Identification of genomic differences between laboratory and commercial strains of *Saccharomyces cerevisiae*

by

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Declaration

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Thesis summary

The yeast *Saccharomyces cerevisiae* is used in many industrial applications including beer brewing, bread making, and winemaking. Winemaking yeast strains have the ability to convert grape sugars into alcohol and other metabolites consistent with good wine. An exploratory comparative approach was undertaken to identify the genes and corresponding proteins that give wine yeast strains of *S. cerevisiae* their distinctive phenotype, with a focus on studying genes that provide tolerance to ethanol.

A genomic and proteomic approach has been used to identify potential 'wine specific' genes. By using amplified fragment length polymorphism (AFLP) techniques, it has been demonstrated that commercial winemaking strains have genetic sequences within their genome that may have arisen from other *Saccharomyces* sensu stricto yeasts. This is the first known report of a wine strain having *Saccharomyces kudriavzevii* genetic sequences encoded within its genome.

To further explore the phenotypic characters distinguishing wine yeast strains from other *S. cerevisiae* strains, a comparative proteomics approach was taken. A proteomics platform using two-dimensional gel electrophoresis (2D gels) has elucidated target proteins for future research, including a glycolytic protein, Tdh3p (glyceraldehyde 3-phosphate dehydrogenase), as well as a one-carbon pool protein, Shm2p (serine hydroxymethyltransferase). The latter protein was characterised

further to determine its possible role and function in wine strains, with results indicating a potential role in wine flavour. It has also been shown that certain wine strains may have different mechanisms for transcription/translation control of *SHM2*.

Using the comparative proteomic approach above, no differences were seen between laboratory strains and wine strains after exposure to an ethanol stress. To ascertain the genes that enable *S. cerevisiae* strains to counteract the high ethanol concentrations encountered during grape juice fermentation, a continuous culture approach was utilised. Ultimately, this will reveal genes that are important to *S. cerevisiae* strains to acclimatise to a high ethanol environment, as opposed to a short-term ethanol stress. The continuous culture approach identified 34 genes that significantly changed expression in the ethanol-containing cultures, suggesting their involvement in ethanol tolerance of *S. cerevisiae*.

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Abbreviations

2D gel	Two-dimensional polyacrylamide gel electrophoresis
AFLP	amplified fragment length polymorphism
APAF	Australian Proteome Analysis Facility
AWRI	Australian Wine Research Institute
cDNA	complementary deoxyribonucleic acid
DIG	digoxigenin
DNA	deoxyribonucleic acid
FBA1	fructose bis-phosphate aldolase
GC-MS	gas chromatography - mass spectrometry
HPLC	high performance liquid chromatography
HSP	heat shock protein
HSTF	heat shock transcription factor
IEF	isoelectric focusing
ITS RFLP	internal transcribed spacer restriction fragment length polymorphism
MALDI-TOF	matrix-assisted laser desorption ionisation time-of-flight spectrometry
MAP	mitogen activated protein
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
NIRS	near infra-red spectroscopy
ORF	open reading frame
PCA	principle component analysis

PCR	polymerase chain reaction
PI	isoelectric point
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RP	ribosomal protein
RPM	revolutions per minute
SAGE	serial analysis of gene expression
SC	synthetic complete
SDS	sodium dodecyl sulphate
SGD	*Saccharomyces* Genome Database
SHM2	serine hydroxymethyltransferase
SPI1	stationary phase induced
STRE	stress response element
TBS-T	tris buffered saline – Tween 20
TDH3	glyceraldehyde 3-phosphate dehydrogenase
TRIS	tris(hydroxymethyl)aminoethane
WUGSC	Washington University Genome Sequencing Centre
YPD	yeast extract/peptone/dextrose medium

CHAPTER 1

Introduction

1.1. INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is an important model organism for studying gene expression and function. As a result, there has been a great deal of research carried out with this organism with regards to its life cycle, mating abilities, genome evolution and gene expression. In fact, the *S. cerevisiae* laboratory strain S288C was the first eukaryote to have its genome completely sequenced and available to the public (Cherry *et al*., 1997).

Yeast have been one of the most exploited organisms used in human societies throughout the ages. Beer brewing, sake making, baking, and winemaking industries all use *S. cerevisiae* in the production of goods for human consumption. Each of these activities use yeast strains that have been selected over time for their particular properties, such as tolerance to a high ethanol concentration (Attfield, 1997; reviewed in Casey and Ingledew, 1986), the ability to use certain sugars (Bell *et al*., 2001), and osmotolerance (Blomberg and Adler, 1989). It has been shown that considerable differences exist between the genome of commercial strains and laboratory strains of *S. cerevisiae* (Azumi and Goto-Yamamoto, 2001; Bidenne *et al*., 1992; Carlson and Botstein, 1983; de Barros Lopes *et al*., 1999; Hauser *et al*., 2001; Perez-Ortin *et al*., 2002) but the genes and proteins that generate the different phenotypes remain to be established.

The yeasts used to ferment grape juice to wine encounter a number of stresses including a high sugar concentration (200 g/L or more), low pH (ca 3-4), and a high ethanol concentration at the end of fermentation (often greater than 15%). It would be valuable to identify and characterise the genes and corresponding proteins that give rise to the wine strain phenotype, including those involved in stress tolerance mechanisms of wine strains. Hauser *et al*. (2001) have identified several genes that differed in expression between a wine and a laboratory strain under standard laboratory growth conditions, while Rossignol *et al*. (2003) have extended this research to investigate the transcriptional differences between strains during fermentation in a synthetic wine medium. By integrating the outcomes of this research, together with all the available published data, the information could then enable the accurate monitoring of yeast performance during fermentation, allow the prediction of wine yeast strain attributes, and permit the selection and production of new and improved strains for commercial use.

1.2. SIGNAL TRANSDUCTION PATHWAYS ARE ACTIVATED UNDER STRESSFUL CONDITIONS

Yeast cells have the ability to elicit a response to external stimuli, such as environmental changes and signal molecules (eg. pheromones), by inducing genes in response to the changing surroundings. The receptors that sense these stimuli and the associated transcription factors that activate a response are essential for cell survival under stressful conditions (Brewster *et al*., 1993). Signalling pathways are activated by different stimuli, but commonly comprise a receptor to sense the stimuli, and an

intracellular factor that promotes the initiation of a cellular response. The assembly and regulation of signalling components that control the intensity and specificity of a signal is an emerging area of investigation and that the complete pathways have not yet been revealed (Dohlman and Thorner, 2001). One signalling pathway of relevance to wine-related studies is the high osmolarity glycerol (HOG) pathway. The phosphorylation of protein kinases in this cascade regulates transcription factors that initiate transcription of genes necessary for a stress response (Figure 1). When activated independently, three MAP kinase kinase kinases, Ssk2p, Ssk22p, and Ste11p, are able to phosphorylate a MAP kinase kinase, Pbs2p, which in turn activates by phosphorylation a single MAP kinase Hog1p. The activated Hog1p, by mechanisms currently not fully understood, induces the expression of genes. It appears though that two transcription factors, Msn2p and Msn4p, are involved in binding to stress response elements in the promoter region of stress-related genes to activate the response (Martinez-Pastor *et al*., 1996).

It has been shown that MAP kinases function identically in two separate MAPK cascades, or operate independently in a single cascade. For example, the HOG pathway, invasive growth, and the pheromone response pathways all share the MAPKK kinase Ste11p (O'Rourke and Herskowitz, 1998). However, a second MAP kinase, Fus3p, only functions as a MAP kinase in the pheromone response pathway (Lee and Elion, 1999). To support the concept of independent MAP kinases, Maeda *et al*., (1995) demonstrated that Pbs2p was activated differently depending on the upstream signal. Both a putative transmembrane osmosensor Sho1p, and

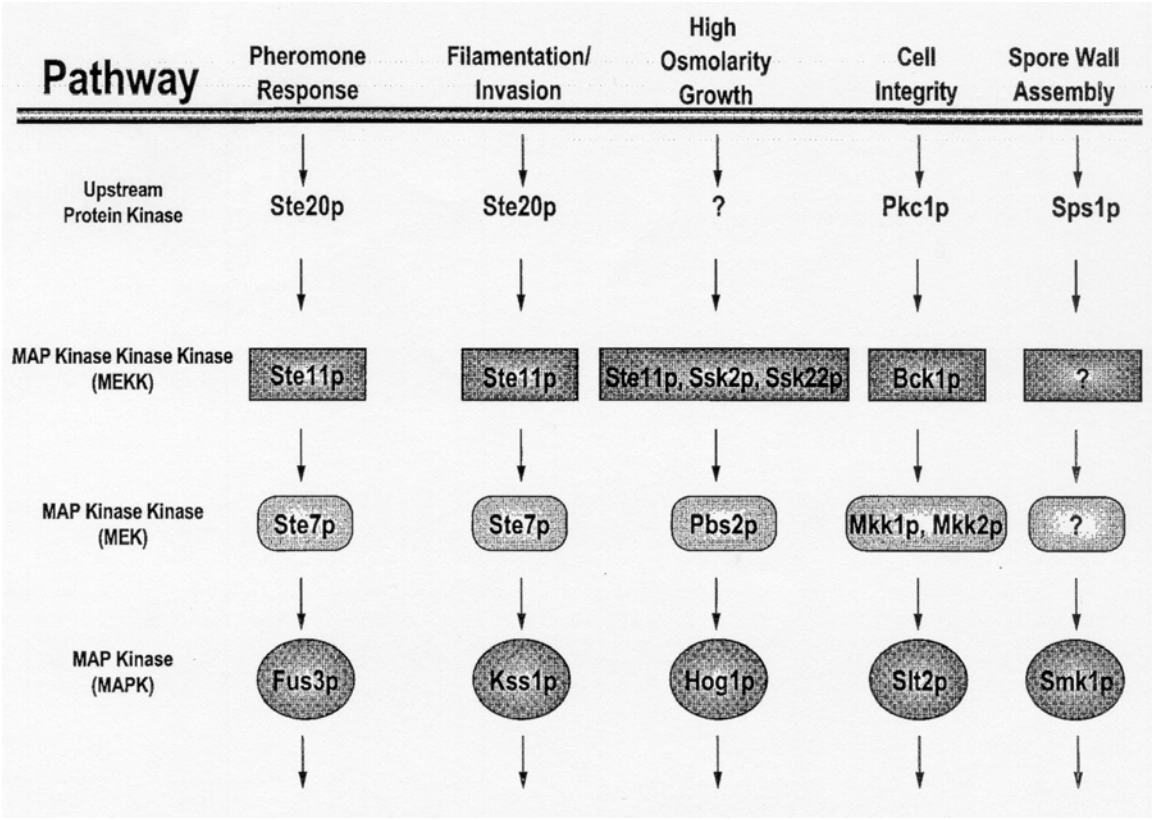


Figure 1: Mitogen-activated protein kinase pathways in yeast cells that are activated in different environmental conditions. Taken from Gustin *et al*. (1998).

MAPKKK's Ssk2p and Ssk22p under the control of the *SLN1-SSK1* two component osmosensor, are osmosensors in the cell membrane that send two different upstream signals to Pbs2p. In addition, it has been shown that by deleting the *PBS2* gene, there was a decreased or delayed response in proteins known to be upregulated under an osmotic shock (Akhtar *et al*., 1997). It should be noted that the research in each of these published studies has used laboratory strains, and the mechanisms of signal transduction in wine strains may differ, thereby resulting in their unique phenotypes.

1.3. THE RESPONSE OF *SACCHAROMYCES CEREVISIAE* AFTER ENCOUNTERING A STRESSFUL ENVIRONMENT

1.3.1. Stress related genes

The change of external environment for a yeast cell can result in many changes in gene expression as a result of a signal being sent from the cell membrane to the nucleus. A study using DNA microarrays looking at various environmental stresses such as temperature shock, chemicals and nutrient depletion showed changes in expression of approximately 900 out of the 6200 genes in *S. cerevisiae* (Gasch *et al*., 2000). The gene expression patterns of yeast exposed to these different environmental stresses were remarkably similar. For each stress, approximately 50 genes with a stress response element (STRE) promoter sequence, recognised by the transcription factors Msn2p and Msn4p, were induced. After a temperature shift from 25^0C to 37^0C, approximately 600 genes were repressed. Two distinct clusters were evident amongst the repressed genes. The first of these include genes for RNA processing, translation initiation, nucleotide biosynthesis, cell growth and other

metabolic processes. The second cluster involved genes almost exclusively encoding ribosomal proteins, and have been shown previously to be regulated by the transcription factor Rap1p (Li *et al*., 1999). Of the induced genes, only 40% have a defined function and consist of genes involved in cell wall modification, protein folding and degradation, DNA damage repair, and intracellular signaling. It should be noted that a similar data set has been seen with other stresses, such as hydrogen peroxide treatment, nitrogen starvation, and hyper- and hypo-osmotic shock (Causton *et al*., 2001).

The ability of commercial yeast strains such as brewing and winemaking strains to endure stress during fermentation is well documented (Attfield, 1997; reviewed in Casey and Ingledew, 1986). One established response by *S. cerevisiae* to endure stress is the induction and expression of heat shock proteins (HSPs). HSPs can aid in the folding and unfolding of proteins by binding to polypeptide chains, and facilitate in the assembly of multiple peptides to form larger structures. They are normally present at low levels and their quantities increase during stress to stabilise proteins (Parsell and Lindquist, 1993). HSPs have been shown to be highly expressed in the presence of elevated temperatures (hence their name), (Brosnan *et al*., 2000; Lindquist and Kim, 1996; Sanchez and Lindquist, 1990; Sanchez *et al*., 1992), solvent tolerance (Alexandre *et al*., 2001; Aranda *et al*., 2002; Betz *et al*., 2004; Carrasco *et al*., 2001; Garay-Arroyo *et al*., 2004; Palhano *et al*., 2004; Rossignol *et al*., 2003), and most other stresses (Causton *et al*., 2001; Gasch *et al*., 2000). Furthermore, it has been illustrated that the HSP70 genes that are induced with

exposure to heat stress are also induced with a high ethanol concentration (Piper *et al*., 1994). Moreover, HSPs are produced during early stationary phase of yeast growth in alcoholic fermentation (Piper *et al*., 1994; Werner-Washburne *et al*., 1996). These studies are relevant for commercial wine yeast strains where one of the major end products of grape juice fermentation is ethanol.

Ethanol as an extracellular stress is of interest to the wine industry as it can be one of the major reasons for stuck wine fermentations. The complete genetic and cellular response of yeast to an ethanol stress has not been described, although recent published studies by Alexandre *et al*., (2001) and Chandler *et al*., (2004) have examined the genetic response of a laboratory strain to a 7% and a 5% ethanol stress respectively. The genes up-regulated after an ethanol stress in these studies include metabolic genes, ionic homeostasis related genes, protein destination genes, and stress response genes such as heat shock protein genes. Other genes are also implicated to play a role in ethanol stress from studies at the genetic level of the inherent high ethanol tolerance of sake yeast. Using microarrays that contain all 6,200 open reading frames of the *S. cerevisiae* laboratory strain S288C, Ogawa *et al*., (2000) identified several genes whose expression was elevated in the ethanol-tolerant sake strain, but not in the laboratory strain. Those induced were a glycerol synthesis gene (*GPD1*), a gene with cytoplasmic catalase activity (*CTT1*), iso-2-cytochrome c (*CYC7*), a heat shock protein (*HSP12*), and a gene that encodes a protein located in the cell wall with an unknown molecular function (*SPI1*). This is consistent with other studies looking at heat, peroxidase, osmotic and ethanol

stresses, where it has been concluded that these environmental stress response genes are activated by different transcription factors depending on the environmental stress (Alexandre *et al*., 2001; Gasch *et al*., 2000; Rep *et al*., 1999). In addition, the identity of genes with a role in ethanol tolerance has been investigated using *S. cerevisiae* mutant strains obtained by transposon mutagenesis (Takahashi *et al*., 2001). Disruption of *BEM2* (encoding a GTPase activating protein), *PAT1* (a topoisomerase II-associated protein), *ROM2* (GDP-GTP exchanging factor for Rho1p), *VPS34* (required for vacuolar protein sorting), and *ADA2* (component of the histone acetyltransferase complex) produced ethanol sensitive mutants that could not grow in medium containing 6% ethanol. These mutants were also sensitive to Calcofluor white, a drug that affects cell wall structure, and zymolyase, showing the necessity of these genes to maintain cell wall integrity after an ethanol stress. By identifying genes induced with an ethanol stress response, the behaviour of wine yeast strains during grape juice fermentation and how these strains are able to tolerate the high ethanol concentrations toward the end of fermentation may be better understood.

1.3.2. Proteins that are differentially expressed after stress.

After a yeast cell experiences an environmental stress, many processes occur such as transcription, translation, post-translational modifications, and folding and cleaving of proteins. Methods such as two-dimensional gel electrophoresis (2D gels), antibody probing, and the latest technology of protein kinase chips (Zhu *et al*., 2000) are invaluable to analyse protein biological activity. The regulation of osmotolerance

in yeast is well characterised as a result of much investigation using these strategies. 2D gel experiments have shown that many proteins were induced more than eight fold in the presence of a high salt concentration (Blomberg, 1995). These proteins were assigned as predominantly glycolytic enzymes, and comprise those required to produce glycerol (Gpd1p) and trehalose (Tps1p) in order to rebalance osmolyte levels (Andre *et al*., 1991; Blomberg, 1995). Other proteins that changed expression include glycolytic proteins such as Tdh3p, Eno1p, and Pdc1p, a heat shock protein Ssa1p, and Act1p, which is involved in cell structure. In addition, a study by Norbeck and Blomberg (2000) found that the modified expression of these genes is dependent on protein kinase A (PKA) activity. An osmotic stress also fully repressed the expression of PKA-dependent glycolytic proteins (Pgi1p and Gdh1p), and a protein in methionine metabolism (Sam1p), while partly repressing other PKA-dependent glycolytic proteins (Pdc1p and Hxk2p), and a heat shock protein (Ssb1p). This is consistent with the previous findings by Gasch *et al*. (2000) who studied the mRNA expression of stressed yeast cells by microarray experiments.

In comparison, it has been shown that the level of messenger RNA in the cell is not necessarily directly proportional to the amount of protein produced by the transcript (Gygi *et al*., 1999). 150 proteins were identified by using 2D gel electrophoresis and mass spectrometry (hence looking directly at the outcome of the message) and compared to serial analysis of gene expression frequency tables that display corresponding mRNA levels. It was noted that some proteins varied up to 20-fold in

expression compared to the calculated mRNA level, showing the importance of post-translational regulation in controlling protein expression.

1.3.3. Cell membrane and lipid modifications of yeast under stress

The yeast cell membrane and cell wall have been shown to reconfigure in response to stress. It has been reported that yeast adjust to elevated levels of extracellular ethanol by altering the fatty acid composition of the membrane (Alexandre *et al*., 1994; Chi and Arneborg, 1999; Guerzoni *et al*., 1999; Heipieper *et al*., 2000). Chi and Arneborg (1999) reported that a strain highly tolerant to ethanol incorporated significantly more long-chain fatty acids into total phospholipids than a less ethanol tolerant strain, and that the ethanol tolerant strain had the ability to increase membrane fluidity in response to ethanol by increasing the proportion of unsaturated fatty acids. It has also been reported that when phosphatidylcholine (a component of the yeast phospholipid cell membrane) is added to sake yeast cultures, the ethanol tolerance of sake yeast was enhanced (Shin *et al*., 1995). An interesting observation of relevance to winemaking is the finding that ethyl esters in lipid extracts accumulate when yeast are exposed to 6% ethanol (Guerzoni *et al*., 1999). Ethyl esters are an important sensory component of wine, and if produced during fermentation as ethanol concentrations rise, may be important in the total composition of the wine after fermentation. Apart from compositional changes in the membrane, the activity of the H^+ATPase increases in response to ethanol or heat shock, resulting in enhanced proton efflux that enables the stress-induced permeability of the membrane to be countered (Coote *et al*., 1994).

1.3.4. Physiological changes to the yeast cell after stress response

The yeast stress response leads to a number of physiological changes. Glycerol is one of the first compounds to accumulate in cells exposed to high ethanol, heat shock, and increased salt levels, and has been shown to maintain turgor pressure and the structural composition of yeast cells (Ansell *et al*., 1997; Blomberg and Adler, 1989; Rep *et al*., 1999). It has also been suggested that accumulated trehalose within the cell can function as an anti-stress compound (Lucero *et al*., 2000; Mansure *et al*., 1994). This finding is supported by Ogawa *et al*. (2000) who noted a substantial increase in intracellular glycerol and trehalose in two sake strains able to tolerate ethanol concentrations of up to 20%. To support the idea of trehalose as a protectant in the presence of high ethanol, this study showed an induction of the trehalose-6-phosphate synthase (*TPS1*) and trehalose-6-phosphatase (*TPS2*) genes with the addition of 10% ethanol to the growth medium. Both of these genes contain multiple stress response elements in their promoter regions (Winderickx *et al*., 1996). Furthermore, this study showed ethanol tolerant strains isolated from a sake mash were resistant to K1 killer toxin and zymolyase, suggesting a change in the conformation of the cell wall with increased ethanol tolerance. The gene encoding a cell wall protein that binds to glucans and covers the cell wall, *SPI1*, was found to be induced strongly in the ethanol tolerant strains under stress conditions, and it was hypothesized that an increased level of Spi1p may provide resistance to killer toxin, zymolyase, and increased concentrations of ethanol (Ogawa *et al*., 2000). Contradictory to these studies, research by Swan and Watson, (1999) did not show any consistent relationship between trehalose accumulation and the stress tolerance

of yeast cells. These authors noted that an ethanol and heat tolerant strain had cellular membranes with high levels of oleic acid, indicating the importance of this fatty acid to the ethanol and heat stress response (Swan and Watson, 1999).

1.4. COMPARING STRAINS

The development of DNA microarrays has been revolutionary for the comparative analysis of *S. cerevisiae* strains and may be used to decipher genetic differences that exist between phenotypes (reviewed in Duggan *et al*., 1999). The genetic variation between a commercial wine strain and the laboratory strain S288C has been examined using microarrays, with most of the S288C genes present in the wine strain (Hauser *et al*., 2001). The results of this study showed that more than 40 genes significantly differed in their regulation in the wine strain, and where analysed, these were attributed to small variations in the promoter region or changes in gene copy number. Two genes that were studied further were *SSU1*, which encodes a plasma membrane protein involved in sulphite resistance and was shown to be upregulated in the wine strain. The other gene, *YHB1*, encodes a flavohaemoglobin whose expression is related to the level of oxygen and was not detected in the wine strain by microarray analysis. This suggests that these two genes may be linked to the physiology of this wine strain, reflecting the adaption of this strain for wine fermentation (Hauser *et al*., 2001).

It has been shown that microarrays can also be used to monitor the genome content of different strains (Winzeler *et al*., 1999). To assess this idea, genomic DNA of a

clinical yeast isolate taken from an AIDS patient was digested and probed against an array containing each open reading frame of S288C. By hybridising several molar amounts of each genome to arrays, it was shown that this isolate was missing a number of S288C genes, as well as displaying several differences in gene copy number. Furthermore, these experiments illustrated the polymorphic nature of the telomeric regions of each strain, with 55% of changes located within 50kb of the telomeres of chromosomes (Winzeler *et al*., 1999).

Amplified fragment length polymorphism (AFLP) is also an effective differential technique for comparing genomes. This technique involves three main steps: 1) the digestion of DNA by restriction endonucleases and ligation of oligonucleotide adapters, 2) selective amplification of digested fragments using PCR, and 3) analysis of amplified fragments using gel electrophoresis (Vos *et al*., 1995). It has been utilised for applications in molecular clinical microbiology (Arnold *et al*., 1999; Jiang *et al*., 2000), bacterial epidemiology (Aarts *et al*., 1998; Janssen *et al*., 1997), and yeast strain comparative studies (Azumi and Goto-Yamamoto, 2001; de Barros Lopes *et al*., 1999; Ganter and de Barros Lopes, 2000). By using AFLP analysis, it is possible to discriminate yeast species and yeast strains based on their genome sequence. Azumi and Goto-Yamamoto (2001) and de Barros Lopes *et al*. (1999) used AFLP analysis to group *Saccharomyces cerevisiae* industrial strains such as wine and baking strains, while also grouping laboratory strains together. Identifying the DNA sequence of the polymorphic fragments could lead to the isolation and

discovery of genetic sequence variations that result in the physiological differences in strains.

It is also possible to compare yeast strains with distinctive phenotypes using a proteomics approach. Joubert *et al*. (2000) have compared a lager brewing yeast and type strains of *S. cerevisiae, S. monacensis, S. carlsbergensis, S. bayanus* and *S. pastorianus* to discover the ancestors of the commercial lager strain. By identifying protein spots that had varying intensities, this group were able to conclude that the lager yeast strain was a hybrid of a *S. cerevisiae*-like parent while the other parent was best represented by a particular *S. pastorianus* strain (Joubert *et al*., 2000). It should be noted however that a comparative analysis of two *S. cerevisiae* strains has not been reported in the literature. Commercial winemaking strains of *S. cerevisiae* having distinctive phenotypes that allow them to undergo grape juice fermentation, while other *S. cerevisiae* strains such as a laboratory strain, S288C, cannot do this as efficiently. Therefore, with the advent of new technologies, it may be possible to identify phenotype-linked genes that exist between two *S. cerevisiae* strains with almost identical genomes.

CHAPTER 2

Materials and Methods

2.1. PREPARATION OF DNA

For all strains (Appendix 3), DNA was purified using mechanical breakage with glass beads (Ausubel, 1994). Yeast cells were disrupted using a Mini-Beadbeater® (BioSpec) for 3 minutes with glass beads. DNA was purified using phenol:chloroform:isoamyl alcohol (25:24:1), and a dried pellet resuspended in sterile MilliQ water. All media and reagents are listed in Appendix 1.

2.2. COMPARING STRAINS USING AFLP

Amplified fragment length polymorphism (AFLP) was used to identify polymorphic fragments between strains. The AFLP reactions were performed as previously described (de Barros Lopes *et al*., 1999; Vos *et al*., 1995). Initially, primers *Pst1*-AA (FAM) (5'-GACTGCGTACATGCAGAA-3'), *Pst*-AC (HEX) (5'-GACTGCGTACATGCAGAC-3') and *Pst1*-AT (TET) (5'-GACTGCGTACATGCAGAT-3') were used in combination with *Mse1*-C (5'-GATGAGTCCTGAGTAAC-3') (Appendix 2). 20 µl of each reaction mix was evaporated off and resuspended in 2 µl of 1x gel loading buffer (98% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Samples were then denatured at 90°C for 3 minutes and placed directly on ice for a further 3 minutes. All 2 µl was loaded onto a 6% polyacrylamide Hoefer sequencing gel (0.1 mm

thick). The gel was run at constant power (40W) for 140 minutes. Once electrophoresis was complete, the gel plates were separated with the gel adhering to the small glass plate. The gel was silver stained according to the Silver Sequence™ DNA Sequencing System Technical Manual (TM023, Promega) with some modifications. Briefly, the gel was fixed for 30 minutes in 10% glacial acetic acid. After washing the gel 3 times for 2 minutes each in water and drained, the gel was stained (1 g/L silver nitrate, 5.5% formaldehyde) for 30 minutes. At the completion of staining, the gel was rinsed in water for 5 seconds before placing it into developing solution (30 g/L sodium carbonate, 2 mg/ml sodium thiosulphate, 5.5% formaldehyde) until bands could be visualised. Development was stopped using 10% glacial acetic acid, and the gel then rinsed thoroughly with Milli-Q water (Millipore, USA). Gels were then scanned while adhered to the glass plate on a HP ScanJet 6100C/T scanner.

2.3. CLONING OF POLYMORPHIC BANDS

Polymorphisms in commercial strains were identified by visually comparing each strain on the same gel to the sequenced laboratory strain, S288C. The polymorphic band was excised with a sterile scalpel and the gel fragment crushed in a 0.5 ml eppendorf tube with a yellow micropipette tip in the presence of 15 µl of sterile Milli-Q water. The tubes were then subjected to 7 freeze-thaw cycles from liquid nitrogen to a 37°C water bath to extract the DNA from the gel. After pelleting the polyacrylamide, 2 µl of the supernatant was used for PCR to check if the DNA had been extracted efficiently. The PCR products were then confirmed to be of correct

size when matched to the original polymorphic fragment on an agarose gel. Once polymorphic bands had been checked for correct size, the bands were blunt-end cloned into the vector pGEM-T Easy Vector® (Promega, Appendix 6). 10 μL of PCR product was evaporated down to 1.5 μL and added to the ligation mix. The ligation mix contained 2x ligation buffer (Promega), 1U of T4 DNA ligase, and 50 ng pGEM-T Easy Vector®. This ligation mix was then incubated at 4°C for 16 hours. 5 μl of ligation reaction was added to JM109 high efficiency competent *Escherichia coli* cells as per instructions (Stratagene). After incubating transformation plates for 17 hours at 37°C, white colonies (indicative of the presence of a DNA insert) were picked. Plasmid DNA was isolated using standard protocols (Sambrook and Russell, 2001). Each was checked for the correct insert by PCR using the original AFLP primers above.

2.4. SEQUENCING OF POLYMORPHIC FRAGMENTS

Sequencing of polymorphic fragments was carried out at the Nucleic Acid and Protein Chemistry Unit of the University of Adelaide. 30 ng of plasmid DNA was placed in a sequencing master mix (8 μl Terminator Ready Reaction Mix, 9.6 pmol T7 primer). Thermal cycling was as follows: (96°C x 10s, 50°C x 5s, 60°C x 4 min) x 25 cycles, then held at 4°C. The resultant amplification mix was ethanol precipitated (3 M sodium acetate (pH 4.6), 100% ethanol), the pellet washed with 70% ethanol, and vacuum centrifuged to dryness. This sample was rehydrated in sterile water, loaded onto a sequencing gel, and run on an Applied Biosystems 373

DNA Stretch sequencer. The bands were assigned and a sequence generated automatically using ABI software.

2.5. COMPARING POLYMORPHIC FRAGMENTS TO S288C DATABASE

Once the polymorphic sequences were analysed and amended for correct base incorporation, the sequence was probed against the *Saccharomyces* Genome Database (SGD, www.yeastgenome.org) using the BLAST software with default parameters. For further comparisons, the Washington University Genome Sequencing Centre (WUGSC) BLAST search engine was used for other *Saccharomyces* species (Cliften *et al*., 2001; http://genome.wustl.edu), and the European Bioinformatics Institute WU-Blast2 protein database was used for comparing protein sequences translated from the polymorphic DNA sequences against other species (http://www.ebi.ac.uk/blast2/).

2.6. VECTORETTE PCR TO ESTABLISH FULL GENE SEQUENCE

Vectorette PCR was used to acquire upstream and downstream sequence of fragment 1116-2 (Gecz *et al*., 1997; Munroe *et al*., 1994). Briefly, 10 μg of genomic DNA was digested with *EcoRV* and *Dra1* and the prehybridised sequences AJHBUB1 (5'GAAGGAGAGGACGCTGTCTGTCGAAGGTAAACGGACGAGAGAAGGG AGAG3') and AJHBUB2 (5'CTCTCCCTTCTGCGGCCGCAGTTCGTCAACATAGCATTTCTGTCCTCTC CTTC3') were ligated. This was achieved by heating the two oligos to 100°C for 10 minutes in the presence of 2x SSC buffer (0.3 M NaCl, 0.03 M Na citrate, pH 7.0)

and 1 M Tris [pH 8.0], then slowly cooling at room temperature to 25°C. The adaptor sequences were then ligated to 1 μg of digested DNA with 1 unit of T4 DNA ligase. This product was then PCR amplified using adaptor-specific primer AJHBUB3 (5'GCGGCCGCAGTTCGTCAACATAGCATTTCT3') and a primer designed within the digested sequence. The PCR product was then gel isolated with a QIAGEN gel purification kit and sequenced. The sequences obtained were then probed against multiple databases, including the S288C, EMBL and EMBLNEW databases.

2.7. INTERNAL TRANSCRIBED SPACER (ITS) AMPLIFICATION AND RFLP OF RDNA.

Ribosomal DNA was analysed using an ITS PCR/RFLP method (Esteve-Zarzoso *et al*., 1999). Genomic DNA was amplified using the primers ITS1 5'-TCCGTAGGTGAACTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3'. The fragment was then digested with *Hae*III and resolved on a 1.5% agarose gel.

2.8. SOUTHERN ANALYSIS

Southern analysis was used to confirm the presence of isolated polymorphic sequences from commercial strains. Initially, 800 ng of genomic DNA was restriction digested with *Eco*R1 and *Hind*III in separate reactions and run on 1% agarose gels respectively. Each gel was then denatured twice in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes each with gentle agitation at room temperature. After rinsing with Milli-Q water, the gel was neutralised twice in

neutralising solution (0.5 M Tris-Cl (pH7.4), 3 M NaCl) for 15 minutes each at room temperature. The gel was then placed upside down on a sheet of 3MM Whatman filter paper soaked in 20x SSC, with the ends of the filter paper resting in a 20x SSC reservoir. A piece of H Bond membrane wetted in 2x SSC was then placed on top of the gel, and topped with three sheets of wetted filter paper and 8 cm of paper towel with a weight on top to maintain downward pressure on the gel/membrane. The transfer was left overnight, with the wells marked the following morning. The membrane was then rinsed in 6x SSC and placed on filter paper wetted with 6x SSC. The DNA was cross-linked to the membrane on both sides using a UV cross-linker (Uvitec).

The membrane was placed between 2 pieces of plastic mesh and put in a hybridisation bottle. Prehybridisation was carried out in 20 ml of Easy Hyb solution (Roche) at 42°C for 2 hours. A digoxigenin (DIG) probe prepared earlier was heated for 10 minutes at 95°C, cooled on ice, and extra Easy Hyb added to a total of 6 ml. The contents of the hybridisation bottle were removed and the DIG probe solution added, then left overnight at 42°C in a hybridisation oven to hybridise. The membrane was washed twice with 2x SSC / 0.1% SDS at room temperature, followed by two washes in 0.5x SSC / 0.1% SDS at 68°C and one wash in washing buffer (1x Maleic acid buffer, 0.03% Tween 20). The membrane was then blocked in blocking buffer (10x block solution in Maleic acid buffer) for 45 minutes at room temperature with gentle agitation. Once blocked, the membrane was incubated for 60 minutes in fresh blocking buffer with 2.5 µl anti-DIG-alkaline phosphatase

added. The antibody solution was removed and the membrane washed twice in 100 ml of washing buffer for 15 minutes, followed by equilibration in 50 ml of CSPD® (Roche Molecular Biochemicals) detection buffer for 5 minutes. The membrane was placed in a plastic bag and 0.5 ml of enhanced chemifluorescence (ECF) detection solution (Amersham Biosciences) carefully dribbled over the entire membrane, sealed, and incubated for 5 minutes at room temperature. The bag was the sealed and incubated for 10 minutes at 37°C to develop. The membrane was then scanned on a Amersham Biosciences STORM 860 phosphoimager.

2.9. METABOLOME FERMENTATIONS AND ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATGRAPHY (HPLC)

To determine the metabolic differences that may exist between a laboratory strain S288C, a commercial wine strain AWRI 838 and a commercial sake strain Kyokai 7 after fermentation, HPLC was used. Briefly, 1 x 10^6 cells/ml were inoculated in Synthetic Complete (SC) medium with 80 g/l glucose and grown anaerobically at 28°C at 200 rpm until all glucose was consumed. The yeast were removed by centrifugation, and the supernatant was stored at -20°C until ready for analysis. HPLC analysis was carried out using an Agilent 1100 series HPLC system. Pretreatment of a OA-HY organic acids column (51272, Merck) was done with polypore organic acid mix (Activon BLPPH-GU). The mobile phase was 0.005 M H_2SO_4 using an injection volume of 10 ml; the flow rate was 0.6 ml/min and the column temperature was 65°C. The flow rate was maintained at 0.6 ml/min and the

eluant detected at 214 nm using a photodiode array detector and a refractive index detector.

2.10. ISOLATING TOTAL PROTEIN EXTRACTS FROM YEAST

This protein extraction protocol is adjusted from Garrels *et al*. (1994). Yeast cells were grown in 50 mls of minimal media to 1 x 10^7 cells/ml and centrifuged to collect cells. The cells were washed with 10 mls of ice cold Milli-Q water, pelleted, and resuspended in 150 µl of lysis buffer (20 mM Tris-Cl (pH 7.6), 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 mM EDTA, 0.1% deoxycholate) containing 2µl Protease Inhibitor Cocktail for fungal and yeast cells (AEBSF 100 mM, 1,10-Phenanthroline 500 mM, Pepstatin A 2.2 mM, E-64 1.4 mM; P8215, Sigma). This mix was then transferred to a 1.5 ml screw cap eppendorf containing 0.28 g of acid-washed glass beads (425-600 micron). This slurry was vortexed for one minute and then placed on ice for one minute, repeating six times with tubes on ice between vortexing. The tubes then had a small hole poked in the bottom with a 25g needle, placed on top of a fresh sterile eppendorf tube, and centrifuged for 30 seconds at 4000 rpm to push the protein mix through. 1/10 volume of ice-cold RNase/DNase mix (0.5 mg/ml DNase 1, 0.25 mg/ml RNase A, 25 mM $MgCl_2$) was added to each tube, mixed, and placed on ice for 10 minutes. Protein mixes were stored at 4°C to avoid continual freeze-thaw cycles in the freezer, which may degrade proteins and give rise to poor quality 2D gels. Protein concentration was then measured by Bradford assays and checked further on coomassie stained 4-20% iGel polyacrylamide gels (Gradipore).

2.11. TWO-DIMENSIONAL GEL ELECTROPHORESIS (2D GELS) TO IDENTIFY DIFFERENCES IN YEAST PROTEOMES

2.11.1. Waite Campus Gels

For 2D gel analysis using our own facilities, yeast at $1x10^7$ cells/ml were taken and the protein extracted as above (adapted from Garrels *et al*., 1994). 250 μg of protein was taken and added to a total volume of 350μL with rehydration stock solution (8 M Urea, 2% CHAPS, 0.5% or 2% IPG buffer). First dimension strips of pH 3-10 NL were used initially, however to separate individual proteins more accurately (Wildgruber *et al*., 2000), pH 3.5-4.5, 5-6, 4-7 and 6-11 strips were used. The strips were then gently caressed over the suspension in 18 cm 'coffin-like' wells to absorb the liquid into the agarose strip and a thin layer of mineral oil added. Programs used for isoelectric focusing of proteins were dependent on the pH of the strip and are outlined in Appendix 5. Active rehydration was done at 20°C with 50μA of current applied per strip.

Once isoelectric focusing was complete, strips were placed in 10 mls of equilibration buffer (50 mM Tris-Cl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS) containing 10 mg/ml dithiothreitol to enable protein reduction and shaken for 15 minutes at 25 rpm. The strips were then removed and placed in a further 10 mls of equilibration buffer containing 12.5 mg/ml iodoacetamide for alkylation of proteins. After 15 minutes at 25 rpm, the strips were placed on top of a 12% polyacrylamide gel and sealed with an agarose overlay. The gels were run in a Hoefer DALT tank (Amersham Biosciences) at 100 mA each in SDS-PAGE running buffer until the

bromophenol blue dye reached the end of the gel. The gels were then either silver stained according to Heukeshoven and Dernick (1988), colloidal coomassie (modified from Neuhoff *et al*., 1988) or SYPRO Ruby® (Bio-Rad) stained.

2.11.2. Australian Proteome Analysis Facility (APAF) 2D gels

The gels produced at APAF were run with modifications to the above protocol. 500 μg of protein sample was added to a total of 150 μl of thiourea/urea rehydration buffer (2 M thiourea, 7 M Urea, 4% CHAPS (w/v), 1% DTT (w/v), 2% ampholytes 6-11) and cup loaded on pH 6-11 strips (prehydrated overnight in rehydration buffer). A Pharmacia Biotech Multiphor II system powered by a Consort E752 Microcomputer electrophoresis power supply was used for isoelectric focusing and run for 31,900 Volthours at 20°C. The following program was used: 100V x 3h, 300V x 3h, 600V x 2h, 1000V x 2h, 2500V x 1h, 5000V x 5h. When the focusing was complete, the strips were equilibrated in equilibration buffer for 20 minutes and then placed on top of a 12% polyacrylamide gel. The strips were sealed with 0.5% agarose with bromophenol blue, and the gels run in a Protean II Multi-Cell electrophoresis unit maintained at 4°C. The gels were run at 50 mA per gel using a Bio-Rad PowerPac 3000 until the dye reached the end of the gel. Gels were then removed and placed into fixative solution (40% methanol, 10% acetic acid) for one hour, the fixative removed, and replaced with SYPRO Ruby® stain and left overnight. After destaining three times in destain solution (10% methanol, 7% acetic acid), the gels were scanned on a Molecular Imager® FX scanner (Molecular

Dynamics). To isolate and identify proteins, the gels were placed in colloidal coomassie solution and left overnight.

2.11.3. Silver Stain

For silver staining (Heukeshoven and Dernick, 1988), the gels were placed in fixative (40% ethanol, 10% acetic acid) overnight, and following a brief 5 minute wash, gels were placed in sensitising solution (0.3% sodium thiosulphate pentahydrate, 30% ethanol, 6.8% sodium acetate) with gentle agitation for 60 minutes. To prepare for the silver stain, gels are thoroughly washed through 6 x 10 minute washes in Milli-Q water and then the silver nitrate solution (0.1% silver nitrate, 0.05% formaldehyde) added and left for 30 minutes. The gels were then briefly rinsed with 3x 20 second washes of Milli-Q water, followed by the addition of developer (3% sodium carbonate, 0.025% formaldehyde) and left until even protein staining was observed. To stop development, the gels were washed briefly with Milli-Q water and 1% glycine added.

2.11.4. Colloidal Coomassie Staining

To visualise proteins before extraction and identification, 2D gels were firstly stained with SYPRO Ruby®, then placed into colloidal coomassie stain (modified from Neuhoff *et al*., 1988). Approximately 550 ml of Milli-Q water was placed in a 1 L beaker, warmed in a microwave, and 170 g of ammonium sulfate dissolved until crystals disappeared completely. 330 ml of methanol was then added very slowly while stirring. When all traces of ammonium sulfate precipitation had dissipated, 36

ml of orthophosphoric acid was added slowly and the solution left stirring for five minutes. Once cooled to room temperature, 10 ml of coomassie stock solution (10 g coomassie brilliant blue G-250 in 100 ml of methanol) was added. The colloidal coomassie stain was left to stir for a short time, but no more than 10 minutes to reduce colloids combining and precipitating out. The gels were then subjected to this stain overnight or longer, then rinsed briefly in 1% acetic acid until clear blue spots were seen with little background interference.

2.12. WESTERN BLOT ANALYSIS

Western blot analysis was carried out as described elsewhere (Sambrook and Russell, 2001). Total protein extracts were mixed with 5x SDS-PAGE sample buffer, vortexed, boiled at 100°C for 5 minutes and allowed to cool to room temperature before loading on the gel. 4-20% gradient Tris-glycine polyacrylamide gels (Gradipore) were run in SDS-PAGE running buffer at 100 Volts (constant voltage), 60 mA, until the dye front had run off the end of the gel.

Wet transfer of proteins onto nitrocellulose membrane (BA83, Schleicher and Schuell) was done using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell following the manufacturer's instructions. Immediately after the SDS-PAGE gel had finished running, the gel was equilibrated in cold Western transfer buffer (25 mM Tris-base (pH 8.0) 192 mM glycine, 20% (v/v) methanol) for 15 minutes. The fibre pads (Bio-Rad), filter paper (3MM, Whatman) and nitrocellulose were also soaked in Western transfer buffer for 15 minutes. The transfer cassette was assembled as

described by the manufacturer. Transfer was conducted at 100 Volts (constant voltage) for 1 hour in Western transfer buffer.

After transfer, the membrane was covered with Ponceau S solution (0.1% (w/v) Ponceau S, 5% (v/v) acetic acid) for five minutes and rinsed with MilliQ water until the background was removed to ensure that the proteins had transferred. This is referred to as “Schiff staining” and ensures that equal amounts of protein have been loaded (hence not relying purely on absorbance readings of protein concentration, eg. Bradford assays). The remaining Ponceau S was washed from the membrane with TBS-T. The membrane was blocked in blocking buffer either overnight at 4°C or for 2 hours at room temperature, with gentle rocking. This was followed by a rinse and 4x 10 minute washes in TBS-T. The blot was probed with the primary antibody using various dilutions in blocking buffer for at least 1 hour at room temperature. The membrane was then washed with 4x 10 minutes in TBS-T. The secondary antibody was also diluted in blocking buffer and the membrane was incubated for at least 1 hour at room temperature. The membrane was then washed with 2x 10 minutes in TBS-T, followed by 2x 10 minute rinses in MilliQ water.

To develop the blot, the membrane was incubated in the presence of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Promega) in alkaline phosphate buffer (100 mM Tris-HCl (pH 9.0), 150 mM NaCl, 1 mM $MgCl_2$) until bands appeared. The membrane was gently blotted with filter paper and allowed to dry before scanning using a HP ScanJet 6100C/T scanner.

2.13. PROTEIN IDENTIFICATION

2.13.1. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF)

Proteins of interest were excised with a sterile scalpel and put into either individual eppendorf tubes or separate wells in a 96-well plate. 120 μL of wash solution (50% v/v acetonitrile, 25 mM NH_4HCO_3, pH 7.8) was added and the tube or plate shaken at 37°C for 10 minutes. The solution was removed, wash solution again added, shaken, and drained. This was repeated three to four times until no blue colour of the coomassie stain could be seen in the gel piece, followed by drying in a speedvac for 15 minutes. When dry, 8 μl of sequencing grade trypsin (Promega) at 15 ng/μl in 25 mM NH_4HCO_3 (pH 7.8) was added to each sample and incubated at 37°C for 16 hours. Extraction of the peptides was achieved by adding 8 μl of extract solution (10% v/v acetonitrile, 1% TFA v/v trifluoroacetic acid), the plate or tube sonicated for 20 minutes in a water bath sonicator, and the resultant peptide solution removed with a sterile pipette tip. Peptides were then purified, concentrated and desalted with Millipore Zip-Tips, mixed with matrix (8 mg/ml α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile, 1% v/v TFA), and spotted onto a MALDI target. Once air-dried, samples were analysed with a Micromass® MALDI time of flight spectrometer. The spectra were acquired in reflectron mode in the mass range 750 to 3500 Da. Peptide masses were searched against *Yeast* using ProteinProbe® on MassLynx® and positive identification made based on sequence coverage, number of missed cleavages, and how well the molecular weight and isoelectric point match to the original position of the isolated protein from the 2D gel.

2.13.2. Tandem Mass Spectrometry (MS/MS)

Proteins to be identified by MS/MS were excised from the gels, washed and tryptic digested as above. The resulting peptides were purified using a Geloader microcolumn to concentrate and desalt the sample. The peptides were then analysed by electrospray ionisation MS/MS using a Micromass® Q-TOF MS. The instrument was operated in manual nanospray mode, with samples sprayed from borosilicate capillaries. Data was initially acquired (MS analysis) over the mass-to-charge m/z range 400-1600 Th to select peptides for MS/MS analysis. After peptides were selected, the instrument was switched to MS/MS mode and data collected over the m/z range required by the precursor mass and charge state, with variable collision energy settings.

2.14. RNA EXTRACTIONS

This procedure was adjusted from Chandler *et al*. (2004). 100 mls of yeast culture grown to 1 x 10^7 cells/ml was collected, washed with ice-cold DEPC-treated water, and stored at -80°C until ready for extractions. Tubes were then thawed on ice with the addition of 0.28 g of chilled acid-washed glass beads, 300 µl of RNA buffer (0.5 M sodium chloride, 0.2 M Tris-Cl (pH 7.5), 10 mM EDTA) and 300 µl of phenol/chloroform/isoamyl alcohol (25:24:1) with brief shaking. Tubes were then vortexed 3x for 1 minute each, placing on ice between vortexing. After centrifuging for 2 minutes at 12,000 rpm, the upper aqueous phase was carefully removed from the protein interface into a new sterile eppendorf tube. 3 volumes of 100% chilled ethanol were added and placed at –80°C overnight. The following morning the tubes

were centrifuged at 4°C at 12,000 rpm for 2 minutes and the supernatant discarded. The pellet was washed in 100 µl of 70% ethanol, spun down again, and the supernatant removed with care so as not to dislodge the pellet. After air-drying in a laminar flow for 10 minutes, the pellet was resuspended in 25 µl of DEPC-treated water. DNase reaction mix (2.5 µl of 1 M Tris (pH 7.5), 20 µl of 25 mM $MgCl_2$, 2 µl of RNase-free DNase (10 U/µl), 0.5 µl of RNasin (40 U/µl)) was added, flick mixed, and incubated at 37°C for 30 minutes. After incubation, 2 µl of 2 M sodium acetate (pH 4), 50 µl of DEPC-treated water saturated phenol, and 10 µl of chloroform/isoamyl alcohol (49:1) were added and placed on ice for 10 minutes. Tubes were then microfuged at 14,000 rpm for 5 minutes and the supernatant transferred to a fresh tube with the addition of 15 µl 3 M sodium acetate (pH 5) and 1 ml 100% ethanol before mixing and putting tubes at –80°C for 20 minutes. A pellet was obtained by centrifuging at 14,000 rpm for 10 minutes at 4°C and the fluid discarded carefully. The pellet was then washed with 70% ethanol, centrifuged, the supernatant removed and the pellet air-dried. The final pellet was resuspended in 12 µl of DEPC-treated water, with 1 µl visualised on a 1.6% MOPS/formaldehyde agarose gel, and the concentration determined by spectrophotometry by measuring the absorbance ratio of 260nm and 280nm (A_{260}/A_{280}).

2.15. cDNA SYNTHESIS

In a 0.2 ml PCR tube, 5µg of total RNA was combined with 1 µl each of gene specific primers (10 µM), 1µl of 10 mM dNTPs mix, and DEPC-treated water to 10 µl, then incubated at 65°C for 5 minutes. Tubes were placed on ice for 3 minutes to

cool, followed by the addition of 10 µl of cDNA synthesis mix (2µl of 10x RT buffer, 4µl of 25 mM $MgCl_2$, 2µl of 0.1 M DTT, 1µl of RNaseOUT^{TM} (40U/µl), 1µl of SuperScript^{TM} III RT (200U/µl)). The 20 µl was gently mixed, collected by brief centrifugation, and incubated at 50°C for 50 minutes. The reactions were terminated at 85°C for 5 minutes, chilled on ice, and collected again by centrifugation. 1 µl of RNase H (10U/µl) was added to each tube and incubated for a further 20 minutes at 37°C. cDNA reactions were then stored at –20°C until ready for PCR.

2.16. REAL-TIME QUANTITATIVE PCR

To compare gene expression between two different samples, real-time quantitative PCR was utilised. Initially, a standard curve with each primer set was established using 10-fold dilutions. Real-time PCR reactions contained 1 µl of cDNA sample, 0.6 µl of forward primer (10 µM), 0.6 µl of reverse primer (10 µM), 7.8 µl of sterile MilliQ water, and 10 µl of 2x SYBR Green PCR mix (AB Gene). Each reaction was flick mixed, spun down, and placed in a Corbett 4000 quantitative real-time PCR machine. The reaction times were as follows: 95°C x 15 min; (95°C x 30 sec, 58°C x 30 sec, 72°C x 30 sec) x 40 cycles; 72°C x 5 min; melt curve 50°C to 95°C, 1°C per 5 sec. The melt curve confirms the correct melting temperature (T_m) for one PCR product. Using the standard curve for each primer set an equation was derived (based on the equation y=mx+c, where m is the average slope of the curve for each dilution), with the fold-change in expression between two samples reported.

2.17. CONTINUOUS FERMENTATIONS IN THE PRESENCE OF ETHANOL

2.17.1. Yeast strain and media used

Strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) from the EUROSCARF deletion collection was used. Synthetic Complete (SC) media with all amino acids was used for continuous fermentation.

2.17.2. Continuous fermentation

Steady-state continuous fermentation was carried out using 1.5L Applikon fermenters (School of Chemical Engineering, University of Adelaide, Australia). Triplicate fermentations were under the control of the FC4 DOS Software and the FC-4 controller. Cultures were grown at 28°C and pH 5.0, with stirring at 800 rpm and sparging sterile air at 0.5L per minute. When the turbidity within the fermenter was on the increase (eg. cell numbers are increasing), the feed rate of fresh medium was increased to dilute the turbidity back to its setpoint. When the turbidity of the culture fell, the feed rate was lowered so that turbidity was restored to its setpoint. This enabled the cells to achieve a relatively homogeneous population over time at the same cell phase and cellular state. After 10-14 generations, cells were harvested and the SC Complete media was changed to SC Complete media supplemented with 6% ethanol (v/v). The dilution rate was maintained at 0.35 h^{-1} for SC media without ethanol, while the dilution rate was lowered to 0.08 h^{-1} with SC 6% (v/v) ethanol-supplemented media. Cells were harvested again after 10-14 generations, snap-frozen in an ice-ethanol bath and stored at -80^{0}C until ready for RNA isolation.

2.18. MICROARRAY ANALYSIS

2.18.1. RNA purification for cDNA microarray analysis

To purify high quality RNA for cDNA synthesis, fluorescent labeling and microarray analysis, the QIAGEN RNeasy Mini protocol for RNA cleanup was used. Briefly, total RNA was prepared as normal with some modifications. After the first air-drying of the nucleic acid pellet, the pellet was resuspended in 100 μl of RNase-free water. 350 μl of Buffer RLT (QIAGEN) and 250 μl of 100% ethanol were added and mixed thoroughly. This mix was then added to a RNeasy mini column (QIAGEN) and centrifuged for 15 seconds at 12,000 rpm, with the flow-through discarded. 350 μl of Buffer RW1 (QIAGEN) was added to wash the column and centrifuged for 15 seconds at 12,000 rpm, with the flow through discarded. 10 μl of DNase 1 (27 Kunitz units) was added to 70 μl of Buffer RDD (QIAGEN), pipetted directly onto silica-gel membrane, and left at room temperature for 15 minutes. 350 μl of Buffer RW1 (QIAGEN) was then added to the column, followed by 15 seconds of centrifugation at 12,000 rpm and the flow through again discarded. The column was transferred to a new collection tube, with two washes with 500 μl of Buffer RPE (QIAGEN), the column centrifuged and flow through discarded again. The column was then transferred to a new collection tube, and centrifuged briefly to remove any contaminating Buffer RPE (QIAGEN). Elution of RNA was carried out by adding 45 μl of RNase-free water directly onto the membrane in the column, which was then centrifuged and the eluant collected. A further 45 μl of RNase-free water was run through the column to increase yield.

To reduce the volume of total RNA for cDNA synthesis, the RNA was precipitated by adding 9 μl of 3 M sodium acetate (pH 5.2) and 180 μl of 100% ethanol. This mix was incubated at –80°C for 1 hour and pelleted by centrifugation at 12,000 rpm for 15 minutes at 4°C. The pellet was washed with 70% ethanol, and the pellet air-dried for an hour. The dried pellet was resuspended in 22 μl of RNase-free water, with 1 μl used for spectrophotometric measurements (A_{260}/A_{280} ratio), and 1 μl used for gel analysis to ensure quality and quantity was high.

2.18.2. Microarray cDNA and dye coupling preparation

For microarray analysis, procedures for cDNA synthesis and dye coupling were as follows. Total RNA (20 μg) was taken up to a total volume of 20 μl with RNase-free water. 2 μl of anchored polyT(V)N (2 μg/μl) was added and the mixture incubated at 70°C for 10 minutes, then placed on ice. 6 μl of 5x Superscript II buffer, 2 μl of 0.1 M DTT, 2 μl of Superscript II (200 U/μl) and 0.6 μl of aminoallyl dNTP mix (25 mM dATP, 25 mM dGTP, 25 mM dCTP, 10 mM dTTP, and 15 mM dUTP) were added, mixed, and incubated at 42°C for 150 minutes. The RNA was hydrolysed by adding 10 μl of 0.25 M NaOH, 10 μl of 0.5 M EDTA (pH 8) and incubated at 65°C for 15 minutes. This reaction was neutralised by adding 15 μl of 0.2 M acetic acid. The resultant cDNA was purified using a QIAquick PCR purification kit. Briefly, the cDNA mix was mixed with 300 μl of Buffer PB (QIAGEN), then applied to the QIAGEN column and centrifuged at 6500 rpm for 1 minute. The eluant was re-passed through the column, followed by washing 2x with 600 μl of Buffer PE (QIAGEN), with the residual buffer removed by centrifuging again at 6500 rpm for

1 minute. The sample was eluted into a clean tube with 90 μl of sterile Milli-Q water. The purified cDNA was dried under reduced pressure then dissolved in 9 μl of 0.1 M $NaHCO_3$ (pH 9). The mixture was added to a Cy3 or Cy5 dye, mixed, and left to incubate at room temperature for one hour in the dark. The labeled cDNA was mixed with 41 μl of Milli-Q water, then purified using the QIAquick PCR purification kit as above. The purified labeled cDNA samples appeared purple after being dried under reduced pressure, indicating a successful synthesis and coupling. The samples were stored at room temperature in a dark cupboard with alfoil covers.

Labeled cDNA was mixed with 0.7 μl of 25 mg/ml yeast tRNA, 4 μl of 2 mg/ml polyA and 20 μl of 1 mg/ml Cot-1 DNA. The mix was dried under reduced pressure then dissolved in 14 μl of formamide and 14 μl of 6.25x SSC. The mixture was heated to 100°C for 3 minutes, transferred to ice, then 0.2 μl of 10% SDS added before the solution was applied to the centre of a 50 x 24 mm cover slip. The array (pre-blocked by incubating in 95°C Milli-Q water for 90 seconds) was lowered onto the cover slip and incubated at 42°C overnight in a humidified chamber.

Prior to scanning the arrays, the slides were washed for 1 minute in 0.5x SSC with 0.01% SDS, 3 minutes with 0.5x SSC, 3 minutes with 0.06x SSC, and finally rinsed 5x with Milli-Q water. The slides were then dried in a centrifuge at 800 rpm for 5 minutes and scanned immediately using a Genepix 4000 scanner (Axon Instruments, USA).

2.18.3. Data and statistical analysis (bioinformatics)

Microarray images were analysed using the SPOT software (CSIRO Image Analysis Group, Australia), and results tabulated by the University of Adelaide Microarray Analysis Group (Ashley Connolly, University of Adelaide). For up-regulated genes, a region spanning 800 bp upstream of start sites was recovered using the Regulatory Sequence Analysis Tools (RSAT) database (http://rsat.ulb.ac.be/rsat/). Upstream sequences were then searched for specific sequence motifs using MATCH at TRANSFAC (http://www.gene-regulation.com/). The sensitivity cut-off for detecting consensus sequences was chosen to minimise false positive matches.

2.19. NEAR INFRA-RED SPECTROSCOPY (NIRS)

2.19.1. Anaerobic fermentations

Single gene deletion strains of the parental strain BY4742 (S288C background) were taken from the deletion collection (EUROSCARF), inoculated in duplicate in 1 ml of SC medium, and grown to stationary at 28°C. 20 µl of each culture were then inoculated into 2 ml of SC medium in 96-well plates in duplicate and sealed with a breathable film. The 96-well plate was then placed in an anaerobic hood and left non-shaking until each microfermentation reached dryness (no detectable glucose by Clinitest (anhydrous Benedict's reagent) after 48 hours). Each strain was transferred to a 1.5 ml eppendorf tube, the yeast removed by centrifugation, and the supernatants analysed by NIRS.

2.19.2. Spectra analysis

The visible (VIS) and NIR spectra (400 - 2500 nm) were acquired with an *FOSS-NIRSystems6500* (Foss NIRSystems, Australia), in transmittance mode and with a 1 mm path length. Samples were temperature equilibrated at 33°C in the instrument before scanning, as temperature effects need to be accounted for in NIRS calibration methods. A total of 1050 data points were collected.

2.19.3. Multivariate data analysis

Spectra was exported on NSAS format into The Unscrambler software (CAMO, Norway) for multivariate analysis. Principal Component Analysis (PCA) is a mathematical procedure for resolving sets of data into orthogonal components whose linear combinations approximate the original data to any desired degree of accuracy. PCA was used to derive the first 20 principal components from the spectral data and used in further analysis to examine the natural groupings of the samples as well as to detect outlier samples. Before performing PCA, all the sample spectra were pre-processed using the second derivative to reduce baseline variation and enhance the spectral features. In order to visualise the relative distribution of the different yeast strains, samples were graphically displayed by means of the first pair of components, the second pair, and the third pair of components.

CHAPTER 3

Identifying genome variation in *Saccharomyces cerevisiae* strains

3.1. INTRODUCTION

Saccharomyces cerevisiae wine yeast strains have been selected over thousands of years of winemaking for properties that include fast growth in high-sugar grape juices, high yield and tolerance to ethanol and, more recently, the biosynthesis of flavour and aroma compounds at concentrations beneficial for wine quality. However, few of the genes that contribute to these important properties have been elucidated. The identification of DNA sequences that are unique and characteristic to wine strains may provide an insight into the genetic basis for these phenotypes. Ultimately, this will provide increased knowledge of how wine strains are able to undergo grape juice fermentation.

The budding yeast *Saccharomyces cerevisiae* S288C was the first eukaryote to have its genome completely sequenced (Cherry *et al.*, 1997). The publishing of the S288C genome has promoted a great deal of research with this organism with regards to its life cycle, mating abilities, genome evolution and gene expression. It has been shown that considerable genetic variation exists between the genome of S288C and commercial strains of *S. cerevisiae* (Azumi and Goto-Yamamoto, 2001; Bidenne *et al*., 1992; Carlson and Botstein, 1983; de Barros Lopes *et al*., 1999; Hauser *et al*., 2001; Perez-Ortin *et al*., 2002; Winzeler *et al*., 1999). In this chapter, the sequenced

strain S288C is compared with commercial strains of *Saccharomyces cerevisiae* using amplified fragment length polymorphism (AFLP). Using this technique, in combination with silver-stained gels for the isolation of polymorphic fragments in commercial strains, it is envisaged that novel sequences within commercial strains will be isolated and identified. These polymorphic sequences can then be investigated further for their possible role in providing the 'wine strain phenotype'.

3.2. RESULTS

3.2.1. Phenotypic differences exist between strains

Using fluorescent AFLP, it has been shown that commercial wine strains are genetically similar to each other, yet distinct from other commercial strains (Azumi and Goto-Yamamoto, 2001; Heinrich *et al*., 2005). Figure 2 shows the grouping of commercial strains with similar phenotypic properties (eg. high ethanol tolerance, the ability to consume high amounts of sugar), as well as the grouping of commonly-used laboratory strains that do not have these attributes of commercial strains.

To demonstrate further that the phenotype exhibited by wine strains is related to genotype, plate assays with wine strains and the laboratory strain S288C were conducted. Figure 3 shows two commercial strains used in the AFLP study (3.2.2) are more tolerant than S288C to high concentrations of glucose and ethanol using a simple plate assay using SC medium with 200 g/l glucose and 10% (v/v) ethanol. This may reflect the environment from where these wine strains were isolated, such as high sugar grape juices (Fleet and Heard, 1993). It is noted these plate assays are

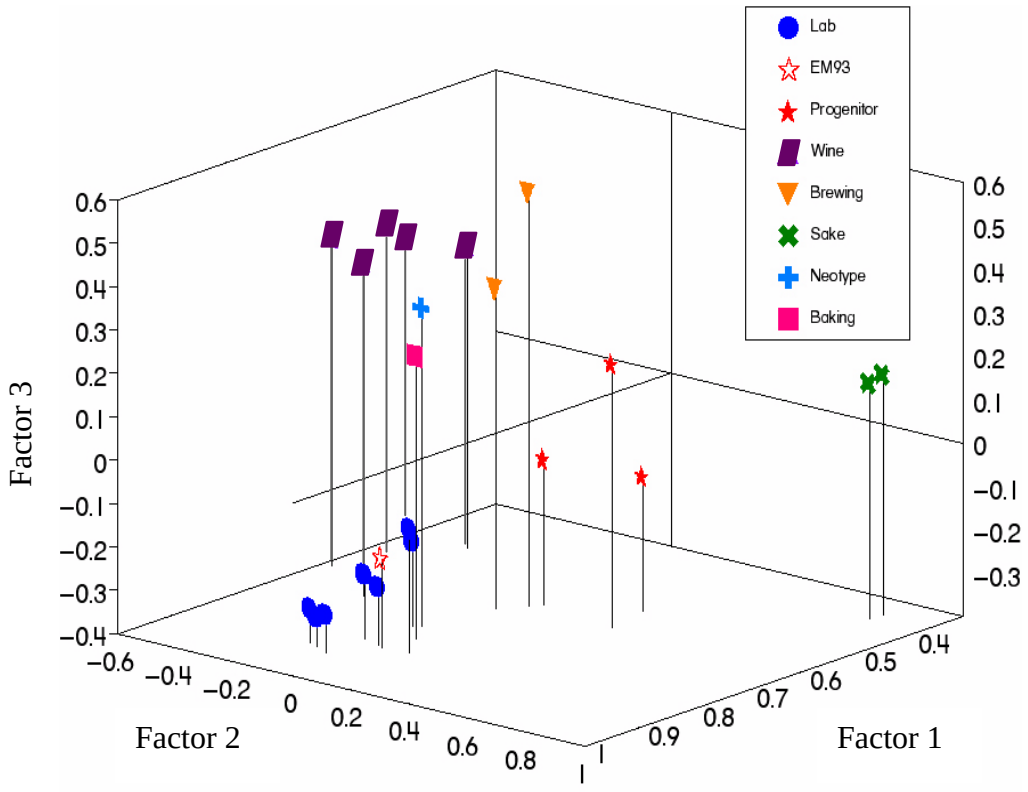


Figure 2: Principle component analysis (PCA) plot of *S. cerevisiae* laboratory and commercial strains. The PCA plot summarises the number of polymorphic sequences between strains based on pairwise correlation coefficients calculated from the presence-absence matrix of AFLP bands. The first three PCA axes are presented, as these were the only axes with eigen values over 1.

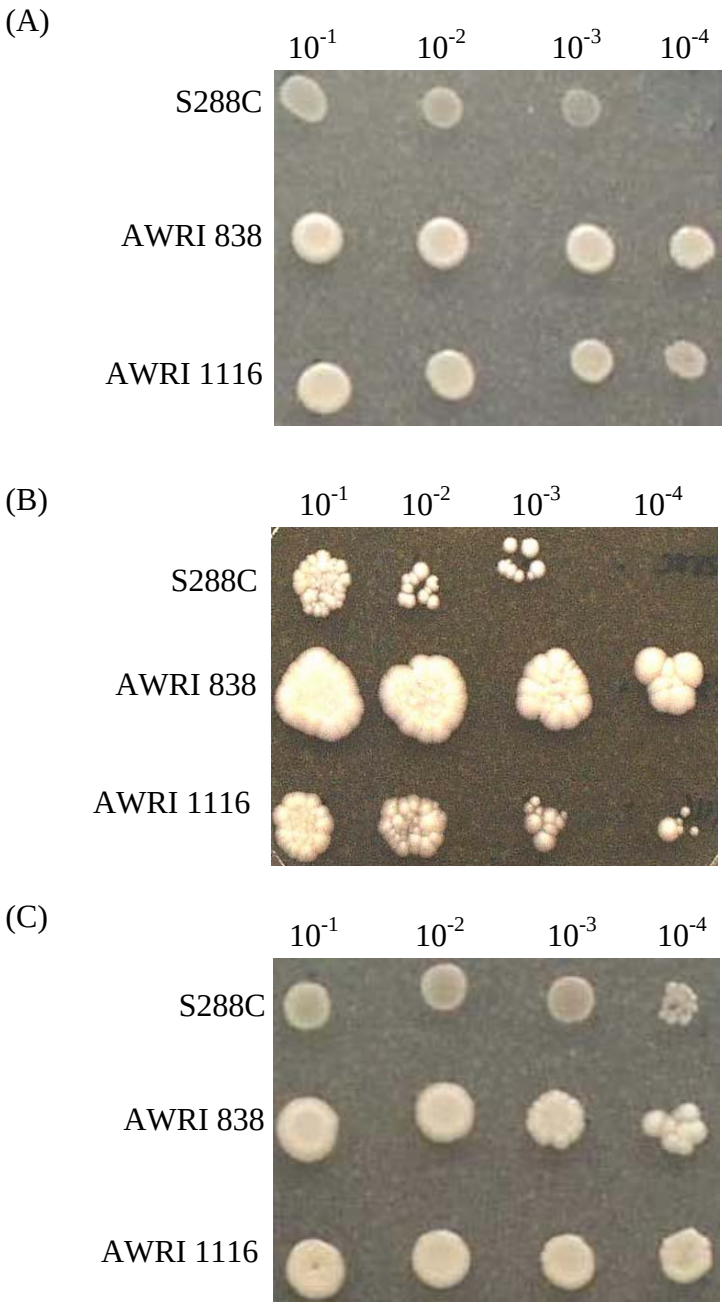


Figure 3: Demonstration of the higher stress tolerance of wine strains AWRI 838 and AWRI 1116 to growth on SC medium containing (A) 200 g/l glucose and (B) 10% (v/v) ethanol. A control plate (C) has been shown which contains 20 g/l glucose and 0% (v/v) ethanol. Cultures were plated in 10-fold serial dilutions at exponential phase (1 x 10^7 cells/ml) and plates incubated overnight at 28ºC.

not a true representation of the wine environment, however they provide an indication of the phenotypic properties of a wine strain. The phenotypic differences that exist between the wine and laboratory strains in this study do reflect the widely recognised dogma for the phenotypic differences between these strains and provided confidence to proceed with a comparative genomics approach using AFLP to isolate and identify genes specific to wine yeast strains.

3.2.2. AFLP reveals novel sequences in wine strains.

AFLP was used in an attempt to identify key genetic elements in commercial wine strains that give rise to their unique properties. Using a combination of oligo primer sets (Appendix 2), a total of 27 polymorphic bands were isolated from silver-stained gels. The results of subsequent sequencing (Appendix 4) and probing against the *Saccharomyces* Genome Database (SGD) can be seen in Table 1. It should be noted that commercial wine strain sequences are not publically available like the laboratory strain S288C. Nineteen sequences had between 95% and 100% identity to the corresponding sequences in S288C, which indicates that a polymorphism has occurred at the restriction site in order to visualise these bands as putative novel sequences. Eight bands had less than 95% identity to the S288C sequences, with several of these investigated further. Fragments 1116-1, -2, -3, -4, and 939-1 (Appendix 4) were chosen for further studies based on their reduced homology to sequences in the S288C database.

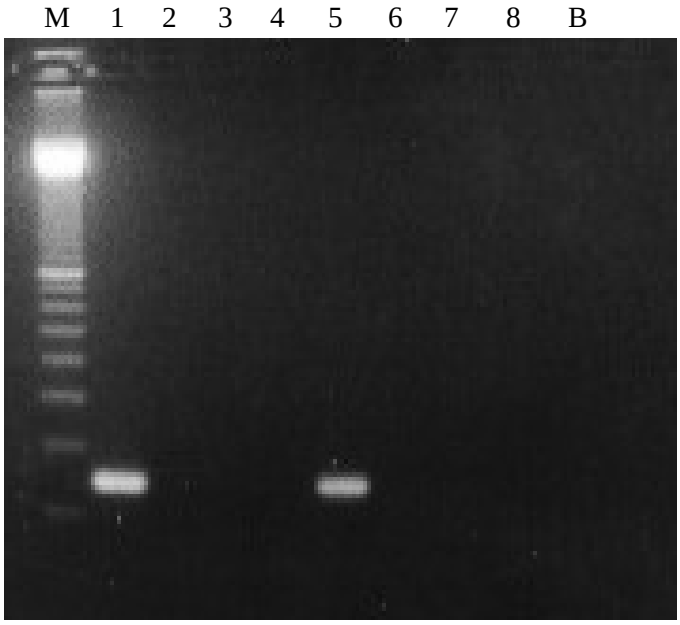
Table 1: Sequence similarity between fragments isolated by AFLP in commercial strains and the *Saccharomyces* Genome Database (SGD).

Fragment number	Yeast	% similarity to SGD	Homologous gene name
838-1	AWRI 838	95%	5’ of *APC11*
838-2	AWRI 838	99%	YIL121W
939-1	AWRI 939	60%	3’ of *CDH1*
939-2	AWRI 939	97%	*HOS3*
939-3	AWRI 939	97%	YGR237C
939-4	AWRI 939	98%	5’ of YAL064W-B
939-5	AWRI 939	100%	YPR158W
939-6	AWRI 939	100%	*PIF1*
1017-1	AWRI 1017	90%	YML125C
1017-2	AWRI 1017	96%	5’ of *MAK10*
1017-3	AWRI 1017	97%	YKL187C
1017-4	AWRI 1017	97%	3’ of *YPS7*
1017-5	AWRI 1017	98%	*CDC4*
1017-6	AWRI 1017	98%	*TAF14*
1017-7	AWRI 1017	98%	*SPT6*
1017-8	AWRI 1017	99%	*CCT5*
1017-9	AWRI 1017	99%	*PCM1*
1017-10	AWRI 1017	100%	3’ of YDL218W
1116-1	AWRI 1116	77%	*KIN1*
1116-2	AWRI 1116	79%	YGR131W
1116-3	AWRI 1116	79%	YIL137C
1116-4	AWRI 1116	85%	*UGP1*
1116-5	AWRI 1116	85%	YMR310C
1116-6	AWRI 1116	86%	5’ of *YOX1*
1116-7	AWRI 1116	98%	*CNE1*
1116-8	AWRI 1116	98%	YGR043C
1116-9	AWRI 1116	98%	5’ of *SOD2*

To determine the origin of these sequences and confirm the presence of the sequence in other yeast, primers specific to the sequences of interest were used to probe the original yeast from which they were isolated, as well as other sensu stricto yeast. The PCR results indicate that 1116-2 and 939-1 are present in two wine strains AWRI 1116 and AWRI 939 respectively (Figure 4). An interesting observation from the PCR results was the amplification of the 1116-2 sequence in the newly described sensu stricto species *Saccharomyces kudriavzevii* (Naumov *et al*., 2000). Previous studies using fluorescent AFLP as a molecular typing technique suggest that AWRI 1116 is an *S. cerevisiae* strain (de Barros Lopes *et al*., 1999). The results in this study imply that AWRI 1116 is a *S. cerevisiae* / *S. kudriavzevii* hybrid yeast. To examine this possibility, further sequence surrounding 1116-2 upstream and downstream was obtained using vectorette PCR.

The 1116-2 sequence was extended upstream and downstream to a total of 911 bp, which incorporated 340 bp of upstream sequence, a 525 bp open reading frame, and 46 bp of downstream sequence (Figure 5). The 911 bp sequence was then probed against all available databases to check for homologous sequences, with the results showing a 100% identity match to an *S. kudriavzevii* contig sequence (Washington University Genome Sequencing Centre (WUGSC) database; Cliften *et al*., 2001). To determine the origin and presence of this *S. kudriavzevii* sequence isolated from AWRI 1116, Southern analysis was used. Using a variety of yeast strains, genomic DNA was prepared from each yeast, digested with a restriction enzyme (*Eco*R1 or *Hind*III), the resultant fragments run on a gel and hybridised to a nylon membrane.

(A)



(B)

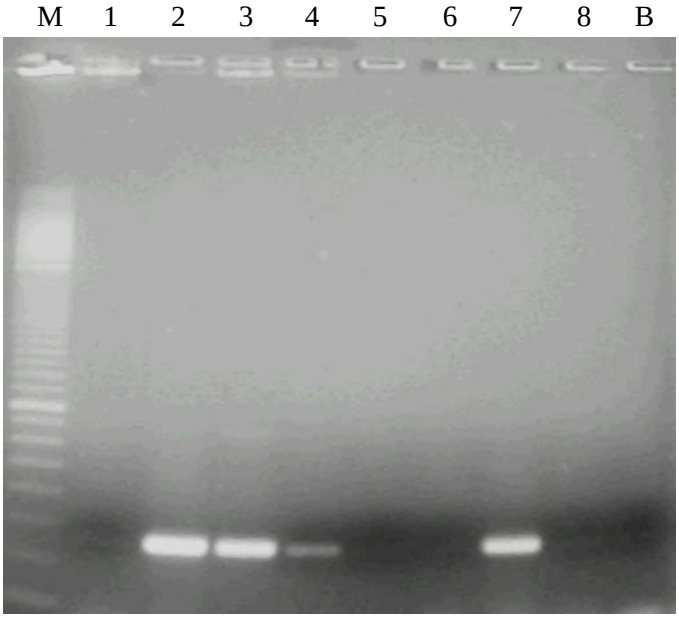


Figure 4: PCR analysis of *Saccharomyces* sensu stricto species for the presence of the (A) 939-1 and (B) 1116-2 fragments. Genomic DNA was isolated from stationary phase cells using standard protocols (Sambrook and Russell, 2001). Lane M: 100bp Marker (Invitrogen); Lane 1: Clone with 939-1 fragment; Lane 2: Clone with 1116-2 fragment; Lane 3: AWRI 1116 (new DNA preparation); Lane 4: AWRI 1116 (old DNA preparation); Lane 5: AWRI 939; Lane 6: *S. mikatae*; Lane 7: *S. kudriavzevii* NCYC 2889; Lane 8: *S. paradoxus*; Lane B: Blank.

5'
GTCGGTGATCAGTGCCGCTAAAATATTGCGCATGTGTTTTCAAGGATTTTTAGCCTTTTACGAACAAT
TTGGCGGCAGGCCGTTTAGAATCTGTTGAAGATTCCTTTTACGAATGACAAAGCTGTGTGTCAGCCAA
GCGGCCCAAAAGCAAGATCTGTATTGCGCCTAGGGTGTAGATAGATATAAGATCAAAATTATTGAATA
CATATCAGTATAAACTTGTAGTAGTTTGTGTTAATTCTTATTCGTGTAATGAGAGTAAGTGAAAAGAG
ACTATCAGGCTTTTGGAAGACTTTGTATTCACACAATCGCGATAATAACAATTAAATAACAACAATAT
ATGTTATCTGCTGCAGACAACCTAATACGCATTGTGAGCGCTGTTTTCCTCATCATATCGATAGGCCT
AATCGGCGGCTTGATGGACACACAAACAAACGGTAGTTCCCGAGTGAACTTTTGTATGTTTGCCGCGG
CTTACGGTCTAATCACTGATTCATTTTACGGGTTTTTGGCAAATTTTTGGTCATCGTTAACATATCCG
GCAATTTTGCTTGTTCTGGATTTTCTAAATTTTCTATTTACGTTTGCAGCAGCTACTGCTTTAGCCGT
CGGTATAAGATGCCATTCGTGCAAAAACAAAACTTACCTGGAGCAGAATAACATCACTCAAGGTTCAA
GTTCCAGATGTCATCAATCACAAGCTGCTGTAGCATTTTTCTATTTTTCCTGTTTCCTGTTTCTGATT
AAAGTGACGGTTTCGGTAGCTGGTATGATGCAAAATGGCGGATTTGGCTTTAATACCGGTTACGGTAG
AAGAAGGGCAAGAAGACAGATGGGAGTGCCCACCATTTCTCAAGTTTAGGTCAGCCAGGTCAAACGAA
AGGCGCTCGGTATTTGAAGGAAAGGAC
3′

Figure 5: Extended sequence of fragment 1116-2 using vectorette PCR. This sequence includes the promoter region (340 bp), an open reading frame (525 bp, shown in blue), and downstream sequence (46 bp). The 1116-2 fragment isolated by AFLP is underlined.

A DIG (digoxigenin)-labelled DNA Molecular Weight Marker VII (Roche) was used. A DIG probe of the 1116-2 fragment from AWRI 1116 (Table 1) was prepared, hybridised to the membrane, and detected using a chemiluminescent assay. The results in Figure 6 show the 1116-2 fragment was present in AWRI 1116, *S. kudriavzevii* NCYC 2889, and the tri-brid CID1 (Groth *et al*., 1999). The size of this fragment is approx 2600bp, which is close to the expected band size from theortical *Eco*R1 restriction digests of upstream and downstream sequence of AWRI 1116-2 in *S. kudriavzevii* of 2593bp. However, this fragment was not present in UVAFERM CEG (Lallemand). This was an unexpected result, as AWRI 1116 is thought to be the progenitor strain of UVAFERM CEG (Manfred Grossman, personal communication). To check the similarity of AWRI 1116 and UVAFERM CEG, and to determine if genome rearrangement of UVAFERM CEG had taken place, the genetic composition of these strains was examined further.

3.2.3. Confirming AWRI 1116 is a progenitor strain of UVAFERM CEG

The possibility of a commercial winemaking strain being a naturally occurring hybrid is of great interest for the wine industry and for ecological yeast research. Although UVAFERM CEG was not used in the initial AFLP screen for novel genes, this strain was expected to have the 1116-2 sequence and be genetically similar to AWRI 1116, as both arise from the Epernay region in France and have been listed as Epernay 2 (Manfred Grossman, personal communication). To test if the gene containing the 1116-2 fragment had undergone horizontal gene transfer, primers were targeted to amplify sequences in genes on both sides of the 1116-2 fragment, as

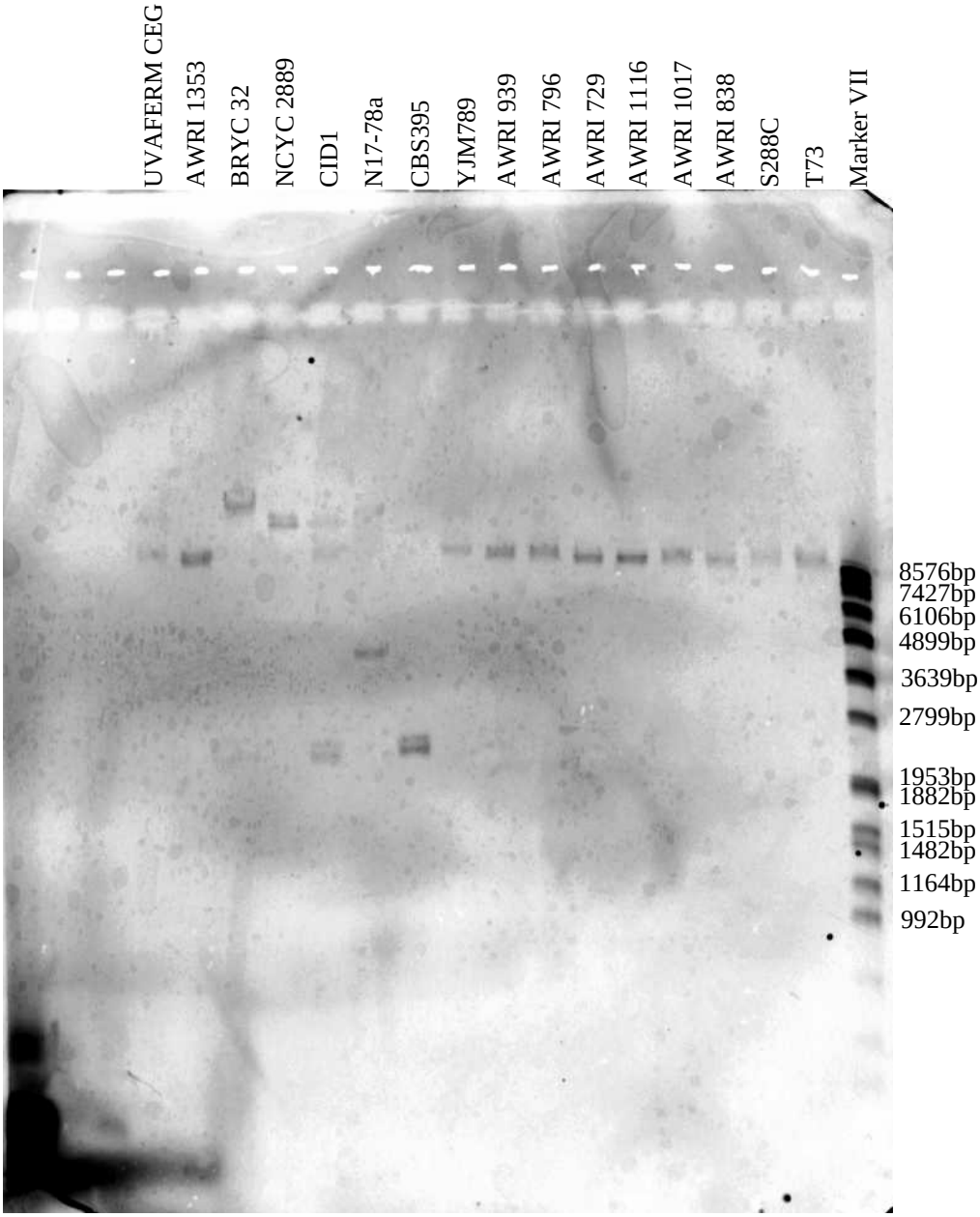
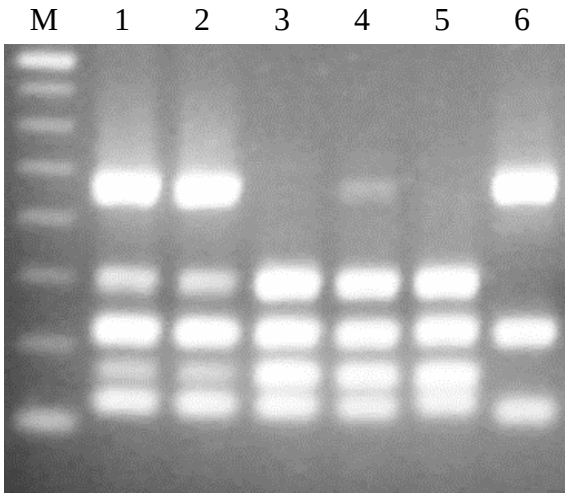


Figure 6: Southern analysis of genomic DNA from several *Saccharomyces* strains for the presence of the 1116-2 fragment. A digoxigenin probe of the 1116-2 fragment was hybridised to *Eco*R1 restriction enzyme digested genomic DNA, followed by hybridisation of an anti-DIG-alkaline phosphatase secondary antibody. The marker is DIG-labelled DNA Molecular Weight Marker VII (Roche). The chemiluminescence after development was scanned by a STORM 860 phosphoimager.

well as primers at the ends of Chromosome XII and Chromosome III (each homologous to genes either side of the 1116-2 *S. kudriavzevii* open reading frame). If only *S. kudriavzevii* sequences amplify in AWRI 1116, there may be a single horizontal gene transfer. If both *S. kudriavzevii* and *S. cerevisiae* sequences above amplify, it is more likely to be a hybrid strain of the two species. All sequences were amplified in both AWRI 1116 and UVAFERM CEG, hinting that these strains were hybrids with two sets of chromosomes: *S. cerevisiae* and *S. kudriavzevii*. To ensure we are dealing with a single strain, UVAFERM CEG was colony purified. PCR using 1116-2 fragment specific primers and internal transcribed spacer restriction fragment length polymorphism (ITS RFLP) were performed on these colonies. ITS RFLP is able to distinguish between *Saccharomyces* species based on restriction enzyme sites in the ITS region. Restriction digest fragments are run on an agarose gel, with each *Saccharomyces* sensu stricto species having a distinct banding pattern (Figure 7). Interestingly, the ITS RFLP results of UVAFERM CEG colonies show there are two distinct patterns – one with a predominant *S. cerevisiae* pattern and less intense *S. kudriavzevii* pattern (CEGSc), the other with a predominant *S. kudriavzevii* pattern and less intense *S. cerevisiae* pattern (CEGSk). Although the non-quantitative nature of PCR is recognised, these two patterns were consistently obtained. PCR using 1116-2 fragment specific primers indicated the colonies with the CEGSk ITS RFLP pattern were positive for fragment 1116-2, whereas the colonies with the CEGSc pattern were 1116-2 negative.

(A)



(B)

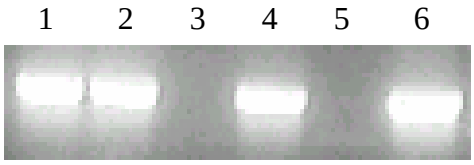


Figure 7: Analysis of *S. kudriavzevii* sequences using (A) ITS PCR RFLP and (B) PCR using specific primers to amplify the *S. kudriavzevii* sequence. Strains analysed were UVAFERM CEG (Lane 1), CEGSk (2), *S. cerevisiae* type strain CBS 1171 (3), CEGSc (4), S288Ca (5), and *S. kudriavzevii* type strain NCYC 2889 (6).

The hybrid nature of strain AWRI 1116 was not perceived in the initial AFLP experiments. To study this further, AFLP patterns of AWRI 1116, the UVAFERM CEG colonies, *S. kudriavzevii* and *S. cerevisiae* strains were compared. The results indicate that AWRI 1116 and the UVAFERM CEG isolates produce most of the AFLP fragments generated by other *S. cerevisiae* strains (Figure 8), suggesting that they possess most or all of the *S. cerevisiae* genome. The AFLP fingerprints of AWRI 1116 and the colonies with the CEGSk pattern also possess distinct fragments that are consistent with these DNAs being derived from *S. kudriavzevii*. This amounts to approximately 10% of the amplified bands in AWRI 1116 and the CEGSk pattern colonies. The results indicate that these yeast are unequal hybrids between the two related *Saccharomyces* sensu stricto species, although it is not possible to determine exactly how much of the genome is derived from each yeast as these species share many AFLP fragments (de Barros Lopes *et al*., 2002). The AFLP fragments amplified in the CEGSc pattern colonies were a subset of the AWRI 1116 and UVAFERM CEG fingerprints, with the *S. kudriavzevii* specific fragments absent. Therefore, although this yeast does possess *S. kudriavzevii* DNA as demonstrated by the rDNA ITS analysis, the CEGSc colonies may have lost a greater part of this second genome. It could also be suggested that CEGSc colonies have undergone loss of the repeats in the rDNA ITS region, as loss of ribosomal RNA genes is not uncommon in species hybrids (Casaregola *et al*., 2001).

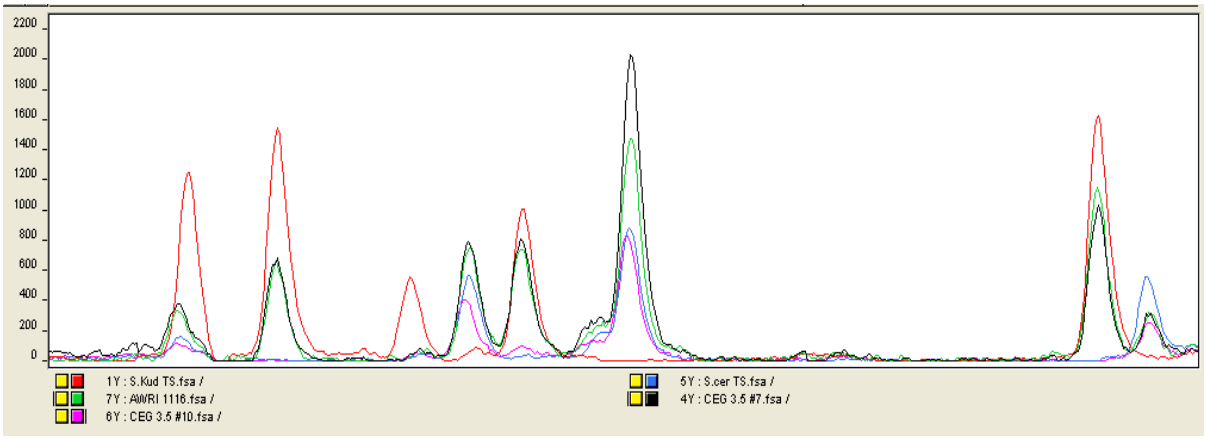


Figure 8: AFLP electropherogram of *S. cerevisiae* type strain S288Ca (blue), *S. kudriavzevii* type strain NCYC 2889 (red), AWRI 1116 (green), CEGSc (pink) and CEGSk (black). AFLP was performed as per Materials and Methods section 2.2, with fragments analysed using the GeneScan® Analysis Software (Applied Biosystems).

3.3. DISCUSSION

The physiological variability between industrial strains and the sequenced laboratory yeast is evident. These traits include the ability to undergo grape juice fermentation, increased stress resistance, the synthesis of distinct metabolites, and homothallism. The genetic basis for these altered phenotypes include translocations that lead to altered gene expression and chimeric proteins (Perez-Ortin *et al*., 2002), the presence or absence of telomeric repeated genes, including several sugar utilisation loci (Carlson *et al*., 1981; Michels *et al*., 1992; Pretorius *et al*., 1986) and the *RTM* (resistance to the toxicity of molasses) genes (Ness and Aigle, 1995) and single nucleotide changes, such as in the *HO* gene (Meiron *et al*., 1995). Generally, however, the genetic basis for the phenotypic variation that exists between *S. cerevisiae* strains remains to be established. Comparison of the known sequence of S288C with that of strains from different habitats may make it possible to search for adaptation by querying the genome directly. Winzeler *et al*., (1998) have used DNA microarrays to identify genes that are missing or present at higher or lower copy number in the clinical isolate strain YJM789. Using this method the authors were able to show that deletion of *PDR5*, a multi-drug resistance gene, was responsible for cyclohexamide sensitivity in strain YJM789. Genome and transcriptome comparisons between a laboratory strain and a commercial wine yeast have also been made (Hauser *et al*., 2001). In addition, Cavalieri *et al*. (2000) have demonstrated allelic variation between vineyard isolate populations using genome-wide transcriptional profiling. Unfortunately, the use of microarrays is limited to the study of already identified sequences. The finding that almost 1% of the

approximately 6200 genes in a laboratory strain are missing from the clinical isolate (Winzeler *et al*., 1999), and the reduced complexity of the AFLP fingerprint of the sequenced laboratory strains compared to several of the other strains studied, indicates that more *S. cerevisiae* genes remain to be identified.

Using AFLP as a measure of genetic variability between *S. cerevisiae* strains, it was found that the commercial wine strains and natural isolates exhibited genetic differences to S288C. It was assumed that, given the small genome size of *S. cerevisiae* and the large percentage of coding DNA, characterising the variable AFLP fragments would be useful for identifying novel genes in unsequenced industrial strains. Of the 27 different sequences identified in this study, 19 are similar to regions of known *S. cerevisiae* ORFs. Five of the 27 fragments show substantial nucleic acid sequence variation (between 77% and 85% similarity) to known ORFs posted on the SGD, while a sequence isolated from AWRI 939 shows little homology to *S. cerevisiae* (60%). These sequences appear to have distinct origins. Fragments 1116-2, 1116-3, 1116-5 and 1116-6 are identical to sequences in *S. kudriavzevii* and were most likely acquired by horizontal gene transfer. Allopolyploidy in *Saccharomyces* sensu stricto species has been previously described (de Barros Lopes *et al*., 2002; Delneri *et al*., 2003; Masneuf *et al*., 1998; Nguyen *et al*., 2000). For example, lager brewing strains, generally referred to as *Saccharomyces pastorianus* or *Saccharomyces carlsbergensis*, are hybrids between *S. cerevisiae* and a *Saccharomyces bayanus*-like yeast. In addition, a cider yeast has been shown to possess DNA from three species: *S. cerevisiae*, *S. bayanus* and *S.*

kudriavzevii (de Barros Lopes *et al*., 2002; Groth *et al*., 1999). While a hybrid wine yeast has also been described previously (Masneuf *et al*., 1998), this is the first reported example of a wine yeast possessing *S. kudriavzevii* DNA. Also, unlike the other hybrids studied to date, only a minor fraction of non-*S. cerevisiae* DNA is present in AWRI 1116, so the initial hybrid seems to have undergone extensive DNA loss. The commercial wine yeast UVAFERM CEG, believed to be derived from AWRI 1116, is a mixture of at least two strains, one very similar to AWRI 1116 and a second that has undergone further loss of *S. kudriavzevii* DNA.

Two fragments, also isolated from strain AWRI 1116, show 77% and 85% sequence similarity to regions in the *KIN1* and *UGP1* genes respectively. The *UGP1* gene (UDP-glucose pyrophosphorylase) is involved in both glucose metabolism and protein glycosylation, where it is thought to be essential for the proper formation of the cell wall. The *KIN1* gene encodes a protein kinase of unspecified function (Lamb *et al*., 1991). Whereas the predicted amino acid sequence of the *UGP1* fragment is similar to the sequence in the laboratory strain (96% identity), the *KIN1* homologue shows only 79% sequence identity. Unlike the *S. kudriavzevii* fragments described above, these sequences do not show identity to other *Saccharomyces* sensu stricto sequences, and therefore their derivation remains unknown. One possibility is that they originate by gene duplication and sequence divergence. Not only have specific genes, especially in subtelomere regions, been shown to be duplicated, the sequencing of the *S. cerevisiae* genome has provided evidence for an ancient autopolyploidy event that has resulted in approximately 13% gene duplication in the

sequenced laboratory strain (Wolfe and Shields, 1997). Interestingly, both the fragments showed slightly higher similarity to the *S. bayanus KIN1* and *UGP1* homologues than to the *S. cerevisiae* genes, thereby suggesting a common ancestor or preceding mating event between these two strains. Alternatively, the fragments are derived from an as yet uncharacterised species.

Although strain AWRI 939 produces an AFLP fingerprint that is the most different to S288C, the polymorphic fragments sequenced from this yeast were generally very similar to the sequenced yeast (97-100%). One of the fragments, however, showed only 60% similarity to a non-coding region in S288C. Although not characterised further, it is clear that sequence disparity in non-coding regions could alter gene expression and by doing so, modify the properties of particular strains.

In yeast, laboratory strains may not require loci that are needed for success in other habitats where adaptive mechanisms to environmental changes are necessary and competition with other species for resources is prevalent. Mechanisms of adaptation to different environments involve the expansion and contraction of the genome, potentially by either gene duplication or hybridisation. Following gene duplication, one of the gene copies is freed from stabilizing selection, creating the potential for evolution of a new function (Ohno, 1970). DNA alterations can influence either the protein activity or its expression. Similarly, allopolyploidy, which is widespread in plants and vertebrates, is predicted to improve adaptability under selective

conditions (Matzke *et al*., 1999). The variability in hybrid genomes caused by DNA instability enhances the genetic diversity.

3.4. CONCLUSION

The genome sequence of several eukaryotic organisms, including *S. cerevisiae*, *Giardia lamblii, Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans* and humans has been completed or is close to completion. One of the challenges in genome research is to relate the phenotypic variation that exists within a species to genetic variation. In this study, AFLP was used to determine the genetic diversity that exists between strains of *S. cerevisiae*. Isolating and sequencing polymorphic AFLP fragments has proven useful in identifying sequences not present in laboratory strains. Furthermore, AFLP has been used to further classify a commercial winemaking strain as a hybrid between *S. cerevisiae* and a recently classified species *S. kudriavzevii* (Naumov *et al*., 2000). At this stage, it is not clear whether these hybrids have advantageous winemaking properties. Studying the consequences of these genome changes will be useful for further understanding genotype-phenotype relationships.

CHAPTER 4

Comparative proteomics: Identifying differentially expressed proteins between two *Saccharomyces cerevisiae* strains

4.1. INTRODUCTION

Extensive phenotypic variation can exist between individuals of a single species. Elucidating the genetic basis for this variation, especially in the post genome era, has received considerable attention in view of its importance in a broad range of objectives including predicting human disease, defining biodiversity and improving agricultural yield. There is clear evidence that differences exist in the genome of winemaking strains compared to other strains of *S. cerevisiae* (Azumi and Goto-Yamamoto, 2001; Carlson and Botstein, 1983; de Barros Lopes *et al*., 1999; de Barros Lopes *et al*., 2002; Hauser *et al*., 2001; Heinrich *et al*., 2005; Perez-Ortin *et al*., 2002), however, how this relates to the phenotype and physiological processes within the cell is unclear.

As described in Chapter 3, there are many genome differences between laboratory and commercial strains, but determining the role or function of these sequences is difficult. The DNA comparisons do provide evidence that novel sequences exist in wine strains. To determine whether such sequences translate to changes in protein expression, or the synthesis of novel or modified proteins, a comparative proteome

approach was taken. There have been initial explorations by many groups to define the proteome of yeast under defined environmental conditions. These studies have lead to the construction of a two-dimensional polyacrylamide gel (2D gel) protein database of *S. cerevisiae* (Boucherie *et al*., 1996), the protein expression response of yeast subjected to environmental stresses (Akhtar *et al*., 1997; Hu *et al*., 2003; Norbeck and Blomberg, 1996), and a comparison between a hybrid lager strain and its parental species (Joubert *et al*., 2000; Joubert *et al*., 2001). More recently, the proteome of an indigenous wine strain has been reported (Trabalzini *et al*., 2003a), as well as the adaptive response of the same wine strain to glucose exhaustion in the surrounding medium (Trabalzini *et al*., 2003b).

In this study, a proteomic approach was taken to compare protein expression between laboratory and commercial strains of *S. cerevisiae*. It was expected that such an approach would identify differences in post-translational modification and proteins not present in the sequenced laboratory strain. By using an exploratory comparative proteomics approach, we hoped to begin to gain an insight into which factors play an important role in defining wine strain phenotypes. If this approach is successful, multiple wine strains could be compared and the persistent differences selected, thereby identifying wine strain-specific proteins. Furthermore, the proteins expressed in each strain after an ethanol stress were investigated. Ethanol produced during fermentation does not have the same impact on wine strains in terms of growth as it does in laboratory strains. It is anticipated that wine strains have evolved over time to catabolise the high sugar and tolerate the high ethanol

encountered during grape juice fermentation. This ability is even more important now as Australian winemakers are inclined to leave grapes on the vine longer to produce highly flavoured, 'fruit-driven' wines. A consequence of this increased ripening is grapes with elevated sugar levels, which generally result in higher ethanol wines after fermentation. Therefore, to focus on ethanol as an environmental stress encountered in grape juice fermentation, the proteomic response of laboratory and wine strains exposed to an ethanol stress will be compared.

4.2. RESULTS

4.2.1. Phenotypic differences demonstrate genetic variation in wine strains

4.2.1.1. Plate Assays – tolerance plates

To demonstrate the greater ability of wine strains to cope with certain environmental stresses compared to laboratory strains, plate-based stress assays were performed. Important parameters that are synonymous with wine yeast in their particular environment were chosen, such as temperature extremes, osmotic stress and ethanol stress.

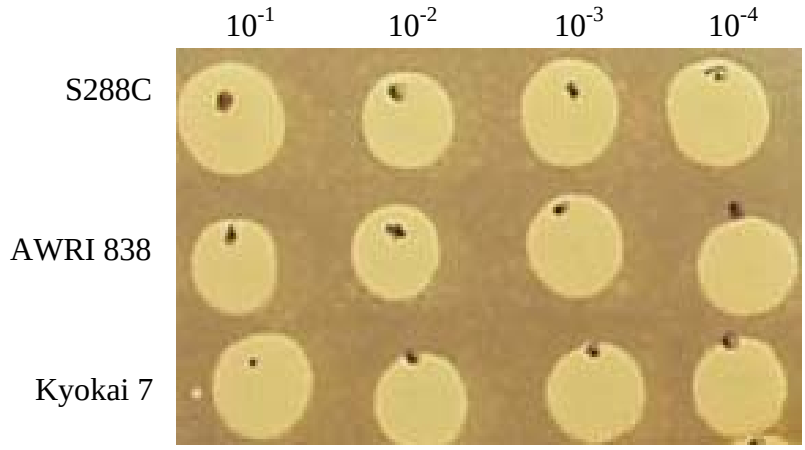
To investigate growth ability at different temperatures, agar plates with serial dilutions of each strain were incubated at temperatures between 4ºC and 40ºC. The results showed no differences in growth between strains (data not shown). To show the ability of wine strains to withstand a high concentration of glucose, SC medium plates containing between 100 g/l and 500 g/l glucose were used. Figure 3 shows that at the highest dilution wine strains are able to grow, whereas the laboratory

strain S288C was unable to grow. No strains were able to grow at the highest glucose concentration (500 g/l). Nevertheless, S288C was able to grow in the presence of 200 g/l sugar with lower dilutions. This result was surprising as it has been hypothesized that many laboratory strains cannot undergo grape juice fermentation due to the high concentration of sugar at the beginning of fermentation (Fleet and Heard, 1993). However, the strains show clear growth differences when exposed to ethanol. Upon increasing the ethanol concentration from 2.5% to 12.5% (v/v) in SC medium agar plates, AWRI 838 and a sake strain, Kyokai 7, used due to its predicted ability to withstand a high concentration of ethanol in sake mash, were more tolerant to ethanol than the laboratory strain S288C (Figure 9). Therefore, from these plate assay results, ethanol was chosen as the environmental stress to study using a comparative proteomics approach.

4.2.1.2. Metabolic differences exist between laboratory and commercial strains

Wine strains have been used in grape juice fermentation over thousands of years for their favourable properties, including the production of aroma and flavour compounds consistent with wine. Other strains have been tested in winemaking, however they do not derive the same metabolic profile that winemakers and consumers alike would prefer (Fleet and Heard, 1993). To characterise some of these metabolic differences between the wine and non-wine strains used in this study, the supernatant from small-scale fermentations of SC medium (80 g/l glucose) was analysed with high performance liquid chromatography (HPLC). Each strain was able to ferment all available glucose (unlike if more wine-like conditions were used

(A)



(B)

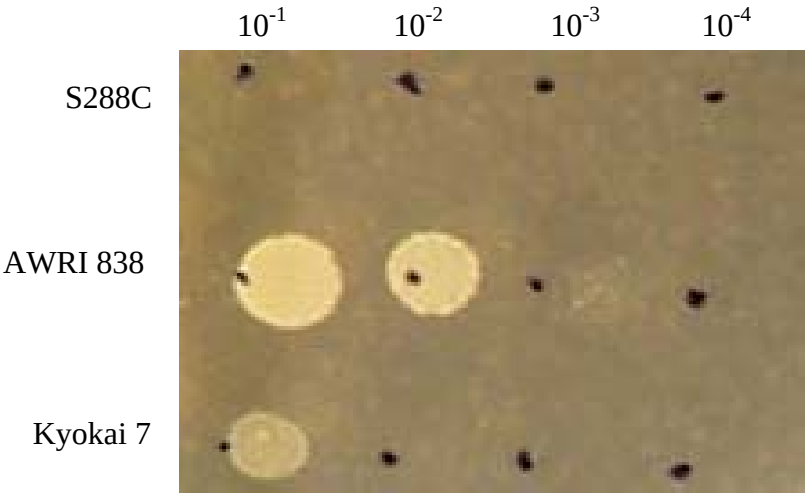
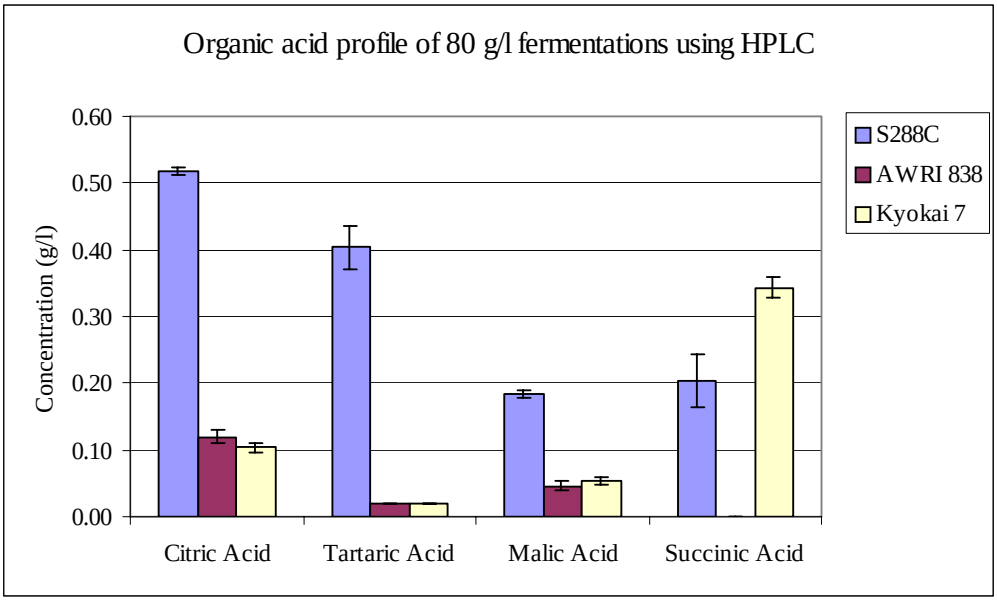


Figure 9: Comparison of ethanol tolerance displayed by different strains using SC medium agar plates containing (A) 2.5% (v/v) ethanol and (B) 12.5% (v/v) ethanol. Both the 0% (v/v) and 2.5% (v/v) ethanol plates displayed similar growth patterns for all yeast. Serial dilutions of each culture at mid-exponential phase (1 x 10^7 cells/ml) were spotted and incubated overnight at 28ºC.

as laboratory strains cannot ferment 200 g/l glucose) and produce a range of organic acids and wine-related compounds (Figure 10).

One of the more noticeable wine-related results is the lower production of acetic acid by the wine and sake strains. Although there are larger quantitative differences in other metabolomic compounds, acetic acid is a particularly unfavourable aroma attribute in wine in high concentrations and has generally accepted maximum limits in practice of approximately 0.8 g/l and a legal maximum limit of 1.5 g/l (Rankine, 1989). Glycerol is also an important wine component with respect to mouth-feel, and is present in higher concentrations in the metabolome of both wine and sake strains. The HPLC profile also showed that the wine and sake strains did not appear to produce as many organic acids (tartaric acid, lactic acid, malic acid) as the laboratory strain. It is possible that the organic acids in wine strains are metabolised further and therefore are not detectable in this study. This is only a preliminary observation based on the organic acids present at high enough concentrations to be able to measure. Overall, these results suggest that wine strains have a set of novel genes or expression patterns that contribute to these differences in metabolic profile. It will be important to elucidate the genetic basis for these strain-specific differences in order to understand how the wine strain genotype relates to its phenotype.

(A)



(B)

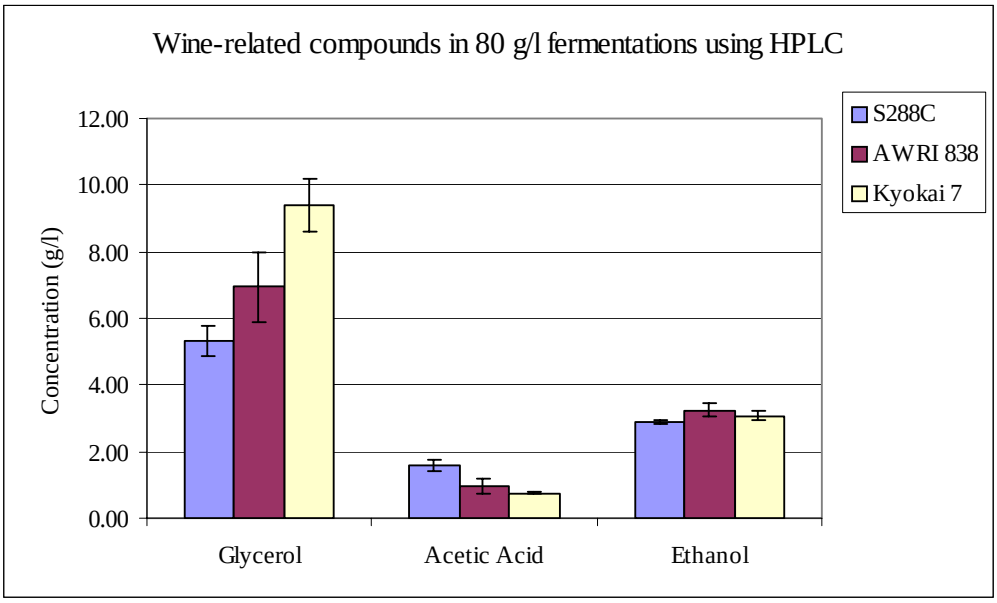


Figure 10: Metabolomic differences that exist between a laboratory strain (S288C), a winemaking strain (AWRI 838), and a sake strain (Kyokai 7). The organic acid profile (A) and other wine-related compounds (B) were analysed by HPLC performed by the Analytical Service at the AWRI.

4.2.2. Proteomic comparison shows protein expression differences between strains

4.2.2.1. Global proteome differences appear minimal between strains

2D gels provide a useful tool for comparing the expression of many proteins between strains. They are also serviceable for quantifying the number of proteins that change expression after an ethanol stress. This technique was applied to uncover differentially expressed or novel proteins in wine strains that might contribute to the wine strain phenotype. It is noted however that most hydrophobic proteins are excluded using 2D gel electrophoresis (Boucherie *et al*., 1995b; Hoving *et al*., 2002; Wildgruber *et al*., 2002), therefore membrane proteins that could be important for ethanol stress may not be identified in this study. Initially, total cell protein extracts taken from AWRI 838 (wine strain) and S288C (laboratory strain) grown in SC medium to mid-exponential phase were compared. To visualise the highest number of expressed proteins by these strains, pH 3-10 non-linear focusing strips were used. These focusing strips are the widest pH range available and can resolve up to 1200 proteins per gel (Boucherie *et al*., 1995b; Amersham Biosciences). After focusing the proteins for 33,860 Volthours, the strips were run on 12% non-gradient gels, developed and scanned. The resultant protein maps of each stain were superimposed and reproducible expression differences recorded. Despite the gels each having close to 800 distinct spots, there were minimal reproducible differences seen between strains (Figure 11). This result indicates that, although phenotypically dissimilar, the proteome between the strains (at least that of the proteins detectable using this technique) appears comparable. We sought to isolate, identify and characterise the

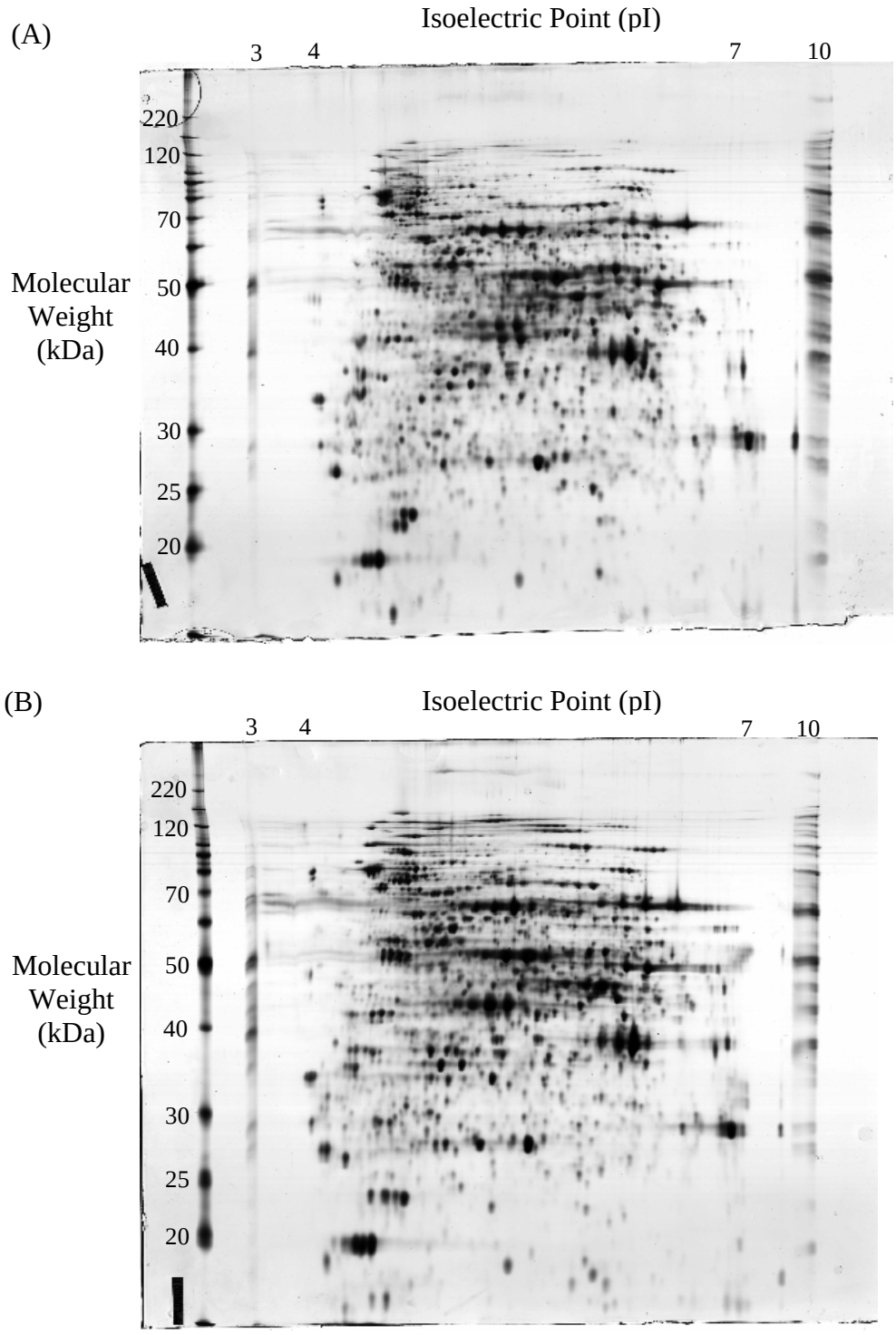


Figure 11: Proteomic comparison of (A) S288C and (B) AWRI 838 using 2D gels. The pH range was 3-10 NL (expanded 4-7 region), with molecular weight ranging from 220 kDa to 20 kDa. Total protein extracts were run on 12% non-gradient polyacrylamide gels and developed with silver stain.

proteins comprising these distinct spots in order to reveal proteins that may provide the phenotypic diversity between wine and laboratory strains. Several high-intensity stained proteins common to both wine and laboratory strains were isolated to obtain "landmark" proteins that could be used to orientate each gel and possible identify closely-located proteins (Table 2; Boucherie *et al*., 1995b). It was however noted that the protein maps resulting from the pH 3-10 non-linear focusing strips were crowded with protein spots and further resolution was required. Therefore, an attempt to expand the observed proteome and identify more protein expression differences was undertaken.

4.2.2.2. Expanding the observed proteome reveals more differences in protein expression

As there were many expressed proteins revealed by each strain using the pH 3-10 non-linear strips, it was possible that larger protein spots were masking lower abundant proteins with altered expression. To explore this possibility and to get better resolution of the proteome, smaller pH ranges were chosen. Initially, pH 3.5-4.5, 5-6, 4-7, and 6-11 narrow range strips were used and run once again on 12% non-gradient gels. After close inspection of these gels for protein expression differences and possible modifications, most protein expression differences between strains appeared to occur on the pH 6-11 gels (Figure 12). In addition, an ethanol stress applied to each strain did not show any reproducible significant (greater than two-fold) expression differences (data not shown). Nevertheless, the differences

Table 2: Proteins that were more highly expressed or modified in wine strain AWRI 838 compared to the laboratory strain S288C.

Observed MW (Da)	Observed pI	Protein identified as:	Fold higher in AWRI 838	Method of identification
35,000	6.6	Tdh3p	530[1] (46%)[2]	MALDI
35,000	6.7	Tdh3p	not present in S288C[3]	MALDI
35,000	6.8	Tdh3p	736 (48%)	MALDI
34,000	6.8	Tdh3p	not present in S288C	MALDI
33,000	6.7	Tdh3p	not present in S288C	MALDI
55,000	7	Shm2p	visible, but not detected in S288C	MALDI
55,000	7.2	Shm2p	115 (14%)	MALDI
55,000	7.4	Shm2p	not present in S288C	MALDI
55,000	7.6	Shm2p	not present in S288C	MALDI
35,000	6.6	Gre3p	visible, but not detected in S288C	MALDI
33,000	6.6	Mdh1p mod?	possible modification present 736 (48%)	MALDI
27,000	7.7	Por1p	not present in S288C	MALDI
27,000	7.2	Por1p mod?	similar spectra to POR1, 990 (41%)	MALDI
28,000	10.8	Rps4Ap	not present in S288C	MALDI
15,000	10	Rpl12p	not present in S288C	MS/MS
14,000	10.5	Rps19p	not present in S288C	MALDI & MS/MS
13,000	10.4	Rps31p	not present in S288C	MS/MS
14,000	10.6	Htb1p	not present in S288C	MS/MS
40,000	5.6	Fba1p mod?	not present in S288C	MALDI
40,000	5.4	Fba1p	Landmark protein	MALDI
47,000	6.2	Eno1p	Landmark protein	MALDI
60,000	5.8	Pdc1p	Landmark protein	MALDI

[1]Indicates the number of pixels present in AWRI 838.
[2]Indicates the percentage increase in pixel intensity in AWRI 838 compared to S288C.
[3]not present in S288C, but is present in AWRI 838.

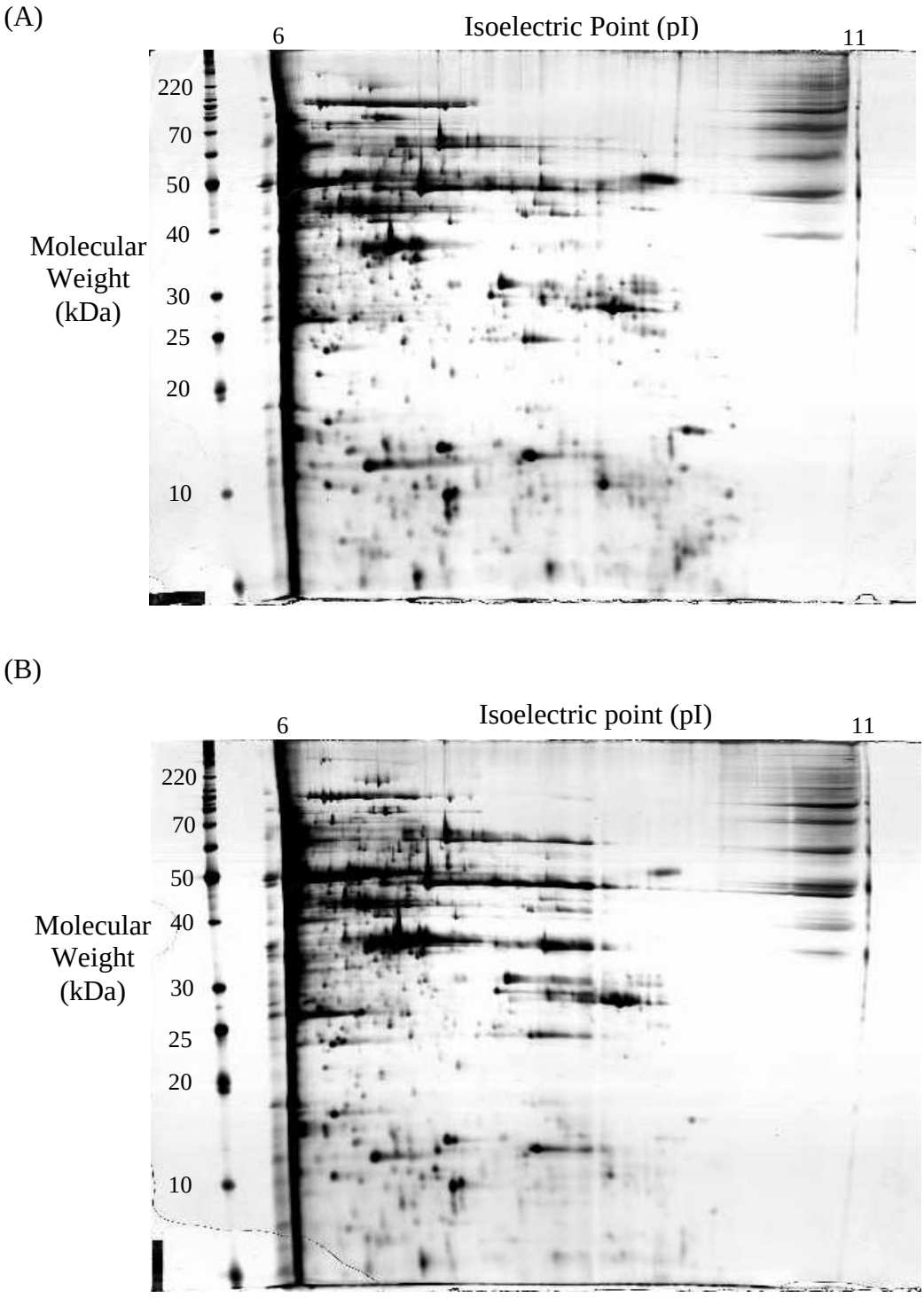


Figure 12: Proteomic comparison of (A) S288C and (B) AWRI 838. The pH range was 6-11, with molecular weight ranging from 220 kDa to 20 kDa. Total protein extracts were run on 12% non-gradient polyacrylamide gels and developed with silver stain.

between strains on the pH 6-11 gels warranted further investigation by isolation and identification of proteins to elucidate wine strain-specific proteins.

4.2.2.3. Identification of proteins that appear wine strain-specific

It can be difficult to get clear, reproducible 2D gels in the basic protein range (Hoving *et al*., 2002; Wildgruber *et al*., 2002). Therefore, due to the clarity and number of protein expression differences seen in the basic pH range, an attempt to isolate and identify proteins of interest from silver-stained gels was undertaken. Previous research has optimised the procedure for isolation of proteins from silver-stained gels (Shevchenko *et al*., 1996b), with subsequent identification by matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry. The resultant spectrum of a peptide fingerprint produced from the MALDI-TOF can then be probed against the MassLynx® *Yeast* database and the protein identified based on spectrum alignment. To use this technology in Australia, a collaboration with the School of Botany, University of Melbourne was established. However, using these procedures, proteins of interest from pH 6-11 silver-stained gels could not be identified.

To overcome this obstacle, protein extracts were taken to the Australian Proteome Analysis Facility (APAF) for 2D gel analysis, protein isolation and subsequent identification. During this visit, total protein extracts from each yeast strain were run in triplicate on Multiphor 2D gels, repeated on two different days (ie. 6 gels for each strain). Gels were then digitally montaged using ImageMaster (Amersham

Biosciences), creating an averaged gel of each yeast sample. Differentially expressed proteins and proteins that appeared modified were isolated (Figure 13), tryptic digested, and analysed by MALDI-TOF. Using the MassLynx software (Micromass), the peptide spectra were compared to theoretical digests and the proteins identified (Table 2). Although there is a large standard deviation due to pixel intensity between gel scans, the computer generated results were confirmed by eye and any insignificant results were discarded.

Tdh3p (glyceraldehyde 3-phosphate dehydrogenase) and Shm2p (serine hydroxymethyltransferase) were not detected on S288C gels, yet were present with five and four isoforms respectively on gels with AWRI 838 proteins. In addition, Gre3p (aldose reductase) and Rps4Ap (component of small ribosomal subunit) were not detected on S288C gels, yet in contrast were highly expressed in AWRI 838 (Figure 13).

A number of proteins were unable to be identified using the MALDI-TOF approach. To overcome this, tandem mass spectrometry (MS/MS) was used, which is a more powerful protein identification technique compared to MALDI-TOF. This approach provides sequence information as opposed to peptide fingerprints, and is therefore more specific for identification of unknown proteins. A further four proteins more highly expressed in AWRI 838 were identified using MS/MS (Table 2), which included three components of small ribosomal protein subunits (Rpl12p, Rps19p and

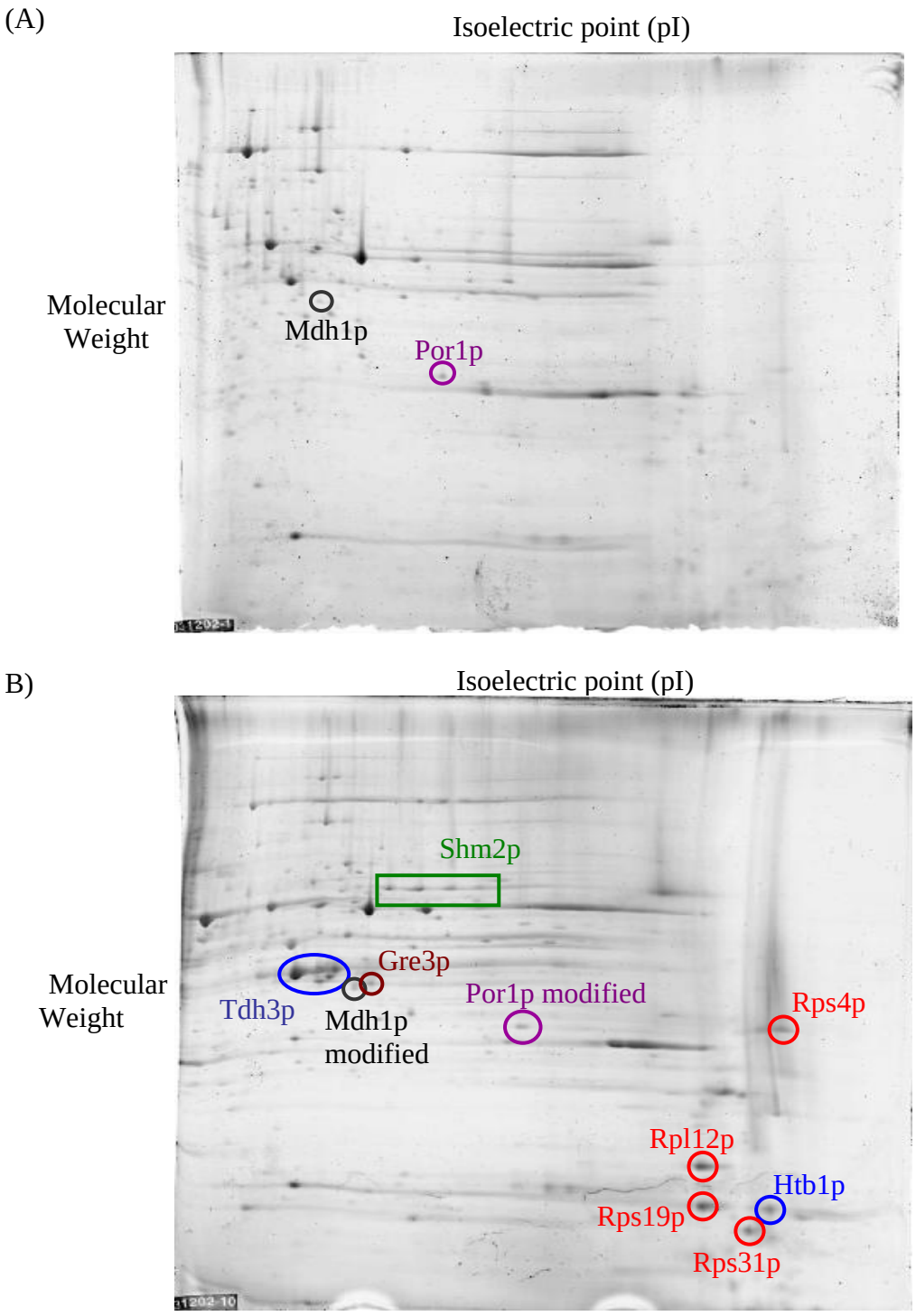


Figure 13: Proteomic comparison of (A) S288C and (B) AWRI 838 using 2D gels at APAF. Each figure is a montage of triplicate gels from duplicate protein extractions. The pH range was 6-11, with molecular weight ranging from 220 kDa to 20 kDa (no markers used at APAF). Total protein extracts were run on 12% non-gradient polyacrylamide gels and developed with SYPRO Ruby®.

Rps31p), and a DNA-binding histone protein, Htb1p or Htb2p (nearly identical sequence).

4.2.2.4. Protein modifications are present in AWRI 838

As mentioned previously, the advantage of proteomics is the possible identification of protein modifications. Proximal modifications are generally amino acid substitutions and can be visualised on 2D gels by the subtle alteration of the isoelectric point and molecular weight, while other modifications such as the addition or loss of a functional group induce greater distance shifts on a 2D gel. Confirmation of any modification is then determined by comparing MALDI-TOF spectra from the corresponding unmodified proteins in each strain with the possibly modified protein to identify common and novel peptide peaks. This putatively identifies the protein of interest, and provides an indication of the nature of the modification. Two possible modifications in AWRI 838 were seen using pH 6-11 gels at APAF based on resultant MALDI-TOF spectra: a modification of Mdh1p (mitochondrial malate dehydrogenase) with a lower molecular weight but the same isoelectric point, and Por1p (voltage-dependent ion-selective mitochondrial membrane porin) with altered isoelectric point and molecular weight. It is anticipated that this is a true reflection of the protein modifications that occur in AWRI 838 compared to S288C, and not a result of sample preparation as both yeast protein preparations were performed simultaneously.

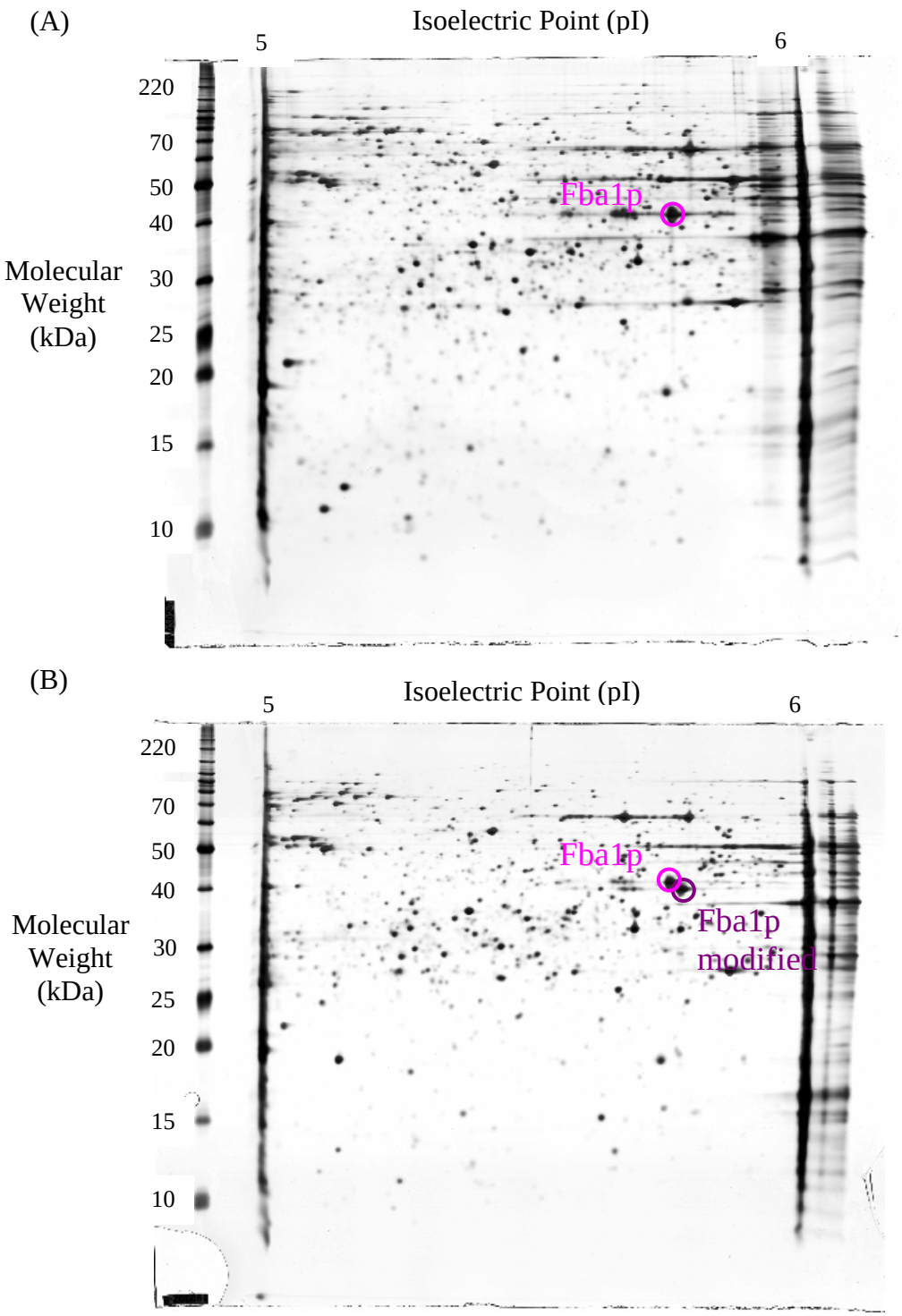


Figure 14: Proteomic comparison of (A) S288C and (B) AWRI 838 using 2D gels. The pH range was 5-6, with molecular weight ranging from 220 kDa to 20 kDa. Total protein extracts were run on 12% non-gradient polyacrylamide gels and developed with silver stain. The modified Fba1p is highlighted.

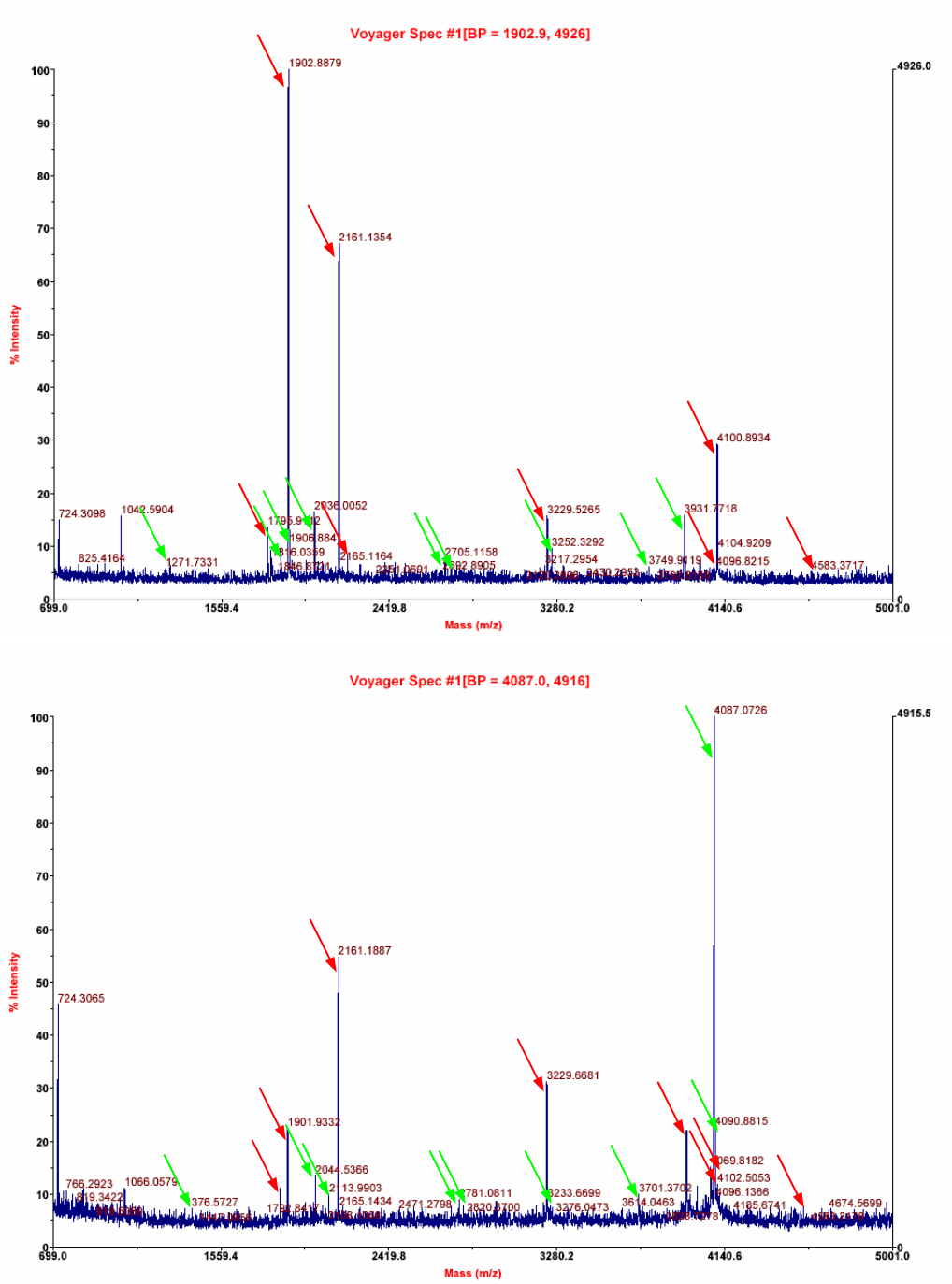


Figure 15: MALDI spectra of trypsin digests with Fba1p (top) and modified Fba1p (bottom). Common peaks are highlighted in red, while peaks unique to each spectra are highlighted in green. Peptides were searched for matches to proteins using the *Yeast* database available from MassLynx.

One putative modification present on pH 5-6 gels of AWRI 838 protein preparations was to the fructose bis-phosphate aldolase protein (Fba1p) (Figure 14). This was assumed due to its proximal location to Fba1p, which was identified by comparing the two maps of each strain with the 2D gel map of *S. cerevisiae* (Boucherie *et al*., 1996). The suspected modified Fba1p protein in AWRI 838, as well as the Fba1p present in both S288C and AWRI 838 strains (matched to 2D gel database), was isolated and tryptic digested. Half of the sample was run through an HPLC, the other half was subjected to peptide fingerprinting by MALDI-TOF. Spectra from both methods returned a similar result, with common peaks between Fba1p in both strains, as well as peaks unique to each protein (Figure 15). The peak intensity is not considered significant due to variation in efficiency of tryptic digestion and peptide extraction. The spectra obtained from MALDI-TOF were analysed for possible modifications such as phosphorylation, methylation, glycosylation, sulphation, acetylation and myristylation based on the size to charge ratio differences of the spectra, with no matches to any known modifications seen. It is possible that the unique peaks in the modified Fba1p are a combination of the possible modifications listed above. Alternatively, as the complete genetic sequence of AWRI 838 is unknown, the modified Fba1p may be produced from a second *FBA1* allele with a different coding sequence obtained through genetic recombination.

4.3. DISCUSSION

With phenotypic variation existing between commercial wine strains and the well-studied laboratory strain S288C, it was necessary to confirm some of these

differences in relation to the fermentation properties of these strains. These properties include the ability of wine strains to withstand high sugar and ethanol concentrations at the beginning and end of fermentation respectively, as well as the production of metabolites consistent with wine. To measure these parameters in the laboratory, plate assays investigating individual stresses and metabolic fingerprinting to identify wine strain-specific metabolites were utilised. It was clear from the plate assays that wine strains do exhibit phenotypes, in particular ethanol tolerance, that are different to the well-studied laboratory strain, and which are likely to be of benefit during winemaking. It was anticipated the sake strain (Kyokai 7) could be used to identify ethanol-related proteins in this study, as it is well established that sake mashes using this strain can reach alcohol contents higher than 16% (Kodama, 1993). However, the rice wine environment, in the presence of other organisms such as *Aspergillus oryzae*, is very different to plate assays, and may explain why there was not the anticipated increased ethanol tolerance by Kyokai 7. From AFLP analysis, the genetic variation between AWRI 838 and Kyokai 7 is seen to be high (de Barros Lopes *et al*., 2002), so it is likely that such genetic variation would also lead to phenotypic differences between these strains despite their common ability to withstand higher ethanol concentrations than S288C.

Although the proteome of a wine strain (a spontaneous yeast isolated from a fermentation that produces quality wines) has been reported (Trabalzini *et al*., 2003a), no proteins specific to wine strains that impart their phenotype were elucidated. Using 2D gels, there appeared to be protein expression and protein

modification differences between the wine and laboratory strains used in this study. These include higher expression of Tdh3p, Shm2p, Rps4Ap, Rpl12p, Rps19p, Rps31p, and Htb1/2p in the wine strain, as well as the presence of a putative modified Fba1p, Mdh1p, and Por1p in the wine strain. Although there is no obvious metabolic link between these proteins and the wine strain phenotype, each gene product may have a role in cellular functions such as metabolism or stress response. In addition, it was valuable to increase the resolution of total protein extracts from each strain to visualise more proteins per yeast protein extraction. To achieve this goal, pH 5-6 gels were utilised where a putative modification of Fba1p was seen. Although the results show many different peptides exist between the two Fba1p protein samples, there were enough common peptides in each protein to provide a confident match to Fba1p. This result, combined with the observed differential expression of glycolytic enzyme Tdh3p, may provide an insight into wine yeast metabolism, in particular possible variations to the glycolytic pathway in wine strains.

An interesting observation using 2D gels was the presence of two modified proteins of a mitochondrial origin, the mitochondrial malate dehydrogenase (Mdh1p) and a voltage-dependent ion-selective mitochondrial membrane porin protein (Por1p). These modifications in AWRI 838 might indicate a variance in mitochondrial role and function that has evolved due to exposure of this wine strain to a grape juice environment. Grapes used for winemaking typically have 5-8 g/l malic acid (Rankine, 1989), which creates an acidic environment, one that is generally made

less acidic via the malolactic bacterial fermentation (Fleet and Heard, 1993). In general, metabolism of extracellular malic acid by *S. cerevisiae* does not occur due to lack of an efficient uptake system and a converting enzyme such as malic enzyme or malate dehydrogenase. To counteract this, Volschenk *et al*., (2001) have overexpressed in wine yeast two malate permease genes, thereby enabling degradation of 8 g/l malic acid in synthetic must. It has also been reported that both expression of the malate dehydrogenase gene, *MAE1*, and activity of the encoded enzyme are increased under anaerobic conditions (Boles *et al*., 1998; ter Linde *et al*., 1999), thereby keeping within the physiological conditions encountered by wine strains during fermentation. It could be postulated that the wine strain AWRI 838 is able to utilise malic acid more effectively with the modified Mdh1p seen on 2D gels than the laboratory strain used in these experiments. This is supported by the metabolic profile of wine strains that had lower concentrations of malic acid present in the final metabolome after fermentation.

It has been reported in the literature that significant changes in gene expression occur after exposure of *S. cerevisiae* strains to an ethanol stress (Alexandre *et al*., 2001; Aranda *et al*., 2002; Betz *et al*., 2004; Carrasco *et al*., 2001; Garay-Arroyo *et al*., 2004; Palhano *et al*., 2004; Rossignol *et al*., 2003). It has also been reported that there are many changes to the proteome of a wine strain in glucose-depleted medium, mainly in carbohydrate metabolism, protein folding, and protein synthesis genes (Trabalzini *et al*., 2003b). Based on these reports, more differences in protein expression between strains after an ethanol stress than those actually observed here

might have been expected. There may be a number of reasons for the differences between these studies, including the concentration and time of exposure of cells to ethanol. We anticipated seeing protein expression changes after an ethanol stress to the yeast that related to the gene expression studies listed above. However, this was not forthcoming from the proteomics techniques used in this study. Possible reasons for this include the protein isolation procedures used, as well as the properties of the isoelectric focusing gels. Moreover, 2D gels do not display low abundant proteins effectively, thus it is difficult to visualise and subsequently identify these proteins (Shevchenko *et al*., 1996a). It may be that low abundant proteins such as signalling molecules have markedly changed expression levels between strains and yet are undetectable by the methods used here. Alternative strategies being implemented by other research groups include isotope-coded affinity tagged (ICAT) chromatography, isoelectric fractionation before 2D gel analysis (Corthals *et al*., 1997; Pedersen *et al*., 2003) and the changing of 'standard' protein solubilisation buffers to allow more proteins to be resolved (Rabilloud *et al*., 1997) and to increase their abundance for subsequent isolation and identification. If this could have been done in this study, a more accurate picture of protein expression differences between *S. cerevisiae* strains could have been achieved.

From the literature cited above, it was also expected that ribosomal proteins would be expressed less during an ethanol stress. However, in this study, there appeared to be no alteration in protein expression in either strain after an ethanol stress to the cells. It has been shown that an environmental stress has a larger impact on

ribosomal protein transcription than on rRNA transcription, which is predominantly due to transcriptional silencing of ribosomal protein genes and rapid turnover of their transcripts after stress (Li *et al*., 1999). It could be suggested that wine strains do not perceive the ethanol stress in the same manner as laboratory strains and therefore do not undergo transcriptional silencing to the same extent as laboratory strains. In addition, the wine strain may be preparing for an environmental stress such as an ethanol stress encountered during fermentation by producing ribosomal proteins before entering stationary phase, thereby providing an advantage to the cell in terms of stress response or energy preservation.

4.4. CONCLUSION

The aim of this exploratory study was to identify differences in protein expression between laboratory and wine strains. Ultimately, it was hoped that this would lead to development of methods for a large-scale comparative analysis and the identification of genes that are responsible for impacting on wine strain phenotypes, especially with regard to ethanol tolerance. Chapter 4 has demonstrated that a comparative proteomic approach has been advantageous for isolating and identifying some of these proteins differentially expressed between strains. Moreover, the power of proteomics has been displayed by the discovery of three possible protein modifications that would not have been seen using methods such as gene expression microarrays. It would be beneficial to further characterise the role and function of the differentially expressed proteins in wine strains, and to determine how relevant

these proteins are in winemaking conditions. This is investigated further in Chapter 5.

CHAPTER 5

Characterising the role and function of proteins that are differentially expressed in wine strains

5.1. INTRODUCTION

The use of proteomics as a tool for identifying protein modifications and protein expression differences between *S. cerevisiae* strains has been established in Chapter 4. An advantage of this approach over transcriptome analysis is that it allows the identification of proteins that are unique to wine strains and which may therefore contribute to the favourable properties of these organisms in grape juice fermentation. It also permits the identification of post-translational modifications that would be undetected by microarrays. By using this platform, the characterization of isolated proteins will in turn allow identification of related structural or regulatory genes, which may contribute to the wine strain phenotype.

Techniques such as quantitative real-time PCR and Northern analysis can be used to rapidly examine gene expression differences between strains under different environmental conditions, hence permitting further characterization of known genes and their possibly altered role in wine strains. Similarily, Western analysis can be used to observe or confirm protein expression differences that may exist between strains. If it is assumed that the number of gene transcripts in each strain also

correlates to the amount of protein synthesised therein, this will permit the confirmation of results obtained with proteomics in Chapter 4. Furthermore, it will allow the screening of many wine strains in various environmental conditions (eg. high ethanol) in order to determine the relevance of the more highly expressed proteins in wine strains. However, it has been implicated that the correlation between mRNA and protein levels using SAGE and 2D gel electrophoresis techniques respectively is insufficient to predict protein expression from quantitative mRNA data (Gygi *et al*., 1999). In contrast, Futcher *et al*. (1999) determined that the level of transcripts produced by a yeast cell equate to the number of proteins synthesised. If the latter were true in this study, the monitoring of other wine strains for the gene of interest under different environmental conditions could be achieved using quantitative real-time PCR.

Ultimately, the study of genes with different patterns of expression amongst wine strains may provide an explanation for the variation in physiological behaviour of wine yeast and the advantages that wine strains have over other *Saccharomyces* strains in highly stressful environments such as high sugar grape juices and high ethanol wine. Therefore, Western blotting and transcriptional profiling using quantitative real-time PCR was used in this study to confirm the protein results achieved using 2D gels. Furthermore, once confirmation of the more highly expressed proteins of interest in the wine strain had been obtained through correlation with gene expression data, the genes of interest were investigated further. Specifically, the degree of similarity in the sequence and pattern of expression of

corresponding structural genes was compared across a number of wine strains. In addition, the method of near infra-red spectroscopy (NIRS) was used to determine whether a high-throughput strategy could be developed to identify differentially expressed genes that influence the yeast metabolome, and by doing so, contribute to wine flavour. In this study, gene deletion mutants were used in order to determine whether a single gene affects the metabolome after fermentation by *S. cerevisae*, thereby elucidating a potential role for the gene in wine flavour. Although the findings are preliminary, the ability to identify yeast 'flavour genes' is of considerable importance to the wine industry, and the experiments described in this study for *SHM2* could be used in large-scale studies.

5.2. RESULTS

5.2.1. Confirmation of increased Tdh3p expression in AWRI 838 using Western blot analysis

Before further study of the genes encoding the differentially expressed proteins described in Chapter 4, it was necessary to confirm the proteomic data upon which identification was based. A convenient method for comparing protein expression between two samples, using either total cellular protein extracts or purified protein fractions, is Western blot analysis (Sambrook and Russell, 2001). To allow the use of this method to confirm increased glyceraldehyde 3-phosphate dehydrogenase (Tdh3p) expression in AWRI 838, an antibody to Tdh3p was acquired (kindly provided by Daniel Gozalbo, University of Valencia; Delgado *et al*., 2001). Unfortunately, antibodies to the other differentially expressed proteins still need to

be produced. Total cellular protein extracts from cells grown in minimal medium were prepared using the same procedure as the proteomic study. Each extract was run on a 4-20% iGel (Gradipore), transferred to a nitrocellulose membrane, and probed with the Tdh3p antibody. *TDH1*, *TDH2*, and *TDH3* deletion strains (using strain BY4742, Euroscarf), in conjunction with a glyceraldehyde 3-phosphate dehydrogenase enzyme (GAPDH, Sigma), were used as controls. As expected, the *TDH3* deletion strain did not exhibit an intense band in the 35kDa range that correlates with the GAPDH positive control. Deletions in the two related genes *TDH1* and *TDH2* produced a protein of the expected size. By using two different total protein isolations from two independent yeast cultures, it was shown and confirmed that Tdh3p was more highly expressed in AWRI 838 compared to S288C (Figure 16).

5.2.2. Real-time PCR suggests post-transcriptional control may exist in wine strains

To support the proteomics data obtained in Chapter 4 quantitative real-time PCR was used. Moreover, this technique provides the ability to screen other wine strains in various environmental conditions and determine the importance of the gene of interest amongst wine strains. RNA was extracted from yeast cultures at the same cell number in SC medium as the protein extracts were taken in Chapter 4 (ie. at mid-exponential phase). After standardising the amount of total RNA between samples, cDNA was synthesised and used in real-time PCR reactions. Reproducibility of each transcript sample was demonstrated by running triplicate

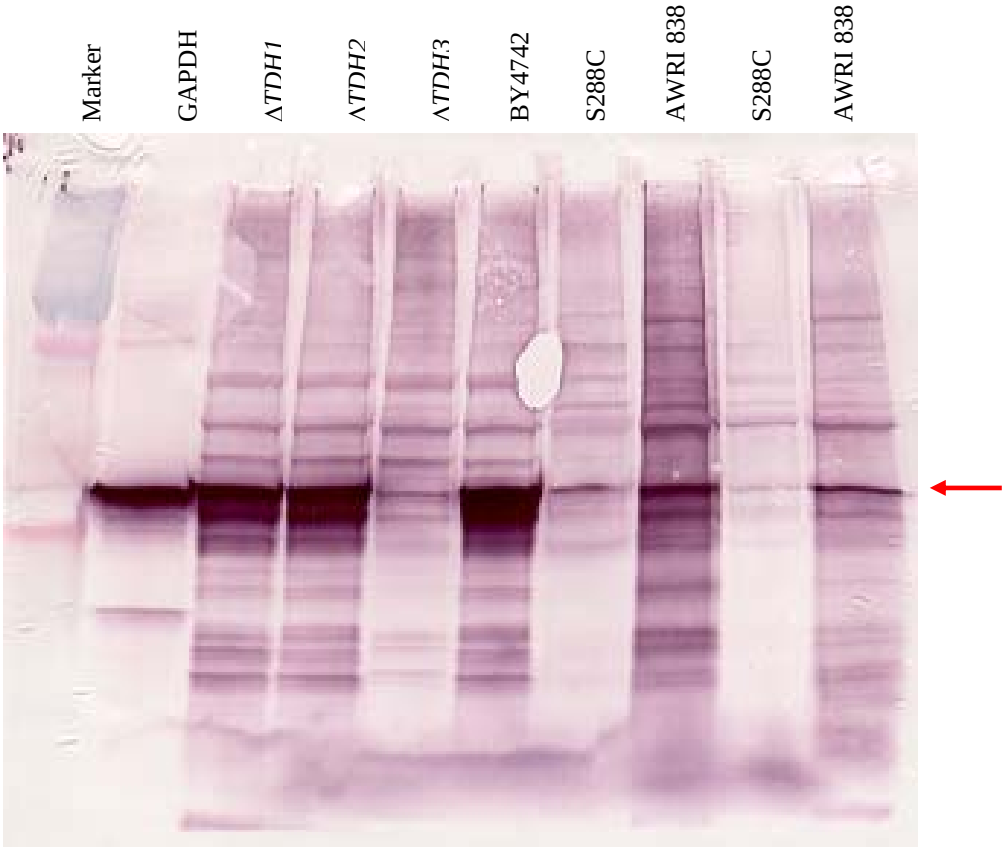


Figure 16: Western blot analysis of the total protein extracts from *TDH* deletion strains (BY4742), S288C and AWRI 838. Protein extracts from cells grown in SC minimal medium were taken at 1 x 10^7 cells/ml, run on a 4-20% iGel (Gradipore), transferred to a nitrocellulose membrane, and probed with a Tdh3p antibody. The red arrow indicates the band size for Tdh3p and the positive control (GAPDH). Schiff staining ensured protein loading was the same (data not shown).

samples from different RNA preparations isolated from the same strain. Using the resultant amplification curves and incorporating the standard curves for each primer set, it was shown that AWRI 838 had five times more copies of *TDH3* transcript than S288C. This correlates to the proteomics data from Chapter 4, which displayed significantly higher expression of Tdh3p in AWRI 838 compared to S288C.

However, of the genes analysed, *TDH3* was the only gene to have a greater than two-fold transcript number in AWRI 838 than S288C, which does not correlate with the 2D gel protein expression data. *GRE3*, *SHM2*, *HTB1* and the ribosomal subunit protein genes showed no significant difference in gene expression between AWRI 838 and S288C (data not shown).

Previous research has determined the gene expression differences that exist between a wine strain, T73, and the laboratory strain S288C using microarray analysis (Hauser *et al*., 2001). The microarray data showed that expression of *SHM2* in T73 was more than 2000-fold higher than seen for S288C. In the proteomics results of this study (Chapter 4), it was shown that the wine strain AWRI 838 had a higher protein expression level of Shm2p than that of S288C (some isoforms of Shm2p were not present in S288C), however the transcript abundance between these strains using real-time PCR showed no significant differences. Based on these results, the transcription of the *SHM2* gene in T73 was investigated in parallel with AWRI 838 and S288C in this study. The results showed a 16-fold increase in the *SHM2* transcript in T73 compared to S288C. It was of interest to see wine strain AWRI 838

possess higher levels of the *SHM2* protein and yet display no transcriptional difference relative to S288C, while for wine strain T73 there was an increase in *SHM2* transcript abundance at the same time point of fermentation. In addition, it was demonstrated that other wine strains also had higher *SHM2* transcript abundance at the same time point of fermentation (see 5.2.4.4). This suggests that *SHM2* is affected post-transcriptionally in AWRI 838, and may have different *cis*- or *trans*-acting elements compared to T73, which was observed to have higher *SHM2* expression using real-time PCR. To confirm the original proteomics data had not provided false leads regarding the higher expression of Shm2p, the 2D gels were repeated.

5.2.3. Replication of 2D gels confirms Shm2p and Tdh3p are expressed more highly in wine strain AWRI 838 than S288C

As the gene transcript data did not correlate well with the 2D gel protein data for all but one of the proteins of interest (Tdh3p), before analyzing the possible role of these genes during fermentation, the proteomic experiments were repeated. For this set of 2D gels, protein from biological replicates (three independent cultures) was extracted to ensure that any differences detected were solely due to the strain. Each sample was again run in triplicate, montaged, and protein expression differences recorded. In confirmation of previous data, both Tdh3p and Shm2p had two isoforms in AWRI 838 that were not present in S288C (data not shown). Also, the Mdh1p that appeared to be modified in the initial 2D gel experiments was present again in AWRI 838 gels but not in the S288C gels. Unfortunately, the changes identified in

the previous study were not consistently observed. Although the reason for this is not fully known, it may be that minor changes in growth conditions and phase affect their expression.

Tdh3p expression was confirmed by Western blot analysis, but attempts to discover the mechanism of higher Tdh3p expression in AWRI 838 compared to S288C were not successful. This result was obtained by comparing the promoter sequences of the sequenced 'wine *TDH3* gene' to the 'laboratory *TDH3* gene' sequence in the S288C database, with no clear evidence in the sequence information for the Tdh3p expression (data not shown). The sequencing of the *SHM2* promoter region in AWRI 838 was also carried out and compared to the S288C database, with seven base changes observed (see 5.2.4.3). Therefore, the enzyme Shm2p, which is involved in conversion of serine to glycine in the folic acid biosynthesis pathway, was considered worthy of further investigation as enzymes involved in the one-carbon pool are likely to play a role in central metabolism.

5.2.4. Further examination of SHM2

5.2.4.1. Near infra-red spectroscopy reveals *SHM2* impacts on the metabolome

Serine hydroxymethyltransferase (*SHM2*) is a cytoplasmic enzyme involved in the conversion of serine to glycine and plays an important role in the one-carbon pool. To determine if the *SHM2* gene is required for growth in minimal medium, a deletion strain for this gene was compared to a wild-type laboratory strain. The results showed no discernable differences in growth rate, cell morphology, or final

cell number (data not shown), which suggested this gene does not play an essential role in central metabolism of laboratory strains. As subsequent metabolic pathways lead to compounds that may contribute to wine sensory properties, and there was no impact of gene deletion on phenotype detected, the potential for this gene having an impact on the final metabolome was investigated.

To demonstrate how the metabolic profile of deletion strains can be compared to a wild-type strain, Allen *et al*. (2003) have previously used GC-MS to discriminate deletion strains with various functions that are expected to impact on the metabolome. The results showed a clear discrimination between each of the 27 deletion strains tested, with genes of similar function grouping more closely than those with very different functions. To study the possibility of *SHM2* influencing the formation of wine sensory compounds, near infra-red spectroscopy (NIRS) was chosen. NIRS separates a complex matrix based on the chemical bonds that exist in the sample media, and is a rapid technique that, in contrast to other techniques such as GC-MS, requires minimal sample preparation. Briefly, 250 mL fermentations were undertaken in SC medium with 8% glucose. When all glucose had been consumed, the yeast was pelleted, and the supernatant analysed by NIRS. Using principle component analysis (PCA) plots, the results showed the separation of the *shm2*Δ strain from the wild-type BY4742 laboratory strain (S288C background), suggesting that different profiles of metabolites were being released by the two strains (Figure 17). To support this finding, a deletion strain with a gene of similar function, *MUP3*, a methionine permease gene, was also analysed for comparison to

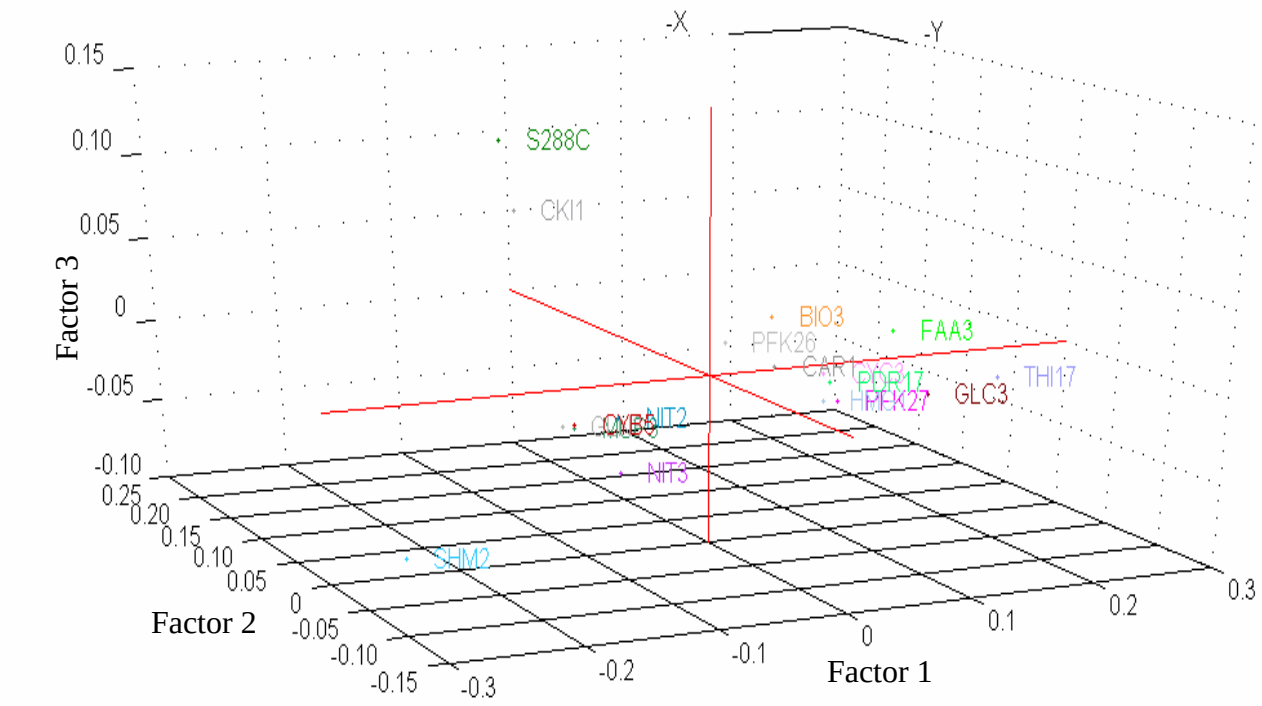


Figure 17: Principle Component Analysis (PCA) plot showing the separation of the *shm2* strain from S288C using near infra-red spectroscopy (NIRS) to analyse culture supernatants from each strain. This technique separates different strains based on -OH, C-H, and N-H vibrational bonds.

the wild-type and shown to be more similar to the *shm2*Δ strain than the wild-type by NIRS. To illustrate how different the metabolic profile of these two deletion strains are to the wild-type, similar experiments with the same strains used by Allen *et al*. (2003), which used GC-MS to discriminate 27 deletion strains, were carried out to validate the method and to check for metabolic differences in the fermentations using NIRS. With the exception of the *ure2*Δ strain, every strain trialed was seen to fall between the *shm2*Δ strain and the wild-type (Figure 17). This confirmed that deletion of the *SHM2* gene resulted in a metabolic profile distinct from that of the wild-type and other deletion strains. It could then be expected that yeast strains, including wine yeast strains, with altered gene expression of *SHM2* could produce a unique metabolic profile during or after fermentation. The strategy developed here, although preliminary, will be important for future studies, as it can be adapted for high-throughput metabolic studies for many genes of interest.

5.2.4.2. Southern analysis shows multiple alleles are not present in the wine strain genome

The potential effect of changes in gene expression of *SHM2* on metabolites and the apparent differences in regulation of the gene in AWRI 838 and T73 lead to a further study of the regulation of this gene. For this, the sequence of the *SHM2* gene, especially the possible regulatory sequences, was determined in the laboratory and wine strains. It was possible sequencing *SHM2* in each of S288C, AWRI 838 and T73 would resolve the mechanics of the higher expression of *SHM2* in T73, and possibly Shm2p synthesis in AWRI 838. Due to the aneuploid nature of wine strains,

it was necessary to ensure there was only one allelic copy of *SHM2* being sequenced. To demonstrate this, Southern analysis was implemented. Briefly, genomic DNA was prepared from each yeast strain, digested with a restriction enzyme, the resultant fragments run on a gel and transferred to a nylon membrane. A digoxigenin-labelled probe for the *SHM2* gene was prepared, applied to the membrane, and detected using a chemiluminescent assay. The resultant Southern blot suggested that it is unlikely that multiple copies of *SHM2* were present throughout the genome of wine strains, since the banding patterns are equivalent to that of the S288C strain (Figure 18). The largest band present in all strains at approximately 11,700 bp was *SHM1* (calculated by restriction analysis using SGD). Having confirmed that all strains were likely to only carry one *SHM2* allele, the sequencing of this gene in each strain was undertaken.

5.2.4.3. Sequencing of the *SHM2* gene shows seven base substitutions in wine strains

Sequencing of the *SHM2* gene was carried out to help define the mechanism behind the differences seen between laboratory and wine strains in gene and protein expression. Primers for sequencing were designed to amplify 370 bp of upstream sequence from the initial ATG, the *SHM2* coding sequence, and 167 bp of termination sequence following the STOP codon. Each primer was designed to amplify from within the genes upstream and downstream of *SHM2* to encapsulate any regulatory sequences to *SHM2*. This approach was performed to ensure a high likelihood that all promoter and terminator sequences would be incorporated. Strains

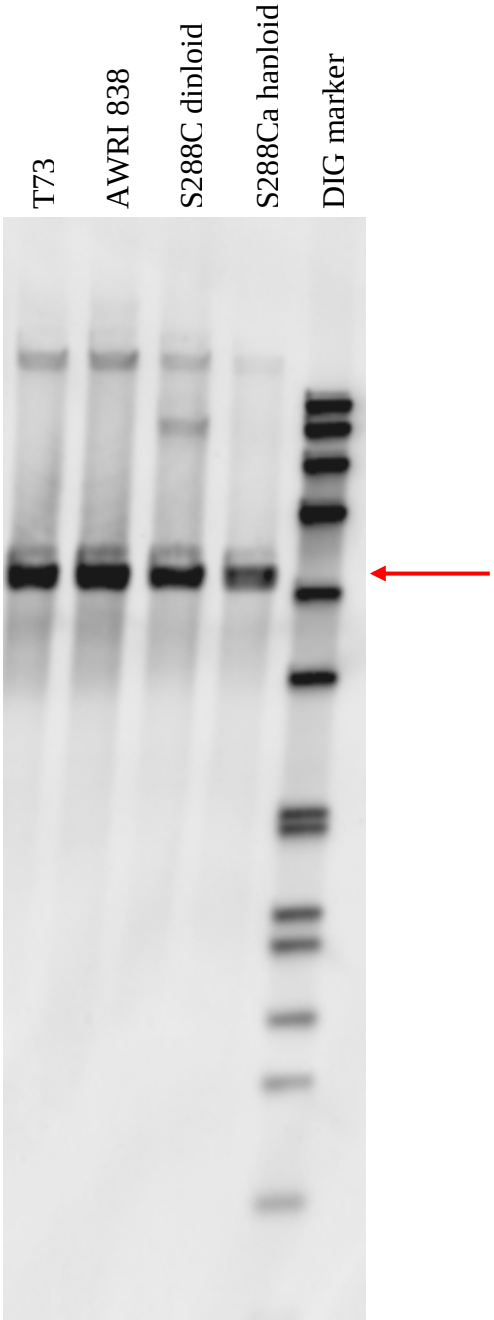


Figure 18: Determining *SHM2* gene copy number using Southern analysis. Genomic DNA of each strain was restriction digested with *Nco*I and fixed to a nylon membrane. After hybridising a DIG-linked probe for *SHM2*, the membrane was developed using chemiluminescence. Using SGD, the arrow indicates the size of the expected fragment generated by the *Nco*I digest.

S288C, AWRI 838 and T73 were sequenced, revealing the identical sequence in both AWRI 838 and T73. Seven differences were found upon comparison of the sequence of the wine strains and the laboratory strain: two in the promoter (C-70A, T-66G), four within the *SHM2* coding sequence, all of which are silent mutations (C192T, G350A, C473T, C1133T), and one in the terminator (A75G), (although the silent mutations will not change the translated amino acid coding sequence, it cannot be ruled out that they do not affect transcript levels). As for the remaining changes, the substitution in the termination site occurred after the predicted polyA tail and is therefore unlikely to have an effect on the cessation of *SHM2* transcription. The significance of the base differences in the promoter were examined by interrogation of the wine yeast sequences using the *Saccharomyces cerevisiae* Promoter Database (SCPD). After examination of all base substitution permutations, these searches revealed the C-70A resulted in the loss of a possible heat shock transcription factor (HSTF) binding site in the wine strains. This notion requires confirmation through mutation analysis to see if this base substitution has an effect on *SHM2* gene expression. Given that AWRI 838 and T73 have the identical sequence, it is unlikely that the differences in *SHM2* expression seen between these strains are the result of a *cis*-acting element. Therefore, it is likely that the expression differences are the result of *trans* factors.

5.2.4.4. Wine strains exhibit differential *SHM2* gene expression in varying environmental conditions

To determine the prevalence of *SHM2* gene expression in multiple wine strains and what impact it may have on wine strain phenotype, several wine strains were taken for analysis. Eight wine strains were grown in two different media, SC medium and yeast extract-peptone-dextrose medium (YPD). These two media were chosen as the proteomics study used SC medium for yeast growth, while the Hauser *et al*. (2001) research that saw high *SHM2* expression with strain T73 used YPD. The strains were chosen based on different regions from where they were isolated and their genetic dissimilarity (de Barros Lopes *et al*., 1999). Total RNA was extracted from cells at 1 x 10^7 cells/ml. After making cDNA, equivalent amounts were added to quantitative real-time PCR reactions, and the amplification curves from quantitative real-time PCR were normalised to actin (*ACT1*) expression. *ACT1* was used as it has been shown in the literature to not change its expression after the cell encounters a stressful environment (Gasch *et al*., 2000; Causton *et al*., 2001; Chandler *et al*., 2004), therefore it can be utilized to equate cDNA concentrations in two different samples. The results showed no significant gene expression differences between strains after growth in SC medium. However, when cells were grown in YPD, seven wine strains showed greater than two-fold expression of *SHM2* compared to the laboratory strain (Table 3). Interestingly, the only wine strain not to have greater than two-fold expression of *SHM2* transcript was AWRI 838, which corresponds well to the previous quantitative real-time PCR experiments used to support the proteomics data (5.2.2).

Table 3: *SHM2* gene expression differences between wine strains and S288C using real-time PCR. All gene transcripts have been normalised to actin (*ACT1*) in the same strain. Yeast were grown in YPD medium and RNA extracted with cells at mid-exponential phase (1 x 10^7 cells/ml).

Strain	*SHM2* expression relative to S288C (x-fold)
AWRI 729	12.8
AWRI 796	13.8
AWRI 825	3.0
AWRI 838	1.2
AWRI 844	2.8
AWRI 870	5.0
AWRI 1017	2.5
T73	3.7
S288C	1.0

5.3. DISCUSSION

The ability of *S. cerevisiae* wine strains to undergo grape juice fermentation and produce favourable sensory compounds is well established. However, the genes and proteins that are responsible for wine strain phenotypes have not been elucidated. The aim of this chapter is to develop methods to identify 'wine genes' and to characterise genes that are potentially associated with the wine strain phenotype. It was anticipated that by taking a comparative proteomics approach, it would be possible to elucidate yeast genes that have an important role in winemaking, in particular those genes that might play a role in ethanol stress resistance and in the synthesis of compounds that impact on wine flavour.

In Chapter 4, the use of 2D gels showed a complex matrix of total cellular protein could be separated to create protein maps for isolation and identification of abundant proteins. In this chapter, Western analysis and real-time PCR was used to confirm the proteomics data and to determine the prevalence of highly expressed genes in other wine strains. Tdh3p expression differences between the wine and laboratory strain used in this study were confirmed by Western analysis. However, the differences in Shm2p, Gre3p, Rps4Ap, Rpl12p, Rps19p, Rps31p, and Htb1/2p expression were not as easily confirmed, with disparity between transcriptional profiling data and protein expression at the same cellular state during fermentation. By repeating the 2D gels with duplicate protein samples for each strain, it was shown that Tdh3p and Shm2p expression were higher in the wine strain AWRI 838. In addition, the putative Mdh1p modification in AWRI 838 was also reproducible. It

remains unclear why other proteins in the initial 2D gel experiments were not reproducible, and it implies that previously published experiments that did not use appropriate replication will need to be repeated. It could be hypothesized that the cell phase and cell number at the time of protein isolation for each strain was not simultaneous (despite a relatively homogeneous yeast population). Future proteomic experiments using 2D gels will incorporate triplicate fermentations from three independent starter cultures to ensure a complete representation of each biological sample of interest. The proteins that show significant expression differences between strains can then be investigated further.

In this study, it was shown that wine strain AWRI 838 had both higher protein and gene expression of the glycolytic gene glyceraldehyde 3-phosphate dehydrogenase (*TDH3*) using proteomics and quantitative real-time PCR respectively. There has been extensive research into glycolytic enzymes, including attempts to increase the rate of fermentation, as well as the formation of ethanol by increasing glucose flux through glycolysis with the overexpression of a combination of glycolytic enzymes (Hauf *et al*., 2000; Schaaff *et al*., 1989). These alterations resulted in large increases in enzyme activity, but only marginal increases in sugar utilisation rate and ethanol production, suggesting that there were no rate-limiting or 'bottleneck' enzymes in the glycolytic pathway. However, it is noted that this work was carried out in a laboratory strain.

One protein that was confirmed to be more highly expressed in wine strain AWRI 838 in this study by Western analysis, real-time PCR and proteomics was Tdh3p. McAlister and Holland (1985) implied the yeast cell does not require *TDH1* and *TDH2* for cellular growth. Other research that has been carried out on *TDH* gene expression has shown they are differentially expressed in cells exposed to an environmental stress such as glucose starvation or heat-shock. Boucherie *et al*. (1995a) showed the presence of a polypeptide synthesised from *TDH1* as cells entered stationary phase and glucose became limited, while *TDH2* was repressed by a mild heat shock. This suggests these polypeptides have different roles in the cell depending on cellular state. In addition, the study of *TDH* gene expression has been expanded to a wine strain during fermentation. It has been shown that the *TDH* genes had their maximal expression at mid-exponential phase, with *TDH2* and *TDH3* having the highest level of gene transcripts among glycolytic genes during wine fermentation (Hauser *et al*., 2001; Puig and Perez-Ortin, 2000). As grape juice fermentation can be a particularly stressful environment for yeast, the increased expression of Tdh3p in AWRI 838 in mid-exponential phase in these experiments may indicate a preparative step for this strain to counteract ensuing stresses. If this expression was constitutive, it may also provide tolerance to the osmotic stress as a result of high sugar concentrations at the start of fermentation. Further research on the protein expression of Tdh3p, as well as the modified Fba1p (4.2.2.4), will be required to confirm this hypothesis relating to glycolytic pathways in wine strains.

The one-carbon pool is pivotal in cellular metabolism, and the supply of one-carbon units is flexible dependent on which and how many one-carbon units there are in the cell (Kastanos *et al*., 1997). It is the metabolic pathways in the one-carbon pool that initiate the formation and synthesis of important compounds such as formate, which is a precursor for many other metabolic pathways in the yeast cell. It was observed in this study that both *TDH3* gene transcript and Tdh3p protein expression levels were higher in wine strain AWRI 838 compared to the laboratory strain S288C. In contrast, further investigation of the *SHM2* gene showed that the Shm2p protein was more abundant in AWRI 838 compared to S288C, yet at the same time the level of *SHM2* transcript did not alter between strains. Expanding the study of this gene to include many other wine strains showed that the level of transcript for *SHM2* increased in each strain by as much as 15.5-fold. This indicates that the *SHM2* gene could play an important role in wine strains, and as the *shm2*Δ strain did not affect growth phenotype, it was hypothesized that *SHM2* may be involved in contributing to wine sensory compounds. The enzyme Shm2p catalyses the conversion of L-serine and tetrahydrofolate (THF) to produce glycine and 5,10-methylene-THF. 5,10-methylene-THF is a precursor to the one-carbon pool, which is required for a number of anabolic processes within the cell including the synthesis of purines, pyrimidines, amino acids and lipids. It is also of relevance with respect to human disease and disorders, as disruption of the genes in folate-related metabolic pathways severely affects phenotype (reviewed in Lucock and Daskalakis, 2000). McNeil *et al*. (1994) showed that deleting the *SHM2* gene had no effect on the growth requirements of the cell (which we also noted using the *SHM2* deletion strain). In

addition, Schlupen *et al*. (2003) have shown the disruption of the *SHM2* gene in *Ashbya gossypii* results in an accumulation of glycine, which can then be further utilised for increased riboflavin synthesis.

To elucidate the possible reasons for the higher protein expression of Shm2p in wine strain AWRI 838 compared to the laboratory strain S288C, as well as the higher gene expression in wine strain T73 (Hauser *et al*., 2001), sequencing of the *SHM2* gene in each strain was performed. Inspection of the sequences manifests a variation in the *SHM2* DNA sequence between the wine strains AWRI 838 and T73 compared to S288C (C-70A and T-66G). Evidence exists in the literature that changes to the DNA sequence of the 5' non-translated region effect not only the expression of genes, but also the targeting of gene products to organelles within a cell in yeast (Chatton *et al*., 1988; Natsoulis *et al*., 1986; Wang *et al*., 2003), *Arabidopsis* (Mireau *et al*., 1996), and humans (Tolkunova *et al*., 2000). As there is no other ATG sequence before the starting ATG of *SHM2* in either the laboratory or wine strains, it is anticipated this change will not affect targeting or transcription rate of any secondary *SHM2* transcripts from the *SHM2* gene. Overall, this research has been unable to discover the possible reason for the difference in transcription between the two wine strains.

An important outcome of this research has been the discovery of two *S. cerevisiae* wine strains having different transcription/translation control of the same gene. This is evidenced by the differences in *SHM2* transcript abundance in wine strains AWRI

838 and T73, yet the DNA sequence being identical in each strain. It should be noted that we have only sequenced 370 bp of promoter sequence and 167 bp of termination sequence, so additional promoter elements may lie upstream (despite designing the primers well into the genes both upstream and downstream of *SHM2*). These results indicate a *trans*- regulatory factor is different between two strains. Further characterisation of *SHM2* in AWRI 838 and T73 is required to determine the importance of this control in terms of gene expression in each strain. It may also reveal how and why *SHM2* is expressed differently between strains and if it affects the wine strain phenotype significantly in terms of the production of wine sensory compounds as the metabolome studies suggest.

To identify the possible role of differentially expressed genes between strains in terms of wine flavour, near infra-red spectroscopy (NIRS) was used. This technique was chosen based on its ability to determine metabolic differences after fermentation using strains with a single gene deleted (Allen *et al*., 2001). It was shown that deletion of the *SHM2* gene resulted in significant variation to the metabolome compared to the wild-type. In addition, when compared to 27 other single gene deletion samples, the *SHM2* gene deletion was the most distant to the wild-type. Together, these results suggest that *SHM2* does have a role in the production of the final metabolome during yeast fermentation. It will be important to study this gene further with respect to its role in determining wine flavour. Furthermore, this study did uncover the use of NIRS for high-throughput chemometric experiments to determine the role of a single gene during yeast fermentation. By utilising the

techniques described in this chapter, it may be possible to screen multiple yeast strains with single gene deletions to establish a possible role in wine flavour for each gene during grape juice fermentation.

5.4. CONCLUSION

The research presented in this chapter demonstrates how proteomics can be used to elucidate differentially expressed proteins between strains for further characterisation of their role and function in wine strains. Two proteins confirmed to be more highly expressed in the wine strain AWRI 838 compared to the laboratory strain S288C were the glycolytic enzyme Tdh3p, and the one-carbon metabolism enzyme Shm2p. Further characterisation of the *TDH3* gene showed that the higher Tdh3p expression observed in AWRI 838 was due to increased transcription, as observed by quantitative real-time PCR. This study also revealed that increased expression of *SHM2* in two wine strains might be related to the synthesis of the final metabolome of wine produced using these strains, which was demonstrated with increased *SHM2* expression with many wine strains and supported by NIRS metabolic profiling. We have shown that by using a multi-faceted approach with biochemical, genetics, transcriptomics, proteomics and chemometric methods, it is possible to expose genes with possible wine-related phenotypes. In addition, investigation of the proteome with 2D gels in conjunction with transcriptional profiling with quantitative real-time PCR has revealed differences in transcriptional/translational control between *S. cerevisiae* wine strains. By using a combination of genetic and proteomic resources, as well as more recent

methodologies such as near infra-red spectroscopy for metabolic profiling, it was possible to elucidate important genes that contribute to the wine strain phenotype and provide wine strains with their unique properties. This study therefore demonstrated that an exploratory comparative approach can be utilised to determine genetic differences between strains of the same species. Further characterization of these genes will need to be carried out in different environmental conditions, different grape juices and varying fermentation parameters implemented to achieve a complete overview of the role and function of genes influencing wine strain phenotype.

CHAPTER 6

Acclimatisation of *Saccharomyces cerevisiae* to ethanol in continuous culture

6.1. INTRODUCTION

Yeast have been exploited by humans for thousands of years in industrial processes such as bread making, beer brewing, the production of wine from grape juices, and, more recently, the bioconversion of various sugar sources to fuel ethanol. As ethanol is accumulated in each of these industrial processes, it may reach levels that are deleterious to the yeast in terms of growth capabilities and survival. This makes ethanol and its effect on yeast growth during fermentation a principal focus for many research groups, as yeast strains used for the above applications are, in general, able to resist high concentrations of ethanol and continue to utilise sugars for growth and energy production.

A number of research groups have identified genes that undergo a transcriptional response following an ethanol stress (Alexandre *et al*., 2001; Aranda *et al*., 2002; Betz *et al*., 2004; Carrasco *et al*., 2001; Chandler *et al*., 2004; Garay-Arroyo *et al*., 2004; Palhano *et al*., 2004; Rossignol *et al*., 2003). Other groups have isolated ethanol-tolerant mutants after continued exposure of cells to ethanol and analysed their gene expression compared to the wild-type (Ogawa *et al*., 2000; Sharma *et al*., 2001; Takahashi *et al*., 2001). The existing ethanol stress data from the literature

cited above shows a large number of stress-induced genes (eg. *HSP12*, *HSP26*, *HSP104*) containing stress response elements (STRE's), which are able to bind the Msn2p/Msn4p transcription factors, as well as other transcription factors such as Hsf1p and Yap1p. In addition, a number of genes such as ribosomal protein synthesis genes are generally repressed (Alexandre *et al*., 2001; Chandler *et al*., 2004). The role of the majority of the ethanol-controlled genes has not been elucidated. Moreover, the proteomics data described in Chapter 4 suggests that increases in gene expression do not necessarily lead to equivalent increases in the amount of protein.

While the above-mentioned studies add valuable information for discovering the genes involved in the ethanol tolerance of yeast, they do not necessarily reflect the grape juice fermentation environment. During fermentation, grape sugars are converted to, amongst other metabolites, ethanol, which accumulates in wine over the course of fermentation. As this accumulation of ethanol is gradual, the yeast are able to continuously acclimatise to the surrounding change in environment. Thus it would be judicious to discover the genes that are not only expressed after short-term ethanol stress, but genes that are continually expressed in the accumulation and continual presence of ethanol. Ultimately, the transcriptional response by cells exposed to an ethanol environment will relate more closely to the yeast contending with the accumulation of ethanol over time in industrial applications compared to the stress response genes identified to date through short-term ethanol exposure to cells. Therefore, the aim of this study is to identify key genes that permit the yeast to

acclimatise to ethanol using continuous culture, as opposed to short-term ethanol stress. These experiments are designed to compare results to the existing literature, and at the same time learn more about gene expression of *S. cerevisiae* in fermentation conditions that are encountered in natural environments such as grape juice fermentation.

6.2. RESULTS

6.2.1. 6% ethanol inhibits growth in batch culture

To identify the genes responsible for ethanol tolerance, a continuous culture approach was taken. It was hoped this technique would help elucidate the genes required for continued growth and survival of industrial strains in a high ethanol environment. For this approach, an ethanol concentration for continuous culture needed to be chosen that would affect cellular growth. The ethanol concentration chosen needed to provide reproducible effects between replicates, while not being toxic and resulting in wash out of cells from the fermenter. To find this ethanol concentration, triplicate fermentations were carried out with the deletion strain collection parent BY4742 (S288C background) in SC media (2% glucose) containing 0, 2, 4, 6, 8 or 10% ethanol and cell growth monitored by optical density for 174 hours. The strain BY4742 was chosen so that for future experiments with genes of interest identified in this study, the deletion strain collection could be utilised in a strain from the same background. It was observed that 6% ethanol slowed the growth rate significantly, requiring 17 hours for stationary phase to be reached as opposed to 10 hours for the 0% ethanol control fermentations (Figure 19).

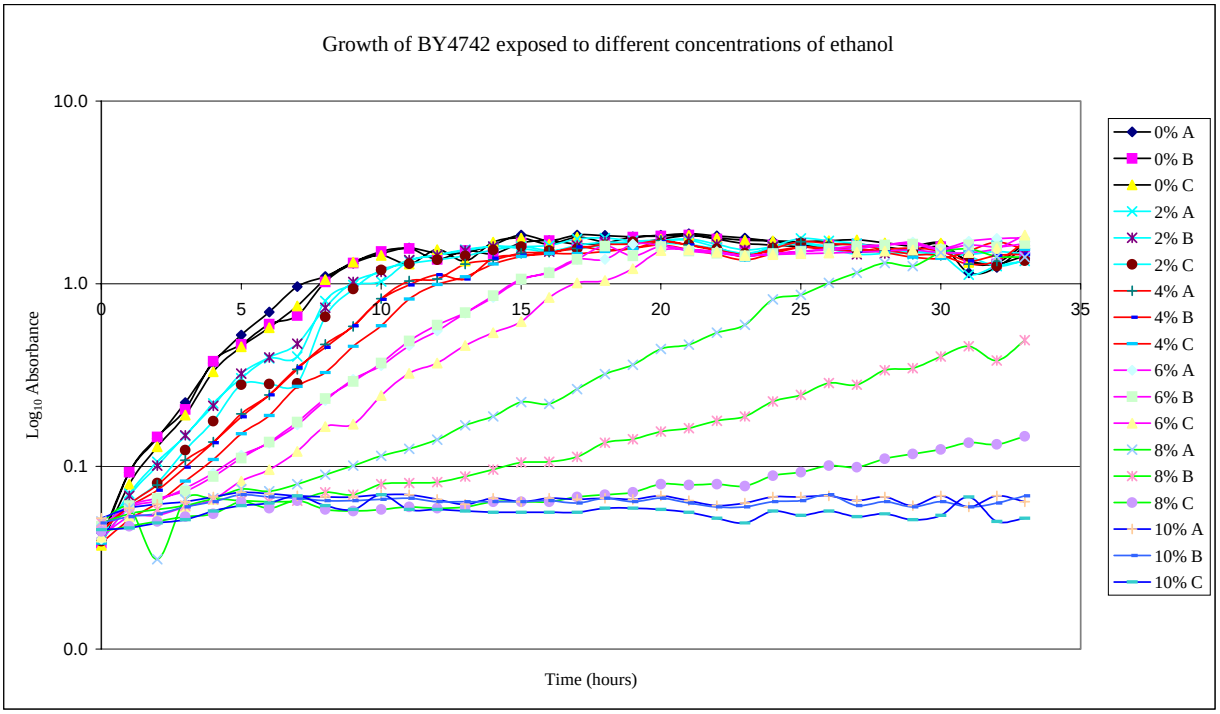


Figure 19: Fermentations of the parent strain (BY4742) from the EUROSCARF deletion collection to select the concentration of ethanol to be used in continuous culture. Varying concentrations of ethanol were added to each culture at 1 x 10^7 cells/ml, in triplicate (A, B, C) and optical density measured every hour for 34 hours.

The triplicates at 6% ethanol showed better agreement than that seen for the 8% ethanol treatment, in which growth was variable (not reproducible) between replicates. Therefore, to ensure consistency between replicates when determining genes involved in ethanol tolerance, 6% ethanol was chosen for the continuous culture experiment below.

6.2.2. 6% ethanol effects gene expression in continuous culture conditions

In order to distinguish between genes that are required for long-term exposure in a high ethanol environment as opposed to short-term stress response genes, a continuous culture technique was utilised. In this particular experiment, the turbidity of the culture was kept constant by manipulating the rate at which fresh medium was fed into the fermenter. When the turbidity within the fermenter was on the increase (eg. cell numbers are increasing), the feed rate of fresh medium was increased to dilute the turbidity back to its setpoint. When the turbidity of the culture fell, the feed rate was lowered so that turbidity was restored to its setpoint. This enabled the cells to achieve a relatively homogeneous population over time at the same cell phase and cellular state. Using 1.5 litre Applikon fermenters at the School of Chemical Engineering, University of Adelaide, the deletion strain parent BY4742 was continuously cultured for 10-14 generations in triplicate fermenters and the cells harvested. The same batch of cells was then exposed to 6% ethanol for another 10-14 generations, and cells harvested again. Using microarrays, the profile of gene expression after ethanol exposure was compared to that of the initial cell population without ethanol exposure.

Microarrays revealed that 34 genes were induced by two-fold or more and three genes were repressed greater than two-fold after acclimatisation to 6% ethanol (Table 4 and 5). The most induced gene after acclimatisation was *HSP12* (8.34-fold), with the most repressed gene being *YHB1* (2.36-fold). Interestingly, 34 transposon ORFs appeared to be induced (data not shown), although the homology of these sequences to each other may account for the high number of transposons identified as induced.

During such long-term fermentations as described above, it is possible that contamination by other yeasts in the laboratory or bacteria may have occurred. Secondly (and less likely), the initial yeast inoculated (BY4742, *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) could have mutated or reverted from its original markers. To ensure the yeast cells at the completion of the experiment were the inoculated BY4742 strain (and not a contaminant), final samples were plated onto minimal media plates and checked for retention of the correct markers. The results showed the correct markers for BY4742, and no contaminants were present (data not shown).

6.2.3. Promoter analysis

It was important to elucidate transcriptional elements that have a role in controlling the transcription of genes in stressful environments. Ultimately, by finding these promoter elements, a better understanding of the transcriptional response and how the yeast adapts to the extracellular stress can be obtained. To determine if known

Table 4: Genes induced after acclimatisation to 6% ethanol using strain BY4742 grown in SC complete medium. * Indicates putative STRE in promoter region, in both orientations (Marchler *et al*., 1993).

Open reading frame	Gene	Fold Change	Function
YFL014W	*HSP12**	8.34	Heat shock protein
YBR072W	*HSP26**	6.30	Heat shock protein
YER150W	*SPI1**	5.19	Stationary phase induced
YJL079C	*PRY1**	3.62	Unknown, pathogen related in yeast
YHR055C	*CUP1-2*	3.30	Metallothionein, binds copper
YPR030W	*CSR2*	3.25	Unknown, cell wall organisation
YDR380W	*ARO10*	3.16	Phenylpyruvate decarboxylase
YPR052C	*NHP6A*	3.15	Non-histone protein, chromatin binding
YML123C	*PHO84**	3.14	Phosphate and manganese transporter
YGL062W	*PYC1**	3.14	Pyruvate decarboxylase
YHR053C	*CUP1-1*	2.95	Metallothionein, binds copper
YKR093W	*PTR2*	2.81	Small peptide transporter
YBR105C	*VID24*	2.80	Vacuolar targeting, import and degradation
YBR067C	*TIP1*	2.69	Temperature shock induced protein
YMR096W	*SNZ1*	2.57	Protein binding, pyridoxine metabolism
YPR180W	*AOS1*	2.48	Activation of Smt3p, protein sumoylation
YDR034W-B	*	2.46	Unknown
YJR148W	*BAT2*	2.41	Branched-chain amino acid transaminase
YNL160W	*YGP1**	2.39	Secreted glycoprotein
YHR049W	*FSH1*	2.39	Family of serine hydrolases
YBR018C	*GAL7**	2.39	Galactose-1-phosphate uridyl transferase
YAL053W	*	2.37	Unknown
YNL125C	*ESBP6*	2.35	Monocarboxylate permease (putative)
YER062C	*HOR2*	2.35	Glycerol-3-phosphatase
YPR194C	*OPT2*	2.31	Oligopeptide transporter
YBR054W	*YRO2*	2.26	Homology to Hsp30p

Table 4 continued

Open reading frame	Gene	Fold Change	Function
YGL255W	*ZRT1*	2.25	High-affinity zinc transport protein
YDR040C	*ENA1**	2.21	Plasma membrane ATPase
YBR172C	*SMY2*	2.21	Unknown
YMR195W	*ICY1**	2.20	Unknown
YLR089C	*ALT1**	2.19	Unknown
YKR067W	*GPT2*	2.18	Glycerol-3-phosphate o-acetyltransferase
YIL136W	*OM45**	2.05	Unknown
YDR399W	*HPT1*	2.00	Hypoxanthine guanine phosphoribosyltransferase

Table 5: Genes repressed after acclimatisation to 6% ethanol using strain BY4742 in SC complete medium.

Open reading frame	Gene	Fold Change	Function
YGR234W	*YHB1*	2.36	Flavohaemoglobin
YOR153W	*PDR5*	2.22	Pleiotropic multidrug resistance transporter
YHR183W	*GND1*	2.06	6-phosphogluconate dehydrogenase

transcriptional elements were present amongst induced and repressed genes after long-term exposure to ethanol, 800 base pairs of upstream sequence from the ATG of each gene were analysed by the MATCH database at TRANSFAC (http://www.gene-regulation.com/pub/programs.html#match). The analysis showed that putative STRE's, which bind transcription factors such as Msn2p and Msn4p, had binding sites present in 14 of the 34 induced genes (Table 4). Five of these 14 genes with putative STRE's in their promoter region, namely *HSP12, HSP26, SPI1*, *PYC1*, and *YGP1* have also been shown to be induced after one hour of 5% ethanol stress (Chandler *et al*., 2004). There were no other significant numbers of transcription factor binding sites in the induced genes detected by the computer program, and likewise for the three repressed genes.

As there was such a low number of genes changing expression under the longer term ethanol stress in this study compared to the existing short term ethanol shock data, it was imperative to determine whether these genes had any common novel promoter elements. This may determine which genes are under the control of stress responsive-like elements in an ethanol-containing environment, and which genes have transcriptional elements that are more specifically controlled to counteract the stressful external environment. For this study, a motif discovery tool called the Multiple EM for Motif Elicitation (MEME) software program was utilised (http://meme.sdsc.edu/meme/website/meme.html). This software has been used successfully to identify novel promoter elements that are essential for gene expression in stressful environments (Gasch *et al*., 2000; Chandler *et al*., 2004). 800

base pairs of the promoter region upstream of the ATG of genes that changed expression more than two-fold were probed against the MEME database. The default parameters were used, and the statistical significance of each putative promoter element analysed by MAST (the statistics program for MEME). MAST also produces a motif diagram and detailed annotations of the motif sequence and alignment across promoters of multiple genes. The results show that the *CUP1-1* and *CUP1-2* promoter sequences contain elements that are common in both genes (TCCAAAGCAGTAAAAGGATTCACCAGGGTTTGGAATCTGAT, AAACTCC GACGACAACAATGATGCAGATTATAGTCAGTCGGCCGAGCCCA). This was not unexpected, as the two genes are identical (100% identity), but did provide evidence that the MEME program could identify promoter elements. When investigating other genes for novel promoter elements however, there appeared to be no common sequences present in the up- or down-regulated genes after a long-term ethanol stress that would represent a transcriptional element.

6.3. DISCUSSION

In this study, the complete transcript profile of a *Saccharomyces cerevisiae* strain in continuous fermentation exposed to 6% ethanol has been investigated. Initially, the most striking result was the low number of genes that vary by two-fold as opposed to findings in the existing ethanol stress literature. There were 34 genes induced by greater than two-fold and only three genes repressed, which is a lower number of genes compared to previous data (Alexandre *et al*., 2001; Chandler *et al*., 2004). This may suggest that if the yeast has time to acclimatise to an ethanol stress, the

number of genes induced or repressed to counteract the stress is lower. It has previously been shown that a short-term environmental stress to the cell provokes a large transcriptional response (Alexandre *et al*., 2001; Causton *et al*., 2001; Chandler *et al*., 2004; Gasch *et al*., 2000). Interestingly, when *S. cerevisiae* cells have been exposed continually to a saline stress, the number of induced genes increases over time (Yale and Bohnert, 2001). The type of transcripts induced altered over time, changing from amino acid metabolism and the transport and destination of protein synthesis genes to detoxification-related responses and fatty acid biosynthesis genes. In this research however, a time-course approach was not taken, and the transcriptional response of yeast over time to continuous exposure to an ethanol stress is unknown. However, it is clear from this study that long-term exposure of *S. cerevisiae* to ethanol reduced the number of genes transcribed compared to short-term exposure of ethanol to cells. Genes with the greatest variation in transcription levels are discussed further below.

Both Alexandre *et al*. (2001) and Chandler *et al*. (2004) have seen significant repression of protein synthesis-related genes after a short-term ethanol stress. Furthermore, the latter group has seen nearly 50% of the ribosomal protein genes that were repressed after 60 minutes continue to be repressed after three hours of sustained ethanol exposure. A similar reduction in ribosomal protein genes was not observed in this experiment after 10-14 generations. It could be hypothesized that protein synthesis is turned off when cells experience a sudden change in their environment (Herruer *et al*., 1988; reviewed in Warner, 1989, Warner, 1999), yet

once the cell has acclimatised to its surroundings, protein synthesis is able to be restored. Interestingly, the proteomics data in Chapter 4 revealed a commercial wine strain, AWRI 838, had higher expression of ribosomal protein genes compared to the laboratory strain S288C. Although there is no other evidence in the literature, it could be hypothesized that wine strains are able to tolerate high ethanol environments by not reducing protein synthesis gene expression, hence permitting the cell to continue normal metabolic activities.

It should be noted that normalisation at the RNA level for microarray analysis occurred in these experiments, whereas some of the other ethanol studies in the literature normalise based on the same cell number. There are limitations however that arise from the latter approach. By using the same cell number, the investigator relies on the same cell breakage between samples, and RNA extraction efficiency during RNA isolation must be the same. It has previously been shown that the effect of an environmental stress is far greater on ribosomal protein transcription than on rRNA transcription, and is predominantly due to transcriptional silencing of ribosomal protein genes and rapid turn over of their transcripts after stress (Li *et al*., 1999). It has also been demonstrated that this repression of ribosomal protein genes is temporary and basal levels are reached after 60 minutes or return to a non-stressful environment (Eisen *et al*., 1998), perhaps explaining why it was not noted in this study.

The observation that heat shock protein genes (HSPs) were the two most induced genes in this study is consistent with previous findings. HSPs have been expressed at high levels in many ethanol stress experiments and have been seen to be expressed by strains that encounter a high ethanol environment such as wine (Aranda *et al*., 2002; Rossignol *et al*., 2003) and lager strains (Brosnan *et al*., 2000). Interestingly, lager strains exhibit a stress response by inducing HSPs early in industrial ferments, and this response is repressed as fermentation proceeds (Brosnan *et al*., 2000). This may be a reflection of the acclimatisation response seen in the continuous culture used in this study, compared to the large number and high intensity of HSPs expression after short-term ethanol stress (7% ethanol for 30 minutes (Alexandre *et al*., 2001); 5% ethanol for 180 minutes (Chandler *et al*., 2004)). In contrast to the lager strain, Rossignol *et al*. (2003) showed a wine strain induced HSPs at the end of the growth phase and remained highly expressed throughout fermentation, with *HSP26* the most highly induced. This suggests that HSPs do play a role in providing ethanol tolerance to the yeast cell, and their expression is likely to be dependent on ethanol concentration and accumulation rate during fermentation. It should be noted that these studies with lager and wine strains to date have been from stationary cultures, unlike the exponential growth phase cells used in the research presented here, but highlights the usefulness of the continuous culture experiment results in this study. Furthermore, there is still a great deal of research that needs to be carried out to establish the role of HSPs in continual high ethanol environments such as grape juice fermentation.

In this study, *SPI1* was induced 5.19-fold in the ethanol exposed cells. *SPI1* encodes a cell wall protein that binds to β-1,6-glucans and β-1,3-glucans, and therefore may act as some form of protectant to the cell during stress by covering the cell wall. Alexandre *et al*. (2001) have examined the transcriptome of a laboratory strain after a 30 minute 7% ethanol stress, but did not see *SPI1* gene expression change after stress. Further studies using a 5% ethanol stress for double the time of exposure, 60 minutes, have shown *SPI1* induced 23.5-fold (Chandler *et al*., 2004). In addition, an ethanol tolerant mutant of an industrial sake strain has shown increased expression of *SPI1* compared to the wild-type strain (no quantification details given, Ogawa *et al*., 2000). These results, in combination with the data obtained in this chapter, suggest that *SPI1* gene expression is required over longer periods of ethanol exposure. It could be hypothesized that *SPI1* gene expression may be involved in acquiring the yeast ethanol tolerance by coating the cell wall with additional Spi1p protein, thereby reducing the loss of viability (Simoes *et al*., 2003). Further studies are required to monitor the presence of Spi1p with continued ethanol stress, and what particular role and function this protein has, if any, on protecting the cell during an environmental stress.

An interesting addition in the study by Chandler *et al*. (2004) was to extend the length of 5% ethanol exposure from 60 minutes to 180 minutes. This may provide leads as to which genes are required for longer periods of exposure to ethanol stress compared to short-term exposure. Three genes were seen to be highly induced at the 180 minute time point as well as after 10-14 generations in continuous culture in this

study: *YRO2* (4.5-fold and 2.26-fold respectively), *SNZ1* (3.1-fold and 2.57-fold), and YLR089C (3.3-fold and 2.19-fold). The gene products of YLR089C and *YRO2* have no established function, but *YRO2* has homology to *HSP30*, encoding a heat shock protein that was induced in both Alexandre *et al*. (2001) and Chandler *et al*. (2004) data. Interestingly, *SNZ1*, a stationary phase-induced gene that is involved in the cellular response to nutrient limitation and growth arrest, was actually repressed in the Chandler *et* al. (2004) data at 60 minutes by 3.5-fold. Together with *YRO2* and YLR089C, these genes may assist in providing yeast with their ethanol tolerant properties.

Previous investigations have shown using microarrays that transposons are up-regulated after a stress (Alexandre *et al*., 2001; Morillon *et al*., 2000). In this study, it was shown that 34 transposon elements were up-regulated by as much as 4.4-fold. One possible explanation could be that transposons have very similar sequences to each other, therefore if only one transposon is up-regulated, it will hybridise to other transposon oligos (25 mer) hybridised on the chip. To counteract this possibility, the DNA sequence of each of the transposons were aligned and the likelihood of cross hybridization occurring verified. The results showed that two transposons, *YCL019W* and *YMR050C*, were almost identical (results not shown). However, the next most similar transposon sequences were only 60% similar. It is possible then that the results described here are a direct result of many transposons up-regulated after ethanol acclimatisation. It has been proposed by Morillon *et al*. (2000) that

yeast activate Ty transcription and retrotransposition to undergo adaptive mutations that may allow the cell to adapt and survive in their stressful environment.

6.4. CONCLUSION

Previous studies have shown that there is a large transcriptional response after short-term exposure of yeast cells to ethanol. Using cDNA microarrays, it has now been shown that long-term exposure of *S. cerevisiae* to ethanol in an otherwise nutrient rich environment results in a considerable reduction of gene expression. Importantly, this study has eliminated the possibility of differences in cell state during fermentation becoming an issue in such studies through use of a turbidostat, which kept the cells at mid-exponential phase. Several of the induced genes have stress response elements in their promoters, and are consistent with existing ethanol stress data. In addition, genes that are not associated with a classical stress response are likely to be fundamental to the cell for providing continued ethanol tolerance as opposed to a short-term ethanol shock. The role and function of these genes will need to be further investigated with deletion and overexpression studies to determine their role in ethanol tolerance.

CHAPTER 7

Overall Conclusions and Future Studies

The phenotypic differences that exist between well-studied laboratory strains and commercial wine strains of *Saccharomyces cerevisiae* have been established. However, what needs to be elucidated is the genetic origins of this variation between *S. cerevisiae* strains. This will promote a greater understanding of how wine strains are able to proliferate and survive during grape juice fermentation which other strains are unable to accomplish. For biotechnological applications, it may also lead to the identification of genes responsible for producing metabolic components that are consistent with good wine quality. The research undertaken demonstrates how new technologies are invaluable for identifying differences between evolutionary similar strains. It also provides evidence for the genes involved in long-term ethanol stress, an environmental condition that is universal for commercial strains of *S. cerevisiae* used in winemaking.

Genetic differences exist between laboratory and commercial strains of *S. cerevisiae*

The use of AFLP as a tool to identify unique genetic sequences in wine strains was successful. Due to the relatively large amount of coding sequence in yeast, an unexpected finding was the number of non-coding sequences isolated from the wine

strains. Further research may ascertain the biological importance of these sequences, however this is not practically possible at this stage. One sequence in particular became the focus of attention after it was revealed to be of non-*S. cerevisiae* origin. The identification of a naturally occurring hybrid winemaking strain between *S. cerevisiae* and *S. kudriavzevii* identified in this study is believed to be the first of its type. Karyotyping is necessary to confirm the chromosomal complement of these hybrids. It raises the question as to how many other wine strains, previously classified as *S. cerevisiae*, are indeed classified correctly, as new species are uncovered over time (Naumov *et al*., 2000). Cross-checking of many genetic sequences from each *Saccharomyces* species may help to answer this question.

Proteomics can be used to identify wine-related proteins

From the existing literature, this is the first known report of a proteomic comparison between two *S. cerevisiae* strains. The 2D gels supported the genetic study by showing differences exist between *S. cerevisiae* strains. By isolating and identifying these differences, it suggests that a modified glycolytic pathway exists in a commercial winemaking strain with the presence of a modified Fba1p and the overexpression of isoforms of Tdh3p. This may explain the ability of wine strains to utilise glucose more efficiently and ferment high sugar grape juices to dryness unlike the well-studied laboratory strains. Another observation using 2D gels linked the higher expression of a one-carbon gene in a wine strain to the strain's metabolome. The study of the *SHM2* gene and its presence in many wine strains indicated a correlation exists between its gene product and the metabolic profile after

fermentation. To investigate these hypotheses further, experiments that overexpress the wine strain genes of interest in the laboratory strain will determine if this provides an advantage, both to the cell in terms of growth and to the resultant metabolites produced during fermentation. Despite the *SHM2* gene having nearly identical sequences between laboratory and wine strains, it may be the base substitutions or other secondary factors that affect transcription. This could be tested by a series of mutations at these sites in the promoter of the *SHM2* gene in both laboratory and wine strains.

Gene expression is reduced by *S. cerevisiae* after acclimatisation to ethanol

It has been shown that *S. cerevisiae* respond to short-term environmental stress with many changes in gene expression (Alexandre *et al*., 2001; Causton *et al*., 2001; Gasch *et al*., 2000). In this thesis, it has been demonstrated that long-term exposure of ethanol to an otherwise nutrient-rich environment dramatically reduces the number of genes expressed. It is anticipated that these are the genes required in wine strains for tolerance to a high ethanol environment, which would be required by wine strains through grape juice fermentation. Future research will study the function of these genes more closely and identify their role in high ethanol environments. This will be done through deletion and overexpression of the most induced and repressed genes to determine their importance to the cell in similar experiments used in this thesis. Finally, although this study provided a valuable link between stress studies and the winemaking situation, it will be necessary to determine the transcriptional response of yeast that are exposed to a constant

addition and accumulation of ethanol in the surrounding medium to simulate fermentation more closely. This will ultimately impart new knowledge of the gene expression a wine strain undertakes during the fermentation process.

Concluding remarks

As the winemaking process becomes more systematic and technical, along with the adoption of scientific knowledge to the art of winemaking, it will be important to provide the wine industry with sound biological knowledge of yeast strains used in the fermentation process. To do this, it is imperative we learn more about the genes and/or corresponding proteins that gives a wine strain its particular phenotype. In this thesis, an exploratory comparative approach has elucidated a possible basis for the wine yeast phenotype, including the identification of a hybrid winemaking strain and the first known proteomic comparison of *S. cerevisiae*. In addition, the genes involved in ethanol tolerance, an important winemaking strain characteristic, have been further defined. Further research will permit the wine industry to utilise this new found knowledge for the prediction of wine yeast attributes, it will improve yeast fermentation properties and performance, and will also permit the tailoring of wine styles through controlled yeast fermentation.

Appendix One

REAGENTS AND SOLUTIONS USED IN THIS STUDY

Minimal Medium

Difco Yeast Nitrogen Base without Amino Acids or Ammonium Sulphate 6.67 g

D-glucose 20 g

Ammonium Sulphate 5 g

Milli-Q H_2O water up to 1000 ml

SC Medium

Difco Yeast Nitrogen Base without Amino Acids or Ammonium Sulphate 6.67 g

D-glucose 20 g

Ammonium Sulphate 5 g

Synthetic Complete Dropout Mix (below) 0.67 g

Difco Agar (for agar plates) 20 g

Sodium Hydroxide (agar plates only) 1 pellet

Milli-Q H_2O water up to 1000 ml

Synthetic Complete Dropout Mix

Adenine hemisulphate 2 g

Arginine HCl 2 g

Histidine HCl 2 g

Isoleucine 2 g

Leucine 4 g

Lysine HCl 2 g

Methionine 2 g

Phenylalanine 3 g

Serine 2 g

Threonine 2 g

Tryptophan 3 g

Tyrosine 2 g

Uracil 1.2 g

Valine 9 g

Omit appropriate components to prepare required dropout mix.

Yeast Extract / Peptone / Dextrose (YPD) Medium

Yeast extract 10 g

Peptone 20 g

D-glucose 20 g

Difco agar (for agar plates) 20 g

Milli Q H_2O up to 1 L

1x AFLP gel loading buffer

98% formamide

10 mM EDTA

0.05% bromophenol blue

0.05% xylene cyanol

AFLP gel developer

Sodium carbonate 30 g/L

Sodium thiosulphate 2 mg/ml

5.5% formaldehyde

20x SSC

3 M Sodium chloride

0.3 M Tri-sodium citrate

Adjusted to pH 7.0

Denaturing solution

0.5 M Sodium hydroxide

1.5 M Sodium chloride

Neutralising solution

0.5 M Tris-Cl (pH 7.4)

3 M Sodium chloride

10x Maelic acid solution

0.1 M Maelic acid

0.15 M Sodium chloride

CSPD detection buffer

0.1 M Tris-Cl

0.1 M Sodium chloride

Lysis Buffer

20 mM Tris-Cl (pH 7.6)

10 mM sodium fluoride

10 mM sodium pyrophosphate

0.5 mM EDTA

0.1% deoxycholate

SDS-PAGE sample buffer (5x strength)

500 mM dithiothreitol

10% (w/v) SDS

40% (v/v) glycerol

300 mM Tris-Cl (pH 6.8)

0.025% (w/v) bromophenol blue

Colloidal coomassie stain

40% (v/v) ethanol

7% (v/v) acetic acid

0.1% (w/v) Brilliant blue G-250

Fixative Solution

40% ethanol

10% acetic acid

Sensitising solution

0.3% sodium thiosulfate pentahydrate

30% ethanol

6.8% sodium acetate

Silver nitrate solution

0.1% silver nitrate

0.05% formaldehyde

Developer solution

3% sodium carbonate

0.025% formaldehyde

Destain solution

20% ethanol

7% acetic acid

Rehydration stock solution (urea)

Urea 4.8 g

CHAPS 0.2 g

DTT 100 mg

IPG Buffer (same pH range as strip) 50 μl

Bromophenol blue a few grains

Milli Q H_2O up to 10 ml

Stored in aliquots at -20^0C

Rehydration stock solution (thiourea)

Thiourea 1.52 g

Urea 4.2 g

CHAPS 400 mg

DTT 100 mg

IPG Buffer (same pH range as strip) 200 μl

Bromophenol blue a few grains

Milli Q H_2O up to 10 mls

Stored in aliquots at -20^0C

12% 2D polyacrylamide gels

5x Tris-Cl (pH 8.8) 93.3 ml

40% bis-acrylamide (C 2.6) 140 ml

10% ammonium persulphate 770 µl

TEMED 77 µl

Milli-Q H_2O 233.3 ml

Tris-Cl, acrylamide and water were mixed and degassed for 20 minutes, followed by APS and TEMED addition. Poured with gradient gel pourer.

1.5 M Tris-Cl (pH 8.8)

Tris base 181.5 g

Milli Q H_2O 750 ml

HCl adjust to pH 8.8

Milli Q H_2O up to 1 l

SDS equilibration buffer

1.5 M Tris-Cl (pH 8.8) 6.7 ml

Urea 72.1g

Glycerol 69 ml

SDS 4 g

Bromophenol blue a few grains

Milli Q H_2O up to 200 mls

Stored at -20^0C

SDS-PAGE running buffer

Tris base 60.5 g

Glycine 288 g

SDS 20 g

Milli Q H_2O up to 20 L

Agarose sealing solution

SDS-PAGE running buffer 25 ml

Agarose 200 mg

Bromophenol blue a few grains

Gel storage solution

1.5 M Tris-Cl (pH 8.8) 500 ml

10% SDS 20 ml

Milli Q H_2O up to 2 L

Western transfer buffer

25 mM Tris base (pH 8.0)

192 mM glycine

20% (v/v) methanol

The pH should be 8.3. Do not add acid or base to adjust the pH.

TBS-T (Tris buffered saline with Tween 20)

To prepare 500 ml of 10x stock solution:

137 mM NaCl

2.7 mM KCl

25 mM Tris base (pH 8.0)

Tween 20 5 ml

Adjust the pH to 8.0 with HCl

Milli Q H_2O up to 500 ml

Blocking buffer

5% (w/v) dry skim milk powder in 1x TBS-T

Ponceau S solution

0.1% (w/v) Ponceau S

5% (v/v) acetic acid

RNA buffer

0.5 M sodium chloride

0.2 M Tris-Cl (pH 7.5)

10 mM EDTA

Alkaline phosphatase buffer

100 mM Tris-HCl (pH 9.0)

150 mM NaCl

1 mM $MgCl_2$

DNase reaction mix

1 M Tris (pH 7.5) 2.5 μl

25 mM M_gCl_2 (Perkin Elmer) 20 μl

RNase-free DNase (10 U/μl) 2 μl

RNasin (40 U/μl, Promega) 0.5 μl

PCR master mix

10x Buffer 2 μL

$MgCl_2$ 1.6 μL

dNTPs 3.2 μL

Forward primer 2 μL

Reverse primer 2 μL

DNA 1 μL

Taq polymerase 0.1 μL

Milli Q H_2O 8.1 μL

Quantitative real-time PCR master mix

2x SYBR Green Mix (AB Gene) 10 μL

Forward primer 0.6 μL

Reverse primer 0.6 μL

DNA 1 μL

Milli Q H_2O 7.8 μL

Restriction digests

Restriction buffer 2 μL

Restriction enzyme 0.2 μL

DNA 5 μL

Milli Q H_2O 12.9 μL

Appendix Two

OLIGONUCLEOTIDE PRIMERS USED IN THIS STUDY

Pst1-AA (FAM)	GACTGCGTACATGCAGAA	Forward primer for AFLP amplification
Pst1-AC (HEX)	GACTGCGTACATGCAGAC	Forward primer for AFLP amplification
Pst1-AT (TET)	GACTGCGTACATGCAGAT	Forward primer for AFLP amplification
Mse1-C	GATGAGTCCTGAGTAAC	Reverse primer for AFLP amplification
AJHBUB1	GAAGGAGAGGACGCTGTCTGTC GAAGGTAAACGGACGAGAGAA GGGAGAG	First oligo for prehybridised sequence of adaptor for vectorette PCR
AJHBUB2	CTCTCCCTTCTGCGGCCGCAGTT CGTCAACATAGCATTTCTGTCCT CTCCTTC	Second oligo for prehybridised sequence of adaptor for vectorette PCR
AJHBUB3	GCGGCCGCAGTTCGTCAACATA GCATTTCT	Adaptor-specific primer for vectorette PCR
ITS1	TCCGTAGGTGAACTGCGG	Forward primer for internal transcribed spacer of rDNA
ITS4	TCCTCCGCTTATTGATATGC	Reverse primer for internal transcribed spacer of rDNA
AJH3	ATGTAAGTAATGGAAATGGT	Forward primer for Clone 1 amplification
AJH4	CAAACTCCCAGTCTCCCAGT	Reverse primer for Clone 1 amplification
AJH7	ACGTCTTCTTAGGTAAGGGC	Forward primer for Clone 6 amplification
AJH8	TGCAGACCAGTCCTCGT	Reverse primer for Clone 6 amplification
AJH13	GCTGCCGCTGGCGACCCTT	Forward primer for Clone 130 amplification
AJH14	GCCCTTTACCACACATT	Reverse primer for Clone 130 amplification
AJH15	CACTGAGTTCCGGTTCCGTAA	Forward primer for Clone 317 amplification
AJH16	CTCCAGCGTGGTCACACGATA	Reverse primer for Clone 317 amplification
AJH17	AACCTAATACGCATTGTGAGC	Forward primer for Clone 179 amplification
AJH18	CCGTAAAATGAATCAGTGATT	Reverse primer for Clone 179 amplification

Appendix Three

STRAINS USED IN THIS STUDY

Strain	Strain details	Reference/Source
S288Ca	FY833 *MAT*a *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63*	Winston *et al*., 1995
S288C diploid	FY2 (*MATα ura3-52*) x FY67 (*MAT*a *trp1Δ63*)	Winston *et al*., 1995
AWRI 729	Commercial wine yeast, UC Davis, USA	ATCC / AWRI culture collection
AWRI 796	Commercial wine yeast, identical to R107	AWRI culture collection
AWRI 825	729 yeast, Dept of Agriculture, W.A., Australia	AWRI culture collection
AWRI 838	Commercial wine yeast, isolate of EC1118	Lallemand / AWRI culture collection
AWRI 844	Commercial wine yeast, isolate of L2056	Lallemand / AWRI culture collection
AWRI 870	Winery isolate, N.S.W., Australia	AWRI culture collection
AWRI 939	*S. cerevisiae* sake yeast	de Barros Lopes *et al*., 1999 / AWRI culture collection
AWRI 1017	Commercial wine yeast, isolate of R2	Petaluma, S.A. / AWRI culture collection
AWRI 1116	729 yeast, Epernay, France	de Barros Lopes *et al*., 1999 / AWRI culture collection
T73	Wine strain isolated from Valencia region, Spain	Lallemand
Kyokai 7	Sake yeast, ATCC 26422	ATCC
UVAFERM CEG	Commercial wine strain	Lallemand
NCYC 2889	*S. kudriavzevii* type strain	National Collection of Yeast Cultures, UK
CBS 1171	*S. cerevisiae* neotype strain	Centraalbureau voor Schimmelcultures
BY4742	Parent strain of deletion collection, *MATα*	EUROSCARF
Δ*TDH1*	Deletion strain of *TDH1* from parent strain BY4742	EUROSCARF
Δ*TDH2*	Deletion strain of *TDH2* from parent strain BY4742	EUROSCARF
Δ*TDH3*	Deletion strain of *TDH3* from parent strain BY4742	EUROSCARF

Appendix Four

POLYMORPHIC SEQUENCES ISOLATED BY AFLP

838-1
TGCAGACACGAAAGACACAAAAGTTATCTCGGCAGTTTACCCTTTATATCGAATGC
AGGCGTAGACAAAAGTGCGTTGCATAACTTACCAAGGTTA

838-2
TGCAGACAAATTGGCTTTGACTAGGACCGAGAGTGTAAAGCCAGAACCGGAGATAA
CCGCTCCGCCTCACTCACGCTTTTCCCGTTCTTTCAAGACAGTGTTA

939-1
TGCAGATTTTCATGCAGCGTCGAGCTGCTAAACGTCACAACTATTTGACGTACATT
CTGCAAAAGTATGTCGACTTCGCACGGACCATTATGCTTGCTTTACTACTTATTTT
GCTTTCGACTTGTTGAGTCCGAAAAGCTGTTCCTGGGTACCAATAGAAAATCAGCT
GAAATCTTCTCAACGAAAAAGTAAAATAAACTTGGCAGCATGCGTTA

939-2
TAAGGCTCTACAGGGNAACAATNGGGGCCATTGAAACTGGTGTTGACTCAATTTTC
AAGGGACCATCTGCA

939-3
TGCAGATGAATATAAAGATAAGTACGCTAAGATTGTAAAAAGTGCTTGAAGCTCAA
AAATATGTATTCCCTTA

939-4
TAACCCGGAAGTTATTAGACTCTTGGTGTTGCCAGTTTCGGATACAGAAACAACAC
TACTGCTGTGACCAATCACATCGGTCGCGGAAGCCGTCTGTGTTTCAGCATGATTG
AATCTTGAAATTGAAGAGGTGACTACTGTTTTCGCCTCAGCAGCTCCAGTACTGGT
AGTTGTCTCGGCAGCTCCAGTATTGGTTGTTGTCTCACTGGTAGCACTGTTCATTT
TAGAGCTGACAGACTCTTCATTCGTAGCCTGTGGCCTCCATGTAGGATAGACCGTA
ACAACATCATTCACAGTAGCCGTGGCCGTCGAAACAATGGCAGGTGAAGCAGTTTC
GGAACACACACCAGATTCGCAGGAAGTTAACAGTAACTAGCGTAGTTTGTTGCCTC
GATTCTGTGGTGGAAATAGGGCACCATGTCGTGTTATTCTGTGGTAACGCCCGTTA

939-5
TGCAGACAGTTTTGATAAGATTTATGGCGTTATGTGGATTGAAGTACCTTTCAATG
GCAATGGCTTACAGAATGATAGCGCCGTTA

939-6
TGCAGAATGACCAAGATGACAGTAACTTGAATCCCCATAATGGTGTGAAAGTCAAG
ATACCGATTTGCTTA

1017-1
TGCAGAACTGGTGTGATACCGGAACCACCGGCTACAATGCCTAAGTGCTTGGAGGA
GTTTGGTTCGTAGTTCAAAGCACCTATTGGCCCCTTA

1017-2
TAACATCCGAACACCAAATATGCGTGGGCGCCAAACAAAGAGACAATGTAATGAGA
CAATGTACTGCTTGTAAACTATAACAGTATCAGCTCAATTTCAATGCCATCTACTC
CAGATATCGTTGCGATTGTCCCTACAAGCCGCCTACAACGGCGTTATTGTTGTAAG
CTGGTAAAATGCAGTGCGTTCCCCCGTTGTGAGTTCGGGCCAAAATTACTTGGCTG
GGAGTTCCGTCCGTACAGTTGTGGCTTGCTGCCTAGACTTAGGCGGACGCGGCGGC
AATTACCCCTGGGTTCTCTCTAGAGCTGGAGAGACATTCTTGGTAACCGAAGGGAA
AACCCGGAAAGAAAATTTGGAATCCCGTTA

1017-3
TGCAGACCGTTTCGGCGACCATAACTTGGGTGATGATGATGATGCTGATTTCGAAA
AACGGGTGAATCGTAATGAAATAACCGCTATCGACAACAGTAGTTCTGCCAATAAT
ACAGATGTCACAGGTAGCACGAGTGATAGAACAGAGCTAAGTCACCCCGACGTGAC
ACCGAAAGATTCAAATGGACCTGTTA

1017-4
TAACCCAAACAATGAGATCTTCATTCTTTTTTTTACCTCAGCTATGACTTGCAGTG
CTTTGTATCATACCGCTCGTTTGGTCGTGCTATTTGTAACAATGAACCTCGTTACC
CCGAACAAAAAAAAAATGAGATATTGAACGCCGCAGCTCATCATGGTAGAATGATGT
AGCTAATACCAAAATAAGTTATATTTAGAATGTTGGATATATCAGTTGCGTTAGAC
TAATCGTTCTAGAAGGATACAGAACTTGGAAAGATCTTTGAACGTATCGTGACTGC
GTGCTTGCTTTATTTGCCGTTGATTTTTTGCTATGACATGCCTAATTTTATGGTAT
CTGTGGCTCCATATCAACTCTTCCAGTTA

1017-5
TGCAGACGGTTCAATAAGGGGTTGGGACGCAAACGACTACTCTAGAAAATTTTCCT
ACCATCATACCAATTTGAGTGCAATTACCACATTTTATGCATCGGATAATATTTTG
GTGAGTGGGTCGGAAAATCAGTTCAATATCTATAATCTACGGAGTGGGAAATTGGT
CCACGCAAATATTCTAAAAGATGCTGATCAGATTTGGTCGGTTA

1017-6
TGCAGAACCAAAGGCGAAGAGAGCTAAGACGGGCAGTGCATCTACCGTGAAAGGGA
GCGTCGACCTAGAAAAATTAGCGTTCGGATTGACTAAACTAAATGAAGATGACCTG
GTTGGTGTTGTTCAAATGGTTACCGACAATAAAACACCAGAAATGAACGTGACGAA
TAATGTTGAAGGGGGTGAATTTATAATTGACCTGTATAGTTTACCTGAGGGATTAT
TGAAAAGTCTATGGGACTACGTTA

1017-7
TAACTAGAGGCGGTTTATTAGGGAATTCTTGAGCAGCTCTTTCGGAATTCTGATAA
CGGATAGCGACTTCGTCCTCAACGTAAATGATTGGAATAGTATGTCCTCTACTGTC
GACGATTTGCTTCTTATGTAGAACTTCTTGTAATCTTTTGTAAAATTTTTGAGTCT
TTGGGTTAGGGCCATTGATTCCGATGGCATTCGGTTGACAGCTTTGAATGATATTA
TCCAAGGTGTCTTCAAATTTTTCAGGATTCGTCTTATCAAATGGATTGTCGACAAT
CTTGTAATCTCTTATAAAATCACCCTTTCTGTTGACGTTAGACAACAATTATAGCG
TCGGCTCCGAATCTACCCTGTCCACANGTTA

1017-8
TACAGACTTATGAACAAGATAAGTTCAAAGAAATGACAGACGATGTGAAGAAAGCG
GGCGCAGATGTTGTTATATGCCAATGGGGGTTTGACGATGAGGCCAATCATCTACT
TCTACAGAATGATTTACCTGCCGTAAGATGGGTAGGTGGCCAAGAACTAGAACACA
TTGCCATTTCCACAAACGGTCGCATTGTTCCAAGATTTCAAGACTTGTCTAAGGAT
AAATTAGGTACATGTTCCACAATTTACGAGCAGGAGTTTGGTACTACTAAGGATCG
TATGCTGATTATCGAGCAAAGTTA

1017-9
TAACGACAGGTCCGATGTTCCAGAACTGTTGAATTTTGAATGCGGTGCGGATTATG
TGAAGACTAACCAGAGATTACCCAAGGGTCTTTCTCCATCCTCGTTTGATTCGCTA
TATTGCTCCTTTGATGGTGACGCAGACAGGGTTGTGTTCTACTATGTCGACTCAGG
ATCAAAATTTCATTTGTTGGATGGTGACAAAATTTCCACTTTGTTTGCAAAGTTCT
TGTCTAAACAACTAGAATTGGCACACCTAGAACATTCTTTGAAGATTGGTGTTGTG
CAAACCGCCTATGCAAACGGCAGTTCCACCGCTTACATAAAAAATACGTTGCACTG
TCCCCGTGTCTTGCACTAAGACAGGTTGTTA

1017-10
TAAGCAAAGAAAAGAATAAGAAAAAGAAAAAGTATGTGAAGGGTATAGACGAACAA
AAAGGAAATCATTGAATTTATGGCATTTGATTGACGTTCCTGTTTGGTTCTGCA

1116-1
TGCAGACATCAAATATGTAAGTAATGGAAATGGTCACCAGGCGCAACAAAAAGAGA
GACAACCAGAATCTGAAAATCCGCCTAGAGAGAATGCACAGAAGTCGAATACGTCC
AGCCAGGGTCGAGCTCCCTCTTCTCAGGGCATGCCTAAACAGTTCCATAGAAAATC
ACTGGGAGACTGGGAGTTTGTTGAAACGGTGGGCGCAGGTTCTATGGGAAAGGTTA

1116-2
TGCAGACAACCTAATACGCATTGTGAGCGCTGTTTTCCTCATCATATCGATAGGCC
TAATCGGCGGCTTGATGGACACACAAACAAACGGTAGTTCCCGAGTGAACTTTTGT
ATGTTTGCCGCGGCTTACGGTCTAATCACTGATTCATTTTACGGGTTTTTGGCAAA
TTTTTGGTCATCGTTA

1116-3
TGCAGAATGGTACTGCTGTCCCCGTTTCTGTCTATGCGCCCTGGGATATCACTAAC
GCAGCATTCACCTTAGACACAATCCAGAAATATCTACCTCTTTTGGAGTCTTATTT
CAAGTGTTCGTATCCTCTGCCCAAGTTGGATTTCGTTCTACTGCCATACTTA

1116-4
TGCAGACCAGTCCTCGTCTTCAATGCTCCAAGCTTTGCAAATTACCAGTAACAACG
ACGTTTTCTAATATGGAACCGTTTGGAATATCTATTTTGTGACCGTCTGAGCAAAC
AATAATGACAGTGCCCCTCAAGGTGACGCCCTTACCTAAGAAGACGTTACCGGTAA
TGGTCAAGTGGTCTAGTTCAACAATCTTCGGGATGTGAGGGATTCTTGCGTTA

1116-5
TGCAGAATCAGTTGGTGCCGATAAACGCAAGAGTCACGATCGACACAATAACAAGA
AAAATCGTACCCCCTCAGGAGGCCTACGGTGATTTTATAGGATTGGACTCGCACTA
TGGATACCACACTCGTATAGCGTCATCATTCACAGACTTGTTCATGAAGGGCCCCT
TA

1116-6
TGCAGATGGCTAGAAGACTTCTGCAACTGTATGTCCATCTTCATTCGTGGGAACTC
CAAGCGCGAACAATCTGTATGCTGTTTCGGGCTACTTATTGCTCCATACGGTTTGT
AATGGACACGGACTCTTGCATTTGCTATATATATGCCTTA

1116-7
TGCAGAAACTGATCCTGGTGTTCTTTCTGTCAAGAAGATATACACCTACTATAAGA
AGCACGGCTATGCTACTGAGGTGATGGCGGCTTCCTTTAGAAATCTAGGCGAGTTG
AAGGCCTTAGCGGGGATCGATGACATGACTCTACCTCTGAACATTTTGGAACAGTT
ATATGAGTCCACAGACCCTATTGAAAATATCTTGAACACTGAAACTGCTAAGCAGG
AAGGTGTTGAGAAAGTCACTTTCATTAGCGACGAACCCCACTTTAGATATGTCCTA
AATGAAGATCAAATGGCTACAGAAAAACTATCTGATGGCATCAGAAAATTCTCTGA
AGACATTGAAGCGCTATATAAGCTGGTTA

1116-8
TGCAGAACGTGGTTGTGGCCAGCAGATGCCAGGGCTGATAAATAATGCCAAGCACA
AAGGTCCAGGCGAACCCAATGAAATCATAAATCCCAATTACATGGGGGAATGGCAT
CCACCGGAAATTGAAAACCCGCTATACTACGAAGAGCAGCACCCATTGCGCATCGA
AAACGTTATCAGTGGTGTGATCCTCGAATTTTGGAGTGGATCTCCAAACATGTTGA
TAACCAACATTTATGTTGGTAAAAATGTAACAGAG

1116-9
TAAGGTCGTAAACGACTGCTTACTCAGGGNAGATCTCCGAAAACGGCAATTGCTGT
GCA

Appendix Five

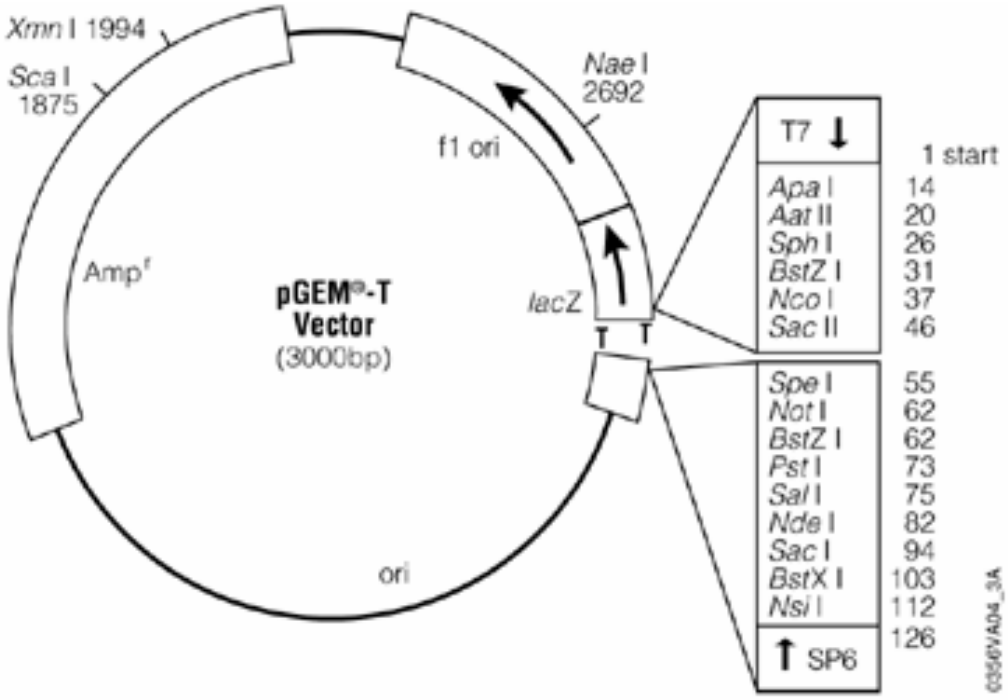
ISOELECTRIC FOCUSING PROGRAMS FOR DIFFERENT PH STRIPS

pH of IEF strip	Program used for IEF
3.5-4.5	30V x 12 h (active rehydration); 500V x 1 h; 1000V x 1 h; 8000V x 7.5 h. Total: 61,860Vhrs
5-6	30V x 12 h (active rehydration); 500V x 1 h; 1000V x 1 h; 8000V x 7.5 h. Total: 61,860Vhrs
4-7	30V x 12 h (active rehydration); 500V x 1 h; 1000V x 1 h; 8000V x 7.5 h. Total: 61,860Vhrs
6-11	*Waite Campus:* 30V x 12 h (active rehydration); 500V x 1 h; 1000V x 1 h; 8000V for 32,000Vhrs. Total: 33,860Vhrs *APAF:* 100V x 3h; 300V x 3h; 600V x 2h; 1000V x 2h; 2500V x 1 h; 5000V x 5h. Total: 31,900Vhrs
3-10 NL	30V x 12 h (active rehydration); 500V x 1 h; 1000V x 1 h; 8000V for 32,000Vhrs. Total: 33,860Vhrs

Appendix Six

pGEM-T vector used for cloning of AFLP polymorphic fragments.

As *Taq* polymerase was used in PCR reactions, T-A cloning was possible at the linearisation site in the multiple cloning site of pGEM-T.



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