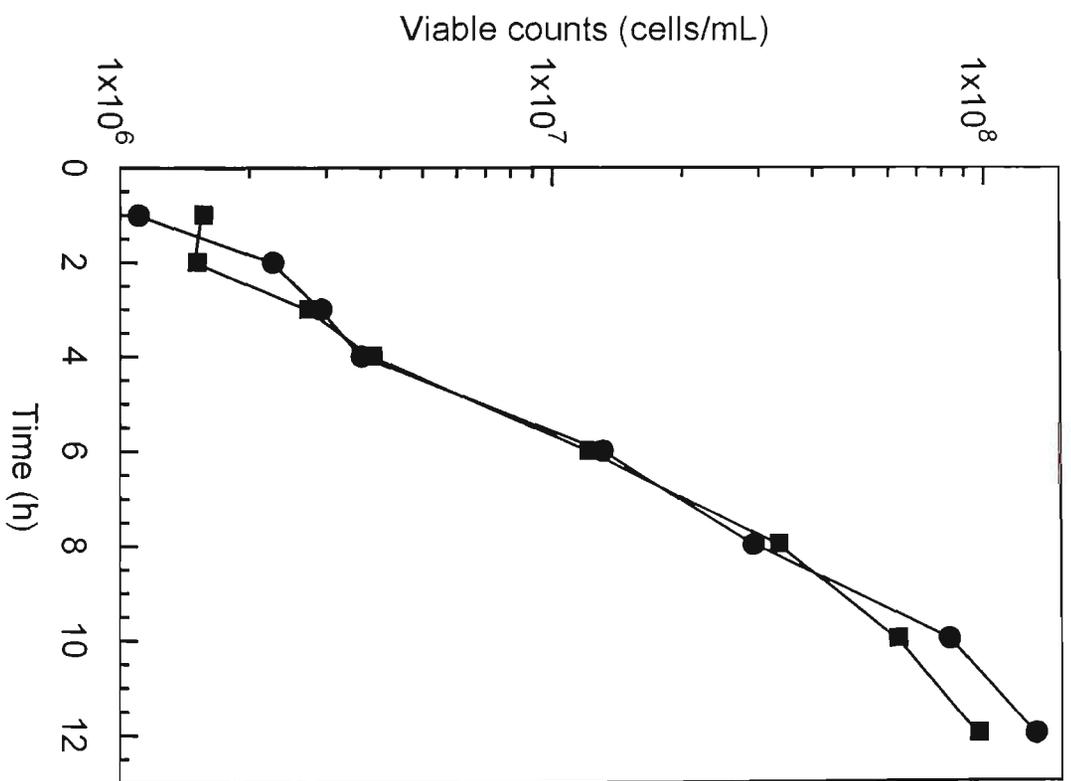
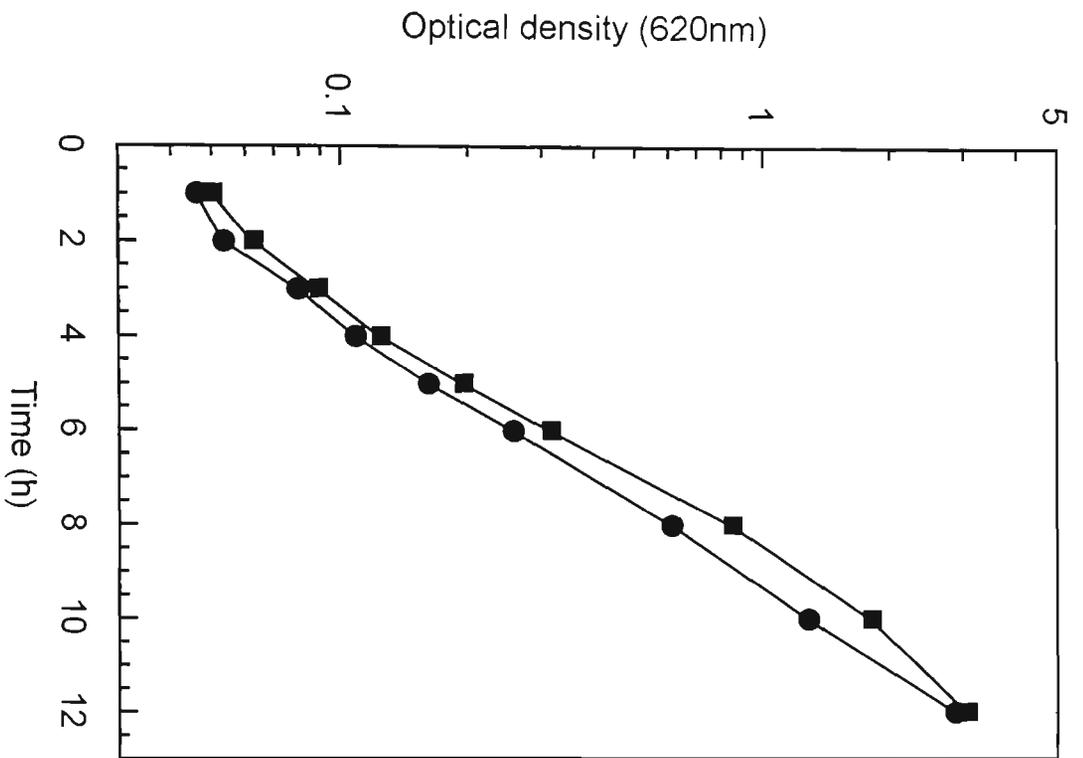
#### **PMY1.1 and SUB61**

To determine the ability of strains PMY1.1 and SUB61 to recover from a non-lethal step change in ethanol concentration, the two strains were inoculated from an overnight culture into fresh YEPD medium containing an ethanol concentration of 4% (v/v) (Figure 3.2). An ethanol concentration of 4% (v/v) was used in this case since it has previously been shown to induce a 4-5 hour lag period in *S. cerevisiae* (Stanley *et al.* 1997). Specific growth rates and doubling times were calculated from viable count data (Table 3.1).

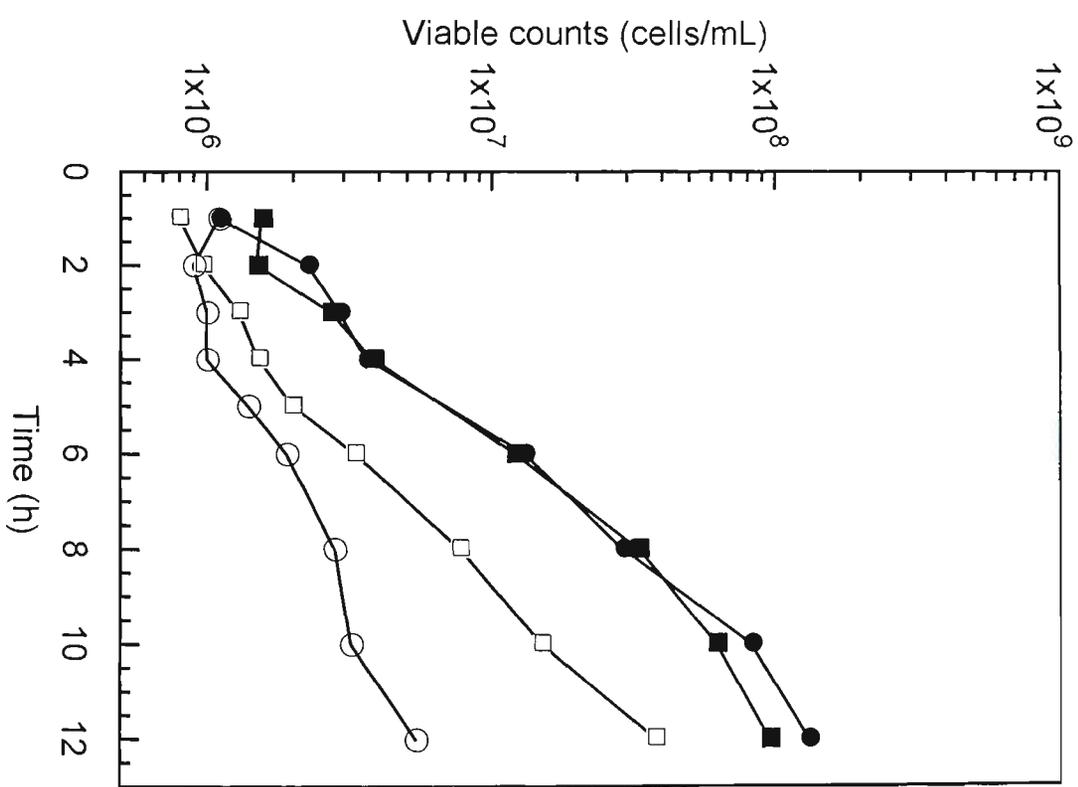
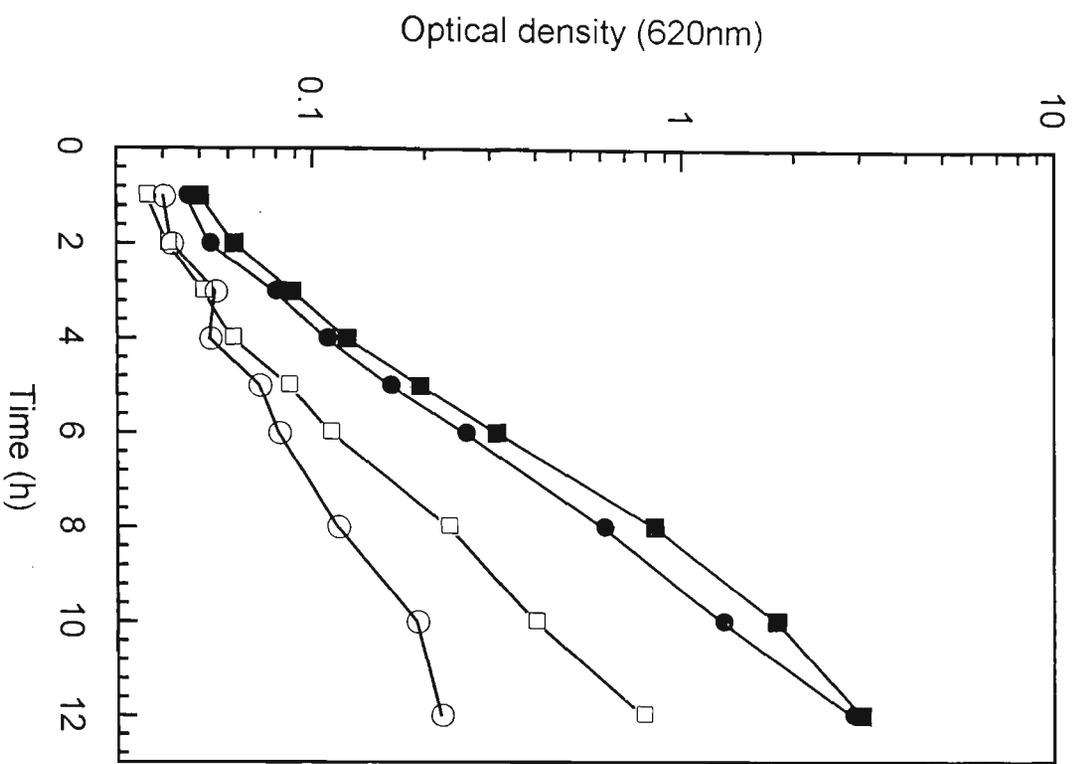
The control cultures without ethanol for both strains had a specific growth rate of 0.5 h<sup>-1</sup> and doubling times of 2 hours. The specific growth rate and doubling time of strain PMY1.1 grown in the presence of 4% (v/v) ethanol was 0.4 h<sup>-1</sup> and 2.47 hours respectively. By comparison, strain SUB61 had a specific growth rate of 0.18 h<sup>-1</sup> and doubling time of 5.46 hours in the presence of 4% (v/v) ethanol. These results suggest that strain PMY1.1 is better able to adapt and recover from ethanol stress than strain SUB61 and for this reason strain PMY1.1 was chosen as the strain to be used for the experimental work in this project.

### **3.2.2 Effect of ethanol concentration on the lag period of strain PMY1.1**

*S. cerevisiae* strain PMY1.1 was considered to be more suitable for ethanol stress experiments than strain SUB61 because of its higher level of intrinsic ethanol tolerance. To determine the most suitable ethanol concentration for the stress experiments in this project, strain PMY1.1 was investigated in the presence of a range



**Figure 3.1:** Growth of *S. cerevisiae* strains PMY1.1 (■) and SUB61 (●) in YEPD medium. Cells from overnight parent cultures were inoculated into YEPD medium and incubated at 30°C/200 rpm.



**Figure 3.2:** Effect of 4% (v/v) ethanol on the growth of *S. cerevisiae* strains PMY1.1 (■, □) and SUB61 (●, ○). Cells from overnight parent cultures were inoculated into YEPD medium only (closed symbols) or YEPD medium containing 4% (v/v) ethanol and incubated aerobically at 30°C/200 rpm.

STRAIN	ETHANOL % (v/v)	GROWTH RATE (h <sup>-1</sup> )	GENERATION TIME (h)
PMY1.1	0	0.5	2
	4	0.4	2.47
SUB61	0	0.5	2
	4	0.18	5.46

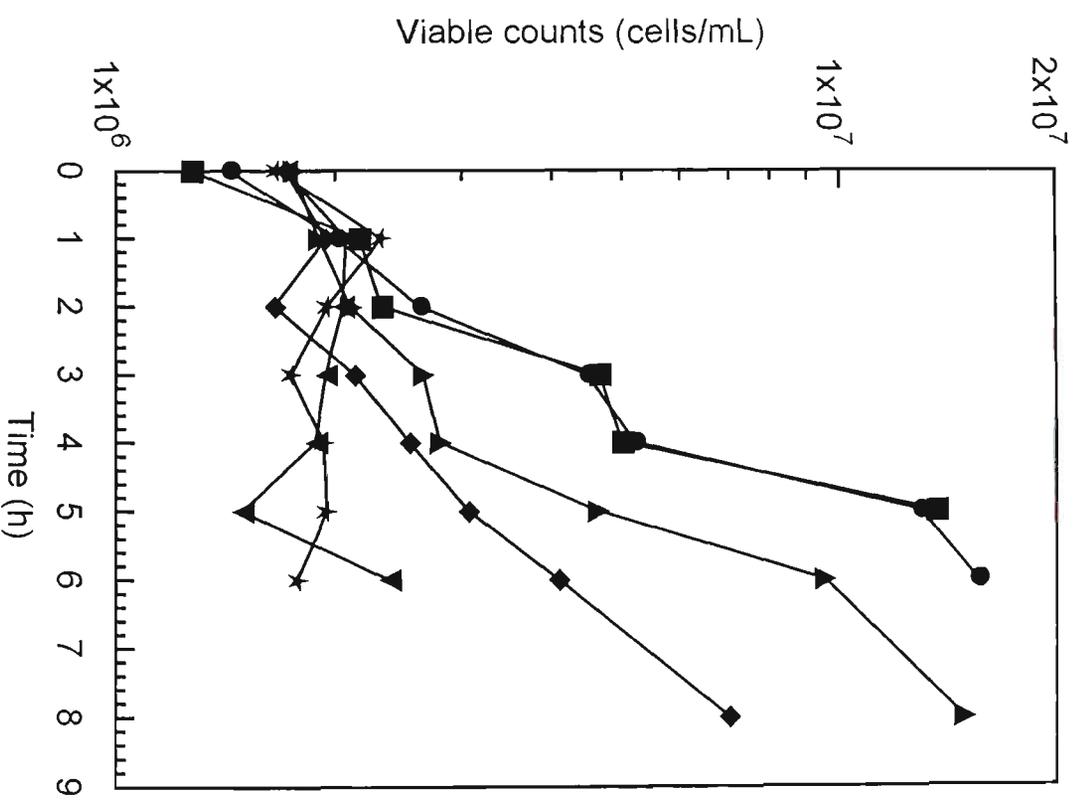
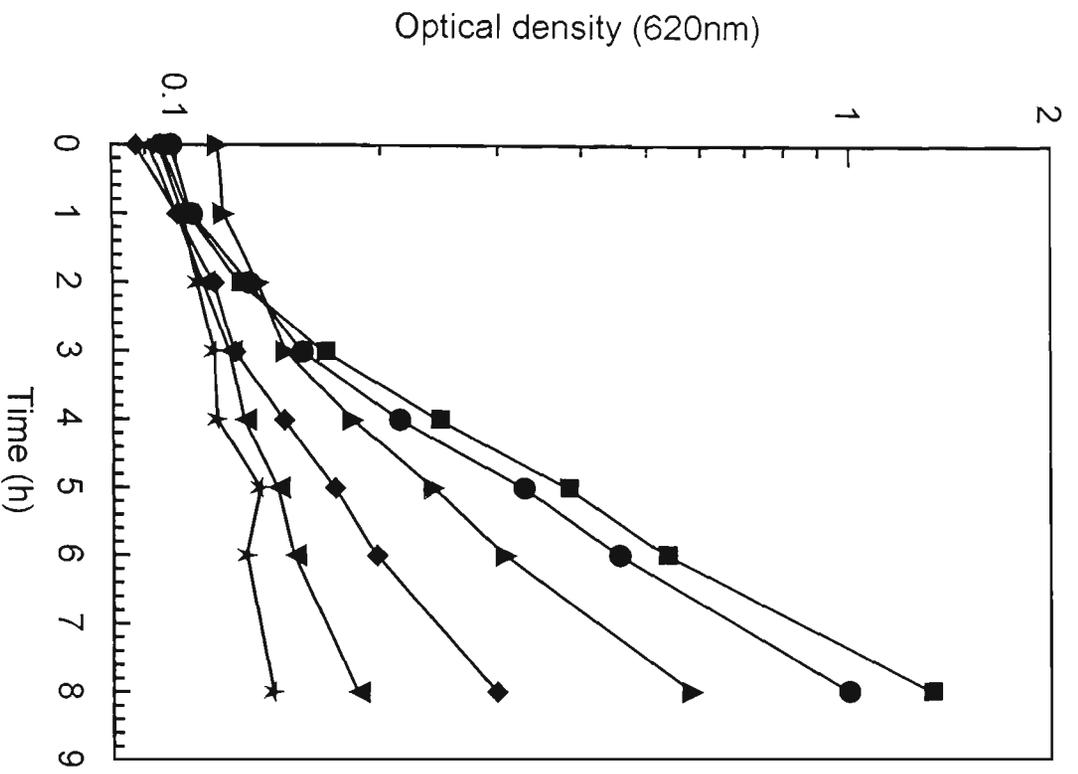
**Table 3.1:** Effect of 4% (v/v) exogenous ethanol on the specific growth rates and generation times of *S.cerevisiae* strains PMY1.1 and SUB61 in YEPD medium.

of ethanol concentrations. The ideal concentration being that which induced a non-lethal lag period of around three to five hours, thus allowing sufficient time for the collection of a suitable number of samples during the adaptation period. A further condition being that after adapting to the ethanol, the stressed cultures should be able to achieve a reasonable exponential growth rate.

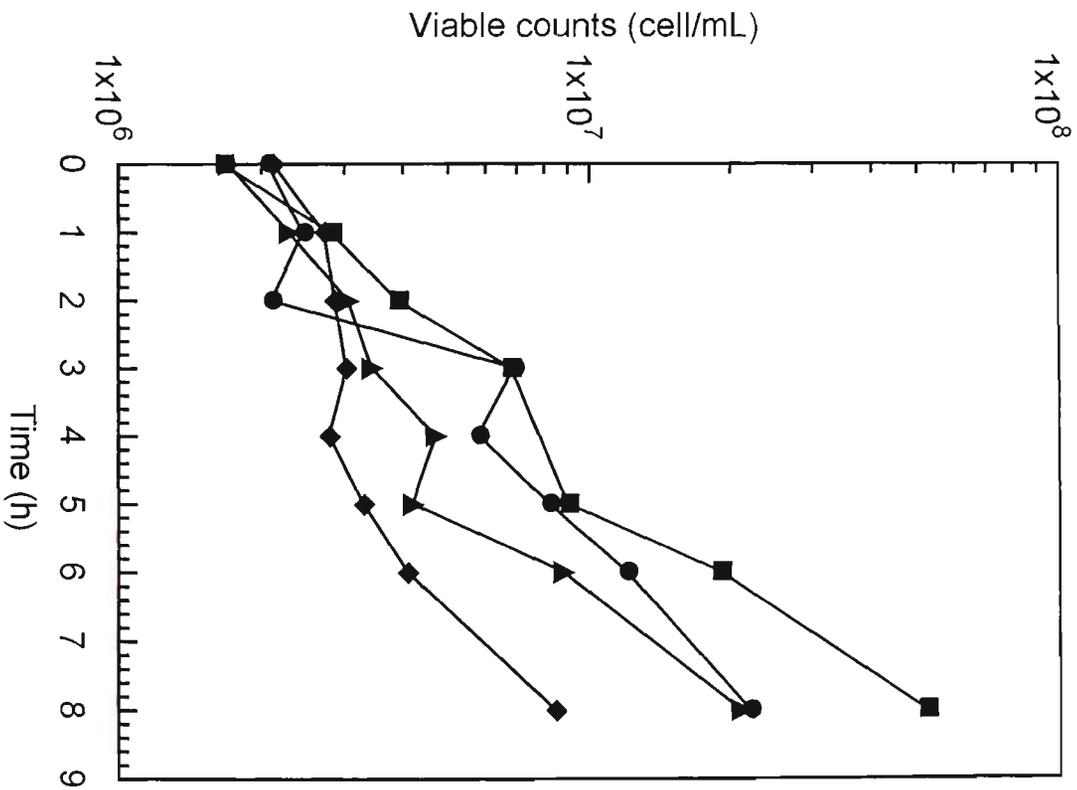
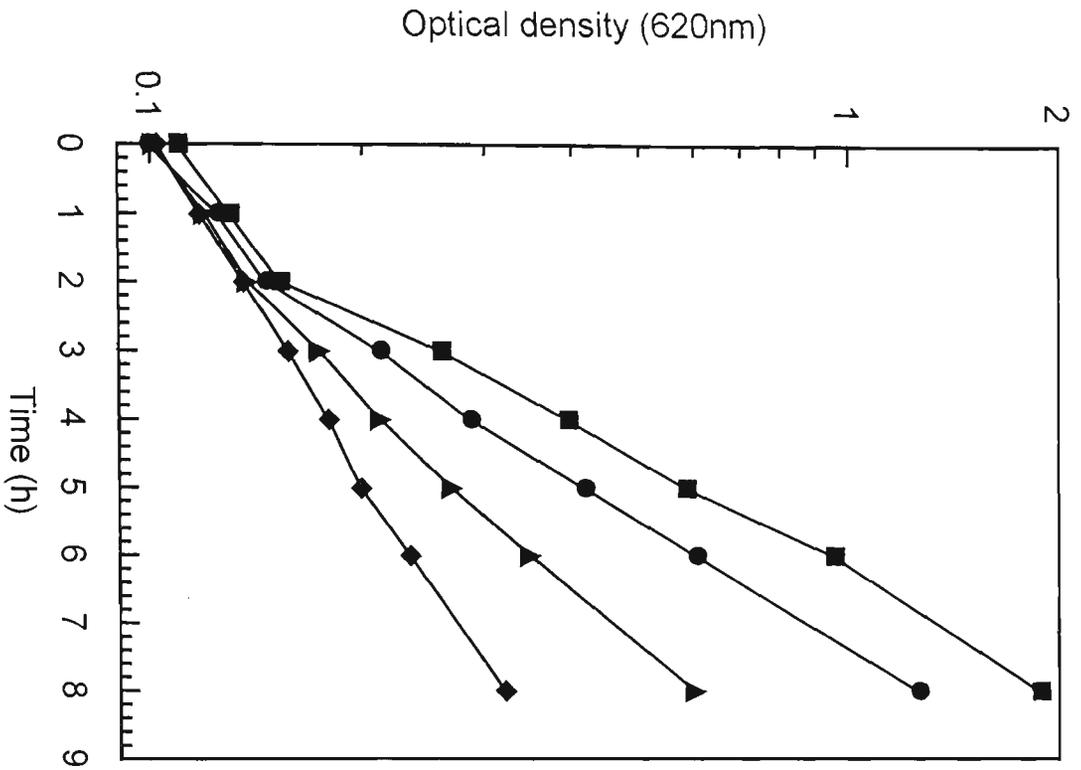
Preliminary experiments with *S. cerevisiae* PMY1.1 indicated that ethanol concentrations in the range 2-10% (v/v) would induce a lag period without being lethal (data not shown). These experiments were refined to better characterize the length of the lag period following inoculation of strain PMY1.1 into YEPD medium containing various ethanol concentrations.

Strain PMY1.1 was grown in the presence of 2%, 4%, 6%, 8%, or 10% (v/v) ethanol and its growth compared to a control culture without ethanol (Figure 3.3). In a second experiment, strain PMY1.1 was grown in the presence of 3%, 5% and 7% (v/v) ethanol and its growth compared to a control without ethanol (Figure 3.4). The culture lag period, specific growth rate and generation times (Table 3.2) were calculated as shown in Figures 3 and 4, Appendix III. Cultures subjected to an ethanol stress below 4% did not have a sufficiently long lag period for the collection of a sufficient number of samples for RNA preparations during the adaptation phase (sample collections being approximately 1 hour apart). On the other hand, cultures containing 7% ethanol or greater had considerably longer lag periods but could only manage low specific growth rates once the cells had adapted to the ethanol stress. Cultures grown in the presence of 4%, 5% and 6% (v/v) ethanol fulfilled the requirement to induce a lag period of around 3-5 hours.

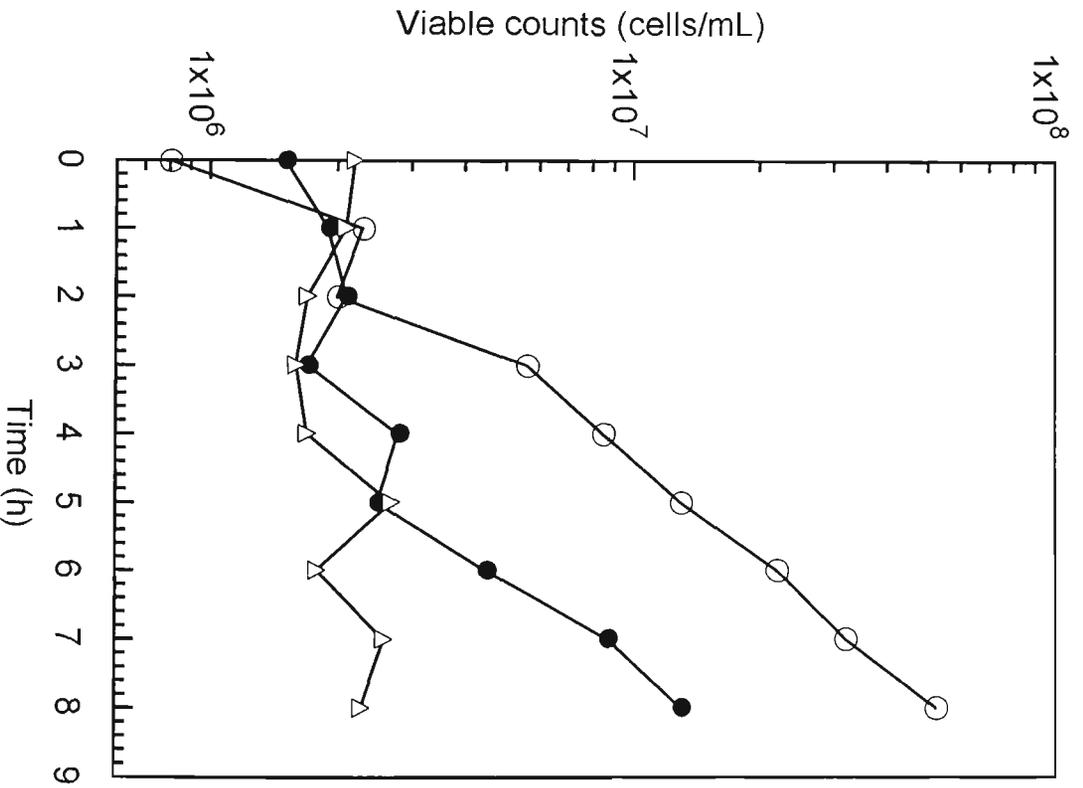
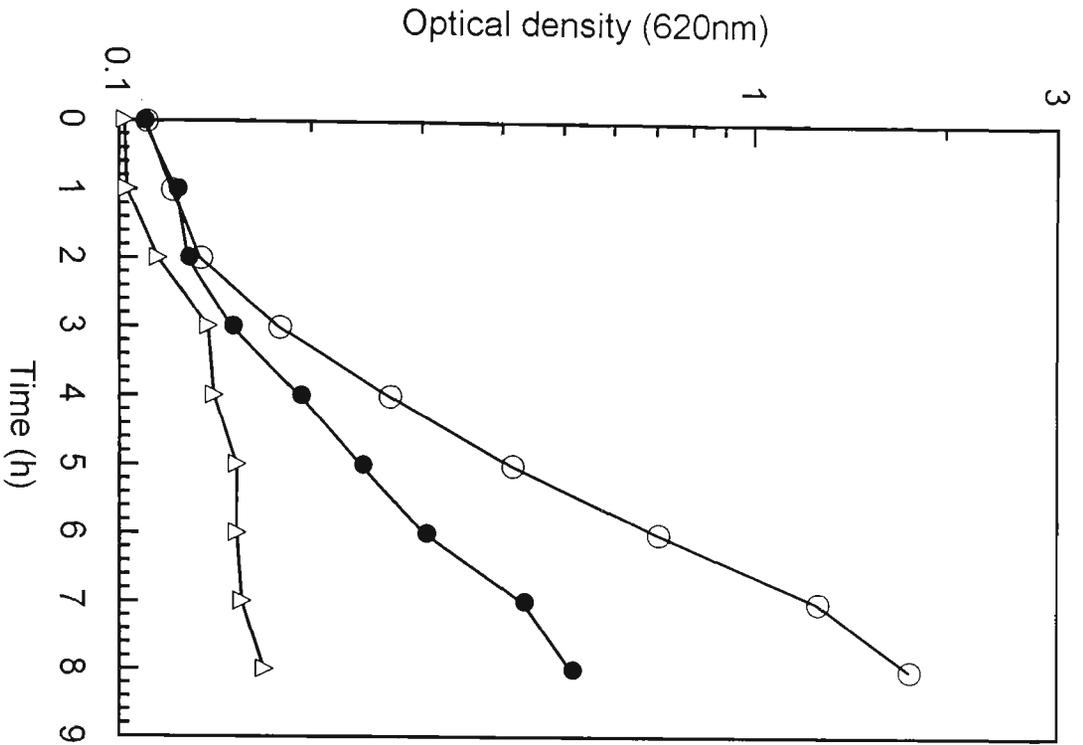
Given the above, *S. cerevisiae* PMY1.1 cells inoculated into YEPD medium containing 5% ethanol was thought to provide sufficient stress for a 3-5 hour lag period, yet permit the cells to grow at a reasonable specific growth rate once adapted to the stress. This was verified in a subsequent experiment where YEPD medium containing 0%, 5% and 10% ethanol was inoculated with strain PMY1.1 from the same overnight culture (Figure 3.5). The culture lag period, specific growth rate and generation time (Table 3.2) was calculated as shown in Figure 2, appendix III. In all



**Figure 3.3:** Effect of 2-10% (v/v) ethanol concentration on the growth of *S. cerevisiae* PMY1.1. Cells from an overnight parent culture were inoculated into YEPD medium only (■) or YEPD medium containing 2% (●), 4% (▲), 6% (◆), 8% (▼) or 10% (★) (v/v) ethanol and incubated aerobically at 30°C/200 rpm.



**Figure 3.4:** Effect of 3-7% (v/v) ethanol on the growth of *S. cerevisiae* PMY1.1. Cells from an overnight parent culture were inoculated into YEPD medium (■) or YEPD medium containing 3% (●), 5% (▲) or 7% (◆) (v/v) ethanol and incubated aerobically at 30°C/200 rpm.



**Figure 3.5:** Effect of 5% and 10% (v/v) ethanol on the growth of *S. cerevisiae* PMY1.1. Cells from an overnight parent culture were inoculated into YEPD medium (O) or YEPD medium containing 5% (●) or 10% (Δ) (v/v) ethanol and incubated aerobically at 30°C/200 rpm.

ETHANOL (%)	LAG PERIOD (h)	GROWTH RATE (h <sup>-1</sup> )	GENERATION TIME (h)
EXPERIMENT 1			
0	0.75	0.38	2.64
2	0.75	0.38	2.64
4	2.5	0.35	2.9
6	3.5	0.3	3.35
8	5	0.22	4.6
EXPERIMENT 2			
0	0.75	0.39	2.6
3	1.5	0.35	2.8
5	2.25	0.34	2.9
7	3.6	0.32	3.3
EXPERIMENT 3			
0	1	0.45	2.24
5	4	0.42	2.4

**Table 3.2:** Effect of ethanol concentration on the lag period, growth rate and generation time of *S. cerevisiae* PMY1.1 in YEPD medium.

three cultures the population of viable cells either increased or was constant, suggesting cell viability was not compromised by even a 10% step change in ethanol concentration. Contrary to the culture containing 10% ethanol, the culture containing 5% ethanol was able to adapt sufficiently to the stress in around 4 hours for exponential growth to commence with a specific growth rate only slightly below that of unstressed cultures (Table 3.2). A step change in ethanol concentration of 5% (v/v) was therefore chosen as the stress protocol for future experiments.

### **3.2.3 The relationship between optical density and viability of cells during lag phase**

Optical density readings measuring the turbidity of cultures do not fully reflect the growth response of the cells. This is particularly an issue for the ethanol-stressed cultures since the lag phase is not properly characterized by optical density readings. For example, between 2 and 5 hours post-inoculation, the growth rate of the culture grown in 5% (v/v) ethanol appeared to be greater according to slope of the optical density plot compared to the corresponding cell viability data determined by plate counts (Figure 3.5). Since the plate counts show that cell division had not yet occurred during this period, the discrepancy was thought to be due to an increase in optical density readings as cells increased in size and formed buds prior to cell division. Thus, although optical density readings provide immediate results, they cannot distinguish between an increase in cell size and cell division. Such readings were considered inadequate for accurately measuring the cell population, especially where cell division is arrested during the stress adaptation period. While plate counts provide measurements of viable cell numbers, they are time consuming to prepare, which is logistically difficult given the sampling time frame of the experiments, and it takes around 48 hours to obtain the results.

The approach to measuring cell population in this thesis combined the advantages of both optical density and viable plate techniques thus overcoming their respective shortfalls. Correlation curves relating optical density readings of a culture to the respective cell population were experimentally established. The benefit of correlation charts is that optical density readings could be measured as the experiment progressed

and, using the correlation charts, such measurements could then provide a good estimate of the viable cell population.

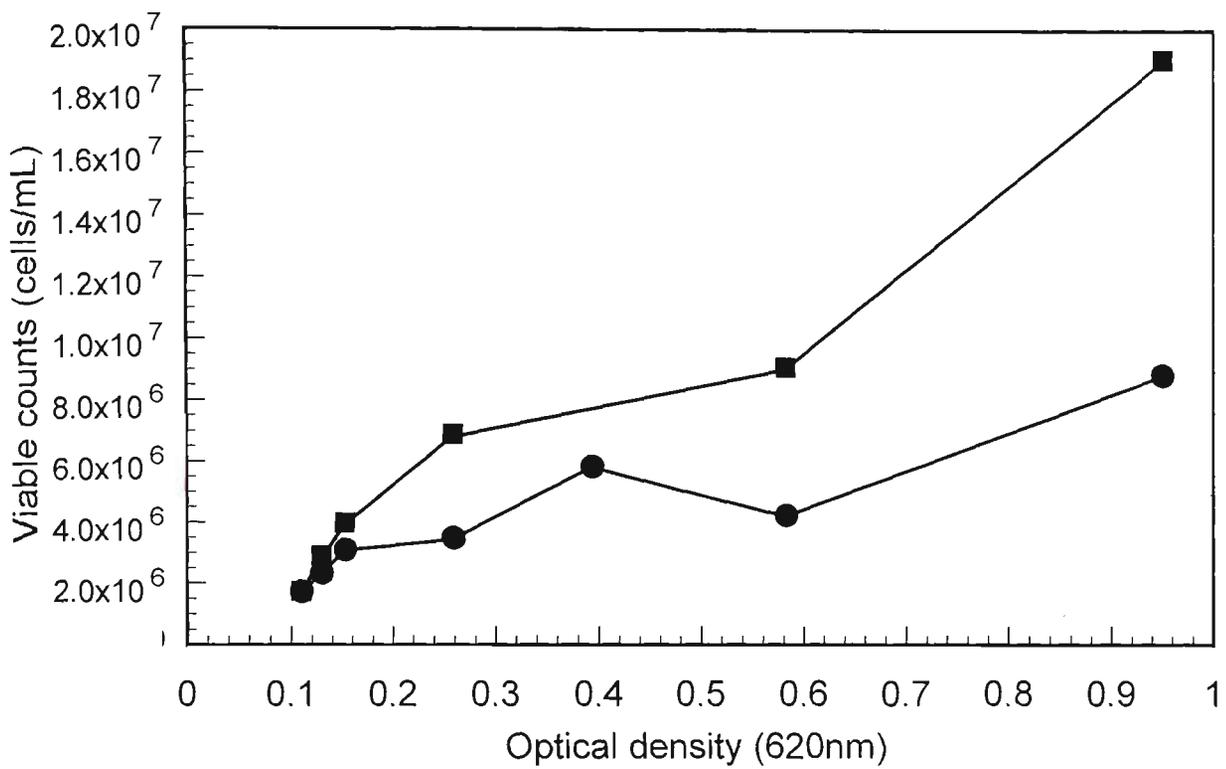
The relationship between optical density and cell number as depicted by the correlation curves, was not the same for ethanol-stressed cultures and the unstressed (control) cultures (Figure 3.6). This was problematic since optical density readings were used as a basis for estimating cell number when harvesting cells for RNA isolation. Therefore, combined data from three previous growth experiments, for each set of culture conditions, were used to prepare separate correlation curves for unstressed and stressed (5% ethanol) cell cultures relating optical density and viability (Figure 1, Appendix III). The correlation curves were used to directly estimate viable cell numbers from optical density readings during growth experiments in order to obtain cell pellets containing around  $10^8$  cells for RNA isolation.

### **3.3 DISCUSSION**

#### **3.3.1 Effect of ethanol on the growth and metabolism of *S. cerevisiae***

The experiments described in this chapter demonstrate that exposure to critical ethanol concentrations has a negative effect on yeast growth as reported many times by other workers, see reviews by Casey and Ingledew (1986); D'Amore *et al.* (1990); Ingram and Buttke (1984); Jones (1989); Mishra (1993); Thomas and Rose (1979). *S. cerevisiae* is a highly ethanol-resistant organism, able to grow in concentrations of 8-12% (v/v) ethanol and survive exposure to 15% (v/v) (Ingram and Buttke 1984) however, this tolerance is strain dependent. For example, in a recent study of 3 industrial wine strains grown for 2 hours in the presence of 10% ethanol, there was a 25% reduction in viability in one strain whilst the other three continued growing, although at a reduced rate (Ivorra *et al.* 1999).

Since the aim of this project was to identify ethanol-stress-related genes in *S. cerevisiae*, it was important from the outset to work with a yeast strain that had reasonable tolerance to ethanol. For this reason two laboratory strains SUB61 and PMY1.1 were tested for ethanol tolerance. Strain PMY1.1 proved to have a higher



**Figure 3.6:** Relationship between optical density and viable counts when *S. cerevisiae* PMY1.1 is grown in the presence or absence of 5% (v/v) ethanol. Cells from an overnight parent culture were inoculated into YEPD medium (■) or YEPD medium containing 5% (●) (v/v) ethanol and incubated at 30°C/200 rpm

level of intrinsic ethanol tolerance than SUB61 and was consequently chosen as the strain to be used for the ethanol stress experiments described in this thesis.

Ethanol at low concentrations, below 2% (v/v), did not have any measurable effect on the growth of strain PMY1.1 (Figure 3.4) yet the growth rate was very low at 10% (v/v), although viability was maintained. By comparison, Holzberg *et al.* (1967) found that there was no apparent growth inhibition of an oenological *S. cerevisiae* strain in batch fermentations where endogenous ethanol concentrations were below 3% (v/v). Using the same strain in a continuous fermentation subjected to step changes in ethanol concentration, there was little effect on growth rate below 3% (v/v) ethanol but the strain was unable to grow above a critical level of 9% (v/v) ethanol. By comparison, the brewing strain *Saccharomyces carlbergensis* LAM 1068 strain was unable to grow in cultures containing 12% (v/v) ethanol (Toda *et al.* 1987). Beaven *et al.* (1982) found that growth of *S. cerevisiae* strain NCYC 431 was retarded by 3% (v/v) ethanol and cell viability declined rapidly in the presence of 9% (v/v) ethanol, although 15% (v/v) ethanol was required to reduce viability in *S. cerevisiae* strain CCY (Sajbidor and Grego 1992).

A closer comparison to the work described in this thesis can be made with the experiments of Lloyd *et al.* (1993). *S. cerevisiae* HSc with a reportedly above average ethanol tolerance (Rose 1980) was inoculated into rich medium containing 0%, 2.5%, 5% and 10% (v/v) ethanol. The strain failed to grow with 10% ethanol but generation times for cells grown in the presence of 0%, 2.5% and 5% (v/v) ethanol, were 1.4, 1.5 and 2.2 hours respectively. Corresponding lag periods calculated from the above published work were approximately 5, 10 and 15 hours respectively. At comparable ethanol concentrations, *S. cerevisiae* PMY1.1 grew more slowly than *S. cerevisiae* HSc. For example, PMY1.1 exhibited generation times of between 2.4 and 2.9 hours when grown with 5% ethanol (Table 3.2), but PMY1.1 was able to maintain viability in the presence of 10% (v/v) ethanol and adapted more rapidly to ethanol stress than *S. cerevisiae* HSc. Compared to the lag period of 15 hours for the reported strain, the growth lag period exhibited by strain PMY1.1 in the presence of 5% (v/v) ethanol was 2-4 hours (Table 3.2). Thus, compared to strain HSc, strain PMY1.1 grew strongly in rich media, retained viability when grown in the presence of ethanol concentrations up

to 10% (v/v), adapted rapidly to a 5% (v/v) ethanol stress and after adaptation to the stress, could achieve a reasonable growth rate.

*S. cerevisiae* PMY1.1 appears to have a comparable level of ethanol tolerance to the *Saccharomyces* species in the cited literature although the varied growth responses of different *Saccharomyces* strains makes it difficult to characterize a typical physiological response of *Saccharomyces* species to ethanol stress.

### 3.3.2 Yeast adaptation to ethanol

The influence of ethanol on microbial growth rate and viability is well documented but there are few reports of its influence on the length of time cultures need for adaptation to the presence of non-lethal ethanol concentrations i.e. the ethanol-induced growth lag period. Walker-Caprioglio *et al.* (1985) reported that *S. cerevisiae* inoculated into an aerobic rich medium in the presence of 1% to 8% (v/v) ethanol exhibited increased growth lag periods and reduced growth rates with increasing ethanol concentration; this effect was even more pronounced in defined media. However, the data presented by these authors did not accurately define and measure the lag period and early stages of exponential growth and their conclusions were therefore subject to doubt. The culture inoculum size of  $1 \times 10^4$  cells mL<sup>-1</sup> was determined using a Coulter counter but subsequent cell density measurements were made using a Klett-Summerson photoelectric calorimeter. This instrument was unsuitable for measuring very low cell numbers since the first data points for all cultures except one were 25 Klett units; 50 Klett units were equivalent to  $2 \times 10^6$  cells mL<sup>-1</sup>. Therefore inoculation levels below approximately  $1 \times 10^6$  cells mL<sup>-1</sup> could not be detected by the calorimeter until the cell population reached  $1 \times 10^6$  cells mL<sup>-1</sup>. Given the insensitivity of the calorimeter and that most of the cultures were inoculated at cell populations well below  $1 \times 10^6$  cells mL<sup>-1</sup>, the growth lag period of the cultures was not sufficiently defined by these authors to make useful comparisons with other lag phase studies.

The above publication by Walker-Caprioglio, Rodriguez and Parks (1985) highlights the difficulties of determining accurate cell numbers during lag phase and the importance of developing a method for accurately measuring cell growth. Problems

such as these were avoided in this project by using initial cell densities of around  $1 \times 10^6$  cells  $\text{mL}^{-1}$  (which can be accurately read using a spectrophotometer) and then using correlation curves relating optical density to viable plate counts to translate the optical density reading into population estimates. Thus optical density measurements could be used to estimate viable cell number during the progress of experiments by reference to correlation curves. It was necessary to prepare correlation curves separately for unstressed and ethanol-stressed cultures since the relationship between optical density and viable counts was different for both conditions. This probably reflects the influence that ethanol has on the progress of cells through the cell cycle and possibly also the effects of ethanol on cell size. This could be further investigated by using phase contrast microscopy to determine the budding index of unstressed and ethanol-stressed cultures and a cell channelizer to monitor cell size.

Comparisons to published work are often difficult to interpret because of the many different experimental conditions that affect growth of yeast cells during an ethanol-induced lag phase. Some of these experimental variables such as the inoculum size and media type have been reported to influence the length of an ethanol-induced lag; the lag period is reduced when inoculum size is increased and when cells are grown in rich media in comparison to defined media. For example, when *S. cerevisiae* strain X2180-1A was subjected to a step change in ethanol concentration of 4% (v/v) in rich medium there was a lag period of 3.6 hours. In this case the inoculum size was  $5 \times 10^4$  cells  $\text{mL}^{-1}$  but when increased to  $5 \times 10^6$  cells  $\text{mL}^{-1}$  the lag period was reduced to 2.6 hours. However, when the same number of cells were inoculated into a defined medium containing 4% (v/v) ethanol the lag periods were 4.86 and 3.4 hours respectively (Stanley, Hobley and Pamment 1997). The inoculum size-dependence of yeast cultures for ethanol-stress-adaptation is an important observation indicating that extracellular factors may play a role in adaptation. Washed inocula were used in the experiments of Stanley, Hobley and Pamment (1997) therefore conditioning factors in the parent culture were not transferred and could not contribute to any extracellular factors influencing ethanol-stress adaptation. Given this inoculum protocol, the lag reducing effect of increasing inoculum size observed by Stanley, Hobley and Pamment (1997) must have been the result of secreted factors in ethanol-stressed cultures. This raises the possibility that ethanol-stressed cells secrete growth-

activating factors into the media and may interact with other cells to influence their stress adaptation rate.

### **3.3.3 Experimental systems used to examine the response of *S. cerevisiae* to ethanol stress**

Although there are many references citing the effect of ethanol stress on yeast cells there are few experiments that examine the adaptation of cells to ethanol stress. Much of the early published work was carried out by exposing cells to a relatively high ethanol concentration for a short period of time i.e. ethanol shock. In contrast to these experiments, the ethanol-induced growth lag phase described in this project is a period for observing adaptative changes of cells exposed to ethanol. In ethanol shock experiments in which yeast cells were exposed to 12% (v/v) ethanol for 3-60 minutes (Rosa and Sa-Correia 1991), and 14% (v/v) or 20% (v/v) ethanol for 30-60 minutes (Costa *et al.* 1997), the high ethanol concentration was lethal, killing a percentage of cells. In the ethanol stress experiments for this project, an objective was to determine a sublethal ethanol concentration that would provide a level of stress sufficient to induce a 3-5 hour lag period whilst maintaining cell viability. These conditions subject the cells to a growth inhibitory stress but are not too severe such as to prevent adaptive metabolic and molecular events to take place.

The choice of 5% ethanol as stressor in this project satisfied the above requirement and is consistent with the findings of Piper *et al.* (1994) who observed that there was a critical threshold of 4% ethanol for detection of hsp's by protein pulse labelling in *S. cerevisiae* BJ2168. The integrated heat shock-responsive *UBI4* promoter-*lacZ* fusion of strain PMY501, a derivative of PMY1.1, was induced 5.2 fold when grown in the presence of 4-6% ethanol suggesting that *UBI4* encoding ubiquitin may be an ethanol-stress response gene (Piper *et al.* 1994). In addition, Northern blots of *S. cerevisiae* PMY3 (pUP41a) RNA probed for ethanol-stress response Hsp12 mRNA showed that maximal expression of Hsp12 occurred when the strain was grown in the presence of 6% ethanol. The induction by 4-6% ethanol of the *UBI4* promoter and transcription of ethanol-stress response gene *HSP12* in a strain derived from PMY1.1 is consistent with results described in this chapter. This also supports the choice of 5% (v/v) ethanol as a stressor for the work described in later chapters.

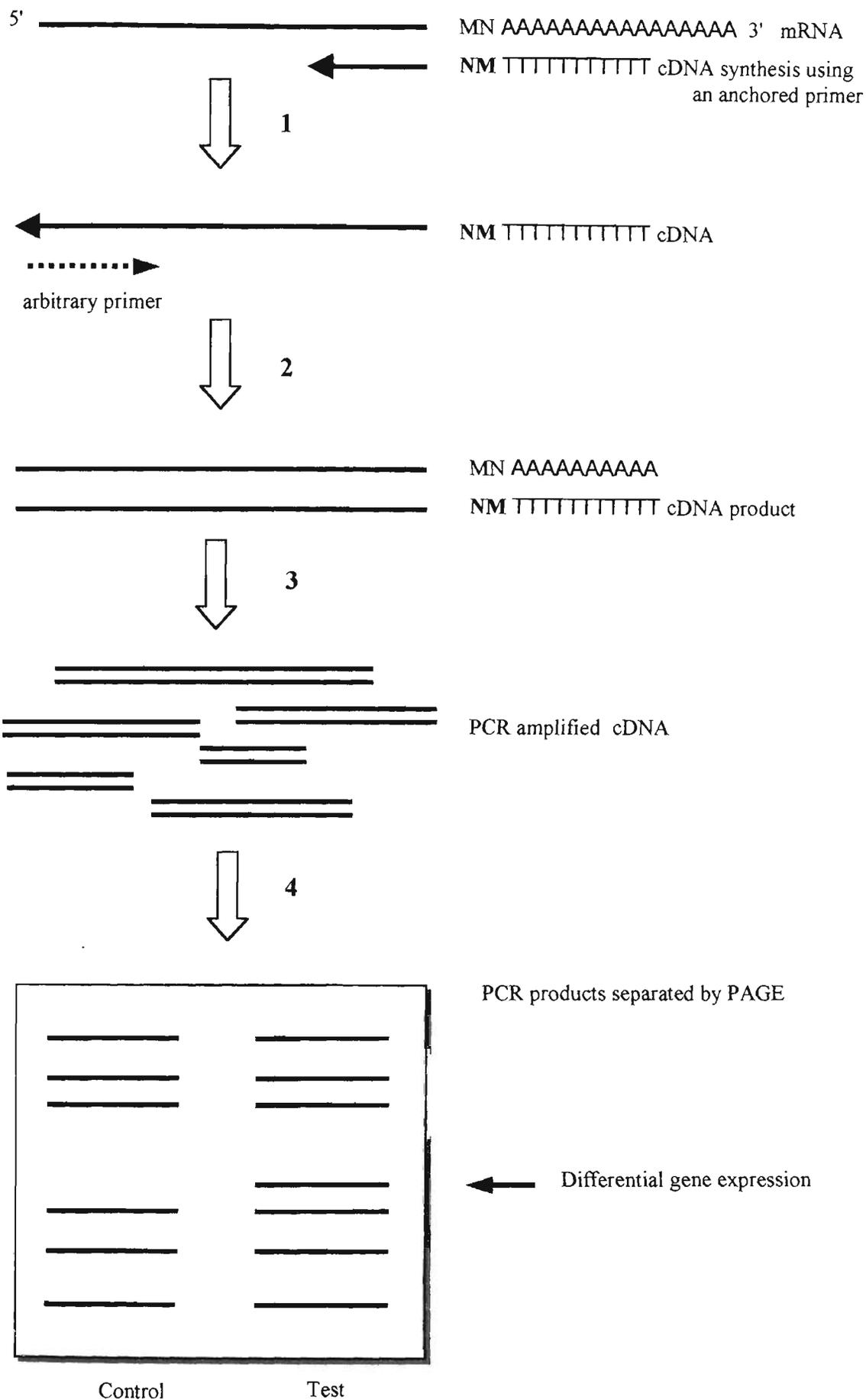
## CHAPTER 4

# IDENTIFICATION AND PRELIMINARY CHARACTERISATION OF ETHANOL-STRESS RESPONSE GENES USING DIFFERENTIAL DISPLAY

### 4.1 INTRODUCTION

The status of *S. cerevisiae* as a model eukaryote for molecular genetic studies coupled to the accessible bioinformatics resources of the SGD and the ease of growing yeast cells make *S. cerevisiae* an ideal candidate for gene expression analysis using Differential Display. Differential Display is sensitive, highly reproducible, uses only a small amount of RNA and does not require prior knowledge of mRNA sequences, neither does it require complex or expensive equipment. It was therefore the method of choice for the analysis of gene expression in the *S. cerevisiae* ethanol-stress response.

Differential Display is a method of comparing the transcriptional profiles of two or more cell populations, developed by Liang and Pardee (1992) to resolve and clone differentially expressed genes from a pair of mammalian cell populations using PCR. It involves creating a set of cDNAs that represent the mRNA population of a cell (or tissue), then resolving these cDNAs using PAGE (Figure 4.1). Each cDNA band on the resultant gel should represent a species of mRNA. Liang and Pardee (1992) achieved this by synthesizing cDNAs from mRNAs by reverse transcription using a set of oligonucleotide primers targeting the 3' polyadenylate tail of the mRNAs. The primer sequence, critical to the success of the method, consisted of the nucleotide sequence 5' T<sub>11</sub> NM where NM are two anchoring nucleotides complementary to the ultimate and penultimate nucleotides of mRNAs adjacent to the PolyA tract. A set of nine such primers with various combinations of anchoring



**Figure 4.1:** Differential Display flow chart. 1. cDNA is synthesized from polyadenylated mRNA from test and control total RNA samples using each of the 3' poly T primer with additional anchoring nucleotides. 2. The cDNA templates are then PCR amplified using the same poly T primer and one of a range of 5' arbitrary primers ( 3.) and PCR products are resolved on non-denaturing PAGE gels (4.) . Bands appearing differentially in test samples are regarded as putatively up-regulated genes.

nucleotide sequences will theoretically anneal to the whole mRNA population i.e. each individual anchored primer targets a subset of mRNAs. Nine sets of cDNAs prepared from these primers were then PCR-amplified using the original set of anchored primers in combination with each of 24 arbitrary tenmer upstream primers. Non-stringent PCR conditions allowed the arbitrary oligonucleotides to anneal to one or more positions on some of the cDNA species and thus generate a series of short partial cDNA sequences up to 500 bases long that were representative of the whole mRNA population. This procedure was undertaken with mRNAs from two cell populations and their expression compared by resolving the products in parallel on a polyacrylamide sequencing gel. Differences in banding patterns between cDNAs from test and control samples corresponded to differences in gene expression. Bands of interest were removed, re-amplified and cloned. Identification of the genes from which the bands originated was achieved by reference to known sequences in GenBank and the cloned products were used as probes for Northern analysis to confirm differential expression.

Whilst the Liang and Pardee (1992) method proved to be successful, there have been reports that Differential Display generates numerous false positive results (Li *et al.* 1994; Liang 1996; Liang *et al.* 1993). False positives are apparently differentially expressed genes that cannot be confirmed in subsequent analyses and may constitute more than 70% of the results (Nishio *et al.* 1994; Sun *et al.* 1994). Li, Barnathan and Kariko (1994); Liang, Averboukh and Pardee (1993); Luce and Burrows (1998); Sompayrac *et al.* (1995); Sung and Denman (1997); Zhao *et al.* (1996) and others have recommended procedures to try to overcome this problem but in most cases they involved extra steps and added expense. Problems leading to generation of false positives may include: lack of sensitivity of the validation procedures compared to the original Differential Display, the heterogeneity of gel bands in Differential Display gels and PCR vagaries that necessitate numerous repeat experiments. Another means of potentially generating misleading results in Differential Display analysis arises as a result of equalizing RNA from test and control sources as in the original Liang and Pardee (1992) protocol. Equalizing RNA may be appropriate when the test conditions do not alter net RNA synthesis relative to the control, however if test conditions perturb net RNA synthesis such an approach is likely to generate false positives or perhaps “false negatives”. Differences in RNA level due to test treatments may have

gone unnoticed in some of the reported work and the amounts of RNA may have been equalized without due consideration to the implications for the generation of false positives. This is supported by the observation of Liang (1996) that the rate of generating false positives varied between experiments and false positives were more prevalent when a cell line or tissue was treated with a test compound than when different cell lines or tissues were compared. To minimize the likelihood of generating false positives for work described in this thesis and to streamline the identification and validation procedures, several modifications were made to the original Liang and Pardee (1992) protocol, principally:

- RNA templates used in Differential Display and for validation were equalised by cell number rather than RNA quantity
- Differential Display was performed over a time-course rather than using a single time-point
- Differentially expressed bands were removed directly from Differential Display gels dried on glass plates rather than being transferred to filter paper
- Differentially expressed bands were sequenced directly using modified upstream primers rather than subcloning
- Northern analysis was standardized using gene-specific oligonucleotide probes rather than using cloned bands as probes
- Validation by Northern and RT-PCR analyses was performed over a time-course rather than at a single time-point

The aims of the work described in this chapter were:

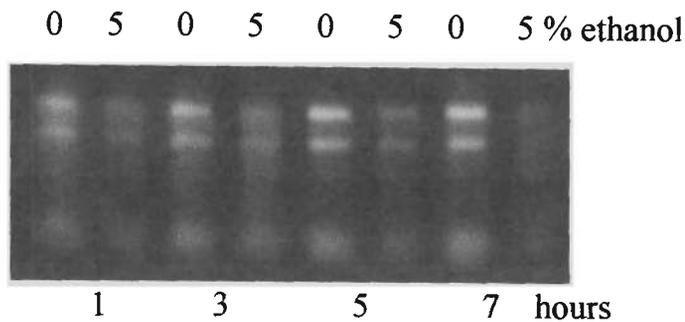
1. To develop an RNA isolation method to provide high quality templates suitable for Differential Display and subsequent RT-PCR,
2. To develop a Differential Display method suitable for yeast that would minimize the number of false positives generated,
3. To use Differential Display to identify genes upregulated in *S. cerevisiae* during its adaptation to ethanol stress,
4. To streamline validation procedures for differentially expressed genes.

## 4.2 RNA PREPARATION

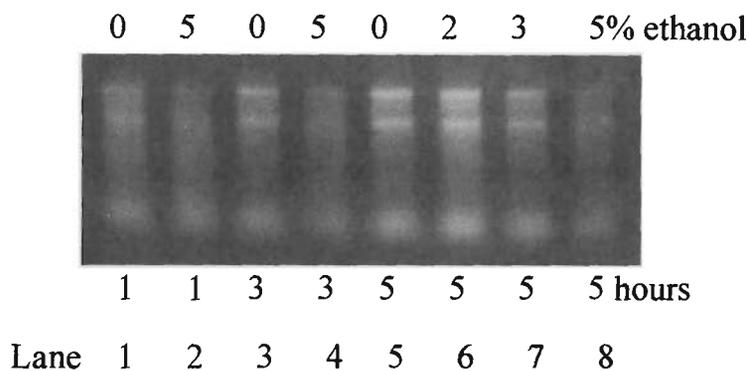
### 4.2.1 The effect of ethanol stress on the level of cellular rRNA

The traditional approach to Differential Display requires equalising RNA quantity from the test and control treatments. This is usually achieved by measuring the absorbance of each RNA sample at 260nm, determining the concentration and preparing test and control RNA solutions of equal concentration. Initial experiments in work for this thesis showed that when stationary phase cells were transferred into fresh YEPD medium containing 5% (v/v) ethanol there was a reduction in the amount of RNA obtained compared to unstressed cells. The amount of RNA isolated from cultures stressed with 5% or more ethanol was found to be consistently reduced in comparison to unstressed cultures even though the same number of cells were present in both samples (Figure 4.2). By comparison to 5% (v/v) ethanol however, the addition of 2% or 3% (v/v) ethanol did not appear to influence RNA integrity or quantity appreciably (Figure 4.3, lanes 5-8). Thus if RNA concentrations were equalized in test (5% ethanol) and control samples, the concentration of mRNA in the test samples would be disproportionately increased compared to unstressed controls. A similar bias towards mRNA early in the time course could occur if RNA were equalised across the time course. The amount and integrity of RNA isolated from unstressed cultures was also consistently reduced early in the time course compared to later in the time course (Figure 4.3, lanes 1, 3 and 5). Equalisation of RNA would be likely to bias the outcome in favour of genes expressed in stressed conditions and early in the time course, leading to the generation of false positive results. The decision was made therefore, to base the Differential Display experiments on the comparison of mRNA populations from equal numbers of viable cells instead of equalizing the amounts of RNA. This strategy was expected to reduce the number of false positive results generated.

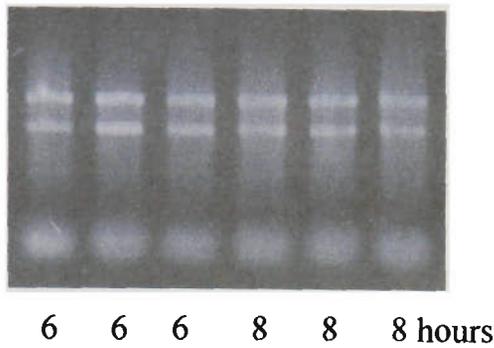
Since RNA samples were to be balanced by cell number rather than by RNA concentration it was important that the RNA isolation technique was consistent between samples. To test this, RNA isolations of two series of cell pellets were performed, each series consisting of three individual tubes of  $10^8$  harvested cells. After RNA isolation and DNase treatment little variation in RNA concentration or



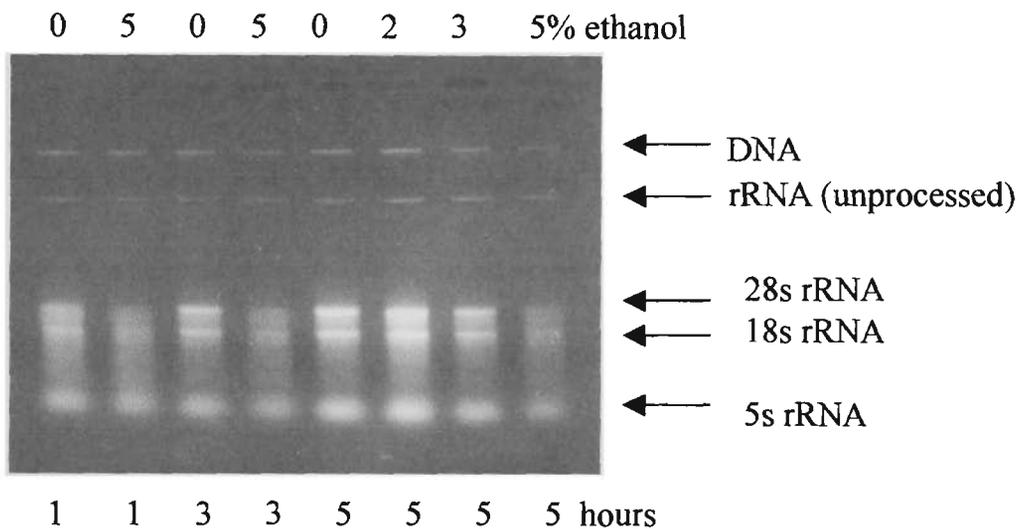
**Figure 4.2:** RNA level is reduced in ethanol-stressed cells. Cultures were grown in the presence (stressed) or absence (unstressed) of 5% ethanol and harvested 1, 3, 5 or 7 hours post-inoculation. Cell pellets of  $10^8$  cells were subjected to RNA isolation and DNase-treatment. RNA was resuspended in 25  $\mu$ L of water and 1  $\mu$ L was visualised on a 1.2% agarose gel.



**Figure 4.3:** RNA level and integrity is reduced in unstressed cells early in the time-course. RNA isolated from  $10^8$  cells harvested from unstressed cultures grown for 1 hour (lane 1) shows reduced concentration and integrity compared to unstressed cultures grown for 3 or 5 hours (lanes 3 and 5). Compared to cells grown for 5 hours without ethanol (lane 5), RNA level and integrity was not affected appreciably by the addition of 2% or 3% ethanol (lanes 6 and 7) unlike cells grown with 5% ethanol (lane 8).



**Figure 4.4:** RNA preparations from  $10^8$  cells taken from exponential phase cultures grown for either 6 hours or 8 hours to demonstrate consistency of the RNA isolation method used for work described in this thesis. Three cell pellets from each time-point were subjected to RNA isolation and DNase-treatment. RNA was resuspended to a volume of 50  $\mu\text{L}$  and 5  $\mu\text{L}$  was visualised on a 1.2% ethidium bromide-stained agarose gel.



**Figure 4.5:** The relatively constant amount of DNA present in RNA preparations indicates that the extraction of nucleic acids was independent of ethanol stress and culture incubation time. The bands labeled unprocessed rRNA were present in all RNA preparations, were not removed by DNase treatment but disappeared following RNase treatment and were therefore presumed to be unprocessed rRNA.

integrity was apparent when the RNAs were resolved on an agarose gel (Figure 4.4). Thus the RNA extraction protocol used for these experiments gave reproducible yields.

There was a possibility that the physiology of ethanol-stressed cells may lead to a reduction in the efficiency of isolating RNA compared to unstressed cells. For example, it was thought that possible changes in cell wall or membrane characteristics due to ethanol-stress could have a negative impact on the RNA isolation procedure. However, the amount of DNA present following RNA isolation was relatively constant regardless of the presence of ethanol or the incubation time of the cultures, whilst the amount of rRNA and its integrity varied considerably (Figure 4.5). Thus, the amount of nucleic acids extracted from ethanol-stressed cells was similar to that from unstressed cells, therefore the efficiency of the RNA isolation procedure was considered to be independent of ethanol-stress and culture incubation time.

It was important that RNA for Differential Display was free of contaminating genomic DNA as PCR amplification of genomic DNA leads to spurious results. In the original Liang and Pardee (1992) paper this was achieved by DNase treatment. For work described in this thesis, DNase treatment was followed by acid phenol extraction to eliminate remaining fragments of DNA. RNA prepared in this way gave good, reproducible results when used in Differential Display, Northern analysis and RT-PCR work.

### **4.3 THE IDENTIFICATION OF PUTATIVE ETHANOL-STRESS RESPONSE GENES USING DIFFERENTIAL DISPLAY**

The Display Systems Differential Display™ Kit containing primers from Operon Technologies Inc. was used for the work described in this chapter; this kit is widely used, locally supplied and was kindly recommended by C. Gross at University of New England, Armadale, NSW.

An exhaustive Differential Display (i.e. adopting sufficient primer pairs to detect most changes in gene expression under a given set of conditions) would require the use of 9 downstream primers and 24 upstream primers amounting to 216 primer pair reactions.

Such an approach would take a great deal more time and resources than was available for the work described here. Additionally, because of reports that differential display tends to generate a large number of false positive results it was decided to focus on using fewer primer pairs and developing an approach that would generate less data but the quality of the results would be more reliable. Thus the work described here was not an attempt to identify all or most ethanol-stress-induced genes but rather to identify some genes that were clearly upregulated in response to ethanol stress.

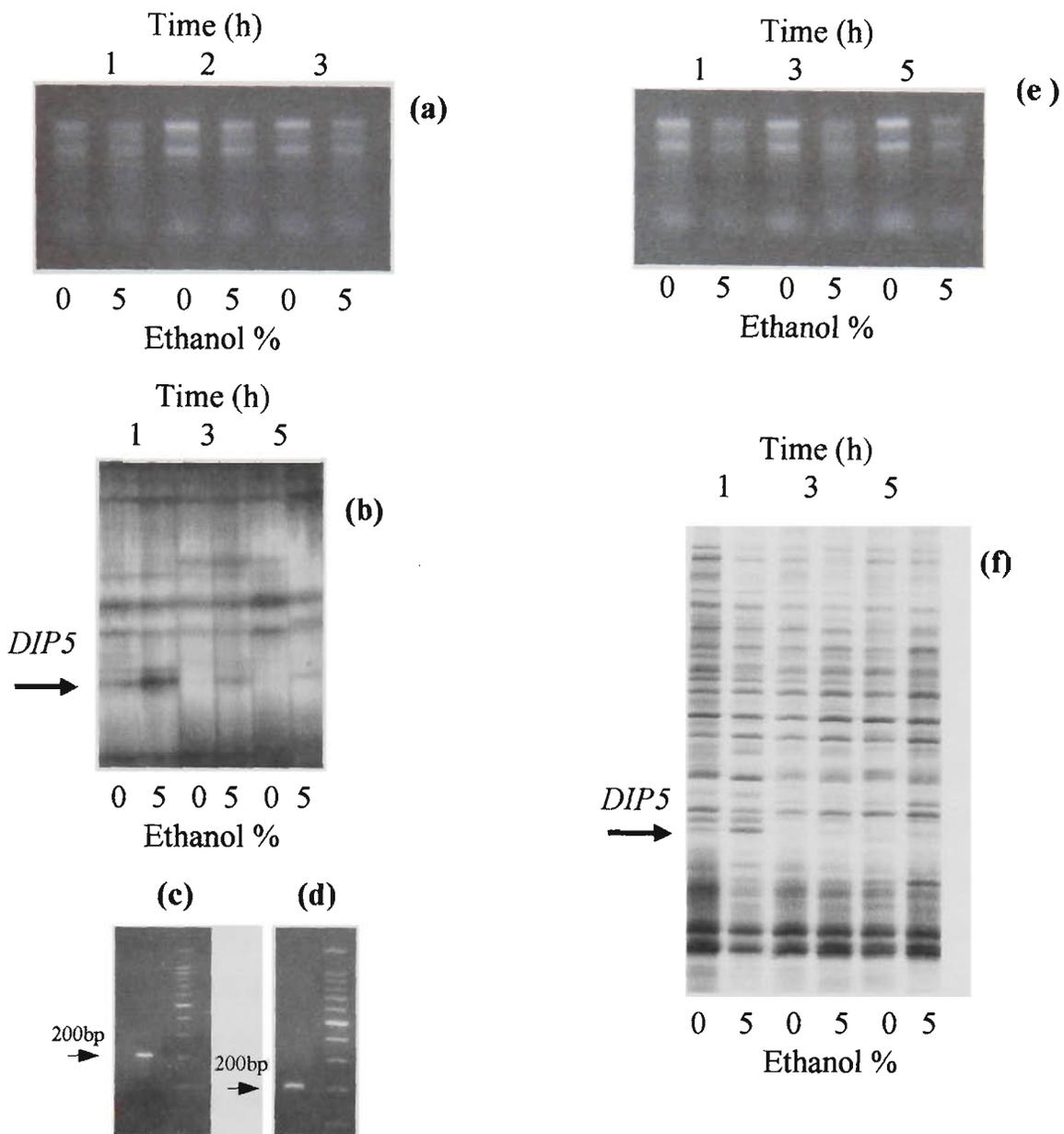
By using templates from samples collected at three or four time points during the stress adaptation period, either six or eight templates (stressed and unstressed) could be compared simultaneously on Differential Display gels. Any cDNA band appearing differentially in stressed samples more than once across the time course was therefore regarded as a putative ethanol-stress response gene but cDNA bands appearing only once in the time course were regarded as being potentially false positives and therefore were not analyzed further. This strategy reduced the need for replicates and generated temporal data not usually obtained in Differential Display experiments. The Differential Display experiments described here were limited to the use of nine upstream primers with only two randomly chosen downstream primers, amounting to approximately 1/12 of the possible primer options. From the 18 primer pairs and using sets of 6 or 8 templates, 7 Differential Display gels were sufficient to identify upregulated Ty2 and Ty1 elements and 4 putative ethanol-stress-induced genes *YGP1*, *DIP5*, *YER024w* and *MNN4*. Primers used in this work are listed in Table 1, Appendix II. A summary of the results of Differential Display experiments and the genes identified is given in Table 4.1.

#### **4.3.1 *DIP5*: a putative ethanol-stress response gene**

RNA for the Differential Display experiment in which *DIP5* was identified as a putative ethanol-stress response gene was prepared from stressed and control cultures harvested after 1, 2 and 3 hours growth (Figure 4.6a). cDNA was synthesized from each of the 6 RNA preparations using the downstream primer D1 and Differential Display performed using the D1/U20 primer combination. A differentially expressed band was revealed on the autoradiograph in samples from

GENE	PRIMERS	PEAK EXPRESSION (h)	DD BAND SIZE (bp)	VALIDATION METHOD	TRANSCRIPT SIZE (bp)
YGP1	D9 & U20 or UX20	2,3	250	NORTHERN & RT-PCR	1400
DIP5	D1 & U20 or UX20	1	200	NORTHERN & RT-PCR	2300
YER024w	D8 & U12 or UX12	1	375	RT-PCR	N/A
MNN4	D9 & U20 or UX20	1	300	RT-PCR	N/A
Ty2	D2 & U17 or UX17	1,2	200	N/A	N/A
Ty1	D1 & U23 or UX23	5	350	N/A	N/A

**Table 4.1:** Summary of Differential Display experiments to identify and validate ethanol stress-response genes. Differential Display using the primer pairs shown above indicated that the genes *YGP1*, *DIP5*, *YER024w*, *MNN4* and the Ty2 and Ty1 transposable elements were up-regulated in PMY1.1 cultures in the presence of 5% ethanol. The Differential Display gel bands ranged in size from approximately 200-375 bp. Bands representing *YGP1* and *DIP5* were differentially expressed in repeated experiments with a peak in expression 2-3 hours and 1 hour post-inoculation respectively. The up-regulation of *YGP1* and *DIP5* in ethanol stressed cultures was validated by Northern and RT-PCR analyses. Transcript sizes on Northern were 1400 bp and 2300 bp respectively. The up-regulation of *YER024w* in ethanol-stressed cultures was validated by RT-PCR. The up-regulation of *MNN4* in ethanol-stressed cultures could not be verified. Verification of Ty1 and Ty2 upregulation in response to ethanol stress was not attempted.



**Figure 4.6:** Identification of the differentially expressed Differential Display band representing the *DIP5* gene: **(a)** DNase-treated RNA for Differential Display. Each lane carries RNA from an equal number of either stressed (5% ethanol) or unstressed (0% ethanol) cells at a given time point over a 3 hour time-course. **(b)** Part of a Differential Display gel from which the band indicated was removed. **(c)** The excised band from **(b)** was re-amplified with the same primer pair as for Differential Display and the PCR products re-amplified with extended primers **(d)**. The 200bp band from **(d)** was sequenced and found to represent the gene *DIP5*. **(e)** For a repeat experiment, DNase-treated RNA was prepared from equal numbers of cells harvested over a 5 hour time-course. **(f)** A repeat Differential Display gel prepared using a gel comb with separated teeth rather than the sharks-tooth comb used in **(b)** was prepared from RNA templates in **(e)**.

stressed cultures grown for 1 hour and also, more faintly, in samples from stressed cultures grown for 2 and 3 hours (Figure 4.6b).

Negative controls were routinely prepared concurrently. There were generally no visible products present in PCR-amplified negative control cDNA reactions from which Reverse Transcriptase was omitted although occasionally a few products were visible that did not resemble the Differential Display bands. Results from negative controls indicated that genomic DNA was not being amplified and that therefore the gel bands were generated from mRNAs.

The differentially expressed gel band (Figure 4.6b) was excised and re-amplified with the same primer pair (Figure 4.6c). DNA from the same isolated gel band was then used as template for another round of PCR with the same downstream primer and the extended upstream primer UX20 (Table 2 appendix II) (Figure 4.6d). A band measuring approximately 200 bp was purified and sequenced. Sequence data is presented in Figure 1, Appendix IV. To test the reproducibility of the modified Differential Display procedure, a repeat Differential Display reaction was performed using the same D1/U20 primer combination with another set of templates prepared from cells grown for 1, 3 and 5 hours (Figure 4.6e). In this instance, a gel comb with separated teeth was used instead of the usual sharks-tooth comb and revealed a similar expression pattern to the previous gel (Figure 4.6f). A differentially expressed band was present in samples taken at 1 hour from stressed cultures and faintly in samples from stressed cultures grown for 3 and 5 hours.

A BLASTn sequence alignment revealed that the differentially expressed band possessed homology to a region on the left arm of *S. cerevisiae* chromosome XVI between coordinates 43011 and 43185 with a probability of  $4.5e-11$ . This region corresponds to the 3' region of *DIP5* (*YPL265w*) that encodes the dicarboxylic amino acid permease responsible for high affinity transport of aspartate and glutamate (Regenberg *et al.* 1998). The ORF is situated on chromosome XVI between the coordinates 41043 and 42869. The *DIP5* gene sequence submitted to Genbank in 1996 consists of 3205 nucleotides, of which 966-2792 is designated as coding sequence. The sequenced differentially expressed band aligned with a region 139

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1   gctctcttat caattatgta agtgcttcta tactatttac ctaagataag
51  aaaaaaaaaa gcaattcaaa attaagctta tcttgacagc ggggctggtt
101 tgtttctaga agacaaaaag tggggaatca tttttacgta actccccctg
151 ataagaagga ctcacatcct tataggtacg ataaagaatg gttgtatctt
201 tcctattttt cgaaatcggtt atcttatata gttgaaactac tacggttaaa
251 aagcttaagc ctcagccctc ttagtcaaac ttcttttttg aaggcaccag
301 ggtgcataaa agtgcgtcta ttgtttccca gtggaactct gttgagatag
351 cgatgtttgt ttttttttca cttaacggca accaataccg atagcgacgt
401 cgctggcagt gttagagtgc cgtagggcgt cgctagatgg cacggcactg
451 attgcggcgg gagtcgctag gcggtgatgc atttccgcac agggaccaga
501 ggaagcttcc caggcggtga cagtaagtga actcattatc atgtcttctc
551 caaaacattc gtgacatcta gtcatgctcc tcgcaattca ctccgattgg
601 tatagctttt tcggtagttt tagctactat gcttagggga aagaggagaa
651 accgtaccgt cagtctcagt caaaaaattt tgatattcaa tctgatagca
701 aagttggaac ttggggttat ctggcccttt tttgttatca tattcgtata
751 cccaacaaca tatcggttcc accggctcct tttatatata aaagacgatg
801 tgtagatgca ctcgagtatt cttggagaac gtaacttgta ttgagctaga
851 gtgctggata aagtaccaca tactaacggt cttttataga gccaaacata
901 attcttttgc acttcaata taaggtaaca gtgaaacaca ggaaaaaag
951 aactaactct aagtaatgaa gatgcctcta aagaagatgt ttaccagcac Start codon
1001 gtctctcctg aactcttctt ctcttgacag tgatcatgac gcttactatt
1051 cgaacaacaaa tcctgacaat tccctgtaa aggagcaaga aatctataac
1101 attgacctgg aagaaaaaca tgtgtcctct cgttcatcca cctctaac
1151 accttcagca agggcagact ctttcgcagt tccagatggt aaagacgaaa
1201 acacgcggtt gaggaagat ttaaaggcaa gacatatttc tatgatcgcc
1251 attggtggtt cattaggtac aggtctgctt ataggtacag gtaccgectt
1301 attgacgggt ggtccggttg cgatgttaat tgcatatgcc tttgtcggcc
1351 ttttagtctt ttacaccatg gcctgtcttg gtgaaatggc ttcttacatt
1401 ccattggatg gttttacaag ttatgcctca cgttacgtgg atcctgcatt Northern probe
1451 aggttttgct attggttata cttacctttt caaatatttc atcttacctc
1501 ccaaccaact tactgctgct gctttggtca ttcaatattg gatcagcaga
1551 gaccgtgtta accctggtgt gtggattact atattcttgg ttgttattgt
1601 cgcttaacaat cctgcgggtg taaaattctt tgggtgaatt gaattttggt
1651 tgtccagttt caaagctatg gtaatgttgg gtctaactct gtactattt
1701 attattatgc ttggtggagg tcctaaccat gaccgcctag ggtttagata
1751 ctggcgtgat cctggtgcgt tcaagaata ttcgacggt atcactggtg
1801 gtaaaggtaa atttgtttcg ttcggtgctg ttttcgttta cagtcttttc
1851 agttacacgg gtattgaatt gacaggatc gtttgtctg aagctgagaa
1901 tccaagaaaa agtgttccaa aggcaattaa attgacagtt taccgatca
1951 ttgtttttta cctatgcacc gttttccttt tgggtatgtg cgttgcatc
2001 aatgaccctc gtttactttc cacaaaaggt aagagtatgt ctgctgcggc
2051 atctccattc gtggttgcca tcaaaaactc aggtatgaa gtcttacctc
2101 atatcttcaa tgettggtgc ttggttttcg ttttcagtc ttgtaactca
2151 gattgttacg tttcttccag aaatttataat gcggtggcaa ttgatggtaa
2201 agcgcctaaag atcttcgcta agacaagtag atggggtggt ccttacaatg
2251 cttaataact ctccgtgctg ttttgtggct tggcgtacat gaatgtgtct
2301 tcaggatcag caaagatttt caactacttt gttaacgttg tttctatggt
2351 cggaatcttg agttggatca ccattttaat tgtttacatc tacttcgata RT-PCR primer
2401 aagcctgcgc tgctcaagg attgacaaat caaaatttgc ttatgtcgtc
2451 cctggccaac gttatggtgc ttattttctt ttattcttct gcattttgat
2501 tcttttaate aaaaacttca ctgttttcct aggtcataaa ttgattata
2551 aaacattcat caccgggtat attggcctgc ctgtctatat cttttcttgg RT-PCR primer
2601 gctggttaca aattgatata caaaaccaa gtgataaagt ctaccgacgt
2651 ggatttgtag acatttaagg aaatatacga tagagaagaa gaagagggaa
2701 gaatgaagga ccaagaaaag gaagagcgtt taaaaagtaa cggtaaaaaat
2751 atggagtgtg tctatgaaaa atttttgggt aatatcttct agatgccttt Termination codon
2801 ttaatcaaag ccttagggca acatgaaact atattcatcc aatcaaaaaa
2851 atgagcacia gaatttctct ttgatcattt ttcttttacc tttataaca
2901 atcgttatca acatttttta tttccagtc taccacagca ttattgcata Differential Display
2951 attaatttac aggaagattt tcttgaaaaa atagaagaa aaataccat band alignment
3001 tttttaagg aaaataaaa ttatatatta catacattc tataaacag
3051 tttaattca aaaatataa caaaaacata aaaataagaa aagttcaagt ARS-consensus
3101 cttcgttatt ttctgtttgt ggagtaatcg atcttattaa catatatgtg Term.codons(proposed)
3151 gagacttttc aaatatagcc acctgcaaca tggaaccata gtgtacaggt putative polyA signal
3201 atcac (GenBank 1996)

```

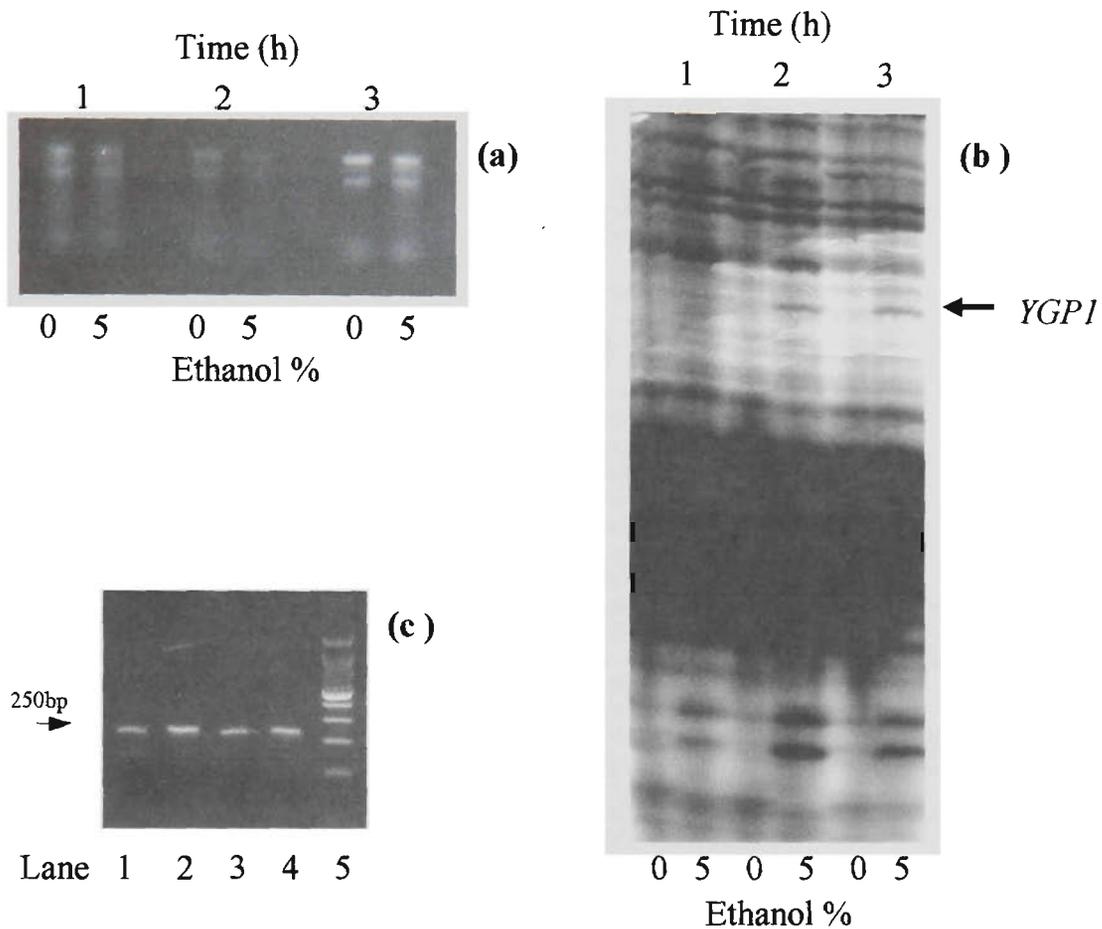
**Figure 4.7:** *DIP5* gene sequence. The *DIP5* gene sequence submitted to Genbank in 1996 by MIPS on behalf of the European yeast chromosome XVI sequencing project shows the Differential Display band alignment, RT-PCR primer sequences, Northern probe sequence, start and termination codons and other putative gene features. The *DIP5* (*YPL 265w*) ORF on chromosome XVI is 1827 nucleotides in length.

nucleotides downstream of the *DIP5* terminator codon, continuing 177 nucleotides downstream (Figure 4.7).

### 4.3.2 *YGPI*: a putative ethanol-stress response gene

RNA for the Differential Display experiment in which *YGPI* was identified as a putative ethanol-stress response gene was prepared from ethanol-stressed and control cultures grown for 1, 2 and 3 hours post-inoculation, and DNase-treated (Figure 4.8a). cDNA synthesized from each of these 6 RNA preparations using the downstream primer D9 was then PCR-amplified in Differential Display reactions using D9 and the upstream primer U20. Following autoradiography two differentially expressed gel bands were found to be present in samples from stressed cultures grown for 2 and 3 hours respectively (Figure 4.8b). Repeated Differential Display reactions using the same primer pair with another set of templates from cells harvested 1, 3, 5, and 7 hours post-inoculation produced similar results (data not shown). The differentially expressed bands in lanes 4 and 6 of Figure 4.8b derived from cultures grown 2 and 3 hours respectively, were excised from the gel and re-amplified with the same primer pair D9/U20 (lanes 1 and 2, Figure 4.8c) followed by re-amplification using the extended upstream primer UX20 (lanes 3 and 4 Figure 4.8c). For both time points a band of approximately 250 bp was visible when the PCR products were resolved on a 2% agarose gel (Figure 4.8c). Both bands were purified and sequenced. Sequence data is shown in Figure 2, Appendix IV.

A BLASTn sequence analysis of both bands against the *S. cerevisiae* genome revealed their homology to a region on the left arm of chromosome XIV between the coordinates 337609 and 337791 with a high probability of  $2.6e^{-68}$  (the probability that the similarity is a chance occurrence is  $2.6 \times 10^{-68}$ ). This region corresponds to the 3' untranslated region of the *YGPI* (*YNL160w*) gene encoding the secreted glycoprotein gp37 (37kD). This protein is produced in response to nutrient limitation and is possibly involved in cellular adaptations prior to stationary phase (SGD). The *YGPI* ORF of 1065 nucleotides is situated on chromosome XIV between the coordinates 336544 and 337608. The *YGPI* structural gene sequence submitted to Genbank by Klionsky, Destruelle and Holzer (1996) is 2165 nucleotides



**Figure 4.8:** Identification of the differentially expressed Differential Display Band representing the *YGP1* gene. **(a)** DNase-treated RNA for Differential Display. Each lane represents an equal number of either stressed (5% ethanol) or unstressed (0% ethanol) cells harvested over a 3 hour time-course. **(b)** Differential Display reactions separated on a 6% acrylamide gel revealed differentially expressed bands in ethanol-stressed samples derived from cultures grown for 2 and 3 hours post-inoculation. **(c)** Both bands were excised and re-amplified with the same primer pair (lane 1, 2 hours and lane 3, 3 hours). The PCR products of approximately 250bp were then re-amplified with the extended primer (lanes 2 and 4). Lane 5 contains a 100bp DNA ladder in which the intense band measures 500bp. The bands in lanes 2 and 4 were sequenced and found to be derived from the gene *YGP1*.

```

1  attcgtactc tattgcatct tcaaagtcgc aagaatctca gtaggagtg
51  aagccggctt ctcgatgcta cacttctcga tgctacagtt caacatcgat
101 actttgaaga aagaaagcgc ctatttatatc tcttttacc c tattagtaat tata box
151 aattagggaaa aagggagaaa aaagtacctc actaaaaaaa ccatcatctc
201 tgaaaatataa aaagcttgat agagggtgac atttgctaga acttctgctg tata box
251 tgttctcttg ggttattgct cttattgaat atcccctcta tttctttctt
301 gcttgtaaaa aatcagctca aaaaacatct acaggattaa tcgtcagtta
351 agtaatacag taatagaaa tgaaagtcca agttgtttta tctgcccttt Start codon
401 tggcatgttc atctgccgtc gtcgcaagcc caatcgaaaa cctattcaaa Northern probe
451 tacagggctg ttaaggcatc tcacagtaag aatatcaact ccactttgcc
501 ggcattggat gggcttaact cttagcaatg tacctacgt aatggaacaa
551 acagtactac caatactact actgccgaaa gcagtcgaatt acaaatcatt
601 gtaagaggtg gtcaagtacc aatcaccaac agttctttga cccacacaaa
651 ctacaccaga ttattcaaca gttcttctgc tttgaacatt accgaattgt RT-PCR primer
701 acaatgtttg ccgtgttgtt aacgaaacga tccaagataa gtcacccgcc
751 ggtgccgttg ttgttgccaa cgccaaatct ttggaagctg tctcattctt
801 cttctctatc atttttgaca ccgaaaagcc tattgttgtc actgaagatt
851 ccgcttatgc cattccagtc gtaacaata agaacgtac caaacgtggt RT-PCR primer
901 gtcttgcctg tcacttctga caaattagtg tactccgggt tcttactctc
951 acctactgct tgttcttacg gtgctggtt gectgttgc atcgttgatg
1001 accaagacga agttaaattg ttcttcgatg cttctaagcc aactttaatc
1051 tcttctgact cgattatcag aaaggaatac agtaacttca ctactcctta
1101 tgggtctatta gaaaacggtg ttccaattgt tccaattgtc tatgacgggtg
1151 gttactcttc cagtttgatt gactccttga gttctgccgt tcaaggtttg
1201 gttgttgttt cttctggttc taccaactca acctcatcta ctattgaaag
1251 cactgaaatc ccagtcgat atgctcaagc taacactcca ttaacttta
1301 ttgacaacaa agatgttcca aagaacgctg tgggtgctgg ttacctatcc
1351 ccaattaagg cccaaatctt gttgtccatt gctgccgtta atggtgtcac
1401 ctccaagtcc gctctggaaa gcattttccc atgaactgat agatattaaa Termination codon
1451 tctagcgaag catagagatt ctttttttac cagtcatcat aaccattatt Differential Display
1501 ttacaaaatt tcacgcgcaac agcttttatt ttcgttcgta ataggaacca band alignment
1551 tacttcccat taaagcagta tgattgtttt atcaccttt ttattgttat
1601 ttaagacata ccccaaataa ttaattaatt aattgatgaa agaatcatat
1651 aaattcttgg ccatacatat tttttatta tcttttgcta catgaccat Termination codons
1701 aggtgaatat cttcccgggg tgacactcca ctgaagtggg aaaaaaagaa (proposed)
1751 aagttttaa acattgctca ccttaagtct cgaaggtcta gcattcacta
1801 cctgtaagtg tcaagacccc acaggtatac atgatacaat aaagaacaa PolyA signal
1851 tgtccgtata attgtataca ttttactaca gataaggat ctcaccccct
1901 tcttttgtgt ctaagtgaaa tcttctgata ttttagtgt tttatagcgt
1951 ccattgtttta aactagaacg gcaaaatagt agttgttgta aacgcttatt
2001 ttcgagacat caggtaaaaa gatagttaaa tcttacaatc aatgcatgta
2051 gtgagtgccg tttatcaata gtttttaaca attcccattt tgaaagaagc
2101 ccacaacaac cctagtagtt cgttatatac gttatgatgg tcaagtgtca
2151 cagtacggaa agctt

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(GenBank 1993)

**Figure 4.9:** *YGP1* gene sequence. The *YGP1* gene sequence (submitted to Genbank in 1993 by H. Holzer) shows the Differential Display band alignment, RT-PCR primer sequences, Northern probe sequence, start and termination codons and other putative gene features. The *YGP1* (*YNL 160w*) ORF on chromosome XIV is 1065 nucleotides in length.

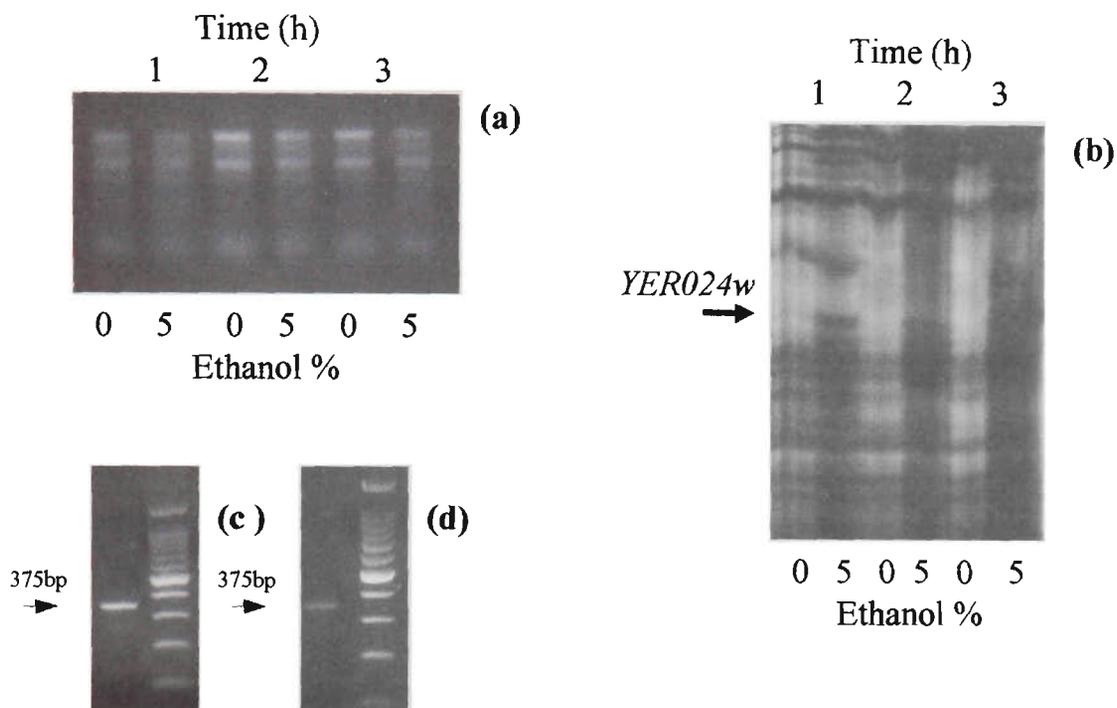
in length, of which 370-1434 is designated as coding sequence. Sequences from differentially expressed bands from both time points aligned with the *YGP1* gene, 6 nucleotides upstream of the terminator codon (1432-1434) and continuing downstream for 189 nucleotides to a point 225 nucleotides upstream of the polyA signal (1839-1844) (Figure 4.9).

### 4.3.3 *YER024w*: a putative ethanol-stress response gene

RNA for the Differential Display experiment in which *YER024w* was identified as a putative ethanol-stress response gene was prepared from the same stressed and control cultures harvested 1, 2 and 3 hours post-inoculation as used to identify *DIP5* (Figure 4.10a). Differential Display using the D8/U12 primer combination revealed a differentially expressed band on the autoradiograph in samples from stressed cultures grown for 1 hour and, at a lower level, from stressed cultures grown for 2 and 3 hours (Figure 4.10b). The band was re-amplified with the same primer pair (Figure 4.10c) followed by re-amplification with D8/UX12 (Figure 4.10d) and a band of approximately 375 bp was purified and sequenced. Sequence data is presented in Figure 3, Appendix IV.

A BLASTn sequence analysis revealed that the differentially expressed band aligned with a region of the right arm of *S. cerevisiae* chromosome V between coordinates 204998 and 205087 with a probability of  $1.8e-14$ . The region represents the 3' region of the hypothetical ORF *YER024w* that belongs to the carnitine/choline acetyltransferase family with similarity to *S. cerevisiae* carnitine N-acetyl transferase at protein level (SWISS-PROT). *YER024w* is located close to the centromere on chromosome V between the coordinates 202191 and 204962. The putative differentially expressed band aligned with the 3' end of the 2772 nucleotide proposed coding sequence of this ORF 32 nucleotides upstream of the terminator codon and continued for 125 nucleotides downstream (Figure 4.11).

The sequence data for this differentially expressed band clearly aligned with the ORF *YER024w* although the sequence quality was not as good as for previous bands. Under the ethanol stress conditions of the experiments described here the *YER024w* mRNA



**Figure 4.10:** Identification of the differentially expressed Differential Display band representing the *YER024w* ORF. **(a)** DNase-treated RNA for Differential Display. Each lane represents an equal number of either stressed (5% ethanol) or unstressed (0% ethanol) cells harvested over a 3 hour time-course. **(b)** A differentially expressed band was excised from the Differential Display gel and re-amplified with the same primer pair **(c)** and the extended primer for sequencing **(d)**. The band of approximately 375bp as estimated from the 100bp DNA ladder was sequenced and proved to be derived from the *YER024w* ORF.

```

1  atgtcaagcg gcagtactat tgtttcgtct gacaagtcgg ggcgaacatt Start codon
51  taagcatgag gaagaactac ccaagttacc attacctaaa ctctgtgaca
101 ctttgcagcg tctgaaggag agtttagagc cattgtatta tgctgatggt
151 tactatacaac atcctttaga tccagaacaa attgagaagc tgcatccat
201 aataagagat tttgaagaga atcccgtaag tgagaagctg caatctaaac
251 tacagagcta ccatgacact agggattgct accttgatga attacactta
301 gacatcaata accagacctc cactagggag attcaggacg atgtcctacc
351 caggaatccc ttcttggttc tcgcagatga cgctttgcc aacatcactc
401 aagctgatag gtcagccgct ctagtgcact ctgcccagc gtttatttct
451 gctttgaaac aagatttggt gccaccagat atcaatgcc ccaatggcaa
501 accactgtca atggtccat ttctgaaact gttcggctact actcgtagtc
551 ctgtttttca gcgcggtgag gttgaaaatt ttgatttga caaaccttac
601 acagcatctg atttagaaga ccctgattat tcaagcagat aggacgataa
651 cgacgaaccc acccaaaaag actttgatga ccgtaaaaga aaacacgaag
701 aagacatatt cactggcaat ggtataacta taaaagaca tccggatagc
751 aaacacatct tgatcattct caggggccaa tactatacac tggaaagtact
801 tgattcgacg aataaaatta tatacactgc tgcagaatta accacaattt
851 tcaaccatat aataaaagat tcttctggca ttgaaaatc aactgcgtta
901 ggtagtttga cctctcactc ttttagaaac tggaaatatg caaggaagag
951 actacagaaa aggtatccta atgaattaca tcgcatagac tctgctctgt
1001 tctgtttaag gctggagca tcacaagaag aaactaccaa tgatggcgat RT-PCR primer
1051 gataccgctg atatcagtc aatgtttaac aggactatca ctgagcgaga
1101 caagaaatgt acttccgcca attgtaagag agtgttttac ggtacttcta
1151 taataaatag caaagccat caagtgggtt catgtgtttc tcgttggtat
1201 gataaattac agttaagttg cactgcagat gcaaaagcca cagtgtattg
1251 ggattccttc acatgtgacg ggtcagtcgt gcttcggtt acttctgaaa RT-PCR primer
1301 tttatacaga atcagtcctt agattagcta gagacgttaa cgccggtgac
1351 ccacagtttt cactctggcc taacgtgaca caaatggatc ctgaaactaa
1401 aaaactgatg acggcgacca taagtgccga cggcggaggc ctttctgaaa
1451 ttgaccctaa gttggttgta aataaaatcg attggtcttt cagtaacatc
1501 ctgaacacac atgtccattt gtcagaaacc aaactagctg atctgatttc
1551 caagtatgat attgttcgcy cttctattcc attgggcaga aggtcagctc
1601 aaagattagg agtcaagccc gattcagatg ttcaagtacg ttacaaata
1651 gctcactatg ctttgatagg aagaatggtg tttggattag aaccagtatc
1701 cacacgtggt ttcaaaaact cgagatcacc ttttattaac attcaaagtc
1751 aagcgcgtgt ggaactatgt caattgttta tttctagctc cattgatggg
1801 acagataaat tagacaaatt tatccaaacc tgcgaaacac ataataacat
1851 ggttaaacat gcaaagtcg ggttaggta tgaaaaaac ttcaatgcat
1901 tgaatatctc cttcaaattt catgaccatt ttggtatcca ctaagtgggt
1951 gatgaatcat ctgctgcgaa agatcttttc gaaaatccac ttgttttacc
2001 tttctcacag cctgagttga ttgttgccaa ctgtggtaat gctgctacca
2051 cgacatttgg tataactcct gctgtacctc atggctttgg aatagggtac
2101 atcattaaag atgatcaagt agatttgacc gtgacatcgc aatttcgaca
2151 ggtgacaggy ttaatgttta tgtaagtgt ggtgttaggc gaaattcgtt
2201 cctactggag gatgtcacgc ggcacttctc ataataaaac tggcgtgaag
2251 atcagtcacg tggtcgataa attgtatgaa atggacaatg cagtcaataa
2301 tctccgaaa cgtaacggcc atacagtcaa tggctcccgc aaaacatctt
2351 cttcatcaca ggtaaatgtg aacaggtatg gcgggttttt cgatttgtaa
2401 ggccacattg acagtaggaa tataatctaaa acaccatcga tgaagaattt
2451 gcagaaaacc ttcaatggct tgaccatgag cgcagacaat gatcattcaa
2501 gttccgcaat ttcggttccc acagaaaagg aaaaattaaa tacaggccat
2551 gaaattttac agatccaacc gcgggaagtg gcaagtaatg gtttgaagc
2601 agatgatgag acagatattg aaatagttgc tggcaatgct gatggcacat
2651 cctcttcgcy atcttcagct acatctctaa attccaagaa acgtaacgct
2701 atcaactcaa gattcगतat tgattttgac cgcagtcgtg tggttagaaa
2751 ggtggcgacc ttagatcaat aagaacgctc tttgtttatc tatttattac Termination codon
2801 tagcattatg cgtaagcttg gcgtgatgtg atatatactt gttatatgaa Differential Display
2851 gtatatcaat atgctttaat ttatgcttat tatttgcctc catgtcagta band alignment
2901 acccgacca gattgccaat tgaaaaaatt ttcatagaat agcccgatgc Termination codons
2951 tgatcatcag taaaaagac agagatttca tctcgacctg taccaggagc (proposed)
3001 taagcaaga gggcggaaag agcgtaatac accgttaaca tcgcgatta
3051 gaggtagaca tgagtgactt ac

```

(SGD 1997)

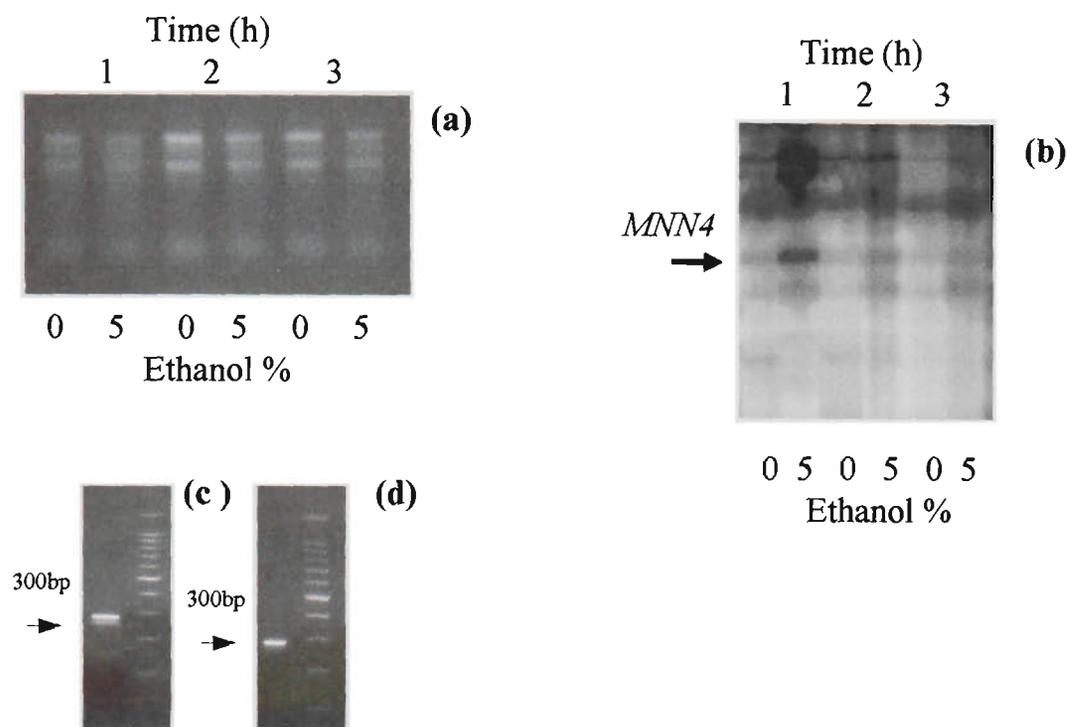
**Figure 4.11:** *YER024w* ORF sequence. The *YER024w* ORF submitted as part of the complete DNA sequence of chromosome V by F.S.Dietrich *et al.* shows the Differential Display band alignment, RT-PCR primer sequences, start and termination codons and other putative gene features. The *YER024w* ORF on chromosome V is 2772 nucleotides in length.

is approximately 125 nucleotides longer than the designated ORF sequence in SGD and NCBI but there is no empirical data in the literature or SGD related to transcript size of this gene. There are several proposed alternative terminator codons indicated in Figure 4.11.

#### 4.3.4 *MNN4*: a putative ethanol-stress response gene

RNA for the Differential Display experiment in which *MNN4* was identified as a putative ethanol-stress response gene was prepared from the same stressed and control cultures harvested 1, 2 and 3 hours post-inoculation as used for the identification of *DIP5* and *YER024w* (Figure 4.12a). Differential Display reactions were performed using primer D9 and upstream primer U20. Following autoradiography a differentially expressed band was visible in samples from stressed cultures grown 1 hour and faintly in samples from stressed cultures grown 2 and 3 hours (Figure 4.12b). Two bands were present following re-amplification with the same primer pair D9/U20 (Figure 4.12c). When removed from the agarose gel, the less intense lower molecular weight band failed to re-amplify therefore only the more intense, higher molecular weight band of approximately 300 bp was processed further. This band was used as template in another PCR reaction using the primer pair D9/UX20 (Figure 4.12d) and the PCR products were purified and sequenced. Sequence data is shown in Figure 4, Appendix IV.

A BLASTn alignment revealed that the differentially expressed band aligned with a region on the left arm of *S. cerevisiae* chromosome XI between coordinates 66269 and 66493 (beginning at 66493) with a probability of 4.0e-78. The band aligned with a region within the *MNN4* gene (*YKL200c*, *YKL201c* and additional sequences). The protein encoded by the *MNN4* gene, Mnn4p, is required for phosphorylation of N-linked oligosaccharides in *S. cerevisiae* (Odani *et al.* 1996). *MNN4* is situated on the Crick strand of chromosome XI between coordinates 63927 and 67463 (beginning at 67463) and consists of two adjacent ORFs, both on the Crick strand, *YKL200c* and *YKL201c*. The putative differentially expressed band aligned with an internal region of the *YKL200c* ORF beginning 615 nucleotides downstream of the start codon and continuing for 224 nucleotides to an adenosine-rich region (Figure 4.13).



**Figure 4.12:** Identification of the differentially expressed Differential Display Band representing the *MNN4* gene. **(a)** DNase-treated RNA for Differential Display. Each lane represents an equal number of either stressed (5% ethanol) or unstressed (0% ethanol) cells harvested over a 3 hour time-course. **(b)** A differentially expressed band was excised from this Differential Display gel and re-amplified with the same primer pair **(c)**. A lower molecular weight band was also visible on the 2% agarose gel but the most dominant band of approximately 300bp was re-amplified with the extended primer for sequencing **(d)**. The sequenced band proved to be derived from the *MNN4* gene.

1 **atg**cttcagc gaatatcadc taaacttcac aggcggttct tatctggcct **Start codon of MNNA**  
51 gctgcgtgtc aagcactacc cattaaggcg cttctcctt ccaactgattc  
101 tactgcagat catcattata acgtttatct ggtcaaattc accgcagcgt  
151 aacggacttg ggcgggacgc tgattacctt ctaccaaatt acaacgaact  
201 tgacagtgat gatgattcct ggtagatcat cctgacttcg tctttcaaaa  
251 acgatcgcaa gatccagttc gctaagacat tatacgaaaa tttaaaattc  
301 ggcaccaacc ctaaatgggt caatgaatat actctgcaa atgacctgct  
351 ctcggtcaaa **atg**ggccctc gaaagggcag taagctcgaa tccgtggatg **Start codon of former**  
401 agttgaagtt ttacgacttc gaccctcgtc tcacgtggtc cgttctgctg **ORF YKL200c**  
451 aaccatttgc aaaataatga cgcagatcag ccagaaaagt tacccttttc  
501 atggtacgac tggacaacct tccacgagct gaataagctg atttccatga  
551 ataaaactgt tctgccttgc aattttcttt tccagtcgct tttcgacaaa  
601 gagtcttag aggccattga gacagagctc ggcgaaactt tgttctata  
651 cgaaagacca aagtacgctc agaaactgtg gtacaaggcc gctagaaaacc  
701 aggacagata caaagactca aaggaactaa aaaagcattg ttccaagcta  
751 ttcactccag acggg**catgg** **ctctcctaag** **ggtttaagat** ttaatacgc RT-PCR primer  
801 atttcaaaata aaggagctgt atgataaagt tagaccgaa gtttaccat  
851 tgcaggcaag aaactacatt ttgact**acac** **agtgcaccc** **actatcc** RT-PCR primer  
901 tccatcatcg aatcagataa ttccacgata caagtccctt tgcaaaactga  
951 aaaatcaaaa aacttgggtgc aat**ccggcct** **gttcagga** **tatattaatg**  
1001 **ataacattaa** **ttctacgaac** **aagagaaaga** **aaaataaaca** **ggcagtagaa** **Differential Display**  
1051 **ttcaaccata** **acaggctttt** **ccaggaattc** **gtcaataacg** **accaagttaa** **band alignment**  
1101 **ctccctatac** **aaactggaaa** **ttgaagaaac** **tgataaattc** **acttttgata**  
1151 **aagatttggg** **ttatttatcc** **ccttcggatt** **tcaagttcga** **tgccctccaaa**  
1201 aaaattgaag agtttagagga acagaagaaa ctctatccgg acaaattttc  
1251 cgctcataat gagaattatc tgaacagttt gaagaattcc gtaaagacaa  
1301 gccctgcatt gcaaaagaaag ttcttctatg aggcctgtgc cgtgaagcaa  
1351 tataaaggta tggggttcca tctgtgacaag aggttcttca atgttgatac  
1401 attaatacaat gataaacaag aataaccaggc tagattgaac tcaatgatca  
1451 gaacattcca aaagtttact aaagccaacg gcatcatatc ttggttgtct  
1501 cacggaacgc tgcacggcta tctttacaat ggaatggctt tcccttggga  
1551 taacgatttc gacttgcaaa tgcccattaa gcatttaca ttgctcagtc  
1601 aatacttcaa ccaatctctt atattggaag acccaagaca gggtaatgga  
1651 cgttatttcc tagacgtcag cgactccttg acagtaagaa ttaacggtaa  
1701 cggtaaaaac aatatcgatg caagattcat tgacgtcgac accggccttt  
1751 acattgatat taccggctca gctagcactt ctgcccttag tagggattac  
1801 ttgaattctt atattgaaga ggcgttgcaa gaggaacatt tggatatcaa  
1851 taatatccct gaatcgaacg gtgagaccgc tactttgccc gacaaagtag  
1901 atgatgggtt agtcaatatg gctacactaa acatcactga gctacgtgat  
1951 tacattacca gcgacgaaaa taaaaatcat aaaagagtc ccaactgatac  
2001 tatattgaaa gatcttttga aaaaggaact ggaagagtta ccaaagtcta  
2051 agaccattga aaacaagttg aatcctaaac aaagatattt tctcaacgaa  
2101 aaacttaaac tttaacaattg tagaacaac ctttttaact cgttcgagga  
2151 actatctccc ttaatcaata ctgttttcca tgggttgcca cgttgattc  
2201 ctacagaca tacctactgc ttgcacaatg aatatcatgt acctgataga  
2251 tatgcatthg atgcttaca aaatactgct tatttgccc aatttagatt  
2301 ttggttcgac tagcaggggt taaagaaatg cagtaatatt aattcatggt  
2351 atccaaacat cccagattt aattcatgga atccgaacct cttgaaagaa  
2401 atatcgtcta cgaattttga gtcgaaactt tttgattcca acaaagtctc  
2451 tgaatactct ttcaaaaacc tatccatgga tgatgttcgc ttaatttata  
2501 aaaatattcc aaaagctggc tttatcgagg tatttactaa ctgtacaat  
2551 tccttcaatg tcaactgcata taggcaaaaag gaattggaaa tcaactactg  
2601 ccaaaacctg acattttatg aaaaaaagaa attattacat caattgcgca  
2651 ttaatgttgc tctaagttga agctcccctg caaaggacc atttctttt  
2701 ggttatgaaa aagctatgtg gaaggattta tcaaaatcta tgaaccagac  
2751 tacattagat caagttacca agattgttca tgaagaatat gtcggaaaaa  
2801 ttattgatct gtccgaaagt ttgaaataca ggaatttttc acttttcaac  
2851 attacttttg atgaaactgg aacaactcta gatgataaca cagaagatta  
2901 tactcctgct aatactgttg aagtaaatcc tgttgatttt aatcaaat  
2951 taaactttag tagcaactcc tttttggatt taaattcata tggtttagac  
3001 ctttttggcg caactttatc cgacgttaac agaaagggtat tcaaatggt  
3051 tgataaggac cctattatg tatacagga ctatgcttat gccaaagttac  
3101 ttgaagaaag aaagcggagg gagaagaaga agaaggagga agaggagaag  
3151 aagaagaagg aagaagagga aaagaaaaag aaggaagaag aagaaaaaga  
3201 aaagaaggaa gaggaagaga agaaaaagaa ggaagaagaa gagaagaaaa  
3251 agaaggaaga agaagaaaaag aagaagcagg aggaagagga gaaaaagaag

continued overleaf

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3301 aaggaagaag aagagaagaa gaagcaggaa gaaggagaaa agatgaagaa
3351 tgaagatgaa gaaaataaga agaataaga tgaagaaaag aagaagaacg
3401 aagaagagga aaaaaagaag caggaagaga aaaacaagaa gaatgaagat
3451 gaagaaaaga agaagcagga agaggaagaa aagaagaaga acgaagaaga
3501 ggaaaaaaag aagcaggagg aggggcacag caattaa
```

(SGD 2001)

**Figure 4.13:** *MNN4* gene sequence. The *MNN4* (*YKL201c*) gene (submitted by Odani *et al.*, 1996) to the DDBJ/EMBL/GenBank databases in 1996) shows the Differential Display band alignment, RT-PCR primer sequences and start codons. The *MNN4* ORF on chromosome XI is 3537 nucleotides in length and consists of the *YKL201c* and former *YKL200c* ORFs.

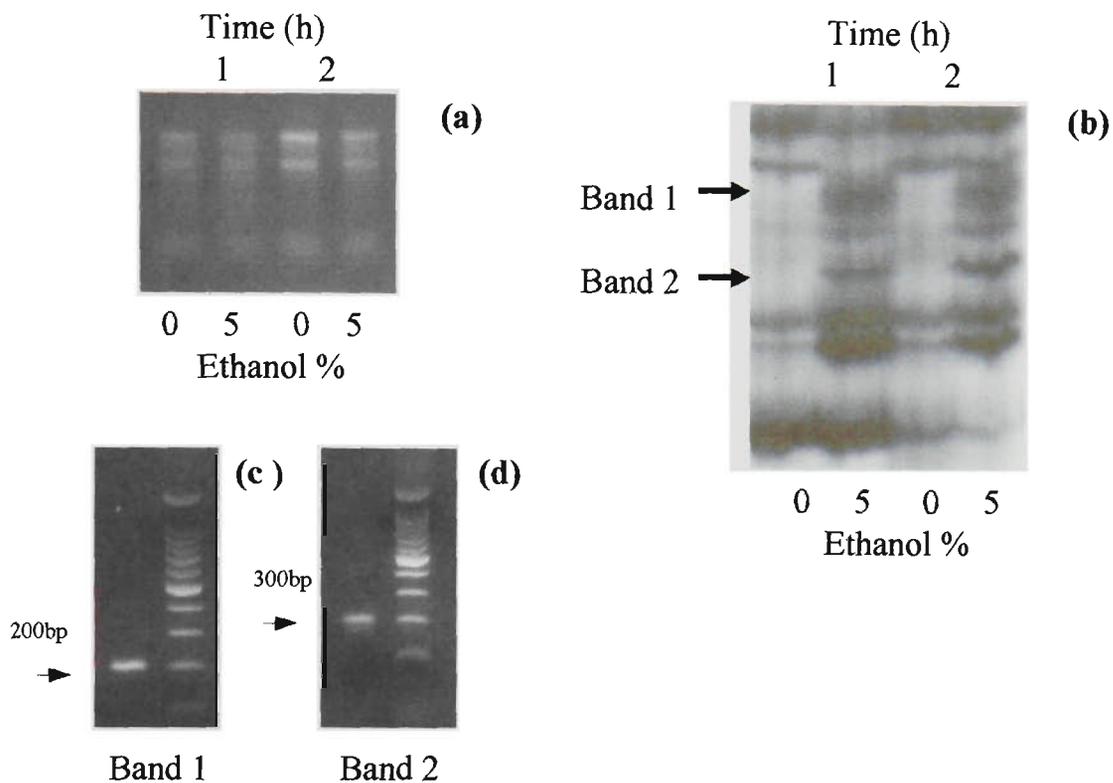
### 4.3.5 Putative ethanol-stress-induced Ty elements

RNA for the Differential Display experiment in which the putative ethanol-stress-induced Ty2 element was identified was from the stressed and control cultures harvested 1 and 2 hours post-inoculation (Figure 4.14a). Differential Display reactions were performed using primers D2/U17. Two series of strongly differentially expressed bands were visible on the autoradiograph in samples from stressed cultures grown for 1 and also for 2 hours (Figure 4.14b). Two bands from a single time-point from stressed cultures grown 1 hour (arrowed) were removed from the gel and re-amplified with the same primer pair followed by re-amplification with D2/UX17 (Figure 4.14c and d). Both bands appeared to be approximately 200 bp long and were purified and sequenced. Sequence data is presented in Figure 5, Appendix IV.

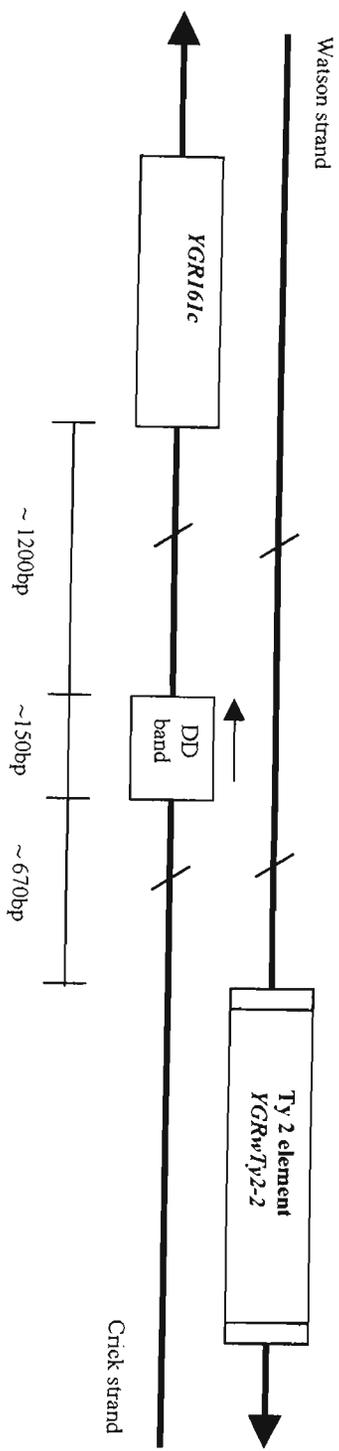
A BLASTn sequence analysis of the two differentially expressed bands revealed that they both aligned to a region between the coordinates 805616 and 805757 on the right arm of *S. cerevisiae* chromosome VII with a probability of  $8.2e-39$ . This sequence corresponds to a region between a hypothetical ORF *YGR161c* and the LTR (long terminal repeat) of a Ty2 element (Figure 4.15).

Another Ty element was identified from RNA prepared from stressed and control cultures grown for 1, 3 and 5 hours (Figure 4.16a) and amplified using the D1/U23 primer combination. Autoradiography revealed a strongly differentially expressed band derived from the stressed culture grown 5 hours post-inoculation (Figure 4.16b). After re-amplification with primers D1/UX23 in duplicate, bands of approximately 350 bp (Figure 4.16c) were sequenced. Sequence data is presented in Figure 6, Appendix IV.

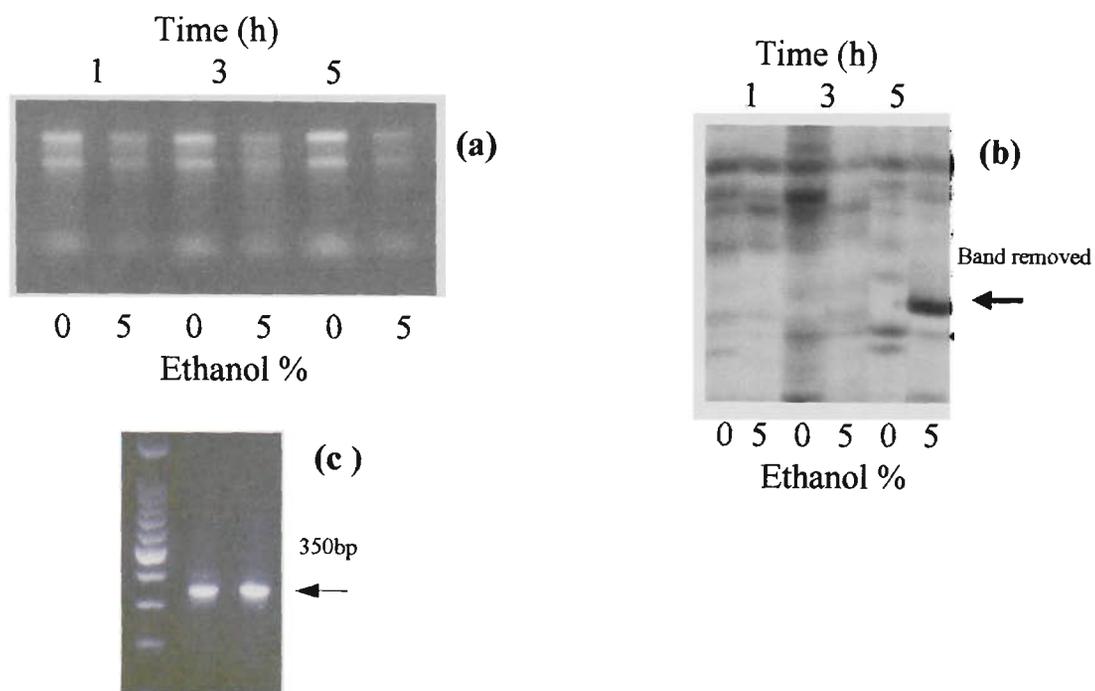
A BLASTn sequence analysis revealed that the duplicate PCR-amplified bands aligned to an internal region of a Ty1 element on chromosome XVI with a probability of  $2.0e-56$  as well as aligning to Ty elements on a further 13 chromosomes with probabilities ranging from  $3.5e-30$  to  $2.0e-56$ .



**Figure 4.14:** Identification of the differentially expressed Differential Display band related to a Ty2 element. **(a)** DNase-treated RNA for Differential Display. Each lane represents an equal number of either stressed (5% ethanol) or unstressed (0% ethanol) cells harvested over a 2 hour time-course. **(b)** Two bands derived from stressed cells were re-amplified with the same primer pair followed by re-amplification with an extended primer for sequencing **(c)** and **(d)**. Both bands were of approximately 200bp. The nucleotide sequence of both bands was the same and derived from a region close to a Ty 2 element.



**Figure 4.15:** The relationship of a differentially expressed Differential Display band to the full length Ty 2 element, *YGRwTy2-2*, on the Watson strand and the hypothetical ORF *YGR161c* on the Crick strand of chromosome VII of *S. cerevisiae*. Sequence data obtained from SGD. Not drawn to scale.



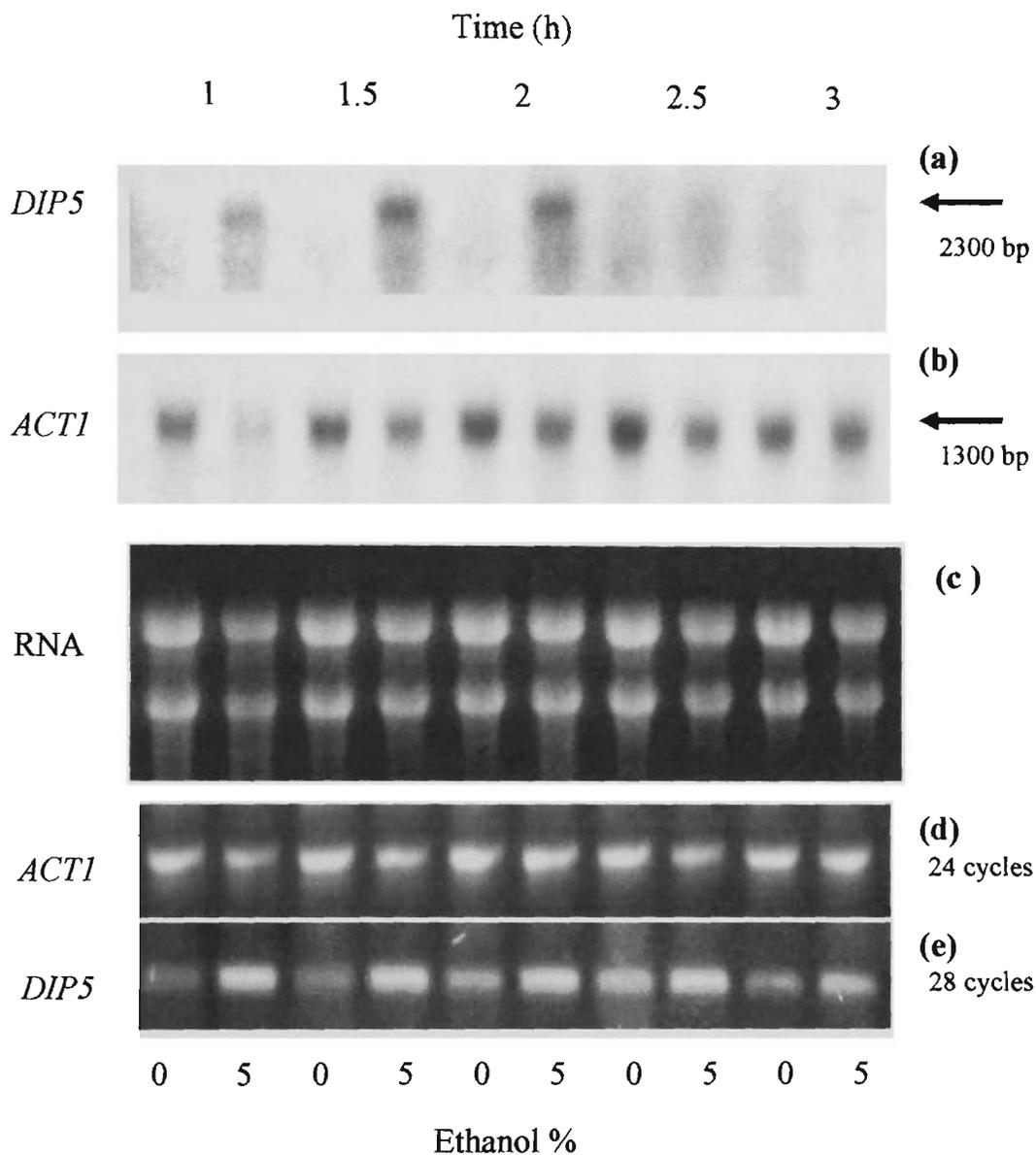
**Figure 4.16:** Identification of the differentially expressed Differential Display band related to a Ty1 element. **(a)** DNase-treated RNA for Differential Display. Each lane represents an equal number of either stressed (5% ethanol) or unstressed (0% ethanol) cells harvested over a 5 hour time-course. **(b)** A band derived from stressed cells grown for 5 hours post-inoculation was re-amplified in duplicate with the same primer pair followed by re-amplification with an extended primer for sequencing **(c)**. The duplicate bands were of approximately 350bp and the nucleotide sequence indicated that they bore relationship to a Ty1 element.

#### 4.4 NORTHERN AND RT-PCR ANALYSES TO VALIDATE DATA FROM DIFFERENTIAL DISPLAY EXPERIMENTS

Differential Display is a highly sensitive technique for identifying changes in gene expression but there have been numerous reports that a high proportion of putative differentially expressed genes could not be validated. Thus, any data generated using Differential Display requires careful verification. For work described in this chapter two approaches, Northern and RT-PCR analyses, were used to test the validity of the findings that *DIP5*, *YGPI*, *YER024w* and *MNN4* are ethanol-stress response genes. In each case the housekeeping gene, *ACT1*, was used as a control. For Northern and RT-PCR analyses RNA templates were derived from the same ethanol-stressed and unstressed cultures unless otherwise stated. Thus temporal expression information derived from Northern and RT-PCR was equivalent. However, for validation purposes, RNA was harvested at more time points than for Differential Display thus validation time-courses contained more information than Differential Display time-courses. Negative controls for RT-PCR in which Reverse Transcriptase was omitted from the cDNA reaction, were routinely included in PCR reactions for the same number of cycles as for the putative genes. The absence of bands in negative controls indicated that RT-PCR bands were derived from cDNA rather than genomic DNA. RT-PCR products were routinely sequenced and searched by BLASTn sequence analysis to verify their origin. The sequences of oligonucleotides used for Northern analyses and as RT-PCR primers are presented in Tables 3 and 4 respectively, Appendix II.

##### 4.4.1 Is *DIP5* a real ethanol-stress response gene?

To validate upregulation of *DIP5* expression in response to ethanol stress Northern analyses were performed using RNA from cultures harvested at half-hourly intervals between 1 and 3 hours post-inoculation. They were probed with a radiolabeled *DIP5*-specific oligonucleotide probe (Table 3 Appendix II) (see Figure 4.17a) then stripped and re-probed with an *ACT1*-specific probe (Figure 4.17b). Cerenkov counts indicated that the percentage incorporation of the radioactive nuclide was 28% for the *DIP5* probe and 14% for *ACT1*.



**Figure 4.17:** Validation of *DIP5* as an ethanol stress-response gene by Northern and RT-PCR analyses. **(a)** Northern analysis was performed by first probing with a *DIP5*-specific oligonucleotide probe. The stripped blot was re-probed with a probe for the housekeeping gene, *ACT1* **(b)**. RNA used for the Northern analysis **(c)** was also used for RT-PCR analysis. The templates were PCR-amplified with both *ACT1*- **(d)** and *DIP5*-specific Primers **(e)** for 24 and 28 cycles respectively. Both Northern and RT-PCR showed that *DIP5* was clearly up-regulated in ethanol-stressed cells.

quality. The level of *DIP5* expression was greatest in cultures grown 1.5 to 2 hours post-inoculation. By comparison, *ACT1* expression was relatively constant across the time-course but clearly reduced in stressed cultures. The amount of RNA was also at reduced levels in stressed cultures (Figure 4.17c).

The *DIP5* transcript was approximately 2300 bp and *ACT1* approximately 1300 bp. The size of the *DIP5* ORF is 1826 nucleotides but the Differential Display band aligned with the 3' untranslated region of the gene thus transcription of *DIP5* extended more than 250 nucleotides downstream of the termination codon (Figure 4.7). The length of the primary transcript was therefore at least 2100 nucleotides. The band of approximately 2300 nucleotides representing *DIP5* mRNA identified by Northern analysis may include a polyA tract of approximately 200 nucleotides. However, the Differential Display band did not extend as far as the putative polyA signal indicating that the primary transcript detected here was shorter than expected. One possible explanation for this is that RNA Polymerase II efficiency is compromised due to ethanol stress.

The downstream region of the *DIP5* ORF is distinguished by an autonomously replicating sequence (ARS) consensus sequence (TTTTATGTTTT), in a complementary orientation to the *DIP5* ORF (Figure 4.6). This feature falls within the region represented by the Differential Display band, however, the significance of this feature, if any, in relation to transcription or ethanol stress is not known. An ARS element is also present downstream of the *ARO8* gene encoding the Aromatic Aminotransferase I involved in aromatic amino acid deamination (Iraqi *et al.* 1998). RT-PCR analysis was carried out using the same RNA samples as for Northern analysis prepared from stressed and control cultures grown between 1 and 3 hours. RT-PCR reactions were performed using gene-specific RT-PCR primers (Table 4 Appendix II) for 24 cycles (*ACT1*) and 28 cycles (*DIP5*) under identical PCR conditions and separated on 1.5% agarose gels (Figure 4.17d and e, back). To amplify comparable PCR products from the same cDNA templates a greater number of PCR cycles were required to amplify *DIP5* products than *ACT1* since the level of *ACT1* expression was greater overall than *DIP5* expression. *DIP5* expression level was greatest in stressed cultures and the pattern of expression was the same as for Northern analysis. The level of *ACT1* expression was likewise greatest in unstressed

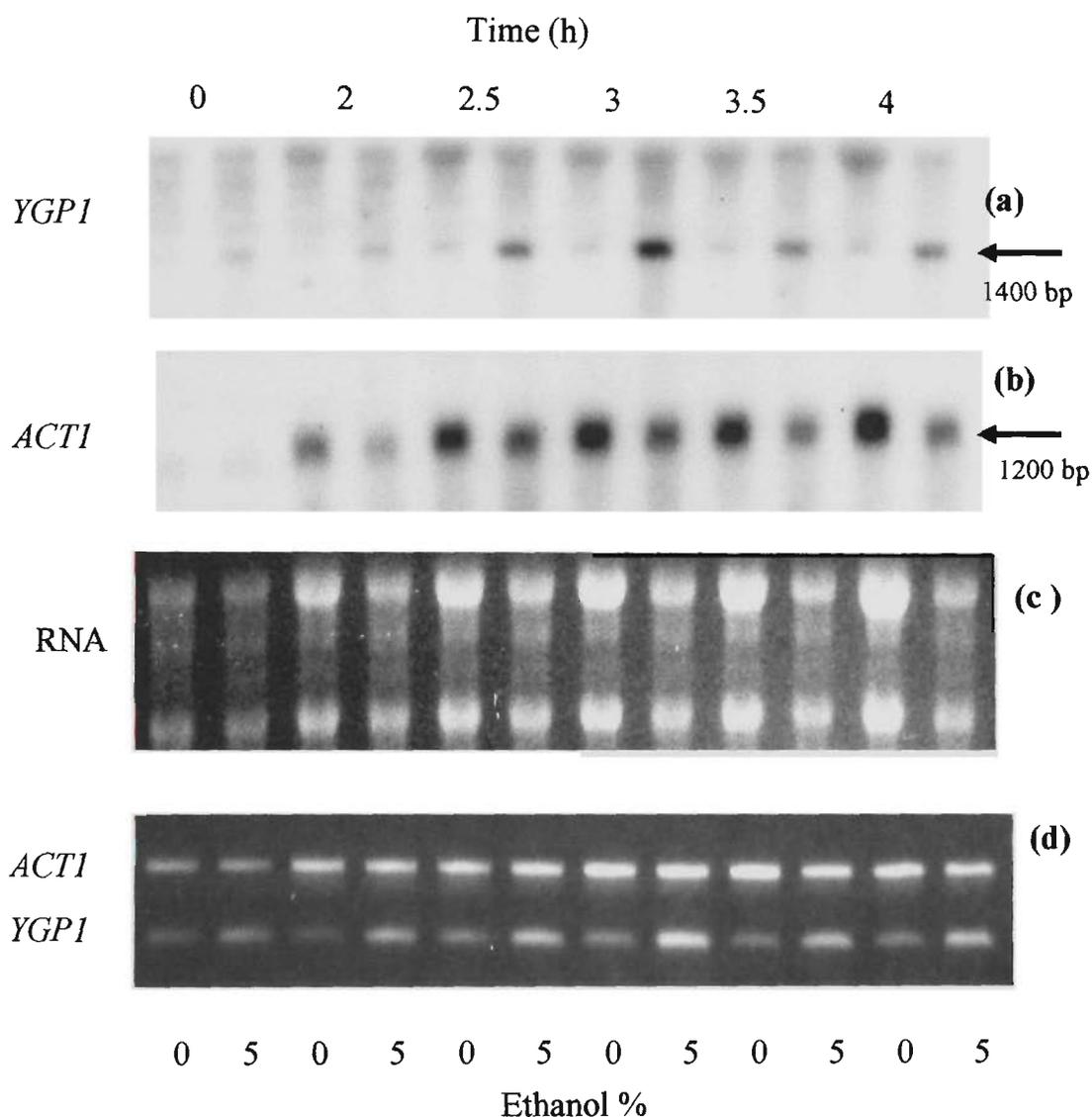
cultures and was consistent over the time-course. The *ACT1* and *DIP5* RT-PCR products were of the expected size and were sequenced and verified by BLASTn sequence analysis (data not shown).

#### 4.4.2 Is *YGPI* a real ethanol-stress response gene?

To validate the upregulation of *YGPI* in response to ethanol stress Northern and RT-PCR analyses were performed on RNA prepared from stressed and control cultures harvested at half-hourly intervals between 0 and 4 hours post-inoculation. The Northern blot was probed with a radiolabelled *YGPI*-specific oligonucleotide probe (Table 3 Appendix II) Figure 4.18a, overleaf, stripped and re-probed with an *ACT1*-specific probe (Figure 4.18b). Cerenkov counts indicated that the percentage incorporation of the radioactive nuclide was 32% for the *YGPI* probe and 49% for *ACT1*. The *YGPI* transcript measured approximately 1400 bp and the *ACT1* transcript approximately 1200 bp. The level of *YGPI* expression was greatest in cultures grown for 3 hours post-inoculation in comparison to *ACT1* expression that was relatively constant across the time-course but consistently reduced in stressed cultures. RNA levels were also lower in stressed cultures (Figure 4.18c). Northern analysis using two alternative oligonucleotide probes targeting both 5' and 3' ends of the *YGPI* mRNA identified a band of the same size (data not shown).

A *YGPI* transcript of approximately 1400 bp was identified in the Northern analysis described here and in the Northern analysis of stationary phase cells (Riou *et al.* 1997). The *YGPI* ORF is 1065 nucleotides long and the Differential Display band was derived from approximately 200 nucleotides of the 3' untranslated region of the transcript (Figure 4.9). A putative polyA signal is situated approximately 400 nucleotides downstream of the termination codon suggesting that an unprocessed transcript would be approximately 1465 nucleotides long. The reason for the small variation between experimental and theoretical transcript size is not known.

RT-PCR analysis was similarly performed to confirm the results from Northern analysis. RT-PCR reactions using *YGPI* and *ACT1*-specific primers were PCR-amplified for 22 cycles and the products separated on a 1.5% agarose gel (Figure 4.18d). The RT-PCR primers (Table 4 Appendix II) generated *YGPI* and *ACT1*



**Figure 4.18:** Validation of *YGP1* as an ethanol stress-response gene by Northern and RT-PCR analyses. (a) Northern analysis was performed by first probing a blot prepared from stressed and unstressed cultures harvested over a 4 hour time-course beginning at time 0, with a *YGP1*-specific oligonucleotide probe. The stripped blot was re-probed with an oligonucleotide complementary to the housekeeping gene, *ACT1* (b). RNA used for the Northern analysis (c) was also used for RT-PCR analysis. The templates were PCR-amplified with both *ACT1* (d) and *YGP1*-specific primers (e) for the same number of cycles. Both Northern and RT-PCR showed that *YGP1* was clearly up-regulated in ethanol-stressed cells.

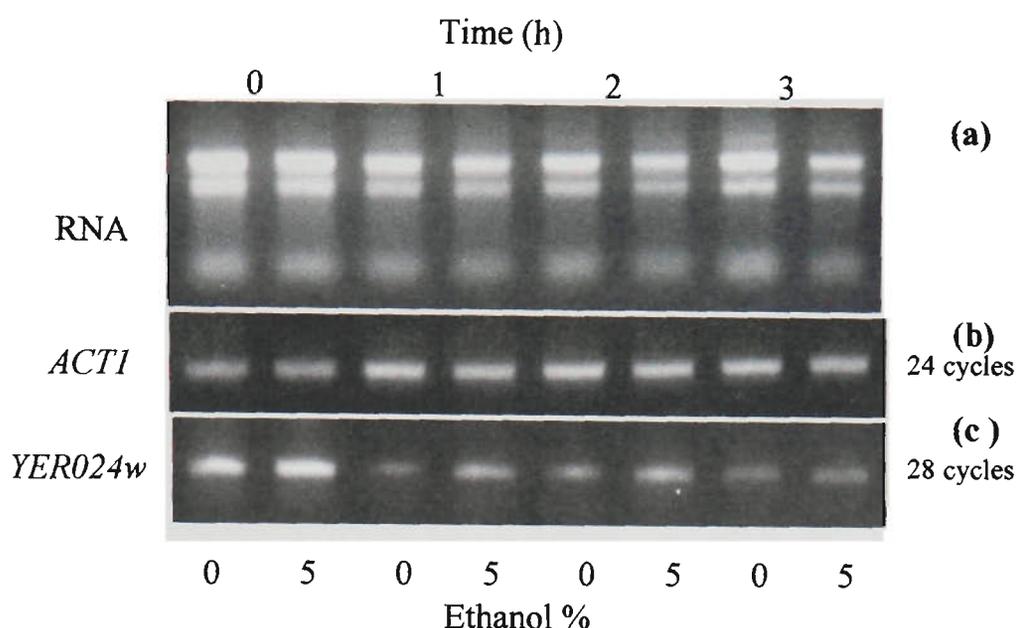
products of expected size that were verified by sequencing (data not shown) and BLASTn sequence alignments. The RT-PCR analysis produced similar results to Northern analysis; *YGP1* expression was at its highest level in stressed cultures grown for 2.5 to 3 hours post-inoculation whilst *ACT1* expression was consistent across the time-course although reduced in stressed cultures in comparison to unstressed controls.

#### 4.4.3 Is *YER024w* a real ethanol-stress response gene?

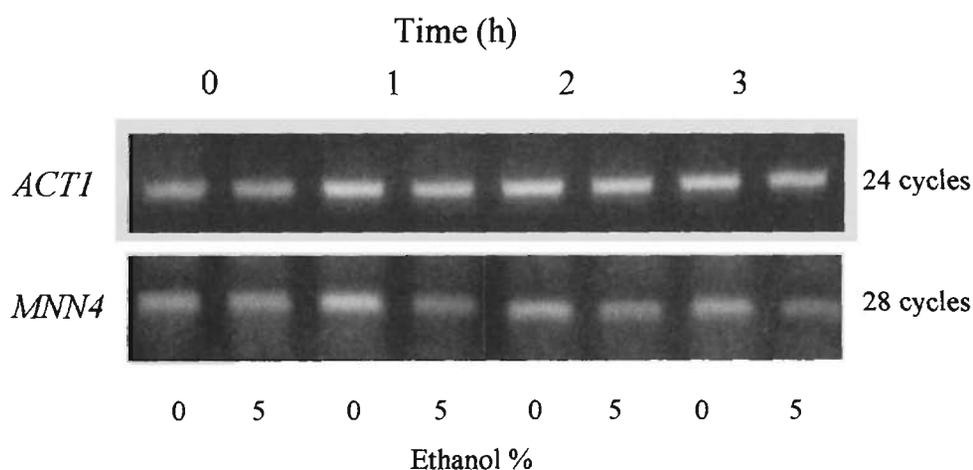
Since the results from RT-PCR analyses were consistently comparable to results obtained from Northern analysis of *YGP1* and *DIP5*, it was decided that RT-PCR would suffice as a means of validating the status of *YER024w* as an ethanol-stress response gene. RNA from stressed and unstressed cultures grown for 3 hours and harvested at hourly intervals was used for RT-PCR (Figure 4.19a). Each RNA sample was derived from  $10^8$  cells except for the RNA samples from cultures harvested immediately after inoculation (Time 0) which were prepared from  $2 \times 10^8$  cells. RT-PCR reactions were PCR-amplified for 28 (*YER024w*) and 24 (*ACT1*) cycles since the overall level of *ACT1* expression was higher than that of *YER024w*. The expression level of *YER024w* in stressed cultures was greater than the level in unstressed cultures at the 0, 1 and 2 hour time-points in comparison to *ACT1* although *ACT1* expression level was greater in unstressed cultures but relatively constant across the time-course (Figure 4.19b). *YER024w* is present at time zero and diminishes over the time-course (Figure 4.19c) in contrast to *ACT1* which is relatively weakly expressed at time zero. RT-PCR products were of the expected size and were sequenced and verified (data not shown).

#### 4.4.4 Is *MNN4* a real ethanol-stress response gene?

To validate upregulation of *MNN4* in response to ethanol stress, RT-PCR analysis was performed on RNA from cultures grown for 3 hours post inoculation (Figure 4.19a). Expression of *MNN4* was considerably lower than the *ACT1* control and required 40 PCR cycles compared to 24 cycles for *ACT1*. As for *ACT1*, *MNN4* was more highly expressed in the unstressed samples and therefore could not be confirmed as an ethanol-stress response gene (Figure 4.20). Because of the low level of *MNN4*



**Figure 4.19:** Validation of *YER024w* as an ethanol stress-response gene by RT-PCR analysis. Templates derived from RNA shown above were PCR-amplified using *YER024w*-specific primers and primers for the housekeeping gene, *ACT1* for 24 and 28 cycles respectively. *YER024w* was more highly expressed in samples derived from stressed cells than in control cells.



**Figure 4.20:** *MNN4* could not be confirmed as an ethanol stress-response Gene using RT-PCR. The same templates as used for *YER024w* RT-PCR were also amplified with *MNN4*-specific PCR primers for 28 cycles. *MNN4* was not up-regulated in samples derived from ethanol-stressed cells.

transcripts in cells harvested for this work it was unlikely that Northern analysis would produce more informative data. The apparent ethanol-stress-induced-upregulation of *MNN4* detected using Differential Display is therefore probably a false positive although a more sensitive method such as real-time PCR may give greater definition to its expression.

#### **4.4.5 Ty elements in the *S. cerevisiae* ethanol-stress response**

No verification work was undertaken for Ty elements because of the technical difficulties involved. The sequence of all *S. cerevisiae* Ty elements is similar and the position and copy number of the various Ty elements in the *S. cerevisiae* database may well be different to that of PMY1.1. Considering also that Ty elements are mobile and may influence the expression of neighboring genes, further analysis of the regulation of Ty elements during adaptation to ethanol-stress response was considered to be beyond the scope of this project and was not undertaken.

### **4.5 DISCUSSION OF RESULTS**

#### **4.5.1 Reduction in RNA level due to ethanol stress**

Ethanol stress consistently reduced the amount of rRNA retrieved from equivalent numbers of cell. It may be argued that ethanol stress affects the cells in a way that interferes with the RNA isolation procedure e.g. making cells refractory to rupture and therefore decreasing the efficiency of RNA isolation. In addition the lower yields of RNA may reduce the efficiency of precipitation. Thus concentration influences would be likely to bias levels of rRNA in favour of the unstressed control samples. However, the level of DNA present in RNA isolation preparations was relatively consistent in each set of RNA isolation preparations (see Figure 4.5) suggesting that growth in the presence of ethanol did not influence cell rupture or precipitation of nucleic acids to a large extent. In addition, the expression of the housekeeping gene *ACT1*, was relatively constant across the time-courses in Northern and RT-PCR analyses, although slightly reduced in stressed cells. At the same time the level of expression of ethanol-stress response genes *DIP5*, *YGP1* and *YER024w* was greater in stressed cells and thus may actually be under-represented. In view of these results it

may be argued that ethanol stress leads to a reduction in the level of rRNA in *S. cerevisiae* cells independently of mRNA levels.

Since the bulk of cellular RNA is rRNA the reduced level of RNA in ethanol-stressed cells suggests translational capacity may be impaired. This may be a contributing factor to the reduced growth rate and increased length of the lag period in ethanol-stressed cultures. There have been no previous reports of a reduction in the level of ribosomal RNA in yeast following exposure to ethanol although ethanol is reported to influence rRNA levels in other species. For example, the rate of RNA synthesis is retarded in *E. coli* exposed to ethanol (Ingram and Buttke 1984) and ingestion of a high level of alcohol by laboratory rats leads to a rapid decline in levels of RNA, DNA and mitochondrially encoded rRNA in left ventricle heart tissue (Kou and Cohen 1998).

In yeast, some stress conditions lead to decreased rRNA levels. For instance, heat shock causes a decrease in rRNA levels due to inhibition of the initiation of rRNA gene transcription (Veinot-Drebot *et al.* 1989) and a failure of the secretory pathway leads to severe repression of rRNA and ribosomal gene transcription (Nierras and Warner 1999). The level of rRNA synthesis is also growth stage-specific; rRNA as well as tRNA levels decrease with declining growth rate (Waldron and Lacroute 1975) and rRNA transcription declines in late log phase when the number of ribosomes per cell falls to less than 25% of the maximum value (Ju and Warner 1994). These authors concluded that the reduction in translational capacity was due to the cell "sensing unfavourable conditions". However, in light of the results of the ethanol-stress experiments described in this thesis, the decline in rRNA observed by Ju and Warner (1994) may be as a result of the accumulation of endogenous ethanol in late log phase cultures.

The addition of ethanol to yeast cultures also leads to a reduction in protein synthesis. The addition of 6.4% (v/v) ethanol led to a 40% decrease in protein synthesis in *S. cerevisiae* when measured by the cellular uptake of tritiated valine into newly synthesized proteins (Girbes and Parilla 1983). The authors suggested that the inhibitory action of ethanol might be due to its interaction with ribosomes. The inhibitory effect of ethanol on protein synthesis is not universal however. At ethanol

concentrations of 4% and 10% (v/v) the synthesis of heat shock proteins Hsp104, Hsp70 and Hsp26 are induced (Piper *et al.* 1994). Thus, whilst there may be a net reduction in protein synthesis, there is an increase in the synthesis of at least some stress response proteins.

From results presented in this thesis it also appears that rRNA is degraded when yeast cells experience ethanol stress. One possible explanation for this observation is that RNase activity is upregulated in response to the presence of stressful amounts of ethanol.

#### **4.5.2 Modified Differential Display**

Differential Display was successfully performed to identify genes upregulated during the adaptation of *S. cerevisiae* to ethanol stress. Using the modified approach to Differential Display described in this thesis the number of false positive results was reduced in comparison to previous reports: only one false positive cDNA was generated. The expression level of most genes as represented by gel banding patterns was found to be similar overall between test and control samples with relatively few bands appearing to be up- or down-regulated across the time-course. This confirmed that using equal cell numbers for RNA isolation did not unbalance the amounts of mRNA in the samples.

Although Differential Display is a sensitive and reproducible method for comparing the gene expression of related cells, there have been many reports of false positives generated (Li, Barnathan and Kariko 1994; Liang 1996; Liang, Averboukh and Pardee 1993). These false positives may constitute more than 70% of the results (Nishio, Aiello and King 1994; Sun, Hegamyer and Coburn 1994). Liang and Pardee recognized this problem and made improvements to the original protocol but still only 5 from 15 bands identified by Differential Display could be confirmed (Liang, Averboukh and Pardee 1993). Further modifications have been proposed to speed up the procedure and reduce the level of false positives (as noted in section 4.1). The modifications to Differential Display described in this thesis are improvements that extend that process and introduce several features to improve the outcome in terms of the quality of the data.

One factor that is likely to contribute to the generation of false positives is that the classical approach to Differential Display equalizes amounts of total RNA from test and control samples. As rRNA constitutes a high percentage of total RNA this procedure rests on the assumption that inherent rRNA levels are not affected by the growth conditions or experimental treatments and are a true reflection of mRNA levels. The suitability of this approach was questioned for work described in this thesis since, under ethanol stress, the amount of rRNA (as visualized on an ethidium bromide-stained agarose gel) in stressed cells was greatly reduced in comparison to unstressed cells. It is therefore likely that equalizing the amounts of total RNA would bias the results in favour of generating false positives as mRNA levels from the stressed culture would be artificially boosted. For this reason it was decided to compare cDNA profiles from equal cell numbers rather than equal amounts of RNA. Using this strategy, the level of overall expression was found to be similar when samples from stressed and unstressed cultures were compared on Differential Display gels indicating that although the level of rRNA was lower in stressed samples the level of mRNA remained relatively constant. Uniform and reliable Differential Display results have been achieved using this strategy and three out of the four gel bands analyzed were subsequently validated as ethanol-stress induced. It should be noted however that there were a number of differentially expressed bands that were downregulated in ethanol-stressed samples that have not been processed further and it is possible that the analysis of these bands would identify genes that are switched off in the presence of ethanol.

Another way of minimizing false positives is by performing Differential Display over a time-course (Burn *et al.* 1994; Sompayrac *et al.* 1995). The appearance of a band of interest repeated across the time-course gives increased confidence in the validity of the data and reduces the need for replicates. However, one limitation of the strategy of investigating only Differential Display bands that occur at more than one time point is that transiently expressed genes may not be identified. In the case of *MNN4*, the one putative ethanol-stress response gene that was not validated in this work, differential expression across the time-course on the Differential Display gel was not as clear as for the other genes. One benefit of time-course Differential Display was that it contributed data on temporal gene expression that was comparable to results from Northern and RT-PCR; differentially expressed bands of the same molecular

weight from different samples/time points were found to be have derived from the same gene e.g. *YGPI*.

False positives may also be generated from contaminating chromosomal DNA in RNA preparations, acting as template in PCR reactions (Liang 1996). An extra acid phenol extraction during RNA preparation was introduced into the protocol to minimize this problem and Differential Display and RT-PCR negative controls indicated that only cDNA and not gDNA was amplified.

Heterogeneous gel bands in Differential Display gels can create problems at the subcloning and characterisation stage of analysis. These bands consist of two or more co-migrating cDNA species that may become apparent when re-amplified and visualised following resolution on an agarose gel. This problem occurred when the differentially expressed band representing *MNN4* was re-amplified. Only the most intense of the two bands generated was analysed (the second band was relatively faint) and results suggest it was a false positive.

Closely migrating bands of similar size is problematic when Differential Display gels are transferred to paper since neighboring bands may be cut from the paper. To minimize the chance of excising neighboring bands that would be PCR-amplified with the band of interest, Differential Display gels were dried on the glass sequencing plates. This meant that band removal was more precise and direct than from gels transferred to paper, and the excised band solution did not require clean-up and precipitation of DNA. Additionally, to confirm that the correct band had been removed a second film was routinely exposed to the gel following band removal.

Using a non-denaturing (urea-free) system (Bauer *et al.* 1993) rather than the traditional denaturing gels enabled the gels to be run at low voltage. Thus inexpensive, non-specialized glass plates cut for the sequencing apparatus could be utilized. The absence of urea also enabled PCR-amplification of DNA directly from the gel band solution without prior purification. DNA could be successfully removed from gels and re-amplified after storage on the glass at room temperature for several months.

The purification and analysis of Differential Display bands was streamlined by re-amplifying the bands using primers carrying extra sequence to enable direct PCR sequencing (Wang and Feuerstein 1995). PCR products were purified at each stage using agarose gel electrophoresis. These modifications allowed separation of target Differential Display bands from other heterogeneous amplification products without recourse to cloning and thus it substantially reduced post-Differential Display gel analysis. Sequence data generated in this way could readily be used to identify genes in SGD.

In recent years the approach used for analysis of gene expression has shifted to gene array technology. However, the approach to Differential Display described here will be useful for groups unable to access this newer technology or groups hopeful of finding novel genes in species for which commercial gene arrays are not presently available (e.g mouse and human full-genome arrays are not yet available). The issue of equalizing cell number rather than total RNA, for analysis of differential gene expression, may be important for groups working in any system using Differential Display, Northern analysis or gene array technology. The comparison of transcript populations from equal cell numbers allowed comparison of mRNA profiles, independently of any effect of the experimental conditions on total RNA content.

#### **4.5.3 Streamlining validation procedures for differentially expressed genes**

Northern and RT-PCR analyses gave similar results for validation of putative ethanol-stress response genes. Both approaches were found to be useful for observing the expression patterns of genes, particularly when performed over a time-course that was more extensive and defined than the one for Differential Display. The two techniques have different strengths and weaknesses: RT-PCR produces results more quickly than Northern analysis, it does not require the use of radioactive labeling and the products can be verified directly by sequencing. However, only Northern analysis can detect transcript size.

Typically, validation of upregulated Differential Display bands would utilise a labeled probe made by amplifying the upregulated band using the same primer pair as used in the Differential Display (Liang 1996). Alternatively, subcloned Differential Display bands would be removed from plasmids, labeled and used as probes. The use of the entire Differential Display band as a probe for these methods would require considerable optimisation for each probe due to differences in length and melting temperature. For the work described in this thesis, oligonucleotide probes of a specific length and melting temperature were used, enabling hybridization conditions to be standardized in Northern analysis. Careful sequence alignments of the oligonucleotide against the whole *S. cerevisiae* genome using modified search parameters enabled probes to be designed that were highly gene-specific and comparable in specificity to the RT-PCR primers. The alternative method of probing Northern blots with radiolabelled cloned or PCR-amplified Differential Display bands would have required optimisation of hybridisation conditions for each gene.

#### **4.5.4 Ethanol-stress response gene transcripts**

The ethanol-stress response genes *DIP5*, *YGPI* and *YER024w* were found to be differentially expressed using a modified time-course Differential Display. They were identified with a high level of confidence by a BLASTn sequence alignment of the differentially expressed band sequence with the *S. cerevisiae* genome using the SGD and validated using Northern and/or RT-PCR analyses performed over a time-course. From comparison with data in the published literature the *DIP5* and *YGPI* gene transcripts visualised by Northern analysis approximated the expected size. *YER024w* was not analysed by Northern Blotting and therefore the transcript size was not determined.

#### **4.5.5 The putative ethanol-stress response gene MNN4**

Attempts to validate the ethanol-stress-induced upregulation of the *MNN4* gene by Northern analysis (data not shown) and RT-PCR were unsuccessful; the gene appeared to be constitutively expressed. The sequence data from this Differential Display band was of high quality and it aligned with a region of the *MNN4* gene

sequence with high probability. However, it is unlikely that this Differential Display band represents the 3' end of a full transcript as it appeared to have been internally primed from the Differential Display upstream primer. In addition, the pattern of expression was not as clear across the time-course on the Differential Display gel as had been seen for other differentially expressed bands. It is possible that a less intense lower molecular weight band that was also PCR-amplified from the Differential Display gel band (Figure 4.12c) may have been derived from an alternative putative ethanol-stress response gene, as has already been discussed in section 4.5.2.

The gene sequence data and annotation for this gene is confusing and some changes have been made to the *MNN4* gene record in SGD: the YKL200c ORF was originally annotated as the *MNN4* gene ORF and the YKL201c ORF has only recently been included with it. YKL201c now encompasses both sequences following results published by Odani *et al.* (1996).

#### 4.5.6 Ty elements

The sequences of two differentially expressed bands that were removed from the same lane both aligned with high probability to a region of chromosome VII that was between a Ty 2 element and the ORF YGR161c. Since the sequences from the two upregulated bands of slightly different mobility were virtually identical, the slight difference in mobility on the Differential Display gel could be due to differences in secondary structure or additional nucleotides on one of the bands. The orientation of the sequenced bands on the Crick strand upstream of YGR161c and also upstream of the Ty element means that these bands were not derived from either but rather may represent a small ORF situated between the two genes. Ty element insertions are known to influence the transcription of adjacent genes (Ribeiro-dos-Santos *et al.* 1997). The *S. cerevisiae* genome is host to many copies of Ty 1-4 retrotransposons that are similar in overall structure and consist of an internal domain flanked by two LTRs (long terminal repeats) analogous to the LTRs of retroviruses. Ty1 is the most highly repeated Ty element in common laboratory strains of *S. cerevisiae* with typically 25-30 copies per haploid genome. By comparison, there are 5-15 copies of Ty2, the second most common transposable element in most laboratory strains

(references in review by Boeke and Sandmeyer (1991)). The likelihood that these mobile elements are situated in the same location in all *S. cerevisiae* strains and whether the element would be so located in PMY1.1 is not known. For this reason the attempted validation of the Differential Display result was not undertaken despite the growing body of literature relating transposable elements to gene expression generally and the yeast stress response specifically.

Another pair of sequenced differentially expressed bands with similar mobility that were derived from the same sample aligned with high probability to a highly conserved internal region of a number of Ty elements in the database (Figure 4.16). Although upregulation of this element may be a valid stress response result and some supporting literature suggests that Ty transcription occurs in *S. cerevisiae* under stress conditions, no further work on this Differential Display sequence was undertaken since validation of the result was beyond the scope of this thesis.

#### **4.5.7 The influence of mRNA stability and copy number on the interpretation of the Differential Display results**

Differential Display has been criticized for preferentially detecting highly expressed genes (Bertioli *et al.* 1995). It is relevant therefore to report on the apparent half-life, transcriptional frequency and copy number of the ethanol-stress response genes and putative ethanol-stress response genes identified in this thesis and to comment on criticisms of the technique by Bertioli *et al.* (1995).

Although the abundance of mRNAs was not measured for the yeast cultures grown under the conditions described in this thesis, the high sensitivity of the Differential Display method used here is supported by the genome-wide yeast expression analysis of Hostege *et al.* (1998). These authors used *S. cerevisiae* Affimetrix GeneChip™ high-density oligonucleotide arrays (HDA) to compare the transcription profiles of various temperature-sensitive transcriptional mutants to that of their parent strains. This strategy provided a measure of the kinetics of mRNA decay and an apparent half-life for each mRNA species could be calculated from the data. Their results include calculated mRNA expression levels of *YGP1*, *DIP5*, *YER024w* and *MNN4* in

mid-log phase cells of *S. cerevisiae* strain SC288c. *YGP1* was present at the level of 14.3 copies/cell, *DIP5* at 1.3 copies/cell, *YER024w* at 0.6 copies /cell, *MNN4* at 0.3 copies/cell and *ACT1* (used as a control in Northern and RT-PCR analyses described in this thesis) was present at 21.1 copies/cell (Table 4.2).

Liang and Pardee (1992) demonstrated using  $^{35}\text{S}$  as a label, that Differential Display could detect rare mRNA species with a copy number of around 30 per cell and the use of  $^{33}\text{P}$  has since increased the sensitivity of the method. It is not known if the expression level of the ethanol-stress response genes during ethanol stress was in excess of the Hostege *et al.* (1998) figures but detectable levels of ethanol-stress response mRNAs in unstressed control cultures were frequently visible, particularly on Differential Display gels and in RT-PCR analyses. In contrast to the 30 mRNA molecules/cell detected by Liang and Pardee (1992) the Affimetrix GeneChip™ HDA performed by Hostege *et al.* (1998) can detect as few as 0.1 mRNA molecules/cell. HDA is no doubt a great deal more sensitive than Differential Display, nevertheless, the HDA data for the ethanol-stress-induced genes suggests they may have been expressed at a low level and that Differential Display as performed for the work described in this thesis was quite sensitive.

Hostege *et al.* (1998) reported that 80% of the yeast transcriptome is expressed at a low level of 0.1-2 copies/cell. This is comparable to the dogma that most mRNA species are rare: 90% of mRNAs are expressed at the level of 15 or less copies/cell in mammalian cells (Table 4.3). Bertioli *et al.* (1995) however, using Differential Display to investigate differentially expressed genes induced in plants by plant pathogens, failed to detect target mRNAs that constituted less than 1.2% of the total mRNA, equivalent to intermediate to abundant mRNAs with 300-12000 copies/cell (Table 4.3). Using the data from Hostege *et al.* (1998) as a yardstick, the genes identified in this thesis as ethanol-stress response genes are therefore classed as rare transcripts with a cellular abundance of between 1.3 and 14.3 mRNA copies/cell. The Differential Display method used for the work described in this thesis was therefore a great deal more sensitive than that performed by Bertioli *et al.* (1995). Their criticism that Differential Display preferentially detects highly expressed genes is not well

GENE	EXPRESSION LEVEL (copies/cell)	MRNA HALF-LIFE (min)	TRANSCRIPTIONAL FREQUENCY (mRNAs/h)
<i>DIP5</i>	1.3	23	2.3
<i>YGP1</i>	14.3	24	22.5
<i>YER024w</i>	0.6	17	1.2
<i>MNN4</i>	0.3	18	0.6
<i>ACT1</i>	21.1	17	45.5

**Table 4.2:** The expression level of ethanol stress-response genes *DIP5*, *YGP1* and *YER024w*, putative ethanol stress-response gene *MNN4* and control gene *ACT1*. Data is from mid-log yeast cultures grown in YEPD medium (Hostege *et al.* 1998).

EXPRESSION CLASS	EXPRESSION LEVEL(copies/cell)	mRNA SPECIES IN CLASS	MEAN % OF EACH SPECIES IN CLASS
Abundant	12,000	4	3.3
Intermediate	300	500	0.08
Rare	15	11,000	0.004

**Table 4.3:** The abundance of mRNA species and classes in a typical mammalian cell. (Table modified from (Alberts *et al.* 1989) and (Bertioli *et al.* 1995)).

founded and their results may reflect their RNA isolation technique rather than the Differential Display technique itself.

The half-life of the ethanol-stress response gene transcripts identified in this thesis range from 17 to 24 minutes under non-stressed conditions (Hostege *et al.* 1998) and they are therefore classified as moderately stable mRNAs. By comparison, the half-life of 20 different *S. cerevisiae* mRNAs determined by Herrick *et al.* (1990) ranged between 2.5 to 45 minutes. *ACT1* was one of the most stable mRNAs with a half-life of 23 minutes (Herrick, Parker and Jacobson 1990) compared to 17 minutes in the Hostege *et al.* (1998) experiments. The half-life of the ethanol-stress-induced genes has not been determined under ethanol-stress conditions but the published figures for the half life of these gene transcripts in unstressed cultures suggest that transcripts with a moderate half-life were detected by Differential Display in the work described in this thesis. However, particularly in ethanol-stressed cultures, careful harvesting of cells and prompt RNA isolation were clearly critical to the success of this Differential Display project.

#### **4.5.8 Prior phenotypic studies of *DIP5*, *YGP1* and *YER024w***

The roles of the ethanol-stress response genes *DIP5*, *YGP1* and *YER024w* have not previously been studied in relation to ethanol stress. However, some work has been undertaken to determine their phenotype by knockout and/or overexpression studies. For example, the uptake of amino acids by the dicarboxylic amino acid permease has been extensively studied by deletion and overexpression of *DIP5* in cells grown in a variety of media. Deletion resulted in a several hundred-fold reduction in uptake of L-aspartate and L-glutamate (Regenberg *et al.* 1998) whereas *DIP5* overexpression led to an increased uptake of the dicarboxylic amino acids as well as alanine, glycine, serine, glutamine and asparagine (Regenberg *et al.* 1999). The *YER024w* null mutant is viable (Smith *et al.* 1999; Winzeler *et al.* 1999) and has been tested under a range of growth conditions and a *YGP1* knockout has been constructed by interrupting the *YGP1* gene by insertion of the *URA3* gene (Destruelle *et al.* 1994). This *YGP1*<sup>-</sup> strain was used as a negative control in Western analysis of the protein product gp37 but its growth phenotype was not reported.

In view of the high level of ethanol-stress-induced expression of *YGPI* compared to the other ethanol-stress response genes and *ACT1*, and the limited amount of data on its phenotype, it was decided to concentrate on investigating the *YGPI* phenotype by knockout and overexpression studies. This would be achieved by constructing a *YGPI* knockout strain and a *YGPI* rescue of the knockout, with *YGPI* behind an inducible promoter to study overexpression, in a PMY1.1 background, and testing the performance of these strains under ethanol stress.

## CHAPTER 5

# ANALYSIS OF PHYSIOLOGICAL ROLE (S) OF *YGP1*

### 5.1 INTRODUCTION

The roles of the three ethanol-stress response genes identified in the previous chapter, *YGP1*, *DIP5* and *YER024w*, have not previously been studied in relation to ethanol stress although some investigation of their phenotypes have been undertaken, as discussed in the previous chapter. Due to time limitations only one of the above three genes could be analysed to determine its phenotype. In view of the high level of expression of *YGP1* in response to ethanol stress described in the previous chapter, it was decided to concentrate on exploring the phenotype of this gene by knockout and over-expression studies.

The PMY1.1Δ *YGP1* knockout strain was created and its phenotype analysed. To test whether over-expression of *YGP1* would alter the growth profile of PMY1.1, a rescue strain was constructed by transforming the *YGP1* knockout strain with the Genestorm™ pYES2/GS expression vector carrying the *YGP1* ORF insert ( Figure 2.1). Genestorm™ pYES2/GS expression vectors are expression tested in *E. coli* and *S. cerevisiae* prior to delivery and contain a C-terminal V5 epitope tag for protein detection and a C-terminal polyhistidine tag (His 6) for protein purification. This vector requires that its host be *ura3<sup>-</sup>* and that it be grown on a uracil-free medium for plasmid maintenance. The *YGP1* gene was inserted behind the *GALI* promoter, thus galactose was required for induction. A control strain, PMY1.1Δ*YGP1* carrying the Genestorm™ plasmid without the *YGP1* insert, was also constructed for comparison with the *YGP1* knockout and *YGP1* rescue strain during growth experiments.

The aims of this chapter were to:

1. Delete the *YGP1* gene from *S. cerevisiae* PMY1.1 and compare the growth of this knockout strain to that of the wildtype during adaptation to ethanol stress,
2. Return the *YGP1* gene to the *YGP1* knockout on an expression plasmid behind an inducible promoter to observe the effect of induced early expression of *YGP1* on growth during adaptation to ethanol stress.

## **5.2 CONSTRUCTION OF A *YGP1* KNOCKOUT USING *kanMX* MODULE REPLACEMENT**

The *kanMX* module was designed and constructed for yeast knockouts by (Wach *et al.* 1994) and the plasmid containing the *kanMX* module was kindly donated for this work by Dr Paul Vaughan, Division of Molecular Science, CSIRO, Parkville, Australia. The *kanMX* gene replacement strategy is represented in Figure 2.2. PCR primers were designed to target the flanking regions of the *kanMX4* module using the published sequence in (Wach *et al.* 1994) with overhangs of approximately 40 nucleotides that were complementary to the flanking regions of the *YGP1* ORF. PCR products obtained by PCR amplification of the *kanMX* module were then used to transform *S. cerevisiae* strain PMY1.1.

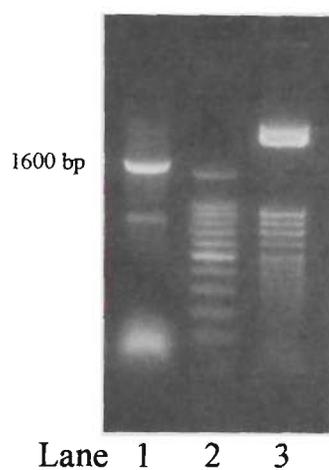
### **5.2.1 Selection, validation and stability of the *YGP1* knockout strain**

The *kanMX* module was PCR-amplified from the pFA6*kanMX4* plasmid using primers YGP1-KO5' and YGP1-KO3' complementary to the flanking regions of the *kanMX* module (Table 5.1). The PCR products were resolved on a 0.8% agarose gel producing a band of the expected size (approximately 1600 bp) (see Figure 5.1). PMY1.1 was transformed by homologous recombination with 2.5 µg of the above PCR products using the lithium acetate method of (Gietz and Schiestl 1995). The cells were plated on YEPD Geneticin plates and incubated at 28°C for 4 days and a single geneticin-resistant transformant colony was streaked onto a fresh YEPD Geneticin plate.

1.	YGP1-KO5'	5' AAGTACGTAGTTGTTTTATCTGCCCTTTTGGCA TGTTACAGCTGAAGCTTCGTACGC 3'
2.	YGP1-KO3'	5' AGTTCTAGAGAAAATGCTTTCCAGAGCGGACTT GGAGGTGCATAGGCCACTAGTGGATCTC 3'
3.	YGP1-PF (-125)	5' CTGCTGTGTTCTCTTGGGTT 3'
4.	YGP1-PR (+72)	5' AAGCTGTTGCGTGGAAATTT 3'
5.	KMX-PF	5' TCGACATCATCTGCCCAGAT 3' *
6.	YGP1-330F	5' GTACAATGTTGCCCGTGTTG 3'
7.	YGP1-531R	5' CAGAAGTGACGGACAAGACA 3'

\* Wach *et al.* (1994)

**Table 5.1:** Oligonucleotides used for replacement of the *YGP1* ORF and primers to determine correct insertion of the *kanMX* module. Oligonucleotides **1** and **2** are the 5' and 3' primers used for removal of the *kanMX* cassette from *pFA6kanMX4* for insertion into PMY1.1. Oligonucleotides **3** and **4** are primers to target the flanking regions of the *YGP1* ORF and oligonucleotide **5** targets a region of the *kanMX* cassette. Oligonucleotides **6** and **7** are primers targeting *YGP1* internal sequences (also used for RT-PCR).



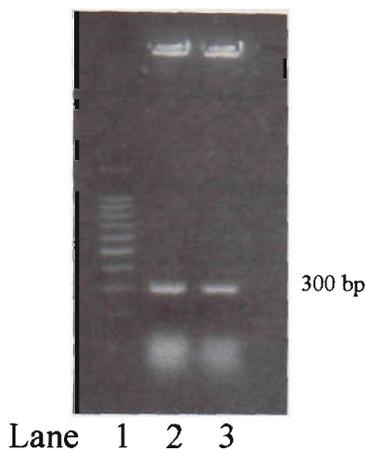
**Figure 5.1:** Ethidium bromide-stained gel of PCR-amplified *kanMX* module. Lane 1: *kanMX* module DNA PCR-amplified from pFA6*kanMX4* as a 1600 bp fragment and resolved on a 0.8% agarose gel, lane 2: 100 bp DNA ladder (Promega), lane 3: Probase 50 ladder (Progen).

The successful replacement of the *YGP1* gene by the *kanMX* module was confirmed using colony PCR of a transformant colony and the PCR products were sequenced to verify gene replacement. The presence and position of the *kanMX* module in two individual colonies was first tested by colony PCR using three primers in the same reaction tube. The primers were complementary to upstream and downstream sequences flanking the *YGP1* ORF: YGP1-PF (-125), YGP1-PR (+72) and the KMX-PF primer (Table 5.1) complementary to internal sequences of the *kanMX* module (see Figure 5.2). Colony PCR using transformant cells as a source of template was expected to generate products of 299 bp and 1834 bp, however only the smaller product of approximately 300 bp was visible when PCR products were resolved on a 1.5% agarose gel (Figure 5.3). Sequencing of the 300 bp PCR product from both ends nonetheless confirmed that the *kanMX* module had been inserted into the *YGP1* gene locus: the PCR product generated was identical to the anticipated 299 bp product of the YGP1-PR and KMX-PF PCR primers. The expected larger 1834bp product was probably not produced in the reaction because it was out-competed by the smaller product in the PCR reaction. To confirm this, colony PCR was performed using a transformant colony and a PMY1.1 wildtype colony as sources of template with primers YGP1-PF and YGP1-PR. In this setting products of the expected size were generated: 1834 bp from the transformant colony (length of the *kanMX* module plus flanking sequences) and approximately 1280 bp from the PMY1.1 wildtype (length of the *YGP1* ORF plus flanking sequences) (Figure 5.4).

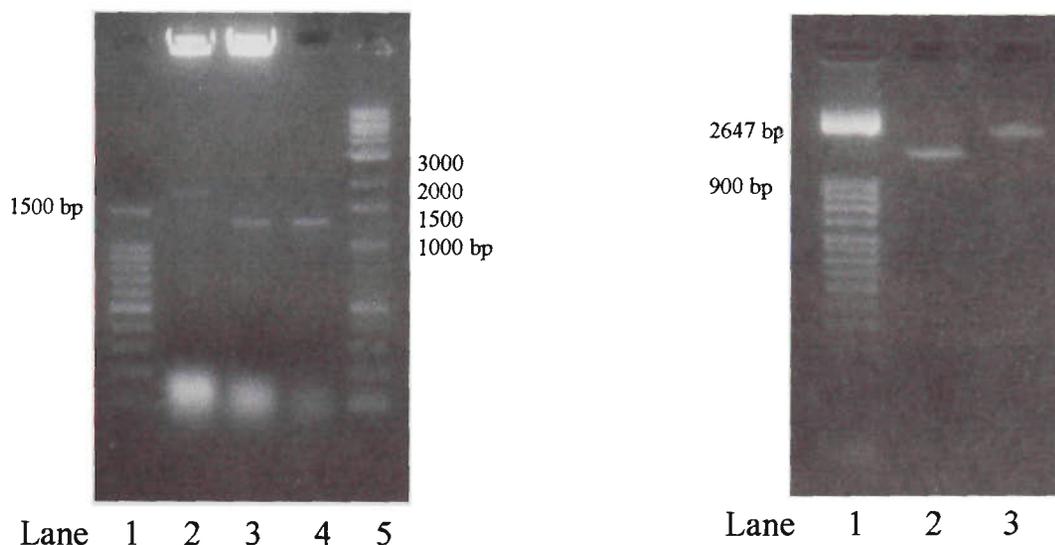
To further confirm the presence of the *kanMX* module in the transformant, the strain was grown in liquid media and genomic DNA isolated from the cells was PCR-amplified using the *YGP1* ORF positioning primers, YGP1-PF and YGP1-PR (Table 5.1). A PCR product of approximately 1800 bp was generated (Figure 5.5) and sequenced from the YGP1-PF (-125) primer. The sequence data could be read approximately 200 bases into the *kanMX* module indicating that the *kanMX4* module had been correctly inserted in place of the *YGP1* gene.

As further validation of the *YGP1* knockout, colony PCR was used to target genomic DNA in cells from a PMY1.1 $\Delta$ *YGP1* colony and cells from a PMY1.1 control colony using the *YGP1* RT-PCR primers (Table 5.1). Colony PCR generated a PCR product





**Figure 5.3:** Colony PCR of transformant colonies using positioning primers resolved on a 1.5% agarose gel. Lane 1: 100 bp ladder (Promega), lanes 2 and 3: PCR products from separate colonies representing a fragment of the *kanMX* marker



**Figure 5.4 (left):** Colony PCR performed on a transformant colony and wildtype colonies as controls using flanking positioning primers. PCR products were resolved on a 1.5% agarose gel. Lane 1: 100 bp ladder (Promega), lane 2: 1800 bp fragment from transformant colony (*YGPI* knockout), lanes 2 and 3: band approximately 1280 bp from wildtype colonies, lane 4: 1 Kb ladder (New England BioLabs)

**Figure 5.5 (right):** PCR products from DNA isolated from PMY1.1  $\Delta$  *YGPI* and PMY1.1 wildtype using primers flanking the *YGPI* ORF. Lane 1: Probase 50 DNA ladder (Progen), lane 2: band representing the *YGPI* ORF generated from PMY1.1 wildtype, lane 3: band representing the *kanMX* marker generated from PMY1.1  $\Delta$  *YGPI*.

of the expected size from the wildtype but no amplified PCR product from PMY1.1 $\Delta$ *YGP1* (data not shown).

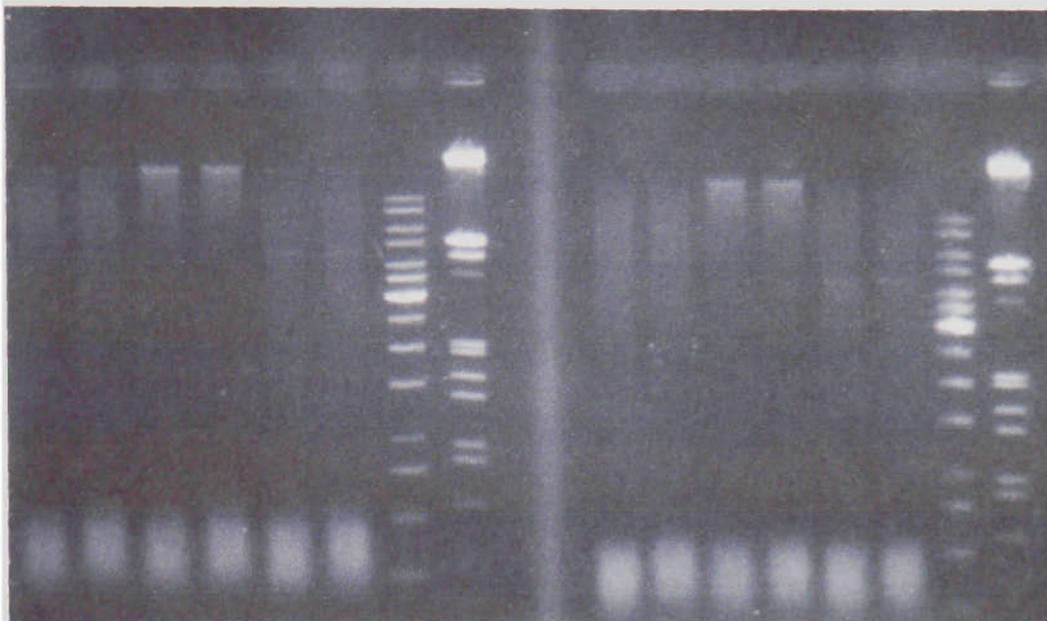
To test the stability of the knockout mutant, serial dilutions of PMY1.1 and PMY1.1 $\Delta$ *YGP1* cultures, grown overnight in non-selective YEPD medium, were plated onto YEPD Geneticin and YEPD plates and incubated for 48 hours at 28°C. Viable counts for the knockout were the same on YEPD as on YEPD Geneticin. The YEPD Geneticin plates did not support growth of PMY1.1 wildtype lacking geneticin resistance (Table 5.2). Thus the *kanMX* marker was not lost in the knockout and the knockout was therefore regarded as a stable construct.

### **5.2.2 Southern analysis of PMY1.1 and PMY1.1 $\Delta$ *YGP1* to confirm replacement of *YGP1* with the *kanMX* cassette**

A Southern analysis of PMY1.1 $\Delta$ *YGP1* and PMY1.1 wildtype was performed to confirm the replacement of *YGP1* by the *kanMX* module in the PMY1.1 genome. Genomic DNA was isolated from both strains and digested in single digests with the restriction enzymes *EcoR1* and *Sal1* and double digests with *Spe1* and *Bgl2*. Each of the three restriction digests was expected to remove either the *kanMX* module or the *YGP1* ORF as a single band, but of different size. The digests were separated on duplicate 1% agarose gels (Figure 5.6, over) and transferred to nylon membranes. A *YGP1* probe was prepared from the 221bp *YGP1* RT-PCR products obtained in prior experiments. The RT-PCR products were gel purified and DIG-labeled using DIG High Prime. The labeled probe was purified and its concentration estimated to be approximately 50 pg  $\mu$ L<sup>-1</sup> using DIG colour detection quantification strips. A *kanMX* probe was prepared by PCR amplifying the module from the pFA6*kanMX4* plasmid using the YGP1-KO5' and YGP1-KO3' primers (Table 5.1) whilst incorporating DIG-labeled dUTP. The DIG-labeled *YGP1* and *kanMX* probes were hybridized to duplicate blots. Following detection using the DIG detection system, *YGP1* was present as a single band only for the wildtype (Figure 5.7a) and the *kanMX* module was present as a single band only for the knockout strain (Figure 5.7b). *YGP1* was therefore replaced by *kanMX* in the PMY1.1 $\Delta$ *YGP1* genome.

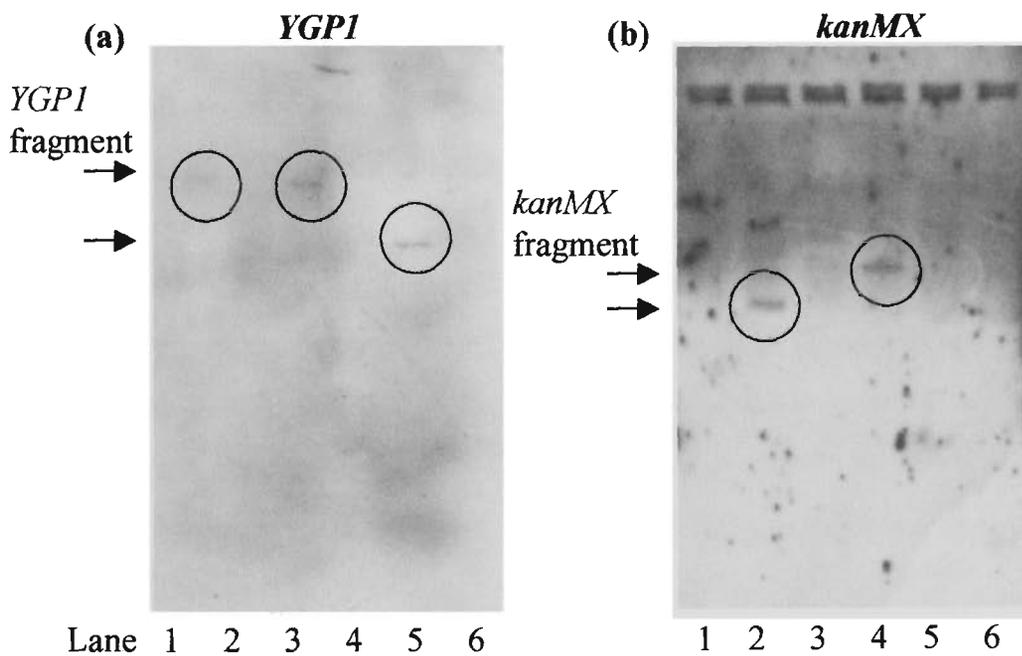
STRAIN	MEDIUM	VIABLE COUNTS (cells/mL)
PMY1.1 $\Delta YGP1$	YEPD	$7 \times 10^7$
PMY1.1 $\Delta YGP1$	YEPD Geneticin	$8.4 \times 10^7$
PMY1.1	YEPD	$6.7 \times 10^7$
PMY1.1	YEPD Geneticin	no growth

**Table 5.2:** Stability of the  $\Delta YGP1$  mutation in PMY1.1



Lane 1 2 3 4 5 6 7 8                      1 2 3 4 5 6 7 8

**Figure 5.6:** Genomic DNA from PMY1.1 wildtype and PMY1.1 $\Delta$  *YGP1* digested with restriction enzymes and resolved on duplicate 1% agarose gels. Lanes 1, 3 and 5 contain digested DNA from PMY1.1 wildtype, lanes 2, 4 and 6 are from PMY1.1  $\Delta$  *YGP1*. DNA in lanes 1 and 2 are digested with *EcoR1*, lanes 3 and 4 are digested with *Sal1* and lanes 5 and 6 were digested with *Spe1/Bgl2*. Lane 7: 1Kb DNA ladder, lane 8: Lambda Phage DNA digested with *HindIII/EcoR1*.



**Figure 5.7:** Southern blot from restriction digests of genomic DNA of PMY1.1 wildtype and PMY1.1 $\Delta$  *YGP1* shown in Figure 5.6 probed with a *YGP1*-specific probe (a) and a *kanMX*-specific probe (b). Lanes 1, 3 and 5 contain DNA digests of the wildtype strain and lanes 2, 4 and 6 contain digests of the knockout.

### 5.2.3 Characterising the *YGPI* phenotype during ethanol stress

To test the effect of removal of *YGPI* on the adaptation rate of PMY1.1 during ethanol stress, PMY1.1 and PMY $\Delta$ *YGPI* were grown in YEPD medium in the presence of 0%, 5%, 7%, 10% and 12% (v/v) ethanol, Figure 5.8a (0%, 7%, 12%), and Figure 5.8b (0%, 5%, 10%). On the evidence shown and in repeat experiments there appeared to be no difference in the growth profiles of the two strains.

To test whether or not *YGPI* influences viability of PMY1.1 at otherwise lethal ethanol concentrations the growth of the knockout and wildtype was further tested in the presence of a lethal ethanol concentration. The strains were pre-adapted to ethanol by growth in YEPD medium in the presence of 5% (v/v) ethanol then transferred to fresh YEPD medium containing 15% (v/v) ethanol and the viability of the cultures was monitored by plate counts. The number of viable cells declined over a period of time but no discernible difference in viability between the response of the knockout and wildtype to the lethal concentration of ethanol was observed (Figure 5.9).

## 5.3 THE *YGPI* RESCUE FOR CONTROLLED EXPRESSION OF *YGPI* IN ETHANOL-STRESSING CONDITIONS

Using an alternative system in which *YGPI* could be induced under the control of an exogenous promoter immediately following inoculation into ethanol-containing medium, the knockout strain PMY1.1 $\Delta$ *YGPI* was transformed with a GeneStorm™ pYES2 expression vector carrying the *YGPI* ORF behind the *GALI* inducible promoter. In this system induction of *YGPI* was expected to occur within a few minutes of the transfer of cells to medium containing galactose (Invitrogen Technical Services Data Sheet).

### 5.3.1 Selection, validation and growth of pYES2 transformants

PMY1.1 $\Delta$ *YGPI* was transformed with pYES2/*YGPI* to generate a rescue strain and with pYES2/GS (without gene insert) as a rescue control strain.

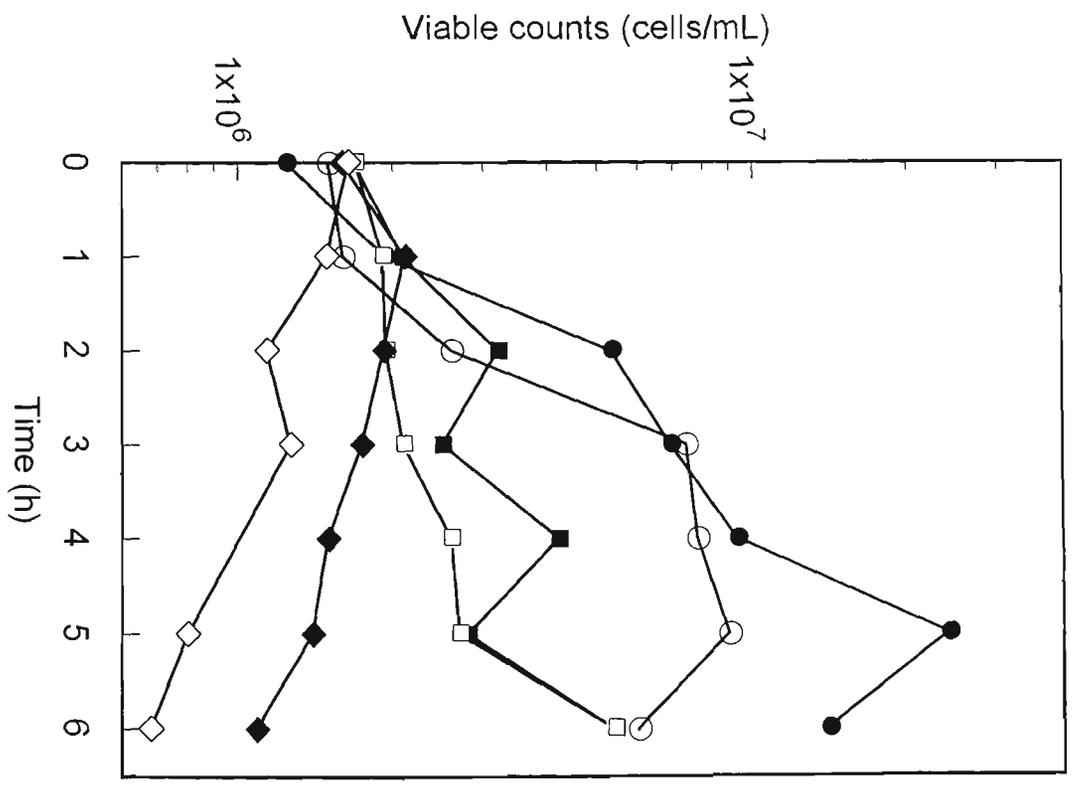
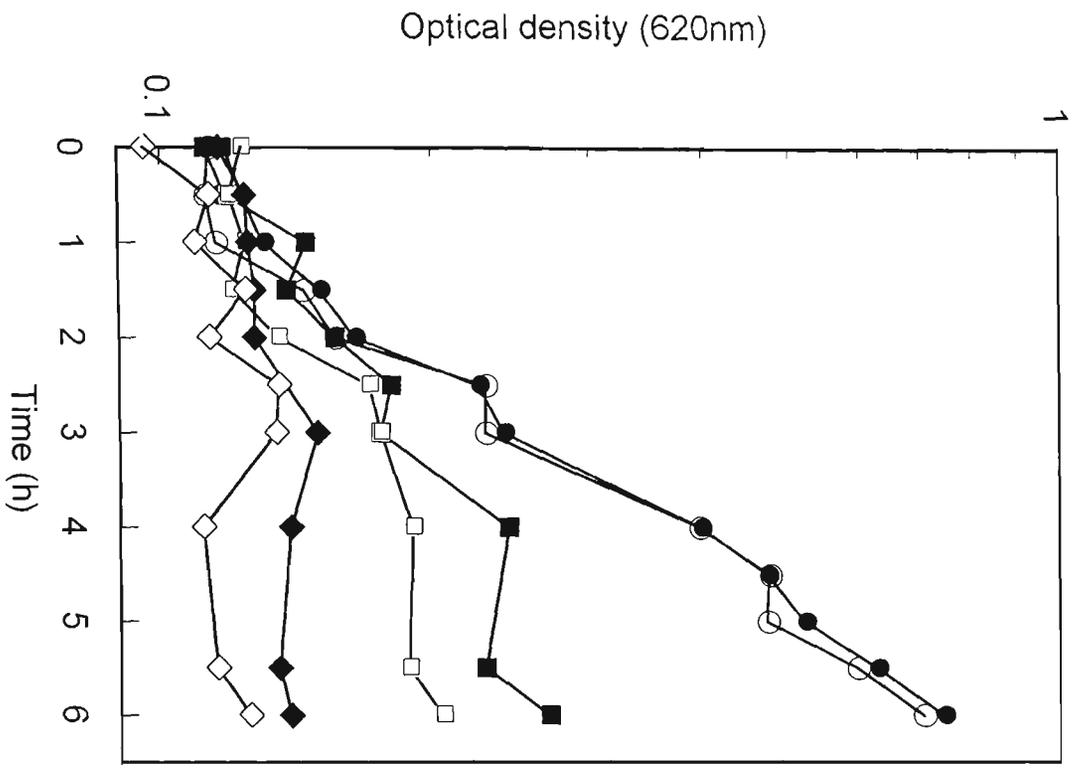
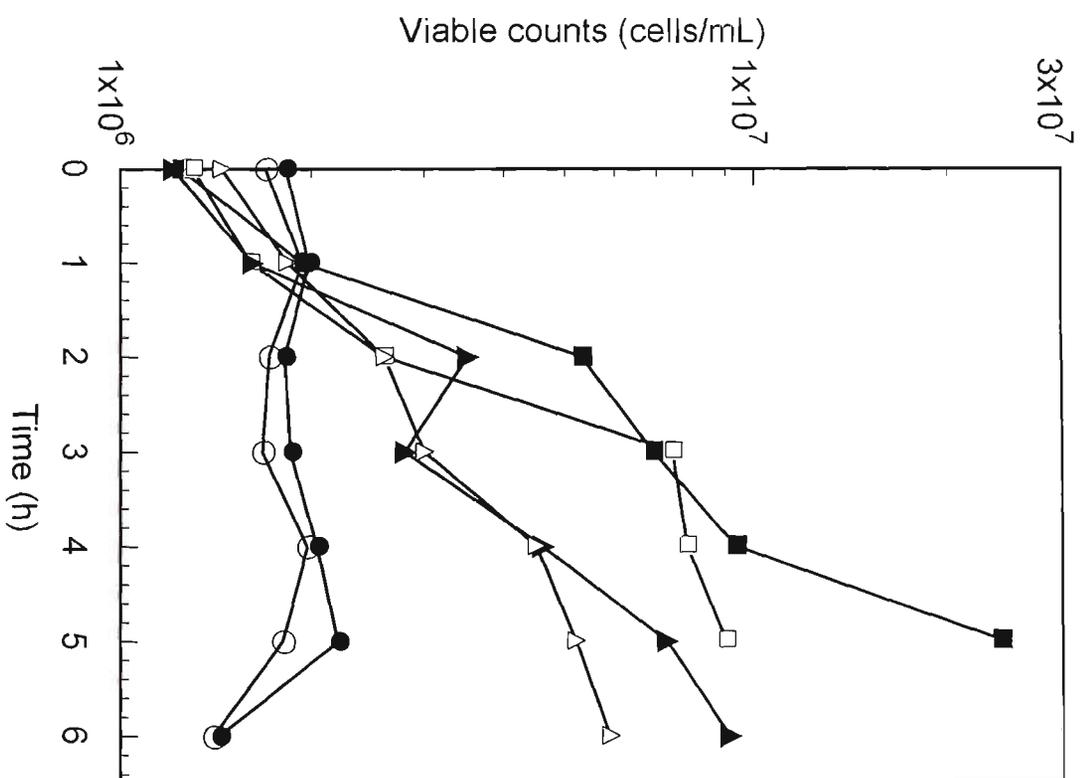
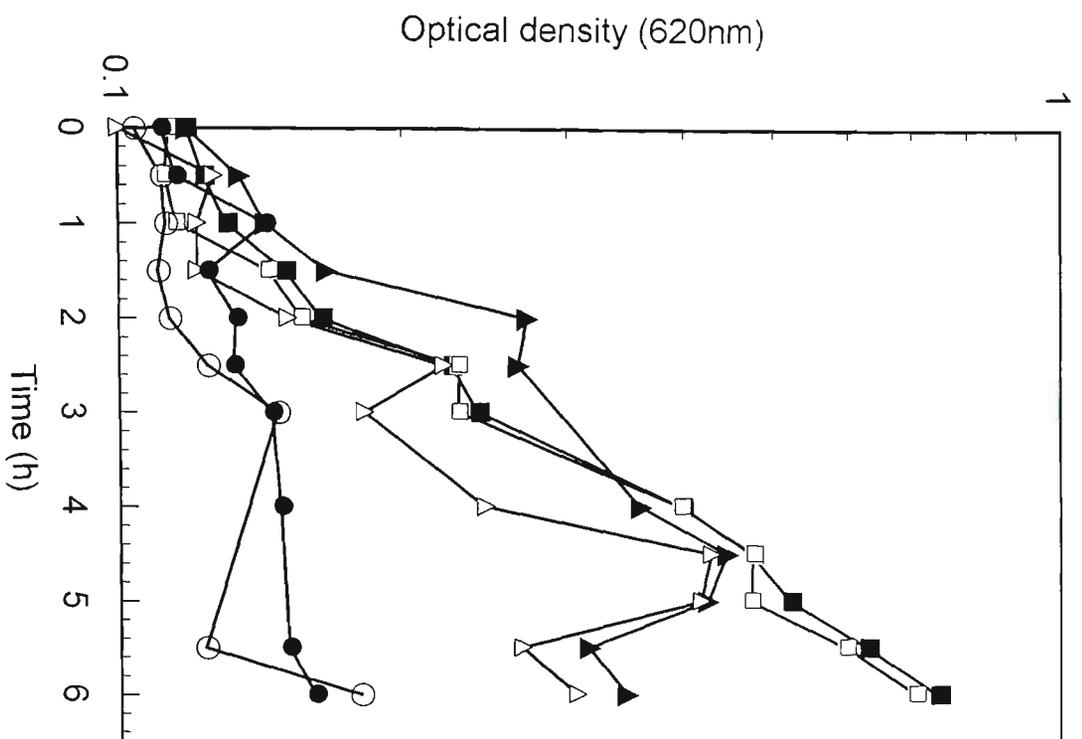
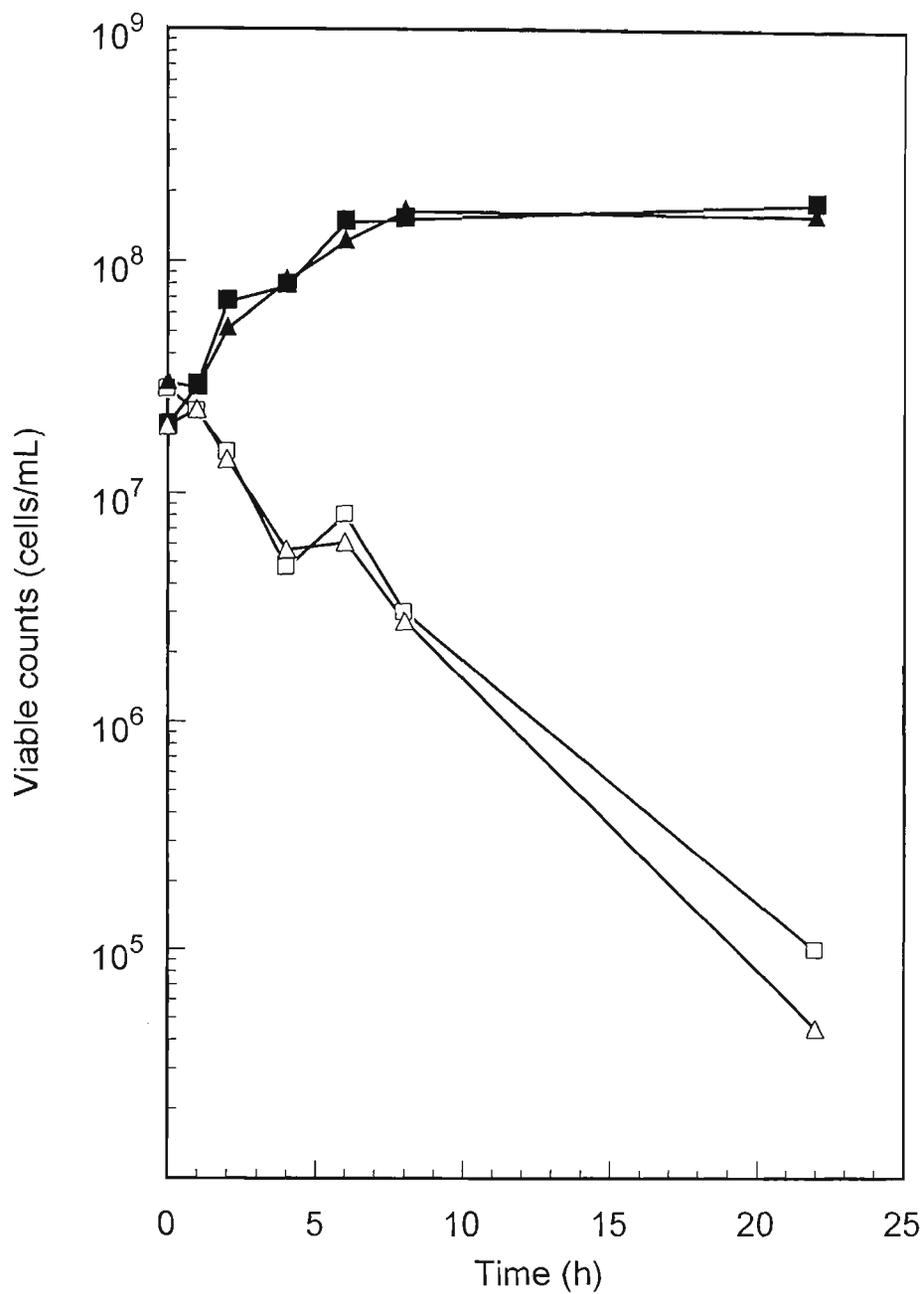


Figure 5.8a : Growth of PMY1.1 wildtype (filled symbols) and PMY1.1Δ YGP1 (open symbols) in the presence of 0% (●, ○), 7% (■, □) and 12% (◆, ◇) ethanol (experiment A).



**Figure 5.8b:** Growth of PMY1.1 wildtype (filled symbols) and PMY1.1Δ *YGPI* (open symbols) in the presence of 0% (■, □), 5% (▲, Δ) and 10% (●, ○) (v/v) ethanol.

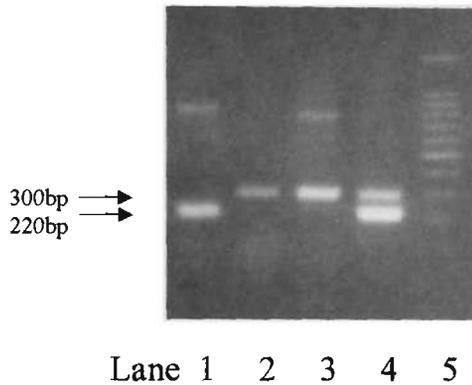


**Figure 5.9:** Death curves of *S. cerevisiae* strains PMY1.1 wildtype (■, □) and PMY1.1 $\Delta$ *YGP1* (▲, △) in the presence of a lethal ethanol concentration. Cultures of wildtype and knockout strains were pre-adapted to growth in the presence of 5% (v/v) ethanol (closed symbols) then exposed to a lethal ethanol concentration of 15% (v/v) (open symbols).

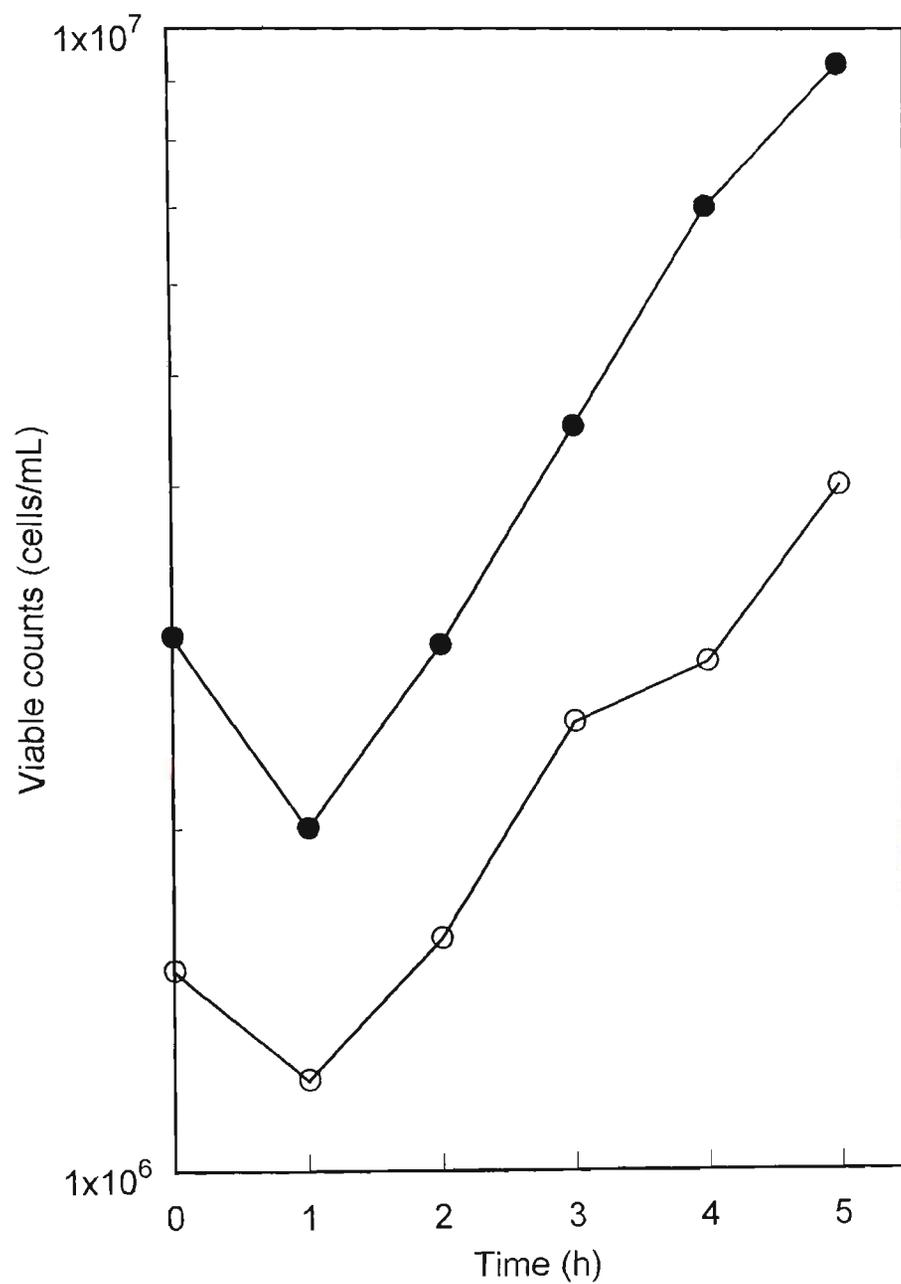
The protocol of Gietz and Schiestl (1995) was used (see section 2.3.9) to transform  $1 \times 10^9$  cells with 0.5  $\mu\text{g}$  of plasmid DNA. Transformed PMY1.1 $\Delta$ *YGP1* cells were plated on uracil-free minimal medium plates containing 200 mg/L geneticin and incubated for 5 days at 28°C. Individual colonies were streaked onto fresh selective media plates and incubated for 48 hours at 28°C.

To confirm that the desired plasmid was present in the transformants, colony PCR was performed on colonies of PMY1.1 (wildtype), PMY1.1*YGP1::kanMX* (knockout), PMY1.1*YGP1::kanMX* pYES2/*YGP1* (rescue) and PMY1.1*YGP1::kanMX* pYES2/GS (rescue control). The reactions were carried out with four primers in each tube: the *YGP1* primers (oligonucleotides 6 and 7, Table 5.1) to target the *YGP1* ORF, whether present on the chromosome or plasmid, as well as the *YGP1* knockout downstream external positioning primer YGP1-PR (+72) and KMX-PF (see Figure 5.2) to target *kanMX* sequences. The PCR products resolved on a 1% agarose gel confirmed the presence of the *YGP1* ORF in the rescue strain as well as the *kanMX* module. (Figure 5.10). The *YGP1* PCR product in the wildtype, rescue strain and rescue control strain was 221 bp as expected and the *kanMX* PCR product from the knockout and rescue strain was 299 bp (i.e. both PCR products were generated from the *YGP1* and *kanMX* sequences in the rescue strain). In addition the *YGP1* PCR product from the rescue strain was more intense suggesting that multiple copies of plasmid DNA were present as starting template.

To determine if growth of the rescue strain was seriously limited by the energy requirement of carrying a multicopy plasmid, it was grown under non-inducing conditions and its growth compared to that of the knockout (Figure 5.11). When grown on minimal medium with glucose as carbon source, the growth of the rescue strain was comparable to the knockout thus the energy requirement for carrying the plasmid was considered to be negligible under the experimental conditions described.



**Figure 5.10:** Colony PCR to confirm the presence of pYES2/*YGP1* in PMY1.1*YGP1::kanMX* pYES2/*YGP1*(rescue strain). *YGP1*-specific primers and *kanMX*-specific primers were used in all PCR reactions with the strains indicated. The 220 bp product represents *YGP1* sequence and the 300 bp product represents part of the *kanMX* marker. Lane 1: wildtype, lane 2: knockout, lane 3: knockout carrying pYES2/GS plasmid only, lane 4: knockout carrying plasmid containing *YGP1* gene (rescue), Lane 5: 100 bp ladder (Promega).



**Figure 5.11:** Growth of PMY1.1Δ *YGP1* (●) and the PMY1.1Δ *YGP1* strain carrying the pYES2/*YGP1* plasmid (rescue) (○) under non-inducing conditions. Cultures were grown in glucose minimal medium at 30°C/200 rpm.

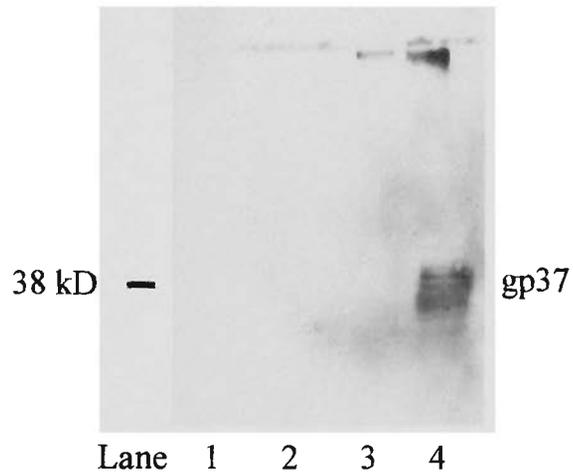
### 5.3.2 Confirmation of expression of gp37 by Western analysis

*YGPI* expression in the rescue strain was confirmed by Western blotting. Protein extracts from cells of the rescue strain and rescue control strain grown for 20 hours in the presence of galactose to induce *YGPI* expression, were de-glycosylated and separated on a 10% polyacrylamide gel with pre-stained mid range protein size standards (BioRad). Resolved proteins were then transferred to a nitrocellulose membrane and immunoblotted using the Anti-V5-HRP Antibody (Invitrogen). A protein band of approximately 38 kD, the expected size of the product of *YGPI*, gp37, was identified that was present in the sample from the de-glycosylated rescue strain but was not present in a sample that was not de-glycosylated or in the rescue control. (Figure 5.12).

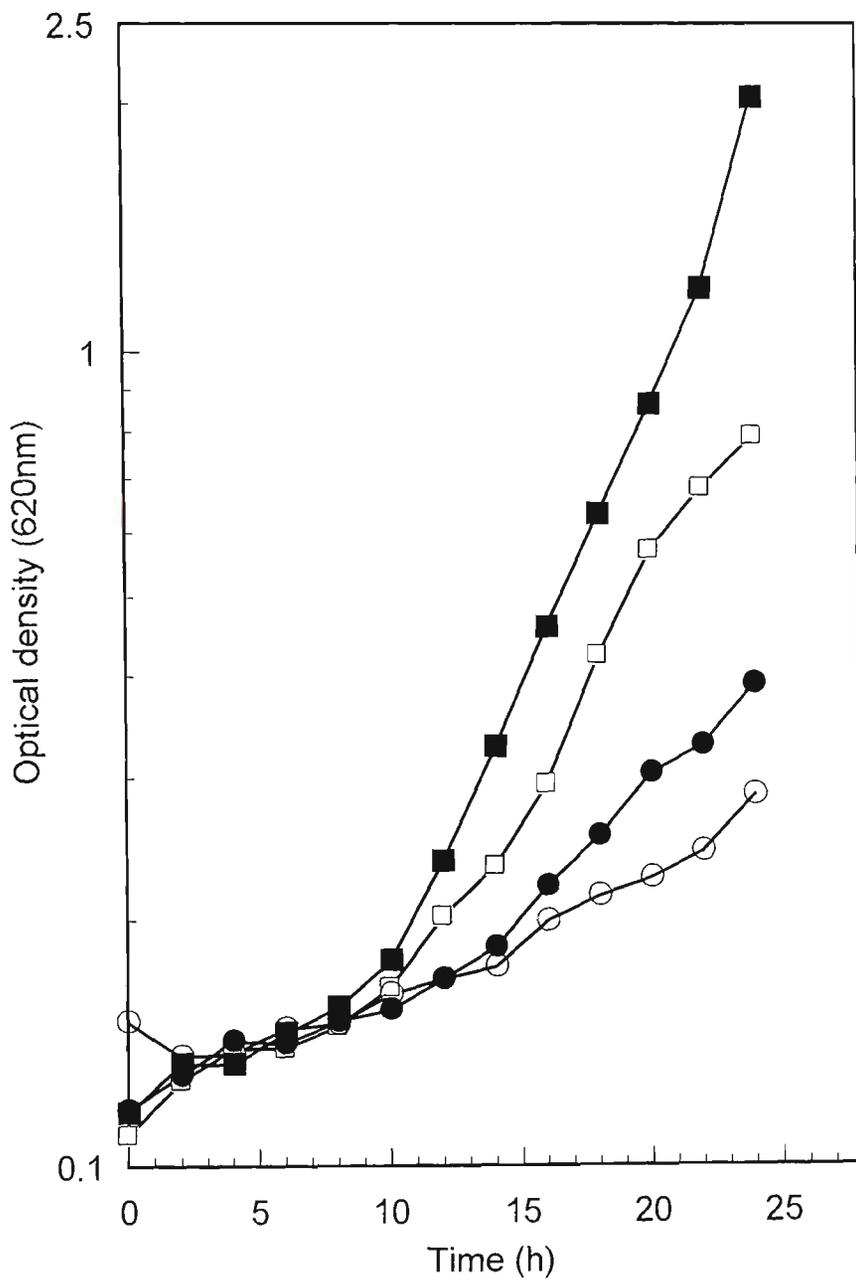
### 5.3.3 Growth of plasmid-bearing strains during ethanol stress

All previous work for this thesis was based on growth of PMY1.1 or PMY1.1 $\Delta$ *YGPI* in YEPD medium (with or without ethanol). However, there were two constraints preventing the use of YEPD medium for the following experiments: maintenance of the pYES2 plasmid requires a uracil-free medium and glucose is a repressor of the *GALI* promoter. Therefore growth of the rescue strain was compared to that of the rescue control PMY1.1 $\Delta$ *YGPI* pYES2/GS (transformed with the plasmid without insert) using a minimal medium that was free of both uracil and glucose. Raffinose was used as a carbon source in this medium. For *YGPI* induction, cells were transferred from raffinose to a raffinose/galactose medium. The *YGPI* rescue and *YGPI* rescue control were grown in minimal medium with 4% raffinose to an approximate OD<sub>620</sub> of 2. The strains were then inoculated to an OD<sub>620</sub> of 0.1 into fresh minimal medium containing 4% raffinose, 2% galactose and 5% ethanol (v/v) for stressed cultures.

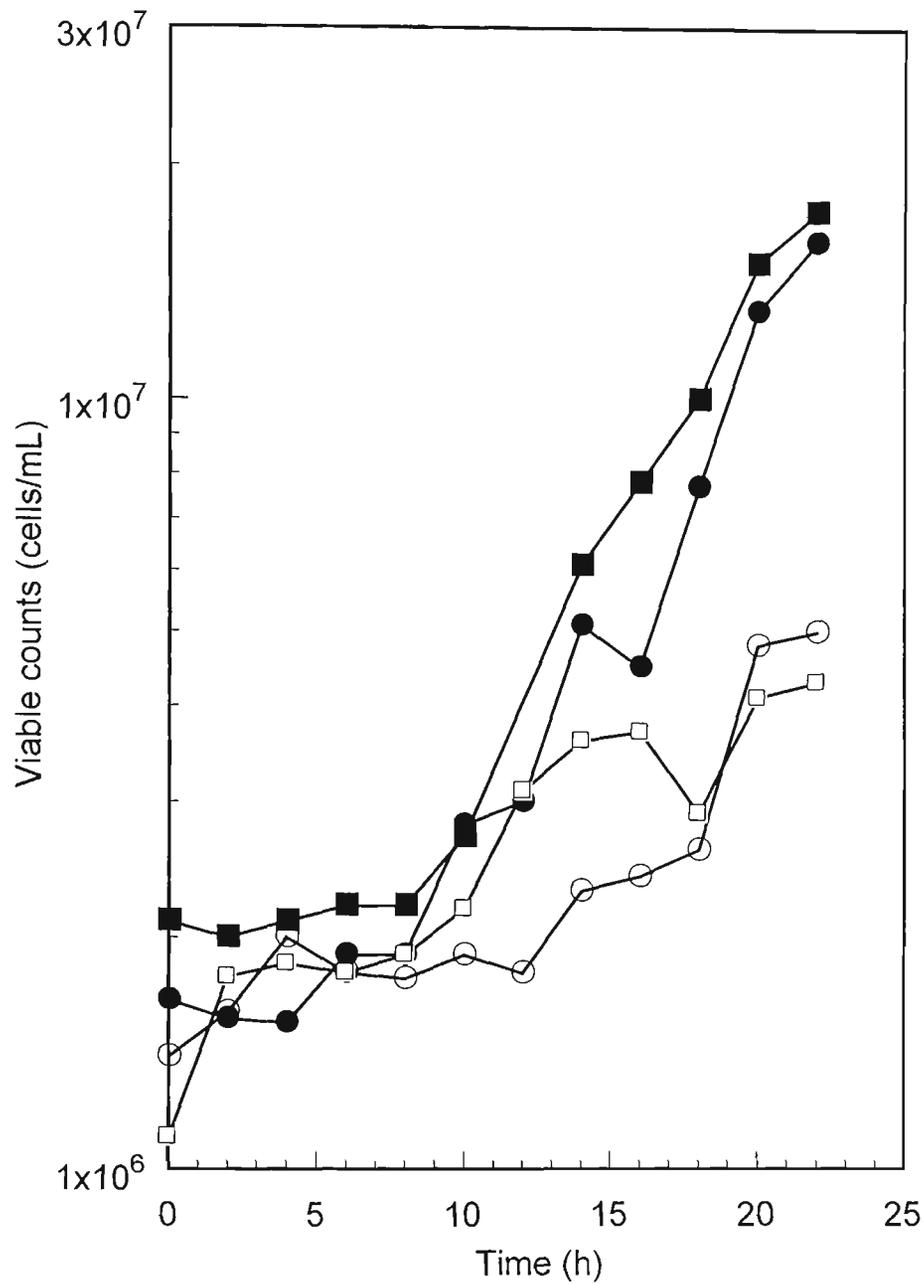
When inoculated to an OD<sub>620</sub> of 0.1 into 4% raffinose minimal medium, growth of the rescue strain was very slow and unstressed control cultures required 10-12 hours to exit lag phase. Growth of the same strain in minimal medium containing 4% raffinose and 2% galactose was even slower and ethanol-stressed cultures were still in lag phase



**Figure 5.12:** Western analysis of PMY1.1 *YGP1::kanMX* pYES2/*YGP1* (rescue strain) and PMY1.1 *YGP1::kanMX* pYES2/GS (rescue control strain) for confirmation of production of gp37 in the rescue strain. Lanes 1 and 2 contain proteins from the rescue control strain, lanes 3 and 4 contain proteins from the rescue strain, lanes 2 and 4 were de-glycosylated prior to electrophoresis. Both strains were grown in a galactose medium to induce expression from the plasmid.



**Figure 5.13:** Growth of PMY1.1 $\Delta$ YGP1 pYES2/YGP1 (rescue strain) in 4% raffinose minimal medium (■, □) and 4% raffinose 2% galactose minimal medium (●,○) in the absence (filled symbols) and presence (open symbols) of 5% (v/v) ethanol.



**Figure 5.14:** Growth of PMY1.1ΔYGP1 pYES2/YGP1 rescue strain (■, □) and PMY1.1ΔYGP1 pYES2/GS rescue control (●, ○) in 4% raffinose 2% galactose minimal medium in the absence (closed symbols) and presence (open symbols) of 5% (v/v) ethanol.

after 24 hours incubation (Figure 5.13). When the rescue strain PMY1.1 $\Delta$ *YGP1* pYES2/*YGP1* and the rescue control strain were grown under inducing conditions in the presence of galactose there was no difference between their adaptation rates to ethanol-stress over a 24 hour period (Figure 5.14).

## 5.4 DISCUSSION

### 5.4.1 The *YGP1* phenotype

The phenotype of the *YGP1*<sup>-</sup> strain was not established by the experiments described here. Deletion of the gene did not result in a significant difference in the growth profile compared to the wildtype, either when grown in the presence of a stressful concentration of ethanol or without ethanol. Pre-adaptation of the knockout and wildtype to 5% ethanol and a subsequent exposure to a lethal ethanol concentration did not result in any difference in the death rate between the two strains.

It is possible that failure to detect a *YGP1* phenotype using a *YGP1* knockout may be due to the presence of another gene able to compensate for the role of *YGP1*. (Wolfe and Shields 1997) showed that *YGP1* is in a block of genes on chromosome 14, which have been retained subsequent to a putative ancient gene duplication event. The homologous block on chromosome VIII contains a *YGP1* homologue, the sporulation-specific *SPS100* gene (Law and Segall 1988) that exhibits 50% identity to *YGP1* at amino acid level and 67% overall similarity. It was thought that *SPS100* could conceivably compensate for *YGP1* in the ethanol-stress response since it is also reportedly induced by ethanol (Moskvina *et al.* 1998). However, investigation of the lag phase level of expression of *SPS100* under ethanol stress conditions by RT-PCR analysis did not indicate that *SPS100* was compensating for *YGP1* in the *YGP1* knockout. *SPS100* appeared to be more highly expressed in unstressed cultures (data not shown).

In this experiment, failure to detect increased *SPS100* expression in an ethanol-stressed lag phase PMY1.1 culture may be due to different growth and stress conditions compared to the (Moskvina *et al.* 1998) experiments. These authors

exposed exponential phase *S. cerevisiae* W303-1A cells grown in a minimal medium to 7% ethanol, therefore the strain, growth medium, growth phase and ethanol concentration were different to the experiments described in this thesis.

When *YGP1* was returned to the *YGP1*<sup>-</sup> strain on a plasmid behind an inducible promoter, early induction of *YGP1* did not influence the growth of the strain compared to the isogenic *YGP1*<sup>-</sup> strain carrying the plasmid only. There was no significant difference in the growth profiles of the rescue strain and rescue control strain in the presence of 5% (v/v) ethanol or without the addition of ethanol. However, it is possible that further experiments in which a higher ethanol concentration is used as stressor and a higher inoculum size is used to reduce the lag period, may reveal differences in adaptation rate between the knockout and rescue strains.

The results obtained using transformed *YGP1*<sup>-</sup> knockout strains should be regarded cautiously however. Since these strains were tested on a raffinose/galactose minimal growth medium that can only be slowly utilized by *S. cerevisiae*, adaptation times were extremely long. Glucose could not be used as the carbon source for *YGP1* induction since the galactose promoter is glucose repressed. In addition, maintenance of the plasmid requires growth in a uracil-free medium therefore a uracil-free minimal medium was used. For these reasons the growth of the plasmid-bearing strains could not be directly compared to growth of the wildtype and knockout in YEPD medium where the growth lag period under ethanol stress was only 3-4 hours.

#### **5.4.2 Further experiments that may reveal the role of *YGP1* in the ethanol-stress response**

Using simple growth experiments to determine phenotype is limited since small differences in cell vitality will go unnoticed in the absence of an appropriate selection pressure, therefore more sensitive and a broader range of methods of determining phenotype should be developed. For example, if *YGP1* provides a competitive advantage compared to the *YGP1*<sup>-</sup> background when grown under ethanol-stress conditions, this may be determined in a growth competition experiment. An

experiment could be carried out in which equal cell densities of knockout and wildtype are grown together in rich medium and also minimal medium in the presence and absence of 5% (v/v) ethanol. Cells should be subcultured into fresh medium every 24 hours and plated onto YEPD and YEPD Geneticin plates and the proportion of each strain present determined from the viable counts since both strains can grow on YEPD but only the knockout can grow in the presence of geneticin. A similar experiment could be carried out using rescue and rescue control strains grown in minimal medium in the presence of 5% (v/v) ethanol.

A novel evolutionary approach developed by (Ferea *et al.* 1999) has revealed patterns of gene expression that change over many cell generations and has lent support to the ethanol-stress-related expression of *YGP1* and *DIP5*. The authors performed a microarray experiment to detect changes in expression profiles that occurred in cultures grown aerobically in carbon limiting chemostats for either 500 or 250 generations. RNA from these evolved strains was compared to the parent. *YGP1* and *DIP5* were among the 1.4% (88/6,124) of genes that showed consistent expression alteration of at least 2 fold between the evolved strain and parent. Both were less strongly expressed in the evolved strain than the parent and were clustered by expression pattern with genes involved in glycolysis and ethanol utilization. Genes involved in respiration were more strongly expressed in evolved strains than in the parent. Physiological data also showed that strains under carbon limitation evolved to be more efficient users of glucose by using respiration to yield a 4 fold greater cell number, a 3 fold greater biomass but around 10 times less ethanol. It is therefore understandable and in agreement with the results presented in this thesis that *YGP1* and *DIP5*, which are up-regulated under ethanol stress conditions, would not be under selection pressure for increased expression when endogenous ethanol levels are low (0.02 g/L as determined by the authors). The *YGP1* expression level was either unchanged or lower in the carbon limited evolved strains and *DIP5* expression was consistently lower in the evolved strains than in the parent. However, this is not as would be expected from the work of (Destruelle *et al.* 1994) who demonstrated that expression of *YGP1* was induced in response to glucose limitation.

A similar approach to that of (Ferea *et al.* 1999) using a non-lethal ethanol stress as selection pressure would be likely to identify the genes that are expressed in response

to ethanol stress and those whose promoters are responsive to ethanol either directly or by induction of stress response regulatory factors. This experiment should also identify whole genome patterns of expression that enable yeast to adapt to ethanol stress and indicate the metabolic responses required for adaptation and would complement the experimental work described in this thesis.

Given the likely extracellular location of the *YGPI* gene product, gp37 (Destruelle, Holzer and Klionsky 1994; Pardo *et al.* 1999), any extracellular role of gp37 in the yeast ethanol-stress response should be further investigated. By adding gp37, purified from the expression plasmid, to the growth medium of the *YGPI* knockout, the influence of this protein on the ethanol adaptation rate could be determined. By using washed and unwashed inocula from cultures of wildtype and knockout, together with protein analysis, it should be possible to determine whether gp37 is extracellular. Further experiments under a range of conditions, including biochemical tests, would be necessary to investigate a possible role for gp37 in intercellular interactions.

In summary, the results from this chapter indicate that *YGPI* does not influence the yeast adaptive response to ethanol stress but the results should be interpreted with caution since only a limited number of growth conditions were imposed on the *YGPI* knockout and the *YGPI* controlled expression strain. It is possible that *YGPI* may have a marginal influence on yeast adaptation to ethanol and a more comprehensive approach may clarify the role of *YGPI*, if any, in the ethanol-stress response but further experimentation is required to confirm or disprove this.

## CHAPTER 6

# THE POSSIBLE ROLES OF *YGP1*, *DIP5* AND *YER024w* IN THE YEAST ETHANOL-STRESS RESPONSE

### 6.1 INTRODUCTION

The ethanol-stress response genes *YGP1*, *DIP5* and *YER024w* are upregulated in *S. cerevisiae* strain PMY1.1 cells during adaptation to ethanol stress. Whether or not these genes have a specific role in the yeast ethanol-stress response is yet to be elucidated. There was no significant difference in the growth profiles of the wildtype, *YGP1* knockout or *YGP1* rescue strains grown either in the presence or absence of ethanol. *DIP5* and *YER024w* will be examined in future studies.

Failure to identify a phenotype for *YGP1* was not entirely unexpected since single gene knockouts in unicellular organisms such as yeast, as well as multicellular organisms such as mouse, frequently fail to produce a detectable phenotype, even when the organism is exposed to a range of conditions. For example, in a EUROFAN functional analysis report of 21 novel genes on *S. cerevisiae* chromosome XIV, *kanMX* knockouts with green fluorescent protein inserts failed to produce a significant GFP fluorescent signal in 15 of the 21 knockouts (Brachat *et al.* 2000). In a whole genome approach, only 40% of 2046 yeast gene knockouts showed a quantitative growth defect in either rich or minimal media (Winzeler *et al.* 1999). However, when seven selection protocols were used to test the growth profiles of 268 Ty insertion gene knockouts on chromosome V more than half (157 of 268) showed a reduction in fitness (Smith *et al.* 1999). Thus, it is likely that a more comprehensive approach to testing the level of *YGP1* expression under a greater range of growth and stress conditions would lead to detection of a *YGP1* phenotype but further experimental investigation was beyond the scope of this project. Therefore, to define the possible roles of *YGP1*, *DIP5* and *YER024w* with a view to designing targeted future

experiments, a literature search and database analysis were undertaken, taking into account both experimentally obtained data, predictions and information deduced from computer analyses.

## **6.2 A PUTATIVE PHENOTYPE FOR *YGPI***

*YGPI* encodes a highly glycosylated secretory protein gp37 (37 kDa) reported to be regulated by nutrient availability (Destruelle *et al.* 1994). The protein has been localized to the periplasm and is one of the most highly glycosylated yeast proteins on a weight-to-weight basis, having an estimated 90 mannose residues per carbohydrate side chain (Destruelle, Holzer and Klionsky 1994). Gp37 is degraded following addition of glucose to stationary phase cells (catabolite inactivation) and the authors propose that, given the periplasmic or extracellular location of gp37, this degradation may represent a novel type of nutrient regulation, presumably by recycling amino acids and glucose. This is one possible role of gp37 in yeast cells and, in the following, I will argue that it may be involved in a variety of cellular activities such as cell wall stabilisation, general stress tolerance, nutrient limitation response, in particular nitrogen limitation, and progress through the cell cycle.

### **6.2.1 Predictions regarding *YGPI* regulation and function**

The YPD Protein Report [<http://www.proteome.com/databases/YPD/reports>] classifies the cellular role of *YGPI* as involved in the “cell stress” response and predicts an N-terminal protein modification site that is a potential Cdc28 target, a potential transmembrane domain and 15 potential N-glycosylation sites, although the glycosylation type is unknown. Related *S. cerevisiae* proteins are sporulation specific Sps100p and two *S. cerevisiae* asparaginases, Asp3dp and Asp3bp. Pedant MIPS [<http://pedant.gsf.de/>] places *YGPI* in a putative functional category of “sporulation and germination, organization of cell wall and amino-acid degradation” and predicts a transmembrane region/signal peptide from nucleotide 1-20. Similar predictions are made by SWISS-PROT/ExPasy [<http://www.expasy.ch>] including a potential 14 to 16 N-glycosylation sites, a signal sequence from nucleotides 1-19, a propeptide from nucleotides 20-37 and the mature protein from nucleotides 38-354. Apart from these protein features predicted by the databases, gp37 has three putative Protein Kinase C

phosphorylation sites (ser/thr X Arg/Lys, where X is any amino acid) as described by Gentzsch and Tanner (1996).

Protein sequence comparisons also indicate that gp37 may have an asparaginase capability. Sequence alignments using PFAM domains [<http://www.sanger.ac.uk/cgi-bin/Pfam>], PROSITE motifs [<http://www.expasy.ch/prosite>], BLOCKS [<http://www.blocks.fhcrc.org/>] and ProDom [<http://protein.toulouse.inra.fr/prodom/doc/prodom.html>] reveal that gp37 is similar to asparaginase and asparaginase/glutaminase proteins. A ProDom protein sequence alignment of 15 members of a family of asparaginases including two in yeast, plus *YGPI* and *SPS100* indicates a possible consensus sequence at position 128-139 of the *YGPI* protein sequence. If the *YGPI* protein does have asparaginase activity it may be used to remove amine groups from asparagine to produce aspartate. Aspartate and glutamate are good nitrogen sources for *S.cerevisiae* (Walker 1998) and are preferentially utilized in industrial fermentations (Boulton 1991). This would also tie in with the ethanol-stress-induced up-regulation of *DIP5* as an importer of aspartate and glutamate and suggest a role for *YGPI* under conditions of nitrogen limitation.

## 6.2.2 *YGPI* expression

### 6.2.2.1 Cell surface

Pardo *et al.* (1999) proposed that *YGPI* may be involved in cell wall construction since gp37 was identified as a protein secreted during protoplast regeneration. In a novel approach to studying the cell wall, the authors collected proteins secreted from regenerating protoplasts into the regenerating medium, visualized them on silver stained 2D electrophoresis gels and identified them by N-terminal sequencing. However, the sequence data obtained for gp37 was for an internal region of the mature protein. The authors suggest this may be due to post-translational modifications similar to that of at least one other secretory glycoprotein, Hsp150 (Russo *et al.* 1992). The reported gp37 molecular weight, 58 kDa and isoelectric point, 4.22 are different to the 37 kDa and 4.61 predicted by Pedant MIPS and Proteome, supporting the argument that post-translational modifications may occur under the conditions of the experiment. It is possible that the size and mobility

discrepancy may be due to glycosylation since a de-glycosylation step was not performed prior to electrophoresis. Considering that gp37 has 14 possible glycosylation sites with an estimated 90 mannose residues per side chain (Destruelle, Holzer and Klionsky 1994), the molecular weight could be as high as 265 kDa, although it is unlikely to be that great.

One of the proposed functions of cell wall glycosylation is signaling between cells. The high level of *YGPI* mRNA in high cell density cultures, prepared by concentrating log phase cells to an OD<sub>650</sub> of approximately 20 (Wang *et al.* 1997) is consistent with gp37 having a role in cell-cell interactions. In a comprehensive yeast two-hybrid analysis, Gp37 has been reported to interact with one protein of unknown function, *YDR105c*, with 30% overall identity to human membrane protein TDE (tumor differentially expressed) (Ito *et al.* 2001).

Although gp37 has been localized to the periplasm it is likely to pass through the cell wall since proteins up to M<sub>r</sub> 200,000 can diffuse freely through yeast cell walls (De Nobel and Barnett 1991). Protein oligomerization, glycosylation and mannosylation influence the passage of proteins through the cell wall and the wall itself is a dynamic framework that changes in composition and porosity according to environmental changes and stage of growth (De Nobel *et al.* 1990). For example, when cells are switched to a low pH, *YGPI* and three other cell wall-related genes are induced (Kapteyn *et al.* 2001). Under these conditions, the authors found GPI-dependent proteins were attached to the cell wall by a novel alkali-labile  $\beta$ 1,3-glucan linkage and proteins usually linked by an alkali-sensitive linkage were more efficiently retained in the wall, thus modifications to cell wall protein linkages may also be regulated by environmental conditions.

Although it is not known if gp37 is physically linked to the cell wall, *YGPI* is one of only five cell wall-related genes found to be down-regulated by activation of the cell wall integrity signaling pathway (Jung and Levin 1999). This pathway is controlled by Rho1 GTPase, the regulatory subunit of glucan synthase which activates protein kinase C (*PKCI*), which in turn activates a MAP kinase cascade. The pathway is induced in response to elevated temperatures and hypo-osmotic shock and the loss of

Pkc1p activity leads to cell lysis. The authors looked for genes controlled by this pathway by comparing an *MKK1* over-expression strain to a wildtype strain. The majority of cell wall related genes upregulated by this pathway coded for GPI-anchored proteins suggesting that activation of the pathway results in structural changes that strengthen the cell wall. Why *YGP1* is down-regulated by this pathway is unknown, but it seems that gp37 and/or its carbohydrate modifications are not required under the conditions of the experiment. It is possible that down-regulation of *YGP1* by activation of the cell wall integrity signaling pathway may reflect the culture growth conditions since the experimental cultures were grown in a raffinose/galactose medium for induction of the plasmid-encoded *MKK1*. By contrast the low pH experiments conducted by Kapteyn *et al.* (2001) in which *YGP1* was upregulated were conducted using a glucose carbon source and suggests that *YGP1* expression may be regulated by glucose.

#### 6.2.2.2 Diauxic shift

Destruelle, Holzer and Klionsky (1994) investigated the level of *YGP1* expression at various stages of growth and reported that *YGP1* mRNA was at a basal level during logarithmic growth but increased during the diauxic shift. This observation was supported using Northern analysis (Wang, Nishikawa and Isono 1997) and microarray analysis (De Risi *et al.* 1997) that showed a greater than 8-fold increase in *YGP1* mRNA during diauxic shift. By tagging *YGP1* with an invertase reporter gene, Destruelle, Holzer and Klionsky (1994) were able to follow expression of this gene during growth. These workers found that invertase activity continued to increase throughout the diauxic lag phase reaching a peak that was 50-fold higher than basal 60-80 hours after respiratory growth had reached a plateau. The authors concluded that *YGP1* was glucose repressed and not induced until the glucose concentration of the medium fell below 1%, however *YGP1* induction did not occur when cells were shifted directly to a low glucose medium. Results presented in this thesis complement the findings of Destruelle, Holzer and Klionsky (1994) and De Risi, Iyer and Brown (1997) in that *YGP1* mRNA was virtually absent in ethanol-stressed cultures 5 hours post-inoculation, the time when they exit lag phase and enter log phase (Figure 4.18). However, results presented here also show that *YGP1* is not glucose repressed since it is induced during ethanol stress in the presence of a high glucose concentration. It is

therefore proposed that the increased expression of *YGP1* observed as cells approach diauxic shift is not due to a drop in glucose concentration, as concluded by (Destruelle, Holzer and Klionsky 1994), but rather is a response to the presence of ethanol. An alternative explanation for the high level of *YGP1* mRNA during lag phase and the diauxic shift is that its expression is simply growth rate-dependent.

### 6.2.2.3 Nutrient limitation or slow growth?

*YGP1* is reported to be expressed at an elevated level in nitrogen- or phosphate-limited cultures, suggesting a general nutrient limitation response (Destruelle, Holzer and Klionsky 1994). In order to determine whether the increased level of *YGP1* expression was in response to nutrient limitation or to slow growth that is associated with nutrient limitation, the authors limited auxotrophic amino acids to slow cellular growth in a non-limiting glucose culture. *YGP1* expression did not increase under slow growth conditions in the presence of 1% glucose, therefore Destruelle, Holzer and Klionsky (1994) concluded that the expression of *YGP1* is regulated in response to specific nutrient levels and not by the growth rate. The authors also concluded that *YGP1* expression is subject to glucose repression but found that it was not fully de-repressed by *SNF1*, a protein kinase required for expression of all glucose-repressed genes, and therefore is partially independent of the *SNF1* general glucose repression pathway. The level of expression of *YGP1* in a *Snf1* mutant depleted of glucose, was around 50% of its expression in the *SNF1* wildtype and 17-fold greater than basal, suggesting that other repressors/activators are responsible for full expression of *YGP1* under low glucose conditions. The authors suggested that *YGP1* is regulated by an alternative regulatory mechanism that senses and responds to multiple environmental signals and that *gp37* may play a role in cellular adaptations prior to stationary phase.

Taking into account the results presented in this thesis, the Destruelle, Holzer and Klionsky (1994) findings could be interpreted differently however. Since *YGP1* expression increases at periods when there are high levels of endogenous ethanol in the growth medium (i.e. diauxic shift) *YGP1* expression could be primarily responsive to the presence of ethanol with glucose repression playing only a minor role.

#### 6.2.2.4 General nutrient limitation?

The work of Destruelle, Holzer and Klionsky (1994) on *YGPI* was extensive but in the light of the findings recorded in this thesis, interpretation of their results regarding nutrient limitation should be re-appraised. One reason Destruelle, Holzer and Klionsky (1994) concluded an increase in *YGPI* expression was a response to nutrient limitation is that its expression increased in a glucose minimal medium culture as it approached stationary phase, that corresponded to less than 1% glucose in the medium. In a similar experiment to investigate *YGPI* expression in response to nitrogen limitation, *YGPI* was not expressed when a histidine auxotroph strain was grown with a range of low concentrations of histidine. Although the growth rate of the nitrogen-limited cultures was unaffected by the level of histidine, they exited log phase earlier than the control, presumably due to nutrient limitation. However, when asparagine (0.002%, 0.02% or 0.2%) was the sole nitrogen source in a non-limiting glucose defined medium, *YGPI* expression rose to a high level but did not correlate with exit from log phase or glucose level. In cultures containing 0.02% or 0.002% asparagine, *YGPI* was expressed at a level approximately 200-fold above basal therefore *YGPI* is induced by asparagine-limitation and its expression level is not correlated to the level of glucose in the medium. Although the cultures exited log phase in an asparagine concentration-dependent manner suggesting nutrient limitation, the expression of *YGPI* was independent of the change in growth phase and was greatest in the culture with an initial asparagine concentration of 0.02%. Thus, *YGPI* is induced under low nitrogen conditions that may be specific to asparagine since it is not induced when histidine is limiting. The response to other limiting nitrogen sources has not been reported.

A similar pattern of *YGPI* expression was seen in phosphate-limited (0.002 mM, 0.02 mM, 0.2 mM and 2 mM) defined medium cultures (Destruelle, Holzer and Klionsky 1994). The highest level of *YGPI* expression was exhibited by the culture containing 0.2 mM phosphate (approximately 1200-fold above basal level) whilst the culture containing 2 mM phosphate exhibited a comparatively low level of *YGPI* expression. The growth rate was initially the same for all cultures but they exited log phase in a phosphate concentration-dependent order, the most phosphate-limited first. Thus the expression of *YGPI* did not influence growth rate or exit from log phase.

In cultures grown with 2% ethanol as carbon source, *YGP1* expression reached approximately 100 times basal level but only after the ethanol concentration had fallen to approximately 0.8% (Destruelle, Holzer and Kliensky 1994). This pattern was repeated in cultures grown with 2% glucose as carbon source. *YGP1* expression exceeded the basal level when the glucose concentration had fallen to around 1%, corresponding to entry into respiratory growth phase and when the endogenous ethanol level had reached approximately 0.8% (Destruelle, Holzer and Kliensky 1994). These experiments and those showing a high level of *YGP1* expression at the diauxic shift suggest that *YGP1* may be induced in response to general carbon source depletion or that its expression is related to respiratory growth and/or stationary phase.

#### **6.2.2.5 Stationary phase**

Further to the work of Destruelle, Holzer and Kliensky (1994), Riou *et al.* (1997) examined the expression of stationary phase and nutrient limitation-responsive genes including *YGP1*, *HSP26* and *HSP30*. In a Northern analysis of wine yeasts grown under wine production conditions the authors demonstrated that *YGP1* was expressed from transition phase or entry into stationary phase and throughout stationary phase. Of 19 previously reported stationary phase or nitrogen limitation-induced genes, *YGP1* was the only gene for which expression remained constant throughout stationary phase and only *YGP1*, *HSP26* and *HSP30* mRNA could be detected at the end of the fermentation (110 hours). *YGP1* was expressed at a level 76-fold greater in stationary phase than its basal level during exponential growth, in comparison to a greater than 100-fold increase in expression of *HSP26* and *HSP30*. The authors considered that the promoters of these three genes would be suitable for heterologous gene expression during the stationary phase of wine fermentations.

#### **6.2.3 *YGP1* transcript stability**

Whether the high level of *YGP1* mRNA throughout stationary phase is due to a constant rate of transcription or greater stability of the transcripts is unclear. *YGP1* has an apparent mRNA half-life in mid log phase of 24 minutes which is close to 19 minute average for mRNA molecules in *S. cerevisiae*, and its transcriptional frequency is 22.5 mRNAs/hour as determined by Holstege *et al.* (1998) in a genome-

wide *S. cerevisiae* expression analysis. However, *YGP1* mRNA level is reportedly 50-fold higher in stationary phase than log phase (Destruelle, Holzer and Kliensky 1994). It is possible that this increased level of mRNA reflects increased transcript stability since Boucherie (1985) reported that the amount of mRNA produced by cells in stationary phase was 10% of that in log phase. Given the high expression level of *YGP1* through stationary phase and the extracellular location of gp37 it may have an important role in maintaining cell viability during nutrient limitation and when cell wall remodelling is required.

In summary, *YGP1* expression is induced when yeast cells grown in minimal medium approach diauxic shift and this is coincident with endogenous ethanol accumulation. *YGP1* is also induced in asparagine-limited and phosphate-limited glucose cultures and when cultures enter stationary phase or commence respiratory growth. *YGP1* expression was not tightly correlated to glucose concentration, being only partially glucose repressed, neither was its expression correlated to growth rate or exit from exponential growth. Since ethanol inhibits some glucose transporters, presumably reducing the amount of glucose transported into the cell, glucose catabolite repression probably has less influence on ethanol-stress-related genes than on many other genes. As concluded by Destruelle, Holzer and Kliensky (1994), the regulation of *YGP1* may be complex since it responds to a number of environmental signals.

Thus it is perhaps not surprising that neither *YGP1* deletion nor over-expression had any appreciable effect on growth rate in the presence or absence of ethanol. Although an increase in growth rate has traditionally been used as a measure of yeast "fitness" it is not the only physiological parameter for assessing cellular changes that may benefit cells in stressful conditions. For example, cells increase in stress tolerance when the Ras/cAMP signaling pathway, that is required for growth, is inactivated (Boy-Marcotte *et al.* 1987). Since *YGP1* has a high profile at diauxic shift, during stationary phase, when adapting to ethanol stress and when carbon-, phosphate- or asparagine-limited, it may be an important general stress- and nutrient limitation-response gene. It seems likely that ethanol is one of the triggers for this response and the high expression of *YGP1* during adaptation to ethanol suggests a link to respiratory growth. The inhibitory effect of ethanol on glucose transporters may lower the level of glucose entering the cell and allow some genes involved in

respiratory growth to be derepressed. Destruelle, Holzer and Klionsky (1994) found that the degradation of gp37 that occurs in the presence of glucose, was reduced following the addition of the translational inhibitor cycloheximide, suggesting that levels of the protein may be post-translationally regulated by protease activity. Degradation of gp37 in the presence of glucose may indicate it has an important role in stationary phase when glucose is absent. It may be required for energy storage, strengthening of the cell wall, exit from stationary phase or commencement of proliferative growth after stationary phase.

### **6.3 REGULATION OF *YGP1***

#### **6.3.1 The evolution in *YGP1* expression during carbon limitation**

A novel microarray experiment performed by Ferea *et al.* (1999) compared gene expression of evolved strains that had been previously grown aerobically under carbon limitation for several hundred generations, with the expression profiles of the parent cultures. These experiments revealed that *YGP1* and *DIP5* were among less than 2% of genes that evolved an altered expression pattern, being less strongly expressed in the evolved strain than the parent strain and clustering with genes involved in glycolysis and ethanol utilization. The evolved strains used respiration for more efficient users of glucose than the parent strain but they produced around 10 times less ethanol (0.02 g/L). Since *YGP1* and *DIP5* are upregulated during ethanol-stress conditions, they would presumably be under little selection pressure for increased expression when endogenous ethanol levels are low. This data demonstrates that yeast cells can adapt to low carbon growth conditions over many generations by increasing the flux of carbon through the respiratory pathway and it suggests that *YGP1* and *DIP5* are not regulated by respiratory growth but may be ethanol-regulated.

#### **6.3.2 What is known about the *YGP1* promoter?**

The pleiotropic nature of *YGP1* expression suggests that more than one regulator controls its induction and there is some experimental evidence to support this. In

addition, a search of the *YGP1* promoter region revealed numerous putative regulatory sequences that may influence *YGP1* expression (Figure 6.1).

### 6.3.2.1 Putative stationary phase elements

A search by Wang, Nishikawa and Isono (1997) of the upstream 500 bp of genes that are known to be up-regulated in the post-diauxic phase revealed that 22 out of the 42 did not have stress response elements (STRE). Instead of STRE, 16 had AAAGG or CCTTT sequences that the authors suggested may be stationary phase response elements. The *YGP1* promoter has 4 of these putative stationary phase elements, 3 AAAGG and 1 CCTTT motifs as well as 2 STREs (AGGGG). Since *YGP1* is expressed at a high and sustained level throughout stationary phase it is appropriate that its promoter contains several of these putative stationary phase response elements.

### 6.3.2.2 Msn2p and Msn4p-dependent STRE regulation

In a *MSN2/4* double mutant, the level of expression of a STRE-driven LacZ reporter was found to be as low in the presence of 7% ethanol, heat shock and low pH as an unstressed control and ten-fold lower than the wildtype (Martinez-Pastor *et al.* 1996). Thus, activation of STRE-containing genes in the presence of ethanol is at least partially dependent on Msn2p and/or Msn4p.

PKA, the cAMP-dependent protein kinase, is essential for growth and prevents induction of the general stress response via transcription factors Msn2p and Msn4p. Thus PKA and these transcription factors are antagonistic; growth is reduced when Msn2p and Msn4p are expressed (Smith *et al.* 1998). The authors suggest that this antagonism may explain the pleiotropic effects of PKA, including growth regulation, stress response and carbohydrate storage. The interplay of these two regulatory systems may also influence *YGP1* expression and explain its complex expression pattern and indicates that growth experiments may not be appropriate for determining the phenotype of stress-related genes.

-1000 AGCGTGCTATTTTTTAAAAAGGGCAAATTTAGGAATGTCTGACGGAATCCTAATCA  
TCTTTGGCTTTTCCCTTCACTCCCTTCTTTTACCGACGATGGTTTGAAGGAAAGAGAAAA  
TGAGCAATAGGAAAATTCAGTAGTCGATTTCCATTTCAATTCCTATTGGGTAATGTGGCG  
GCGAGGGCTAAACATTCGGTATCATTGATTCAATGTTCTACAATGTTCAAATTCTCGGAT  
GGGTATCCCTAAATTGGGATCTCTTTCCAGAGGAAGGGGCCTTGATGTGGATTGAAGACAC  
ATGAGTTTTTTTCTCACCGTCGAAACTTTTGCTT TAAAACATCGGCCATTCGTTGG  
TTTATTATGTGACTTTTTTGTGACTTTTTTTTTTTCGAAGTTTTCTCGTCAATCCGCGG AGA  
TCCGGCGGGCGAAAAGTCTCTCACTTTTACCCAATCGGGGCATGTTATGTGGACATGTTAT  
GTGCCAAGAGACACATGAGCCCAAAAAGGGTACTGTCTAAATAGTTTAATACGA  
ATCGAAACTTCTCGAAGAAGGGGCCGAAAATTAGCGCAGAAGGACGAAAGTTTATACTT  
TTCTAATGGAGAGGCTCAGGAGCTGAAAGCATATATTCGTACTCTATTGC -350 bp  
ATCTTCAACTCCGAAGAATCTCAGTAGGAGTGAAGCCGGCTTCTCGATGCTACAGTTCAAC  
ATCGATACTTTGAAGAAGAAAGCGCCTATTATATCTCTTTTACCCTATTAGTAATAATTAG  
GAAAGGGAGAAAAAAGTACCTCACTAAAAAAACCATCATCTCTGAAATATAAAAGCTT  
GATAGAGGGTGACATTTGCTAGAACTTCTGCTGTGTTCTCTTGGGTTATTGCTCTTATTGA  
ATATCCCTTCTATTCTTTCTTGCTTGTAATAAATCAGCTCAAAAAACATCTACAGGATTA  
ATCGTCAGTTAAGTAATACATAATAGAAA ATG start codon

**TATAA, TATAT:** putative tata boxes

**TAATTAGGA:** predicted Mcm1p binding site

: predicted Reb1 binding site

**AGGGG, CCCCT:** STRE (stress response element)

**AAAGG, CCTTT:** putative stationary phase response elements (Wang *et al.* 1997)

**TCCGCGG.** : PDRE (pleiotropic drug response element) (De Risi *et al.* 2000)

**CGAAAATTAG:** potential Rlm1p binding site (Jung and Levin 1999)

**ATCCCTAA (TA/TAGGGAT):** PDS (post diauxic shift element) (Boorstein and Craig 1990)

**GATAA/GATAGA:** GATAA nitrogen regulation element (Bysani *et al.* 1991)

**CGAAACTTT:** HSE (heat shock element) (Craig *et al.* 1992)

**Figure 6.1:** The 1000 bp promoter region of *YGPI* indicating putative regulatory elements. The -350 bp of the promoter region used in a *YGPI/SUC2* fusion by (Chang *et al.* 2001; Destruelle *et al.* 1994) and the regulatory elements contained in it are also indicated.

### 6.3.2.3 The transcription factor Mcm1p and cell cycle regulation

A search of the *YGP1* promoter extending 800bp upstream using MatInspector V2.2 Quandt *et al.* (1995) predicted 2 Mcm1p-binding sites and the Transfac programme [<http://transfac.gbf-braunschweig.de/TRANSFAC>] recognised the Mcm1p and Reb1p transcription factor binding sequences in the *YGP1* promoter. Mcm1p plays a direct role in the induction of genes during G2 and M (Althoefer *et al.* 1995). It also combines with other DNA-binding factors to direct cell cycle expression of some early G1 phase genes, M phase genes and genes at the M/G1 boundary (references in Cho *et al.* (1998)). Mcm1p is part of a positive feedback loop for induction of transcription of the mitotic cyclin *CLB2* following activation by Clb2p-Cdc28p.

More than 10% of yeast protein-coding genes are cell cycle-regulated including *DIP5* in M phase and *YGP1* at the M/G1 boundary (Spellman *et al.* 1998). *YGP1* is strongly cell cycle-regulated at a level that is comparable to the cyclin *CLN2*. The clustering algorithm of Eisen *et al.* (1999) that clusters genes by expression patterns derived from whole-genome microarray experiments can indicate gene function, since genes that cluster together are presumably involved in common processes. Although there is no direct evidence that *YGP1* is regulated by Mcm1p, the majority of the 34 genes including *YGP1* that peaked in expression at the M/G1 boundary contain Mcm1 binding sites in their promoter region. All known MCM genes involved in DNA replication were also contained in this cluster (Spellman *et al.* 1998).

In a library of genes that Mcm1p binds upstream of *in vitro*, many belong to two classes, cell cycle and cell surface e.g. the G1 cyclin *CLN3*, the plasma membrane H<sup>+</sup>ATPase, *PMA1* and the secreted heat shock glycoprotein *HSP150* (Kuo and Grayhack 1994). Numerous cell cycle-regulated nutrient-related genes involved in membrane transport of amino acids, ammonia, sugars and iron reach peak expression during M and M/G1. Other genes that reach peak expression at this stage include genes involved in budding, secretion, glycosylation and synthesis of lipids and cell wall components (Spellman *et al.* 1998). Glycosylation and secretion are coordinated with cell cycle progression since expression of *ALG7*, the first gene in the dolichol pathway for N-glycosylation of proteins is directly linked to START-1 arrest suggesting that this gene may be critical in determining the proliferative potential of

cells (Pretel *et al.* 1995). Any failure in the secretory pathway leads to severe repression of transcription of both rRNA and ribosomal protein genes (Nierras and Warner 1999) and an important early response to the addition of glucose to glucose starved G0-arrested cells is the induction of N-glycosylation genes. Thus, in light of the indirect evidence, it is likely that *YGP1* is regulated via its Mcm1-binding site at the M/G1 boundary of the cell cycle and it may be required for progress through START. However, this hypothesis requires experimental verification.

#### **6.3.2.4 Nitrogen catabolite repression and GATAA elements**

The *YGP1* promoter also contains 1 GATAA element suggesting regulation by nitrogen availability. GATAA elements in *S. cerevisiae* are responsible for regulated expression of nitrogen catabolic genes and are highly sensitive to nitrogen catabolite repression (NCR) mediated by transcriptional activators and repressors (for reviews see Grenson (1992) and Horak (1997)). NCR occurs when preferentially utilized nitrogen sources such as aspartate, glutamate or ammonia are available and genes for uptake and utilization of poor nitrogen sources are repressed. When only poor nitrogen sources are available the genes for their utilization are expressed at a higher level. The promoters of several genes involved in nitrogen catabolism have GATAA elements and two or more of these elements are required for high level expression (Bysani *et al.* 1991).

#### **6.3.2.5 The PDS (post diauxic shift element) and HSE (heat shock element)**

The *YGP1* promoter contains a PDS element (post diauxic shift element) (Boorstein and Craig 1990) that was first identified in the promoter of *SSA3* (HSP70). This element is capable of activating a heterologous promoter following the diauxic shift and in stationary phase. Furthermore, the PDS element interacts positively with the heat shock element, HSE, to mediate high levels of *SSA3* transcription in response to nutrient limitation and lowered levels of intracellular cAMP. Since the *YGP1* promoter is predicted to feature at least one HSE and *YGP1* is reported to be induced in response to a temperature increase (Jung and Levin 1999), this type of coregulation could also apply to *YGP1*.

### 6.3.3 *YGP1* regulation: experimental evidence

It is clear that expression of *YGP1* may be regulated via a number of regulatory elements. Whilst some of these may seem likely regulators in view of the known expression patterns of *YGP1*, whether or not they are functional is still a matter for speculation. However, there is some experimental evidence to confirm *YGP1* regulation under certain environmental conditions that can provide insights into its role in yeast cells and in the ethanol-stress response. Known *YGP1* regulators and signaling pathways from experimental data are shown in Table 6.1, overleaf.

#### 6.3.3.1 *YGP1* expression in the multidrug resistance response

*YGP1* was one of only 26 genes to be upregulated by overexpression of *PDR1* and *PDR3*, the transcription factors that activate genes in the pleiotropic drug resistance response and one of only 15 with a PDRE (Pleiotropic Drug Resistance Element) in its promoter (Figure 6.1). To investigate gene expression related to the multidrug resistance response, De Risi *et al.* (2000) performed a microarray experiment using strains overexpressing the *PDR1* and *PDR3* (Pleiotropic Drug Resistance) homologous transcription factors compared to a strain that did not express the transcription factors. The signal from upregulated genes in cells overexpressing Pdr1p and Pdr3p was therefore amplified. Targets of the transcription factors were genes involved in transport, membrane lipids, cell wall biosynthesis, nutritional stress and cell defence as well as the well-known ABC transporters (multidrug ATPases). Several of the genes identified by this approach are able to reduce the intracellular accumulation of hydrophobic compounds or modify the passive diffusion of hydrophobic drugs across the membrane. The regulation of *YGP1* by Pdr1p/Pdr3p in the cellular defence against drugs is significant in light of its induction by ethanol stress and suggests *YGP1* expression may lead to changes in the characteristics of the cell membrane or cell wall to limit entry of drugs such as ethanol.

YGP1 INDUCTION BY	INDUCING CONDITIONS	FOLD INDUCTION	TRANSCRIPTIONAL MEDIATOR	GENES NOT REQUIRED FOR RESPONSE	REFERENCE
high osmolarity	0.7 M NaCl 0.95 M sorbitol	3.2 1.8	MSN2/MSN4 NA	HOG1 NA	(Rep et al. 2000)
low external pH	pH5.5 - pH3.5	1.6		SIT2	(Kapteyn et al. 2001)
ethanol	5% (v/v)	upregulated			this thesis
G1 arrest	deletion CDC25	>200	CDC28?	CDC25	(Chang et al. 2001)
G1 arrest	deletion RAS2	>200	CDC28?	RAS2	(Chang et al. 2001)
heat shock	25-37°C and 33-29°C	upregulated	MSN2/MSN4		(Gasch et al. 2000)
oxidative stress	H <sub>2</sub> O <sub>2</sub>	upregulated	MSN2/MSN4		(Gasch et al. 2000)
carbon limitation	<1% glucose or 0.8% ethanol	upregulated		SNF1	(Destruelle et al. 1994)
asparagine limitation	0.02% (w/v)	200			(Destruelle, Holzer and Klionsky 1994)
phosphate limitation	0.2 mM phosphate	1200			(Destruelle, Holzer and Klionsky 1994)
diauxic shift	diauxic shift	>8			(De Risi et al. 1997)
stationary phase	45 hr - 96 hr culture	76			(Riou et al. 1997)
stationary phase	96 hr culture	155.3			(Chang et al. 2001)
cell cycle	M/G1 boundary	9.334			(Spellman et al. 1998)
cell wall integrity	overexpression <i>MKK1</i>	down regulated	<i>RLM1, PKC1, MPK1</i>		(Jung and Levin 1999)
multidrug resistance	overexpression <i>PDR1-3/PDR3-7</i>	>2	<i>PDR1-3/PDR3-7</i>		(De Risi et al. 2000)

**Table 6.1:** *YGP1* induction and signaling pathways

### 6.3.3.2 *YGP1* regulation by the yeast cell wall integrity signaling pathway

Yeast cell wall remodeling that occurs during growth and environmental change is monitored and regulated by the cell wall integrity signaling pathway controlled by Rho1 GTPase (see section 6.2.2 also). The function of one target of this pathway, the transcription factor Rlm1p that is activated by Mpk1p, was investigated in a filter-based mini array comparing the expression profiles of cells in which signaling from the pathway was either strongly or normally active. Using this approach five genes including *YGP1* and its homologue, *SPS100*, *CTT1* (encoding cytoplasmic catalase T) and *PGK1* (3-phosphoglycerate kinase) displayed reduced expression when the cell wall integrity signaling pathway was active. Whilst the benefit to the cell of negative regulation of *YGP1* by Mpk1p is unclear, it is possibly an energy saving strategy since both *YGP1* and *SPS100* are highly glycosylated and the glycolytic enzyme *PGK1* is also down regulated. *YGP1* has a putative Rlm1 binding site at -428 (TGAAAATTA) and is shown to be down regulated via the transcription factor Rlm1p (Jung and Levin 1999) although, in view of the putative protein kinase C binding site on gp37, the protein may be directly phosphorylated by this kinase.

### 6.3.3.3 *MSN2/MSN4*-dependent expression in response to heat shock and oxidative stress

Using microarray experiments Gasch *et al.* (2000) measured changes in mRNA profiles as cells responded to temperature shocks, hydrogen peroxide, the superoxide-generating drug menadione, the sulfhydryl-oxidising agent diamide, the disulfide-reducing agent dithiothreitol, hyper- and hypo-osmotic shock, amino acid starvation, nitrogen source depletion and progression into stationary phase. Under the conditions of these experiments *YGP1* was *MSN2/MSN4*-dependent for heat shock and oxidative stress exhibiting a greater than 6-fold increase in expression following a 37°C temperature shock but whether *MSN2/MSN4* are involved in *YGP1* expression under other environmental conditions is unknown.

#### 6.3.3.4 Is *YGP1* expression *CDC28*-dependent?

To determine whether entry into stationary phase was linked to nutrient limitation or slow growth, Chang *et al.* (2001) used temperature-sensitive Ras pathway mutants to arrest cell growth despite a nutrient rich growth medium. To act as an indication of stationary phase expression, the mutants also expressed *YGP1* carried on a plasmid as a *SUC2/YGP1* fusion protein in a *suc<sup>-</sup>* background. Thus the level of *YGP1* expression could be determined by using an invertase assay to measure the amount of *SUC2* gene product present. Inactivation of the *RAS1* gene led to a greater than 200-fold increase in *YGP1* expression as measured by invertase assay, suggesting that *YGP1* is negatively regulated by the ras/cAMP signaling pathway and that its expression is linked to growth rather than nutrients. By contrast, a *cdc28* mutant that arrests growth in G1 did not demonstrate any increase in *YGP1* level, whilst a *cdc25* mutant, that also arrests in G1, and the wildtype registered high *YGP1* expression implying that *CDC28* is required for *YGP1* expression during stationary phase.

The *SUC2/YGP1* fusion construct used for the above experiments by Chang *et al.* (2001) was also used by Destruelle, Holzer and Klionsky (1994) in work relating to *YGP1* expression during stationary phase, diauxic shift and following nutrient limitation, as described in sections 6.2.2.1 to 6.2.2.5. In the experiments described above, the level of *YGP1* expression measured as invertase activity was used as a reporter of the stationary phase status of cultures. Chang *et al.* (2001) reported that *YGP1* is not induced by heat shock, high osmolarity, oxidative stress or ethanol stress but is induced greater than 150-fold when cultures have grown for 4 days (stationary phase). This fusion construct for *YGP1* expression contains only 350 bp of the *YGP1* promoter region therefore it can be deduced that the regulatory element for induction of *YGP1* in stationary phase is present within that range. As a corollary, other regulatory elements further upstream are presumably involved in regulation of *YGP1* following exposure to the other stresses listed, since *YGP1* has been reported to be upregulated in response to such stresses in this thesis and by other workers. The *YGP1* promoter contains 3 putative stationary phase elements, 2 stress response elements, 1 post diauxic shift element and 1 pleiotropic drug response element upstream of -350 bp (Figure 6.1). Thus it is not surprising that Chang *et al.* (2001) failed to detect the full extent of the *YGP1* stress response.

### 6.3.3.5 The rye (defective for the regulation of *YGP1* expression) mutants

To find novel genes that might provide information regarding entry into stationary phase, Chang *et al.* (2001) generated mutants that were selected on the basis of their aberrant *YGP1* expression. In these mutants, *YGP1* was expressed at a high level during log phase. They were defective for entry into stationary phase following nutrient starvation and were unable to survive prolonged periods of nutrient deprivation. In addition the expression of *ACT1* remained at an uncharacteristically high level during stationary phase and the *rye* mutants exhibited abnormal cell morphologies during exponential growth. Cells were often elongated, sometimes carried multiple buds and in liquid medium were prone to flocculate.

Mutated genes from the above work were identified by complementation, deleted and the knockout strain transformed with the *SUC2/YGP1* fusion construct for monitoring *YGP1* expression. Three of the RYE genes encoded RNA polymerase II-associated proteins, Ssn/Srb proteins that regulate gene expression by modulating the activity of the RNA polymerase II holoenzyme towards a particular set of promoters. The authors suggest that the Ssn/Ssb proteins are required for proper entry into stationary phase and that the holoenzyme may be a target of the signaling pathway that coordinates cell growth and nutrient availability (Chang *et al.* 2001). If this is so, regulation of *YGP1* is also linked to cell growth and nutrient availability and since *YGP1* expression is cell cycle-regulated with peak expression at the M/G1 boundary, *YGP1* probably plays an important part in cell growth.

### 6.3.3.6 Response of *S. cerevisiae* to short-term ethanol stress

In a recent microarray experiment, Alexandre *et al.* (2001) investigated the response of early exponential phase *S. cerevisiae* cells grown in rich medium, to a 30 minute incubation in the presence of 7% ethanol. Numerous novel ethanol-stress response genes were identified including *YGP1*, some of the oxidative stress response genes, most of the heat shock proteins, genes involved in energy metabolism including trehalose, glycerol and glycogen metabolism, protein destination and ion homeostasis. The induction of *YGP1* as an early ethanol-stress response gene confirms the findings of this thesis. There was a similar proportion of genes upregulated by short-term

ethanol stress as there were downregulated. One third of the downregulated genes encoded ribosomal proteins and included genes involved in growth and protein synthesis. This ethanol shock experiment identified immediate and possibly transiently expressed yeast ethanol-stress response genes as is evidenced by the failure to detect genes involved in cell wall biosynthesis or lipid metabolism, known to be ethanol stress targets. The large number of hsp's induced in this early ethanol-stress response suggests that protein refolding is a necessary early response to ethanol. Another early response appears to be rapid metabolism of trehalose, glycerol and glycogen. The authors were unable to identify any ethanol-stress-specific signaling pathways but induction of several HOG-dependent genes involved in glycerol metabolism indicates that at least some ethanol-stress related genes are regulated via the HOG pathway.

#### **6.3.4 A possible role for *YGPI* in yeast cells**

Empirical evidence indicates that *YGPI* encodes a secreted, highly glycosylated protein. The gene is positively regulated by the pleiotropic drug resistance transcription factors Pdr1p and Pdr3p, the stress response transcription factors Msn2p/Msn4p for heat shock and oxidative stress and is negatively regulated by the transcription factor Nlm1p in the yeast cell wall integrity signaling pathway. In addition, *YGPI* appears to be downregulated by the Ras/cAMP signaling pathway and may be positively regulated by *CDC28* for entry into stationary phase. This information is limited considering the many putative regulators of *YGPI*, its pleiotropic expression pattern and the recent publication of numerous full genome microarray experiments. Thus, although the role of *YGPI* is unclear, it seems likely to be involved in cell wall strengthening or remodeling in response to environmental signals such as heat shock or the presence of amphipathic compounds such as ethanol that can penetrate and disrupt the phospholipid bilayer of the plasma membrane. *YGPI* probably has a similar role during cell growth when the cell wall stretches and becomes porous and in stationary phase when the cell wall is less porous and resistant to lysis. Given the complex regulation of *YGPI* it seems likely that it is an important gene whose regulation is fine-tuned to give maximum cell wall protection. Perhaps it has different properties depending on its regulation such as variable extents of glycosylation and possibly phosphorylation. The role of gp37 in the response to

nutrient limitation is even less clear but an asparaginase activity is a possibility although its putative role as an asparaginase remains to be tested.

### **6.3.5 The nutrient limitation effect of ethanol and role of *YGPI* in the ethanol-stress response**

Regulation of *YGPI* during the cell cycle appears to be important for entry of cells into stationary phase and for their survival when deprived of nutrients. *YGPI* regulation may even influence progress through START since expression of *YGPI* peaks at the M/G1 boundary of the cell cycle. By implication, one of the effects of ethanol stress may be inhibition of cell cycle regulators and would help explain the slow growth of ethanol-stressed cells and their nutrient deprived phenotype.

The presence of ethanol at the cell surface may also lead to nutrient deprivation since it is reported to inhibit the general amino acid permease, *GAPI* (Leao and Van Uden 1984) and some hexose transporters leading to a reduced uptake of glucose (Leao and Van Uden 1982). This is not the first time that ethanol stress and nutrient limitation have been linked. Nutrient limitation rather than ethanol toxicity due to endogenous ethanol has previously been proposed as the major factor responsible for a decline in fermentative activity in the early stages of fermentation (Dombek and Ingram 1986). The wider substrate range of *DIP5* reported recently by Regenber *et al.* (1999) suggests it may respond to nutrient limitation and its up-regulation in response to ethanol stress suggests it may have a special role in nitrogen import and compensate in part for loss of *GAPI* activity.

The precise role of *YGPI* in the ethanol-stress response is still not clear but it is involved in many of the protective processes of the cell such as the general stress response, the response to nutrient deprivation, multidrug resistance and cell wall integrity. In addition, *YGPI* clearly has an important role during stationary phase and for progress through the cell cycle. The activation and functioning of these protection mechanisms in the presence of ethanol makes intuitive sense since ethanol is well known for its toxic and disruptive effects.

## 6.4 THE POSSIBLE ROLE OF *DIP5* IN THE ETHANOL-STRESS RESPONSE

### 6.4.1 What is known about *DIP5* from the literature?

The *S. cerevisiae* ORF *YPL265w* encodes *DIP5*, the dicarboxylic amino acid permease. *DIP5* is one of a group of 24 amino acid permeases of the AAP family that includes transporters found in bacteria and fungi. The *S. cerevisiae* amino acid transporters are thought to be H<sup>+</sup>-symporters (Andre 1995). The AAP family is part of a larger group, the Major Facilitator Super-family (MFS) comprising permeases with 12 transmembrane domains that includes five transporter families: the hexose transporters, the MFS drug-resistance family, purine and pyrimidine transporters and carboxylic acid transporters. These transporters probably have a common ancestor.

The protein encoded by *DIP5* has high transport capacity and high affinity for L-glutamate and L-aspartate (K<sub>t</sub> of around 50 μM and 40 μM respectively) and cells expressing *DIP5* can grow on either amino acid as sole nitrogen source (Regenberg *et al.* 1998). *DIP5* deletion results in a several hundred-fold reduction in uptake of L-aspartate and L-glutamate in cells grown on proline (i.e. in the absence of nitrogen catabolite repression - NCR), or with ammonia as nitrogen source. However, *DIP5*-dependent uptake of aspartate and glutamate is lower in ammonia-grown cells than proline-grown cells suggesting regulation by NCR. Therefore the authors state that *DIP5* is “moderately regulated” by nitrogen source. NCR reduces the level of nitrogen catabolic enzymes and some of the amino acid permeases through repression of their genes.

*DIP5* and the general amino acid permease *GAP1*, are the main glutamate uptake systems expressed in yeast since *gap1/dip5* mutants are unable to grow on glutamate as sole nitrogen source. *DIP5* overexpression in yeast grown on proline, led to increased uptake of glutamate and aspartate as well as alanine, glycine, serine, glutamine and asparagine (Regenberg *et al.* 1999) suggesting that *DIP5* substrate specificity may not be as narrow as at first thought.

By comparison, *GAP1* imports almost all amino acids including glutamate and aspartate with a  $K_m$  of 0.8 mM and 1 mM respectively. The amino acid permease encoded by *AGP1* also has a wide range of substrates with particular affinity for glutamine and asparagine. *GAP1* is most active in cells growing on poor nitrogen sources such as proline. It is not active in the presence of ammonium ions due to downregulation by NCR as well as nitrogen catabolite inactivation (NCI), inhibition of the permease itself (Grenson 1992). It is thought that on ammonium-based medium, more permeases are expressed, but those that are active on proline medium, such as *GAP1*, *AGP1* and *DIP5*, play a less important role in ammonia-grown cells since they are to various extents under NCR (Regenberg *et al.* 1999). Promoters that are regulated by nitrogen source have a common sequence motif, (T/A)GAT(A/T)(A/G), a GATA factor binding site that binds the transcription factor Gln3p. The *DIP5* promoter contains nine of these elements at positions -225, -243, -424, -615, -740, -810, -882, -915 and -954, as well as two STRE and 1 STRE-like element.

#### 6.4.2 What is known about *DIP5* expression?

Microarray gene expression experiments indicate that, like *YGP1*, *DIP5* is induced at diauxic shift with a 2.5-fold increase in transcript level (De Risi, Iyer and Brown 1997) and is cell cycle-regulated, peaking in expression in M phase (Spellman *et al.* 1998). *DIP5* has been clustered by expression pattern with many cell cycle-regulated nutrient-related genes involved in transport of amino acids, ammonia, sugars and iron across the cell membrane, nearly all of which reach peak expression during M or M/G1. Like *gp37*, the *DIP5* protein product has a putative N-terminal modification site that is a possible *CDC28* phosphorylation site [<http://www.proteome.com/databases/YPD/reports>]. It is possible that *DIP5* expression is linked to progression through START since Dudani and Prasad (1983) observed a reduction in amino acid uptake in G1-arrested cells that was only evident in *cdc28* mutant cells arrested at their non-permissive temperature. *Cdc28* arrests cells at START but in G1-arrested *cdc4* and *cdc7* mutant cells, amino acid uptake was unaffected. This is similar to the evidence for regulation of *YGP1* expression by *Cdc28* but not *Cdc25* (as described in section 6.3.3.4), therefore both *YGP1* and *DIP5* may be regulated by *Cdc28*, although not necessarily directly, and therefore may be involved in growth and progression through G1 of the cell cycle.

*DIP5* mRNA is 11-fold more abundant in cells grown on YEPD than glucose minimal medium, but *GAP1* mRNA is 11-fold more abundant in cells grown on minimal medium compared to rich medium (Wodicka *et al.* 1997). This finding demonstrates that *DIP5* regulation is the reverse of that of *GAP1* even though both have been shown to be similarly regulated by NCR, however, it is clear that there are other factors apart from NCR that regulate *DIP5* expression. Several stress proteins were also expressed in cells grown in minimal medium suggesting that this medium elicits a stress response but despite the presence of several STREs in its promoter, *DIP5* was only induced at a low level in minimal medium.

*DIP5* was among 1.4% (88/6,124) yeast genes that showed a consistent change in expression between evolved strain and parent in evolution experiments under carbon limitation (Ferea *et al.* 1999). *DIP5* was consistently lower in expression in evolved strains than in the parent. Expression of genes involved in respiration increased in evolved strains since the cultures turned to respiration for more efficient use of glucose, but they produced approximately ten-fold less ethanol. Since *DIP5* is upregulated under ethanol-stress conditions, its expression may not be required at ethanol levels as low as 0.02 g/L measured in experiments described by Ferea *et al.* (1999). Evolution of a low level of expression of *DIP5* and *YGP1* suggests they are not essential for growth under carbon limitation or for respiratory metabolism but instead may be regulated by ethanol.

In respiratory deficient petites, *DIP5* was upregulated along with genes involved in the flux and conversion of metabolites generated by the peroxisomal  $\beta$ -oxidation of fatty acids to intermediates of the TCA and glyoxylate cycles (Epstein *et al.* 2001). In this work the authors used microarray to compare the expression of respiratory deficient petite mutants (*Rho*<sup>o</sup>) and the isogenic *Rho*<sup>+</sup> wildtype when treated with the inhibitor of oxidative phosphorylation, antimycin. The results of this experiment and accompanying microscope data indicate that respiratory deficiency, but not inhibition of ATP synthesis, induces genes for peroxisomal activities and metabolite restoration (anaplerotic) pathways that would be expected to mitigate the loss of a complete TCA cycle in mitochondria. The increased expression of *DIP5* in respiratory deficient petites compared to the wildtype suggests *DIP5* is not essential for respiratory

metabolism and supports the conclusions drawn from the expression evolution experiments of Ferea *et al.* (1999) discussed above. On the other hand, *DIP5* may be required for import of glutamate and/or aspartate for use in anaplerotic biosynthetic reactions that take place in peroxisomes.

In order to investigate the signaling pathways involved in respiratory deficient petite gene expression, Epstein *et al.* (2001) generated petite strains with null alleles of *RTG1*, *RTG2* and *RTG3*, genes known to control signaling between mitochondria, peroxisomes and nucleus. The authors used microarray to compare expression of these knockout strains to the petite parent strain. The RTG genes regulate genes in the retrograde pathway and are responsible for the expression of several genes encoding TCA cycle enzymes in cells with dysfunctional mitochondria (Liao and Butow 1993). *DIP5*, *GAP1* and *AGP1* were upregulated in RTG signaling mutants along with *PUT1* and *CAR2*, the products of which break down proline and arginine respectively, presumably to generate glutamate that could not be generated in a *Rho*<sup>o</sup> strain. The upregulation of *DIP5* as an importer of glutamate, presumably destined for the TCA cycle, was almost independent of the RTG genes suggesting it is controlled by an alternative signaling pathway that may act as upstream modulator of *RTG1*, *RTG2* and *RTG3* in the retrograde response pathway. Glutamate synthesised from TCA cycle intermediate,  $\alpha$ -ketoglutarate is a potent inhibitor of RTG-dependent gene expression therefore glutamate levels may constitute a key signal in the retrograde response pathway (Epstein *et al.* 2001). Although *DIP5* regulation bears similarities to that of *GAP1* and *AGP1* regarding NCR, its induction by an alternative signaling pathway suggests a different and specific role for *DIP5* in the import of nutrients used for biosynthesis and cellular metabolism.

*DIP5* was expressed at a higher level in cells grown on the fatty acid oleate (Kal *et al.* 1999) than on glucose (Velculescu *et al.* 1995), thus *DIP5* may also be required for catabolic activities such as  $\beta$ -oxidation of fatty acids, as well as the anaplerotic activities described above. For cells grown on oleate the pattern of gene expression was remarkably similar to that of genes upregulated in respiratory deficient petites in the experiments of Epstein *et al.* (2001). Growth on oleate induced genes encoding enzymes involved in peroxisomal function and biosynthesis,  $\beta$ -oxidation,

gluconeogenesis, mitochondrial functions, glyoxylate cycle, communication between peroxisomes, cytosol and mitochondria including transport of NADH and acetyl-CoA as well as several stress proteins. Acetyl-CoA generated in peroxisomes by  $\beta$ -oxidation, leaves the peroxisome as succinate generated by the glyoxylate cycle or as acetylcarnitine; the former route appears to predominate in oleate-grown cells. However, a role for *DIP5* in the cellular activities involved in growth on oleate is not clear.

To investigate the regulation of genes expressed in oleate-grown cells, a yeast strain with mutations in genes encoding the transcription factors Pip2p and Oaf1p, that control transcription of major enzymes in peroxisomal metabolism, was compared to the wildtype grown on either oleate or glucose (Kal *et al.* 1999). *DIP5* was upregulated in the *pip2/ oaf1* mutant grown on the fatty acid oleate suggesting that *DIP5* is negatively regulated by these factors in oleate-grown cells and is required for import of amino acids when  $\beta$ -oxidation in peroxisomes is inhibited. Therefore, under the experimental conditions described in this section, glutamate and aspartate may only be required by cells in the early stages of  $\beta$ -oxidation to prime the pathway and “top up” the TCA cycle.

### 6.4.3 *DIP5* regulation

*DIP5* appears to be regulated by the Tor proteins that are thought to primarily regulate protein translation but also mediate nutrient-sensing pathways. Tor1p and Tor2p are homologous phosphoinositol kinases that control translation initiation, early G1 progression and also play a role in entry into stationary phase (Barbet *et al.* 1996). In order to define some of the nutrient-sensitive pathways in yeast, Hardwick *et al.* (1999) used rapamycin, an inhibitor of the Tor proteins to mimic nutrient deprivation in cells grown in rich medium. Rapamycin induces severe cellular responses such as G1 cell cycle arrest, translation arrest, glycogen accumulation, sporulation, autophagy and repression of rRNA synthesis (references contained in Hardwick *et al.* (1999)). Microarray combined with biochemical methods identified a number of upregulated genes that would normally be repressed by NCR in the presence of a high quality nitrogen source such as ammonia, glutamine or asparagine (Hardwick *et al.*

1999). Amongst these were *DIP5* and several other specific permeases as well as the general amino acid permease *GAP1* and *AGP1*.

The nitrogen discrimination pathway genes, activated when rapamycin inhibits Tor regulation, are under direct control of the Ure2p/Gln3p complex and when a high quality nitrogen source is present Ure2p binds the GATA-binding transcription factor Gln3p, inhibiting transcription. The authors suggest that Tor-dependent, rapamycin-induced changes to Ure2p were due to dephosphorylation of Ure2p, leading to expression of genes usually repressed by Ure2p. The results of this experiment indicate that *DIP5*, as well as several other amino acid permeases, are controlled by the TOR regulatory pathway. Hardwick *et al.* (1999) suggest that the Tor proteins directly modulate the glucose activation and NCR pathways and pathways that respond to the diauxic shift such as glycolysis and the TCA cycle.

*DIP5* is therefore regulated via the TOR pathway that modulates nitrogen catabolite repression by its influence on Gln3p/Ure2p and genes expressed at the diauxic shift. This pathway is also thought to regulate entry into stationary phase and appears to partially control the switch from fermentation to respiration. *DIP5* is not as highly regulated by nitrogen source as *GAP1* and therefore presumably has a separate role, possibly involving anaplerotic functions. In addition *DIP5* appears to be cell cycle-regulated suggesting it may be required for cell growth in G1.

#### **6.4.4 What role does *DIP5* play in the ethanol-stress response?**

The role of *DIP5* in the ethanol-stress response is unclear although early work by Horak *et al.* (1978) hints at a possible link between *DIP5* and ethanol stress. These authors found that glutamate uptake was stimulated in cells pre-incubated with glucose prior to addition of antimycin (an inhibitor of oxidative phosphorylation) but this effect was more striking when cells were pre-incubated with ethanol. Thus, when oxidative phosphorylation is inhibited and there is a high requirement for glutamate as a TCA intermediate, presumably incubation with ethanol would induce *DIP5* and consequently increase glutamate import. In this model, inhibition of the general amino acid permease *GAP1* by ethanol would mean that *DIP5*, induced in the presence of ethanol, would be responsible for high capacity import of the dicarboxylic

amino acids glutamate and aspartate. Glutamate and glutamine derived from glutamate play a central role in nitrogen and carbon metabolism since they are precursors for the synthesis of other amino acids and are also required for nucleic acid synthesis. The linking of a possible requirement for glutamate uptake to exposure of cells to ethanol would explain why *DIP5* is induced by ethanol stress.

As already stated, *DIP5* also has a high affinity for aspartate and this amino acid can be channeled into the TCA cycle following transamination to produce oxaloacetate. In addition, if *gp37* has asparaginase activity this would not only enable a phenotype to be assigned to *YGP1*, it would also assign a role to this gene in the ethanol-stress response and connect its function with that of *DIP5*.

A mechanism by which *DIP5* activity may be increased during ethanol stress is related to activation of the plasma membrane  $H^+$ ATPase in the presence of ethanol and the subsequent efflux of protons. *DIP5* and other amino acid permeases are not distributed evenly in the plasma membrane but clustered around proton pumps, presumably to take advantage of protons outside the cell that can be used by these  $H^+$  symporters for amino acid import. Thus, increased activity of the plasma membrane  $H^+$ ATPase may lead to a higher level of permease activity and, in addition to a higher level of ethanol-stress-induced *DIP5* transcription, there may also be increased activity of the *DIP5* permease itself following ethanol stress.

## **6.5 THE POSSIBLE ROLE OF *YER024w* (*YAT2*) IN THE ETHANOL-STRESS RESPONSE**

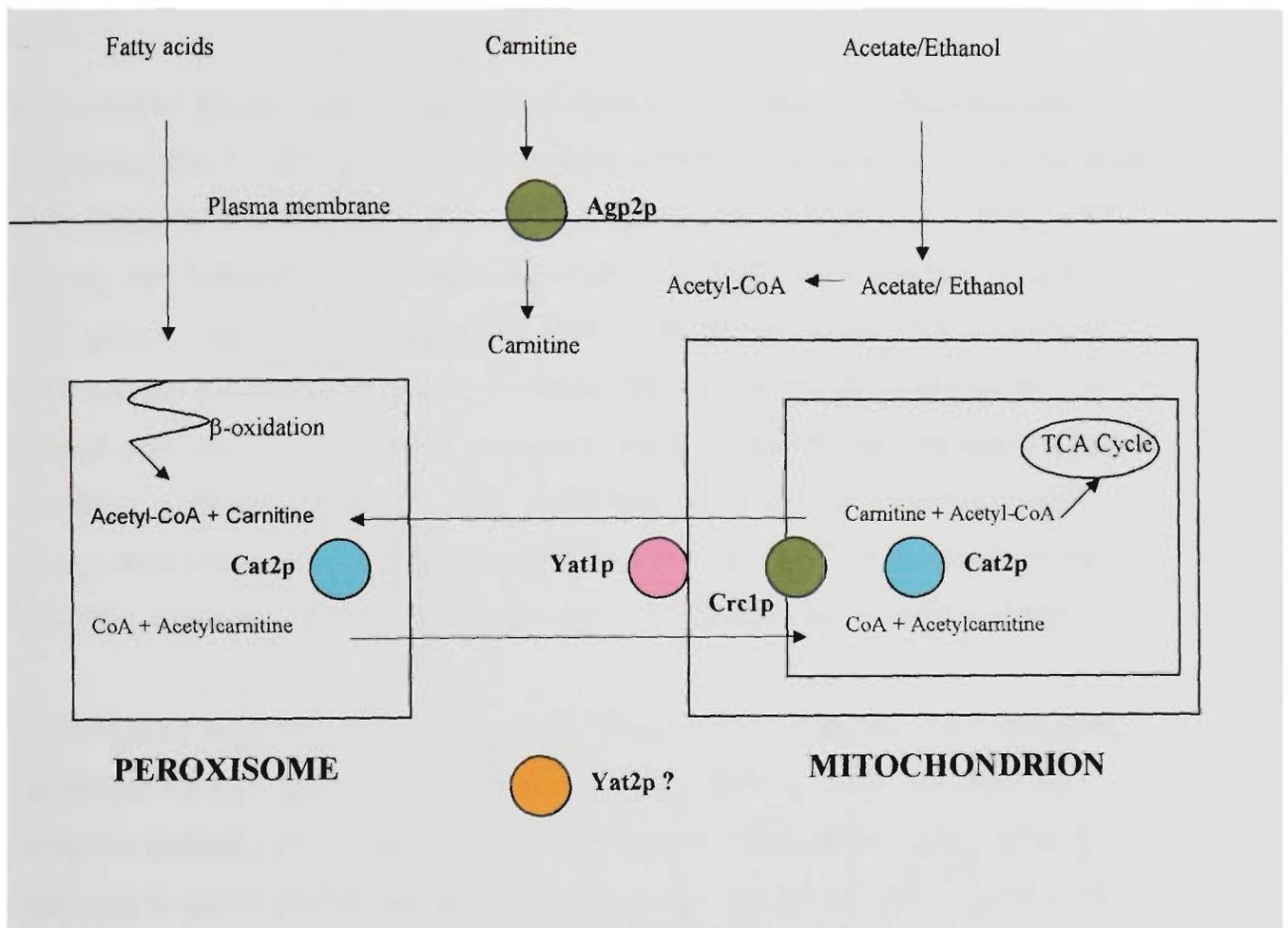
### **6.5.1 What is known about *YER024w* (*YAT2*) function, expression and regulation?**

*YER024w* encodes a carnitine acetyltransferase (CAT) that has recently been named *YAT2* by Swiegers *et al.* (2001) because of its homology to *Yat1p*, the carnitine acetyltransferase associated with acetyl transfer across the outer mitochondrial membrane (Schmalix and Bandlow 1993). The acetyltransferase encoded by *YAT2* contributes significantly to total cellular CAT activity in cells grown on ethanol (Swiegers *et al.* 2001). A third carnitine acetyltransferase, *CAT2*, is associated with

peroxisomes and the inner membranes of mitochondria. Swiegers *et al.* (2001) showed that all three CATs are essential for growth on non-fermentable carbon sources in a strain with a disrupted *CIT2* gene encoding the first enzyme in the glyoxylate cycle. The roles of the CAT proteins are shown in a diagram by Swiegers *et al.* (2001), Figure 6.2.

There are two pathways for the transfer of acetyl-CoA generated in peroxisomes by  $\beta$ -oxidation of fatty acids (Schmalix and Bandlow 1993). In the first pathway, known as the carnitine shuttle, the peroxisomal CAT is thought to catalyse the transfer of acetyl groups from acetyl-CoA to carnitine to form acetylcarnitine for transport across the mitochondrial membrane. The mitochondrial CAT catalyses the reverse reaction whereby carnitine is recycled and acetyl-CoA is released into the mitochondrial matrix for entry into the TCA cycle. L-carnitine, required for transfer into mitochondria of activated acyl groups of peroxisomal or cytoplasmic origin, is not synthesised by *S. cerevisiae* but is present in yeast extract (Swiegers *et al.* 2001) and imported into the cell by a member of the amino acid permease family encoded by *AGP2* (van Roermund *et al.* 1999). The second pathway for utilization of acetyl-CoA is the glyoxylate cycle that takes place partly in peroxisomes (Kunau *et al.* 1995). Deletion of *CIT2* encoding glyoxylate cycle-specific citrate synthetase that catalyses the condensation of acetyl-CoA with oxaloacetate to form citrate, inactivates the second pathway and generates a carnitine-dependent yeast strain in which all acetyl-CoA is transferred to the mitochondria via the carnitine shuttle.

*YAT2* contributes around 50% to total cellular CAT activity in wildtype cells grown in minimal medium containing 3% ethanol as carbon source, but when *CIT2* and *YAT2* are disrupted, cells are unable to grow on ethanol, oleate, acetate and glycerol, but grow normally on glucose (Swiegers *et al.* 2001). All three CAT genes are essential for growth on non-fermentable carbon sources in the *cit2* background but are unable to cross-complement each other. Deletion of *YAT2* does not prevent growth on non-fermentable carbon sources and has no discernible phenotype when grown on rich or minimal medium (Winzeler *et al.* 1999). The ability of the *yat2* strain to grow on non-fermentable carbon sources but inability of the *cit2/yat2* strain to grow under the same conditions suggests that *YAT2* is required for utilization of non-fermentable



**Figure 6.2:** Cellular location of the proteins involved in carnitine-dependent metabolic activities taken from Swiegers *et al.* (2001).

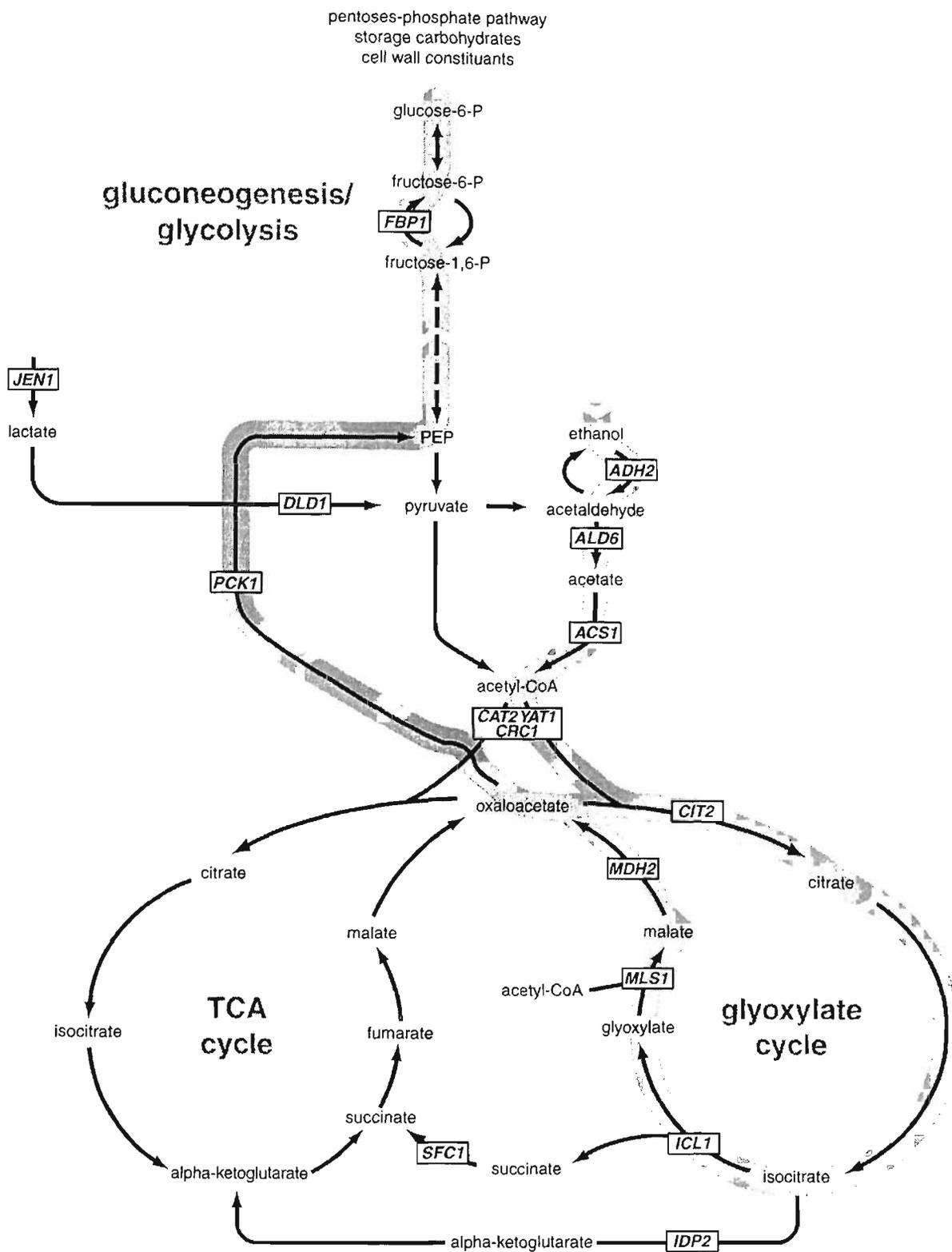
carbon in the absence of a functional glyoxylate cycle. Since the three CATs do not complement each other they are presumably all required for growth on non-fermentable carbon sources in the absence of the glyoxylate cycle pathway.

Yat2p has no distinct signal sequences to direct it to any specific cell compartment suggesting that *YAT2* might encode a cytosolic carnitine (Swiegers *et al.* 2001) similar to the carnitine acetyltransferase in *Aspergillus nidulans* (Semple *et al.* 1998). CAT activity was detected in *S. cerevisiae* by Atomi *et al.* (1993) at a very low level in cells grown on glucose but greater than 90% of that activity occurred in the cytosol. CAT activity has also been reported to occur in the cytosol of glycerol-grown cells (Kispal *et al.* 1991). Acetyl-CoA is required in the cytosol for the first step in lipid and sterol synthesis (Lehninger *et al.* 1993), therefore Yat2p may transport acetyl groups from acetyl-CoA generated in peroxisomes or acetyl-CoA generated by the decarboxylation of pyruvate, the latter being more likely to avoid futile cycling.

*YAT2* was co-regulated with a group of 142 genes in cells exposed to cell damaging conditions such as carcinogenic alkylating agents, oxidising agents and ionizing radiation (Jelinsky *et al.* 2000). The nature of *YAT2* and its upregulation following exposure to agents such as these suggests that it may be required for lipid and sterol synthesis for membrane repair. *YAT2* was upregulated 10-fold during diauxic shift (De Risi, Iyer and Brown 1997) and was one of 30 genes co-repressed in a *cat8* null mutant in a glucose depleted culture at the diauxic shift (Haurie *et al.* 2001), suggesting that *YAT2* is positively regulated by the transcriptional activator Cat8p. As well as being responsible for the derepression of numerous genes involved in ethanol utilization at the diauxic shift (Haurie *et al.* 2001), including *ADH2* (Walther and Schuller 2001) and gluconeogenic genes (Hedges *et al.* 1995), Cat8p is essential for growth on non-fermentable carbon sources (Randezgil *et al.* 1997). The genes for utilization of ethanol controlled by Cat8p are shown in a diagram by Haurie *et al.* (2001), Figure 6.3.

### **6.5.2 What role does *YAT2* play in the ethanol-stress response?**

From published data and experimental data presented in this thesis, the role of *YAT2* in the ethanol-stress response appears to involve the transport of activated acetyl

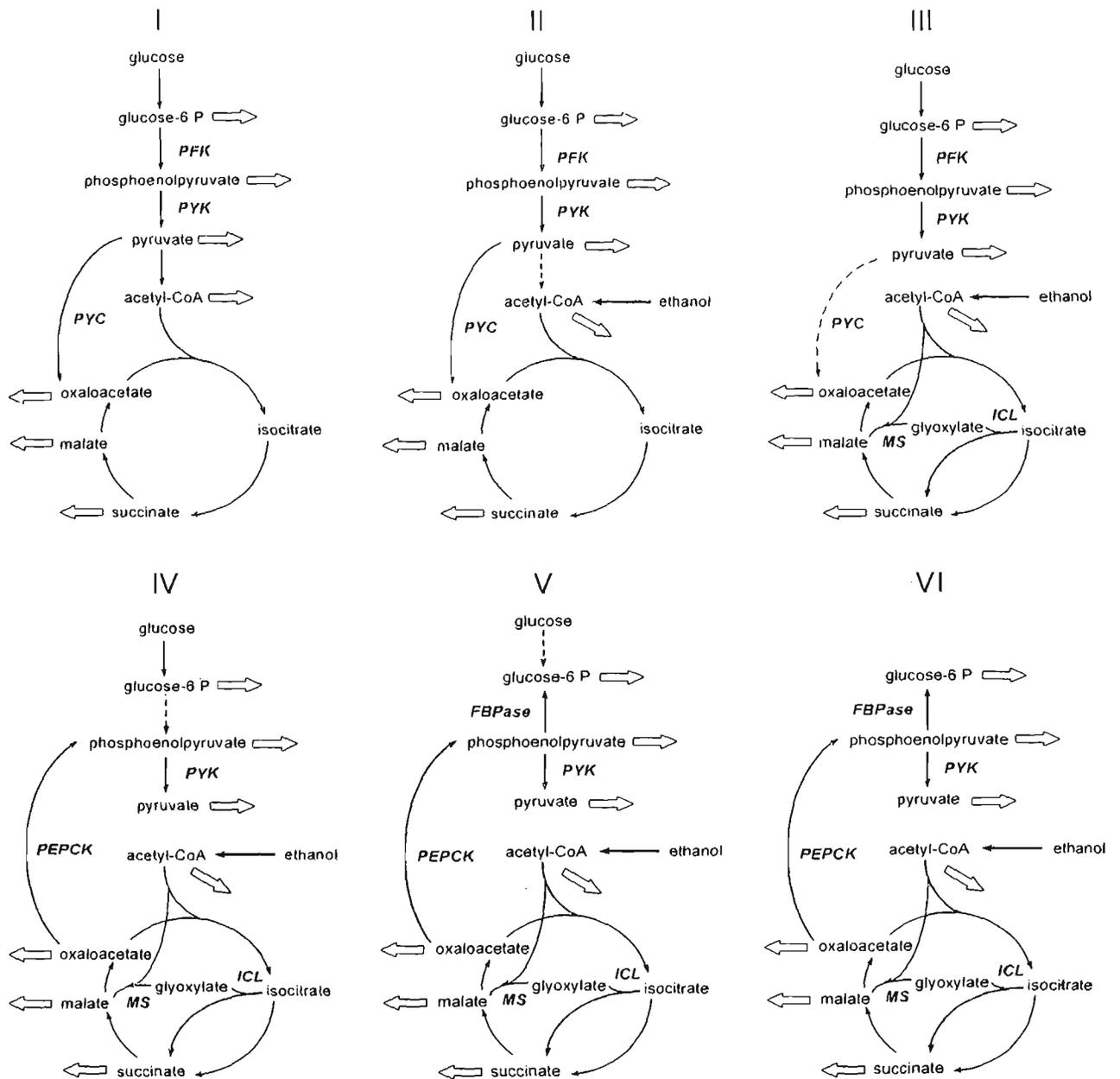


**Figure 6.3:** Diagram showing metabolic pathways essential for ethanol utilisation. Genes controlled by Cat8p are identified by name in boxes (Haurie *et al.* 2001). Reproduced with the kind permission of the authors.

groups in the cytoplasm of *S. cerevisiae*. The destination of *YAT2*-bound acetyl groups could be either the peroxisomes for entry into the glyoxylate cycle, the mitochondria for entry into the TCA cycle or to enzymes involved in biosynthesis of fatty acids and sterols in the cytoplasm.

The source of acetyl-CoA for this activity must be from either pyruvate or the  $\beta$ -oxidation of fatty acids or from the oxidation of ethanol to acetaldehyde, acetate and finally acetyl-CoA. Work described in this thesis identified *YER024w* (*YAT2*) as upregulated in cells growing in rich glucose medium in the presence of ethanol, as cultures exit the lag phase. This suggests that the source of acetyl-CoA, at least in this experimental setting, is pyruvate generated by glycolysis. However, a high level of CAT activity has been observed in cells grown on a mixture of ethanol and glucose and in cells grown on ethanol alone (Claus *et al.* 1983). Therefore, I would argue that acetyl-CoA can also be generated from the oxidation of ethanol under the conditions described and that this acetyl-CoA could presumably be utilised in fatty acid synthesis. However, this may lead to the utilization of ethanol for gluconeogenesis thus generating a futile cycle when glucose is available.

Although it is well known that many of the enzymes required for respiration, ethanol oxidation and gluconeogenesis are repressed in the presence of glucose, (De Jong-Gubbels *et al.* 1995) have demonstrated that under some conditions at least, concurrent utilization of ethanol and glucose occurs. These authors investigated the activity of key enzymes and the growth efficiency of carbon- and energy-limited aerobic *S. cerevisiae* chemostat cultures, grown on mixtures of ethanol and glucose. A diagram representing the increasing contribution of ethanol to carbon metabolism in a mixed ethanol and glucose culture as the ethanol fraction increases is shown in Figure 6.4. Situations I and VI represent the carbon flux in single substrate cultures growing on glucose or ethanol respectively. At low levels of ethanol, activity of the glyoxylate cycle enzymes malate synthase and isocitrate lyase was detected and thought to represent the utilization of ethanol as a source of acetyl-CoA for the TCA cycle and for fatty acid synthesis. At higher ethanol concentrations the synthesis of some TCA intermediates occurred, also with acetyl-CoA as precursor but using the glyoxylate cycle. Finally, at even higher ethanol to glucose ratios, the gluconeogenic



**Figure 6.4:** Diagram representing the increasing contribution of ethanol to carbon metabolism in carbon-limited cultures (De Jong-Gubbels *et al.* 1995).. Reproduced with the kind permission of the authors.

enzymes PEP carboxylase and fructose-1,6-bisphosphatase (FBPase) were derepressed allowing the synthesis of PEP and glucose-6-phosphate from ethanol. The expression of gluconeogenic enzymes and their antagonists simultaneously may lead to futile cycling but in a yeast strain grown on glucose with constitutive expression of gluconeogenic enzymes behind a non glucose-repressible promoter, futile cycling did not occur (Navas *et al.* 1993). In these constructs the presence of the gluconeogenic enzymes FBPase and phosphoenolpyruvate carboxykinase (PEPCK) had no significant effect on the rate of glucose consumption but CO<sub>2</sub> and ethanol production did not increase with glucose consumption suggesting that changes in glucose metabolism occur and/or ethanol is utilized. The authors suggest that one possible reason for the evolution of such complex regulation of gluconeogenic enzymes might be that when a nitrogen source is limiting for growth, proteins not in active conformation might be recycled to replenish amino acids, via the process of catabolite inactivation.

As well as the influence of glucose on gene expression, the influence of ethanol on enzymes, transporters and many other aspects of cellular metabolism may impact on glucose derepression that occurs in the presence of ethanol. For example, ethanol denatures some glycolytic enzymes at relatively high concentrations *in vitro* (Millar *et al.* 1982) and at physiological concentrations (Brown *et al.* 1981) and therefore may be capable of inhibition of these enzymes *in vivo*, particularly for glycolytic enzymes present in the plasma membrane. The large number of hsp's induced in cells exposed to 7% ethanol for 30 minutes (Alexandre *et al.* 2001), suggests that protein denaturation is an important early effect of ethanol exposure. Some glycolytic enzymes have homologues that may be induced in the presence of ethanol. For example, a second functional pyruvate kinase, Pyk2p, does not cause any of the deleterious effects expected from futile cycling, when overexpressed in cells grown on ethanol, suggesting that *PYK2* may be expressed under conditions of low glycolytic flux (Boles *et al.* 1997).

In addition, the general amino acid permease *GAP1* is inhibited by ethanol yet ethanol activates the plasma membrane H<sup>+</sup>ATPase. The presence of ethanol also leads to a release of glucose residues from trehalose as well as its synthesis (Alexandre *et al.* 1998) and ethanol leads to an increase in the synthesis of glucose-rich proteins such as

*YGP1* that are exported to the periplasm and/or the cell surface. In addition, in ethanol-limited cultures glucose seems to be exported out of the cell, presumably by the Hxt5p transporter (Diderich *et al.* 1999). The combined effects of ethanol on cells could therefore result in modulating the influence of glucose on some glucose-repressible genes allowing their expression, even in the presence of relatively high glucose concentrations. This might also explain some of the nutrient limitation phenotypes of ethanol-stressed cells and the pleiotropic effects of ethanol stress on yeast cells.

*YAT2* has a disease related human homologue, *CPT2*, the mitochondrial carnitine palmitoyltransferase implicated in myoglobinuria (Andrade *et al.* 1998). The importance of carnitine in the mammalian system is indicated by research investigating mitochondrial function and ambulatory activity in aged rats fed a dietary supplement of acetyl-L-carnitine (Hagen *et al.* 1998). Aged rats fed the supplement were found to have increased cellular respiration and cardiolipin levels and those treated for 1 month were twice as active as before the dietary supplements were given. The authors assert that age-related decline in carnitine, cardiolipin and ambulatory activity can be reversed by acetyl-L-carnitine addition to the diets of aged rats.

## CHAPTER 7

# CONCLUDING REMARKS AND FUTURE STUDIES

Differential Display, with the modifications described in this thesis, has been successfully used to identify ethanol-stress response genes. Although there are now *S. cerevisiae* whole genome microarray experiments being performed that generate large amounts of data, Differential Display is still the method of choice for identification of novel genes in most species including human, where the entire transcriptome is not yet commercially available as microchip or filter arrays.

The approach adopted in this thesis for characterising phenotypes of genes, involved the use of laboratory based approaches (e.g. construction of knockout strains) and analysis using bioinformatics resources. The handling of large amounts of expression data by clustering, sequence comparisons and pattern analysis was, until recently, perceived as a barely valid experimental procedure that could only be used as a guide for laboratory experiments. Current publications suggest however, that expression profiling is the method that holds the most promise for future functional analysis (Hughes *et al.* 2000). Some authors have suggested that genes will be characterised by the expression profile of the knockout strain thus generating a profile that will serve as a fingerprint for comparison to a comprehensive database of fingerprints. For example, pathways involved in responses to drugs or involved in disease states could be determined using this approach.

### 7.1 CONCLUSIONS

Ethanol stress leads to numerous deleterious effects on yeast cells that have been described in the literature. In addition to previously published findings, ethanol stress was found, in the work described in this thesis, to reduce the amount of rRNA in

stressed cells. This is probably a major factor in the slow growth of ethanol-stressed cells. *YGP1*, *DIP5* and *YAT2* are expressed in response to ethanol stress and their likely roles in the ethanol-stress response have been deduced, as far as is possible from information in the literature and databases. There are many yeast genes that have not been allocated a phenotype but few of these have such detailed expression data available as *YGP1*. *YGP1* is clearly involved in modifications to the cell surface during periods of high stress resistance such as stationary phase and is important for cell cycle progression in G1 of the cell cycle where bud initiation occurs. *YGP1* is also highly expressed during nitrogen (asparagine) limitation and phosphate limitation and is therefore likely to have a role in the passage of cells into stationary phase. The very high level of *YGP1* expression in response to phosphate limitation requires further investigation. *DIP5*, the dicarboxylic amino acid permease, is involved in importation of aspartate and glutamate, probably for anaplerotic processes in the early stages of fermentations in a rich growth medium. *YAT2*, one of the three known *S. cerevisiae* carnitine acetyl transferases is probably responsible for transport of acetyl-CoA in the cytoplasm, principally for the biosynthesis of fatty acids and sterols which are required for plasma membrane modifications during ethanol stress.

The expression of *YAT2* for transport of acetyl groups in ethanol-stressed cells strongly suggests that acetyl-CoA is generated by utilization of ethanol and leads to the conclusion that ethanol as well as glucose are used as substrates when cells are adapting to ethanol stress. Therefore the proposed role of *DIP5* in anaplerotic reactions and *YAT2* involvement in transfer of acetyl-CoA for use in biosynthetic processes recommends the products of these genes for overexpression studies.

In conclusion, the aims of this project to identify and characterise novel ethanol-stress response genes have been fulfilled. *YGP1*, *DIP5* and *YAT2* have been identified and confirmed as novel ethanol-stress response genes and the growth phenotype of *YGP1* has been investigated using knockout and overexpression constructs. The *YGP1* growth phenotype was not determined using the procedures described and this will be the subject of future studies using a range of different growth and stress conditions.

## 7.2 FUTURE EXPERIMENTAL WORK

### 7.2.1 Experiments to clarify the roles of ethanol-stress response genes

#### 7.2.1.1 Further physiological experiments to determine the *YGPI* phenotype

In addition to the growth experiments discussed in section 5.4.2, the following further experiments to investigate the *YGPI* phenotype should be carried out.

1. In light of information from bioinformatics resources that included a putative asparaginase activity for *YGPI*, and the importance of amino acid metabolism in the ethanol-stress response, it would be of interest to investigate whether *YGPI* has asparaginase activity. The PMY1.1 *YGPI* knockout and PMY1.1 *ASG1/ASG2* (aspariginases) knockouts could be grown on minimal media with asparagine as nitrogen source to determine if *YGPI* is able to influence asparagine uptake.
2. Since expression of the N-glycosylated protein *YGPI* and its homologue *SPS100* are downregulated by the cell wall integrity signaling pathway (Jung and Levin 1999), the nature of cell wall glycosylation and mannosylation during ethanol stress should be investigated. Alcian blue staining could be used to screen for the loss of mannosyl phosphate groups on N- and O-linked chains at the cell surface (Klis 1994).
3. To investigate the reason for the high level of *YGPI* expression in phosphate limiting cultures (greater than 1000-fold increase above basal) (Destruelle, Holzer and Klionsky 1994), growth of *YGPI* knockout and wildtype strains in phosphate-limited cultures should be tested.
4. Since *YGPI* is cell cycle-regulated and may be linked to passage through G1 by *CDC28*, a protein analysis of gp37 should be undertaken to determine whether *YGPI* is phosphorylated. Protein analysis should be accompanied by microscopic examination of wildtype and *YGPI* knockout cells through the cell cycle.

### 7.2.1.2 Experiments to investigate the *DIP5* and *YAT2* phenotypes

1. Further experiments should be undertaken with *DIP5* and *YAT2* knockout and rescue strains in physiological experiments using a range of growth media. These experiments would ideally be performed in chemostat cultures and explore such parameters as nitrogen limitation.

### 7.2.2 Further characterisation of the *S. cerevisiae* ethanol-stress response

1. To obtain greater definition of the *S. cerevisiae* ethanol-stress response, the total cellular gene expression under the ethanol-stress conditions described in this thesis could be investigated. This could be achieved using a microarray and/or proteome analysis. Results from such work would provide a detailed picture of changes in flux in metabolic pathways and enable specific genes to be targeted for knockout and/or overexpression studies.
2. An almost complete set of the knockout strains of *S. cerevisiae* ORFs is now available. These knockouts could be grown under ethanol stress in microtitre format and analysed spectrophotometrically to reveal genes essential for growth in the presence of ethanol.
3. The metabolic responses of yeast cells to ethanol stress could be further analysed using a metabolome approach whereby metabolites generated in the knockout cultures described above could be determined and compared with metabolite profiles for the wildtype.
4. Genes required for growth of yeast cells during ethanol stress could also be identified by generating ethanol-sensitive mutants then identifying the mutated genes by complementation. An experiment such as this has recently been performed by Takahashi *et al.* (2001) in which five genes were identified. The influence of these genes on ethanol tolerance could be further investigated by microarray analysis of knockouts compared to wildtype strains.

5. A novel approach to mutagenesis involving transforming yeast cells with oligonucleotides or Ty transposons could be combined with expression evolution as described by (Ferea *et al.* 1999) under ethanol stress in continuous cultures to generate new yeast strains able to tolerate ethanol. Ethanol-tolerant strains would then require genetic characterisation to identify ethanol-tolerance genes and promoters.
  
6. Using published ethanol-stress data and the ethanol-stress-induced genes identified in this thesis, a computer-based promoter analysis should be undertaken to identify common *cis* regulatory elements in promoters of ethanol-stress response genes. Any promoter elements thus identified could be further investigated using a yeast 2-hybrid interactome approach.

# APPENDIX I

## SOLUTIONS AND BUFFERS

**Amino acid and uracil stock solutions:** 20 mg/mL stocks of uracil, leucine and histidine were prepared by dissolving amino acids separately in sterile distilled de-ionized water. Uracil stocks were autoclaved, stored at room temperature and shaken prior to use. Leucine and histidine stocks were autoclaved and stored at 4°C. Uracil was used at a final concentration of 16 µg/mL when spread on plates or 20 µg/mL in liquid media. Leucine and histidine were used at a final concentration of 40 µg/mL when spread on plates. Leucine was used at a final concentration of 100 µg/mL in liquid media and histidine was used at a final concentration of 20 µg/mL in liquid media (Kaiser *et al.* 1994).

**Ampicillin stock:** A 20 mg/mL stock solution was prepared by dissolving ampicillin (Sigma) in distilled de-ionized water and was filter sterilized using a 0.22 µm filter. Aliquots of 1 mL were stored in microfuge tubes at -20°C and used at a final concentration of 100 µg/mL.

**Acid phenol (water buffered phenol) (pH5)** was prepared using Special Grade phenol (Wako Pure Chemical Industries Limited), 0.1% (w/v) 8-hydroxyquinoline and DEPC-treated water. Equal volumes of phenol and DEPC-treated water were mixed with the 8-hydroxyquinoline in a baked bottle with a stirring bar for ten minutes then allowed to separate. The aqueous (top) phase was removed and discarded and an equal volume of water added to the remaining phenol. The procedure was repeated until the water phase was at pH 5.0. The acid phenol was stored with a layer of DEPC-treated water at 4°C.

**Buffered phenol, pH 7.5,** was prepared using Special Grade phenol (Wako Pure Chemical Industries Limited), 0.1% (w/v) 8-hydroxyquinoline and RNA lysis buffer (see below). Equal volumes of phenol and 5 x RNA lysis buffer were added to the 8-hydroxyquinoline and mixed with a stirring bar in a baked bottle for 10 minutes. The

two phases were then allowed to separate. The aqueous phase (lower) was removed and discarded and an equal volume of 1 x RNA lysis buffer was added to the phenol and mixed with a stirring bar for 10 minutes. After the phases had separated the aqueous upper phase was removed and discarded. This procedure was repeated using 1 x RNA lysis buffer until the aqueous phase measured pH 7.5 when tested with pH paper. The phenol was stored with a layer of 1 x RNA buffer at 4°C.

**Differential Display loading solution** contained 0.2% (w/v) bromphenol blue, 0.2% (w/v) xylene cyanol, 20% (w/v) Ficoll and 10 mM EDTA dissolved in distilled de-ionized water and filter sterilized into a sterile bottle.

**DIG antibody solution** was prepared by diluting DIG Anti-Digoxigenin-AP, FAB fragments conjugated to alkaline phosphatase (Boehringer Mannheim) 1:10,000 in DIG blocking buffer (see below) in an RNase-free container for immediate use.

**DIG blocking buffer** was prepared as recommended by the suppliers by diluting DIG Blocking Reagent (Boehringer Mannheim) 1:10 with Maleic acid buffer (see below) in an RNase-free container and used immediately.

**DIG detection buffer** contained 100 mM Tris-HCl and 100 mM NaCl dissolved in DEPC-treated water in baked glassware. The pH was adjusted to 9.5 with concentrated HCl prior to autoclaving.

**DIG washing buffer** was prepared by adding Tween 20<sup>®</sup> (0.3% v/v) to Maleic acid buffer (see below) in an RNase-free container and was used immediately.

**0.5 M EDTA** was prepared by dissolving EDTA in distilled de-ionised water, stirring vigorously over gentle heat for several hours. The solution was cooled and the pH adjusted to 8.0 using 2 M NaOH prior to autoclaving.

**Ethidium bromide** for non-denaturing RNA and DNA agarose gels was prepared as a 10 mg/mL stock by dissolving ethidium bromide in distilled de-ionized water over gentle heat. Ethidium bromide was stored in a light proof bottle at 4°C and added to

the cooled gel prior to pouring at a final concentration of 1  $\mu\text{g}/\text{mL}$ . Ethidium bromide solution for denaturing formaldehyde agarose gels was prepared as a 1 mg/mL stock using DEPC-treated water in baked glassware. It was stored at 4°C in a light-proof baked bottle and 1  $\mu\text{L}$  was added to samples prior to electrophoresis to a final concentration of approximately 0.03  $\mu\text{g}/\mu\text{L}$ .

**Geneticin** stock solution of approximately 100 mg/mL was prepared by adding 1 mL of sterile distilled de-ionized water to 100 mg geneticin in the suppliers' bottle and shaking to dissolve. The stock solution was stored at 4°C and used at a final concentration of 0.2 mg/mL.

**4 M Lithium chloride** was prepared by dissolving 1.69 g lithium chloride in DEPC-treated water to a final volume of 10 mL and was autoclaved in a baked bottle. The solution was stored at 4°C.

**25 mM Magnesium chloride** (Perkin Elmer) used in PCR reactions was supplied with the enzyme, Amplitaq™ DNA Polymerase.

**Maleic acid buffer** contained 0.1 M maleic acid and 0.15 M NaCl and DEPC-treated water. The pH was adjusted to 7.5 with 2 M NaOH prior to autoclaving.

**Methylene blue stain** for RNA markers was prepared by dissolving 0.04% (w/v) methylene blue in 0.5 M sodium acetate. The pH was adjusted to 5.2 with glacial acetic acid and the solution was filter sterilized through a 0.22  $\mu\text{m}$  filter into a baked bottle.

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

**PAGE electrode buffer (10 x)** contained 144.2 g glycine, 30.3 g Tris base and 10 g SDS dissolved in 1 L distilled water. It was not necessary to adjust the pH of the

dissolved reagents or autoclave the solution. The 10 x solution was diluted with distilled water immediately prior to use.

**PAGE 1.875 M Tris-HCl gel buffer (5 x)** was prepared by dissolving 56.8 g Tris base in distilled water, adjusting the pH to 8.8 using 5 N HCl and making up to 250 mL prior to autoclaving. The buffer was stored at 4°C and diluted with distilled water immediately prior to use.

**PAGE 1.25M Tris-HCl stacking gel buffer (10 x)** was prepared by dissolving 37.8 g Tris base in distilled water, adjusting the pH to 6.8 using 5 N HCl and making up to 250 mL prior to autoclaving. The buffer was stored at 4°C and diluted with distilled water immediately prior to use.

**PCR buffer (10x)** (Perkin Elmer) was for PCR was supplied with the enzyme Amplitaq™ DNA Polymerase and contained 100 mM Tris-HCl (pH 8.3) and 500 mM KCl and was stored at -20°C.

**Phosphate buffer** contained 0.4 M NaH<sub>2</sub>PO<sub>4</sub>. The pH was adjusted to 6.8 with 2 M NaOH prior to autoclaving.

**Polyethylene glycol 50% (w/v)** was prepared by dissolving polyethylene glycol 8000 in distilled de-ionized water to a concentration of 0.5 g/mL and filter sterilized through a 0.45 µm membrane filter.

**Protein loading solution (2 x)** contained 0.3 M Tris-HCl (pH 6.8), 10% (w/v) SDS, 25% (v/v) β-mercaptoethanol, 50% (v/v) glycerol, 0.01% (w/v) bromphenol blue dissolved in distilled water. The buffer was stored at -20°C and diluted 1:1 with protein samples.

**Protein Transfer buffer** contained 48 mM Tris base, 39 mM glycine, 20% methanol and 0.037% SDS prepared in distilled water and used immediately. The pH of the reagents when dissolved did not require adjustment.

**RNA lysis buffer (5 x)** (Ausubel *et al.* 1997) contained 2.5 M NaCl, 1 M Tris base and 50 mM EDTA in distilled de-ionized water. The pH was adjusted to 7.5 with HCl and the buffer was filter sterilized through a 0.22  $\mu\text{m}$  membrane filter into a baked bottle.

**RNA formaldehyde gel loading solution (6 x)** contained 10% (w/v) bromphenol blue dissolved in 50% (w/v) sterile glycerol solution prepared with DEPC-treated water in a baked bottle and stored in a light proof bottle at 4°C.

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

**RT-PCR gel loading solution (6 x)** contained RNA loading solution (above) diluted 1:10 in a 20% (w/v) Ficoll solution. The solution was stored at 4°C.

**SDS 10% ( w/v)** was prepared by dissolving sodium dodecyl sulfate in distilled de-ionized water with gentle heat. The solution was filter sterilized through a 0.45  $\mu\text{m}$  membrane filter into a baked bottle.

**2 M Sodium acetate** was prepared by dissolving sodium acetate in DEPC-treated water in a baked bottle. The pH was adjusted to 4.0 with glacial acetic acid prior to autoclaving.

**3 M Sodium acetate** was prepared by dissolving sodium acetate in as little distilled de-ionized water as possible in baked glassware. The pH was adjusted to 5.3 with approximately 35% (v/v) glacial acetic acid. The solution was filter sterilized through a 0.22  $\mu\text{m}$  membrane filter.

**SSC (20 x)** contained 3 M NaCl and 300 mM tri-sodium citrate dissolved in distilled de-ionised water. The pH was adjusted to 7.0 with HCl and 0.1% (v/v) DEPC was added prior to autoclaving.

**TAE buffer (10 x)** contained 400 mM Tris base, 200 mM sodium acetate anhydrous, 20 mM EDTA dissolved in DEPC treated water. The pH was adjusted to between 7.2-7.4 with glacial acetic acid prior to autoclaving. The 10 x stock solution was diluted in distilled and de-ionized DEPC-treated water prior to use with RNA.

**TBE buffer (10 x)** contained 0.89 M Tris base, 0.89 M boric acid and 20 mM EDTA. The 10 x stock solution was diluted in distilled and de-ionised water prior to use.

**TBS (Tris Buffered Saline Solution)** contained 50 mM Tris base and 150 mM NaCl. The pH was adjusted to 7.5 with 5 N HCL and the solution was used immediately.

TBS blocking buffer with 1% gelatin was prepared by adding 1% gelatin (w/v) to TBS, warming gently to dissolve and allowing to cool to room temperature prior to use.

**TBST buffer (Tris Buffered Saline Solution with Tween-20)** was prepared by adding 0.02% Tween-20 to TBS buffer.

**1 M Tris** was prepared by dissolving Tris base in distilled de-ionized water in baked glassware. The pH was adjusted to 7.5 with glacial acetic acid and the solution was filter sterilized through a 0.22  $\mu\text{m}$  membrane filter.

## ENZYMES, MOLECULAR WEIGHT STANDARDS AND MOLECULAR BIOLOGY KITS

**Restriction enzymes:** *EcoR*1 (MBI Fermentas), *Sal*1 (MBI Fermentas) *Spe*1 (New England Biolabs), *Bgl*1 (MBI Fermentas).

**Reaction enzymes:** Amplitaq (Perkin Elmer), RNase-free DNase I (Boehringer Mannheim), RNase A (Epicentre Technologies), T4 Polynucleotide kinase (Amersham Pharmacia Biotech), Endonuclease H (Boehringer Mannheim), Superscript<sup>TM</sup> II RNase H<sup>-</sup> Reverse Transcriptase (Gibco BRL), Recombinant RNasin<sup>®</sup> Ribonuclease Inhibitor (Promega), ABI Prism Cycle Sequencing.

**Molecular weight standards:** RNA Markers, 0.28-6.58 kb (Promega), 100bp DNA Ladder (Promega), Probase 50bp DNA Ladder (Progen), 1kb DNA Ladder (New England Biolabs), Pre-stained Broad Range Protein Standards (BioRad).

**Molecular biology kits:** Wizard<sup>TM</sup> PCR Preps DNA Purification System (Promega), Differential Display<sup>TM</sup> Kit (Display Systems), Bandpure<sup>TM</sup> DNA Purification Kit (Progen), MasterPure<sup>TM</sup> Yeast DNA Purification Kit (Epicentre Technologies), Super Signal<sup>®</sup> West Pico Trial Kit (Pierce).

## SUPPLIERS

**Advanced Biotechnologies Limited**, Epsom, Surrey, UK  
**Amersham Pharmacia Biotech**, New Jersey, USA  
**Ashwood Refrigeration**, Wetherill Park, Australia  
**Bartelt Instruments Pty Ltd**, Heidelberg, Victoria, Australia  
**Beckman Instruments Gmbh**, Munchen, Germany  
**Bio-Rad Laboratories**, Richmond, CA, USA  
**BDH (Merck Pty Ltd)**, Kilsyth, Victoria, Australia  
**Boehringer Mannheim GmbH**, Mannheim, Germany  
**Brand Gmbh**, Wertheim, Germany  
**B. Braun Biotech International**, Melsungen, Germany  
**Bresatec Pty Ltd**, Adelaide, South Australia  
**Denver Instruments Company**, Colorado, USA  
**Difco Laboratories**, Detroit, Michigan, USA  
**Display Systems Biotechnology Incorporated**, Los Angeles, CA, USA  
**Epicentre Technologies**, Madisom, WI, USA  
**Eppendorf**, Engelsdorf, Germany  
**Gallenkamp**, Leicestershire, UK  
**Gibco BRL**, see Invitrogen  
**Gilson Medical Electronics**, Villiers-le-Bel, France  
**Hanna Instruments**, Padova, Italy  
**Hofer Scientific Instruments**, see Amersham Pharmacia Biotech  
**ICN Biomedicals Incorporated**, Aurora, Ohio, USA  
**Invitrogen**, Mulgrave, Victoria, Australia  
**LKB Biochrom**, Cambridge, England  
**LKB-Producter AB**, Bromma, Sweden  
**MBI Fermentas**, Vilnius, Lithuania  
**Millipore Corporation**, Bedford, MA, USA  
**MJB Research Incorporated**, Watertown, Mass, USA Pharmacia  
**New England Biolabs**, Beverly, MA, USA  
**New Brunswick Scientific**, Edison, NJ, USA  
**Operon Technologies Incorporated**, (Qiagen Operon, California, USA)  
**Oxoid Limited**, Basingstoke, Hampshire, England

**Pacific Oligos Pty Ltd**, Toowong, Queensland, Australia  
**Perkin Elmer**, Boston, Massachusetts, USA  
**Pharmacia LKB**, California, USA  
**Pierce**, Rockford, Illinois, USA  
**Plastek Scientific**, Upper Beaconsfield, Victoria, Australia  
**Polaroid Corporation**, Cambridge, Mass, USA  
**Progen Industries Limited**, Darra, Queensland, Australia  
**Promega Corporation**, Annandale NSW, Australia  
**Ratex Instruments Pty ltd**, Boronia, Victoria, Australia  
**Roche Molecular Systems Incorporated**, Branchburg, NJ, USA  
**Sigma Chemical Company**, St Louis, MD, USA  
**Thermoline Australia Scientific Instruments Pty Ltd**, (U-lab, Eltham, Australia  
**Wako Pure Chemical Industries Limited**, Osaka, Japan  
**Wallac**, Turku, Finland

## APPENDIX II

### PRIMERS AND PROBES

**Table 1:** Differential Display primer combinations used in this work

<b>Downstream</b>	<b>Upstream</b>
D1: 5' TTTTTTTTTTTAA	U20: 5' GATCAAGTCC U23: 5' GATCACGTAC
D2: 5' TTTTTTTTTTTAC	U2: 5' CTGCTTGATG U17: 5' ATCTGACTG
D3: 5' TTTTTTTTTTTAG	U2: 5' CTGCTTGATG U17: 5' GATCTGACTG
D4: 5' TTTTTTTTTTTCA	U4: 5' GATCGCATTG U14: 5' GGAACCAATC
D5: 5' TTTTTTTTTTTCC	U4: 5' GATCGCATTG U14: 5' GGAACCAATC
D6: 5' TTTTTTTTTTTTCG	U7: 5' GATCATGGTC U18: 5' TCGATACAGG
D7: 5' TTTTTTTTTTTTGA	U10: 5' TACCTAAGCG U12: 5' GATCTAACCG
D8: 5' TTTTTTTTTTTTGC	U10: 5' TACCTAAGCG U12: 5' GATCTAACCG
D9: 5' TTTTTTTTTTTTGG	U20: 5' GATCAAGTCC U24: 5' CTTTCTACCC
(DisplaySystems )	

**Table 2:** Modified upstream primers used to PCR-amplify Differential Display gel bands prior to sequencing

UX12: 5' GGAGAAGCACGATCTAACCG

UX17: 5' GGCATAGCTCGATCTGACTG

UX20: 5' GCGATAGCTCGATCAAGTCC

UX23: 5' GCAGTTGCACGATCACGTAC

**Table 3:** Gene-specific oligonucleotide probes for Northern analysis

*YGP1*: 5' TTCGATTGGGCTTGCGACGACGGCAGATGAA

*DIP5*: 5' CTAATGCAGGATCCACGTAACGTGAGGCAT

*ACT1*: 5' CGGTTTGCATTTCTT GTTCGAAGTCCAAGG

**Table 4:** RT-PCR primers used to target transcripts of *YGP1*, *DIP5*, *YER024w*, *MNN4* and *ACT1* genes. Primer numbers signify distance from the start codon.

YGP1-330F	5' GTACAATGTTGCCCGTGTTG 3'
YGP1-531R	5' CAGAAGTGACGGACAAGACA 3'
DIP5-1433F	5' ATAAAGCCTGCCGTGCTCAA 3'
DIP5-1592R	5' GCCAATATACCCGGTGATGA 3'
YER024-1037F	5' CCAATGATGGCGATGATACC 3'
YER024-1272R	5' TAAACCGAAGCACGACTGAC 3'
MNN4-766F	5' CATGGCTCTCCTAAGGGATT 3'
MNN4-896R	5' GATAGTGGATGCGACTGTGT 3'
ACT1-431F	5' AGGTATCATGGTCGGTATGG 3'
ACT1-809R	5' CGTGAGGTAGAGAGAAACCA 3'

**Table 5:** Oligonucleotide primers used to amplify the *kanMX* module and create the *YGP1* knockout

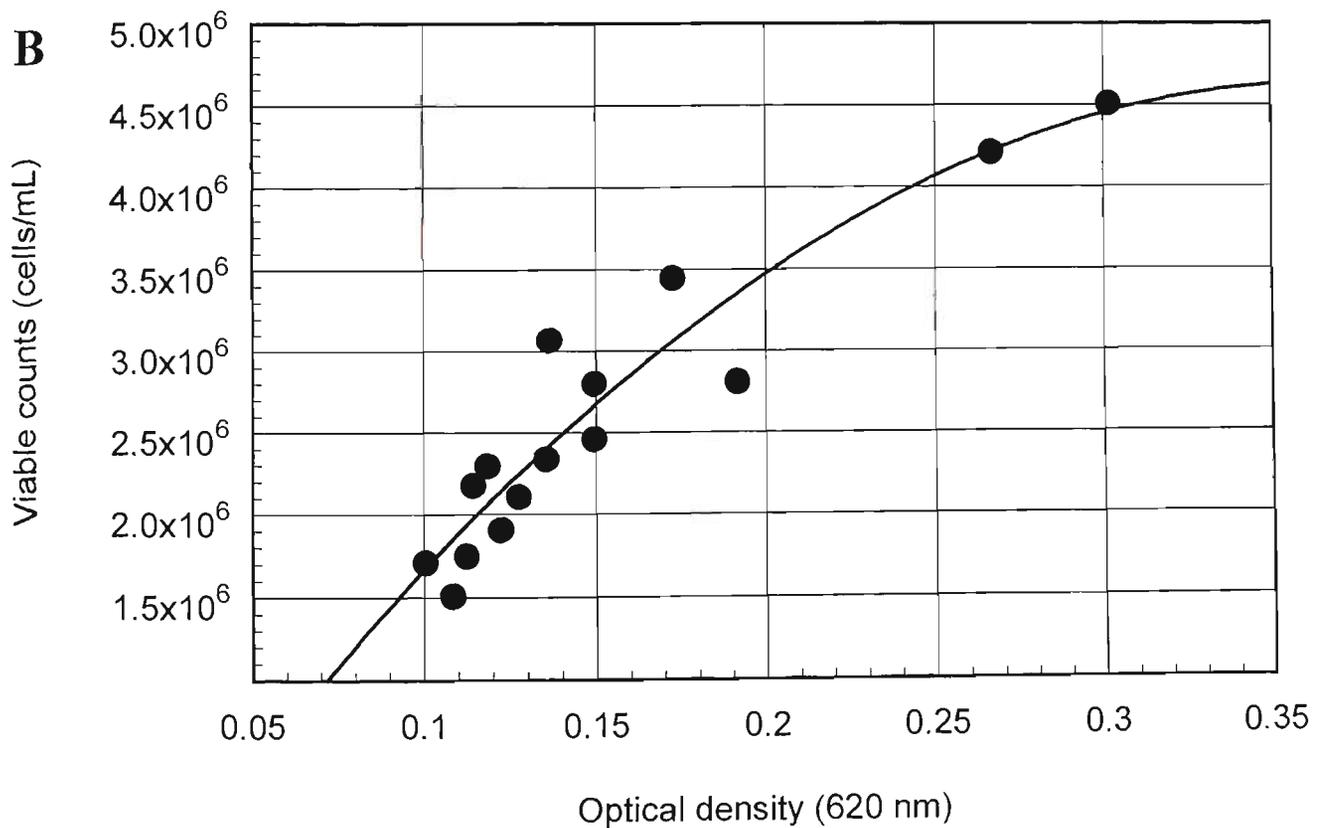
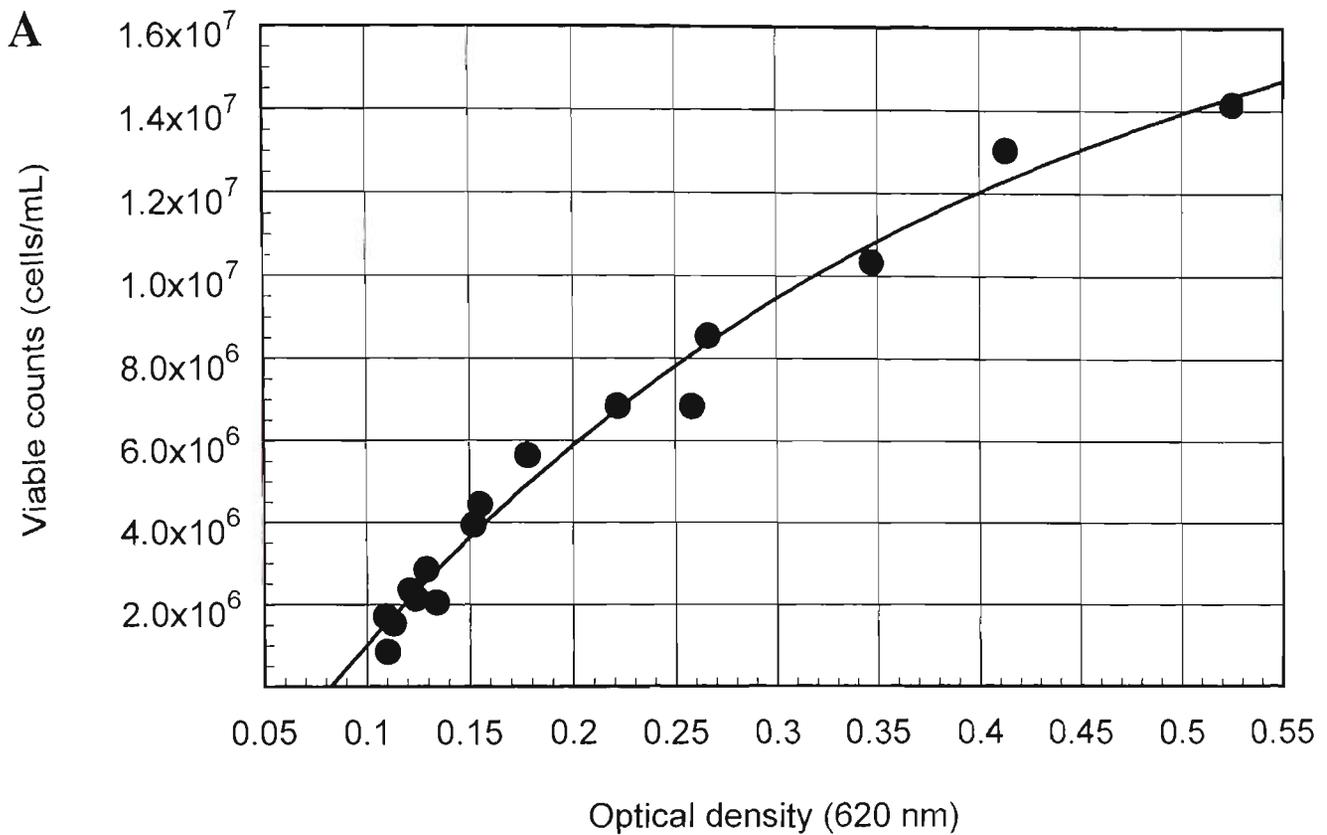
YGP1-KO5'	5' AAGTACGTAGTTGTTTTATCTGCCCTTTTGGCA TGTTACAGCTGAAGCTTCGTACGC 3'
YGP1-KO3'	5' AGTTCTAGAGAAAATGCTTTCCAGAGCGGACTT GGAGGTGCATAGGCCACTAGTGGATCTC 3'

**Table 6:** Oligonucleotide primers used to confirm knockout constructs. *YGP1*-PF was complementary to a region 125 nucleotides upstream of the knockout cassette, *YGP1*-PR was complementary to a region 72 nucleotides downstream of the knockout cassette and *KMX*-PF was complementary to a region within the *kanMX* gene.

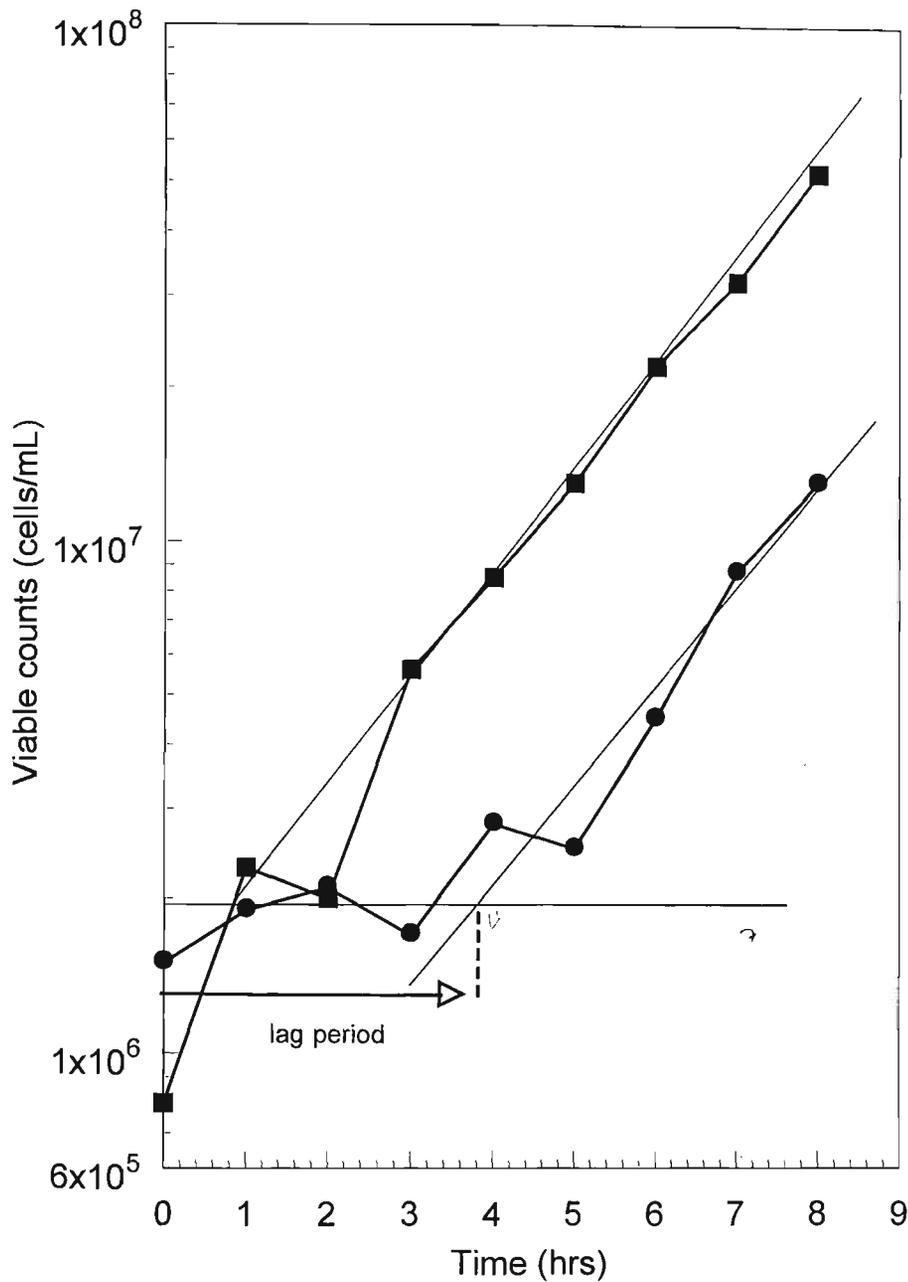
YGP1-PF (-125)	5' CTGCTGTGTTCTCTTGGGTT 3'
YGP1-PR (+72)	5' AAGCTGTTGCGTGGAATTT 3'
KMX-PF	5' TCGACATCATCTGCCAGAT 3' <sup>1</sup>

<sup>1</sup> (Wach *et al.* 1994)

## APPENDIX III



**Figure 1:** Correlation curves showing the relationship between viable counts and optical density of *S. cerevisiae* PMY1.1 grown in YEPD medium in the absence or presence of 5% (v/v) exogenous ethanol. Cells from overnight parent cultures were inoculated into YEPD medium only (A) or YEPD medium containing 5% (v/v) ethanol (B). Data from 3 separate experiments is included.

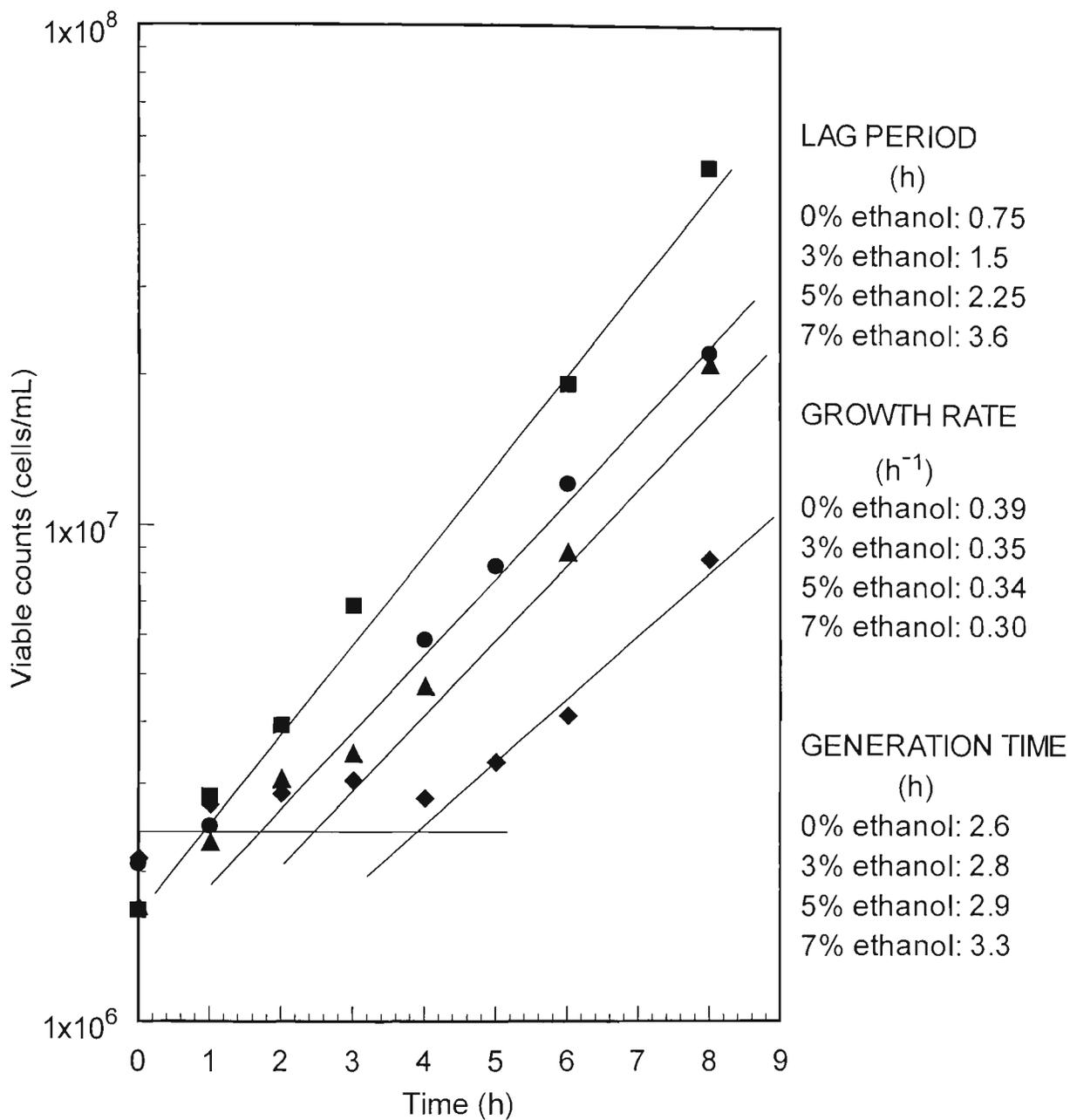


LAG PERIOD (h)  
 0% ethanol: 1.0  
 5% ethanol: 4.0

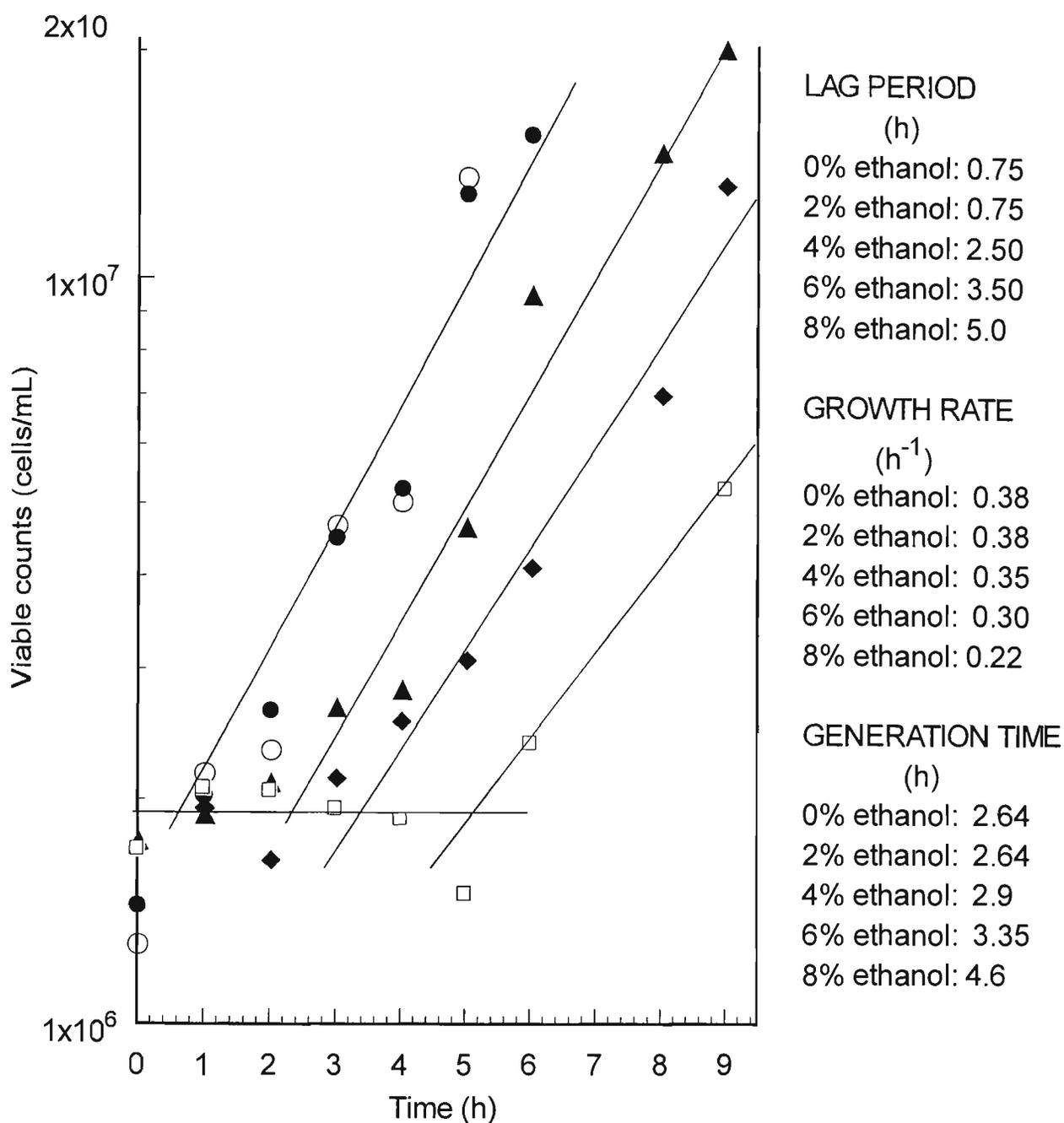
GROWTH RATE ( $h^{-1}$ )  
 0% ethanol: 0.45  
 5% ethanol: 0.42

GENERATION TIME (h)  
 0% ethanol: 2.24  
 5% ethanol: 2.40

**Figure 2:** Determination of lag period, growth rate and generation time for *S. cerevisiae* PMY1.1 grown in YEPD medium in the absence (■) and presence (●) of 5% (v/v) ethanol.

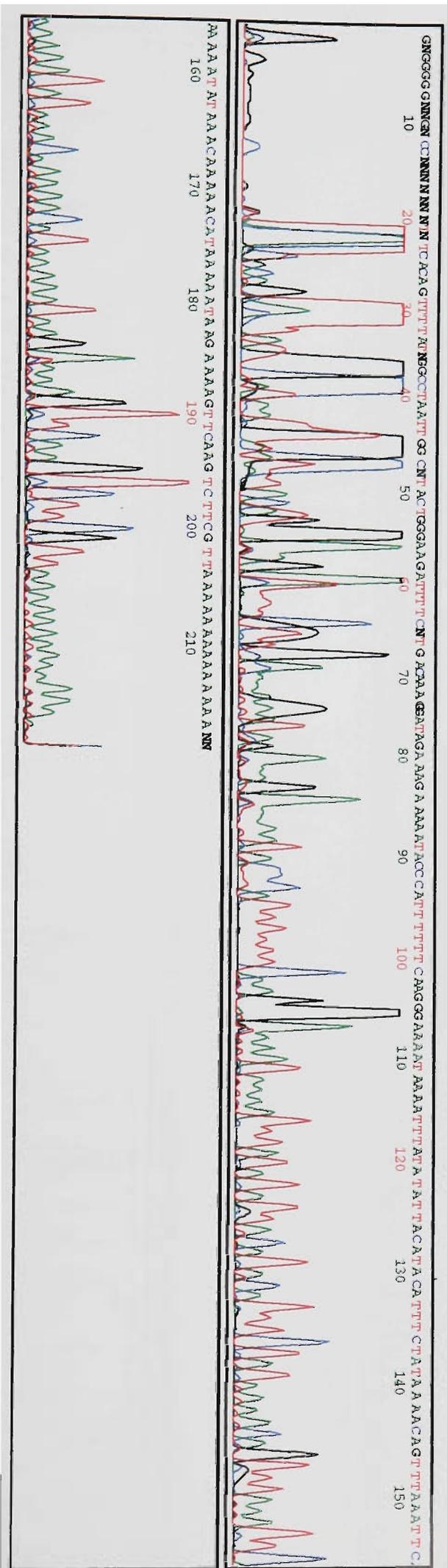


**Figure 3:** Determination of lag period, growth rate and generation times of *S. cerevisiae* strain PMY1.1 grown in YEPD medium in the absence (■) or presence of 3% (●), 5% (▲) or 7% (◆) (v/v) ethanol.



**Figure 4:** Determination of growth rate, lag phase and generation time of *S. cerevisiae* PMY1.1 grown in YEPD medium in the absence (O) or presence of 2% (●), 4% (▲), 6% (◆) or 8% (□) (v/v) ethanol.

## APPENDIX IV



**Figure 1 :** Sequence electropherogram of Differential Display band found to have derived from *DIP5*.

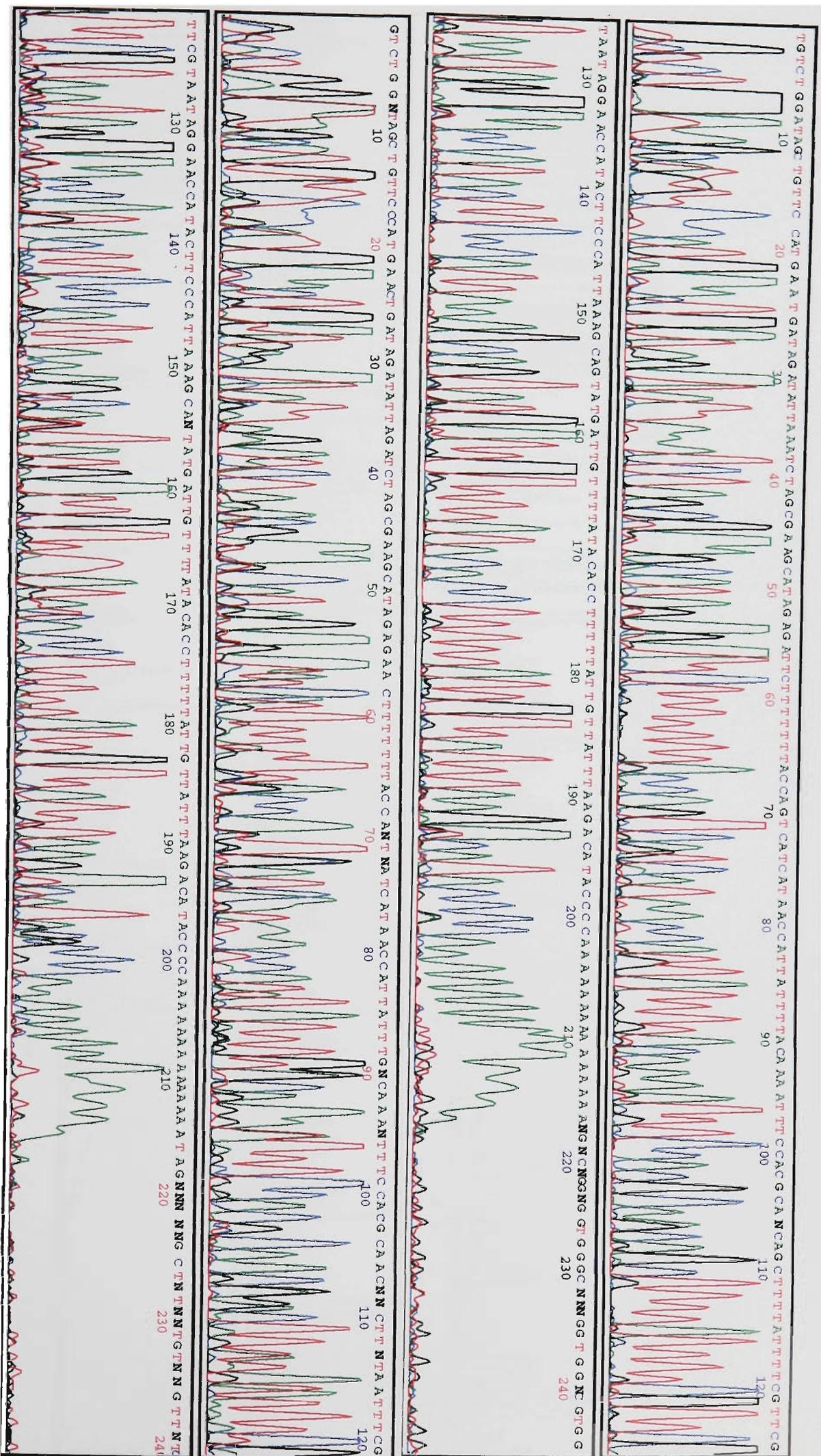


Figure 2: Sequence electropherograms of Differential Display bands found to have derived from *YGP1*.

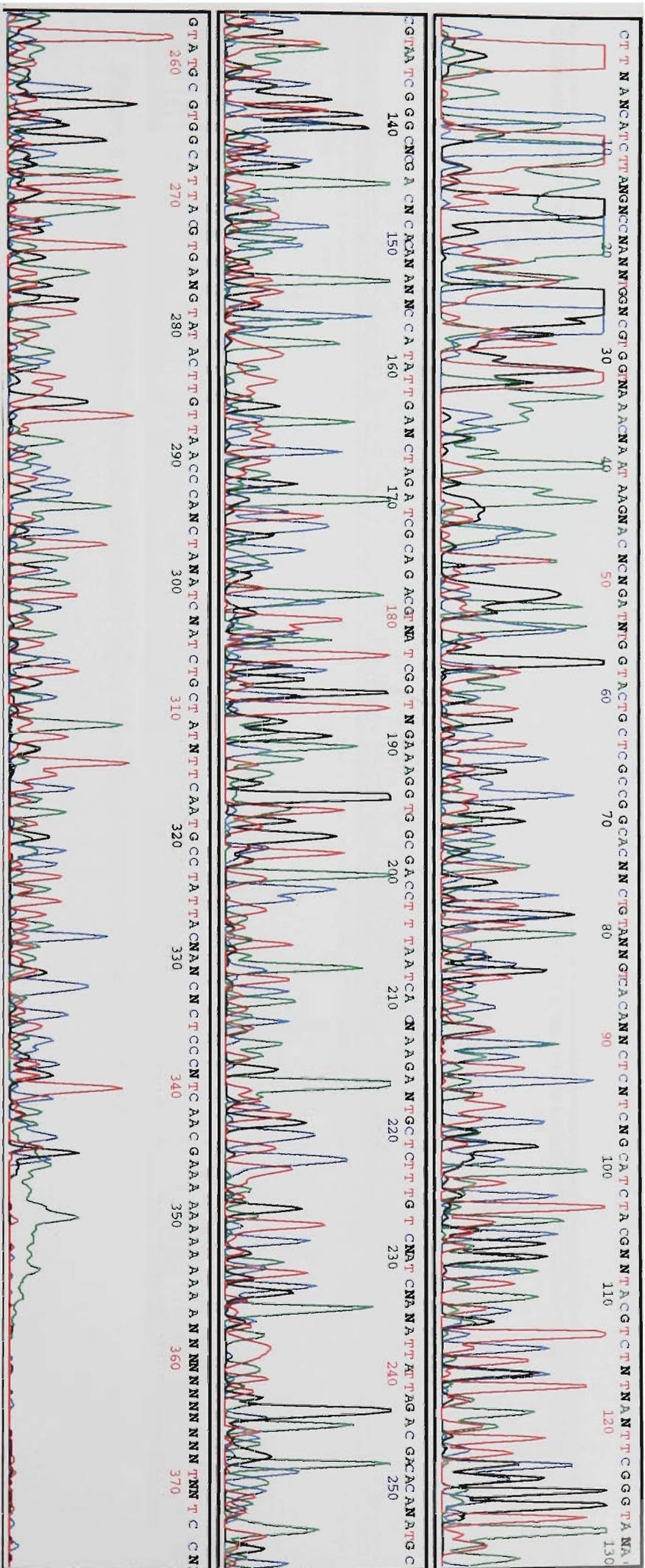


Figure 3: Sequence electropherogram of Differential Display band found to have derived from YER024w.

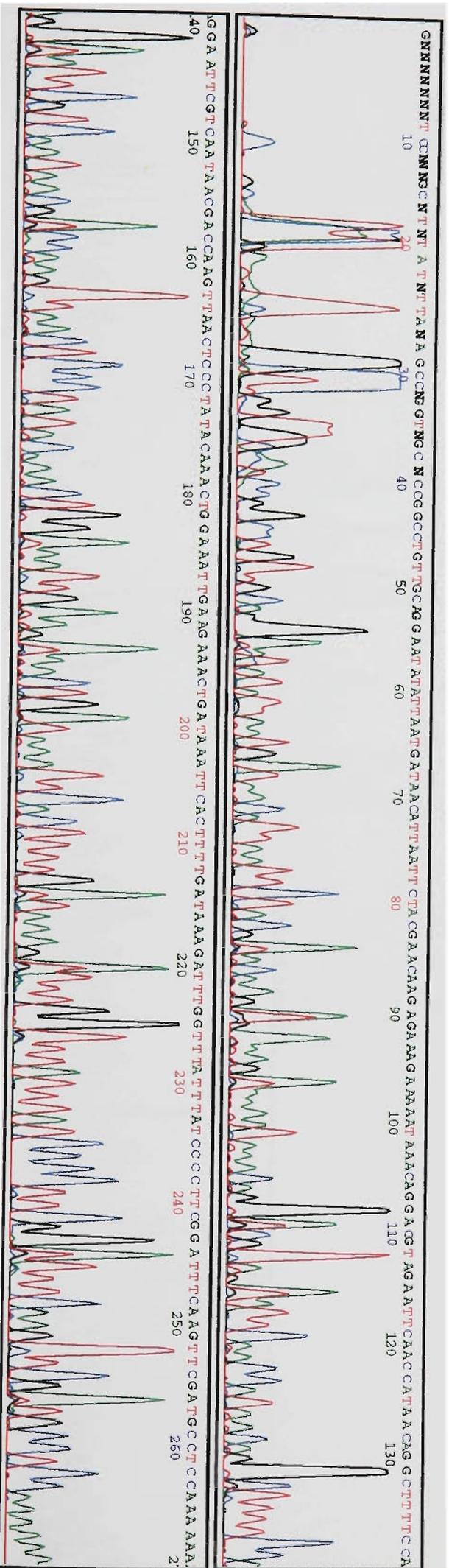


Figure 4: Sequence electropherogram of Differential Display band found to have derived from *MNV4*.

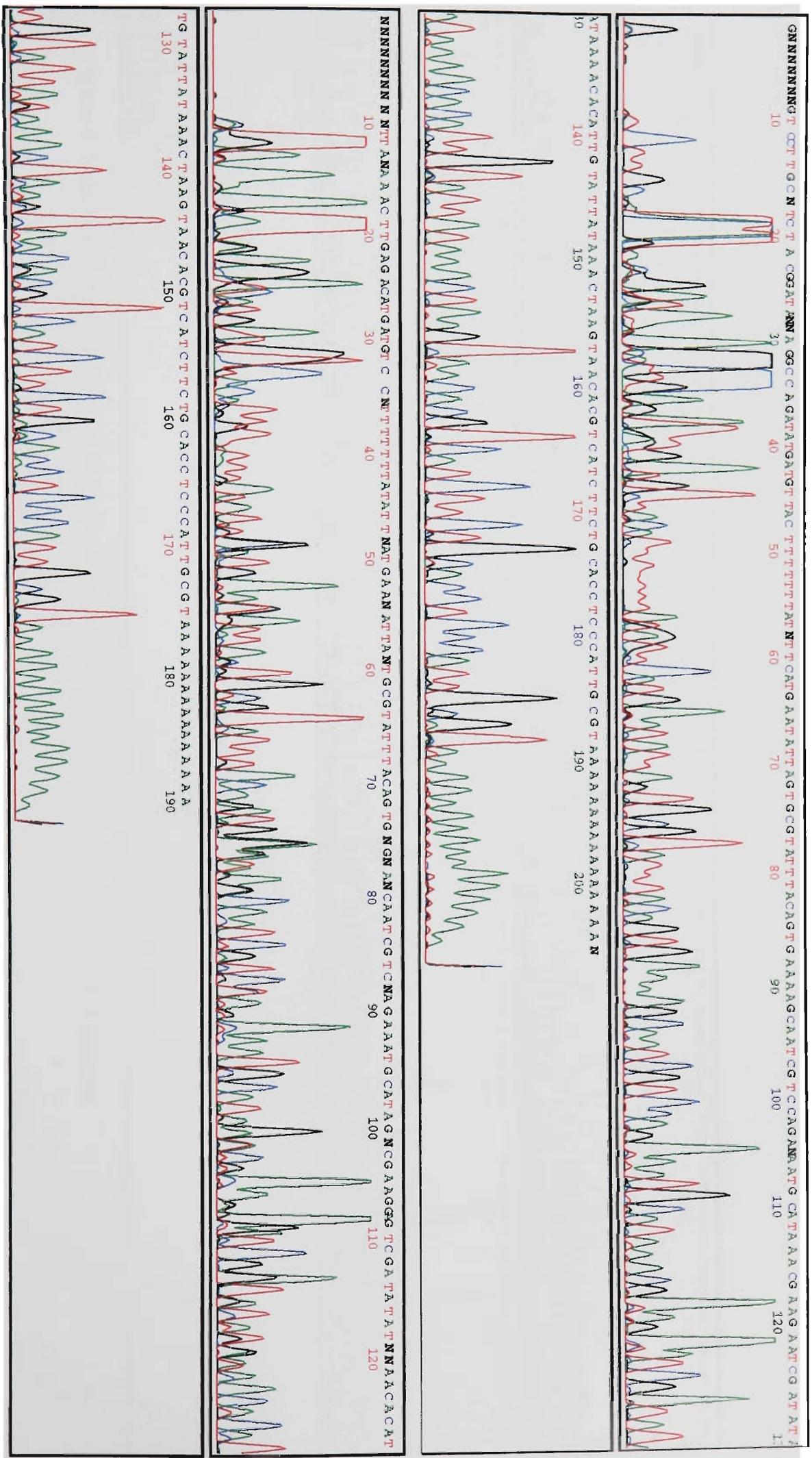


Figure 5: Sequence electropherograms of Differential Display bands found to have derived from Ty2 elements (upper sequence, band 1 and lower sequence, band 2).

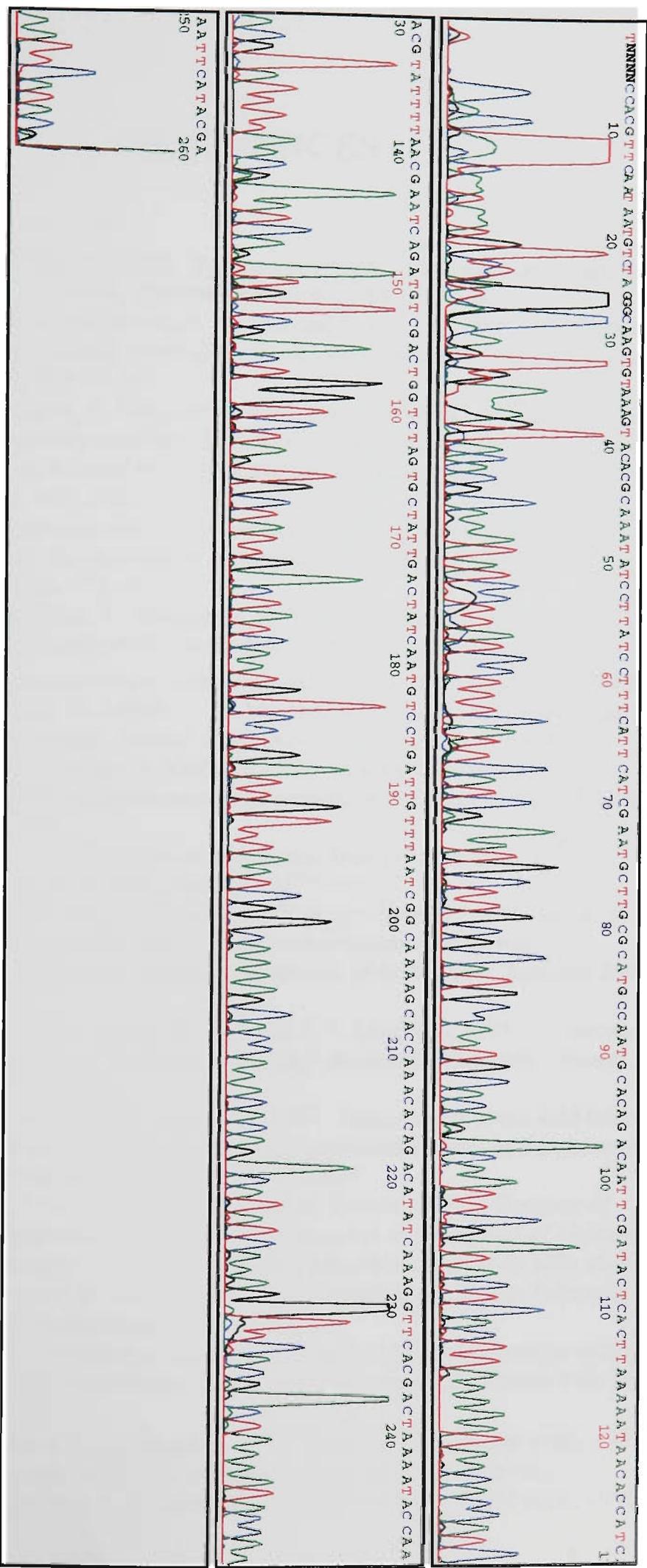


Figure 6: Sequence electropherogram of Differential Display band found to have derived from a Ty1 element

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